THE PATHOLOGY OF LARYNGEAL HEMIPLEGIA IN THE HORSE

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THE PATHOLOGY OF LARYNGEAL HEMIPLEGIA IN THE HORSE

VOLUME I

ABSTRACT

A review of the literature on equine laryngeal hemiplegia indicated that further investigations were warranted, in order to define more clearly the underlying pathogenic processes. This investigation was undertaken on one Standardbred and fourteen Thoroughbred horses, of which four were clinically affected with laryngeal hemiplegia, five subclinically affected and six were apparently normal. Their left and right recurrent laryngeal nerves were examined in detail, as were hindlimb nerves from two of the clinically affected animals. A variety of histological techniques were employed, including light and electron microscopy of resin embedded nerve, and single teased fibre preparations. Quantitative data obtained from these investigations was evaluated statistically. Some intrinsic laryngeal muscles, and the *extensor digitorum longus* muscle were investigated. In addition, the motor nucleus of the recurrent laryngeal nerve fibres, the *nucleus ambiguus* , and long central nerve fibre tracts were examined.

The results demonstrated that the neuropathy of equine laryngeal hemiplegia is characteristic of a distal axonopathy. Rather than being a disease of the left recurrent laryngeal nerve, as it has previously been considered, a generalised neuropathological process affecting long and large diameter nerve fibres was found. The primary site of the lesion was shown to be the axon rather than the myelin sheath. Distal axonal atrophy was demonstrated. As well as the involvement of peripheral nerve fibres in the disease process, some evidence for the involvement of long fibres of the central nervous system was found.

In addition, an investigation of the pathology associated with stringhalt, another nervous condition of horses, was performed on one animal. It was demonstrated that this was also a distal axonopathy, although a number of differences from idiopathic laryngeal hemiplegia were observed. It was an acute rather than a chronic process, and lacked any involvement of central fibres.

In conclusion, it was suggested that a number of previously postulated causes for equine laryngeal hemiplegia could be dismissed, on the basis of the finding of a generalised distal axonopathy. This included those which suggested mechanical damage to the left recurrent laryngeal nerve, such as stretch or compression. In light of the findings, the most likely aetiology underlying the pathology of equine laryngeal hemiplegia was considered to be an acquired or inherited metabolic defect affecting energy production in the axon, with the resultant inability to support the distal areas of long, large diameter nerve fibres.

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PART I

INTRODUCTION

INTRODUCTION

Respiratory diseases are second in importance only to lameness in the horse (British Equine Veterinary Association, 1965). One of the most important of these diseases is laryngeal hemiplegia, a condition that is often referred to as roaring. This disease has blighted the Thoroughbred virtually since the inception of the breed, and according to Weiss (1937), 5% of all Thoroughbreds develop the condition. The clinical signs exhibited by laryngeal hemiplegic horses result from paresis or paralysis of the left *cricoarytenoideus dorsalis* muscle, and subsequent loss of control of movement of the arytenoid cartilage. During exercise this results in inspiratory stridor and dyspnoea, and because of laryngeal airway obstruction, a reduction in the important ability of the animal to perform athletically.

Although the cause of the laryngeal paralysis is known in some few animals to be related to traumatic or irritant damage to the recurrent laryngeal nerve, or to plant or chemical intoxications, there is no history of any of these aetiological factors in the majority of affected animals. Veterinarians have traditionally referred to these latter cases as idiopathic laryngeal hemiplegia (Cook, 1976), although this term is not strictly correct. An idiopathic disease is defined in the Concise Oxford Dictionary (1964) as a disease which is not preceded or occasioned by another. Black's Medical Dictionary (1965) takes a wider view, and states that idiopathic is a term applied to diseases indicating that their cause is unknown. The word hemiplegia is defined by Black's Medical Dictionary as a paralysis

affecting one side of the specified system. Paralysis usually refers to a loss of motor function, due to interference with the nervous system, whereas paresis is used when the loss of motor function is only partial. In some cases, the condition seen in horses is hemiplegia, while in others strictly speaking it is a hemiparesis. Laryngeal hemiplegia, as a term for this condition is imprecise, as it wrongly conveys the impression that the underlying nervous dysfunction is limited to one side of the larynx, and even then the side involved is not specified. In this thesis, the term idiopathic laryngeal hemiplegia will be used in preference to laryngeal hemiplegia only when it is necessary to distinguish between laryngeal hemiplegia in which the causal factor is known, and other cases of the disease.

Clinical signs of laryngeal hemiplegia are usually manifest in young adults (Williams, 1902; Hoare, 1915; Cook, 1970; Marks *et al*, 1970b; Mason, 1973). In a recent study in Thoroughbreds, which included in its analysis a comparison of the age distribution of the population from which the horses involved in the study were taken, the relative risk of becoming a roarer was greatest at 2 years of age (Goulden & Anderson, 1981). An interesting aspect of this disease is that it almost always occurs on the left side, and is associated with atrophy of the left intrinsic laryngeal muscles, secondary to a neuropathy of the left recurrent laryngeal nerve (Hoare, 1915; Cole, 1946; Cook, 1970; Marks *et al*, 1970a; Mason, 1973; Duncan *et al*, 1974).

The purpose of this thesis is to accurately define the nature and distribution of pathology in the recurrent laryngeal nerves of idiopathic laryngeal hemiplegic horses, as a basis to allowing a better understanding of the pathogenesis of the disease. A synopsis of the present knowledge on the anatomy, pathology and suggested aetiologies is presented and critically discussed.

ANATOMY

The left and right recurrent laryngeal nerves differ in their anatomical arrangement. The left nerve branches from the vagus more distally than the right, and passes around the aorta before it ascends the neck to the larynx (Getty, 1975). Both recurrent laryngeal nerves are of considerable length, coursing from the central motor neurones in the midbrain to the muscles they innervate in the larynx. The left nerve can measure up to 100 cm, distal to its separation from the vagus in the thoracic cavity (Cole, 1946). When the vagal course is included, the total axonal length may be up to 250 cm, and as such approximately 100% longer than any other motor nerve in the horse, with the exception of its right counterpart (Duncan & Griffiths, 1973). Because of the different levels of branching of the left and right recurrent laryngeal nerves from the vagus, the left can be up to 31 cm longer than the right (Cole, 1946).

PATHOLOGY

The underlying involvement of the laryngeal muscles and the nerves innervating these muscles, in laryngeal hemiplegia, has been appreciated since early in the 19th century. Bouley (1825) reported the observation in a roarer, of compression of the left recurrent laryngeal nerve by enlarged lymph nodes in the area of the thoracic inlet. In the same year, Dupuy (1825), reproduced the roaring sound in a horse, and wasting of the associated laryngeal muscles, by division or compression of the vagal or recurrent laryngeal nerves. Detailed *post mortem* inspection of the laryngeal muscles of a horse with laryngeal hemiplegia was performed by Percivall (1840), who found the left *cricoarytenoideus dorsalis* to be very pale and approximately half its usual size. The *cricoarytenoideus*

lateralis, the *ventricularis*, the *vocalis* and the *arytenoideus transversus* muscles were reported to be "altogether colourless and scarcely recognisable as muscles", while the muscles of the right side were "unusually red and strong".

The pathological changes present in the left recurrent laryngeal nerves of horses with laryngeal hemiplegia were investigated by Cadeac (1897), who noted a distally distributed degeneration of nerve fibres, with the proliferation of Schwann cells. Although the lesions were found to be limited to the distal extremity of the left recurrent laryngeal nerve, changes were found at a distance of 30-40 cm from the larynx. In contrast to more recent findings (*vide infra*), Cadeac found the changes to be most marked in the fascicles composed of mainly small myelinated nerve fibres. This was perhaps merely a reflection of the less advanced techniques available at this time, for the study of nervous tissue.

In 1933, Argyle found a 5 cm constricted segment of mid-cervical left recurrent laryngeal nerve, in a horse with laryngeal hemiplegia, an observation not verified by other workers. Atrophy of the left laryngeal muscles was also noted. An attempt to locate the central nucleus of the nerve fibres of the recurrent laryngeal nerves, in the *medulla oblongata*, was also made by Argyle (1934). He stated that the nucleus of the vagal nerve was examined in horses affected with laryngeal hemiplegia, and degeneration of cell bodies of this nucleus observed, on the left side. It is unclear whether the nucleus identified and examined was actually the *nucleus ambiguus*, the nucleus of the motor fibres of the recurrent laryngeal nerve, or the dorsal vagal nucleus.

The first detailed pathological investigation of laryngeal hemiplegia was undertaken by Cole (1946). More recently, several researchers have used modern light and electron microscopic techniques to study affected muscle and nerve from laryngeal hemiplegic horses (Gunn, 1972; Duncan & Griffiths, 1973; Gunn, 1973; Duncan *et al*, 1974; Duncan, 1975; Duncan *et al*, 1978). Pathological features characteristic of neurogenic atrophy were found in those muscles of the larynx innervated by the left recurrent laryngeal nerve. These changes included fibre type grouping, variation in fibre size, the presence of atrophic and hypertrophic fibres, the demonstration of centrally placed nuclei, fascicular atrophy, increased fat and connective tissue within and between fascicles, and a loss of myelinated nerve fibres in intramuscular nerves.

A large number of horses with no clinical or endoscopic evidence of laryngeal hemiplegia have shown neurogenic atrophy of those laryngeal muscles innervated by the left recurrent laryngeal nerves, and have been referred to as subclinical cases (Cole, 1946; Gunn, 1972; Duncan & Griffiths, 1973; Duncan, 1975). Atrophy of the laryngeal muscles was demonstrated in 27% of larynges examined by Cole (1946), but only 26% of these animals showed clinical signs of laryngeal hemiplegia. In a more recent survey of 44 horses, 14 (32%), were found to have these so called subclinical lesions (Duncan & Griffiths, 1973). According to Cole (1946), no clinical signs were observed if there was less than 50% atrophy of the *cricoarytenoideus dorsalis* muscle.

It appeared that the adductor muscle, the *cricoarytenoideus lateralis,* was more severely affected than the abductor, the *cricoarytenoideus dorsalis* , in both clinical and subclinical cases of laryngeal hemiplegia (Gunn, 1972; Duncan & Griffiths, 1973; Gunn, 1973; Duncan *et al*, 1974; Duncan, 1975). In some of these cases there was also early evidence of neurogenic atrophy in those muscles supplied by the

right recurrent laryngeal nerve. This took the form of fibre type grouping and the presence of atrophied, angular fibres (Cole, 1946; Gunn, 1972; Duncan & Griffiths, 1973; Gunn, 1973; Duncan, 1975).

In addition to studying the laryngeal muscles, Cole (1946) examined the recurrent laryngeal and vagal nerves at a number of levels. Marked degeneration of nerve fibres, with proliferation of Schwann cells and endoneural fibrosis were noted, particularly in the distal part of the left recurrent laryngeal nerve. The degeneration decreased in severity in a proximal direction, with only a few fibres affected at the level of the thoracic inlet, and only rarely any changes in the region of the aortic arch. In two cases, a small number of nerve fibres were also affected in the right recurrent laryngeal nerve. Cole, like Argyle (1933), found degeneration more often in those fascicles consisting of small myelinated fibres.

Employing more sophisticated techniques for the study of peripheral nerve, namely single nerve fibre preparations and plastic embedded sections for light and electron microscopy, a substantial amount of information was obtained regarding the pathological alterations present in the recurrent laryngeal nerves in equine laryngeal hemiplegia by Duncan and coworkers in Glasgow (Duncan & Griffiths, 1973; Duncan *at al*, 1974; Duncan, 1975; Duncan *et al*, 1978). Normal equine recurrent laryngeal nerve was shown to consist of a variable number of fascicles containing medium sized myelinated fibres, with an occasional smaller diameter fibre present. In man, it has been demonstrated that considerable plexus formation of the fascicles occurs along the length of the recurrent laryngeal nerves (Sutherland & Swaney, 1952). Using retrograde horseradish peroxidase tracer techniques in the cat, it was shown that the nerve fibres to the adductors and abductors of the larynx are diffusely arranged in this nerve (Malmgren

et al, 1977). Collections of small diameter myelinated fibres, often present in separate fascicles, were shown to be sensory fibres to the oesophagus and trachea (Duncan, 1975). The presence of unmyelinated fibres was demonstrated, and confirmed with electron microscopy. Renaut bodies (*vide infra*) were a common finding in normal recurrent laryngeal nerve, but were observed more frequently in animals affected with laryngeal hemiplegia. In a number of other species, including the rat (Dalqvist *et al*, 1982), man and the giraffe (Harrison, 1981), a greater number of large fast conducting fibres were found in the left recurrent laryngeal nerve than in the right. It was postulated that these large fast conducting fibres in the left nerve, allow the nerve impulses to travel faster on the left side, and thus compensate for its greater length. A total of 88 larynges were examined by Duncan (1975), of which nine were from clinical cases of laryngeal hemiplegia and 40 (45.5%) were from subclinical cases. The 39 normal animals were mainly ponies. Six of the cases, said to be typical of the disease spectrum, were discussed in the report by Duncan *et al* (1978). Of these six animals, three were from clinical cases of laryngeal hemiplegia in Thoroughbreds, aged 5, 6 and 13 years and all were over 16 hands (160 cm) in height. The remaining three cases were subclinical and were also Thoroughbreds. They were aged 5-6 years of age and each was over 15.2 hands (155.5cm) in height. The recurrent laryngeal nerves were sampled at a number of sites between the larynx and the aortic arch. The vagal nerves were also collected, as were distal samples of other peripheral nerves, including the ulnar, tibial, digital and phrenic.

Light microscopy of the recurrent laryngeal nerves revealed pathological alterations at all levels of the left nerve, and at the distal level of the right. The most obvious change was a progressive distal loss of large myelinated fibres in the left recurrent laryngeal nerve, with no

observable decrease in fibres in the right nerve. Clusters of regenerating fibres and "onion bulbs" were seen frequently at all levels of the left recurrent laryngeal nerve and in the distal right nerve, while evidence of degeneration was only rarely observed. No abnormalities were detected in any of the other peripheral nerves examined.

Ultrastructurally, the above changes were confirmed, and the presence of numerous Bungner bands, the remnants of axonal degeneration were noted. As observed by light microscopy, active axonal degeneration was not a common finding. However, the accumulation of various axonal organelles, including mitochondria, dense bodies, tubule-like structures, and disorientated neurofilaments was seen on occasions in axons possessing split myelin sheaths, in massively swollen axons and in demyelinated axons.

Single fibre preparations demonstrated changes indicative of chronic demyelination and remyelination. These took the form of variation in internode length, due largely to the presence of short intercalated segments. However, there was little evidence of active demyelination. Although axonal degeneration was observed only occasionally, some fibres were seen to show distal degeneration and proximal paranodal demyelination. In two cases of laryngeal hemiplegia, distal tapering of consecutive internodes was a frequent finding. Few fibres with uniformly short internodes were seen. Such changes are characteristic of regenerating fibres. Intercalated segments were rare in fibres from the tibial and median plantar nerves of one clinical case, and the distal recurrent laryngeal nerves of two control ponies revealed only two intercalated segments from 100 teased fibres.

Fibre diameter histograms confirmed the distal loss of myelinated fibres, and indicated a marked absolute shift to smaller diameter fibres in the distal nerve. These changes extended proximal to the level of the aortic arch in one case of laryngeal hemiplegia.

In summary, there was a progressive distal loss of myelinated fibres in the left recurrent laryngeal nerve, selectively affecting the larger diameter fibres. Regenerative clusters and "onion bulbs" were present, at all levels of the left recurrent laryngeal nerve and in the distal right nerve. In general, all of the above pathological changes were observed in both clinical and subclinical laryngeal hemiplegia, but to a lesser degree of severity in the latter cases.

The predilection of pathological changes for the distal left recurrent laryngeal nerve was noted by Cadeac (1897), Cole (1946), Duncan *et al* (1974) and Duncan *et al* (1978). The latter investigation also demonstrated the presence of these alterations in the recurrent laryngeal nerves of subclinical as well as clinical cases of laryngeal hemiplegia.

While the demonstration of "onion bulbs" in affected recurrent laryngeal nerve has only been reported in recent years (Duncan *et al* , 1974; Duncan, 1975; Duncan *et al*, 1978), the proliferation of Schwann cells was noted in earlier studies (Cadeac, 1897; Cole, 1946). The significance of these "onion bulbs" is discussed by Duncan *et al* (1978), and the specificity of large "onion bulb" formations for a process of chronic demyelination and remyelination was noted. Teased fibre studies have shown that in peripheral nerve with "onion bulb" formations, fibres with signs of demyelination and remyelination predominate (Madrid *et al*, 1977). This situation existed in the recurrent laryngeal nerves from laryngeal hemiplegic animals (Duncan *et al*, 1978).

Changes were also observed in the right recurrent laryngeal nerve. However, involvement of only the distal portion of the right nerve was demonstrated, and even then although regenerating clusters and "onion bulbs" were observed, there was no obvious loss of myelinated fibres. This right sided nerve involvement substantiates the finding of neurogenic atrophy in the right sided laryngeal muscles of some affected animals (Gunn, 1973; Duncan *et al*, 1974; Duncan, 1975).

As indicated by Duncan *et al* (1978), the involvement of both left and right recurrent laryngeal nerves in the disease process must form the basis of any hypothesis on the pathogenesis of the condition of laryngeal hemiplegia in horses. Duncan and his coworkers also emphasised that interpretation of the high incidence of abnormalities suggestive of demyelination and remyelination in teased fibres, must be approached cautiously in the presence of obvious axonal degeneration, as evidenced by the marked fibre losses present. The authors felt that the teased fibre results confirmed the chronic nature of the lesion, especially in the presence of only occasional active demyelination.

The significance of the tapering of internodes observed in two cases was discussed with respect to the occurrence of similar findings as a feature of the normal ascending recurrent laryngeal nerve in rabbits (Williams & Kashef, 1968). They stated that tapering of internodes has also been seen in association with compressive nerve lesions in guinea pigs (Ochoa & Marotte, 1973). However, in affected recurrent laryngeal nerves the tapered internodes were not found in the region of the aortic arch where compression of the nerve has been suggested as a pathogenic factor *(vide infra)*.

AETIOLOGY

Laryngeal hemiplegia is a disease of horses that has been known for many years, and because of the importance of the condition and its unknown pathogenesis, there have been a great many theories offered as to its cause. Any hypothesis regarding the aetiology of this disease must satisfactorily explain the various clinical and pathological characteristics of the condition.

In a limited number of cases of laryngeal hemiplegia, the cause is readily determined. Causal factors which can produce lesions of the recurrent laryngeal or vagal nerves were outlined by Goulden & Anderson (1981b). These include irritant perivascular or perineural injections (Marks *et al* , 1970a) accidents to the neck (Gilbert, 1972), guttural pouch mycosis (Cook, 1976), neoplasia (Hoare, 1915; Cook, 1976), organophosphate intoxication (Rose, 1978; Rose *et al* , 1981), lead poisoning (Hoare, 1915), vitamin deficiency (Loew, 1973; Cymbaluk *et al*, 1977), and plant poisoning (Hoare, 1915; Williams, 1945; Kral, 1951; Schebitz, 1964; Neal & Ramsey, 1972). In a study of 127 horses with laryngeal hemiplegia, Goulden & Anderson (1981b) incriminated the above factors in only 11%. They suggested that in the New Zealand environment at least, it was unlikely that any of these factors were important in the aetiology of most cases of this disease.

The commonly considered causes of the so called idiopathic laryngeal hemiplegia can be divided into four main groups:

(i) Mechanical causes
(ii) Bacterial or viral agents
(iii) Plant and chemical intoxications
(iv) Vitamin deficiencies.

In addition there are a number of factors which are considered to predispose an individual to the disease. These include breed, heredity, size, sex, management, conformation, climate and geography.

Each of the possible causes is discussed in the following pages.

Mechanical causes

BIOMECHANICAL THEORY OF STRETCH TO THE LEFT RECURRENT LARYNGEAL NERVE

Tension and stretch of the recurrent laryngeal nerve have long been considered relevant to the aetiology of laryngeal hemiplegia (Martin, 1887). Hypotheses as to the cause of this stretching included; backward movement of the heart at exercise (Argyle, 1934); overextension of the head (Frank, 1953); a combination of both of these situations (Cook, 1976); and the increase of neck length during growth (Martin, 1887).

In 1970, Rooney and Delaney postulated a biomechanical theory, of stretch to the left recurrent laryngeal nerve being the cause of equine laryngeal hemiplegia. This hypothesis was based on premises regarding the nature, incidence and pathology of the disease. Rooney and Delaney concluded that due to the anatomical differences in the course and length of the left recurrent laryngeal nerve, as compared with the right, the left nerve is placed under considerable tension during movement of the neck. They considered this tension to be exacerbated in the individual with a long neck or large body, thus providing an explanation for the size predisposition of the disease. According to Rooney and Delaney this tensile force could produce "necking" of the nerve and compression of the associated blood vessels, resulting in vascular insufficiency and

subsequent nerve damage. It was stated that the "necking" would occur in the middle third of the nerve. This theory was discussed by Marks *et al* (1970b), who suggested that tension so produced could be exacerbated by enlarged lymph nodes, excessive pericardial fat or backward displacement of the heart.

One of the basic premises of this hypothesis involved an incorrect interpretation of Cole's work (1946), on the pathology present in diseased recurrent laryngeal nerves. Rooney and Delaney state, "Based on the work of Cole, the primary site of damage to the left recurrent nerve is interpreted to be in the middle third of the nerve." What Cole actually found was, "Marked degeneration and fibrosis occurred in the peripheral portion of the left recurrent nerve", not the middle third. Moreover, both Cole, and more recently Duncan *et al* (1978) have shown that the distal nerve pathology observed in this disorder can extend proximally, at least to the area of the aortic arch. Thus the distribution of the recurrent laryngeal nerve pathology is not that which would be expected if "necking" of the nerve at its midpoint was the cause. Furthermore, this theory of stretch to the left recurrent laryngeal nerve provides no explanation for the pathological changes present in the distal right recurrent laryngeal nerve (Duncan *et al*, 1978).

From a purely mechanical point of view, the hypothesis that tension on the nerve would produce "necking" at the midpoint, is also incorrect. Any material which is fixed at two points, when subjected to tension will tend to give way at the fixed points, not the middle of the length of material (Soden & Kershaw, 1974). The recurrent laryngeal nerve should behave in this way, with the two fixed points being the larynx and the aortic arch. Nevertheless, when "necking" does occur as a result of stretch, the location of the "neck" cannot be predicted, because of the lack of precise uniformity along the length of any material (Alexander, 1981).

Peripheral nerves characteristically possess considerable strength and elasticity, due to the anatomical arrangement and properties of the components within the nerve trunk (Sunderland, 1965). Moreover, peripheral nerves have at least three mechanisms which allow considerable stretch to occur before deformity or pathological changes result. Firstly, the whole nerve pursues an undulating course in the surrounding tissues. Secondly, the nerve fascicles describe a wavy course along the nerve, and thirdly, the nerve fibres undulate along the fascicles. While these mechanisms do protect the nerve from stretch, beyond a certain limit damage will occur. When this happens the cross-sectional area of the nerve is decreased, leading to compression of the contents and impairment of blood supply and of nerve conduction. With increasing tension, nerve fibres may rupture within the fascicles, and later the perineurium, and hence the fascicles may be torn. The site of these ruptures tends to be at widely spaced intervals along the nerve (Sunderland, 1965), not at the midpoint as was suggested by Rooney & Delaney (1970).

Factors which may jeopardise nerve fibres by lowering the threshold at which stretch begins include adhesions, changes in the connective tissues of the nerve that decrease elasticity, and any deformity which imposes a longer course on the nerve (Sunderland, 1965).

COMPRESSION OF THE RECURRENT LARYNGEAL NERVE IN THE AREA OF THE AORTIC ARCH

Mechanical damage to the recurrent laryngeal nerve, due to compression, has been mentioned as a cause of laryngeal hemiplegia by a number of authors (Bouley, 1825; Fergusson, 1838; Haslam, 1893; Vermeulen, 1913; Hoare, 1915; Argyle, 1934; Hutyra *et al*, 1938).

It has been suggested that a discrete compression of the left recurrent laryngeal nerve may occur as the nerve passes around the aortic arch, possibly leading to a blockage of axoplasmic flow, and resultant axonal degeneration (Duncan *et al*, 1978). Ultrastructurally, the accumulation of neurofilaments or tubules and other organelles has been demonstrated in diseased left recurrent laryngeal nerves by these authors. They interpreted these findings as indicating a derangement in axonal transport, possibly due to compression of the nerve. However, the accumulation of axonal organelles was by no means a frequent finding, and is not specifically related to nerve compression. In addition, these authors found, in one of nine cases of laryngeal hemiplegia, numerous Renaut bodies and thickening of the perineurium in the region of the aortic arch. They also found Renaut bodies in greater numbers in diseased than in healthy recurrent laryngeal nerves.

Renaut bodies occur in a subperineural position. They are approximately spherical, whorled and cell sparse in appearance. Ultrastructurally, they have been shown to consist of fibroblasts and loosely arranged and randomly oriented collagen (Asbury, 1973). The exact function of these bodies remains uncertain, but it has been postulated that they cushion the nerve fibres from compressive forces (Renaut, 1981). The significance of Renaut bodies however, must be considered carefully, as they are particularly common in the horse, and are at best nonspecific indicators of neuropathological disease. Notwithstanding this, they do appear to occur more frequently in nerves which are subjected to compression, whether it be from natural or pathological causes (Asbury, 1973).

The fact that certain of the laryngeal muscles are affected preferentially during the disease process of laryngeal hemiplegia (Duncan *et al*, 1978), was taken as providing further evidence for the involvement of compression of

the recurrent laryngeal nerve in the pathogenesis of the condition. These authors suggested that the damage occurred only to those nerve fibres closest to the point of compression. If grouping of the nerve fibres into adductor and abductor areas within the recurrent laryngeal nerve took place, selective involvement of nerve fibres innervating particular muscles would occur. However, in man (Sunderland & Swaney, 1952), and the cat (Gacek *et al*, 1977), it has been shown that nerve fibres to the adductor and abductor muscles are mixed within the recurrent laryngeal nerves, and so the opportunity for selective compression would not occur. The repeated division of fascicles within nerve trunks, to form plexuses along the length of the nerve is the usual situation within nerves (Sunderland, 1965), and was observed in human laryngeal nerve (Sunderland & Swaney, 1952). This plexus formation was seen to redistribute adductor and abductor fibres so that they were intermixed in different fascicles when the recurrent laryngeal nerve was extralaryngeal in position. The anatomical arrangement observed would preclude selective damage to fibres innervating adductor or abductor muscles (Sunderland & Swaney, 1952).

Haslam (1893) claimed that in horses affected with laryngeal hemiplegia there was a ribbon-like flattening of the recurrent laryngeal nerve as it passed between the trachea and the aorta (Haslam's anomaly). He suggested that compression of the nerve resulted in this anatomical variation, and was a possible cause of laryngeal hemiplegia. More recently, a detailed examination of five clinically normal horses with Haslam's anomaly, revealed no signs of pathology in the recurrent laryngeal nerves. Neither was there any clinical or endoscopic evidence of laryngeal paralysis in association with these cases (Mason, 1973). If compression of the nerve at this site is a cause of the nerve damage, one would expect pathological changes associated with the recurrent laryngeal nerve as it passes around the aorta, in many cases of laryngeal hemiplegia.

This however is not the case. While there is a distally progressive loss of myelinated fibres in affected recurrent laryngeal nerve, fibre density approached the normal values in almost all diseased recurrent laryngeal nerve at the level of the aortic arch. Furthermore, other observed pathological changes were mild at this site when compared with more distal levels of the nerve (Duncan *et al*, 1978). Of particular importance is the fact that a compressive lesion of the left recurrent laryngeal nerve provides no explanation for the presence of pathological alterations in the distal right nerve, and the right laryngeal muscles.

Over the years other compressive lesions have been suggested as contributing to the production of laryngeal hemiplegia. These include aneurysm of the aortic arch (Hutyra *et al*, 1938), enlarged lymph nodes (Hutyra *et al*, 1938; Schebitz, 1964), tumours (Hoare, 1915), abscesses (Hutyra *et al*, 1938), enlarged thyroid glands (Vermeulen, 1913), and dilation of the oesophagus (Hutyra *et al*, 1938).

Bacterial and viral agents

The observation has often been made that roarers are initially identified clinically, immediately following a respiratory tract infection, and the conclusion drawn is that respiratory tract disease is causatively associated with the laryngeal hemiplegia (Bouley, 1825; Fergusson, 1938; Argyle, 1934; Frank, 1953; Quinlan, 1957; Maguire, 1958; Mahaffrey, 1962; Schebitz, 1964).

Many organisms have been implicated as causing the damage to the left recurrent laryngeal nerve. These include the influenza virus (Frank, 1953; Marks *et al*, 1970a), rhinopneumonitis virus (Marks *et al*, 1970a), *Streptococcus equi* (Hoare, 1915; Schebitz, 1964), *Bacterium viscosum equi* (Hutyra *et al*, 1938), *Actinobacillus equi* (Hutyra *et al*, 1938), and *Trypanosome equiperdum* (Hutyra *et al*, 1938).

The association of recurrent laryngeal nerve paresis with previous respiratory infections has been noted in man (Fex & Elmqvist, 1973). A high incidence of the condition following a few weeks after the occurrence of "Hong Kong flu", was noted in several parts of Europe in the winter of 1969/1970. However, in some of those affected, other nerves were also involved, including the facial, vagal, hypoglossal, phrenic and cranial laryngeal nerves. Complete recovery was not seen commonly, and if it occurred recovery was prolonged. The viral infection was thought to be the cause of the condition.

It has usually been considered that micro-organisms initiate the damage to the recurrent laryngeal nerve by the enlargement cf lymph nodes and subsequent compression of the nerve (Bouley, 1825; Fergusson, 1838; Hutyra *et al*, 1938; Schebitz, 1964), or by the production of neurotoxins (Hutrya *et al*, 1938). An attempt to explain the left sided nature of laryngeal hemiplegia was made by Hutyra *et al* (1938), who postulated that the left recurrent laryngeal nerve had a greater susceptibility to these bacterial toxins. It has also been suggested that *Streptococcus equi* may elicit an allergic response in horses previously sensitised to this organism (Neal & Ramsey, 1972).

In a survey of 127 cases of laryngeal hemiplegia, Goulden & Anderson (1981b) reported obvious signs of recent respiratory infection in only a few of the animals examined, and endoscopic evidence of recent infection in less than one third. Taking into consideration the widespread nature of respiratory infections, and the fact that most of the animals in this series were young Thoroughbreds, a group of horses in which upper respiratory tract infection is very common (Bryans & Gerber, 1972), the apparent association of respiratory infection and laryngeal hemiplegia could be purely coincidental.

The hypothesis that respiratory infections cause laryngeal hemiplegia fails to explain the predisposition of the condition for larger horses. All sizes and breeds of horse contract respiratory infections yet mostly large horses develop laryngeal hemiplegia. In addition, it does not explain the greater incidence of the disease in the male, or the assymetry of the lesion (Marks *et al*, 1970b). However, the possibility still remains that toxins produced in small amounts by infectious agents, may have an affinity for long nerve fibres, and therefore produce damage to the recurrent laryngeal nerves.

The local spread of an infectious process to involve the recurrent laryngeal nerve is unlikely as the perineurium provides an effective barrier to the spread of local inflammatory processes. Histologically, it was seen that undamaged perineurium was very resistant to even severe and prolonged infection (Sunderland, 1965). A further protection against the involvement of the recurrent laryngeal nerve in localised infectious processes of the respiratory tract, is the protected course of the nerve in the area of the larynx (Quinlan *et al*, 1982). In this region the recurrent laryngeal nerve and its branch to the *cricoarytenoideus dorsalis* muscle are separated from the lumen of the larynx by the laryngeal cartilages and muscles.

Plant and Chemical Intoxications

PLANT INTOXICATIONS

The prolonged ingestion of certain plants, especially those of the genus *Lathyrus* and *Cicer arietinum* (chick peas) have long been associated with the development of laryngeal paralysis (Fleming, 1889; Argyle, 1934; Hutyra *et al*, 1938; Schebitz, 1964; Cook, 1970). In addition lucerne has been said to produce such a condition (Fleming, 1889; Hutyra *et al*, 1938). Feeding experiments with *Lathyrus*

sativus (Indian vetch) indicated that prolonged ingestion of the plant was needed to produce laryngeal paralysis, and on average only 33% of horses receiving a regular diet of vetch developed this condition. The toxic compound was shown to be an alkaloid, lathyrin (Hutyra *et al*, 1938). Atrophy of the muscles of the left side of the larynx was reported by Argyle (1934) as a feature of the intoxication with *Lathyrus sativus*, but no description of the pathology present in the recurrent laryngeal nerves has been recorded. While these plant poisonings do appear to lead to a left laryngeal paralysis, they are apparently unrelated to the majority of cases of idiopathic laryngeal hemiplegia, and according to Cook (1970) constitute less than 5% of all cases of laryngeal hemiplegia in horses.

CHEMICAL INTOXICATIONS

Lead poisoning

In early considerations of the aetiology of laryngeal hemiplegia, the occurrence of laryngeal paralysis as part of the syndrome of lead poisoning in the horse was noted (Fleming, 1889; Argyle, 1934; Hutyra *et al*, 1938). This paralysis was said to result from degenerative changes throughout the entire length of the vagal nerve, but particularly in the terminal part of the left recurrent laryngeal nerve (Hutyra *et al*, 1938).

It has been shown that horses are sensitive to lead intoxication at low dosages over prolonged periods, and that forage contamination is the usual source of the lead (Krigman *et al*, 1980; Burrows, 1982). The main clinical signs of lead poisoning in horses are related to impaired peripheral nerve function, especially of motor nerves. Involvement of the recurrent laryngeal nerves, with resultant laryngeal paresis is a common feature of the condition (Fleming, 1889; Burrows, 1982).

The pathogenesis of the peripheral neuropathy produced by lead intoxication is uncertain, and conflicting views are held regarding the relative involvement of segmental demyelination and axonal degeneration (Lampert & Schochet, 1968; Dyck *et al*, 1977; Bennington, 1978; Krigman *et al*, 1980). While some studies have led to conclusions of a primary effect on Schwann cells and the myelin sheath (Lampert & Schochet, 1968; Dyck *et al*, 1977), others have demonstrated axonal degeneration (Fullerton, 1966; Krigman *et al*, 1980). A recent proposal for the pathogenesis of the condition suggested that the initial lesion was a lead induced alteration of the blood-nerve barrier, resulting in increased permeability and hence endoneural oedema. Three mechanisms by which the oedema could damage nerve fibres were suggested. Firstly, oedema could lead directly to myelin degeneration. Secondly, in conjunction with minor trauma, oedema could contribute indirectly to the neuropathy. Thirdly, the oedema could secondarily alter the microcirculation within fascicles and cause focal ischaemia (Ohnishi *et al*, 1977).

While a small proportion of cases of laryngeal paralysis in horses may be due to lead poisoning, this does not appear to be an aetiological factor in the majority of cases (Goulden & Anderson, 1981b).

Vitamin Deficiencies

Thiamine deficiency (vitamin B1) was proposed as a possible cause of laryngeal hemiplegia in horses by Loew (1973). This proposal was based on the laryngeal involvement observed in vitamin B1 deficiency in man (beriberi), and bracken fern poisoning in cattle. It was postulated that at some early critical period of growth, thiamine nutrition of the horse proves inadequate, especially in those larger or faster growing animals, with a correspondingly higher thiamine requirement. An attempt to explain the reported

association of excessive training of young horses with the appearance of laryngeal hemiplegia (Marks *et al*, 1970a) was made on the basis of a proposed higher thiamine requirement with such a training regime.

A survey of the thiamine status of 47 horses was undertaken in order to test this hypothesis (Cymbaluk *et al*, 1977). The horses involved were in three groups; 12 mixed breed animals, 23 Standardbred and Thoroughbreds, and 12 clinical cases of laryngeal hemiplegia. The only statistically significant finding was a lower mean plasma thiamine concentration in the affected animals when compared with the unaffected mixed breed group. The results of this investigation do not conclusively implicate thiamine deficiency in the pathogenesis of laryngeal hemiplegia. The expected significant difference between the thiamine levels in the unaffected Standardbred and Thoroughbred group and the affected animals was not found. In addition, the number of animals in each group was small.

In support of the postulated association between the excessive training of young horses with the production of a thiamine deficient state is the report of thiamine deficiency in man, occurring with periods of intense physical activity (Dyck, 1982). An outbreak of beriberi occurred among schoolboys in Japan, and was traced to thiamine deficiency, caused by a diet not supplemented with vitamins, during prolonged periods of intense physical activity.

The laryngeal form of bracken fern poisoning in cattle was offered by Loew (1973) in support of the involvement of thiamine deficiency in laryngeal hemiplegia in horses. Although this intoxication is attributed to thiamine destruction in the horse, an "aplastic anaemia factor" is said to be involved in ruminants (Evans, 1964). The laryngeal symptoms observed in calves with bracken fern

poisoning, have been demonstrated to result from haemorrhage and oedema in the area of the larynx (Evans, 1964). Neurogenic atrophy of the laryngeal muscles has not been reported.

In man, chronic thiamine deficiency (beriberi), has been shown to produce a distal neuropathy of sensory and motor nerves, affecting the legs initially, with distal muscle wasting and sensory loss (Cavanagh, 1964; Cavanagh, 1979). In more severely affected individuals there is involvement of the recurrent laryngeal nerves, with paralysis of the left vocal cord and hoarseness (Cavanagh, 1964). Clinically, laryngeal paralysis may only become evident late in the course of the disease, yet it has been reported from a pathological point of view that the vagal, recurrent laryngeal and phrenic nerves were affected before the distal limb nerves (Blackwood *et al*, 1971). Similarly, thiamine deficiency in animals leads to degeneration of distal nerve fibres, especially of the hindlimbs, and of some spinal tracts (Cavanagh, 1964; Collins *et al*, 1964). The pathological alterations observed in thiamine deficiency of rats have been shown to affect the axons of myelinated fibres primarily, followed by changes in myelin sheath contour, with subsequent focal demyelination (Collins *et al*, 1964).

The possible role of thiamine deficiency in equine laryngeal hemiplegia was discussed by Duncan *et al* (1978). While confirming that the neuropathy of laryngeal hemiplegia, like that of thiamine deficiency was a disease of the "dying back" type, these authors felt that some of the pathological findings observed in the condition of laryngeal hemiplegia were contrary to those seen with thiamine deficiency. They found no evidence of a generalised polyneuropathy in horses affected with laryngeal hemiplegia, as is seen in man and animals with thiamine deficiency (Cavanagh, 1964; Collins *et al*, 1964). Frequent evidence of demyelination and remyelination were seen in recurrent

laryngeal nerve fibres from animals with laryngeal hemiplegia, while it was stated that such changes were not common in thiamine deficient nerves (Duncan *et al*, 1978).

It is possible however that these differences in the pathology of thiamine deficiency and laryngeal hemiplegia are merely reflections of the anatomical peculiarities of the horse, and the chronic nature of this neuropathy of horses.

Deficiencies of several other of the B-group vitamins have produced polyneuropathies affecting distal long nerve fibres (Cavanagh, 1977; Weller & Cervos-Navarro, 1977; Bennington, 1978). In addition to thiamine, the vitamins deficiencies involved are those of nicotinic acid, pantothenic acid, pyridoxine, riboflavin and vitamin B12.

Factors Predisposing to Equine Laryngeal Hemiplegia

Clinical observation of cases of laryngeal hemiplegia over the years has indicated that the following factors may predispose individuals to the disease:

(i) Breed
(ii) Heredity
(iii) Size
(iv) Sex
(v) Management
(vi) Conformation
(vii) Climate and geography.

BREED

A variable breed prevalence exists for equine laryngeal hemiplegia, which seems likely to be a reflection of the size differences between breeds. Goulden & Anderson (1981a) showed a similar prevalence of the disease in Thoroughbreds and Standardbreds, contrary to the commonly held impression that the condition is seen more frequently in Thoroughbreds.

However, as only 10 Standardbred animals were examined the authors felt that the results should be interpreted with caution. Popular opinion also supports a high incidence of laryngeal hemiplegia in heavy-weight crossbred horses (Hopkin, 1888; Hoare, 1915; Dornblaser, 1967; Cook, 1975). It is generally considered that the smaller breeds are less susceptible to the disease (Hoare, 1915; Dornblaser, 1967; Marks *et al*, 1970a).

HEREDITY

An hereditary predisposition to laryngeal hemiplegia has been held to exist since the earliest records of the disease, and is a view still held by many (Hopkin, 1888; Fleming, 1889; Smith, 1926; Saks, 1927; Lee, 1932; Argyle, 1933; van Lent, 1933; Hutyra *et al*, 1938; Quinlan, 1957; Cook, 1970). However, the opposite view has also been expounded (Williams, 1901; Williams, 1902; Kuhn, 1938), and was justified by the lack of any definitive evidence for genetic transmission of the condition, even with attempts at analysis of pedigree information of direct descendants of a roarer (Kuhn, 1938).

Although the inheritance of laryngeal hemiplegia has not been convincingly demonstrated, this opinion was accepted and the disease was listed as inherited after a Royal Commission on Horse Breeding in Britain in 1889 (Argyle, 1933; Cook, 1970). The British Horse Breeding Rules of 1948, and the Horse Breeding Act of 1958 also implicated laryngeal hemiplegia as being an inherited condition. More recent legislation by the British Veterinary Association (Horse Breeding Policy and Legislation, 1977) placed laryngeal hemiplegia high on a list of "probable" inherited diseases, and referred to it as being "probably a commonly occurring recessive gene in the Thoroughbred".

Anecdotal evidence of the genetic basis of laryngeal hemiplegia abounds in the literature. Of the famous early racehorses which suffered from the condition, many are recorded as having passed the propensity to develop laryngeal hemiplegia on to their descendants. An often quoted example is that of "Ormonde", a well known winning horse owned by the Duke of Westminster, and affected with laryngeal hemiplegia, as was his dam "Lady Agnes" (Hopkins, 1888; Fleming, 1889). Another well documented family line bearing the condition comes from the French Government Studs information (Fleming, 1889). The stallion "Eastham" left a long line of roarers including "Chasseur", and a grandson "Carnassier". The latter was the sire of "Ganymede", also a roarer.

Descendants of "Ganymede" included "Quebec" and "Troarn", both roarers and both with many offspring as roarers, nine and seven respectively. The proportion of roaring offspring born to roaring sires was reported by differing sources as being between 33-66%, and transmission of the condition was said to be more certain when both parents were affected (Fleming, 1889). Whereas anecdotal evidence should not be ignored, it falls far short of acceptable scientifically measured information.

There has been some documented scientific evidence in support of laryngeal hemiplegia being an inherited disease (Smith, 1926; Saks, 1927; Smith, 1927; Crew & Smith, 1930; van Lent, 1933; Cook, 1970; Cook, 1975a; Cook, 1981). An investigation of the inbreeding coefficient of normal and laryngeal hemiplegic Clydesdales, was carried out by Smith (1926, 1927). The results of this analysis indicated that animals affected by the disease were considerably more inbred than the unaffected animals. He concluded that laryngeal hemiplegia was a recessive defect, and was present in more than one family line. Saks (1927) researched the occurrence of the disease in

Russian Thoroughbreds, and concluded that laryngeal hemiplegia was inherited as a simple recessive defect. A study of the incidence of the disease in Belgian draught horses (van Lent, 1933), including more than 1500 stallions, found a high incidence of offspring with laryngeal hemiplegia, in many family lines.

Cook (1970, 1975b, 1976, 1981) is an advocate of the inherited nature of laryngeal hemiplegia in horses. He considered that the results of a series of 85 test matings supported the heritable nature of the condition, and an autosomal recessive mode of inheritance. The mating of normal animals, and of normal to affected animals supported this theory, but the results obtained from mating affected animals contradicted the view. When mating homozygous recessive animals, the genetic status proposed for affected horses, one would expect 100% of the offspring to be affected. However, the value obtained by Cook, in his admittedly limited number of affected animal matings was 50%. This view of laryngeal hemiplegia being inherited as an autosomal recessive appears to be a gross over-simplification of the possible genetic basis for the disease. It takes no note of degrees of penetrance or expressivity, or even multifactorial influences that may be expected in a disease of this type. From the prevalence figures quoted above, it is clear that laryngeal hemiplegia cannot be inherited as a simple recessive or dominant.

However, the possibility of a genetic influence acting through conformational features should be considered (Hopkins, 1888; Cook, 1975b). Factors such as neck length and body size, which influence the susceptibility of an individual to laryngeal hemiplegia (Goulden & Anderson, 1981a) are inherited conformational characteristics (Cook, 1975b). In the 19th century it was noted that as breeding improved the size and conformation of the Thoroughbred, there was a concurrent increase in the tendency to develop laryngeal hemiplegia (Hopkins, 1888).

SIZE

It is widely accepted that larger horses are the most prone to laryngeal hemiplegia (Dornblaser, 1967; Cook, 1970; Marks *et al* , 1970b; Duncan *et al* , 1978). Many authors have considered the disease to be more common in heavier individuals (Hobday, 1935; Tagg, 1935; Dornblaser, 1967; Cook, 1976), an observation substantiated by Goulden & Anderson (1981a). Statistical analysis of data obtained by the latter authors showed that heavier Thoroughbreds were more susceptible to laryngeal hemiplegia. In addition, matched pairing of data demonstrated that this difference was not due to a greater number of the affected animals being male.

It has also been stated that tall horses are more likely to develop this disease (Dornblaser, 1967; Marks *et al*, 1970a). Both Cook (1976) and Goulden & Anderson (1981a) found that the majority of horses with laryngeal hemiplegia were greater than 160 cm in height. The latter authors confirmed that affected horses were significantly taller than unaffected horses, by analysis after matched pairing for age and sex.

SEX

The tendency for laryngeal hemiplegia to occur more commonly in male than in female horses has often been noted (Percivall, 1840; Hopkins, 1888; Williams, 1901; Wright, 1963; Marks *et al*, 1970a; Cook, 1976; Goulden & Anderson, 1981a).

Two studies have attempted to take into account the distribution of the sexes in the population at large, when considering the differences in the incidence of laryngeal hemiplegia (Williams, 1901; Goulden & Anderson, 1981a). Both of these investigations found males to be six times more susceptible to the disease than females, thus indicating a male predisposition to the condition.

MANAGEMENT

Many management factors have been thought to predispose to laryngeal hemiplegia in horses. These include; poorly ventilated, hot stabling conditions (Fleming, 1889; Lee, 1932); excessive and premature training (Fleming, 1889; Lee , 1932; Marks *et al*, 1970a); use of a tight bearing rein in conjunction with the use of a horse for show jumping (Fleming, 1889; Mahaffrey, 1962). One author reported that laryngeal hemiplegia has never been observed in unhoused horses (Williams, 1901), while another felt that those animals turned out to grass were more likely to develop the disease (Hopkin, 1888).

CONFORMATION

Certain conformations have, rightly or wrongly, become associated with a tendency to develop laryngeal hemiplegia. Conformational characteristics which have been implicated include; animals with long, light necks (Fleming, 1889; Quinlan, 1957); those with flat sides, narrow chests and elbows inclined inwards (Fleming, 1889); horses with a lack of width between the eyes, associated with a narrow intermandibular space (Fleming, 1889; Quinlan, 1957; Dornblaser, 1967; Marks *et al*, 1970b); and long fronted or long necked individuals, with a relatively great distance from the base of the heart to the head (Marks *et al*, 1970b).

It seems possible however, that the appearance of the condition of laryngeal hemiplegia in a horse of a particular shape is purely coincidental, with the exception of those conformational features that may affect the length of the recurrent laryngeal nerves.

CLIMATE AND GEOGRAPHY

The influence of climate has long been considered to play an important part in the incidence of laryngeal hemiplegia (Hopkin, 1888; Fleming, 1889; Williams, 1902; Lee, 1932; Hutyra *et al*, 1938; Cook, 1970; Mason, 1978).

It has been said that it is a condition of the temperate climate, but not of the tropics (Hopkin, 1888; Fleming, 1889; Hutrya *et al*, 1938; Cook, 1970), being common in Britain, Europe, North America and South Africa yet uncommon in Arabia, Australia, New Zealand, Kenya and other tropical countries (Cook, 1970). With respect to New Zealand and Australia at least, these facts are incorrect (Goulden & Anderson, 1891a).

Some authors thought that temperature influenced the incidence of the condition. Some felt that a cold, moist climate which predisposed to respiratory disease in general, increased the likelihood of occurrence of laryngeal hemiplegia (Fleming, 1889). Others felt that the condition was associated with extreme heat (Hart, 1887).

Geographical differences which may affect the prevalence of the disease could be due to any of a number of factors, including climate, the frequency of respiratory disease, prevalent management techniques and the composition of the local population of horses.

In view of the widespread occurrence of the disease throughout the world, it is unlikely that climate and geography are important aetiological factors.

PART II

PERIPHERAL NERVE PATHOLOGY IN EQUINE LARYNGEAL HEMIPLEGIA

1. PERIPHERAL NERVE AND ITS REACTION TO DISEASE

INTRODUCTION

In order to understand the pathological processes affecting peripheral nerves, knowledge of the unique structural, functional and metabolic features of the component neurones is necessary. To fulfill the role of communication between distant regions of the body, and cope with the demands of growth, the cell processes of neurones may be extremely long. In the evolution of the wide variety of animal species, this has meant the development of cell processes of enormous length in some nerves. In equine recurrent laryngeal nerve for example, the axonal process of the motor neurone can be up to 250 cm long (Duncan & Griffiths, 1973). The synthetic metabolic processes for the entire neurone are located solely in the cell body. Supply of these synthesised materials along the axon, relies on axonal transport. Thus, when the peripheral nervous system is affected by mechanical interference, genetic defects, nutritional deficiencies or intoxication, the cell may no longer be able to cope with the demands imposed upon it by its structure, and pathological processes may ensue.

Peripheral neuropathy has been defined as deranged structure and function of peripheral motor, sensory and/or autonomic neurones, involving either the entire nerve fibre or selected areas (Dyck, 1982). These diseases present variable clinical and pathological pictures, depending on features such as; the severity of the disease process; its rate of progression; the anatomic structures affected; the population of neurones or Schwann cells involved;

the level damaged within the neurone; and the basic pathogenic process. The underlying cellular and molecular mechanisms producing peripheral neuropathies are varied and complex, and are only incompletely understood (Dyck, 1982). While the two major components of peripheral nerve, axons and Schwann cells, may be affected separately by a disease process, because of their complex structural and functional relationship, rarely will abnormalities involve either exclusively (Aguayo *et al*, 1979). Axonal alterations can lead to secondary myelin damage and *vice versa* (Schroder, 1975). The pathological findings in peripheral neuropathies are also affected by the considerable capacity of the nerve fibre to regenerate and of the Schwann cell to remyelinate its associated axon. However, a complete return to normal structure does not usually occur, resulting in alterations characteristic for regenerated and remyelinated nerve. These include alterations of shape, thickness and length of newly formed myelin sheaths and increased populations of Schwann cells (Schroder, 1975).

Knowledge of the normal structure of nerve fibres is necessary in order to recognise pathological changes. The node of Ranvier and paranodal area (Fig. 1) tend to be more complex in large diameter myelinated fibres than in small ones (Williams & Landon, 1963; Berthold, 1978). The myelin sheath is fluted in the paranodal region, and the grooves thus formed are filled with Schwann cell cytoplasm rich in mitochondria, probably reflecting the high metabolic requirements of this region (Weller & Cervos-Navarro, 1977). This fluting can result in bulbous expansions of the paranodal area, with assymetry of the bulbs on either side of a node of Ranvier (Williams & Kashef, 1968). There is a marked constriction in axon diameter in the nodal area, this being a particular feature of large diameter fibres (Hess & Young, 1952). In small axons the cross-sectional area at the node is one third of that of the internode, while in large axons the reduction is to

one fifth to one sixth of normal. Thus, it is likely that in the more constricted nodal area of large fibres there will be an increased axoplasmic flow rate (Landon & Hall, 1976).

PATHOLOGY OF PERIPHERAL NERVES

Classification of Pathology

When considering the pathological processes affecting peripheral nerve the first distinction to be made is whether the disease process is intrinsic to the axons and Schwann cells, or whether it is initiated from outside the neuronal components of the nerve. In the latter case causative factors such as trauma, ischaemia, compression and inflammation may be involved (Dyck, 1982). Regardless of the aetiology, peripheral nerve reacts morphologically to disease in only a limited number of ways. Historically, the degnerative processes affecting nerve fibres have been divided into three categories;

1. Wallerian degeneration
2. Axonal degeneration
3. Segmental demyelination

In the first two there is predominant involvement of the axon, while in the third the major effect is on the Schwann cell and myelin sheath. In many disorders however, a mixture of axonal degeneration and segmental demyelination occurs (Webster *et al*, 1967; Dyck, 1973, 1975a).

WALLERIAN DEGENERATION

Wallerian degeneration, first described by Waller in 1950, refers to the degenerative changes which occur in the distal portion of a nerve following transection, or some other traumatic insult. Traditionally, the term Wallerian degeneration is used to refer to the process as it occurs

in peripheral nerve. Wallerian type changes occur in the distal parts of transected nerve fibres in the central nervous system, but there are some differences in the reaction due to the absence of a Schwann cell sheath.

Following transection, degeneration of the axon and myelin sheath occurs, affecting all sizes and types of nerve fibres. Early changes seen ultrastructurally are the paranodal accumulation of mitochondria, dense bodies and lamellar bodies (Ballin & Thomas, 1969a), followed by granular disintegration and disappearance of the neurofilaments and microtubules (Lee, 1963). The axon becomes more irregular in shape and fragments, the first breaks occurring near nodes of Ranvier. This is accompanied by paranodal retraction of the myelin. The axon and myelin sheath then break down into shorter segments, the boundaries of which are marked by Schmidt-Lantermann incisures. The ellipsoids thus formed, consist of axonal fragments surrounded by myelin, and are progressively digested into rows of smaller ovoids and lipid droplets (Blackwood *et al*, 1971). This latter process of degradation occurs in Schwann cells and macrophages (Nathaniel & Pease, 1963). The lamellar nature of the myelin is preserved for the first 6 days, and is then replaced by homogenous lipid droplets. Complete disappearance of the myelin debris may take several months. Some cells retain this debris even during regeneration (Nathaniel & Pease, 1963). Many of these changes can be appreciated by light microscopy and teased fibre preparations.

AXONAL DEGENERATION

In contrast to Wallerian degeneration axonal degeneration of individual nerve fibres within a peripheral nerve may occur, unassociated with local trauma. Causes include chemical intoxications, vitamin deficiencies, genetic and metabolic abnormalities. This situation is thought to be indicative of metabolic derangement of the whole neurone,

and is generally manifest in the distal axon (Bennington, 1978). As with Wallerian degeneration, linear rows of myelin ovoids and balls form (Fig. 2A), but in addition, irregularities of the myelin sheath and secondary demyelination and remyelination may occur at more proximal levels where the axon is still intact. A major difference from Wallerian degeneration is the tendency for selective involvement of certain populations of nerve fibres, rather than involvement of them all. Ultrastructural alterations, which are not a feature of Wallerian degeneration, may be present within the preserved axon. In the chronic, slowly evolving neuropathies of this type the most obvious finding may be the loss of nerve fibres, more pronounced distally. In longstanding neuropathies this fibre loss is evidenced by marked increases in endoneurial cell content, indicating previous Schwann cell proliferation. Histologically, there is a gradation of changes varying from severe fibre degeneration and loss distally, to apparently normal nerve proximally (Bennington, 1978). In particularly chronic disease processes of this type, the pathological manifestation may be axonal atrophy rather than axonal degeneration. These conditions are usually inherited, and are assumed to result from inborn errors of metabolism. Gradual loss of nerve fibres is seen, with only the rare occurrence of actively degenerating fibres, possibly due to axonal shrinkage. Paranodal and segmental demyelination are often observed, and because of the chronic nature and likelihood of repeated episodes of segmental demyelination, "onion bulb" formations (*vide infra*) may also be found. Included in this group of conditions are Friedreich's ataxia and peroneal muscular atrophy (Dyck, 1975a). Neuronal degeneration, with subsequent axonal degeneration is thought to play a part in a number of disorders including amyotrophic lateral sclerosis and Werdnig-Hoffman disease. However, the separation of neuronal degeneration with axonal degeneration, can be difficult in some diseases (Dyck, 1975a).

SEGMENTAL DEMYELINATION

Segmental demyelination refers to the segmental loss of myelin, either paranodal or internodal, with preservation of the axis cylinder (Fig. 2B). Two categories of this disease process exist;

(i) Primary segmental demyelination, which involves a selective abnormality in the development or maintenance of myelin.

(ii) Secondary segmental demyelination, which is thought to occur subsequent to axonal abnormalities (Dyck *et al*, 1971a).

Primary segmental demyelination

Primary segmental demyelination can be further subdivided on the basis of the specific pathogenesis. In one group of segmental demyelinating disorders there is a presumed metabolic disturbance of the Schwann cell leading to degeneration of its myelin sheath, while in others the disease process is immune mediated, and the myelin sheath is invaded and digested by macrophages (Waksman & Adams, 1955; Schroder, 1975; Weller & Cervos-Navarro, 1977).

The earliest change observed in segmental demyelination is widening of the nodal gap. The terminal myelin loops detach from the axolemma, the enclosed fingers of Schwann cell cytoplasm swell and myelin debris appears. There is loosening and disruption of the myelin lamellae with the formation of vesicular and osmiophilic fragments and ovoids within the Schwann cell cytoplasm. Within several days, macrophages appear and phagocytose partially digested myelin debris (Cragg & Thomas, 1964; Peterson & Murray, 1965). This progressive loss of myelin occurs in the territory of the affected Schwann cell, and may be restricted to the paranodal region or spread to involve an entire internode (Dinn, 1970). The distribution of demyelinated regions on nerve fibres is random, with no

clustering on particular fibres as occurs with secondary demyelination. Many internodes may be affected, but the individual lesions may differ in age and severity (Bennington, 1978). Proliferation of Schwann cells occurs (Allt, 1976).

Once remyelination commences, usually within a few days of the start of demyelination, recovery is generally rapid and complete (Bennington, 1978). Initially, Schwann cell proliferation occurs in the immediate area of the demyelinated axon. One Schwann cell becomes associated with the bare axon and proceeds with the spiral formation of new myelin (Dinn, 1970). Early in the course of segmental demyelination, the presence of axons devoid of myelin may be observed. Generally, unmyelinated axons do not exceed 3 µm in diameter, and so it has been assumed that any axons larger than this without a myelin sheath are demyelinated (Ochoa & Mair, 1969a; Weller & Cervos-Navarro, 1977). With paranodal demyelination, two mechanisms of repair have been observed. When the nodal gap is less than 15 µm, the existing Schwann cells reconstitute the sheath. When the gap is greater than 15 µm, a short intercalated internode of myelin with its own associated Schwann cell is formed (Cavanagh & Jacobs, 1964). When a whole internode is demyelinated, reconstitution of the myelin sheath occurs by the formation of several short internodes, each with its own Schwann cell (Bennington, 1978). The resulting short thinly myelinated segment remains as a permanent record of previous demyelination recognisable in teased fibre preparations (Morgan-Hughes, 1968; Dinn, 1970). In transverse sections segmental demyelination and remyelination is apparent as fibres with disproportionately thin myelin sheaths (Weller & DasGupta, 1968). During remyelination, the myelin lamellae are not completely compacted, and the associated Schwann cell cytoplasm may be more abundant than usual (Allt, 1969). In large myelinated fibres especially, the

myelin sheath is never completely restored to its usual thickness, nor is it in regenerated fibres (Schroder, 1975). Because of the presence of short intercalated internodes in remyelinated nerve, the mean internode length is decreased and the overall internode length is more variable (Schroder, 1975).

Secondary segmental demyelination

The process of secondary segmental demyelination is discussed in the section "The Schwann cell in health and disease".

Other Pathological Alterations in Peripheral Nerve

DEGENERATION AND REGENERATION

Degenerating myelinated fibres

Teased fibres consisting of linear rows of myelin ovoids and balls, are characteristic of Wallerian and axonal degeneration, and constitute category E of Dyck's classification (1975a), as depicted in Fig. 20. Initially degeneration is apparent as fibres with irregular beading of the myelin sheath, and with subsequent division of the fibre into large segments of myelin. There is progression to linear rows of myelin ovoids of varying sizes as the myelin segments are degraded further. Finally, the fibres appear as clumps of myelin lying in rows, separated from each other by thin Schwann cell bands (Dyck *et al*, 1968). The segmentation of myelin into ovoids at 2-7 days after injury begins paranodally, and the boundaries of the ovoids are formed by Schmidt-Lantermann clefts (Ohmi, 1961; Lee, 1963). This process of myelin degeneration has been reported to take from 14 days (Guth, 1956), to as long as 3 months (Barton, 1962; Lee, 1963). Initially, the myelin ovoid undergoing digestion in the Schwann cells has the same lamellar pattern as the intact myelin sheath.

There is subsequent degradation to homogenous lipid droplets (Dyck, 1975a). The distinction between axonal degeneration and segmental demyelination of fibres is based on two observations

(i) The type and size of myelin breakdown products, which appear as small balls in segmental demyelination, and large ovoids in axonal degeneration.

(ii) The distribution of degeneration of the myelin sheath along the nerve fibre. The whole length of the teased fibre is involved in axonal degeneration, but not in segmental demyelination (Madrid & Wisniewski, 1977).

Axonal swellings

Axonal swellings are prominent ultrastructural features of a number of exerimental and naturally occurring neuropathies (Lampert, 1967). Swellings occur in the distal and proximal stumps of transected nerve, degenerating axons, the tips of regenerating axons and dystrophic axons (Lampert, 1967; Griffin *et al* , 1977). Ultrastructurally, these swellings differ from one another. In transected nerve they are filled with mitochondria, membranous dense bodies and vesicular organelles. In swollen degenerating axons there is granular disintegration of neurofilaments, enlarged mitochondria, clumped axonal debris and occasionally disintegrating membranous dense bodies. In regenerating axon tips, the accumulated organelles tend to be less numerous than in reactive axonal enlargements. However, dystrophic axons can be almost indistinguishable from the reactive swellings of transected nerve (Lampert, 1967). A number of the toxic distal axonopathies, including acrylamide (Prineas, 1969b), and hexacarbon intoxication (Spencer & Schaumburg, 1976), are associated with the formation of axonal swellings containing numerous neurofilaments. The accumulation of axonal organelles has also been reported in ischaemic lesions of peripheral nerve (Korthals *et al* , 1978). Lampert (1967) emphasised

that this accumulation of organelles within axons is a nonspecific reaction to axonal damage. It is widely accepted that, regardless of the initiating factors, the organelle accumulation seen in damaged nerve is a consequence of interruption of the bidirectional axoplasmic transport systems (Martinez & Friede, 1970; Ochs, 1975; Griffin *et al*, 1977).

Ultrastructural axonal alterations

In disease processes of primary axonal degeneration, ultrastructural alterations to the axon may be observed, before overt fibre degeneration is obvious. The particular changes seen vary with the different neuropathies, but in general they tend to be nonspecific changes indicative of early primary axonal disease.

The accumulation of neurofilaments and/or microtubules is seen in organophosphate (Prineas, 1969a), acrylamide (Prineas, 1969b) and hexacarbon intoxication (Spencer *et al*, 1980). Increases in vesicular or membranous organelles and mitochondria have been reported in organophosphate intoxication (Prineas, 1969a), isoniazid intoxication (Chua *et al*, 1980), thiamine deficiency (Collins *et al*, 1964; Prineas, 1970) and uraemic neuropathy (Dyck *et al*, 1971a). The redistribution of axonal cytoskeletal organelles, the microtubules and neurofilaments, has been reported with a number of neuropathies. These include hexacarbon intoxication and giant axonal neuropathy of man (Asbury *et al*, 1972). In ßß-iminodipropionitrile intoxication the neurofilaments are displaced to a peripheral position within the axon, while mitochondria, smooth endoplasmic reticulum and microtubules are located at the centre (Papasozomenos *et al*, 1981). In the distal axonopathy associated with ingestion of *Karwinskia humboldtiana*

(buckthorn), the microtubules and other organelles become peripherally placed (Heath *et al*, 1982). In organophosphate intoxication, margination of the microtubules was also noted (Prineas, 1969a).

Additional findings in isoniazid intoxication are vacuoles occurring between the axon and Schwann cell, with altered arrangement of the microtubules and smooth endoplasmic reticulum (Jacobs *et al*, 1979b). In thiamine deficiency in rats, redundant projections of membrane are seen in the axoplasm (Collins *et al*, 1964). Clumping of the neurofilaments and microtubules has been reported in uraemic neuropathy (Dyck *et al*, 1971a).

Axon-Schwann cell networks

Axon-Schwann cell networks can be seen ultrastructurally in transverse and longitudinal sections of nerve. A detailed account of the formation and possible function of these complexes was given by Spencer and Thomas (1974), and is illustrated in Fig. 3. Organelles observed in these complexes include clear and dense core vesicles, membranous dense bodies, mitochondria, glycogen-like granules and glycogen filled mitochondrial remnants. These networks occur to a limited extent in normal nerve, especially in the paranodal area, but in situations of axonal pathology are seen more frequently and not restricted to this region (Spencer & Thomas, 1974; Spencer & Schaumburg, 1976; Berthold, 1978). They have been found in a wide variety of neuropathies, including acrylamide (Prineas, 1969b), isoniazid (Schlaepfer, 1964), thallium (Spencer & Schaumburg, 1976), and hexacarbon intoxications (Spencer & Thomas, 1974), thiamine deficiency (Collins *et al*, 1964; Prineas, 1970), human uraemic neuropathy (Dyck *et al*, 1971a) and in the proximal stumps of transected nerves (Morris *et al*, 1972). A common feature of these diseases is the presence of abnormal organelles. In the toxic distal axonopathies, the axon-Schwann cell networks are observed most frequently

proximal to the site of fibre degeneration (Spencer & Thomas, 1974). It has been postulated that these networks reflect an aspect of the symbiotic relationship of the axon and Schwann cell, whereby the Schwann cell selectively phagocytoses abnormal axoplasmic contents. It is considered to be a nonspecific reaction of the Schwann cell to axonal damage, and a sensitive indicator of minimal or early axonal disease.

Axonal regeneration

Although nerve fibres possess a considerable capacity for regeneration it is a slow process and recovery is frequently incomplete. Following Wallerian degeneration of peripheral nerve the degree of recovery depends on factors such as the site of the lesion, the age of the individual, the approximation of transected ends and the amount of soft tissue injury (Bennington, 1978). In axonal degeneration recovery is dependent upon certain influences such as the cause, severity and duration of the disease process. The conditions for regeneration are usually optimal, as the basement membrane is intact and the columns of proliferating Schwann cells, the bands of Bungner, act as guiding pathways for the regenerating axons (Schroder, 1975). Fine axon sprouts move distally from the proximal stump into the denervated Schwann cell band, at 2-5 mm per day. Subsequently, these become remyelinated by proliferating Schwann cells (Bennington, 1978).

A number of characteristics of regenerating nerve fibres facilitate their identification in histological preparations of peripheral nerve. These include:

(i) The myelin sheaths of regenerated nerve fibres remain thinner than normal (Cragg & Thomas, 1964; Schroder, 1972).

(ii) A proportion of small axons have inappropriately thick myelin sheaths (Schroder, 1972). It has been suggested that these small axons with disproportionately thick myelin sheaths, represent regenerating axons which have failed to reinnervate endorgans, and are undergoing axonal atrophy. Ultrastructural evidence in support of this hypothesis is the small axonal calibre, the frequent occurrence of loops and folds of the myelin sheath, and the presence of lysosome-like dense bodies in some axons (Schroder, 1972).

(iii) A number of relatively large diameter axons have no myelin sheaths (Schroder, 1972).

(iv) Uniformly short internodes are present on regenerated nerve fibres (Vizoso & Young, 1948).

(v) Frequently in these fibres the perinuclear and periaxonal Schwann cell cytoplasm is increased, and unusually large Schmidt-Lantermann incisures with wide periaxonal cytoplasm are present (Schroder, 1975).

Following Wallerian and axonal degeneration, several regenerating axon sprouts from a single nerve fibre remain closely associated with each other. These are termed regenerating clusters (Fig. 4), and appear hisologically as discrete groups of three or more small myelinated fibres (Ochoa & Mair, 1969b; Madrid *et al*, 1977). Often the fibres comprising the cluster possess disproportionately thin myelin sheaths and are surrounded by abundant Schwann cell cytoplasm. These alterations indicate recent regeneration and remyelination (Dyck, 1966). Regenerating clusters are seen commonly in a variety of neuropathies, especially if there has been repetitive injury to nerve fibres (Weller & Cervos-Navarro, 1977). Their frequency increases with age (Ochoa & Mair, 1969b).

Mast cells

Mast cells are seen only occasionally in the endoneurium of normal human nerve, but are common in nerve from the cat, mouse and rat. They are recognisable by their numerous filapodia and cytoplasmic inclusions, and the absence of basement membrane (Fig. 5). Increased numbers of endoneurial mast cells have been reported in association with peripheral nerve pathology. In Wallerian degeneration, for example, mast cells may increase in number four to five times (Bennington, 1978).

The Schwann cell in health and disease

Schwann cells constitute 90% of the cellular content of the endoneurial compartment, and are recognisable by their association with a basement membrane. The remaining endoneurial cells are mostly fibroblasts and rarely mast cells (Ochoa & Mair, 1969b). Schwann cells ensheath every axon, both myelinated and unmyelinated, in the peripheral nervous system. A single internode on a myelinated fibre represents the domain of one Schwann cell (Fig. 6). It is usual for myelinated fibres to occur in single units, but they occur sometimes as conglomerates with unmyelinated fibres (Ochoa & Mair, 1969b). In normal nerve, all Schwann cells are associated with axons. The repeating unit of myelin, the lamella, is made up of two layers of fused Schwann cell membrane (Fig. 7), appearing ultrastructurally as the major and minor dense lines (Bennington, 1978). Schmidt-Lantermann incisures, thought originally to be artefactual, are funnel shaped clefts occurring along the myelin sheath. The lamellae split to enclose a small amount of Schwann cell cytoplasm, producing spiral tracts of cytoplasm through the sheath. The larger the myelinated fibre, the greater the number of incisures per internode. They are also more numerous in young and in regenerated nerve fibres (Hiscoe, 1947), and are thought to provide connecting pathways from the adaxonal to the outer layer

of Schwann cell cytoplasm (Weller & Cervos-Navarro, 1977). Certain inclusions occur normally in the cytoplasm of Schwann cells, particularly in the nuclear and paranodal regions (Dyck, 1975a; Schroder, 1975; Landon & Hall, 1976). Ultrastructurally, the Π or protagon granule (Fig. 8) is a membrane bound, ellipsoidal or comma shaped structure composed of parallel lamellae (Gamble & Eames, 1966; Powell *et al*, 1978). Myelin-like or μ granules, appear in two forms, either as a whorl of myelin with no central axoplasm (Fig. 10) or as homogenous lipid droplets (Fig. 11) (Dyck, 1975a). Although these structures do occur in normal nerve, they are seen more frequently in degenerating, regenerated or remyelinated nerve fibres (Schroder, 1975; Landon & Hall, 1976; Powell *et al*, 1978; Jacobs *et al*, 1979a). It has been stated that Π granules are probably the result of a degenerative phenomenon, although apparently not specific for any one disease of peripheral nerve (Dyck, 1975a).

Schwann cells are known to proliferate in response to degeneration and demyelination of nerve fibres. In most peripheral neuropathies affected nerves show increased numbers of nuclei, being those of the increased Schwann cells, macrophages, fibroblasts and mast cells (Dyck, 1975a). With repeated episodes of damage there are repeated waves of proliferation. Schwann cells possess high cell surface activity, tending to form complexes in contact with other Schwann cells. They are involved in "onion bulb" formation and will surround collagen to form collagen pockets (Fig. 14) (Ochoa & Vial, 1967; Bennington, 1978).

The proliferation of Schwann cells, regularly follows Wallerian or axonal degeneration. This proliferation occurs within the basement membrane tube, to form continuous longitudinal columns of cells, termed bands of Bungner (Fig. 9). These provide guiding pathways for regenerating axons (Nathaniel & Pease, 1963; Thomas, 1964). The bands

decrease in size with time if they remain denervated, although they can accept regenerating axons for several years after injury (Young, 1949). The Bungner bands of myelinated fibres tend to be of relatively larger diameter, and have cell processes of irregular shape with occasional remnants of myelin and lamellated inclusions. In addition, they show excess folding of the basement membrane, a change associated with loss of the axon and myelin. The Schwann cell bands resulting from the degeneration of unmyelinated fibres are smaller, consist of plate-like Schwann cell processes and are often arranged in groups. Folded basement membrane is also a feature of these structures (Ochoa & Mair, 1969b). It has been demonstrated in humans that bands of Bungner of both types increase in frequency with age, indicating progressive fibre degeneration in the nerves of older individuals (Ochoa & Mair, 1969b).

Ultrastructural alterations to the basement membrane, which surrounds all Schwann cells and their processes, are sometimes seen in degenerative and demyelinative conditions (Fig. 12). Even in normal nerve, folded empty profiles of basement membrane, indicating previous degeneration of a myelinated fibre, have been observed (Dyck, 1966).

Secondary segmental demyelination

The interactions of axons and Schwann cells are important in the normal function of peripheral nerves and in the pathological processes of the various acute and chronic neuropathies. While, in the past it has been assumed that the presence of segmental demyelination was indicative of disordered Schwann cell function, it has become increasingly evident that the axonal degeneration of a variety of neuropathies is accompanied by segmental demyelination, secondary to the axonal changes (Dyck, 1982). Evidence of myelin abnormalities, such as irregularity of the myelin sheath, segmental demyelination and the presence of intercalated internodes is reported with

neuropathies where the predominant finding has been one of axonal degeneration (Collins *et al*, 1964; Hopkins, 1970; Dyck *et al*, 1971; Dyck & Lais, 1973). Although the mechanism of the paranodal segmental demyelination seen with axonal degeneration is poorly understood, it occurs in uraemic (Dyck *et al*, 1971a), alcoholic (Collins *et al*, 1964), diabetic (Bennington, 1978) and thiamine deficiency neuropathies (Collins *et al*, 1964), peroneal muscular atrophy (Madrid *et al*, 1977), Friedreich's ataxia (Dyck & Lais, 1973), amyotrophic lateral sclerosis (Dyck, 1975a), the prolonged constriction of nerves (Baba *et al*, 1982), acrylamide (Hopkins, 1970) and hexacarbon intoxications (Spencer & Schaumburg, 1977a).

The evaluation of teased fibre preparations from patients with uraemic neuropathy led Dyck and coworkers (1971a) to conclude that the observed irregularities of myelin, paranodal and segmental demyelination were secondary to axonal changes. This hypothesis was based on the following evidence;

(i) Degeneration of myelinated and unmyelinated fibres had occurred, and was more severe distally.

(ii) The paranodal and segmental demyelination seen was not randomly distributed, but occurred especially on certain fibres. This was confirmed statistically.

(iii) Ultrastructural abnormalities including clustering of neurofilaments, and the accumulation of mitochondria and lamellar dense bodies were observed in preserved axons.

(iv) The regression of the number of myelin lamellae against axis cylinder circumference indicated a selective decrease in axonal volume.

The stages observed in the process of axonal degeneration by Dyck and coworkers (1971a), were excessive irregularity of

the myelin sheath, retraction of myelin at the nodes, multiple regional segmental demyelination and finally, degeneration of the fibre (Dyck *et al*, 1971a). The occurrence of remyelinated or intercalated internodes on nerve fibres was taken to indicate that the process was chronic in nature, and that affected axons did not necessarily degenerate. The presence of intercalated internodes on a single teased fibre was regarded as evidence of previous axonal abnormality.

Similar findings were reported on the analysis of teased fibres from patients with another primary axonal disorder, Friedreich's ataxia (Dyck & Lais, 1973). Also, in peroneal muscular atrophy of man, segmental demyelination is observed, with ultrastructural alterations indicative of axonal disease, and the clustering of intercalated segments on particular fibres (Madrid *et al*, 1977). Similarly, in diabetic neuropathy, the distal loss of nerve fibres, together with segmental demyelination is seen, especially in those cases which are chronic in nature (Bennington, 1978).

In a number of axonal disorders with segmental demyelination, the finding of single teased fibres with preservation of proximal regions of the fibre, and axonal degeneration in distal regions of the same fibre (type I in Dyck's classification, 1975a), has been reported. Often, there is paranodal demyelination in the preserved proximal portions, supporting the hypothesis that segmental demyelination occurs secondary to axonal pathology. These fibres have been noted in uraemic neuropathy (Dyck, 1971a), thiamine deficiency (Takahashi & Nakamura, 1976), acrylamide intoxication (Hopkins, 1970) and the prolonged constriction of nerves (Baba *et al*, 1982).

In the neuropathy of acrylamide intoxication, intercalated segments were seen most frequently at an intermediate level on nerves. Because most nerve fibres have degenerated in distal regions of the nerve, these segments are seen only rarely at this level. Proximally, the degree of pathology is less severe, and so these intercalated segments are also infrequently observed (Hopkins, 1970). The high incidence of secondary segmental demyelination in chronic axonal degenerations, compared with the infrequent occurrence with acute conditions has been noted by a number of authors (Dyck, 1975a; Spencer & Schaumburg, 1977; Bennington, 1978).

It can be seen therefore, that although one can divide the pathogenic processes affecting myelinated fibres into those predominantly affecting the axon, and those with major involvement of myelin, the finding of segmental demyelination in a neuropathy does not necessarily indicate a primary disorder of the Schwann cell or its associated myelin sheath.

"Onion bulbs"

"Onion bulb" formations (Fig. 13) are seen as concentrically layered Schwann cell processes surrounding a core (Webster *et al*, 1967; Dyck, 1975a; Raine, 1977; Weller & Cervos-Navarro, 1977; Bennington, 1978). The layers of the "onion bulb" are known to be processes of Schwann cells, as ultrastructurally they are observed to possess basement membrane (Webster *et al*, 1967; Dyck, 1975a). The spaces between Schwann cell processes are occupied by longitudinally oriented collagen fibres (Webster *et al*, 1967). "Onion bulbs" are usually seen surrounding thinly myelinated fibres (Webster *et al*, 1967; Ballin & Thomas, 1969b). Less commonly, a number of other structures are observed within these formations. They include normal myelinated fibres, demyelinated fibres, unmyelinated fibres,

bands of Bungner and combinations of the above (Webster *et al*, 1967; Madrid *et al*, 1977). Early "onion bulb" formations consist of myelinated fibres surrounded by only one or a few Schwann cells and their processes, and may be seen in pathological situations or rarely in normal nerve (Dyck, 1966; Politis *et al*, 1980).

The presence of "onion bulb" formations in peripheral nerve is not specific for a single disease entity. Rather, it is a standard pathological reaction to repeated insults to myelinated fibres in the peripheral nervous system, and occurs in many diseases (Dyck, 1975a; Raine, 1977; Bennington, 1978). Some controversy exists as to whether the repeated events are degeneration and regeneration, or segmental demyelination and remyelination of nerve fibres. The most widely accepted view is that repeated episodes of segmental demyelination and remyelination are the initiating events in "onion bulb" formation (Webster *et al*, 1967; Ochoa & Mair, 1969b; Dyck, 1975a; Schroder, 1975; Madrid *et al*, 1977; Bennington, 1978). Remyelinated axons are said to be more susceptible to demyelination than normal axons, perhaps explaining the repeated episodes of demyelination on particular internodes, necessary to produce "onion bulbs" (Ludwin, 1981). The association of segmental demyelination and remyelination with "onion bulb" formation does not preclude the possibility that the process of demyelination is secondary to a disease process in the axon. Thus, the classification of a neuropathy as one of primary segmental demyelination and remyelination, can not be made solely on the basis of the presence of these formations (Spencer & Weinberg, 1978). Generally, "onion bulbs" occur commonly in neuropathies which are chronic in nature (Ochoa & Mair, 1969b; Thomas, 1970; Schroder, 1975).

Collagen pockets

Small bundles of collagen fibres, which indent Schwann cells or are surrounded by mesaxon-like arrangements (Fig. 14), are termed collagen pockets (Gamble & Eames, 1964; Ochoa & Vial, 1967; Ochoa, 1976). They are thought to result from Schwann cell processes surrounding already formed collagen (Gamble & Eames, 1964). While some workers observed these structures exclusively in association with unmyelinated fibres (Ochoa & Vial, 1967; Thomas, 1973), others reported their occurrence in Schwann cell processes not containing axons (Gamble & Eames, 1964). They occur frequently in some neuropathies, especially if chronic in nature (Thomas & Olsson, 1975). In such conditions, it is thought that the collagen fibres replace degenerated unmyelinated fibres (Thomas, 1973). The evidence offered for this theory was their similarity in size to unmyelinated axons and their increased frequency with age and in some neuropathies. Thus, while collagen pockets do occur in healthy nerve, their presence in increased numbers suggests the loss of unmyelinated fibres.

INTERNODE LENGTH

A direct relationship between internode length and fibre diameter has been noted by a number of authors (Hiscoe, 1947; Vizoso & Young, 1948; Lascelles & Thomas, 1966; Dyck, 1975a; Weller & Cervos-Navarro, 1977). At the time of onset of myelination the population of Schwann cells is stable. They are already spaced out along the fibres with the initial length of approximately 300 µm. During growth, there is no increase in the number of Schwann cells, and so the internodes must increase in length passively (Hiscoe, 1947; Vizoso & Young, 1948; Lascelles & Thomas, 1966). It is thought that large diameter fibres commence myelination earlier than small ones, and consequently are subject to more elongation of their internodes with growth (Vizoso & Young, 1948; Lascelles & Thomas, 1966). Thus,

in a young growing animal, internode length will be less than in the adult (Hiscoe, 1947). Although internode length is constant along a nerve fibre (Weller & Cervos-Navarro, 1977), there is considerable variation between fibres of the same diameter in different nerves (Vizoso, 1950; Dyck, 1975a; Friede *et al*, 1981; Friede & Bischausen, 1982).

The presence of short intercalated internodes has long been noted in normal and diseased nerve (Renaut, 1881; Vizoso & Young, 1948; Lubinska, 1958). A detailed study of these internodes was reported by Lubinska (1958), who found their infrequent occurrence in apparently normal nerve with increased frequency in older animals. It is now recognised that these intercalated internodes mark the positions of previous demyelination and remyelination. When partial demyelination of an internode occurs, the formation of short internodes (50-150 µm) is observed, whereas if an entire internode is demyelinated, several consecutive longer intercalated internodes are seen. These are approximately 300 µm in length, and when summed usually equal the normal internode length for that fibre. Sometimes the thickness of the myelin sheath differs between intercalated internodes of a fibre or adjacent fibre, indicating lesions of different ages.

The effect on internode length of demyelinating conditions, and of axonal degeneration and regeneration was investigated by Fullerton and coworkers (1965). In the demyelinating conditions investigated, increased variability of internode length was noted. This was related to the presence of short intercalated internodes amongst longer normal ones. Once remyelination is well advanced the myelin sheath can be restored to near normal thickness, and the finding of an internode length inappropriately short for the thickness of the fibre may be the only distinctive feature of the remyelinated internode (Ludwin, 1981). In degeneration and regeneration, the maturity of an animal has an effect on internode length. In adults the regenerated fibres

possess uniformly short internodes of approximately 300 µm. Whereas, in young growing animals the internode length is greater due to passive increases in length of the newly formed internodes by growth (Vizoso & Young, 1948).

RELATIONSHIP OF AXONAL DIAMETER AND MYELIN SHEATH THICKNESS

A direct correlation between myelin thickness and axonal diameter has been claimed by a number of workers (Dyck, 1966; Friede & Samorajski, 1967; Dyck *et al*, 1971a; Williams & Wendell-Smith, 1971; Schroder, 1972). However, in a recent study it was found that in large axons the thickness of the myelin sheath did not increase with axonal calibre (Friede & Bischausen, 1982). The volume of myelin was shown to have a precise linear relationship with the product of axonal circumference and internode length. Thus, the deviation in sheath thickness for large fibres merely resulted from analysis without reference to internode length.

In pathological states of peripheral nerve there is deviation from the usual regression line obtained for axon calibre and myelin sheath thickness, in a particular nerve. In a number of neuropathies there is alteration of axonal calibre, which would directly affect the relationship of the two parameters. The myelin sheath possesses a greater ability to adapt to changes in axonal size than was thought possible, adjustment occurring by slippage of myelin lamellae (Friede & Miyagishi, 1972). It has been demonstrated that when the volume of axoplasm increases in swollen fibres, outward slippage of myelin lamellae occurs, resulting in a sheath with fewer turns of lamellae. With a shrinking axis cylinder contraction of the sheath occurs by inward slippage of lamellae, resulting in increased thickness of the myelin sheath relative to axonal calibre (Martinez & Friede, 1970; Friede & Miyagishi, 1972). Thus, in conditions with axonal atrophy, with

preservation of the myelin sheath, the ratio of myelin sheath thickness to axonal diameter is increased (Dyck *et al*, 1971a; Long *et al*, 1980; Baba *et al*, 1982). In conditions with axonal swelling, this ratio is decreased.

The relationship of axonal calibre and myelin sheath thickness has also proved useful in the identification of remyelinated internodes, especially in the early stages of remyelination. Generally, these internodes are of small diameter and possess a small number of myelin lamellae per micron of axis cylinder perimeter (Dyck, 1975a; Weller & Cervos-Navarro, 1977). In regenerated fibres also, there is deviation from the normal regression line for the relationship between axonal calibre and myelin thickness. The regression line increases more gradually than normal due to the presence of a number of small axons with disproportionately thick myelin sheaths. These fibres are thought to be regressing after failing to reach an endorgan (Schroder, 1972).

AGE RELATED CHANGES OF PERIPHERAL NERVE

The presence of pathological alterations in apparently normal nerve, and the increased occurrence of these abnormalities with increasing age has been reported in man and animals. Many investigations have been carried out in order to establish the prevalence of these abnormalities in normal subjects of differing age groups (Cottrell, 1940; Lubinska, 1958; Lascelles & Thomas, 1966; Ochoa & Mair, 1969b; Arnold & Harriman, 1970; Gutrecht & Dyck, 1970; Stevens *et al*, 1973; Griffiths & Duncan, 1975; Braund *et al*, 1982a; 1982b). With this information it is possible to provide a normal range of aging changes for a given nerve, and so interpret findings more accurately.

In newborn animals, the diameter of nerve fibres has been shown to be less than that of adults. In man, nerve fibres reach their adult size by 5-10 years of age (Cottrell, 1940; Gutrecht & Dyck, 1970; Stevens *et al*, 1973), and in the dog, by 1 year of age (Braund *et al*, 1982b). Internode length however, is said to continue to increase until growth ceases, although in one study, adult values for internode length were attained before growth was completed (Stevens *et al*, 1973).

A number of pathological changes are found in the nerves of older individuals. These are as follows;

(i) There is an increase in the number of intercalated internodes, indicating demyelination and remyelination (Lascelles & Thomas, 1966; Stevens *et al* 1973).

(ii) There is a slight increase in active axonal degeneration (Arnold & Harriman, 1970; Griffiths & Duncan, 1975; Braund *et al*, 1982a). Less than 3% of fibres are affected by the above two processes in dogs less than 10 years of age. However, in older dogs, 11% of fibres may be involved (Braund *et al* , 1982a). Similar levels are reported in normal and aged human nerve (Stevens *et al*, 1973).

(iii) Most authors conclude that there is a decrease in total fibre content and density with age (Corbin & Gardner, 1937; Lascelles & Thomas, 1966; Swallow, 1966; Ochoa & Mair, 1969a; Stevens *et al*, 1973; Tohgi *et al* , 1977). Others however dispute this (Griffiths & Duncan, 1975; Braund *et al*, 1982b).

(iv) There is an increase in regenerating clusters, excess basement membrane, inappropriately thin myelin sheaths and myelin debris in Schwann cell cytoplasm (Ochoa & Mair, 1969a; Braund *et al*, 1982b).

(v) Internode length varies with age. In older nerves internode length decreases and its variability increases (Vizoso, 1950; Lubinska, 1958; Lascelles & Thomas, 1966; Stevens *et al*, 1973; Braund *et al*, 1982a). These changes are thought to result from the occurrence of segmental demyelination and remyelination, with the formation of short intercalated internodes, and to a lesser extent from regenerated nerve fibres with uniformly short internodes.

(vi) There is an increase in the number of myelin lamellae per µm of axis cylinder perimeter with age. This has been said to indicate axonal atrophy, possibly resulting in secondary segmental demyelination (Dyck, 1975a). In addition, the vulnerability of long nerve fibres in this aging process has been noted, with a greater degree of pathology occurring distally (Cottrell, 1940; Takahashi, 1964).

An increase in the frequency of Renaut bodies with age in peripheral nerve has been reported in man (Dyck, 1975a) while no such increase was noted in a recent study in dogs (Braund *et al*, 1982a). According to Asbury (1973), these structures are not observed in the newborn.

The cause of these age related changes is poorly understood, but in man minor compressive changes occurring throughout life, vascular alterations and the aging of neurones with possible axonal atrophy have been implicated (Dyck, 1975a; Braund *et al*, 1982a).

The Role of Axoplasmic Transport in Peripheral Neuropathy

Axoplasmic transport is the movement of materials, synthesised in the cell body of the neurone, into and along the axons and dendrites. It is the process which supplies the axon with materials necessary for function and the maintenance of structural integrity (Ochs & Worth, 1978). Since axoplasmic transport is important in the function of nerve fibres, it is not surprising that the possibility of impaired transport playing a role in the pathogenesis of peripheral neuropathies, has been suggested. Indeed, it is likely to play an important part in the clinical disease of many peripheral neuropathies, although not necessarily a primary or direct role (Smith, 1981). There are at least three subdivisions within the system of axoplasmic transport. These are fast anterograde, slow anterograde and the retrograde systems. The fast axonal transport system involves movement of mainly membranous components. The slow system moves a high proportion of cytoplasmic proteins. The retrograde system moves some of the materials involved in fast anterograde transport in the opposite direction (Grafstein *et al*, 1970; Ochs, 1972; Olsson *et al*, 1978; Droz & Chretien, 1980; Grafstein & Forman, 1980; Landon, 1981). Fast axoplasmic transport is critically dependent on a continued supply of ATP from oxidative phosphorylation. This substrate is produced locally along the length of the axon (Ochs & Hollingworth, 1971; Ochs, 1972).

Impaired axoplasmic transport has been thought likely to occur in a variety of neuropathies which interfere with axonal integrity, including many of the distal axonopathies (Smith, 1981). The swelling of axons and accumulation of organelles, that occurs when nerve fibres are damaged (Lampert, 1967), has been attributed to dysfunction of the axoplasmic transport systems. The corresponding accumulation of organelles in a variety of neuropathies, does lend support to the involvement of defective axonal

transport in these diseases. Again, it should be emphasised that these accumulations may occur secondary to some other axonal pathology.

Investigations of axonal transport in peripheral neuropathies should attempt to determine whether transport is altered early in the course of the disease, before overt axonal alterations are seen. Only then would it be possible to differentiate primary abnormalities in axonal transport from interruption secondary to severe pathological changes in the nerve fibres (Droz & Chretien, 1980; Pleasure, 1980). Interference with axoplasmic transport has been demonstrated in a number of neuropathies of man and animals, including diabetic and uraemic neuropathies, peroneal muscular atrophy and a number of the toxic distal axonopathies. However, in the majority of investigations of the latter conditions, there was no clear evidence that abnormalities of transport were early initiating events in the pathogenesis of the neuropathy. The slowing of transport was usually minor, did not precede marked axonal changes, and there was no correlation with the severity of the neuropathy (Pleasure *et al*, 1969; Bradley & Williams, 1973; Brimijoin, 1982). One exception is ßß-iminodipropionitrile neuropathy, in which a total block of the slow axonal transport of neurofilament proteins has been reported, with no effect on fast transport (Griffin *et al*, 1978; Griffin & Price, 1980). Of the toxic distal axonopathies, the neuropathy induced by the hexacarbon compounds does show impaired fast axoplasmic transport, related in degree to the severity of the neurological deficit. The changes in fast transport are probably not primary, but appear to be related to the accumulation of neurofilaments. Axonal degeneration and impairment of fast axoplasmic transport were both related to the swellings, and thus it was considered that obstruction of transport reached a critical point in some of these swellings, resulting in degeneration distal to them (Mendell *et al*, 1976; Mendell & Sahenk, 1980).

New evidence for the involvement of impaired transport in axonal degeneration, has been gained by the study of the transfer of materials from the anterograde to retrograde transport systems in the distal axon. Results of studies of experimental diabetic neuropathy in rats, have suggested that impairment of the turnaround of fast transported materials in the distal axon could precipitate distal axonal degeneration (Sidenius & Jakobsen, 1981). Other investigations into acrylamide and hexacarbon neuropathies (Sahenk & Mendell, 1981), have provided evidence for a decrease in the bidirectional transport rate in the distal axon, with an intact turnaround process. These findings correlated with the differences in severity of the neuropathies observed.

Thus, while axonal flow probably is affected in a number of peripheral neuropathies, it is not known whether there is a direct effect on the transport mechanisms, whether there are disturbances of local metabolism upon which axoplasmic transport relies, whether decreased availability of materials for transport from the cell body occurs, or whether there is increased demand in the periphery of the nerve fibre (Schroder, 1975).

APPROACH TO INVESTIGATING A PERIPHERAL NEUROPATHY

In determining the techniques to be used for the investigation of a peripheral neuropathy, the functional and structural peculiarities of the peripheral nervous system must be considered. Neurones can be extremely long, they vary considerably in diameter, and are involved in close interactions with Schwann cells. Thus, a variety of techniques are necessary to determine the pathological changes which may occur. In this study light microscopy, teased fibre preparations and electron microscopy were used. Quantitative evaluation of these preparations was also performed.

Sampling

Peripheral nerve can be sampled for histological investigation by biopsy or at *post mortem*. A greater amount of tissue is available at autopsy, but the methods suitable for the study of autopsy material are limited. Because of the effects of *post mortem* autolysis the best method of obtaining nerve samples for the detection of fine structural details is by biopsy. Extreme care in the handling of nerve, during collection and preparation is essential, in order to avoid the introduction of artefact. The nerve chosen for investigation should be only moderately involved in the disease process, as if severely affected, little information will be obtained about the pathogenesis of the condition. Similar consideration should be given to the level of sampling of a nerve trunk. It should be remembered that the level of biopsy may alter the resultant nerve fibre diameter distributions, due to the effect of distal tapering of fibres, or extensive branching (Thomas, 1970; Braund & Vandevelde, 1978). The length of nerve taken should be sufficient to enable preparations for light microscopy, electron microscopy and teased fibre studies.

Fixation

Probably the most suitable fixation schedule for peripheral nerve, which enables processing for all of the above mentioned techniques, involves glutaraldehyde and osmium tetroxide. Glutaraldehyde improves the appearance of the axoplasm, particularly the neurofilaments and microtubules, while osmium tetroxide produces satisfactory preservation of the myelin sheath (Lampert, 1967). In nonsurvival situations, fixation by perfusion, or initial *in situ* fixation are the methods of choice, as they minimise handling of the nerve and result in optimal preservation (Thomas, 1970).

Light Microscopy

Embedding the processed tissue in epoxy resins provides material both for examination by light and electron microscopy. During embedding it is essential that specimens are accurately oriented, so true transverse and longitudinal sections can be obtained. With light microscopy of transverse and longitudinal sections, the morphology of myelinated nerve fibres and other components of the nerve can be observed. In addition, the populations of myelinated fibres can be investigated quantitatively. The Schwann cell population may be used as a means of measuring previous nerve damage, and has been said to be more reliable than myelinated fibre population or size distribution, as the latter may return to near normal values, while the Schwann cell population remains elevated (Thomas, 1970). In comparison, nerve that has been fixed in formalin and embedded in paraffin wax, cannot be depended upon to provide a preparation adequate for detailed assessment of nerve fibre changes. In some studies in which both methods have been used, no abnormalities were observed in paraffin embedded nerve, while on examination of resin embedded material, pathological changes were obvious (Ochoa & Mair, 1969a). Silver staining of paraffin embedded sections has been widely used for selective axonal staining, but is now less frequently used because of its lack of specificity, inconsistency of staining, the uncertainty of staining all myelinated fibres and the improved resolution obtained with combined light and electron microscopy of resin embedded material (Dyck, 1975a). On examination of transverse sections of this material by light and electron microscopy, it has been seen that most myelinated fibres are not circular in outline. Experimental work (Karnes *et al*, 1977; Arbuthnott *et al*, 1980), has shown that this shape is likely to represent the *in vivo* shape of nerve fibres, rather than result from changes due to processing.

It is important that changes resulting from artefact are recognised as such, and not interpreted as pathological. The usual reasons for artefactual changes are the crushing of fresh nerve or poor tissue processing. Most crushing damage results from handling the nerve with forceps, or touching it with swabs during its removal. In transverse sections the fascicles appear compressed and angular, and the large myelinated fibres in particular are distorted. Poor processing results in the shrinkage of axons and Schwann cell processes, splitting and disruption of the myelin lamellae, especially in the thicker myelin sheaths.

Teased Fibre Preparations

The process of teasing single nerve fibres has major advantages over other light microscopic techniques in diagnostic power, and in improved understanding of the pathogenesis of disease processes. Descriptive grading of teased fibre appearance, and measurement of internode length provides much information on the specific processes of axonal degeneration and regeneration, and segmental demyelination and remyelination occurring in a nerve, and the activity and severity of these processes (Dayan, 1979). Single teased fibre preparations are the only reliable way of adequately examining lengths of individual nerve fibres, over more than one or two internodes. They allow visualisation of the territory supplied by each Schwann cell, as delineated by the nodes of Ranvier. Even the best oriented longitudinal section seldom shows more than two internodes in the same plane, and so teasing is necessary to study the distribution of axonal or myelin sheath changes along a single nerve fibre (Bennington, 1978). One can define the morphology of nodes of Ranvier, irregularities in the myelin sheath, and quantitative studies can be undertaken on internode length measurements. In addition, it is a much easier and more reliable technique for the detection of segmental demyelination and

remyelination. The use of a grading system to classify the pathological changes observed on teased fibres, has allowed the assessment of the degree of peripheral nerve pathology present (Dyck, 1975a). Provided that the teased fibres are obtained without preselection, this method of assessing the degree of pathology has been said to be the most sensitive indicator of peripheral nerve abnormality (Dyck, 1975b). The classifications used are descriptive, and only those criteria with a small "between observers" difference are used (Fig. 20). It is possible to recognise fibres with areas of paranodal or segmental demyelination, remyelinated or intercalated segments, degenerating fibres, other irregularities of the myelin sheath and in some instances regenerated fibres. Regenerated fibres do not have a separate category in Dyck's classification (1975a), but can be recognised by their inappropriately short uniform internode lengths (Fullerton *et al*, 1965). Sometimes a unique morphological feature may be present, such as the enlargements seen in giant axonal neuropathy of man, and the experimental hexacarbon neuropathies (Bennington, 1978). Again, it is important to recognise artefact and minimise its occurrence by careful preparation. Excessive stretch on a nerve during biopsy or teasing may produce separation of fibres at nodes of Ranvier or Schmidt-Lantermann incisures. It can also lead to a beaded appearance, which could be mistakenly attributed to pathological change. Any abnormalities seen at the ends of teased fibres should be disregarded, as they could result from damage by forceps during teasing (Dyck, 1975a). The incidence of segmental demyelination and remyelination have been shown to be more accurately defined by teased fibre morphological studies than by transverse sectioning, an expected finding in view of the greater ease of detection of these changes in teased fibres (Braund *et al*, 1982a; 1982b). The appearance of a single myelinated fibre with a disproportionately thin myelin sheath in a transverse section of nerve could equally represent a remyelinated or a regenerated nerve fibre.

Only in teased fibre preparations can the distinction between these two changes be made. In regenerated fibres uniformly short internodes will be observed, while with remyelination variations in internode length and myelin sheath thickness over consecutive internodes will be present (Bennington, 1978). In teased fibre preparations, degenerating axons are readily recognised, whereas isolated fibre degeneration can easily be missed in transverse and longitudinal sections. Sometimes the artefact produced in transverse sections, by poor techniques of fixation and cutting resemble the appearance of myelin ovoids and balls. Similarly, a superficial longitudinal section of a fibre may incorrectly give the appearance of linear rows of myelin ovoids and balls (Dyck, 1975a).

Electron Microscopy

Assessment of the fine structural detail of peripheral nerve by electron microscopy, should only be considered in conjunction with a light microscopic survey of resin embedded sections. There are few pathological findings of significance that will be observed ultrastructurally, in the absence of changes at the light microscopic level (Bennington, 1978). A recent study was undertaken to assess the relative advantages of examining peripheral nerve by light or by electron microscopy (Sugimura *et al*, 1980). With light microscopy more extensive sampling was possible, and more fibres could be evaluated, but there were certain structures which could not be observed adequately and measurements that could not be made or were less accurate than at the ultrastructural level. Electron microscopic examination of nerve enables the definition of the ultrastructural components of the nerve, and estimation of the axon to myelin sheath ratio. However, fewer fibres are observed and the risk of biased sampling increased with electron microscopy alone. Many workers have recognised the necessity of employing electron microscopy

in gaining accurate information regarding myelin sheath thickness and axis cylinder size. The estimation of myelin thickness by counting the number of lamellae, avoids error which can be introduced by the presence of separation of myelin lamellae or Schmidt-Lantermann incisures (Bischoff, 1965). While some shrinkage of nerve fibres has been observed at the ultrastructural level, as a result of fixation and subsequent processing of nerve, it has been concluded that no appreciable change in morphology occurs, as all components of nerve fibres are affected to a similar degree (Arbuthnott *et al*, 1980). The preservation of nerve fibres in electron microscopy is considered to be adequate if no splitting of the myelin sheath has occurred, apart from the Schmidt-Lantermann incisures, if the axolemma adheres closely to the inner turns of the myelin sheath, and if the fine structure of mitochondria, neurofilaments and microtubules is preserved. Some splitting of the myelin is likely in the largest fibres with the thickest myelin sheaths (Arbuthnott *et al*, 1980). Unmyelinated nerve fibres may be visible on light microscopy, but can only be examined in detail by electron microscopy. Observation of the fine structure of axonal organelles may provide information linking structural changes of the various subcellular components with underlying biochemical disturbances, as well as providing an extremely sensitive method of detecting minimal pathological change (Dayan, 1979). Again, care must be taken not to mistake damage induced by inadequate care in the collection and processing of the nerve, for true pathological changes.

Morphometry

The use of quantitative histological techniques in the study of peripheral nerve can be extremely helpful in identifying subtle changes early in the course of disease, and in characterising the distinct patterns of nerve fibre

pathology seen with some disorders (Bennington, 1978). The detection of slight decreases in nerve fibre populations can be very difficult without employing quantitative methods. Even the most skilled observer is unlikely to detect changes in nerve fibre populations of less than 20-30% (Dayan, 1979). "Thick" and "thin" transverse sections of resin embedded nerve are the most suitable for accurate quantitative studies. From these studies information is obtained regarding the numbers and size distributions of myelinated fibres; the internode lengths and their variability; and the relationship of the number of myelin lamellae to the perimeter of axis cylinder (Dyck, 1975a). Although active fibre degeneration is easily recognised morphologically, the alterations occurring with incomplete regeneration and repair are most accurately defined quantitatively. Furthermore, the site of degeneration along the nerve fibre may be indicated by quantitative studies.

FIBRE DIAMETER DISTRIBUTIONS

Measurements made from transverse sections of resin embedded nerve provide detailed information on myelinated fibre diameter distributions and their density. For these purposes resin embedded sections are essential to avoid the shrinkage which occurs with paraffin processing. Changes in the fibre diameter distributions are observed in diseased nerve. While alterations in fibre density will indicate the presence and extent of fibre loss with conditions of axonal degeneration, alterations in the fibre spectrum may indicate selective loss of large or small diameter fibres in particular. Interpreting the results of these quantitative studies relies on a knowledge of the normal fibre population for a particular nerve, at a particular level, in an individual of a particular age. It is important when measuring individual fibres to ensure that no fibres are included which are sectioned obliquely,

or show artefactual changes (Dyck *et al*, 1968). Karnes and coworkers (1977) demonstrated that in carefully oriented transverse sections of nerve, the mean diameter calculated from the measurement of circumference was the best among the various estimates of myelinated fibre diameter, and thus was the most suitable method for use in investigations of nerve morphometry. In addition, as a parameter it was found to be less likely to be influenced by artefact introduced by oblique sectioning and other shape deformations, than was diameter (Pullen, 1982). However, Friede & Bischausen (1982) found that basing the estimations on measured noncircular axons, as above, or on estimated circular axons, did not significantly alter the trends observed or their interpretation.

INTERNODE LENGTH

Quantitative studies of teased fibre preparations, involving the measurement of internode lengths and the calculation of their variability, have been found to be a useful means of investigating and defining the nature of the pathological processes observed in peripheral neuropathies (Lascelles & Thomas, 1966). Information is obtained regarding the presence of previous degeneration and regeneration, and of previous demyelination and remyelination in the nerve (Dyck, 1975a). In a normal nerve fibre the internode length is reasonably constant along the fibre (Stevens *et al*, 1973). Axonal degeneration, and subsequent regeneration results in uniformly short internode lengths, on even the largest diameter fibre, and so internode length is decreased (Hiscoe, 1947; Vizoso & Young, 1948). Whereas, with segmental demyelination and remyelination the internode length may be decreased, and the variability of internode length markedly increased, due mainly to the presence of short intercalated segments of varying lengths. Analysis of the distribution of abnormal internodes amongst the nerve fibres, to show either random or nonrandom

distributions, will help differentiate primary segmental demyelination from secondary segmental demyelination, consequent to primary axonal abnormalities (Dyck *et al*, 1971a). Again, it is important to know the normal values for internode length and variation in internode length, and the changes which occur with age.

THE RELATIONSHIP BETWEEN AXONAL SIZE AND MYELIN SHEATH THICKNESS

The ratio of myelin thickness to a measurement of axonal size (e.g. axonal perimeter or cross-sectional area), may provide information regarding the underlying pathogenic mechanism of a disease process. For example, in some primary axonal diseases, shrinkage of the axis cylinder occurs, initiating secondary myelin changes, and is detected by an increase in this ratio (Dyck *et al*, 1971a). Accurate estimation of the thickness of the myelin sheath is obtained by counting the number of myelin lamellae on electron micrographs. Because the profile of the nerve fibres can be quite irregular, measurement of the axon perimeter is more accurate than estimation of the diameter (Friede & Samorajski, 1967).

An in-depth investigation of peripheral nerve, as detailed above, may not allow specific definition of the pathological process in question. Nevertheless, significant gains in understanding of the disease may be achieved. Adequate investigation of peripheral nerve should provide accurate information on the presence and severity of a peripheral neuropathy; on the type of fibre degeneration; on specific histological abnormalities; and possible pathogenic mechanisms (Dyck, 1975b). Disadvantages in employing these techniques in the study of peripheral nerve pathology do exist. As with all histological techniques there can be considerable sampling error. While there may be a diversity of functional disturbances at the cellular level, the tissue

reacts in only a limited number of ways, and thus specific diagnosis can not be made. The presence of age related changes, incidental lesions and a range of normal variation in individuals may make the interpretation of results difficult. In addition, while these techniques do provide a considerable amount of reliable information, an enormous input is required, particularly if a large number of nerve samples are examined.

2. MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The 15 horses used in this study were donated to the Massey University Large Animal Hospital for euthenasia. Of these animals six were normal , five were subclinical cases of laryngeal hemiplegia and four were clinically affected. Control animals were those which showed no clinical signs of laryngeal hemiplegia, no endoscopic evidence of left sided laryngeal assymetry or asynchrony, and no or negligible evidence of pathology in the intrinsic laryngeal muscles or recurrent laryngeal nerves. The subclinical cases were diagnosed after finding neurogenic atrophy on microscopic examination of the laryngeal muscles, and evidence of obvious pathological change in the left recurrent laryngeal nerve. Endoscopic examination of the subclinical group demonstrated a degree of left arytenoid assymetry and/or asynchrony in two of these horses, while the larynges of the three remaining subclinical cases appeared to be functioning normally. The clinical group consisted of those horses which made an abnormally loud inspiratory noise at exercise, and showed left sided laryngeal paralysis endoscopically. All of the horses were entire or gelded males, and were Thoroughbreds, with the exception of horse 2, a Standardbred.

TABLE 1: EXPERIMENTAL ANIMALS

DISEASE STATUS	CASE NUMBER	AGE (years)	HEIGHT (cm)
CONTROL	1*	0+	104
	2*	1	143.5
	3*	1	-
	4*	1	146.5
	5*	2	-
	6	3	163.5
SUBCLINICAL	7	2	161
	8	>7	156
	9	9	-
	10	10	168
	11	12	160
CLINICAL	12*	2	157.5
	13	8	163
	14	9	170
	15	22	162.5

- Measurement not obtained
* Entire male
+ This individual was 1 day old

SAMPLING METHOD FOR RECURRENT LARYNGEAL AND VAGAL NERVES

Pieces of nerve of 1.5 to 3 cm in length were collected from the recurrent laryngeal nerves at three sites. These were:

(i) Immediately caudal to the larynx (distal sample)

(ii) At the estimated midpoint of the nerve (midcervical sample)

(iii) At 2-4 cm distal to its separation from the vagal nerve (proximal sample).

The midpoint of the nerve was estimated using umbilical tape, to trace externally the route of the recurrent laryngeal nerve from the larynx to its point of division from the vagal nerve. The length of tape was then halved, and the level marked on the skin.

Samples of the vagal nerve were also collected from each horse, from the following sites:

(i) At the level of the midpoint of the recurrent laryngeal nerve.

(ii) Immediately proximal to the separation of the recurrent laryngeal nerve from the vagus.

All of the above sites are illustrated in Fig. 15.

The intrathoracic sections of nerve were collected immediately *post mortem* . The remainder of the samples were removed surgically from the anaesthetised animal. Acetyl promazine[1] was used for premedication at a dose rate of 25mg/400kg. This was followed 15-30 minutes later by anaesthetic induction, using glycerol guaiacolate[2] at 50g/400kg and thiopentone sodium[3] at 2g/400kg. Anaesthesia was maintained with an oxygen and halothane mixture, delivered through a circle system.

The animal was placed in right lateral recumbancy to enable collection of the cervical left recurrent laryngeal nerve and vagal nerve sections. The same procedure was repeated with the animal in left lateral recumbancy, and the corresponding right recurrent laryngeal nerve and vagal nerve samples were obtained.

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1. Acetyl promazine injection, active ingredient acepromazine maleate. The Boots Co. Ltd, Nottingham, England.

2. Equinesin, active ingredient guaiaphenesin. South Island Chemicals Ltd, Christchurch, New Zealand.

3. Intraval sodium, active ingredient thiopentone sodium. May & Baker Ltd, Dagenham, England.

For sampling of the nerve at the level of the larynx a skin incision was made parallel and ventral to the linguofacial vein, directly over the lateral surface of the larynx. Dissection was continued medially between the fascia surrounding the vein and the omohyoid muscle, haemostasis being achieved by means of electrocautery. This dissection exposed the thyroid gland with its associated blood vessels, and the caudal region of the larynx (Marks *et al*, 1970b). The thyrolaryngeal artery and the laryngeal and cranial thyroid veins run cranially from the thyroid gland towards the caudal border of the dorsal cricoarytenoid muscle. The recurrent laryngeal nerve was almost invariably found associated with these vessels, usually on their medial side in loose connective tissue (Fig. 16).

Surgical exposure of the recurrent laryngeal and vagus nerves in the midcervical region was performed through a 10 cm incision immediately dorsal to the jugular furrow. A plane of dissection was established between the jugular vein and the omohyoid muscle, exposing the ventral border of this muscle. Directly underlying the ventral region of the muscle was the carotid artery, with its associated nerve trunks. Incision of the omohyoid muscle, and then of the carotid sheath revealed the carotid artery, with the vagal nerve lying on its dorsomedial surface. The recurrent laryngeal nerve was found on or slightly below the ventral surface of the artery, in loose connective tissue. The exact position of the nerve varied slightly between individuals (Fig. 17).

Once the nerve was identified, fine forceps and iris scissors[1] were used to expose it. Care was taken not to touch the nerve at any stage or to interfere with its blood supply. Once sufficient surrounding tissue had

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1. Suture and dissecting forceps, 9 cm, serrated ends, stainless steel. Straight iris scissors, sharp points, 11 cm, stainless steel. C.W. Dixey & Son Ltd, London, England.

been dissected away, *in situ* fixation of the nerve was carried out, using 2% glutaraldehyde[1]. This ensured a minimum of artefact, the nerve still possessing an intact blood supply at the time of fixation, while being held at its normal resting length. After 3 minutes of *in situ* fixation, sharp dissection including as little surrounding tissue as possible, was employed to remove a 1.5-3 cm length of the nerve (Dyck & Lofgren, 1966).

Once the appropriate samples had been taken from the anaesthetised animal, a deeper plane of anaesthesia was obtained using the oxygen/halothane mixture. The animal was then exsanguinated by severing the carotid arteries at the level of the third or fourth tracheal ring. The left forelimb and the first five or six ribs were removed immediately after death, allowing access to the intrathoracic course of the recurrent laryngeal and vagal nerves. The recurrent laryngeal nerve was sampled as it curved around the aortic arch, and a 1.5-3 cm length of the vagal nerve was collected immediately proximal to the separation of the recurrent laryngeal nerve. *In situ* fixation was not practical at this level because of the vast volumes of fixative that would have been required to cover the nerves. Instead, a method of weighted fixation was used, to ensure straightening of the nerve fibres within the nerve trunk, during the initial period of fixation. Hooked aluminium weights were used, 200 mg for recurrent laryngeal nerves, and 500 mg for vagal nerves. Once the nerve was exposed, a 1.5 metric nylon suture was placed around the nerve trunk, using an attached atraumatic needle. A hooked aluminium weight was then placed 2-3 cm distal to the suture (Fig. 18). Placing gentle traction on the suture, and using fine, sharp scissors the length of nerve was dissected out, and suspended immediately in the fixative (Dyck & Lofgren, 1966). The sampling procedure was repeated on the right

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1. Appendix 1.

side after removal of the forelimb and the first two ribs. The recurrent laryngeal nerve was sampled as it curved around the costocervical or subclavian arteries, and the vagal nerve sampled proximal to separation of the recurrent laryngeal nerve. The samples collected *post mortem* were then processed in the same way as those taken from the anaesthetised animal. Intralaryngeal nerve samples were removed immediately *post mortem* , from two additional animals. One was a 145 cm, 1 year old, male Standardbred, and the other was a 147.5 cm, 3 year old, male Standardbred. The branch of the nerve innervating the *cricoarytenoideus dorsalis* was identified and removed on both left and right sides, as were the branches to the *cricoarytenoideus lateralis* muscles.

After 3 minutes of fixation the nerve was divided into two parts, one for processing for light and electron microscopy, and the other for teased fibre preparations. The nerve was placed on a sheet of dental wax[1] and cut with a new razor blade[2]. This technique ensured minimum crush artefact to individual nerve fibres. At least a 1 cm length was taken for teased fibre studies, and the remainder used for microscopic examination after embedding in polarbed or epon resin.

PROCESSING FOR TEASED FIBRE PREPARATIONS
(Katrak *et al*, 1980; Nukada *et al*, 1981).

The recurrent laryngeal and vagal nerves were fixed in 2% glutaraldehyde for 10 and 12 minutes respectively. This time included the initial 3 minutes of *in situ* fixation. The samples were then transferred to 0.1M

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1. Ash, high stability modelling wax, Amalgamated Dental Trade Distributors Ltd, London, England.

2. Gem Scientific, duridium style, single edge razor blades.

cacodylate buffer for at least 30 minutes, until the specimens for light and electron microscopy were ready for the next stage of processing. Connective tissue attached to the nerve trunk was dissected away using fine forceps, during the period of this buffer wash. The next stage of processing involved 90 minutes of immersion in 1% osmium tetroxide (Appendix 1). Initially the sample bottle was shaken vigorously to aid penetration of the osmium, and then placed on a mixer, in a fume hood. A repeat wash of 30 minutes in 0.1M cacodylate buffer was carried out, with a change of buffer after 15 minutes, to ensure elimination of all of the osmium residue. The nerves were held in 66% glycerol overnight at a temperature of 60^{o}C. After being transferred to 100% glycerol the samples were stored in a refrigerator until required.

TEASED FIBRE PREPARATIONS

Teasing of nerve fibres was carried out on a glass microscope slide, under a dissecting microscope [1], at a magnification of 40x, using curved forceps[2] and a fine pointed needle when necessary. With the length of nerve in a drop of glycerol, the epineurium was stripped off, thus separating the nerve trunk into fascicles. The perineurium was then removed, exposing the nerve fibres and endoneurium. The fascicles were separated into small strands of nerve fibres, which were divided repeatedly until individual fibres were isolated. If the fibre contained less than five nodes of Ranvier along its length, it was discarded. The fibre was transferred to a clean glass slide, laid edge to edge with the one being used for teasing. The fibre was grasped at one end and slid along the slide, through a small drop of glycerine at

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1. Olympus dissecting microscope, Tokyo, Japan.
2. Number 7 forceps, 4.5 inch, curved, fine pointed. Dumoxel, A. Dumont & Sons.

the edge, and onto the next slide. Once in the centre of the clean slide it was released (Dyck, 1975a). Ten such fibres were placed on each slide for grading and measuring (Fig. 19). To dry off excess glycerine, the slides were kept at 60°C for 24-48 hours and then mounted in glycerine jelly.

One hundred teased fibres were obtained from each nerve sample, without preselection and in approximately equal numbers from all fascicles. The single fibres were graded descriptively according to the system described by Dyck (1975a), and depicted in Fig. 20.

Internode lengths were measured using an ocular micrometer. For each teased fibre the mean internode length, and coefficient of variation of internode length were calculated. Both the length calculations and grading of internodes were compared in terms of total nerve fibres and total internodes. The "one-sample runs test" was used to determine whether abnormal internodes were randomly distributed or occurred in groups along particular nerve fibres (Siegel, 1956).

In the teased fibre statistical analyses, comparisons of mean internode length were made on the basis of the disease status of the animal, the side of origin of the nerve sample, and the level of the sample of recurrent laryngeal nerve. The significance of differences ($P < 0.1$) was determined by the "Kolmogorov-Smirnov" test. Teased fibres from the 1 day old foal were not included in this analysis. As well as overall comparisons, the analyses were performed separately for small and large nerve fibres. For the distal nerve samples a small fibre was arbitrarily defined as having a mean internode length of less than 800 µm, and a large fibre as having a mean internode length of 800 µm or more. At the proximal level of nerve sampling the breakpoint between small and large fibres was defined as 975 µm.

PROCESSING FOR LIGHT AND ELECTRON MICROSCOPY
(Katrak *et al*, 1980; Nukada *et al*, 1981)

The nerve samples were further fixed in 2% glutaraldehyde for 90 minutes, and then transferred to 0.1M cacodylate buffer for 30 minutes. During this latter period the specimen was divided into pieces approximately 1-2 mm long. The nerve was placed on a sheet of dental wax in a pool of buffer and examined under a dissecting microscope, at a magnification of 40x. Adherent connective tissue was removed and a cross-section of the entire nerve trunk taken for subsequent transverse sectioning. The remainder of the sample was divided into fascicles or groups of fascicles, to be cut into 1-2 mm cubes. To preserve orientation of the nerve fibres, the length of fascicle taken was slightly greater than its width. Identification of the long axis of the nerve was therefore facilitated, when embedding the tissue blocks in resin. The samples were then transferred to 1% osmium tetroxide for 90 minutes. The sample bottle was shaken vigorously by hand for 10 seconds, to aid penetration of the osmium tetroxide into the specimen. The next step in the processing schedule was a 30 minute wash in cacodylate buffer, with a change of buffer during the 30 minute period. An *en bloc* method of staining was used, involving 30 minutes in a 1% phenylenediamine solution, freshly prepared for each set of samples processed. After staining, the tissue samples were dehydrated in a graded series of alcohols, for a period of 10 minutes in each of 80%, 95%, 100%, and a second 100% alcohol. This was followed by two 10 minute periods in propylene oxide [1], and then overnight immersion in a 50:50 mixture of propylene oxide and polarbed [2].

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1. Propylene oxide, BDH Chemicals Ltd, Poole, England.
2. Appendix 2.

The following morning the residual polarbed was poured off and fresh 100% polarbed added, to cover the specimens, for 60 minutes. The samples were embedded in flat rubber moulds[1] in epon or polarbed resin. Several blocks were embedded for each sampling level, including a transverse section of the whole nerve trunk, several fascicles transversely oriented and one longitudinally. Orientation was checked under a dissecting microscope. The moulds were then placed in an oven at 60°C for polymerisation of the resin to occur. After 120 minutes, when the resin was sufficiently viscous to prevent drifting of the specimens, the orientation was checked and corrected if necessary. The resin was left to cure at 60°C for 48 hours.

PREPARATION OF SECTIONS FOR LIGHT MICROSCOPY

"Thick" sections for light microscopy were cut[2] at 1.5 µm using a glass knife, and were not stained before mounting, as phenylenediamine had been incorporated into the processing schedule. These sections were examined under a light microscope in order to detect any morphological changes, and to measure the myelinated fibre populations present at each level of the nerve.

Using a prism projection microscope, tracings of the transverse nerve sections were made on to paper, in order to estimate the density of myelinated fibres per mm^2 of transverse fascicular area. Transverse sections of the whole nerve were traced initially, at low magnification (x100 or x250), showing fascicular outlines. This was followed by high power projection (x650 magnification), to enable measurement of individual nerve fibres. Fibres from several fascicles were traced at the higher

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1. Silicone rubber mould, Polaron Equipment Ltd, Watford, England.
2. LKB 8806A Ultratome 3, LKB, Bromma 1, Sweden.

magnification. Fibre diameters and fascicular areas were calculated from the tracings with the use of a digitiser[1]. Nerve fibres not measured because of artefact were included in the estimation of the total number of fibres per mm^2 of fascicular area. Diameter histograms were then plotted.

The diameter distributions of myelinated nerve fibres, for the various sample sites in affected and unaffected animals were compared using the Kolmogorov-Smirnov "two sample" test (Katrak *et al*, 1980). This test is based on the agreement between the two cumulative frequency distributions, with the statistic being a function of the maximum difference between the two distributions, over the common diameter intervals measured. Small (≤ 9.5 µm) and large (> 9.5 µm) diameter fibres were considered separately.

ELECTRON MICROSCOPY

After viewing the "thick" sections by light microscopy, the area of the block to be used for "thin" sectioning was chosen. Silver to grey ribbons were cut, using a diamond knife, and transferred to unsupported copper grids[2]. These were then stained with uranyl acetate and lead citrate[3].

The "thin" sections were examined on a Philips 200 electron microscope, in order to detect any morphological changes. Electron micrographs were taken of a number of fibres (up to 75 per sample), as encountered serially in X and Y traverses of the grid, to ensure that no fibre was

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1. Hipad TM Digitiser, Houston Instrument, Austin, Texas.
2. Square 400 mesh, unsupported copper grids, Polaron Equipment Ltd, Watford, Englad.
3. Appendix 3.

included more than once. The axis cylinder perimeter was measured from the micrograph in μm, using programmed digitisation and calculation. Major dense lines, indicating the number of myelin lamellae were counted from the micrograph for each fibre, under a dissecting microscope. The regression analysis of the axis cylinder perimeter (logarithm to base e) and the number of myelin lamellae was then determined.

3. RESULTS

LIGHT MICROSCOPY

Morphology

The complete record of observations made by light microscopy of each site of both laryngeal nerves from the 15 horses, is to be found in Appendix 6.

Two basic forms of pathological change were observed.

(i) Loss of myelinated fibres

This was divided into four categories; no loss (-); a slight loss (+); a mild loss(++); and a severe loss (+++), as illustrated in Figs. 22 and 23 and tabulated below (Table 2). There was a corresponding graded increase in endoneurial nuclei.

(ii) Individual fibre pathology

These were myelinated fibres with disproportionately thin myelin sheaths, regenerating clusters, "onion bulb" formations and myelinated fibres with myelin debris in their Schwann cell cytoplasm. These structures were observed to be rare (+, 1 or 2 in a transverse section of a nerve); occasional (++,

1 or 2 per fascicle); moderate (+++, 5-10 per fascicle); and numerous (++++, more than 10 per fascicle).

TABLE 2: MYELINATED FIBRE LOSS IN EQUINE RECURRENT LARYNGEAL NERVE

NERVE LEVEL	GROUPS OF HORSES					
	SUBCLINICAL			CLINICAL		
	DEGREE LOSS	NO. AFFECTED	NO. EXAMINED	DEGREE LOSS	NO. AFFECTED	NO. EXAMINED
L distal	++	5	5	+++	4	4
L midcervical	+	3	5	++	3	3
L proximal	-	0	5	-	0	4
Rt distal	+	2	5	+	3	4
Rt midcervical	-	0	5	+	1	4
Rt proximal	-	0	5	-	0	4

The loss of myelinated fibres was most severe in the distal left recurrent laryngeal nerve of the clinical group, with progressive decrease in severity in a proximal direction (Fig. 22). In fact, at the proximal level of this nerve no loss of fibres was apparent. This same gradation in the loss of myelinated fibres was present in the subclinical group, although less severe than the clinical. Similarly, in the right recurrent laryngeal nerve, a distally distributed loss of fibres was apparent in the clinical and subclinical groups, but was considerably less marked than on the left side (Fig. 23).

No loss of myelinated fibres was observed in the limb nerves from the two clinical horses studied. Nor was a loss apparent in any of the samples from the control group of horses.

The frequency of individual nerve fibres with pathology, which included thinly myelinated fibres, regenerating clusters, "onion bulbs" and myelin debris, is recorded in Table 3, and illustrated in the recurrent laryngeal nerves in Figs. 24-28, and in hindlimb nerves, in Figs. 29-33.

TABLE 3: THE FREQUENCY OF INDIVIDUAL NERVE FIBRE CHANGES IN EQUINE RECURRENT LARYNGEAL AND LIMB NERVES

NERVE	GROUPS OF HORSES		
	CONTROL	SUBCLINICAL	CLINICAL
LARYNGEAL			
L distal	++	+++	++
L midcervical	++	+++	++++
L proximal	++	++++	++ to ++++
L vagus 1*	-	++	++ to ++++
L vagus 2**	-	++	+++
Rt distal	+ to ++	++	++ to ++++
Rt midcervical	+	++ to ++++	++ to ++++
Rt proximal	+	+ to ++	++ to +++
Rt vagus 1	-	- to +	+ to ++
Rt vagus 2	+	-	-
LIMB			
Extensor digitorum longus branch			++ to +++
superficial peroneal			++ to +++
common peroneal			+ to ++
tibial			++ to +++
median			++

* Vagal nerve proximal to separation of the recurrent laryngeal nerve.

** Vagal nerve at the midcervical level.

The proximal to distal increase in severity of pathology, as seen with fibre loss, was also observed in the frequency of these pathological changes. One exception to this trend was the distal left recurrent laryngeal nerve of the clinical group. Few myelinated fibres remained in this nerve to take part in these changes. A slight proximal to distal increase in severity of pathology was also observed along the peroneal nerve.

A number of other changes were observed which indicated active isolated fibre pathology. Fibres with split myelin sheaths, usually containing dense granular material in the split (Fig. 34), were seen rarely in the subclinical and control left distal, subclinical and clinical right distal, and subclinical right midcervical recurrent laryngeal nerves. Swollen fibres with dense axoplasm and attenuated myelin sheaths (Figs. 35 and 36) were observed in all groups in the left distal nerve, in the clinical left midcervical nerve, clinical right distal nerve, clinical and subclinical right midcervical nerve, subclinical right proximal recurrent laryngeal nerve, and the clinical vagal nerve proximal to separation of the recurrent laryngeal. Often these swollen fibres were at the centre of "onion bulb" formations. Collapsed myelin sheaths occupying Schwann cells, were seen rarely in the subclinical left distal recurrent laryngeal nerve and clinical left vagus proximal to separation of the recurrent laryngeal nerve.

Renaut bodies were present at each level of the recurrent laryngeal nerve, in some members of each group. Within a group, at a particular level there was usually considerable variation in their number and form. There did not appear to be any correlation between the level of sampling and the extent of these structures, nor between the severity of pathology observed and their development.

Renaut bodies were observed in the 1 day old foal (Fig. 37). In one instance a Renaut body, not in a subperineurial position was seen (Fig. 38).

Transverse sections of the right midcervical recurrent laryngeal nerve of the 1 day old foal (Fig. 39), and of the right proximal nerve sample of a clinical animal were ribbon-like in appearance (Haslam's anomaly), being only one fascicle in width.

Morphometry

DENSITY OF MYELINATED NERVE FIBRES IN EQUINE RECURRENT LARYNGEAL NERVE

The density of myelinated fibres in the left and right recurrent laryngeal nerves of the control, subclinical and clinical laryngeal hemiplegic animals, at the three sampling levels investigated, are recorded in Table 4.

TABLE 4: DENSITY OF MYELINATED FIBRES (NO./MM^2) IN EQUINE RECURRENT LARYNGEAL NERVE

NERVE LEVEL	GROUPS OF HORSES					
	CONTROL		SUBCLINICAL		CLINICAL	
	DENSITY*	S.D.**	DENSITY	S.D.	DENSITY	S.D.
L distal	2664	85	1644	78	695	37
L midcervical	2558	161	2224	133	1520	80
L proximal	2575	63	2097	97	1952	48
Rt distal	2940	105	1859	78	1658	37
Rt midcervical	2657	145	2066	56	1748	50
Rt proximal	2944	127	1789	81	2038	34

* Mean fibre density
** Standard deviation

Comparisons of the control with the two affected groups, at each particular sampling site, revealed a decrease in fibre density associated with disease. These differences were examined by the students t test, and in all instances proved to be significant at the 1% level. The loss of fibres was most severe in the distal left recurrent laryngeal nerve of the clinical laryngeal hemiplegic animals. A gradation in the extent of the decrease in fibre density, from the control through subclinical to clinical groups, was apparent at all sampling sites, with the exception of the proximal right recurrent laryngeal nerve.

There was a proximal to distal decrease in myelinated fibre density within the two diseased groups, with the exception of the left and right proximal levels of the subclinical group.

Comparison of the left and right samples of corresponding levels, within the clinical group, indicated a more severe loss of fibres on the left side, significant at the 2% level.

SIZE OF MYELINATED NERVE FIBRES IN EQUINE RECURRENT LARYNGEAL NERVE

Fibre diameter distributions in equine recurrent laryngeal nerve

The distributions of the populations of myelinated fibres in the recurrent laryngeal nerves of control, subclinical and clinical laryngeal hemiplegic horses, are presented as histograms in Figs. 40-42. The mean fibre diameters and their standard deviations are recorded in Table 5.

TABLE 5: MEAN MYELINATED FIBRE DIAMETER (μm) IN EQUINE RECURRENT LARYNGEAL NERVE

NERVE LEVEL	GROUPS OF HORSES					
	CONTROL		SUBCLINICAL		CLINICAL	
	FIBRE DIAMETER	S.D.	FIBRE DIAMETER	S.D.	FIBRE DIAMETER	S.D.
L distal	8.4	1.4	7.4	1.9	4.4	2.0
L midcervical	8.8	1.5	7.9	1.6	6.3	1.9
L proximal	8.6	1.4	9.2	1.7	9.2	1.7
Rt distal	8.5	1.9	9.5	1.7	9.2	2.0
Rt midcervical	8.5	1.7	9.7	1.4	9.5	2.1
Rt proximal	8.7	1.9	9.6	1.9	9.3	1.8

Histograms of the recurrent laryngeal nerves from the control horses, in all instances, contained a major peak of fibres at 8-11 μm, which constituted the majority of the population of nerve fibres. In addition, there was a considerably smaller peak at 2.0-4.5 μm, which was variable in height, and appeared to be more obvious at proximal levels of the nerve.

Comparison of the fibre diameter histograms of the distal left recurrent laryngeal nerves of control animals with the clinicals, showed a marked shift to smaller fibre diameters in the latter group, with almost complete disappearance of the large fibre peak. There has been a loss of large diameter fibres, and a corresponding relative increase in small diameter fibres. The subclinical animals also showed a shift to smaller fibre diameters, although less severe than that of the clinical group. The selective loss of large diameter fibres was reflected by the decreased mean fibre diameter in the subclinical and clinical groups at this level (Table 5), as was the more extensive involvement of the clinical animals. These differences were significant at the 1% level.

At the midcervical level of the left recurrent laryngeal nerve, the loss of large fibres was apparent, although less so than at the distal level, in both clinical and subclinical groups. Again, this, and the greater involvement of the clinical animals was reflected by the decrease in the mean fibre diameters, significant at the 1% level.

At the proximal level of the left recurrent laryngeal nerve, no loss of large myelinated fibres was apparent in the subclinical and clinical groups. In fact, the major peak of large diameter fibres was shifted to slightly greater diameters in the affected groups when compared with the controls. The mean myelinated fibre diameters of the latter groups were correspondingly higher. The same difference was observed in the control group relative to the diseased groups at all three levels of the right recurrent laryngeal nerve.

Comparisons of fibre distributions of distal left with distal right recurrent laryngeal nerves showed a marked shift to smaller fibre diameters on the left in both clinical and subclinical groups, although less marked in the subclinical animals. This was reflected by the correspondingly lower mean fibre diameter on the left. A similar, although less obvious change on the left side was also seen at the midcervical levels of these two groups. At the proximal level the fibre diameter distributions on the left side were almost identical to those on the right.

Comparisons of the histograms of the left recurrent laryngeal nerves of the clinical animals, at the three sampling levels, showed the selective loss of large myelinated fibres to be progressively more severe in a proximal to distal direction. This was also true for the subclinical animals, although differences were not as obvious. A similar, but considerably less extensive

distally graded shift to smaller fibre diameters was observed in the right recurrent laryngeal nerve, in both diseased groups.

Comparison of the fibre diameter distributions of the left and right sides, and at all levels of the recurrent laryngeal nerves of the control group, did not reveal any noticeable differences.

Fibre diameter distributions in intralaryngeal recurrent laryngeal nerve

The mean myelinated fibre diameters and standard deviations of the intralaryngeal adductor and abductor branches of the left and right recurrent laryngeal nerves are shown in Table 6, and their distributions in fig. 43.

TABLE 6: MEAN MYELINATED FIBRE DIAMETER (µm) IN INTRALARYNGEAL BRANCHES OF THE EQUINE RECURRENT LARYNGEAL NERVE

NERVE	SIDE	MEAN FIBRE DIAMETER	STANDARD DEVIATION
adductor branch	L	7.5	1.8
	Rt	10.1	1.7
abductor branch	L	9.4	1.6
	Rt	10.1	1.1

With the exception of the branch of the recurrent laryngeal nerve innervating the left *cricoarytenoideus lateralis* muscle, an adductor, the fibre diameter distributions appeared similar to those of the whole recurrent laryngeal nerves. However, the presence of the small peak at 2-4 µm was obvious only in the branch to the left *cricoarytenoideus dorsalis*, the abductor muscle. The histogram for the branch innervating the left *cricoarytenoideus lateralis*

muscle, showed changes characteristic of the disease process of laryngeal hemiplegia. That is, there was a shift to smaller diameter fibres. However, when the distributions of the right adductor and abductor branches were compared, there was a shift to slightly smaller diameters in the major peak of the latter branch. That is, the branch of the recurrent laryngeal nerve innervating the *cricoarytenoideus lateralis* muscle contained relatively more large fibres than the branch innervating the *cricoarytenoideus dorsalis* . This difference in the two distributions was significant at the 1% level in the large fibre population.

TEASED FIBRE PREPARATIONS

Morphology

The percentage of abnormal teased myelinated fibres in the recurrent laryngeal nerves from control, subclinical and clinical laryngeal hemiplegic horses are presented in Appendix 8, and summarised in Table 7.

TABLE 7: PERCENTAGE OF ABNORMAL TEASED MYELINATED FIBRES IN EQUINE RECURRENT LARYNGEAL NERVES

NERVE LEVEL	GROUPS OF HORSES					
	CONTROL		SUBCLINICAL		CLINICAL	
	MEAN %	RANGE	MEAN %	RANGE	MEAN %	RANGE
L distal	4.4	(1-8)	51.8	(24-72)	61.8	(43-83)
L midcervical	2	(1-3)	21	(4-35)	49.7	(42-56)
L proximal	0.8	(0-3)	11.5	(0-30)	8.8	(2-24)
Rt distal	2.4	(0-5)	6.6	(0-16)	14.3	(7-27)
Rt midcervical	1	(0-2)	4.8	(1- 9)	7.2	(3-14)
Rt proximal	1.6	(0-4)	7.8	(1-19)	11.5	(0-30)

Three major trends are apparent.

(i) The percentage of abnormal fibres is considerably higher in the subclinical and clinical groups than is the controls. Indeed, the subclinical group appears to be intermediate between the control and clinical groups, with the exception of the left proximal recurrent laryngeal nerve level.

(ii) The level of the nerve sample also appears to influence the percentage of abnormal fibres. These increase from the proximal through midcervical to the distal level of the left recurrent laryngeal in all three groups of horses. While this distal increase in severity of pathology is most obvious in the clinical and subclinical groups, it is also present in the controls.

(iii) There is a difference in the percentage of abnormal teased fibres between the left and right recurrent laryngeal nerves. This is most obvious when comparing the left and right distal and midcervical samples of the subclinical and clinical groups.

The percentage of abnormal fibres in limb nerves from two of the clinical laryngeal hemiplegic horses varied from 8% in the tibial nerve, to 18% in the deep peroneal nerve, with a mean value of 13.3%. Within the peroneal nerve there appears to be a distal increase in the percentage of abnormalities, from the proximal level of the common peroneal (12%) to the superficial (15%) and deep peroneal (18%) nerves. This degree of abnormality approximates that of the proximal left and right recurrent laryngeal nerve samples of the clinical group.

The percentage of the different teased fibre abnormalities or grades (after Dyck, 1975a), are shown in Table 8, and the different types are depicted in Figs. 44-53.

TABLE 8: GRADES (after Dyck, 1975a) OF ABNORMAL FIBRES IN EQUINE RECURRENT LARYNGEAL AND LIMB NERVES

NERVE LEVEL	ABNORMAL FIBRE TYPES (mean %)						
	B	C	D	E	F	G	I
CONTROL							
left distal		1.0	0.4	0.8	2.0		0.2
midcervical	0.3	0.3		0.5	1.0		
proximal				0.6	0.2		
right distal			0.4	0.4	1.6		
midcervical		0.2			0.8		
proximal		0.4			1.2		
TOTAL	0.03	0.31	0.14	0.38	1.14		0.03
SUBCLINICAL							
left distal	0.4	3.8	9.2	7.4	30.2	0.8	
midcervical	0.4	1.8	2.0	5.2	11.0	0.2	0.4
proximal	0.3			3.3	2.3	0.8	
right distal		1.0	0.4	0.6	4.2	0.4	
midcervical			0.2	1.2	3.4		
proximal		0.2	0.8	0.8	6.0		
TOTAL	0.17	1.17	2.17	3.07	10.45	3.45	0.07
CLINICAL							
left distal		2.0	5.0	1.3	53.0	0.8	
midcervical		13.0	3.0	1.0	42.7	1.7	
proximal		0.5	0.3	0.5	7.3	0.3	
right distal		1.0		1.5	9.5	2.3	
midcervical		1.0		1.5	8.0	0.5	
proximal				1.0	8.5		
TOTAL		0.96	1.30	1.13	20.57	0.87	
LIMB NERVES							
median	3.0	1.0			4.0	8.0	
deep peroneal	1.5	1.0	0.5	1.0	5.5	3.5	
superf. peroneal		3.0		3.0	9.0		
common peroneal					10.0	2.0	
tibial					5.0	3.0	
TOTAL	1.0	1.0	0.17	0.83	6.50	3.33	

In all groups of horses the predominant abnormality observed was that of short intercalated internodes (F). However, many more of these internodes were present in the clinical (20x) and subclinical (10x) groups than in the controls.

The control group contained few abnormal fibres. As well as remyelinated internodes (F), axonal degeneration (E) and demyelination (C), were observed, and were more common than fibres with both demyelination and remyelination (D).

In the subclinical group, degenerating fibres (E) and those with focal myelin thickenings (G) were approximately one third as common as intercalated internodes. Less frequently seen were fibres with areas of demyelination and remyelination (D), and those with demyelination alone (C).

In the clinical group, demyelination (C), demyelination and remyelination (D), degeneration (E) and focal thickening of myelin (G) were all present, far less frequently than those with remyelinated internodes (F). These former abnormalities were relatively less frequent in the clinical than the subclinical group. Also in this group, while intercalated internodes were the predominant change, the relative levels of the other grades were not constant between the different sampling levels. In the left distal nerve sample, 10 times as many F fibres were seen than the next most common change, demyelination and remyelination (D). Whereas, in the midcervical nerve sample, fibres with demyelination (C) were almost one third as common as those with remyelination (F).

The relative extent of different teased fibre abnormalities also differed between the various sampling levels of the clinical and subclinical animals. In the subclinical group, as in the clinical, the most commonly observed

change was that of intercalated internodes (F). However, the relative frequencies of the other grades was greater in these horses. This was particularly apparent in the left distal and midcervical samples. The major difference between the subclinical and clinical groups lay in the higher proportion of axonal degeneration (E) in the latter. In addition, the amount of demyelination and remyelination (C and D) in the left distal nerve of the subclinical animals, was noticeably greater than the same level of the clinical group.

Morphometry

Detailed statistics for the internode lengths of the recurrent laryngeal nerves of control, subclinical and clinical laryngeal hemiplegic horses are presented in Appendix 9. A summary of the internode length statistics from the total fibre population, which illustrated the trends present in all fibre and internode analyses, is presented below (Table 9).

TABLE 9: MEAN INTERNODE LENGTH AND VARIATION IN EQUINE RECURRENT LARYNGEAL NERVE

NERVE	CONTROL		SUBCLINICAL		CLINICAL	
	meanIL* (µm)	CV+ (%)	meanIL (µm)	CV (%)	meanIL (µm)	CV (%)
left distal	849	9.3	640	28.4	577	30.8
proximal	968	7.1	928	9.4	1001	10.5
right distal	819	8.3	868	10.7	819	12.6
proximal	973	7.8	997	10.6	930	11.3

* Internode length

\+ Coefficient of variation

The mean internode lengths of each nerve sample from the left and right recurrent laryngeal nerves of the control group was compared with each of the diseased groups, using the Kolmogorov-Smirnov test (Appendix 9). Differences in the mean internode length, significant to the 5% level were as follows:

(i) The internode length decreased in a proximal to distal direction in all three groups.

(ii) In the control horses, the mean internode length of the distal left nerve was greater than that of the right.

(iii) In contrast, in the subclinical and clinical groups, the mean internode length of the left distal nerve was considerably less, and the coefficient of variation higher, than on the right.

(iv) The mean internode length of the distal left nerve of the control group was greater than that of the subclinicals, which was greater than that of the clinicals. There was a corresponding increase in the coefficient of variation from the control, through subclinical to clinical groups. Although the internode lengths of the proximal left, and both levels of the right recurrent laryngeal nerve did not differ significantly, the coefficient of variation did increase in the affected groups.

The distribution of abnormal internodes on the teased fibres was evaluated by a "one-sample runs" test, and a probability that abnormal internodes were distributed randomly obtained. A 1% level of significance was used. In the subclinical and clinical groups, the abnormal internodes, in the left and right recurrent laryngeal nerves, showed a nonrandom distribution. That is, they were clustered on particular fibres. This was true for both distal and proximal sampling levels. The control

group was not included in this analysis because of the insignificant number of abnormal internodes present in the recurrent laryngeal nerves of these horses.

ELECTRON MICROSCOPY

Morphology

Detailed data concerning the electron microscopic study of each of the recurrent laryngeal nerve samples from 13 horses are recorded in Appendix 10, and summarised below.

The pathological changes observed in the light microscopic examination of the nerves were confirmed. The gradation of myelinated fibre loss, and of the individual fibre changes ("onion bulb" formations, regenerating clusters, thinly myelinated fibres and myelin debris) were also seen at this higher level of resolution (Figs. 54 & 60). In addition, many components of the nerve, not visible, or not able to be accurately defined, at the light microscopic level were identifiable by electron microscopy.

The denervated Schwann cells, bands of Bungner, were recognised by the presence of a surrounding basement membrane (Fig. 54D). As would be expected, their frequency reflected the loss of myelinated fibres. They increased in number between the right and left recurrent laryngeal nerves, from proximal to distal levels of both left and right nerves, and from subclinical to clinical laryngeal hemiplegic animals. Branches of the peroneal nerve examined also contained bands of Bungner, at approximately the same frequency as seen in the proximal left and right recurrent laryngeal nerve samples.

Recent nerve fibre degeneration, as evidenced by a collapsed myelin sheath without an axis cylinder (Fig. 55A), was also observed at all levels of the left and right recurrent laryngeal nerves. They were seen in all three groups of horses. They varied in frequency from one to several in a particular section, generally being more common in the subclinical group. Similarly, from one to several of these structures were seen in sections from the branches of the peroneal nerve examined from two of the clinically affected animals.

Other degenerating myelinated fibres were seen, with a small axis cylinder containing dense axoplasm, a disintegrating myelin sheath and an excess outer layer of Schwann cell cytoplasm. These fibres were seen in the left and right recurrent laryngeal nerves of subclinical and clinical animals, and varied in frequency from one to several in any one section.

Fibres with split myelin sheaths were observed rarely in the distal left recurrent laryngeal nerves of control and subclinical animals. The splits contained densely packed organelles including mitochondria, vesicles, membranous dense bodies and floccular material.

The presence of myelinated fibres with clumped floccular material in an adaxonal position, was noted on a number of occasions (Fig. 56). In all cases, the axis cylinder was markedly reduced in size. These fibres were seen in the left and right recurrent laryngeal nerves in the subclinical group, and one was observed in the distal right nerve of a clinically affected animal.

Myelinated fibres with disproportionately thick myelin sheaths relative to their axonal calibre (Fig. 57), were found in the left recurrent laryngeal nerves of subclinical and clinical animals, and to a lesser extent, in the right nerve. This abnormality was also noted in a branch of the peroneal nerve from a clinically affected horse.

The presence of increased numbers of accumulated axoplasmic organelles, mainly mitochondria and membranous dense bodies (Figs. 55B), was observed in the left and right recurrent laryngeal nerves of subclinical, and to a lesser extent clinical animals. Less commonly, the accumulated organelles were microtubules and neurofilaments.

Markedly enlarged nerve fibres, distended with abnormal organelles, were seen in three of the left distal nerve samples, one from each group of horses. In all cases, the swollen fibres were surrounded by concentrically arranged Schwann cell processes, that is they were part of "onion bulb" formations. One such "onion bulb" contained two of these fibres. On one occasion an attenuated myelin sheath surrounded the axon (Fig. 59A-C), while in the remainder the fibre was demyelinated. The accumulated organelles varied between these fibres (Figs. 59D-F), but included dense membranous bodies, vesicular and tubular structures.

Projections of adaxonal Schwann cell cytoplasm into the axoplasmic compartment, were seen in the left and right recurrent laryngeal nerve, in particular in the subclinical group. In these animals, the projections were seen in many of the samples. Often the fibres showed other abnormalities, including being part of "onion bulbs", containing myelin debris in their Schwann cell cytoplasm, accumulated organelles or a thin myelin sheath. Several well developed axon-Schwann cell networks (Fig. 55C) were also noted in the left and right recurrent laryngeal nerves of clinical and subclinical animals. One such fibre contained densely packed disoriented neurofilaments in the axoplasm (Fig. 55D). In another thinly myelinated fibre a dense body was present in the network. Both adaxonal Schwann cell projections, and fully developed axon-Schwann cell networks were seen in branches of the peroneal nerve from clinically affected animals.

Margination of the microtubules in a subaxolemmal position, with clustering of the remaining microtubules, was seen occasionally in the distal left and right recurrent laryngeal nerves of some of the subclinical and clinical groups of horses.

Excess convoluted basement membrane, usually around Schwann cell processes of "onion bulbs" and regenerating clusters, was observed in the left and right recurrent laryngeal nerves, predominantly in clinical animals. Occasionally a basement membrane profile was observed lying free in the endoneurium. Excess basement membrane was also observed around Schwann cell processes, Schwann cells without axons, and the Schwann cell of a thinly myelinated fibre in the peroneal nerve of clinically affected horses.

Redundant loops of myelin were seen occasionally, associated with nerve fibres of the left and right recurrent laryngeal nerves from clinically and subclinically affected horses. These usually involved small myelinated fibres. They were also observed on several myelinated fibres from the peroneal nerves of clinically affected horses.

An abnormal axoplasmic organelle, in the form of a clumped floccular body with a concentrically layered outer membranous area, was observed on two occasions in subclinically affected animals. One was from a proximal left recurrent laryngeal nerve sample, and the other from the right midcervical level.

An unusual nerve fibre was observed in the midcervical right recurrent laryngeal nerve from a control horse (Fig. 58). This thinly myelinated fibre possessed a distended adaxonal area containing mainly vesicular structures, and with two separate apparent axonal compartments. Such a pathological change is of uncertain significance, and

has not previously been reported, at least in the literature examined.

In the branch of the peroneal nerve innervating the *extensor digitorum longus* muscle of a clinically affected horse, a large myelinated fibre was seen with loosely compacted inner myelin lamellae. In addition a small fibre with excess Schwann cell cytoplasm was observed.

In the common peroneal nerve from a clinical animal, an attenuated Schwann cell process contained a loculated myelin breakdown product (Fig. 61). Excess convoluted basement membrane surrounded this process.

Morphometry

The regression analyses of axon size, as indicated by axis cylinder perimeter (log to base e), against the number of myelin lamellae surrounding that axon are presented as scatter graphs and regression lines in Figs. 62-67. At each level the regression lines for the subclinical and clinical groups were compared with those of the controls, to determine whether axonal atrophy was present in the two affected groups. A more gradual slope of the line, and/or lower position of the line was interpreted as indicating axonal atrophy. Both situations represent nerve fibres with a smaller axon than is normal, relative to the number of myelin lamellae. A more gradual slope of the line was seen in the clinical animals in the left midcervical and proximal, and in the right distal and midcervical recurrent laryngeal nerves. This difference in slope proved to be significant at the 1% level, using the students t test. A more gradual slope was also seen in the subclinical horses in the left proximal, and in the right distal and midcervical recurrent laryngeal nerves. A lower position of the regression line, without lessening of the slope, again significant at the 1% level, was seen in the distal left recurrent laryngeal nerve from the clinically affected [illegible]

4. DISCUSSION

LIGHT MICROSCOPY

Morphology

For many years it has been known that pathology of the left recurrent laryngeal nerve is involved in the aetiology of equine laryngeal hemiplegia (Cadeac, 1897; Cole, 1946; Duncan *et al*, 1974; Duncan, 1975; Duncan *et al*, 1978). Both Cole (1946) and Duncan (1975) showed that this pathology was associated with a loss of myelinated nerve fibres, most obvious in the distal part of the nerve. The present study confirmed these observations, but in addition, has demonstrated that a similarly graded, although less severe loss of myelinated nerve fibres also occurred in the right recurrent laryngeal nerve of laryngeal hemiplegic horses. Previous researchers (Duncan, 1975; Duncan *et al*, 1978), found no loss of these fibres in the right nerve, although individual fibre pathology (Cole, 1946; Duncan, 1975; Duncan *et al*, 1978), and neurogenic damage to some right intrinsic laryngeal muscles had been observed (Duncan, 1975; Duncan *et al*, 1978). Moreover, as has been reported previously by these authors, the results of the present investigation confirmed that loss of myelinated fibres is more severe in the clinically affected laryngeal hemiplegic horses, than in subclinically affected animals.

Considerable differences in the frequency and distribution of individual nerve fibre pathological changes exist between the present results and those of previous workers (Duncan, 1975; Duncan *et al*, 1978). Regenerative activity, in the form of regenerating clusters and "onion bulb" formations, was observed frequently at all levels of the left and the distal right recurrent laryngeal nerve in subclinical and clinical laryngeal hemiplegic horses by Duncan (1975) and Duncan and coworkers (1978). In the present study however, a proximal to distal increase in the occurrence of these

changes was recorded in both left and right recurrent laryngeal nerves of similarly affected horses. While this pathology was more severe in the left nerve, it was not confined to the distal part of the right nerve, as suggested by Duncan and coworkers (1978). Even in the control animals, a low level of these pathological changes was noted, and followed the same distribution observed in the affected animals. As with the loss of myelinated fibres, there was a gradation in severity of pathology from the subclinical to clinical animals. The low frequency of these changes in the distal left recurrent laryngeal nerve of the clinical horses probably reflected the severe myelinated fibre loss at this level, and the consequent lack of fibres to be thus affected.

The demonstration in both left and right recurrent laryngeal nerves, of many "onion bulbs" which result from repeated insult to the nerve fibres over a period of time, suggests that the factor or factors initiating the pathology of equine laryngeal hemiplegia occur repetitively.

The presence of regenerating clusters in the left recurrent laryngeal nerve at the level of the aortic arch, the left vagus, and at the thoracic inlet in the right recurrent laryngeal nerve of clinical and subclinical laryngeal hemiplegic horses indicates a more extensive nerve involvement than reported by previous authors (Duncan, 1975; Duncan *et al*, 1978).

As observed by Duncan (1975) and Duncan and coworkers (1978), evidence of active nerve fibre degeneration was not a common finding. It was seen at various levels of the left and right recurrent laryngeal nerves of the subclinical and clinical laryngeal hemiplegic animals, and in the distal area of the left nerve of control horses. The distribution of this active fibre degeneration followed a similar pattern to the other nerve changes characteristic of laryngeal hemiplegia. For this reason, it seems likely that these changes represent part of the disease process.

The presence in other long peripheral nerves from laryngeal hemiplegic horses of pathological changes, similar in type and distribution to the pathology seen in the recurrent laryngeal nerves, represents a major divergence from the findings of other workers (Duncan, 1975; Duncan *et al*, 1978). These authors found no evidence of disease in a number of peripheral nerves examined. In the present study, pathological changes were observed in the long hindlimb nerves of laryngeal hemiplegic horses. The type and frequency of these changes approximated that demonstrated in the more proximal levels of the right recurrent laryngeal nerve. Therefore, it seems likely that these changes are part of a general disease process, clinically typified by laryngeal hemiplegia. The extent of the pathology observed was in keeping with the length of these nerves relative to the recurrent laryngeals.

In human recurrent laryngeal nerve, the repeated division of fascicles to form plexuses (Fig. 68), occurs along the length of the nerve (Sunderland & Swaney, 1952). Different fascicular patterns, in transverse sections of nerve taken only a few millimetres apart, and the presence of connective tissue septa within fascicles, found in the present study, indicated that a similar arrangement occurred in equine recurrent laryngeal nerve. This arrangement makes the separation of adductor and abductor fibres into separate fascicles unlikely. Such a separation has been suggested as a possible reason for the apparent susceptibility of the adductor fibres to damage, such as produced by compression (Duncan *et al*, 1978). Moreover, the observed arrangement of widely separated small fascicles, rather than large tightly packed ones, according to Sunderland (1965), would render the nerve less susceptible to injury by compression.

In contrast to the findings of Duncan and coworkers, who considered that Renaut bodies were more common in diseased

than in healthy recurrent laryngeal nerve, the present study showed no apparent association between these structures and the disease status of the nerve. Two aspects of Renaut body occurrence differed from that mentioned in the literature;

(i) Their presence in the 1 day old foal refuted the observation that these structures do not occur in the newborn (Asbury, 1973). Although present in this animal, they were not as large as those normally seen in adult horses.

(ii) In one instance, a Renaut body was observed lying in the endoneurium and surrounded by nerve fibres. This situation has not previously been noted.

Haslam's anomaly, a ribbon-like flattening of the recurrent laryngeal nerve as it passes around the aortic arch in the horse has been observed by a number of authors (Haslam, 1893; Mason, 1973). An aetiological association of this so called anomaly with equine laryngeal hemiplegia has been postulated (Haslam, 1893). An identical arrangement of the nerve was observed on two occasions in the present investigation, and in both instances it occurred in proximal areas of the right recurrent laryngeal nerve. It was unassociated with laryngeal hemiplegia. This observation supports the opinion of Mason (1973), who considered that there was no relationship between this anomaly and laryngeal hemiplegia.

Morphometry

A progressive distal decrease in the total number of myelinated fibres in the left recurrent laryngeal nerves of subclinically and clinically affected horses was reported in the only previous morphometric investigation of myelinated fibre density alterations in equine laryngeal hemiplegia (Duncan, 1975; Duncan *et al.* 1978). In addition to this distally graded loss of myelinated fibres in the left

recurrent laryngeal nerve, the present study also showed a similar, but less severe, change in the right nerve. Thus, it was demonstrated that the loss of nerve fibres associated with equine laryngeal hemiplegia, is widespread throughout both left and right recurrent laryngeal nerves. It should also be noted that, while no loss was obvious visually in some samples, the more sensitive quantitative methods did provide evidence of a significant decrease in fibre density. This is in accord with the conclusion of Dayan (1979), who found that a considerable percentage of fibres must be lost before the loss can be detected, even by a skilled observer.

The presence of a significant amount of regenerative activity in many of the samples from affected animals, confused the observed pattern of alterations to the fibre density. Regenerating clusters, with several fibres replacing the one lost, gave the false impression of less fibre loss than had actually occurred. Since regenerating clusters were frequently seen in the more proximal levels of the nerve in both affected groups of horses, the true extent of fibre loss was masked to some extent at these levels. For similar reasons, the frequent occurrence of regenerating clusters may account for the exceptions in the distal to proximal gradation of fibre loss in the subclinically affected animals.

The greater decrease in fibre density in the longer left recurrent laryngeal nerve, may reflect the influence of nerve fibre length on susceptibility to the pathological process of laryngeal hemiplegia, and was also noted by Duncan and coworkers (1978).

A previous light microscopic investigation of the fibre diameter distributions of the recurrent laryngeal nerves from horses with laryngeal hemiplegia, showed a marked shift to smaller fibre diameters in the most distal parts of the left recurrent laryngeal nerve, and to a lesser extent at

the proximal levels of the nerve in clinically affected animals (Duncan, 1975; Duncan *et al*, 1978). A loss of large fibres was also noted in subclinically affected horses, in the distal area of the left nerve.

The fibre diameter spectrum observed in the control animals, in the present investigation, is typical of that reported for the recurrent laryngeal nerve, in the horse (Duncan, 1975) and in other species (Harrison, 1981). The major peak of medium to large myelinated fibres constitutes the motor fibres to the laryngeal muscles, and in these animals, was of 8-11 µm. However, a bimodal distribution was evident in most of the histograms, particularly at more proximal levels of the nerve. The population of small diameter fibres are believed to consist mainly of sensory fibres to the trachea and oesophagus (Duncan, 1975). The observation of fewer of these fibres in the distal areas of the recurrent laryngeal nerve supports the above suggestion. However, the presence of these small fibres in the intralaryngeal branches indicates that some few reach the laryngeal muscles. In the right recurrent laryngeal nerve of clinical and subclinical animals, where the fibre diameter histograms appeared normal, the major fibre peak was of a significantly larger diameter than in the controls. This value, of approximately 10-13 µm, is similar to that reported previously for equine laryngeal nerve (Duncan *et al*, 1978). The reason for this shift to larger diameter fibres in these areas of affected horses, is probably related to the different age distributions of the animals in the control and the diseased groups. The former group contained a 1 day old foal, at which age the myelinated fibres would not have attained their adult calibre (Cottrell, 1940; Gutrecht & Dyck, 1970; Stevens *et al*, 1973; Braund *et al*, 1982b).

In both clinically and subclinically affected laryngeal hemiplegic horses, the loss of fibres demonstrated, was shown to involve selectively those of large diameter. This was influenced by nerve fibre length, as not only was it

more obvious in the distal areas of the nerve, it was also greatest on the left side. The extent of the alteration in fibre diameter spectra was less severe in the subclinical horses. In the left proximal and all levels of the right recurrent laryngeal nerve, the mean fibre diameter of the control group was less than that of the affected animals. However, if the bias to smaller diameters in the control group, introduced by inclusion of the 1 day old foal, was removed, this difference may have been reversed.

Thus, by quantitative means, this study has confirmed the findings of Duncan (1975), that the pathological process of equine laryngeal hemiplegia involves a selective loss of large myelinated fibres, with a distal pattern of distribution.

In other species it has been demonstrated that the left recurrent laryngeal nerve contains relatively more large diameter fibres than its right counterpart, presumably to increase the speed of impulse conduction and compensate for the greater length of the left nerve (Harrison, 1981). However, in the present study, comparison of corresponding levels of the left and right recurrent laryngeal nerves of the control animals did not reveal any differences in the large fibre populations.

Distal tapering of nerve fibres has been shown to occur (Williams & Wendell-Smith, 1971), and a comparison of distal and proximal levels of the recurrent laryngeal nerves of control animals was expected to illustrate this. However, the proximal and distal mean fibre diameters and distributions were similar.

The apparent susceptibility of the adductor muscle, the *cricoarytenoideus lateralis*, to atrophy in equine laryngeal hemiplegia has been reported (Gunn, 1972; Duncan & Griffiths, 1973; Duncan, 1975). Moreover, large diameter fibres are known to be more vulnerable to this disease (Duncan *et al*,

1978). In an attempt to determine whether the susceptibility of the *cricoarytenoideus lateralis* muscle to this disease was related to the fibre diameter of its nerve supply, a comparison of the distribution of fibre diameters in its nerve with that of the *cricoarytenoideus dorsalis* was performed. As only two animals were included in this study the results must be interpreted cautiously. A comparison of these distributions in the right sided nerves to the *cricoarytenoideus lateralis* and *cricoarytenoideus dorsalis* muscles, revealed that the nerve to the adductor muscle contained more large diameter fibres. Thus, it is possible, indeed likely, that the susceptibility of the *cricoarytenoideus lateralis* to the diesease process of laryngeal hemiplegia is related to the greater number of large diameter fibres in its nerve. However, examination of the left nerves revealed a shift to smaller diameter fibres in the nerve supplying the *cricoarytenoideus lateralis* muscle. This shift is characteristic of that seen along the course of the recurrent laryngeal nerves in animals with laryngeal hemiplegia. This finding implies that the nerve fibres to this muscle were probably affected by this disease process.

TEASED FIBRE PREPARATIONS

Morphology

This investigation reports the first comprehensive examination of quantitative teased fibre preparations of the recurrent laryngeal nerves from laryngeal hemiplegic horses. One other group of researchers (Duncan, 1975; Duncan *et al*, 1978) have previously reported teased fibre observations in these nerves.

Teased fibre preparations represent the only reliable technique of examining a length of an individual nerve fibre over several consecutive internodes. Thus, they provide

a more sensitive assessment of the disease status of that nerve fibre than other histological techniques. Moreover, use of Dyck's system (1975a) to grade the teased fibres provides a clear distinction between changes indicative of primary demyelination and those of axonal degeneration. It also allows a more accurate estimation of the degree of pathology present in a nerve.

There was considerable variation in the percentage of abnormal fibres observed. This depended on the disease status of the animal, and the level and side of the recurrent laryngeal nerve sampled. The percentage of abnormalities increased from control through subclinical to clinical laryngeal hemiplegic animals. Within each group there was a proximal to distal increase in the percentage of abnormal fibres, which was more marked on the left side than on the right. The percentage of fibres affected in the control horses was similar to that found in ponies by Duncan (1975), and "normal" peripheral nerve of other species (Stevens *et al*, 1973; Braund *et al*, 1982a).

The observation that intercalated internodes were the predominant pathological change in teased fibre preparations of the recurrent laryngeal nerve of laryngeal hemiplegic animals, is in accord with the reports of Duncan and coworkers. This finding could suggest that segmental demyelination is the primary neuropathological lesion of equine laryngeal hemiplegia. However, other evidence, presented in the morphometric teased fibre section of this thesis, indicates that this demyelination occurred secondary to axonal changes.

Duncan and coworkers found little evidence of active axonal degeneration in teased fibre preparations of the animals they studied. A similar result was recorded in the present study, for most of the animals investigated. However, in some of the subclinical group more than 10% of fibres were undergoing active degeneration, whereas in the control

animals, usually only 1% or less of fibres showed this change. Although, these changes could be due in part to age, active axonal degeneration is usually present at only a low frequency in the nerves of older individuals (Braund *et al*, 1982a). Thus, it seems likely that isolated active axonal degeneration was occurring as part of the disease process of laryngeal hemiplegia.

At the time of the examination, relatively more active pathological changes were occurring in the distal levels of the left recurrent laryngeal nerve in the subclinical horses than in those clinically affected with laryngeal hemiplegia. In the latter animals, there were more active changes present in the midcervical recurrent laryngeal nerve than in the area close to the larynx. Conversely, as would be expected in a disease process which appears to initially affect the distal ends of long nerve fibres, the most chronic changes were seen in the distal samples of the left nerve.

The percentage of abnormal teased fibres observed in the long hindlimb nerves from two clinical laryngeal hemiplegic animals, was approximately the same as that seen in the left recurrent laryngeal nerve at the level of the aortic arch, and at all levels of the right nerve. In addition, some proximal to distal increase in the percentage of abnormal teased fibres was seen within the limb nerves. This is in contrast with the findings of previous workers (Duncan, 1975; Duncan *et al*, 1978), who found no significant changes in these nerves, and suggests that the disease process associated with laryngeal hemiplegia may also affect other long peripheral nerves.

Morphometry

A detailed analysis of internode lengths in the recurrent laryngeal nerves of control, subclinical and clinical laryngeal hemiplegic horses was undertaken in the present investigation in an attempt to determine the primary nerve lesion in this disease. It was important to assess whether the initiating lesion involved the Schwann cell, or the axon itself.

Increased variation of internode length was demonstrated in a previous study of teased fibre preparations in equine recurrent laryngeal nerve (Duncan, 1975; Duncan *et al*, 1978). This increase in variability, together with a decrease in the mean internode length in the nerves of the diseased animals was demonstrated in the present study. Such changes indicated that demyelination and remyelination had occurred (Fullerton *et al*, 1965). These changes followed the same pattern of distribution observed for the other pathological alterations mentioned in previous sections. The increase in severity from subclinical to clinical, from right to left, and from proximal to distal was again observed.

The clustering of abnormal internodes on particular nerve fibres, as observed in this investigation, has been interpreted previously as evidence for a primary pathological change affecting the axon being responsible for the changes in the myelin sheath (Dyck *et al*, 1971a). The term secondary segmental demyelination was introduced to describe this process, and has been demonstrated in a number of human neuropathies, including those associated with uraemia (Dyck *et al*, 1971a), and Friedreich's ataxia (Dyck & Lais, 1973). The frequent occurrence of demyelination and remyelination which was observed in diseased recurrent laryngeal nerve, has been shown in these human disorders to reflect the chronic nature of the disease processes (Dyck, 1975a; Spencer & Schaumburg, 1977). While the predominance of demyelination and remyelination could be interpreted as

indicating a primary disease of the Schwann cell, it is more likely that it is related to the chronic nature of the disease. Thus, the results indicated that while the predominant change was one of demyelination and remyelination, it was likely that this occurred secondary to underlying axonal pathology.

The relatively minor proximal to distal decrease in mean internode length in the left and right recurrent laryngeal nerves of the control animals probably reflected distal tapering of the nerve fibres (Williams & Wendell-Smith, 1971). It would be expected that with a decrease in fibre diameter a corresponding decrease in internode length would occur. However, such a decrease in fibre diameter in more distal areas of the recurrent laryngeal nerves was not demonstrated by analysis of the fibre diameter distributions. The marked proximal to distal decrease in mean internode length associated with disease was probably due to a number of factors. These include firstly, the distal tapering of nerve fibres as mentioned above; secondly, the presence of intercalated internodes, indicative of demyelination and remyelination; thirdly, the presence of regenerated fibres with uniformly short internodes; and finally the distally graded loss of large myelinated fibres demonstrated by light microscopic morphometric techniques. This causes the population of nerve fibres with long internodes to be reduced, and hence the mean internode length for the nerve will be decreased.

Mean internode length in the distal left recurrent laryngeal nerve was significantly larger than that on the right in control horses. In other species, a higher proportion of large nerve fibres in the left recurrent laryngeal nerve has been demonstrated (Harrison, 1981; Dahlquist, 1982). This would explain the longer internode lengths found on the left nerve, but light microscopic quantitative studies mentioned previously in this thesis, did not reveal a higher proportion of large fibres on the left. An alternative

explanation for the longer internodes in the left, is the greater passive increase in the length of this nerve as a result of growth (Lascelles & Thomas, 1966).

In view of the direct relationship between fibre diameter and internode length, and the selective vulnerability of large myelinated fibres reported previously (Duncan *et al*, 1978), and demonstrated in the present study, separate analyses of the large and small teased fibre populations may have been expected to reveal preferential involvement of the large fibres. However, probably because of the extensive loss of these fibres in affected nerves, such selective involvement could not be demonstrated. In addition, the frequent occurrence of intercalated internodes, to a lesser extent regenerated internodes in diseased nerve, made the accurate separation of fibres into small and large populations difficult.

ELECTRON MICROSCOPY

Morphology

The electron microscopic examination of recurrent laryngeal nerve confirmed the distal distribution of nerve fibre pathology in equine laryngeal hemiplegia, as seen in light microscopic and teased fibre preparations. It also confirmed similar findings reported in a previous electron microscopic investigation of equine recurrent laryngeal nerve in this disease (Duncan *et al*, 1978). In addition, a variety of features not seen at the light microscopic level were observed. For example, remnants of past nerve fibre degeneration, bands of Bungner, could be visualised, and as would be expected, were more common in the clinical than subclinical animals, and in the distal part of the nerve. Numerous bands of Bungner had previously been noted in nerves from laryngeal hemiplegic horses by Duncan and coworkers (1978).

A distinctive finding of the electron microscopic investigation of the recurrent laryngeal nerves, was that primary axonal pathology was more obvious than was indicated by light microscopy. Recent active axonal alterations, were evidenced by a number of pathological changes. These included large lamellated myelin ovoids occupying Schwann cells, fibres with small densely packed axons, fibres with split myelin sheaths, and fibres with small axons and clumped floccular adaxonal material. These were seen at all levels of left and right recurrent laryngeal nerves, but were relatively more common in the subclinically affected horses. This finding contrasts with the previous report by Duncan and coworkers (1978), in which active axonal degeneration was not seen commonly at the electron microscopic level. As observed by these authors, a number of fibres seen in cross section to be swollen to many times their normal size, were also demonstrated in the present investigation. These contained a variety of normal and abnormal axoplasmic organelles, and were noticed in the distal parts of some of the left recurrent laryngeal nerves, of all three groups of horses. They probably represent a local derangement of axoplasmic transport, which has resulted in the accumulation of organelles and axonal swelling. The presence of these fibres in "onion bulb" formations indicated that there had been repeated insults, either insufficient to produce degeneration, or with degeneration and subsequent regeneration.

Axonal atrophy was evidenced by the presence of fibres with disproportionately thick myelin sheaths relative to their axonal calibre, and fibres with redundant loops of myelin. A number of the latter appeared in regenerating clusters, and probably represented fibres regressing after failing to reinnervate an endorgan. Shrinkage of the axon, with consequent inward slippage of the myelin lamellae, and thus increased thickness of the myelin sheath, has been shown to occur in a number of human neuropathies including Friedreich's ataxia (Dyck & Lais, 1973) and uraemic neuropathy (Dyck *et al* , 1971a).

In addition to more obvious signs of axonal pathology a number of changes were seen that were more subtle indicators of axonal disease. These included accumulated axoplasmic organelles, adaxonal Schwann cell projections, axon-Schwann cell networks, margination of microtubules and abnormal axoplasmic organelles. Again these changes were seen in the left and right recurrent laryngeal nerves, and were more frequent in the subclinical group of horses. The finding that active nerve fibre degeneration was consistently more prominent in the subclinical animals, suggests that the disease process of laryngeal hemiplegia is more active in this group. This finding is consistent with that recorded in the section on teased fibre preparations.

The active fibre degeneration and more subtle axonal changes which were observed in the control animals, occurred at a much lower frequency than in the affected horses. These may represent "normal" wear and tear on the nerve, or in some instances perhaps represent an early part of the disease process of equine laryngeal hemiplegia.

As has been shown in the light microscopic and teased fibre preparations, the type and degree of pathology in the peroneal nerve from clinical horses, was similar to that present in the proximal left and the right recurrent laryngeal nerves.

Morphometry

While electron microscopic examination of equine recurrent laryngeal nerve has been reported previously (Duncan *et al*, 1978), morphometric analysis was not performed. The evidence presented in previous sections of this thesis, indicated that the disease process of laryngeal hemiplegia was chronic and primarily axonal in nature, and had a predominantly distal distribution. Thus, the disease may be classified as a distal axonopathy. In man, some of the chronic distal

axonopathies have been shown to be associated with atrophy of the distal axon (Dyck *et al*, 1971a; Dyck & Lais, 1973). This atrophy can only be detected accurately by comparison of axonal size and myelin sheath thickness. For this reason, such a comparison was performed.

The results showed that axonal atrophy was present at a number of levels in the recurrent laryngeal nerves of subclinical and clinical laryngeal hemiplegic animals. The distal distribution of the pathology of laryngeal hemiplegia, demonstrated in light microscopic and teased fibre preparations would suggest that any axonal atrophy, which may be involved in the pathogenesis of the nerve degeneration, would also show a similar pattern. Such a distal distribution was apparent in the clinically affected horses. However, initial examination of the regression analyses of the subclinical group did not reveal this pattern, as their distal and midcervical left recurrent laryngeal nerve samples showed no significant differences when compared with the controls. This may be explained by the presence of a considerable amount of regenerative activity in these samples. "Onion bulbs" and regenerating clusters, with their associated thinly myelinated fibres, were common findings, and could mask the presence of fibres showing axonal atrophy. In the subclinical group, it is possible that less axonal atrophy was present than in the clinically affected horses, and thus the thinly myelinated fibres could more readily conceal the presence of atrophic fibres. When significant differences were found, this population of thinly myelinated fibres would tend to lessen these differences, and thus, the degree of axonal atrophy present in the nerves of horses affected by laryngeal hemiplegia, is probably more severe than is reflected by the regression lines derived in this study.

SUMMARY

It is concluded from this study that equine laryngeal hemiplegia is a distally distributed polyneuropathy, which is primarily axonal, and of an apparently chronic nature.

A distal distribution of the pathology was demonstrated by light microscopic, teased fibre and electron microscopic preparations and confirmed quantitatively. This preferential susceptibility of long nerve fibres at their most distal parts was manifest in two ways. Firstly, the proximal to distal increase in severity of pathological changes was observed within a nerve trunk, and was particularly noticeable in the left recurrent laryngeal nerve, the longest nerve in the horse's body. Secondly, a comparison of the degree of pathology in nerve trunks of differing lengths, demonstrated the direct relationship between nerve length and degree of pathology. Thus, the left recurrent laryngeal nerve, which can be 30 cm longer than the right, was predictably more severely involved. The extent of pathology in the distal hindlimb nerves examined was also consistent with this pattern.

The techniques of peripheral nerve examination used in this investigation, provided evidence for the primary axonal nature of the pathology of this disease. Light microscopic morphometric examination of resin embedded nerve demonstrated selective loss of large myelinated fibres in particular. Although demyelination and remyelination were prominent findings in the teased fibre preparations, the grouping of abnormal internodes on particular fibres indicated that the demyelination had occurred secondary to some underlying axonal pathology. Electron microscopic examination revealed active axonal degeneration, as well as more subtle indications of early axonal disease. In addition, morphometric electron microscopic techniques demonstrated axonal atrophy in the recurrent laryngeal nerves of laryngeal

hemiplegic animals. Changes indicative of axonal pathology were also present in hindlimb nerves examined. Thus, the results of this study have provided a considerable amount of evidence for the primarily axonal nature of the disease process in equine laryngeal hemiplegia.

The frequent finding of "onion bulb" formations at certain levels of affected recurrent laryngeal nerves, both in this and previous studies (Duncan, 1975; Duncan *et al*, 1978), indicates that the disease process of equine laryngeal hemiplegia is a chronic one. Rather than a single insult to peripheral nerve, it is likely that there are repeated episodes of damage to the nerve fibres, a prerequisite for the formation of these structures (Dyck, 1975a; Raine, 1977; Bennington, 1978).

The presence of fibres which were greatly distended with accumulated axonal organelles, fits the current hypothesis regarding the pathogenesis of distal axonopathies (Spencer *et al*, 1976). A direct local effect of the damaging influence on the axon itself, with consequent interference with energy metabolism and axoplasmic transport, is thought to precipitate axonal degeneration distal to a point of damage. The swollen axons observed may be due to local interference with axoplasmic transport, and as such probably represent a local area of axonal damage.

A similar pattern of pathology to that shown to occur in equine laryngeal hemiplegia, is observed in a number of human diseases. In uraemic neuropathy there is a distal loss of nerve fibres, secondary demyelination, ultastructural axonal alterations and axonal atrophy (Dyck *et al*, 1971a). Similar findings have been reported for Friedreich's ataxia (Dyck & Lais, 1973). In peroneal muscular atrophy, a distal neuropathy, ultrastructural alterations indicative of axonal disease are present, and demyelinated and remyelinated segments are clustered on

particular nerve fibres (Madrid *et al*, 1977). In diabetic neuropathy also, there is a distal loss of myelinated fibres, with segmental demyelination particularly in chronic cases (Bennington, 1978). However, the pathology of equine laryngeal hemiplegia is not identical to any of the above diseases. Although the fundamental pathological changes are similar, they differ in extent and distribution. These differences may be largely due to the relative nerve lengths of the two species.

PART III

MUSCLE PATHOLOGY IN EQUINE LARYNGEAL HEMIPLEGIA

Introduction

Muscle histopathology is a useful adjunct to the investigation of a neuropathy because it demonstrates the effects of denervation on the muscle. Usually this denervation is partial, with changes occurring in those muscle fibres belonging to the motor unit innervated by the damaged motor axon (Buxton, 1980). The affected muscle fibres shrink, and thus the sarcolemmal nuclei appear more numerous. Adjacent muscles fibres appear normal in size or even hypertrophied. The denervated muscle fibre stimulates neighbouring healthy nerve fibres to send out fine axonal sprouts to reinnervate the denervated fibres, a process known as collateral sprouting. A characteristic feature of denervation is fibre type grouping, which occurs because the denervated muscle fibres in the vicinity of a surviving nerve fibre will be reinnervated by collateral sprouts from this fibre, and thus will assume the same metabolic type, as determined by the innervating nerve fibre. In a myosin ATPase stained frozen section, this appears as the grouping of fibres of the same histochemical type, and is pathognomonic of reinnervation. The term neurogenic atrophy is used to describe the resultant morphological alterations in muscle.

In the present investigation the secondary effects of denervation were examined in muscles supplied by the recurrent laryngeal nerves. This enabled a comparison to be made between the degree of pathology observed in the recurrent laryngeal nerve and the muscles it supplied. A muscle innervated by another long peripheral nerve was also examined.

Materials and Methods

Collection and processing of the laryngeal muscles

The left and right *cricothyroideus*, *cricoarytenoideus lateralis* and *cricoarytenoideus dorsalis* muscles were removed at *post mortem*. These muscles were dissected carefully from the laryngeal cartilages, and their weights were recorded. The *cricothyroideus* muscle was collected, as it is the only intrinsic laryngeal muscle not supplied by the recurrent laryngeal nerve, but by the external branch of the cranial laryngeal. The *cricothyroideus lateralis* and *cricoarytenoideus dorsalis* muscles were selected, as early indicators of neurogenic atrophy in the course of laryngeal hemiplegia (Duncan & Griffiths, 1973). Muscles were taken from the left and right sides to enable comparison. In addition the *extensor digitorum longus* muscle was sampled, as representing another muscle of the body supplied by a long nerve.

Once the weights of the laryngeal muscles had been recorded a block of tissue was taken from each for frozen transverse sectioning. Within an hour of death of the animal the muscle was frozen by immersion in isopentane[1], which was sitting in liquid nitrogen. The frozen block of muscle was mounted on a chuck, using an embedding medium for frozen tissue specimens[2]. Transverse sections were cut[3] at a thickness of 10 μm. Two sections were taken from each muscle, one for histochemical staining with myosin adenosine triphosphatase (myosin ATPase), and the other for staining with haemotoxylin and eosin (Appendix 4). The muscle sections were then examined under a light microscope for pathological changes.

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1. Isopentane, Koch-Light Laboratories Ltd, Buckinghamshire, England.
2. Tissue-Tek 2, Lab Tek Products, Naperville, U.S.A.
3. Lipshaw Cryotome, electric microtome, Lipshaw Manufacturing Co., Michigan, U.S.A.

In order to provide an assessment of the degree of pathology present in each muscle, the following grading system was used (Anderson, 1984).

Mild pathology (+)

1. Rounding of, and an apparent increase in the number of sarcolemmal nuclei.
2. The occurrence of a higher than normal number of nuclei within the body of muscle fibres.
3. Minor variation in fibre size and fibre type grouping.

Moderate pathology (++)

1. Fibre atrophy and hypertrophy.
2. An apparent increase in the amount of endomysial and perimysial connective tissue.
3. Appearance of grouped atrophy.

Marked pathology (+++)

1. Widespread atrophy and hypertrophy of fibres.
2. Marked endomysial and perimysial fibrosis.

Severe pathology (++++)

1. Widespread fatty and fibrous replacement of muscle fibres
2. Pyknotic nuclear clumps.

Results

Details of the pathological changes for the individual muscles are presented in Tables 10, 11 and 12.

TABLE 10: MUSCLE PATHOLOGY IN CONTROL HORSES.

HORSE NO.	MUSCLE	DEGREE OF PATHOLOGY	
		LEFT	RIGHT
1	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	-	-
	cricoarytenoideus dorsalis	-	-
	extensor digitorum longus		-
2	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	+	-
	cricoarytenoideus dorsalis	-	-
	extensor digitorum longus		-
3	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	+	-
	cricoarytenoideus dorsalis	-	-
	extensor digitorum longus		-
4	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	++	-
	cricoarytenoideus dorsalis	-	-
	extensor digitorum longus		-
5	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	-	-
	cricoarytenoideus dorsalis	-	-
	extensor digitorum longus		-
6	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	+	-
	cricoarytenoideus dorsalis	-	-
	extensor digitorum longus		-

\- no pathology
\+ mild pathology
++ moderate pathology
+++ marked pathology
++++ severe pathology

TABLE 11: MUSCLE PATHOLOGY IN SUBCLINICAL LARYNGEAL HEMIPLEGIC HORSES

HORSE NO.	MUSCLE	DEGREE OF PATHOLOGY	
		LEFT	RIGHT
7	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	+++	+
	cricoarytenoideus dorsalis	+++	-
	extensor digitorum longus		-
8	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	++	-
	cricoarytenoideus dorsalis	+	-
	extensor digitorum longus		-
9	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	+++	-
	cricoarytenoideus dorsalis	+++	-
	extensor digitorum longus		-
10	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	+++	-
	cricoarytenoideus dorsalis	++	-
	extensor digitorum longus		-
11	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	++	-
	cricoarytenoideus dorsalis	+	-
	extensor digitorum longus		+

- no pathology
+ mild pathology
++ moderate pathology
+++ marked pathology
++++ severe pathology

TABLE 12: MUSCLE PATHOLOGY IN CLINICAL LARYNGEAL HEMIPLEGIC HORSES

HORSE NO.	MUSCLE	DEGREE OF PATHOLOGY	
		LEFT	RIGHT
12	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	++++	++
	cricoarytenoideus dorsalis	++++	-
	extensor digitorum longus		-
13	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	++++	+
	cricoarytenoideus dorsalis	++++	-
	extensor digitorum longus		++
14	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	++++	++
	cricoarytenoideus dorsalis	+++	+
	extensor digitorum longus		+
15	*cricothyroideus*	-	-
	cricoarytenoideus lateralis	++++	+
	cricoarytenoideus dorsalis	++++	+
	extensor digitorum longus	+	+
	tibialis cranialis	+	-
	gastrocnemius	-	-
	biceps femoris	-	-

- no pathology
+ mild pathology
++ moderate pathology
+++ marked pathology
++++ severe pathology

Cricothyroideus muscle

No pathological changes were observed in the *cricothyroideus* muscle from any of the horses studied.

Left cricoarytenoideus lateralis

Some pathology was observed in the left *cricoarytenoideus lateralis* muscles in all horses studied, with the exception of two control animals. One of these was the 1 day old foal. Pathological changes were mild to moderate in the other controls, moderate to marked in the subclinicals and severe in the clinicals (Figs. 69-72).

Right cricoarytenoideus lateralis muscle

No pathology was observed in the right *cricoarytenoideus dorsalis* muscle of any of the control animals. However, one subclinical had mild changes present, and all four clinical horses had changes which varied from mild to moderate (Fig. 73).

Left cricoarytenoideus dorsalis muscle

No pathological changes were detected in the left *cricoarytenoideus dorsalis* muscles of the control horses. Mild to marked pathology however, was observed in the muscles from the subclinical animals. In three of the four muscles from the clinical laryngeal hemiplegic horses, severe neurogenic atrophy was observed (Fig. 74). In the remaining clinical case, a somewhat lesser, but nevertheless obvious degree of pathology was seen.

Right cricoarytenoideus dorsalis muscle

No pathological changes were detected in the right *cricoarytenoideus dorsalis* muscles from control or subclinically affected animals. However, mild changes were present in these muscles from two of the clinical laryngeal hemiplegic horses, while morphology was normal in the remaining two.

Extensor digitorum longus muscle

No signs of pathology were seen in the *extensor digitorum longus* muscles from the control horses (Fig. 75), or from four of the five subclinical animals. In the remaining subclinical horse, mild pathological changes (Fig. 76) which included low myosin ATPase fibres in groups of over 20, were observed. In contrast to this, three of the four clinically affected horses demonstrated signs of neurogenic pathology (Fig. 77). Mild changes were present in the muscles of two horses. Fibre type grouping, of 100+ fibres, and variation in fibre size, were seen in these animals. Moderate pathology, with the additional finding of grouped atrophy, was detected in the other.

Other hindlimb muscles were studied in one clinical laryngeal hemiplegic horse, including the *tibialis cranialis*, *gastrocnemius* and *biceps femoris*. Of these, mild changes were seen in only the left *cranialis tibialis* muscle.

Discussion

The pathology observed in the intrinsic laryngeal muscles of the subclinical and clinical laryngeal hemiplegic horses is in agreement with the findings of previous workers (Gunn, 1972; Duncan & Griffiths, 1973; Gunn, 1973; Duncan *et al*, 1974; Duncan, 1975; Anderson, 1984). Changes indicative

of neurogenic atrophy were seen in the laryngeal muscles innervated by the recurrent laryngeal nerve. The existence of a group of horses without clinical or obvious endoscopic evidence of laryngeal hemiplegia, but with neurogenic changes in the muscles innervated by the left recurrent laryngeal nerve was noted. The adductor muscles, the *cricoarytenoideus lateralis*, was more severely affected than the abductor, the *cricoarytenoideus dorsalis*, a feature which was more apparent in the less severely affected subclinical animals. Right recurrent laryngeal nerve involvement was also evidenced, by early changes in the right *cricoarytenoideus lateralis* muscle in particular.

A previous study (Duncan, 1975), reported the examination of other muscles supplied by long nerves, and found no abnormalities. In the present investigation, the *extensor digitorum longus* muscle contained mild or moderate evidence of neurogenic pathology, in one of the five subclinical, and three of the four clinical horses, while no abnormalities were detected in the control group. The degree of pathology observed in this hindlimb muscle approximated that seen in the muscles innervated by the right recurrent laryngeal nerve. The finding of neurogenic atrophy, particularly in association with clinical laryngeal hemiplegia, of another muscle supplied by a long peripheral nerve, suggests that this disease may in fact be a generalised polyneuropathy, rather than a specific disease of the recurrent laryngeal nerve.

There appears to be a correlation between the overall degree of muscle pathology present within a group, that is control, subclinical or clinical, and the degree of recurrent laryngeal nerve pathology, as detailed in Part II of this thesis. The clinical laryngeal hemiplegic horses, with severe muscle changes correspondingly show marked alteration in the left recurrent laryngeal nerve. This contrasts with the control group, in which little muscle damage is present, and only occasional signs of pathology present in the

recurrent laryngeal nerve. The subclinical group is intermediate between these two extremes.

The degree of pathology present in the laryngeal muscles is a direct reflection of the extent of nerve fibre damage and loss in the distal sample of the recurrent laryngeal nerve. This is illustrated in the clinical horses. Severe changes were present in the left laryngeal muscles of all, as was severe involvement of the distal left recurrent laryngeal nerve. The variation in the degree of nerve pathology at more proximal levels of the left nerve did not produce corresponding variation in the extent of muscle damage.

Thus, it appears that early in the course of laryngeal hemiplegia, there is loss of almost all large myelinated nerve fibres in the distal area of the left recurrent laryngeal nerve. This results in a correspondingly severe neurogenic atrophy of the laryngeal muscles, manifest clinically as left laryngeal paresis or paralysis. The damaging influence affecting the recurrent laryngeal nerve however, seems to be an ongoing process, rather than relatively static as seen in the muscle. With time it appears that more proximal levels of both left and right recurrent laryngeal nerves are involved in the disease process. The distal left recurrent laryngeal nerve samples from the 2 and the 22 year old laryngeal hemiplegic horses are very similar in appearance, that is almost completely devoid of myelinated fibres and correspondingly, the degree of muscle pathology is severe in both. However marked differences are present in more proximal levels of the recurrent laryngeal nerve, and reflect the ongoing process of axonal degeneration with time.

PART IV

THE CENTRAL NERVOUS SYSTEM IN EQUINE LARYNGEAL HEMIPLEGIA

INTRODUCTION

The first reported investigation of the central nervous system of horses with laryngeal hemiplegia was performed by Argyle, in 1934. He examined the *medulla oblongata* in order to ascertain whether any changes were present in the nucleus of origin of the nerve fibres innervating the intrinsic laryngeal muscles. He described the way in which he identified the nucleus of the vagus in normal horses, and then in two animals with laryngeal hemiplegia. Degeneration was observed in a proportion of cells of this nucleus only on the left side. However, it was not clear whether the nucleus examined was actually the *nucleus ambiguus*, or the dorsal motor nucleus of the vagal nerve. In a more recent study of laryngeal hemiplegia, Duncan (1975) examined the motor nuclei of the recurrent laryngeal nerves in three clinical cases of the disease. No significant findings were observed. In view of this lack of specificity concerning the nucleus examined by Argyle (1934), and the small number of cases examined by Duncan (1975), it was proposed to further examine the central nuclei of the recurrent laryngeal nerve fibres. The cell bodies of these fibres are located in the *nucleus ambiguus* in the *medulla oblongata* (Gacek, 1975).

A second site within the central nervous system of horses with laryngeal hemiplegia was also examined. As discussed in previous chapters there is a distal distribution of nerve pathology in the recurrent laryngeal nerves of affected horses, suggesting that the condition may be a distal axonopathy. Often in diseases of this type, long fibres of the central nervous system are also affected distally

(Spencer & Schaumburg, 1976). To determine whether this occurred in laryngeal hemiplegic horses, the distal regions of long nerve fibres within the central nervous system were examined.

As a prerequisite for this study, the relevant anatomy and changes which may be expected in the area following neurogenic disease must be known. These are briefly reviewed.

Nucleus ambiguus

The *nucleus ambiguus* is a narrow column of motor neurones in the lateral region of the *medulla oblongata* (Bowsher, 1962; Crosby *et al*, 1962; Getty, 1975). It extends from the caudal end of the medulla, where it meets the cervical spinal cord, to the rostral end of the glossopharyngeal nucleus (Getty, 1975). The nucleus is made up of clusters of cells interspersed by more scattered cells, and thus is readily observable only at some levels (Crosby *et al*, 1962). The *nucleus ambiguus* lies ventral or ventromedial to the spinal trigeminal nucleus (Bowsher, 1962; Getty, 1975), a relationship which is particularly helpful in identification. These anatomical features were reported for a variety of species, including the horse (Getty, 1975). Other structures within the *medulla oblongata* which assisted in identifying the *nucleus ambiguus* were the hypoglossal nucleus, the dorsal motor nucleus of the vagus, the solitary tract, the lateral reticular nucleus and the olive. The position of the *nucleus ambiguus* in relation to the above structures is illustrated in Fig. 79.

A localisation of the neurones of the *nucleus ambiguus* into regions supplying specific intrinsic laryngeal muscles or groups of muscles has long been proposed (Crosby *et al*, 1962). Early evidence for such a distribution was obtained by means of nerve sectioning experiments, or by direct

electrical stimulation of nerve cells (Furstenberg *et al*, 1955). In the cat and the monkey, it was shown that the cell bodies innervating the *cricothyroideus* muscle were most rostrally placed, those innervating the adductor muscles were intermediate, and the abductor group was in a caudal position in the nucleus. Recent investigators have used horseradish peroxidase to trace retrograde axoplasmic flow from the muscles injected with the substance, to the cell bodies which supply them, in the *nucleus ambiguus* (Gacek, 1975; Hinrichson & Ryan, 1981; Pasaro *et al* , 1983). In the main, these workers verified earlier findings that motor neurones supplying the intrinsic laryngeal muscles in the cat and rat were located throughout the whole length of the *nucleus ambiguus* (Kalia & Mesulam, 1980; Hinrichson & Ryan, 1981; Pasaro *et al*, 1983). The medium sized cells in the rostral third of the nucleus innervated the *cricothyroideus* and *cricoarytenoideus dorsalis* muscles. These were more compactly arranged than the large neurones in the caudal two thirds of the nucleus, which supplied the adductor muscles and the *cricoarytenoideus dorsalis* muscle. It was noted that there was overlapping of the cells representing different muscle groups (Hinrichson & Ryan, 1981; Pasaro *et al* , 1983). The innervation of the intrinsic laryngeal muscles was found to be entirely ipsilateral (Gacek, 1975; Hinrichson & Ryan, 1981; Pasaro *et al*, 1983). Conflicting results were obtained in the cat by Gacek (1975), using the same horseradish peroxidase tracer techniques. He proposed a dual innervation of the *cricothyroideus* and *cricoarytenoideus dorsalis* muscles, with cell bodies in two separate nuclei, the *nucleus ambiguus* and rostral to it, the retrofacial nucleus. However, subsequent studies have not confirmed the presence of a separate retrofacial nucleus in the cat (Kalia *et al*, 1980; Pasaro *et al*, 1983) or the rat (Hinrichson & Ryan, 1981).

The localisation of the motor neurones of particular groups of laryngeal muscles in the *nucleus ambiguus* has been used to support the hypothesis of a central lesion being responsible for impaired function of individual or groups of laryngeal muscles. The occurrence of overlap between the groups of motor neurones, noted previously, could preclude this possibility.

It has been shown that following axotomy the nerve cell body undergoes marked changes in morphology and metabolism. The light microscopic manifestations of this usually reversible sequence of alterations is known as chromatolysis, or the "axon reaction". Consistent features of this process are the apparent disappearance of the Nissl substance (granular endoplasmic reticulum) from the cell body, migration of the nucleus to an eccentric position and swelling of the cell (Kirkpatrick, 1968; Cragg, 1970; Liebermann, 1971; Engh & Schofield, 1972; Graftstein, 1975; Prineas & Spencer, 1975). The term chromatolysis really only refers to the changes in the Nissl substance, while "axon reaction" refers to the overall process. Recovery of normal morphology is usual and occurs over a period of weeks or months (Liebermann, 1971). Ultrastructurally the Nissl bodies were shown to consist of concentrations of granular endoplasmic reticulum, with many polyribosomal complexes apparently lying free between cisterna of the endoplasmic reticulum. In chromatolytic neurones, there was disorganisation of the ordered cisternal arrays of endoplasmic reticulum, with replacement by short cisternal lengths. There was an apparent net loss of granular endoplasmic reticulum membrane, and dispersion of the remaining granular endoplasmic reticulum and free polyribosomes to the cell periphery. The proportion of free to membrane bound ribosomes increased (Liebermann, 1971). Recovery was associated with reconstitution of the granular endoplasmic reticulum (Prineas & Spencer, 1975).

In addition to these structural alterations to the neuronal cell body there are metabolic changes. Evidence from chemical and autoradiographic studies of ribonucleic acid (RNA) and protein metabolism in chromatolytic neurones supports the hypothesis of the primarily anabolic nature of this response (Liebermann, 1971; Grafstein, 1975; Prineas & Spencer, 1975). Following axotomy it has been demonstrated that there is increased nuclear RNA synthesis, increased nucleolar RNA content, and increased rate of passage of newly synthesised RNA from the nucleus to the cytoplasm. These changes were followed closely by increased cytoplasmic RNA content, increased cytoplasmic protein synthesis and increased cytoplasmic protein content (Watson, 1968).

The intensity of the alterations observed varies with the type of neurone, the age of the individual, the species of animal and the length of intact axon. The response varies from no structural changes at all, through to cell death (Cragg, 1970). The closer the site of injury to the cell body, the more pronounced the "axon reaction". Similarly, repetitive injury will enhance the morphological alterations (Engh & Schofield, 1972; Prineas & Spencer, 1975). Cell death occurs in some affected cells, and is seen more frequently in young animals (Cragg, 1970; Liebermann, 1971). Cells in an extreme chromatolytic state, or with organelle free cytoplasm are thought to be those which will eventually die (Liebermann, 1971).

Investigations of cell body responses in distal axonopathies have in the past revealed normal morphology, or only minor nonspecific changes, even in the presence of severe distal axonal degeneration (Cavanagh, 1964). It was considered that the distal distribution of the axonal degeneration in these conditions makes the chromatolytic response less frequent and less obvious than expected. However Prineas (1969b) observed changes said to be "not unlike

chromatolysis" in acrylamide intoxication. A number of other human peripheral nueropathies with distally distributed lesions have also been reported as producing changes resembling the "axon reaction". These include the neuropathies associated with alcoholism, thiamine deficiency, porphyria and uraemia (Prineas & Spencer, 1975).

Recently, investigators have reconsidered the involvement of the cell body in distal axonopathies (Cavanagh, 1982a; Sterman, 1982a; 1982b; 1983). They considered it unlikely that the cell body, which plays such a critical role in maintenance of the axon, should be unaffected by axonal disease. In these recent investigations of the toxic distal axonopathies produced by acrylamide (Cavanagh, 1982a; Sterman, 1982b) and 2,5-hexanedione (Sterman, 1982a; 1983), a spectrum of morphological changes resembling the "axon reaction" were demonstrated. Sterman concluded that the alterations seen were consistent with an axonal site of toxic action, and that an intact cell body would be expected to modify its metabolism to repair toxin induced damage to, or depletion of, materials in the axon. The perikaryal response seen was different for the two toxic chemicals, suggesting specific cell body reactions with different distal axonopathies (Sterman, 1982a; 1982b; 1983).

The nature of the signal which initiates the "axon reaction" has not been clearly defined. In his review of the subject, Cragg (1970) concluded that probably more than one mechanism is involved. The most likely theories offered were loss of a trophic influence from the periphery, or loss of repressor substances which inhibit anabolic activities of the cell body.

Long spinal tracts

The dorsal column of the spinal cord is made up of the gracile and cuneate tracts. These contain long ascending sensory fibres from the hind and forelimbs respectively, which originate in the dorsal root ganglia and terminate in the gracile and cuneate nuclei in the dorsal *medulla oblongata*. Fibres from the proprioceptive endings in muscle, below the level of the fifth thoracic segment, synapse with a group of large cells at the base of the dorsal horn of the spinal cord. These secondary cells send their axons cranially in the dorsal spinocerebellar tract, which ascends in the dorsolateral column of the white matter of the spinal cord to the cerebellum. Above the fifth thoracic segment the primary proprioceptive fibres from muscle do not synapse with secondary cells in the spinal cord, but course cranially to the lateral cuneate nucleus. This nucleus is located in the *medulla oblongata*, lateral to the gracile and cuneate nuclei. Thus, while proprioceptive fibres from the hindlimbs synapse in the spinal cord, those from the forelimbs run directly from their muscle of origin to the lateral cuneate nucleus (Bowsher, 1962).

Evidence of axonal pathology in these long spinal tracts manifests as axonal spheroids, on haematoxylin and eosin staining, which represent swollen degenerating/regenerating axons (Lampert, 1967). The presence of these structures has been demonstrated in normal humans (Brannon *et al*, 1967), and animals (Dayan, 1971), including the horse (Puscher *et al*, 1971). Their frequency is known to increase with age (Dayan, 1971).

MATERIALS AND METHODS

At *post mortem* the brain was removed from 14 experimental animals. These animals were divided into three groups, control, subclinical and clinical. As detailed in Part II

of this thesis, classification was based on the presence or absence of clinical signs of laryngeal hemiplegia, and on the finding of obvious intrinsic laryngeal muscle or recurrent laryngeal nerve pathology. The *medulla oblongata* was obtained and fixed in 10% formol saline for at least 2 weeks. Tissue blocks were taken from three sampling sites in the *medulla oblongata*, on both left and right sides (Fig. 78). The blocks were embedded in paraffin wax,[1] and sections cut at 3 µm. These were stained with haematoxylin and eosin, and luxol fast blue with cresyl violet (Appendix 5). Haematoxylin and eosin staining will demonstrate the presence of inclusion bodies, cellular infiltrates and blood vessel changes, while luxol fast blue-cresyl violet stain will reveal any alterations to the Nissl substance of the neuronal perikarya. The cresyl violet stains the nucleus, cell body and part of the dendritic tree, while the myelin sheath is stained by the luxol fast blue (Prineas & Spencer, 1975).

Nucleus ambiguus

To assist in confirming the identification of the relatively illdefined *nucleus ambiguus*, sections of the medulla were examined from a horse with a known degenerative lesion of the right vagal and recurrent laryngeal nerves. This 4 year old, male Anglo-Arab presented clinically with regurgitation of food from both nostrils, dysphagia and right sided laryngeal paralysis. At *post mortem* a *Gastrophilus* larva was found in the roof of the right guttural pouch, lateral to the articulation of the hyoid bone, directly overlying the internal carotid artery. Two cranial nerves were seen coursing through this area, the vagal and accessory nerves. Histological examination revealed severe Wallerian degeneration in these nerves. In addition mild changes were present in the hypoglossal nerve. Thus, with the known presence of axonal damage close to the neuronal cell body, it was expected that features of the "axon

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1 Gurr's paraffin wax, melting point 56°C, Searle Scientific Services, High Wycombe, Bucks, England.

reaction" would be observed in the right sided nuclei of affected nerves, including the *nucleus ambiguus*. The sections of the *medulla oblongata* were viewed under a light microscope. The *nucleus ambiguus* was identified and examined for pathological changes.

Long spinal tracts

The distal portions of the long fibre tracts were examined in sections of the caudal *medulla oblongata*. The number of axonal spheroids present in a transverse section of a tract or nucleus was recorded.

RESULTS

Nucleus ambiguus

Sections of the *medulla oblongata* from the horse with known degeneration of the right vagal and recurrent laryngeal nerves were examined at the levels depicted in Fig. 78. These demonstrated changes consistent with axonal damage in several nuclei. Neurones showing chromatolysis, or more strictly speaking, the "axon reaction", were present in the dorsal vagal nucleus and the group of cells identified as the *nucleus ambiguus* (Fig. 80), on the right side, but not on the left. While this evidence does not definitively identify the collection of neurones as the *nucleus ambiguus*, the morphology of the cells, and their position relative to other nuclei and tracts within the *medulla oblongata* is in agreement with descriptions of the *nucleus ambiguus* in other species (Crosby *et al*, 1962; Pasaro *et al*, 1983). Thus, it was concluded that the collection of cells believed to represent the *nucleus ambiguus*, and identified as such in Fig. 79, was in fact this nucleus.

The *nucleus ambiguus* was identified on the left and right sides of the *medulla oblongata* in all the other experimental animals. At this level, the nucleus appeared as a scattered group of 5-15, medium to large multipolar neurones. No changes resembling the "axon reaction" or neuronal degeneration were observed in the sections examined. Because of the diffuse nature of this nucleus, the accurate counting of cells was not possible. However, a subjective assessment revealed no differences between the nuclei on the left and right sides of any individual (Figs. 81-84).

Long spinal tracts

Examination of the long ascending fibre tracts in the region of the caudal *medulla oblongata* revealed the presence of axonal spheroids in all animals. The most common site for these structures was the lateral cuneate nucleus (Table 13), as illustrated in Figs. 85 & 86. In addition, axonal spheroids were observed rarely in the dorsal spinocerebellar tracts of two young, "normal" horses and two old laryngeal hemiplegic animals.

TABLE 13: THE FREQUENCY OF AXONAL SPHEROIDS IN THE LATERAL CUNEATE MUSCLES

HORSE NO.	DISEASE STATUS	AGE (yrs)	FREQUENCY OF AXONAL SPHEROIDS	
			LEFT	RIGHT
1	Normal	0	0	1
2		1	5	3
3		1	3	7
4		1	3	3
5		2	8	3
6		3	50+	40
7	Sub-clinical	2	50+	50+
8		>7	18	50+
10		10	50+	50+
11		12	22	23
12	Clinical	2	8	50+
13		8	27	23
14		9	20	50+
15		22	7	23

DISCUSSION

Nucleus ambiguus

Although distal axonal pathology was severe in the left recurrent laryngeal nerves of the clinical cases of laryngeal hemiplegia, and less severe but still considerable in the subclinical cases, no morphological alterations were noted in the parent cell bodies of the *nucleus ambiguus*. In addition, there was no apparent loss of neurones from the nucleus as a result of degeneration, which occurs subsequent to a severe "axon reaction". In view of the fact that transection of an axon usually leads to a sequence of structural changes in the cell body, features of this change, such as dissolution of the Nissl substance, migration of the nucleus to an eccentric position and swelling of the cell, or the degeneration and loss of neurones, could be expected in the parent cell bodies of horses with severe recurrent laryngeal nerve pathology. The fact that this did not occur may be related to the distance between the cell body and the damaged axon. In the recurrent laryngeal nerve of horses, this distance is considerable. Even at the level of the aortic arch, where mild pathology is present, the distance from the cell body may be in excess of 150 cm (Cole, 1946; Duncan & Griffiths, 1973). Thus, Argyle's finding (1934) of left sided degeneration of cell bodies in the nucleus giving rise to the nerve fibres of the recurrent laryngeal nerve was not confirmed by the present investigation. Indeed the absence of any pathological alterations in the *nucleus ambiguus* is in accord with Duncan's findings (1975) in three laryngeal hemiplegic animals. This lack of any morphological evidence of cell body involvement in the pathological process of laryngeal hemiplegia, adds support to the theory that the primary damaging influence is on the axon itself, and not on the cell body with secondary distal axonal compromise. However, the lack of pathological changes in the *nucleus ambiguus* of laryngeal hemiplegic

animals is in conflict with the findings in the toxic distal axonopathies of acrylamide and 2,5-hexanedione (Sterman, 1982a; 1982b; 1983; Cavanagh, 1982a). In these latter conditions alterations similar to the "axon reaction" have been observed. In view of the acute nature of these toxic distal axonopathies, it is more likely that the "axon reaction" would be present, than in the chronic disease of laryngeal hemiplegia, where little active axonal degeneration is apparent at any one time. The experimental animals used in the toxic studies were rats, and so the distance from the axonal damage to the cell body would be small when compared with the length of the equine recurrent laryngeal nerve. Thus, it would be more likely that the "axon reaction" should occur in these distal axonopathies in the rat.

Long spinal tracts

Axonal spheroids, apparent histologically as eosinophilic masses, were observed in the lateral cuneate nuclei of all affected animals investigated. A previous extensive investigation of the equine central nervous system (Mayhew *et al*, 1978) reported the finding of up to five such spheroids in the lateral cuneate nuclei of normal horses. When axonal spheroids were present in diseased states, for example equine degenerative myeloencephalopathy, they were more numerous and there was usually an associated loss of neuronal cell bodies, astrogliosis and accumulation of a green-brown pigment, mainly in macrophages.

Examination of Table 1 reveals that axonal spheroids occur frequently in the lateral cuneate nuclei of all horses affected clinically or subclinically with laryngeal hemiplegia. Moreover, these structures were infrequent in the "normal" animals, with one exception. The presence of numerous spheroids in this 3 year old control animal suggests the possibility that an undefined nervous disease

was present in this case. However it can also be seen from Table 1 that all of the "normal" horses were young, and with only two exceptions, the clinical and subclinical laryngeal hemiplegic animals were older. Therefore, with the known increase in incidence of spheroids with age (Dayan, 1971), evidence for the apparent correlation of the frequent occurrence of spheroids with laryngeal hemiplegia must be interpreted with caution. However, when axonal spheroids were present in clinically and subclinically affected laryngeal hemiplegic animals, they did appear in the distal extremities of the long fibre tracts, the equivalent position of the nerve fibre pathology in the left and right recurrent laryngeal nerves. The occurrence of large numbers of spheroids in the two younger animals, one of which was clinically affected and one subclinically affected with the disease, suggests the presence of spheroids as part of the disease process of laryngeal hemiplegia. While these long central fibre tracts, in which the spheroids occur, do not approach the considerable length of the recurrent laryngeal nerves, they may not be very dissimilar in length from those distal nerves of the hindlimb, innervating the muscles which showed evidence of neurogenic pathology, as described in the preceding chapters. However, a postulated association of large numbers of spheroids with laryngeal hemiplegia, does not explain their presence in one young "normal" animal. Thus, it must be emphasised that these results do not allow any firm conclusions to be drawn. However, the possibility cannot be dismissed that the frequent occurrence of axonal spheroids in the lateral cuneate nuclei of horses affected with laryngeal hemiplegia, represents a central nervous system component of this apparent distal axonopathy, warranting further investigation of long central tracts in this disease.

PART V

THE PATHOLOGY OF STRINGHALT

INTRODUCTION

Stringhalt, a disease of horses characterised by exaggerated flexion of the hindlimbs, has long been recognised in epidemic and sporadic forms (Reakes, 1916; Kerrigan, 1917; Seddon & Belschner, 1926; Barry, 1956; Wheat, 1972; Lamont, 1977; Pemberton, 1979; Pemberton & Caple, 1980; Read & Kirk, 1983; Cahill *et al*, 1985). The pathology is poorly understood, but recent reports have shown both a peripheral axonal degeneration and lesions of the spinal cord (Lamont, 1977; Pass, 1978; Hartley, 1979; Munday, 1979; Pemberton & Caple, 1980). Although the aetiology of this disease is as yet unknown, a neurotoxic pathogenesis is considered to be the most likely (Seddon & Belschner, 1926; Barry, 1956; Lamont, 1977; Pemberton, 1979; Pemberton & Caple, 1980; Read & Kirk, 1983).

Traditionally, stringhalt has been thought of as a disease of the hindlimbs, but forelimb involvement (Reakes, 1916; Cahill *et al*, 1985), and signs of laryngeal dysfunction (Reakes, 1916; Kerrigan, 1917; Barry, 1956; Pemberton & Caple, 1980) may also be observed. Involvement of such divergent anatomical areas of the body suggests that the pathogenesis involves a polyneuropathy. To further define the pathological basis of stringhalt, a detailed investigation was undertaken on an affected 5 year old, 160 cm Thoroughbred gelding. This animal was 1 of 21 involved in an outbreak of the disease in New Zealand (Cahill *et al*, 1985). When examined initially, 12 weeks after onset, it showed severe clinical signs of stringhalt, and atrophy of the distal hindlimb muscles. Coincident with the onset of the disease, it had been noted that the horse called less often than usual, and its whinny became quiet and hoarse.

MATERIALS AND METHODS

Endoscopic examination

At the time of the initial examination, and immediately prior to destruction, 7 weeks later, the symmetry and movement of the larynx were observed with a fibreoptic endoscope, with the horse in a standing position.

Pathology

Muscle samples were collected immediately *post mortem* from the larynx, forelimbs and hindlimbs, and frozen within 1 hour of removal. All intrinsic laryngeal muscles were removed in their entirety and weighed. The forelimb muscles sampled were the long head of the *triceps brachii*, *extensor carpi radialis*, *flexor carpi ulnaris* and *flexor carpi radialis*. Muscles sampled from the hindlimbs included the *vastus lateralis*, *biceps femoris*, *gastrocnemius*, *extensor digitorum longus*, *tibialis cranialis* and *peroneus tertius*. The muscle samples were processed, sectioned and stained by the methods described in Part III of this thesis. The degree of pathology present in each muscle was assessed according to the grading system adapted from Anderson (1984), and presented in Part III.

Peripheral nerve samples were collected from the sites illustrated in Figs. 15 & 87. In addition, the tibial nerve was sampled at the level of the junction of the *gastrocnemius* muscle with its tendon. The only forelimb nerve collected was the median, at a site medial to the elbow joint. Excluding the intrathoracic segments of the recurrent laryngeal and vagal nerves, all nerve samples were taken from the anaesthetised animal. These samples were then processed as described in Part II of this thesis, for examination by light and electron microscopy, and for teased fibre preparations. Quantitative investigations

including internode length estimations and myelinated fibre diameter distributions, were also performed, as described in Part II.

The central nervous system was removed and fixed in 12.5% formol saline. Selected areas from a variety of sites in the hindbrain and spinal cord were embedded in paraffin, and 3 µm sections stained with haematoxylin and eosin, and luxol fast blue and cresyl violet.

RESULTS

Endoscopic findings

At the initial examination a visual comparison of the left and right arytenoid cartilages revealed that left abduction during inspiration was less than the right. Between respirations, the left cartilage was more upright and closer to the midline. At the subsequent examination, 7 weeks later, inspiratory abduction of the left arytenoid cartilage was absent, whereas the right cartilage appeared to hyperabduct. The positioning of the left arytenoid cartilage between respirations was unchanged.

Muscle pathology

At *post mortem*, the *cricoarytenoideus dorsalis* and *cricoarytenoideus lateralis* muscles appeared paler than normal.

TABLE 14: WEIGHTS OF THE LARYNGEAL MUSCLES (g) IN STRINGHALT

MUSCLE	LEFT	RIGHT
cricoarytenoideus dorsalis	5.77 (8.17)*	5.60 (8.70)
cricoarytenoideus lateralis	3.21 (3.04)	4.09 (3.79)
cricothyroideus	5.77 (5.76)	6.56 (5.74)
arytenoideus transversus	2.75 (2.38)	2.80 (2.72)
ventricularis	1.30 (2.23)	1.68 (2.65)
vocalis	4.21 (5.10)	4.20 (5.73)

* Muscle weights of Thoroughbred horses (Quinlan *et al*, 1975)

Individual muscle weights (Table 14) showed a marked decrease in size of the *cricoarytenoideus dorsalis* muscles, and a lesser decrease in *ventricularis* and *vocalis* . Histopathological changes indicative of neurogenic disease were observed in all intrinsic laryngeal muscles from both sides, but were more severe in those from the left. These changes and their severity are summarised for each muscle in Table 15, and depicted in Figs. 88-91.

The most severe changes were noted in the *cricoarytenoideus dorsalis* , *cricoarytenoideus lateralis* , *ventricularis* and *vocalis* muscles, and involved fatty and fibrous replacement of muscle fibres (Fig. 88). As observed in Fig. 89, the right sided muscles were less severely affected than their left counterparts. However, even in these severely affected muscles, small bundles of hypertrophic fibres also existed. The least affected intrinsic laryngeal muscle was the right *arytenoideus transversus* , in which the majority of fibres were normal except at the edges of fascicles, where angular atrophic fibres were observed. No abnormalities were noted in either *cricothyroideus* muscle. Myosin ATPase staining showed fibre type grouping indicative of reinnervation, in the left and right *cricoarytenoideus lateralis* and left *vocalis* muscles only.

TABLE 15: MUSCLE PATHOLOGY IN A CASE OF STRINGHALT

MUSCLE	DEGREE OF PATHOLOGY	
	LEFT	RIGHT
LARYNGEAL		
cricothyroideus	-	-
cricoarytenoideus lateralis	++++*	+++*
cricoarytenoideus dorsalis	++++	++++
ventricularis	++++	+++
vocalis	++++*	+++
arytenoideus transversus	+++	++
HINDLIMB		
vastus lateralis	-	-
biceps femoris	+*	++*
gastrocnemius	++	++
peroneus tertius	++*	++*
extensor digitorum longus	++*	+*
tibialis cranialis	+++*	+++*
FORELIMB		
triceps brachii	-	-
extensor carpi radialis	+*	-
flexor carpi ulnaris	++*	-
flexor carpi radialis	-	++*

- no pathology
+ mild pathology
++ moderate pathology
+++ marked pathology
++++ severe pathology
* fibre type grouping present

Hindlimb muscles showed similar histopathological changes (Fig. 90) to those seen in the laryngeal musculature. However, degenerative changes tended to be less severe than in the larynx, while more frequently observed fibre type grouping indicated increased reinnervation of previously denervated muscle. This fibre type grouping was present in all of the distal hindlimb muscles examined, with the exception of the *gastrocnemius*. In general, the more distal muscles showed more severe pathological changes. Forelimb muscles showed only mild lesions indicative of neurogenic disease, but fibre type grouping was observed in the more distal muscles sampled (Fig. 91).

Pathology of peripheral nerves

LIGHT MICROSCOPY

All samples of recurrent laryngeal nerves examined in transverse sections of epoxy resin stained sections, showed a loss of myelinated fibres, with a distally graded increase in severity (Figs. 92-94). The fibre loss was more marked in the left nerve than in the right. Immediately caudal to the larynx, the left nerve contained only a few myelinated fibres (Fig. 93). There was also widespread myelin debris present in Schwann cells and macrophages, both of which were present in increased numbers. Again, this change was progressively more severe in distal areas of the nerve. The extent of degenerative changes at the distal end of the right recurrent laryngeal nerve appeared similar to those of the midcervical level of the left nerve, while the severity of changes at the right midcervical area was similar to those at the proximal level of the left nerve.

Regenerating clusters of nerve fibres, and fibres with disproportionately thin myelin sheaths in relation to their axonal calibre, were more common in the proximal parts of affected nerves (Fig. 97). "Onion bulb" formations, usually surrounded thinly myelinated fibres, and were also more frequently observed in proximal areas (Fig. 97).

Renaut bodies, a common feature of normal equine peripheral nerve, were observed at all levels of the left and right recurrent laryngeal nerves, with the exception of the left nerve at the level of the aortic arch.

Transverse sections of the right vagus showed two peripherally placed fascicles of medium diameter myelinated fibres, with pathological changes. Although there was no loss of nerve fibres, fibres with abnormally thin myelin sheaths, early "onion bulb" formations and an occasional regnerating cluster were observed.

The hindlimb nerves exhibited similar pathological changes to those observed in the recurrent laryngeals (Fig. 95). The same distally progressive increase in severity was present, although overall the pathology was less severe. While not present in every section, Renaut bodies were a common finding. The median nerve of the forelimb also showed similar changes, but without noticeable loss of myelinated fibres (Fig. 96).

TEASED FIBRE PREPARATIONS

Teased myelinated fibres, graded according to the scheme of Dyck (1975a), showed a high incidence of abnormalities in all nerves examined. This ranged from 34% in the branch of the peroneal nerve innervating the *biceps femoris*, to 83% in the left recurrent laryngeal nerve immediately caudal to the larynx (Table 16).

TABLE 16: GRADING OF TEASED FIBRES OF RECURRENT LARYNGEAL AND LIMB NERVES FROM A HORSE WITH STRINGHALT (Dyck, 1975a)

SAMPLE	% OF FIBRE TYPES*							
	A	C	D	E	F	G	I	ABNORMAL
RECURRENT LARYNGEAL NERVES								
left distal	17	12	5	61	3	2		83
left midcervical	42	7		50	1			58
left proximal	55	9		29	2		5	45
right distal	65			35				35
right midcervical	57	1		42				43
right proximal	62	4		30(7)	2		2	38
LIMB NERVES								
left tibial	58	2		40(5)				42
right tibial	53	1		46				47
left superficial peroneal	48	2		50				52
left branch deep peroneal	38			61(13)		1		62
right branch deep peroneal	36	1		63(11)				64
left branch to EDL	64			36(11)				36
right branch to EDL	40			60				60
rt motor branch deep peroneal	42			54(9)	2	1		57
left common peroneal	44			54(7)		2		56
right common peroneal	46			54				54
left branch to BF	54		2	44				46
right branch to BF	66	2	2	30				34
right median nerve	48	5		38	4	2	3	52

EDL *extensor digitorum longus* muscle

BF *biceps femoris* muscle

*A, normal appearance. C, segmental demyelination. D, segmental demyelination and remyelination. E, axonal degeneration. F, segmental remyelination. G, focal thickenings of myelin. I, fibre with several proximal internodes with or without proximal paranodal or segmental demyelination, and distal to those degeneration (Dyck, 1975a).

( ) E fibres with large myelin ovoids

The predominant abnormality observed was that of axonal degeneration (E), of which two variations were observed. The majority of fibres classified as type E had small fragments of myelin debris scattered along the vestiges of each myelinated fibre (Fig. 98). In contrast, seven of the nerves studied contained, in addition, continuous rows of large myelin ovoids, as depicted in Fig. 99, and in parenthesis in Table 3.

Changes indicative of demyelination (type C) and remyelination (type F), and combinations of these two processes (type D) were observed, but less frequently than type E changes. The demyelination was usually paranodal in distribution (Fig. 100), while remyelinated segments appeared as short intercalated internodes.

Only three nerves contained fibres with type I change, in which there is paranodal demyelination or normal nodal architecture proximal to myelin ovoids (Fig. 101). Paranodal demyelination was usually observed in these fibres.

ELECTRON MICROSCOPY

Ultrastructural examination of nerves extended the light microscopic findings. Denervated Schwann cells, bands of Bungner, (Figs. 102 A & C) were seen in all nerves with the exception of the median and the branch of the peroneal innervating the *biceps femoris*. Myelin debris was seen within Schwann cell cytoplasm, Schwann cell processes and in macrophages. This ranged from large lamellated myelin ovoids to complex osmiophilic structures of varying morphology, often with a multiloculated appearance (Fig. 102 A-d). In addition smaller elongate structures, known as protagon granules (Fig. 103D), and lipid droplets or μ bodies (Fig. 102F) were commonly seen in Schwann cell cytoplasm. A small proportion of fibres with disproportionately thick myelin sheaths relative to their

axonal calibre were interpreted as atrophic fibres (Fig. 103A).

Regenerative features included unmyelinated axonal sprouts (Fig. 102C & D) and clusters of regenerating fibres (Fig. 103A). Excess convoluted basement membrane was seen in association with these, and with thinly myelinated fibres (Fig. 103D-F). Less commonly seen were early "onion bulb" formations. Regenerating axonal sprouts in some instances had a lucent floccular appearance, while others contained densely packed disoriented neurofilaments (Fig. 102D). Other abnormalities noted were the presence of dense membranous bodies (Fig. 103B), the margination of microtubules (Fig. 102E), and a distension of the adaxonal Schwann cell cytoplasm (Fig. 102F).

MORPHOMETRY

Fibre diameter distributions

The distributions of the populations of myelinated fibres in the recurrent laryngeal nerves from the horse with stringhalt are presented as histograms in Fig. 104. The populations were analysed statistically at distal, midcervical and proximal sampling levels in both left and right nerves. The mean fibre diameters are recorded in Table 17.

TABLE 17: MEAN MYELINATED FIBRE DIAMETERS (μm) IN THE RECURRENT LARYNGEAL NERVES FROM A HORSE WITH STRINGHALT

NERVE LEVEL	FIBRE DIAMETER	STANDARD DEVIATION
L distal	6.4	1.8
L midcervical	7.3	2.3
L proximal	7.4	2.7
Rt distal	7.5	2.3
Rt midcervical	7.8	1.3
Rt proximal	6.4	1.8

At all levels of the left and right recurrent laryngeal nerves, the myelinated fibre diameter distributions differed markedly from those of the control animals (Part II, Figs. 40-42 and Table 5). In all instances there was a shift to smaller diameters, as well as alteration of the shape of the distributions. The normal major peak of large fibres was reduced in height, while the lower peak of small fibres was increased. This gave the distributions a more bimodal appearance than is normal for recurrent laryngeal nerve. This selective loss of large diameter fibres was reflected by the decrease in mean fibre diameter (Table 17), significant at the 1% level in all instances using the students t test. There appears to be a proximal to distal increase in severity of large fibre loss, and more marked involvement of the left nerve than the right.

When compared with the histograms and mean fibre diameters for laryngeal hemiplegic horses (Part II, Figs. 40-42 and Table 5), while there was a similar selective loss of large diameter fibres, it was more extensive along both the left and right recurrent laryngeal nerves in stringhalt.

Internode lengths

Detailed statistics for the internode lengths of the recurrent laryngeal nerves from the horse with stringhalt are presented in Appendix 11. A summary of the data, illustrating the obvious trends, is presented below (Table 18).

TABLE 18: MEAN INTERNODE LENGTH OF NORMAL (A) FIBRES OF RECURRENT LARYNGEAL NERVE FROM A HORSE WITH STRINGHALT

NERVE LEVEL	MEAN IL * (µm)	CV ** (%)
left distal	732	10.9
left proximal	1069	7.3
right distal	755	9.8
right proximal	935	8.3

* IL internode length
** CV coefficient of variation

There was a decrease in mean internode length in recurrent laryngeal nerve from the horse with stringhalt, when compared with values for the control animals (Appendix 9), in the distal sampling levels of both left and right sides. There was also a proximal to distal decrease in internode length in recurrent laryngeal nerve affected with stringhalt, on both left and right sides, although the decrease was slightly greater on the left. The increased variability of internode length, noticeable only at the distal sampling levels, was not as severe as that seen in laryngeal hemiplegia.

Central nervous system pathology

The brain and spinal cord were essentially normal, except for a small number of axonal swellings (spheroids) at various levels between the *medulla oblongata* and *cauda equina*. In the latter, three spheroids were present in the ventral corticospinal tract of one side, and one on the other side. At the level of the cranial cervical spinal cord, one spheroid was present in the gracile and one in the spinocerebellar tract. Sections from the *medulla oblongata*

demonstrated the presence of three spheroids in the gracile tract and nucleus.

DISCUSSION

The pathological investigation of this animal with stringhalt, indicated the existence of a distal axonopathy, preferentially involving the longest nerves. The degree of pathology was greatest in the nerve known to be the longest in the horse (Duncan & Griffiths, 1973), the left recurrent laryngeal, and was least in the shortest nerves studied. Moreover, in the affected nerves, a proximal to distal increase in the severity of damage was noted. The predominant finding in all histological preparations was one of axonal degeneration, indicating a primary effect of the disease on the axon. However, there did not appear to be any involvement of long nerve fibres within the central nervous system.

Histological examination of muscle sections, demonstrated neurogenic atrophy of varying degrees. The degree of muscle pathology followed the same pattern observed in the nerve sections, and was directly related to the length of nerve supplying the particular muscle.

The 4 month duration of the disease was in keeping with the pathological findings in the peripheral nerves. Of the type E degenerated fibres observed in teased fibre preparations, the vast majority consisted of filamentous strands of tissue with small remnants of myelin debris spaced out along them. Thus, it seemed that consequent to the initial axonal degeneration, the process of myelin degradation and the removal of debris was almost complete, a process known to take as long as several months (Nathaniel & Pease, 1963). Light and electron microscopic findings also confirmed the predominance of these chronic changes. However, in all histological preparations, a low incidence

of active axonal degeneration was also present. This was indicated by;

(i) type E teased fibres composed of large myelin ovoids, fibres which indicate an early stage of fragmentation of the myelin sheath and axis cylinder in the process of axonal degeneration (Dyck *et al* 1968).

(ii) the ultrastructural observation of Schwann cells occupied by large myelin ovoids, with the preserved lamellar pattern of myelin, a pattern supposedly retained for approximately 6 days after degeneration commences (Nathaniel & Pease, 1963).

(iii) apparently degenerating axonal sprouts, seen on electron microscopic examination.

(iv) the type I teased fibres, with distal degeneration, and usually proximal paranodal demyelination on the same fibre. These fibres have been reported in a number of distal axonopathies, including uraemic neuropathy (Dyck *et al*, 1971a), thiamine deficiency (Takahashi & Nakamura, 1976) and acrylamide intoxication (Hopkins, 1970). It has been suggested that these fibres have been sampled at the site in the fibre, distal to which degeneration has occurred. Proximal to this point paranodal demyelination may be seen, secondary to the primary pathological changes within the axon (Dyck *et al*1971a).

A number of ultrastructural alterations were observed, which supported the primary axonal nature of the disease. These included;

(i) degeneration of axonal sprouts, unmyelinated and myelinated fibres.

(ii) the accumulation and rearrangement of axoplasmic organelles. These are reported in a number of distal

axonopathies, including hexacarbon intoxication, giant axonal neuropathy and buckthorn intoxication (Asbury *et al*, 1972; Spencer *et al*, 1980; Heath *et al*, 1982).

(iii) projections of adaxonal Schwann cell cytoplasm extending into the axoplasm and a distension of the adaxonal Schwann cell cytoplasm. These may indicate attempts by the Schwann cell to sequestre excess or abnormal organelles from the axon (Spencer & Thomas, 1974; Spencer & Schaumburg, 1976). More extensive axon-Schwann cell networks are particularly well developed in the toxic distal axonopathies.

(iv) large numbers of collagen pockets. These are seen in many chronic neuropathies, and are thought to reflect the loss of unmyelinated fibres (Thomas, 1973).

(v) increased numbers of μ and Π granules in Schwann cell cytoplasm, features of axonal degeneration and regeneration and of remyelination (Schroder, 1975; Landon & Hall, 1976).

The ability of the peripheral nerve to regenerate, even in the presence of continued degeneration, was reflected by the frequent appearance of regenerating clusters. However, there was some evidence to indicate that not all the attempts at regeneration were successful. Continued degeneration and demyelination were apparent in some fibres of the clusters. Some of these fibres contained recent myelin ovoids in their Schwann cell cytoplasm, and in others accumulated organelles, indicative of local deranged axonal transport were seen. The presence of atrophic nerve fibres in regenerated nerve has been said to represent the regression of regenerated fibres, following their failure to reinnervate the endorgan (Schroder, 1972). The atrophic fibres observed in this investigation probably resulted from the above process.

The quantitative techniques used on the recurrent laryngeal nerves confirmed that the pathology of stringhalt involved a distally distributed, selective loss of large diameter myelinated fibres. In addition, they demonstrated that while similar in some aspects, the pathology resulting from stringhalt and laryngeal hemiplegia are different. Stringhalt appeared to involve a more acute pathological process, affecting more extensive lengths of vulnerable nerve fibres. Thus, the clinical signs are more widespread in stringhalt. The amount of regenerative activity present was considerably greater than seen in laryngeal hemiplegia. Regenerating clusters were more evident in proximal areas of affected nerve, and thus masked some of the fibre loss, and increased the population of small diameter fibres.

Evidence of segmental demyelination and remyelination was observed in the form of paranodal demyelination or intercalated internodes, and was most obvious in the recurrent laryngeals. Presumably these changes were consequent to axonal pathology, representing secondary segmental demyelination as seen in the type I fibres. The incidence of secondary segmental demyelination in peripheral neuropathies has been reported to be related to their duration, a higher incidence occurring in chronic conditions (Dyck, 1975a; Spencer & Schaumburg, 1977; Bennington, 1978). The incidence of segmental demyelination and remyelination in equine laryngeal hemiplegia (Duncan *et al*, 1978) was considerably greater than was seen in the nerves from this horse with stringhalt. Presumably this is a reflection of the more chronic nature of the former disease. Structures generally associated with repetitive episodes of segmental demyelination and remyelination of nerve fibres, "onion bulbs" were also noted in a number of the nerves examined (Webster *et al*, 1967; Dyck, 1975a; Schroder, 1975; Madrid *et al*, 1977).

Renaut bodies, structures known to be particularly prominent in equine peripheral nerve (Renaut, 1881), were observed frequently, but not invariably in all peripheral nerves examined. They did appear more consistently in sections of the recurrent laryngeal nerves. In a recent investigation they were reported to be "not prominent" in any of the peripheral nerves, other than the recurrent laryngeal (Duncan, 1975), a finding not reflected by the present study. The incidence of these structures did not appear to be related to the degree of pathology present.

The abundance of fibres with disproportionately thin myelin sheaths in a number of the recurrent laryngeal and limb nerve samples could indicate regenerated or remyelinated nerve fibres. It seems likely that they represent the former, because of the low incidence of demyelination and the high incidence of degeneration noted in teased fibre preparations.

Recovery from stringhalt probably depends on regeneration of axons, and thus will be a protracted process, particularly when one considers the large distance axons must regenerate. For instance, in this case, regenerating clusters were observed at the aortic arch. From here they would have to grow along the length of the recurrent laryngeal nerve to reinnervate the appropriate intrinsic laryngeal muscle. Moreover, while regeneration of nerve fibres, and thus recovery of function is possible, there may be lasting changes in the muscles. Alterations in fibre types could occur, and permanently affect muscle function. In a competitive animal such as the racing Thoroughbred, this may result in impairment of performance.

The findings from this pathological investigation of a case of stringhalt, indicate that this disease falls into the group of conditions known as distal axonopathies. For confirmation of these findings, detailed investigations of more horses with stringhalt should be performed, as it is possible that more than one pathological process may cause the characteristic clinical signs of this disease.

PART VI

FINAL DISCUSSION

The results of this pathological investigation indicate that equine laryngeal hemiplegia may be classified as a distal axonopathy. This group of conditions includes many naturally occurring and toxic neuropathies of man and animals. Characteristically, these polyneuropathies are bilaterally symmetrical, with distal axonal degeneration of the longest and largest fibres of peripheral nerves and central nervous system tracts (Cavanagh,1964; Spencer & Schaumburg, 1976; Spencer & Schaumburg, 1978a). In addition to axonal degeneration, segmental demyelination and remyelination are sometimes seen. The myelin sheath degeneration is secondary to axonal changes (Spencer & Schaumburg, 1978a), and the extent to which it occurs varies directly with the rate of axonal damage (Spencer & Schaumburg, 1976). Differences in the nature of the degenerative processes occur within this group of diseases; some pursue a slow course, while others are rapidly progressive; some predominantly affect sensory nerves while others affect mainly motor nerves; some are predominantly centrally located while others are peripheral conditions; and some are progressive while others are reversible (Spencer *et al*, 1979).

The term "dying-back" was first introduced to describe this group of conditions (Cavanagh, 1964). While it provided an adequate description of the distribution of changes in nerve trunks, it inaccurately described the development of changes in individual nerve fibres. In the toxic distal axonopathies a multifocal distribution of axonal pathology rather than terminal, and centripetal spread of degeneration occurs (Spencer & Schaumburg, 1976). It appears that there is simultaneous degeneration of a

distal length of nerve fibre, commencing at one of the sites of axonal pathology. To more accurately describe this process, the term distal axonopathy was introduced (Spencer & Schaumburg, 1976).

This study of laryngeal hemiplegia demonstrated these pathological characteristics of distal axonopathies. Although the pathology observed was not bilaterally symmetrical in the recurrent laryngeal nerves, explained by the difference in their lengths, preferential involvement of the longest and largest diameter nerve fibres, and a proximal to distal gradation of pathology occurred. Changes in the hindlimb nerves and muscles, and possibly in the long fibre tracts of the central nervous system indicate the existence of a polyneuropathy, suggesting a more widespread distribution of the neuropathy of equine laryngeal hemiplegia than has previously been observed. The common factor in these pathological changes, is the selective involvement of the distal ends of long nerve fibres.

Many naturally occurring and experimental conditions of man and animals result in distal axonopathies. Toxic, metabolic and genetic diseases may be involved. Some of the toxins capable of producing these diseases are the hexacarbon compounds (Spencer *et al*, 1980), carbon disulphide (Cavanagh, 1979), acrylamide (Hopkins, 1970), isoniazid (Jacobs *et al*, 1979), the organophosphates (Bouldin & Cavanagh, 1979a, 1979b), thallium (Cavanagh, 1979), arsenic (Cavanagh, 1979), and the nitrofurans drugs (Cavanagh, 1979). Distal axonopathies associated with metabolic dysfunction include deficiencies of several of the B group vitamins (Collins *et al*, 1964; Prineas, 1970), uraemia (Dyck *et al*, 1971a), diabetes mellitus (Sugimura *et al*, 1980), porphyria, alcoholism and malnutrition (Dyck, 1975a). Genetically determined disorders associated with this pathology are Friedreich's ataxia (Dyck & Lais, 1973; Schoental & Cavanagh, 1977; Cavanagh, 1979), peroneal muscular atrophy (Cavanagh, 1964), and giant axonal

neuropathy (Asbury *et al*, 1972). Other naturally occurring conditions of man are amyotrophic lateral sclerosis (Cavanagh, 1964; 1979) and Werdnig-Hoffman's disease (Cavanagh, 1964; 1979). The occurrence of naturally occurring distal axonopathies in animals has also been recently documented, and include canine giant axonal neuropathy (Duncan & Griffiths, 1977; Duncan, 1980), distal denervating disease (Griffiths & Duncan, 1979) and sensory neuropathy of Dachsunds (Duncan & Griffiths, 1982).

The pathogenesis of distal axonal degeneration is as yet unknown, but many theories have been proposed to explain why the axon responds in a stereotyped manner to such a wide variety of apparently unrelated conditions. It is extremely unlikely that any one pathogenic mechanism is responsible for the distal axonal degeneration seen in this varied group of neuropathies. While sharing many features in common, differences do exist between individual diseases, particularly in the ultrastructural alterations observed. These structural changes have been said to be a reflection of biochemical alterations, and thus it is probable that several or many subcellular biochemical lesions can produce a distal axonopathy (Thomas, 1980). The particular initiating lesion, whether initiated by neurotoxic, genetic or metabolic means, will be determined by the causative agent, and the selective vulnerability of the neurone or region of the axon to the agent.

The current most popular theory on the pathogenesis of distal axonopathies is one of direct local axonal damage. The theory that these diseases might be initiated by such damage along the entire length of the axon, was prompted by the demonstration of the multifocal nature of the axonal pathology, not confined to the extremity of the nerve fibre (Spencer & Schaumburg, 1976; 1978b; Cavanagh, 1979; Spencer *et al*, 1979). Spencer and coworkers (1979) postulated that the substances which produce distal

axonopathies may act at related sites in metabolic pathways, particularly those concerned with energy production. It was assumed that the enzymes required for these metabolic processes were manufactured in the cell body and transported to the axon. Under normal conditions, the axonal demand for these enzymes could be met by the cell body. However, with depletion or inactivation of these enzymes along the entire length of the axon, the demand would exceed the ability of the cell body to resupply them. While supply may be adequate for the proximal regions of the nerve fibre, the distal areas would remain deficient in the essential substances. Thus, depletion of energy production in the distal axon may interfere with axoplasmic transport in these regions, and precipitate axonal degeneration. The selective vulnerability of long and large diameter axons was explained by the relatively greater metabolic demands of this population of nerve fibres. It was suggested that with continued intoxication the progression of pathology to involve more proximal regions of nerves, could be explained by continued depletion of the enzymes, outstripping the supply from the cell body.

A similar theory, based on the hypothesis proposed by Schoental & Cavanagh (1977), also implicating abnormal energy production, was based on the cofactor requirements of the neurone. These authors postulated that cofactors were inactivated by toxic substances, metabolic or genetic factors, along the length of the axon, with their replenishment dependent on supply of the cofactor or its precursors from the cell body. With these increased demands placed on the cell body, sufficient cofactor would be supplied to proximal regions of the axon only, leaving the distal areas depleted. This may then result in inadequate production of energy, for processes such as axoplasmic transport and impulse transmission, with subsequent degeneration of the distal axon.

Another proposal for the pathogenesis of the neurofilamentous toxic distal axonopathies is that the neurofilaments themselves are the target site for the neurotoxins within the axon. It has been suggested that the hexacarbon compounds, carbon disulphide and acrylamide may bind to the neurofilaments, and so alter their function (Savolainen, 1977). As the neurofilaments move along the axon by slow axoplasmic transport, there is progressive intramolecular and intermolecular crosslinking of the neurofilament proteins, induced by the neurotoxin, until no further movement of the continuously growing aggregate is possible (Graham, 1980; Graham & Abou-Donia, 1980; Graham *et al*, 1980; Anthony *et al*, 1983). The axonal degeneration seen is an indirect consequence of the accumulation of neurofilaments with obstruction at the nodes of Ranvier, probably interfering with the flow of essential nutrients to the more distal regions (Cavanagh, 1982b). Although this process may be important in some of the human distal axonopathies, the accumulation of neurofilaments was not a prominent feature of the pathology of equine laryngeal hemiplegia.

The conclusion that the neuropathy of laryngeal hemiplegia is a distal axonopathy, explains the vulnerability of animals with certain physical characteristics. Large horses, known to be particularly susceptible to laryngeal hemiplegia (Hobday, 1935; Tagg, 1935; Dornblaser, 1967; Marks *et al*, 1970a; 1970b; Cook, 1975b; Goulden & Anderson, 1981a), would be expected to have longer recurrent laryngeal nerves, and thus be predisposed to this disease of long nerve fibres. Similarly, the left sided nature of the clinical manifestations of the disease can be explained by the pathological process affecting the distal ends of the longest nerve fibres in the horse. The early involvement of the *cricoarytenoideus lateralis* muscle may also be explained by the fact that total axonal volume is important in the vulnerability of nerve fibres to distal axonal degeneration. Long and large diameter axons have

a marked susceptibility (Spencer & Schaumburg, 1978a; 1978b; Bouldin & Cavanagh, 1979a; 1979b; Spencer *et al*, 1979; Sumner, 1978). This characteristic was considered as a possible reason for the selective vulnerability of the *cricoarytenoideus lateralis* muscle early in the disease process of laryngeal hemiplegia. It was postulated, and subsequently demonstrated, on a limited number of samples, that the nerve innervating this muscle had a higher proportion of large diameter fibres than the *cricoarytenoideus dorsalis* muscle. Thus, in a disease process preferentially affecting large diameter fibres, one would expect the *cricoarytenoideus lateralis* muscle to be more susceptible.

A new insight into the aetiology of laryngeal hemiplegia can be gained by its classification as a distal axonopathy. Of great significance, is the evidence that the disease is a generalised neuropathy affecting widely divergent anatomical areas. Thus, many of the aetiologies postulated for laryngeal hemiplegia in the past, which specifically consider damaging influences acting on the left recurrent laryngeal nerve, can be dismissed. This includes compression of this nerve as it passes around the aortic arch (Duncan *et al*, 1978), and stretch of the nerve (Rooney & Delaney, 1970). The remaining possible aetiologies considered in Part I of this thesis, including bacterial and viral agents, plant and chemical intoxications and vitamin deficiencies remain as conceivable aetiological factors of this disease.

Infections may result in the pathological lesions of a distal axonopathy, either by the production of neurotoxins, or by increasing the requirements for various enzymes or cofactors, necessary for normal axonal function. However, no such association has been recorded in other species.

Certain plant and chemical intoxications are known to produce laryngeal paralysis in the horse (Fleming, 1889; Argyle, 1934; Hutyra *et al*, 1938; Schebitz, 1964; Cook, 1970; Rose *et al*, 1981). Although these are apparently not related to the widespread disease of idiopathic laryngeal hemiplegia (Goulden & Anderson, 1981a), they may provide insight into the pathology produced by neurotoxic compounds in the recurrent laryngeal nerves. Of the toxic distal axonopathies, involvement of the recurrent laryngeal nerve has been reported with organophosphate (Wells & Bradley, 1976; Bouldin & Cavanagh, 1979a; Bouldin & Cavanagh, 1979b), and acrylamide intoxication (Hopkins, 1970).

Some organophosphates have delayed neurotoxic activity, with the production of a predominantly motor, distal neuropathy, selectively affecting large diameter, long fibres of peripheral and central nervous systems (Spencer & Schaumburg, 1976; Schoental & Cavanagh, 1977; Cavanagh, 1979). A specific instance of organophosphate poisoning of horses was reported in Australia by Rose and coworkers (1978; 1981). Several foals developed signs of dyspnoea and inspiratory stridor, with endoscopic evidence of laryngeal paralysis. Pathological studies performed on these foals showed evidence of recent active axonal degeneration, affecting most fascicles of both left and right recurrent laryngeal nerves. The loss of myelinated fibres was most severe in the distal left recurrent laryngeal nerve, with only a mild loss in the right nerve. In a chronic case studied there was evidence of neurogenic atrophy in the *cricoarytenoideus dorsalis* and *cricoarytenoideus lateralis* muscles. While the clinical signs were only referable to recurrent laryngeal nerve paralysis, it is possible that the long limb nerves were also affected distally. However, other peripheral nerves were not examined.

Involvement of the recurrent laryngeal nerves in organophosphate poisoning, has also been reported in pigs (Wells & Bradley, 1976). While axonal degeneration was detected in the distal parts of long nerve fibres in the peripheral and central nervous systems, these changes were most severe in the distal limb nerves. Moreover, the initial clinical signs were referable to hindlimb abnormalities.

In a study of acrylamide intoxication in baboons, it was noted that in all treated animals the bark became hoarse or disappeared (Hopkins, 1970). The main pathological change observed was axonal degeneration of motor and sensory nerves. There were marked decreases in the numbers of myelinated nerve fibres, with selective involvement of large diameter fibres, and the long nerves to distal muscles were most severely affected. The involvement of the recurrent laryngeal nerves was used to confirm the predominantly distal distribution of the lesion. At the level of the larynx very few myelinated fibres remained in the recurrent laryngeal nerve, yet at the proximal level of the aortic arch, the loss of myelinated fibres was not severe. Single fibre studies demonstrated some fibres which were degenerating distally and preserved proximally, although usually with some evidence of demyelination or remyelination in the preserved proximal part of the fibre. Intercalated segments, indicative of previous paranodal demyelination and remyelination, were seen frequently at an intermediate level of the recurrent laryngeal nerves. These changes were only rarely encountered proximally, and distally where very few fibres remained to be thus affected. The tendency for demyelination to occur in proximal parts of nerves affected with distal axonopathies was noted, and it was suggested that this paranodal demyelination was probably secondary to an axonal change that preceded complete degeneration (Hopkins, 1970). The pathological changes and their distribution in the recurrent

laryngeal nerves of the acrylamide intoxicated baboons were similar to those in the recurrent laryngeal nerves of laryngeal hemiplegic horses (Duncan & Griffiths, 1973; Duncan *et al*, 1978). The results of the present investigation confirm this, and in addition demonstrate that similar pathological alterations occur in stringhalt.

The main difference between the distal axonopathies of toxic aetiology and the neuropathy of equine laryngeal hemiplegia lies in the distribution of pathological changes. In all of these diseases there is preferential involvement of the distal parts of the longest nerves. The distal axonopathies of man, and species other than the horse, appear clinically as diseases of the hindlimbs, because the longest nerve fibres innervate this area. In contrast, in the horse distal axonopathies are manifest as laryngeal dysfunction, for a similar reason. It seems possible therefore, on the basis of the findings of this investigation, that the differences between laryngeal hemiplegia in horses, and distal axonopathies in other species, may be largely due to the differences in the relative lengths of the recurrent laryngeal and other peripheral nerves, and spinal tracts between the species.

As previously stated in this thesis, deficiency of B vitamins can result in a distal axonopathy (Cavanagh, 1964; 1979). In the horse, since the recurrent laryngeal nerves are much longer than any other nerves in the body (Cole, 1946), it seems likely that a B vitamin deficiency could produce damage mainly affecting these nerves. Demyelination and remyelination, prominent features of equine laryngeal hemiplegia are also known to occur in thiamine deficiency (Collins *et al*, 1964). Indeed, in this deficiency these processes are also thought to follow primary axonal changes (Collins *et al*, 1964). Thus, in light of present evidence, a role for thiamine deficiency in the aetiology of equine laryngeal hemiplegia cannot be dismissed.

Factors predisposing to the disease of laryngeal hemiplegia, such as size, conformation and breed may influence the length of the recurrent laryngeal nerves, and consequently affect their susceptibility to the effects of a distal axonopathy. In addition, a genetic predisposition to the disease, as seen in a number of naturally occurring human distal axonopathies could exist. Management regimes which may place additional enzyme or cofactor requirements on already vulnerable long nerve fibres, could influence the degree of pathology present in a distal axonopathy. For example, regimes such as excessive and premature training of young animals could increase the demand for vitamins.

It appears likely from the results of this study that the distal axonopathy of idiopathic laryngeal hemiplegia is chronic in nature, and has an underlying axonal atrophy. In human conditions of this sort, interference with the metabolism of the nerve fibre is thought to be a likely cause (Dyck, 1975a). Inherited or acquired influences may result in deficiency of certain enzymes or cofactors, necessary for local energy production in the nerve fibre, and thus interfere with axonal transport. This could interrupt the supply of essential nutrients to distal parts of the fibre, initially precipitating axonal atrophy and eventually leading to degeneration. Inherited deficiency of an enzyme or cofactor, involved in the production of energy essential for normal function of the nerve could occur. Alternatively, an acquired deficiency of a cofactor, such as that occurring with low intakes or high requirements of the B group vitamins could exist. It is of note that the Thoroughbred horse has not evolved but has been selectively bred by man. The production of an animal of greater speed has led also to an animal of greater size, with correspondingly longer recurrent laryngeal nerves. The normal metabolic processes may no longer be able to support the energy requirements of such long nerve fibres, and the selection pressure of nature against such a defect has been removed by man.

A weakness of the present study is the difference that existed in the ages of the control and diseased groups of animals. This was necessitated by the inability to obtain sufficient older animals without the pathology of laryngeal hemiplegia. Moreover, only a limited number of nerves other that the recurrent laryngeals were examined. However, interpretations were made according to the limitations these factors imposed.

In addition to idiopathic laryngeal hemiplegia, this thesis contains studies on another equine disease which involves the recurrent laryngeal nerves. This disease, stringhalt, proved to be a widespread polyneuropathy, involving laryngeal, forelimb and hindlimb nerves. A number of similarities with the pathology of idiopathic laryngeal hemiplegia were observed, in that the pathology showed a preference for the distal ends of long nerve fibres. Moreover, the primary changes were axonal in nature, selectively affecting large diameter myelinated fibres. In addition, the left recurrent laryngeal nerve was the most severely affected, followed by the right nerve, and then those to the distal hindlimb muscles. Thus, stringhalt can also be classified as a distal axonopathy. However, a number of differences in the pattern of the pathological alterations exist between the two diseases. More active axonal degeneration, and less evidence of demyelination and remyelination was evident in the relatively acute process of stringhalt. Nor was there a central component observed in this disease. While the overall distal distribution of pathology was similar in the two diseases, more severe and widespread changes were seen along the recurrent laryngeals and in the limb nerves in stringhalt. It seems likely that these apparent discrepancies are due to differences in the severity of the initiating insult, and the duration of the two conditions. In stringhalt, the process is acute and severe, and occurs over a limited period of time. The underlying similarity of the

pathological changes of stringhalt and idiopathic laryngeal hemiplegia lend support to the hypothesis that the latter condition should be classified as a distal axonopathy. In view of the association of stringhalt with the plant *Hypochaeris radicata* , in certain seasonal and climatic conditions, an underlying toxic aetiology is possible. However, the occurrence of the disease in drought situations, and on poor soil and pasture, may implicate a deficiency, such as of the B vitamins, as a possible cause. Possibly even a combination of both factors may be involved.

The present study has provided evidence of the existence of two hitherto unidentified distal axonopathies of horses. These conditions have similar neuropathological lesions to certain human diseases, and in the future could provide important models for the study of the distal axonopathies of man.

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