QUANTITATIVE ASPECTS OF BLOOD VESSELS
AND PERINEURrium IN DIABETIC NEUROPATHY

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ABSTRACT

The cause of diabetic polyneuropathy is at present unknown. Abnormalities of the vasa nervorum are well documented, but a causal relationship between their occurrence and that of diabetic polyneuropathy remains equivocal. Recent studies have reported findings that support this hypothesis, in particular the claim that the number of "closed" endoneurial capillaries is greater in diabetic neuropathy than in control subjects, this being positively correlated with the severity of neuropathy. As these studies were undertaken on elderly diabetic patients (mean age > 50 years), accompanying vascular disease would not be unexpected. Perineurial basal laminal thickening has also been observed in patients with diabetic neuropathy, but its relationship to concomitant microangiopathy has not so far been documented.

I have examined sural nerve biopsies from a series of 27 younger diabetic patients with symmetric distal sensory and autonomic neuropathy (mean age 39.8 years). Morphometric observations on endoneurial capillaries and perineurial cells were compared with results from age-matched organ donor control cases and patients with type I hereditary motor and sensory neuropathy (HMSN I). Perineurial basal lamina thickness was significantly greater in the diabetic cases than in the organ donor control group.

Capillary luminal area did not differ between the 3 groups and the presence of "closed" capillaries in the diabetic cases was not confirmed. Capillary density was also comparable between the diabetic and organ donor cases, but was reduced in patients with HMSN I, this being related to hypertrophic changes and a resultant increase in fascicular area. The finding that transverse capillary endothelial cell area, and nuclear and pericapillary basal laminal area were all increased both in patients with diabetes and HMSN I make it difficult to present these abnormalities as evidence in favour of a vascular basis for the neuropathy as HMSN I is a condition in which a vascular basis can be discounted.
Published material presented in this Thesis


Declaration of author's contribution

All of the morphometric studies and experimental work was performed by the author. The content of the majority of this thesis has been published (see above).

Clinical data on the diabetic patients from King's College Hospital were provided by Dr PJ Watkins.
Acknowledgements and dedication

The research described in this thesis was conducted in the Department of Clinical Neurosciences, Royal Free Hospital School of Medicine, London, under the supportive and enthusiastic supervision of Professor PK Thomas. I am indebted to Dr Rosalind King for numerous reasons, including the fact that she taught me everything I know about electron microscopy. I would also like to thank Jane Workman for her invaluable technical advice and patience, and John Muddle for instruction in the use of the Kontron MOP Videoplan, and the time he dedicated to writing the specific morphometric computer programs for this project. I am also grateful to Dr PJ Watkins (King's College Hospital) for supplying biopsy material.

To the other members of the Department, both present - Isla, Andrea, Vin - and past - Adrian, David, Eric, Noshir - to name but a few, I express my sincerest gratitude for their friendship and encouragement at all times.

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# LIST OF ABBREVIATIONS

The following is a list of abbreviations used throughout this thesis:

- **AGEP**: Advanced Glycosylation End-Product
- **AR**: Aldose Reductase
- **ARI**: Aldose Reductase Inhibitor
- **ATP**: Adenosine Triphosphatase
- **BM**: Basement Membrane
- **BNB**: Blood-Nerve Barrier
- **cAMP**: cyclic Adenosine 3',5'-Monophosphate
- **CMT**: Charcot-Marie Tooth
- **CNS**: Central Nervous System
- **CNTF**: Ciliary Neurotrophic Factor
- **EAN**: Experimental Allergic Neuritis
- **ECM**: Extra Cellular Matrix
- **EDTA**: Ethylenediaminetetraacetic Acid
- **EM**: Electron Microscope
- **FGF**: Fibroblast Growth Factor
- **GAG**: Glycosaminoglycan
- **GBM**: Glomerular Basement Membrane
- **HMSN I**: Hereditary Motor & Sensory Neuropathy (type I)
- **HRP**: Horse Radish Peroxidase
- **hRPE**: human Retinal Pigment Epithelium
- **IDDM**: Insulin Dependent Diabetes Mellitus
- **IGF-I**: Insulin-Like Growth Factor-I
IL-1: Interleukin-1
IP₃: Inositol 1,4,5-triphosphate
MNCV: Motor Nerve Conduction Velocity
NADP: Nicotinamide Adenine Dinucleotide Phosphate
NCV: Nerve Conduction Velocity
NEG: Non-Enzymatic Glycation
NGF: Nerve Growth Factor
NIDDM: Non-Insulin Dependent Diabetes Mellitus
NO: Nitric oxidase
PAS: Periodic Acid-Schiff
PCBM: Perineurial Cell Basement Membrane
PDGF: Platelet Derived Growth Factor
PKC: Protein Kinase C
PMP22: Peripheral Myelin Protein 22
PNS: Peripheral Nervous System
SCa: Slow Component a
SCb: Slow Component b
TGF beta: Transforming Growth Factor beta
TJ: Tight Junction
TNF: Tumour Necrosis Factor
CHAPTER 1. INTRODUCTION

1A DIABETES MELLITUS

1A.1 Glucose regulation

Glucose regulation in the body is controlled by what may be described as a closed-loop feedback system. The islets of Langerhans in the pancreas contain two types of secretory cells, beta and alpha cells producing insulin and glucagon respectively. Both of these hormones affect the production of glucose by the liver where it is stored in the form of glycogen.

The concentration of glucose in the blood directly affects and regulates the secretion of both insulin and glucagon. Insulin increases the production and glucagon the breakdown of liver glycogen. Insulin also affects the utilisation of glucose by tissues such as fat and muscle. In other tissues, however, most notably the brain, glucose uptake is not insulin-dependent. In healthy individuals a compensatory reduction in the secretion of glucagon and increase in that of insulin accompany the development of elevated plasma glucose levels, thereby counterbalancing the possible development of hyperglycaemia and maintaining the plasma glucose at a near-normal concentration.

There are a number of factors that can contribute to glucose intolerance by affecting either tissue sensitivity to insulin or insulin secretion. Ageing may result in resistance or decreased sensitivity of tissues to insulin action. Measurements of insulin binding to cells in the elderly has generally revealed no defect. Insulin resistance has therefore been related to a defect in the cellular effects of insulin action beyond the receptor site; it has been shown that there is diminished insulin-stimulated glucose transport in isolated adipocytes from elderly patients in comparison to that of young controls (Fink et al., 1984). Elderly patients with insulin resistance additionally appear to have diminished β-cell sensitivity to glucose (Chen et al., 1985).

Other factors that may induce resistance to insulin are obesity, decreased physical activity, starvation, stress hormones and decreased insulin secretion. β-cell damage or dysfunction causes glucose intolerance by decreasing insulin secretion. Other factors with a similar effect are a low carbohydrate diet, starvation or inhibitors of insulin release such as catecholamines.
1A.2 Clinical classification of diabetes mellitus

Diabetes mellitus is a common disease and with its vascular complications is a major contributing cause of myocardial infarction, cerebrovascular disease and peripheral vascular disease. It may also lead to end-stage renal disease and blindness (Halter & Porte, 1987).

There are two common forms of diabetes, juvenile and maturity-onset, differentiated by obvious clinical differences and the age of diagnosis, in addition there are several other less frequently occurring types such as gestational diabetes. In 1979 a classification system was devised for diabetes by the National Diabetes Data Group of the National Institute of Health. Clinical characteristics were defined for the 2 major types of diabetes mellitus Insulin-Dependent Diabetes Mellitus (IDDM), Type I, or juvenile-onset diabetes and Non Insulin-Dependent Diabetes Mellitus (NIDDM), Type II, or maturity-onset diabetes.

IDDM is characterised by an absolute deficiency of insulin subsequent to the development of gross pancreatic beta-cell damage and necrosis which are believed to result from either the direct effect of viruses or chemical toxins or an autoimmune mechanism. The secretion of glucagon is also abnormal as increased production is not observed in response to a hyperglycaemic stimuli. Familial trends indicate the presence of a clear genetic component, however, environmental influences must play an important role as less than 10 percent of first degree relatives are affected.

Patients with IDDM experience a sudden onset of the disease, severe hyperglycaemia and a rapid progression to ketoacidosis and death unless treated with insulin. The term Juvenile-Onset diabetes is derived from the age of diagnosis as about 50 percent of patients have been diagnosed before the age of 21 years, the peak incidence occurring near puberty. Patients are characteristically lean and prone to ketosis.

Although the beta-cells of individuals with NIDDM retain the ability to synthesise insulin, levels fluctuate from below to above normal values resulting in the loss of homeostatic control of blood glucose levels: the direct effect of intravenous glucose to stimulate insulin secretion is markedly impaired in NIDDM patients with fasting hyperglycaemia (Ward et al., 1984) and a major diagnostic criterion for and characteristic feature of NIDDM is the presence of hyperglycaemia in the fasting state (American Diabetes Association Clinical Education Programme,
The effects of this defect are exacerbated by the development of
target-organ resistance to insulin; hepatic sensitivity to insulin is diminisheid
leading to an increased rate of glucose production. Plasma glucose levels in
individuals with NIDDM therefore have to increase considerably, as a result of
reduced β-cell sensitivity, in order for the production of insulin to be sufficient
to compensate for the decreased hepatic sensitivity.

Many of the characteristics of NIDDM are the opposite of IDDM, although both
are associated with retinopathy, microvascular disease, neuropathy,
nephropathy and arteriosclerosis. NIDDM usually has a slow onset. The
patients are not prone to ketosis and do not therefore depend upon insulin
therapy for survival; many patients, however, require insulin to control
hyperglycaemia.

Genetic, dietary and environmental factors are believed to be of causal
importance in the development of NIDDM. More than 20 percent of first degree
relatives are affected. Obesity is a major clinical feature of individuals with
NIDDM. Socioeconomic factors also appear to play a large role determining the
age of onset and the frequency of NIDDM, probably due to nutrition levels and
resultant body weight. Autoimmune phenomena are not increased in patients
with NIDDM.

Above average mortality related to diabetes has been estimated to be increased
two to threefold in those under 40 years of age and as high as elevenfold for
patients aged 15 years or less. Sixty percent of the death certificates
mentioning renal or cardiovascular disease carry an associated diagnosis of
diabetes in the USA. Clinical expression and development of various
complications are a function of duration of disease. Cardiovascular disease is
the major cause of death in NIDDM patients as opposed to renal failure in IDDM
cases (Carter Center of Emory University, 1985).

Numerous and variable types of peripheral neuropathy are associated with
diabetes mellitus resulting in the production of a very complex situation
regarding the classification of neuropathies within this disease. This is further
complicated by the fact that mixed syndromes are frequently observed within
one individual. The most feasible classification system to date is that advocated
by Thomas (Thomas, 1973a) whereby diabetic neuropathies are broadly
subdivided into two groups (1) the predominantly sensory and autonomic
symmetrical polyneuropathies and (2) focal and multifocal neuropathies. The
first category includes sensory or sensorimotor polyneuropathy and autonomic neuropathy; the latter, cranial neuropathy, trunk and limb mononeuropathy and proximal motor neuropathy or diabetic amyotrophy.

The symmetrical nature of the neuropathies in the first division suggest a metabolic cause whilst it seem likely that vascular lesions may be of importance in the aetiology of focal neuropathies. It is possible that a degree of overlap may exist between the types of pathological processes i.e. nerves compromised by a metabolic disturbance may be more susceptible to vascular anomalies, leading to a discernible overlap between syndromes.

1A.3 Incidence of diabetic neuropathy

The annual incidence of neuropathy appears related to the duration of diabetes, the prevalence being 7.5% at the time of diagnosis and rising to approximately 50% after 25 years known duration (Pirart, 1978). Susceptibility is related to the sex of the individual, the risk being slightly greater in males.

1A.4 Clinical features

Quantitation of thermoreception thresholds and autonomic function can be used as measures of the integrity of small calibre myelinated and unmyelinated fibres. Clinical manifestations include loss of temperature and pinprick sensibility, pain and burning dysaesthesiae and autonomic dysfunction.

Quantitation of vibration sense and sensory nerve conduction may be used for the evaluation of large fibres; amplitude represents the summated activity of the larger (A alpha) myelinated fibres and maximal conduction velocity reflects the function of the largest, fastest conducting fibres. Abnormalities in vibration perception have been correlated with electrophysiological measurements and structural changes in late diabetes (Consensus Statement, 1988) and abnormalities in sensory nerve conduction have been strongly correlated with sural nerve myelinated fibre density in early diabetic neuropathy (Veves et al., 1991). Examples of observable alterations include reductions in conduction velocity and diminished amplitude and increased temporal dispersion of the sensory action potentials.

In a study of 73 symptomatic patients with different syndromes of diabetic polyneuropathy and 33 asymptomatic diabetics, electrophysiological tests and autonomic function were more abnormal in the symptomatic group. Additionally, patients with foot ulceration were associated with the greatest
degree of electrophysiological abnormality (large fibres) whilst the ratio of autonomic to electrophysiological abnormality was greatest in patients with painful neuropathy (small fibres) (Young et al., 1980)

Small fibre damage has been stated to be the salient change in all diabetic polyneuropathies, abnormalities in cold and/or warm thresholds being found to constitute the earliest evidence of neurological deficit, with the involvement of large fibres to a greater or lesser degree (Young et al., 1986). It has also been suggested that somatic neuropathy illustrated by alterations in nerve conduction precedes abnormalities of autonomic function, the latter being associated predominantly with severe defects of nerve conduction. The need for further studies is clearly indicated concerning the differential vulnerability of different fibre sizes in diabetes and the associated responsible factors. Regardless of which fibre type is the first to be affected in diabetic neuropathy, it has been concluded that sensory nerve conduction abnormalities are an early manifestation of nerve damage in diabetic neuropathy and that conventional electrophysiological tests in the lower limbs are therefore a reliable indicator of structural abnormalities in early subclinical diabetic neuropathy.

Typical diabetic peripheral neuropathy is distal, symmetrical and mixed sensorimotor in type (Ellenberg, 1976), with decreases in both sensory and motor NCV. In newly diagnosed cases of diabetes, improvement in conduction velocity has been observed following treatment (Fraser et al., 1977).

1A.4(i) Distal sensory and autonomic polyneuropathy

Distal sensory and autonomic polyneuropathy is the commonest form of diabetic neuropathy. The correlation between the two distinct components has not been revealed to any real extent; in some patients autonomic features predominate, whereas in other cases the reverse is true. In a fairly comprehensive study it was demonstrated that the majority of patients with symptomatic autonomic neuropathy had an associated sensori-motor neuropathy, whereas only 60% of patients with symptomatic sensorimotor neuropathy had additional autonomic involvement, the latter being invariably present where sensory polyneuropathy is advanced (Tackmann et al., 1981). Associated autonomic changes include pupillary and lacrimal gland dysfunction, cardiovascular disturbances, thermoregulatory disorders, alimentary tract disorders, genito-urinary disturbances, unawareness of hypoglycaemia and disturbances of respiratory control.
Sensory symptoms vary in intensity. In mild cases they may be manifested as numbness, tingling or burning paraesthesiae in the feet and less frequently the hands; aching pains in the legs and feet and impairment of cutaneous sensibility in the legs and occasionally the fingers. A severe neuropathy may give rise to a symmetrical distal loss of deep pain sense, joint position sense and vibration sense, these changes being most pronounced in the lower extremities. Tendon reflexes are lost and proprioceptive sensory ataxia may lead to the development of an ataxic gait. In more severe cases sensory loss may develop over the anterior aspect of the lower trunk.

The development of perforating ulcers in the feet is a troublesome complication. Loss of pain sensation is the primary factor involved in their occurrence. Other contributing determinants may be ischaemia resulting either from atherosclerosis or small vessel disease and the increased liability of tissue to infection in the diabetic state. Anhidrosis, a component of autonomic dysfunction, may also contribute to the formation of ulcers as it leads to dry skin that cracks and fissures easily. Abnormal foot posture with clawing may also develop, and in rare instances, neuropathic arthropathy.

Regarding sensory and autonomic polyneuropathy, there is a typical distal "length-related" pattern of sensory disturbances in the limbs which implies that longest nerve fibres are the most vulnerable. It is not possible to deduce the underlying pathology from these observations as both the summation of multifocal lesions distributed along the peripheral nerve trunks and a distal axonopathy of the "dying-back" type, which may be due to defective axonal transport or impaired synthetic mechanisms leading to loss of functional maintenance of those parts of the cells most distal from the perikaryon, result in the production of a similar pattern.

1A.4(ii) Small fibre neuropathy

Small fibre neuropathy is a diabetic syndrome that may produce symptoms after only a few years of hyperglycaemia and result in varying degrees of pain and temperature sensory loss. Dysfunction of the autonomic nervous system is regularly associated. Large fibre functions are affected to a minor degree compared to the severity of the pain and temperature sensory loss. This type of neuropathy may progress to other forms.
1A.4(iii) **Acute painful neuropathy**

This is an uncommon form of diabetic neuropathy. Severe constant pain is experienced in the lower extremities, being maximal in the feet. The hands may also be affected. Sensory impairment, maximal distally, is slight. This syndrome is often preceded by quite severe weight loss and recovery by weight gain.

1A.4(iv) **Ataxic neuropathy**

Patients exhibit involvement of the large sensory fibres revealed by impairment of touch/pressure, vibration and joint position sense. In severe cases the lower limbs are affected by sensory ataxia. Instability and body sway correlates with the severity of the neuropathy. So far involvement of small fibres has been excluded.

1A.4(v) **Proximal motor neuropathy**

This syndrome also known as "diabetic amyotrophy" involves very little sensory loss, the predominant effect being proximal leg weakness and atrophy. Two distinct disorders in diabetic patients produce these clinical symptoms. One is an acute asymmetrical proximal focal or multifocal motor neuropathy, probably caused by vascular lesions in the major nerve trunks (Asbury, 1977). The second is a slowly progressive proximal motor neuropathy resulting in symmetric or occasionally asymmetric proximal hip and leg muscle weakness. Muscles supplied by the femoral, obturator, sciatic and gluteal nerves are commonly involved. The site of nerve involvement is presumed to be in the lumbrosacral plexus and proximal limb nerves.

1A.4(vi) **Focal and multifocal neuropathies of diabetes**

Cranial mononeuropathies, truncal neuropathies, proximal asymmetrical motor neuropathies, compression neuropathies and entrapment neuropathies are amongst some of the relatively characteristic neuropathic syndromes associated with diabetes mellitus, although none is unique to diabetic patients.

1A.5 **Major non-neuropathic complications of diabetes**

The associated complications of diabetes mellitus, which are non-specific and may result in increased morbidity and mortality, include nephropathy, arteriosclerosis, retinopathy and capillary microangiopathy. Although it cannot be concluded that these affiliated complications are directly attributable to hyperglycaemia or insulin deficiency evidence has been produced of a link
between the incidence of these pathological changes and the degree of hyperglycaemia (Raskin et al., 1983; Sosenko et al., 1984).

1A.5(i) **Nephropathy**

There are two major forms of nephropathy associated with diabetes; nodular glomerulosclerosis and tubular nephrosis. Proteinuria, white blood cells and granular casts are revealed in urine samples and the levels of these indicators rise as the disease advances. Infection frequently develops in the kidney and/or the urinary tract and may lead to significant impairment in the kidney function. Renal disease occurs in 50% of all diabetics who survive for 20 years or more and tends to be progressive and irreversible once it has reached an advanced stage. Approximately 40% of deaths in long-term IDDM patients (20-30 years duration) can be related to renal failure.

1A.5(ii) **Arteriosclerosis - Macrovascular disease**

Coronary artery disease is the major cause of death in diabetics (Carter Centre of Emory University, 1985). The pathological changes seen in the larger arteries and coronary circulation of diabetic patients are identical to those of nondiabetic patients with the same syndrome, however, the incidence is greater in the diabetic population and the age of onset is younger (Strandness et al., 1964). The degree of vascular disease has been shown to correlate with duration of the disease and patient age.

Arteriolosclerosis is also common in the diabetic population. Morphological examination reveals concentric hyaline thickening of the arterioles, widening of the endothelium and in severe cases partial or complete occlusion of the lumen by a PAS-positive plaque. Due to its staining properties, it is considered that this material contains glycoproteins and is similar to basement membrane which also stains Periodic acid-schiff (PAS)-positive; however the specific nature of the plaque is unknown. Advanced vascular disease in the lower legs is common and often severe in diabetics. This feature in combination with the effects of loss of pain and touch sensation due to concomitant neuropathy, which it has been suggested may itself result from arteriosclerosis and microangiopathy (LoGerfo & Coffman, 1984) often results in gangrene or death of the affected tissue (Strandness et al., 1964).
Diabetes is the leading cause of blindness in the USA, retinopathy being one of the most characteristic associated complications. The retinal changes can be classified into two types; retinitis proliferans and background retinopathy.

Retinitis proliferans accounts for approximately 25% of the cases of diabetic retinopathy. Small vessels originating from the optic disc or other area of the retina grow along the surface of the retina and often into the vitreous. This is followed by infiltration with fibrous tissue. Retinal separation and haemorrhage may occur on contraction of the scar tissue causing severe or total loss of sight. Associated microaneurysms and exudate deposition often occur. Retinitis proliferans is most frequent in IDDM patients presumably as a feature of the duration of disease and the period of time required for development of the retinopathy. Oedema and/or plaque formation may also result in diabetic maculopathy.

Background retinopathy is associated with oedema, microaneurysms, exudates and haemorrhages. Tiny aneurysmal dilatations of the capillaries (30 to 90 µm in diameter), arterioles, or rarely, venules are often the first lesions in the retina. Rupture, bleeding or leakage of plasma proteins from these aneurysms result in the subsequent deposition of PAS-positive hyalinised material and lipids. Similar aneurysms to those associated with diabetic retinopathy are found to occur with other disorders but are rarely as numerous or as frequent as in the diabetic population.

Varying hypotheses have been proposed with regard to the causal agent, examples being capillary basement membrane thickening, hyaline deposition, a disorder of polysaccharide metabolism, venous stasis and the loss of pericytes. No conclusive aetiological mechanism has been demonstrated so far, although a relationship has been suggested between diabetic control and the incidence of retinopathy and recent work has suggested that aldose reductase-linked destruction of pericytes may be the initial link in the chain of events which lead to the formation of microaneurysms, intraretinal haemorrhages, chronic occlusion of capillary beds, subsequent ischaemia and the resultant growth of new vessels (Tokahashi et al., 1992).

The prognosis for vision restoration depends on the site of the new vessels, being better where the lesions are located peripherally as opposed to centrally. Treatment is photocoagulation therapy; the laser is directed primarily at
abnormal areas and new vessels in the retina. More recently the method has been used to produce a number of small scars, ranging from 300 to 1000 across the retina on the basis that a reduced retinal oxygen demand will reduce any new vessel growth.

Cataracts are another very common complication in diabetes. Two types are found; senile cataracts which occur predominantly in older patients and are no different to those found in elderly nondiabetic individuals (Straatsma et al., 1985) with the exception that the frequency is greater and the cataracts appear to mature more rapidly, and metabolic or "snowflake" cataract.

Cataracts are more prominent in patients with IDDM than NIDDM and have been correlated with the degree of glycaemic control, similar metabolic cataracts being induced by experimental hyperglycaemia (Kinoshita, 1974). Aldose reductase, an enzyme which reduces glucose to the sugar alcohol sorbitol, is present in lens tissue. It has been proposed the accumulation of intracellular sorbitol is instrumental in the development of this form of diabetic cataract as similar cataracts have been induced by high extracellular levels of glucose. This theory has been further consolidated by the effects of aldose reductase inhibitors in experimental diabetes (Beyer-Mears & Cruz, 1985).

1B NON-NEURAL COMPONENTS OF THE HEALTHY PNS

Peripheral nerves are composite structures. In 1876 Key and Retzius (Key & Retzius 1876) described and named three component connective tissue sheaths, the epineurium, the perineurium and the endoneurium.

1B.1 The epineurium

The epineurium is the outermost sheath and surrounds the entire nerve. Its components blend loosely with the surrounding connective tissues allowing the nerve a degree of mobility. At irregular intervals along its length it is anchored by blood vessels. The epineurium is chiefly composed of collagenous fibrils whose diameter, in human peripheral nerves, ranges from 60 to 110nm (Gamble & Eames, 1964). It also contains elastic fibres and associated microfibrils (Thomas, 1963) that are usually orientated in a longitudinal direction, often adjacent to the perineurium, mast cells and macrophages in numbers similar to those found in loose connective tissue in general, and variable amounts of fat.
1B.2 The perineurium

Large nerve trunks consist of a number of funiculi or fascicles. Peripheral nerves are composed of one or more of these fascicles. Fascicles are bounded by the perineurium or perineural sheath, a multilayered structure composed of alternating concentric lamellae of contiguous flattened polygonal perineurial cells and fine collagen fibrils (Shanthaveerappa et al., 1962; Thomas, 1963; Akert et al., 1976). The number of these lamellae, which may be as great as ten or twelve, vary according to the size of the fascicle, larger fascicle having a correspondingly greater number of layers.

Perineurial cells are bounded on both endoneurial and epineurial aspects by basal lamina (basement membrane) of varying thickness; a PAS-positive substance which separates all connective tissue space from bordering fat and non-connective tissue cells (Gersh & Catchpole, 1949). This basement membrane (BM) may reach a quite substantial thickness, 0.5 μm having been reported in human peripheral nerves (Gamble & Eames, 1964).

Because of their flattened shape, perineurial cells have a very large surface area to volume ratio. The volume of cytoplasm is substantially increased at the perinuclear zone which is the chief location of cytoplasmic organelles including endoplasmic reticulum and mitochondria. Glycogen particles are also abundant. The cytoplasm also contains bundles of filaments that are similar in appearance to those of actin in smooth muscle cells. This has led to the proposal that the perineurium may possess contractile properties and play a role in the flow of endoneurial fluid (Ross & Reith, 1969).

The perineurium encloses the endoneurium and acts as a perifascicular diffusion barrier isolating and maintaining the endoneurial microenvironment by preventing or delaying the entry of extraneural substances (Olsson & Reese, 1971). This property has been attributed to the presence of "tight junctions" (zonulae occludentes) (Reale et al., 1975). Tight junctions occur where contiguous perineural cells overlap or interdigitate at their borders (Thomas, 1963), and link the closely juxtaposed cell membranes (Burkel, 1967; Thomas & Jones, 1967; Akert et al., 1976) which, in these regions, are devoid of the normal investment of BM.

The ultrastructure of tight junctions has been revealed by freeze-fracture as consisting of a system of networks of numerous branching and anastomosing ridges on the P fracture face with complimentary grooves on the E face.
Together these features constitute the morphological basis of the diffusion barrier (Beamish et al., 1991). Tight junctions have been demonstrated as being efficient in preventing the passage of compounds which are sufficiently small to penetrate openings of a diameter of 2nm in gap junctions (Goodenough & Revel, 1970).

Perineurial cells also posses gap junctions or maculae occludentes. Under freeze-fracture examination these are seen as a collection of particles on the P face with corresponding depressions on the E face. Gap junctions represent sites of intercellular communication and allow the transference of certain inorganic ions and small molecules between neighbouring cells, indicating a degree of metabolic co-operation (Spray, 1985). They may also serve to mechanically tether the cells together as they have been identified on the lateral process linking concentric lamellae (Thomas, 1963).

A prominent feature of perineurial cells is the possession of numerous cytoplasmic pinocytotic vesicles/caveolae which open onto both the endoneurial and epineurial aspects of the cell (Gamble & Eames, 1964), (Akert et al., 1976), (Latker et al., 1985). Histochemical studies have revealed the presence of high levels of oxidative and dephosphorylating enzyme activity including that of Adenosine triphosphatase (ATPase), 5-nucleotidase, glycerophosphatase and creatine phosphatase within these pinocytotic vesicles (Shanthaveerappa & Bourne, 1962), and Na\(^+\)-K\(^+\)-ATPase activity has been detected in the innermost perineurial layers of rat sciatic nerve (Llewelyn et al., 1987b; Powell et al., 1991). These observations imply that a transport system exists across the sheath (Oldfors & Johansson, 1979; Oldfors, 1981b) and that the perineurium may potentially function as a metabolically active diffusion barrier, the innermost lamellae being the key region (Shanthaveerapa et al., 1962), and play a regulatory role in the movement of sugars and amino acids into and out of the endoneurial compartment. It may also participate in the removal of any excess endoneurial fluid entering via the vasa nervorum. Perineurial cells have also been ascribed a possible role in the removal of endoneurial debris.

The fact that the salient prominent structural specialisations seen in tissues where such transmembranous transport activity is high, such as in the kidney tubules and associated capillary networks, are lacking in perineurial cells suggests that the activity of this transperineurial transport system is relatively low and therefore unlikely to circumvent the diffusion barrier properties of the perineurial cell tight junctions and BMs.
The perineurial lamellae are separated by connective tissue spaces containing bundles of collagen fibrils, of a diameter ranging between 40 and 80 nm, orientated in circular, longitudinal and oblique directions to produce a lattice-like construction (Akert et al., 1976). These collagen fibrils are larger than those in the endoneurium but smaller than those in the epineurium (Thomas, 1963; Gamble & Eames, 1964). The thickness of the connective tissue spaces varies considerably, tending to be thinnest towards the innermost layers of the perineurial sheath.

The extracellular spaces in the perineurium also contain a few elastic fibres and the occasional fibroblast, recognizable by the lack of BM (Burkel, 1967). The presence of mast cells (Olsson, 1968a) macrophages and small unmyelinated axons has also been documented. Perineurial cells periodically branch to produce processes which traverse the connective tissue spaces and connect with adjacent lamellae. Occasionally the connective tissue space may be obliterated by fusion of the abutting BMs of cells from different layers. Continuities exist between the endoneurium and epineurium where the perineurium is traversed by blood vessels connecting the epineurial and endoneurial vasculature.

The perineurial sheath persists along the entire length of the peripheral nervous system (PNS) extending as far as the nerve terminals. The number of component layers reduces distally, the smallest nerve branches being surrounded by a single layer of perineurial cells. This structure has been termed the "sheath of Henle". At unencapsulated endings and at neuromuscular junctions the sheath is open-ended (Burkel, 1967) providing a region for communication between the connective tissues of the epineurium and the endoneurium and a possible site of entry for toxins. In contrast, the sheath of Henle is continuous with the capsules of muscle spindles and encapsulated end organs (Shanthaveerappa & Bourne, 1962). At both types of terminal end the perineurial cells remain separated from the Schwann cells and their associated axons by a small connective tissue space.

It has been suggested that perineurial cells may be epithelial in nature due to the fact that they possess intercellular tight junctions. This is an unlikely scenario as numerous other properties of the sheath belie this interpretation, for example, epithelia are bounded by BM on only one surface (Shanthaveerappa et al., 1963) and the presence of unit collagenous fibrils in the interlaminal spaces typical of perineurial cells is a feature which is never encountered in true epithelia.
Perineurial cells, perhaps best described as auxiliary non-conductive nervous tissue cells, have been shown to actually stem from fibroblasts (Thomas & Jones, 1967). Tissue culture studies have revealed that Schwann cells cultured with normal nerve cells do not provide the perineurial ensheathment commonly observed when fibroblasts are added to the culture (Bartlett Bunge et al., 1980).

1B.3 The vasculature of peripheral nerves

The endoneurium contains a microvascular plexus, rich in anastomoses, whose function is concerned with the exchange of gases, nutrients and metabolic waste products between the blood serum and endoneurial matrix. The vasa nervorum is also involved in the regulation of the chemical composition of the endoneurial microenvironment, and endoneurial fluid volume and pressure. As in other organs, nutrients and metabolites are transferred between the blood and the tissue across the endothelial cell lining of the vessel (Adams, 1942; Lundborg & Brânemark, 1968). Axons receive the majority of their metabolic requirements from their remote cell bodies via axonal transport, and as such the intrafascicular plexus chiefly supplies the non-neural parenchymal intrafascicular components i.e. fibroblasts and Schwann cells.

The blood supply is obtained from adjacent regional large vessels that arrive at intervals along the nerve and bifurcate into ascending and descending nutrient arteries. These join an extensive longitudinal anastomotic network of arterioles and venules that constitute the epineurial and perineurial vascular plexuses. The perineurium is traversed obliquely by numerous precapillary vessels or arterioles, of a diameter usually less than 50μm, which then join the endoneurial vascular network (Lundborg, 1979). The vessels that pass through the perineurium often carry with them a "perineurial sleeve" (Thomas, 1963; Burkel, 1967), formed from prolongations of the innermost layers of the perineurium. This sheath persists for a short distance into the endoneurium, but does not come into close contact with the vessel.

The nature of the constituent vessels of the endoneurial vascular plexus has been disputed. Bennet and colleagues (Bennet et al., 1959) suggested that a "true" capillary has a less than 50% investment by pericyte processes, and that more than that indicates that the vessel is probably a postcapillary venule. According to this criterion the majority of vessels in the endoneurial vascular plexus of the guinea pig have been designated as small venules (Waksman, 1957).
A more recent study on the size and structure of endoneurial vessels in the sciatic nerve of the rat indicated that the same observation is true of this species (Bell & Weddell, 1974a). The same vessels were, however, also shown to possess alkaline phosphate activity. This enzyme plays a role in phosphate reactions for energy production to support transport or carrier mechanisms, a fact substantiated by localisation of alkaline phosphatase activity, at electron microscopic (EM) level, to the pinocytotic vesicles of vessel endothelium.

The known fact that conventional postcapillary venules do not posses alkaline phosphatase activity, this being a feature of 'true' capillary beds, in conjunction with the barrier properties of the endoneurial vascular plexus implies that it is more reasonable to designate the majority of vessels in the rat sciatic endoneurial network as large capillaries which posses an unusually complete investment of pericytes, as opposed to postcapillary venules. Occasional arterioles and "true" venules are seen. A similar network is present in the cat and man with the exception that the proportion of smaller capillaries is slightly greater.

The majority of endoneurial vessels are arranged in a direction parallel to the long axis of the nerve; a few occasionally run obliquely or transversely (Bell & Weddell, 1984b). The capillaries in peripheral nerves are larger and more widely spaced than those in muscle (Bell & Weddell, 1984a). The endoneurial capillary plexus in man is thought to drain directly to vessels situated outside the perineurium as few "true" venules are found in the endoneurium. It has been suggested that small terminal nerve branches that lack an endoneurial blood supply acquire their nutrients via the perineurium which possesses a degree of permeability to certain compounds (Oldfors, 1981b). Very small nerves may also conceivably receive nutrients in this manner.

The spatial distribution of capillaries in rat sural nerve fascicles has been shown to be uneven, particularly so in large fascicles where capillary density is greatest in the subperineurial zone (Nukuda et al., 1965; Manis & Low, 1988). This observation may help to explain why fascicular degeneration is centrally located in some ischaemia-induced peripheral neuropathies.

The components of PNS endoneurial capillaries are identical to those of capillaries in other tissues; endothelial cells, pericytes and basal lamina. They also possess an intrinsic ultrastructural feature that is of enormous significance; the endothelial cells are bound together by tight junctions (Olsson & Reese,
fusions of the outer dense layer of the plasmalemma of each cell, providing an effective barrier for the endoneurium against higher molecular weight material from the blood (Olsson & Reese, 1971). Bell and Weddell found varying types of interendothelial junctions in rat sciatic nerve, including tight junctions (pentalaminar with fused outer leaflets), punctate tight junctions (outer leaflets sealed only at points), and gap junctions (outer leaflets closely approximated but separated by a narrow gap containing a dark amorphous material) (Bell & Weddell, 1984a). No transendothelial channels or fenestrations have been found in endoneurial capillaries.

The blood/nerve barrier in other parts of the nervous system has been shown to be less effective. Plasma proteins and exogenous tracer molecules have unimpeded access to the endoneurial compartment in spinal nerve roots, the autonomic ganglia and dorsal roots. Vessels in these zones of the PNS lack the aforementioned "tight junctions" and often possess fenestrations (Olsson, 1971). Tight junctions are similarly absent in perineurial and epineurial vessels, "open" junctions having been described in mouse sciatic nerve (Olsson & Reese, 1971), and tracers such as horse radish peroxidase pass freely across the endothelial cell layer. Fenestrations have also been described in epineurial vessels (Bell & Weddell, 1984a; Bell & Weddell, 1984b).

The endothelial cells of endoneurial capillaries possess an active vesicular transport system (Arvidson, 1984), which operates from both the luminal to abluminal side (Karnovsky, 1967; Bruns & Palade, 1968), and from the interstitium to the vessel lumen. The presence of Na\(^{+}\)-K\(^{+}\)-ATPase activity has recently been demonstrated in both the abluminal and luminal plasmalemmas of these cells in rat sciatic nerve (Powell et al., 1991) confirming the presence of an active transport system.

The presence of anionic sites of varying density have been demonstrated on the endothelial plasma membranes and BMs of the vasa nervorum; diaphragms of vesicles, transendothelial channels and luminal endothelial processes proving highly anionic, abluminal membranes the least anionic, with luminal membranes and the basal laminae of endothelial cells and pericytes being situated somewhere in between (Bush & Allt, 1990). These anionic sites probably function as charge filters and in this way restrict the passage of substances across the peripheral nerve barrier. Tight junctions do not posses an anionic charge, suggesting their barrier function is solely size dependent.
Pinocytotic transport across endothelial cells has been shown to be minimal where vascular barriers exist; the brain endothelium shows very few vesicles and the endothelial cells of endoneurial vessels contain fewer vesicles than those of epineurial and perineurial vessels (Bush & Allt, 1990). Due to the presence of the tight blood/nerve barrier (BNB) in peripheral nerves it is likely that the primary function of vesicles in the vasa nervorum is the absorption of nutrients or chemical messengers for the direct use of the endothelial cells themselves as opposed to the transendothelial transport of plasma constituents. The use of vesicles by the endothelial cells within a local controlling mechanism seems appropriate, as any regional imbalances in the ionic or osmotic milieu of the endoneurial compartment needs to be rapidly corrected for maintenance of functional integrity.

Numerous anastomoses exist between and within the intrafascicular endoneurial plexus and the interfascicular epineurial nutrient vascular plexus (Lundborg & Brånemark, 1968). These physical attributes and the resulting adaptability of the microcirculation allow the vascular network to act as an in situ reservoir and confer a considerable degree of resistance to ischaemia upon peripheral nerves. A relatively large number of nutrient arteries can be occluded without producing any change in microvascular flow within the endoneurial plexus. The blood flow of peripheral nerves is not believed to be an autoregulated system (Low & Tuck, 1984), and is instead reliant on extraneural factors such as blood viscosity and pressure.

As a result of the crucial role played by the vasa nervorum in regulating the chemical environment of the endoneurial compartment, vascular abnormalities may have a severe effect on nerve physiology. Changes in endoneurial fluid composition and volume, such as those that occur in oedematous conditions, may aggravate any concurrent neuropathy. In some neuropathies metabolic waste products may accumulate to such a degree that they attain neurotoxic concentration.

1C.1 THE ENDONEURIUM - THE NERVE MICROENVIRONMENT

The endoneurial cellular components of the PNS are surrounded by extracellular fluid, a mucopolysaccharide fibre-reinforced gel containing various ions and molecules, which forms the "microenvironment". The neural elements consist of myelinated and unmyelinated axons. In human sural nerve fascicles, 40 to 50% of the endoneurial tissue is composed of non-neural cellular elements (Poduslo, 1984). These components primarily consist of fibroblasts, Schwann
cells, and the endothelial cells and pericytes of blood vessels. Macrophages and mast cells (Olsson, 1968a) may also be found. The endoneurial fluid comprises as much as 20 to 30% of the tissue volume. These proportions and the density of the constituent non-neural cellular components vary between different regions of the same nerves and between different nerves. Variations are also apparent with age, species, and pathological conditions.

A continuous exchange of materials occurs between the endoneurial matrix and the blood. Various compounds are also released into the endoneurial compartment by its cellular components. The nerve microenvironment is maintained by the structural and regulatory functional properties of the perineurium, the endoneurial vasa nervorum, and the anatomical structure of the proximal and distal nerve ends. Together these features provide an effective barrier between the endoneurium and external tissues and fluids, enabling the fascicular contents to maintain homeostasis, under most conditions, regardless of any changes in extracellular fluid composition, such as those that arise in cases of local inflammation.

The peripheral nervous system (PNS) contains no lymphatic channels. When patent blue dye is injected epineurally, it is removed within a few hours by the lymphatic system and can be traced to regional lymph nodes. When the same dye is injected endoneurally, it spreads rapidly along the nerve for a distance of 4 to 6 cms where it remains unchanged for up to 6 hours. Pale staining may still persist after 2 or 3 days. The actual mechanism of dye removal from the endoneurium remains to be studied.

1C.1 Mast cells, macrophages, fibroblasts & Renaut bodies

Endoneurial fibroblasts are generally angular in shape, with a correspondingly angular nuclei. They possess elongated cellular processes, have no BM and are the main producer of endoneurial collagen (Salonen, 1987). They tend to be located subperineurially and within endoneurial septa and generally respond to pathological conditions by proliferation. There is a resident population of macrophages in peripheral nerve which are sometimes evident along the length of blood vessels or subperineurally. With inflammation monocytes enter from the bloodstream.

Mast cells possess no BM and have numerous filopodia or microvilli and are commonly found in association with capillaries and beneath the perineurium. In human peripheral nerves, the granules within these cells contain dense cores
and scroll like formations, the ultrastructural appearance of which may occasionally be hidden by their dense osmophilia. Mast cells are often difficult to distinguish from other cells in haematoxylin and eosin stained sections, however, Toluidine blue reveals their presence as the granules appear brilliantly metachromatic, a feature which is resistant to fixation and dehydration through alcohols. The number of endoneurial mast cells varying according to animal species (Olsson, 1968a).

Mast cells are able to influence the nerve microenvironment in many different ways as they are rich in heparin, histamine and many other potent biological compounds which they are capable of liberating into the endoneurium under pathological conditions by the process of degranulation (Olsson, 1968a), an effect that is enhanced by their proliferative activity in many neuropathies. New evidence has revealed that mast cells may play a role in demyelinating disease, as mast cell proteases are capable of dissolving myelin proteins and myelin basic protein and myelin protein $^2$ ($P_2^$) are capable of stimulating mast cell degranulation (Johnson et al., 1988).

Renaut corpuscles or bodies have a roughly spherical outline in transverse section and are composed of whorling filamentous strands associated with pale staining nuclei. They are generally located in a subperineurial position and extend along the axis of the nerve for a considerable distance, terminating in a rounded bulb. They seem to be bound both to the endoneurium and to the inner surface of the perineurium as they are disrupted if the perineurium is stripped away. By electron microscopy they are seen to be composed of a few cells, mostly coiled fibroblasts, embedded in a mucopolysaccharide matrix consisting of occasional loosely arranged, randomly orientated collagen fibrils and large amounts of fine fibrillar material.

Renaut bodies are histological curiosities. Their number increases with advancing age in healthy nerves, and in certain neuropathies, especially in nerve trunks naturally subjected to compression, such as adjacent to limb joints (Neary et al., 1975). Renaut proposed that they represent natural cushions that protect fibres from compression.

1C.2 Components of the extracellular endoneurial matrix

The extracellular matrix not only mechanically supports the cells which it surrounds, but also regulates their functions - migration, adhesion, differentiation, polarisation and synthesis, through specific interactions mediated
by cell-surface receptors such as integrins and cell surface proteoglycans. Extracellular matrix (ECM) molecules of the peripheral nervous system can be placed into one of three categories, collagens and related molecules, noncollagenous glycoproteins and proteoglycans (Carbonetto, 1984).

1C.2(i) Collagens

The collagens are members of a family of structural proteins which share certain common components and function as the major conformational components of the ECM. Structural differences determine the functional properties of the genetically distinct classes, designated types I to XI (Martin et al., 1985). Only types I, III, IV, V (Pleasure, 1984) and VI are found in peripheral nerves. Collagens have the formation of a semi-rigid triple helix, made possible by the characteristic amino acid sequence of the constituent $\alpha$-chains, i.e. the presence of glycine, the smallest amino acid, in every third position in the polypeptide chain. Variations in length, primary structure and the presence and localisation of both globular domains and interchain disulphide-bonding confer the supra-molecular assembly and resulting specialised functions upon the different collagen types. Covalently cross-linked disulphide bonds contribute appreciably to the insolubility of collagens (Kefalides, 1973) and may be responsible for their low turnover (Heathcote & Grant, 1981) and structural rigidity (Smaje et al., 1980).

Collagen IV, is a non-fibril forming BM collagen whose presence within the PNS is restricted to the BMs of Schwann cells, vessel endothelial cells and perineurial cells (Shellswell et al., 1979). Collagen type V is a fibrillar collagen. Although not confined to BMs, it is particularly abundant at these locations.

Collagens I, III & VI are also fibrillar collagens. Collagen VI has the supramolecular architecture of beaded filaments 2-3nm in diameter, pairs of "beads" being distributed at intervals of 90-110nm. Lateral aggregation of these beaded filaments in some circumstances may lead to the formation of 100nm cross-striated periodic filaments which are thought to be analogous with Luse bodies (fibrous long-spacing collagen). Luse bodies occur in healthy nerves but are much more common under pathological conditions where they are usually located underneath the perineurium.

Large cross-banded fibres are characteristic of type I collagen, whilst collagen types III and V form fine reticular fibrils. Approximately 49% of the total protein content of whole peripheral nerve is composed of collagens I and III, the relative
proportions of which have been disputed. Junquiera and colleagues concluded that type I and III are exclusively located within the epineurium and endoneurium respectively (Junquiera et al., 1979). Salonen and colleagues later revealed that both types occur in both of these regions, although type I predominates in the endoneurium (Salonen, 1987). These collagen types are also located between the perineurial cell layers where they form interstitial collagen fibrils.

The fibres (40-65nm diameter) of the endoneurium are considerably smaller than those of both the epineurium and the perineurium (Gamble & Eames, 1964) and are orientated in a predominantly longitudinal direction, along the axis of the nerve, often forming aggregations around endoneurial capillaries and myelinated nerve fibres. Two sheaths of collagen fibrils, called the sheaths of Key and Retzius (Key & Retzius, 1876) and Plenk and Laidlaw (Plenk, 1927; Laidlaw, 1929) exist in close association with the Schwann cell basal lamina. The former outer layer consists of longitudinally orientated collagen fibrils which are continuous across the nodes of Ranvier, the latter sheath lies immediately adjacent to the Schwann cells' BM, is comprised of a network of fine argyrophilic fibres and indents at areas of nodal constriction. The lattice-like arrangement of collagen fibrils within these sheaths contrast with the less compactly arranged connective tissue elements elsewhere in the endoneurium although endoneurial collagen fibrils do exhibit a tendency to group into bundles surrounded by small clefts of intrafascicular space. The collagenous components of peripheral nerves confer a degree of resistance to stretch and compression.

Fibroblasts are the major producers of endoneurial fibrillar collagen (Salonen, 1987). Procollagen is secreted by exocytosis from fibroblasts and assembled into fibrils in the endoneurial compartment. It has been suggested that cells other than fibroblasts, such as Schwann cells, may be capable of extracellular fibrillogenesis, the resulting microfibrils being supplied with additional tropocollagen by fibroblasts. This hypothesis was first proposed by Nathaniel and Pease (Nathaniel & Pease, 1963). Following nerve crush injury the cytoplasmic content of Schwann cells associated with degenerating fibres is reduced and, in consequence, the Schwann cell plasmalemma retracts from its BM. Proliferating Schwann cells growing within these old empty tubes of redundant BM generate new BMs in close association with their cell membranes and small unit collagenous fibrils, presumably therefore of Schwann cell origin, appear in the space between the new and the old basal laminae (Thomas, 1964).
Tissue culture studies have confirmed this theory, cultured Schwann cells producing a population of uniform, thin cross-banded fibrils that, as they are believed to be composed of collagen I and III raises the possibility that each fibril is a mixture of both types. Typical large collagen fibrils are formed following the addition of fibroblasts to these cultures confirming the suggestion that these cells are required for final polymerisation, contributing the necessary enzymes for conversion of procollagen to tropocollagen or for fibril assembly (Bartlett Bunge et al., 1980). Fibroblasts are unable to synthesis collagen IV, this type being manufactured only by those cells invested in basal lamina (Bartlett Bunge et al., 1980).

1C.2(ii) The architecture and function of basement membranes

The non-collagenous glycoprotein, which include fibronectin, laminin and entactin, and proteoglycan components of the ECM within the PNS are primarily located within BMs, although fibronectin also exists as insoluble interstitial extracellular fibrils which occur in both the epineurium and endoneurium but are particularly abundant in the perineurium and at the nodes of Ranvier of nerve fibres (Pateau et al., 1980).

Basement membranes were first described by Todd and Bowman (Todd & Bowman, 1945) who defined them as being a distinct form of connective tissue immediately underlying epithelia. The introduction of the periodic acid-Schiff (PAS) method (Gersh & Catchpole, 1949) later revealed that BMs effectively separate all non-connective parenchymal cells and fat from adjacent connective tissue spaces; fibrous extracellular connective tissues elements including elastic fibres, unit collagenous fibrils, microfibrils and connective tissue cells i.e. fibroblasts are devoid of this layer.

BMAs, as viewed under the electron microscope, closely follow the contours of the cell membrane and consist of three distinct regions (Inoue & LeBlond, 1989), the lamina lucida or rara, the lamina densa and the lamina reticularis. The lamina lucida is an electron-lucent layer that lies immediately adjacent to the cell membrane and is crossed by electron-dense filamentous strands running in an approximately perpendicular direction to the plasma membrane. These filaments seem to be attached to the plasmalemma of the cell by their distal ends and in continuity with the electron dense elements of the lamina densa at their proximal ends (Inoue, 1989). The thickness of the lamina lucida varies with fixation, being most prominent following glutaraldehyde fixation and reduced after formaldehyde fixation, however, a thin layer is always present
which has been said to correspond to the surface glycoprotein coat of the associated cell (Inoue, 1989). The lamina densa is a more distal electron dense layer which at high magnification appears as a compact three-dimensional network of dense cords (Inoue, 1989). The outermost layer, the lamina reticularis, is a transitional zone between the lamina densa and the connective tissue. It contains delicate anchoring fibrils which traverse the layer perpendicular to the plasma membrane, elastic microfibrils and fine interstitial fibrils. The lamina reticularis is absent in some BMs, e.g. those of blood vessels.

The major functions of BMs, those of cell support, passive selective molecular sieving/ultrafiltration and the regulation of cell migration, growth and differentiation are dependent on the architectural properties and charge of its structural components and the binding of cell surface receptors to site-specific determinants in these matrix elements. The composition of the endoneurial extracellular microenvironment is therefore directly affected by the basal lamina as it forms a barrier to the passage of macromolecules between all intrafascicular non-connective and perineurial cells and the endoneurial matrix.

Collagen V has been described primarily as a cell surface-associated or pericellular matrix component as once secreted it is retained in the vicinity of the cells membrane and is believed to contribute to the rigidity of the BM (Gay et al., 1981).

Collagen type IV (MW 500 kDa) is a major component of BMs. It is composed of three disulphide-linked polypeptide α-chains; two α1(IV) and one α2(IV), in a triple helical conformation (Charonis & Tsilisbary, 1992). The carboxyl terminal ends of the molecules form the globular noncollagenous domains known as NC1. Collagen type IV forms natural polymers by the interaction of its NH₂-terminal and COOH-terminals and the lateral association of constituent chains and self-assemblies to form a covalently stabilized three dimensional polygonal network.

Fibronectin is another recognised component of BMs (Vaheri & Mosher, 1978). Fibronectin is a high molecular weight adhesive glycoprotein (~440,000) and is synthesised by a number of parenchymal and connective tissue cells. The molecule consists of two similar or identical subunits of polypeptide chains which are held together by disulphide bonding near their carboxyl terminals. Its dimeric and multifunctional properties give it the ability to mediate important biological functions such as cell-cell and cell-matrix adhesion and cell-to-cell
interactions (Yamada & Olden, 1978; Hynes & Yamada, 1982). Certain specific domains within the molecule are implicated in the covalent binding of fibronectin to receptors in plasma membranes; fibronectin is a peripheral membrane protein of fibroblasts (Yamada, 1983). Other regions are responsible for binding to ECM molecules; fibronectin is known to bind to collagen types I, II, III, IV and VI, glycosaminoglycans (GAG) and proteoglycans such as heparin, heparan sulphate and fibrin (Tarsio et al., 1985).

Laminin is a ubiquitous component of BMs (Timpl et al, 1979). The conformation of laminin is structurally distinct from that of fibronectin, however it also possesses the ability to mediate cell-matrix adhesion. It is a flexible cruciform molecule that consists of seven globular regions connected by rod-like segments. The globular regions account for its functional abilities and contain binding sites for heparin, proteoglycans i.e. heparan sulphate, collagen type IV and a plasma membrane receptor, laminin therefore contributes greatly to the formation of the BM. Laminin self-assembles via terminal-domain interactions, to form a second polymer network with the BM (Yurchencho & Schittny, 1990).

Each molecule is composed of three genetically different polypeptide chains, one B1 or S chain, one A, M or K chain and one B2 chain which are connected to each other by several disulphide bonds. Variations in the distribution of laminin types within BMs have been demonstrated using monoclonal and polyclonal antibodies reactive with different epitopes of the laminin molecule (Leu et al., 1986). Perineurial cells and Schwann cell BM laminins have been shown to possess different populations of chains. Laminin in the BMs of perineurial cells contains a large proportion of A & S chains whilst in the BMs of Schwann cells it has a larger proportion of B1 and M chains. B2 chains are common to both, suggesting that BM functions may be customised according to the nature of the cell type. Fibronectin is synthesised by both fibroblasts and endothelial cells within the PNS, whilst laminin is produced only by endothelial cells (Foidart et al., 1980).

Entactin (thought to be analogous with nidogen) is a dumbbell shaped molecule which has a molecular weight of approximately 150kDa (Paulsson et al., 1986). It is chemically and immunologically distinct from laminin and constitutes approximately 10% of the protein content of BMs (Carlin et al., 1981). Entactin/nidogen has been localised to the endoneurial compartment and BMs of Schwann cells. Immunolocalisation of this molecule at electron microscope level has revealed the greatest density of staining at the plasmalemma-BM interface,
suggesting that it may play a role in cell adhesion (Carlin et al., 1981).

Entactin/nidogen has a strong self-aggregating tendency and an affinity for laminin and type IV collagen, immunohistochemical studies have revealed that entactin/nidogen only occurs in BMs in co-distribution with laminin (Timple et al., 1983b). Entactin plays a bridging role between the two major BM proteins, the COOH-terminal globule of the entactin dumbell reacting with both the central domain of laminin and collagen IV. Laminin can interact directly with type IV collagen, however, entactin/nidogen bridging is the major (highest affinity) interaction.

Proteoglycans are compounds which result from the covalently-linked association of proteins and GAGs. GAGs are high polyanionic compounds that possess the ability to produce an increase in osmotic pressure in the surrounding tissues. Proteoglycans have also been identified as BM components. They often contain varying amounts of sulphate and as such possess a strong negative charge that may help to restrict the penetration of the BM by anionic molecules. Proteoglycans are also capable of restricting the movement of extracellular compounds as a result of their considerable size. In skin these molecules play an important role as part of the support matrix of connective tissue, and may be related to cell-cell, and cell-matrix interactions; however, little is known about the functions of proteoglycans in peripheral nerves (Poduslo, 1984).

Heparan sulphate proteoglycans, a class of macromolecules characterised by a protein core covalently bound to heparan sulphate chains have been shown to be a component of the Schwann cell basal lamina (Eldridge et al., 1986). Heparan sulphate proteoglycan can bind to itself through a core-protein interaction to form dimers and oligomers, and can bind laminin and type IV collagen through its glycosaminoglycan chains (Yurchencho & Schittny, 1990). Two new BM components have recently been identified, an amyloid P (Inoue et al., 1986) and protein BM-40 (Lankat-Buttgereit et al., 1988). In the past it was generally believed that the components of the BM were layered across its span, the lamina densa being composed of collagen IV and the lamina lucida of laminin which was believed to aid adhesion of the basal lamina to the cell membrane (Foidart et al., 1980; Bunge & Bunge, 1983) and fibronectin (Madri et al., 1980), with heparan sulphate proteoglycan existing at the interface.
This theory has since been contested by Inoue and colleagues. They proposed that the cords of the lamina densa and the narrow electron dense filomentous strands which traverse the lamina lucida contain a core filament of collagen IV (Inoue, 1989), the resulting network of fine collagen type IV threads forming a loose meshwork which serves as a scaffolding to which other components of the basal lamina, i.e. laminin (Bignami et al., 1984), fibronectin and entactin are attached (Laurie et al., 1980); laminin, fibronectin and heparan sulphate proteoglycans were immunologically co-localised with collagen IV (Inoue, 1989). Additional evidence is provided by the fact that treatment of BMs with purified plasmin, a proteolytic enzyme capable of effectively digesting laminin and fibronectin, removes a plasmin-sensitive outer sheath, leaving only a network of fine filaments (Inoue, 1989).

In support of Inoue's theory, Desjardins and Bendayan found collagen type IV, laminin and entactin to occur in co-localisation across the entire width of the glomerular basement membrane (GBM) in rats. With reference to heparan sulphate proteoglycans, although the presence of these components has been cited within the glomerular lamina densa in both rats (Chakrabarti et al., 1989) and humans (Veves et al., 1992), it is generally agreed that the density of these sites is considerably greater within the lamina rara interna and externa of GBMs (Kanwar & Farquhar, 1979a; Desjardins & Bendayan, 1990). The structural arrangement of components may, however, vary between different BMs.

The mesh-like molecular sieves formed by collagen type IV and laminin, in exclusion from other components of the BM, is probably insufficiently tight to restrict the passage of albumin, although it is probably able to exclude large macromolecules. Heparan sulphate proteoglycans, as a result of their size, high anionic charge and relatively exposed nature, may play a role of some importance in charge-dependent permaselectivity and the retardation of transport of macromolecules.

Heparan sulphate polyanionic chains possess very large spheres of hydration, leaving very little free water space between the macromolecules. This permits the passage of only very small macromolecules across the BM (Yurchencho & Schittny, 1990). They may also provide a receptor site for chemical messages from other cells (Eldridge et al., 1986). The GAG composition of BMs may affect the deposition and three-dimensional organisation of BM collagens as GAGs have both the ability to induce conformational changes in proteins and to influence the deposition of collagen fibrils (Kanwar & Farquhar, 1979).
Although the porosity and charge characteristics of the BM may partly determine the permeability of the capillary wall, the endothelial cells themselves provide the major barrier limiting permeation of macromolecules in most capillary beds.

1C.3 Endoneurial fluid

The intrafascicular endoneurial fluid contains water and various ions, i.e. sodium, potassium and chloride, in addition to other soluble compounds. It has also been shown to contain serum plasma proteins such as albumin (Mata et al., 1987), compounds which were previously considered to be unable to permeate the barriers of the PNS. Endoneurial fluid is derived from the endoneurial microvasculature and its production is determined by two major forces, net hydrostatic pressure and net osmotic pressure. The primary influence is capillary hydrostatic pressure which forces fluid out of the microvessels. This is opposed by the colloid osmotic pressure of the blood which regulates resorption of fluid from the endoneurial compartment.

Under normal conditions there is a general overproduction of endoneurial fluid. The intrafascicular pressure is subsequently greater than that of the surrounding connective tissue (Low et al., 1977), which explains why the endoneurial contents of peripheral nerve fascicles herniate when surgical incisions are made in the perineurium (Sunderland & Bradley, 1952). Endoneurial fluid in the PNS is translocated in both centrifugal and centripetal directions. The primary route is centrifugal (proximo-distal), towards the nerve terminal, as revealed by the movement of experimentally injected radioactive or coloured compounds (Weiss et al., 1945). The effects of gravity and the observable increase in extracellular space distally (Stevens et al., 1973) support the theory that this is the major directional route of endoneurial fluid which is believed to leave fascicles at the open terminal ends of nerve branches (Saito & Zacks, 1969; Malmgren & Olsson, 1978) or in the preterminal region where the number of perineurial cell layers is reduced.

The rate of centrifugal flow is generally slower than that of centripetal flow, the exception occurring in oedematous conditions where the rate of the former system may be increased to aid in the removal of excess fluids via the nerve roots where it may gain access to the cerebrospinal fluid and its sink mechanism. Despite the efficacy of the perineurial barrier, it is theoretically possible that a small amount of endoneurial fluid may be translocated transperineurially, especially under pathological conditions, being reabsorbed into extraneural blood vessels or lymphatics. The preterminal regions of nerves,
which possess relatively thin perineurial sheaths, and the areas of the perineurium surrounding entering blood vessels are the most likely regions of fluid escape.

**1D NEURAL COMPONENTS OF THE HEALTHY PNS**

**1D.1 Classification of peripheral nerve fibres**

Peripheral nerves contain two types of axon, myelinated and unmyelinated. Both forms are associated with satellite cells known as Schwann cells. These axons are elongated cellular processes that extend from neuronal cell bodies situated in the grey matter of the ventral lamina of the brain stem and the ventral horns of the spinal cord and from other more peripherally located neurons in the spinal and cranial sensory ganglia and in autonomic ganglia. Many of these axons are therefore partially located within the central nervous system (CNS). The only fibres which exist entirely within the PNS are the postganglionic autonomic fibres, most of which are unmyelinated, and the peripherally-directed processes of dorsal root ganglion cells.

**1D.2 The myelinated fibre**

In human peripheral nerves, the diameter of myelinated fibres ranges from between 1 to 20μm. The external surface of these axons are closely enveloped by a longitudinal chain of Schwann cells that form a surrounding compact tubular sheath of myelin, a proteophospholipid. Continuous spirals of Schwann cell cytoplasm, known as Schmidt-Lanterman incisures, connect the inner adaxonal and the outer abaxonal Schwann cell cytoplasmic compartments of the Schwann cell. The myelin sheath runs along the entire length of the axon, commencing close to the cell body, although excluding the initial segment, and ending 1 to 2 μm from the terminal point of the cell process in the periphery. The only interruptions occur at the nodes of Ranvier. The outer surface of the Schwann cell is covered by a basal lamina which forms an external sheath along the entire length of the fibre and is continuous across the nodes of Ranvier.

The axon is bounded by a membrane called the axolemma. The axoplasm is an electron-lucent amorphous cytoplasmic fluid which contains various filamentous structures; microtubules, neurofilaments and microfilaments, and membranous organelles including numerous characteristically elongated mitochondria, 0.1-0.3μm in diameter and up to 10μm or more in length and smooth endoplasmic reticulum. The internal organisation of the axoplasm is therefore relatively simple compared to that of the cytoplasm within the neuronal cell body, which possesses the majority of the neuron's population of protein
producing organelles i.e. ribosomes, rough endoplasmic reticulum and Golgi apparatus.

Microtubules are the largest filamentous structures, being approximately 25nm in diameter. These are cylindrical structures, composed of tubulin, which possess lateral projections of up to 100nm in length. The cross-sectional density of microtubules in small unmyelinated axons (50-100\(\mu\)m\(^2\)) is considerably greater than that in large myelinated axons (10-20\(\mu\)m\(^2\)) (Friede & Samorajski, 1970). Microtubules form part of the cytoskeleton of axons and are involved in fast axonal transport mechanisms.

Neurofilaments are another major component of the axoplasm. These are similarly orientated in a longitudinal direction and have a diameter of approximately 8-10nm. Their length remains undetermined. Every 50nm or so along the length of these filaments one to eight radial spokes, roughly 10nm wide and 30 to 60nm long, extend laterally to connect with groups of adjacent neurofilaments. When viewed in transverse section they produce a regular polygonal lattice-like arrangement. Neurofilaments occur at a density of 100-300\(\mu\)m\(^2\) axonal cross-sectional area. Neurofilaments are also thought to be involved in axonal transport. Axoplasm also contains a much smaller filamentous component, 7-10nm in diameter, the microfilament. These structures are believed to be composed of actin and appear homogenous with the thin filaments of muscle cells.

The nucleus of the Schwann cell is elongated in appearance and usually located around the midpoint of the internode where it is surrounded by an accumulation of cytoplasm that often indents the myelin. The perinuclear region of cytoplasm is rich in mitochondria, lipid vacuoles, rough endoplasmic reticulum, Golgi bodies and centrioles and as such is a site of intense metabolic activity. The Schwann cell of a normal myelinated axon may also contain \(\pi\) or Reich granules that occur both singly and in clusters. These perinuclear cytoplasmic lamellar bodies are approximately 1\(\mu\)m in length and have two components; flattened laminations of osmiophilic membranes with a smaller periodicity, 5-6nm, than that of myelin at 17nm and an amorphous element (Thomas & Slatford, 1964).

Considering that the number of Reich granules increases with advancing age and in certain neuropathies, in association with the fact that they are uncommon in Schwann cells associated with unmyelinated fibres (Martinez et al., 1978), has led to the proposal that they may be a product of a degenerative
phenomenon of myelin, produced in response to metabolic injury or damage by toxic agents. However, as they are also common in normal healthy tissue it is more generally assumed that they are secondary lysosomes of no pathological significance.

Marchi-positive Elzholz or \( \mu \) granules are another form of dense inclusion frequently found within the paranodal Schwann cell cytoplasm under abnormal conditions. These are globular bodies of unsaturated lipid with a lamellar structure similar to that of myelin but with a smaller periodicity.

The myelin sheath is a highly organised structure consisting of spirally arranged lamellae derived from the apposed and compacted membranes of the Schwann cell. Each lamellar unit is composed of a light and a dark layer. The light electron-lucent area is divided by a narrow and slightly darker band called the intraperiod line. The myelin sheath is composed of alternating bands of lipid and protein. The lipid forms a bimolecular leaflet, the hydrophobic ends being orientated in towards the centre and the hydrophilic or polar ends outwards towards the protein layers. This explains the characteristic banded appearance of myelin seen in transmission electron microscopy following fixation with osmium tetroxide as the protein and polar ends of the lipid molecules, which are derived from the cytoplasmic aspect of each pair of membranes, bind to the heavy atomic nucleus of the osmium to produce the electron-dense dark bands. The lighter electronlucent bands represent the hydrophobic chains in the lipid and are derived from the apposed outer surfaces of each pair of membranes. Large myelinated fibres in healthy nerves posses correspondingly thicker myelin sheaths than their smaller counterparts.

When teased preparations are examined, myelinated nerve fibres are seen to be regularly indented along their length. These structures, the "nodes of Ranvier" (Ranvier, 1871), represent complete interruptions in the myelin sheath with exposure of the axolemma. Axonal narrowing occurs at the nodes. The width of the nodal gap correlates with the diameter of the fibre. The segments of fibre between the nodes are referred to as internodes and designate the territory of each individual, adjacent Schwann cell. In mammalian fibres internodal lengths range between 200 to 1,500\( \mu \)m. A close and direct relationship exists between internodal length and fibre diameter, the largest calibre fibres possessing the longest internodes. Internodal length is remarkably consistent along the length of any fibre, any shorter or intercalated internodes being attributable to remyelination subsequent to Schwann cell damage.
The terminal ends of the myelin sheath in small fibres approach the nodal axon at an acute angle. In larger fibres the myelin terminates in bulbous expansions. The paranodal regions of the sheath are highly crenated, the axon inside appearing fluted as it conforms to the shape of the surrounding myelin sheath. Accumulations of Schwann cell cytoplasm, rich in mitochondria, fill the resultant external troughs in the myelin sheath restoring the fibre to its cylindrical shape. In most peripheral nerves the proximal paranodal bulb is larger than the distal.

When seen in longitudinal section, the major dense lines in the myelin lamellae open up as they approach the nodal axolemma to form terminal "cytoplasmic pockets". The myelin lamellae are firmly attached to the juxtanodal axolemma at their ends. In the largest fibres, only some of the loops may make contact with the axon. The Schwann cell cytoplasm extends past the terminal ends of the myelin sheath to form a collar of fine processes which irregularly overlap with the corresponding collar from the Schwann cell of the next internode. It has been suggested (Williams & Landon, 1964) that the large accumulation of mitochondria within the paranodal Schwann cell cytoplasm may provide the source of energy-rich ATP-ase needed by the axolemma ionic pump for maintenance of nodal axolemma polarisation. The space contained immediately within the collars, surrounding the axolemma and bounded on either side by the myelin terminal ends is called the nodal gap. Numerous finger-like Schwann cell nodal processes arise from the nodal collars and extend into the nodal gap to form specialised contact points with the axolemma.

The nodal axolemma, as demonstrated by freeze-fracture studies, possesses a dense population of large intramembranous particles in the outer cytoplasmic leaflet, 1200μm⁻² compared to that of the internodal or juxtanodal membranes at 100μm⁻² (Kristol et al., 1978). It has been suggested that these may correspond with sodium voltage-sensitive channels.

The nodal gap contains a moderately electron dense granular material, "gap substance" whose polyanionic components provide a cation exchange reservoir (Landon & Langley, 1971) which could be of importance in regulation of the ionic movements which accompany the propagated nerve action potential; GAGs, which are known to possess cation-binding and exchange properties, have been identified as constituents of gap substance. The gap substance probably acts as an ion exchange buffer, maintaining and concentrating a high, but osmotically inactive, level of sodium ions immediately outside the nodal axolemma (Landon & Langley, 1971). During the passage of an action potential
potassium ions pass out of the axon via the axolemma. The properties of the gap substance are likely to additionally limit diffusion of these potassium ions away from the node.

The myelin sheath of nerve fibres may deviate from the usual regular and cylindrical appearance to produce outfoldings or more rarely aberrant lamellae which thrust out from the sheath into the surrounding Schwann cell cytoplasm. The latter appear as myelin bulbs when viewed in transverse section. Redundant myelin loops may also bulge into the axon. These structures appear to have no pathological significance.

1D.3 The unmyelinated axon

Small unmyelinated axons exist between the larger myelinated fibres of peripheral nerves. Unmyelinated axons pass through the nerve trunk in groups of 8-10 within a common chain of Schwann cells. Each axon is separated from its neighbour by a surrounding Schwann cell process. Schwann cells that surround unmyelinated axons have been retermed Remak cells by some authors such as Ochoa (Ochoa, 1976). The whole assembly of Schwann cell and axons is conveniently called a Remak fibre.

In adult somatic nerves the entire surface of unmyelinated axons is usually invested by an encircling Schwann cell process whose apposing cell surfaces may overlap, but rarely by more than one complete turn. At no point along its course is an unmyelinated axon exposed to the endoneurium, the axon remaining ensheathed by the Schwann cell basal lamina in regions where it is not entirely encompassed by the Schwann cell process. The occurrence of numerous spirals of Schwann cell process around an unmyelinated fibre is a feature of a myelinating axon.

"Collagen pockets", first described by Gamble and Eames (Gamble & Eames, 1964), are formed by the encircling of several collagen fibrils by one or more turns of a Schwann cell process and are common features in mature nerves. These pockets, which are only made by satellite cells associated with unmyelinated axons, are frequently lined by Schwann cell basal lamina. It has been suggested that the collagen fibrils within these pockets may have a skeletal function and have been actively enveloped by the Schwann cells (Gamble & Eames, 1964). An alternative hypothesis is that Schwann cells synthesise collagen fibrils to fill the spaces vacated by degenerated unmyelinated axons (Thomas, 1973b); collagen pockets are more numerous in
old and pathological nerves. It has also been suggested that the primary function of collagen pockets may be as anchoring structures as they are usually 6µm or less in length and occur at intervals of around 20µm (Carlsen & Behse, 1980).

In human sural nerve the length of Schwann cell associated with unmyelinated fibres ranges from between 200 to 500µm. The axoplasmic density of neurofilaments in unmyelinated fibres is similar to that of their myelinated counterparts, ranging from 100 to 300 µm⁻². The concentration of microtubules, however, is significantly greater in unmyelinated axons at 50 to 100 µm⁻².

Schwann cells associated with unmyelinated axons have elongated bacilliform nuclei which are longitudinally orientated along the length of the fibre. Perinuclear cytoplasm contains numerous mitochondria, occasional rough endoplasmic reticulum, Golgi bodies, microtubules and intermediate filaments in addition to prominent dense bodies and lysosomes. The cytoplasm in regions remote from the nucleus usually contains only microtubules, intermediate filaments and mitochondria and is therefore often indistinguishable from the axons. It is believed that all Schwann cells stem from the same cell type, a theory which is supported by the fact that the Schwann cells formerly associated with unmyelinated fibres may myelinate regenerating axons (Aguayo et al., 1976).

1D.4 Morphometry of peripheral nerve

The diameter of myelinated fibres in human peripheral nerves ranges from between 2 to 22µm (O'Sullivan & Swallow, 1968). There are two distinct populations of myelinated nerve fibres in both the radial and sural nerve, designated All and Alll, resulting in a bimodal distribution of fibre sizes with peaks at 3µm to 6µm (Alll) and 9µm to 13µm (All). Assessment of the percentage of fibres falling above and below the trough of the histogram, at 7µm to 8µm in the sural nerve, is useful in quantitative observations.

The axon provides the stimulus for the Schwann cell to produce myelin (Simpson & Young, 1945) and there is a definite relationship between myelin thickness and axon diameter, the number of myelin lamellae produced being determined by information transferred to the Schwann cell from the axon. The two populations of myelinated nerve fibre possess different myelin/axon relationships, the All group of fibres having relatively thicker myelin sheathes than the Alll fibres. Myelinated fibre density in the sural nerve of an adult
control individual ranges from between 7,500 to 10,000 mm$^{-2}$. In control sural nerve unmyelinated fibre axonal diameter ranges from between 0.5 to 3.5 μm. A unimodal curve is apparent in frequency distribution histograms, the peak occurring at approximately 1.5 μm in adults.

Evaluation of the density of myelinated nerve fibres supplies us with an indication of the severity of the neuropathy. Appraisal of the diameter of the remaining fibres is additionally useful, an observable alteration in distribution, such as an increase in the ratio or number of small myelinated fibres informs us that these additional axons probably represent regenerating myelinated fibres.

Another useful parameter for neurological comparisons is the g ratio: axonal diameter divided by total fibre diameter. It was predicted by Rushton (Rushton, 1951) that a g ratio of 0.6 to 0.7 would be optimal for conduction velocity and although for smaller fibres the g ratio does deviate from this theoretical optimum, for fibres of a larger calibre the ratio does approach 0.6 to 0.7. An abnormally high g ratio indicates remyelination or regeneration.

It is common for a mild peripheral neuropathy to develop with advancing age, with evidence of segmental demyelination and remyelination and axonal degeneration and regeneration. The cause of this neuropathy is undetermined; it may be due to distal axon degeneration resulting from neuronal aging or be primarily due to local factors, for example ischaemia, but emphasises the need for age-matching individuals in comparative investigations.

1D.5 The choice of nerve for biopsy

There are several criteria which should be met when selecting a suitable nerve for an investigative biopsy. Firstly, the nerve should be affected by the neuropathic process; the sural nerve due to its distal location is particularly susceptible to peripheral neuropathy. Secondly, it should be readily accessible for biopsy and constant in location; at the level between the Achilles tendon and lateral malleolus the sural nerve conforms to both of these requirements.

Thirdly, the chosen nerve should not be exposed to trauma during the course of daily life; the sural occupies a relatively protected position. Whilst it is true that subcutaneous fibres in the foot are slightly susceptible to damage by shoes and everyday trauma which may lead to retrograde changes in the nerve at more proximal levels, this problem occurs more frequently in other superficial nerves supplying distal regions of the body. Finally, it should be readily accessible to
neurophysiological study prior to biopsy; measurements of sural nerve conduction velocity (NCV) are easy to perform.

In conclusion the sural nerve appears to be the ideal choice of nerve for studying symmetric distal sensory and autonomic diabetic neuropathy. It is a sensory nerve, supplying the skin of the lateral aspect of the heel, the lateral side of the foot and fifth toe, and the lateral and posterior part of the lower one third of the leg; it is also involved in the nervous supply of the ankle, subtalar and calcaneocuboid joints. It contains an additional population of unmyelinated autonomic fibres. It is also one of the best documented of those nerves commonly chosen for biopsy.

The superficial branch of the radial where it emerges from under the brachioradialis tendon and becomes subcutaneous is also a common choice for biopsy, particularly in cases where biopsy of the sural nerve is unadvisable due to concurrent oedema, venous stasis, peripheral vascular disease or infection and subsequently impaired healing. The morphometry of the radial nerve has been fairly well documented and NCV studies are simple and easy to perform.

1E PATHOLOGICAL ALTERATIONS IN THE PNS

1E.1 Demyelination

The ultrastructural pathology of distal diabetic polyneuropathy consists of a combination of varying degrees of primary segmental demyelination and axonal degeneration (Thomas and Lascelles, 1966). Primary segmental demyelination occurs as a result of a disturbance in Schwann cell metabolism or of myelin itself. Two useful models are experimental allergic neuritis (EAN) and experimental diphtheritic neuropathy as they both represent a relatively 'pure' example of a demyelinating disease with little axonal involvement. The major difference between them is that demyelination in the latter case results from an apparent disturbance in Schwann cell metabolism whilst in the former the process is performed by immunocompetent cells, the Schwann cells themselves playing a 'passive' role.

Lesions in EAN are predominantly located in the dorsal spinal roots, spinal ganglia and ventral roots with peripheral nerves appearing relatively spared. This phenomenon of selective vulnerability has been attributed to the comparatively greater permeability of vessels at affected sites (Allt et al., 1971). Cellular infiltrates within these lesions occur most frequently around intraneural vessels.
The myelin sheaths of fibres within the immediate vicinity appear disorganised and are surrounded by numerous macrophages containing myelin debris. Demyelination may be initiated at any point along the internode by invading mononuclear cell attack although nodal gap widening also occurs and can be attributed to a generalised response by Schwann cells to immune attack.

Diphtheritic neuropathy can be induced by two in vivo methods; systemically by the parenteral injection of Corynbacterium diphtheria toxin and locally by injecting the diphtheria toxin directly into peripheral nerve. Demyelination is non-inflammatory and independent of the immune response (Waksman, 1957). There is usually little or no involvement of axons except at very high doses where extensive axonal degeneration may occur as a direct effect of the toxin on the axon.

Diphtheria toxin specifically inhibits the transfer of amino acids from soluble RNA to growing polypeptide chains (Pleasure et al., 1973). One of the properties of proteins is their ability to stabilise membrane systems. In diphtheria intoxication replacement of the membrane proteins is prevented due to inhibition of the synthesis of myelin proteolipid and basic proteins, causing the membranes to lose their stability and break down. This mechanism explains the latent period between the injection of toxin and the initiation of demyelination; the delay is related to the half life of the myelin basic proteins and the time they take to lose their stability. The process of demyelination, as it results from primary Schwann cell dysmetabolism, commences at the nodal regions.

Widening of the nodal gap is the first observable occurrence in primary segmental demyelination (Morgan-Hughes, 1968). Many internodes along the same fibre may be affected simultaneously. The Schwann cell microvilli become disrupted, the terminal myelin loops detach from the axolemma and the nodal Schwann cell processes appear swollen with myelin debris derived from the breakdown of the terminal loops and inner myelin lamellae. As the process of demyelination continues the terminal myelin loops retract further and there is a concurrent increase in the amount of myelin debris within the paranodal Schwann cell cytoplasm. Following withdrawal of the Schwann cell cytoplasm from the node it remains invested only by a layer of basement membrane.

Demyelination may sometimes be restricted to the paranodal region or may affect the entire segment. In the latter case the myelin lamellae along the length
of the internode separate and become disrupted, resulting in the formation of ovoids which are ultimately degraded to vesicular and lamellated osmiophilic fragments within the Schwann cell cytoplasm (Webster et al., 1961). The area of sheath closest to the Schwann cell nucleus remains intact for the longest period. Large numbers of macrophages appear in the tissue several days after the commencement of myelin disintegration and phagocytose the partially digested myelin debris, often in conjunction with a thin envelope of Schwann cell cytoplasm.

The process of remyelination, which commences a few days after the onset of demyelination, is initiated by the division and proliferation of Schwann cells which migrate along the denuded axon. The areas of axon ensheathed by these cells effectively defines the dimensions of the new internodes; nodes of Ranvier will form where adjacent cells abut. Spirals of loose Schwann cell membranes are formed around the denuded axons which subsequently compact, due to elimination of the intervening Schwann cell cytoplasm, to produce myelin lamellae with a regular repeat period. Once remyelination begins, recovery is usually rapid and complete.

If demyelination has occurred only at the paranodal regions of an internode, a new short internode of myelin will form in this region, however, if demyelination has occurred along the entire length of the internode, several short myelinated internodes will be formed in its place, each with its own Schwann cell nucleus. The new myelin sheath is thinner, less circular and has fewer myelin lamellae than the original sheath.

Secondary demyelination occurs as a result of changes in axonal calibre, most frequently atrophy. Remyelination occurs if the alterations in axonal dimensions are short-lived or very slow. The myelin sheath has an insulatory function, therefore demyelination and nodal gap widening with myelin loss will have detrimental effects on nerve fibre electrophysiology, as manifested by the development of clinical signs and symptoms. Conduction block is related to focal demyelination.

1E.2 Axonal degeneration

The ultrastructural changes that characterise pathological axonal degeneration are largely commensurate with those of Wallerian degeneration: axonal degeneration subsequent to nerve transection (Ballin & Thomas, 1969). The process commences with retraction of the axonal terminal processes and their
ensuing disintegration. The resulting debris is consumed by phagocytic cells. Concurrent with these changes, swelling of the nodal and paranodal regions of preterminal nodes of Ranvier takes place due to the interruption of axonal transport. Accumulation of axonal neurofilaments, mitochondria, multivesicular bodies and membranous dense bodies are apparent within these regions of axonal constriction, particularly on the distal side of the following nerve section.

The next stage in the sequence of events includes the degeneration or degradation of microtubules, fragmentation of neurofilaments, and mitochondrial swelling. The axon subsequently becomes packed with granular debris. The volume of cytoplasm in the Schmidt-Lanterman incisures increases, making them more readily visible. At the nodes of Ranvier, the nodal Schwann cell microvilli and the myelin loops become detached from the axolemma and the myelin retracts (Ballin & Thomas, 1969). The axon then ruptures at the nodes of Ranvier, the myelin breaking down concomitantly with the axon and sealing off at either end of the sections to produce ellipsoids. Interruptions of the myelin sheath also occur in regions adjacent to the Schmidt-Lanterman incisures. This process occurs within the persisting Schwann cell basal laminal tubes.

The ellipsoids subsequently degenerate into smaller ellipsoids, finally forming spherical ovoids. The process of myelin degeneration in Wallerian degeneration and non-inflammatory degeneration is performed by lysosomes in the Schwann cells. The myelin debris is subsequently transferred to vacuolated macrophages which enter the endoneurium for a transient period. In more proximal regions, secondary demyelination may occur, the axon, although appearing atrophic, remaining relatively intact. The resulting "unemployed" Schwann cells, which resemble small lymphocytes due to their rounded shape and size, with the exception that they are surrounded by BM, remain in the endoneurium. Severe lesions and those occurring in close proximity to the neuronal cell body may lead to chromatolysis of the perikaryon and cell death, implying metabolic derangement of the whole neuron which manifests itself as a dying-back axonopathy. Nerve conduction is relatively normal until it fails completely and muscles become denervated and undergo atrophy. Unmyelinated fibre degeneration proceeds in a similar manner.

Regenerative phenomena involve the proliferation of Schwann cells and resultant production of bands of Büngner within the old basal laminal tubes. Regenerating axonal sprouts which fail to attain appropriate distal connections degenerate. Recovery which is precipitated by the reinnervation of denervated peripheral
structures may be only partial depending on the patients' age, the basis of the neuropathy and its degree of severity.

1F. **THE BARRIER PHENOMENON**

The endoneurial blood-nerve barrier (BNB) in conjunction with the tight perineurial cell seal around PNS fascicles serves to maintain the balanced endoneurial milieu necessary to support the rapid, complex and repeated ion exchanges upon which nerve function relies.

The chemical nature and electrical charge of osmotically active moieties are of great importance regarding their method of transportation across the blood nerve barrier. The variety of mechanisms utilised include diffusion, facilitated transport which is mediated by the actions of saturable stereospecific carrier molecules (Goldstein & Betz, 1986), energy-dependent active transport which occurs against a concentration gradient and pinocytosis or vesicular transport. There are two conflicting views concerning the concept of vesicular transport. One proposes that the process is active and involves the fusion and fission of macromolecules, the other that the process is passive and that seemingly 'free' vesicles are in fact connected to the cell surface via a nodular branching network of tubules which terminate in pores of various diameters (Bundgaard et al., 1979). Glucose is the primary metabolic substrate in the PNS and is taken up by facilitated transport (Rechtand et al., 1985).

1F.1 **The perineurial component**

The existence of the perineurial barrier can be demonstrated experimentally by both morphological and neurophysiological methods. In the former system, a tracer is injected around the nerve and after a suitable delay and appropriate fixation, its position is visualised within the nerve by microscopy. In the latter system, a substance which possess the capacity to reduce either the resting or the action potential is applied to the surface of the nerve, and comparisons made of the time delay between the application and effect of the substance on desheathed and normal nerves (Martin, 1964). A limitation of this method is that only substances which eventually penetrate the perineurium may be used.

Pinocytosis is a common phenomenon in the perineurial cell and vesicular transport may counteract the effect of the tight junctions to a certain degree. The basement membrane acts as a macromolecular filter and helps to retard or block the further passage of substances across the perineurium (Palade & Bruns, 1968). Continuities exist between the endoneurium and epineurium where the
perineurium is traversed by blood vessels and may provide a possible route of entry to the endoneurium (Burkel, 1967).

The component of the barrier mechanism which effectively prevents the penetration of a tracer may vary according to its chemical nature. Tracers used in studies of perineurial diffusion barrier efficacy have ranged from inorganic ions to macromolecules and experimental animal models have included mice, rats and rabbits with similar results in each. The compounds used generally fall into one of three categories with regard to their penetrative ability.

The first group contains compounds which appear to be totally excluded by the perineurium, and include dyes with a high-affinity for serum proteins i.e. albumin, such as Evans-blue (Olsson & Reese, 1971b; Seneviratne, 1972) trypan blue (Emirouglu, 1955), lithium carmine (Weiss & Röhlich, 1954), methyl and methylene blue (Martin, 1964) and fluorescein isothiocyanate (Olsson, 1966b). It also contains several protein tracers including horseradish peroxidase (HRP) (MW 40 kDa) (Olsson & Reese, 1969, 1971b), ferritin (MW 500 kDa) (Waggener et al., 1965), cytochrome C (MW 12 kDa) (Malmgren & Brink, 1975), sodium fluorescein (Malmgren & Olsson, 1980), microperoxidase (2 kDa) (Towfighi & Gonatas, 1977), fluorescent diaminoacridine (Aker, 1972) and the ionic tracer lanthanum nitrate (Ghabriel et al., 1989).

These compounds may traverse several layers of the perineurium before their passage is finally halted (Olsson & Reese, 1971b), a feature largely attributed to their relative size. Penetration of the electron microscopic tracer ferritin in rat sciatic nerve was seen to be restricted to the outermost layer of the perineurial sheath, with only a minimal degree of vesicular uptake (Waggener et al., 1965), (Oldfors, 1981b); the marker being localised within the BMs of the respective cells.

The smaller molecules, HRP and microperoxidase, penetrate the barrier to a slightly greater extent, their presence having been documented within the extracellular spaces of the outermost layers of the perineurial sheathes in both rat (Towfighi & Gonatas, 1977) and mouse (Olsson & Reese, 1971b) sciatic nerves. At an ultrastructural level HRP activity was localised to both the basal laminae and vesicles of the outermost perineurial cell layers, however, the small amounts of the tracer transferred across the perineurial cells by vesicular transport (Oldfors & Sourander, 1978) does not reach a significant level.
The isolated presence of HRP activity in endoneurial phagocytes has been presented as evidence that HRP is capable of totally penetrating the perineurial barrier (Oldfors & Sourander, 1978), however, this observation was probably attributable to the presence of endogenous peroxidase activity; HRP activity has been documented in the occasional endoneurial macrophage in control mice (Sima & Robertson, 1978a). Studies involving HRP are additionally complicated by the fact that HRP may induce anaphylactic or toxic reactions which may in turn increase vascular permeability (Olsson & Reese, 1971b).

The location of the ultimate barrier to these various protein tracers appears to be the same, the innermost layer or layers of the perineurial sheath, presumably due to the presence of tight junctions (Olsson & Reese, 1971), appearing to constitute a rigid barrier which prevents access to the endoneurium (Olsson & Reese, 1969, 1971b).

The second category consists of substances which are able to diffuse slowly across the perineurium and eventually penetrate the endoneurium. These compounds tend to have a smaller molecular weight than members of the first category and include small water solutes and ions. Agents included in this group include potassium, sodium, calcium chloride, barium and sucrose amongst others (Feng & Lui, 1949; Crescitelli, 1951; Olsson et al., 1971a). The selective permeability of the perineurium to members of this group is believed to depend upon the actual physical dimensions of the intercellular routes and the size of the molecules.

The third category contains substances that, due to their extremely low molecular weight, are able to freely diffuse across the perineurium i.e. oxygen and carbon dioxide. As the perineurial barrier of peripheral nerves has been shown to be relatively resistant to ischaemia (Lundborg et al., 1973) and post-mortem changes, fresh autopsy material is suitable for use in studies of human perineurial permeability. Söderfeldt and colleagues have revealed that the human PNS possesses similar barrier properties to those of experimental animals, entry of topical applications of Evans blue labelled albumin and HRP into the endoneurium of femoral nerves being prevented by the perineurium in tissue samples obtained within the first day after patient death (Söderfeldt et al., 1973). Persistence of barrier function following death suggests a high structural to metabolic ratio of function.
The restrictive permeability of the perineurium plays the role of providing a compartment of limited volume and leakage whose composition is regulated by the combined activity and attributes of the perineurial cells and the endothelium of endoneurial capillaries.

1F.2 The vascular component

Over the years various tracers have been used to investigate the permeability characteristics of the blood-nerve barrier (BNB) in peripheral nerves. Many of the earlier studies involved the use of trypan blue, Evans blue, fluorescent (fluoresceinisothiocyanate, FLA) or radioactive labelled plasma protein albumin (MW 69 kDa) (Olsson, 1966b; Olsson & Reese, 1969, 1971b; Brightman et al., 1970). The development of more sensitive techniques utilising tracers including ferritin (Shinowara, 1982), dextrans of various molecular masses labelled with fluorochrome (Hulström et al., 1983) and fluorescein (Waris & Tervo, 1980), HRP (Karnovsky, 1967), microperoxidase (Michel et al., 1984), sodium fluorescein (MW 376 Da) (Malmgren & Olsson, 1980; Waris & Tervo, 1980) and diaminoacridine (MW 259.7 Da) (Aker, 1972) has overcome the limitations of macroscopic detection of tracers by light microscopy and led to a more detailed definition of the BNB.

The intravenous injection of these numerous tracers has revealed a largely similar pattern of vessel permeability resulting in gross staining, over time, of all parenchymatous organs with the exception of the brain and spinal cord (Brightman et al., 1970; Goldstein & Betz, 1986). Within the PNS the tracers are located, almost immediately following admininstration, in the lumena of peripheral nerve blood vessels. Extravasation of the tracers from epineurial and perineurial vessel is indicated by the presence of rapidly spreading activity in the surrounding connective epineurial and perineurial tissues (Olsson, 1966b, 1968b, 1971c; Olsson et al., 1971a), and probably occurs via fenestrations or vesicular transport (Olsson & Reese, 1971b). The temporary fusion of plamalemma vesicles across the width of endothelial cells may provide another route of exit by the production of transient intra-endothelial channels. Extravasated proteins are reabsorbed into the lymphatic system and by this route conveyed back to the blood.

The amount of these tracers found within the endoneurial compartment is generally neglible as a result of the competence of the BNB in peripheral nerves (Olsson, 1968b; Olsson et al., 1971a). The efficacy of the BNB has, however, been found to be species dependent; intravenously injected albumin...
whilst it is unable to penetrate the BNB in the rat (Olsson, 1966b; Dyck et al., 1980a) and the mouse (Olsson & Reese, 1969, 1971b) is capable of penetrating the endoneurial compartment in the rabbit, guinea pig, cat and monkey (Olsson, 1967).

The development of the HRP technique by Karnovsky and colleagues (Karnovsky, 1967), and subsequent histological refinements (Malmgren & Olsson, 1978) has enabled enzymes with peroxidase activity to be visualised at both light and electron microscopical levels. Studies utilising other compounds with peroxidase activity, i.e. cytochrome C (MW 12 kDa), catalase and lactoperoxidase have been more limited. Intravenously injected HRP is able to penetrate the capillary endothelial barrier of muscle, via pinocytosis/vesicular transport, within 11 to 15 minutes (Karnovsky, 1967). The BNB of peripheral nerves both in mice (Olsson & Reese, 1971b) and rats (Dyck et al., 1980a), is impermeable to HRP as a result of the lack of pinocytotic transport and the presence of endothelial tight junctions. A degree of vesicular HRP transport has been proposed in both of these species (Dyck et al., 1980a; Arvidson, 1984) the presence of HRP within endoneurial macrophages being observed soon after injection of the tracer (Arvidson, 1977). As previously explained it is possible that this result is artefactual.

Microperoxidase is able to penetrate the capillary endothelium of muscle within 1 minute (Wissig, 1979) the most rapid route being through "open" type junctions. By comparison, the BNB within the peripheral nerves of mice has been found to be generally impermeable to the much small fluorescent tracer sodium fluorescein (MW 376 Da) (Malmgren & Olsson, 1980) although a limited degree of penetration is observable at high doses with long survival times. Substantial variations in penetration were also found to exist between individual nerves and fascicles within a multi-fascicular nerve. The BNB of the femoral and sciatic nerves of the rabbit have been found to be similarly impermeable to the fluorescent tracer diaminoacridine (MW 259.7 Da) (Aker, 1972).

Although their entry may be delayed, small water solutes and ions such as lanthanum (MacKenzie et al., 1987) are generally able to penetrate the BNB of mammalian peripheral nerves; penetration of the endoneurium compartment by radiolabelled $^{24}$Na and $^{36}$Cl ions in the PNS of the rabbit is less rapid than that seen in other soft tissues including muscle (Manery & Bale, 1941; Welch & Davson, 1972). The passage of polar non-electrolytes appears to be size-dependent, the speed of equilibration of endoneurial fluid content with
[\textsuperscript{14}C]lulin, [\textsuperscript{51}Cr]EDTA and [\textsuperscript{14}C]urea plasma content in the rabbit sciatic nerve increasing with decreasing molecular weight respectively (Bradbury & Crowder, 1976). In conclusion, the BNB of peripheral nerves in the mouse, rat and rabbit appears to be very efficient, providing an effective largely size-dependent barrier to the penetration of experimental tracers with the exception of very small water soluble non-electrolytes and ions. The BNB barrier in the guinea pig and cat appear to be considerably less effective.

1F.3 Pathological alterations in the barrier function

1F.3(i) The perineurium

The perineurium plays a role of primary importance in the regulation of the endoneurial microenvironment, acting as a macromolecular diffusion barrier and conferring an important degree of protection upon peripheral nerve fibres to disease promoting agents including toxins, antigens and viruses (Waggener et al., 1965). Pathological alterations in the barrier function may therefore facilitate the entry of precluded agents which may then exert a detrimental influence on the nerve parenchyma. The type and degree of physical or metabolic trauma inflicted upon a nerve are of significance with respect to whether or subsequent alterations in perineurial permeability occur.

The permeability properties of the perineurium appear relatively resistant to both compressive (Rydevik & Lundborg, 1977) and stretching forces (Weerasuryia et al., 1979). Perineurial permeability, however, has been shown to be readily increased at the site of experimental crush injury (Olsson & Kristensson, 1973). Various mediators of the inflammatory response, including histamine, appear to have no effect on the barrier function of the perineurium; although pus cells may completely encircle a nerve they appear unable to penetrate the perineurium.

Injections of various local anaesthetics (Myers et al., 1986b), collagenase and chymopapain are capable of inducing toxic injury in the PNS with resulting alterations in perineurial barrier function. Local applications of hypertonic electrolytic and nonelectrolytic solutions, i.e. sodium chloride and sucrose respectively, are also capable of increasing the permeability of the perineurial barrier (Kristensson & Olsson, 1976; Weerasuryia et al., 1979).

The perineurial component of barrier function appears more resistant to ischaemia that the vascular component. The peripheral nerves of rabbits have been shown to remain impervious to local injections of protein tracers even following 24hrs of complete ischaemia, the integrity of the perineurial barrier
being breached only after a period of 48hrs (Lundborg et al., 1973). As a result of this phenomenon fresh autopsy material is suitable for use in studies on human perineurial permeability (Söderfeldt et al., 1973).

The development of an effective perineurial permeability barrier is a maturational process. Numerous perineurial cell layers in the peripheral nerves of immature animal species lack tight junctions. Similar observations have been described in the peripheral nerves of human fetuses (Gamble & Breathnach, 1965). The efficiency of the adult perineurial diffusion barrier is achieved after a postnatal period of several weeks (Oldfors & Sourander, 1978). This deficiency may contribute to the development of pathological lesions in immature animals by allowing extraneurial agents, e.g. viruses, access to the endoneurium via diffusion between adjacent cells. The exceptions to the rule are animal species which produce precocious offspring; peripheral nerves in the guinea pig possess a perineurium with mature diffusion barrier properties from birth (Kristensson & Olsson, 1971b).

The presence of a pathological influence during the development and maturation of the PNS may also interfere with the permeability properties of the perineurium. Severe protein-malnutrition during this period in the rat has been shown to produce a long-standing functional deficiency in the perineurial barrier function (Oldfors & Sourander, 1978). It would be of interest to determine whether the same results occur in developing human nerves under similar conditions.

1F.3(ii) The vasculature of the PNS

The relationship between the mechanisms of pathological processes and vascular permeability in peripheral nerves vary. The localisation of parenchymatous lesions induced by pathological blood-borne agents i.e. toxins, may be directly influenced by variations in indigenous vascular permeability or conversely, the pathological process may produce a direct increase in vascular permeability, resulting in extravasation of serum components and subsequent oedema.

The blood-nerve barriers of dorsal and ventral spinal nerve roots, dorsal root ganglia (Olsson, 1968b), and to a certain degree, those of autonomic ganglia have been shown to be highly permeable in comparison to that of the central nervous system (CNS) and other areas of the PNS including peripheral nerves. This phenomenon is presumably responsible for the selective neurotoxicity to doxorubicin (Adriamycin), an antimitotic drug, seen in the dorsal root and
autonomic ganglia which results in the production of neuronal death (Cho et al., 1977). A range of pathological factors, including industrial agents, metals and drugs, are believed to gain access to the ganglia via this same vascular route.

Interspecies variations exist in the susceptibility of peripheral nerves to certain diseases. The distribution of lesions in diphtheritic and allergic neuritis differ between the rabbit and guinea pig (Waksman & Adams, 1956), the greater resistance to the disease in the rabbit being attributed to the rabbits more efficient BNB.

Compression, section, ligation, inflammation, ischaemia, radiation and perfusion with hypertonic solutions are all capable of producing increases in vascular permeability in peripheral nerves. Following experimental nerve section, crush or ligature there is a vigorous increase in the permeability of blood vessels in the immediate vicinity of the trauma (Mellick & Cavanagh, 1967; Olsson, 1966b). If the trauma is sufficiently severe, similar increases occur at sites distal to the injury.

Degranulating mast cells release vasoactive amines such as histamine. In non-nervous tissue, chemical mediators of inflammation including bradykinin, histamine and prostaglandin possess the capacity to increase vascular permeability dynamically via the formation of large interendothelial gaps following the contraction and subsequent separation of endothelial cells. The inhibition of these alterations by $\beta_2$-stimulators such as terbutaline and isoprenaline, without normalisation of increased blood flow, suggests that the action of inflammatory mediators on endothelial cells is receptor-mediated (Arfors et al., 1979).

Although they have no effect on the blood-brain barrier, vasoactive amines may contribute to the development of alterations in the BNB of the PNS by a similar action; the permeability of endoneurial vessels has been shown to be increased by histamine and 5-hydroxytryptamine, mediators of acute inflammation (Olsson, 1966a), and the local injection of histamine into rat sciatic nerve results in an increase in endoneurial fluid pressure and fascicular area (Powell et al., 1980).

Lundborg studied the effects of temporary ischaemia with the use of the intravenous tracer Evans blue (Lundborg, 1975) and found that endoneurial extravasation of the dye only commenced after 8hrs of ischaemia and reached a fairly extensive level after 10hrs. The effects of acute graded compression on
the peripheral nerve BNB has been studied in the rabbit, endoneurial protein extravasation and vasogenic oedema being produced following direct compression of between 200 to 400mmHg for a period of 2 hours (Rydevik & Lundborg, 1977).

Under certain conditions radiation injury may also produce vasogenic endoneurial oedema (Lundborg & Schildt, 1971), as may perfusion with hypertonic solutions. Perfusion of rabbit sciatic nerve with a 5% or greater concentration of sodium chloride results in endoneurial extravasation of proteins. If administration of the tracer is delayed for a period of 30 minutes post-perfusion, the permeability properties of the endoneurial vessels appear to return to normal. In contrast, permeability changes following similar osmotic disruption of the perineurial diffusion barrier appear irreversible (Kristensson & Olsson, 1971).

1F.3(iii) The effects of alterations in the peripheral nerve barrier.

Oedema, defined as an abnormal accumulation of fluid within a tissue, results from an increase in vascular permeability and occurs in various tissues under numerous pathological conditions. The flow of plasma from the blood into the tissue is increased and a subsequent accumulation of exudates occurs in the tissue. Care must be taken, when viewing tissue sections, that artifactual tissue separation produced during histological preparation is not incorrectly mistaken for oedema. Oedematous fluid generally accumulates around endoneurial vessels and beneath the perineurium. In contrast to artifactual interstitial spaces, endoneurial clefts occupied by oedematous fluid are stained by protein stains. Under oedematous conditions nerve fibres appear widely spaced and transverse fasicular area is substantially greater than that of similarly processed control tissue.

BNB alterations with subsequent oedema and the endoneurial accumulation of proteinaceous material and perivascular and endoneurial cellular infiltrates are known to occur in certain human neuropathies, e.g. leprosy and inflammatory demyelinating neuropathies. It is uncertain whether endoneurial oedema in peripheral nerves, characteristic of some neuropathies, and the accompanying increase in endoneurial fluid pressure, is capable of directly causing fibre damage.

There are various forms of oedema. Vasogenic oedema is characterised by increased vascular permeability to protein molecules and has been shown to occur, for example, in EAN (Levin et al., 1974) and following trauma (Olsson,
Increased endoneurial concentrations of protein macromolecules result in a rise in endoneurial fluid osmotic pressure associated with an influx of water and a subsequent increase in endoneurial fluid pressure. The production of a protein-rich endoneurial environment in vasogenic oedema may have both detrimental and beneficial effects. Whilst collagen production may be stimulated and result in fibrosis, the components of the endoneurium are bathed in what is to all purposes a "tissue culture medium", supplying ideal conditions for the proliferation of mast cells, fibroblasts and Schwann cells and subsequent repair of the nerve.

Axons possess the capacity to absorb constituents from the endoneurial microenvironment that then enter the retrograde axonal transport system and are conveyed back to the perikarya (Kristensson & Olsson, 1971a). It has been suggested that vasogenic oedema may stimulate axonal outgrowth by the delivery of chemical messengers to the nerve body (Sparrow, 1981a; Gainer & Fink, 1982).

In direct contrast to the situation in vasogenic oedema, vascular permeability to proteins is unaltered in nonvasogenic oedema. In the PNS, nonvasogenic oedema may be preferentially extracellular, as is the case in galactose neuropathy (Malmgren et al., 1979) or intramyelinic, as found in hexachlorophene intoxication (Powell et al., 1978). In the latter case oedema results from a direct toxic effect on cellular membranes resulting in myelin degeneration, with the splitting of lamellae and formation of water-filled vacuoles.

Oedema in traumatic neuropathies most probably occurs as a consequence of direct physical damage to the vasa nervorum in conjunction with the effects of histamine liberated by disrupted mast cells. Under different pathological conditions, the factors responsible for the production and maintenance of increased vascular permeability may be concordantly variable.

Breakdown of the peripheral nerve BNB may be attributed to one of two processes; the activation of intracytoplasmic vesicles or opening of endothelial tight junctions. Heavy metal poisoning with, for example, lead, tellurium, mercury or cadmium (Lampert et al., 1970), results in peripheral nerve oedema due to the leakage of osmotically active plasma constituents either through or between damaged endothelial cells. Enhanced vesicular transport is the mechanism most frequently associated with the extravasation of detrimental
agents (de la Motte & Allt, 1976), i.e. in mercuric chloride intoxication (Ware et al., 1974). The opening of tight junctions, although known to occur, for example in ligated nerve (Fukuhara et al., 1979) and experimental lead neuropathy (Myers et al., 1980), is a less frequent phenomenon (Weerasuriya et al., 1979). Fenestrations may occur in chronic conditions (O'Hara & Ikuta, 1985). The loss of functional 'charge filtering' anionic sites from endothelial cell membranes, similar to that known to occur in regions of experimentally enhanced permeability in the CNS (Nagy et al., 1983), may also result in increased permeability in the PNS.

Peripheral nerve oedema may be relatively persistent due to the lack of a lymphatic drainage system (Sunderland, 1965). A certain amount of oedematous fluid is probably resorbed into the blood by pinocytosis or tubule formation across endothelial cells. The bulk of oedematous fluid is probably removed via translocation in a centifugal direction, the primary route of fluid movement in the PNS, although, as previously stated, under pathological conditions centripetal flow may become increasingly significant.

An additional tranperineurial route of exit may exist either via vesicular transport; extravasated serum proteins have been located between perineurial cell layers in EAN (Allt, 1972), or thorough imperfection in the perineurial barrier where the vas nervorum enter and leave; Evans blue is able to escape the endoneurium compartment at endoneurial pressures of 5mm Hg via these junctions. It has been suggested that the production of perineurial peristaltic waves may aid the removal of oedematous fluid (Ross & Reith, 1969), however, subsequent studies have failed to find any evidence in support of this theory.

Numerous investigation have been performed, using experimental model of diabetes, in order to determine whether diabetes produces any effect on the barrier properties of peripheral nerves; the widths of both perineurial cell (Johnson et al., 1981c) and endoneurial capillary (Guy et al., 1984) BMs are characteristically and significantly increased in the peripheral nerves of individuals with diabetes mellitus, and it has been proposed that these alterations may arise in response to abnormal permeability, a theory discussed in greater detail at a later point.

Conflicting evidence has been produced; whilst both perineurial and endoneurial vascular permeability to the macromolecule fluorescent labelled albumin have been reported to be increased in experimental alloxan-diabetes (Seneviratne,
and vascular permeability to be increased in both streptozotocin-diabetic and genetically diabetic BB-rats (Williamson et al., 1987), other studies have failed to detect increased permeability across either barrier in both streptozotocin (Sima & Robertson, 1978a) and alloxan-diabetic rats (Sima & Robertson, 1978a) and in the genetically diabetic db/db mouse (Sima & Robertson, 1978a; Carson & Hanker, 1980); the perineurial and endothelial cell barriers in BB-rats (Sima & Hay, 1981) and streptozotocin-diabetic rats (Sima & Robertson, 1978a) proving impermeable to tracers as small as microperoxidase (MW 2 kDa) although permeability to the small water soluble molecule \([^{14}C]\)mannitol has been stated as increased (Rechtand et al., 1987).

The majority of experimental physiological evidence to date therefore suggests that permeability to large molecules is not increased in diabetic peripheral nerves. The possibility does remain that disruption of the endoneurial microenvironment may still occur as a result of an increase in permeability to small water soluble molecules (Rechtand et al., 1987).

With reference to vascular abnormalites in human diabetes mellitus, increases in capillary permeability have been documented in the diabetic kidney (Churg et al., 1962), retina (Kohner et al., 1967), skeletal muscle (Trap-Jensen, 1970) and skin (Parving, 1976; Bollinger et al., 1982; O’Hare et al., 1983) and linked with microangiopathy and peripheral neuropathy (Valensi et al., 1991).

Disrupted perineurial cell tight junctions, similar in appearance to those seen in galactosaemic rats (Beamish et al., 1992) and experimentally osmotically disrupted epithelial tight junctions (Wade & Karnovsky, 1974), have been documented in human diabetic nerves (Beamish et al., 1991).

Abnormal perineurial and endoneurial concentrations of plasma proteins including albumin, IgG and IgM have been reported in the peripheral nerves of diabetic patients (Ohi et al., 1985; Graham & Johnson, 1985; Poduslo et al., 1988) and used as testimony in favour of a breakdown in the BNB. However, albumin has also been immunohistochemically identified as a normal component of endoneurial fluid in the peripheral nerves of rats (Mata et al., 1987). In addition, although after a single intraperitoneal injection immunoglobulins are excluded from the endoneurium, repeated injections yield endoneurial labelling (Seitz et al., 1985). These results suggest that the permeability barrier to serum proteins may not be as absolute as once believed and that, whilst it may serve to limit the entry of these proteins, endoneurial accumulation may occur over time as a
result of their high serum concentration. This fact may be of considerable significance if it allows the exposure of endoneurial cellular elements to abnormal serum components under pathological conditions.

1G PATHOLOGY AND PATHOPHYSIOLOGY OF ANIMAL MODELS

Animal models of both experimentally and genetically induced diabetes have been used extensively to study alterations in the peripheral nervous system.

1G.1 Chemically-induced diabetes

The two most widely used chemically-induced diabetic animals are the streptozotocin- and the alloxan-diabetic rat. Alloxan is a pyrimidine with structural similarities to uric acid and glucose and streptozotocin is isolated from *Streptomyces achromogenes*. Both compounds are cytotoxic to pancreatic beta cells (Rakieten et al., 1963). Although their precise mechanisms of action are not fully understood, it is thought that they are related to the structural properties of the compounds. Beta cells possess the specialised ability to rapidly recognise and metabolise the substrate glucose. Streptozotocin is a glucose with a highly reactive side chain and alloxan is relatively similar structurally to glucose. Following their intravenous injection the extremely rapid binding of these agents suggests a degree of selective pancreatic attraction similar to that of glucose.

Streptozotocin damage is evident between 30 to 120 minutes after administration and irreversible alloxan damage within one hour. The diabetogenic property of alloxan was first discovered in 1943 and the earliest reports of streptozotocin-diabetes in rats and dogs were made in 1963. Rabbits and guinea pigs appear to be insensitive to streptozotocin.

Over the years a large volume of literature has accrued on the effects of both alloxan and streptozotocin administration and it has come to be generally accepted that, due to its more specific action, streptozotocin is the more effective agent; specific and irreversible lesions are produced in other organs, including the kidney, if the dose of alloxan given is larger than that required to induce diabetes.

The discovery of alloxan and streptozotocin was greeted with great enthusiasm as their mode of action, pancreatic lesions with concomitant beta cell destruction, were seen to mimic the situation in insulin-dependent human diabetes mellitus. The nerves of streptozotocin and alloxan diabetic rats also
possess an abnormal resistance to ischaemic conduction failure, a phenomenon known to exist in human diabetes (Jaramillo et al., 1985). It was hoped that these animals could provide valuable, relatively permanent cases of diabetes for morphological and electrophysiological studies, as a single intraperitoneal injection of either substance, preferably in the fasting state, is capable of producing longstanding experimental diabetes in rats for a period of one to two years without the need for insulin therapy (Zemp et al., 1981).

Regarding the pathology of peripheral nerves in experimental diabetes, reports have been variable and conflicting, however, it is generally agreed that any evidence of demyelination is insignificant (Sharma & Thomas, 1974; Brown et al., 1980; Sharma et al., 1981, 1985).

Degenerative changes have been reported in the plantar nerves of streptozotocin-diabetic rats (Brown et al., 1980), but these probably relate to an increased vulnerability to compression, a phenomenon known to occur in diabetes mellitus. The few studies that have reported the presence of axonal degeneration in both alloxan-diabetic (Powell et al., 1977) and streptozotocin-diabetic rats (Bestetti et al., 1981) involved animals that had been diabetic for at least 12 months. In such cases the significance of these observations is unclear as a relatively high proportion of the observed alterations were probably age-related (Grover-Johnson & Spencer, 1981).

It has also been concluded that there is no loss of myelinated fibres, although a small but significant degree was reported in one study (Sharma et al., 1985). Small but significant reductions in the calibre of myelinated fibres compared with age-matched controls have been reported by some (Sharma et al., 1977; Yagihashi et al., 1990a) but not all authors (Sharma & Thomas, 1974).

It has been suggested that this axonal 'dwindling' may be due to shrinkage resulting from immersion in a hyperosmolar solution, the same phenomenon being seen to occur in the peripheral nerves of cats after the infusion of hypertonic dextrose (Dyck et al., 1981). If this is the causative mechanism, neurofilament density should be seen to be increased, however, as has not been found to be the case in galactose intoxication, a model in which axonal dwindling occurs in association with endoneurial oedema (Nukuda et al., 1986), it can be argued that there is no osmotic basis for any reduction in axonal calibre.
Slight but significantly reduced NCVs are evident in both alloxan-diabetic (Sharma & Thomas, 1974; Moore et al., 1981b; Thomas et al., 1981) and streptozotocin-diabetic rats (Sharma & Thomas, 1974; Thomas et al., 1981; Mayer & Tomlinson, 1983b). As skeletal growth retardation is a feature of untreated diabetes (Sharma et al., 1981, 1985), the maturation of nerve fibres is likely to be correspondingly impaired (Sharma et al., 1977). Reductions in NCV are probably therefore related partly to a slight reduction in fibre size in comparison with control animals; a small increase in NCV occurs in rats up to the age of nine months as nerve fibre diameter increases with maturation, and rats made diabetic at the age of nine months show only a slight reduction in NCV (Thomas et al., 1981) and nerve fibre size (Wright & Nukuda, 1995), and partly to direct metabolic effects.

It has been suggested that retrograde axonal transport may act as a messenger and indirectly control axonal protein synthesis at the neuronal soma. The transport velocity of axonal structural proteins, slow component a, is reduced (Jakobsen, 1980) as a consequence of impaired retrograde axonal transport. This may therefore result in retardation of axonal growth compared to other cellular elements of the peripheral nerve.

Continuous subcutaneous insulin infusion (McCallum et al., 1986), or regular treatment with insulin (Sharma et al., 1985) in experimental diabetes is capable of completely correcting deficiencies in body weight with varying degrees of correction in fibre size and skeletal growth and restoring NCV to near normal values in both alloxan (Preston, 1967) and streptozotocin-diabetic rats (Greene et al., 1975). This evidence provides further testimony in favour of the theory that electrophysiological abnormalities in these animals are related to maturational retardation as opposed to a direct toxic effect of the diabetes-inducing agent.

Reports of endoneurial oedema and increased fascicular area in experimental diabetes are inconsistent, some workers having described an increased water content (Jakobsen, 1978; Tomlinson et al., 1986; Anand et al., 1988) whilst others have concluded that endoneurial oedema is a rare occurrence (Powell et al., 1981a). Variations in results may be due to the severity of diabetes or dehydration of the animals. The significance of endoneurial oedema in those animals where the reports were positive is questionable as the severity was too modest to result in damage to the nerve fibres.
In contrast to the condition in human diabetes, Schwann cell and endoneurial capillary basal laminal hypertrophy are rarely observed in experimentally diabetic animals (Sharma & Thomas, 1974), although capillary BM changes have been reported in alloxan-diabetic dogs (Bloodworth et al., 1969). The incidence and appearance of tight junctions between the endothelial cells of the endoneurial vasculature is also normal (Sharma & Thomas, 1974). Glycogen is also seen to accumulate in the Schwann and perineurial cells, a phenomena unique to the diabetic condition.

In view of the obvious structural abnormalities seen in the PNS of human diabetics and the unestablished presence of minor abnormalities in the PNS of experimentally-diabetic animals, the relevance of extrapolation of causative aetiologies in the latter condition to that of human diabetes mellitus should be viewed with considerable caution.

1G.2 Genetically determined diabetes

The homozygous db/db diabetic mouse, a trait inherited as an autosomal recessive pattern with full penetrance provides one model of diabetes (Hummel et al., 1966). Obesity due to abnormal fat deposition is a major physical manifestation in these animals and precedes the development of hyperglycaemia, glycosuria and polyuria.

It has been suggested that abnormalities in the db/db mouse reflect a defective peripheral utilization of glucose; plasma insulin levels are near-normal and concurrent with continued excessive glyconeogenesis and reduced utilization of glucose. As such, the diabetic mutant mouse has been classified as being analogous with human Type II non-insulin dependent diabetes mellitus. Minor motor disabilities in these animals have been attributed to the presence of a neuropathy (Hanker et al., 1980), however, it seems likely that these findings may merely be a direct consequence of obesity.

Although peripheral myelinated fibre size in these animals is generally agreed to be less than that of age-matched controls (Hanker et al., 1980; Sharma et al., 1983) there is little or no evidence of either segmental demyelination, axonal degeneration or myelinated fibre loss in the peripheral nerve trunks (Hanker et al., 1980; Sharma et al., 1983). The relatively minor quantitative findings in these animals must therefore be interpreted with caution as it is often difficult to distinguish between morphological changes due to disease and those caused by age.
Microangiopathy, a fairly ubiquitous feature of human diabetes, is also lacking in the db/db mouse, the endoneurial vessels expressing only occasional and mild degrees of basal lamina hypertrophy (Carson & Hanker, 1980). NCV in the db/db mouse is reduced in comparison with control animals (Sima & Robertson, 1978b). The early onset of this deficit is likely to be metabolic in nature as the level of accompanying morphological changes are insufficient to account for this change. Analogous with the situation in chemically-induced experimental diabetes, the pathological alterations of established human diabetic neuropathy (Thomas & Lascelles, 1966) are not reflected in the db/db mouse and in reality it is not unfair to state that this animal does not provide a very good comparative model for analogy with human diabetes.

A spontaneously diabetic inbred strain of Chinese hamsters also exists. In this instance the inheritance is polygenic, the severity of the disease varies between the animals and in relation to the reduction in pancreatic insulin secretion (Gerritsen & Blanks, 1974; Grodsky et al., 1982). Motor nerve conduction velocity (MNCV) is reduced by 16 to 22% in mature animals in comparison with controls (Kennedy et al., 1982). The occurrence of pathological abnormalities in the peripheral nerves of these animals remains under dispute. Mild degrees of demyelination and remyelination, and degeneration and regeneration have been reported in chronically ill animals (Schlaepfer et al., 1974). In contrast, a subsequent study failed to find evidence of any such changes despite the presence of reduced NCVs (Kennedy et al., 1982). Again, this animal is not a suitable model for experimental comparison due to the lack of peripheral sensory or motor neuropathy.

Autosomal-recessive inherited diabetes, resulting from hypoplasia of the islets of Langerhans, has been identified in dogs (Engerman & Kramer, 1982). These animals, once diabetic, become hyperglycaemic, ketoacidotic, lipaemic, hypcholesterolaemic and hypoinsulinaemic. They often develop cataracts and are insulin dependent. Although electrophysiological, morphometric and biochemical abnormalities of the PNS have not been extensively investigated, Steiss and colleagues demonstrated a reduction in motor and sensory NCVs in diabetic dogs that exhibited no signs of neuropathy (Steiss et al., 1981).

Morphologically, no significant levels of difference have so far been found between the tibial nerves of diabetic and control animals. This is not the case with the plantar nerves, segmental demyelination and axonal degeneration proving statistically greater in the diabetic dogs (Braund & Steiss, 1982). This
feature could be the effect of greater susceptibility of diabetic nerves to compression, the nerves in question supplying the sole of the foot. Further studies need to be performed in order to deduce whether this animal model could provide suitable experimental comparision with human diabetes mellitus.

1G.3 The BB-Wistar rat

The spontaneously diabetic IDDM BB Wistar rat was first discovered in 1974 and is proposed to exhibit many of the features encountered in human IDDM (Marliss et al., 1982). The rats are not obese and any abnormalities would be free of the question of cause or potentiation by chemical diabetes-inducing agents. Numerous investigations of pathological abnormalities known to be associated with diabetes have been performed using this animal, including retinopathy (Sima et al., 1983; Chakrabarti et al., 1985) metabolic derangements (Marliss et al., 1983), microangiopathy (Sima & Hay, 1981) and morphometric and functional deficits in the PNS.

The development of diabetes in these animals is genetically determined and mediated by selective destruction of the pancreatic beta cells by an immune mechanism (Marliss et al., 1982). The animals develop hyperglycaemia, polyuria, glycosuria, ketonuria, weight loss and become dehydrated. Profound hypoinsulinaemia (Marliss et al., 1983) precedes the rapid onset of diabetes and if insulin supplementation is not subsequently initiated the animals become ketoacidotic and die.

1G.3(i) Structural and morphometric changes in the PNS

Conflicting reports have been made regarding pathological alterations in the peripheral nerves of this animal model. Sima described progressive deleterious alterations in untreated animals with the irreversible loss of junctional contacts between the paranodal terminal myelin loops and the paranodal axolemma (Sima, 1985), the extent of which was seen to correlate with the duration of disease. It was proposed that this phenomenon arises as a result of the abnormal function or loss of the cell adhesive and/or substrate adhesive molecules that constitute the junctional complexes. With advanced axoglial dysjunction, the paranodal myelin was seen to retract (Sima & Brismar, 1985).

Sima also reported frequent evidence of axonal atrophy in the sural nerves of BB Wistar rats with advanced diabetes. This process was stated as being most pronounced in distal sensory fibres with predominant selective loss of myelinated fibres (Sima, 1980). With increasing duration of diabetes the
process was seen to progress in a proximal direction. As a result of these observations he described the changes in the peripheral nerves of the BB Wistar rats as being consistent with a central-peripheral distal axonopathy and consequently classified the condition as a distal symmetric, mainly sensory polyneuropathy.

In contrast to these findings, Mendell and workers failed to demonstrate any evidence of the prevalent level of axonal degeneration described by Sima (Mendell et al., 1981). In addition, segmental degeneration was not evident to any significant extent and myelinated fiber density was not reduced in comparison to control animals.

1G.3(ii) Functional abnormalities

The BB Wistar rat exhibits a significant deficit in NCVs; after 1 year's duration of the disease NCVs will realise only 60% of the normal value.

Sima and colleagues suggested that depressed excitability due to metabolic changes at the nodes of Ranvier may be the responsible agent (Sima & Hay, 1981). Nodal junctional contacts are believed to maintain the high concentration of electrical activity and $\text{Na}^+$ permeability of the nodal gap by preventing the lateral migration of nodal axolemmal $\text{Na}^+$ channels into the internodal region. It was proposed that with the progressive loss of these channels via axoglial dysjunction, the electrical capacity of the node may be reduced, as the excitation of the nodal membrane and size of the current depends directly upon nodal sodium permeability and the opening up of the channels and resultant inward flow of ionised sodium.

In conclusion, it was proposed that a combination of reduced sodium permeability (Brismar et al., 1983), as a consequence of reduced sodium channel density, and increased axoplasmic sodium concentration, values of 40mmol being recorded in contrast to normal levels of less than 10mmol (Brismar, 1983), may account for the impaired action current, decreased excitability and low conduction velocities characteristic of diabetic fibres. Sima also attributed a certain degree of nerve dysfunction to axonal atrophy and loss of the conducting fibres, particularly in the case of chronic diabetes.

It has also been suggested that high axoplasmic ionised sodium levels may be responsible for reported ultrastructural intra-axonal oedema and swelling (Sima & Brismar, 1985), and additionally potentiate a decrease in sodium permeability as
the accumulation of this ion can be correlated with impaired sodium-potassium ATP-ase activity (Greene et al., 1984), a phenomenon also reported in the BB-rat (Brismar & Sima, 1981), (Greene et al., 1987c).

Sima and colleagues proposed that axoglial dysjunction, similar to that which they described in the BB Wistar rat may account for human diabetic neuropathies. It is possible that such a mechanism may be responsible for the small component of NCV abnormalities in human diabetics that is seen to reverse following improved glucose control.

Deficits in NCV in the BB-rat have also been reported in the apparent absence of any accompanying causal morphological abnormalities suggesting that other factors must be involved (Brismar et al., 1983). Maturational arrest as a result of the diabetic condition may be partly responsible, however, other contributary elements must be at play as NCV in this model has also been found to be significantly reduced compared to weight-matched controls (Mendell et al., 1981).

The fact that pathological structural abnormalities of the severe degree characteristic of human diabetic neuropathy never develop in this model suggests that it is a metabolic neuropathy, with hyperglycaemia the significant predisposing factor. Additional factors interacting with hyperglycaemia must contribute to the production of the structural damage in human diabetic neuropathy. Alternatively, it is possible that the shorter duration of the disease in the BB rat compared to that in humans may be responsible for the lack of analogous detrimental abnormalities and the mechanism at play in the BB rat may lead to the development of demyelination in the long-term. In line with all of the animal models of diabetes available to date, the significance of extrapolation of information gleaned from experimental studies using this model to the aetiology of the disease in their human counterparts remains questionable.

1H PATHOLOGICAL ALTERATIONS ASSOCIATED WITH HUMAN DIABETIC POLYNEUROPATHY

Pryce (Pryce, 1893) was one of the earliest workers to report on degenerative changes in the peripheral nerves of patients with diabetes mellitus. The most common form of diabetic neuropathy is a distal symmetric primarily sensory polyneuropathy which predominantly involves the sensory and autonomic functions. All the diabetic patients involved in this study had this form of neuropathy. The major ultrastructural observation in diabetic polyneuropathy is
axonal loss, subsequent to atrophy and Wallerian-type degeneration, affecting both myelinated and unmyelinated axons (Thomas & Lascelles, 1966; Behse et al., 1977).

A proximal to distal spatial pattern of fibre degeneration and loss is evident in the peripheral nerves of both neuropathic and non-neuropathic diabetic patients (Dyck et al., 1986), and is indicative of a primary dying-back axonopathy, the longest myelinated axons being affected preferentially (Thomas & Lascelles, 1966). A certain degree of fibre loss has been reported in the spinal roots, particularly the dorsal roots (Greenbaum et al., 1964). Segmental demyelination is a prominent feature. The degree of fibre loss and segmental demyelination correlates approximately with clinical severity as evidenced by a reduction in NCV.

The nature of the primary lesion in the PNS has been disputed over the years. It has been suggested that degeneration of the anterior horn and lumbar dorsal root ganglion cells constitutes the primary lesion (Greenbaum et al., 1964). This theory has fallen into disfavour following the revelation that the degree of fibre loss in the peripheral nerves is often substantially greater than that of neuronal loss within the ganglia. In certain neuropathies segmental demyelination has been proved to be a secondary effect of primary axonal degeneration (Thomas, 1971). The relationship between these two processes in diabetic polyneuropathy is less clear.

In 1986 Fraser and colleagues (Fraser et al., 1986) concluded that selective damage to the myelin sheaths with axonal sparing was the salient change, a theory later supported by Dolman (Dolman, 1963) upon finding degeneration of the myelin sheath to be a noticeably more prominent occurrence than axonal degeneration in the peripheral nerves of the 36 diabetic patients included in her study.

Bischoff, the first researcher to undertake an ultrastructural study of diabetic neuropathy (Bischoff, 1965), concluded that a metabolic lesion of Schwann cells was the primary defect (Bischoff, 1968). The reported occurrence of segmental demyelination but not axonal loss within the nerves of non-neuropathic diabetic patients served to substantiate this theory (Chopra et al., 1969). In a series of later studies segmental demyelination was found to be a universal feature of diabetic nerves (Chopra & Fannin, 1971), (Behse et al., 1977).
Other research has implicated axonal degeneration as the primary lesion. As the result of a later study, Bischoff revised his initial theory upon finding axonal degeneration to be a common feature in early diabetic neuropathies (Bischoff, 1968). The presence of entirely unaffected fibres adjacent to those undergoing segmental demyelination led Dyck and colleagues to support the theory that demyelination was a secondary effect of axonal degeneration, as the distinct focality of these pathological alteration precluded their attribution to generalised alterations in the endoneurial milieu (Dyck et al, 1986).

Thomas and Lascelles (Thomas & Lascelles, 1966) concluded that diabetic neuropathy is constituted by a combination of segmental demyelination and axonal degeneration having found segmental demyelination to be a universal feature with a prevalent degree of axonal loss, particularly in the severe chronic cases. There was no evidence to indicate whether axonal loss was secondary to segmental demyelination or had occurred independently.

It is likely that the effects of diabetes mellitus on axonal and Schwann cell function are unrelated, no conclusive correlation having been found between demyelination and axonal loss (Behse et al., 1977) these abnormalities appearing to be independently occurring processes (Thomas & Eliasson, 1984). Disparities between the evidence submitted in support of either axonal degeneration or segmental demyelination as the primary pathological changes in diabetic polyneuropathy may reflect differences in patient selection or, more probably, tissue sampling, evaluation and the judgemental opinion of the observer.

Where the neuropathy has advanced to a chronic level, fibre density may be substantially reduced. Coincident Schwann cell proliferation leads to the production of flattened columns of adjacent Schwann cells processes known as bands of Büngner. Axonal regeneration may be a prominent feature in chronic cases of polyneuropathy (Llewelyn et al., 1991a) and is indicated by the presence of numerous unmyelinated axonal sprouts and regenerative clusters of both unmyelinated and myelinated fibres. Axonal regeneration is often seen to fail at a later stage for reasons that at present are unknown.

Hypertrophic changes, resulting from repeated episodes of demyelination and remyelination in longstanding diabetes, may lead to the formation of onion bulbs. These consist of concentric whorls of Schwann cell processes surrounding a central myelinated fibre or group of unmyelinated axons (Ballin & Thomas, 1968; Vital et al., 1973). Schwann cell hyperplasia has been
described in approximately 60% of patients with diabetic neuropathy (Lapresle, 1968). Abnormal numbers of Reich or \( \pi \) granules are often present within the Schwann cells.

Other diabetes-related pathological alterations include an increase in the deposition of endoneurial collagen (Thomas & Eliasson, 1984) and in endoneurial area (Behse et al., 1977). Hyperplasia of the basal laminal zone of endoneurial vessels was first described by Fagerberg (Fagerberg, 1959). This PAS-positive material was later identified as reduplicated basal laminae (Bischoff, 1967). Whilst vascular basal laminal hyperplasia is a common feature in chronic neuropathies, its occurrence is statistically greater in diabetes mellitus (Vital et al., 1974). Endoneurial endothelial cell swelling and proliferation has also been described (Dyck et al., 1986a, 1986b). Perineurial cell basal laminal thickening is another characteristic feature of diabetic neuropathy (Johnson et al., 1981) and is sometimes accompanied by a prominent degree of perineurial calcification (R.H.M. King et al., 1988). Calcium deposits are also frequently found within the reduplicated basal laminal zones of the vasa nervorum (King et al., 1989).

1J THE PATHOGENESIS OF HUMAN DIABETIC POLYNEUROPATHY

The aforementioned multiplicity of syndromes that are associated with diabetes suggests that the disease has a multifactorial basis. Biochemical abnormalities due to insulin deficiency or hyperglycaemia are cardinal features of diabetes mellitus. It seems likely that the majority of neuropathies associated with diabetes mellitus result from the combined effects of altered nerve metabolism, initiated by insulin deficiency and/or hyperglycaemia, in conjunction with unspecified genetic and/or environmental factors (Greene et al., 1985, 1988a; Winegrad, 1987). It has been demonstrated that acute experimental diabetes produces a significant alteration in peripheral nerve substrate and energy metabolism (Greene & Winegrad, 1981), most probably as a result of altered \( \text{Na}^+\text{-K}^+\text{ATP-ase} \) activity (Greene & Lattimer, 1984b).

The majority of workers have concluded that there is a relationship between the degree of hyperglycaemic control in diabetes mellitus and the manifestation of neuropathy (Greenbaum, 1964; Pirart, 1965), with poorly controlled patients possessing the severest degree of damage. However, cases will always be found of well-controlled diabetics with neuropathy and poorly controlled individuals with an apparent lack of complications leading to a less conclusive correlation between diabetic control and the occurrence of neuropathy (Mulder et al., 1961). The results of the recently published Diabetes Control and Complications Trial
Lasker, 1993) have now firmly established a relationship between hyperglycaemia and neuropathy.

Two varying hypotheses exist regarding the events related to the occurrence of metabolic disturbances produced by hyperglycaemia. The first proposes that the metabolic alterations lead directly to pathological alterations of the nerve fibres. The second that metabolic disturbances result in tissue alterations, for example in the nerve vasculature, which then lead to the observed pathological changes in nerve fibres. Studying pathological alterations in human diabetic peripheral nerves may help to reveal the nature of the pathogenetic mechanism or mechanisms responsible for the development of neuropathy.

It seems likely that certain focal lesions within peripheral nerves are of ischaemic origin, for example those of the third cranial nerve; others may be related to the abnormal susceptibility of diabetic nerves to the effects of compression injury. The pathological basis of diabetic polyneuropathy appears much more complex and, as iterated by Greenbaum (Greenbaum, 1964b), has led to the evolution of numerous and conflicting aetiological theories over the years.

An ischaemic basis was proposed as early as 1929 by Woltman and Wilder (Woltman & Wilder, 1929). This theory was supported at a later date by Fagerberg (Fagerberg, 1959) and Weller (Weller, 1965). Dolman was unable to demonstrate any correlation between peripheral nerve and vascular lesions in the series of 36 diabetic patients included in her study (Dolman, 1963). Similarly, Greenbaum (Greenbaum, 1964b) failed to find evidence of a relationship between the severity of neuropathy and abnormalities of the vasa nervorum. As such both Dolman and Greenbaum rejected the theory of vascular occlusion as the causative agent in favour of the theory of a metabolic defect due to inadequate control (Greenbaum et al, 1964) a proposal since supported by numerous other workers (Pirart, 1965; Clements, 1979; Winegrad et al., 1983).

Interest has, however, recently been revived in the vascular theory by Dyck and colleagues (Dyck et al., 1985a). Mechanisms which have been proposed to be of pathogenetic significance in the production of diabetic neuopathies are listed overleaf and discussed individually.
METABOLIC
- Vitamin deficiency
- Lipid abnormalities
- Diketone toxicity
- Sorbitol accumulation
- Myo-inositol deficiency
- Disturbed protein metabolism
  - Impaired synthesis of structural proteins
- Abnormal neurotrophic responses

MECHANICAL
- Abnormal susceptibility to pressure

VASCULAR
- Atherosclerosis
- Microangiopathy
- Intraluminal fibrin deposition

1J.1 Metabolic hypotheses

1J.1(i) Vitamin Deficiency

It has been hypothesised in past years that diabetic neuropathy may be produced by a B group vitamin deficiency. In thiamine deficiency there is a lack of conversion of pyruvate to acetyl coenzyme A (CoA) due to the absence of the coenzyme thiamine pyrophosphate. Pyruvate metabolism abnormalities are known to occur in diabetes, however, no correlation has been found between this dysfunction and neuropathy (Thompson et al., 1960). The administration of thiamine, vitamin B12 or pantothenic acid has also failed to produce any improvement during therapeutic trials on diabetic subjects (Schuman et al., 1954). The theory of vitamin deficiency as a causative agent has been generally discarded.

1J.1(ii) Lipid Abnormalities

The PNS myelin phospholipid and galactolipid content has been shown to be reduced in the spontaneously diabetic BB Wistar rat suggesting an abnormal maturation and/or turnover of myelin lipids (Hofteig et al., 1983). As galactocerebroside is an important constituent of paranodal axoglial contacts (Saida et al., 1984) such alterations may result in loss of these contacts and ensuing degenerative changes.
Other reported anomalies include a reduction in the content of phosphatidylinositol-phosphatidylserine, cholesterol, cerebroside and most phospholipids. These have been correlated with diminished myelin volume in human diabetic nerves (Brown et al., 1979). Myelin viscosity and the levels of endoneurial cholesterol esters are markedly elevated, the latter presumably as a result of myelin degradation. It is unknown whether these changes are of aetiological importance.

1J.1(iii) Diketone Toxicity

The experimental administration of y-diketones results in the development of a peripheral neuropathy (Divincenzo et al., 1977). It remains unclear whether the endogenous production of neurotoxic diketones in diabetic subjects is a realistic proposition. It is true that diketones with profiles that differ from those usually found in healthy controls do exist in diabetic individuals (Zlatkis et al., 1980), but no correlation has been found between their occurrence and that of neuropathy.

1J.1(iv) Sorbitol Accumulation

Under normal metabolic circumstances, glucose is primarily oxidised to carbon dioxide and water with the resultant production of energy stored as adenosine triphosphate (ATP). In a hyperglycaemic state, excess glucose enters tissues which are freely permeable to glucose and hence exposed to ambient blood glucose levels. These include lens, retina, kidney, blood vessels, islet cells and nerves (Winegrad et al., 1983), all of which bear the brunt of diabetic manifestations. Muscle and adipose tissue require insulin for glucose penetration. The polyol pathway exists in various tissues, including aorta, retina and renal glomeruli, and is activated upon saturation of the normal glycolytic route.

The polyol pathway involves two enzymes, the first of which is NADPH-requiring aldose reductase (AR), which reduces free glucose to its sugar alcohol sorbitol. AR possess broad substrate specificities for many other aldo-sugars, including galactose, which it converts to the corresponding carbohydrate derived polyols. Its affinity for galactose is greater than that for glucose, so galactose-intoxicated animals convert more galactose to galactitol than glucose to sorbitol. Sorbitol dehydrogenase is the second enzyme and converts the polyols to their corresponding keto-sugars i.e. sorbitol to fructose. Although sorbitol dehydrogenase also possess a broad substrate specification the catabolism of galactitol is significantly slower than that of sorbitol.
It is now generally accepted that the polyol pathway also exists in the PNS, AR being located within the cytoplasm of Schwann cells and sorbitol hydrogenase in that of axons; during the acute phase of Wallerian degeneration associated with Schwann cell proliferation and axonal degeneration, AR activity has been shown to persist or be slightly increased whilst sorbitol dehydrogenase activity almost completely disappears (Gabbay & O'Sullivan, 1968a). Recent immunolocalisation of AR at the electron microscope level has confirmed that AR is primarily located within Schwann cells associated with myelinated fibres in the rat PNS (Powell et al., 1991). The AR content of Schwann cells related to unmyelinated fibres appeared very low.

Sugar alcohols penetrate cell membranes poorly and once formed are trapped intracellularly, their only routes of disposal being conversion to the respective keto-sugar or slow leakage from the cell. Fructose may be converted to lactate or stored as glycogen. However, the rate of conversion of fructose to lactate has been reported to be very slow in normal sciatic nerve suggesting that nerve, unlike skeletal muscle, contains little fructokinase, the enzyme involved in the next stage of glycolytic breakdown. Abnormal accumulations of glycogen and Lafora body-like polyglucosans, which are probably derived from glycogen deposits, have been described in the myelinated axons of both streptozotocin- (Powell et al., 1981a) and alloxan-diabetic rats (Polwell et al., 1977) and undoubtably occur as a result of endoneurial polyol accumulation.

Diabetic nerves produce far greater amounts of sorbitol than normal nerves when incubated at high glucose levels, suggesting an intrinsic alteration in diabetic nerves which results in the shunting of glucose away from normal metabolism and along the polyol pathway (Gabbay, 1969).

A hypothesis exists which proposes that, in the diabetic state, hyperglycaemic stimulation of the polyol pathway leads to an accumulation of endoneurial nerve glucose, fructose and sorbitol (Gabbay et al., 1966), consequent metabolic interference, as opposed to gross osmotic changes, resulting in dysfunction of the host cell and the production of diabetic neuropathy.

It is an established fact that polyol accumulation is responsible for at least one non-neuropathic complication of diabetes mellitus, namely cataracts. Experimental sugar cataracts in rats can be produced by glucose, galactose and xylitol intoxication. The high concentration of aldose sugars in the aqueous humour of these animals in conjunction with their ability to penetrate the lens
results in excess intracellular concentrations of these sugars which are then converted by AR to the respective sugar alcohols. The parallel movement of water into the lens fibre with resultant lenticular swelling, vacuole formation and hyperhydration can be directly attributed to the osmotic effects of accumulated polyol pathway intermediates (Gabbay, 1973). Eventually there is precipitous loss of osmotic integrity as a result of the swelling and electrolyte imbalance, and a massive influx of sodium ions and water occurs resulting in lenticular opacity, most probably due to macroaggregation or precipitation of the normally translucent lenticular proteins.

An enzyme of the glucuronic acid-xylulose shunt, NADP-L-hexonate dehydrogenase, which although it has many similarities to AR generally has a poor ability to convert aldoses to their respective sugar alcohols, is able to form sorbitol at extremely high glucose concentrations. Whilst the presence of this enzyme has been demonstrated in some tissues affected by diabetes, i.e. the kidney & retina (Gabbay & O'Sullivan, 1968a), it does not exist in peripheral nerve or lens; sorbitol accumulation and its prevention at these sites can be entirely attributed to the action and inhibition, respectively, of AR. The fact that sugar cataracts in galactosaemic and diabetic rats can be delayed or prevented by the administration of AR inhibitors (ARI) (Peterson et al., 1979; Kinoshita et al., 1979; Beyer-Mears & Cruz, 1985) serves to prove that polyol accumulation is the causative mechanism.

Support for the theory that polyol accumulation is responsible for the production of diabetic neuropathy requires evidence of enhanced PNS polyol pathway activity and polyol accumulation. AR activity has been shown to be significantly increased in the lens, kidney, sciatic nerve, skeletal muscle, retina and spinal cord of the BB diabetic rat (Ghahary et al., 1991), although it is not entirely understood whether this occurs as a result of increased production and hence activity of AR protein, levels of AR mRNA have been shown to be increased in the kidneys (Ghahary et al., 1991), or increased activation of present levels of AR by high intracellular concentration of glucose, accompanied by changes in enzyme kinetics.

The db/db mouse provides a small degree of support for the theory of sorbitol accumulation as the aetiological mechanism in human diabetic neuropathy, the lack of pathological alterations in this model being attributed to a concomitant absence of sorbitol accumulation, as although the polyol pathway does exist in this species, flux along this route is low (Llewelyn et al., 1991a).
Significant increases in nerve glucose, sorbitol and fructose content have been demonstrated in both the alloxan- (Gabbay et al., 1966), (Stewart et al., 1967) and streptozotocin-diabetic rat (Gabbay, 1973; Yue et al., 1982; Anand et al., 1987), and spontaneously diabetic GK (Goto-Kakizaki) rat compared to age-matched controls (Suzuki et al., 1990). Galactitol (dulcitol) accumulation has also been reported in galactosaemic rats (Gabbay & Snider, 1972; Mizisin & Powell, 1993).

Reported reductions in NCV (Sharma & Thomas, 1974; Greene et al., 1975; Mayer & Tomlinson, 1984a) and axonal calibre in experimentally-diabetic animals and motor NCV in the diabetic GK rat (Suzuki et al., 1990) may be attributable to concomitant enhanced polyol pathway activity; a statistically significant correlation has been demonstrated between motor NCV and nerve sorbitol content in streptozotocin-diabetic rats (Kato et al., 1990).

The fact that insulin therapy results in the concurrent restoration to near normal levels of both NCV and nerve glucose, sorbitol and fructose levels in streptozotocin-diabetic rats with well-controlled diabetes, and a reduction in sciatic nerve AR activity in the BB rat (Ghahary et al., 1991) favour the existence of a causal relationship between sorbitol accumulation and acute functional Schwann cell damage. The possibility cannot be excluded that these results may arise from the correction of other associated metabolic alterations by insulin therapy.

Polyol accumulation has also been documented in human diabetic tissue with markedly elevated levels of sorbitol being found in peripheral nerves obtained post mortem (Ward et al., 1972; Mayhew et al., 1983). Dyck and colleagues found endoneurial concentrations of fructose and sorbitol in one third of the diabetic patients included in their study to be above the highest levels for the controls (Dyck et al., 1980b). The assumption that the clinical, neurophysiological or pathological findings resulted from these changes was precluded by the size of interpatient variations.

Confirmation of high PNS sorbitol content in human diabetes has been provided by two more recent studies (Sima et al., 1988c; Dyck et al., 1988) and, despite their previous failure to find any relationship between elevated sorbitol and fructose levels and the presence or severity of neuropathy (Dyck et al., 1980b), in this study Dyck and colleagues observed a negative correlation between nerve sorbitol content and myelinated fibre density (Dyck et al., 1988).
The advanced stages of galactose neuropathy are associated with extensive primary demyelination, Schwann cell morphological alterations, axonal degeneration and abnormalities in NCV (Gabbay & Snider, 1972; Powell et al., 1981a; Forcier et al., 1991; Mizisin & Powell, 1993). Endoneurial galactitol accumulation has been causally linked to the development of experimental galactose neuropathy (Gabbay & Snider, 1972), removal of galactose from the diet resulting in lowered galactitol levels and improved motor NCV. The site of galactitol accumulation is uncertain. AR is located in Schwann cells and galactitol would therefore be expected to accumulate within these cells (Gabbay, 1973); however, the lack of correlating ultrastructural changes in Schwann cells (Sharma et al., 1976; Powell et al., 1981a) has led to the proposal that galactitol may slowly leak out of Schwann cells and accumulate in the endoneurium, its passage out of the endoneurial compartment being delayed by the blood-nerve barrier.

Endoneurial accumulation of this osmotically active polyol (Sharma et al., 1976) leads to the production of nonvasogenic nerve oedema. The blood/nerve and perineurial barriers to protein tracers have generally been reported as remaining intact in galactosaemic rats (Malmgren et al., 1979; Powell et al., 1981b), although a recent study did report increased permeability to $[^{123}]$I-albumin (Chang et al., 1987). Conservation of PNS barrier properties probably aids the endoneurial accumulation of sugar alcohols.

Endoneurial oedema in galactosaemic rats is associated with increased endoneurial pressure (Myers et al., 1979; Mizisin et al., 1986) and a slight but significant increase in endoneurial water content and fascicular area (Sharma et al., 1976). Perineurial ATPase plays a role of considerable importance in the normal regulatory capacity of the perineurium (Shanthaveerapa & Bourne, 1962), helping to counteract the osmotic effects of intracellular proteins by actively pumping sodium out of the cell. The perineurium in galactosaemic rats shows a significant increase in the density of caveolae/vesicles (Beamish et al., 1992), indicating that it may indeed be actively involved in the removal of endoneurial oedematous fluids and therefore in playing a role in the regulation of the endoneurial environment.

The rigidity of the perineurium (Low et al., 1980), aids the production of elevated endoneurial pressure when oedematous increases in nerve volume occur over a few minutes, i.e. in acute conditions such as cold injuries and burns (Myers et al., 1980). Substantial increases in endoneurial fluid pressure in
these cases results in deformation of the transperineurial vessels from their usual cylindrical shape (Myers et al., 1986a) or capillary collapse. Subsequent restriction of the endoneurial blood supply may lead to ischaemia and hypoxia resulting in detrimental structural and functional cellular alterations within the nerves. It has been hypothesised that the level of endoneurial oedema in galactosaemic rats may be sufficient to produce neurological deficits as result of this chain of events, despite the failure to detect any reduction in endoneurial blood flow (Tuck et al., 1984).

The accumulation of galactitol is analogous that of sorbitol and fructose in experimental diabetes and galactosaemic rats have subsequently been used as an experimental model of diabetic neuropathy. It has been proposed that the common aetiological mechanism may be involved in the production of peripheral neuropathy, a theory supported by reports of reduced endoneurial blood flow (Monafo et al., 1988), and increased sciatic nerve water content, wet weight (Anand et al., 1988) and extracellular space (Jakobsen, 1978), in streptozotocin-diabetic rats. However, not all workers have found evidence of endoneurial oedema in this model (Powell et al., 1981a).

Evidence of sorbitol accumulation in diabetic neuropathy playing a similar role to that of galactitol in the aetiology of the galactosaemic neuropathy requires the demonstration that ARIs are able to prevent sorbitol accumulation and acute and chronic nerve damage despite persistent hyperglycaemia.

In conflict with this theory, endoneurial polyol accumulation in galactosaemic rats has been shown to be significantly greater than in experimentally diabetic animals (Sharma et al., 1976) due to the slower rate of metabolism of galactitol by sorbitol dehydrogenase (Stewart et al., 1967). Analogies between the two conditions may not therefore be particularly relevant as the effects of polyol accumulation in galactose-intoxication, i.e. marked endoneurial oedema, are not reflected in experimental diabetes.

Additionally, in chronic neuropathies, due to the very low elastic modulus of peripheral nerve (Low et al., 1980), progressive oedema produces only a small increase in endoneurial fluid pressure as a result of a concomitantly large deformation of the nerve (Myers et al., 1980). Increases in endoneurial pressures are therefore rarely in excess of 6mmHg (Low et al., 1980), partially due to time-dependent increases in perineurial compliance, and as normal endoneurial capillary pressure is in the region of 10-15mmHg the production of
ischaemia due to constriction of endoneural blood flow seems unlikely to be a factor in the production of human diabetic peripheral neuropathy.

Another possible aetiological mechanism associated with endoneurial polyol accumulation and nonvasogenic oedema in diabetes mellitus that does not rely upon the gross physical effect of these changes, is the possible osmotic disruption of perineurial and epithelial tight junctions permitting the entry of precluded agents that may be causal factors in the development of a neuropathy. The distal concentration of these putative neurotoxins, consequent upon the proximal-distal centripetal flow of endoneurial fluid, would result in the longest axons, which would have the greatest exposure, degenerating first and the production of a distal symmetrical neuropathy of the kind typically associated with diabetes mellitus.

Despite reports of fenestrated endothelial vessels (Powell et al., 1985), disrupted perineurial tight junctions (Beamish et al., 1991) and overhydration of peripheral nerves in human diabetic neuropathy (Griffey et al., 1988) it is generally agreed that peripheral nerve endoneurial oedema is not a prominent feature of human diabetic neuropathy (Behse et al., 1977) and, as previously mentioned, there is little evidence to substantiate the theory that the permeability barrier of peripheral nerves is altered, as result of oedema or any other mechanism, in experimental diabetes. Disrupted perineurial tight junctions have also been found in the genetically diabetic db/db mouse (Schiavinato et al., 1991). Far from confirming the theory of osmotic disruption, this finding partially refutes it as flux along the polyol pathway in this animal is low and sorbitol accumulation and endoneurial oedema does not occur (Llewelyn et al., 1991a).

Differences in peripheral nerve ATPase activity have been detected between streptozotocin-diabetic rats and galactosaemic rats (Lambourne et al., 1987). Perineurial ATPase activity has been found to be reduced (Llewelyn & Thomas, 1987a), and abnormalities in intramembranous particle density and distribution have been noted in the perineurial sheaths (Gabriel et al., 1986a) of streptozotocin-diabetic rats. Intramembranous particles represent integral membrane proteins, some of which are likely to be ATPase molecules.

The presence of diminished levels of perineurial ATPase may lead to the endoneurial accumulation of sodium and water and the production of oedema. The severe degree of endoneurial oedema seen in galactosaemic rats compared
with streptozotocin-diabetic rats would imply a comparatively greater degree of perineurial ATPase activity in the latter. The differences between galactosaemic and streptozotocin-diabetic rats, in both actual perineurial ATPase activity levels and the discordance between the expected and actual levels of perineurial ATPase activity in these animals, that suggests that different pathological processes are at work in the two models, indicate that the galactosaemic rat may not be a very reliable model of diabetic neuropathy.

As a result of all these discrepancies the relevance of comparisons between pathological occurrences in oedematous animal models, such as galactosaemic rats, and human diabetes remains unclear. Whilst endoneurial sugar alcohol accumulation is a feature of both human diabetes and galactosaemic rats, it is probable that in the former case these agents exert their detrimental pathogenetic effect, if any, in a manner distinct from the production of endoneurial oedema formation.

Notwithstanding all of these facts, a variety of inhibitors, including alrestatin, sorbinil, statil, tolrestat, ponalrestat and ICI 105552, have been used both in experimental studies and human trials in attempts to validate the theory of polyol accumulation. ARIs are capable of ameliorating the accumulation of sugar alcohols in both diabetic and galactosaemic rats (Dvornik et al., 1973). The endoneurial accumulation of polyols, water and electrolytes, and the incidence of Schwann cell injury in galactosaemic rats has been shown to be reduced following ARI administration (Mizisin & Powell, 1993), evidence in support of the polyol pathway theory.

Improvements in NCV have been achieved with ARIs in both animals and humans, with concurrent prevention or reduction in the accumulation of polyol pathway intermediates in the nerves of streptozotocin-diabetic rats (Yue et al., 1982; Cameron et al., 1986; Kato et al., 1991) and prevention or delay of the development of functional and structural neuropathy in the BB rat (Sima et al., 1990).

It has been proposed that ARI administration in early experimental diabetes produces improvement in NCV by restoring normal nerve growth; maturational retardation is a known side-effect in streptozotocin-induced diabetic rats (Cameron et al., 1986). This theory was supported by a reported increase in the NCV of the slower conducting motor fibres of the interosseous nerve with ARI treatment (Mayer & Tomlinson, 1983b), however, similar improvements were
not found in a recent parallel study (Cameron & Cotter, 1992b). Discrepancies between these reports have been attributed to methodological differences and variations in the age of the animals used; the rats in the positive report were younger and therefore in a more rapid growth phase.

Clinical trials with ARIs have often yielded conflicting results, even where the same ARI and preparation have been utilised. Improvements in NCV have been reported following treatment with alrestatin (Gabbay et al., 1979) in as many as 40% of patients with diabetic neuropathy (Culebras et al., 1981) and in a double-blind placebo trial involving 30 patients with clinical neuropathy Fagius and his group demonstrated distinct improvement only in the group that received oral alrestatin (Fagius et al., 1981). By contrast no noticeable benefits were produced in the 9 diabetic patients included in a placebo crossover study using the same ARI (Handelsman & Turtle, 1981).

Slight, although highly statistically significant, increases in NCV were achieved with sorbinil in a randomised double-blind crossover study involving clinically non-neuropathic diabetics (Judzewitsch et al., 1983). Subsequent short-term (Fagius et al., 1985) and prolonged (Sima et al., 1988c) clinical trials with ARIs have produced small but significant improvements in electrophysiological indexes which were associated, in the latter trial, with reported increases in nerve fibre regeneration and myelinated fibre density, and a reduction in nerve sorbitol content, data consistent with the polyol pathway hypothesis. The treatment of diabetic patients with an ARI has also been shown to reduce excess nerve water content (Griffey et al., 1988).

By contrast Jennings and colleagues found no evidence of improvements in measures of neuropathy throughout a clinical trial with sorbinil (Jennings et al., 1990) in agreement with similar studies involving older patients with chronic neuropathy (Lewin et al., 1984; O'Hare et al., 1988) and individuals with asymptomatic neuropathy (Martyn et al., 1987), although they did report attenuation of abnormal platelet aggregation and arrested progression of microalbuminuria.

As a result of these numerous trials it is now generally accepted that diabetes-related NCV deficits may be ameliorated by ARI treatment (Jaspan et al., 1983). It has been further postulated that extension of the period of ARI treatment may result in resolution of the clinical symptoms and neurological deficits in established cases, and ARI treatment if initiated sufficiently early in
the course of diabetes may retard development of a neuropathy. Conclusive evidence of ARI administration in clinical trials resulting in improvements in symptomatic neuropathy remains to be presented. In a more recent 12-month clinical trial with tolrestat, an ARI which apart from a possible risk of liver damage appears to be free of major side effects, 80% of the diabetics showed a small improvement of autonomic neuropathy (Giugliano et al., 1993).

One factor which may account for the ability of ARIs to prevent sugar cataracts in rats but apparent inability to improve neuropathy in diabetic patients relates to the relative concentrations of polyols within the two tissues. Hexokinase is an enzyme which catalyses the transfer of a phosphate group from adenosine triphosphate to D-glucose. Hexokinase activity is very low in lens tissue resulting in substantial accumulations of sorbitol and fructose under hyperglycaemic conditions and subsequent osmotic changes.

Despite the fact that the ARI Statil has been reported to prevent both sorbitol and water accumulation in the sciatic nerves of streptozotocin-diabetic rats (Tomlinson et al., 1986) and the concentration of sugar alcohols found in some tissues such as renal papilla (Gabbay et al., 1968b) in experimental diabetes and galactosaemia have been reported to reach osmotically significant levels, the exceedingly high levels of polyol pathway intermediates found in the lens (Kinoshita et al., 1979) are never obtained in other tissues, concentrations of sorbitol in the nerve being measured in micromoles as opposed to millimoles in the lens (Stewart et al., 1967).

As a result, it is unlikely that any significant osmotic effect will be produced from sorbitol accumulation in nerves, especially so considering the fact that sorbitol accumulation is primarily intracellular (Gabbay, 1973) whilst oedematous fluid is anatomically confined to the extracellular space (Jakobsen, 1978). The previously mentioned effect of Statil on the water content of peripheral nerves may result from prevention of the deleterious effects of sorbitol accumulation on the normal mechanism of water extraction from the endoneurial compartment. Additionally, nerve water content is not universally increased even when impulse conduction is slowed (Greene, 1983): endoneurial oedema was found in only 2 of the 27 streptozotocin-diabetic rat sciatic nerves examined by Powell and colleagues, despite the fact that most of the animals were severely diabetic and that 11 of the rats had cataracts (Powell et al., 1981a). It is more probable that sorbitol accumulation in the nerve produces secondary disturbances, for example in myo-inositol metabolism (Finegold et al., 1983).
The beneficial results of ARI administration in experimental diabetes have usually been achieved in animals that are markedly hyperglycaemic, and may therefore be directly related to this condition. In addition, despite the fact that Ponalrestat treatment for a period of 4 months served to prevent completely the characteristic nerve conduction slowing and structural abnormalities known to occur in the BB diabetic rat (Sima et al., 1990), detrimental alterations were seen to occur after 6 months of treatment suggesting that additional pathological mechanisms were beginning to exert an effect. As such ARI treatment may not solve the whole problem and prove ineffective. Maturation deficits could not account for the axonal atrophy seen to develop at this time as the animals in whom 4 months treatment proved effective were similarly stunted. One possible mechanism may relate to nonenzymatic glycation of long-lived axonal proteins consequent upon severe and continuous hyperglycaemia (Williams et al., 1982).

The most convincing evidence of the beneficial effects of ARI administration in experimental diabetes comes from experiments on animals with early diabetes and related to the prevention or reversal of early changes. The efficacy of ARIs in preventing deficits in the longer term (Willars et al., 1988) or correcting established abnormalities (Cameron et al., 1989) has been questioned. Irreversible structural changes have usually occurred in the nerves of diabetic patients before they present with a clinical neuropathy, a fact which may produce discrepancies in the effectiveness of ARI treatment in human and experimental diabetes. Studies with ARIs in newly diagnosed diabetic patients with peripheral NCV deficits but without clinical neuropathy might provide a better test of the polyol pathway hypothesis.

The dose of ARIs used in rat and human trials generally differs by an order of magnitude, being greatest in the former. Administration of the ARI ponalrestat at dose levels comparable to the upper limit used in human clinical trials, produced a significant reduction in sciatic nerve sorbitol content in early experimental diabetes but failed to alleviate the reduction in NCV normally achieved with the high dose levels usually employed (Cameron & Cotter, 1992b). This suggests that the degree of AR inhibition necessary to block the polyol pathway and reverse NCV deficits may not have been achieved in human clinical trials.

The effectiveness of a compound may also vary between species and tissues, rat lens, human lens and human platelet ARs express differences in susceptibility
to inhibition by ARIs (Kador et al., 1980). Clinical trials have also revealed the presence of other obstacles to the adoption of ARI treatment in human diabetes mellitus. Sorbinil has been shown to be a 10 times more potent inhibitor of human AR in vitro than alrestatin (Kinoshita et al., 1979) but a high frequency of adverse reactions to the drug have been observed with hypersensitivity symptoms such as rash, fever, lymphadenopathy and abnormal liver function tests (Handlesman & Turtle, 1981).

Inhibition by sorbinil of the polyol pathway in tissues other than nerve has been shown to inhibit cataract formation, preserve lens growth, cell hydration and protein components, reduce glomerular sorbitol accumulation and prevent galactosaemic retinal capillary thickening (Frank et al., 1983; Robison et al., 1983). However, to date none of the available ARIs have been proven to confer any statistically consistent benefit in diabetic neuropathy.

The cumulative doubts raised by many of these observations in conjunction with the difficulty in isolating any probable effects of sorbitol accumulation from those due to the metabolic alterations of diabetes which cannot be explained solely by sorbitol accumulation serve to complicate evaluation of the polyol theory.

In addition to reduced myo-inositol and increased sorbitol content, a reduction in cyclic adenosine 3',5'-monophosphate (cAMP) levels have been demonstrated in the nerves of streptozotocin-diabetic rats, all three abnormalities being normalised by ARI administration with a significant improvement in MNCV. The administration of illoprost, a prostacyclin analogue, produced comparative improvements in MNCV and cAMP levels without any noticeable effect on sorbitol and myo-inositol abnormalities (Shindo et al., 1992). This implies a possible role for cAMP in peripheral nerve dysfunction and the aetiology of diabetic neuropathy, independent of the effects of myo-inositol and sorbitol content. The mechanism of cAMP reduction and the relationship between cAMP and Na^+\text{-}K^+\text{-}ATPase activity remain to be elucidated.

1J.1(v) Myo-inositol Deficiency

In developed countries the average daily intake of myo-inositol ranges between 0.5 to 1g, of which only a small proportion is excreted in the urine and virtually none in faeces. Humans also possess the ability to synthesize the compound and to date there have been no documented cases of myo-inositol deficiency found in humans.
**Myo-inositol** is an important constituent of phospholipids in mitochondria, endoplasmic reticulum and cell membranes. In nerve, free myo-inositol is the precursor of phosphatidylinositol and energy-related phosphoinositides in the BB diabetic rat have been found to be influenced by the concentration of myo-inositol in the surrounding tissue. Myo-inositol uptake in peripheral nerve is mediated by a high-affinity Na\(^+\) gradient-dependent active carrier-mediated transport system, the concentration of free myo-inositol in mammalian peripheral nerve is approximately 50 times greater than that in plasma (Greene et al., 1975).

Na\(^+-\)K\(^+\)-ATPase, a membrane associated enzyme, plays an important role in maintaining a transmembranous sodium gradient in nerves; intraaxonal Na\(^+\) concentrations reflect an equilibrium between the passive influx of Na\(^+\) and its active outwards transport. A reduction in the axolemma sodium gradient is associated with the development of subthreshold nodal membrane potentials that block conduction in large myelinated axons and result in decreased current generation during nerve excitation and the production of slow composite NCV.

Lipids and inositol lipids have been implicated as endogenous activators of Na\(^+-\)K\(^+\)-ATPase in certain mammalian tissues and microsomal Na\(^+-\)K\(^+\)-ATP-ase activity is activated by the specific action of phosphatidylinositol (Simmons et al., 1982). Phosphoinositides in peripheral nerves may therefore be involved in the regulation of ionic transfer across the axolemma in nerves (Hendrickson & Reinertsen, 1971) and alterations in nerve inositol and inositol phospholipids content may play a role in the production of defects in the sodium pump and subsequently reduced NCVs. Nerve impulse transmission has been shown to be accompanied by an increase in the rate of incorporation of myo-inositol into axolemmal phospholipids (Hawthorne & White, 1973).

Alterations of inositol phospholipid metabolism in the PNS may result from (1) changes in the activities of enzymes involved in their metabolism, (2) defects in inositol transport into and across the endoneurium and (3) by changes in the rate of transport of enzymes and substrates from the cell body to the axons.

Hyperglycaemia is a condition which competitively inhibits the uptake of myo-inositol into peripheral nerves (Greene & Lattimer, 1983a), presumably on the basis of the steric structural similarity between myo-inositol and the pyranose form of D-glucose, the intraendoneurial transport of inositol is reduced by 40% in diabetic nerves (Gillon & Hawthorne, 1983).
Theoretically, if myo-inositol content is diminished in diabetic nerves, resulting perturbations in membrane phosphoinositide may cause a reduction in enzymatically determined Na\(^+\)K\(^+\)-ATPase activity and a consequent reduction in the transmembranous sodium gradient. This would not only impair nerve impulse conduction (Brismar & Sima, 1981) but possibly further blunt sodium gradient-dependent myo-inositol uptake (Greene & Lattimer, 1983a) forming a self-reinforcing pathophysiological cycle (Greene & Lattimer, 1984b).

It is believed that reduced nerve myo-inositol content exerts its influence on Na\(^+\)-K\(^+\)-ATP-ase activity via decreased protein kinase C (PKC) activity (Lattimer et al., 1989); the major metabolic pathway for myo-inositol is its reversible incorporation into the phosphoinositides which are linked to important cell regulatory mechanisms involving PKC activation, calcium mobilization and prostaglandin metabolism via inositol 1,4,5,triphasphate (IP\(_3\)) and diacylglycerol.

PKC is an important membrane protein that phosphorylates a broad range of proteins in the presence of calcium and phospholipid and is hence involved in numerous fundamental cell processes. It has been proposed that the \(\alpha\)-subunit of Na\(^+\)-K\(^+\)-ATPase undergoes kinase-mediated phosphorylation distinct from its actual catalytic site, implying that PKC may play a regulatory phosphorylating role on Na\(^+\)-K\(^+\)-ATPase (Greene et al., 1987a).

The metabolic turnover of phosphoinositol releases two important intracellular mediators, IP\(_3\) and diacylglycerol which modulate PKC activity. Due to its high calcium requirement, at physiological intracellular concentrations of free calcium, PKC would be expected to be virtually inactive. Diacylglycerol greatly enhances the affinity of PKC for calcium, activating it at physiological calcium concentrations. IP\(_3\) has been implicated in the release of calcium from intracellular storage sites and augmented IP\(_3\) mediated calcium mobilisation is thought to activate a whole range of other calcium-dependent processes within cells.

Impaired phosphoinositide turnover in diabetic peripheral nerve, as a result of depleted myo-inositol content, may cause diminished diacylglycerol and/or IP\(_3\) release, decreased activation of PKC and a resultant decrease in Na\(^+\)-K\(^+\)-ATPase activation via a phosphorylation mechanism. The addition of exogenous diacylglycerol amongst other PKC agonists, to the nerves of alloxan-diabetic rabbits in vitro has been shown to normalise completely the ouabain-inhibitable respiratory component without affecting respiration in normal healthy nerve
These data are compatible with the theory that direct phosphorylation of Na\(^+\)-K\(^+\)-ATPase or an associated regulatory membrane protein by PKC may be modulated by myo-inositol metabolism.

Peripheral nerve Na\(^+\)-K\(^+\)-ATPase is fundamental to both substrate metabolism and energy. Perturbations in Na\(^+\)-K\(^+\)-ATPase activity may therefore have widespread consequences. The composite steady state rate of energy utilisation in axons and Schwann cells of alloxan-diabetic rabbits is strikingly decreased (Greene & Winegrad, 1981), suggesting that the rates of specific energy requiring processes that operate in normal nerve are decreased in diabetes and may potentially contribute to the structural abnormalities that are believed to underlie neurological deficits; it has been proposed that axonal atrophy in diabetic neuropathy may be a consequence of slowed axonal transport secondary to a Na\(^+\)-K\(^+\)-ATPase defect (Medori et al., 1988).

If the theory of myo-inositol depletion as the primary instigator of pathological changes in diabetic neuropathy is to be believed, corroborative evidence of concurrent reductions in myo-inositol content, Na\(^+\)-K\(^+\)-ATPase activity and NCV in experimental diabetes is required.

Diminished NCV (Greene et al., 1982), ouabain-sensitive Na\(^+\)-K\(^+\)-ATPase activity (Das et al., 1976; Greene & Lattimer, 1983b) and myo-inositol content (Clements & Stockard, 1980; Mayer & Tomlinson, 1983a; Anand et al., 1988) have all been demonstrated in peripheral nerve tissue of untreated streptozotocin-diabetic rats, including cases where plasma concentrations of the compound have been found to be normal. Similar reductions in myo-inositol content have been found in the peripheral nerves of alloxan-diabetic rabbits (Greene & Winegrad, 1981; Greene & Lattimer, 1984b) and reductions in MNCV in the spontaneously diabetic GK rat has been linked to depleted nerve myo-inositol levels (Suzuki et al., 1990).

Decreased Na\(^+\)-K\(^+\)-ATPase activity in the BB Wistar rat has been shown to parallel decreases in nerve myo-inositol levels, vigorous insulin therapy at an acute phase of diabetes restoring myo-inositol concentrations and Na\(^+\)-K\(^+\)-ATPase activity to near normal levels and partially correcting nerve conduction and evoked action potential amplitudes (Greene et al., 1984). The less reversible component of NCV slowing probably reflects early structural changes. The results of these studies seem to support the hypothesis that reduced MNCV in experimental diabetes may be largely attributable to low nerve
myo-inositol content. The more recent discovery that sciatic nerve Na\(^{+}\)-K\(^{+}\)-ATPase activity is unaltered in the diabetic mutant (db/db) mouse has cast some doubts on the obligatory involvement of Na\(^{+}\)-K\(^{+}\)-ATPase defects in diabetic neuropathy (Bianchi et al., 1987).

The correction of peripheral nerve abnormalities in experimental diabetes by the administration of dietary myo-inositol supplements would provide further evidence in favour of the myo-inositol deficiency hypothesis; however reports to date have produced conflicting testimony. Most observations on changes in NCV in experimental diabetes have been performed on rats during the period of rapid growth, rats continue growing until approximately 9 months of age. This fact complicates interpretation of the results. Nevertheless, dietary supplementation with myo-inositol and consequent normalisation of peripheral nerve myo-inositol content and treatment with insulin to normalize the hyperglycaemia have both been found to correct abnormalities in NCV (Greene et al., 1975, 1982; Mayer & Tomlinson, 1983a), and prevent the associated fall in nerve Na\(^{+}\)-K\(^{+}\)-ATPase activity (Simmons et al., 1982; Greene & Lattimer, 1983). A daily dietary supplementation of myo-inositol in the region of 1% dry food weight proved sufficient to produce these improvements; higher doses at 3% produced deleterious effects (Greene et al., 1975).

Conversely, Jefferys (Jefferys et al., 1978) and Thomas and colleagues (Thomas et al., 1981) found myo-inositol supplements of similar levels to confer no beneficial effect whatsoever on peripheral nerve abnormalities. Anomalies between the negative results of these studies and the positive findings of others were attributed to the fact that mature rats were used in both of these studies and the dose of streptozotocin used to induce diabetes by Jefferys and colleagues was smaller than that often reported. Consequently, prior to myo-inositol supplementation the degree of hyperglycaemia in these animals was comparatively low, abnormalities in NCV and myo-inositol content being correspondingly small.

Another obstacle to the acceptance of the myo-inositol depletion theory relates to experimental galactose neuropathy in which, despite the fact that endoneurial myo-inositol content is reduced, Na\(^{+}\)-K\(^{+}\)-ATPase activity is seen to be increased (Llewelyn et al., 1987b).

Low myo-inositol content in the nerves of untreated experimental animals is now a well documented fact, but the same feature in human diabetics has been
disputed. Increased concentrations of urinary myo-inositol have been demonstrated in untreated human diabetics (Clements & Reynertson, 1977) suggesting a resultant reduction in size of the body pool and myo-inositol content in the sciatic nerves, removed post mortem (Mayhew et al., 1983), and sural nerves (Greene et al., 1987b) in diabetic patients to be reduced. Other workers have found no differences between healthy controls and human diabetics with regard to either sural nerve myo-inositol and scyllo-inositol content (Ward et al., 1972; Dyck et al., 1980b; Dyck et al., 1988) or plasma or erythrocyte myo-inositol levels (Gregersen et al., 1983). Dyck and colleagues were unable to demonstrate any direct relationship between diabetic neuropathy and myo-inositol content in human diabetic nerves (Dyck et al., 1980b).

The beneficial results achieved with dietary supplementation of myo-inositol in experimental diabetes, notwithstanding the failure to demonstrate conclusively a reduction in nerve myo-inositol content in human diabetes, led to the theory that similar effects may be produced in human diabetic patients and as such a number of clinical trials have been performed. Myo-inositol is readily absorbed from the intestines and has been widely demonstrated as nontoxic in both diabetic and normal healthy individuals, the exceptions being cases where the oral intake has been exceedingly high or where delayed excretion, as in the case of terminal renal failure, has led to very high serum concentrations.

The results achieved in human therapeutic trials have proven inconclusive, no improvements being noted in the majority of cases (Gregersen et al., 1978, 1983). However, statistically significant positive results have been reported in one trial (Clements et al., 1979). There are several factors which may explain the lack of concordance found between the effects of myo-inositol dietary supplementation in experimental and human diabetes.

Species differences may always play a role where comparisons are being made between experimental and human therapeutic trials. The actual daily intake of myo-inositol expressed in mg/kg body weight is not comparable between the animals and human subjects, due to the much larger daily proportion of food ingested to body weight ratio in the rats. The amounts of myo-inositol given in the human trials ranged from 500mg twice a day to 2000mg three times a day; 45g/day would have to be given to the patients to achieve an intake that corresponds with that of the experimental animals. Possibly as a result of this the plasma concentrations of myo-inositol in the human subjects never achieved as high a level as that found in the rats (Gillon et al., 1983).
Another possible explanation could be that the patients who took part in the clinical trials were under conventional treatment to normalise their hyperglycaemia, and their blood glucose levels would therefore be lower than those found in the experimental animals. Finally, the animals used for the trials were all young; many of the patients who took part in the human trials were middle aged or older and as such may have had the disease for several years. It is likely that irreversible structural alterations would have taken place in their nerves which would make them unresponsive to treatment. A true comparison of the effects of dietary supplementation with myo-inositol between experimental and human diabetes can only be made where the trial patients are young, untreated, have only recently become diabetic and are given higher dose of the compound.

The trial performed by Gregersen and colleagues (Gregersen et al., 1978) provides the best comparison as the individuals involved were young, had no clinical symptoms of neuropathy and had a disease duration of less than 10 years; additionally the dose of myo-inositol used was the highest of any clinical trial. Nonetheless, Gregersen was unable to demonstrate any significant improvement in nerve action potential, motor conduction velocity, sensory conduction velocity or vibratory perception threshold. To date no significant evidence has been presented to substantiate the myo-inositol deficiency hypothesis by myo-inositol supplementation in human diabetes.

Work has been performed which suggests that a link may exist between the reduced myo-inositol levels and sorbitol accumulation in the nerves of experimentally diabetic animals, as the administration of sorbinil, an aldose reductase inhibitor, has been shown to not only prevent increases in sorbitol concentrations but to also prevent myo-inositol depletion, even in the presence of continuing hyperglycaemia (Finegold et al., 1983). Nerve fructose levels were only partially reduced by sorbinil administration (Finegold et al., 1983), suggesting that the polyol pathway may have been only partially blocked by the action of sorbinil. An alternative explanation is that, despite complete blockade of the polyol pathway, nerve fructose levels may still be elevated as a result of increased nerve glucose levels interfering with fructose phosphorylation.

Greene and Lattimer reported that a prevention in the fall of nerve myo-inositol content by aldose reductase inhibitors was shown to prevent the reduction in nerve Na^+-K^+-ATPase activity that has been linked to conduction slowing (Greene & Lattimer, 1984a). A later study failed find any significant correction
of MNCV and myo-inositol content (Kato et al., 1991), differences probably being attributable to variations in diabetic conditions and treatment schedules. The proposal that axonal atrophy in diabetic neuropathy is a consequence of slowed axonal transport secondary to a Na\(^+\)-K\(^+\)-ATPase defect resulting from perturbations in myo-inositol metabolism (Medori et al., 1988) was supported by normalisation of axonal transport after ARI treatment (Tomlinson et al., 1984), amelioration of these abnormalities being attributed to normalisation of nerve myo-inositol content and the following cascade of events.

Hyperglycaemic stimulation of the polyol pathway may play a role in the development of metabolic, functional and structural abnormalities in diabetic animals as a consequence of impaired Na\(^+\)-K\(^+\)-ATPase activity, possibly in conjunction with the effects of polyol accumulation; extrapolation of this evidence to human subjects suggesting a similar aetiology (Sima, 1985; Greene et al., 1987c). The fact that the administration of myo-inositol to correct reduced myo-inositol nerve content in streptozotocin-diabetic rats serves to normalise diabetic nerve dysfunction without influencing nerve sorbitol concentration (Greene et al., 1975, 1982) supports the theory that polyol pathway effects in nerve might be mediated primarily by secondary alterations in myo-inositol metabolism, by a biochemical mechanism which at present remains undetermined. ARI administration may serve to interrupt the diabetic pathophysiological cycle of myo-inositol by a mechanism which still remains to be elucidated.

Structural and functional Schwann cell and axonal abnormalities that are known to occur in experimentally diabetic rats include slowed motor NCV, impaired axonal transport, reduced Na\(^+\)-K\(^+\)-ATPase activity, diminished Na-dependent amino-acid uptake, an attenuated trans-axolemmal Na\(^+\) gradient, and nodal and paranodal swelling (Greene et al., 1982; Tomlinson & Mayer, 1985; Greene et al., 1987c; Simpson & Hawthorne, 1988; Greene et al., 1990; Kim et al., 1991).

The facts that (1) myo-inositol content has not been universally found to be decreased in diabetic nerves and that (2) amelioration of nerve dysfunction following ARI administration has been achieved in both experimental (Miwa et al., 1989) and human (Dyck et al., 1988) diabetes without any obvious effects on nerve myo-inositol content, provide arguments against the "myo-inositol depletion hypothesis".
It is possible that myo-inositol depletion may play a role at an early stage of both experimental and human diabetes, before the development of any structural alterations. Direct evidence has recently shown that myo-inositol depletion is capable of impairing important cellular physiological functions; physiological dysfunctions induced in nontransformed human retinal pigment epithelial (hRPE) cells cultured in media containing hyperglycaemic concentrations of glucose were seen to be normalised by the addition of ARIs or myo-inositol (DelMonte et al., 1991). Conclusive evidence in support of myo-inositol depletion playing a primary causative role in the production of human diabetic neuropathy remains limited to date.

1J.1(vi) Disturbed Protein Metabolism - Impaired Synthesis of Structural Proteins

Impaired protein synthesis in the neuronal cell body resulting in the diminished delivery of structural proteins to the distal portions of the axons and distal axonal degeneration has been a further hypothetical mechanism for diabetic neuropathy. This hypothesis is supported by several facts. Experimental animals, made diabetic during the prepuberal period, show a retardation in nerve fibre growth (Sharma et al., 1981). A similar result is produced by a severe protein restriction in the diet of experimental rats (Oldfors & Ullman, 1980), leading ultimately to distal axonal degeneration (Oldfors 1981a). Thomas and workers found an analogous reduction in the uptake and incorporation of $[^3\text{H}]$-leucine into protein by dorsal root ganglia in streptozotocin-diabetic rats (Thomas et al., 1984).

Axonal degeneration may result from non-enzymatic glycation of axonal structural proteins interfering with their normal polymerisation and assembly (Williams et al., 1982), although the actual effects of this process on axonal integrity is unclear.

An intimate relationship is thought to exist between the pathological processes of disease and disturbances in axonal transport, as changes in axonal intercellular protein transport are evident, in both toxic and metabolic neuropathies in animals, before the manifestation of any clinical signs of neuropathy or the development of pathological alterations in nerve fibres (Jakobsen et al., 1978). It is however, not generally proposed that these abnormalities play a primary causative role in the development of neuropathy, but rather exist as a link in the chain of events leading to this eventuality (Tomlinson & Mayer, 1984).
Sidenius and Jacobsen (Sidenius & Jakobsen, 1982) demonstrated that there is a reduction both in synthesis and transport velocity of anterograde slow component a (SCa) in streptozotocin experimental diabetes. SCa transports tubulin and the neurofilament polypeptide triplet to the main structural components of nerve fibres, microtubules and neurofilaments that are largely responsible for determining axonal calibre. The other two anterograde components, slow component b (SCb) and fast axonal transport remain unaffected.

The change in SCa is preventable with the use of insulin therapy (Sidenius & Jakobsen, 1982). The reduction in SCa transport in the sciatic nerves of streptozotocin-diabetic rats appears to correlate with the degree of axonal dwindling found by some to occur in this model. In support of this idea, the alloxan-diabetic rabbit, an animal model which exhibits no signs of nerve fibre pathology, appears to have an unaffected SCa transport function.

Myo-inositol treatment and ARI administration have been shown to prevent the development of defects of orthograde axonal transport in experimental diabetes (Mayer & Tomlinson, 1983a) suggesting that polyol pathway related effects, such as myo-inositol depletion, may play a contributary role to the production of diabetic neuropathy by this route. Alternatively, alterations in SCa transport may be a secondary effect of the disease related to a reduction in the amounts of trophic factors in the PNS, such as Nerve Growth Factor, and a subsequent decrease in the retrograde transport of these agents.

Unused or degraded materials that have been synthesised in the axons are delivered to the cell soma by the retrograde transport mechanism for recycling or disposal. It has been suggested that this conveyor belt acts as a 'feedback mechanism' and helps the cell to regulate its anabolic and catabolic activities with reference to the demands of the distal terminals. It is therefore possible that the level of neuronal perikaryal protein synthesis may be controlled by retrograde axonal transport. This has lead to the suggestion that diminished delivery of retrograde components and their reduced rate of axonal transport may be a primary defect in diabetic neuropathy and lead to diminished levels of protein synthesis (Jakobsen et al., 1983), a decrease in the delivery of slowly transported structural proteins to the axon (Jakobsen & Sidenius, 1980) and the retardation of nerve fibre growth in diabetic peripheral neuropathies occurring as a secondary effect.
Abnormal neurotrophic responses

Retrogradely transported neurotrophic factors, such as nerve growth factor (NGF), are required for both the development and maintenance of NGF-sensitive sensory and autonomic neurons and axonal processes. The application of NGF to the cut proximal ends of transected rat sciatic nerves prevents the otherwise normal death of a significant number of neurons in the dorsal root ganglion (Rich et al., 1987). The production of NGF by the target organs of autonomic and sensory neurons is suppressed following developmental innervation. Conversely, denervation enhances the production of NGF. Subsequently, the amount of NGF synthesized by these target organs correlates with the density of innervation by NGF-sensitive neurons (Thoenen et al., 1987).

Schwann cells also possess the ability to manufacture and express neurotrophic factors and their receptors, including NGF (Bandle et al., 1987), ciliary neurotrophic factor (CNTF) (Lin et al., 1990) and IGF-I (Hansson et al., 1986), during development and regeneration (Sobue et al., 1988; Scarpini et al., 1988).

The characteristic distal dying-back pattern associated with diabetic peripheral sensory and autonomic neuropathy could theoretically arise as a result of the diminished manufacture, release or transport of neurotrophic factors, or an abnormality in the normal cellular response to these agents consequent upon altered neuronal signal transduction due to chronic hyperglycaemia and/or insulin deficiency.

Although it may purely be a reflection of the established diabetes-related reduction in axonal transport of proteins, glycoproteins and neurotransmitters, the retrograde transport of NGF in streptozotocin-diabetic rats has been shown to be diminished (Jakobsen et al., 1981). Growth factors regulate neuronal gene expression and hence protein synthesis in the cell body and thereby play a role of considerable importance in the survival and maintenance of the neuron and axonal process. Downregulation of neurofilament gene expression following reduced delivery of neurotrophic factors to the cell body may result in axonal atrophy. Reduced axonal diameters with concomitant slowing of nerve conduction velocities have been reported in both diabetic patients and experimentally diabetic animals (Moore et al., 1980).

Despite the fact that NGF levels were shown to be increased in most NGF-producing tissues in streptozotocin-diabetic rats, a dramatic reduction in the levels of NGF was found in the superior cervical ganglion which contains a
population of NGF-dependent neurons, possibly as a result of reduced retrograde transport of NGF (Hellweg & Hartung, 1990). NGF levels have been found to be reduced in sympathetically innervated target organs in streptozotocin-diabetic rats (Hellweg & Hartung, 1990) and in the serum of individuals with diabetic neuropathy, being greatest in patients with the greater neurological impairment (Faradji & Sotelo, 1990). These observations provide evidence in support of the theory that a disrupted neurotrophic mechanism may play an aetiologically important role in the production of diabetic neuropathy.

Abnormalities in the production of neurotrophic factors and their receptors by Schwann cells may also be of pathogenetic significance as, although their levels of synthesis and expression respectively is low where axonal contact exists (Johnson et al., 1988), they are important in normal neuronal maintenance.

The production of neurotrophic agents and their receptors by Schwann cells is upregulated following loss of axonal contact (Taniuchi et al., 1986; Heumann et al., 1987). Abnormalities in the normal response of Schwann cells as part of the regenerative process, may be impaired in diabetes mellitus, Schwann cell NGF receptor expression has been shown to be increased in other human neuropathies involving active degeneration (Sobue et al., 1988), and could be causal in the production of the known diminished regenerative capacity of peripheral nerves associated with this disease.

1J.2 Mechanical hypotheses

Mulder and workers (Mulder et al., 1961) proposed that some focal neuropathies in diabetics may result from an abnormally high susceptibility of the nerve to compression resulting from external pressure or entrapment. It is not known whether entrapment neuropathies in non-diabetics differ pathologically from those occurring in diabetics.

1J.3 The vascular hypothesis

1J.3(i) Atherosclerosis

Atherosclerosis is a common disorder of large and medium sized arteries. It is characterised by the formation of yellowish plaques of cholesterol, lipids and cellular debris in the inner layers of the walls. Subsequent thickening, fibrosis and calcification results in narrowing of the lumen and reduced circulation in the areas and organs supplied by the vessel. The pathogenesis of atherosclerosis is not clearly understood, possible contributory factors are believed to be injury to
the arterial endothelium, proliferation of smooth muscle in the vessel wall and hyperlipidaemia due to dietary excess, faulty carbohydrate metabolism or a genetic defect. Atheromatous lesions are the major cause of coronary heart disease leading to angina pectoris and myocardial infarction, amongst other cardiac disorders. Atherosclerosis is associated with aging and has been connected to obesity, hypertension and diabetes.

Woltman and colleagues (Woltman et al., 1929) were among the first to propose atherosclerosis as the cause of diabetic neuropathy. The reliability of their observations has been questioned as the nerve specimens used were largely obtained at autopsy from patients with longstanding diabetes or from limbs amputated for arteriosclerotic gangrene, and as such atherosclerosis would have been expected to be frequently encountered. Additionally at least some of the described infarcts were in reality Renaut corpuscles, anatomical structures found in healthy nerves. Later studies resulted in the revival of this theory following the discovery that, although not a major clinical component, peripheral neuropathies do occur in non-diabetic patients with large vessel occlusive vascular disease (Eames et al., 1967) presumably due to resultant ischaemia (Chopra et al., 1969).

This theory has since fallen into disfavour following the demonstration that large vessel occlusive disease does not play an important role in the production of peripheral nerve damage in diabetic neuropathy; the NCV deficits seen in patients with diabetic neuropathy are markedly greater than those of non-diabetic patients with lower limb ischaemic vascular disease (Chopra & Hurwitz, 1969) and correlate histologically with a comparatively greater degree of segmental demyelination (Chopra & Hurwitz, 1967). Additionally, no evidence has been found to date of a correlation between chronic vascular disease and diabetic neuropathy (Dolman, 1963; Greenbaum et al., 1964; Thomas & Lascelles, 1966).

1J.3(ii) Microangiopathy

Capillary basement membrane thickening is the salient feature of diabetic microangiopathy (Siperstein et al., 1968) and is ubiquitous within diabetic tissues (Aegenaes & Moe, 1961) having been described in virtually every capillary bed, including those of skin, pancreas, adipose tissue, skeletal muscle, kidney and peripheral nerve. Local variations in thickness have been found to occur, suggesting that involvement may not be global.
It is probable that an abnormal metabolic process resulting from the altered environmental milieu in diabetes mellitus provides the primary mechanism responsible for the development of associated microvascular disease, as nondiabetic donor kidneys develop typical lesions when transplanted into diabetic individuals (Barbosa et al., 1994). It has been suggested, however, that a contributary genetic component may exist, certain individuals possessing an inherited propensity to both diabetes mellitus and microangiopathy.

Over the years abnormalities of the vasa nervorum have been proposed by some researchers as being of aetiological significance for diabetic polyneuropathy. Fagerberg (Fagerberg, 1959) first drew attention to thickening and hyalinisation of the walls of diabetic endoneurial capillaries by PAS-positive material, which was later found at electronmicroscopic level to correspond to reduplicated layers of basement membrane (Bischoff, 1967), with concomitant luminal narrowing. He concluded that as the occurrence of diabetic retinopathy and nephropathy, both of which are known to be of vascular origin, generally paralleled that of angiopathy and neuropathy in diabetic individuals, the latter complication was also likely to have a vascular basis.

Considerable interest has been shown in this theory over the years, however, not all investigations have produced confirmatory evidence of diabetic microangiopathy in the PNS; Chopra and colleagues found only a slight degree of endoneurial vessel wall hyperplasia in 3 of the 11 diabetic patients included in their study whilst the vessels of the remaining 8 patients appeared normal. PAS(+)ve staining was not observed and none of the vessels were occluded (Chopra et al., 1969).

In later studies by Vital and colleagues, it was reported that marked thickening of endoneurial capillary basement membranes of patients under 40 years of age occurs only in individuals with a diabetic neuropathy. In young diabetic patients numerous capillaries with normal or only marginally increased basal lamina thicknesses were found, although in many cases the associated endothelial cells appeared swollen and abnormally rich in organelles (Vital et al., 1976, 1977). The absence of abnormalities in endoneurial capillary structure has also been cited in painful diabetic neuropathies (Brown et al., 1976).

The majority of studies have, however, confirmed the fact that endoneurial vessel basal lamina hyperplasia is a characteristic feature of diabetic nerves (Lapresele, 1968; Bischoff, 1973). In a more recent comparative study of nerve
biopsies from seven diabetic and seven non-diabetic patients, Powell and colleagues (Powell et al., 1985) reaffirmed the proposal that endoneurial capillary basement membranes are thicker in diabetics as significant differences were found in all 7 case subjects studied.

Microangiopathy and associated endoneurial capillary basal lamina hyperplasia is not a phenomenon unique to diabetes mellitus, but is rather a common feature in chronic neuropathies. Nethertheless its occurrence and extent has been quantitatively proven, using the method employed by Jordan and Perley (Jordan & Perley, 1972) on muscle capillaries, to be statistically significantly greater in diabetic neuropathies than in nondiabetic neuropathies (Vital et al., 1973, 1974).

Further support for the theory that diabetic polyneuropathy may be of vascular origin is supplied by observations on experimental diabetes in animals. Microangiopathy has been reported, by some authors, in the PNS of chronic alloxan-diabetic rats (Powell & Myers, 1984), and the diabetic BB-Wistar rat, the latter being a model in which focal ischaemic lesions have been documented in the peripheral nerves of the proximal lower limb (Sima & Thiebert, 1982).

A relationship between impaired circulation and peripheral nerve disorders has been known to exist for some considerable time. Nerves contain the enzyme systems for glycolysis, the Krebs cycle and electron transport, and energy is derived for the generation, maintenance and restoration of the resting potential by aerobic metabolism. The levels of oxygen required by healthy nerve even when increased by activity, are comparatively small in relation to the levels demanded by other tissues. Charge is continually lost at rest due to constant transmembranous leakage of sodium and potassium along their respective ionic concentration gradients. Energy must therefore be utilised to restore and maintain these gradients. The cessation of aerobic metabolism does not directly interrupt the generation of action potential, but will result in a gradual decrease in resting potential consequent upon the uncompensated leakage of sodium and potassium. Under these conditions the process of glycolysis is utilised in an attempt to overcome the decrease in resting potential. In conclusion the functional activity of the peripheral nervous system is rendered relatively resistant to ischaemia as a result of the low levels of energy required, the presence of the glycolysis system, the existence of energy stores which enable continued activity under anoxic conditions, and the removal of postassium from the immediate environment.
The major peripheral nerves are supplied with blood by nutrient arteries arising from adjacent major arteries. Although these enter the nerve trunks at multiple points, long stretches of nerve exist which lack a nutrient artery. The extensive longitudinal anastomosis of the epineurial arteries and vasa nervorum fully compensate for this anatomical feature and provide a more than adequate supply of blood to all parts of the nerve (Adams, 1942). As a result of this feature, the effect of occlusion of epineurial arteries by disease or surgical processes are limited and nerve damage minimalised (Smith et al., 1977). The susceptibility of different peripheral nerves to ischaemia varies in accordance with the pattern of their nutrient and epineurial vessels.

Numerous physiological studies using experimental animals have been performed over the years in order to determine the nature of ischaemic changes in peripheral nerves. Peripheral nerves contain different populations of fibres which vary in calibre, myelination, physiological characteristics and functions, including ionic concentrations, the nature of the axolemma and local metabolic activity. Consequently, the effects of ischaemia on the amplitude, afterpotential, refractory period and conduction velocity of the action potential of the nerves may be considerably selective and may result from alteration in all or only one group of fibres.

*In vitro* anoxic conditions are known to produce a decrease in amplitude and conduction rate and an increase in threshold and refractory period in nerve trunk compound action potentials (Gerard, 1930), with unmyelinated fibres appearing to possess a greater resistance to ischaemia than their myelinated counterparts possibly as a result of their smaller metabolic requirements and better local diffusion.

Using a variety of different experimental animal species Wright demonstrated that all agents which effectively block aerobic metabolism produce similar results, the severity of damage varying with temperature and the metabolic rate of the nerve (Wright, 1946). The findings of these in vitro studies are consistent with the theory that ischaemia exerts its effect by causing impairment of the metabolic processes responsible for maintenance of the ionic gradient and by the resultant production of local accumulations of potassium and depolarisation.

Morphological observations on experimental ischaemic insult have demonstrated that the production of severe histological ischaemic necrosis of nerve has only
proved reproducible with the occlusion of a minimum of two major nutrient vessels and in these cases muscle necrosis is an accompanying factor (Fowler & Gilliatt, 1981). The occlusion of anastomotic arterioles by ligation of a nerve may produce local histological changes in the proximal region, as may the stripping of superficial epineurial vessels. Severity of the lesions is dependent on collateral blood supply and the degree of diffusion from surrounding tissues. Peripheral nerve function may be permanently impaired and histological damage occur if ischaemia is sufficiently severe or prolonged.

Nerve fibre damage may not be demonstrable even after ligation of numerous arteries (Fowler & Gilliatt, 1981), implying that although endoneurial blood flow may be greatly reduced, the intraneural plexuses are able to provide sufficient supply to significantly delay or prevent ischaemic damage. This apparent absence of any abnormality after partial obliteration of the vascular supply has been shown to be deceptive, as whilst nerve conduction appears normal under resting conditions, rapid stimulation of the nerve demands significantly more energy than can be produced by the reduced blood supply.

The salient pathological occurrences at the ischaemic core include a patchy distribution of mixed Wallerian degeneration and to a lesser degree local primary demyelination with myelin thinning and paranodal demyelination (Nukuda & Dyck, 1984). The proximal borders of acute ischaemic lesions characteristically contain enlarged dark axons densely packed with organelles. This may be a result of either disrupted axonal flow or the selective redistribution of axoplasmic organelles towards the lesion. At the distal borders axon attenuation may be observed and at the most distal affected regions axons may undergo either further degeneration or, conversely, regeneration as the presence of a restricted blood supply may not necessarily impede nerve regeneration. In severely affected regions or if ischaemia is prolonged all cellular components may become necrotic. Endoneurial collagenization may also occur due to the collateral blood supply. The affected musculature may undergo gangrene.

The production of local ischaemic lesions in conjunction with varying degrees of alteration in the blood/nerve barrier were reported after the intra-arterial injection of arachidonic acid, a compound which produces platelet aggregation and vasoconstriction (Parry & Brown, 1981). Ischaemic fibre degeneration due to multiple capillary occlusion alone has also been reported as a result of intra-arterially injected polystyrene microsphere embolization (Nukuda & Dyck, 1984), a method which produces selective ischaemic damage without arterial
occlusion and the development of foot or leg gangrene.

*In vivo* studies of the effects of impaired vascular supply to the cat sciatic nerve have demonstrated that nerve conduction is lost after 30 minutes duration of complete ischaemia (Bentley & Schlapp, 1943). A definitive duration of anoxia sufficient to induce structural alterations has not so far been determined, a period of 30 minutes may prove sufficient in the retina, whilst several hours may be required in peripheral nerves. Nerves appear more resistant to ischaemia than muscles as a period of only 2 hours of ischaemia in the latter tissue is capable of producing degenerative changes. Human peripheral nerves are considerably longer and greater in diameter than those of most experimental animals, therefore, local diffusion is less effective and the occlusion of fewer nutrient arteries may be sufficient to produce comparable levels of ischaemic damage.

Factors which may contribute to the production of hypoxia, which has been considered to be of primary importance in the development of nerve damage (Tuck et al., 1984), include blood hyperviscosity, reduced oxygen tension and increased resistance to blood flow. The occurrence of such rheological alterations in the diabetic state has been investigated in order to determine the relative contributions, if any, of such vascular changes to the aetiology of the disease process.

High blood viscosity slows blood flow and results in stagnation in the microcirculation. Theoretically, subsequent local hypoxia and lactic acidosis may lead to microvascular damage. Plasma viscosity, haematocrit, erythrocyte aggregation and erythrocyte deformability are the major determinants of whole blood viscosity. Haematocrit, the measurement of the packed cell volume of red cells expressed as a percentage of total blood volume, is increased in diabetes mellitus. Its contribution to increased blood viscosity in diabetes can be discounted as corrections for haematocrit abnormality have not revealed any observable degree of normalisation in blood viscosity (Lowe et al., 1980).

Decreased red cell deformability is critically dependent on the quality of metabolic control in diabetes and to a lesser extent on the onset, duration and complications of the disease. Hyperlipidaemia is a common condition in diabetes mellitus. A definite correlation has been demonstrated between membrane cholesterol/phospholipid ratio, plasma cholesterol and membrane fluidity (Bryszewska et al., 1986); the erythrocyte membrane contains appreciable levels of cholesterol in (Rice-Evans & Chapman, 1981). The effects of high serum
cholesterol (Jennings et al., 1991) in conjunction with glycosylation (McMillan et al., 1978) may cause a reduction in red cell deformability and impede their ability to transverse capillaries. Decreased erythrocyte deformability has been associated with diabetes in some (Einst & Matrai, 1986) but not all (Bareford et al., 1986) reports.

Blood viscosity may be increased by elevated glucose concentrations. Increased and abnormal plasma protein levels have been associated with diabetes in some (Shmid-Schönbein & Volger, 1970; McMillan, 1974) but not all investigations (MacRury & Lowe, 1990). This abnormality may contribute to increased plasma viscosity by the promotion of erythrocyte aggregation, a feature known to occur in adult diabetes. An increased propensity to platelet aggregation is also associated with diabetes mellitus (Bastyr et al., 1989). In one study platelet aggregation was found to be enhanced only in diabetic patients with neuropathy, and not in those with no evidence of clinical complications (Ford et al., 1992).

Hyperfibrinogenaemia has been shown to occur in human diabetes (Barnes et al., 1977). Fibrinopeptide A is cleaved from the A-alpha chain of fibrinogen by the action of thrombin and thus forms the first step in the conversion of fibrinogen to fibrin; as such it is a sensitive indicator of \textit{in vivo} thrombin activity. Plasma fibrinogen A levels have been shown to be significantly elevated in types I and II diabetes, with and without complications, implying that coagulation abnormalities are not the result of existing complications (Ford et al., 1991), and that an increased susceptibility to thrombosis in diabetes may play a role in the development of diabetic angiopathy.

In view of the results of these studies it is not surprising that whole blood viscosity has been found to be elevated in diabetic patients with a peripheral neuropathy, (Barnes et al., 1977; Lowe et al., 1980). Although Barnes and colleagues (Barnes et al., 1977) failed to find evidence of similar rheological alterations in diabetic patients without complications, more recent studies have revealed the existence of raised blood viscosity in individuals without any clinically detectable retinopathy, neuropathy or any other vascular complications (Lowe et al., 1980; MacRury et al., 1991), a fact which complicates the issue of the importance of rheological changes in the production of diabetic neuropathy.

The development of diabetic microvascular complications has been shown to be associated with abnormal rheological indices by some (Lowe et al., 1986b) but
not all workers (MacRury et al., 1991), however, to date a specific relationship between diabetic neuropathy and these abnormalities has not been demonstrated.

Glycated haemoglobin is present in greater concentrations in diabetic animals than controls (Low et al., 1984). The non-enzymatic glycation of haemoglobin results in an alteration in its dissociation constant. The oxygen affinity of glycated haemoglobin is subsequently greater than that of its non glycosylated counterpart which may contribute to reduced endoneurial oxygen tension in diabetic nerves. This possible effect is countered by an increase in erythrocyte 2,3-diphosphoglycerate resulting in little or no difference between erythrocyte oxygen affinity between diabetic and healthy animals (Samaja et al., 1982).

Local pressure gradients are necessary to deliver blood to tissue. The majority of studies investigating a diabetes-related increase in vascular resistance have been performed on animal models. Reduced nerve blood flow and endoneurial oxygen tension have been demonstrated in the sciatic nerves of streptozotocin-diabetic rats (Tuck et al., 1984), the former feature being attributed to an increase in resistance to flow resulting from microangiopathy with a concomitant reduction in the number or calibre of endoneurial capillaries and blood hyperviscosity. Erythrocyte adhesion to endothelial cells could also increase endoneurial vascular resistance in the PNS (Barnes et al., 1977).

Sural nerve endoneurial oxygen tension values have been shown to be below those of dorsal foot veins in diabetic patients with chronic sensorimotor neuropathy (Newrick et al., 1986), the nerve venous oxygen gradient in diabetics being the reverse of that in healthy individuals. It is possible that this unphysiological oxygen gradient may arise as a result of arteriovenous shunting around peripheral nerves similar to that known to occur in the diabetic foot (Watkins & Edmonds, 1983) and conceivably lead to endoneurial hypoxia. The fact that the mean age of the diabetic patients used by Newrick et al. was appreciably greater than that of the controls casts a shadow of doubt over the significance of these findings. No indication of any statistically significant difference between the two groups was given for endoneurial oxygen tension. Additionally, the number of controls used in this study was very small. The presence of reduced oxygen tension in human diabetic neuropathy requires further investigation before any definite conclusions can be drawn.
Abnormalities of platelet function is a common feature in diabetic patients and may contribute to the production of diabetic neuropathy (O'Malley et al., 1975). No direct evidence in support of this theory has so far been presented. Increased platelet aggregability and stickiness in the diabetic condition may be of considerable pathological importance in a capillary network affected by abnormal arteriovenous shunting, a phenomenon which, as previously stated, is known to occur in diabetes.

Oxygen supplementation produced a correction in nerve conduction velocity and endoneurial concentrations of glucose and polyol pathway end-products to levels approaching those found in healthy rats without a similar normalisation of plasma glucose, suggesting a specific peripheral nerve rather than a generalised effect (Low et al., 1984). Total correction was not expected as the persisting hyperglycaemic environment would still be exerting a detrimental effect (Dyck et al., 1981).

In contrast to an earlier report which stated that increased endoneurial vascular resistance in streptozotocin-diabetic rats is preceded by a period of hyperaemia (Tilton et al., 1989), Cameron and colleagues demonstrated a reduction in sciatic nerve endoneurial blood flow in this animal model with a duration of diabetes ranging from as little as 1 week to 4 months (Cameron et al., 1991a). Conduction velocity in the sciatic nerve was correlated with blood flow. The discrepancy in results between these two studies has been attributed to the different techniques used to measure blood flow, Tilton et al. using the microsphere entrapment technique and Cameron et al. using microelectrode polarography monitoring and hydrogen clearance.

Similar reductions in blood flow have been observed in normal rats rendered hyperglycaemic by glucose infusion supporting the hypothesis that hyperglycaemia-induced blood flow reductions and resultant endoneurial hypoxia play a contributory role in the production of nerve conduction velocity deficits in experimental diabetes. Treatment of streptozotocin-diabetic rats with guanethidine, resuting in the production of a functional adrenergic sympathectomy, produced near-normalisation of reduced endoneurial blood flow and conduction velocity (Cameron et al., 1991c), the remaining deficit in blood flow probably being attributable to other features of experimental diabetes such as increased blood viscosity.
Another recent study involving the use of the streptozotocin-diabetic rat model, whilst corroborating Tuck’s findings of reduced intraneural oxygen tension, failed to produce evidence of diminished endoneurial blood flow (Zochodne & Ho, 1991). Anomalies in the results of such investigation in experimental diabetes in conjunction with the fact that comparative levels of degeneration and demyelination to those found in human diabetes do not occur in this animal model (Sharma & Thomas, 1974) cast doubts as to the relevance of any conclusions drawn from these results concerning the pathogenesis of human diabetic neuropathy.

The demonstration of impaired exercise-induced conduction velocity increment in diabetic neuropathy was interpreted as possibly being due to impaired nerve blood flow (Tesfaye et al., 1992), based on earlier observations of epineurial nutrient artery disease in diabetic neuropathy (Korthals et al., 1988; Tesfaye et al., 1990). Utilising the procedure of micropipette insertion as a method of recording local microcirculatory pressure, Tooke could find no differences between the values for human diabetics and controls, except at extreme flow rates (Tooke, 1980).

Theoretically, increased blood viscosity, decreased oxygen tension and reduced blood flow may contribute to the pathogenetic processes involved in the production of diabetic neuropathy. Conclusive evidence of the existence of any of these abnormalities and any pathogenetic contribution remain to be proved.

It is recognised that certain vascular conditions may result in the production of an ischaemic environment within the PNS. Large vessel occlusive disease or embolization is one such example, acute occlusion resulting in either spontaneous activity of nerve fibres or loss of function. The femoral and iliac are the most commonly affected arteries. Multiple embolization may occur affecting more than one vessel simultaneously. Various factors, including patient age, contribute to the magnitude of the resultant damage. Although an acute or transitory period of ischaemia, for example 30 minutes, is sufficient to induce changes, subsequent recovery is rapid, permanent neurological damage occurring only if arterial flow is not reestablished within a few hours.

Necrotizing angiopathy is another condition which is capable of producing ischaemic damage in peripheral nerves. It occurs in polyarteritis nodosa, rheumatoid arthritis and Wegener’s granulomatosis and involves the occlusion, at multiple sites, of 50 to 400μm diameter epineurial arterioles resulting in a
multiple mononeuropathy, and capillary disease. A morphological study of resultant lesions in the sciatic nerve revealed depletion by axonal degeneration of myelinated and unmyelinated fibres of all calibres in multifocal central fascicular regions (Dyck et al., 1972). Diffusely scattered, organelle rich, swollen degenerating axons were apparent distally. Axons appeared more vulnerable to the effects of ischaemia than Schwann cells. Many other conditions affecting both small and large vessels are also associated with the production of ischaemic nerve damage.

Focal third, fourth and sixth cranial mononeuropathies are commonly associated with diabetes. Of these, third nerve palsy is the most frequent condition, occurring most often in patients over the age of 50 years and being rare in younger individuals and children. Oculomotor nerve dysfunction is usually complete, resulting in loss of ocular mobility; however, pupillary sparing is regularly reported. A gradual improvement with complete recovery within 3 to 5 months is the norm. Non-inflammatory focal centrofascicular demyelinating lesions of the third cranial nerve in its intracavernous portion have been described in two studies (Dreyfus et al., 1957; Asbury et al., 1970).

The observed pupillomotor sparing in conjunction with the centrofascicular nature of the lesion suggests that these fibres are peripherally located. Extensive hyalinisation of intraneural blood vessels was reported in these cases and despite the fact that no causative occluded vessels were found either within the epineurium or the vasa nervorum, it is generally agreed that the pathological appearance of these lesions in addition to an absence of nuclear chromatolysis of the cell bodies and the abrupt onset suggests an ischaemic origin. The prompt and complete clinical recovery of an earlier identical palsy in one of the patients is not typical of ischaemic peripheral nerve lesions and the underlying demyelinating pathology that explains this rapid recovery has also been shown to be atypical of vascular lesions in nerves (Fujimura et al., 1991). The precise vascular events responsible for putative ischaemia is these cases remains undetermined.

The pathology of lesions in asymmetric diabetic neuropathies has also lead to the proposal of an ischaemic basis (Raff et al., 1968). Extensive fibrotic thickening and hyalinisation of small arteriolar and capillary walls were described in a detailed pathological study of lower limb multiple mononeuropathy (Raff & Asbury, 1968). The evidence provided by these studies is not wholly reliable since, as previously mentioned, certain structures described as infarcts have
since been reinterpreted as Renaut corpuscles. Although no quantitative relationship between fibre and vessel alterations was ratified, the sum of evidence favoured an ischaemic cause.

Following the general acceptance that both diabetic asymmetric and cranial neuropathies have an ischaemic origin (Dreyfus et al., 1956; Raff & Asbury, 1968; Raff et al., 1968; Asbury et al., 1970), certain researchers have resurrected the theory proposed by Woltman and Wilder in 1929 (Woltman & Wilder, 1929) that a similar aetiological mechanism may be responsible for the development of distal diabetic polyneuropathies.

On the basis of a pathologic study of experimental ischaemic neuropathy (Dyck et al., 1984), Dyck and colleagues have formulated criteria for ischaemic nerve damage (Dyck et al., 1985c, 1986a). They stated that the degenerative process should commence at a proximal position in the nerve and be multifocal with maximal degeneration distally. Both large and small fibres are affected, axonal degeneration being the predominant and salient change with secondary segmental demyelination. Axonal sprouting from disease-transected fibres and varying degrees of recovery may occur (Nukada & Dyck, 1984). If these criteria are well corroborated, a statistical correlation between the severity of vessel pathology and fibre pathology is indisputably apparent, and no other pathological process capable of producing the observable fibre damage is concurrent, they suggested that it is not unreasonable to propose an ischaemic basis for a disease of unknown cause.

Dyck and colleagues concluded that the pathology of diabetic distal polyneuropathy fulfilled their previously defined criteria for ischaemic nerve damage, (Dyck et al., 1986a, 1986b) citing capillary damage as the causative agent (Dyck et al., 1985a, 1985b, 1986b). Axonal degeneration and secondary demyelination are cardinal features of both diabetic neuropathy and ischaemia. They stated that the presence of multifocal and proximal fibre loss in mildly affected cases was inconsistent with the theory of a dying-back neuropathy or a disease of Schwann cells, and that the spatial distribution of fibre loss was consistent with the multifocal pattern found in experimental ischaemic injury (Dyck et al., 1986a, 1986b).

Experimental evidence which endorses this theory was provided by the demonstration that in a chronically hypoxic atmosphere, non-diabetic rats develop reversible reductions in NCV and resistance to ischaemic conduction...
failure (Low et al., 1986a), abnormalities which are known to be associated with diabetes mellitus. The fact that the development of retinopathy, nephropathy and polyneuropathy are statistically associated and microangiopathy is known to play a causal role in the production of the former two complications, suggests that it is not inconceivable that microangiopathy may play a similar role in the latter situation (Pirart, 1978).

Johnson and colleagues evaluated the histopathology and myelinated fibre densities of proximally (lumbrosacral trunk), intermediate (posterior tibial nerve) and distally (sural) located nerves obtained at autopsy from a series of 16 diabetic individuals and found a pattern of focal demyelinating lesions (Johnson et al., 1986). They concluded as a result of these findings, the lack of evidence of diffuse demyelination, the size and wedge shape of the lesions and the presence of similar focal lesions in a series of sural nerve biopsies obtained from 16 patients with vasculitis that the lesions were of ischaemic origin, the responsible vessels being epineurial and arteriolar or larger in size. Other studies have revealed similar cases of focal lesions in diabetic distal symmetric neuropathy which have been attributed to ischaemia (Sugimura & Dyck, 1982; Johnson, 1983a), the slow progressive summation of these lesions producing the characteristic pattern of neuropathy seen to occur in this form of diabetic neuropathy.

Additional support for their theory was supplied by a more recent study in which Dyck and colleagues claimed that the number of endothelial nuclei and percentage of "closed" endoneurial capillaries in the nerves of neuropathic diabetic patients is significantly greater than that in non-neuropathic diabetics and age-matched controls (Dyck et al., 1985b). Despite the fact that previously undertaken studies have failed to detect any specific or reproducible correlation between the presence of microvascular disease and diabetic neuropathy (Dolman 1963; Greenbaum et al., 1964; Reske-Nielsen & Lundbaek, 1968), Dyck and colleagues reported a statistically significant positive correlation between these abnormalities and the severity of neuropathy as assessed by an "index of pathology" which combined abnormalities in surviving fibres in addition to myelinated fibre loss (Dyck et al., 1985a, 1985b), reviving the theory that microangiopathy may play a role of primary importance in the pathogenesis of diabetic neuropathy via the production of ischaemia.

The existence or lack of such a correlation does not conclusively validate the theory of cause and effect either way, as the lack of an exact correlation may be
due to sampling problems. Conversely, a close correlation may result from either the presence of neuropathy and microangiopathy as independent manifestations of the diabetic condition or the latter existing as a secondary effect of the former.

To date, researchers have so far failed to establish firmly and conclusively the presence or absence of a causal relationship between microvascular changes and distal symmetrical polyneuropathy in diabetes mellitus. One of the primary aims of this study is to determine conclusively whether microvascular disease of the vasa nervorum in patients with distal symmetrical diabetic polyneuropathy is of any aetiological significance in the development of the neuropathy.

1J.3(iv) Intraluminal Fibrin Deposits
Another vascular theory has been proposed in a study which documented the discovery of deposits of occlusive endoneurial capillary plugs of degranulated platelet intraluminal fibrin and occasional infiltration of the vessel walls in nerve biopsies from diabetic patients (Timperley et al., 1985). Similar plugs have also been observed in the BB Wistar rat (Sima & Thibert, 1982). It was hypothesised that the plugs were a result of alterations in endoneurial pressure produced by abnormal vascular permeability or possible metabolic changes (Timperley et al., 1976; Williams et al., 1980). Excessive deposition of fibrin may result from abnormalities of the clotting system or defects in fibrinolysis, abnormalities of which have been demonstrated in diabetics (Fearnley et al., 1963); however, this hypothesis remains unsubstantiated. Some of the patients included in this study had acute illnesses with disseminated intravascular coagulation, others were cases of chronic neuropathy. Other studies have failed to find evidence of such intravascular thrombi (Johnson et al., 1986).

1L HEREDITARY MOTOR AND SENSORY NEUROPATHY TYPE I
Due to the hereditary nature of this disease, and consequent elimination of any uncertainty regarding a causal agent, HMSN I patients provide a good disease control, with characteristic endoneurial capillary BM thickening, for comparative studies.

Hereditary motor and sensory neuropathy type I (HMSN I) (Thomas et al., 1974) is the commonest variant of Charcot-Marie-Tooth (CMT) disease and is the demyelinating hypertrophic form, the neuronal form being designated HMSN type II. The onset of HMSN I usually occurs during the first or second decades and commences with foot deformity or difficulty in walking. The progression of
the disease is slow, leading to distal lower limb weakness and wasting amongst other symptoms. In its advanced state similar changes may arise in the upper limbs.

In 1983 Nukuda and colleagues performed a morphometric study on sural nerve biopsies from patients with HMSN I. They found axonal transverse area to be reduced in correlation to the length of the myelin spiral, a feature which suggests a primary axonal atrophy with subsequent secondary segmental demyelination and remyelination. The occurrence of more marked effects distally supported this theory, but it now seems unlikely in view of the demonstration that the disorder is related to mutations in the gene for peripheral myelin protein 22 (PMP22). Repeated episodes of segmental demyelination and subsequent remyelination lead to the development of hypertrophic changes with the formation of "onion-bulbs" composed of concentrically arranged Schwann cells processes that surround the remaining nerve fibres (Thomas et al., 1975).

The numbers of Schwann cells is greatly increased and so is that of fibroblasts although to a less prominent degree. Intrafascicular area is increased. The epineurium appears unaffected by the process. The number of myelinated fibres is significantly reduced, those that remain often possess an abnormally thin myelin sheath. Onion bulb formations without axons at their centres may be present.

All the fascicles appear affected to the same degree. The perineurium appears generally unaffected with the exception that the basal laminal zone is thicker than that seen in healthy "normal" nerves. The endoneurial capillaries have a significantly thickened basal membrane in comparison to control normals. The inheritance is usually autosomal dominant. In most patients, HMSN 1a (CMT type 1A) is associated with a 1.5 megabase segmental DNA duplication in region p11.2-p12 of chromosome 17 (Roa et al., 1983; Vance et al., 1989) that includes the gene encoding for the 22-kDa peripheral nerve myelin protein (PMP-22) (Snipes et al., 1993).
Certain abnormalities of the structural components of endoneurial capillaries, including basement membrane thickening (Timperley et al., 1985; Powell et al., 1985; Yasuda & Dyck, 1987; Malik et al., 1989a, 1992) and endothelial cell hyperplasia (Timperley et al., 1985; Dyck et al., 1985b; Powell et al., 1985; Yasuda & Dyck, 1987; Malik et al., 1989a, 1992) are undisputed characteristic features of diabetic polyneuropathy, whilst other reported alterations, such as endothelial cell hypertrophy (Timperley et al., 1985; Powell et al., 1985) and luminal closure (Dyck et al., 1985b, 1986a; Yasuada & Dyck, 1987) remain in dispute.

The potential contribution of these changes, if any, with reference to the causation of diabetic polyneuropathy is unclear. Of primary concern in relation to this thesis is the recent revival of the theory that ischaemia, consequent on endoneurial capillary closure, may be responsible for diabetic polyneuropathy, the number of closed endoneurial capillaries having been correlated with the severity of neuropathy (Dyck et al., 1985b, 1986a; Yasuada & Dyck, 1987).

With the exception of the retention of abnormally persistent and often circular, Schwann cell basal laminal tubes following axonal degeneration (King et al., 1989), perineurial cell basal laminal hyperplasia is the most characteristic pathological feature of diabetic polyneuropathy, providing a useful marker for distinguishing diabetic from non-diabetic peripheral neuropathies (Johnson et al., 1978, 1981; Johnson & Doll, 1984).

The primary aims of the research undertaken in this investigation were (1) to establish the prevalence of purported abnormalities of the vasa nervorum and perineurium within human diabetic peripheral nerves, and (2) to determine whether structural changes are present that would suggest an ischaemic basis for diabetic polyneuropathy either as a result of endoneurial capillary closure or capillary loss with a consequent reduction in the density of vascularisation, both of which could potentially lead to a reduction in endoneurial oxygen tension and hypoxia. An additional aim was to determine, both by qualitative and quantitative assessment, whether any correlation exists between perineurial cell and endoneurial capillary basal laminal thickening, with a view to elucidating whether they share a similar causation.
CHAPTER 2. MATERIALS

2A SUBJECTS - CLINICAL DATA

All 27 diabetic patients included in this study (13 male, 14 female; mean age 39.8 years, range 23-57 years) had clinical and electrophysiological evidence of a distal symmetric predominantly sensory polyneuropathy. None had evidence of focal cranial, thoracoabdominal or limb neuropathy. In 17 patients there was significant autonomic dysfunction, either on clinical assessment (symptoms of postural hypotension, diabetic diarrhoea and gustatory sweating) or on formal testing (a drop of 30mm Hg or more in systolic blood pressure on standing, and heart rate variation on deep breathing of less than 10 beats/min). It is recognised that some of those without autonomic symptoms may have had abnormal functional tests had they been performed. The clinical and biochemical data, at or near the time of nerve biopsy, are given in Table 1. Twenty-five patients had insulin-dependent diabetes and all were receiving highly purified porcine or bovine insulin; two patients had non-insulin-dependent diabetes.

Absent dorsalis pedis pulses were documented in four patients (Cases 14, 18, 21, 23; Table 1); none had symptoms of claudication. Patient 16 had absent distal pulses in both legs and claudication and required a below knee amputation on one side. Five patients (patients 8, 9, 21, 23, 24) had neuropathic foot ulceration and two of these (patients 21, 23) underwent below knee amputation because of persistent infection. One patient (patient 13) had neuropathic arthropathy at the ankle.

Comparative tissues from nine organ donor control cases (5 male, 4 female, mean age 44.7 years; range 28-55 years) were used in this study. None of these individuals were known to have had neuropathy or disorders in which peripheral neuropathy may occur.

Biopsy material was also obtained from nine patients (6 male, 3 female; mean age 32.7 years, range 11-57 years) with hereditary motor and sensory neuropathy Type I (HMSN I). The criteria for diagnosis were those of Harding and Thomas (Harding & Thomas, 1980). In most cases, inheritance was of the autosomal dominant pattern. These patients were included as they provided a disease control group, of an appropriate age range, who had a defined disorder for which a vascular pathogenesis can be discounted.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Duration of diabetes (years)</th>
<th>Autonomic neuropathy</th>
<th>Retinopathy</th>
<th>Proteinuria</th>
<th>Plasma creatine (μmol/l)*</th>
<th>HbA₁ (%)</th>
</tr>
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<td>F</td>
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<td>NR</td>
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</tr>
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</table>

+ = present; 0 = absent
* = patients not included in perineurial basal lamina study.
2B PRIMARY FIXATIVES AND EMBEDDING MEDIA

2B.1 Primary fixatives and buffers

2B.1(i) PIPES Buffer:
100mls of PIPES buffer were made by adding 3.024g of piperazine-$N,N$-bis[2-ethane sulphonic acid] to 50ml of distilled water. When the solid had completely dissolved, 15mls of 1M sodium hydroxide were added and the solution made up to 100ml total volume with the addition of distilled water.
The pH of this solution was 7.6.

2B.1(ii) Phosphate Buffer:
The following ingredients were combined to give a pH 7.4 stock solution;
- Sodium chloride - 0.7g
- Sodium dihydrogen orthophosphate ($\text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O}$) - 0.265g
- Disodium hydrogen orthophosphate ($\text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O}$) - 3.1g
- Distilled Water - 100ml

A stock solution of 10% glutaraldehyde was diluted with this buffer to give a final working concentration of 2.5% glutaraldehyde.

2B.1(iii) Sodium Cacodylate Buffer
The following components were combined;
- Sodium cacodylate - 42.8g
- Distilled water - 100ml
- HCl - 0.9ml

The resulting 0.2M stock solution was pH adjusted if necessary to 7.4.

2B.1(iv) Karnovsky’s Fixative - K1:
The following components were added;
- Paraformaldehyde - 14g
- Distilled water - 70ml
- 1M sodium hydroxide - 21 drops

The mixture was heated to a temperature of between 50-60°C until the paraformaldehyde had dissolved and then cooled to room temperature to prevent precipitation on addition of the following ingredients;
- 10% glutaraldehyde - 170ml
- 0.2M sodium cacodylate buffer - 105ml
- Calcium chloride - 175mg

2B.1(v) Karnovsky’s Fixative - K2:
- K1 - 20ml
- 0.2M sodium cacodylate buffer - 75ml
- 20% dextran (MW 15000-20000) - 5ml
2B.2 **Secondary fixative**

A solution of 1% osmium tetroxide was made in 100ml of distilled water and the mixture subjected to ultrasonic vibrations until the solid had dissolved. From 1981 3g of potassium ferricyanide were added. This solution was added to the appropriate buffer in the ratio 1 : 1, for example:

\[
\frac{0.2M \text{ sodium cacodylate buffer (pH 7.4)}}{\text{Sucrose}} = \frac{100ml}{20g}
\]

Resulting in final working concentrations of 1% osmium tetroxide, 10% sucrose in 0.1M sodium cacodylate buffer.

2B.3 **Embedding media - Araldite**

The ingredients were added in the following order, stirring thoroughly at each step.

- **Araldite CY212 (epoxy resin)** - 10.00ml
- **Dodecyl succinic anhydride (DDSA) [hardener]** - 9.00ml
- **Dibutylphthalate [plasticiser]** - 0.25ml
- **Nadic methyl anhydride (MNA) [accelerator]** - 1.00ml
- **Benzyl dimethylamine (BDMA) [accelerator]** - 0.40ml

2B.4 **Embedding media - Durcupan**

The ingredients were combined in the order shown below, stirring thoroughly after each addition.

- **Durcupan A/M (epoxy resin)** - 10.00ml
- **Durcupan B (hardener)** - 10.00ml
- **MNA** - 0.20ml
- **BDMA** - 0.50ml
- **Dibutylphthalate** - 0.25ml

2C **HISTOLOGICAL STAINS**

2C.1. **Light microscopy - thionin**

The following components were added and stirred over a gentle heat until the solid had completely dissolved. The resulting purple/pink solution was then filtered and stored in a refrigerator at 4°C between use.

- **Thionin** - 2.0g
- **Distilled water** - 50.0ml
- **0.1N sodium hydroxide** - 12.5ml
- **90% ethanol** - 62.5ml
2C.2 Light microscopy - acridine orange

The components listed below were combined and stirred until the solid had completely dissolved. The solution was filtered and stored in a refrigerator at 4°C between use.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>Acridine orange</td>
<td>1.3g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100.0ml</td>
</tr>
<tr>
<td>0.1N sodium hydroxide</td>
<td>25.0ml</td>
</tr>
</tbody>
</table>

2C.3 Electron microscopy - methanvl uranyl acetate

The stain was prepared in a 10ml glass bottle. Extreme care was taken at all times to ensure minimal contamination by dust and other solid particles. The bottle was cleaned by filling with methanol and putting it in an ultrasonic cleaning bath several times before the stain was prepared. 1.25g of uranyl acetate was weighed out on lint-free paper and put in the bottle. 10ml of 100% methanol was added and the solution put back into the ultrasonic bath until the solid had dissolved to produce a transparent yellow solution. To further reduce the chance of particulate contamination of the sections at EM level, the stain was spun in a centrifuge at 3000rpm for a period of 5 minutes each time it was used. The stain was kept in the dark at 4°C.

2C.4 Electron microscopy - lead citrate

The same precautions were taken in the preparation of this solution as for that of the uranyl acetate. The bottle was cleaned with distilled water in an ultrasonic bath. The ingredients are as listed below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Lead citrate</td>
<td>25.0mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10.0ml</td>
</tr>
<tr>
<td>10N sodium hydroxide</td>
<td>0.1ml</td>
</tr>
</tbody>
</table>

The sodium hydroxide was placed in the bottle, followed by a small volume of the distilled water. The lead citrate was then added. This procedure helps the lead citrate to dissolve. The remainder of the distilled water was then added and the solution put back in the ultrasonic bath until no solid particles were apparent. Before use the stain was spun in a centrifuge at 3000rpm for 5 minutes.
CHAPTER 3. METHODS

3A BIOPSIES

Fascicular biopsies of the sural nerve were taken from a site immediately posterior to the lateral malleolus in 23 of the diabetic patients, with informed consent and the approval of the Ethics Committee at King's College Hospital. Sural nerves were removed immediately following amputation in three patients (16, 21 & 23). Radial nerve biopsy was performed on patient 18.

Sural nerve fascicular biopsies from the retromalleolar site were similarly obtained from the 9 HMSN I patients.

The fascicular biopsies were performed under local anaesthetic with 1% lignocaine (Antigen Ltd, Ireland). Following removal the tissue was immediately placed on a sterile swab soaked in saline. Before fixation it was attached to a small piece of card by means of its own adhesive properties, to keep it straight. The time period between removal of the specimen and immersion in the first fixing solution was never more than 4 minutes.

Total sural nerve biopsies were taken from the same site and processed in the same way from the nine organ donor cases. This material was taken with informed consent from the next of kin and the approval of the Ethics Committee at the Royal Free Hospital at the time of organ donation. In all except one case the tissue was removed during life before ventilatory arrest. In the remaining instance, the sural nerve was removed within 45 minutes of circulatory occlusion. It was considered justifiable to include results from this subject as they were well within the normal range obtained from the other cases in the group.
3B TISSUE PROCESSING

3B.1 Primary fixation

Nerve specimens biopsied after October 1977 were protein fixed by immersion in a solution of 3% distilled glutaraldehyde in piperazine-\(N,N\)-bis[2-ethanesulphonic acid] (PIPES) buffer (Baur & Stacey, 1977) containing 2% sucrose (see section 2.2.1.) for a period of 3 hours at 4°C. Tissue from biopsies performed before October 1977 were protein fixed in one of the following:

a) 2.5% distilled glutaraldehyde in 7% sucrose phosphate buffer at 4°C for 3 hours.

b) 2.5% distilled glutaraldehyde in 10% sucrose 0.15M sodium cacodylate buffer at 4°C for 3 hours (section 2.B.1).

c) \(K_2\) at 4°C for 3 hours (section 2.B.1).

In each case the tissue was removed from the fixative after a minimum period of 1 hour, carefully detached from the card and cut into sections of equal length varying between 3mm to 4mm, depending on the length of the biopsy specimen, and then replaced in the fix. This procedure was performed to enhance penetration of the fixative by reducing the percentage of perineurial surface area, a natural barrier preventing free diffusion of substances between the endoneurium and epineurium, per total surface area of each length of tissue. If fresh nerve is cut into these lengths, it is difficult to avoid traumatic damage as fresh myelin is very fragile. The tissue was then washed for a minimum of half an hour, before secondary fixation, in a solution of the appropriate buffer, the optimum time being 1 hour.

3B.2 Secondary fixation

Specimens from biopsies performed after October 1977 were postfixed in a solution of 1% osmium tetroxide in PIPES buffer with 2% sucrose at a temperature of 4°C for a period of 3 hours (Langford & Coggeshall, 1980). From 1981 this recipe was amended to include the addition on 1.5% potassium ferricyanide. This component helps to preserve the cell membranes and enhances staining of sections for examination under the electron microscope. Tissues processed before October 1977 were postfixed in either:

a) 1% osmium tetroxide in 7% sucrose phosphate buffer at a temperature of 4°C for 3 hours following primary fixation in solution a), or

b) 1% osmium tetroxide in 10% sucrose sodium cacodylate buffer (see section 2.B.2) at a temperature of 4°C for 3 hours following primary fixation in either solution b) or solution c).

All fixative solutions had a comparable osmolarity and pH.
3B.3  **Dehydration**

Following an initial rinse in 15% alcohol, the tissues were dehydrated through a series of graded alcohols at room temperature (RT) for the time periods stated below.

- 15% ethanol - 2 x 5 minutes
- 30% ethanol - 2 x 10 minutes
- 50% ethanol - 2 x 15 minutes
- 70% ethanol - 2 x 30 minutes
- dehydrated absolute ethanol - 3 x 20 mins. + 2 x 60 mins.

The advantage of using dehydrated absolute ethanol (obtained with the addition of Linde sieves) over merely absolute ethanol is that it ensures removal of the maximum amount of water from the tissue and consequently enhances the penetration of the water immiscible embedding resin. Myelin is very susceptible to incomplete dehydration, any remaining water trapped between the layers of the myelin sheath may result in disruption and splitting of the sheath during resin embedding and the subsequent production of unwanted artifacts.

3B.4  **Embedding**

Following dehydration, the nerve specimens were embedded in resin. Two different embedding media were used, Araldite (see section 2.B.3) before January 1986 and Durcupan (see section 3.B.4) after that date. The same ratios of each resin were used in conjunction with 1-2 epoxypropane (propylene oxide), the intermediary between ethanol and the resins, and the tissues soaked in these solutions for the time periods shown below.

- Propylene oxide - 2 x 15 minutes at RT
- Propylene oxide : resin - 1:1 - 60 minutes at RT
- Propylene oxide : resin - 1:3 - Overnight at RT
- Complete resin - Overnight at 4°C

The nerve specimens were then embedded in freshly made resin, in a plastic mould and aligned for transverse sectioning. The resin blocks were polymerised overnight in an oven at a temperature of approximately 64°C, evacuated to 260 mmHg. Using a vacuum oven aids penetration of the nerve specimen by the viscous resin.
3C HISTOLOGICAL TECHNIQUES

3C.1 Sectioning and staining for the light microscope (LM)
Semi-thin, 0.5 μm, sections (green-mauve) were cut using freshly made glass knives on either a Sorval Porter-blum Ultramicrotome MT-2, a Reichert-Jung Ultracut or Reichert-Jung Ultracut E. The sections were floated off on a bath of 10% acetone and transferred to a drop of the same solution on a clean glass slide. This drop was then gently evaporated off until the sections were completely dry and attached to the slide. They were stained with thionin (Sievers 1971), (see section 2.C.1) by flooding the slide with the solution which was then heated gently. The slide was then washed in distilled water, blotted dry on fibre free paper and warmed to remove all the water.

Counterstaining was performed by covering the sections in a drop of acridine orange (Sievers, 1971), (see section 2.C.2). This solution was gently warmed by holding the slide over a naked flame for a period of approximately 8 seconds. Distilled water was then used to wash the sections and the slide again blotted dry and gently warmed to ensure removal of any remaining water. The slides were then covered with a cover slip using a small amount of fresh resin which was polymerised in an oven at a temperature of 37°C overnight. The sections were then ready for examination using either a Leitz Ultraphot or a Zeiss Axiophot light microscope.

3C.2 Sectioning for the electron microscope (EM)
HR25 copper/rhodium grids were cleaned in an ultrasonic bath in a dilute solution of Lypsol followed by a brief period in a series of graded alcohols (15%, 30%, 50%, 70% & dehydrated absolute ethanol). They were then blotted dry on fibre free paper and stored in a dust-free environment until needed.

Sections of a thickness varying between 60nm to 90nm (silver) were cut using either a fresh glass knife or a diamond knife on either a Sorval Porter-Blum Ultramicrotome MT-2, a Reichert-Jung Ultracut or Reichert-Jung Ultracut E and floated off on a bath of 10% acetone or distilled water. The sections were expanded with xylol vapour (xylene) and picked up on the rhodium side of the grids. The grids were placed on fibre-free paper to dry and left in a grid box to dry completely overnight.
3C.3 Uranyl acetate and lead citrate staining techniques

Sections cut and mounted on grids for examination at EM level were stained by immersing the grids in a drop of fresh uranyl acetate solution (see section 2.3.3) for a period of 8 minutes. The grids were washed by dipping them in and out of the solutions detailed below 50 times, breaking the meniscus of the solution on each count.

i) 100% methanol
ii) 70% methanol
iii) 50% methanol
iv) Distilled water

The grids were then blotted dry on lint-free paper and covered with a drop of fresh lead citrate solution (see section 2.C.4) for a period of 8 minutes. They were washed by the method stated above for the same number of counts but in different solutions as stated below,

i) 0.002M sodium hydroxide solution
ii) Distilled water
iii) Distilled water
iv) Distilled water

The grids were then blotted dry and left overnight before examination using a JEOL 100CX electron microscope.

3D PHOTOGRAPHIC TECHNIQUES

3D.1 Fascicular photographs

Semi-thin sections, stained with thionin and acridine orange, were examined under a light microscope. Exposures were taken of 37 fascicles from the diabetic group, 15 fascicles from the HMSN I group and 10 fascicles from the control group on black and white 35mm Agfa-ortho 25 Professional film, using either the x 6.3, x10 or x25 lens on the Leitz Ultraphot or the x10, x20 or x40 lens on the Zeiss Axiophot.

The film was developed in Agfa-Gevaert Rodinal film developer (diluted 1:10, Rodinal:water) under red light, at a temperature of 20°C for a period of 5 minutes, with continuous agitation for the first minute and 20 seconds agitation in each subsequent minute. Development was halted by replacing the Rodinal with a solution of 3% acetic acid for 1 minute. The film was fixed in Agfa Structurix G328 (diluted 1 part fix + 4 parts water) for 5 minutes and finally washed in running water for 30 minutes. The film was left to dry in a drying cabinet.
When the film was dry, 20.3cm x 25.4cm photographs were printed on Agfa-Gevaert Rapitone P 1-3 paper using a De Vere 54 Varicon Enlarger. The photographic paper was developed by immersion in Agfa-Gevaert Rapidoprint G182b activator and fixed by two subsequent immersions in Agfa-Gevaert G386b rapid-fixer. The prints were washed in running water for half an hour before being dried. These processes were performed under yellow light.

The photographs were labelled with the patients' name and identification number and each fascicle given a number for reference. The magnification was calculated and all endoneurial microvessels classifiable as capillaries on the criteria of Bell and Weddell (Bell & Weddell, 1984b) were identified and numbered, their centres being denoted by the application of a black pen mark. Blood vessels situated within the perineurium were excluded.

3D.2 Electron micrographs of blood vessels

Thin sections stained with uranyl acetate and lead citrate were examined in the electron microscope. Individual fascicles were identified and their blood vessels located with the aid of the corresponding fascicular photograph. Micrographs (Agfa-Gevaert Scientia film, 6.5cm x 9cm) were taken of each numbered capillary, totalling 369 vessels from the diabetic group, 166 vessels from the HMSN I group and 97 vessels from the organ donor control group. The magnification ranged from x 1600 to x 6000 depending on the size of the vessel.

The film was developed under red light in a 1 + 15 dilution of Studinol and water at a temperature of 20°C for a period of 2 minutes. The process was then halted by immersing the film in a solution of 3% acetic acid for half a minute and the film fixed for 5 minutes in a solution of 1 + 4 Structurix and water plus 25mls of Aditan per litre of diluted fix. It was finally washed in running water for half an hour.

After being left overnight in a drying cabinet, 24cm x 30.5cm prints were made of each exposure on Agfa-Gevaert rapitone P 2-3 paper using a De Vere 54 Varicon Enlarger. The prints were developed as stated in section 3.D.1. The micrographs were labelled with the patients' identification number, fascicle number and vessel number. Different coloured indelible pens were used to demarcate the perimeters of the basal laminal area, the endothelial cells and the lumen as shown in figure 1.
FIGURE 1  Diagram of a transverse section through an endoneurial capillary to illustrate the measurements obtained.  N = endothelial cell nuclei; P = pericyte process.  The small black arrow indicates the perimeter of the lumen, the large black arrow the perimeter of the endothelial cell layer and the white arrow the perimeter of the basal laminal zone.

- Basal laminal area
- Endothelial cell area
- Area of lumen
3D.3  **Electron micrographs of the perineurium**

Thin sections stained with uranyl acetate and lead citrate were examined in the electron microscope. Fifteen sites were chosen at random around the perimeter of each fascicle (fig. 2) and the full thickness of the perineurium recorded on electron micrographs at a magnification of × 8300. The schematic representation of these sites in figure 2 is not intended to imply that they were evenly spaced around the fascicle. The method of film development and subsequent printing on 24cm x 30.5cm Agfa-Gevaert Rapitone P 2-3 paper was the same as for that of the blood vessel micrographs in section 3.D.2.

Invariably more than one micrograph was needed to span the entire width of the perineurium at each site, a montage being constructed from the resulting prints which showed all the layers of the perineurium from those most proximal to the endoneurium to those most distal. The final magnification of these prints was calculated and the montages labelled sections 1 to 15 for each individual fascicle.

Eighteen pencil lines, 1cm apart, were drawn across these montages, in a direction perpendicular to the layers of perineurial cells and crossing the entire width of the perineurium. These lines were divided into two sets of 9 and the lines numbered consecutively 1-9 within each set. A table of random numbers was then used to select 10 numbers and the correspondingly numbered pencil lines on the montages were overdrawn in ink (fig. 3). The schematic representation of these lines in the appropriate figure does not therefore imply that these lines were equidistantly spaced. Measurements of perineurial cell basal laminal thicknesses were made along the 10 ink lines as described in part 3.E.3.

Perineurial cell basal laminal width was investigated in 34 fascicles from 24 of the diabetic patients (11 male, 13 female, mean age 39.2 years), 10 fascicles from 7 patients with HMSN I (5 male, 2 female, mean age 32.1 years) and 16 fascicles from the 9 organ donor control cases (5 male, 4 female, mean age 44.7 years). A larger number of fascicles were included in the morphological investigation of endoneurial capillaries than in that of perineurial cell basal laminal width as those fascicles that were either so small as to preclude clear demarcation of 15 separate sections across the perineurium or had a disrupted or damaged perineurium were excluded in the latter investigation.
FIGURE 2 Diagram of a transverse section through a fascicle to illustrate the measurements obtained. Measurements of perineurial cell basal lamina thickness were acquired from electron micrograph montages of 15 randomly chosen sites across the perineurium, depicted above by numbered boxes.
FIGURE 3 Diagram of a transverse section through the perineurium to illustrate the measurements obtained. En = endoneurium; Ep = epineurium; N = perineurial cell nuclei; P = perineurial cell. The black arrows indicate basal laminal zones. Numbering of perineurial cell layers down the right side of the diagram corresponds with data in Table 4. Numbering along the top of the diagram refers to the 10 randomly spaced lines as described in section 3.D.3.
3E MORPHOLOGICAL TECHNIQUES

All morphological analysis was performed using a Kontron MOP Videoplan Interactive Image Analysis System with a computer assisted digitising tablet.

3E.1 **Analysis of blood vessel distances**

The light microscope photographs of the fascicles were placed on the digitising tablet and direct measurements made of fascicular endoneurial area, defined as that area demarcated by the innermost perineurial layer. The number of capillaries within the endoneurium was counted and fascicular area per capillary and capillary density derived from these two pieces of information.

Intercapillary distance between the centre of each vessel and that of its nearest neighbour was directly measured as was the shortest distance between the centre of each capillary and the innermost layer of perineurium. These results were statistically analysed by means of a Mann-Whitney U-test. Percentage frequency histograms were drawn for each parameter for the three groups, diabetic, HMSN I and control, and medians and inter-quartile ranges deduced.

3E.2 **Analysis of blood vessel structure**

Direct measurements were made of the perimeter of the basal laminal zone (PER BL), the perimeter of the endothelial cells (PER CL), the perimeter of the lumen (PER LU), the number of nuclei (NUCLEI) and the number of pericytes (PERICYT) from electron micrographs of each blood vessel using the digitising tablet and pen (fig. 1).

From these measurements the computer was able to derive the area of the basal laminal zone (AREA BL) defined as that area between PER BL and PER CL; the area of the endothelial cells (AREA CL) defined as that area between PER CL and PER LU and the area of the lumen as that area within the line designated PER LU. Percentage frequency histograms were drawn for each parameter, and medians and inter-quartile ranges calculated. A Mann-Whitney U-test was performed to illustrate differences in these measurements between the three groups under investigation.
3E.3 Analysis of perineurial cell basal laminae

Ten perineurial cell layers were numbered on each montage of electron micrographs. The layers adjacent to the epineurium were designated numbers 1, 2 and 3, number 1 being the outermost layer. The four most centrally situated layers of the perineurium were numbered 4, 5, 6 and 7 and the innermost endoneurial layers were numbered 8, 9 and 10, with 10 being the innermost layer. Where there were less than 10 layers of perineurial cells, for instance 8, the peripheral outer and inner layers were labelled 1, 2, 3 and 8, 9, 10 respectively and the two remaining central layers designated 5 and 6.

Direct computer measurements were made, using the digitising tablet and pen, of the thickness of the basal lamina on both the epineurial and endoneurial aspects of each numbered perineurial cell layer at the 10 points where the cells were crossed by the previously described ink lines (section 3.4.3) crossed the cells. This process was repeated along each of the 10 marked lines and performed on all 15 montages per fascicle resulting in the production of 150 measurements of basal lamina thickness at each of the 20 designated points.

Statistical tests were performed on the resulting measurements of basal laminal thickness for both the epineurial and endoneurial aspects of each of the 10 perineurial cell layers. Medians were calculated and frequency distribution histograms plotted to illustrate the results. Statistical analyses were performed on the data from each of the three groups in order to determine whether any significant variation exists between the inner and outer basal lamina thicknesses of each numbered perineurial cell, and whether basal laminal thickness appears to be dependent upon or related to the position of the cell within the perineurium.

Statistical comparisons of basal lamina thickness were also made between all the endoneurial aspects of the 10 cellular layers and all the epineurial aspects, in order to determine whether one side of the perineurial cells has a predominantly thicker basement membrane than the other, regardless of the cells' position within the perineurium.

Intragroup statistical analysis was performed on equivalent measurements of endoneurial and epineurial basal laminal thickness for each layer in order to determine whether any significant difference existed between the 3 experimental groups.
Fascicular area was directly measured for each of the 34 diabetic, 16 HMSN I and 10 control fascicles. The total number of perineurial cell layers present in each of the 15 montages per fascicle was counted and the mean and median number of layers calculated for statistical analysis.

The number of degenerative or necrotic perineurial cells was also directly measured. Degenerative cells were defined as those showing evidence of generalised atrophy with disruption and disintegration of the cell membrane often accompanied by the production of an abnormally wide gap between the cell membrane and the basal lamina. Necrotic cells were identified by the presence of cellular debris between adjacent paired basal laminae in the absence of an intervening perineurial cell. The number of such perineurial cells was counted and expressed as a percentage of the total number of perineurial cells recorded on the 15 montages per fascicle. Mean and median values were calculated and the results statistically evaluated.
CHAPTER 4. RESULTS

4A QUANTITATIVE OBSERVATIONS

4A.1 Endoneurial capillaries

The results for the three groups of subjects studied are given in Tables 2 and 3. As most measured variables were not distributed normally, all statistical analyses have been performed by means of a U-test, unless otherwise stated. This test compares the median and interquartile range of the two groups of measurements without the prerequisite of Gaussian distribution.

4A.1(i) Basal laminal zones

The median value for the area of the basal lamina zone in the diabetic subjects was 172.7μm². This was slightly greater than the value for the HMSN I cases, at 152.8μm², although not significantly so. The median basal laminal areas for both the diabetic and HMSN I cases were significantly greater (p < 0.01) than the value for the control group, 67.6μm². Frequency distribution histograms illustrate the difference in distribution between the three groups (fig. 4). This pattern is repeated in the basal laminal thickness measurements. Whilst the median thickness in the diabetics was 4.65μm, marginally greater than that of 4.08μm recorded for the HMSN I cases, the difference was not statistically significant. However, both of these thicknesses were significantly greater (p < 0.01) than that of the control group at 2.28μm. A frequency distribution histogram serves to substantiate these observations (fig. 5).

4A.1(ii) Endothelial cell area

The median area occupied by the endothelial cells in the HMSN I subjects was 43.5μm² and this was significantly greater (p < 0.01) than the values obtained for the other two groups. The diabetic median area value of 31.7μm² was also significantly greater (P < 0.01) than that of 24.8μm², the figure calculated for the donor control subjects. These trends are reflected by a relative frequency histogram (fig. 6).
<table>
<thead>
<tr>
<th></th>
<th>Diabetic patients</th>
<th>Control subjects</th>
<th>HMSN I patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>27</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>No. of fascicles</td>
<td>72</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td>No. of blood vessels</td>
<td>368</td>
<td>166</td>
<td>97</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>39.8</td>
<td>44.7</td>
<td>32.7</td>
</tr>
<tr>
<td>range (years)</td>
<td>23-57</td>
<td>28-55</td>
<td>11-57</td>
</tr>
<tr>
<td>Basal laminal area (μm²)</td>
<td></td>
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<tr>
<td>median</td>
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<td>67.6</td>
<td>152.8</td>
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<tr>
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<tr>
<td>Basal laminal thickness (μm)</td>
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<tr>
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<td>Endothelial cell area (μm²)</td>
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<tr>
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<td>Luminal area (μm²)</td>
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<td>3.1-12.7</td>
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<td>No. of pericyte processes</td>
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<td>3</td>
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<tr>
<td>interquartile range</td>
<td>2-5</td>
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Table 2  Morphometric analysis of endoneurial capillaries in the peripheral nerves of diabetic, organ donor control and hereditary motor and sensory neuropathy Type I cases.
### Table 3
Morphometric analysis of endoneurial capillaries in the peripheral nerves of diabetic, organ donor control and hereditary motor and sensory neuropathy Type I cases.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic patients</th>
<th>Control subjects</th>
<th>HMSN I patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>27</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>No. of fascicles</td>
<td>72</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td>No. of blood vessels</td>
<td>368</td>
<td>166</td>
<td>97</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>39.8</td>
<td>44.7</td>
<td>32.7</td>
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<tr>
<td>range (years)</td>
<td>23-57</td>
<td>28-55</td>
<td>11-57</td>
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<td>Fascicular area (μm²)</td>
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<td>66,490</td>
<td>112,100</td>
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<td>30,680-102,200</td>
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<tr>
<td>interquartile range</td>
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<tr>
<td>No. of capillaries per fascicle</td>
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<td></td>
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</tr>
<tr>
<td>median</td>
<td>4.5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>interquartile range</td>
<td>3-8</td>
<td>2-7</td>
<td>3-8</td>
</tr>
<tr>
<td>Endoneurial area per capillary (μm²)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>14,850</td>
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<td>25,630</td>
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<tr>
<td>interquartile range</td>
<td>11,460-22,230</td>
<td>11,230-20,460</td>
<td>19,010-28,820</td>
</tr>
<tr>
<td>Capillary density (No. mm⁻²)</td>
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<tr>
<td>median</td>
<td>65.50</td>
<td>66.51</td>
<td>39.10</td>
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<tr>
<td>mean</td>
<td>69.88</td>
<td>69.24</td>
<td>44.89</td>
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<td>Nearest-neighbour distance (μm)</td>
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<td>median</td>
<td>86.1</td>
<td>82.8</td>
<td>101.1</td>
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<tr>
<td>interquartile range</td>
<td>44.2-125.9</td>
<td>28.1-119.1</td>
<td>40.1-148.4</td>
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<td>Shortest distance of capillary to perineurium (μm)</td>
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<tr>
<td>median</td>
<td>20.3</td>
<td>40.1</td>
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<tr>
<td>interquartile range</td>
<td>10.2-76.6</td>
<td>7.5-78.7</td>
<td>10.1-111.9</td>
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</table>
FIGURE 4  Frequency distribution histograms of capillary endothelial cell basal laminal area in the nerves of (a) control; (b) diabetic and (c) HMSN I cases.
FIGURE 5 Frequency distribution histograms illustrating capillary endothelial cell basal laminal thickness in diabetic, donor control and HMSN I specimens.
FIGURE 6 Frequency distribution histograms of endoneurial capillary endothelial cell area in (a) control; (b) diabetic and (c) HMSN I nerves.
4A.1(iii) **Endothelial cell nuclei number**

Two of the groups, the diabetic and the HMSN I patients, were recorded as having the same median value of 2 with regard to the number of endothelial cell nuclei seen in transverse section. This figure was significantly greater (p < 0.01) than that observed in organ donor control cases and is illustrated by frequency distribution histograms (fig. 7). If these parameters are calculated per unit length of endothelial cell perimeter, the median values for the HMSN I and diabetic patients are again significantly greater (p < 0.05) than that for the control group. When this method of evaluation was adopted the value for diabetic nerves proved significantly greater than that for the HMSN I nerves (p < 0.05).

4A.1(iv) **Luminal area**

The median luminal area in the diabetic patients was 5.3\( \mu \text{m}^2 \). This was marginally greater than the value for the control patients, which was 4.9\( \mu \text{m}^2 \) and slightly less than the value of 6.2\( \mu \text{m}^2 \) calculated for the HMSN I cases. However, none of these slight differences proved to be statistically significant as illustrated by a frequency distribution histogram (fig. 8). The lower end of the range was expanded (fig. 9) to reveal that organ donor control subjects were the group with the highest proportion of capillaries with the smallest luminal area.

4A.1(v) **Number of pericyte processes**

There was no difference in the median number of pericyte processes seen in transverse section with reference to the diabetics and organ donor controls. However, the former did possess a significantly higher inter-quartile range (p < 0.01) as illustrated in figure 10. The HMSN I patients had a significantly lower median value of 2 compared with that of 3 for the other two groups. When corrected for length of endothelial cell perimeter, the figures yielded the same statistical results.

4A.1(vi) **Fascicular area**

The median value for fascicular area was 77,110\( \mu \text{m}^2 \) in the diabetic patients, which was marginally higher than 66,490\( \mu \text{m}^2 \), the organ donor control median, although not significantly so. The figure of 112,100\( \mu \text{m}^2 \) for the HMSN I patients was greater than that for the other two groups, although not significantly so, this fact being attributable to hypertrophic changes associated with this condition (fig. 11).
FIGURE 7 Frequency distribution histograms of endothelial cell nuclei number in endoneurial capillaries in (a) control; (b) diabetic and (c) HMSN I nerves.
FIGURE 8 Frequency distribution histograms of endoneurial capillary lumen area in (a) control; (b) diabetic and (c) HMSN I nerves.
FIGURE 9 Frequency distribution histograms of endoneurial capillary lumen area in (a) control; (b) diabetic and (c) HMSN I nerves.
FIGURE 10 Frequency distribution histograms of the number of pericyte processes associated with endoneurial capillaries in (a) control; (b) diabetic and (c) HMSN I nerves.
FIGURE 11 Frequency distribution histograms of fascicular area in (a) control; (b) diabetic and (c) HMSN I nerves.
There was no significant difference in the median number of blood vessels per fascicle between the three groups. The median endoneurial area per capillary in the HMSN I nerves was 25,630\(\mu\)m\(^2\) which was significantly greater (p < 0.01) than the values for the control and diabetic patients at 15,270\(\mu\)m\(^2\) and 14,850\(\mu\)m\(^2\) respectively.

Welch's t-test, which does not assume equal variance or populations with equal standard deviations, was used to compare capillary density. The mean value of 44.89\(\mu\)m\(^3\) in the HMSN I cases was found to be significantly smaller than that of 69.88\(\mu\)m\(^3\) (p = 0.0003) in the control cases and 69.24\(\mu\)m\(^3\) (p = 0.0001) in the diabetic patients. There was no significant difference between the organ donor control and diabetic subjects.

These results were confirmed by a Mann-Whitney U-test, the median value for capillary density in the HMSN I cases, at 39.10\(\mu\)m\(^3\), proving significantly smaller than both the median values of 66.51\(\mu\)m\(^3\) (p = 0.0038) for the diabetic group and 65.50\(\mu\)m\(^3\) for the control group (p = 0.0027). No significant difference was found between the median values for the control and diabetic groups. These results are illustrated by figure 12.

The intercapillary nearest-neighbour distances were of an equivalent level in the diabetic and control patients. In the nerves of HMSN I patients the median distance, at 101.1\(\mu\)m, was significantly increased (p < 0.01) compared with the figures recorded for diabetic and control patients, 86.1\(\mu\)m and 82.8\(\mu\)m respectively. These results reflect the hypertrophic nature of the pathology of HMSN I (fig. 13). The shortest distance between capillary and perineurium was not significantly different between the three groups, however, it was apparent that the majority of fascicular blood vessels are located in the subperineurial zone of the endoneurium (fig. 14).
FIGURE 12 Peripheral nerve fascicular capillary density in diabetic neuropathy, organ donor control and HMSN I cases.
FIGURE 13 Frequency distribution histograms of nearest-neighbour intercapillary distance in (a) control; (b) diabetic and (c) HMSN I nerves.
FIGURE 14 Frequency distribution histograms of the shortest distance between endoneurial capillary and perineurium in (a) control; (b) diabetic and (c) HMSN I nerves.
4A.2 Perineurial cells - intragroup analysis

The results for the 3 case groups studied are given in Tables 4 and 5.

4A.2(i) Endoneurial and epineurial aspects of the perineurial cells

As basal lamina thickness observed in the relative frequency histograms was not normally distributed, statistical analysis was performed on this variable by means of a Kolmogorov-Smirnoff test.

Significant differences (p<0.01) were observed between the distribution of endoneurial and epineurial basal lamina thicknesses in perineurial cell layers 4, 7, 9 and 10 in the organ donor control group and in layers 7, 8 and 10 in the diabetic patients, in both cases cells from the central and outer zones of the perineurium. In the HMSN I cases (p<0.01) a similar difference was found only in perineurial cell layer number 10, the outermost layer. However, when the measurements of basal lamina thickness on the epineurial aspects of all 10 perineurial cell layers were combined and statistically compared with that of the combined endoneurial aspects, no significant difference was recorded in any of the three experimental groups.

To determine whether perineurial cell basal lamina thickness is related to the cells' location within the perineurium, statistical comparisons were made between the innermost, central and outermost layers for each group. This was performed by combining the endoneurial and epineurial basal lamina thicknesses for each perineurial zone; outer, central and inner, cellular layers included in this analysis being 1 & 2, 5 & 6 and 9 & 10 respectively.

In all three groups, diabetic, organ donor control and HMSN I, no significant differences were found in basal laminal thickness between any of the three zones with the exception of the centrally located cells in the diabetic patients which had significantly thicker basement membranes (p<0.01) than those of the innermost layers. However, when median values of epineurial and endoneurial aspects of basal lamina thickness are plotted against the position of the cell within the perineurium (fig. 15) a general pattern becomes apparent, the basement membrane of the central cells appearing thickest and those of the innermost zone the thinnest. This trend is least obvious in the control group and most pronounced in the diabetic group.
<table>
<thead>
<tr>
<th></th>
<th>Diabetic</th>
<th>Control</th>
<th>HMSN I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases (no. of fascicles)</td>
<td>24 (34)</td>
<td>9 (16)</td>
<td>7 (10)</td>
</tr>
<tr>
<td>Number of male (M) and female (F) patients</td>
<td>11 M, 13 F</td>
<td>5 M, 4 F</td>
<td>5 M, 2 F</td>
</tr>
<tr>
<td>Number of perineurial cell layers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>8.50</td>
<td>8.69</td>
<td>10.30</td>
</tr>
<tr>
<td>median</td>
<td>8.00</td>
<td>8.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Percentage number of degenerate or necrotic cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>19.47</td>
<td>1.11</td>
<td>4.15</td>
</tr>
<tr>
<td>median</td>
<td>17.89</td>
<td>1.15</td>
<td>3.14</td>
</tr>
<tr>
<td>Fascicular area ($\mu m^2$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>112,340</td>
<td>97,658</td>
<td>135,281</td>
</tr>
<tr>
<td>median</td>
<td>84,774</td>
<td>88,148</td>
<td>109,207</td>
</tr>
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</table>

**TABLE 4** Morphometric analysis of peripheral nerve perineurial cells from organ donor control cases and diabetic and hereditary and motor sensory neuropathy Type I patients.
<table>
<thead>
<tr>
<th>Perineurial cell layer number</th>
<th>Epineurial (Ep) or endoneurial (En) aspect</th>
<th>Median thickness of perineurial cell basal lamina (μm)</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>1</td>
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<tr>
<td></td>
<td>En</td>
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<tr>
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<td>Ep</td>
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<td></td>
<td>En</td>
<td>0.143</td>
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<tr>
<td>4</td>
<td>Ep</td>
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<td>En</td>
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<td>5</td>
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<tr>
<td>6</td>
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<tr>
<td></td>
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<td></td>
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</table>

**TABLE 5** Morphometric analysis of perineurial cell basal laminae in peripheral nerve in diabetic neuropathy, hereditary motor and sensory neuropathy Type I and organ donor control subjects.
FIGURE 15 Plot of median basal lamina thickness in relation to the epineurial and endoneurial aspects of each perineurial cell studied and its position within the perineurium in diabetic, organ donor control and HMSN I cases.
4A.3 Perineurial cells - intergroup analysis

4A.3(i) Diabetic patients and organ donor controls

The diabetic patients had significantly greater (p<0.01) values for basal lamina thickness than the organ donor control group with regard to both endoneurial and epineurial aspects of all ten perineurial cell layers (fig. 16-20).

4A.3(ii) Diabetic patients and HMSN I patients

The diabetic patients had significantly thicker (p<0.01) basal laminae than the HMSN I patients with regard to the majority of endoneurial and epineurial aspects of the perineurial cells (figs. 16-20). The exceptions were the epineurial aspects of cell layers 7 and 10 (figs. 19 and 20) and the endoneurial aspects of cell layers 4 and 7 where no significant difference was found (figs. 17 and 20).

4A.3(iii) HMSN I patients and organ donor controls

No significant difference was recorded between the HMSN I and organ donor control groups with reference to basal lamina thickness on the epineurial aspects of perineurial cell layers 2, 3, 6 and 8 and the endoneurial aspects of layers 2, 3, 5, 6 and 10 (figs. 16-19). The organ donor control cases had significantly thicker (p<0.01) basal laminae than the HMSN I patients on the epineurial side of perineurial cell 1 (fig. 16). The HMSN I group had significantly greater (p<0.01) values for basal lamina thickness than the control group on the epineurial aspects of cell layers 4, 5, 7, 9 and 10 and on the endoneurial aspects of perineurial cells 4, 7, 8 and 9 (figs. 17-20).

When the median values of perineurial cell basal lamina thickness are plotted in relation to the position of the cells within the perineurium, the diabetic patients are revealed as having greater values than the control and HMSN I cases, the difference being most noticeable in the central perineurial region (fig. 15).

4A.3(iv) Correlation of perineurial cell basal lamina thickness and age

The median values for perineurial cell basal lamina thickness were calculated for each individual patient and the results were plotted against age (fig. 21). The correlation coefficient (r) was 0.4875 in the diabetic patients which proved significant (p=0.0035). The correlation coefficient for the HMSN I cases and was 0.7634 which also proved significant at p=0.0102. There was no correlation (r=-0.0662) between the two parameters in the organ donor controls.
FIGURE 16 Relative frequency histograms of basal lamina thickness on (a) the epineurial and (b) endoneurial aspects of perineurial cell layer 1; and (c) the epineurial and (d) endoneurial aspects of perineurial cell layer 2 in diabetic, organ donor control and HMSN I cases.
FIGURE 17 Relative frequency histograms of basal lamina thickness on (a) the epineurial and (b) endoneurial aspects of perineurial cell layer 3; and (c) the epineurial and (b) endoneurial aspects of perineurial cell layer 4 in diabetic, organ donor control and HMSN I cases.
FIGURE 18 Relative frequency histograms of basal lamina thickness on (a) the epineurial and (b) endoneurial aspects of perineurial cell layer 5; and (c) the epineurial and (b) endoneurial aspects of perineurial cell layer 6 in diabetic, organ donor control and HMSN I cases.
FIGURE 19 Relative frequency histograms of basal lamina thickness on (a) the epineurial and (b) endoneurial aspects of perineurial cell layer 7; and (c) the epineurial and (b) the endoneurial aspects of perineurial cell layer 8 in diabetic, organ donor control and HMSN I cases.
FIGURE 20 Relative frequency histograms of basal lamina thickness on (a) the epineurial and (b) endoneurial aspects of perineurial cell layer 9; and (c) the epineurial and (b) the endoneurial aspects of perineurial cell layer 10 in diabetic, organ donor control and HMSN I cases.
FIGURE 21 Plot of median perineurial cell basal lamina thickness against age with first order regression in (a) control; (b) diabetic and (c) HMSN I cases.
4A.3(v) Fascicular area and correlation with age

The median values for fascicular area for the diabetic and organ donor control groups were of a similar order, 84,774μm² and 88,148μm² respectively. The value for the HMSN I group was greater at 109,207μm²; however, no statistically significant difference was found between any of the three groups. The values obtained for these smaller sets; 34 fascicles from the diabetic patients, 16 fascicles from the HMSN I group and 10 fascicles from the organ donor controls, confirm the results of the earlier statistical analyses which were performed on a larger number of fascicles, 72 from the diabetic group, 34 from the organ donor control group and 15 from the HMSN I group.

Regarding the relationship between age and fascicular area, when these two variables are plotted against each other (fig. 22) for each experimental group, the correlation coefficient (r) for the diabetic patients was 0.337 which is not significant at p<0.01. The values of r = -0.267 for the organ donor controls and r=0.539 for the HMSN I cases were additionally not found to be significantly different (p<0.01).

4A.3(vi) Fascicular area and number of perineurial lamellae

When fascicular area is plotted against the number of perineurial cell lamellae, (fig. 23) the calculated r values of 0.0012 and 0.6297 for the control and HMSN I groups respectively show no significant correlation (p>0.01), although the correlation for the HMSN I patients proved significant at p=0.0510. However the correlation for the diabetic group of patients (r=0.4072) reached a higher level of significance at p=0.0168.

The median number of perineurial lamellae in the HMSN I group of patients was 10.0. This was significantly higher (p<0.0001) than 8.0, the value calculated for both the diabetic and organ donor control groups. Welch's t-test was performed on mean values of perineurial cell laminae number. At 10.21 the difference between the value for the HMSN I group and those of 8.61 for the organ donor controls and 8.42 for the diabetic group again reached a high level of significance (p<0.0001). There was no significant difference between the control and diabetic groups (p<0.01).
FIGURE 22 Plot of mean fascicular area against age in (a) controls; (b) diabetics and (c) HMSN I patients. Bars show standard deviations.
FIGURE 23 Plot of fascicular area against the number of perineurial cell lamellae with 1st order regression in (a) control; (b) diabetic and (c) HMSN I cases.
4A.3(vii) Degenerate or necrotic cells

The mean percentage number of degenerate or necrotic cell layers was calculated for each individual fascicle and the results plotted against the mean number of perineurial cell lamellae (fig. 24). The diabetic patients had a considerably greater percentage of degenerate or necrotic perineurial cells than the organ donor controls. The values for the HMSN I patients fell between those of the other two groups.

Welch's t-test was performed to compare the mean percentage number of degenerate or necrotic cells. The value for the diabetic patients was 19.47% which was significantly greater than both the values of 1.11% for the organ donor controls ($p<0.0001$) and 4.15% for the HMSN I patients ($p<0.0001$). At $p<0.01$ no significant difference was found between the means for the control and HMSN I group, however, when $p=0.0302$ a significant difference was shown.

A Mann-Whitney U-test was also performed on the data. The median value of 17.89 for the diabetic patients again proved significantly greater than 3.14 ($p<0.0001$) and 1.15 ($p<0.0004$) the figures for the HMSN I and control group respectively. The difference between the median values for the HMSN I and organ donor control groups approached significance at $p<0.01$ and was significant at $p=0.0123$.

4A.3(viii) Comparison of perineurial cell and capillary basal laminae

From the scatter plot of median endothelial cell basal lamina thickness against median perineurial cell basal lamina thickness (fig. 25) it can be deduced that diabetic patients have far greater values for both of these parameters compared with the organ donor control cases, the figures for the HMSN I patients falling between the values for the aforementioned groups.

Statistical comparison of median values of capillary basal lamina thickness revealed that the values for the diabetic patients and the HMSN I patients at were both significantly greater ($p\leq0.0001$) than that of the organ donor controls. There was no significant difference ($p<0.01$) in measurements of median capillary basal lamina thickness between the diabetic and the HMSN I group.
These findings contrasted with the results for median perineurial cell basal lamina thickness as no significant difference (p<0.01) was found between the values for the HMSN I cases and the organ donor controls. The median value for the diabetic group was significantly greater than those of both the HMSN I group (p<0.0005) and the organ donor controls (p<0.0001).

Statistical intergroup comparisons of maximum values for perineurial cell basal lamina thickness and maximum capillary basal lamina thickness corresponded with those for the median values (p<0.01).

No significant correlations (p<0.01) were observed within any of the three groups when the median and maximum values of endoneurial capillary basal lamina thickness were plotted against the median and maximum values of perineurial cell basal lamina thickness, respectively (figs. 26-28).
FIGURE 24  Plot of percentage number of degenerate or necrotic perineurial cells against the number of perineurial cell lamellae in organ donor control; diabetic and HMSN I cases.
FIGURE 25  Plot of median thickness of endothelial cell basal laminae against median thickness of perineurial cell basal laminae in organ donor control; diabetic and HMSN I cases.
FIGURE 26 Plots of (a) maximum thickness of endoneurial capillary basal lamina against maximum thickness of perineurial cell basal lamina with 1st order regression and (b) median thickness of endoneurial capillary basal lamina against median thickness of perineurial cell basal lamina with first order regression in organ donor control cases.
FIGURE 27 Plots of (a) maximum thickness of endoneurial capillary basal lamina against maximum thickness of perineurial cell basal lamina with 1st order regression and (b) median thickness of endoneurial capillary basal lamina against median thickness of perineurial cell basal lamina with 1st order regression in diabetic patients.
FIGURE 28 Plots of (a) maximum thickness of endoneurial capillary basal lamina against maximum thickness of perineurial cell basal lamina with 1st order regression and (b) median thickness of endoneurial capillary basal lamina against median thickness of perineurial cell basal lamina with 1st order regression in HMSN I patients.
4B QUALITATIVE OBSERVATIONS

Light microscope examination of individual fascicles revealed a moderate to severe loss of myelinated fibres in both the diabetic (figs 30a & b) and the HMSN I (figs 31a & b) patients compared with the controls (figs 29a & b). Electron microscopy of comparative fascicular sections (figs 32a, b & c) illustrates the gross morphological differences between the three groups. The control nerves (fig. 32a) possessed a normal distribution of both large and small myelinated fibres, each associated with an individual Schwann cell. Unmyelinated axons occurred in small groups, the axons being invested by an encircling cytoplasmic process of the accompanying Schwann cell.

Transverse sections of peripheral nerves from the HMSN I cases (fig. 32c) revealed the characteristic presence of "onion bulbs". These hypertrophic structures result from repeated episodes of segmental demyelination and subsequent remyelination with accompanying Schwann cell proliferation. The onion-bulbs were composed of whorls of concentrically laminated and interdigitated Schwann cell processes which usually surrounded a centrally located myelinated fibre (fig. 33). The Schwann cell processes often contained regenerating unmyelinated axonal sprouts. Occasional onion-bulbs were present which possessed a central band of Büngner as opposed to a myelinated fibre (fig. 34). Active degenerative changes evidenced by axonal accumulations of organelles were occasionally found in the HMSN I tissue (fig. 35). The onion-bulbs were widely separated (fig. 36), the interstitial spaces being occupied by numerous proliferative Schwann cells, often associated with new axonal sprouts and collagen.

Many structures which are encountered in healthy nerve were evident in the diabetic tissue. These included, amongst others, myelin bulbs, aberrant outfoldings of myelin sheath lamellae which have no pathological significance; Reich granules, Schmidt-Lanterman incisures and paranodal regions (figs 37-42). Mast cells (fig. 43a) were occasionally found in close proximity to blood vessels and although these cells are normal endoneurial components of healthy nerve (fig. 43b) they were encountered more frequently in the diabetic nerves. Macrophages (figs 44a & b) were also evident in the endoneurial compartment of the diabetic nerves. Apart from an increase in endoneurial collagen (fig. 32b), the connective tissues in the diabetic nerves appeared normal; no inflammatory infiltrates were seen and there was no evidence of nerve oedema.
The major ultrastructural observation in diabetic nerves was axonal loss affecting both myelinated and unmyelinated axons. Active axonal degeneration was not encountered very frequently, as many of the diabetic patients had an advanced neuropathy and possessed few remaining myelinated fibres. Active fibre breakdown or demyelination was rarely seen. When degenerating fibres were found they appeared packed with membranous dense bodies, distended mitochondria and granular debris (fig. 45) that had accumulated as a result of the cessation of axonal transport.

Abnormal axons were also found that contained dense neurofilamentous aggregations (fig. 46). Active axonal degeneration of Wallerian type was occasionally encountered. Associated Schwann cells contained large accumulations of lipid debris within their cytoplasm (fig. 47).

Vacuolated macrophages containing cellular and lipid debris were occasionally seen in association with degenerating axons. The macrophages were easily identified as, unlike Schwann cells, they do not possess a basement membrane and were often located within a loose fitting sheath of persistent Schwann cell basement membrane. They were sometimes seen to be actively engulfing cellular remnants by surrounding them with finger-like processes (fig. 48).

In some instances macrophages were seen to be engaged in the process of active demyelination. These cells were full of lipid debris, the associated axon, although atrophic, appearing relatively intact (fig. 46).

Following axonal degeneration, a tube of Schwann cell basal lamina persists. In normal healthy tissue this usually has a collapsed (fig. 49) or convoluted (fig. 50) appearance. The tube gradually expands as it becomes filled with proliferating Schwann cells giving rise to columns of cells known as bands of Büngner. These crenated basal laminal tubes were encountered in the diabetic nerves, but more frequently they appeared considerably more rigid and often retained a more circular profile around the bands of Büngner (fig. 51), the enclosed proliferating Schwann cells having produced a new layer of basement membrane which closely adhered to the cell membrane.

Regenerating axonal sprouts were associated with mature bands of Büngner (fig. 52), the stacks of Schwann cell appearing to act as guides. Collagen pockets (fig. 53), formed by the encircling of several collagen fibres by one or more turns of a Schwann cell process, were often encountered in the diabetic
nerves. These pockets were lined by Schwann cell basal lamina and appeared to be an exclusive feature of satellite cells associated with unmyelinated axons (fig. 54). It has been suggested that these structures may have a skeletal function and due to their greater incidence in old and pathological tissue be formed as a result of degenerative changes.

Reich or \( \pi \) granules (fig. 55) are perinuclear lamellar bodies in Schwann cells composed of stacks of osmiophilic membranes together with dense material. The lamellae frequently appear separated, an artifact of tissue preparation consequent upon their inherent instability. These granules occur in normal healthy nerves, but, their number has been stated to be increased in certain neuropathies and with age. It has been proposed that they may be a degenerative product of myelin breakdown. They probably represent secondary lysosomes.

The presence in diabetic nerves of an accumulation of Reich granules (fig. 56), often in conjunction with lipofuscin (fig. 58), within the cytoplasm of a Schwann cell suggests that the cell was probably once associated with a myelinated fibre which has since degenerated. Large numbers of such granules were often encountered in bands of Büngner and in Schwann cells associated with regenerating axonal sprouts (fig. 58).

Occasionally degenerating axons containing accumulations of malorientated neurotubules (fig. 59) or lipid debris (fig. 60) were seen associated with bands of Büngner in the diabetic nerves. Axonal regeneration was a prominent feature of many of the diabetic nerves. It is probable that a considerable number of regenerating axonal sprouts will fail to make appropriate distal connections, and in that in these instances the sprouts will degenerate. Bands of Büngner containing both healthy and abnormal sprouts rich in dense bodies and mitochondria (fig. 61) were sometimes seen in the diabetic nerves.

Some of the Schwann cell processes within the old circular tubes of persistent basement membrane were seen to be associated with single large regenerating axonal sprouts (fig. 62). It seem likely that these axons were destined to become myelinated, a one to one relationship with the satellite cell being the case in these instances. Lone myelinated axons were sometimes seen surrounded by an old basal laminal tube (fig. 63), in other cases the fibre was accompanied by other Schwann cell processes (fig. 64). The old Schwann cell basal laminal tubes appeared to maintain their circular profile despite the fact
that in the early stages of regeneration, they appeared to contain little in the way of cellular processes or collagen (fig. 64).

At later stages of regeneration, clusters of myelinated fibres occurred within the basal laminal tubes (fig. 65) and Schwann cell processes were often seen to line the insides of the tubes, suggesting that it may act as a guide along which the new Schwann cell processes grow (figs 65 & 66). Some of the Schwann cell processes contained small unmyelinated axons (fig. 66) and others collagen pockets (fig. 67).

At these later stages of regeneration, the extracellular spaces within the basal laminal tubes were seen to be tightly packed with collagen fibrils (figs 65, 66 & 67), presumably of Schwann cell origin. Occasionally some cellular debris was found within a basal laminal tube (fig. 68). The basal laminal tubes were often seen to persist even when the clusters were fully mature and contained 5 or more myelinated fibres (fig. 69).

Endoneurial vessels consistently ran in a direction parallel to that of the long axis of the fascicles and were therefore sectioned transversely. Electron micrographs of representative endoneurial capillaries from the diabetic, donor control and HMSN I cases are shown in figures 70-72. The capillary walls of vessels from the diabetic (fig. 71) and HMSN I (fig. 72) patients and the organ donor control cases (fig. 70) were universally composed of a single layer of endothelial cells surrounded by a continuous layer of basal lamina. Hyperplasia of endothelial cells was evident in some capillaries both in the diabetic (fig. 73) and HMSN I (fig. 74) biopsies. Although lipofuscin inclusions were occasionally encountered in the endothelial cells of diabetic capillaries (fig. 75), these cells generally appeared normal in both the diabetic and HMSN I cases.

Additional layers of basal lamina, the number of which varied considerably between individual vessels, were located outside the innermost layer and were interspersed with scattered collagen fibrils (fig. 76). The number of basal laminal layers in the organ donor control cases was generally low, in the magnitude of 3-5 (figs 77a & b). Thickening of the pericapillary basal laminal zone, with reduplication of basal lamina resulting in the production of multiple layers, was evident in both the diabetic (fig. 78) and HMSN I (fig. 79) patients as compared with donor control subjects.
The appearance of the hyperplastic basal laminal zone varied between individual diabetic patients. In the majority of cases clearly identifiable basal laminal layers were apparent in the innermost region of this zone; however with increasing distance from the parent cell the components of the zone assumed a more amorphous appearance (fig. 80). In some instances the entire basal laminal zone appeared fairly amorphous with the exception of the innermost layer (fig. 81). The presence of calcium deposits within the basal laminal zone was a frequent (figs 76, 81 & 82) although not ubiquitous (fig. 83) feature of diabetic vessels. Whilst the degree of basal laminal zone hyperplasia appeared comparable between the HMSN I and the diabetic patients, differences in the general appearance of the zone existed between the two groups. In the HMSN I cases the basal laminal layers tended to be interrupted and broken up into irregular lengths (fig. 84). In contrast, those of the diabetic patients, which appeared to be slightly thicker, were generally more continuous (fig. 85).

Pericytes were found to be a common component of capillary walls in all three groups, diabetic (fig. 71), HMSN I (fig. 72) and organ donor controls (fig. 70). These cells are characteristically located between the basal laminal layers and partially encircle the endothelial cells. Pericytes are generally separated from the endothelial cells by a single layer of basement membrane, and this arrangement was seen to be the norm for the majority of control endoneurial capillaries (figs 77a & b). In the diabetic and HMSN I biopsies, although the pericytes showed no apparent signs of pathological abnormalities, they were frequently seen to be displaced peripherally, presumably as a result of basal lamina reduplication (figs 71, 74 & 78).

The zones of reduplicated basal lamina in the diabetic and HMSN I vessels were often seen to be encircled to varying degrees by the cellular processes of endoneurial fibroblasts (figs 72 & 82), recognisable by their angular shape and lack of basement membrane. Tight junctions were often apparent between endothelial cells (figs 86a & b) in all three groups. No obvious difference was seen between the three groups with respect to lumen area and no evidence of vasculitis or vascular occlusion by platelet "plugs" or fibrin was seen in any of the three groups. Additionally, fenestrated capillaries or inflammatory infiltrates related to vessel walls or in the endoneurium, perineurium or epineurium were not encountered.
The perineurium surrounding sural nerves in the organ donor control cases (figs 87a & b) appeared normal, the layers of flattened perineurial cells showing no signs of pathological alterations. The cells were bounded on both endoneurial and epineurial aspects by a thin layer of basement membrane and were separated by connective tissue spaces containing transversely, longitudinally and obliquely orientated collagen fibres. Basement membranes often have two distinct areas, the lamina densa and, closest to the cell membrane, the lamina rara or lucida. This characteristic appearance was only noted on the endoneurial aspect of the innermost layer of the perineurial sheath, the lamina densa on both sides of the other perineurial cell layers generally extending up to the cellular membrane.

A moderate degree of perineurial cell basal laminal thickening, occasionally accompanied by the presence of intercellular debris, was observed in the sural nerves of HMSN I patients (figs 88a & b).

The degree of perineurial basal laminal hypertrophy in the diabetic cases was extremely marked, the thickened basement membrane usually having a homogeneous (fig. 89) or at times "moth eaten" appearance (fig. 90). Distinctly laminal basement membrane reduplication of the type associated with diabetic blood vessels (fig. 78) was not observed. The degree of basal laminal thickening was more marked in the central layers of the perineurium and never appeared to affect the basement membrane on the endoneurial aspect of the innermost layer.

Evidence of degenerative changes in diabetic perineurial cells was frequently encountered. This took one of two forms, atrophy or cellular disintegration. In the former case a substantial gap between the basal lamina and the cell membrane was seen (fig. 90). Varying degrees of cellular disintegration were observed in the diabetic tissue. Necrotic cells with a discontinuous cellular membrane (fig. 91) were frequently found. Where the process had progressed to a more advanced stage cellular debris was all that remained between adjacent perineurial cell basal laminae (fig. 90). Occasionally pairs of basal lamina were seen to persist in the absence of an intervening cell or any cellular remains (fig. 92). Degenerative changes in the perineurial cells were very rare in the HMSN I patients and even more so in the organ donor control tissue.
Cellular debris (fig. 93) and calcified deposits (fig. 94) were often observed between perineurial cell layers in the diabetic specimens, and were usually located within the central or outer perineurial zones. Some of these calcium deposits exhibited an internal structure of alternating light and dark bands (fig. 95), whilst others appeared as very small amorphous dark bodies (fig. 96). The nature of these calcified granules often caused them to separate from the tissue in thin sections resulting in the production of holes (fig. 95). Myelin figures (fig. 89) were occasionally seen within the cytoplasm of perineurial cells.
Figs 29a & b  Light photographs of transverse sections through sural nerve fascicles from control individuals. Open arrow = perineurium; Ep = epineurium; En = endoneurium; large filled arrow = endoneurial blood vessel; small filled arrow = myelinated fibre. Bars = 100μm. Thionin and acridine orange stain.
Figs 30a & b Light micrographs of transverse sections through sural nerve fascicles from diabetic patients showing loss of myelinated nerve fibres. Subperineurial oedema is evident in (b). Open arrow = perineurium; Ep = epineurium; En = endoneurium; large closed arrow = endoneurial blood vessel; small filled arrow = myelinated fibre. Bars = 100μm. Thionin and acridine orange stain.
Fig. 30a

Fig. 30b
Figs 31a & b  Light photographs of transverse sections through sural nerve fascicles from HMSN type I patients showing loss of myelinated nerve fibres and prominent hypertrophic changes. Large open arrow = perineurium; small open arrow = onion bulb; large closed arrow = endoneurial blood vessel; small closed arrow = myelinated fibre; Ep = epineurium; En = endoneurium. Bars = 50µm. Thionin and acridine orange stain.
Fig. 32 Low power electron micrographs of transverse sections from the sural nerves of an a) control, b) diabetic & c) HMSN I patient. Large filled arrows = myelinated fibre; small filled arrows = unmyelinated axon; open arrows = Schwann cells; (*) = axons. Bars = 5µm.
Fig. 33 Electron micrograph of an onion bulb formation from the sural nerve of an HMSN I patient. The structure is composed of numerous whorls of Schwann cell processes (small arrows) surrounding a central myelinated fibre (large arrow). These processes often contain regenerating unmyelinated axonal sprouts (*). Bar = 2 µm.

Fig. 34 Electron micrograph of an onion bulb formation from the sural nerve of an HMSN I patient. The concentric layers of Schwann cell processes (small arrows) and associated axonal sprouts (*) surround a centrally located band of Bühngner (B). Bar = 2 µm.
**Fig. 35** Electron micrograph of an onion bulb formation from the sural nerve of an HMSN I patient. The axoplasm (A) of the central myelinated fibre contains an abnormal accumulation of organelles which suggests that the axon is degenerating. The surrounding Schwann cell processes (arrows) contain regenerating unmyelinated axonal sprouts (*). Bar = 2μm.

**Fig. 36** Low power electron micrograph of a transverse section through a sural nerve fascicle from a patient with HMSN I. The onion-bulb formations (large arrows) are widely spaced, the intervening areas being occupied by Schwann cells (small arrow), their associated axonal sprouts and collagen (C). Bar = 4μm.
**Fig. 37** Electron micrograph of a myelin bulb (open arrow) related to outpouching of the myelin sheath from a diabetic sural nerve. Large arrow = myelinated fibre; S = Schwann cell; small arrow = Schwann cell basal lamina. Bar = 1μm.

**Fig. 38** Electron micrograph of a Reich granule (R) contained within the cytoplasm of a diabetic Schwann cell. Bar = 0.25μm.
Fig. 39  Electron micrograph of a near-nodal region of a myelinated axon from a diabetic sural nerve biopsy. Small arrows = nodal Schwann cell processes; A = axon; M = myelin; large arrow = Schwann cell basal lamina. Bar = 0.5μm.

Fig. 40  Electron micrograph of a Schmidt-Lanterman incisure in a myelinated axon from a diabetic sural nerve biopsy. A = axon; large filled arrow = myelin; large open arrow = Schwann cell; small filled arrow = Schwann cell basal lamina. Bar = 1μm.
Fig. 39

Fig. 40
Fig. 41  Electron micrograph of a paranodal region of a myelinated fibre in a diabetic sural nerve. The Schwann cell (S) contains a large volume of cytoplasm and numerous mitochondria (*). Large arrow = Schwann cell basal lamina; small arrows = neurotubules; within circle = neurofilaments; A = axon. Bar = 0.5μm.

Fig. 42  Electron micrograph of a myelinated fibre paranodal region and Schmidt-Lanterman incisure from a diabetic sural nerve. Pseudodesosomes (small filled arrow) are evident within the incisure. S = Schwann cell; A = axon; open arrow = myelin; (*) = mitochondria, large filled arrow = Schwann cell basal lamina. Bar = 0.5μm.
**Figs 43** Electron micrographs of mast cells from a) diabetic and b) control sural nerve tissue. Theses cells possess dense metachromatic granules (small arrow) containing scroll-like formations and have numerous pseudopodia (large arrow). Bar = 1μm.

**Figs 44a & b** Electron micrographs of macrophages from the sural nerves of diabetic patients. The cells are characterised by their lack of basement membrane, their angular shape with numerous elongated cellular processes (large arrow), and cytoplasm rich in rough endoplasmic reticulum (small arrow) and ribosomes. Bar = 1μm.
Fig. 45 Electron micrograph of a degenerating axon (A) from sural nerve of a diabetic patient. The axon contains a large amount of cellular debris (D). Bar = 0.5μm.

Fig. 46 Electron micrograph of a demyelinated axon profile (A) from a diabetic sural nerve. A macrophage (M) full of lipid debris (D) can be seen. Lipid debris is also present in the cytoplasm of the associated Schwann cell (S). All three cells are surrounded by a tube of persistent basement membrane (small arrow) and encircled by Schwann cell processes (large arrow). Bar = 1μm.
Fig. 47 An electron micrograph of a degenerating axon and associated macrophage (M) from a diabetic sural nerve. The cytoplasm of the Schwann cell (S) contains a large amount of lipid debris (*). The macrophage on the right contains a centriole (arrow). N = macrophage nuclei. Bar = 1μm.

Fig. 48 Electron micrograph of a macrophage (M) containing lipid debris (*) from a diabetic sural nerve. The cell which lacks its own basement membrane is surrounded by a loose fitting sheath of persistent Schwann cell basement membrane (large arrow). The remnant of the Schwann cell (S) is being actively engulfed and is surrounded by macrophage processes (small arrow). N = macrophage nuclei. Bar = 1μm.
Fig. 49  Electron micrograph of collapsed empty basal lamina (arrow) form the sural nerve of a diabetic patient. Bar = 0.5μm.

Fig. 50  Electron micrograph of an empty convoluted basal laminal (arrow) tube from the sural nerve of a diabetic patient containing two Schwann cell processes surrounded by new basal lamina. Bar = 0.5μm.
**Fig. 51** Electron micrograph of a band of Büngner (B) from the sural nerve of a diabetic patient. The Schwann cell processes are invested by a newly generated layer of basement membrane (small arrow) and encircled by an old persistent sheath of Schwann cell basal lamina (large arrow). Bar = 1 µm.

**Fig. 52** Electron micrograph of regenerating axonal sprouts in the sural nerve of a diabetic patient. The Schwann cell processes surround numerous small unmyelinated axons (A). N = Schwann cell nucleus. Arrow = Schwann cell basal lamina. Bar = 0.5 µm.
Fig. 53 Electron micrograph of collagen pockets (C) from a diabetic sural nerve. The pockets are lined with Schwann cell basal lamina (small filled arrow) and are formed by the encircling of small bundles of collagen fibrils (open arrow) by one or more turns of Schwann cell process (large filled arrow). It has been suggested that these structures may have a skeletal function and have been actively engulfed by the Schwann cell. Bar = 0.25μm.

Fig. 54 Electron micrograph of axonal sprouts in the sural nerve of a diabetic patient. The numerous regenerating axons (A) are surrounded by Schwann cell processes (arrow). Some of the Schwann cell processes have engulfed collagen fibrils to produce collagen pockets (*). Bar = 1μm.
Fig. 55  Electron micrograph of a Schwann cell from the sural nerve of a diabetic patient. The cell contains Reich granules (R), evidence that the Schwann cell was probably once attached to a myelinated axon which has since degenerated. N=Schwann cell nucleus; (*)=unmyelinated axon; arrow=Schwann cell basal lamina. Bar = 1μm.

Fig. 56  Electron micrograph of a degenerated axon. The basement membrane formed by the original satellite cell has persisted as a circular basal laminal tube (arrow). The proliferating Schwann cells (S) growing within this tube have since produced new basement membranes. The fact that the original Schwann cell was previously associated with a myelinated axon is indicated by the presence of large numbers of lipofuscin inclusions (*) and Reich granules (R). Bar = 0.5μm.
Fig. 57  Electron micrograph of a regenerating axon (*) from the sural nerve of a diabetic patient. It is evident, due to the large number of Reich or π granules (R) cellular inclusions, that the upper Schwann cell (S) was originally associated with a myelinated axon. The Schwann cells are surrounded by an original redundant sheath of persistent basement membrane (large arrow). The unmyelinated axon on the right has been sectioned at a near nodal region and is bounded on some sides by Schwann cell processes (small arrow). Bar = 0.5μm.

Fig. 58  An electron micrograph of regenerating axons (A) from the sural nerve of a diabetic patient. The new axonal sprouts are associated with a Schwann cell (S) which was previously associated with a myelinated axon. This fact is substantiated by the presence of large number of Reich granules (arrow) and inclusions of lipofuscin (*) in the cytoplasm of the Schwann cell. Bar = 0.5μm.
Fig. 59 Electron micrograph of a group of Schwann cell processes and regenerating axons from the sural nerve of a diabetic patient. One (open arrow) contains an unusual amount of smooth endoplasmic reticulum. Small arrow = Schwann cell basal lamina; large arrow = axon. Bar = 1μm.

Fig. 60 An electron micrograph of a band of Büngner, containing several small unmyelinated axons (*) and a collagen pocket (diamond), from a diabetic sural nerve. The central large axon contains a large amount of lipid debris (D). Arrow = Schwann cell basement membrane. Bar = 1μm.
Fig. 61  Electron micrograph of a Schwann cell (S), from a diabetic sural nerve, containing regenerating axonal sprouts (A) some of which are rich in dense bodies and mitochondria. It is possible that the degenerating sprouts are dying back as a result of having failed to reach a suitable destination. Arrow = Schwann cell basal lamina. Bar = 1μm.

Fig. 62  Electron micrograph of a large regenerating axon (A) in the sural nerve of a diabetic patient. The Schwann cell (S) is surrounded by a new layer of basement membrane (small arrow) and is growing through an original, larger sheath of persistent basement membrane (large arrow). Bar = 0.5μm.
Fig. 63  Electron micrograph of a regenerated myelinated axon (A) and surrounding Schwann cell (S) enclosed by a sheath of persistent basement membrane (arrow). Bar = 0.5μm.

Fig. 64  Electron micrograph of a regenerating axon (A), from the sural nerve of a diabetic patient, sectioned through the paranodal region. The Schwann cell contains desosomes (open arrow) which connect uncompacted myelin within the Schwann cell (S). Large filled arrow = old basal laminal tube; small filled arrow = new Schwann cell basal lamina. Bar = 0.5μm.
Fig. 65  Electron micrograph of a mature cluster of myelinated fibres (large filled arrows) from the sural nerve of a diabetic patient. Cellular processes of the proliferating Schwann cells (open arrow) are invested by a new layer of basement membrane and are guided by and grow along the old persistent basal laminal tube (small filled arrow). The extracellular space within this tube is densely packed with new collagen fibrils (C). Bar = 1μm.

Fig. 66  Electron micrograph of a mature cluster of myelinated fibres (large arrow) from the sural nerve of diabetic patient. The old basal lamina tube (small arrow) is lined by new Schwann cell processes. Some of the Schwann cell processes contain regenerating unmyelinated axons (*), others are associated with mature myelinated fibres. N = Schwann cell nucleus. Bar = 2μm.
Fig. 67  Electron micrograph of a regenerating cluster of myelinated fibres (MF) in a diabetic sural nerve. Some of the accompanying Schwann cell processes have engulfed collagen fibrils to produce collagen pockets (*). The components of the cluster are separated by newly formed, densely packed collagen fibrils (C) probably of Schwann cell origin. The cluster is surrounded by a rigid circular sheath of old basal lamina (arrow). N = Schwann cell nuclei. Bar = 1μm.

Fig. 68  Electron micrograph of two regenerating cluster of myelinated fibres from the sural nerve of a diabetic patient. The cluster on the right contains numerous Schwann cell processes (large arrows) and a central new myelinated fibre (MF) all surrounded by a sheath of persistent basement membrane (small arrow). The cluster also contains some cellular debris (star). Some of the Schwann cell processes in the cluster on the left contain regenerating unmyelinated axons (*) and collagen pockets (diamonds). N = Schwann cell nucleus. Bar = 2μm.
Fig. 69  Electron micrograph of a mature cluster, containing 8 regenerated myelinated fibres (large filled arrow), from a diabetic sural nerve. To the left of the cluster is a convoluted empty tube of old persistent basement membrane (small filled arrow), and at the top of the picture an old basal lamina tube containing a new Schwann cell process (large open arrow). N = Schwann cell nuclei; small open arrow = Reich granule; diamond = regenerating unmyelinated axon; (*) = newly formed collagen fibrils. Bar = 2μm.
Fig. 70  Electron micrograph of a typical endoneurial capillary from the sural nerve of a normal organ donor control. The endothelial cells (E) which are joined by tight junctions (small filled arrow) are surrounded by a thin layer of basement membrane (large filled arrow) and partially encircled by adjacent pericyte processes (open arrow). N = Endothelial cell nuclei; PN = Pericyte nuclei; L = Lumen. Bar = 2μm.
Fig. 71  Electron micrograph of a typical endoneurial capillary from the sural nerve of a diabetic patient. The endothelial cells (E) are surrounded by numerous layers of reduplicated and persistent basement membrane (star) containing pericyte processes (arrows). The pericyte to the right has been displaced peripherally as a result of basal laminal hyperplasia. N=Endothelial cell nuclei; P=Pericyte nucleus; L=Lumen. Bar = 2\mu m.
Fig. 72 Electron micrograph of a typical endoneurial capillary from the sural nerve of a patient with HMSN I. The endothelial cells (E) are surrounded by pericyte processes (large filled arrows). Tight junctions (small filled arrow) can be seen between some of the endothelial cells. The basal laminal zone (star) which is abnormally thickened is partially encircled by a fibroblast process (open arrow). N = Endothelial cell nuclei; (*) = Lumen. Bar = 1μm.
Fig. 73  Electron micrograph of an endoneurial capillary from a diabetic sural nerve showing increased numbers of endothelial cell nuclei (N). (*) = Basal laminal zone; arrow = Pericyte process. Bar = 2μm.

Fig. 74  Electron micrograph of an endoneurial capillary from the sural nerve of an HMSN I patient, showing numerous reduplicated layers of basement membrane (*). The endothelial cells (E) are surrounded by pericyte processes (arrows) some of which have been peripherally displaced. The number of endothelial cells is abnormally large indicating a degree of hyperplasia. N = Endothelial cell nuclei; L = Lumen. Bar = 3μm.
Fig. 75 Electron micrograph of an endoneurial capillary from a diabetic sural nerve. The hypertrophic basal laminal zone (star) contains numerous deposits of calcium (*). The endothelial cell wall (arrow) of this capillary appears unusually thin in places and one endothelial cell contains abnormal deposits of lipofuscin granules (diamonds). N = Endothelial cell nuclei; L = Lumen. Bar = 2μm.
Fig. 76  Electron micrograph of a typical endoneurial capillary from a diabetic sural nerve, showing an abnormally thickened basal lamina zone (BZ) composed of layers of persistent basement membrane (small arrows) interspersed with collagen fibrils. The basal laminal zone contains numerous deposits of calcium (*). Persistent Schwann cell basal laminal tubes, some of which are empty and some of which contain regenerating axons, can be seen at the top of the figure (large arrows). The lumen of the capillary (L) is open and clearly not obstructed. E = Endothelial cell. Bar = 2μm.
Figs 77a & b  Electron micrographs of endoneurial capillaries from organ donor control sural nerves. The central lumen (L) is surrounded by a single layer of endothelial cells (E) and one or two layers of basement membrane (large arrows). Small arrow = Pericyte process; N = Endothelial cell nuclei. Bar = 2μm.
Fig. 77a

Fig. 77b
**Fig. 78** Electron micrograph of an endoneurial capillary from a diabetic sural nerve. The basal laminal zone (star) is abnormally thickened and contains deposits of calcium (*). The pericyte processes (arrows) have been displaced peripherally by the persistent reduplicated layers of basement membrane. 

N = Pericyte nucleus; L = Lumen. Bar = 2µm.

**Fig. 79** Electron micrograph of an endoneurial capillary from the sural nerve of a patient with HMSN I. A considerable degree of basement membrane (star) hypertrophy is apparent. 

N = Endothelial cell nuclei; arrow = Pericyte process; L = Lumen. Bar = 1µm.
Fig. 80  Electron micrograph of an endoneurial capillary from a diabetic sural nerve, showing abnormal thickening of the basal laminal zone (BZ). The innermost basement membrane layers of this zone are clearly identifiable while the outermost components have assumed a rather amorphous appearance. The cellular process of a fibroblast (large arrow) can be seen to be partially encircling the hypertrophic basal laminal zone. Small arrow = Pericyte process; E = endothelial cell; L = Lumen. Bar = 2μm.
Fig. 81 Electron micrograph of a typical endoneurial capillary from the sural nerve of a diabetic patient. The lumen (L) is occupied by an erythrocyte (E) and surrounded by a single layer of endothelial cells (large arrow). The basal laminal zone (BZ) is abnormally thickened and has a generally amorphous appearance and contains numerous pericyte processes (small arrows) and deposits of calcium (*). A mast cell (M) can be seen situated immediately outside the basal laminal zone. N = Endothelial cell nucleus; PN = Pericyte nuclei. Bar = 2µm.
Fig. 82  Electron micrograph of an endoneurial capillary from the sural nerve of a diabetic patient. The endothelial cells (small filled arrows) are surrounded by an enormously thickened basal laminal zone (star) which is partially encircled by a fibroblast process (open arrow). Calcium deposits (*) are visible in the basal laminal zone. N = Endothelial cell nuclei; large filled arrow = Pericyte process; L = Lumen. Bar = 3μm.

Fig. 83  Electron micrograph of an endoneurial capillary from the sural nerve of a diabetic patient showing a persistent and abnormally thickened basal laminal zone (star). Small arrow = endothelial cell; N = Endothelial cell nuclei; large arrow = Pericyte process; P = Pericyte nucleus; L = Lumen. Bar = 2μm.
Fig. 84 Electron micrograph of endoneurial capillary basement membrane (arrows) from the sural nerve of a patient with HMSN I. The layers of BM appear fragmented. E = Endothelial cell; P = Pericyte process. Bar = 0.5μm.

Fig. 85 Electron micrograph of endoneurial capillary basement membrane (arrows) from the sural nerve of a diabetic patient. The layers of basement membrane appear comparatively thicker and less fragmented than those in the HMSN I case (fig. 84). P = Pericyte process. Bar = 0.5μm.
**Fig. 86a** Electron micrograph of an endoneurial capillary from a diabetic sural nerve. The endothelial cell layer (arrow) is surrounded by concentric layers of reduplicated basement membrane (*) containing pericyte processes. The lumen of the capillary (L) is clearly not obstructed. N = Endothelial cell nuclei; P = Pericyte nucleus; F = Fibroblast. Bar = 2μm.

**Fig. 86b** Enlargement of the boxed area in fig. 86a showing a typical tight junction (small arrow) between adjacent endothelial cells (E). Large arrow = Basement membrane; P = Pericyte process; N = Endothelial cell nuclei. Bar = 0.5μm.
Figs 87a & b  Electron micrographs of transverse sections through the perineurium of sural nerves from control cases. The perineurial cells (large arrows), which contain numerous pinocytotic vesicles, appear healthy and have intact cell membranes. The perineurial layers are interspersed with collagen fibrils (*). The basal laminal zones (small arrows) appear relatively thin. N=Perineurial cell nuclei. Bar = 1μm.
Fig. 87a

Fig. 87b
Figs 88a & b  Electron micrographs of transverse sections through the perineurium from the sural nerves of HMSN I patients. The perineurial cells (large arrows), one of which is conspicuously vacuolated (diamonds), are bounded by layers of slightly thickened basement membrane (small arrows). The intercellular spaces contain collagen fibrils (stars) and cellular debris (*). Bar = 1μm.
Fig. 89 Electron micrograph of a transverse section through the perineurium of a sural nerve from a diabetic patient. The perineurial cells (P) are bounded on both sides by an abnormally thickened and homogeneous basal laminal zone (B). Cellular debris (*) and collagen fibrils (arrows) are present within the intercellular spaces. A myelin figure (M) is present in the cytoplasm of one cell. N=Perineurial cell nuclei. Bar = 1μm.
Fig. 90  Electron micrograph of a transverse section through the perineurium of a sural nerve from a diabetic patient. The perineurial cells (P), which are separated by connective tissue spaces containing collagen fibrils (star), are invested by hypertrophic basal laminal zones (small open arrows) which have a generally homogeneous appearance, however, in places they are punctuated by electron lucent area (large filled arrows) producing a "moth-eaten" effect. The perineurial cells contain numerous small vesicles (small filled arrows). The central perineurial cell is degenerating (*) and the two cells to its right appear atrophic (large open arrows). Cellular debris (diamond) is present within the intercellular layers. Bar = 1μm.

Fig. 91  Electron micrograph of necrotic perineurial cells from the sural nerve of a diabetic patient. The cell membranes (arrow) are discontinuous. The basement membranes (*) are unusually thick. M = Mitochondria; star = Collagen fibrils. Bar = 1μm.
Fig. 92  Electron micrograph of necrotic perineurial cells from the sural nerve of a diabetic patient. The lower two layers of basement membrane contain the remnants of a necrotic cell (star). The central perineurial cell has totally degenerated, all that remains are the two adjacent layers of basal lamina (arrows). Cellular debris (*) is interspersed amongst the collagen fibrils (C) within the intercellular spaces. Bar = 1μm.

Fig. 93  Electron micrograph of a transverse section through the perineurium of a sural nerve from a diabetic patient. The intercellular spaces contain numerous matrix vesicles (small arrows) and cellular debris (*). The basement membranes (large arrows) are abnormally thickened. Longitudinally (Lc) and transversely (Tc) arranged collagen fibrils present within the intercellular spaces produce a lattice-like structure. Bar = 1.5μm.
**Fig. 94** Electron micrograph of a transverse section through the perineurium of a sural nerve from a diabetic patient. The intercellular spaces contain calcium deposits (open arrows). The perineurial cell basement membranes (*) are abnormally thick. Large filled arrows = Perineurial cell; small filled arrows = Perineurial cell vesicles. Bar = 1μm.

**Fig. 95** Electron micrograph of a transverse section through the perineurium of a sural nerve from a diabetic patient. A large number of calcium deposits (stars) are present within the intercellular spaces. Diamond = Collagen fibrils; (*) = Basement membrane; arrows = Perineurial cells. Bar = 1μm.

**Fig. 96** Electron micrograph of a perineurial calcium deposit from the sural nerve of a diabetic patient. Some of these deposits have an internal structure of alternating light and dark bands (large arrow), while others appear as small amorphous dark bodies (small arrow). Bar = 0.5μm.
At present the causation of diabetic polyneuropathy remains unknown. Generally speaking there are two major schools of thought, the first proposes a metabolic origin and the second a vascular basis.

Two major metabolic disturbances are identifiable in peripheral nerve in diabetic subjects. The first is increased flux in the polyol pathway and the second non-enzymatic glycation of peripheral nerve proteins. With regard to the first of these, it has been proposed that hyperglycaemic stimulation of the polyol pathway, which is primarily located within Schwann cells in peripheral nerve, leads to an accumulation of polyols and resultant primary metabolic disruption and dysfunction of the host cell. In support of this theory significant increases in endoneurial polyol content have been documented in both experimental (Gabbay et al., 1966; Anand et al., 1987) and human diabetes (Mayhew et al., 1983; Dyck et al., 1988).

In addition, insulin therapy has been shown concurrently to produce near normalisation both of NCV deficits and elevated endoneurial polyol levels in streptozotocin-diabetic rats (Ghahary et al., 1991), evidence in favour of a causal relationship. In human diabetic neuropathy, one study has reported the existence of a correlation between elevated nerve sorbitol content and neuropathic severity (Dyck et al., 1988). Also Sima and colleagues have reported enhanced regeneration/remyelination following prevention of sorbitol accumulation using sorbinil (Sima et al., 1988c). Nevertheless this theory requires considerable substantiation.

Peripheral nerve myo-inositol has been shown to be reduced in experimentally-diabetic animals (Anand et al., 1988; Tomlinson et al., 1984). Evidence of a similar phenomena in human nerves is limited (Greene et al., 1987b), the majority of studies having found levels to be near-normal (Ward et al., 1972; Dyck et al., 1988). Diminished myo-inositol content may be related to sorbitol accumulation as the administration of an ARI has been proven to ameliorate both of these abnormalities in experimental diabetes despite the presence of continuing hyperglycaemia (Tomlinson et al., 1984).

Theoretically, diminished peripheral nerve myo-inositol content, whether related to abnormalities in the polyol pathway or the steric competitive inhibition of uptake by glucose, may in turn lead to perturbations in phosphoinositide
turnover and reduced diacylglycerol and calcium-dependent protein kinase C (Lattimer, 1989) stimulated Na\(^+\)-K\(^+\)-ATPase activity. The resultant reduction in the efficiency of the Na\(^+\)-K\(^+\) pump would result in intra-axonal sodium accumulation, inactivation of the Na channels and a subsequent reduction in NCV.

In support of this theory, experimental evidence has proved the existence of many of its component abnormalities i.e. Na\(^+\)-K\(^+\)-ATPase activity is decreased (Green & Lattimer, 1983b) and intra-axonal sodium levels increased (Brismar & Sima, 1981b) in experimentally diabetic animals. However, in order ultimately to validate the theory conclusive evidence is required which proves that diminished NCVs can be restored to values approaching those found in normal animals by dietary myo-inositol supplementation. So far related studies have produced conflicting results; some workers having found supplementation to be effective (Greene et al., 1975; Mayer & Tomlinson, 1983a) whilst others could demonstrate no significant improvement (Jefferys et al., 1978; Thomas et al., 1981). Once again no definite conclusion can be drawn regarding the ratification of this theory.

In addition, contrary to the predictions of the sorbitol/myo-inositol hypothesis, tissue protein kinase C activity in the heart (Okumura et al., 1988) and kidneys (Craven & DeRuvertis, 1989) of diabetic rats has been shown to be in actuality enhanced by elevated glucose concentrations. Protein kinase C activity in bovine retinal capillary endothelial cells has been found to be similarly elevated by high glucose concentrations, despite a concurrent reduction in Na\(^+\)-K\(^+\)-ATPase activity (Lee et al., 1989a). In accordance with the sorbitol/myo-inositol theory, ARI treatment with sorbinil was seen to result in restoration of Na\(^+\)-K\(^+\)-ATPase activity; however, protein kinase C activity remained elevated (Lee et al., 1989a). It is therefore seems likely that these two glucose-related metabolic abnormalities may be induced via different biochemical mechanisms. It is possible that the turnover rates of a substrate may be increased despite a reduction in its concentration, and as such despite the apparent reduction in myo-inositol concentration within diabetic tissues, the rate of myo-inositol turnover may actually be increased and hence responsible for enhanced protein kinase C activity.

The second major metabolic abnormality in diabetic nerve is the nonenzymatic glycation of proteins. This is discussed later (Section 5C.1).
The present investigation is largely concerned with diabetes-related alterations in cellular BMs and the endoneurial vasculature, and as such a major proportion of this discussion will revolve around the possible contribution of vascular abnormalities in the aetiology of diabetic neuropathy.

5A PERINEURIAL CELL ABNORMALITIES

5A.1 N°. of perineurial cell layers and fascicular area

The width of the sural nerve perineurial sheath has been reported as being increased in patients with diabetes mellitus (Malik et al., 1992). The number of cellular layers within the perineurial sheath, previously reported to range from between 6 to 10 distinct strata in human sural nerves (Beggs et al., 1989), was found to be very variable in this study, extending from 5 to 16 across the three groups. In concordance with findings in a similar study on dermal nerves (Johnson & Doll, 1984), the number of layers did not appear to be significantly affected by the diabetic state as the mean and median values for the diabetic and organ donor control group were comparable.

Sunderland and Bradley demonstrated the existence of a linear relationship between the diameter of peripheral nerves and the thickness of the encompassing perineurial sheath (Sunderland & Bradley, 1952) an observation which supports the hypothesis that, in order to maintain the same circumferential tension to resist a given inner pressure, the relationship between the diameter of a thin walled tube and the thickness of its wall must be linear.

The thickness of the perineurial sheath is affected by several variables including (1) the number of perineurial cell layers, and the combined width of both (2) the cellular layers and (3) the intervening interlamellar extracellular spaces. Sunderland and Bradley measured the thickness of the perineurial sheath from the inner- to the outer-most perineurial cell layer, therefore encompassing any concomitant variations within the three components of perineurial sheath width that may arise with fascicular area.

The fact that no significant correlation \( p<0.01 \) was found between the number of perineurial cell laminae and fascicular area in any of the three groups included in this study does not necessarily serve to undermine the theory of Sunderland and Bradley, as the contribution of alterations in cellular width or interlamellar space, which contains collagen and elastic fibres and the occasional fibroblast and macrophage (R.H.M. King et al., 1988), were not included by the methodological techniques utilised in this investigation. Despite this fact, the
individual strata of the perineurial sheath did appear to be generally more widely spaced both in the diabetic and HMSN I tissues compared with the nerves from the organ donor control group. Statistical analysis would be required before any definitive statement could be made. The number of perineurial cell layers in the nerves of patients with HMSN I was statistically greater than that in both the diabetic and control nerves and fascicular area was also considerably increased.

Nevertheless, the number of perineurial cell layers does appear to be a primary contributor to perineurial sheath thickness as demonstrated by the fact that the correlation between this variable and fascicular area was seen to approach significance in both the diabetic and HMSN I groups, at $p=0.0168$ and $p=0.051$ respectively. A significant correlation ($p<0.01$) may have been achieved with higher group numbers. It is possible that the lack of any significant correlation in the control group might stem from the fact that the average number of layers in healthy nerves might be routinely greater than that required to contain intrafascicular pressure under normal conditions, acting as a safety mechanism under conditions of acute insult.

Variations may exist between different peripheral nerves with respect to the standard relationship between the number of perineurial cell layers and fascicular area. If true, this phenomenon may partially account for the presence of anomalies between the results of this study and that of Sunderland and Bradley, as the majority of specimens examined in this investigations were sural nerves whilst the study by Sunderland & Bradley involved median, radial, ulnar and sciatic nerves.

The relationship between the thickness of the perineurial sheath and fascicular area has also been found to vary along the length of individual nerves; despite the lack of any corresponding alteration in diameter the thickness of the perineurial sheath was found to vary along the course of the median nerve (Sunderland & Bradley, 1952). This may be a protective measure against the effects of external factors i.e. occurring in the region of joints or may be related to the rate of intrafascicular fluid flow and hence intrafascicular pressure, or a change in the proportion of circular and obliquely orientated pressure resisting fibres to longitudinal fibres in the perineurium. Sampling discrepancies as a result of this phenomenon may be introduced in comparative studies.
5A.2 Perineurial cell BM thickness

In this investigation peripheral nerve PCBM thickness was found to be significantly greater in the diabetic patients than normal control individuals, a finding in support of previous observations in human diabetic dorsal root ganglia (Johnson, 1983b), dermal (Johnson & Doll, 1984) and sural nerves (Johnson et al., 1986; King et al., 1989). It is generally concluded that perineurial cell basement membrane (PCBM) thickening is one of the pathological features that distinguishes diabetic peripheral neuropathies from non-diabetic neuropathies, although the most obvious feature is persistent/rigid parent basal laminae around regenerating axonal sprouts (King et al., 1989). PCBM thickening has not been found to be a characteristic feature in either the sural or dermal nerves of non-diabetic neuropathic patients (Johnson et al., 1981); PCBM thickness in these individuals was found to be approximately equivalent to that of comparative control specimens obtained at autopsy (Johnson & Doll, 1984). Johnson and colleagues stated that the thickness of the sural nerve PCBM in diabetic neuropathic patients is significantly greater than that of age-matched patients with a comparative severity of non-diabetic neuropathies (Johnson et al., 1978).

The observations of this study have generally confirmed these findings as, although the thickness of the PCBM of a few lamellae in the patients with HMSN I was slightly greater than the values for the control group, the situation was reversed with regard to other layers, whilst in the majority of cases no significant difference could be detected between the two groups. By comparison PCBM thickness in the diabetic group was found to be significantly greater than that of the HMSN I patients in all but 4 of the 20 regions measured.

Abnormally thickened diabetic sural nerve PCBM have been described as containing small inclusions of collagen and being more irregular in contour and less electron dense than those of non-diabetics (Johnson et al., 1981). Contrary to these observations PCBM thickening in the dermal nerves of diabetic patients was reported as being uniform and diffuse as opposed to focal, the BM of one lamellae often fusing with that of the adjacent cellular layer (Beggs et al., 1989). Cellular or collagenous PCBM inclusions were rarely seen in these smaller nerves (Johnson & Doll, 1984).

In agreement with Johnson and associates, we found diabetic PCBM thickening to be patchy, focal regions of a comparatively greater depth being interspersed between thinner, uniform stretches of BM thickness. The hypertrophic BMs of
the perineurial sheath in the diabetic group were also frequently found to contain cellular debris and collagen fibrils.

Johnson and colleagues further stated that the PCBMs of diabetic nerves are less electron-dense that those of healthy control nerves (Johnson et al., 1981). This observation was not confirmed; the electron-density of the PCBMs in these two groups was similar, or even marginally greater in the diabetic group.

It is possible that PCBM thickening may be a passive process, resulting from thinning of, or a reduction in, perineurial cell volume, similar to that seen to occur in endothelial cells (Vrako, 1970). The lack of any demonstrable relationship between PCBM thickness and the thickness of the perineurial cell that it encloses serves to disprove this theory (Johnson, 1981).

Sural nerve PCBM thickening in diabetic patients receiving insulin therapy has been shown to be significantly greater than that in individuals receiving other treatments (Johnson et al., 1981). The reason for this finding is unclear. It may be related to the formation of immune complexes within the BM, a theory which is later discussed in greater detail.

Intercellular tight junctions, which are primarily located in the innermost layers of the perineurium (Olsson & Reese, 1971) are believed to be largely responsible for the efficacy of the perineurial permeability barrier. Structural alterations to these tight junctions have been reported in human diabetic sural nerves (Beamish et al., 1990). It is feasible that diabetic PCBM thickening occurs in order to help circumvent any alterations in the perineurial permeability barrier as a result of this mechanism, the abnormally thickened BM serving to delay the passage of, or bind detrimental agents. If this were the case, the extent of PCBM hypertrophy would be expected to be greatest in the innermost region of the perineurium. Alternatively, diabetic PCBM thickening may be a passive process related for instance to accelerated NEG secondary to hyperglycaemia.

No significant difference was found between inner and outer PCBM thickness in human diabetic dermal nerves (Johnson & Doll, 1984). This is not a wholly surprising observation as dermal nerves are very small and usually surrounded by only one to three layers of perineurial cells, and therefore the total thickness of the perineurium may be insufficiently great to result in the production of local variations in perineurial cell BM thickness.
In conflict with both of the aforementioned hypotheses, the results of this study, performed on larger sural nerves, have revealed that the severity of diabetic PCBM hypertrophy is in fact greatest in the central region of the perineurium, and that the innermost perineurial lamellae consistently exhibit the smallest degree of BM thickening.

If PCBM hypertrophy does occur in order to enhance the efficacy of the perineurial barrier or circumvent its deterioration under pathological conditions, these observations imply that the integrity of the tight junctions in the innermost layer of the sheath are conserved, and that the efficacy of the perineurial permeability barrier is primarily disrupted within its central and outer zones.

In general, no significant difference was found between the combined endoneurial and epineurial aspects of PCBM thickness in any of the three groups involved in this study. This finding implies that diabetic PCBM hypertrophy is not specifically aimed at preventing the movement of molecules in any particular direction across the perineurial barrier.

In all three groups, the BM on both sides of all the layers of perineurial cells, with the exception of the endoneurial aspects of the innermost lamellae, were devoid of a lamina lucida, the lamina densa extending right up to the plasmalemma of the perineurial cells. This feature has been previously noted in peripheral nerves (King et al., 1989). The reason for this structural anomaly is unknown.

5A.3 Degenerate/necrotic perineurial cells

The mean recorded percentage of degenerate or necrotic perineurial cells in the sural nerves of the diabetic patients was significantly greater than the corresponding values for both the HMSN I and control groups. The mechanism responsible for cellular degeneration, a process which has previously been noted to occur in the dermal nerves of diabetics (Johnson et al., 1986), is unknown. The degeneration of endoneurial fibroblasts has been documented by Grehl and Schröder in a variety of neuropathies including HMSN, paraproteinaemic neuropathy, chronic inflammatory demyelinating polyneuropathy and polyarteritis nodosa, its ubiquity leading to the suggestion that it is a non-specific feature of peripheral neuropathy (Grehl & Schröder, 1991).
Although the incidence of fibroblast degeneration was not assessed in diabetic neuropathy, the fact that perineurial cells are derived from fibroblasts (Thomas & Jones, 1967; Bunge et al., 1989) implies that the deterioration and loss of perineurial cells may be related to a similar phenomenon. The mechanism may be exacerbated by the diabetic state as the extent of perineurial cell degeneration was significantly greater in the diabetic nerves compared with the HMSN I nerves. It would be of considerable interest to determine whether endoneurial fibroblasts are similarly affected in diabetic polyneuropathy, and to draw comparisons between diabetic and HMSN I patients with reference to the extent of perineurial cell and fibroblast degenerative phenomena.

It is possible that the dimension of the enveloping basal laminal sheath may be directly related to the physiological condition of the parental perineurial cell, as we found that the severest degree of PCBM hypertrophy occurred in the zones of the perineurial sheath that contained the highest proportion of degenerate/necrotic cells. It is feasible that perineurial cells may produce abnormally thickened BMs in order to compensate for their deteriorating functional integrity, a proposal which merits further investigation. It has been suggested that BM thickening in the diabetic kidney is related to its reduced filtering abilities, a proposal which is discussed later in more detail.

5A.4 Sex/age and PCBM thickening

The results of this study conflict with prior testimony regarding PCBM thickening in the PNS and age. No statistical evidence was found in this study of any correlation between sural nerve PCBM thickness and age in the organ donor control group, results which contrast with those of Johnson and Doll who stated that dermal nerve PCBM thickness in healthy individuals increases with age (Johnson & Doll, 1984). These anomalies between the observations of this study and that of Johnson and Doll may be attributable to two facts. Firstly, capillary BM thickness has been found to correlate with age in both the skeletal muscles (Dunn et al., 1979) and peripheral nerves (Sima et al., 1991) of healthy individuals, a statistically significant increment becoming apparent in persons over the age of 40 years. If this phenomenon is similarly true of PCBM thickening, the fact that the organ donor controls in this study were relatively young (mean age 44.7 years; age range 28-55 years) compared with those in the study by Johnson and Doll (mean age 60.5 years; age range 31-82) may have served to mask this trend. Secondly, different nerves were used, introducing a potential source of variation.
In discordance with the situation in healthy sural nerves we found a positive correlation between PCBM thickness and age in the sural nerves of both the diabetic ($p=0.0035$) and HMSN I patients ($p=0.0102$), results which again conflict with those of Johnson & Doll (Johnson & Doll, 1984). It is possible that these observations are attributable similarly to incongruities in the ages of the patients, the mean age of our diabetic group was 39.2 years compared with that of 62 years in Johnson and Dolls' study and to differences in the duration of diabetes. Variability may have also been introduced due to the different nature of the peripheral nerves under investigation. It is unlikely that the difference between the two studies regarding the ratios of diabetic men to women is significant as diabetic PCBM thickening in the PNS has been found to be comparable between men and women, suggesting that the phenomenon is not sex-related (Johnson et al., 1981).

5A.5 **PCBM hypertrophy - correlation with duration of disease**

PCBM thickness was not correlated with the duration of diabetes mellitus in this investigation due to lack of data regarding the latter variable. A positive correlation has previously been described between these two variables in the dermal (Johnson & Doll, 1984) but not sural nerves (Johnson et al., 1981) of diabetic individuals. Tracer studies have revealed that the dermal nerves of rats do not possess a perineurial diffusion barrier (Oldfors, 1981b). If a perineurial permeability barrier is similarly lacking in human dermal nerves, PCBM thickening cannot be triggered as a compensatory mechanism, implying that PCBM hypertrophy is in reality a passive coincidental feature of diabetic neuropathy consequent upon alterations in the diabetic endoneurial milieu. This conclusion is supported by the lack of evidence of any breach in the integrity of the perineurial barrier to large molecules in animal models of diabetes (Jakobsen et al., 1978; Sima & Robertson, 1978a).

It is possible that PCBM thickening in the sural nerve may result from a combination of passive incidental and active compensatory mechanisms, as the sural nerve does possess a perineurial permeability barrier. If this were the case, the extent of PCBM thickening in the sural nerve would be expected to be comparatively greater than that in dermal nerves, a theory which requires validation. Unfortunately, as dermal nerves do not possess an intrinsic vascular bed, their metabolic demands being supplied by centrifugal flow or transperineurial diffusion or transport, the effect of diabetes on endoneurial capillary BM thickening in these peripheral nerves is undeterminable.
5A.6 Perineurial calcification

We found perineurial calcification to be fairly prominent in the sural nerves of the diabetic group, a feature previously noted by Kalimo and colleagues (Kalimo et al., 1981). Frequent, and in some instances extensive, accumulations of extracellular electron dense perineurial deposits have previously been reported within the sciatic and sural nerves of patients with both diabetic (Paetau & Haltia, 1976; R.H.M. King et al., 1988) and non-diabetic neuropathies (Van Lis et al., 1979; King et al., 1988).

The composition of these deposits has been determined by X-ray microanalysis and energy loss spectroscopy and it has been revealed that, although trace amounts of magnesium, sodium and chlorine are present (Paetau & Haltia, 1976) they are predominantly composed of calcium and phosphorus (King et al., 1988) the ratio of which, at 18% and 40% respectively, is approximately the same as that in hydroxyapatite (Paetau & Haltia, 1976). The highly crystalline nature of these deposits as disclosed by electron-diffraction studies (Paetau & Haltia, 1976) and their occasional spicular appearance in electron micrographs (R.H.M. King et al., 1988) complies with this proposal.

The pathogenesis and localisation of metastatic calcification are not entirely understood, but certain local factors are believed to be instigatory in the process. The perineurium possesses several attributes which suggest an intrinsic predisposition to the process. The lowered glomerular filtration rate in renal failure leads to phosphate retention which in turn causes a reduction in plasma calcium. This disturbance in divalent ion metabolism results in an abnormal increase in the calcium phosphorus ion product, metastatic soft tissue calcification occurring when the value of this product exceeds 70 (David, 1975).

Tracer studies have revealed that the innermost layers of the perineurium constitute the tightest region of the barrier and are probably responsible for the fact that the majority of extraneural compounds are unable to totally traverse the perineurial barrier (Olsson & Reese, 1971). Certain small ions are able to gain access eventually to the endoneurium, selective permeability in these cases being believed to depend upon the physical dimensions of the intercellular routes and actual molecular size. Locally applied extraneural phosphate ions are only able to penetrate the endoneurium to a small degree (Causey & Palmer, 1953).

The possibility exists that the efficacy of these barrier mechanisms (Söderfeldt et al., 1973) may lead to the production of abnormally high extracellular
concentrations of phosphate and calcium ions within the central and outer regions of the perineurium, the development of a comparative calcium phosphorus ion product to that associated with metastatic calcification in renal failure (Paetau & Haltia, 1976) and the subsequent formation of hydroxyapatite. The fact that calcified perineurial deposits have only been found to occur between the outer or central layers of the perineurium (Paetau & Haltia, 1976; Van Lis et al., 1979) provides evidence in support of this mechanism.

The results of this study, in agreement with a more recent investigation (R.H.M. King et al., 1988) suggest that perineurial calcification within diabetic nerves occurs predominantly within the central zone of the perineurium. The severest degree of diabetic PCBM hypertrophy found in this investigation was seen to occur within this same region. Abnormalities in the molecular structure and composition of diabetic BMs may result in subsequent alterations in its partially charge-selective, partially physical barrier function. This fact in combination with the exceptional thickness of the PCBM at these sites may further serve to restrict the passage of calcium and phosphate ions and play a contributory aetiological role in their accumulation at these sites.

Local ATPase activity at the site of mineralisation may also participate in the process of metastatic calcification, ATP reacting with free calcium ions to form a complex which is initially hydrolysed to pyrophosphate and subsequently to hydroxyapatite. Local alkaline phosphatase and ATPase activity have both been demonstrated within the perineurium (Shanthaveerapa & Bourne, 1962). The presence of a calcifiable matrix, i.e. elastic fibres, collagen fibres and mucoproteins, has been implicated as an essential factor in metastatic calcification (Neff et al., 1974). Such ECM elements are common intercellular perineurial components.

Van Lis and colleagues presented evidence which suggested that the accumulation of perineurial calcified deposits was immunologically associated with that of lipids (Van Lis et al., 1979). These lipids were believed to correlate structurally with either myelin debris being transported out of the endoneurium following demyelination, pathological changes in the nerves, or interlamellar membranous vesicles from degenerate/necrotic perineurial cells or fibroblasts. It has been proposed that these cellular degradation products may absorb large amounts of calcium ions from the surrounding extracellular matrix. Although quantitative analysis was not attempted in this study, calcium deposits were often seen to be co-localised with cellular debris.
PCBM hypertrophy and the perineurial permeability barrier

The contribution of diabetic PCBM thickening to the development of a neuropathy, if any, is at present undetermined. The abnormal accumulation of BMs around cells must undoubtedly have some effect on their functional role (Abrahamson, 1986; Brownlee et al., 1988).

Abnormal depositions of immunoglobulins (IgM) and complement (C3) have been cited within the PCBMs of human diabetic peripheral nerves. It has been suggested that these may reflect the presence of associated alterations in the perineurial permeability barrier and effectively contribute to NEG-related BM thickening, a theory which parallels the proposed aetiology of diabetic GBM thickening (Miller & Michael, 1976). Loss or degeneration of the perineurial permeability barriers' selectivity may theoretically lead to fibre abnormalities (Powell, 1983; Powell et al., 1985) and axonal loss.

As previously stated there is limited evidence to suggest that diabetes has any effect on the perineurial permeability barrier of peripheral nerves. It seems likely that the nonspecific accumulation of IgM etc within PCBMs is AGEP-associated; however, the presence of these factors does not necessarily imply that any actual penetrative breach of the perineurial barrier has occurred; numerous tracers have been found to penetrate the perineurium of healthy individuals to a considerable depth. As such, the deposition of these factors (Graham & Johnson, 1985) cannot be ascribed as being of any pathogenetic relevance.

It would be interesting to determine whether there is any correlation between the degree of PCBM thickening and the severity of neuropathy in the peripheral nerves of diabetic patients. The demonstration of a positive relationship does not, however, necessarily imply the existence of a cause and effect association, but may merely reflect the coincidental development of these alterations.

DIABETIC MICROANGIOPATHY

Comparative methodological techniques

Tissue processing

Siperstein and colleagues proposed that the physical dimensions of BMs are affected by the method of tissue processing, primary fixation in buffered glutaraldehyde and postfixation in osmium tetroxide as opposed to primary fixation in osmium tetroxide resulting in the appearance of a comparatively thicker capillary BM. In addition it was suggested that differential effects are
produced on normal and diabetic BMs, the former being affected to a significantly greater degree than the latter and as such the prevalence of diabetic capillary BM thickening reported in studies which have employed this methodology will be artifactually lower than those in which the tissue was primarily fixed in osmium tetroxide (Siperstein et al., 1973).

These observations were later questioned by Williamson and colleagues who found that primary fixation with glutaraldehyde as compared to osmium tetroxide resulted in a prevalence of diabetic muscle capillary BM thickening of 65% and 30% via the respective methods, suggesting that glutaraldehyde fixation does not result in reduced sensitivity of differential measurements or selectively enhance BM thickening in control tissues (Williamson et al., 1976b).

5B.1(ii) Quantification

The thickness of interstitial skeletal muscle capillary BMs has been evaluated by a variety of techniques. Siperstein and workers favoured using the mean value of 20 measurements of BM thickness taken across equidistant radiating lines (avoiding any regions which were obviously artifactually thickened as a result of tangential sectioning) on the basis that this method accounts for both focal and segmental variations in BM along the vessels (Siperstein et al., 1968). This method has subsequently been used in the quantification of diabetic capillary BM thickness in both the endoneurium of peripheral nerves in rats (Sharma & Thomas, 1974) and human skeletal muscle (Siperstein et al., 1973).

Williamson and colleagues preferred an alternative technique whereby capillary BM thickness is assessed by the mean value of two minimum measurements taken at points not less than 1cm apart on an electronmicrograph (Williamson et al., 1969). The proposed advantage of this technique is that it avoids overestimation of capillary BM thickness due to tangential sectioning and non-uniform section thickness. This method has also been utilised in the quantification of both human diabetic peripheral nerve endoneurial capillary (Behse et al., 1977) and skeletal muscle capillary (Williamson et al., 1969; Kilo et al., 1972; Jordan et al., 1972) BM thickness.

Taking the mean value of a larger number of measurements of BM thickness would appear to be the more accurate method as the error attributed to the inclusion of obliquely sectioned zones of capillary BM in the determination of BM thickness by this procedure has been shown to be relatively small at approximately 10%, whereas evaluation by minimum thickness may
underestimate BM thickness by as much as 20-40% due to the avoidance of naturally occurring regions of focal or segmental thickening (Siperstein et al., 1973). Discrepancies between these two methods in the estimation of BM thickness was confirmed in a comparative study; the average of the mean values of healthy human muscle capillary BM thickness was evaluated at 0.134 µm whilst the average of the minimum values was 0.087 µm (Williamson et al., 1969).

The generation of computer assisted values of BM thickness, in addition to other morphological variables, have been used in more recent studies. This method has the advantages that it both takes focal variations in BM into account and provides a considerably more accurate value of overall BM thickness. Computised measurements have been used in comparative studies of capillary BM in both skeletal muscle (Ramirez et al., 1991) and peripheral nerves (Malik et al., 1992).

The majority of quantitative and comparative studies investigating microangiopathic and related abnormalities in peripheral nerves have been performed on tissues which have been processed in an equivalent manner to that utilised in this study i.e. primary fixation in buffered glutaraldehyde followed by secondary fixation in osmium tetroxide, dehydration through a graded series of alcohols and embedding in an epoxy resin (Malik et al., 1989a, 1992). In addition measurements of BM thickness have generally been digitally computised. As such the contribution of artifactual physical variations due to differences in processing techniques, and the generation of discrepancies as a result of incongruities between the quantitative methods employed in the origin of conflicting observations between these studies can be largely eliminated.

5B.2 The prevalence of BM hypertrophy

Basement membrane hypertrophy is the most characteristic morphological feature of diabetic microangiopathy and has been reported in heart (Silver et al., 1977), retina (Bloodworth, 1963; Ashton, 1974), skin (Aeganaes & Moe, 1961), kidney (Osterby, 1974; Bendayan, 1985) and skeletal muscle (Siperstein et al., 1968; Kilo et al., 1972; Williamson & Kilo, 1977; Kilo & Williamson, 1979). Abnormal thickening of peripheral nerve endoneurial capillary BMs has also been documented in both human (Vital et al., 1973; Guy et al., 1984) and experimental diabetes (Powell et al., 1977; Sima et al., 1982).
Looking at a specific example, interstitial muscle capillary BM thickness in diabetic patients is significantly greater than that in healthy controls (Zacks et al., 1962; Siperstein et al., 1968; Jordan et al., 1972; Kilo et al., 1972; Ellis et al., 1986), the reported range of mean values in diabetic patients being 0.18-0.204\(\mu m\) compared with that of 0.11-0.14\(\mu m\) in controls. One study reported that the difference between capillary BM thickness in diabetic and healthy subjects only achieved significance in individuals over the age of 40, a fact which was attributed to the dual effects of age and disease (Jordan et al., 1972).

The prevalence of diabetic skeletal muscle capillary BM thickening found by Jordan and colleagues was comparatively low, compared with the 98% reported by Siperstein (Siperstein et al., 1968) 8 out of the 37 diabetics included in the study exhibited no signs of BM hypertrophy (Jordan et al., 1972). These discrepancies might result from variations in experimental methodology. The prevalence of diabetic muscle capillary BM thickening in tissue which has been primarily fixed in osmium tetroxide and BM thickness evaluated by the mean measurement technique was found to be 100% compared with 75% using the same method of tissue processing but the minimum value method of assessing BM thickness, 65% using primary fixation in glutaraldehyde and the mean value of evaluating BM thickness and as little as 45% when the tissue was fixed in glutaraldehyde and BM thickness was determined by measurement of the minimum value (Siperstein et al., 1973).

5B.3 Capillary BM hypertrophy

5B.3(i) Correlation with sex and age

Human skeletal muscle capillary BM thickness has generally been found to correlate positively with age in healthy individuals (Jordan et al., 1972; Dunn et al., 1979) having been reported to be approximately 0.064\(\mu m\) at 11 years old and 0.105\(\mu m\) at the age of 70 (Kilo et al., 1972). Some authors have however failed to find evidence of any such relationship (Siperstein et al., 1968). Sex-related differences were apparent in individuals between the ages of 40 and 60 years, men possessing a considerably thicker capillary BMs (Siperstein et al., 1968), and equivalent values between the two sexes were only attained in post-menopausal women (Kilo et al., 1972).

Conflicting testimony has been provided as to the existence of a similar phenomenon in individuals with diabetes mellitus. Although confirmatory reports have been published (Kilo et al., 1972; Kilo & Williamson, 1979; Dunn et
al., 1979), a significant difference being apparent between patients when
categorised into two groups, those above and those below the age of 40
(Jordan et al., 1972), numerous studies throughout the years have failed to find
any evidence of a relationship between muscle capillary BM thickness and
diabetic patient age, despite a considerable degree of variation in the techniques
employed (Siperstein et al., 1968; Ellis et al., 1986; Ramirez et al., 1991).
These latter findings, and the lack of any apparent sex-related difference, may
be due to masking of age and sex-related effects on capillary BM thickness by
the comparatively greater influence of the disease.

A correlation has also been recently demonstrated between human sural nerve
endoneurial capillary BM thickness and age in healthy controls (Sima et al.,
1991). Malik and colleagues later failed to find any evidence of such a
relationship between these two variables in the sural nerves of clinically mild
IDDM and NIDDM diabetic patients (Malik et al., 1992). As in the situation with
skeletal muscle capillaries, this is probably attributable to masking by the
concurrently greater influence of the disease and its period of duration on the
generation of BM hypertrophy as young IDDM diabetic patients were found to
have a significantly thicker capillary BMs than healthy individuals of an
equivalent age (Vracko, 1982; Sima et al., 1991).

Sharma and Thomas (1974) reported a slight increase in endoneurial capillary
BM thickness in older streptozotocin- and alloxan- diabetic rats; however, the
contribution of diabetes to this phenomenon can be discounted as it is an
accepted fact now that experimental diabetes in animal models, unlike the
situation in human diabetes mellitus, does not result in the production of
capillary BM hypertrophy.

In summary, it is generally agreed that capillary BM thickness in most tissues of
healthy individuals increases with advancing age and that sex-related difference
may also exist. These facts underlie the importance of selecting age- and
sex-matched controls in comparative studies of the effect of diabetes mellitus on
endoneurial capillary BM hypertrophy, considerations which have not always
been taken into account in related studies.

5B.3(ii) Correlation with duration of disease

Kilo and colleagues, in a comprehensive study incorporating diabetic and control
groups composed of approximately 150 individuals, reported the existence of a
highly significant correlation, following the elimination of statistical "outliers",
between muscle capillary BM thickness and the duration of diabetes mellitus (Kilo et al., 1972). However, as the majority of studies, some of which have involved large patient numbers i.e. 50, have produced conflicting testimony (Zacks et al., 1962; Siperstein et al., 1968; Raskin et al., 1983; Ellis et al., 1986; Ramiez et al., 1991) it is generally accepted that there is no relationship between muscle capillary BM thickness and the duration of diabetes. The development of diabetic retinopathy and GBM thickening however related to duration of diabetes mellitus (Osterby, 1974).

58.4 Endoneurial capillary BM hypertrophy

Endoneurial capillaries in this and related studies (Malik et al., 1992) were identified by the presence of a single layer of endothelial cells in the absence of any complete encirclement by other cell types i.e. smooth muscle cells.

Endoneurial capillary BM thickness in the sural nerves of our control group was found to range from 1.83 to 2.97\(\mu\)m, with a median value of 2.28\(\mu\)m. These results are largely comparable with those of previous reports, although a slightly greater range, at 1-4\(\mu\)m, was described by Behse and associates (Behse et al., 1977). The width of the basal laminal zone of endoneurial capillaries in the PNS is therefore considerably greater than that of intramuscular capillaries (Behse et al., 1977).

The results of this study confirm the widely held observation that endoneurial capillary basal laminal hypertrophy is a recognised component of diabetic polyneuropathy (Aeganaes & Moe, 1961; Bischoff, 1965; Vital et al., 1974; Behse et al., 1977; Guy et al., 1984), as in accord with previous studies (Timperley et al., 1985; Powell et al., 1985; Yasuda & Dyck, 1987; Malik et al., 1989a; Britland et al., 1990; Malik et al., 1992) sural nerve endoneurial capillary BM thickness and area in the diabetic group of patients were found to be significantly greater than the corresponding values for the organ donor control group. The correlation is not absolute. Malik and colleagues recently reported on a case of Mendenhall's syndrome, a disease associated with a severe diabetic polyneuropathy, where the microvessels in the sural nerve showed only a very mild degree of microangiopathy despite a concomitant gross loss of myelinated nerve fibres (Malik et al., 1995).
The artifactual contribution, if any, of the effect of discrepancies between the general calibre of endoneurial vessels in the diabetic nerves compared with that in the control nerves can largely be discounted as there appears to be no relationship between vessel calibre and BM thickness; Jordan and colleagues demonstrated that the largest interstitial capillaries in human skeletal muscle do not necessarily possess the thickest BMs (Jordan et al., 1972).

The hypertrophic diabetic endoneurial capillary BMs were seen to have a typically multilayered appearance, the distinct strata being interspersed with zones of extracellular matrix rich in fine collagen fibres. This appearance is characteristic (Vital et al., 1973; Malik et al., 1992). We made no attempt to quantitate the number of concentric layers within the basal laminal zone, the fragmented or compacted nature of the layers of BM in some vessels making this impossible, although we did observe that the number of layers of BM surrounding the diabetic vessels appeared to be substantially greater than that around control vessels. Despite our concerns regarding the accuracy of such comparisons, Behse and colleagues reported that the number of constituent lamellae associated with the diabetic endoneurial vessels is significantly greater than that for controls capillaries, at 11-17 and 5-10 respectively (Behse et al., 1977). The width of the individual layers of diabetic endoneurial capillary BMs has also been reported as increased (Bischoff, 1965), a statement which we found to be true in some instances.

The reported prevalence of diabetic endoneurial capillary BM hypertrophy in the PNS has not been found in all related studies. The reasons for these discrepancies probably parallel those previously stated in explanation of incongruities in the prevalence of diabetic skeletal muscle interstitial capillary BM thickening. For example Behse and colleagues described the presence of BM hypertrophy, constituted either by an increase in the width of the BM or the number of component lamellae, in only half of the 24 diabetic patients included in their study. These findings can be attributed to the fact that comparisons drawn using measurement of the minimum thickness of the BM which, for reasons as stated earlier, may lead to errors in estimation of as much as 20-40% (Behse et al., 1977).

The apparent thickness of capillary walls (endothelial cells and BMs) has been found to be affected by the method of fixation; the width of these variables are significantly greater following immersion as compared with perfusion or in situ fixation as a direct consequence of vessel collapse or vasoconstriction. Area,
however, is unaffected and therefore would appear to be the most appropriate parameter to measure in comparative studies of microvascular disease (Schenone & Dyck, 1986), although basement membrane thickness might seem a more suitable variable for assessment of possible alterations in capillary permeability.

Considering the fact that the peripheral nerves biopsies of all three groups examined in this study were fixed by approximately the same process, statistical analysis was performed on measurements of both BM thickness and area as the degree of collapse was likely to be comparable. It could be argued that the abnormally thickened capillary BMs in the diabetic nerves may be more rigid and subsequently more resistant to collapse than those of the other two groups introducing an element of artifactual error. As the statistical differences between the three groups regarding endoneurial capillary BM area were reflected by those of BM thickness, this does not seem to be a realistic scenario.

58.5 Increased production of BM components

The increased synthesis of BM components is a mechanism which may potentially be of considerable importance in diabetic basal laminal hypertrophy. The distribution and relative proportions of the constituent elements within normal BMs i.e. collagen type IV, laminin, fibronectin and heparan sulphate proteoglycan have been determined (Laurie et al., 1982; Yurchencho & Schittny, 1990) enabling related studies to detect the presence of any abnormalities within diabetic BMs. The majority of studies have confirmed that alterations in these values and ratios do occur in diabetes, although some reports have provided conflicting testimony.

The production of collagen by cultured skin fibroblasts from patients with type I diabetes mellitus, as indicated by the incorporation of radioactive proline into collagen within the medium, increases in proportion to the glucose content of the media, a 75% increase in production being demonstrable at a concentration of 5mg/ml as compared with the rate at the basal physiological glucose concentration of 1mg/ml (Kjellström, 1986). No correlation was found between donor age and collagen production. Although collagen production was also stimulated in the control cultures, this occurred to a considerably smaller degree and only at the higher glucose concentration.

These results suggest that hyperglycaemic blood glucose levels effectively stimulate the production of collagens by diabetic fibroblasts which appear to
have somehow been rendered particularly sensitive to ambient glucose concentrations. The effect on type II cells was negligible or minimal, a fact for which no reasonable explanation could be found. *In vivo*, the rates of both retinal collagen synthesis (Reddi, 1985) and glomerular BM synthesis (Grant et al., 1976; Brownlee & Spiro, 1979; Cohen et al., 1982) have been shown to be accelerated in diabetic rats and the total content of collagen type IV has been found to be increased in diabetic GBMs (Falk et al., 1983a; Taylor et al., 1980).

Further *in vivo* evidence of accelerated collagen synthesis is supplied by the observation that the serum levels of collagen type IV, as determined by the immunological assessment of serum type IV collagen peptide content, are significantly greater in diabetic patients than healthy subjects, reflecting increased de novo synthetic activity within diabetic BMs; excessively synthesised collagen IV molecules overflowing into the blood stream (Matsumoto et al., 1990).

The activity of enzymes which are involved in the synthesis of collagens, including prolyl-hydroxylase (Cohen & Khalifa, 1977a), lysyl-hydroxylase (Cohen & Khalifa, 1977a) and glucosyl transferase (Spiro & Spiro, 1971), the latter two of which catalyse the synthesis of disaccharide units, have also been found to be increased in diabetic renal glomeruli.

Increases in the serum content of collagen type IV peptides (which reflect accelerated collagenesis) were also found to correlate both with the duration of diabetes mellitus and the clinical severity of many complications which are associated with microangiopathic alterations such as nephropathy and retinopathy (Matsumoto et al., 1990), evidence which supports the supposition that hyperglycaemia plays a causal role.

The production of laminin has been reported to be similarly enhanced by diabetes, increased content having been reported in EHS tumours from diabetic (db/db) mice (Rohrbach et al., 1982) and GBMs (Falk et al., 1983a). By contrast, in a concurrent study, the composition of hypertrophic EHS tumour BMs, synthesised as a result of hyperglycaemia secondary to insulin deficiency, was found to be normal (Philajaniemi et al., 1982). Similarly, the levels of mRNA encoding the B1 chain of laminin were found to be unchanged in the kidney cortex of KKAy diabetic mice despite a concommitant increase in BM thickening (Ledbetter et al., 1990).
As mRNA levels encoding collagen type IV were simultaneously increased the relative proportion of these components within the newly synthesised BM must reflect this ratio, i.e., the content of laminin must be comparatively reduced compared with that in normal equivalent BMs, a fact which complies with a previous report of reduced content of laminin within human diabetic GBMs (Shimomura & Spiro, 1987). The glomerular synthesis of fibronectin has also been found to be increased in diabetic kidneys (Roy et al., 1990).

Additional evidence of diabetes-related increases in the production of BM components is supplied by the fact that the expression of mRNA levels for collagen type IV, fibronectin and laminin B₁ by cultured human endothelial cells (Cagliero et al., 1988, 1991) and mRNA for collagen type IV by retinal capillary endothelial cells (G.L. King et al., 1988) is increased in high-glucose media with an accompanying proportionally increased synthesis of these proteins. mRNA levels for collagen type IV have also been found to be increased in concordance with BM thickening in the kidney cortex of KKAy diabetic mice (Ledbetter et al., 1990), and mRNA levels for laminin B₁ have been found to be increased in the kidneys of streptozotocin-diabetic rats (Poulsom et al., 1988; Giambrone et al., 1989).

5C MECHANISMS OF BM HYPERTROPHY

The factors responsible for altered BM metabolism and the molecular basis of basal laminal hypertrophy in diabetes mellitus remain unresolved. The fact that nonvascular and vascular BMs are similarly affected in DM suggests that BM thickening may be a generalised phenomenon of the disease, occurring as a result of factors other than those which exert an exclusive influence on any particular cell type, i.e. endothelial cells.

Ultrastructural investigations of muscle capillary (Barnett et al., 1983) and dermal nerve perineurial cell (Beggs et al., 1989) BM thickness involving identical twins discordant for diabetes mellitus have revealed that dysmetabolism rather than genetic factors play a causative role in BM hypertrophy as BM thickness in both tissues was found to be significantly greater in the diabetic twin, and the development of GBM thickening has been reported in non-diabetic healthy kidneys from living donors or cadavers following their transplantation into diabetic patients (Mauer et al., 1983).

Amelioration of the hyperglycaemic environment in the early stages of diabetes has been found to prevent the progression of hypertrophic microangiopathic BM
abnormalities. The institution of insulin therapy and normalisation of ambient blood glucose levels at the onset of diabetes has been found to be effective in preventing the development of collagenous abnormalities in experimental models (McLennan et al., 1986) including GBM (Cohen & Khalifa, 1977b; Rasch, 1980) and retinal capillary BM thickening (Chakrabarti & Sima, 1987). Pancreatic islet transplants in alloxan-diabetic rats have also been found to prevent hypertrophic changes in muscle capillary BMs (Bell et al., 1980), all evidence that implies hyperglycaemia is of critical importance in the generation of this component of diabetic microangiopathy.

5C.1 **(1) Non-enzymatic glycation (NEG)**

The process of non-enzymatic glycation (NEG) is initiated by the covalent attachment of free uncharged glucose molecules either to the free amino group on the N-terminus of proteins and/or binding to the ε-amino group of sidechain residues i.e. lysine along the polypeptide chains. This results in the fully reversible formation of ketoamine Schiff base adducts (aldimines) which rapidly and spontaneously rearrange to form slightly more stable early glycation or "Amadori" products. The formation of Amadori products is also reversible and as such the content of both Amadori products and aldimines, which reach equilibrium and constant steady-state levels over a period of weeks, is completely dependent upon ambient glucose levels.

In proteins with short half-lives of days or weeks, such as myelin, this is as far as the process of NEG is able to progress. In long-lived proteins, such as collagens and axonal proteins, these early glycation adducts may slowly undergo a complex sequence of dehydrations and condensations known as "Amadori rearrangement" leading to the eventual and irreversible formation of stable, persistent, complex, highly cross-linked, insoluble carbohydrate-protein polymers which are collectively known as advanced glycosylation end-products or AGEPs.

The slow accumulation of AGEPs throughout the life of the protein, which occurs at a rate that is proportional to the equilibrium concentration of Amadori products, occurs over the years and leads to an increase in the incidence of inter- and intraprotein covalent, heat-stable, disulphide cross-linking (Brownlee et al., 1984). The functional capacities of tissue proteins may be modified by NEG due to disorganisation of the core proteins' 3-D structure.

Only one class of AGEPs have been identified to date. These are heterocyclic pyrrole-based structures which appear to result from a reaction between an
Amadori product and an Amadori-derived fragmentation product (Brownlee 1991). 5-hydroxy-methyl-1-alkylpyrrole-2-carbaldehyde or pyralline (AFGP) is a member of this group. The heterocyclic imidazole derivative 2-furoly-4(5)-(2-furanyl)1-H-imidazole (FFI) was proposed as a representative of another class of AGEPs, following its isolation and identification from in vitro NEG proteins (Pongor et al., 1984), believed to result from the condensation of two Amadori products. Ammonia and furosine are produced during the strong acid hydrolysis required to cleave proteins into their constituent amino acids and it has subsequently been revealed that FFI is in fact a product of the reaction between these two compounds and not an AGEP.

It is not easy to quantify the content of AGEPs directly within tissues as treatment to solubilise or degrade insoluble proteins such as collagen results in alteration or destruction of AGEPs due to loss of inherent cross-linking. The majority of studies have therefore evaluated the degree of tissue NEG, by determining the content of glycated amino acid residues, although once again the results may be affected by the loss of sugars during the extraction and preparation processes, especially in the case of insoluble matrix proteins. The extrapolation of information provided by these assays to the aetiology of associated complications in vivo is limited by the fact that they only evaluate the extent of early NEG, as determined by ketoamine adducts, whereas the content of AGEPs is probably of considerably greater pathogenetic importance.

The amount of native protein-linked fluorescence has been utilised as a means of gauging the tissue content of advanced AGE products as by their nature AGE products are fluorescent yellow-brown chromophores (Dominczak et al., 1990), the emission of fluorescence at 440nm following excitation at a wavelength of 370nm being believed to result from the presence of constituent heterocyclic aromatic rings. The fluorescence spectra of collagen adducts in both healthy elderly and diabetic individuals have been shown to be identical to that of in vitro glycosylated collagen (Kohn et al., 1984).

Polyclonal antibodies to specific AGEPs have recently been produced; however, this method of detection also fails to guarantee accurate determination of the AGEP content of tissues as these antibodies are highly specific for particular types of AGE compounds and as such phenotypically different AGEP will not be labelled (Sensi et al., 1991).
5C.1(i) Association with age, hyperglycaemia and diabetes mellitus

The NEG of proteins, particularly those with a long half-life, is a common post-translational modification in normal tissues and occurs as a direct consequence of the glucose-rich milieu which constantly bathes living tissues. NEG is an important mechanism in the process of ageing, as demonstrated by the fact that the degree of in vitro NEG of proteins, which results in the formation of melanoidins via the browning reaction (Kennedy & Baynes, 1984), correlates positively with increasing age (Kohn et al., 1984). As NEG constitutes one of the mechanisms responsible for tissue ageing it is also therefore characteristically progressive and irreversible under normal physiological conditions and commences or accelerates following developmental maturation.

It has been proposed that the formation of AGE products on long-lived proteins in conjunction with their subsequent conformational rearrangement renders them "recognisable" to macrophages and stimulates rapid internalisation and intracellular processing. It has been suggested that this mechanism is mediated via specific high-affinity AGEP-protein cell surface receptors (Haberland et al., 1982a; Vlassara et al., 1985) which appear to be distinct from other senescent protein scavenger-receptors which recognise covalently modified proteins by changes in their charge and/or configuration (Vlassara et al., 1986). Early reversible glycation products on myelin proteins do not elicit such a response in macrophages, in accord with the proposal that a threshold level of modified residues is required to initiate the pathway (Haberland et al., 1982). This is the only route by which NEG-modified proteins may be eliminated from the system.

Hyperglycaemia per se is a condition which is known to enhance the NEG of a variety of structural and circulating proteins, the rate and severity of which is a function of both glucose concentrations and duration of exposure (Tarsio et al., 1985). Even modestly elevated levels of blood glucose are capable of resulting in a quite significant accumulation of AGE products as the rate of the reaction is second order with respect to the glucose concentration (i.e. proportional to the square of this value) (Brownlee, 1991). Considering the fact that the tissues of diabetic patients are commonly exposed to hyperglycaemic conditions during periods of poor control it does not seem unreasonable to propose that the degree of NEG in diabetic patients is likely to accelerate and therefore be comparatively greater than that in age-matched members of the general population.
This theory has been proven correct. The content of NEG-amino acids within peripheral nerves has been found to be markedly increased in experimental diabetes (Vlassara et al., 1981), the major proportion of the near 3-fold increase being constituted by glycated lysine and its associated Amadori products. The levels of NEG plasma fibronectin in alloxan-diabetic dogs have been found to be increased in proportion to blood glucose levels, the process of NEG only occurring on the lysine residues of the protein molecules (Tarsio et al., 1985).

The degree of NEG has also been shown to be enhanced in diabetes mellitus; compound immunoreactivity has revealed that albumin-enriched plasma samples from diabetic patients contain elevated levels of the AGEP pyralline compared with those of control subjects (Hayase et al., 1989) and abnormally large accumulations of AGEPs have been found on long-lived proteins within numerous tissues and organs of diabetic patients. The two most frequently documented examples involve NEG of the lens protein α-crystallins and haemoglobin A, both of which have been proved to be structurally and functionally modified by the process (Stevens et al., 1977, 1978).

NEG of the lens protein α-crystallin, which is an extremely long-lived protein, results in the increased susceptibility of constituent sulphydryl groups to oxidation and the subsequent formation of abnormal intra- and intermolecular disulphide bridging within and between indigenous protein molecules (Cerami et al., 1988). The actual mechanism evoked in this process is not clearly understood, but it is believed that molecular confirmational rearrangement of the protein molecule following NEG may serve to expose the hitherto hidden sulphydryl groups (Monnier et al., 1979) as the administration of disulphide-bond reducing agents in diabetic animals results in a reduction in lens opacification.

It has been proposed that the NEG-associated polymerisation of lens proteins, in association with the more major effects of sorbitol accumulation, may play a small role in the formation of the high molecular weight aggregates instrumental in the development of diabetic (Kinoshita, 1975; Stevens et al., 1978) and experimental sugar cataracts (Monnier et al., 1979). Subsequent studies have provided testimony which conflicts with this proposal, no significant difference having been found between the levels of glycated lens crystallins in both human and experimental diabetes and those in normal controls (Chiou et al., 1981).

In addition to the major haemoglobin (haemoglobin A), the adult human erythrocyte contains a number of minor derivative haemoglobins, haemoglobin
A$_{is}$, haemoglobin A$_{ib}$ and haemoglobin A$_{ic}$, which are sensitive to plasma glucose content and whose concentrations therefore effectively reflect the previous weeks' blood sugar levels. The concentration of these minor haemoglobins in diabetic patients has been found to be elevated, 6 to 12% of the total haemoglobin content occurring as haemoglobin A$_{ic}$ and 2 to 3% as haemoglobin A$_{is+b}$ compared to 3 to 6% and 1 to 2% respectively in normal healthy individuals.

These anomalies presumably reflect the presence of increased concentrations of glycolytic intermediates which possess the two essential requirements for adduct formation during NEG, namely the presence of a phosphate group and a free aldehyde i.e. glucose-6-P, fructose-1-6-P etc (Bunn et al., 1976). The phosphate group functions to position the aldehyde at the correct location on the haemoglobin molecule to allow adduct formation. Corroborative evidence is provided by the observation that an adduct which is analogous with haemoglobin A$_{ib}$ is formed in vitro as the product of a reaction between the amino-terminal of the beta chain of haemoglobin A and phosphorylated hexoses or trioses containing a free aldehyde or ketone group (Stevens et al., 1977).

Haemoglobin is glycated at both intrachain lysine and amino-terminal valine residues (Kennedy & Baynes, 1984). Although the number of NEG lysine residues on the protein significantly exceed those of valine, it is the latter abnormality which is of primary importance in the generation of functional alterations. The rate of adduct formation correlates with the concentration of glycolytic intermediate at a given consistent concentration of haemoglobin. As a result of this process periodic monitoring of minor haemoglobin levels provides an objective method of assessing the degree of carbohydrate control in diabetic patients (Koenig et al., 1976).

5C.1(ii) Correlation with incidence of diabetic complications

Components within numerous tissues and organs whose structure and/or function are known to be pathologically altered by diabetes have been found to undergo accelerated NEG. Examples include basic nerve myelin proteins (Vlassara et al., 1983), haemoglobin (Stevens et al., 1977), red blood cell membrane proteins (Miller et al., 1980), plasma proteins i.e. albumin (McFarland et al., 1979; Guthrow et al., 1979), lens alpha-crystallin (Cerami et al., 1988) and collagens (skin) (Lyons & Kennedy, 1985). This fact seems to suggest that the NEG of both short and long-lived proteins may be of some significance in the aetiology of associated diabetic complications, for example in collagen rich lung
tissue (Perejda & Uitto, 1982) NEG-related abnormalities in collagen and elastin may be responsible for the reduced lung elastic behaviour seen in juvenile diabetes (Schuyler et al., 1976), and NEG of erythrocyte cell membrane proteins may be responsible for diabetes-related alterations in deformability, microviscosity and increased oxygen affinity (McMillan et al., 1978).

A correlation has been demonstrated to exist between both the severity of diabetic neuropathy and the level of glycosylated haemoglobin derivatives (Drury et al., 1982), evidence which, providing that the degree of haemoglobin NEG accurately reflects the extent of generalised NEG within other tissues, supports the theory that accelerated NEG may be of pathogenetic importance. By contrast, no relationship has been found between the extent of muscle capillary BM thickening and HbA$_{1a}$ levels in diabetic patients (Ramirez et al., 1991), (Raskin et al., 1983). In reality this is not an unexpected finding as the short-life span of haemoglobin limits the formation of NEG-related products to reversible early Amadori products, a proposal validated by Dominiczak and workers who reported that the levels of glycosylated haemoglobin derivatives (HbA$_{1a}$) in diabetic patients do not correlate with the tissue content of AGEPs (Dominiczak et al., 1990).

As such, the concentration of HbA$_{1a}$ only effectively reflects the degree of short-term glycaemic control. The accumulation of AGEPs on long-lived proteins is a slow process which occurs over the years and as such is dependent upon the average level of glycaemic control over this period. Consequently, despite the fact that although a diabetic patient may be currently experiencing a period of good control, as reflected by normal levels of HbA$_{1a}$, considerably large accumulations of AGEPs may be present within their tissues, as a result of formation during prior periods of poor control. A true reflection of long-term glycaemic control could be achieved by assessing HbA$_{1a}$ levels but only by taking serial measurements over several years.

These finding do not therefore preclude the possibility that prolonged hyperglycaemia and accelerated NEG in diabetes mellitus are of pathogenetic importance; indeed the magnitude and duration of tissue or organ exposure to a hyperglycaemic milieu has been found to correlate closely with the severity and rate of development of diabetic nephropathy, retinopathy and neuropathy (Brownlee, 1991), but rather suggests that a more accurate and reliable indicator of the overall degree of NEG is required.
The accumulation of fluorescent collagen-linked AGEPs in the skin of diabetic patients has been found to correlate positively both with age and duration of the disease (Monnier et al., 1986; Dominczak et al., 1990). Partial correlation analysis revealed that the duration of diabetes mellitus was considerably more important than the age of the individual (Dominczak et al., 1990), a finding which is probably related to the fact that the cumulative period of hyperglycaemia, during which AGE products accumulate, was probably correspondingly greatest in patients who had been diabetic for the longest periods. The degree of NEG of skin collagen therefore appears to be a suitable and reliable indicator of the overall extent of NEG, serving as a model for other metabolically inert, highly polymerised extracellular macromolecules.

Numerous studies have proved the existence of a correlation between the degree of tissue NEG and the occurrence of known diabetic complications (Vishwanath et al., 1986); a correlation has been demonstrated between the severity of diabetic neuropathy and the degree of lens protein NEG (Sensi et al., 1991). Increased skin collagen-linked fluorescence has been found to correlate with the occurrence and/or severity of diabetic complications including retinopathy (Monnier et al., 1986; Dominczak et al., 1990). In comparison to non-diabetic patients with varying severities of renal dysfunction, the serum concentration of AGEPs and the AGEP content of arterial wall collagen have been found to be comparatively greater in patients with diabetes mellitus (Makita et al., 1991). The suggestion that AGEP formation contributes to the pathogenesis of diabetic nephropathy was supported by the fact that the extent of AGEP accumulation paralleled the severity of renal functional impairment.

Not all of the related studies have produced corroboratory testimony. Collier and colleagues failed to find any relationship between the incidence of human diabetic complications and the thickness and hence collagen content of skin, the assumption being that any increase in collagen content must have been due to NEG (Collier et al., 1989). These results are not too damming as it must be taken into account that the methodology of NEG assessment used in that study was considerably less accurate and more speculative than that utilised in the previously mentioned fluorescence studies.

5C.1(iii) Correlation with BM hypertrophy in diabetes mellitus

Epidemiological and long term clinical studies strongly suggest that hyperglycaemia is associated with an increased risk of microangiopathy (West et al., 1980; Raskin et al., 1983; Sosenko et al., 1984), and generalised capillary
BM thickening is a common feature in both human (Kilo et al., 1972; Williamson & Kilo, 1977; Tilton et al., 1981; Raskin et al., 1983; Sosenko et al., 1984) and experimental (Bloodworth et al., 1969; Yasuda et al., 1984) diabetes of long duration. As such NEG and AGEP accumulation may be the mechanism responsible for these changes and therefore be of major aetiological importance in both the initiation and progression of diabetic microvascular disease with its characteristically thickened BM.

In support of this suggestion, the degree of NEG within the human lens capsule (the BM of lens epithelium) of diabetic patients has been found to be significantly greater than that of age and sex matched non-diabetic patients (Mandel et al., 1983), and the retinal accumulation of AGEPs in streptozotocin-diabetic rats has been shown to be significantly greater than that in healthy animals (Hammes et al., 1991). Additionally, although diabetes of relatively short duration in streptozotocin-diabetic rats results in an increase in the extent of muscle capillary BM NEG, the period appears insufficiently prolonged for hyperglycaemia and NEG to have any physical effect on BM thickness, implying that BM hypertrophy is critically dependent on the accumulation of AGEPs, which occurs at a slow rate, rather than early glycation products (Copeland et al., 1990).

Hyperglycaemia contributes to the development of renal dysfunction in diabetes mellitus; patients who are well controlled are most likely to avoid nephropathy, whilst those with poor glycaemic control are in the highest risk category (Daniels & Hostetter, 1991). Glomerular permeability is characteristically increased in both diabetic animals and humans and to a relatively lesser extent in galactosaemic rats (Daniels & Hostetter, 1991).

The levels of GBM glycated collagens have also been found to be increased in both human (Cohen et al., 1981; Garlick et al., 1988) and experimental diabetes (Cohen et al., 1980; Le Pape et al., 1981) and galactosaemic rats (Daniels & Hostetter, 1991). GBMs contain both lysine and hydroxylysine residues which are both readily susceptible to NEG. The amount of glycated lysine residues were found to comprise a significantly greater percentage of the total content of glycated residues than glycated hydroxylysine in the GBMs of both healthy and diabetic individuals (Garlick et al., 1988). This finding is attributable both to the ratio of the 2 components within BM proteins and the fact that, due to variations in the location of the residues on the polypeptide chain as opposed to in their structure, lysine is more susceptible to NEG than hydroxylysine.

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The degree of GBM NEG has been linearly correlated with age in both experimental (Monnier et al., 1988) and human (Cohen & Yu-Wu, 1983) diabetes and with ambient glucose concentration in diabetic rats (Cohen et al., 1980, 1981). Garlick and colleagues later failed to confirm the findings of Cohen and Wu-Yu, no correlation being detectable in their study between the content of glycated GBM amino acids, namely glucitol-lysine and glucitol-hydroxylysine, and either the length of disease in diabetic patients in age in both diabetic and non-diabetic subjects. They did however find that the severity of NEG in bovine GBMs and lens capsules positively correlate with age (Garlick et al., 1988). It is difficult to explain these discrepancies. They might possibly be due to differences in the age range and developmental states of the two species; specimens of GBM were obtained from both immature calves and mature animals whilst all of the specimens of human GBM were from adults.

A more likely explanation is that the severity of GBM NEG does increase with both age and the duration of diabetes mellitus but this process was masked by the methodological limitations of the experiment which assessed the degree of NEG only as a product of the content of early ketoamine Amadori adducts, the levels of which reach equilibrium after a few weeks, and failed to account for the accumulation of AGEPs which are believed to be the agents of primary importance in this pathogenetic mechanism. This theory, although it provides a possible explanation as to why no correlation was found between the severity of NEG and either age or duration of disease in human subjects does not explain why a positive correlation was found for the bovine specimens, as the same problem should have occurred.

5C.1(iv) Mechanisms of NEG-associated BM hypertrophy

5C.1(iv)A Increased resistance to degradation

The (urea) soluble fraction of GBMs contains glycosaminoglycans and non-collagenous proteins which contain only minimal amounts (2/1000 amino acid residues) of hydroxylysine residues and no hydroxyproline residues. The (urea) insoluble fragment contains the collagenous components of the BM matrix and as such possess a high content of hydroxyproline and hydroxylysine residues, moderate amounts of cystine and comparatively low levels of arginine and alanine (Cohen et al., 1980).

The properties of human collagens undergo modification with advancing age. Their solubility decreases as reflected by increased resistance to enzymatic degradation by collagenase (Kohn et al., 1984), and digestion by pepsin
following acetic acid extraction (Schnider & Kohn, 1981). The NEG of renal GBMS has been shown to render them similarly resistant to enzymatic proteolytic digestion (LePape et al., 1981) to agents including pepsin, papain, trypsin and endogenous glomerular protease (Lubec & Pollack, 1980). The susceptibility of fibrin to proteolytic degradation by plasmin is also known to be reduced following NEG.

It is believed that the stabilization of mature collagens occurs as a result of an increase in the density of inter- and intra-molecular cross-linking consequent upon prolonged NEG and the accumulation of AGEPs, several of the amino acid residues in BM proteins, such as lysine, have a strong receptor-mediated affinity for glucose (Gallop et al., 1972), the resulting ketoamine links serving to mask and protect the lysine-arginine bonds that are preferentially split by protease.

Newly synthesised collagen type I in streptozotocin-diabetic rats, induced by the subcutaneous implantation of polyester fabric and identified by its amino acid content; one third glycine in addition to relatively large amounts of 4-hydroxyproline and hydroxylysine, has been shown to be abnormally resistant to acetic acid extraction and enzymatic digestion by pepsin (Chang et al., 1980; Williamson et al., 1985). Collagen from diabetic patients has also been shown to be considerably more resistant to degradation (Schnider & Kohn, 1981) and less soluble in neutral salt solutions and weak acids (Hamlin et al., 1975) than collagen from age-matched non-diabetics.

As these diabetes-related alterations appear analogous with changes which are characteristic of the ageing process (Bailey et al., 1974), it seems plausible to suggest that the same causal mechanism is at play in both situations, and that NEG and accelerated ageing are responsible for the increased resistance of diabetic proteins to degradation. In support of this proposal the solubility of collagen from streptozotocin-diabetic rats is normalised by treatment with both β-aminopropionitrile (which inhibits lysyl oxidase activity) and D-penicillamine (which complexes lysine and hydroxylysine aldehyde groups) (Chang et al., 1980). These results also suggest that enhanced intermolecular lysine-derived cross-linking in experimental diabetes is mediated by lysyl oxidase activity which catalyses the formation of natural cross-links in collagens. The activity of lysyl oxidase has been reported to be increased in the lungs of rats following the institution of experimental diabetes (Madia et al., 1979).
Cultured vascular endothelium secretes lysyl oxidase (Levene & Heslop, 1977) and synthesises both collagen type IV which is a BM collagen and types I, III and V which are interstitial collagens, although the latter is usually closely affiliated with BMs (Levene et al., 1988). Mediation of the formation of collagenous cross-links is therefore an innate function of vascular endothelium and reliant upon the inherent endogenous production of lysyl oxidase, a fact substantiated by the experimental evidence that the addition of lysyl oxidase inhibitors to the culture media effectively prevents the synthesis of collagenous cross-links (Levene et al., 1986). The hypertrophic BMs characteristic of diabetic microangiopathy may be contributed to by augmented endothelial lysyl oxidase content/activity.

Glycopeptides liberated following the proteolytic digestion of the majority of glycoproteins usually contain only a single carbohydrate unit; however, in some glycoproteins the size, charge or proximity of the component carbohydrates may prevent cleavage of the peptide chains between their sites of attachment (Lubec & Pollak, 1980). NEG may result in a similar effect on customarily readily degradable glycoproteins due to steric or electrostatic interference with proteolytic enzyme activity, a theory which is supported by the finding that the molecular weight of protein fragments following pepsin digestion of skin collagen is increased by diabetes mellitus (Schider & Kohn, 1981).

Leakage of proteolytic enzymes i.e. collagenase inhibitors (Chang et al., 1980) such as α-1-antitrypsin and α-2-macroglobulin (Kilo & Williamson, 1979; Williamson & Kilo, 1976) has been shown to be increased. Additionally, the presence of glucose at concentrations equivalent with those found in diabetic kidneys, which are freely permeable to this substrate, was found to inhibit significantly the activity of α-glucosidase (glucosyl-galactosyl-hydroxylsyne glucohydrolase) (Sternberg et al., 1983). α-glucosidase is involved in the catabolism of BM collagens; the activity of this enzyme is specific for collagen disaccharide units. These are additional mechanisms which, due to a subsequent reduction in the turnover rates of their components, may contribute to the accumulation of matrix elements within diabetic BMs and the generation of their hypertrophic appearance.

Reduced susceptibility of diabetic BMs to proteolytic digestion may be responsible for the reportedly increased width of individual basal laminal layers reported in diabetic hypertrophic peripheral nerve endoneurial capillary BMs (Bischoff 1965).
5C.1(iv)B  Increased synthesis of components

Exactly how high ambient glucose levels cause an upregulation in the transcription of genes coding for BM components remains unknown. Two of the most likely mechanisms are (1) by exerting a direct effect on nuclear gene expression, or (2) by effectively masking recognition of the presence of the BM.

(1) Extra-cellular matrix components are important elements in the regulation of cellular phenotypes and induction of specific gene expression and secretory activity specific cell types (Haitoglou et al., 1992). Glucose-6-phosphate, a particularly potent glycosylating agent has been proposed as being of primary importance in the aetiology of diabetic microangiopathy. This theory arose as the result of comparisons between glycogen storage disease type I and diabetes mellitus as the natural history and histology of renal disease in the two conditions are comparable. Glycogen storage disease is characterised by a deficiency in glucose-6-phosphatase, which despite normal or even low blood glucose concentrations results in elevation of glomerular intracellular glucose-6-phosphate levels to those associated with severe diabetes.

It has been suggested that the formation of AGE products on DNA; Amadori products have been histologically identified in the nucleus of cells from diabetic patients (Kelly et al., 1989) and in vitro studies have revealed that glucose-6-phosphate is capable of initiating AGE product formation on the amino groups of DNA nucleotides, may cause extensive nucleic damage effectively equivalent to the premature onset of cellular senescence (Price et al., 1971). Such alteration may include structural DNA changes, chromosomal mutations, a decline in DNA repair, replication and transcription and altered gene expression. Such irreversible changes may be responsible for the pathological renal abnormalities of both diabetic patients and those with glycogen storage disease type I (Brownlee, 1991). Similar mechanisms may be at play in the production of hypertrophic BMs throughout the body.

(2) Site-specific interactions with cell-surface components on the basal surface of the parent cell are of crucial importance in the adhesion of these cells to the underlying BM. AGEP accumulation may play a role in the aetiology of BM hypertrophy by modifying normal interaction of transmembranous ECM receptors (integrins) with their specific matrix ligands; not only do collagen IV, fibronectin and laminin specific integrins have a β subunit in common, but a common integrin for these three proteins also exists.
Hemidesmosomes are specialised cell-ECM adhesive junction. Although they have been shown to contain a specific intergrin $\alpha_4\beta_4$ heterodimer (Stepp et al., 1990) the exact composition of the site and extracellular matrix ligand for the integrin is unknown. The number of hemidesmosomes has been found to be decreased in *in vitro* recombinations of corneal epithelial cells and denuded BMs when one or the other of the components is derived from a diabetic animal (Azar et al., 1992).

Diabetes related alterations in cell-extracellular matrix (ECM) interactions following either (1) changes in the biochemical composition of the ECM components involved in the formation of hemidesmosome i.e. by NEG, or (2) the occurrence of abnormal proportions of BM elements and hence the composition of the BM around these sites (Karttunen et al., 1986; Tarsio et al., 1987) may lead to a dilution effect or masking of the active sites. Diabetes-induced biochemical alterations of ECM receptors on the cells surface may also be causal, leading to defective recognition of the specific sites for hemidesmosome formation.

Laminin, fibronectin and collagen IV, which are all synthesised by cells with an underlying BM, have been sited as being the elements most likely to be responsible for the attachment of the parent cell to the ECM; plastic petri dishes coated with purified laminin and fibronectin are capable of promoting the attachment of both bovine retinal capillary endothelial cells and pericytes (Mandarino, 1992).

When these cells are cultured for a few days in a medium whose elevated glucose concentration is equivalent to that found in poorly controlled diabetic patients, the ability of the cells to attach to laminin, but not fibronectin, is inhibited. This suggests that recognition of the BM component laminin may be affected by diabetes-related NEG (Mandarino, 1991). The ability of endothelial cells to adhere to both collagen type IV and laminin is also decreased (Charonis & Tsilibary, 1992) following the *in vitro* NEG of these macromolecules (Tsilibary & Charonis, 1990; Haitoglou et al., 1992) presumably due to AGEP-related alterations in the cell-binding domains of the matrix proteins. NEG and AGEP formation on both the cell surface receptor and the matrix molecule itself may interfere with the normal binding of the BM membrane to the cell membrane, this loss of recognition leading to the abnormal synthesis of the constituent elements and possible layering of the basement membrane.
The proliferation rate of retinal endothelial cells and pericytes cultured on NEG collagen type IV or NEG laminin was found to be increased and decreased respectively compared with the rate of multiplication on control type IV collagen or laminin (Charonis & Tsilibary, 1992). As an increased proliferation of endothelial cells and loss of pericytes are characteristic features of diabetic retinopathy, it seems likely that the NEG of BM macromolecules may play a contributory role in the pathogenesis of diabetic retinal microvascular alterations. There are dangers in attributing the genesis of pathological abnormalities to alterations produced in *in vitro* situations, as differences in the cellular environment, which may have a critical effect, are not considered.

Another mechanism that may be instrumental in stimulating the abnormal synthesis of BM components involves NEG-related modification of the proteins involved in intracellular signalling pathways or NEG-associated alterations in the activity of transcriptional factors. Monocytes and macrophages possess high affinity receptors specific for AGE products. Binding of these cell surface integrins to AGE-associated proteins stimulates the cells to produce tumour necrosis factor \(\alpha\) (TNF), insulin-like growth factor I (IGF-I) and interleukin-1 (IL-1) which in turn result in the upregulation of glomerular collagen type IV synthesis (Vlassara et al., 1988b).

Decreased proteoglycan-binding capacity or reduced synthesis of integral proteoglycan components within diabetic BMs may render them functionally defective and hence trigger the compensatory production of other BM matrix elements. It has been proposed that heparan sulphate proteoglycans, via this mechanism, are responsible for down-regulating the synthesis of BMs by the adherent cell (Rohrbach & Martin, 1982; Rohrbach et al., 1982). Recent studies have provided evidence that this mechanism may be responsible for human GBM hypertrophy, as a highly significant correlation was found between the number of GBM anionic sites and GBM thickness (Vernier et al., 1992).

A final possible mechanism involves diabetes-related activation of protein kinase C (PKC) (Lee et al., 1989b); PKC activation is known to lead to modification of transcriptional events.

5C.1(iv)C Abnormal self-assembly of the BM

Self-association and site-specific multiple interactions between the various matrix protein components are responsible for maintenance of the precise, geometrical structure that is integral to the normal functional capacities of BMs,
a proposal that is demonstrated by the fact that the binding of fibronectin to collagen is enhanced in the presence of GAGs and results in the formation of insoluble complexes.

NEG and AGEP formation on these constituent proteins has been found to interfere with these interactions. For example, the increased incidence of both inter- and intra-molecular NEG-associated cross-linking associated has been found to result in the abnormal in vitro ultrastructural assembly of both collagen (Tsilibary et al., 1988; Charonis & Tsilibary, 1992) and laminin (Charonis et al., 1990; Charonis & Tsilibary, 1992) polymers.

NEG was found to produce remarkable alterations in the structure and shape of laminin polymers this was thought to be due to the observable dramatic reduction in the specific association of dimers, the step in the self-assembly process which precedes the development of small oligomers and polymers (Charonis & Tsilibary, 1992). The NC1 domain of collagen type IV is of primary importance in the formation of the ultrastructural network of this component, due to its function in intermolecular lateral association. NEG was found to interfere with this process, and was attributed to the attachment of glucose to lysine number 56 on the constituent α1(IV) chain (Charonis & Tsilibary, 1992). AGE-accumulation has also been found to result in increased intermolecular spacing of type I collagen (Tanaka et al., 1988).

These results require in vivo validation if they are to be realistically assigned a contributory role in the aetiology of BM abnormalities in diabetes for the following reasons. In vitro experimental studies do not take into consideration the influence of the cellular environment in vivo which may be of critical importance in the self-assembly and interactions of these macromolecules; neither do they account for the fact that some of the amino acids within extracted macromolecules which undergo NEG in vitro may be unavailable for NEG in BM in the in vivo situation due to their participation in physiological interactions; the sites at which haemoglobin molecules are glycated varies significantly between in vitro and in vivo conditions.

Variations in the concentrations of GAGs have been reported in skins of alloxan-diabetic rats (Kofoed et al., 1970) and decreased synthesis and pronounced reductions in the macromolecular content of the proteoglycan heparan sulphate have been reported in the GBMs in both experimental and human diabetes (Rohrbach & Martin, 1982; Parthasarathy & Spiro, 1982;
Kanwar et al., 1983; Sternberg et al., 1985; Shimomura & Spiro, 1987; Vernier et al., 1992). In a more recent ultrastructural study, which utilised the electrostatic binding of cationic dyes to polyanionic sulphated GAG chains of proteoglycans in order to visualise the presence of anionic sites within BMs, the staining density in the GBM of diabetic BB-rats was found to be reduced compared with age and sex-matched animals (Chakrabarti et al., 1989), which suggest either diminished synthesis of GAGs or reduced sulphation of GAGs or both.

Ledbetter and colleagues revealed that although the steady-state levels of mRNA encoding for the proteoglycan heparan sulphate were unaltered in the kidney cortex of diabetic KKAy mice compared with normal mice, the ratio of heparan sulphate to collagen type IV content may be reduced in hypertrophic BMs as the levels of mRNA for type IV collagen were significantly increased (Ledbetter et al., 1990).

Although measurements of mRNA levels encoding for the various components of BMs gives an indication of the amount of these elements being synthesised they may not reflect the true composition of the BM due to the effects of NEG on inter- and intramolecular interactions and the resultant stability and turnover rates of the respective protein molecules (Tarsio et al., 1985).

In actuality, reductions have been found both in the amount of sulphate incorporated into proteoglycans (Rohrbach et al., 1982; Cohen & Surma, 1984) and the levels of proteoglycan core protein in BMs of diabetic models (Rohrbach et al., 1982). A reduction in the sialic acid components of glomerular BMs has been noted in both human diabetes and experimental diabetes. The reason for this deficiency remains unknown.

In opposition to the theory that the NEG of GBMs is responsible for the reduction in number of heparan sulphate proteoglycan anionic charge sites in diabetes mellitus, Vernier and colleagues found the density of GBM anionic sites in the group of patients in their study who presented with normal or microalbuminuria but varying severities of NEG-attributed glomerular lesions, to be normal (Vernier et al., 1992).

The co-operative binding of heparan by fibronectin and gelatin (denatured calf collagen) is decreased if either of the latter agents has been glycated in vitro prior to their addition, suggesting that NEG of one or more of elements involved
in the integral formation of insoluble BM complexes in capable of perturbing the self-assembly process (Tarsio et al., 1985). AGE-product cross-linking on laminin also results in decreased binding of both collagen type IV and heparan sulphate proteoglycan (Charonis et al., 1990), and NEG fibronectin exhibits a reduced affinity for collagen (Tarsio et al., 1987).

The possibility also exists that the distribution of components in hypertrophic diabetic BMs may be compositionally abnormal. This theory is supported by the result of two immunocytochemical studies using streptozotocin-diabetic rats in which collagen type IV, present across the entire width of the lamina densa in normal BMs (Yurchencho & Schittny, 1990), was detectable only on the subendothelial side of the lamina densa in thickened glomerular BMs (Bendayan, 1985; Desjardins & Bendayan, 1990). Alternatively, the distribution of collagen type IV within these regions may have been normal, the production of an atypical pattern of immunological staining being due to masking of the antigenic sites by either biochemical alterations due to NEG or other components, structural rearrangement within the BM, or the secretion of a modified form of collagen type IV that was not recognised by the antibody used in the investigation.

5C.1(iv)D NEG-related binding of plasma proteins

NEG and AGE formation on BM constituents may contribute to BM hypertrophy via an additional mechanism as these alterations favour the increased covalent binding of circulating plasma proteins (e.g. albumin and IgG); AGE product precursors on cross-linked long-lived structural proteins i.e. myelin (Brownlee et al., 1986b) and collagen, are capable of chemically "trapping" short-lived, non-glycosylated proteins such as low density lipoproteins (LDL) and IgG (Brownlee et al., 1985). Once incorporated into the BM further AGEs may form on these normally short-lived proteins which then in turn serve as attachment sites for additional molecules of extravasated circulating plasma proteins leading to their abnormal accumulation.

In support of this theory, accumulations of albumin and other plasma proteins have been reported in the dermal capillaries of patients with long-standing diabetes (Chavers et al., 1981) and it has been proposed, following the localisation of bound albumin, IgG and polyvalent immunoglobulins in diabetic kidney glomerular and tubular BMs (Michael & Brown, 1981; Murrah et al., 1984; Melvin et al., 1984) that this mechanism may be of particular relevance in the pathogenesis of diabetic nephropathy. Further support is provided by the
fact that repeated intravenous injections of glycosylated plasma proteins in normal mice results in the production of a similar degree of glomerular BM thickening to that seen in human and experimental diabetes (McVerry et al., 1980).

Serum protein deposition within the relevant BM has been correlated with both renal tubule BM thickening (Murrah et al., 1984) and dermal capillary BM thickening (Chavers et al., 1981), but not gingival capillary BM thickening (Murrah et al., 1984). Considering that diabetes exerts a ubiquitous effect on the thickness of these BMs, this evidence refutes the theory of a causal relationship between abnormal plasma protein deposition and BM hypertrophy; differences may merely reflect the effects of functional and structural variations between BMs within diverse tissues.

5C.2 Enhanced polyol pathway activity

A significant reduction in skeletal muscle capillary BM thickness was reported in type I diabetic patients following the improvement of blood glucose control (Raskin et al., 1983) suggesting that, as NEG-related changes have been shown to be largely irreversible despite the initiation of strict glycaemic control (McLennan et al., 1986) and the process is ongoing in the absence of hyperglycaemia (Brownlee, 1991), an alternative mechanism may have been instrumental in the development of this microangiopathic abnormality.

Aldose reductase (AR) activity has been demonstrated in the majority of tissues and organs which are commonly both associated with diabetic pathophysiological complications and exhibit concurrent characteristic features of diabetic microangiopathy i.e. retina (Akagi et al., 1983), aorta (Clements et al., 1969), kidney, and peripheral nerves (Powell et al., 1991). AR activity has also been found in skeletal muscles although the precise location of the enzyme i.e. within vascular cells or muscle fibres themselves, is not clear (Morjana & Flynn, 1989).

It has been proposed that hyperglycaemia-related augmentation of polyol pathway activity in diabetes mellitus may be responsible for the development of diabetic microangiopathy and possible endothelial cell dysfunction following the demonstration of polyol pathway activity within the endothelial cells of both human umbilical veins (Okuda et al., 1991) and retinal microvasculature (Chakrabarti et al., 1987), and the intracellular accumulation of sorbitol by endothelial cells cultured in media with a high glucose concentration (Koh et al., 1987).
1986; Hawthorne et al., 1989). If this theory is correct then AR inhibition should theoretically prevent BM thickening (Frank, 1986).

Galactose, like glucose, is readily converted by AR to the respective sugar alcohol. Increased flux along the polyol pathway in galactosaemic animals is considered to be of instrumental importance in the development of characteristic retinal capillary abnormalities in galactosaemic rats (Frank et al., 1983). The fact that the components of galactosaemic retinopathy are analogous with pathological and pathophysiological retinal aberrations in diabetes mellitus suggests a common aetiological origin.

This theory is supported by the observation that ARI treatment is capable of effectively preventing retinal capillary BM thickening in both galactosaemic (Robinson et al., 1983, 1986) and experimentally diabetic (Chander et al., 1984; Tilton et al., 1989) rats, and that co-incubation with sorbinil, an ARI, effectively suppresses the increased expression of mRNA for collagen type IV by retinal capillary endothelial cells cultured in media with a high glucose content (King et al., 1988).

The increased in vivo content of both laminin and collagen type IV within the retinal capillary BMs of galactosaemic rats is attenuated by ARI treatment (Das et al., 1990), in confirmation of the previously reported prevention of retinal capillary BM thickening in galactosaemic rats by similar therapeutic measures (Frank et al., 1983; Robinson et al., 1983). These results suggest that hyperglycaemia and augmented polyol pathway activity may lead to the derangement of cellular genetic mechanisms involved in the expression of BM components; however, the precise link between AR activity and the genetic defect is undetermined.

Despite the fact that skeletal muscles are functionally unaffected by diabetes mellitus, the blood vessels within this tissue also exhibit the characteristic features of diabetic microangiopathy (Vracko et al., 1970; Kilo et al., 1972) and as such quantification of muscle capillary BM thickening has also been utilised in related investigations into the determination of the responsible pathogentic mechanism.

Ramirez and colleagues found no evidence that treatment with ponalrestat (an ARI) has any effect on skeletal muscle capillary BM thickening in diabetes mellitus, as throughout the duration of an 18 months clinical trial, despite a
significant and unexplained decrease in BM thickness in both groups, no significant difference was found between the severity of BM thickening in patients receiving the ARI and those receiving a placebo at any measured time point (Ramirez et al., 1991). There are several explanations for these results. (1) Although skeletal muscles are known to contain the enzyme AR, it may not actually be located within the endothelial cells of the constituent capillaries. (2) The dose of ARI used in the trial was insufficient to be of any therapeutic benefit. (3) Endothelial cell AR activity may vary across the range of tissues associated with capillary BM thickening in diabetes; ARI treatment in diabetic rats resulted in a significant reduction in increased permeability to $^{125}$I-labelled albumin in both the retina and kidney but not the aorta (Williamson et al., 1987). (4) Topographic differences in BM structure and function may render them varyingly susceptible to the diverse spectrum of mechanisms that have been proposed as being of possible pathogenetic importance in the generation of diabetic BM hypertrophy. As such the effects of ARI treatment on BM thickness may vary accordingly.

In relation to this last point, the instigation of aggressive insulin treatment and resulting euglycaemia is capable of preventing progressive BM thickening in both the superficial and deep retinal capillary beds of diabetic BB-rats (Chakrabarti & Sima, 1987, 1989). By contrast ARI administration in both hyperglycaemic diabetic BB-rats (Chakrabarti & Sima, 1989) and galactosaemic rats (Frank et al., 1983; Robinson et al., 1983), although it successfully prevents BM thickening in deep retinal capillary beds, has no effect on the concurrent development of this abnormality in the superficial capillary plexus (Chakrabarti & Sima, 1987, 1989).

These results suggest that whilst hyperglycaemia appears to be a factor of primary importance in the aetiology of these abnormalities at both locations, polyol pathway activity only seems to play a contributory pathogenetic role in the deep capillary beds. It has been proposed that topographic and physiological discrepancies between the two capillary beds - the superficial retinal vascular plexus is primarily arterial in origin, whilst the deep capillary bed is a low pressure venous system - may account for variations in the effectiveness of treatment with ARIs.

The relatively greater intravascular pressure in the superficial capillary bed is likely to facilitate glucose permeation and NEG of BM components in association with diminished prostaglandin synthesis and increased secondary collagen synthesis, whilst the greater affinity of glucose for deoxyhaemoglobin compared
with oxyhaemoglobin may reduce the availability of free glucose in the venous system. As such, although hyperglycaemia is a common initiator of capillary BM thickening in both systems, the effect of NEG is probably greater in the superficial capillaries, whilst the polyol pathway may be of paramount importance in the generation of this phenomenon in deep capillaries.

Increases in skeletal muscle capillary BM thickness may be similarly mediated by hyperglycaemia and accelerated NEG rather than the polyol pathway. Considering that retinal capillary BM thickening (Robinson et al., 1986) in diabetic and galactosaemic rats respectively is prevented by ARI treatment, it is not unreasonable to suggest that changes in diabetic muscle capillary BM thickness may not reflect structural and functional abnormalities in the kidney due to variations between the two tissues in the aetiological mechanism at play (Ellis et al., 1986).

Although muscle capillary BM width was found to correlate weakly with GBM thickness in diabetic patients, no correlation was apparent with the renal functional parameters of creatine clearance or urinary albumin excretion (Ellis et al., 1986). Therefore muscle capillary BM thickness appears to be a better indicator of glycaemic control than a predictor of renal and retinal pathology.

Not all investigations have provided confirmatory evidence that ARI are capable of alleviating the ubiquitous generation of the component abnormalities associated with diabetic microangiopathy, i.e. increased BM thickness or permeability.

In favour of the theory, rectification of characteristically enhanced glomerular filtration rates, proteinuria (Beyer-Mears et al., 1986; Goldfarb et al., 1986) and GBM thickening (Tilton et al., 1989) in experimentally diabetic rats have all been reported to be ameliorated by ARI treatment.

Glomerular sorbitol content is elevated in streptozotocin-diabetic rats (Beyer-Mears et al., 1984) implying that enhanced polyol pathway activity may play a possible role in the development of diabetic nephropathy. The abnormally high glomerular filtration rate of diabetic patients participating in a 6 months clinical trial with ponalrestat was reported to be attenuated by ARI treatment (Pederson et al., 1991), implying the existence of a cause and effect relationship. However, the validity of this assumption is undermined by the fact that the collective albumin excretion rate of the group was low, implying that
the patients were as a very low risk of developing renal disease.

By contrast, ARI treatment was found to have no effect on glomerular permeability in galactosaemic rats (Daniels & Hostetter, 1991) and two long-term studies involving streptozotocin-diabetic rats failed to demonstrate the conveyance of any beneficial effects of ARI treatment on either glomerular histology or proteinuria (Daniels & Hostetter, 1989; Mauer et al. 1989) implying that the polyol pathway does not mediate glomerular injury in either diabetic or galactosaemic rats (Engerman & Kern, 1989).

Körner and colleagues again found no evidence that ARI treatment results in any improvement in elevated renal hyperfiltration or albuminuria despite the concurrent prevention of renal cortical sorbitol accumulation in streptozotocin-diabetic rats (Körner et al., 1992). The positive reports in earlier studies (Goldfarb et al., 1986; Tilton et al., 1989) may be attributable to the relatively mild diabetic state of the experimental animals. Also, as data regarding renal tissue sorbitol content were not presented, the establishment of any cause and effect relationship between renal polyol metabolism and diabetic nephropathy was not fully validated.

In conclusion, augmentation of the polyol pathway appears to play a role in diabetic retinal capillary BM thickening within the deep vascular plexus but not the superficial capillary bed. By contrast the polyol pathway does not appear to contribute to diabetic muscle capillary BM thickening. The situation regarding increased glomerular permeability or GBM thickening in both diabetic and galactosaemic rats remains to be validated.

5C.3 Aminoguanidines - NEG versus the polvol pathway

Although aminoguanidine and its analogues possess ARI activity (Kumari et al., 1991b), they are considerably less effective than other established ARIs such as sorbitol and tolrestat, pharmacologically effective doses being measured in millimolar as opposed to nanomolar concentrations respectively (Tilton et al., 1993). In addition to this attribute, aminoguanidine is also an effective pharmacological inhibitor of AGEP formation and accumulation (Bucola et al., 1991; Edelstein & Brownlee, 1992) by selectively blocking reactive carbonyls on early glycation ketoamine Amadori products. It was proposed that this function may be partly attributable to its ARI activity as the products of the polyol pathway i.e. fructose, fructose-6-phosphate and pentoses are considerably more reactive than glucose in the formation of AGEPs (Kumari et al., 1991). In
view of more recently published observations on the potency of the compound with regard to its ARI capacity (Tilton et al., 1993) this does not seem a very realistic scenario.

Aminoguanidine has been found to be capable of preventing both the in vitro and in vivo formation of AGEPs and associated cross-linking (Brownlee et al., 1986a, 1988). At the molecular level it has been shown to prevent NEG-associated alterations in heparan binding to both collagen and fibronectin (Brownlee et al., 1987) and ameliorate in vitro defects in the self-assembly of laminin polymers (Charonis et al., 1990; Charonis & Tsilibary, 1992). The fact that aminoguanidine has no effect on lysyl-oxidase dependent cross-linking serves to disprove the theory that the abnormal degree of intra- and intermolecular cross-linking found within diabetic tissues is mediated by the induction of enzymatic pathways (Madia et al., 1979).

Aminoguanidine therapy has been shown to be of considerable benefit in the prevention of numerous diabetic complications. Aminoguanidine treatment following the initiation of streptozotocin-induced diabetes in experimental rats was reported to result in a small but significant improvement in MNCV defects in conjunction with beneficial effects on myelinated fibre size and atrophy (Yagihashi et al., 1992). These results were attributed to the prevention of AGEP formation on neural skeletal and myelin proteins as demonstrated by attenuation of AGEP-associated fluorescence in treated animals (Yagihashi et al., 1992).

With reference to microangiopathic abnormalities, the instigation of aminoguanidine treatment has been found to result in reductions in the accumulation of plasma proteins on the aortic walls of diabetic rats in addition to attenuation of excessive cross-linking of aortic matrix elements (Brownlee, 1991). It has also been found to prevent the development of detrimental structural vascular alterations in the kidneys (Nicholls & Mandel, 1989; Soulis-Liparota et al., 1991; Ellis & Good, 1991) and retinas (Hammes et al., 1991) of animals with early experimental diabetes. The effect of aminoguanidine in these instances have been largely attributed to its NEG-inhibitory functional capacity (Nicholls & Mandel, 1989; Ellis & Good, 1991) as shown by reported concomitant prevention of AGEP accumulation (Hammes et al., 1991; Brownlee, 1991) and normalisation of both the rate of synthesis of BM components including laminin (Giambrone & Brownlee, 1989), and the susceptibility these BMs to proteolytic degradation.
The results of these studies strongly imply that NEG and AGEP accumulation do play a pathogenetically instrumental role in the generation of diabetic complications and suggest a potential therapeutic role for aminoguanidine and its analogues in their prevention. Possible agents include aspirin which acts by competing with glucose for the same amino groups. Although aspirin has been shown to be effective in reducing the in vitro NEG of certain proteins (Sensi et al., 1987), its clinical use is prohibited due to its serious side effects, as to be effective the drug would need to be taken at fairly high doses throughout the entire life of the patient.

D-lysine, a naturally occurring amino acid which is not incorporated into mammalian proteins, has been implicated as being of potentially greater therapeutic benefit, the reaction between this agent and free glucose effectively eliminating excess circulating glucose from the system and therefore preventing the formation of early glycation products. In vitro studies have confirmed that D-lysine does effectively inhibit the NEG of both soluble and insoluble proteins and limits the formation of AGEPs (Sensi et al., 1989). In vivo studies are now required in order to determine the physiological parameters of the compound i.e. renal handling, intestinal absorption and catabolism etc., whether there are any side-effects and its therapeutic potential and efficacy.

Chronic hyperglycaemia and NEG have been causally linked with the generation of GBM hypertrophy in both experimental (Fox et al., 1977) and human diabetes.

The effects of restoring blood glucose levels to euglycaemic levels have not been found to be so beneficial where the attenuation of pre-existing BM hypertrophy is concerned. The institution of insulin therapy in animals with established diabetes, whilst it was found to reduce the degree of NEG of haemoglobin effectively, had no effect on the extent of collagen NEG (McLennan et al., 1986). In addition, islet transplantation was not found to result in the regression of existing hypertrophic GBMs in animals with experimental diabetes mellitus (Gotzsche et al., 1981).

If NEG is the link between hyperglycaemia and diabetic BM hypertrophy, differences in the relative turnover rates of affected proteins would explain the variation in effect of normalising hyperglycaemia on the extent of protein NEG. AGEP accumulation is a process which, once initiated, is able to progress despite the absence of ongoing hyperglycaemia. It is possible that collagen
molecules may be sufficiently long-lived for this process to have commenced in contrast to haemoglobin molecules, whose short half-life, location within cells which have a rapid turnover rate and subsequent relatively quick natural rate of replacement, effectively restricts the extent of NEG to the production of early glycation products, suggesting that clinical normalisation of NEG-related abnormalities may not be possible in some tissues.

Other studies have reported observations which conflict with this theory. The reversal of dysmetabolism by isogenic pancreatic transplant in patients with diabetes mellitus has also been found to result in normalisation of PCBM thickness in human diabetic dermal and sural nerves (Beggs et al., 1989). In addition, Raskin and colleagues found that meticulous control of blood glucose levels by continuous sub-cutaneous insulin infusion for a period of 2 years resulted in not only a significant reduction in the levels of glycosylated haemoglobin but reductions in skeletal muscle capillary BM thickness to levels comparable with those seen in healthy individuals (Raskin et al., 1983).

It is possible that these results may be related to the relatively rapid turnover rate of muscle capillaries and that the effect of strict glycaemic control is not actually due to regression of existing hypertrophic BMs but prevention of their generation in newly formed vessels. The turnover rates of renal and retinal capillaries may be relatively slower than that of muscle capillaries which may account for the lack of any similar effect on diabetic GBM or retinal capillary BM thickening.

5C.4 Variations between PC and capillary BMs

If hyperglycaemia and its consequent effects, i.e. NEG, are instrumental agents in the production of hypertrophic diabetic BMs the question arises, why does the appearance of these BMs vary between cell types and tissues if a common mechanism of production is involved. The characteristic conformation of thickened diabetic PCBM, whilst often appearing "holey", is more often analogous with that of hypertrophic diabetic GBMs, that is, amorphous and homogeneous in nature. The structure of thickened diabetic endoneurial capillary BMs is by contrast usually markedly multilaminar, these discrepancies suggesting that the mechanism responsible for the BM hypertrophy may differ between the two cell types.

The lamellated appearance, frequent presence of interlamellar lipid and cellular debris, and fragmented and electron-opaque appearance of circumferentially
located layers of BM suggests that capillary BM hypertrophy is due to endothelial cell hyperplasia, the reduplicated appearance resulting from the production of new ensheathments of BM interspersed with cellular debris with each repeated cycle of endothelial cell death and regeneration. The number of layers therefore represent the number of episodes of endothelial cell regeneration (Vracko & Benditt, 1970). This phenomena may be accentuated by the previously mentioned increased synthesis of diabetic BM components in association with their abnormal resistance to proteolytic degradation. An alternative suggestion is that the reduplicated basal laminae result from pericyte turnover (Giannini & Dyck, 1994a, 1994b), a proposal that is discussed more fully later.

If the BMs of endoneurial and transperineurial capillaries, as previously discussed, are more persistent in diabetes mellitus, a feature which appears to be true of Schwann cell basal laminae (King et al., 1989), the resultant progressive accumulation of reduplicated layers of BM around these vessels in conjunction with accompanying collagenisation may account for their characteristic hyperplastic appearances.

This theory was supported by an experiment designed to study the process of angiogenesis in muscle following its excision and immediate reimplantation (Vracko & Benditt, 1970). New capillaries were seen to regenerate within what appeared to be old denuded basal laminal tubes which sometimes contained evidence of cellular debris, repeated episodes resulting in the production of a multilayed hypertrophic BM which was similar in appearance to that of diabetic capillaries. Further evidence that the basal laminal tube used as scaffolding throughout the regenerative process was a remnant derived from the old necrotic vessels was supplied by the fact that in animals given water containing silver nitrate for 20 months prior to surgery and unlabelled water post-operatively only the outer investment of the BM in the regenerated capillaries appeared labelled with silver aggregates at EM level.

It is possible that the presence of anomalies between the structures of hypertrophic capillary and perineurial cell BMs may merely reflect the more static nature of the boundaries of perineurial cells despite the universal mechanism; the homogeneous appearance of PCBM thickening implies that its production is continuous and therefore attributable to simple hypertrophy rather than cellular hyperplasia (Johnson, 1981).
Each laminin molecule is composed of three chains, one B1 or S chain, one A, M or K chain and one B2 chain which are connected to each other by several disulphide bonds. Laminin forms independent networks in BMs. The content of the various BM components may vary in connection with their function (Chardin et al., 1992); Grant and Le Blond found that although laminin, collagen type IV and heparan sulphate proteoglycan are ubiquitous components within BMs, the relative amounts of each vary according to the specific tissue type (Grant & LeBlond, 1988). The isoforms of laminin and collagen type IV may vary between different specific BMs leading to structural diversification (Charonis & Tsilibary, 1992). Related dissimilarities between the BMs of perineurial and endothelial cells may partially explain why the hyperplastic BMs of perineurial cells have an amorphous appearance whilst those of endothelial cells appears reduplicated.

The population of laminin types within the BMs of perineurial and Schwann cell is dissimilar; a large proportion of laminin molecules contain A & S chains in PCBMs whilst Schwann cell BMs contain predominantly B1 and M chain laminin molecules. The component chains of laminin within the BMs of endoneurial vessel endothelial cells is at present unknown. The effect of diabetes mellitus on the production of laminin may be selective according to the constituent chains within the laminin molecule, i.e. the production of laminin molecules with high proportions of A and S chains may be continuously enhanced, whilst that of molecules with high proportions of B1 and M chains is sporadic.

5D DIABETIC NEUROPATHY - A VASCULAR ORIGIN?

5D.1 Ischaemia

Two basic requirements must be fulfilled in order to maintain the functional integrity of peripheral nerve fibres. Firstly, the axonal-nerve cell body connection must be intact and secondly the fibres must receive a continuous and adequate supply of oxygen via the intraneural vascular system.

The PNS is a well vascularised structure with two integrated but functionally independent microvascular systems; the extrinsic and the intrinsic. The extrinsic system is composed of segmentally arranged vessels and the majority of component nutrient arteries are either direct branches of the main arteries of the limbs or subsidiary branches of muscular or periosteal arteries which run across or close to a nerve en route to their destination. These local nutrient vessels, upon reaching the epineurium, divide into ascending and descending branches which then anastomose with the intrinsic system.
The intrinsic system is composed of the epineurial, perineurial and endoneurial plexuses. The epineurial plexus consists of numerous arterioles and venules running in a predominantly longitudinal direction. These form a large number of anastomoses and arteriovenous shunts. The intrafascicular plexus is composed primarily of capillaries which are similarly arranged in a predominantly parallel direction to the axis of the nerve.

No particular direction of flow seems to predominate in any nerve segment, changes in direction occurring at almost every anastomosis, and when a nerve is cut, damaged or ligated, the direction of blood flow in the venules and capillaries of that area often subsequently changes. Additionally, use of the vital microscope has revealed the presence of empty 'reserve' endoneurial capillaries which come into immediate use under traumatic condition (Lundborg, 1975). A major feature of the arrangement of intraneural nutrient vessels is the considerable overlap of supply between vessels entering the nerve at different levels; as such no single vessel dominates the circulation in any given segment.

Due to the specific architecture of the neural vascular system, in the event that any one of the regional nutrient arteries becomes occluded, a preformed collateral pathway is already available to maintain the blood supply. It has been demonstrated that when the tibial nerve of rabbit is cut both proximally and distally, the blood supply received through extrinsic vessels entering along the length of the section is sufficient to prevent any detectable decrease in intraneural blood flow, thus demonstrating the effectiveness of the vascular supply of the PNS (Lundborg, 1975).

The oxygen requirements of peripheral nerve fibres are also quite small, the normal blood supply providing a more than ample margin of safety. Subsequently, even following total disruption of neural blood supply, diffusion from adjacent vascularised tissue may still supply sufficient oxygen for the nerve fibres to continue functioning for some time.

As a result of these features the production of a truly ischaemic experimental lesion has proven problematic and has been found to require the ligation of more than one nutrient artery (Lundborg, 1975). In some instances even this degree of disruption has failed to produce a reproducible and consistent degree of ischaemic damage (Fowler & Gilliatt, 1981). The application of a compression cuff around a limb has been claimed to result in the production of an ischaemic lesion (Mayer & Denny-Brown, 1964), however, it has since been demonstrated
that these lesions are a direct result of the applied pressure which causes displacement of the node of Ranvier and intussusception of the paranodal myelin (Ochoa et al., 1972).

Clinically and physiologically there is ample evidence to support the hypothesis that vascular disorders can lead to the development of ischaemic peripheral nerve lesions. Examples of such conditions include arterial embolism, injuries resulting in trauma to a main vessel, occlusive vascular disease, tourniquet paralysis and polyarteritis nodosa amongst others.

Certain diabetic focal neuropathies, i.e. that of the third cranial nerve (Asbury et al., 1970) have also been attributed to a vascular cause, leading to the suggestion that other diabetic peripheral neuropathies may have a similar basis. Little conclusive evidence has so far been produced in support of this theory with reference to the aetiology of distal sensory and autonomic polyneuropathies.

5D.1(i) Epineurial arteriolar abnormalities

Numerous studies have been performed in order to investigate the role of microangiopathy in the development of diabetic polyneuropathy; however, the part played by any pathological alterations in epineurial arteries has received little specific attention. Vasculopathy with thickening of the vessel walls by a PAS-positive material was proposed as a causal factor by Fagerberg (1959); however, the morphological methodology was not optimal and criteria were not utilised in order to differentiate between arterioles, venules and capillaries.

Thomas and Lascelles, on finding vascular abnormalities to be present in only 2 of the 8 patients (mean age 51.8 years) included in their study, and these being only minor in nature, supported Dolman's earlier conclusion that the correlation that exists, if any, between the severity of vascular lesions and the extent and distribution of demyelination in diabetic nerves is poor at best (Dolman, 1967), as they could find no evidence of any correlation between epineurial arteriolar occlusive vascular disease and the severity of peripheral neuropathy (Thomas & Lascelles, 1966).

Reske-Nielsen and Lundbaek investigated the occurrence of pathological abnormalities in the peripheral nerves of 15 IDDM diabetics. Again no attempt was made to differentiate between the type or location of the vessels involved, but the descriptions strongly imply that the vessels which were primarily
involved were generally epineurial in nature. No correlation was found between
the severity of the neuropathy and the extent of vessel abnormalities which
were stated as being infrequent, focal and variable (Reske-Nielsen & Lundbaek,
1968).

A later more comprehensive study (Korthals et al., 1988) demonstrated that the
intimal area of epineurial arterioles is increased in the sural nerves of diabetic
patients and is largely due to the proliferation of muscle-like cells, a finding in
keeping with the proposal that hyperglycaemia and associated metabolic
alterations induce proliferative cellular changes in the components of vessel
walls. A possible confounding contribution of age-related changes was
dismissed as intimal area was not found to increase with advancing age in the
control group. In addition the fact that medial vessel area in the diabetic
patients was not found to be significantly different to that in the healthy
individuals eliminates any suggestion that the results were due to selective
measurement of larger vessels in the diabetic group.

As the increase in intimal area in the diabetic vessels was only infrequently
reported as being severe enough to result in luminal narrowing or closure it is
not surprising that no significant difference in luminal area was found between
the diabetic and control vessels, or that no correlation was detectable between
intimal area and the severity of neuropathy. As a result of these studies it
therefore appears unlikely that increased intimal area or any other epineurial
arteriolar abnormalities are of pathogenetic significance in the development of
diabetic neuropathy.

5D.1(ii) Endoneurial microvascular abnormalities

Axonal degeneration, with secondary segmental demyelination is the
predominant pathological change produced by ischaemia (Korthals &
Wisniewski, 1975; Nukuda & Dyck, 1984). The resultant lesion commences at a
proximal position, is patchy and multifocal and maximal distally. Endoneurial
collagen is increased and axonal sprouting may occur. Axonal degeneration is
also the primary pathological alteration in diabetic polyneuropathy (Dyck et al.,
1986a).

The concentrations of the minor haemoglobins \( A_{1a} \), \( A_{1b} \) and \( A_{1c} \) are increased in
patients with diabetes mellitus. As in vitro studies have demonstrated that
haemoglobin \( A_{1c} \) possesses a greater affinity for oxygen than haemoglobin A
(Koenig et al., 1976) it is possible that clinically elevated levels of haemoglobin
$A_{ic}$ may contribute to tissue hypoxia (Ditzel et al., 1976). Physiological evidence suggests that this is an unlikely scenario (Bunn et al., 1978) as the in vivo whole blood oxygen saturation curves in both diabetic and healthy individuals are essentially similar.

It has been proposed that, in conjunction with rheological alterations i.e. blood hyperviscosity (Tuck et al., 1984) and the effects of any epineurial and perineurial vascular insults, diabetic endoneurial microangiopathic abnormalities may result in the generation of endoneurial ischaemia, impaired axonal and Schwann cell respiration and protein metabolism and subsequent intrafascicular focal nerve fibre loss (Dyck et al., 1985, 1986a), multiple focal lesions summating to produce the proximo-distal pattern of fibre loss characteristic of diabetic polyneuropathy. A distally-accentuated pattern of fibre loss can also result from a distal axonopathy of "dying-back" type (Said et al., 1983, 1993).

Support for an ischaemic component is provided by the fact that two of the characteristic abnormalities of diabetic peripheral nerves, conduction slowing and resistance to ischaemic failure are partially prevented by oxygen supplementation in experimental diabetes and reproducible in non-diabetic animals by oxygen deprivation (Low et al., 1984), and the fact that the focal and centrifascicular, proximo-distal pattern of fibre loss shown to be associated with some cases of diabetic polyneuropathy is comparable to that seen following microsphere embolisation of endoneurial capillaries (Nukuda et al., 1984). Exactly how much ischaemia realistically contributes to human diabetic neuropathy remains uncertain, especially in the case of generalised sensory/autonomic polyneuropathy (Thomas, 1987). It is not established that rapidly reversible slowing of nerve conduction or increased resistance to ischaemic conduction failure are a prelude to a subsequent degenerative neuropathy.

Abnormalities in fascicular capillaries density due either to capillary loss or increased fascicular area (Jakobsen, 1978) or capillary occlusion, reduced luminal calibre, increased resistance to blood flow and impaired diffusion of oxygen across thickened capillary walls (Vital et al., 1973) are all endoneurial vascular abnormalities which may potentially contribute to intrafascicular hypoxia.
5D.1(ii)A Fascicular area

It has been proposed that the endoneurial accumulation of osmotically active polyols in the diabetic state may not only be of significance in the aetiology of diabetic polyneuropathy via a metabolic pathway but may be instigatory via a vascular mechanism. The first suggests, on the basis that analogies exist between the role played by polyol accumulation in the development of galactose and diabetic neuropathies, that consequent endoneurial oedema and increased endoneurial pressure restricts blood flow within the vasa nervourm leading to ischaemia and cellular dysfunction.

The development of endoneurial oedema would appear to be a feature of primary importance in this sequence of events. Johnson and colleagues found no difference in dermal nerve fascicular area between diabetic and non-diabetic patients matched for age and severity of neuropathy (Johnson & Doll, 1984). Within the PNS, sural nerve fascicular area has not been found to increase with age in either diabetic or healthy individuals (Dyck et al., 1985b) and as such any distortion of the results by age-related variations in fascicular area in the determination of the effects of diabetes within parallel studies is effectively negated. The results of these studies comply with the general observation that sural nerve fascicular area is generally unaffected by diabetes mellitus (Malik et al., 1992), the median value for their diabetic group (77,110μm²) proving only slightly and unstatistically greater than the value for the organ donor control group (66,490μm²). On the other hand, Behse and colleagues found fascicular area to be increased in some cases of diabetic neuropathy (Behse et al., 1977).

Mild peripheral nerve oedema has been documented in streptozotocin-diabetic rats (Anand et al., 1988) and PET studies have demonstrated overhydration of peripheral nerves in diabetic neuropathy that can be corrected by ARIs (Griffey et al., 1988). However, the profound and undisputed extent of endoneurial oedema associated with galactose neuropathy (Sharma et al., 1976; Wadhwani et al., 1989) has not been found to be a component of either experimental diabetic neuropathy (Powell et al., 1981a) or human diabetic polyneuropathy (Thomas & Eliasson, 1984a) and it therefore does not seem unreasonable to eliminate this theory on these grounds.

5D.1(ii)B Capillary density

As fascicular area is relatively unaffected by either age or diabetes mellitus, the principal cause of reductions in endoneurial capillary density can be primarily attributed to capillary degeneration.
The effects of age on sural nerve endoneurial capillary density have not been unequivocally determined; a decrease in density with advancing age has been described in both healthy and diabetic individuals by Dyck and colleagues (Dyck et al., 1985b), whilst Sima and associates later failed to find evidence of any such relationship (Sima et al., 1991).

Similar discrepancies have been voiced regarding the effect of diabetes mellitus on endoneurial capillary density. Sural nerve endoneurial capillary density in diabetic patients has been reported as being significantly reduced and diabetic endoneurial capillary density was found to correlate inversely with myelinated fibre density \( (p < 0.01) \) (Malik et al., 1992). Such an alteration would result in reduced endoneurial blood flow and oxygen tension (Newrick et al., 1986; Malik et al., 1992). Alternatively, reductions in capillary density may be a secondary manifestation in response to nerve fibre loss.

The results of this study comply with the greater consensus that the density of endoneurial capillaries is unaffected by diabetes mellitus (Dyck et al., 1985b; Sima et al., 1991).

It has been suggested that disparities in capillary density may exist between the varying syndromes of diabetic neuropathy, possibly as previously mentioned as a secondary effect, endoneurial capillary density being reported as increased in patients with newly presenting chronic painful neuropathy but reduced in patients with recurrent foot ulceration (Britland et al., 1990), and that the inclusion of patients with a varying range of diabetic syndromes may explain these observational discrepancies (Malik et al., 1992).

5D.1(ii)C Intercapillary distances

The contribution of variations in fascicular area to increased intercapillary distances, for reasons mentioned previously, are negligible. The minimum intercapillary distance in the sural nerves of diabetic patients has been reported to be comparable with that of healthy individuals (Dyck et al., 1985b) and mean intrafascicular capillary distance in the peripheral nerves of the diabetic patients included in this study was not found to differ statistically from that of the control group. It can be concluded that endoneurial capillary degeneration is not enhanced by diabetes mellitus and that the generation of any general reduction in endoneurial oxygen tension as a result of reduced intrafascicular capillary density via either increased fascicular area or enhanced capillary degenerative phenomena is unlikely.
Ischaemic lesions are multifocal, large fascicles and central fascicular areas appearing most susceptible (Korthals & Wisniewski, 1978). This predominantly centro-fascicular pattern of fibre degeneration suggests that these cores are regions of poor perfusion. If the central intrafascicular density of endoneurial capillaries is lower than that within the bordering zones the resulting peripheral-central gradient in endoneurial blood flow and oxygen tension would help to explain the apparent increased vulnerability of fibres within the fascicular core, as the effects of ischaemia within this region would be more pronounced.

In support of this hypothesis intercapillary distances in the centres of fascicles have been reported as being significantly greater than those within the subperineurial zones of the sciatic but not the tibial or peroneal nerves of healthy rats (Nukuda et al., 1965). Subsequent studies have, however, failed to find any evidence of a peripheral-central intrafascicular gradient in either blood flow (Sladsky et al., 1983) or oxygen tension (Tuck et al., 1984) within the sciatic nerves of healthy rats. Two possible explanations for this finding have been proposed. Firstly, that vulnerable levels of the sciatic nerve were not included in these studies (Parry & Brown, 1982) and secondly, as already discussed, that some of the subperineurial capillaries are "reserve" capillaries which only become functional under conditions of extreme metabolic demand (Lundborg, 1975) and as such discrepancies between central and subperineurial capillary density only become apparent under pathological conditions. The latter proposal is supported by the observation that differences between central and subperineurial endoneurial blood flow and oxygen tension were found to become apparent following femoral artery ligation and the generation of a subsequent reduction in blood flow (Sladsky et al., 1983), supposedly as a result of the activation of subperineurial "reserve" vessels by ischaemic insult (Lundborg, 1975).

It is possible that the appearance of centro-fascicular fibre vulnerability may result from the fact that, whilst degenerative phenomena proceed within the central ischaemic core, the comparatively high blood flow and oxygen tension in the subperineurial regions of the fascicles may be sufficient to maintain the integrity of peripherally located fibres. Care must be taken in extrapolating information from one animal species to another as interspecies variations in the anatomical structure of analogous capillary beds have been shown to exist i.e. the vasa nervorum in the guinea pig are primarily composed of venules as opposed to capillaries, a situation which is reversed in rats and human beings (Waksman, 1957).
In an unquantified study of human peripheral nerves, Lundborg reported that centro-fascicular density appeared relatively reduced compared with that in the subperineurial zone (Lundborg, 1979). Although similarly unquantified, the density of endoneurial capillaries within these two regions, in all three groups of individuals, was found to be largely comparable, with the exception of very small fascicles which contained only one or two predominantly peripherally located vessels. These findings, in combination with the fact that the physiological condition of the vessels did not appear to be affected by their location within the endoneurium, i.e. a higher incidence of closed vessels was not found within the subperineurial zone in contradiction to Lundborg’s theory of "reserve" vessels (Lundborg, 1975), imply that the generation of focal ischaemic lesions within peripheral nerves is not related to areas of comparatively poor vasculization.

5D.1(iii) BM hypertrophy

Various components of diabetic microangiopathy have been reported to correlate with the severity of neuropathy (Yasuda & Dyck, 1987; Malik et al., 1989a; Britland et al., 1990). Vital and colleagues observed that endoneurial capillary BM thickening was most prominent in the peripheral nerves of those diabetic patients with the greatest severity of neuropathy (Vital et al., 1973). In two later reports BM thickening was statistically correlated with the severity of myelinated fibre abnormalities in the sural nerves of diabetic patients (Dyck et al., 1985b; Yasuda & Dyck, 1987). This finding was recently confirmed by Malik and associates, BM area being found to correlate inversely with both myelinated fibre density (p<0.05) and motor and sensory NCV (Malik et al., 1992). The mean age of the patients involved in the two of these publications was relatively high, at 61.2 years and 53.2 years (Dyck et al., 1985b; Yasuda & Dyck, 1987). However, as it has recently been revealed that endoneurial capillary BM hypertrophy is not affected by age (Giannini & Dyck, 1994, 1995) the contribution of advanced age in the diabetic patients, and/or age discrepancies between the diabetic and control individuals who took part in these studies to the generation of a statistically significant difference can be discounted.

The existence of a relationship between endoneurial capillary BM hypertrophy and the severity of diabetic neuropathy has not therefore been unequivocally demonstrated as a result of these investigations, and Sima and colleagues reported that they could find no evidence of any correlation between endoneurial capillary BM thickness and the degree of nerve fibre pathology (Sima et al., 277)
1991). The situation therefore remains unclear. Even if a correlation were demonstrated unequivocally between the extent of capillary BM hypertrophy and both the duration of diabetes and neuropathic severity, it does not necessarily imply a cause and effect relationship, but may reflect a similar degree of secondary concurrent effects.

The relatively normal appearance of the endoneurial vasculature, recently noted by Malik and colleagues, in the sural nerve of a patient with Mendenhall's syndrome serves to support this theory (Malik et al., 1995). Mendenhall's syndrome is characterised by a mutation in the insulin receptor gene resulting in lifelong uncontrolled hyperglycaemia. Two conclusions are implied by these observations. Firstly, that chronic hyperglycaemia does not necessarily result in microangiopathic abnormalities. Secondly, that coincident severe neuropathy in the absence of advanced microangiopathy suggests that metabolic disturbances may independently play a primary role in the pathogenesis of diabetic neuropathy.

The abnormal accumulation of BM components may either be responsible for, or the result of, a degree of functional abnormality. One possible consequence is increased rigidity and reduced compliance of the vessel wall which may lead to haemodynamic alterations i.e. increased vascular resistance and restricted fascicular circulation. The effect may be further augmented as a consequence of PCBM thickening and the physical constrictions of transperineurial vessels (Powell, 1983; Powell et al., 1985). This theory is supported by the observation that resting endoneurial blood flow is reduced as a result of streptozotocin-induced experimental diabetes (Tuck et al., 1984; Monafo et al., 1988).

The failure to find any difference between human diabetic subjects and healthy individuals in local nailfold microcirculatory pressure, except at extreme flow rates (Tooke, 1980), conflicts with the expected results if this theory is to be believed, especially considering the fact that concurrent abnormalities in capillary permeability (Bollinger et al., 1982) and vessel morphometry (Redisch et al., 1973) exist within these capillary beds and argues against the contribution of BM hypertrophy to increased vascular resistance in the aetiology of microangiopathic alterations.
5D.1(ii)E Capillary closure

Luminal capillary occlusion may occur as a result of both physiological and non-physiological mechanisms, both of which may lead to the ensuing development of endoneurial hypoxia. Physiologically, closure is shown by the close apposition of the endothelial cell surfaces in the midline of the vessels (Malik et al., 1992), non-physiological closure by fibrin or platelet occlusion. The actions of prostacyclin, a platelet antiaggregant and vasodilator synthesised in endothelial cells (Weksler et al., 1977) are opposed by platelet thromboxane A2. In experimental diabetes the level of thromboxane A2 is increased (Gerrard et al., 1992) and in conjunction with an accompanying reduction in prostacyclin synthesis (Ward et al., 1989) could theoretically lead to increased platelet aggregation and adhesion in microvessels, resulting in microvessel closure, increased intercapillary distance and the production of endoneurial hypoxia (McMannis et al., 1986). It has been demonstrated that the accumulation of AGEPs may occur on endothelial cells, a process which results in the induction of two additive procoagulatory changes in the endothelial cell surface (Brownlee et al., 1991).

Endoneurial microvascular closure has been found to correlate with age in both diabetic and healthy individuals (Dyck et al., 1985b, 1986a; Sima et al., 1991), an observation which stresses the importance of using age-matched control subjects in related studies. Sural nerve capillary closure in both of these groups was also shown to correlate with vascular density, the closed vessels being primarily comprised of post-capillary venules (Sima et al., 1991). This fact, in conjunction with the lack of any obvious endothelial cell abnormalities, implies that closure was the result of a physiological rather than a pathological process, the closed vessels being redundant (the number of venules has been positively correlated with age).

In an early study, Dolman reported that the frequency of closed endoneurial and perineurial vessels appeared to be increased in diabetic peripheral nerves; however, she failed to decipher whether any statistical correlation existed between these alterations and neuropathological severity. Despite this, the fact that she frequently observed healthy vessels within areas of focal demyelination and narrowed vessels in healthy regions of these nerves led her to conclude that the lesions were concurrent and did not imply a cause and effect relationship (Dolman, 1963). Additionally, as she found the incidence of luminal narrowing in non-diabetic hypertensive controls to be comparable to that in the
peripheral nerves of the diabetic patients she concluded that these abnormalities could not contribute to the development of the neuropathy.

Subsequent studies have provided conflicting testimony. Timperley and colleagues found evidence of endoneurial capillary occlusion by fibrin in the sural nerves of 9 out of the 24 diabetic patients involved in their study (mean age 56 years). This observation in addition to the fact that thrombotic vessels were often located within necrotic areas of the fascicle, led them to attribute the concomitant patchy distribution of demyelination to secondary hypoxia (Timperley et al., 1976); these observations must be viewed with a little caution as traumatised fibres produced as a result of 'surgeons error' were identified as degenerating fibres. The lack of similar findings within the sural nerves of the remaining 15 patients was attributed to the focal nature of the lesions and the phasic nature of fibrin deposition and removal, it being suggested that although the lumen of vessels in the sections under observation appeared patent, serial sectioning would probably have revealed occlusion at another point along the length of the nerve.

Dyck and colleagues described an increase in the percentage of "closed" capillaries in human diabetic sural nerves (Dyck et al., 1985b, 1986a). The mechanism of occlusion although stated as non physiological, was not clearly established. The severity of the neuropathy was assessed by the extent of fibre loss and abnormalities of the remaining fibres and expressed in terms of an "index of pathology" (Ip). A strong correlation was reported between the Ip and the incidence of "closed" capillaries \( p<0.008 \) in both the IDDM and NIDDM neuropathic diabetic subjects. In a later study, working with Yasuda, Dyck again reported that the incidence of "closed" endoneurial capillaries was increased in diabetic neuropathic individuals. The fact that they also found endoneurial capillary luminal area in the non-neuropathic diabetic patients to be significantly greater than that in the control subjects, a finding which was presented as further evidence that vessel closure in the neuropathic patients was therefore considered to be of pathogenetic significance with regard to the neuropathy (Yasuda & Dyck, 1987).

The conclusion voiced as a result of these three studies has come under close scrutiny due to the possible contribution of age-related effects. The mean age of the diabetic patients who took part in one of these studies was stated as being greater than twice the value of the control group (Dyck et al., 1986a). Although the same authors had failed to include the age of the subjects in their

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previous paper (Dyck et al., 1985b), the fact that the source of recruitment and number of individuals in each group were largely the same and that the investigations were performed only one year apart, implies that the same specimens had been used. In the most recent study, once again, there was a large discrepancy between the mean age of the diabetic patients (53.2 years) and the control subjects (30.9 years) (Yasuda & Dyck, 1987). In view of these facts, it can be argued that the increased closure of endoneurial vessels in the diabetic nerves may merely reflect an age-related phenomena.

Considering that the diabetic patients were relatively elderly, co-existent vascular disease would not be an unexpected complication and may have resulted in the generation of focal lesions which were superimposed on the generalised neuropathy. The likelihood of this occurrence is supported by the fact that focal sural nerve fibre loss has been found to be apparent in older NIDDM patients but not relatively younger IDDM individuals (Sima et al., 1988a).

The presence of a high incidence of "closed" capillaries was not confirmed by Malik and colleagues (Malik et al., 1989a). In their considerably more accurately age-correlated comparative study the percentage of closed endoneurial capillaries in the sural nerves of the diabetic patients (mean age 46.9 years) was actually found to be slightly less than that of the control individuals (mean age 47.5 years) (Malik et al., 1992). In support of these findings this investigation has failed to find any difference between satisfactorily age-matched diabetic, HMSN type I and organ donor control subjects in the incidence of "closed" endoneurial vessels in either the radial or sural nerve. Completely occluded diabetic vessels were not observed in this study in support of a previous study which similarly failed to find any evidence of endoneurial vascular occlusion by fibrin or platelets (Malik et al., 1992).

Although no significant difference was apparent, the median luminal area of the diabetic endoneurial capillaries (5.3μm²) evaluated in this investigation was found to be marginally greater that in the control tissues (4.9μm²), a finding supported by prior studies (Powell et al., 1985; Yasuda & Dyck 1987; Britland et al., 1990; Malik et al., 1989a, 1992). Although the median value for the HMSN I patients was slightly greater (6.2μm²) than that of the diabetic patients, the difference was not statistically significant and is therefore unlikely to be of aetiological significance.
In contrast to previous reports by Dyck and colleagues, a relatively recent report failed to find any evidence of any correlation between the number of closed endoneurial vessels and the degree of nerve fibre pathology within the sural nerves of diabetic patients, or any relationship between vascular closure and duration of diabetes (Sima et al., 1991). The findings of this study confirm this report and do not support the theory that diabetic microangiopathy is pathogenetically related to nerve fibre pathology via ischaemia and hypoxia.

This statement is further supported by the failure of a recent study to produce evidence of any difference between IDDM patients (both with and without macroangiopathy) and age- and sex-matched control subjects in the patterns of platelet adhesion and aggregate/thrombus formation on both cultured human endothelial cells and fibroblasts (which do not contain von Willebrand factor). No abnormalities in diabetic platelet-vessel wall interactions were detected, even in the presence of macroangiopathy, which suggests that a chronic state of increased platelet reactivity does not exist in diabetes mellitus and this mechanism does not play any contributory role in the development of diabetic vascular disease (Nievelstein et al., 1991).

ARI therapy with sorbinil has been shown to confer a small but significant degree of improvement in NCV slowing (Judzewitsch et al., 1983) and nerve fibre repair and regeneration (Sima et al., 1988c) in neuropathic diabetic patients. Similar treatment does not however result in the production of any significant beneficial effects with reference to endoneurial microvascular abnormalities (Sima et al., 1991), suggesting that detrimental pathological alterations associated with diabetic neuropathies may be more likely to be associated with perturbations in AR activity than microvascular anomalies.

5D.1(ii)F Endothelial cell abnormalities

Endoneurial capillary interendothelial cell tight junctions (TJ) comprise the main component of the blood/nerve barrier in peripheral nerves and, as such, pathological alterations of endothelial cells and any resultant deterioration of the TJs are potentially of considerable aetiological significance in the pathogenesis of diabetic polyneuropathy.

It has been proposed that diabetes mellitus, resultant hyperglycaemic stimulation of the polyol pathway and endoneurial polyol accumulation may lead to the osmotic disruption of epithelial and perineurial tight junctions, facilitating the entry of previously excluded agents which may be instrumental in the
development of a peripheral neuropathy.

In support of this theory, the loss of endoneurial capillary interendothelial cell TJs has been documented in the sural nerves of patients with diabetes mellitus (Sima et al., 1991), and disrupted perineurial TJs, which have been attributed to a similar origin, have been reported in the peripheral nerves of both diabetic patients (Beamish et al., 1991) and diabetic db/db mice (Schiavinato et al., 1991).

The permeability of vessels formed after the onset of diabetes does appear to be abnormal; new retinal capillaries in diabetic patients with proliferative retinopathy are substantially more "leaky" than those formed prior to the onset of the disease (Kohner et al., 1967), and angiogenic vessels within granulation tissue in streptozotocin-diabetic rats have been found to be more permeable than the vessels of the neighbouring skin or muscle from which they were derived (Kilzer et al., 1985) and structurally abnormal vessels within tissues commonly associated with diabetic complications i.e. aorta, kidney and peripheral nerves (Kilzer et al., 1985; Williamson et al., 1990a).

The proposal that hyperglycaemia and increased polyol pathway activity play a causal role in the aetiology of these abnormalities is supported by the fact that ARI therapy is capable of completely preventing hyperpermeability both within granulation tissue vessels in diabetic rats (Williamson et al., 1985) and similar capillaries that have been exposed to hyperglycaemic concentrations of glucose in non-diabetic rats (Williamson et al., 1990a), and retinal capillaries in galactosaemic rats (Lightman et al., 1987), genetically diabetic BB-rats (Williamson et al., 1987) and diabetic patients (Cunha-Vaz et al., 1986).

Evidence that the diabetic condition leads to the generation of similar detrimental effects within the vasa nervorum of peripheral nerves is limited. Although increased vascular permeability of the this capillary bed has been documented in alloxan-diabetic rats (Seneviratne, 1972) and the sciatic nerves of diabetic BB-rats (Williamson et al., 1987), other investigations have found both the perineurial and endothelial cell permeability barriers in BB- (Sima & Hay, 1981) and streptozotocin-diabetic rats (Sima & Robertson, 1978a) to remain functionally competent in preventing the penetration of the small molecular tracer microperoxidase. Furthermore, the presence of serum proteins in the endoneurium of peripheral nerves of patients with diabetes mellitus (Graham & Johnson, 1985) does not necessarily imply that the blood/nerve barrier has been
breached, as serum proteins have also been found in the peripheral nerves of healthy animals (Mata et al., 1987).

The plausibility of the proposed mechanism of interendothelial cell TJ disruption has also been queried. Galactose-intoxication provides an experimental model which demonstrates the pathological effects of augmented polyol pathway activity. Comparisons between diabetic and galactosaemic animals have been made in order to determine whether the polyol pathway plays a similar role in the genesis of diabetic peripheral neuropathies. The relevance of drawing analogies between the two conditions is questionable as neurological defects in galactosaemic animals, in contrast to the situation in diabetic neuropathies, may be mediated by primary Schwann cell damage as a result of the comparatively greater intracellular accumulation of osmotically active polyols.

Regardless of this pathogenetic discrepancy, the galactosaemic model has effectively demonstrated that the osmotic disruption of endothelial cell TJs within diabetic nerves is not a viable proposal as, although the endoneurial concentration of polyols in galactosaemic rats far exceeds that ever encountered in the peripheral nerves of diabetic animals, the interendothelial TJs of the vasa nervorum in galactosaemic rats have been found to remain intact (Forcier et al., 1991).

This study is in support of the observations of Sharma and Thomas, who failed to find any evidence of abnormal endothelial cell TJs in the vasa nervorum in the peripheral nerves of either streptozotocin or alloxan-diabetic rats (Sharma & Thomas, 1974) or any other morphological change that could account for the previously reported increased vascular permeability of this capillary bed in alloxan-diabetic rats (Seneviratne, 1972). No evidence was found of interendothelial TJ disruption in any of the included 368 diabetic endoneurial vessels included in this investigation.

Whilst it is generally agree that human endoneurial capillaries are not normally fenestrated, Johnson reported the presence of occasional fenestrated endoneurial vessels within peripheral nerve biopsies from diabetic neuropathic patients (Johnson, 1977). Fenestrated capillaries were not seen in any of the nerves examined in this study, but very occasional ones were found in some previous material (R.H.M. King, personal observation). Endoneurial capillary endothelial cell hyperplasia, as assessed by endothelial profile and nuclear number, has been found to be a prominent feature of
diabetic microangiopathy (Goldenberg et al., 1959; Timperley et al., 1985; Dyck et al., 1985b; Powell et al., 1985; Yasuda & Dyck, 1987; Britland et al., 1990; Malik et al., 1989, 1992). This conclusion is supported by the results of this study. An occasional contradictory report has been published; Sima and colleagues failed to find any morphological evidence of endothelial cell abnormalities within the vasa nervorum of human diabetic sural nerves, the number of endothelial cell nuclei number in the diabetic patients being comparable with that in the nerves of age-matched controls (Sima et al., 1991).

Lorenzi and colleagues reported that endothelial cells cultured in media with a high glucose content have a decreased growth potential (Lorenzi et al., 1985). They suggested that reduced intraendothelial cellular concentrations of myo-inositol may be causal, associated perturbations in inositol phospholipid metabolism impairing the transduction signals of extracellular trophic factors leading to a subsequent reduction in endothelial cell proliferation (Lorenzi et al., 1985). Alternatively the effects may be mediated by damage to the DNA duplication, a process which affects all phases of the cell cycle. These observations provide a possible explanation for hyperglycaemia-related endothelial cell loss and the generation of acellular capillaries characteristic of certain components of diabetic microangiopathy, but do not account for the degree of endothelial cell hyperplasia in the vasa nervorum associated with diabetic neuropathy, and question the validity of extrapolating conclusions drawn as a result of in vitro experiments to all analogous situations in vivo.

Endothelial cell hyperplasia associated with diabetes mellitus may be related to accelerated NEG as the synthesis and secretion of tumour necrosis factor (TNF), interleukin-1 (IL-1) and insulin-like growth factor-I (IGF-I) by monocytes and macrophages in AGEP-rich tissues is known to stimulate endothelial cell proliferation (Vlassara et al., 1988b) which possesses corresponding cell surface receptors. In accord with this proposal, aminoguanidine treatment has been found to prevent effectively both retinal endothelial cell proliferation and the concomitant accumulation of AGEPs in streptozotocin-diabetic rats (Hammes et al., 1991).

Microvascular endothelial cells are also capable of synthesising basic fibroblast growth factor (FGF), platelet derived growth factor (PDGF) transforming growth factor beta (TGF beta) and IGF-I (Yue et al., 1992) which are all being increasingly recognised as potential angiogenic regulators. Diabetic neuropathy may stimulate endothelial cell hyperplasia via the action of these factors;
following their synthesis, molecules of FGF are bound to the heparan sulphate proteoglycan component of the vascular BM and released following any form of tissue injury or hypoxia, so that they may subsequently exert a local angiogenic effect.

Endoneurial vascular endothelial cell hypertrophy, although not a ubiquitous finding (Yasuda & Dyck, 1987; Malik et al., 1989a, 1992) has also been described in the peripheral nerves of diabetic patients (Timperley et al., 1985; Powell et al., 1985), and statistically confirmed by this investigation.

The diabetic patients from whom specimens were obtained in studies which failed to find any evidence of endoneurial capillary endothelial cell hypertrophy had either clinically mild diabetic neuropathy (Malik et al., 1989a, 1992), or were non-neuropathic (Yasuda & Dyck, 1987; Malik et al., 1989a). It is possible that endothelial cell hypertrophy may only be associated with the more advanced stages of diabetic microangiopathy (Malik et al., 1992).

Endothelial cell hypertrophy and hyperplasia have both been linked with metabolic, haemostatic and pathological abnormalities in diabetes mellitus (Porta et al., 1987). Endothelial cell hyperplasia has been found to correlate with neuropathological abnormalities of myelinated nerve fibres in the sural nerves of diabetic patients (Dyck et al., 1985b; Yasuda & Dyck, 1987) and endothelial cell number to correlate inversely with both myelinated fibre density \( (p < 0.05) \) and with reductions in both motor and/or sensory nerve conduction velocities (Malik et al., 1992).

The number of endothelial cell nuclei has been found to increase slightly with age in the peripheral nerves of both diabetic and non-diabetic individuals (Dyck et al., 1986a). The frequency of large microvessels, with correspondingly greater number of endothelial cell nuclei, also increases with age (Sima et al., 1991). Hence, the extent of endothelial cell hyperplasia in the diabetic patients in two of these studies (Dyck et al., 1985b; Yasuda & Dyck, 1987) may be attributable to the effects of advancing age and not therefore directly related to the severity of neuropathy. Future investigation into the presence of a correlation between endothelial cell abnormalities and myelinated nerve fibre density within the nerves of the three groups of individuals involved in this study would be of interest with regard to the validation of the relationship.
Experimental ischaemia affects all components of the endoneurial capillaries within peripheral nerves (Benstead et al., 1990), including the endothelium, pericytes and BMs. Endothelial cell and BM hyperplasia, swollen and disrupted endothelial cell mitochondria, and increases in luminal area have been documented as a result of experimental ischaemia in the sciatic nerve of the rat (Benstead et al., 1990), these observations implying possible breakdown of endothelial cell function and compromisation or loss of the integrity of the blood/nerve barrier.

Endothelial cells are highly susceptible to ischaemic damage, as indicated by the fact that endothelial cells damage results from a relatively mild degree of ischaemia. Comparative detrimental endothelial cell abnormalities have been observed in the central ischaemic core and less hypoxic regions of the fascicles as indicated by the relatively mild degrees of focal demyelination. These observations in conjunction with the evidence that endothelial cell abnormalities are a component of diabetic microangiopathy have led to the proposal that diabetic polyneuropathy may be of an ischaemic origin. Malik and colleagues observed that the extent of endoneurial capillary disease within the PNS in patients with diabetes mellitus does not appear to correlate with the general degree of microangiopathy; endothelial cell hypertrophy and hyperplasia were evident in the sural nerves specimens but were not apparent in the vessels of either skin or muscles. The degree of BM hypertrophy was also found to be comparatively greater in the peripheral nerves (Malik et al., 1989a). As a result of these obvious inconsistencies it was proposed that endothelial cell abnormalities in the peripheral nerves of neuropathic diabetic patients result from endoneurial hypoxia.

In support of an ischaemic aetiology, endoneurial oxygen tension has been reported to be reduced to below critical levels in streptozotocin-diabetic rats (Low et al., 1984), suggesting that a high proportion of axons and Schwann cells are chronically exposed to hypoxic conditions. If this is true, the endoneurial cells would be forced to utilise the anaerobic glycolytic pathway in order to supplement the process of oxidative metabolism and fulfil their total energy-requirements. The results of in vitro studies have supported this theory; nerve creatine phosphate and lactate in streptozotocin-diabetic rats have been found to be respectively decreased and increased.

Endoneurial intracellular accumulations of glycogen have also been documented in these animals (Powell et al., 1977; Bestetti et al., 1981; Moore et al., 1981a;
Sima et al., 1982). It has been suggested that these glycogen deposits acts as a substrate store for anaerobic respiration and are produced in specific response to hypoxia (Grover-Johnson & Spencer, 1981). Together these physiological abnormalities may contribute to the resistance to ischaemic nerve conduction failure shown by diabetic nerves, a proposal which is supported by the evidence that this phenomenon is attenuated as a result of oxygen supplementation.

Further support a causative role of hypoxia in the production of diabetic neuropathy is provided by the observation that inactivation of the sodium channels and permeability changes in alloxan-diabetic rats (Brismar, 1979) have been found to be reproducible in healthy animals as a result of exposure to anoxic conditions (Brismar, 1981).

The reductions that have been found in the myo-inositol content of peripheral nerves in experimental diabetes (Greene & Winegrad, 1981) may be a direct consequence of endoneurial hypoxia, as the cellular uptake of myo-inositol is an energy-dependent process (Greene & Lattimer, 1982) being mediated by an active transport system involving Na^+-K^+-ATPase, as evidenced by the effects of Na^+ deprivation and ouabain inhibition of Na^+-K^+-ATPase activity (Okuda et al., 1991).

Despite these observations a link has also been found between the polyol pathway and myo-inositol metabolism in endothelial cells: diminished myo-inositol uptake by cells cultured in a hyperglycaemic media was found to be attenuated following the addition of an ARI (Okuda et al., 1991). The relationship between these two pathways remains elusive. It does not seem unreasonable to propose that hyperglycaemic stimulation of the polyol pathway and decreased myo-inositol content are likely to have a direct effect on endothelial cell function (Hawthorne et al., 1989), however, the nature of the alterations is as yet unestablished.

The results of this study have revealed that the microvascular alterations associated with diabetic polyneuropathy and ischaemic neuropathy (Benstead et al., 1990) probably do not have a common origin. No evidence was found of endothelial cell degenerative phenomena in the diabetic biopsy specimens, as had been noted previously by Malik and colleagues (Malik et al., 1992). The endothelial cells of the vasa nervorum in the diabetic nerves appeared healthy, and morphologically indistinguishable from those in the HMSN I and organ donor control specimens. It has been suggested that endothelial cell
abnormalities in acute experimental ischaemia (Benstead et al., 1990) may not parallel those in chronic experimental ischaemia, a theory which remains to be investigated, and considering that diabetes is a chronic disease this may explain the discrepancies that were found between diabetic and experimental ischaemic microvascular abnormalities.

Although endoneurial vascular endothelial cell hyperplasia and hypertrophy are accepted to be commonly associated with diabetic polyneuropathy, these alterations do not imply a change in, or impairment of, barrier function. Endothelial cell area was found to be significantly increased in the diabetic group in this study in comparison to that seen in the control specimens; however, the degree of endothelial cell hyperplasia in the HMSN I cases, a disease in which vascular factors are known to be of no aetiological significance, was found to be significantly greater than that in the diabetic nerves. This implies that endothelial cell abnormalities are not of aetiological significance with regard to the generation of neuropathological alterations, but are merely a co-existent phenomenon. Furthermore, Llewelyn and colleagues (Llewelyn et al., 1988) proved that a focal pattern of degeneration does not necessarily imply an ischaemic cause, as they found the non-uniform focal and patchy pattern of fibre loss in the sural nerves of patients with diabetic polyneuropathy to be indistinguishable from that associated with HMSN I.

Further evidence against the likelihood that diabetic polyneuropathy is of ischaemic origin concerns the presence of obvious differences between the pathology and clinical features associated with human ischaemic and diabetic neuropathies. The pathological pattern of primary demyelination evident in nerve biopsies from patients with chronic obstructive airways disease, which is attributable to prolonged endoneurial hypoxia (Malik et al., 1990), is distinctly different from that associated with diabetic polyneuropathy. In addition, the preferential loss of large fibres and relative sparing of unmyelinated axons in ischaemic vasculitic neuropathy results in a clinically greater loss of motor function than sensory perception with only minor autonomic dysfunction (Fujimura et al., 1991), a situation which is clearly dissimilar to diabetic distal symmetrical polyneuropathy which predominantly involves sensory and autonomic function.

In recent years another mechanistic theory relating to augmented polyol pathway activity and the subsequent generation of ischaemia within the endoneurial compartment has been proposed. Deficits in aortic and arterial
endothelium-dependent relaxation have been reported in experimental diabetes (Meraji et al., 1987; Durante et al., 1988; Cameron & Cotter, 1992a) and have been attributed to hyperglycaemic stimulation of the polyol pathway. This proposal has been substantiated by the evidence that endothelial cells contain the polyol pathway (Hawthorne et al., 1989). ARI therapy effectively normalises this deficiency (Cameron & Cotter, 1992a) and rats rendered hyperglycaemic by glucose infusion exhibit a reduction in blood flow of a similar magnitude to that seen in diabetic rats. Concomitant patchy aortic endothelial cell damage was observed in some cases but was not a ubiquitous finding suggesting that the derangement was primarily of a metabolic origin.

These abnormalities were designated as being the result of the impaired production or release of nitric oxidase (NO), otherwise known as vascular endothelium-derived relaxing factor (Meraji et al., 1987; Durante et al., 1988), as opposed to deficits in the smooth muscle guanylate cyclase system or acetylcholine reception or transduction (Cameron & Cotter, 1992a). This statement is based on the experimental observation that the acetylcholine-induced relaxation of precontracted diabetic arteries is equivalent to that of healthy arteries (Head et al., 1987), although one report suggested that the exposure of healthy rat arteries to elevated glucose concentrations results in impaired acetylcholine induced relaxation (Tesfamariam et al., 1990).

Increased flux along the polyol pathway may affect that production of NO via one of two mechanisms. Firstly, considering the fact that AR requires NADPH as a cofactor, under diabetic hyperglycaemic conditions competition will arise with other NADPH-requiring enzymes. NO synthase, which mediates the synthesis of NO from L-arginine (Moncada et al., 1989), is such an enzyme. Its activity will therefore be reduced leading to a diminished content of NO.

The second mechanism relates to reduced ATP synthesis. When polyol pathway flux is high, glucose is diverted through the pentose phosphate shunt, a process which results in a deficit of 3 molecules of ATP for every glucose molecule passing along this route. Agonist-stimulated NO release requires metabolic energy; the administration of inhibitors of oxidative and glycolytic metabolism in healthy rats was found to result in a reduction of aortic NO synthesis (Griffith et al., 1986; Weir et al., 1991), and as such this deficit in energy metabolism may be sufficient to impair NO production.
The concomitant accumulation of AGEPs on matrix elements within hypertrophic vascular diabetic BMs may contribute to the phenomenon of abnormal endothelium-dependent relaxation by three different mechanisms. Firstly it has been proposed that AGEPs may chemically quench NO in a dose-dependent manner; the levels of accumulated AGEPs in experimentally diabetic animals have been found to correlate well with defects in the vasodilatory response to NO (Bucola et al., 1990). Secondly, reduced microvascular distensibility due to the accumulation of AGEPs within hypertrophic diabetic capillary BMs may physically impair the effects of vasodilatory factors (Kastrup et al., 1987). Finally, the production of oxygen-free radicals as a direct result of protein glycation and subsequent metabolism may also prevent vasodilation (Brownlee et al., 1988).

In addition, endothelial cells express at least 2 cell surface polypeptides, termed AGE-binding proteins, that function as specific acceptor molecules for glucose modified proteins, advanced glucose derivatives or AGEP accumulation on proteins being necessary for recognition (Schmidt et al., 1992). AGEPs bound to these receptor sites are internalised and subsequently degraded and/or trancytosed. It has been suggested that these two polypeptides are subunits of a complex which may play an important role in processes subsequent to binding, such as endocytosis and signal transduction mechanisms. Accelerated NEG may also therefore exert a direct effect on endoneurial vascular function by this process; AGE-endothelial cell interactions are capable of modulating a range of endothelial cell functions, i.e. NO (Bucola et al., 1991).

Tissue protection against oxidative stress and superoxide radicals may be compromised by diabetes mellitus. Superoxide dismutase activity are reduced as a result of the hyperglycaemic stimulation of AR activity, as is and the subsequent breakdown of NO (Gryglewski et al., 1986). To compound the problem further, NEG which is known to be accelerated as a result of the diabetic milieu, is a process which generates oxygen-free radicals.

All of these factors are likely to have a cumulative effect on endothelial function and integrity in the diabetic state, a situation which is exacerbated by the fact that the endothelium from diabetic rats has been found to be abnormally sensitive to superoxide damage. This statement is supported by the observations that oxygen free radicals elicit a reduction in aortic endothelium-dependent relaxation in diabetic rats (Peiper & Gross, 1988) and scavengers of oxygen-derived free radicals are capable of attenuating
endothelial cell dysfunction (Tesfamariam & Cohen, 1993). These numerous mechanisms may contribute to reductions in the levels of NO within diabetic vasculature, an abnormality which may subsequently result in the generation of vasoconstrictive abnormalities and, theoretically, the production of endoneurial ischaemia within the peripheral nervous system.

In support of this hypothesis reduced endoneurial blood flow and hypoxia have been documented in the peripheral nerves of diabetic rats (Tuck et al., 1984), endoneurial oxygen tension has been found to be reduced in diabetic patients (Newrick et al., 1986). Treatment with guanethidine (Cameron et al., 1991a), resulting in the production of a functional adrenergic sympathectomy and Prazosin, an α-adrenoreceptor blocker resulting in the blockage of sympathetic vasoconstrictor tone (Cameron et al., 1991b) have been found to effectively prevent the development of reductions in endoneurial blood flow and NCV deficits in streptozotocin-diabetic rats.

The theory that NO production is attenuated by diabetes mellitus leading to endoneurial hypoxia requires further substantiation as doubts have been expressed regarding the validity of experimental in vitro evidence purporting to show that NO-mediated vascular relaxation is decreased by diabetes (Tilton et al., 1993); the vessels were cultured in media which was lacking in L-arginine or any other N-substituted arginine containing peptides (Meraji et al., 1987; Durante et al., 1988) and the lack of a suitable substrate would therefore effectively exert a rate-limiting effect on the production of NO regardless of the actual concentration of NO synthase. In addition the concentration of glucose used in another investigation far exceeded the maximum that would ever be encountered under physiological conditions (Tesfamariam et al., 1990).

5D.1(ii)G Pericytes

The incidence of pericyte degeneration, identified by the presence of interlaminal cellular debris, and acellular capillaries i.e. ghost vessels primarily composed of a redundant tube of BM devoid of any intact central endothelial cell lining, both previously considered to be characteristic degenerative phenomena unique to diabetic retinopathy, have been reported to be prevalent in the skeletal muscle of the lower extremities of patients with diabetes mellitus (Vracko, 1970; Williamson & Kilo, 1980; Tilton et al., 1981, 1985). This suggests that a common pathophysiological mechanism may be involved in the aetiology of diabetic vascular complications throughout the entire body. The trend for these two abnormalities to increase in a proximo-distal direction (Tilton et al., 1985)
also implies that increases in venous pressure may be of aetiological significance although the pathological mechanism remains unclear.

Two different pathogenetic mechanisms have been proposed as being potentially responsible for pericyte degeneration and the increased incidence of acellular capillaries within diabetic tissue. The first is related to ischaemia. Generalised arteriosclerosis is more prevalent in diabetic individuals than the general population and is a process which may result in chronic ischaemia within the target organ and subsequent necrosis and atrophy of the distal microvasculature as manifested by the presence of acellular capillaries.

In support of this hypothesis: (1) the incidence of medium-sized muscular arterial occlusion has been found to be abnormally high in diabetic patients, (2) the development of analogous detrimental endothelial cell and pericyte alterations to those associated with diabetic microangiopathy have been reported in experimental ischaemia (Benstead, 1990), and (3) hyalinization and precapillary arteriolar obliteration have been documented in the diabetic retina (Ashton, 1953), further support for the theory that focal retinal ischaemia plays a prominent causal role in the generation of diabetic retinopathy (Bresnick et al., 1975).

The second causal mechanism is centred upon hyperglycaemic stimulation of the polyol pathway. Retinal pericytes, which contain AR, accumulate sorbitol when cultured in media with a high glucose concentration. The fact that extensive cellular degeneration was seen to occur after 30 days incubation suggests that sorbitol accumulation leads to the development of detrimental metabolic perturbations which render the pericytes nonviable (Buzney et al., 1977). Further support for this theory was provided by the discovery that ARI treatment prevented characteristic retinal pericyte loss in galactosaemic dogs (Kador et al., 1988). The retinal changes associated with galactose intoxication are akin to those of diabetic retinopathy and as such this mechanism has been theoretically extended in explanation of the comparative degenerative phenomena associated with this syndrome. It is possible that diabetes-related pathological abnormalities in pericytes may lead to endothelial cell damage and the ultimate production of acellular capillaries.

Treatment of streptozotocin-diabetic rats with aminoguanidine, which is a proficient inhibitor of AGEP formation but a poor AR inhibitor, has also been found to result in a significant reduction in retinal pericyte loss in conjunction
with the prevention of retinal AGEP accumulation, implying that the pathological process is multifactorial (Hammes et al., 1991).

Within the PNS, although endoneurial microvascular pericyte degeneration has been reported in the peripheral nerves of galactosaemic rats (Forcier et al., 1991), and attributed to enhanced flux along the polyol pathway following the localisation of the enzyme AR to these cells (Chakarabarti et al., 1987), no evidence has been found that diabetes mellitus exerts any similar deleterious effect on the ultrastructural integrity of endoneurial capillary pericytes (Yasuda & Dyck, 1987; Malik et al., 1990a, 1992). In actuality the situation appears reversed, as the majority of studies have attested to the presence of a prominent degree of pericyte hyperplasia as demonstrated by a quantitatively significant increase in the number of pericyte nuclei in patients with both clinically mild diabetes mellitus \( (p < 0.02) \) (mean value in diabetics 0.83, control 0.59) (Malik et al., 1992) and those with established neuropathy (Yasuda & Dyck, 1987; Malik et al., 1990a). Conflicting testimony has been published, some authors having failed to find evidence of any obvious quantitative structural abnormalities (Sima et al., 1991), but these reports are in the minority. The results of this study comply with the greater body of evidence, as, despite the fact that no significant difference was found between the diabetic and control group with reference to the median number of pericyte processes, the inter-quartile range in the diabetic patients was significantly higher.

The hypertrophic basal laminal zones surrounding a large number of the diabetic endoneurial vessels examined in this study contained evidence of cellular debris which appeared to be of perineurial cell origin. Similar observations have been published by Giannini and Dyck (1994). This fact in association with the increase in perineurial cell process number observed in this investigation and the previously reported evidence of perineurial cell hyperplasia (Yasuda & Dyck, 1987; Malik et al., 1990a, 1992) imply that the marked reduplication of BM characteristic of diabetic endoneurial microvessels may be due to enhanced pericyte as opposed to endothelial cell or macrophage turnover (Giannini & Dyck, 1995). This hypothesis is supported by the finding that evidence of endothelial cell degenerative phenomena or the localisation of macrophages within the vessel walls are infrequently encountered in these same capillaries (Giannini & Dyck, 1995).

If ischaemia is the pathogenetic mechanism at play, the fact that peripheral nerves possess an extremely efficient collateral blood supply may sufficiently
explain the generation of discrepancies between the incidence of these degenerative phenomena within neural and other tissues. Further validation is required of the specific aetiological role played by enhanced AR activity in retinal and muscle capillary pericyte degeneration in order to explain why comparative changes do not occur in the vasculature of peripheral nerves.

Tilton and colleagues (Tilton et al., 1985) measured the pericyte circumferential coverage of skeletal muscle capillaries and found that, despite the fact that the amount of pericyte degeneration was significantly greater in the lower extremities of diabetic patients, the percentage of pericyte circumferential investment was comparable with that in control individuals. This implies that the homeostatic mechanism governing the relative proportions of pericyte to endothelial cells was able to fully compensate for the abnormal degree of pericyte degeneration, a situation which contrast with that in the retina where the regenerative capacity of pericytes appears to be exhausted leading to their selective loss. Although quantification of this variable was not performed in this study, we conclude that the situation within peripheral nerves appears comparable with that in skeletal muscle, as the percentage investment of the endothelial cell surface of endoneurial capillaries by pericyte processes in the diabetic nerves did not appear significantly different to that in the control nerves.

The physiological functions of pericytes are at present poorly understood. It has been suggested that retinal pericytes may prevent the proliferation of associated endothelial cells; when co-cultured, direct contact between the two cell types effectively inhibited endothelial cell multiplication, however, when the cells were separated by a porous membrane, which allowed the diffusion of soluble materials but prevented actual physical contact, endothelial cell proliferation occurred (Orlidge & D’Amore, 1987). It has been suggested that neovascularisation in diabetic retinopathy may occur as a direct result of the selective loss of retinal capillary pericytes (Speiser et al., 1968).

The fact that endothelial cell proliferation is also prevented by direct contact with aortic smooth muscle cells supports the theory that pericytes and the smooth muscle cells perform common functions within capillaries and large blood vessels respectively. Confirmation of this proposal was provided by Tilton and colleagues who demonstrated that the pericytes of skeletal muscle capillaries exhibit contractile responses when stimulated with vasoconstrictive agents (Tilton et al., 1977). Diabetes-related abnormalities in these functions
may potentially result in the generation of haemodynamic changes.

5D.2 Derangement of the blood/nerve barrier

The 3-dimensional self-assembly of BMs, which normally involves precise geometric interactions between type IV collagen, laminin, heparan sulphate proteoglycan and entactin appears disordered by AGEP induced changes in diabetes mellitus and may result in an effective increase in intermolecular pore size and disruption of its size-selective filtration efficiency (Parthasarathy & Spiro, 1982; Brownlee et al., 1984; Deckert et al., 1988).

Despite their thickened appearance, diabetes results in the production of detrimental alterations in the filtration properties of GBMs as evidenced by increased glomerular permeability to macromolecules (Seneviratne 1972); proteinuria has long been regarded as a clinical hallmark of diabetic renal microangiopathy. This phenomenon may be partially explained by the effects of NEG, as the permeability of glycated albumin is significantly greater than that of native albumin (Patel et al., 1991).

The negative charge of the glomerular wall is largely responsible for its filtration selectivity due to effective repulsive charge interactions and steric hindrance of negatively charged molecules such as plasma albumin. A substantial degree of the negative charge of the GBM is conferred by the net anionic charge of the sialic acid and proteoglycan i.e. heparan sulphate, components of the BM (the anionic charge of GAGs largely results from the periodic situation of sulphate residues along the length of the polymers) (Kanwar & Farquhar, 1979a). The content of both sialic acids and heparan sulphate residues have, as previously stated, been found to be reduced in human and experimental diabetes and reductions in heparan sulphate content have been correlated with proteinuria and increased glomerular permeability (Groggel et al., 1988; Vernier et al., 1992) to agents such as ferritin (Kanwar et al., 1982) and $^{125}$I-labelled albumin (Rosenzweig & Kanwar, 1982).

Abnormal NEG-related binding of anionic glycated proteins to diabetic vascular BM matrices may alter the integrity of the charge-selective filtration barriers, a theory supported by the fact that the increased glomerular clearance of IgG must be due to a defect in the GBMs size-selective barrier (Friedman et al., 1983), and that the synthesis of a grossly thickened BM occurs as an attempt to compensate for these changes and restore normal permeability.
It is feasible that analogous alterations in the composition of endoneurial capillary BMs may be of aetiological significance in the production of other diabetic complications via an alteration in the blood/nerve barrier. Increased vascular permeability, as revealed by fluorescein angiopathy, is a prominent abnormality in diabetic retinopathy and has potentially been attributed to a combination of endothelial cell changes and BM leakiness. Tracer studies have revealed that small vessels throughout the rest of the body are also abnormally permeable in patients with long-standing diabetes mellitus (Williamson & Kilo, 1976), the transcapillary escape rate of albumin and IgG (Parving & Rossing, 1973) and sodium fluorescein in dermal capillaries (Bollinger et al., 1982) have both been found to be increased in diabetic tissue. The capillaries of skeletal muscles are also known to be similarly affected (Alpert et al., 1972).

The development of such alterations within the endoneurial vascular plexus of peripheral nerves may play a role in the pathogenesis of diabetic polyneuropathy, via the inadequate or excessive entry or exit of metabolites. This does not seem to be a very likely scenario. The vascular endothelium has been identified as the main component of the capillary permeability barrier and the blood/nerve barrier of the PNS is extremely efficient as a result of the presence of specialised TJs between the endothelial cells of endoneurial capillaries; the BMs of vessels from the diaphragmatic muscles of rats do appear to provide a structural restraint to the transcapillary movement of intravenously injected ferritin molecules, no evidence of accumulations of the marker being found within the basal laminal zones (Bruns & Palade, 1968).

There is limited evidence that any anomaly occurs in the peripheral blood/nerve barrier of diabetic patients as a result of either disruption of these structures or the abnormal transcellular movement of molecules and as such the theory that the hypertrophic BM of endoneurial capillaries is produced as a compensatory measure is not a very probable. Increased IgG and albumin content have been reported in the endoneurium in diabetic neuropathy (Poduslo et al., 1988) but this could be due to augmented transfer of glycated molecules.

It is more likely that in this case capillary BM hypertrophy is purely due to the physical effects of NEG which would render both structural BM proteins and any bound circulating proteins abnormally resistant to catabolism and degradation resulting in their abnormal persistence, their subsequent accumulation resulting in the characteristic reduplicated appearance of endoneurial vessel BMs, the structural hallmarks of diabetic microangiopathy.
Indeed, variations in vessel calibre, with the production of a new investment of basement membrane following each alteration, may be a normal occurrence in healthy nerves, but, due to the fact that in healthy nerves redundant BM is fairly rapidly degraded the process is not obvious.

5D.3 Immune mechanisms

A theory has been proposed that exogenous insulin, introduced as therapy to control the diabetic state, may be perceived as a foreign antigen and in this manner elicit an immune-mediated response which may be instrumental in the development of associated complications such as renal disease.

Another possible immune-mediated mechanism relates to the effects of accelerated diabetic NEG and the accumulation ofAGEPs; Amadori products are poor immunogens (Brownlee et al., 1984). It has been suggested that the presence of both AGEPs in their own right and "trapped" plasma proteins such as albumin and IgG bound to AGE adducts on long-lived peripheral nerve proteins (Brownlee et al., 1983) effectively act as in situ immune complexes which initiate subsequent macrophage scavenger-receptor mediated degenerative phenomena (Vlassara et al., 1985a). The discovery that the major peripheral nerve myelin protein \( P_\theta \) undergoes NEG in experimental diabetes (Vlassara et al., 1983) led to the proposal that NEG may initiate myelin uptake and degradation via macrophage-mediated phagocytosis thereby stimulating or contributing to the process of segmental demyelination, the degree of which has been shown to correlate with the severity and duration of hyperglycaemia (Vlassara et al., 1984). Additional evidence in support of this theory was provided by the detection of abnormal nonspecific deposition of IgM and complement \( C_\gamma \) in diabetic peripheral nerve PCBMs (Graham & Johnson, 1983), IgG, albumin and polyvalent immunoglobulins in human diabetic kidney glomerular and tubular BMs, and parotid ductal and acinar BMs (Murrah et al., 1984), albumin in dermal capillary BMs (Chavers et al., 1981) and activated complement (poly C9) membrane attack complex in diabetic kidneys (Falk et al., 1983b).

The failure to detect insulin or insulin antibodies within BMs, the presence of albumin which is a non-immunologically associated protein and the absence of any detectable anti-BM serum antibodies (Miller & Michael, 1976) or any evidence of the initiation of an immune response, i.e. invasion by inflammatory cells, a typical marker of immune injury, serve to refute the theory that immune-mediated mechanisms are of aetiological significance in the
development of related degenerative or pathological diabetic phenomena.

Recent evidence has, however, been produced which suggests otherwise. Inflammatory infiltrates and vasculitis were seen to be associated with axonal and demyelinating lesions in the proximal nerves of patients with severe proximal diabetic neuropathy but not those individuals with milder forms of the same syndrome, the lesions appearing in the latter case, to resemble those characteristic of distal symmetric sensory polyneuropathy (Said et al., 1994).

These observations imply that metabolic factors may be of primary importance in distal symmetrical sensory polyneuropathy, the more severe forms of proximal diabetic neuropathy being produced by or associated with a superimposed inflammatory process and/or reactive vasculitic ischaemia which is triggered by the initial lesions.

5D.4 Hypertension

Diabetes and hyperglycaemia appears to induce metabolic, functional and structural changes in the vasculature (Tilton et al., 1989; Pugliese et al., 1991) which greatly increase the susceptibility of these vessels to injury by additional diabetes-independent genetic and environmental risk factors (Pugliese et al., 1991) such as hypertension, hypercholesterolaemia, dietary protein intake, etc. (Barbosa & Samer, 1984; Dunn, 1990). Whilst virtually all diabetic subjects develop early vascular functional and structural changes, increases in vascular permeability and haemodynamic changes in certain tissues have been demonstrated soon after the onset of poorly controlled diabetes in both experimental (Williamson et al., 1987; Tilton et al., 1989; Pugliese et al., 1990) and human diabetes (Ohl et al., 1985; Deckert et al., 1989), these only progress to late vascular complications and endstage vascular disease in some cases.

The independent occurrence of diabetes and hypertension, in streptozotocin-diabetic and non-diabetic rats respectively, has been shown to result in increased vascular permeability to $^{125}\text{I}$-albumin within tissues that primarily corresponded with sites of clinically significant vascular disease in human diabetes mellitus, i.e. the eyes and aorta (Tilton et al., 1992). When diabetes and hypertension were combined in the same animals, an additive effect was produced on both enhanced permeability to $^{125}\text{I}$-albumin in the eyes, aorta and granulation tissue, and GBM thickening. The combined effect of these two factors was found to have no impact on vascular permeability in those tissues unaffected by diabetes or hypertension alone, suggesting that the
preferential development of diabetic vascular dysfunction within these tissues is probably related to a greater susceptibility to injury via diverse mechanisms. It is not therefore an unrealistic theory that hypertensive arteriolar pressure may play a role of aetiological significance in the development of generalised diabetic microangiopathy (Parving et al., 1983; Zatz et al., 1986) and augmented vascular permeability (Williamson et al., 1990a). Substantiation of this theory has been provided by numerous studies which have provided evidence of the co-existence of the various component factors in this hypothesis.

Conflicting evidence has been produced regarding the presence of any relationship between the incidence of diabetic muscle capillary BM thickening and hypertension and venous blood pressure, Zacks and colleagues contending that such an association does not exist (Zacks et al., 1962), whilst in a more recent study muscle capillary BM thickening was found to correlate with venous hydrostatic pressure in both diabetic and non-diabetic individuals (Tilton et al., 1989).

The role played by hypertension in the aetiology of diabetic pathological microangiopathic complications in the eye and kidney is less ambiguous, the incidence and severity of diabetic nephropathy and retinopathy both having been repeatedly found to correlate with elevations in blood pressure (Knowler et al., 1980; Parving et al., 1981). Concomitant reductions in glomerular pathology and permeability have been found to accompany alleviation of elevated systemic blood pressure in diabetic patients (Parving et al., 1985; Zatz et al., 1986b).

Daniels and Hostetter (1991), in a study designed to assess the nature of glomerular structural and functional alterations in the permeability barrier related to galactosaemia, recently provided further evidence that hypertension contributes to the development of increased microvascular permeability and BM thickening in diabetes. They found that galactose intoxication resulted in the development of proteinuria, but only at a relatively low level and in the absence of any concomitant GBM thickening, a surprising observation considering the fact that galactose is more reactive than glucose in inducing protein glycation. The glycation of circulating plasma proteins was very rapid and the extent of GBM NEG was increased (Daniels & Hostetter, 1991). The slight increase in glomerular permeability was attributed to a combination of NEG-related functional impairment of the filtrational GBM barrier and enhanced permeability to circulating glycated proteins.
The relatively greater severity of proteinuria accompanying diabetic nephropathy must therefore be related to a pathogenetic factor which is absent in the galactosaemic model. It was suggested that this is a haemodynamic component as glomerular capillary hypertension is known to be associated with diabetic (Zatz et al., 1986b) but not galactosaemic glomerulopathy. It may therefore be concluded that although NEG may contribute to the development of diabetic nephropathy, it is insufficient to account for the extent of glomerular pathology and pathophysiology associated with diabetic renal disease in the absence of any co-existent increase in glomerular micropressure.

5D.4(i) Increased production of nitric oxidase

The nucleophilic hydrazine aminoguanidine and its analogues inhibit both NO synthase activity, as a result of their structural similarity to L-arginine (Corbett et al., 1992), and the action of diamine oxidase, an enzyme which plays a role of instrumental importance in histamine metabolism (Hui & Taylor, 1985).

Treatment with aminoguanidine has also been found to result in the attenuation of haemodynamic vascular anomalies in early experimental diabetes. Aminoguanidine has been shown to counteract effectively the development of increased regional blood flow and enhanced vascular permeability in the sciatic nerve, retina, aorta and kidneys (Ido et al., 1990; Williamson et al., 1990b; Tilton et al., 1990; Itakura et al., 1991; Soulis-Liparota et al., 1991; Corbett et al., 1992).

As evidence has been cited of the concurrent inhibition of NO synthase activity and NO production in these studies (Corbett et al., 1992) the development of the these alterations have been attributed to a diabetes-mediated increase in NO synthase activity. NO is a potent vasodilator which increases blood flow and mediates the effects of endothelium-dependent vasoactive agents such as histamine and bradykinin and hence their functional capacity in the regulation of increases in blood flow and vascular permeability.

A recent study has provided evidence which confirms this hypothesis. Tilton and colleagues found that aminoguanidine and methylguanidine administration were equally effective in normalising augmented vascular permeability in the sciatic nerve, aorta and ocular tissues of streptozotocin-diabetic rats (Tilton et al., 1993). Methylguanidine is a considerably less potent inhibitor of AGEP formation than aminoguanidine; it lacks the structural reactive hydrazine moiety to which this function is primarily attributed, and neither agent possesses a
profound AR inhibitory capacity but are similarly efficacious in the inhibition of NO synthase activity, suggesting that the beneficial effects of therapy in both instances were clearly attributable to repression of NO synthase activity.

These results suggest that diabetes-related increases in the activity of NO synthase are pathogenetically related to early vascular dysfunction and that AGEP formation may be more influential in the generation of end-stage structural vascular alterations. The poor AR inhibitory ability of aminoguanidines also suggests that augmented polyol pathway activity is not of aetiological significance in the development of diabetic microangiopathy.

If NO synthase activity and NO production are increased by diabetic metabolic derangements, vascular endothelial cell contractile function would be expected to be impaired, a feature which has been demonstrated in the aorta of streptozotocin-diabetic rats (Cameron & Cotter, 1992a). Such an effect in arteriolar vessels would result in locally decreased vascular resistance and increased blood flow, the transmission of these effects to vessels further downstream resulting in microvascular hypertension within the terminal capillaries.

5D.4(ii) Thromboxane $A_2$ synthesis

Thromboxane $A_2$ is a short-lived potent vasoconstrictive prostaglandin which acts as a mediator of glomerular intravascular pressure. The potency of this agent and its effect in the generation of microvascular abnormalities have been demonstrated by the evidence that increased thromboxane $A_2$ synthesis positively correlates with proteinuria in experimental nephrotic syndrome (Remuzzi et al., 1985) and the progression of renal disease in hypertensive rats is effectively ameliorated following inhibition of thromboxane $A_2$ synthesis (Purkeson et al., 1982).

Thromboxane $A_2$ has been proposed to play a similar role in the generation of glomerular hypertension and the development of diabetic nephropathy. The glomerular production of this agent has been found to be increased in chronic experimental diabetes (Schambelan et al., 1985), and the specific inhibition of thromboxane $A_2$ synthesis results in a clinical reduction in albuminuria in both diabetic patients (Barnett et al., 1984) and diabetic KKAy mice without any concomitant reduction in blood glucose or levels of glycated haemoglobin (Ledbetter et al., 1990). In the latter study levels of mRNA encoding for collagen type IV, which were significantly elevated in untreated animals, were
also reduced, implying that thromboxane $A_2$ may also have a direct stimulatory
effect on the synthesis of BM elements (Zatz et al., 1986a, 1986b).

5D.4(iii) Innervation of the vasa nervorum

It has been suggested that unlike the situation in most tissues, e.g. heart, the
vascular system of peripheral nerves possesses no autoregulator capacity (Low
& Tuck, 1984), and is therefore generally unable to compensate for local
reductions in blood pressure and oxygen delivery, despite the fact that Lundborg
and Brånemark had previously revealed that stimulation of the lumbar
sympathetic plexus results in a marked constrictional response in the vascular
supply of peripheral nerves, suggesting that they are in fact regulated by
sympathetic innervation (Lundborg & Brånemark, 1968). A recent ultrastuctural
study revealed that the terminal arterioles which supply the endoneurial vascular
plexus are innervated (Beggs et al., 1991) and neurotransmitters which elicit
vasodilator and constriction responses have been localised to the perivascular
fibres of the vasa nervorum (Appenzeller et al., 1984). These observations
suggest that endoneurial blood flow within peripheral nerves may be regionally
regulated to meet the metabolic requirements of individual fascicles.

The incidence of abnormalities of the vasa nervorum has been found to be
greater in the peripheral nerves of diabetic patients than healthy controls, the
discrepancy, although evident in endoneurial capillaries, being most prominent in
transperineurial vessels (Beggs et al., 1991). More than 40% of diabetics have
clinical autonomic dysfunction and the incidence is even greater in individuals
with peripheral somatic neuropathy, implying perhaps that this starts as an initial
autonomic neuropathy contributory to the development of peripheral somatic
neuropathies.

One proposed mechanism is that precapillary denervation affecting the
perivascular innervation of the vasa nervorum leads to the development of
limited vascular reactivity (Kastrup et al., 1987) via the production of structural
and functional microangiopathic alterations (Malik et al., 1989b) which in
association with rheological factors result in the development of hypoxic
conditions and ischaemic lesions within peripheral nerve. It has been deduced
that the endoneurial capillary BM thickening associated with a variety of
inherited and acquired neuropathies (Behse et al., 1977) may similarly result
from primary precapillary denervation (Malik et al., 1992) and capillary
hypertension (Williamson & Kilo, 1977).
Beggs and colleagues, however, were unable to demonstrate any supportive correlation between perivascular denervation and fibre loss and as such the aetiological significance, if any, of perivascular innervation remains unestablished (Beggs et al., 1991). It is possible that the opening of epineurial shunt vessels could direct blood from the intrafascicular compartment of nerve and thus contribute to endoneurial hypoxia.

Diabetic skeletal muscle capillary BM hypertrophy seems to be primarily mediated by hyperglycaemia and accelerated NEG rather the effects of enhanced polyol pathway activity, ARI treatment in patients with diabetes mellitus having been found to be of no beneficial effect in attenuating this abnormality (Ramirez et al., 1991). In addition, the ischaemic pathogenetic mechanism mediated by increased NO synthase activity and subsequently increased microvascular blood flow and hypertension within the relevant capillary bed would have minimal effect in skeletal muscle capillary beds. In explanation of this statement, all the component vessels within the retinal capillary bed are effectively open at all times, therefore any increase in arteriolar blood flow will be directly transmitted to the retinal plexus resulting in retinal capillary hypertension. By contrast, under resting conditions as few as 10% of the constituent vessels of the skeletal muscle capillary bed may contain flowing blood and as such any increases in nutrient arteriolar blood flow in this system may be easily accommodated by recruitment of reserve vessels thereby effectively preventing any appreciable increase in intravascular pressure.

Dolman reported that the incidence of endoneurial and transperineurial vessel cellular hyperplasia and luminal narrowing within the peripheral nerves of both diabetic and non-diabetic hypertensive individuals was comparable, implying that although hypertension may be responsible for the production of these abnormalities they play no pathogenetic role in the generation of neuropathic alterations (Dolman, 1963).
CHAPTER 6. SUMMARY AND FUTURE WORK

Changes in the connective tissue matrix are intimately associated with both human and experimental diabetes mellitus, the hallmark of diabetic connective tissue is the deposition of collagen at numerous periarticular sites and in the skin, and generalised basement membrane hyperplasia of both vascular and non-vascular cells is a characteristic alteration in long-standing diabetes. It has been suggested that they may play a role in the development of functional long-term complications.

Morphometric observations on endoneurial capillaries and perineurial cells from sural nerve biopsies of patients with diabetes mellitus were compared with results from organ donor control cases and from patients with hereditary motor and sensory neuropathy type I. The area of the lumen of capillaries did not differ between the three groups. The area occupied by the capillary endothelial cells in transverse section and the number of endothelial cell nuclei were increased both in the patients with diabetic neuropathy and hereditary motor and sensory neuropathy, as was the thickness of the surrounding basal laminal zone. "Closure" of endoneurial capillaries in diabetic neuropathy, reported in another study, was not confirmed. Capillary density and nearest-neighbour distances were similar in the diabetic and organ donor control cases. Capillary density was reduced in the patients with hereditary motor and sensory neuropathy, this being related to increased fascicular area consequent upon the presence of hypertrophic changes.

The presence of thickening of the pericapillary basal laminal zone and endothelial cell hyperplasia both in diabetic and hereditary sensory and motor neuropathy, the latter being a neuropathy in which a vascular basis can be discounted, makes it difficult to use such changes as an argument favouring a vascular cause for diabetic neuropathy. There were differences in the basal laminal zone between the diabetic and hereditary motor and sensory neuropathy cases suggesting that the reduplicated basal lamina was more persistent in the diabetic patients.

Perineurial cell basal laminal thickness was significantly greater in the diabetic patients as compared both with the HMSN I cases and the organ donor controls. This was most obvious for the intermediate layers of the perineurium. The thickness of the perineurial cell BM in the HMSN I cases was only slightly greater than that in the organ donor controls and the difference was not statistically significant.
The fact that a significant difference was found between the cases of diabetic neuropathy and HMSN I for endoneurial capillary basal laminal thickness but not perineurial cell basal laminal thickness indicates that the thickening of the perineurial basal laminal zone is the more characteristic feature of diabetic neuropathy.

The thickening of the perineurial cell basal laminae was compared with the thickening of the basal laminal zone around the endoneurial microvessels. No significant correlation was found either for the diabetic neuropathy or HMSN I cases or for the organ donor controls. These results suggest that the causative mechanisms are likely to differ, a conclusion supported by the morphological appearances; the basal laminal thickening around the perineurial cells is uniform, whereas that around the capillaries consists of basal laminal reduplication. Atrophy and necrosis of perineurial cells were observed in patients with diabetic neuropathy but rarely in the cases with HMSN I and not in the organ donor cases. This may be similar to the degeneration of endoneurial fibroblasts that has been described as a non-specific finding in neuropathies.

The immunological identification and localisation of collagen types and other connective tissue elements within peripheral nerves at both light and EM level would enable us to determine the molecular nature of both these hypertrophic BMs and the abnormally dense deposits of endoneurial collagen characteristic of diabetic neuropathy and allow us to establish, for example, whether one specific component is largely responsible for perineurial and endothelial cell basal laminal hypertrophy, or whether the production of all the constituent elements is enhanced.

Evidence has recently been produced which suggests that glomerular mesangial expansion in diabetes mellitus may result from the enhanced production of collagen type VI. If collagen type VI can be demonstrated to be a component of the extracellular endoneurial microfibrils within the PNS, this would enable us to determine whether endoneurial collagenization in diabetic neuropathy can be similarly attributed to the increased production of collagen type VI. The ratio of collagens types III and I has been shown to be increased in skin samples of diabetic mice and conjunctival biopsies from diabetic patients. It would be interesting to determine whether a similar phenomenon occurs in the PNS.
The morphology of collagen fibrils has also been shown to be altered by diabetes mellitus, the diameter of these fibrils in both the skin of diabetic patients (Hanna et al., 1987) and the endoneurial compartment of peripheral nerves in the BB-rat (Muona et al., 1989) having been found to be increased. The possibility exists that endoneurial collagen fibrils are composed of a combination of both collagen types I and III. If this scenario is true, the reported increase in diameter may be a direct result of an alteration in the ratio of component collagen types. The immunological identification and localisation of collagen molecules at EM level would enable us to either validate or disprove these theories.

The future immunological detection and localisation of AGEs in diabetic peripheral nerves would also be of interest. If their presence within the BMs of perineurial, Schwann and endothelial cells is proven, this would provide evidence in support of the theory that diabetic basal laminal hypertrophy can be, at least partially, attributed to an abnormal resistance to proteolytic degradation as a result of non-enzymatic glycosylation.

Having published preliminary data which suggested that the degree of PCBM thickening correlates with myelinated fibre loss (Johnson & Doll 1981), in a later investigation Johnson failed to detect any evidence of a relationship between the severity of diabetic neuropathy and PCBM thickness in the human sural nerve (Johnson 1981). The situation may be more clearly resolved by the future determination of myelinated fibre density for the patients included in this study. This would enable us to determine whether any correlation exists between the severity of neuropathy and both PCBM thickening and the components of diabetic microangiopathy, a positive correlation having been previously proposed to exist between endoneurial capillary abnormalities and the "index of pathology" (Dyck et al., 1985b, 1986a).
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