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THE PATHOLOGY OF EQUINE LARYNGEAL HEMIPLEGIA

Thesis submitted for the degree of Doctor of Philosophy in the  
Faculty of Veterinary Medicine, the University of Glasgow.

by

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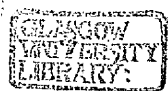
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GENERAL INTRODUCTION

Laryngeal hemiplegia is a common disease of the horse which, due to the resultant narrowing of the rima glottidis, produces a decrease in athletic performance in affected animals. The horse suffers from inspiratory dyspnoea, producing a "roaring" noise on inspiration. Horses producing this classical inspiratory noise have been described as "Roarers".

Although much research has been carried out in attempts to correct the disease surgically, a fully satisfactory operation has yet to be devised. Many suggestions have been made as to the possible aetiology of the condition, but its true nature still remains unknown. By comparison however, little work has been performed in the investigation of the pathology of the disease. In particular, few attempts have been made to apply the new techniques available in neuropathology to the problem of laryngeal hemiplegia and it may be that the information made available by such studies will be of use, in defining the pathogenesis of the disease.

It is the purpose of this study, to examine grossly and histologically the laryngeal muscles and their nerve supply in the horse, from clinically normal horses and from cases of laryngeal hemiplegia.

INTRODUCTION AND REVIEW OF LITERATURE

Equine laryngeal hemiplegia was first described in the veterinary literature at least 100 years ago, and reports of horses with undiagnosed respiratory disease appear as far back as 1772 (Gibson). The true incidence of the disease remains unknown, but there is no doubt that laryngeal hemiplegia is a common disease, and Cook (1965) states that it is probably the commonest cause of unsoundness in the horse in this country. A suggested incidence of 5% in the thoroughbred (Weiss 1937) shows that it is indeed an important disease and a survey by the British Equine Veterinary Association (1965) has shown that it is second only to viral upper respiratory tract infections in total respiratory disease incidence.

The classical condition is unassociated with any other disease, and it is not, as was previously thought, seen in association with guttural pouch mycosis (Cook 1966), nor with generalised systemic poisoning (Hutyra and Marek 1912). It can however be seen in association with soft palate paresis (Cook 1965).

It is generally agreed, that clinically it is almost exclusively the left side which is affected. Early observers noted the following incidence on the left side, Gunther 90%, Cadiot 95% and Fleming 99%. More recently, Cook (1965) reports a left sided incidence of not less than 98% whilst in a later publication one case of bilateral laryngeal paralysis in a horse with evidence of a generalised motor disorder is recorded (1970). A case of right sided paralysis in association with tracheal collapse (Hanselka 1973) has recently been reported but no details of the pathology of the laryngeal muscles or nerves were included.

The disease is certainly known to be related to the size of the horse, Marks et al (1970) stating that in their estimate, over 50% of horses/

horses greater than 17.0 hands suffer from the disease. Cook (1965) in his large series of cases states that 95% of cases occur in horses over 16.0 hands. The disease is practically unknown in ponies (under 14 hands). In Cook's series (1965) at least 80% of the cases were showing clinical evidence of the disease by the age of 6 years and he suggested that the defect may be congenital as some young horses show symptoms as yearlings as soon as they are galloped (1970). Mason (1973) reviewed the incidence of the disease in both sexes, and found that most workers found a higher incidence in geldings and entire males than in mares.

It certainly was of major importance at the end of the 19th century due to the effect of the disease on the large working horse, which was used commercially and as animal of war. In connection with the latter, Sir Frederick Hobday once wrote that "the noise that affected horses would make would be undesirable in a surprise attack on the enemy, and such horses might give the show away".

It is not surprising that at this time more attention was paid to alleviating the condition than to investigating its aetiology. It has often been the case in veterinary medicine that the cure has come before the cause was known. Attention was therefore focussed on a possible surgical cure of the disease.

The Gunther Brothers in Berlin were the first to perform any radical surgery and experimented on horses during the 1850's. Their methods included vocal cord resection and laryngeal ventriculectomy, but their results were poor mainly due to the lack of good anaesthesia and sterile technique, many of their horses dying of sepsis. Later in this country, Fleming (1882) in combination with Professor Moller repeated Gunthers operation, and at first acclaimed it as a great success, but/

but later he was reported as saying that "he wishes to modify the whole operation".

Attempts at suturing the diseased recurrent nerve to the vagus and to the phrenic nerve were first attempted in the horse in 1933 (Ballance) but with little success. The rationale behind this method was sound and the idea of neurorrhaphy of the recurrent nerve both with itself, following injury, and with other nerves, has since been investigated in great detail because of medical interest. The stimulus for such research was provided in the field of laryngeal transplant surgery in man, where the incidence of laryngeal neoplasia is high, and laryngectomy is the common therapy. Replacement of the diseased larynx with a transplant, is surgically feasible but the greatest difficulty behind such a technique is the successful reinnervation of the transplanted larynx. Many different attempts have been made in experimental animals. to solve this problem, involving straight neurorrhaphy of the cut recurrent nerve (Gordon and McCabe 1968, Doyle et al 1967), anastomosis of the vagus with the cut recurrent nerve (Doyle et al 1967), transplantation of the proximal portion of the cut recurrent nerve into the dorsal cricoarytenoid muscle (Doyle et al 1967), transplantation of the phrenic nerve into the same muscle (Taggart 1971) and implantation of a nerve muscle pedicle from the sternohyoid muscle into the dorsal cricoarytenoid muscle (Hengerer and Tucker 1973). In general the results of neurorrhaphy have been poor, failure of the procedure having been ascribed to misdirection of regenerating nerve fibres; (Taggart, 1971) that is the union of abductor to adductor fibres and vice versa. This results in synkinesis, that is, uncoordinated contraction of the abductor and adductor muscles producing a non-functional vocal cord. In the case of the phrenic nerve implantation (Taggart 1971)/

(Taggart 1971) 3 out of 6 dogs achieved abduction synchronous with inspiration, whilst in the sternohyoid implant (Hengerer and Tucker 1973) 3 out of 5 dogs achieved synchronous abduction on inspiration, and on direct stimulation of the nerve supply. It is known that reinnervation of a muscle can occur up to 1 year after denervation (Gutman and Young 1944) but it is felt that such procedures as previously described, would fail in equine laryngeal hemiplegia due to the chronicity of the disease, and the resultant severe muscle atrophy which occurs.

The first surgical method to receive any success in the horse was that performed by Williams in the States in 1910, in which he removed the mucosa from the lining of the left lateral ventricle (ventricle of Morgagni) which caused cicatricial displacement of the vocal cord. He demonstrated the technique to Hobday who modified the technique by cutting through the cricothyroid membrane and removing the lining from both ventricles. Despite the fact that both Williams and possibly the Gunther brothers had pioneered this technique, it was to Hobday that credit was given, and whose name was used to describe an operation which is still the standard technique used today. More recently, Pouret (1966) has modified the standard ventriculectomy by suturing the saccular margins and by shortening and fixing the cricotracheal membrane.

However the need for some alternative operation was pointed out by Marks et al (1970) when they stated that some method was needed to prevent the intrusion of arytenoid cartilage into the rima glottidis. In their experience the Hobday operation significantly improved 80% of their cases but only 10% were completely successful. This low success rate was ascribed to the fact that the arytenoid cartilage which contributes 70% of glottal diameter tends to collapse in laryngeal paralysis and to protrude/



protrude into the airway. This effect is not prevented by the Hobday operation.

The idea of Marks et al (1970) was to replace the abductor muscle (Cricoaarytenoideus dorsalis - C.A.D.) with an elastic prosthesis in combination with a unilateral ventriculectomy, and so to abduct both the vocal cord and the arytenoid cartilage. The authors obtained a 90% success rate on horses operated on for the first time. Modifications of this operation have been reported by Johnson (1970) and Speirs (1972) but post-operative problems notably coughing, have been reported and these have been discussed at some length. (A.A.E.P. Proc. 1973).

The list of suggestions of the aetiology of the condition over the past 100 years is impressive but inconclusive, and the cause of the disease is still unknown. These suggestions have varied from poisoning by plant or bacterial toxins, to mechanical damage, and even to congenital hypoplasia of the left recurrent laryngeal nerve (Greaves 1889). In practical terms, the suggestions as to the aetiology can be divided into two categories.

- 1) Damage to the recurrent laryngeal due to some noxious or infectious agent.

- 2) Mechanical damage to the recurrent laryngeal nerve.

- 1) Many different noxious agents have been suggested, including poisoning by plants such as lathyrus sativus, chick pea or lucerne (Hutyra and Marek 1912), chronic lead poisoning (Hutyra and Marek 1912) or poisoning by neurotoxins produced by Streptococcus equi. Other bacterial and viral toxins have been accused of selectively attacking the left recurrent laryngeal nerve, (Fleming 1908, Hutyra and Marek 1912, Quinlan and Morton 1957 and Schebitz 1964), but with little or no proof. Some authors have indeed/

indeed suggested that infectious conditions of the upper respiratory tract (especially Strangles), predispose to the disease. However, Quinlan et al (1957) in a follow-up study of an outbreak of Strangles in South Africa could find no statistical correlation between infection and recurrent nerve paralysis. In addition, such a theory fails to explain why horses and ponies, which are equally susceptible to infectious diseases, do not show an equal incidence of laryngeal hemiplegia.

The excursion of the left recurrent nerve into the mediastinum has led many to suggest that lesions such as abscess formation may be affecting the nerve (Numann-Kleinpaul and Tabbert 1936). This was suggested particularly in the case of Strangles, in which it was also suggested that enlargement of the anterior mediastinal lymph nodes could have some mechanical affect on the left recurrent nerve.

2) Most of the mechanical theories of the origin of the lesion are based on the different anatomical course of left and right recurrent nerves. This will be discussed in detail later, but the salient differences are that the left nerve, after origin from the left vagus, loops round the aorta before passing up the neck while the right is given off from the right vagus at the level of the second rib, passes behind the costo-cervical artery and ascends the neck. It has been suggested by some authors (Hutyra and Marek 1912, Navez 1913-14) that the pulsation of the aorta together with the backward movement of the heart during exercise (Marks et al 1970) may compress the nerve as it passes between the trachea and aorta. Suggestions that aortic aneurysms may increase this effect are unlikely as these are uncommon in the horse at this site. More recently, Rooney and Delaney (1970) and Marks et al (1970) advanced similar biomechanical theories as to the cause. Both/

Both point out that the axis of the left nerve, which is fixed at the heart and the larynx, differs from that of the right nerve, which is congruent with the cervical spine. Such differences produce an increase in tension on the left nerve when the head is flexed to the right but not on the right nerve when a similar move is made to the left. Rooney and Delaney (1970) suggest that the resulting nerve compression produces a vascular insufficiency rather than direct mechanical damage, and resultant ischaemic nerve damage. Marks et al (1970) base their theory on the fact that the neural pathology seen in roaring is identical to that described in an experimental case where nerves were kept under tension for a long period (Highett and Sanders 1943). The two situations however do not bear comparison as the experimental model referred to by Marks et al involved shortening and stretching of a nerve following section and suture (Highett and Sanders 1943). It is felt by the author that insufficient pathological details of the equine neuropathy were known at this time to state that changes were identical.

Mason (1973) reviewed the possible role of an anomalous course of the left recurrent nerve in the causation of the disease. This anomaly was first noted in 1893 by Haslam, when he described a case in which the left nerve was found to lie lateral to the trachea after looping round the aorta, instead of lying ventral to it. This anomaly was found in 3 horses which "roared" whereas in 3 normal horses, the left nerve was always found ventral and not lateral to the trachea. When found lateral to the trachea, Haslam described the nerve as being flattened and ribbon-like at this point. Argyle (1932) also found this deviation of the nerve/

nerve in both roarers and non-roarers. Haslam in a subsequent paper (1895) reported no deviation of the nerve in 3 cases which had evidence of laryngeal muscle atrophy. Mason (1973) dissected the thoracic pathways of the left recurrent nerve in 33 randomly collected horses and ponies, and the entire course of the nerve in 8 of these. In 5 cases, the course of the left recurrent nerve was anomalous and in one of these the cardiac nerves also had an anomalous course. His description of the laryngeal musculature in these cases is not clear, as he states that there was no evidence of "laryngeal abductor muscle atrophy" but he makes no mention of whether or not he examined these muscles histologically. The examination is incomplete in the light of work by Cole 1944 & 1946, Gunn 1973a and 1973b and Duncan et al 1974 a & b who showed evidence of sub-clinical atrophy histologically. In addition, Duncan (1974 a & b) has given evidence that the adductor muscles may be affected before the abductor muscle, but Mason (1973) makes no mention of examining these. He concludes by stating that it is doubtful whether his and other observations of the anomalous course of the left nerve have any relevance to the aetiology of laryngeal hemiplegia.

Heredity has also been implicated in the causation of the disease and as early as 1889 the R.C.V.S. produced evidence before a Royal Commission on Horse Breeding that "roaring" was possibly heritable. Much of this evidence stemmed from the observation that both Ormonde, one of the greatest race-horses ever, and his dam were roarers, and many of their offspring were found to be afflicted. Weiss (1937) in a limited investigation, suggested that hereditary factors might be important, but Marks et al (1970) suggested that the only role of genetics may be in the inheritance of a certain size and conformation. Any suggestion of/

of hereditary factors being involved would have to be confirmed by very careful statistical analysis of the Stud-Book, and this has not yet been done.

Unlike other earlier authors Vermeulen (1914/15) thought that paralysis of the left side of the larynx was the most obvious symptom of an extensive nervous disorder and that it was associated with thyroid deficiency, because a number of "roarers" had histological evidence of thyroid pathology.

The most recent suggestion as to the aetiology of laryngeal hemiplegia has come from Loew (1973) who suggested that the disease was due to chronic thiamine deficiency. This idea was based on the fact that the laryngeal nerves can be involved in beriberi in man and that thiamine deficient cattle are known to roar. In these cattle, however, laryngeal oedema may be the cause of the inspiratory difficulty. The author is aware of no endoscopic or clinical evidence of bovine laryngeal paralysis. This theory does not explain the remarkable clinical asymmetry of the lesion, and provides no direct evidence that roarers are in fact thiamine deficient. In a later study on normal standard-bred horses, Loew (1973) produced figures for normal blood thiamine levels and found that stallions had a significantly lower level of thiamine than geldings or mares and that horses less than 5 years of age had lower values than older animals.

By comparison with the efforts expended in attempting to find a cure for the disease, few major pathological investigations have been carried out. As Argyle (1932/33) points out, dissection of the recurrent nerves "is a long business and results in considerable fatigue and backache" and this is possibly one of the reasons that has detracted many from carrying out histopathological studies in depth.

Probably/

Probably the earliest reference to the gross pathology of the disease was published by Percivall (1840) in a textbook entitled "Hippopathology". He described a unilateral wasting of the muscles on the left side of the larynx, but more interestingly quotes a Mr. J. Field who produced the clinical signs and pathological symptoms of laryngeal hemiplegia by sectioning the recurrent nerve. This was a piece of evidence that many people later in that century failed to grasp when they were searching for the lesion which produced, to them, a peculiar hemiatrophy of the larynx.

It was not until the beginning of the 20th century that any detailed reports appeared. Thomassen (1901) published a report entitled "Investigations in the pathogenesis of Hemiplegic laryngis in the Horse" in which he described in some detail the pathology of five "roarers". To him can be given the credit for the discovery that the lesion in left recurrent nerve is first seen in its most peripheral portion, that is nearest the larynx. He found that in all cases the medulla and left vagus were normal and that the thoracic portion of the left nerve was never affected, even in chronic cases. In no cases did he ever find any compression of the vagus or recurrent nerve, and in one case he found that the adductor muscles were more wasted than the abductor. In all the other chronic cases the degree of muscle atrophy was equal, but in his fifth case, only the upper part of the adductor muscle was reduced in bulk when compared with the right. He suggested that this case was of reasonably recent onset.

Vermeulen (1914-15) thought that the recurrent laryngeal neuropathy was only part of a generalised nervous disorder and that the degeneration seen in the left recurrent nerve could extend in a central direction and/

found evidence of pathological changes in the posterior third of the nucleus ambiguus, in two chronic roarers. He found a discrepancy in the total number of nerve cells in the nucleus compared to that seen in the normal horse, but gave no other description. He also described various types of pathology of the thyroid gland and said that decreased thyroid function enabled bacteria and toxins to circulate freely in the blood and cause nervous tissue damage.

Argyle (1932-33) was next to publish some careful work on the pathology of "roaring" in which he described marked changes in the nucleus ambiguus. These changes consisted of shrinkage of the neuronal cell body, eccentric nucleoli, loss of Nissl's granules and swelling of the nucleus. Although he found no histological abnormality in the vagi of "roarers" he did find slight changes in the left recurrent nerve of one case at its origin from the vagus. He also noted the anomalous course of the left nerve as described by Haslam (1893) in both "roarers" and "non-roarers" but dismissed its significance in the causation of the disease. He also found a constricted portion of the nerve about half way along the cervical portion of the nerve in one case. However, one of his most useful contributions was a list of suggestions that he made as to further investigation into the disease. He suggested that in all horses which were destroyed because of laryngeal paralysis

- a) a naked eye and microscopic examination should be made of the entire left recurrent nerve.
- b) that the left nerve should be compared with the right nerve.
- c) that the nerve endings should be examined in muscles from left and right sides, especially the abductors.
- d) the medulla should be examined.

Careful/

Careful examination of all these factors is the basic purpose of this thesis.

Cole in 1944 and 1946 published what were and still remain the largest and most comprehensive studies of the pathology of laryngeal hemiplegia. His results were drawn from a study of 174 larynges and their nerve supply, but unfortunately no examination of the brain was included in his series. He described the histological changes in the laryngeal muscles and nerves and laryngeal cartilages and correlated these results with his clinical laryngoscopic and faradic stimulation findings. The changes in the laryngeal muscles from roarers was similar to that described previously, i.e. atrophy with fat and connective tissue replacement. The main changes in the left recurrent nerve were disappearance of the myelin sheath, axonal disintegration, proliferation of Schwann Cells and finally complete fibrosis. The most distal portion of the nerve (i.e. that part nearest the larynx) was most severely affected while only one or two fibres were affected at the thoracic inlet. Degeneration was first seen in the small diameter nerve fibres. Out of the 174 larynges collected 43 showed evidence of pathology of the laryngeal musculature, but only 25% of these showed clinical evidence of laryngeal paralysis. He estimated that horses which had less than 50% atrophy of the left dorsal cricoarytenoid did not show symptoms of laryngeal paralysis.

Such evidence, in addition to the fact that he described three cases with atrophy of the right transverse arytenoid, introduces the concept of sub-clinical disease. That is, 15% of his series of horses with evidence of laryngeal muscle pathology had shown no clinical signs of laryngeal paralysis. This possibility of sub-clinical disease was reinforced by the findings of Gunn (1972 and 1973) who, using the new muscle enzyme/



enzyme histochemical techniques, showed early evidence of neurogenic atrophy in the laryngeal muscle of clinically normal horses. When normal laryngeal muscle is stained for the enzyme myosin ATP-ase activity, a mosaic pattern of high and low fibres is found (Gunn 1972). A similar pattern is found in most but not all skeletal muscles of man and experimental animals (Dubowitz and Brooke 1973). However in some clinically normal horses Gunn found large and small groups of histochemically similar fibres, with the loss of mosaic pattern. This fibre type grouping, as it has been called, has been reported by other workers in equine laryngeal muscle (Duncan et al 1974 a,b) and is indicative of reinnervation of muscle fibres following denervation (Karpati and Engel 1968, Edström and Kugelberg 1969).

Although the right side of the larynx is very seldom affected clinically, Cole (1946) reported three cases in which there was histological evidence of atrophy on the right side. Gunn (1973) found marked reduction in muscle size (indicating atrophy) in some areas of the right transverse arytenoid muscle of a chronic roarer and noted that as in Cole's cases right sided involvement may be seen in cases where the left side is very severely involved. Duncan (1974) has also reported involvement of the right side of the larynx, in some muscles where fibre type grouping was the only evidence of neurogenic disease.

Gunn also noted an increase in the size of homogenous fibre type groups in the right side, when the left side was severely affected (1972). As well as finding abnormal fibre type grouping in adult horses, he demonstrated type grouping in a foal and also in a foetus (1973).

The question of preferential atrophy of the adductor group and abductor laryngeal muscles in neurogenic disease of the larynx, is an interesting one which stems from an old "law" of laryngology known as Semon's/

Semon's law. In 1881, Sir Felix Semon stated that there was "a proclivity of the abductor fibres of the recurrent laryngeal nerve to become affected sooner than the adductor fibres". These observations applied to his own clinical experience in human otolaryngology and to his experimental work in dogs. (see Clarke 1887). The latter involved the faradization of the nerve supply to dogs larynges, shortly after death. Fatigue of the abductors before the adductors was taken to be indicative of greater susceptibility of the former group of muscles.

Although not mentioning Semon's law, Cole (1946) found that the left cricoarytenoid muscle (abductor) and the left transverse arytenoid muscle (adductor) were affected more severely, and before the other muscles, notably the left lateral cricoarytenoid, vocalis and ventricularis (adductor muscles). This order of atrophy confirmed that described in an earlier textbook (Nieberle and Cohrs 1949 ) and by Rooney (1970). However, Clarke (1887) refers to one "roarer" in which the adductors were more atrophied than the abductor, and Thomassen (1901) describes two cases in which the abductor was not so severely affected as the adductor. Cook (1965, 1970) supports the view that Semon's law is applicable to the horse but bases his argument on Cole's (1946) findings. Such estimates of the degree of atrophy in laryngeal muscles were purely subjective, and do not compare favourably with the more objective methods now available for muscle quantitation (Report of Sub-Committee 1968). A more objective method of quantitating muscle atrophy was attempted in this study and will be discussed in detail later.

Idiopathic laryngeal paralysis also occurs in man and such cases represent one-quarter to one-third of the total incidence of human laryngeal paralysis (Scott-Brown et al 1965). It would also appear that in/

in all cases of laryngeal paralysis in man, the left side is affected twice as frequently as the right (Scott-Brown et al 1965), although this has been related to the risk of involvement with mediastinal pathology of the left nerve. Laryngeal paralysis has also been described in other human peripheral neuropathies; that seen in beriberi (Erbslöh and Abel 1970) and in diabetic neuropathy (Shuman and Weissman 1968).

Mason (1973) reintroduced the concept of the anomalous course of the left recurrent nerve at the aortic arch (Haslam's anomaly) but could not correlate this with evidence of atrophy of the left abductor muscle. However, he showed conclusive flattening of the nerve at this point and the presence of Renaut bodies inside the fascicles.

While many aspects about the pathology of equine laryngeal hemiplegia have been described, much remains unknown. Few attempts have been made to investigate the pathology of the disease and Cole's (1946) work has for too long, been the standard text. While his work was admirable in many ways, the advent of the more modern methods of investigation demand that it be updated. The pathogenesis of the disease may also provide much information in the important field of human and animal peripheral nerve disease.

## MATERIALS AND METHODS

Details of the horses and ponies from which larynges were collected

a) Ponies used in parasitological research

A total of 15 larynges was collected from ponies used in research into the pathogenesis of Strongylus vulgaris. As most of these animals were between 6-9 months old, they were used as controls.

b) Horses and ponies killed for humane purposes

The majority of animals in this group were at one time patients in the Surgery Department of the University of Glasgow Veterinary Hospital. The reason for their death or euthanasia is given in table 3. Where possible the horse was endoscoped before death using a Hauptner rigid endoscope.

In some cases it was possible to listen to the horse at exercise but this was impossible in a number of cases due to their ambulatory state. Latterly endoscopy was performed using an Olympus colonoscope (CF - LB2). This has many advantages over the rigid endoscope (Cook 1974).

In most cases the larynx and nerve supply were removed as soon as possible after death but a number of larynges were received many hours later and therefore the histological examination was affected by post-mortem change in these specimens.

c) Clinical case of laryngeal paralysis.

Only four cases of laryngeal paralysis were available for post-mortem (table 1).

Case No. 47988 was bought by the Surgery Department for use as a teaching case. Case No. 49589 died following post-operative complications, and Case No. 52838 was destroyed due to pharyngeal and soft palate paresis. All were suffering from gross inspiratory dyspnoea, and on endoscopic examination, were found to have left sided laryngeal hemiplegia. The chronicity/

chronicity of the disease in each case was uncertain; all showed clinical evidence of atrophy when the left dorsal cricoarytenoid muscle was palpated. Case No. 49589 suffered from laryngeal stridor after trotting for a short while but Case No. 47988 would only "roar" after a short canter. Presumably the previous Hobday operation in the latter case, had partially alleviated the condition. Case No. 52838 was not exercised but was reported to suffer from inspiratory dyspnoea following light exercise. This case also had evidence of a scar on the ventral surface of the larynx and on endoscopy it was noticed that the entire left arytenoid cartilage had been resected.

Case No. 40733 was admitted for destruction due to chronic tendon damage, which prevented it from being exercised. It had been operated on five years previously for laryngeal hemiplegia, and a bilateral ventriculectomy performed.

d) Horses with other abnormal endoscopic findings (Table 2).

Two horses were found to have asymmetrical larynges on endoscopy, although they were not typical or similar to those seen in laryngeal hemiplegia. In both cases it was impossible to exercise the horses due to their condition and enquiry about previous history was not fruitful.

The first case, 57769 was endoscoped shortly before death and was seen to have an asymmetrical larynx with the left vocal cord and arytenoid cartilage in a more abducted position than the right side. In fact the left half of the glottis was held in an almost fixed abducted position. The "grunt-to-the-stick" test which is designed to test adduction (Cook 1965) was not performed. No obvious atrophy of the left CAD could be detected on palpation.

The second case, 57780 was first examined under anaesthesia using a flexible colonoscope. The horse was lying in left lateral recumbency and/

and it was noted that only the left side of its larynx was moving. The right side was stationary. On recovery from anaesthesia the horse was again endoscoped using the fibrescope and during this time the horse was attempting to rise and was making violent respiratory movements. Both sides of the larynx were now seen to be moving, but the left arytenoid and vocal cord were seen to be abducting more than the right side. The animal did not recover and died in extremis.

#### Method of sampling the intrinsic laryngeal muscles

The following muscles were sampled in most cases; the left and right dorsal cricoarytenoid (CA), lateral cricoarytenoid (CAL) and cricothyroid (CT). Such a sampling method allowed comparison of muscles from both sides of the larynx and also of the muscles supplied by the recurrent laryngeal nerve (CAD and CAL) and that supplied by the anterior laryngeal nerve (CT). In addition it allowed comparison of the abductor (CAD) and true adductor (CAL) muscles of the larynx. The third muscle (CT), tenses the vocal cord.

Due to the large size of the abductor muscle (CAD) in comparison with the other intrinsic muscles of the larynx, samples were taken from different areas of this muscle in separate cases and occasionally two areas in the same larynx. In 6 cases, samples were taken from a variety of distal limb muscles to investigate the possibility of any generalised motor disorder.

All muscle samples were removed using either muscle biopsy clamps or artery forceps using a "no touch technique" (Bethlem 1970) and therefore the muscle sample was neither touched, nor stretched. The sample was split into 2 pieces, one being used for enzyme histochemistry, the other fixed in 4% buffered neutral formalin for routine staining.

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In addition, in a number of cases a portion of muscle was taken for examination of intramuscular nerves and their motor endplates, stained using the supravital methylene blue method (appendix).

#### Details of nerves and brains collected

Samples of the recurrent laryngeal nerves were collected for histological examination in 40 cases. In three of the horses suffering from laryngeal hemiplegia (47988, 52838 and 40733) almost the entire lengths of both recurrent nerves were removed. They were not removed from the third clinical case (49589).

On removal, portions were taken from both left and right nerves at the level of the larynx (distal) and at 10 cm intervals along the nerve for a distance of 40 cms. In addition, on the left side, a piece of nerve was taken at the level of the aortic arch. In the remaining clinically normal cases a more limited examination of the nerves was performed and in a number, the separate branches to the abductor muscle and adductor muscle group collected.

In order to compare the recurrent laryngeal nerves with peripheral nerves, samples of the latter, including ulnar, tibial, digital, facial and phrenic nerves were collected in six cases..

In the 4 clinical cases of laryngeal paralysis, the brain stem was available for histological examination of the motor nucleus of the vagus. After fixation for over two weeks in 4% BNF, the medulla was serially sectioned and sections stained with Haematoxylin and Eosin, and sections from one case were stained with Holzer's stain for glial fibres and Nissl's cresyl violet stain.

#### Muscle fixation

a) Histochemistry

A/



A portion of muscle was mounted on a thin strip of filter paper and then dusted lightly with talcum powder. The strip was then dipped in liquid nitrogen ( $-160^{\circ}\text{C}$ ) for 10 secs. and then immediately transferred to the cryostat. The powder reduces the chance of ice artefact by preventing formation of an air bubble, which would delay freezing time of the muscle as it is immersed in the liquid nitrogen.

The muscle blocks were mounted on chucks in the cryostat using a biological glue (Ames OCT compound).

Serial sections were cut at  $10\ \mu$  on a Slee cryostat and mounted on pre-cooled coverslips. The sections were melted by sliding a finger across the underneath surface of the coverslips which were then blown dry for a few minutes.

The sections were then stained immediately for the following enzymes with the exception of those stained for myosin ATP-ase. These were left for two days to increase the adherence of the section to the coverslip and to decrease the wrinkles which occur, (Dubowitz and Brooke 1973).

- a) Myosin ATP-ase
- b) Nicotinamide adenine dinucleotide (NADH)
- c) Succinic dehydrogenase (SDH)
- d) Phosphorylase

The methods used to demonstrate these enzymes are detailed in the appendix.

Cryostat cut sections were also stained with Haematoxylin and Eosin, Periodic Acid Schiff (PAS) and in a small number of cases a modified Gomori trichrome method (Engel and Cunningham 1963).

b) Formalin fixation

Muscle/

Muscle removed shortly after death was always allowed to relax for 15-20 minutes pre-fixation in order to decrease contraction artefact. After relaxation the muscle was pinned out on a small piece of cord, and fixed for 48 hours in 4% buffered neutral formalin. After washing overnight in running tap water, the blocks were double embedded in paraffin wax.

Both TS and LS blocks were cut at 8  $\mu$  stained with Haematoxylin and Eosin, Gomori or Masson trichrome and Periodic Acid-Schiff (PAS).

Sections were dehydrated through an ascending series of alcohols, cleared in xylene and mounted in D.P.X. (Gurr.)

#### Nerve fixation

The nerve samples removed from the recurrent nerves at different levels were cut transversely into 4 parts and fixed.

- 1) in 4% BNF for routine staining
- 2) in Flemings Fluid, for Kultschitzky staining
- 3) in 4% BNF for teasing
- 4) in 3% Glutaraldehyde for fine section preparation

#### 1) Routine staining

The portion of nerve was stretched slightly on a card and allowed to partially dry before fixation in 4% buffered neutral formalin (BNF) for 24 hours. After washing overnight, the nerves were double embedded and 8  $\mu$  thick L.S. sections obtained. Sections were stained with Haematoxylin and Eosin, Solochrome Cyanin, Van Gieson, and Palmgren's silver method for axons.

#### 2) Fixation in Fleming's fluid

A small portion was fixed in Fleming's fluid for 24 hours, after pre-stretching on a piece of card. The sample was then washed overnight and double embedded. Sections 5  $\mu$  thick were cut and stained with Kultschitzky's/

Kultschitzky's haematoxylin. This stain is used for identification of the myelin sheath which stains black (see appendix).

### 3) Teasing

A length of  $1\frac{1}{2}$  - 2 cms was fixed for 24 hours in 4% BNF and then post-fixed for a further 24 hours in 1% osmium tetroxide. After this period the nerve was stored in 70% glycerol until teased, at least 20 fibres of different diameters and from different fascicles being selected. No attempt was made to select abnormal fibres. Final teasing was done in clove oil and the fibres allowed to dry overnight before mounting in DPX. Measurement of internode length/internode diameter was performed on 20 fibres at 100 times and 1000 times magnification, respectively. Three measurements of diameter along the length of each internode (avoiding the paranodal area) were taken and the mean diameter calculated. In all cases the internode length/diameter relationships were computed and the regression coefficient (b) and correlation coefficient (r), calculated. In a number of cases attempts were made to classify the type of change occurring. Three headings were chosen, 1) normal fibres, 2) fibres with evidence of demyelination, 3) fibres with evidence of remyelination.

### 4) Fixation in glutaraldehyde

In a number of the more recent cases, portions of the recurrent laryngeal nerves were taken for fixation in 3% glutaraldehyde. A portion of nerve about 10 cms in length was suspended under slight tension for 2 hours in glutaraldehyde, followed by washing overnight in phosphate buffer. Post-fixation was in 1% osmium tetroxide in buffer for 1 hour, followed by 2 washes in buffer for 15 minutes each. The nerve was then dehydrated in graded alcohols before clearing in propylene oxide. The propylene oxide was drained off and a 50/50 mixture of propylene oxide/epon added. Following rotation overnight, the samples were embedded in capsules (Taab) in/

in epon. Semi-thin (or thick) sections at  $1.5 \mu$  were cut on a pyramitome (LKB.) and stained with toluidine blue.

#### Quantitation of myelinated nerve fibre population

Quantitation was performed on Kultschitzky stained sections, photographed and enlarged to one thousand times original size. Magnification was checked by photographing a 1 mm scale, and checking subsequent enlargement of this with the prints.

Total nerve fibre counts were carried out in six cases at various points on the recurrent laryngeal nerves. These counts did not include the fascicles which contained only or mainly small diameter myelinated fibres as these are thought to be sensory fibres from the oesophagus and trachea (section 1). In three clinical cases it was possible to count the total number of fibres at three points on the course of the left RLN.

Nerve fibre diameter measurements were made in four cases using a millimetre rule. These measurements however were not made at identical positions in each nerve as a number of sections were rejected due to technical problems. In one sub-clinical case the abductor/adductor branches of the left and right RLN's were examined, and in two other normal cases these branches were quantitated, but only from one side in each case.

Fibres which were not completely circular were measured across their lesser diameter; Dyck (1968) has discussed in detail the errors involved in such measurements. Any fibre which had an indefinite outline was counted but not measured.

Measurement of nerve fibre density in two of the clinical cases, in the left RLN at three points on the course of the nerve were also performed./

performed. Fascicular area was measured from traces of the enlarged photographs, using a planimeter, (Haff No. 315), compensating planimeter. Results were expressed as the number of fibres/mm<sup>2</sup> and illustrated graphically.

Details of clinical cases of laryngeal hemiplegia

TABLE 1

<u>Case No.</u>	<u>Type of Horse</u>	<u>Age (Years)</u>	<u>Sex</u>	<u>Height</u>	<u>Reason for Destruction</u>
1) 47988	Carthorse	12	Gelding	16.2 hands	laryngeal hemiplegia
2) 49589	Hunter	9	Gelding	16 hands	" "
3) 52838	Heavy Hunter	8	Gelding	17.2 hands	laryngeal hemiplegia and soft palate paresis.
4) 40733	Hunter	12	Gelding	16.1 hands	laryngeal hemiplegia and tendon damage.

TABLE 2 Horses with possible left sided adductor paralysis

<u>Case No.</u>	<u>Type of Horse</u>	<u>Age (Years)</u>	<u>Sex</u>	<u>Height</u>	<u>Reason for Destruction</u>
1) 57769	T.B.	13	Gelding	16 hands	tendon damage
2) 58877	Hunter	12	Gelding	16.2 hands	" "

TABLE 3 Details of clinically normal horses with pathology of the laryngeal musculature

<u>Case No.</u>	<u>Type of Horse</u>	<u>Age (Years)</u>	<u>Sex</u>	<u>Height</u>	<u>Reason for destruction or cause of death</u>
1) 26443	Hunter	20	Gelding	16 hands	Tendon damage
2) 49696	Hunter	13	Gelding	16 hands	Navicular disease
3) 50316	Hunter	10	Gelding	16 hands	"
4) 49199	Hock	9	Gelding	15 hands	"
5) 45404	Trotter	6	Gelding	16 hands	Fractured pedal bone
6) 49993	Thoroughbred	10	Gelding	15.1 hands	Fractured radius/ ulna
7) 53610	Clydesdale	5	Mare	17.2 hands	Caesarian section
8) 50396	Standard bred	2	Gelding	15.2 hands	
9) 53816	Trotter	20+	Mare	15.2 hands	
10) 54788	Hunter	8	Gelding	16.1 hands	Blind
11) 55336	Thoroughbred	2	Gelding	14.2 hands	Poor conformation
12) 52858	Hack	20	Gelding	15.3 hands	Arthritic hock
13) 56599	Thoroughbred	4	Mare	16.1 hands	
14) 50035	Hunter	1	Filly	14.2 hands	Tendon damage

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TABLE 3 continued

<u>Case No.</u>	<u>Type of Horse</u>	<u>Age (Years)</u>	<u>Sex</u>	<u>Height</u>	<u>Reason for destruction or cause of death</u>
15) 36820	Hunter	9	Gelding	15 hands	
16) 58431	T.B.	7	Gelding	16.1 hands	Nasal osteosarcoma
17) 57374	T.B.	9 months	Colt	14.2 hands	Hock injury
18) 54646	Pony	20	Gelding	14 hands	Arthritic carpus
19) 59190	Clydesdale	8	Mare	16 hands	Grass sickness
20) 57510	T.B.	5	Gelding	17 hands	Tendon damage
21) 58856	T.B.	7 months	Colt	14 hands	Twisted leg
22) 59170	Garron	15	Mare	14 hands	Tendon damage
23) 49733	Hunter	10	Gelding	15 hands	Navicular disease



SECTION 1

THE NORMAL EQUINE LARYNX

INTRODUCTION

In comparison with what is known about the normal anatomy, histology and physiology of the larynx and its nerve supply in other species, comparatively little is known about the normal equine larynx. This is perhaps surprising in view of the fact that the horse and man are only known species in which idiopathic laryngeal paralysis is found. This introduction therefore reviews what is known about the normal anatomy and histology, of the larynx and to nerve supply both in the horse and other species.

In the standing horse the larynx is found to lie between the branches of the mandible. It is related dorsally to the pharynx and origin of the oesophagus and laterally to the parotid and mandibular glands, medial pterygoid, occipito-mandibularis, digastricus stylohyoid and pharyngeal constrictor muscles. Ventrally it is covered by skin, fascia and sterno-hyoid and omo-hyoid muscles. Dorsally the cavity of the larynx communicates with the pharynx and ventrally with the trachea. The body of the larynx is made of cartilages which are connected by ligaments and membranes and moved by extrinsic and intrinsic muscles. The cavity of the larynx is lined with mucous membrane.

There are three single cartilages of the larynx and one pair. The former are the cricoid, thyroid and epiglottic cartilages and the pair of cartilages, the arytenoids. The intrinsic laryngeal muscles which move these cartilages are six in number, and take their name from their origin's and insertions on the laryngeal cartilages. All of the intrinsic muscles and the cricoid and arytenoid cartilages are derived from the 6th branchial arch. The dorsal cricoarytenoid muscle (CAD) is a large fan-shaped muscle lying on the dorsal aspect of the larynx; it originates from the lamina of the cricoid cartilage and inserts onto the muscular process of the arytenoid/

arytenoid cartilage. Contraction of this muscle rotates the arytenoid cartilage and therefore the vocal process and fold are abducted. It is the sole abductor muscle of the larynx.

The lateral cricoarytenoid muscle (CAL) is the direct antagonist of the CAD. It lies medial to thyroid cartilage and in a groove in the cricoid cartilage. It originates from the arch of the cricoid and inserts onto the muscular process of the arytenoid; contraction of it results in rotation of the arytenoid cartilage in the opposite direction to that of the CAD and hence in adduction of the vocal cord.

The transverse arytenoid muscles are jointed by a fibrous raphe at the median ridge of the cricoid cartilage, just dorsal to the CAD muscle. Each takes its origin from the muscular process of the arytenoid and pull from these muscles on this cartilage results in narrowing of the rima glottidis.

The vocalis and ventricularis muscles have similar origins and insertions and together are known as the thyro-arytenoideus muscle. They arise from the cricothyroid membrane and are separated by the laryngeal saccule, before inserting onto the arytenoid cartilage and transverse arytenoid muscle. Together they produce some closing of the rima and slackening of the vocal folds.

The cricothyroid (CT) muscle is found in the lateral groove of the cricoid cartilage and arises from the ventral, posterior edge of this cartilage and inserts onto the posterior border of the thyroid cartilage. Its action is to tense the vocal folds, on contraction the muscle pulling the thyroid and ventral part of the cricoid cartilages together. This produces a rotation of the cricoid through the cricothyroid joint carrying the base of the arytenoid cartilage with it, thus tensing the vocal/

vocal cords. The CT muscle is the only intrinsic laryngeal muscle which does not insert or originate from the arytenoid cartilage.

The normal routine histology and histochemistry of laryngeal muscle have been examined in a number of species in some detail. Histochemistry has been performed on the laryngeal muscle of man (Ganz 1971), horse (Gunn 1972), monkey (Saghal and Hast 1974), cat (Abo-el-Enein 1967), rabbit (Hall-Craggs 1968) and cow (Ganz 1971).

With the progress of knowledge of histochemistry and the techniques necessary to demonstrate specific enzymes at cellular level much has been learned about the functional activity of individual muscle fibres, following determination of their histochemical morphology. While there is a large number of enzyme histochemical methods available in general terms they are used to a) demonstrate aerobic or oxidative metabolism, for example by staining for succinic dehydrogenase (SDH) or nicotinamide adeninedinucleotide (NADH), an enzyme and coenzyme of oxidative metabolism respectively b) demonstrate anaerobic activity, for example by staining for phosphorylase activity, an enzyme involved in the anaerobic degradation of glycogen or c) demonstrate the intrinsic speed of contraction of a muscle fibre by use of the ATP-ase reaction - in general type II fibres are fast and type I fibres are slow, and so indicate the contraction characteristics of the muscle as "fast" if predominantly type II and "slow" if predominantly type I (Barany 1967). Such methods have been applied to both human and animal muscle with differing results.

Muscle fibres in man and experimental animals have often been described as either having been high in aerobic activity and low in anaerobic activity or vice versa, but this generalisation has been criticised by some and the arguments are reviewed authoritatively by Engel (1974) who also discussed the histochemical fibre type nomenclature.

Engel/

Engel suggests that the best available terminology is the type I/ type II classification based on their light and dark staining respectively with myosin adenosine triphosphatase reaction (ATP-ase at pH 9.4). He also notes that it is possible to sub-divide the type II fibres into 11A, 11B and 11C on the basis of the ATP-ase reaction following preincubation at pH 4.2 and 4.6 (Brooke and Kaiser 1970 a & b). Throughout this study therefore muscle fibres were classified according to the routine ATP-ase method and in some cases sub-divided following acid preincubation (see materials and methods).

It is the work of Gunn (1972) which is of the greatest pertinence to the current study. He used three enzyme methods to indicate whether single muscle fibres were high in oxidative activity (staining succinic dehydrogenase activity - SDH), high in anaerobic activity (staining for phosphorylase activity) and either fast or slow twitch fibres (using the myosin ATP-ase reaction). In the CAD muscle of the normal horse Gunn found that 72% of fibres were high in ATP activity, and that 100% of fibres were high in SDH activity. Using these enzyme reactions to classify the fibre types, he found that there were four types of fibre present in normal equine laryngeal muscle, and that it was possible to demonstrate fibres which were high for the ATP, SDH and phosphorylase reactions. This is against the theory of reciprocal reaction of muscles fibres (Dubowitz and Pearse 1960), that is those fibres which are high in ATP activity are automatically high in anaerobic activity (phosphorylase) and low in aerobic activity (SDH). In addition Gunn found some fibres which were low in ATP activity but high in both aerobic (SDH) and anaerobic (phosphorylase) activities.

Results from other species have been broadly similar to Gunn's.

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In both the cat (Edström et al 1974) and the monkey (Saghal and Hast 1974) most of the fibres were type II fibres on the ATP reaction, and indeed in both the cat and monkey the CAL and thyroarytenoid muscles contained 90-100% of type II fibres. Comparing the results of oxidative enzymes is more complex. While most found that in general the laryngeal muscles were high in oxidative activity, there were obvious species differences. In the human and bovine laryngeal muscle (Ganz 1971), there was high oxidative activity in the CAD and thyroarytenoid muscles, and in the monkey, (Saghal and Hast 1974) all the muscles with the exception of the CAL and transverse arytenoid which had a mosaic pattern, were uniformly high in oxidative activity. Hall-Craggs (1968) correlated the histochemistry of rabbit laryngeal muscles with their contraction times, and compared their fibre population types and speed of contraction with the anterior tibial - a "fast" muscle. He concluded that the thyroarytenoid muscle with a homogeneous population of fibres of high oxidative type (SDH) and moderate phosphorylase activity, could exhibit fast activity. This was supported by the fact that its contraction time equalled the anterior tibial and was faster than the CT, which had a similar fibre population type to the anterior tibial. This finding differed from the previous assumption that fibres with intense oxidative activity were slow tonic muscles.

Work in the cat has also showed that differences in the oxidative stains can occur. Edström et al (1974) showed that while most fibres in the laryngeal muscles had a high oxidative activity, type I fibres could be found which were low in SDH but high in NADH, and type II fibres which were high in SDH but low in NADH were seen. In another study on the cat/

cat Abo-El-Enein (1967) found a mosaic pattern in the CAD and CT muscles using the Sudan Black B reaction, but in the CAL all fibres stained darkly. A previous study on diaphragmatic muscle has shown the Sudan Black B reaction to correspond to the SDH reaction and therefore can be taken as a marker of aerobic metabolism (Davies and Gunn 1972).

The nerve supply to the intrinsic muscles of the larynx is derived from the recurrent and anterior laryngeal nerves. It is now well documented that the recurrent laryngeal nerve (RLN) is the source of motor innervation for the CAD, CAL, transverse arytenoid and thyro-arytenoid muscles, while the anterior laryngeal nerve supplies the CT muscle. The extensive literature on this subject has been well reviewed by Arnold (1965).

The nerve cells controlling the motor units of the intrinsic laryngeal muscles, are found in the nucleus ambiguus which is situated in lateral part of the formation reticularis in the medulla oblongata. The motor fibres to all the intrinsic muscles with the exception of the cricothyroid muscle, are found in the left and right recurrent laryngeal nerves, which originate from the vagi at different levels. The right nerve is given off at the level of the second rib, turns around the costo-cervical artery and runs rostrad on the ventral surface of the trachea. In the neck it is found on the ventral aspect of the carotid artery. The left nerve originates from the vagus as the latter begins to cross the aortic arch, at about the level of the third rib. It then passes around the aorta in mid-line direction where it is then found to lie between aorta and trachea, and then runs forward to lie in a similar position to the right nerve. In the horse, both left and right recurrent nerves branch at the level of the larynx and divide to give off branches to the dorsal cricoarytenoid (CAD)/

(CAD) and transverse arytenoid muscles. The remainder of the nerve then passes between the CAD and cricopharyngeus, medial to the thyroid cartilage to supply the remaining adductor muscles (Sissons 1966).

Much controversy has raged about the branching of the RLN to supply the various groups of muscles at the level of the larynx. This controversy arose as it was thought that if branching of the nerve occurred extralaryngeally then one of the two branches, supplying the abductor or adductor muscles might be affected more often e.g. following thyroidectomy. It was thought that such an anatomical arrangement might explain Semon's Law (see introduction). King and Greig (1948) found that in 30% of cases the RLN divided into a medial and lateral branch, the former supplying the abductor, the latter the adductor muscles. Morrison (1952) gave support to this, by stating that a large number of dissections in cadavers identified this division, and while confirming this work Rueger (1972) summarised other supporting evidence. Vogel (1952) demonstrated in the dog that the nerve branched a few millimeters below the cricoid cartilage, the medial branch supplying the CAD muscle. Many authors however disagreed with this, some stating that the nerve branched endolaryngeally (Gisel and Pickler 1956, English and Blevins 1969), while Williams (1954) showed that although the branch may divide outside the larynx, the lateral branch supplied all the intrinsic muscles while the medial branches supplied only the mucosa. In contrast Sunderland and Swaney (1952) found that in 70% of cases there was an extralaryngeal branching, but either branch could carry either adductor or abductor fibres. Most of this work was done in man and only English and Blevins (1969) and Vogel (1952) comment on the innervation in other species. Also Sisson (1966) notes that in the horse the RLN branches extralaryngeally.

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The histology of the nerve and its fibre type composition have been the subject of a number of studies. Much interest was centered around the position of abductor and adductor fibres in the RLN with regard to explanations of Semon's Law. Sunderland and Swaney (1952) disproved Russell's (1892) suggestion that abductor and adductor fibres lay in different fascicles, by showing in the human RLN, that abductor and adductor fibres were constantly changing fascicles and were engaged in plexus formation. In addition the number of fascicles in the nerve constantly changed in number.

The size of fibres in the recurrent laryngeal nerves has been examined by a number of workers but with differing results. Most authors have found the nerve fibre distribution in the rabbit, cat and monkey in the distal part of the nerve to be unimodal (Fernand and Young 1951, Evans and Murray, 1954, and Murray 1957, Lund and Palmer 1969). Most of these authors have suggested that the finding of a unimodal distribution with no fibres above  $14 \mu$  or few below  $6 \mu$  would suggest that there were no large sensory afferent fibres nor small sensory afferents. In turn such muscles were unlikely to have any proprioceptive receptors. Evans and Murray (1954) in fact described two groups of fibres in the rabbits' recurrent nerve near the vagus, one medium sized group, and one smaller group of myelinated fibres. However each group was separate and at the level of the larynx, no small fibres were present these having been sensory fibres from oesophagus and trachea.

However other authors reported different findings in the recurrent laryngeal nerves of man. While Faaborg-Anderson (1957) also found a unimodal distribution in the human recurrent nerve (range  $2-21\mu$ ), Tomasch and Britton (1953) found a bimodal distribution in one recurrent nerve studied. They also noted that the nerve contained two groups of/

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of fibres, one of which was primarily sensory fibres from trachea and oesophagus, but the muscular branches were found to be bimodal in distribution with peaks at 1-2 $\mu$  and 6-7  $\mu$  on the left side and at 1-2 $\mu$  and 4-5 $\mu$  on the right side. In a more detailed study in the cat Abo-el-Enein (1967) found that the fibre diameter spectrum in both left and right nerves was bimodal with peaks at 2-4 $\mu$  and 8-10 $\mu$  (or 10-12 $\mu$ ). Without more information on the neurophysiology of the larynx it is difficult to know what function the various fibres constituting the recurrent laryngeal have. Supra-nodose vagotomy was carried out in the rabbit to determine the number and size of fibres which degenerated in the recurrent laryngeal nerve (Evans and Murray 1954). They found that there was total degeneration of the fibres which made up the larger diameter group and therefore suggested that all these fibres were extrafusal efferent fibres. A number of other studies confirmed these findings but in a study on one intact Macaca mulatta and in another one after unilateral supra-nodose vagotomy, Brocklehurst and Edgeworth (1940) found that 97% of the myelinated nerve fibres in the muscular parts of the recurrent nerve were efferent and 3% were afferent. It therefore seems that considerable species differences can occur. Similar experiments in the horse would be difficult due to the diffuse nature of the nodose ganglion.

At present therefore it can be presumed that the majority of fibres that make up the muscular branches of the recurrent nerve are extrafusal efferent fibres, and a small percentage are likely to be afferent in origin arising from muscle receptors. These branches also contain unmyelinated sympathetic and parasympathetic fibres (Duncan 1974, unpublished observations). Abo-el-Enein (1967) concluded from his study/

study that the fibre spectra in the cat's recurrent nerve included a sufficient range of fibres to include proprioceptive afferent fibres. Proof of this suggestion came from electrophysiological studies in which he recorded discharges in recurrent nerves following stretching of individual laryngeal muscles.

There is probably no more contentious point in laryngeal physiology than the presence or absence of stretch sensitive organs in the intrinsic laryngeal muscles. Several authors including Lucas Keene (1961) and Abo-el-Enein (1967) have reported finding such structures but many authors have maintained that there are no muscle spindles in these muscles (for discussion see Abo-el-Enein (1967)). In his study on the cat, Abo-el-Enein produced ample histological evidence of muscle spindles, spiral and claw endings, tendon organs and free nerve terminals. Spiral endings were far more numerous than spindles. He suggested that due to the longitudinal orientation of spiral endings round muscle fibres, that they would be sensitive to stretch of the muscle fibres and hence could have a proprioceptive function. Proof of this function was found neurophysiologically in that constant tonic activity was found in the laryngeal muscles, such activity arising from either muscle spindles or spiral endings.

## MATERIALS AND METHODS

Laryngeal muscles and nerves were obtained from a large number of horses and ponies (see general materials and methods). However, a large number of these were abnormal and only material in which the laryngeal muscles were thought to be entirely normal was used for discussion in this section. In particular it was not possible to use the recurrent nerves despite their apparent normality where there was any alteration in the muscle histochemical fibre type distribution in the muscles which they were supplying. Normal material therefore came mainly from very young horses or from ponies up to middle age.

The methods used on such material were as described in the general materials and methods. In one pony a complete dissection of the course of both recurrent laryngeal nerves was made and sections of the nerves taken at different levels to check the total number of fascicles throughout the course of the nerve.

An attempt was made to estimate the length of the recurrent laryngeal nerve and other long peripheral nerves in the standing horse. The anatomical points chosen are shown in fig.1. The recurrent laryngeal nerve was estimated to run from medulla to the base of the heart (fig.1 No.1) and then back up the neck to the larynx (fig.1 No.2). The longest forelimb motor nerve was estimated to run from the spine to the carpus (fig.1 No.3) and the extra distance the sensory nerve travelled was from the carpus to fetlock (fig.1 No.4). Similar measurements were made on the hind leg (fig.1 No.5 & 6). These measurements were made on a range of sizes of horses.

## RESULTS

### a) Gross Anatomy

In the one pony in which a detailed dissection of the recurrent laryngeal nerves was made, no difference was found to that previously described (Sisson 1966) in the course of the nerves from their point of origin on the vagus to their termination at the larynx. The position of the left recurrent nerve as it branches from the vagus to run round the aorta is seen in figure 2 and as it passes round the aorta to ascend the neck in figure 3.

In all cases in which a dissection of the recurrent nerves was continued to their point of termination (10 cases) the left and right nerves were seen to branch extralaryngeally, into an abductor and adductor branch (figure 4). Stimulation of each of these branches in turn produced abduction or adduction respectively, of the vocal cord.

### b) Muscle Histology

Routine cryostat-cut Haematoxylin and Eosin stained transverse sections, showed that there was little variation in muscle fibre size in each laryngeal muscle and that most of the fibres were polygonal in outline. There was little endomysial or perimysial connective tissue, and in the latter, blood vessels and myelinated nerve bundles could be seen.

Histochemistry showed that all the muscles had a mosaic pattern when stained for the presence of myosin ATP-ase, phosphorylase and PAS. This pattern was not always so obvious when staining for the presence of the oxidative enzymes, SDH and NADH. In some cases however a mosaic pattern was seen (figs. 5 & 6) but a high percentage of intermediate fibres was also noted. Fibres which were high in SDH especially were found to have concentrations of diformazan granules just below the sarcolemma.

The/

The percentage of type I and II fibres according to their staining for ATP-ase activity, was not quantitated, but in all the muscles examined (the CAD and CAL were the muscles most frequently sampled) there was obvious preponderance of type II/type I fibres possibly in the ratio 60:40 (fig. 7 a,b). This preponderance of acid-labile, alkali resistant fibres was also noticeable when the acid-preincubation technique was applied (fig. 7c). No detailed attempts were made to sub-classify type II fibres on the basis of their ATP-ase reaction following acid preincubation. Whether this division is into type IIA and IIB fibres is uncertain, but in figure 7c it can be seen that there are three types of fibres. The grey fibres appear to correspond to the type I fibres (compare with fig. 7 a and b) but the intensely dark fibres are type II fibres, while the light fibres are also type II fibres. After acid preincubation at pH 4.6, no reversal has taken place, but the reaction appears 'cleaner' and fibre typing is made more easy. This was typical of most cases where this stain was employed where it was apparent that acid preincubation produced better results than the standard alkaline incubation (pH 9.5) produced. However the exact pH at which accurate reversal and sub-division of type II fibres could be reliably reproduced is not known, but complete reversal and inhibition of acid labile type II fibres can be achieved following acid preincubation at pH 3.9.

In one case where serial sections were stained for ATP-ase (routine pH 9.5 plus acid preincubation), PAS, phosphorylase and NADH, a reciprocal reaction was noted in that most type II fibres were noted to be high in phosphorylase and glycogen (PAS) and low in NADH.

### c) Nerve Histology

Sections cut at intervals in both recurrent nerves in which a total dissection/

dissection was performed showed that the number of fascicles varied considerably along the length of the nerve (table 4).

Examination of both left and right vagii just before the origin of the recurrent nerves showed that the bundle of nerves going to make up these latter nerves were found grouped together within the vagus. (fig. 8)

In all cases it was seen that both recurrent nerves contained two types of fascicles, those which contained predominantly small myelinated nerve fibres and those which contained almost exclusively medium to large fibres (fig. 9). Occasionally smaller fibres were noted in the latter but these were thought to be infrequent. In some cases a group of small fibres could be seen to be occupying a fascicle in which most of the fibres were larger, but the anatomical separation of such groups was often distinct (fig. 10b). In other cases, fibrous tissue septae were seen to divide fascicles into two or three compartments, suggesting that possible plexus formation might be occurring. In some normal nerves Renault bodies were seen but these will be discussed later (section 4).

At the level of the larynx, it was noted that the recurrent nerve consisted mainly of the medium-large myelinated fibres (fig. 10c) the smaller fibres presumably having been oesophageal and tracheal branches.

Inspection of the nerve fibre distribution in one normal case (10 cms from larynx) shows that the right recurrent nerve was bimodal with peaks at 5-6  $\mu$  and 12-13  $\mu$  and a range of 3-17  $\mu$  (figure 11). This bimodality was not so noticeable in the left nerve although there was a possible peak at 4-5  $\mu$  within the second peak at 11-12  $\mu$  and a range of 4-16  $\mu$  (fig.11).

The total number of fibres in the left nerve was 761 and in the right nerve 780.

The extrafusal terminal innervation will be discussed in section 4  
as/

as the norm has not yet been defined. No evidence was seen in the routine transverse sections of the presence of muscle spindles or other sensory structures. Also, there was no evidence of these structures in samples stained with methylene blue although the extrafusal motor innervation was clearly seen. However, early electromyographical examination of the intrinsic muscles would suggest that like the cat, tonic activity is present in these muscles and that sensory structures such as spindles or spiral endings must also be present in the equine larynx (Duncan 1975 unpublished observations).

Comparison of the length of the recurrent laryngeal nerve (no distinction was drawn between left and right) and the longest motor nerve in fore and hind limbs, shows that the recurrent nerve is approximately 100% longer than other motor nerves (Table 5). No attempt was made to measure the difference in length of the laryngeal nerves but it was estimated that the left RLN would be about 20-25 cms longer than the right due to its different origin, and course within the thorax.



### DISCUSSION

The gross anatomy of the equine larynx and its nerve supply was found to be similar to previous descriptions. In the normal pony in which both recurrent nerves were fully dissected, the left recurrent nerve did not have an anomalous course at the aortic arch, and there was no evidence of Haslam's anomaly (Mason 1973, see introduction). In all cases examined the recurrent nerve was seen to branch just before the larynx, one branch supplying the abductor muscle (CAD), the other supplying all the adductor muscles (including the CAL) with the exception of the cricothyroid muscle (CT) which was supplied by the anterior laryngeal nerve.

Routine histology of laryngeal muscle showed no difference from other normal skeletal muscles. The histochemistry of the laryngeal muscles showed some common features with previous work in the horse and other species, especially with regard to the percentage of type I and II fibres as judged by the myosin ATP-ase reaction (Gunn 1972, Saghal and Hast 1974, Edström et al 1974). In all the equine muscles examined there was a greater number of type II fibres. Some differences however were found between the results of this and other work on the number of fibres which have high concentrations of oxidative enzymes. While Gunn (1972) found that the normal equine CAD was made up of entirely high oxidative fibres, the present results have shown that a mosaic pattern can be seen in these muscles when stained both for NADH and SDH. Intermediate fibres are however common and often obscure this difference and in many cases a mosaic pattern was not observed. Most fibres which were high in ATP-ase activity were also high in phosphorylase activity and glycogen, but the comparison with oxidative enzymes was often difficult to make. The reliability of the ATP-ase method and its clear distinction of fibre types makes it an ideal reaction with/

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with which to type fibres (Engel 1974). An interesting exercise remains to quantitate the percentage of type II A, B and C fibres in normal laryngeal muscle following acid preincubation (Brooke and Kaiser 1970 a, b).

The histological findings in the right and left recurrent nerves which were sectioned at different intervals, showed that the total number of fascicles is constantly changing. This is similar to the human recurrent nerve where it has been shown that fascicle number frequently changes, with obvious plexus formation (Sunderland and Swaney 1952). They also showed that the adductor/abductor fibres are not found in one position in the nerve but are intermingled and constantly change position. The separation of some fascicles in the equine nerve, by strands of connective tissue might suggest that plexus formation is occurring.

Previous findings, in other species, of division of the recurrent nerve into two i.e. some fascicles with only small fibres, and some with mainly large fibres, has been confirmed in the horse. It was also shown that the majority of the smaller fibres were not present at the level of the larynx and it was therefore suggested that these were sensory fibres from oesophagus and trachea. Histograms of nerve diameter distribution in the fascicles with the larger fibres (laryngeal muscular branches) in one normal pony have indicated that a bimodal distribution may be found.

Muscle spindles or other sensory endings were not seen but this may be due to a lack of complete sampling of the muscles. Equally, spindles may not be obvious on routine transverse section if not sectioned through the lymph space. Identification of such structures requires a more systematic search of the entire muscles, stained either by a gold chloride or silver method.

It/

It is felt that sufficient was known about the normal equine larynx and its nerve supply in order to be able to identify and measure the affects of disease.

a) Left RLN

<u>Point on nerve</u>	<u>No. of fasciculi</u>
Origin	11
10 cms from origin	13
10 cms from larynx	10
60 cms "	13
50 cms "	15
40 cms "	12
30 cms "	18
20 cms "	15
Branch to CAD	4
" " adductors	7

b) Right RLN

<u>Point on nerve</u>	<u>No. of fasciculi</u>
Origin	15
50 cms from larynx	9
40 cms "	8
30 cms "	8
20 cms "	11
Branch to CAD	6
" " adductors	6

TABLE 4 Total number of fasciculi in the recurrent laryngeal nerves of a normal pony at various levels.

Height (hands)	1	2	1+2	3	4	5	6 cms
14.	80	94	174	90	110	94	119
13.3	75	100	175	85	105	87	115
16.	98	116	214	106	132	95	130
15.	85	105	190	97	117	95	123
11.2	60	78	138	75	93	75	99
16.2	100	110	210	103	129	105	139
16.2	98	117	215	110	130	107	141
15.3	98	116	214	105	135	105	138

TABLE 5 Approximate skin/nerve measurements of long peripheral nerves in the horse. For explanation of headings see fig. 1 (1 + 2 = total length of the RLN).



Fig. (1): Skin/nerve measurements made on a range of horses.

- 1) Vagal course of R.L.N.
- 2) Cervical and thoracic course of R.L.N.
- 3) Course of longest forelimb motor nerve.
- 4) Extra course that sensory nerve has to pass.
- 5) Course of longest hind limb motor nerve.
- 6) Same as 4).

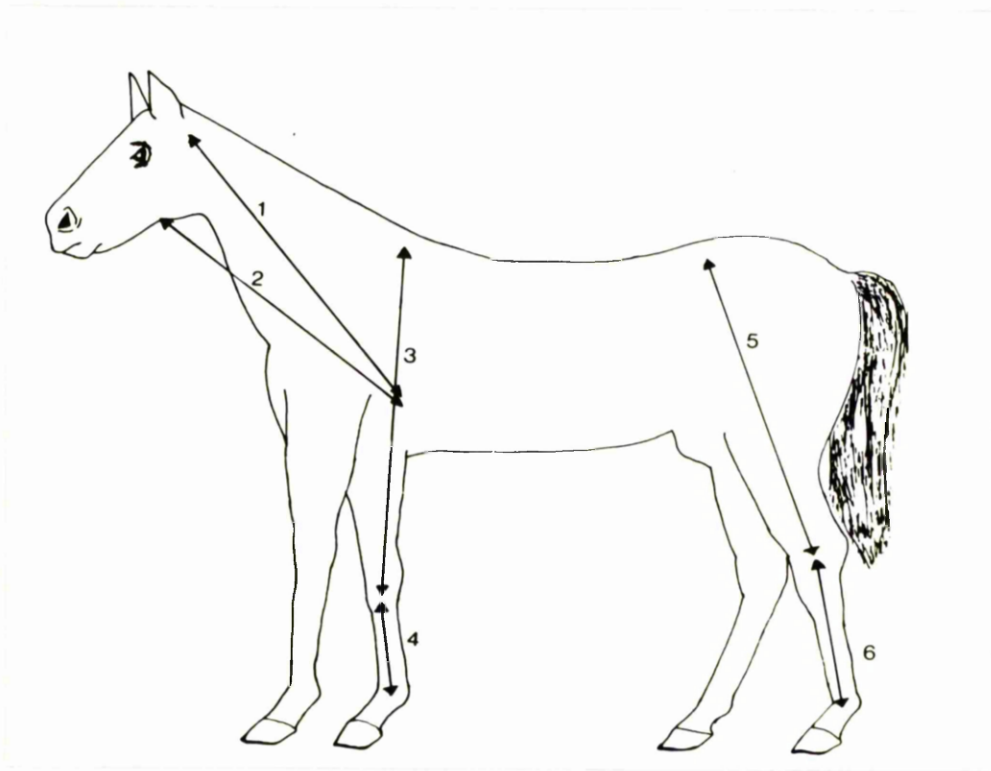




Fig. (2): Gross dissection of the left side of the thorax.

The left R.L.N. (arrow) is seen to leave the left vagus before passing around the aorta (A). The left phrenic nerve runs below the vagus.

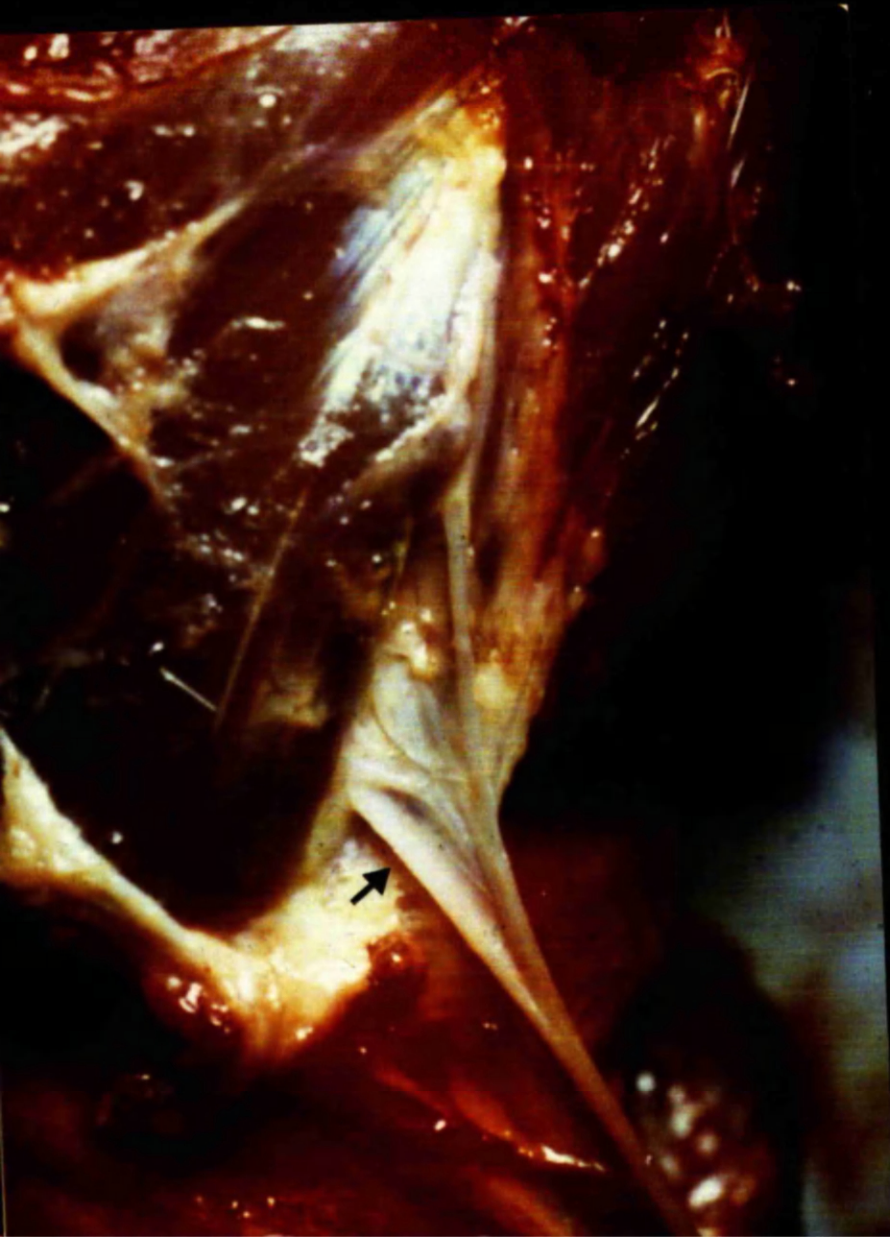
Fig. (3): Gross dissection of the thorax showing the left

R.L.N. (arrow) running round the aorta, which has been sectioned and reflected.



Fig. (4): Dorsal view of the larynx showing the right R.L.N.

branching to innervate the right C.A.D. (arrow). The branch to the transverse arytenoid muscle lies between the abductor branch (arrow) and the branch supplying the remaining adductor muscles.



All muscle sections are cryostat cut transverse sections unless otherwise stated.

Fig. (5): Normal right C.A.D. stained for N.A.D.H. showing a similar result to that seen in fig. (5). Intermediate fibres can also be seen but there is a high proportion of type II fibres.

(X 120)

Fig. (6): Normal left C.A.L. stained for S.D.H. While both type I (dark) and type II fibres can be seen, intermediate staining fibres are also present.

(X 120)

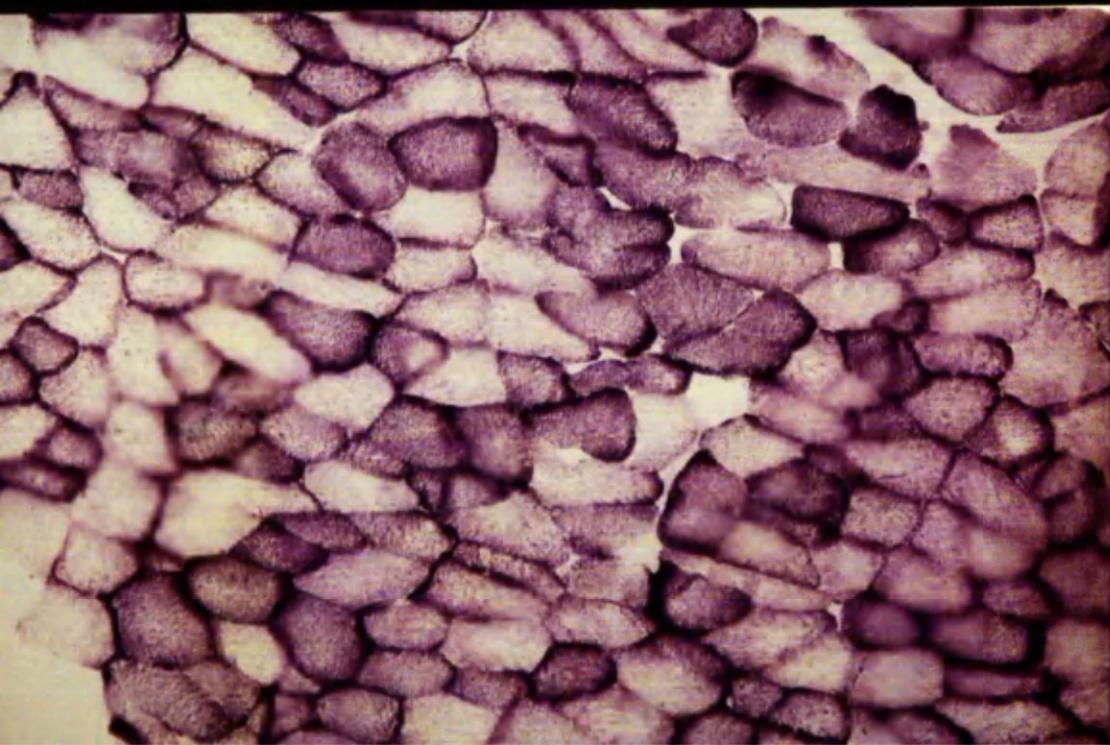
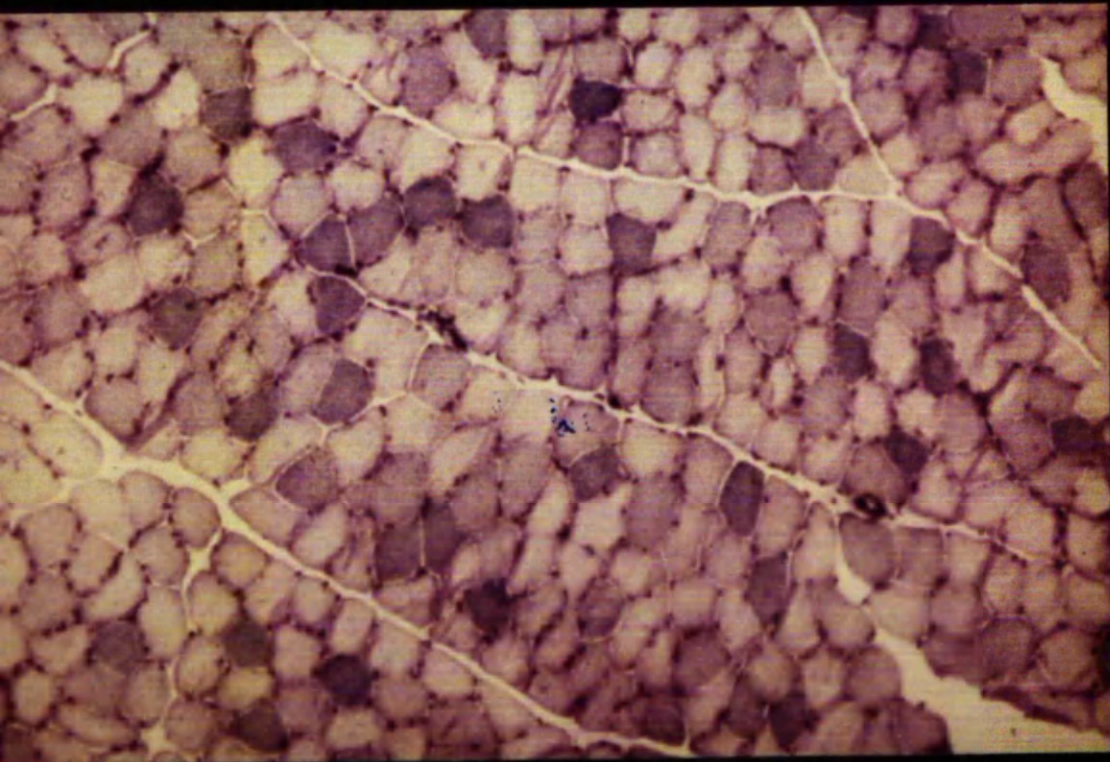


Fig. (7): Serial sections from the normal C.A.D.

- a) routine ATP-ase (pH incubation pH 9.5, without preincubation.
- b) ATP-ase (incubation pH 9.5, following acid preincubation at pH 4.6).
- c) ATP-ase (incubation pH 9.5, following acid preincubation pH 4.1).
- d) Phosphorylase.

There is no reversal of fibre type staining after preincubation at pH 4.6 but the reaction appears 'clearer' than in a). Following preincubation at pH 4.1 however, type I fibres appear dark grey, whilst type II fibres are split into light and dark fibres. It is possible that the former are IIA fibres and the latter IIB fibres. Most of the type II fibres as judged by b) stain darkly for phosphorylase content (d).

(X 240)

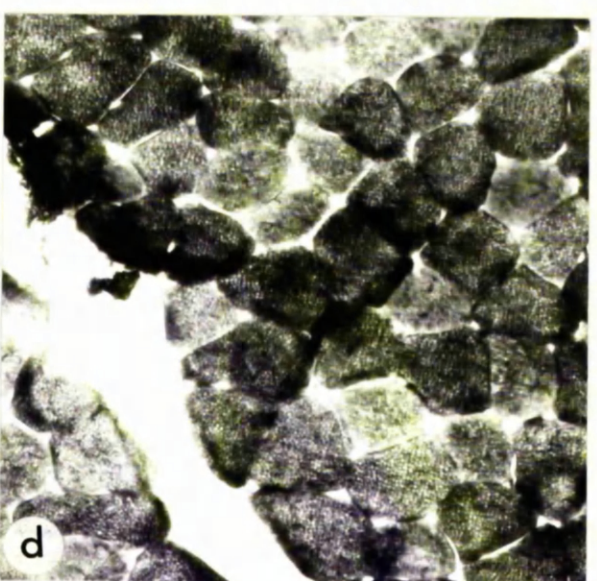
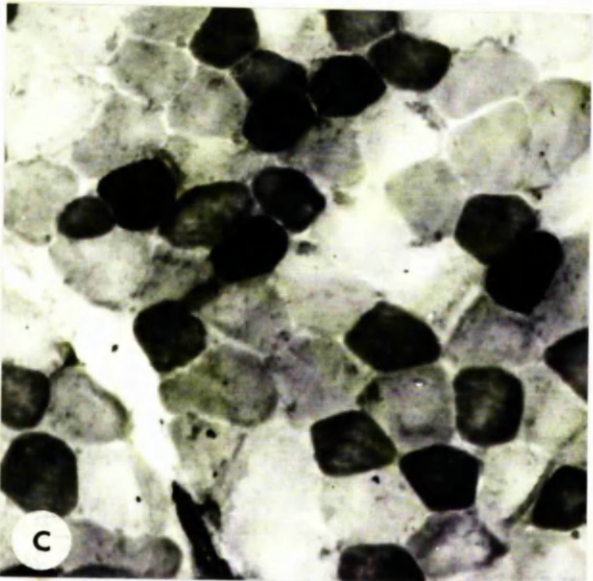
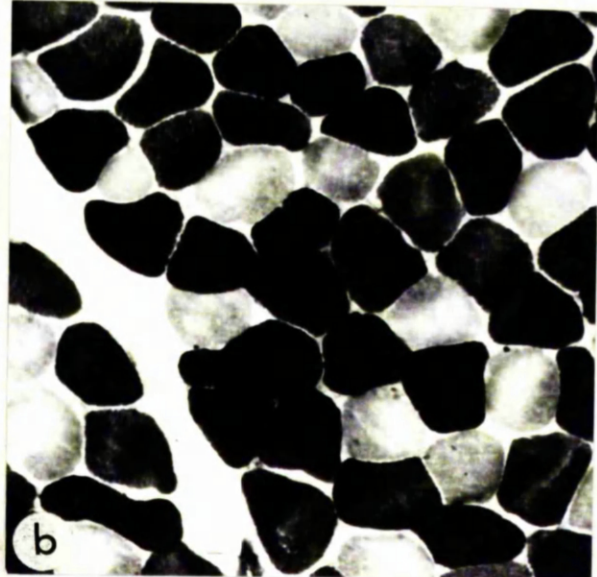
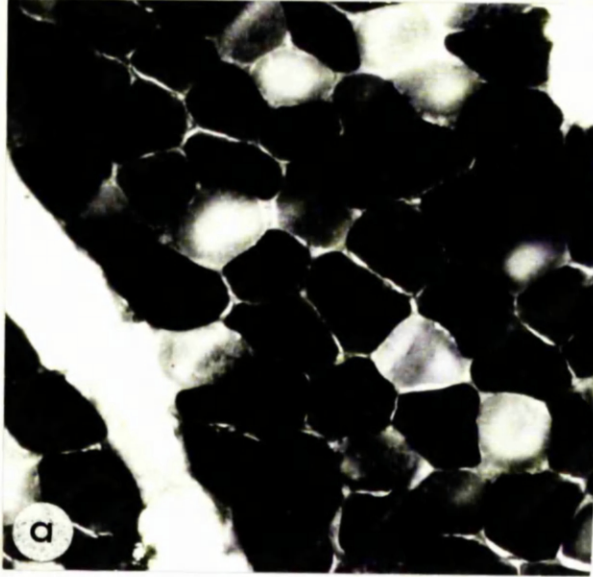




Fig. (8): Left vagus mid cervical region. Note that the larger myelinated axons which are going to make up the left R.L.N. are found to lie in close proximity, separate from most of the non-myelinated fibres.

(Kult. X 120)

Fig. (9): Left R.L.N. from a normal pony, 20 cms. from the larynx. The fascicle to the left is composed mainly of medium/large myelinated fibres whilst that on the right contains mainly small fibres with a few scattered medium sized fibres.

(Kult. X 250)

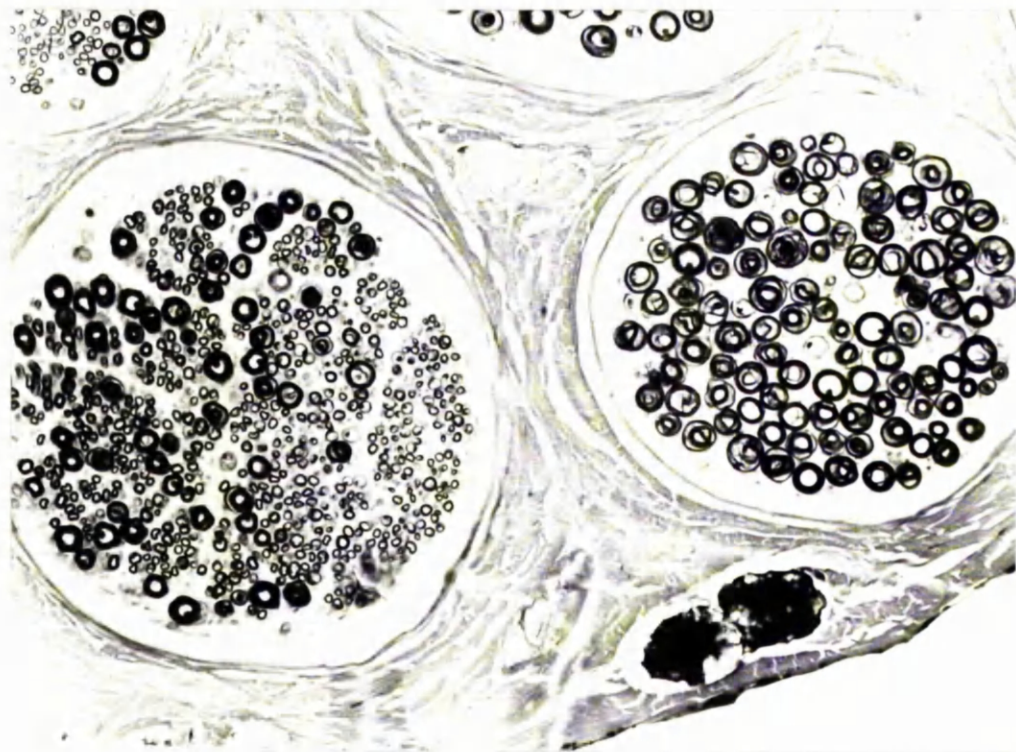
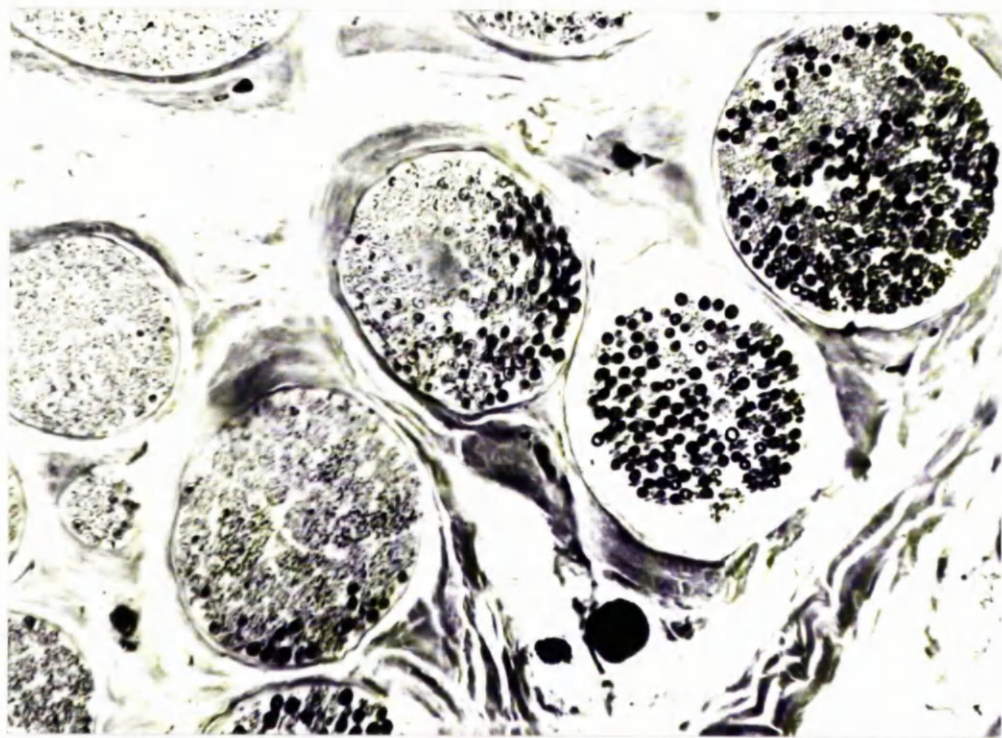


Fig.(10): Left R.L.N. from a normal pony at a) before the aortic arch b) 15 cms from the larynx c) at the level of the larynx. Note that small myelinated fibres which are numerous in a) have decreased in number 15 cms from the larynx (small group which are anatomically separated from the larger fibres - arrow). At the level of the larynx, c), there are few if any small fibres present.

(Kult X 120)

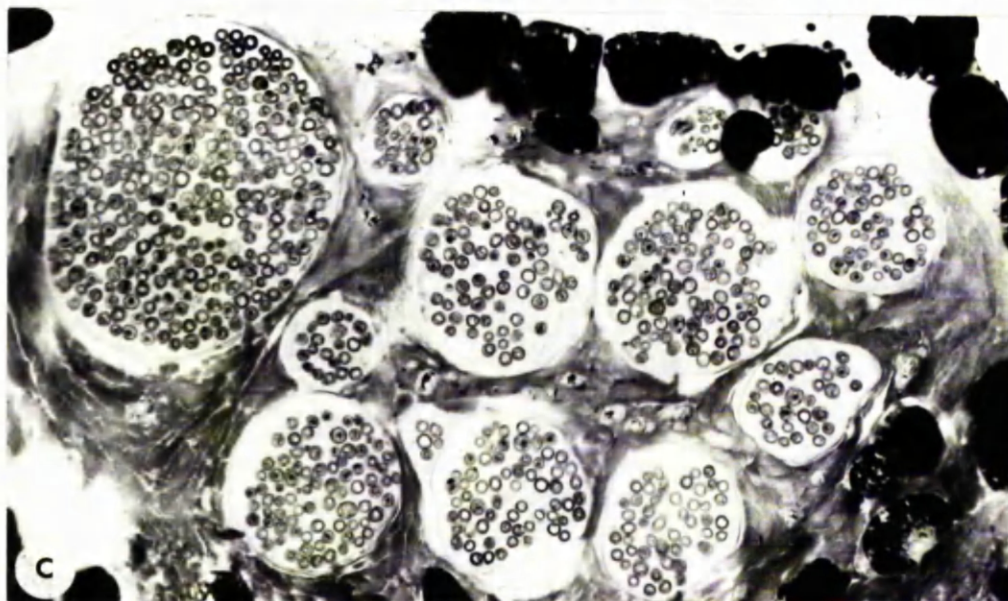
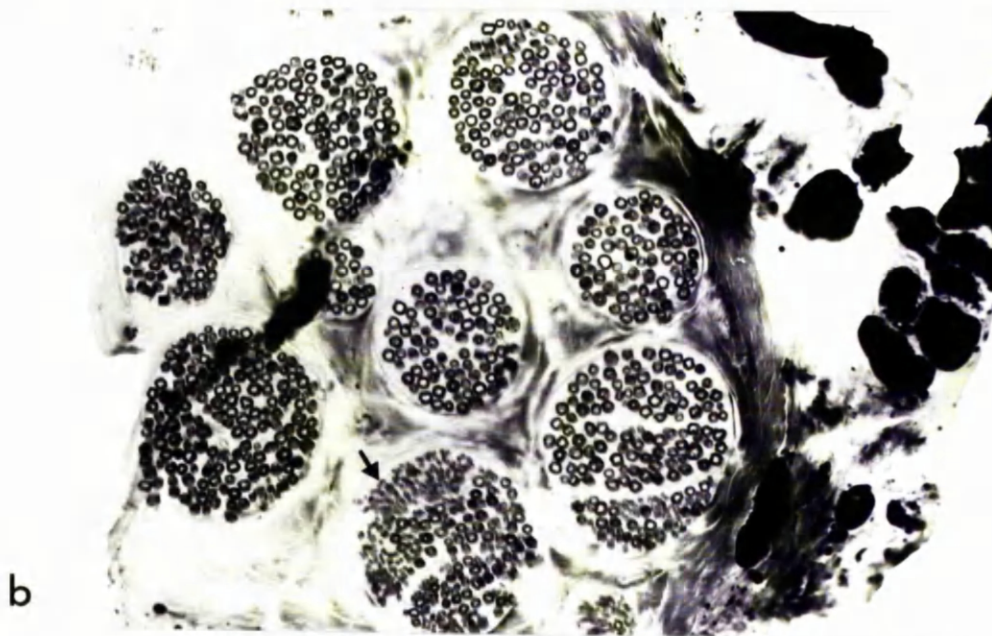
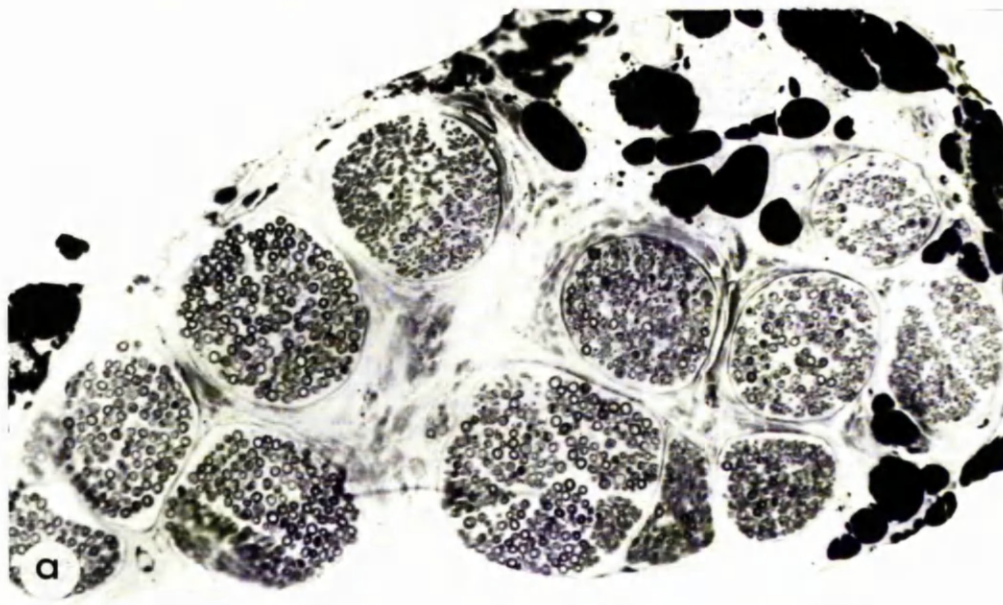
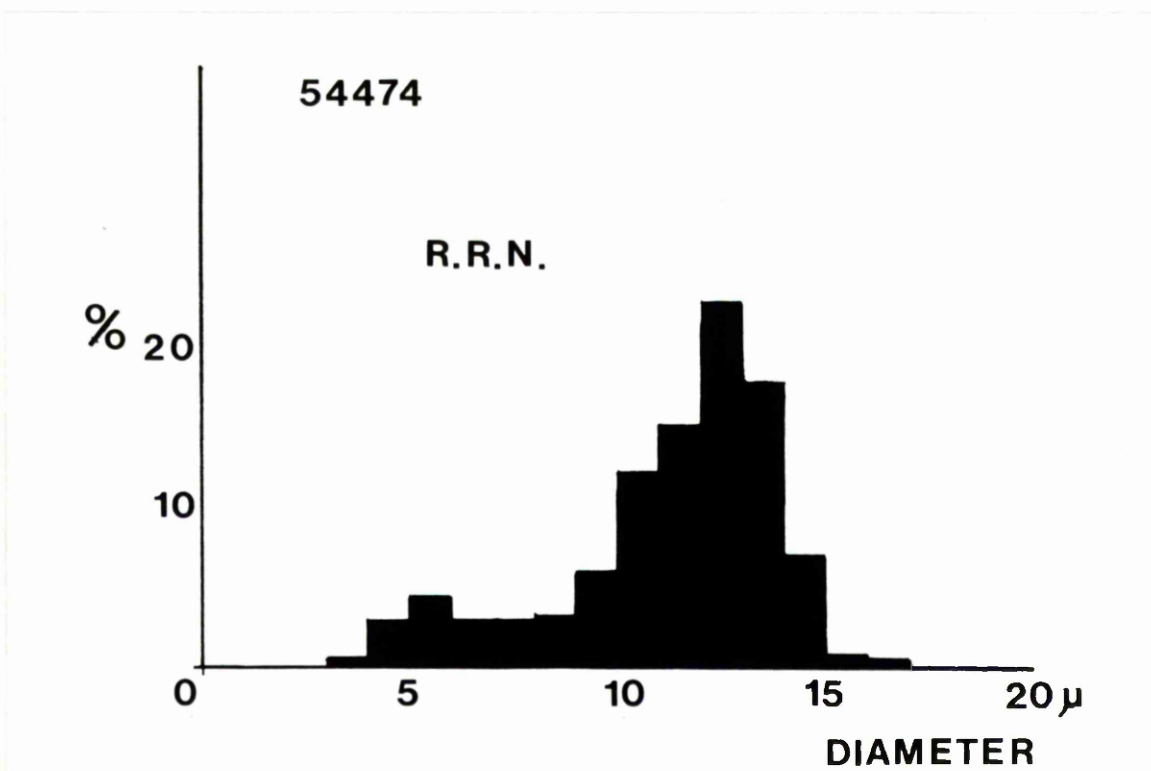
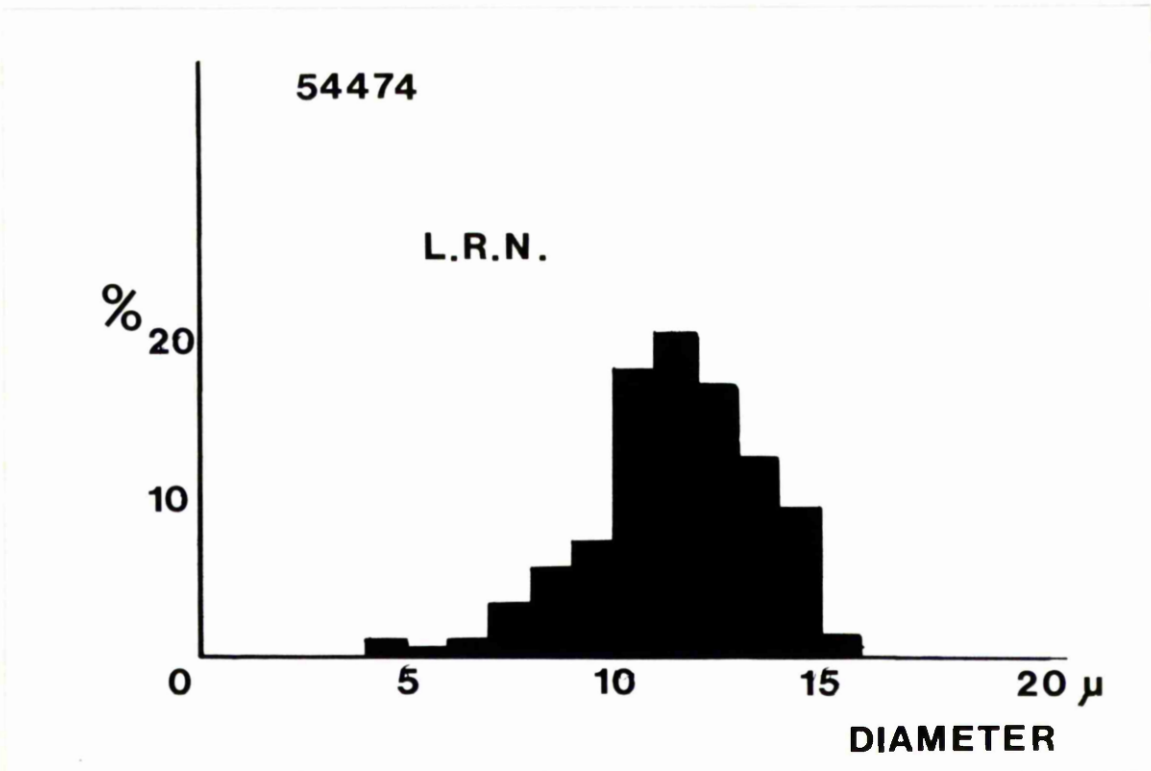


Fig. (11): a) Fibre diameter spectra of the left R.L.N. )  
                  b) Fibre diameter spectra of the right R.L.N.) Normal pony

A bimodal distribution is more obvious in the right R.L.N.



SECTION 2

MUSCLE PATHOLOGY

INTRODUCTION

Muscle reacts in a number of different ways to disease and the resulting pathological changes are often non-specific, when they occur at random. Changes such as variation in fibre size, internal nuclei, nuclear clumps or chains and degeneration, are found in many neuromuscular disorders. However, a specific combination of these changes may be pathognomonic for certain diseases. For example, in human dystrophica myotonica histological confirmation is conclusive if there is evidence of variation in fibre size, sarcoplasmic masses and ringed fibres, internal nuclei and nuclear chains.

It is generally acknowledged that muscular disorders are divided into two categories a) the myopathies b) the neurogenic atrophies. A myopathic disorder refers to a primary disease of the muscle cell itself. Neurogenic muscle disease is the result of any disorder of the lower motor neurons (LMN), which consists of the motor nerve cell with its axons and terminal arborisations. The trophic affect of nerve on muscle is well known (Guth 1968), and any interruption of the nerve supply will result in muscle atrophy. Muscle is one of the few tissues in the body to show such striking clinical atrophy following denervation.

Each LMN innervates a number of muscle fibres and together they form the motor unit. The size of the motor unit varies from muscle to muscle, extraocular muscles which are required to make small exact movements having small motor units, whereas limb muscles have large units. The cell body of the motoneurone lie in the brain or spinal cord while the axon is in the peripheral nervous system.

It is difficult to distinguish between lesions at various parts of the LMN on histological examination of the affected muscle, as the resultant changes are similar. The earliest change in denervation is the presence/



presence of scattered, atrophic, angular muscle fibres. The fibres although atrophic, still retain their cross striations and only lose these a long time after denervation. Often these atrophic fibres are found in groups, the number of fibres within these groups increasing with the severity of the disease. These groups may become so large as to involve entire fascicles (fascicular atrophy). The presence of "small" and "large" group atrophy is often taken as being pathognomonic of denervation (Dubowitz and Brooke 1973). The remaining fibres are often hypertrophied, and it is not uncommon to see adjacent atrophied and hypertrophied fibres in the same fascicle. Increasing duration of this process leads to further atrophy, and in denervation atrophy of a long duration there is a marked increase in endomysial connective tissue and fatty tissue (Sunderland and Ray 1950).

In the early stages of denervation, the sarcolemmal nuclei may assume a tigroid form (Dubowitz and Brooke 1973) in which the chromatin typically appears dispersed throughout the nucleus. Such nuclei are often found together in clumps and later in the disease process are seen as clumps of darkly staining pyknotic nuclei. The position of the nuclei can also change and they may be found centrally or internally. The apparent sarcolemmal nuclear proliferation may only be a relative increase due to the decrease in the sarcoplasm itself. Although the changes described are typical of denervation, at a later stage the histological separation of neurogenic and myopathic processes may become more difficult.

The changes typical of primary muscle disease include muscle fibre necrosis and regeneration, random variation in fibre size, centralisation of sarcolemmal nuclei, vacuolar and hyaline degeneration, and an increase in/

in endomysial connective tissue. However, some or all these changes have been reported in long term neurogenic atrophy. They have been described in experimental denervation by Tower (1935) and Adams (1962) in the cat, where degenerate fibres were found 1 month after neurectomy. In man such changes have been described in numerous diseases affecting the LMN, including chronic spinal muscular atrophy - S.M.A. (Mastaglia and Walton 1971, Pearce and Harriman 1966) in motor neuron disease - amyotrophic lateral sclerosis (Achari and Anderson 1974) and in a long term follow up study on polio sufferers (Drachman et al 1967). Cazzato (1969) has also described myopathic changes in muscle biopsies from patients with numerous human neurogenic diseases, and reviews this aspect in depth.

Other evidence of a myopathic process in denervation has been described. Myopathic potentials on electromyography have been found in some patients with chronic spinal muscular atrophy (Meadows et al 1969) and increased levels of serum CPK, an indicator of muscle damage have been described in motor neuron disease (Achari and Anderson 1974) and in S.M.A. (Mastaglia and Walton 1971).

Dubowitz and Brooke (1973) refer to the "myopathic" change in denervation and suggest that the difference in the changes seen in chronic/acute denervation are due to two factors. In chronic denervation, reinnervation occurs and tends to prolong the process and produce a spectrum of changes (Adams 1962). Secondly in long standing disease there is more fibre breakdown and resultant cellular response, although no reason is given for fibre degeneration occurring. Cazzato (1969) suggested several reasons for the presence of "myopathic" change in chronic denervation 1) partial reinnervation, 2) vascular changes, 3) hypertrophy/

hypertrophy followed by degeneration due to inadequate blood supply, 4) denervated muscle more susceptible to trauma, and 5) patient suffering from both myopathic and neurogenic disease.

The histological appearance of muscle in old age must also be taken into account when looking at the changes in equine laryngeal muscle. Age changes in human skeletal muscle have been well described in two recent papers (Tomlinson et al 1969 and Jennekens et al 1971). Tomlinson et al (1969) were impressed by the similarity of age to change to neurogenic atrophy although there was no direct correlation of these changes with increasing age. Jennekens et al (1971) in a histochemical study of aged muscle showed that there was both neurogenic and myopathic change, with the latter change being more obvious, in two distal limb muscles studied. They suggested that vascular insufficiency was the possible reason for the evidence of myopathic change in distal muscles.

Histochemistry of skeletal muscle biopsies has become increasingly important for three reasons. Firstly to demonstrate the individual fibre types within a muscle and hence any selective involvement of a particular fibre type in a certain disease process. Secondly to show the absence of particular enzymes, such as phosphorylase in McArdle's disease, and thirdly to demonstrate structural abnormalities within the muscle fibre (Engel 1962).

Some of the first experimental studies performed in laboratory animals using histochemical techniques were the investigations on the effect of denervation on the different fibre types as demonstrated by the enzyme stains (Romanul and Hogan 1965, Smith 1965, Engel et al 1966, Karpati and Engel 1968a). One of the main findings of this work was that the most reliable method of classifying fibre types in neurogenic/

neurogenic atrophy was the myosin ATP-ase reaction as it was least affected by denervation. In contrast, other histochemical reactions (Romanul and Hogan 1965) showed that after experimental denervation there was a gradual de-differentiation of fibre types. Fibres which were high in oxidative or glycolytic activity reverse to low activity whilst the remaining fibres were unchanged. The contrast between the fibre types was therefore lost. One other important finding of these initial experiments was the preferential atrophy of the type II fibres in denervation, as judged by the myosin ATP-ase stain (Engel et al 1966, Karpati and Engel 1968a).

While the effect of denervation on the histochemical profile of a muscle was now well established the effect of reinnervation on the muscle's histochemical reactions was unknown. The effect of reinnervation on skeletal muscle was therefore studied in great depth. Much of the interest in this field arose from the observations of Buller et al (1960) who demonstrated that when the nerves innervating the soleus and flexor hallucis longus muscles in the cat (slow and fast muscles respectively) were crossed, then the contractile properties of the muscles changed, the soleus becoming fast contracting and the flexor hallucis longus, slow. Might there therefore be corresponding changes in the muscle enzymes in such muscles? The work of Romanul and Van der Meulen 1967, Dubowitz 1966, and Guth et al 1970, showed that enzyme histochemical changes occurred. It was clearly shown that in the soleus muscle of the cat and rabbit, which is composed almost entirely of type I fibres, clusters of type II fibres were found after reinnervation by the nerve to flexor hallucis longus. In addition, the flexor hallucis or flexor digitorum which normally show a preponderance of type II fibres, showed large/

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large areas of type I fibres after reinnervation by the soleus nerve supply.

This work was developed by Karpati and Engel (1968b) when they showed that reinnervation of a muscle by its own nerve supply, after crushing the nerve on three separate occasions, or by cutting it and resuturing it, could produce marked changes in the fibre type distribution, or fibre type grouping. They also gave evidence that this fibre type grouping was due to collateral sprouting of sub-terminal nerve fibres.:

The elegant work of Edström and Kugelberg (1968, 1969) has added considerably to the knowledge and correlation of the motor unit in normal and reinnervated muscle. They showed that following direct repetitive stimulation of single nerve fibres in L4 and L5 ventral roots, it was possible to map out individual motor units which had become depleted in P.A.S. activity. This demonstrated that fibres of a particular motor unit, while confined in a limited area, were scattered and not clumped. Repetitive stimulation of single nerve roots two months after experimental section and resuture of the nerve supply to the anterior tibiàl muscle, showed that fibre type grouping as demonstrated by SDH reaction, had occurred. Kugelberg et al (1970) also showed that partial denervation of reinnervated muscle could result in atrophy of large groups of histochemically similar fibres.

While fibre type grouping was commonly seen in chronic peripheral neuropathies in man (Engel 1965), and had been adequately demonstrated in experimental conditions, the method by which it was achieved was not proven although Karpati and Engel (1968b) had produced evidence to suggest that it was due to collateral sprouting. However, there was no evidence for this in human peripheral neuropathies. Morris (1969) showed that in the human muscle biopsies which had marked grouping there was a much increased/

increased functional terminal innervation ratio (Coers and Woolf 1959).

It is now well accepted that fibre type grouping due to collateral sprouting is evidence of reinnervation (Engel 1970) but Morris (1970) has shown that reinnervation (as judged by increase in the terminal innervation ratio) may not produce clear cut evidence of fibre type grouping, but instead results in an increase in the number of intermediate staining fibres, these presumably being in the early stage of reinnervation. Intermediate fibres are often seen in sections stained for oxidative enzymes. Their presence makes classification of fibre type difficult and for this purpose oxidative enzyme staining may be less useful than the use of myosin ATP-ase reaction where intermediate fibres are less often demonstrated, (Engel 1974).

The available information on the percentage of the main fibre types in various human muscles has been inconsistent (Brooke and Engel 1969, a, b, Edstrom and Nystrom 1969, Morris 1970), however Jennekens et al (1971 a,b) in studies on autopsy material from patients who had no known neuromuscular disease, were able to give more detailed information. In order to assess the number of histochemically similar fibres which could be found in apposition in health and disease, they introduced the concept of the "enclosed fibre". A fibre was considered to be enclosed if in a transverse section it was completely surrounded by fibres of its own histochemical type. Increase in the number of enclosed fibres of both the main histochemical types (type I and II) was taken to be evidence of fibre type grouping.

In their first study (1971 a), they examined proximal and distal limb muscles from young and middle aged subjects. Of the five muscles examined only the extensor digitorum brevis (EDB) was found to have an increased/

increased number of enclosed fibres, all the other muscles having the normal mosaic pattern. From this data they were able to give some indication of the number of enclosed fibres necessary to indicate abnormality.

This study was compared with a survey of similar muscles from older subjects (1971 b), and while type grouping could now be found in all the limb muscles examined, the EDB was always involved and showed the most severe changes. It was therefore suggested that denervation and reinnervation in this muscle may be a feature of ageing, as correlative evidence of nerve fibre degeneration with age is readily available (see Jennekens et al 1971 b).

The surprisingly high incidence of type grouping in the EDB led Jennekens et al (1972) to look in more detail at this muscle and three other distal limb muscles, from patients of all ages. The EDB was only normal in the first decade of life but from the second decade onwards there was an increasing degree of fibre type grouping. Morphological abnormalities such as small and large group atrophy plus hypertrophy were also seen, and in the older age group, "myopathic changes" were common. By comparison, the other three muscles examined were normal. Again such changes were assumed to be due to chronic denervation and reinnervation.

More recently Johnson et al (1973) have attempted, using data from 36 muscles from six human patients at autopsy, to obtain reliable information on the fibre type constitution of these muscles and their spatial relationship both in health and disease. They postulated, that schematically skeletal muscle fibres were arranged hexagonally and that to obtain one enclosed fibre (Jennekens et al 1971 a), it was necessary on average to have seven histochemically similar fibres lying together (Jennekens/

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(Jennekens et al show only six fibres giving 1 enclosed fibre). While most fibres contained in one motor unit were thought to be scattered, this might not automatically be the case as was shown in the rabbit (James 1971), and therefore not all enclosed fibres were dependent on their numerical proportions within a given muscle. Using their hexagonal model in which fibre distribution was random, Johnson et al (1973) calculated the expected number of enclosed fibres for a complete range of fibre type proportions and compared this with what was actually found. As might have been predicted, the EDB was found to be the one muscle which had a consistently non-random spatial distribution of fibre types. Of the other muscles only the abductor pollicis brevis muscle had any loss of random distribution.

Such fibre type analysis and spatial distribution studies have not been performed in such detail in skeletal muscle of the domestic animals. However Gunn (1972, 1973) produced quantitative evidence of fibre type grouping in the laryngeal muscles of clinically normal horses by measuring the number of type I fibres whose sides lay in contact. In a histochemical study on a clinical roarer he found that there was preferential atrophy of type II fibres. Duncan et al (1973 and 1974) also showed evidence of fibre type grouping in equine laryngeal muscles but these changes were not quantitated. They also showed evidence of morphological abnormalities in these muscles and likened them to the changes described by Jennekens et al (1972) in the human EDB.



## MATERIALS AND METHODS

These were as indicated in the general materials and methods. Details of the histochemical techniques appear in the appendix. In addition to measuring muscle fibre size (Section 3) estimates were made of various changes in the muscles examined.

The following pathological changes were assessed in the laryngeal muscles.

- 1) Degree of muscle atrophy
- 2) Hypertrophy
- 3) Grouped atrophy
- 4) Fascicular atrophy
- 5) Fibre type grouping
- 6) Increased connective tissue
- 7) Increased fat
- 8) Degeneration
- 9) Regeneration
- 10) Cellular infiltrate
- 11) Internal nuclei

Each of the changes was graded on a 0 to +++ basis.

- 0 = no change
- + = little change
- ++ = moderate change
- +++ = severe change
- = no section or preparation

In most cases all the changes were judged on cryostat cut, transverse, H. + E. stained sections, and fibre type grouping in sections stained for myosin ATP-ase. Occasionally however it was necessary to refer to/

to paraffin sections for a more accurate estimate of increase in endomysial connective tissue, and the presence of degeneration and regeneration.

Grouped atrophy was classified as existing, when at least 4 - 6 atrophic fibres lay beside each other.

## RESULTS

Details of the muscle pathology will be sub-divided into various headings depending upon the clinical nature of the cases. That is, those which had clinical evidence of disease, either true laryngeal hemiplegia or adductor paralysis (as judged by endoscopy), will be described under separate headings, as will those which had evidence of sub-clinical disease alone.

In each of these cases, a scoring system was employed in an attempt to estimate the severity of the lesion. The results of this are on pages 89 - 98 .

### 1) Laryngeal muscle pathology in clinically affected cases

The muscle pathology of the four cases examined was more severe in 47988 and 52838, than in 49589 and 40733.

#### a) 52838 and 47988

In both cases there was gross atrophy of the muscles of the left side of the larynx and this was most obvious when one compared the left and right CAD muscles (fig. 12). The left CAD muscle was almost entirely replaced by fat and connective tissue whereas the right CAD is grossly normal.

Histologically the fatty and connective tissue increase was very noticeable. Despite the obvious chronicity of the lesion occasional muscle fibres of normal size were seen but there was no evidence of hypertrophy (fig. 13). Clumps of pyknotic nuclei were evident in 47988 but not in 52838, but in both, chains of rounded plump nuclei with scattered chromatin were seen (fig. 14). These resembled tigroid nuclei as described by Adams et al (1962) and Dubowitz and Brooke (1973).

There/

There was little evidence of myopathic change in either case, with only occasional internal nuclei, but estimation of such change may not be significant at this stage of the disease as very few muscle fibres of any size were present. Intramuscular nerve bundles contained few intact axons and there was marked endoneurial fibrosis.

Enzyme histochemistry was not performed on the muscles from the left side as the tissue would not freeze, but was useful in demonstrating the presence of fibre type grouping in the muscles supplied by the right R.L.N. in case 47988. Sections stained for myosin ATP-ase activity showed that type grouping was more marked in the right C.A.L. than in the right C.A.D. In addition to this, early grouped atrophy was noticeable at the tip of a fascicle in the C.A.L. but there was no obvious atrophy in the C.A.D.

b) 49589 and 40733

The muscles on the left side of the larynx supplied by the R.L.N. were grossly atrophic but some muscle tissue was detectable in both cases.

The histo-pathological changes were very different to those seen in the other two clinical cases. The muscle atrophy was not so severe and there was marked variation in fibre size. Both small group atrophy and fascicular atrophy were prominent but occasional hypertrophied fibres were still present (figs. 15 & 16). Connective tissue and fat were increased in amount (fig. 17) and clumps of pyknotic nuclei were obvious. There was a remarkable number of internal nuclei but only minimal evidence of fibre necrosis.

Two target fibres were found in the left C.A.L. in sections stained for NADH activity in 49589, and targetoid fibres were also seen. Type II fibre predominance was more obvious than type grouping, although small/

small groups of type I fibres could also be seen. Most of the small atrophic fibres were type II. There was no evidence of fibre type grouping in the left CT, the right sided laryngeal muscles, or the distal limb muscle (fig. 18 a/f).

## 2) Muscle pathology in the sub-clinical cases

It was often possible in the more severely affected sub-clinical cases to detect gross evidence of laryngeal muscle atrophy. Such muscles were usually paler and of smaller bulk than the contralateral muscles. In most of the cases where this atrophy was grossly noticeable, the left adductor muscle sampled (CAL) was more severely affected than the ipsilateral abductor (CAD).

Laryngeal muscles were classed as showing histological evidence of sub-clinical pathology when there was atrophy with fibre type grouping, or fibre type grouping alone. A number of older ponies which showed marked variation in fibre size but with no evidence of fibre type grouping were not classed as sub-clinical cases, such variation being possibly directly related to ageing.

Only occasional muscles showed evidence of fibre type grouping alone. In the majority of affected muscles there was also marked variation in fibre size with angulation and atrophy (fig. 19 & 20). This atrophy was not so severe in some cases and only occasional, diffusely scattered, atrophic fibres were seen (fig. 21). Small group atrophy with evidence of hypertrophy was commonly seen, and in the more severely affected cases, fascicular or large group atrophy was frequent (fig. 22). Compensatory hypertrophy in response to the atrophy was common and there was evidence of an increase in perimysial and endomysial connective tissue (fig. 22). Internal nuclei were commonly seen and in severely affected cases there was clumping of pyknotic nuclei. Tigroid nuclei were also common and these were seen singly, in clumps or in chains. Intramuscular nerve/

63

nerve bundles were often difficult to find in affected muscles compared with the contralateral controls and were noted to have a decreased myelinated fibre content and an increase in endoneurial connective tissue (fig. 23).

"Myopathic changes" such as internal nuclei, fibre splitting, fibre necrosis and cellular infiltrate were noted in the more severely affected cases (figs. 24 & 25).

In nearly all the cases the left adductor muscle (CAL) was more severely affected than the left abductor (CAD). This was confirmed by two independent observers. In three sub-clinical cases, very early neurogenic atrophy was present in the right CAL (fig. 26), but never in the right CAD. The left and right CT muscles were always normal.

Nearly all the sub-clinical cases showed evidence of fibre type grouping as demonstrated by the myosin ATP-ase reaction. Fibre type grouping was often more marked in the CAL muscle than in the CAD and this was obvious in one of the foal's which showed evidence of sub-clinical disease, (fig. 27). A number of cases showed a marked type II fibre predominance and without evidence of groups of type I fibres, these could not be described as showing fibre type grouping. However in some muscles there were some large groups of type I fibres despite the large number of type II fibres (fig. 28). Occasional sub-clinical cases also showed evidence of fibre type grouping on the right side, most often in the right CAL muscle (fig. 29). As mentioned in the section on the normal equine larynx, no serious attempts were made to sub-classify the type II fibres using the myosin ATP-ase reaction following acid-preincubation. However, in one case where serial sections were prepared it might be concluded that the majority of fibres were type IIb fibres, with small groups of type I fibres (fig. 30).

Hypertrophic fibres were mainly type II, but occasional type I hypertrophy/

hypertrophy was seen. In nearly every case, the small angular atrophic fibres were high in NADH activity and low in both SDH and phosphorylase activity, (figs. 31 & 32). Type grouping was not obvious in the sections stained for these enzymes, but occasionally was seen in PAS stained sections (fig. 33). Angular fibres, high in NADH activity were evidence of early neurogenic atrophy in the right CAL muscle in one case (fig. 31). Target fibres were very rare, but again were seen in the NADH reactions in one case (fig. 34).

3) Muscle pathology in cases with possible adductor paralysis.

57769

Grossly the left adductor muscles were severely atrophied and pale in colour. In comparison the left abductor muscle (CAD) was almost normal as were all the muscles on the right side of the larynx.

Histologically this difference was even more obvious. The left CAL showed a striking range of changes while the left CAD had only a few scattered angular atrophic fibres (fig. 35). Large group atrophy was obvious in the left CAL and there was marked hypertrophy and replacement by fatty and connective tissue (fig. 36). Internal nuclei were frequent and the "myopathic changes" seen included fibre splitting, necrosis, cellular infiltrate and even regeneration (fig. 36). Clumps of pyknotic nuclei were obvious.

Histochemistry showed that fibre type grouping was marked in the left CAD (fig. 37) and there was a marked type II fibre predominance in the CAL. Subsarcolemmal NADH activity was high in some fibres of the left CAD, (fig. 36) similar to areas seen in the left CAL of one sub-clinical case (fig. 34). The right side of the larynx also showed evidence of grouping but only in the CAL, the CAD having the normal mosaic pattern (fig. 39).

A/

A distal limb muscle examined in this case was normal (fig. 39).

58877

Although the degree of atrophy both grossly and histologically in this case was not so severe as in the preceding case, the difference between left CAD and CAL muscles was notable (figs. 40 & 41).

The left CAD showed marked type grouping with little obvious atrophy while the left CAL showed type II predominance and marked atrophy. Large group atrophy, hypertrophy, increased connective tissue, internal nuclei (fig. 41) and fibre necrosis were all seen in the left CAL.

4) Changes in limb muscles examined.

In no case were the changes seen in these muscles, similar to those in laryngeal muscles of the clinical or sub-clinical cases. The only change which was noted was a marked variation in fibre size in some muscles (fig. 42). This was noted in the distal limb muscles of two cases and also in the proximal limb muscles of two old ponies. In no case was fibre type grouping obvious but it was noticed in the latter two cases that the smallest fibres were predominantly type I fibres (fig. 42).



### DISCUSSION

The pathology of the first two clinical cases (47988 and 52838) was typical of long term neurogenic atrophy, while the third and fourth cases (49589 and 40733), although showing equally severe clinical signs, were less severely affected pathologically. The severity of the lesion is presumably, related to the duration of the denervating process but this is impossible to estimate from the pathology. All were within the same age group (8-12 years).

While there was little obvious muscle tissue on the left side of the larynx, in the first two cases a few normal sized muscle fibres were present. These fibres tended to be adjacent and it is likely that they were within the same motor unit. In man, small muscle fibres may still be present 10 years after denervation (Adams et al 1962).

It is interesting to speculate at what stage of atrophy does a muscle in fact become non-functional? Cole (1946) suggested that a factor of 50% atrophy was required in the laryngeal muscles of the horse before the animal developed a paralysed larynx. Erbslöh (1968) has shown that in amyotrophic lateral sclerosis (A.L.S.) in man, 50% of the muscle tissue must be destroyed before manifest paresis occurs.

Long term neurogenic change can sometimes be mistaken for chronic myopathic change but examination of intramuscular nerve bundles in the 'roarers' showed that there were few surviving axons with marked endoneurial fibrosis thus confirming the neurogenic origin of the lesion.

It is thought that the third and fourth cases (49589 and 40733) demonstrated changes that lay between those seen in two other 'roarers' and those seen in the sub-clinical cases. It was of interest that case 49589 was one of the few cases, either clinical or sub-clinical, in which target-/

target-fibres were demonstrable. Such fibres were first described in detail by Engel (1961) and have since been produced experimentally following denervation and reinnervation, (Dubowitz 1967) and tenotomy (Engel et al 1966). They have been described as a feature of most human chronic denervative processes but not in the more acute denervating diseases such as Werdnig-Hoffman disease, the more severe form of S.M.A. (Dubowitz and Brooke 1973). However, they are extremely uncommon in neurogenic disease in the domestic animals even where reinnervation was established both physiologically and histologically (Griffiths - personal communication). They are not seen in completely denervated animal muscles (Tomonaga 1969) but in such cases, "central areas" with high oxidative enzyme activity have been described (Griffiths et al 1973). These central areas have also been seen in completely denervated equine and canine laryngeal muscle (Duncan - unpublished observations 1973). Whether target fibres represent denervation or reinnervation is still not certain.

The other interesting histological finding in the clinical cases was the involvement of the right side of the larynx in one case. It is uncertain whether or not the right side was involved in the two of the other three cases as the cryostat sections from these muscles contained ice artifact and some showed P.M. change. In the fourth case (40733) the muscles from the right side of the larynx were normal.

The muscle pathology seen in the sub-clinical cases was possibly more informative, as the lesion was more active than that seen in the chronic clinical cases. It was evident from the survey that a wide range of types and ages of horses showed evidence of sub-clinical disease. Of the clinically normal horses, 40% ( 23 ) had histological evidence of laryngeal muscle pathology. Both the sub-clinical and clinical disease was related to/

to the size of the horse. 92% ( 24 ) of horses over 14.2 hands were found to show laryngeal muscle pathology. In this study there was no sex incidence but this would require a larger study. There was no apparent direct correlation between age and sub-clinical disease, but such a process possibly becomes more severe with age as it was very occasionally possible to detect neurogenic atrophy in smaller, aged horses (54646). However it should also be noted that neurogenic atrophy can be found in very young horses, as two horses under 1 year of age were found to be sub-clinical cases (57374 and 58856). In particular, in the second case, 58856, atrophy was severe, and type grouping marked, indicating that the disease was of some duration. A wider survey of foals' larynges would seem to be indicated by these findings, as might be the possibility of a congenital origin of the disease. Nearly all the Thoroughbreds examined were found to have evidence of laryngeal muscle atrophy but of the two which were histologically normal, one was young and stunted, and in the other case, no cryostat sections were available. Of the remaining sub-clinical cases there was no obvious breed predilection and any suggestion as to a breed predilection or heritable basis for this disease will require a very careful genetic study.

The range of changes seen in the sub-clinical cases in the routine light microscopical sections was remarkable, and varied from scattered single fibre atrophy to large group atrophy with compensatory hypertrophy. The similarity of the lesion to that described in the human E.D.B. by Jennekens et al (1972) is striking, and although the changes in the E.D.B. become more severe with age they can be seen in young people. The aetiology of the lesion in the E.D.B. is unknown but the Newcastle workers suggested that the nerve supply to this muscle may be affected by badly fitting shoes/

shoes, or the muscle may be compressed by the tendon of the extensor digitorum longus muscle. Whatever the cause, they suggested that the pathogenesis of the lesion was a chronic denervation and reinnervation of the muscle, and it seems almost certain that a similar process occurs in the equine laryngeal muscles. It would seem that at an early stage of the disease, reinnervation is sufficient to compensate for denervation, i.e. there is no atrophy but fibre type grouping is obvious.

The formation of small fibre type groups may follow denervation of only part of the motor unit, for example when only one or two sub-terminal nerve fibres degenerate (fig.43 a,b). Denervation of this group of reinnervated muscle fibres would lead to small group atrophy. Further denervation of adjacent muscle fibres may lead to an increase in the size of the histochemically homogeneous fibre type group, as the healthy unit responds with collateral sprouts reinnervating the denervated fibres (fig. 43c). It is suggested that in these muscles it may be possible that the whole motor unit is not denervated simultaneously. The histology and histochemistry of the lesion would indicate that denervation primarily at the intramuscular level (i.e. the sub-terminal nerve fibres of the motor unit) may be prominent. In contrast, loss of the entire motor unit and not just occasional sub-terminal nerve fibres would, for example in the case of the reinnervated group (fig. 43c), result in the formation of a group of atrophied histochemically homogeneous muscle fibres (fig. 43d). Such a process must also occur in the laryngeal muscles as similar areas were found, indeed whole atrophic fascicles containing either type I or type II muscle fibres were seen. Kugelberg et al (1970) suggested that the boundaries of reinnervating collateral sprouts seemed to be contained within individual fascicles and that the perimysium may be acting as barrier/

barrier, at least for terminal collaterals. Possible support for a distal progression of the lesion may be drawn from the clinical case in which there was evidence of fibre type grouping on the right side of the larynx. As will be discussed later, it was noticeable in all the clinical and sub-clinical cases that the CAL muscle was more severely affected than the CAD. In the clinical case with right sided involvement and in many of the sub-clinical cases on the left side, fibre type grouping was much more marked in the CAL than the CAD. This difference may be due to an increased 'drop-out' of sub-terminal fibres in the former muscle with subsequent reinnervation and expansion of the groups of histochemically similar fibres (see fig. 27 a,b).

Equally of course, such a process could occur if simple whole motor-units are progressively denervated, but the finding of single scattered angular atrophic fibres in some muscles, suggests that often only part of the motor unit has degenerated. It would also seem likely that if the process is of sufficient chronicity then reinnervation will obscure any atrophy and only fibre type grouping would be observed.

Histochemistry has been useful in demonstrating the types of fibres which are most commonly found in the fibre groups, and which fibre type is most affected by the denervative process. In Gunn's (1973) clinical case he found that there was a preferential atrophy of type II fibres, and this would apply in the present series although atrophic type I fibres could also be seen. Architectural changes in the muscle fibres such as target fibre formation were found only in one sub-clinical case and possible tubular aggregates in one sub-clinical case, and one case of adductor paralysis. Tubular aggregates refer to the areas beneath the sarcolemma which were abnormally high in NADH activity. These were first described/

described by Engel (1964) as mitochondrial aggregates but this name has since been changed, as they have been found to be made up solely of tubular structures. They are found only in IIB fibres (Dubowitz and Brooke 1973) and stain highly for all the oxidative enzymes except S.D.H. They were not stained for this enzyme in the two cases previously mentioned but it is felt that there was an excess amount of "high NADH material" even considering the sub-sarcolemmal position. These structures are thought to be non-specific but have been described in denervated muscle (Price 1973). Pearse and Johnson (1970) also described tubular aggregates from a patient with myopathy and suggested that the histochemical properties of these structures indicated that they were mitochondrial in origin.

While the possible pathogenesis of fibre type grouping has been discussed no account has been given for the cases which showed evidence of type II fibre predominance. Gunn (1972) showed that in the normal CAD of the horse, 72% of the fibres were high in ATP-ase. The relative frequency of the two main fibre types was not calculated in the present study but it was noted that type II fibres were more frequent in both the normal CAD and CAL. It would appear therefore, that if random denervation of these muscles occurs, there would be a greater chance of the reinnervating motor neuron to be a type II neuron than a type I. Such a process of change in a muscle with a higher proportion of one fibre type than the other, may increase the frequency of occurrence of fibre type predominance following reinnervation, rather than fibre type grouping. Selective degeneration of either type I or II neurons may occur, but the occurrence of groups of both fibre types would suggest that both types of neurons are equally susceptible. Morris (1970) noted in human peripheral neuropathies, that grouping of type I fibres is much more common/

common than grouping of other fibres and he suggested that this may be due to type I neurons demonstrating a greater propensity for collateral sprouting. He also described a greatly increased number of intermediate fibres in association with an increased functional terminal innervation ratio, and suggested that such fibres may be type II fibres undergoing reinnervation and transformation to type I fibres. He was using oxidative stains to classify fibre types. In the laryngeal muscles it was often difficult to differentiate fibre types using these stains and it may therefore be, that a number of the intermediate fibres seen were changing their metabolic profile following reinnervation.

Gunn (1973) also indicates that in his one clinical case, the left transverse arytenoid muscle showed a type II fibre predominance, whereas in the contralateral muscle there were some type I fibres. Again this may be due to the original predominance of type II fibres. In addition he notes that in areas of severe atrophy, fibres have a reduction in their previous capacity for aerobic or anaerobic metabolism. This was found experimentally by Romanul and Hogan (1965).

While Gunn was the first to publish evidence of type grouping in equine laryngeal muscle, some of his results could benefit from closer scrutiny. In particular his definition of abnormality may be suspect. If one analyses Gunn's figures, on the evidence presented, one can perhaps draw some further conclusions. In his first series (1972) of 12 horses, three were found to be normal, his definition of normality being that the muscle fibres of the CAD showed little difference between left and right either in fibre type or distribution. The distribution of fibre types was measured by counting the number of ATP-ase low fibres lying in contact. A group was defined as "the number of fibres in the/

the same fascicle that were low in myosin ATP-ase whose sides (rather than angles) were in contact." In his second series (1973) one was a clinical roarer, while of the three fetuses examined, one was described as being abnormal.

The mean group size in both papers was compared (Table 6) Excluding the foetal horses (13,14,15) and the foal (5), the horses classified as abnormal show the following left - right differences in the ascending order - 0.9, 1.8, 1.9, 6.3, 10.0. The first three are admittedly small but could possibly be significant if the figures were based on a large number of groups especially if the variance in the number of fibres per group is small.

Therefore without more data it is difficult to evaluate the statistical significance of some of his cases, and certainly the data is insufficient, mean group size alone being not enough. Evidence of significance using the student's T-test would have been useful as would have been the variance in number of fibres per group. If more data had been provided then perhaps more conclusions could have been deduced.

His finding of type grouping in one foetus, if it were true, would be interesting as it raises the possibility of a congenital nature of the disease. This possibility has already been raised in the current study as two very young horses were affected. However, it may be faulty to apply great significance to the myosin ATP-ase reaction in developing muscle, as Guth and Samaha (1972) have shown that in such muscle, histochemical and biochemical levels of ATP-ase activity do not correspond. In particular slow muscles were found to have low ATP-ase activity on biochemical estimation, but high on histochemical estimation.

It is admitted that a discrepancy of this current work was that neither/



neither the percentage of fibre types nor their distribution was quantitated. In the light of the work by Jennekens et al (1971a) and Johnson et al (1973) it would seem desirable that such an approach should be applied to equine laryngeal muscle. The concept of the enclosed fibre would seem to provide much more data than that provided by Gunn's method of type group quantitation, and it is probably necessary to quantitate the distribution of both types of fibres (Dubowitz and Brooke 1973). It would also be interesting to investigate the hypothetical model of Johnson et al (1973) and see how regularly the fibre type distribution of the laryngeal muscles showed a non-random spatial distribution.

The fact that the right side of the larynx can also be pathologically involved in the disease (Cole 1946, Gunn 1972, Duncan et al 1974) must be highly significant when considering the pathogenesis of the disease. Although the right side of the larynx has not been reported to be affected clinically (apart from one case: Hanselka 1973) it may well be that advanced atrophy of the right side may produce some, if only slight, failure of right sided abduction which is difficult to detect on endoscopy. It is well known that following ventriculectomy, many horses make good progress for a year or two and then regress (Marks et al 1970). This may be due to collapse of the left arytenoid cartilage and/or to some change in the right side. Although it may be more common to find atrophy or type grouping on the right side when the left side is chronically affected, as in the clinical case (Gunn 1972), right sided involvement was also found in six of the sub-clinical cases in the present study. Gunn (1972) also noted that mean group size in the right side of his sub-clinical cases increased when group size was raised on the left. In the sub-clinical cases of this series, evidence of pathology of the right side as judged by either atrophy or fibre type grouping, or both, was/

was found in the middle to old age group and in those in which the changes on the left side were fairly severe. The most obvious case of right side involvement was in one of the "roarers" (47988), where type grouping and atrophy were much more marked in the adductor (CAL) than in the abductor (CAD).

This question of preferential atrophy of either adductor or abductor muscles is puzzling. The scoring system used to quantitate the pathological changes shows that the degree of atrophy was always greater in CAL than in the CAD, or equal to it, but never the opposite. Many observers have described the reverse, (with perhaps one exception; Thomassen 1901) and this aspect will be discussed in more detail when considering the actual sizes of these muscles.

This difference between muscle groups was never more noticeable than in case 57769 in which there was possible endoscopic evidence of adductor paralysis. It is difficult to determine on qualitative histological examination whether or not 50% of the CAL was denervated, but the degree of change was very severe. By comparison the CAD showed minimal atrophy but considerable fibre type grouping and this was reassuring evidence of the neurogenic origin of the lesion in both the CAD and CAL, as in the latter the degree of "myopathic change" was remarkable.

Such evidence of "myopathic change", is presumed to be due to the long duration of the disease. It was most evident in two cases, one "roarer" (49589) and in the possible case of adductor paralysis (57769). However varying degrees of "myopathic change" could be seen in the sub-clinical cases as can be seen from the scoring system. The possible origin of these changes has already been discussed and it is perhaps better to refer to them as the changes seen as part of the picture of a chronic/

chronic neuropathy as suggested by Dubowitz and Brooke (1973). To describe them as "myopathic change" in this context is probably confusing.

The hypothesis that laryngeal hemiplegia is part of a more generalised motor disease, as judged by examination of other skeletal muscles is so far not proven. Distal limb muscles from one roarer, several sub-clinical cases and a proximal muscle from two old ponies has not shown any evidence of neurogenic change of note. Fibre type grouping has not been seen, but in all cases there were marked variations in fibre size, but no evidence of group atrophy. In general the smaller fibres were type I fibres. This may be due either to a selective atrophy with age, or to normal biological variation of fibre size, type I fibres generally being smaller than type II fibres. The latter is perhaps more likely as in man, ageing tends to have a preferential affect on type II fibres (Jennekens et al 1971a). Ageing however in man can be associated with disuse, cachexia, and disseminated neurogenic atrophy and each one of these three factors may play a part in the variation in fibre size in the horse.

Lastly it may be of interest to compare the muscle pathology seen in equine laryngeal hemiplegia with that seen in diseases of the LMN of man, in particular to determine if the pattern of the lesion is similar to any specific human neuropathy. Attempts to compare and perhaps liken two entirely separate diseases may be dangerous, but may be useful in comparing the pathogenesis and aetiology of each disease. Dubowitz and Brooke (1973) have divided the diseases of the LMN into four categories and these are:-

- 1) motor neuron disease
- 2) chronic neuropathy
- 3) simple neuropathy
- 4)/

#### 4) spinal muscular atrophy (S.M.A.)

Although a very general classification, this system allows for some variation in the histological appearance of affected muscle.

It appears that the muscle lesion of laryngeal hemiplegia resembles that seen in chronic peripheral neuropathies of man such as that seen in peroneal muscular atrophy. The distal distribution of this disease plus its histological appearance are similar to the equine laryngeal lesion, especially if type I fibre hypertrophy is a feature only of human chronic neuropathies, as this is occasionally seen in laryngeal muscle. The proximal distribution of the disease as seen in S.M.A. is unlike the equine lesion, as it is a distal phenomenon, and the acute form of S.M.A. (Werdnig-Hoffman disease) is not similar histologically to laryngeal paralysis. Motor neuron disease however does have some similarity in its distal distribution and pathological features. However, if one accepts the proposed pathogenesis of the equine muscle lesion as put forward in this discussion then it may be reasonable to suggest that the lesion is similar to that seen in the chronic peripheral neuropathies of men. Confirmation of this awaits correlation of the results, of both the muscle and nerve pathology.

1972			
	Mean	Group	Size
<u>Horse No.</u>	<u>Left CAD</u>	<u>Right CAD</u>	<u>left minus right</u>
1	3.8	no figures	no result
2	3.6	3.7	-
3	3.6	4.1	-
4	2.0	3.6	-
5	2.2	1.8	0.4*
6	2.1	2.6	- *
7	2.4	1.9	0.5
8	3.4	2.5	0.9
9	13.9	3.9	10.0
10	4.7	2.9	1.8
11	2.1	1.7	0.4*
12	9.8	3.5	6.3
1973			
13	1.2	1.1	0.1*
14	1.4	1.1	0.3
15	1.1	1.1	0.0*
16	3.6	1.7	1.9 Roarer

\* = normal by Gunn's description

- = negative result

TABLE 6

Mean group size of type I fibres in Gunn's 1972 and 1973 series.

CASE NO.	Muscle	Degree of atrophy	Hyper-trophy	Grouped atrophy	Fascic. atrophy	Fibre type grouping	>C.T.	>Fat	Nuclear clumps	Degen.	Regen.	Infiltrate	Internal nuclei
47988	L.CAD	+++	0	+++	+++	-	+++	+++	+	0	0	0	+
	CAL	+++	0	+++	+++	-	+++	+++	++	0	0	0	0
	CT	0	0	0	0	0	0	0	0	0	0	0	0
	R.CAD	0	0	0	0	++	0	0	0	0	0	0	0
	CAL	+	+	+	0	+++	0	0	0	0	0	0	0
	CT	0	0	0	0	0	0	0	0	0	0	0	0
49589	L.CAD	+++	++	+++	++	+++	++	++	++	+	0	0	+++
	CAL	+++	+	+++	+++	+++	+++	+++	++	0	0	+	+++
	CT	0	0	0	0	0	0	0	0	0	0	0	0
	R.CAD	0	0	0	0	0	0	0	0	0	0	0	0
	CAL	0	0	0	0	0	0	0	0	0	0	0	0
	CT	0	0	0	0	0	0	0	0	0	0	0	0
52838	L.CAD	+++	0	+++	+++	-	+++	+++	0	0	0	0	+
	CAL	+++	0	+++	+++	-	+++	+++	+	0	0	0	+
	CT	0	+	0	0	0	0	0	0	0	0	0	0
	R.CAD	+	0	0	0	0	+	0	0	+	0	0	0
	CAL	-	-	-	-	+	-	-	-	-	-	-	-
	CT	-	-	-	-	-	-	-	-	-	-	-	-
E.C.R.	0	0	0	0	-	0	0	0	0	0	0	0	



















CASE NO.	Muscle	Degree of atrophy	Hyper-trophy	Grouped atrophy	Fascic. atrophy	Fibre type grouping	>C.T.	>Fat	Nuclear clumps	Degen.	Regen.	Infiltrate	Internal nuclei
58856	L.CAD	++	0	+	0	+	0	0	0	0	0	0	0
	CAL	++	+	++	0	+++	0	0	0	0	0	0	0
	CT	-	-	-	-	-	-	-	-	-	-	-	-
	R.CAD	-	-	-	-	-	-	-	-	-	-	-	-
	CAL	0	0	0	0	0	0	0	0	0	0	0	0
	CT	-	-	-	-	-	-	-	-	-	-	-	-
59170	L.CAD	NO	NO	ACCIDENT									
	CAL	+	+	+	-	+	0	0	0	0	0	0	0
	CT	-	-	-	-	-	-	-	-	-	-	-	-
	R.CAD	-	0	0	0	-	0	0	0	0	0	0	0
	CAL	-	0	0	0	0	-	0	0	0	0	0	0
	CT	-	-	-	-	-	-	-	-	-	-	-	-
49733	L.CAD	0	0	0	0	-	0	0	0	0	0	0	0
	CAL	+	+	+	0	-	0	0	+	0	0	0	++
	CT	0	0	0	0	-	0	0	0	0	0	0	0
	R.CAD	0	0	0	0	-	0	0	0	0	0	0	0
	CAL	0	0	0	0	0	-	0	0	0	0	0	+
	CT	0	0	0	0	0	-	0	0	0	0	0	0

Fig. (12): a) Dorsal view of the larynx of a clinical case to show the gross atrophy of the left CAD compared with the normal right side.

b) Antero/dorsal view of the larynx of a clinical case (40733) to show the gross atrophy of the left transverse arytenoid (open arrow) compared with the right side. Note also that the left arytenoid cartilage (arrow) is partially collapsed.



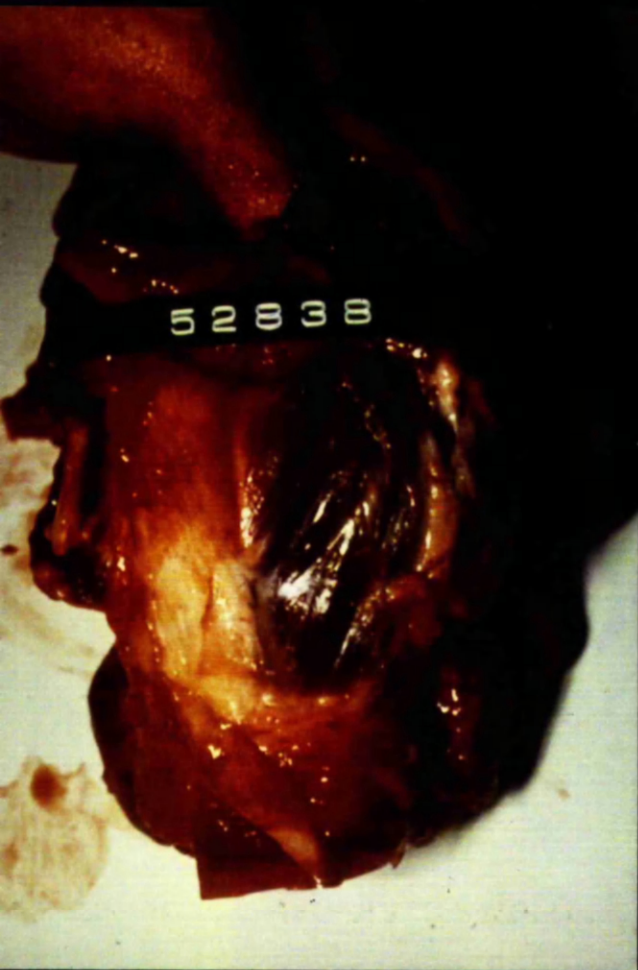


Fig. (13): Chronic neurogenic atrophy in a clinical case (47988). There is a marked increase in fatty and connective tissue with few muscle fibres obvious. However, the muscle fibres of normal size that are present are grouped together.

(Gomori X 35)

Fig. (14): Left C.A.D. of a clinical case (52838). Note the chains of nuclei within atrophic muscle fibres. Most are round and in some a 'tigroid' appearance of scattered chromatin is evident.

(H. & E. paraffin, L.S. X 400)

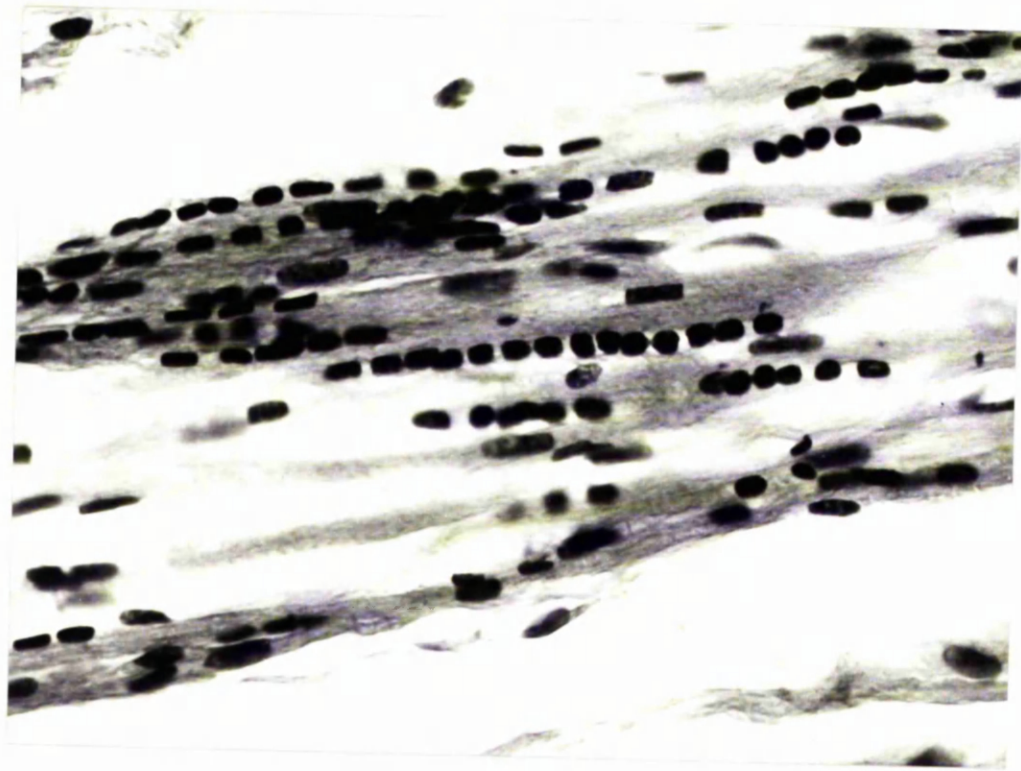
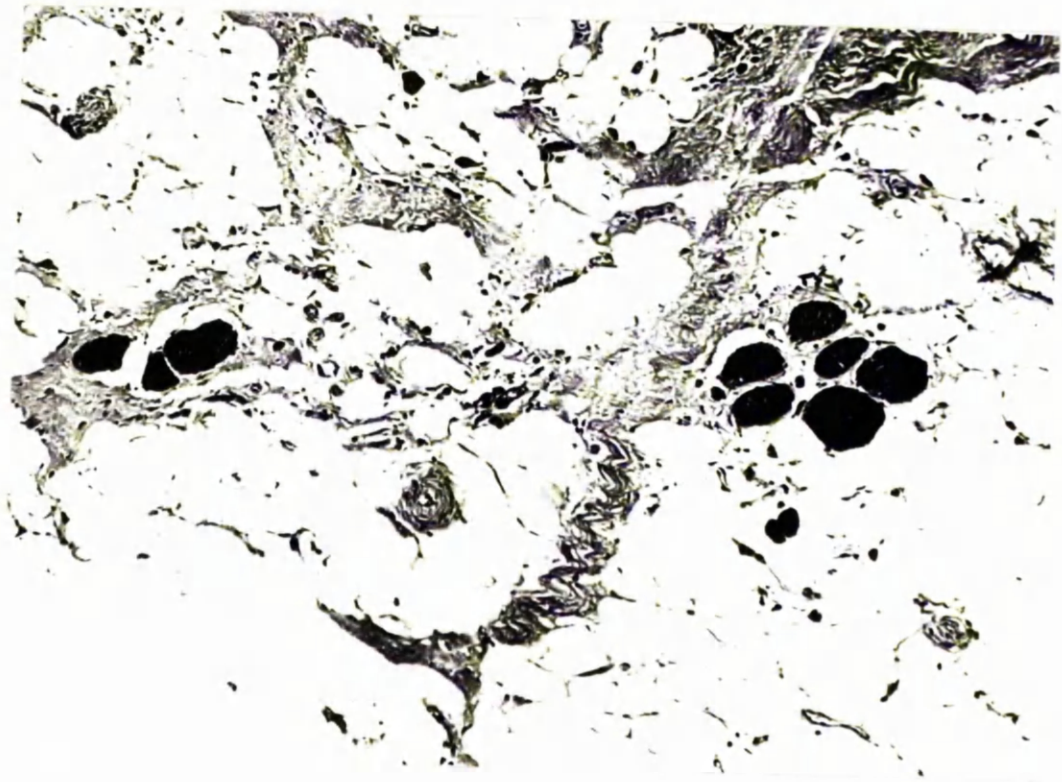


Fig. (15): Left C.A.D. of a clinical case (49589). Atrophy is predominant, but normal and hypertrophied fibres are still present in considerable numbers.

(Gomori, paraffin X 35)

Fig. (16): Left C.A.D. of a clinical case (40733).

Hypertrophic and normal sized fibres are still present but there are a considerable number of internal nuclei.

(H. & E. X 120)

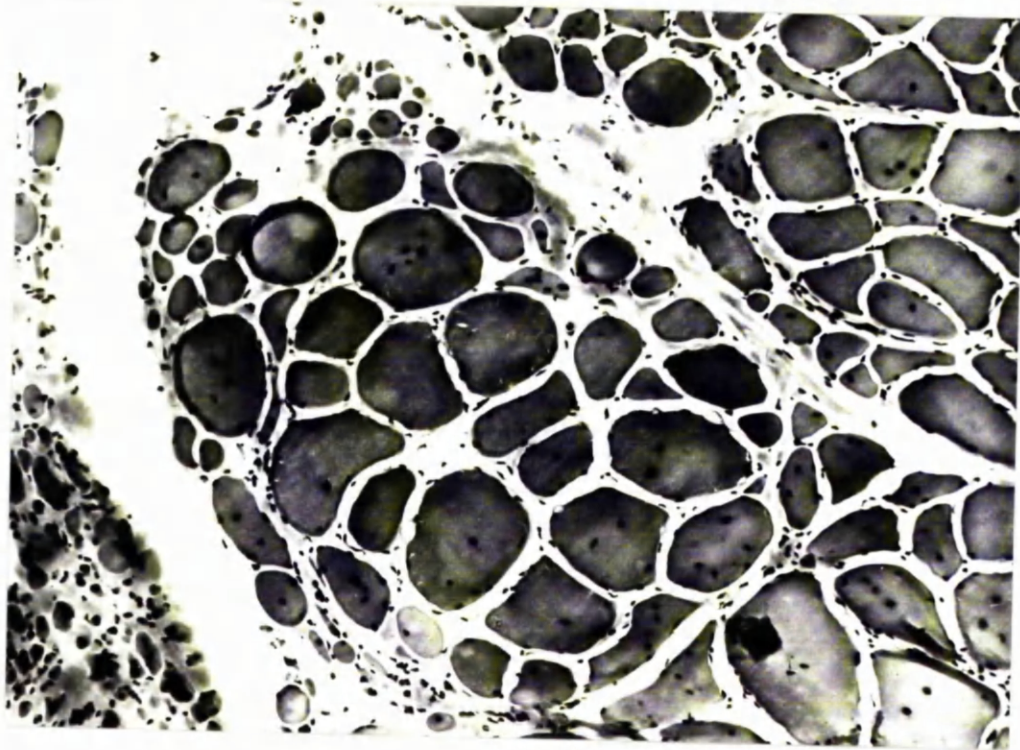
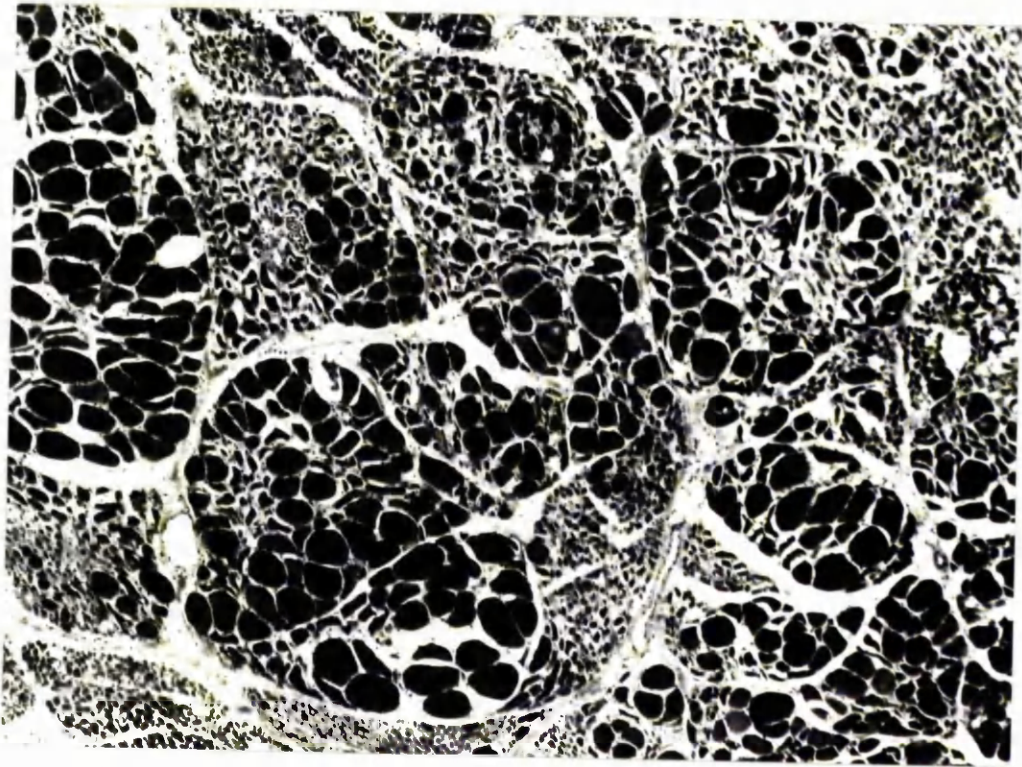


Fig. (17): Left C.A.D. of a clinical case (40733). There is gross atrophy with fatty tissue replacement. Chains of sarcolemmal nuclei can be seen.

(H. & E., paraffin, L.S. X 120)

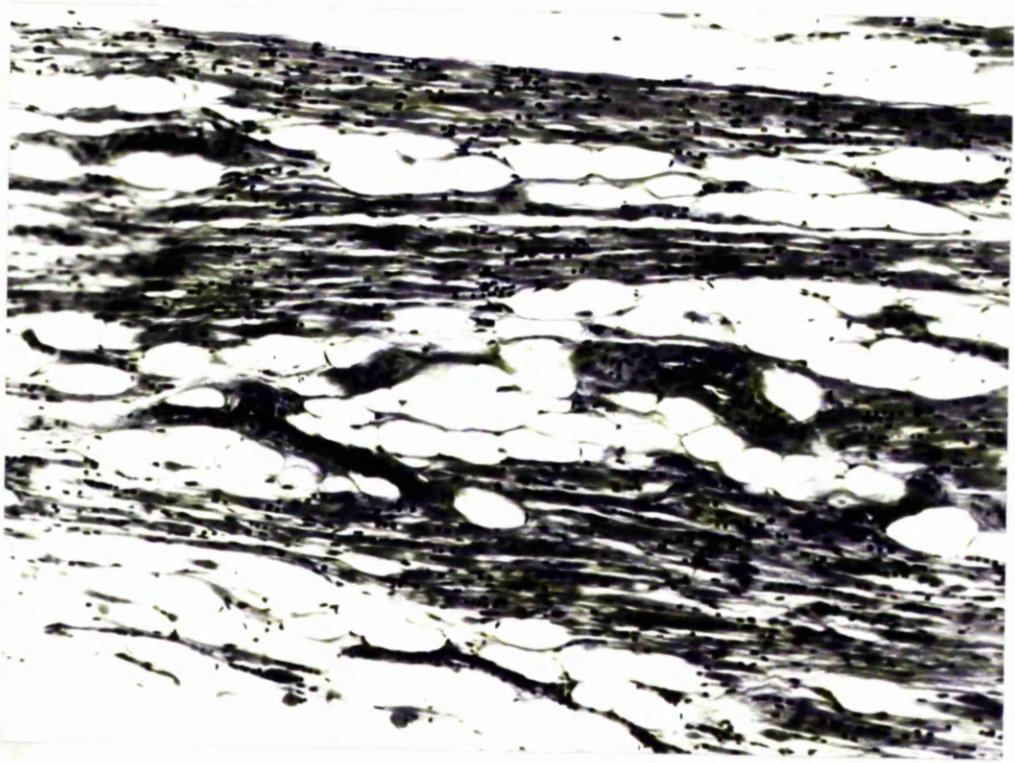


Fig. (18): Areas from six muscles from a clinical case.

a) left C.T.

b) right C.T.

c) right C.A.D.

d) right C.A.L.

e) right transverse arytenoid

f) long digital extensor

All have the normal mosaic pattern. Note the marked type

II fibre predominance in d).

(ATP-ase, acid preincubation pH 3.9 X 24)



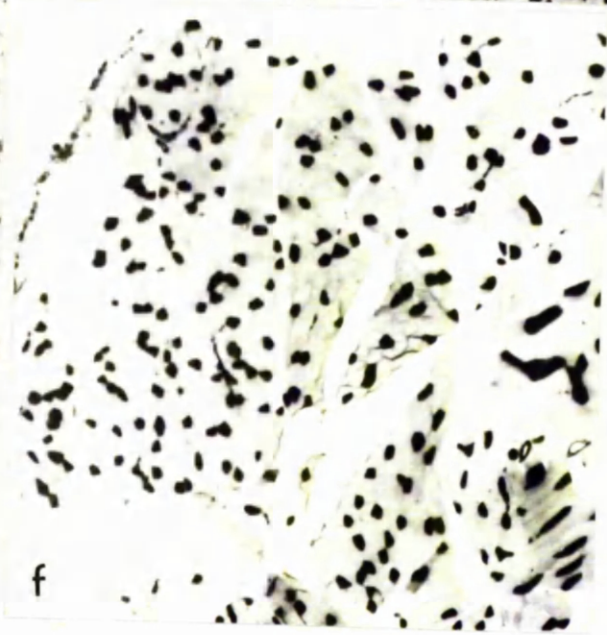
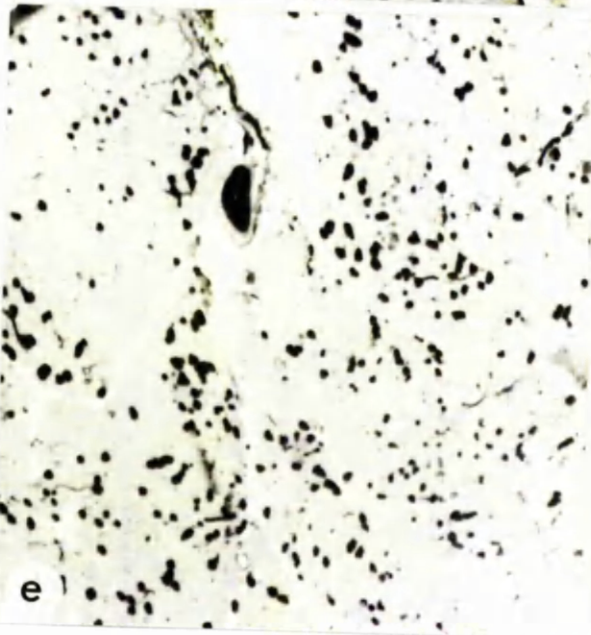
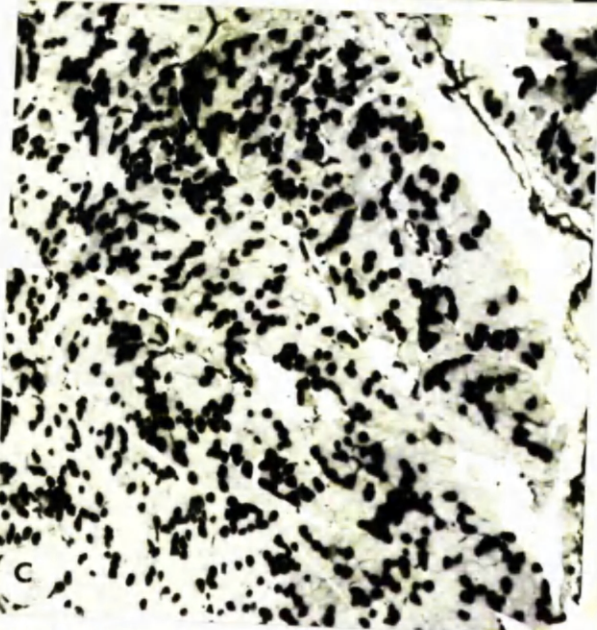
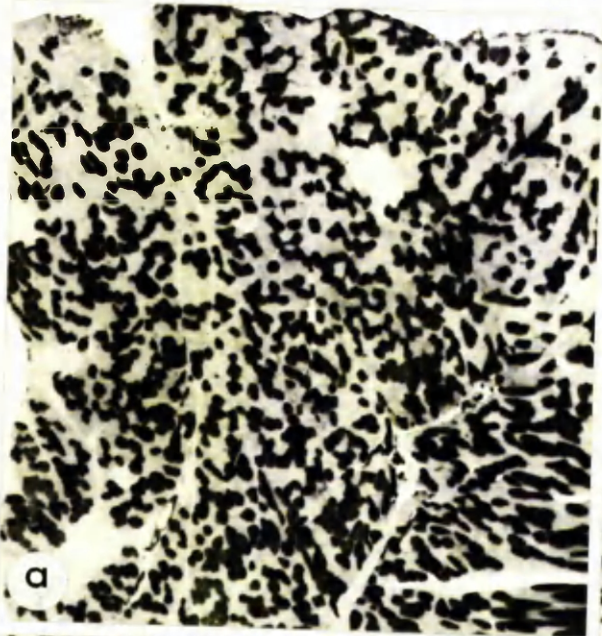


Fig. (19): Left C.A.L. of a sub-clinical case. Considerable atrophy and angulation is present, and a number of fibres are hypertrophic.

(H. & E. X 120)

Fig. (20): Left C.A.L. of one of the young sub-clinical cases (58865) in which there is considerable atrophy.

(H. & E. X 250)

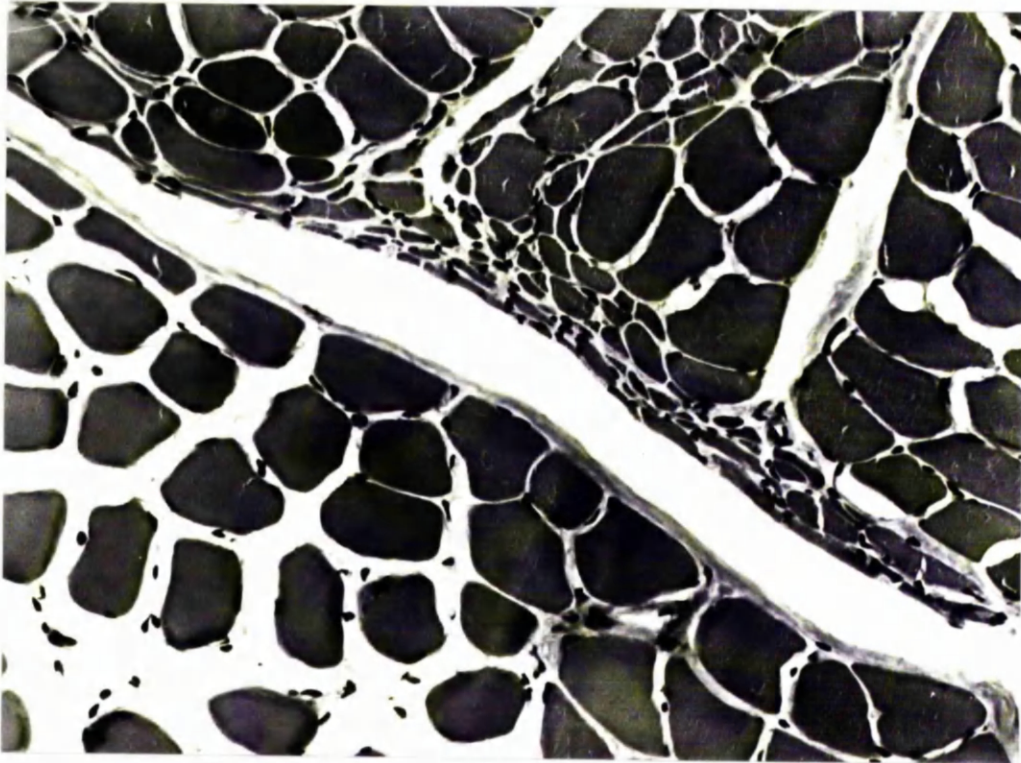
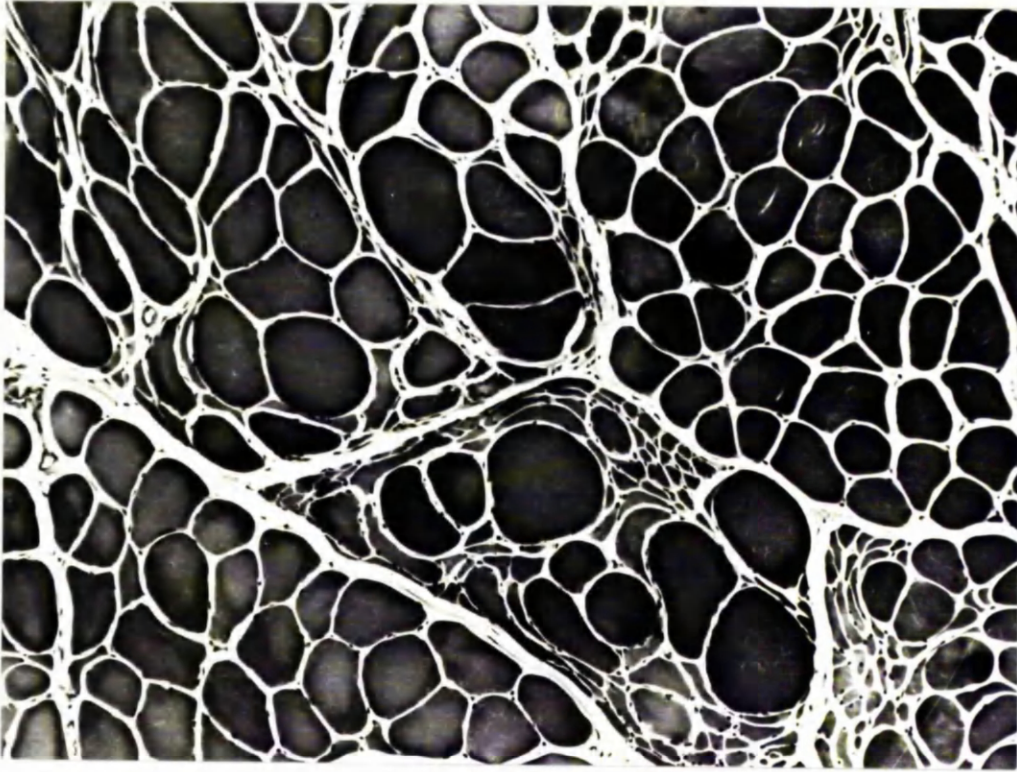


Fig. (21): Left C.A.L. of a sub-clinical case in which there is only minimal atrophy (50035). Only a few atrophic, angular fibres are seen.

(H. & E. X 400)

Fig. (22): Left C.A.L. of a sub-clinical case. Note the fascicular atrophy with endomysial fibrosis. In one fascicle which is almost totally atrophic, one large hypertrophic fibre is still present.

(H. & E. X 120)

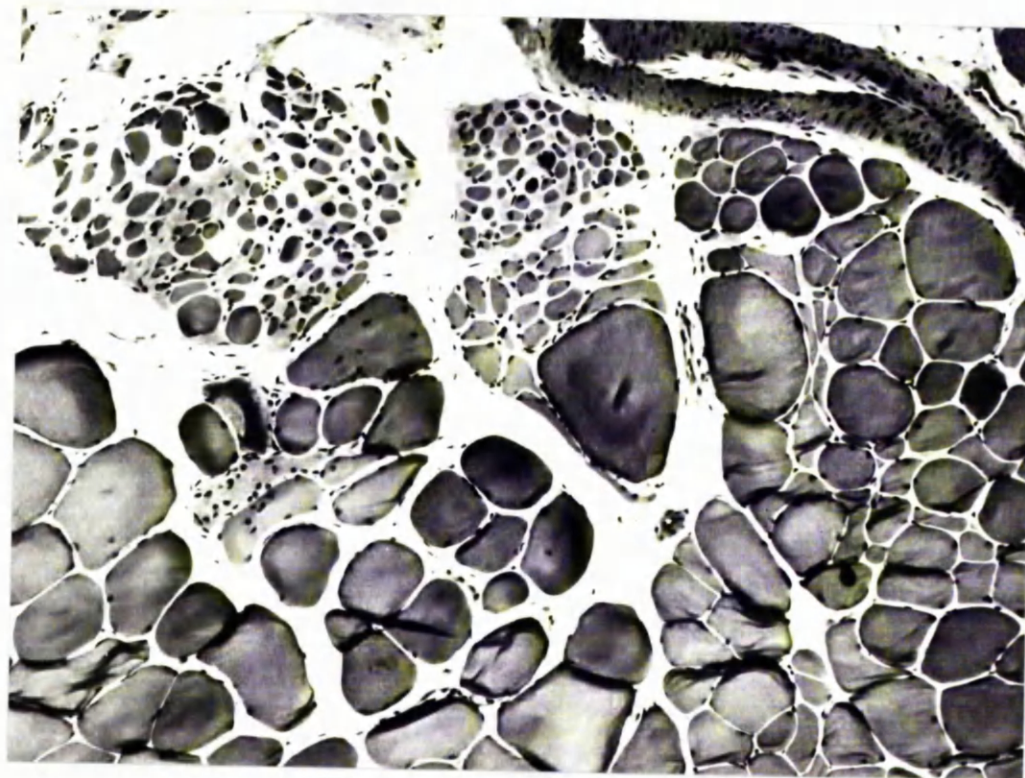
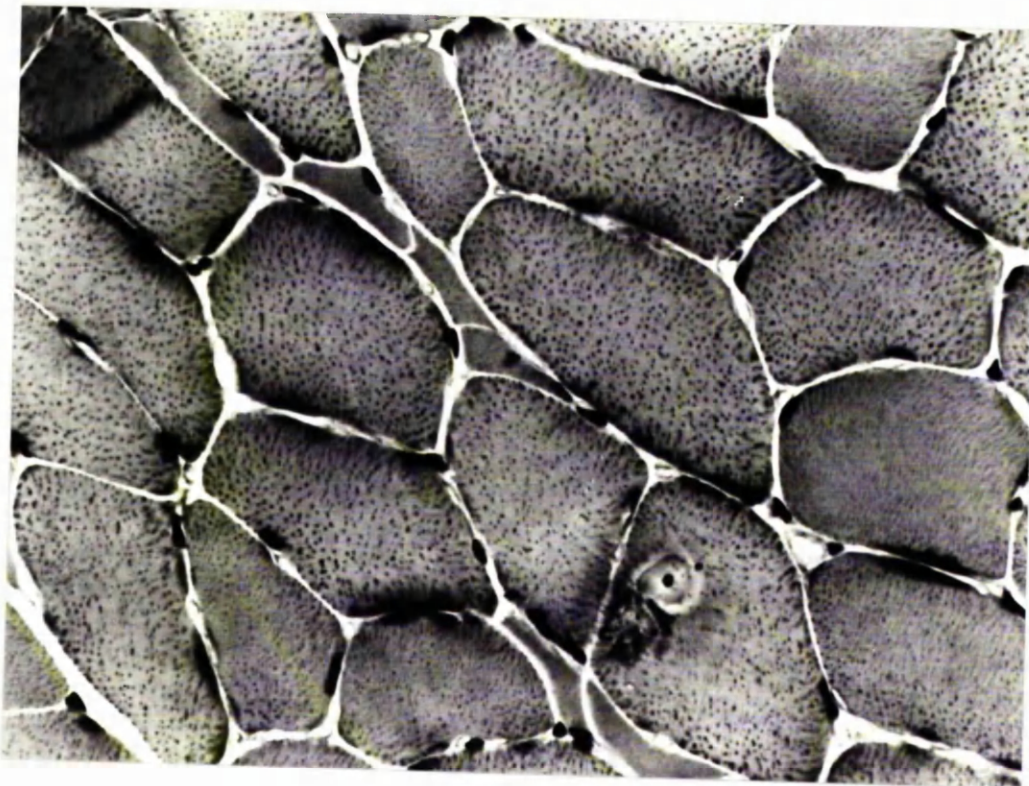


Fig. (23): Intramuscular nerve bundle from the left C.A.L. of a sub-clinical case. There is an increase in endoneurial connective tissue.

(Gomori, paraffin, L.S., X 250)

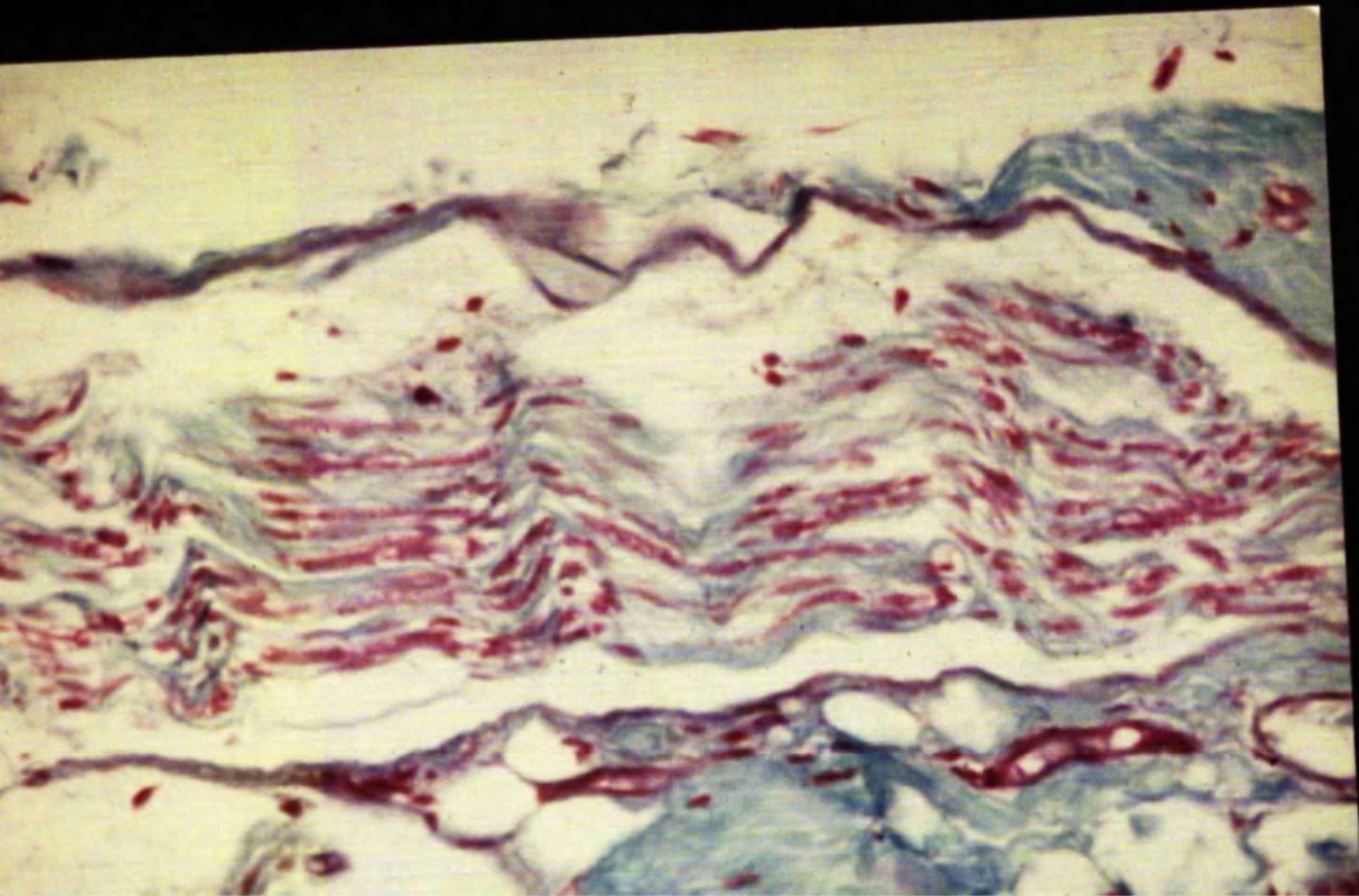


Fig. (24): A range of fibres in the process of splitting was seen (a, b and c). In each case, sarcolemmal nuclei can be seen to lie in the split. In c) the fibre is completely split with a 'nesting' appearance.

(H. & E., a, b, c X 400)

Fig. (25): Necrotic fibre. Note also the increase in internal nuclei.

(H. & E. X 400)



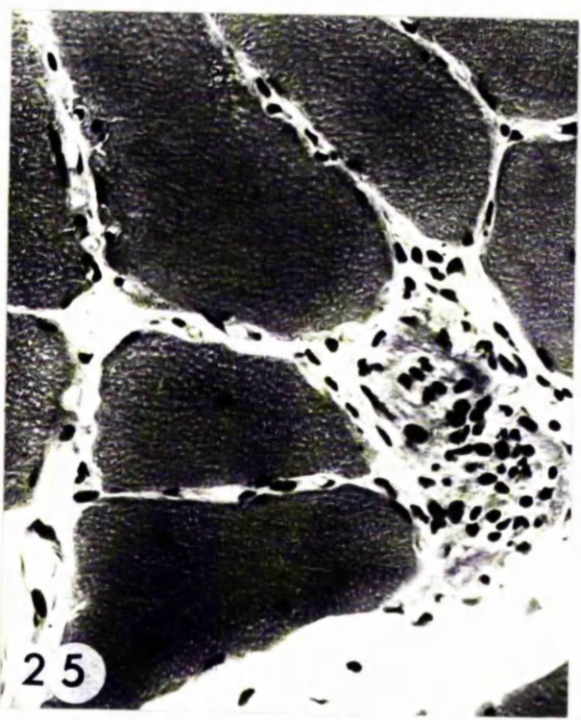
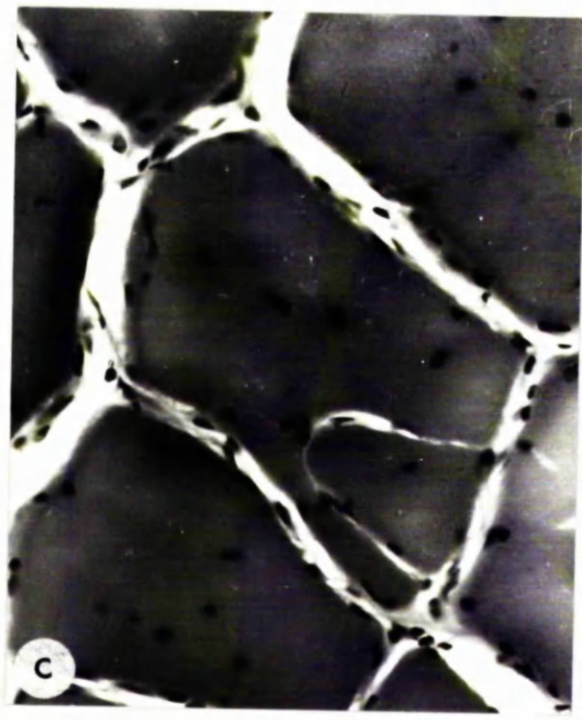
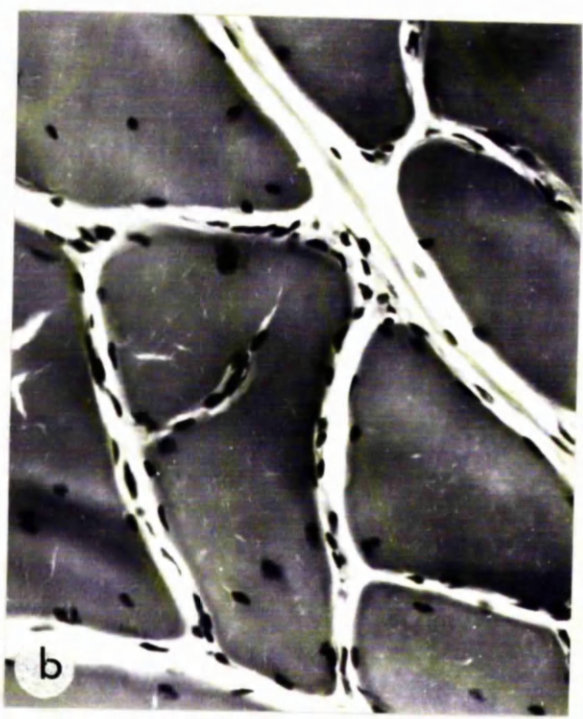
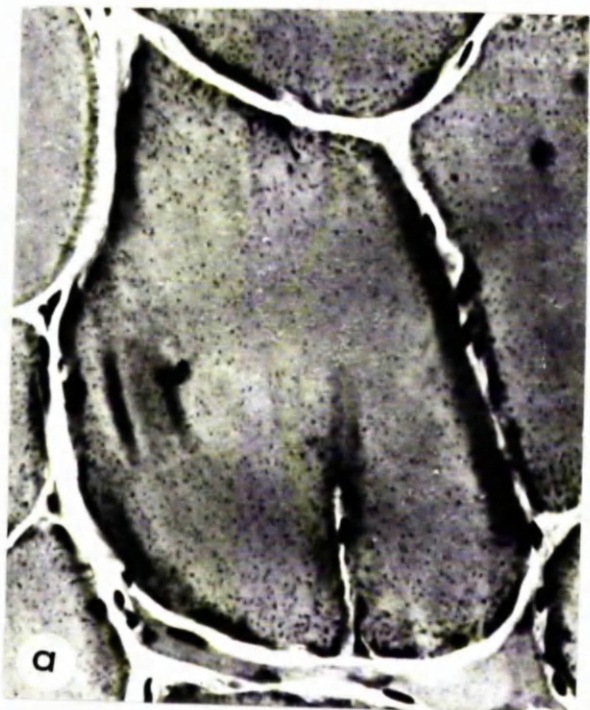


Fig. (26): Right C.A.L. of a sub-clinical case (45404)

showing angulation and atrophy at the tip of a fascicle.

(H. & E. X 250)

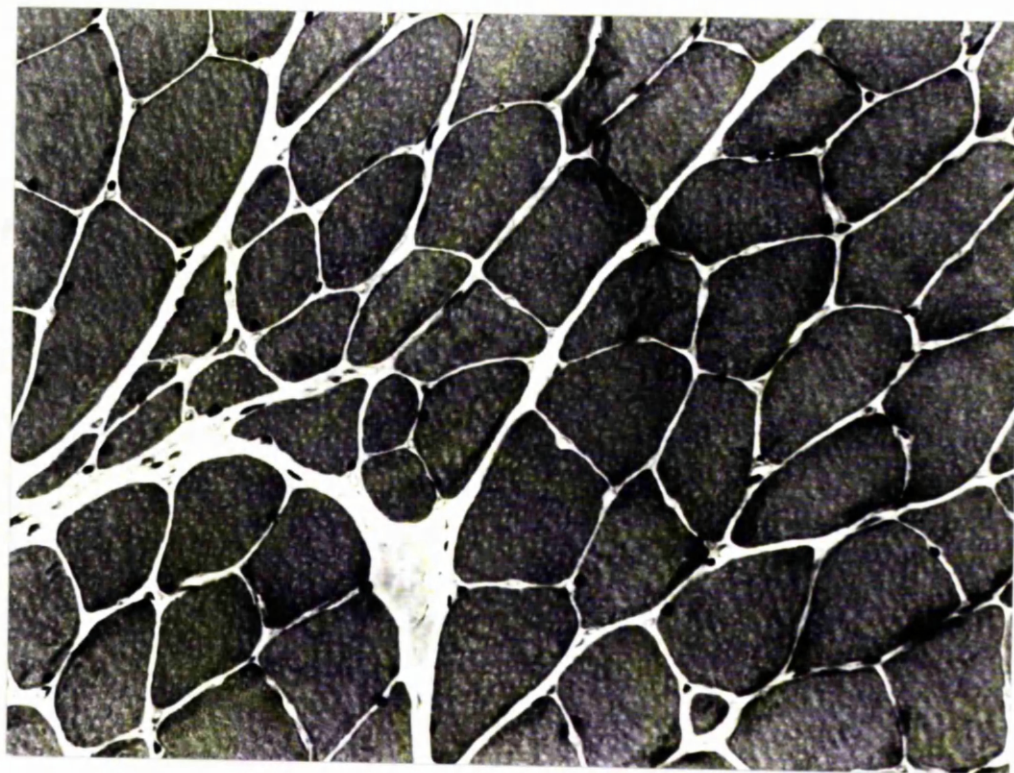


Fig. (27): a) Left C.A.D. of a foal with evidence of sub-clinical disease (58865).

b) Left C.A.L. from same case.

Note that fibre type grouping is more marked in b).

(ATP-ase acid preincubation, pH 3.9 X 24)

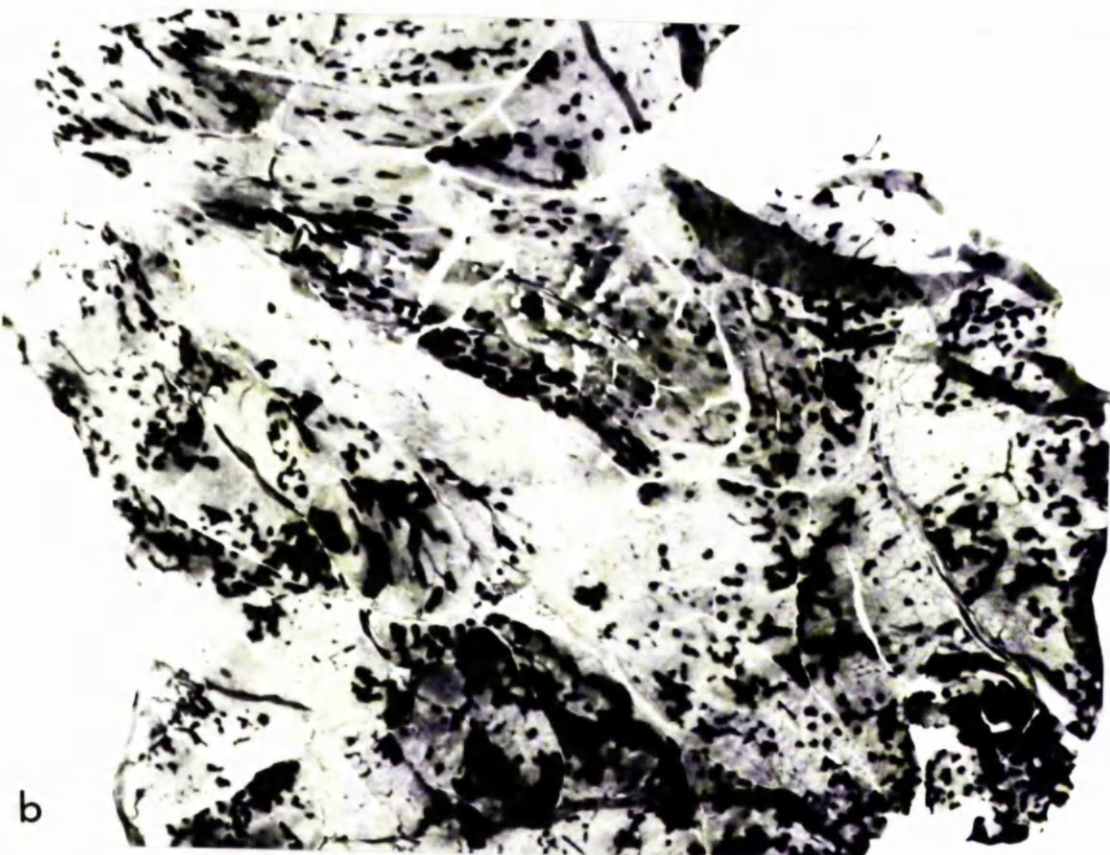


Fig. (28): Left C.A.L. of a sub-clinical case showing marked fibre type grouping (59190). While there are some large groups of type I fibres, the majority of fibres are type II.

(ATP-ase, acid preincubation, pH 3.9 X 24)

Fig. (29): Right C.A.L. of a sub-clinical case (57510).

There are small groups of type I and II fibres.

(ATP-ase, acid preincubation, pH 3.9 X 24)

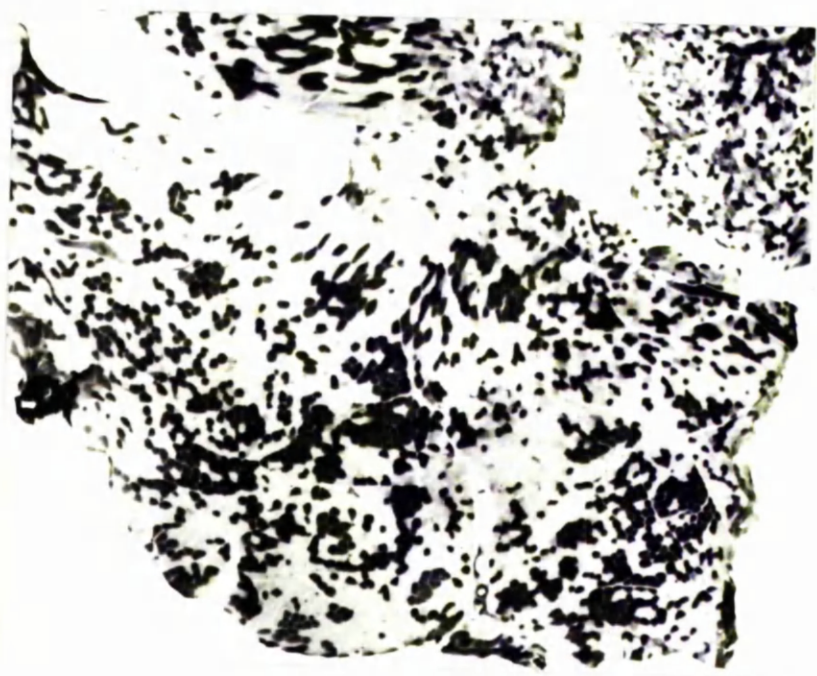
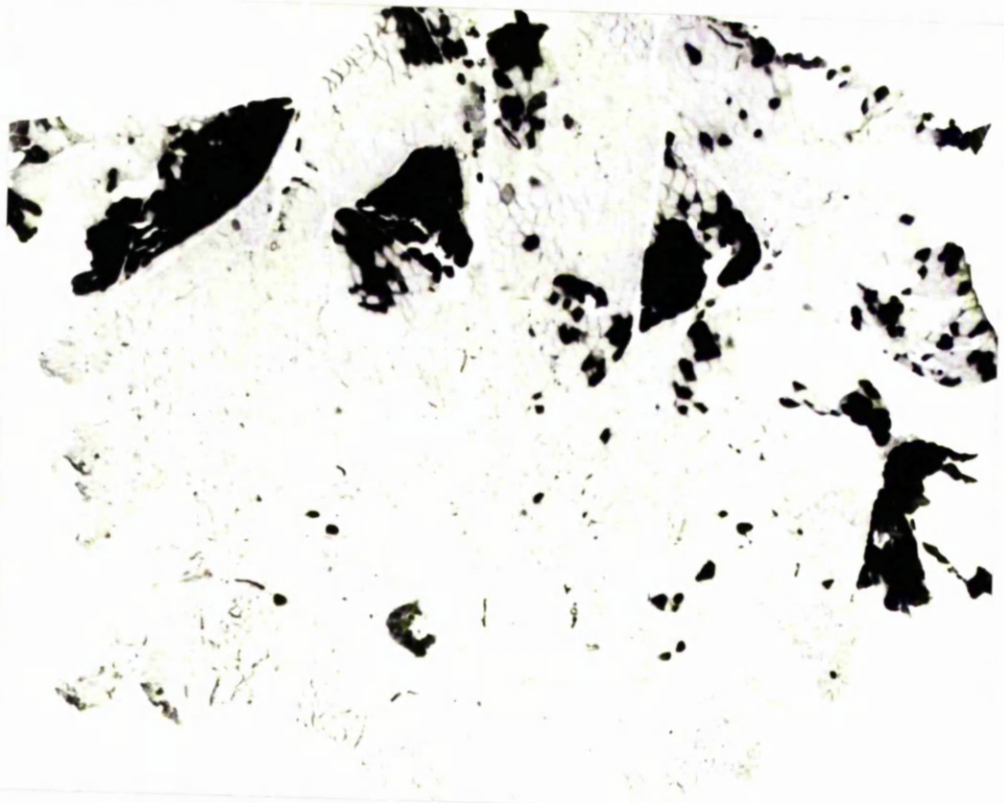


Fig. (30): Serial sections from the left C.A.L. of a sub-clinical case (59170) in which there was minimal type grouping.

a) ATP-ase, routine incubation.

b) ATP-ase, acid preincubation, pH 4.2.

c) ATP-ase, acid preincubation, pH 4.1. (over page)

Note that the slight increase in acidity has increased the contrast between the type I and II fibres considerably. In b) most of the type II fibres appear grey and may all be type IIB fibres. In c) the small groups of type I fibres (dark) are more obvious.

(X 24)





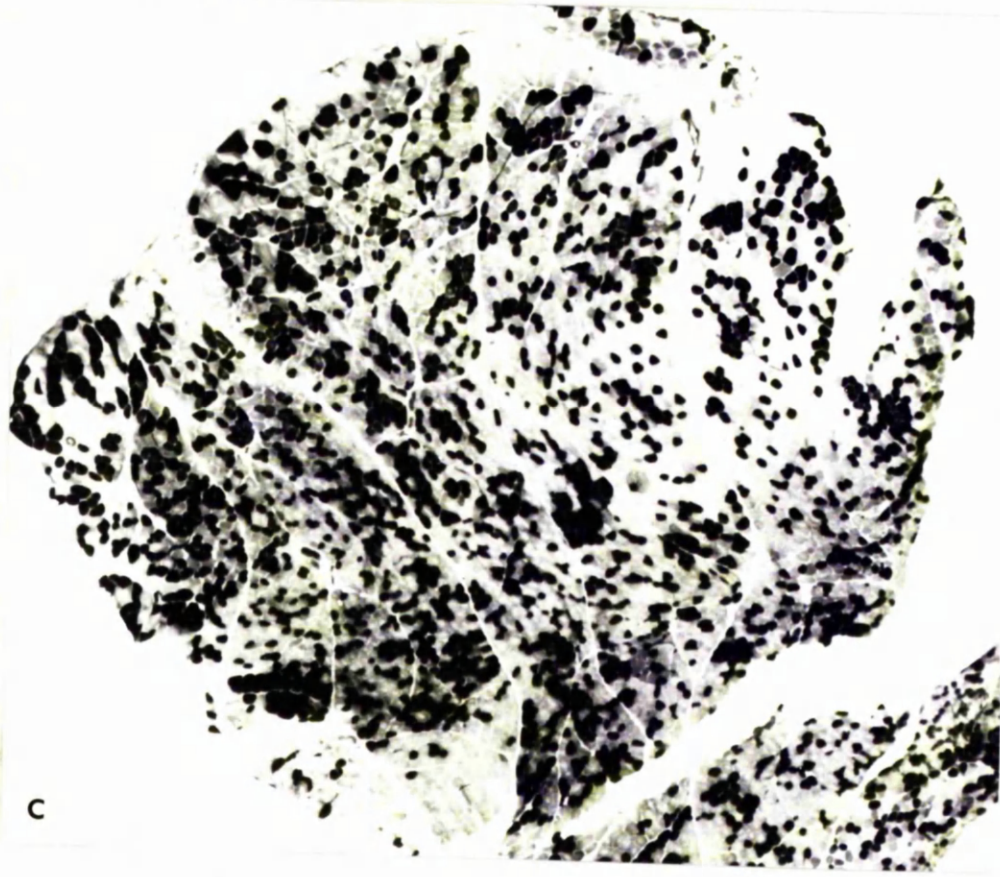


Fig. (31): Sub-clinical case, left C.A.D. The angular,  
atrophic fibres stain high for N.A.D.H.

(N.A.D.H. X 250)

Fig. (32): Sub-clinical case, left C.A.L. The angular  
atrophic fibres stain low for S.D.H.

(S.D.H. X 120)

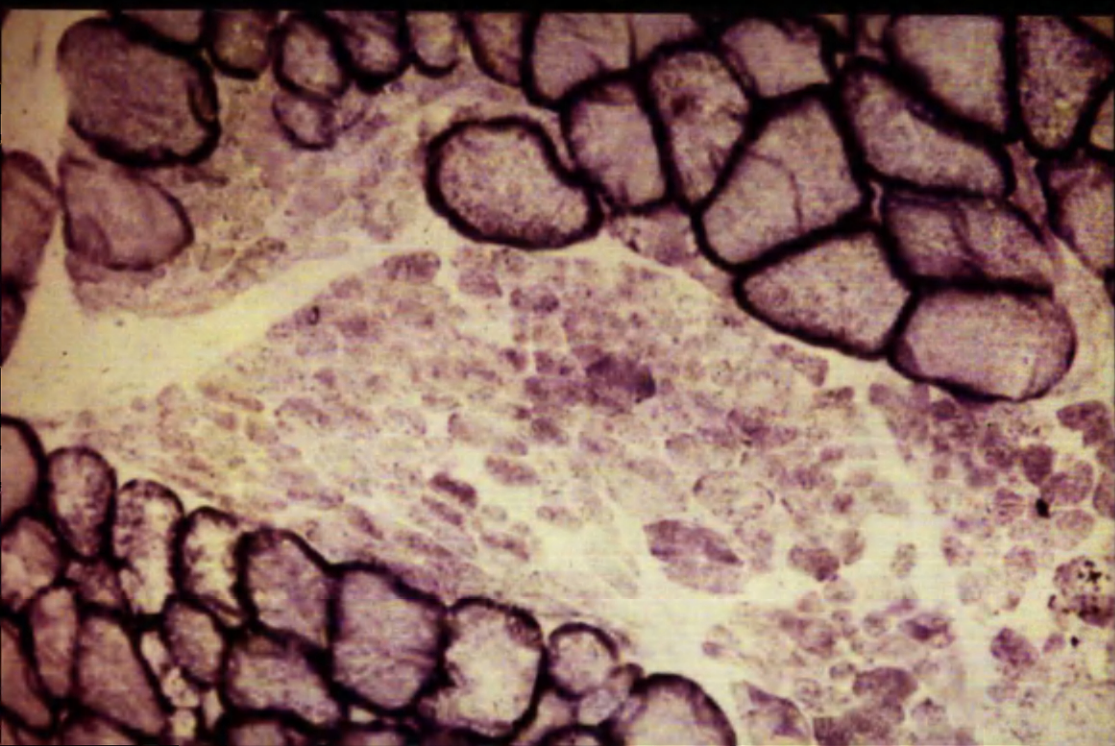
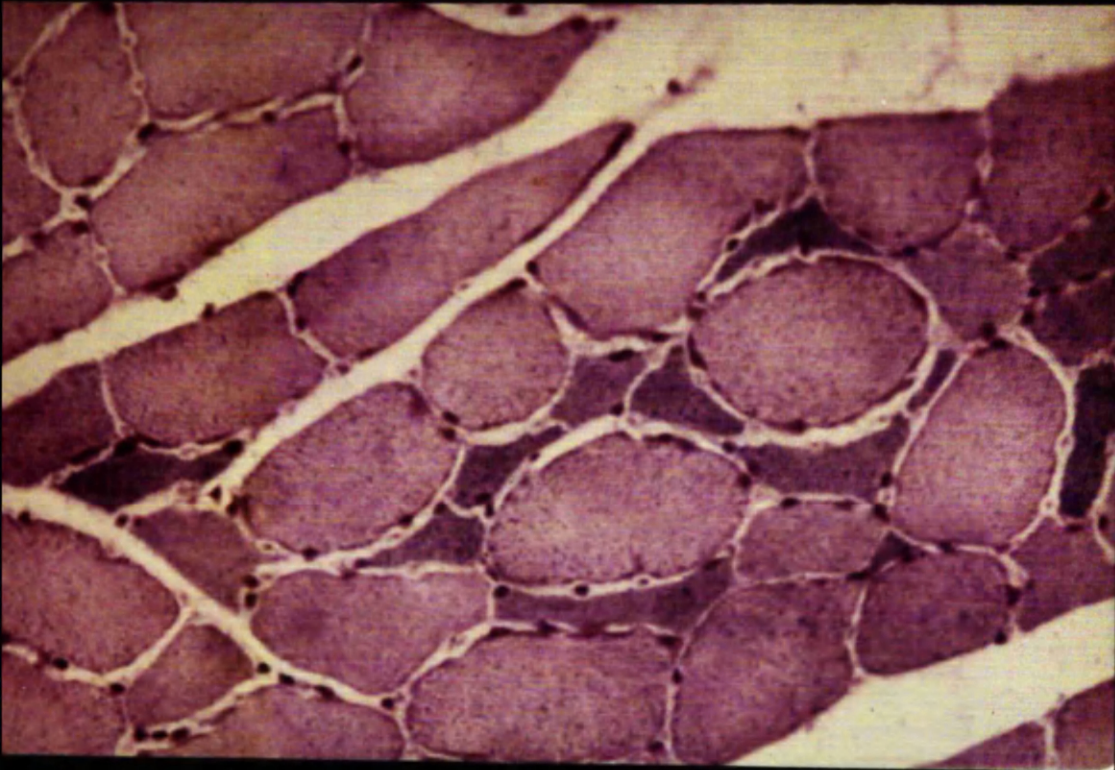


Fig. (33): Left C.A.D. of a sub-clinical case. Fibre type grouping.

(P.A.S. X 120)

Fig. (34): Left C.A.L. of a sub-clinical case. A typical target fibre can be seen. Fibres with abnormally high areas of oxidative activity beneath the sarcolemma can also be seen.

(N.A.D.H. X 400)

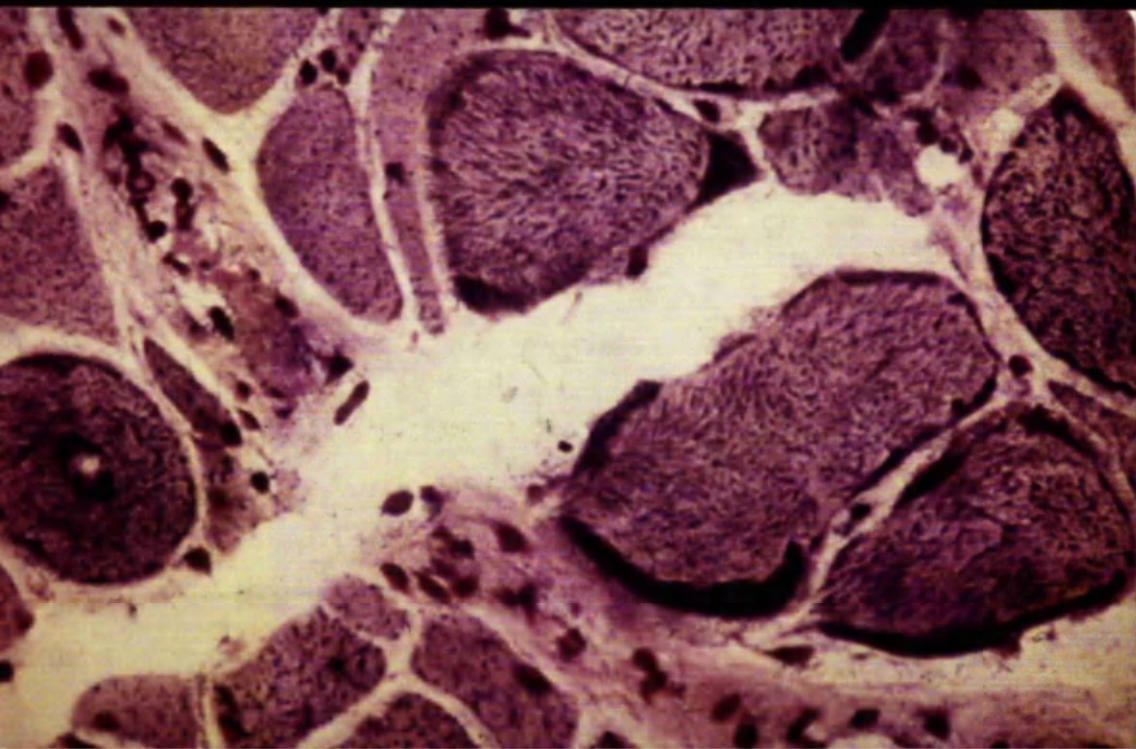
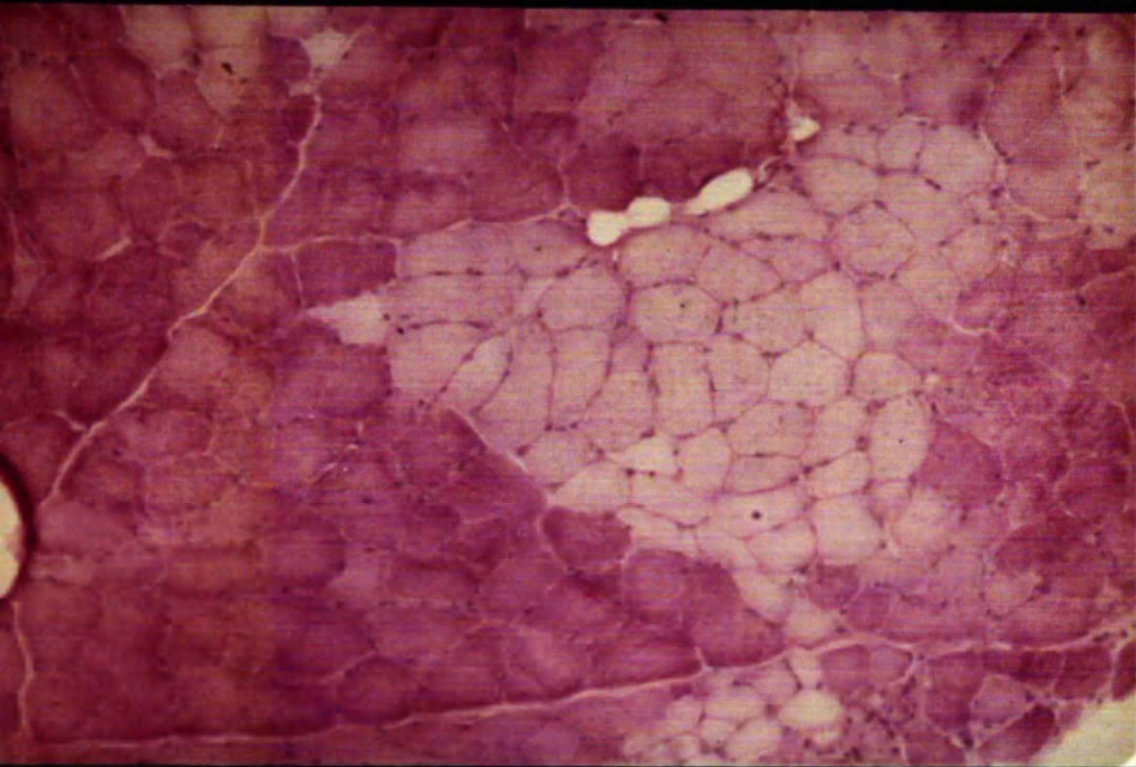


Fig. (35): Left C.A.D. of a case with adductor paralysis

(57769). Note the minimal atrophy. Compare with (36).

(H. & E. X 120)

Fig. (36): Left C.A.L. from same case as (35). Note the marked

atrophy and endomysial and perimysial fibrosis. There

is also some hypertrophy and internal nuclei

(H. & E. X 120)

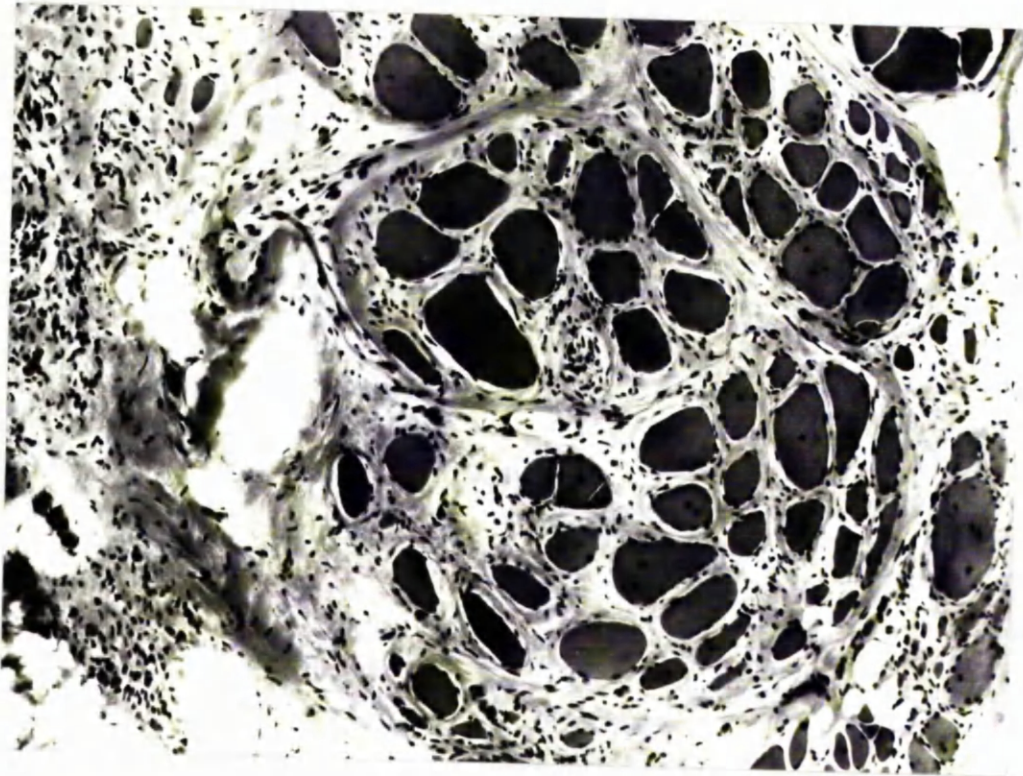
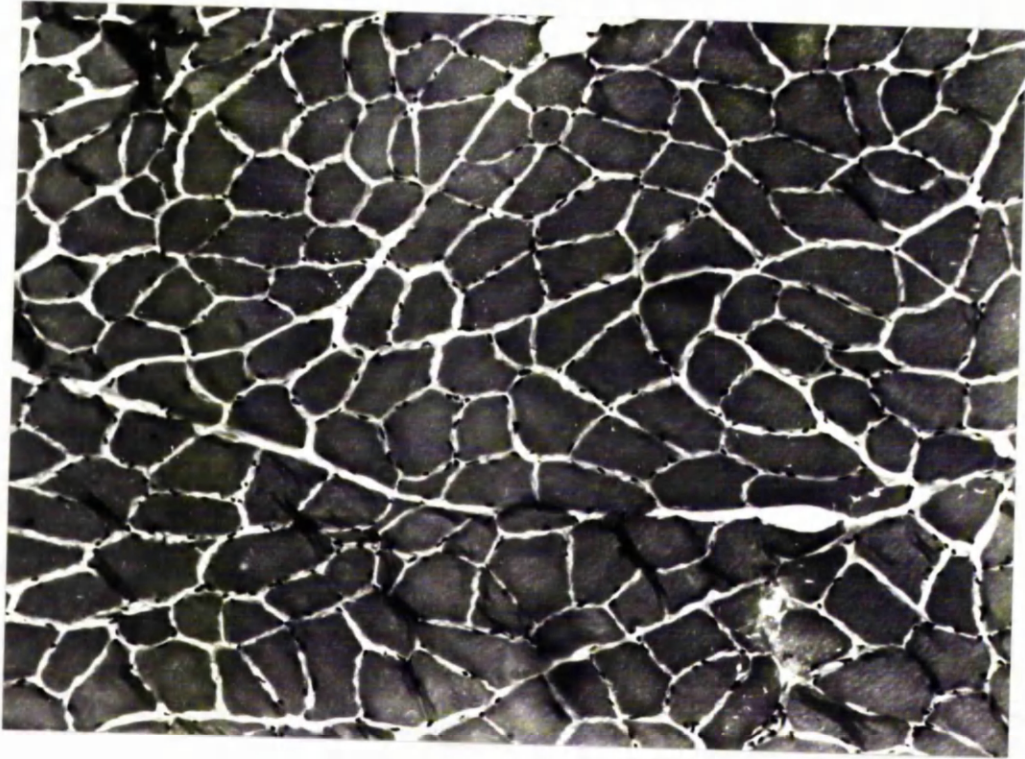




Fig. (37): Left C.A.D. of a case with adductor paralysis  
(57769). Note the obvious fibre-type grouping.  
(ATP-ase, acid preincubation pH 4.6 X 24)

Fig. (38): Left C.A.D. from same case as (37). Areas of  
abnormally high oxidative activity beneath the sarcolemma,  
similar to 'tubular aggregates'.  
(N.A.D.H. X 400)



Fig. (39): Areas from four muscles of a case with adductor paralysis.

- a) Right C.A.L., ATP-ase acid preincubation pH 4.6
- b) Right C.A.L.   "       "       "       pH 4.3
- c) Right C.A.D.   "       "       "       pH 4.6
- d) Extensor carpi radialis ATP-ase acid preincubation  
pH 4.3.

In a) it can be seen that the type II fibres have reversed and split into types IIA (light) and IIB (dark). Grouping of type I fibres (dark) is present. At pH 4.3, (b) complete reversal has occurred with all the type II fibres being light. In contrast to (a) and (b) a mosaic pattern was noted in the right C.A.D., (c) and also in a distal limb muscle (d), where three types of fibres can be seen.

(X 35)

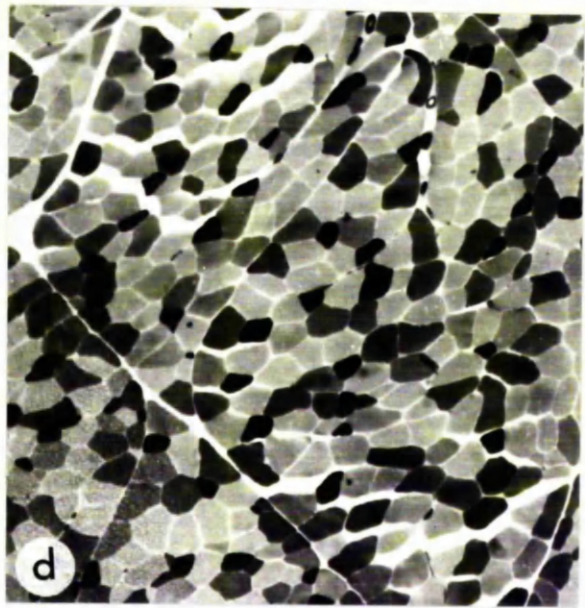
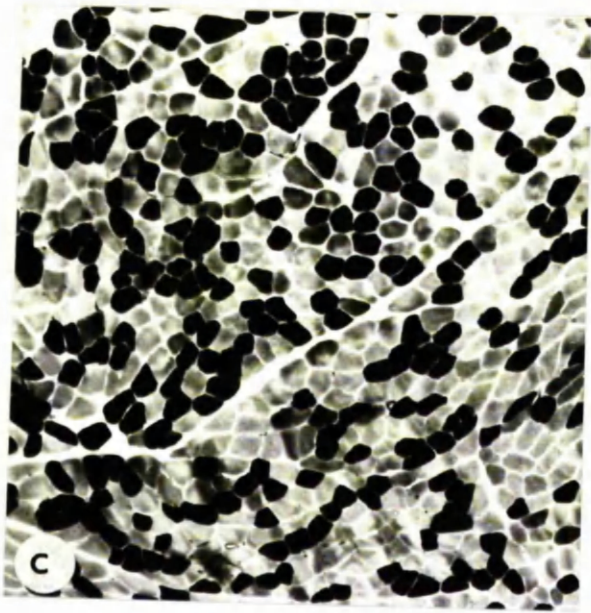
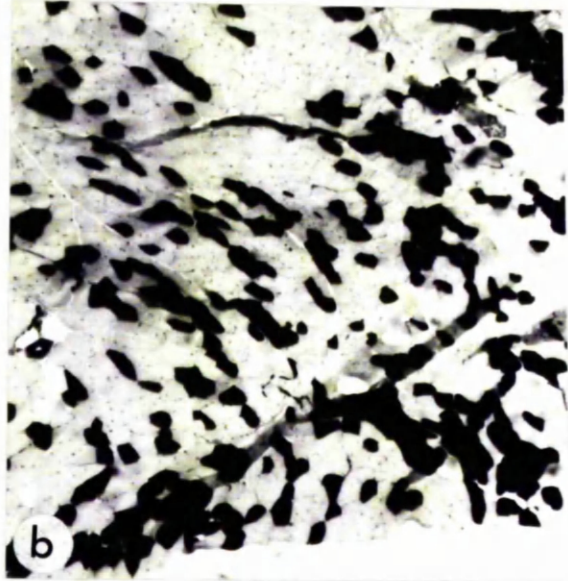
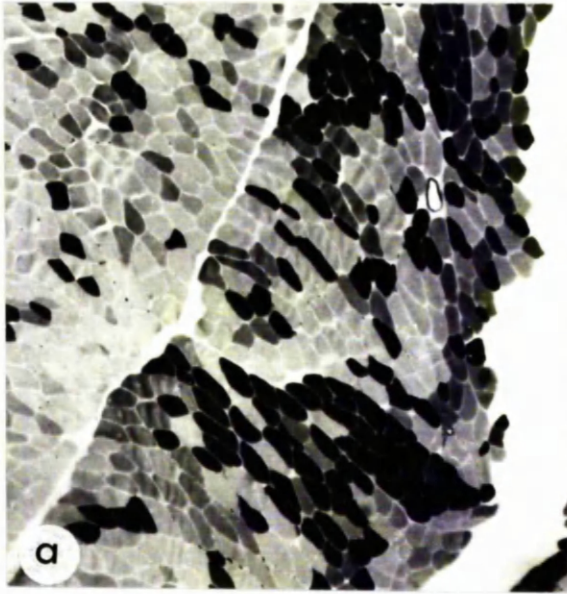


Fig. (40): Left C.A.L. of a case with adductor paralysis  
(58877). Atrophy and hypertrophy are very obvious.  
Internal nuclei are frequent and there are small clumps  
of pyknotic nuclei.

(H. & E. X 120)

Fig. (41): Left C.A.D. of same case as (40). Apart from some  
post-mortem change this muscle is normal. Compare with (41).

(H. & E. X 120)

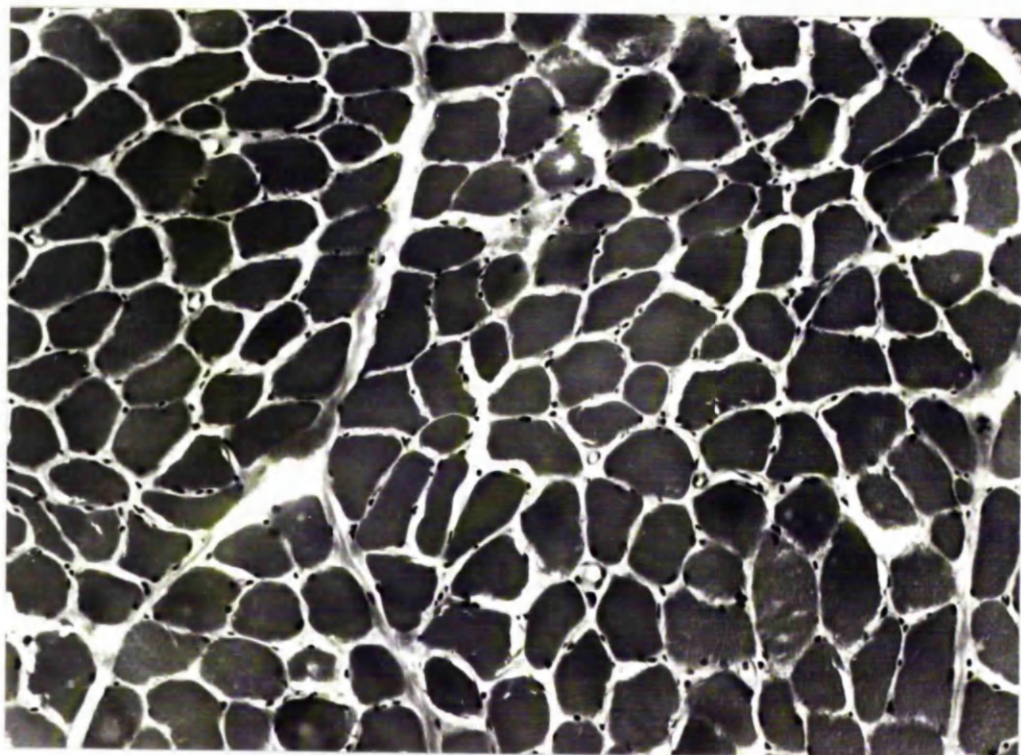
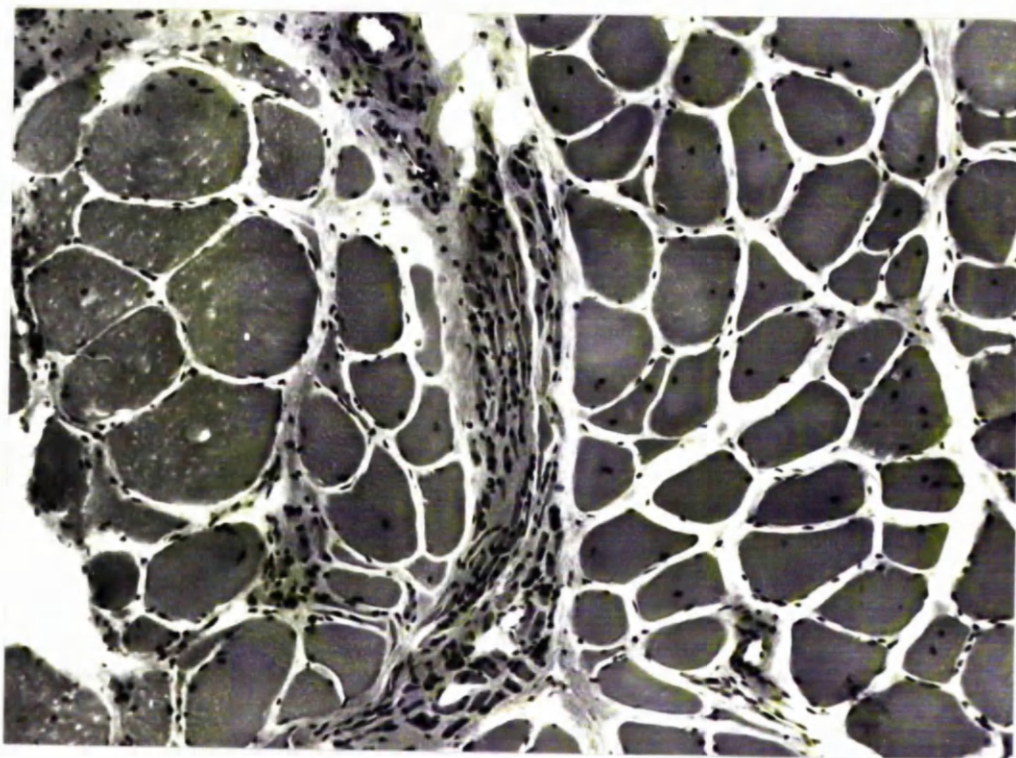


Fig. (42): Distal limb muscle of an aged pony. Note the variation in fibre size, but the absence of fibre type grouping. Most of the small fibres are type I (dark) but the very small angular fibres are both type I and II.

(ATP-ase, acid preincubation pH 4.1 X 120)

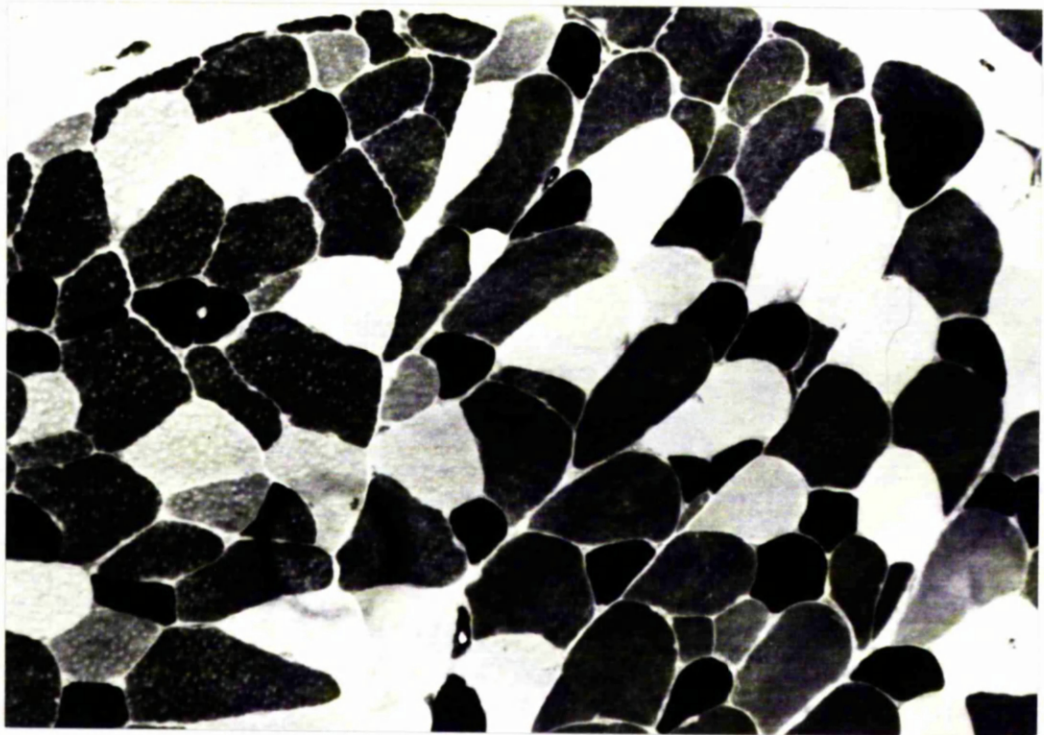
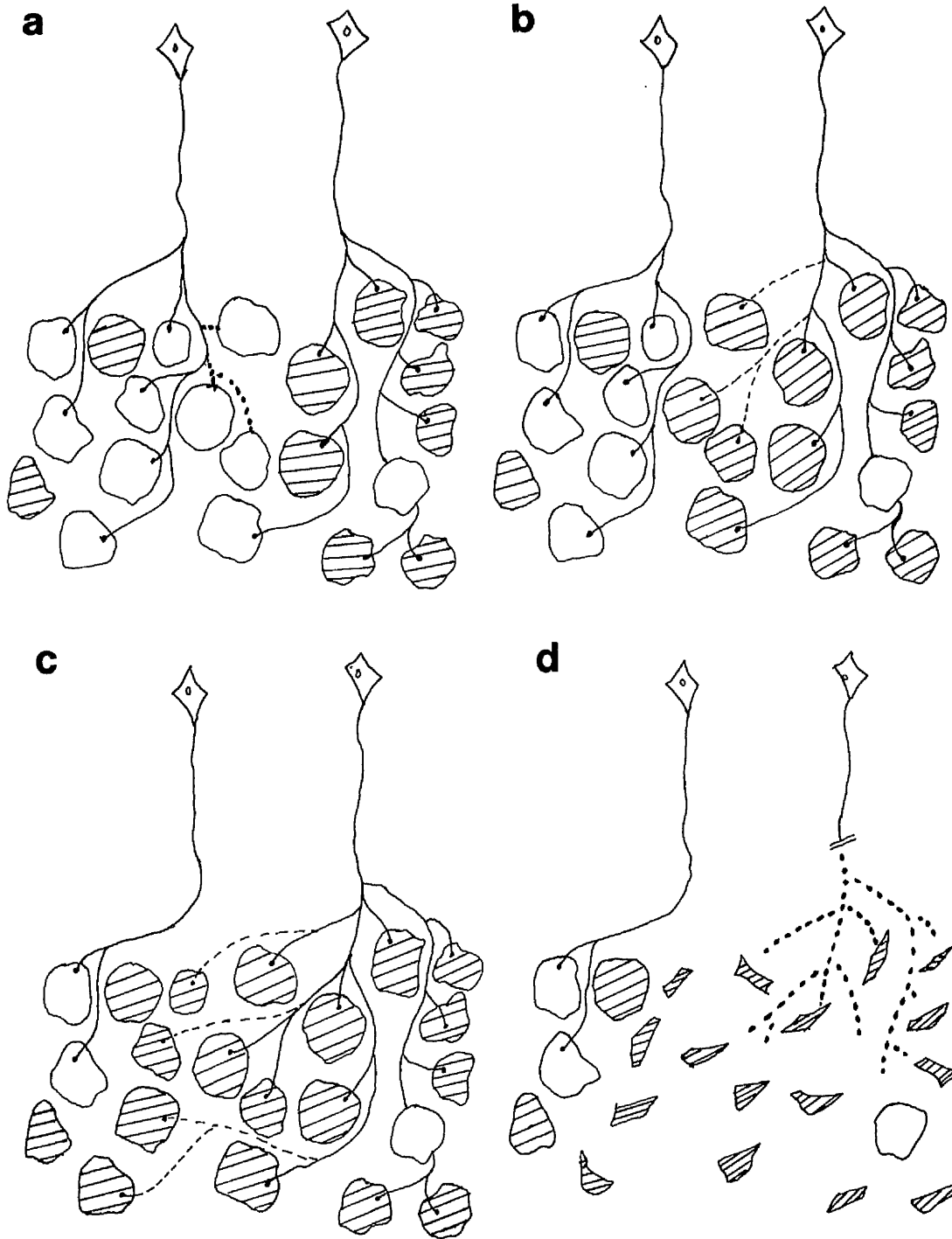




Fig. (43): Schematic representation of the pathogenesis of the lesion in the laryngeal muscles. While most of the changes may take place at the most distal, intramuscular locations, a, b, and c, denervation of entire motor-units more proximally, d, also occurs.



..... degeneration

----- collateral sprouts

SECTION 3

MUSCLE QUANTITATION

INTRODUCTION

As in all biological disciplines, quantitation is playing an increasing part in our understanding of basic disease mechanism of neuromuscular disease. This is becoming especially important in the interpretation of muscle biopsy findings, where the judgement of change can often be subjective. A report by a sub-committee in 1967 (Neurol. Sci. 6, 179-188) suggested various criteria which could be used in the assessment of changes observed in muscle biopsies.

Much emphasis was placed on the estimation of fibre size as one of the most important parameters in the assessment of pathological change. The methods involved in this estimation will be discussed later. They also classified the morphological changes which could be observed within a muscle biopsy and recommended a method for their quantitation.

The quantitative assessment of the morphological changes present in the affected laryngeal muscles has been described in a preceding section. It was decided to make a detailed study of laryngeal muscle fibre area in order to -

- (a) investigate muscle fibre area and obtain accurate values for these in health and disease.
- (b) investigate the validity of Semon's Law in equine laryngeal hemiplegia.
- (c) to devise a sampling technique in muscles affected by neurogenic atrophy.

The reason for questioning the application of Semon's law to equine laryngeal hemiplegia is apparent from the results of the qualitative estimates of the degree of atrophy present in the laryngeal muscles as described in section 2.

The measurement of muscle fibre size is a controversial one as there has/

has been much dispute as to the measurements made and on the material used. Whether or not fibre area or fibre diameter are more accurate measures has been much debated. Fibre diameter, either maximum or minimum diameter, or orthogonal diameters have been studied by a number of authors both for healthy and diseased muscle (Greenfield 1957, Song et al 1963, Brooke and Engel 1969 a,b,c,d, Bell and Conen 1967, Moore et al 1971, Aherne et al 1971, Polgar et al 1973). Those who used muscle fibre area as their method of quantitation include Marin and Denny-Brown 1962, Sissons 1965, Coers & Hildebrand 1965, Aherne 1968, Reniers et al 1970, Reske-Nielsen et al 1970. Gunn (1973), provided valuable information on equine laryngeal muscle fibre area.

It is important to remember that most muscle fibres are not circular (Song et al 1963) and therefore the question is raised of which diameter, the lesser or greater, one should measure. Because of this consideration Song et al (1963) suggested that orthogonal diameters could be used (the maximum and minimum diameters); the maximum diameter is defined as the greatest value of diameter obtained at right angles to the minimum diameter. These could then be used to compute the arithmetic mean. To-date, only Aherne et al (1971) and Polgar et al (1973) have published any major reports using this method. Brooke and Engel (1969 a,b,c,d) while not referring to Song et al (1963) used the latter's minimum diameter explaining it would be the diameter least affected by either kinking or obliquity. Moore et al (1971) adopted this method in their study.

Muscle fibre area has been measured in a number of ways, one of the most common being planimetry (Marin & Denny-Brown 1962, Sissons 1965, Aherne et al 1971, Reniers et al 1970, Reske-Nielsen et al 1970). Aherne 1968/

1968 devised a direct microscopic method of measuring area using the formula -

$$A = L l$$

Where A = cross-sectional area L = mean value of measurements made across the fibres from the most lateral point on one side to the most lateral point on the other and l = mean value of measurements made at random across the fibre. He later revised this method to include estimation of the standard deviation (Aherne et al 1971).

Another method of estimation of fibre area involved projection and drawing of muscle fibres on a piece of paper, cutting out each of the individual fibres which were collectively weighed (Eranko 1955, Bell & Conen 1967 and Gunn 1973). After calibration the mean fibre area can be calculated.

Sissons (1963, 1965) provided much valuable information on the measurement of muscle fibre size. He made measurements both of fibre diameter and area, calculating the latter by projecting muscle fibre images onto squared graph paper, and counting the number of squares within each fibre. He then compared these results with those obtained by planimetry, and while finding a slight difference described this as minimal. The planimetric results were in addition found to be highly reproducible. He pointed out however that it was much more accurate to measure a large number of areas containing a small number of fibres, than a few areas with many fibres. He also found significant differences in fibre size between different blocks from the same muscle.

The number of fibres measured by each of the authors so far mentioned, varied considerably. Most have suggested 200 fibres, picked in a random fashion, would be sufficient, but Moore et al (1971) and Reniers/

Reniers et al (1970) measured the area of up to 2000 fibres in diseased muscle.

It was with this general background that a Sub-Committee met in 1967 to discuss the quantitation of muscle biopsy findings, and although work has been published since then, much of what they discussed is relevant. As was mentioned earlier they pointed out that muscle fibre size was one of the most important quantitative parameters. They recommended that it was best measured in cryostat sections as up to 20% shrinkage can occur in paraffin sections. Other authors have recognised this difficulty and have applied corrections to their results where necessary (Aherne 1968). They discussed the area  $V$  diameter measurement argument and suggested that a single diameter measurement in one plane would be simplest, but it was also suggested that fibre area might be more satisfactory. It was felt that measurements of 150 randomly chosen fibres would be sufficient. They also pointed out the reported difference in size between the two main types of muscle fibre, type I and II, and suggested that such measurements should include equal numbers of each. Suitable histochemical stains would therefore have to be used.

The information so far received on muscle fibre size is not in total agreement, in particular whether muscle fibre area or diameter have normal distributions (i.e. symmetrical Gaussian distribution) in healthy muscles. The distribution of both normal and diseased muscle will be discussed at the end of this section.

### Materials and Methods

Muscle fibre area was measured in six horses, three sub-clinical horses, two clinical cases, and one normal pony (table 7), and the muscles measured were as indicated in the same table. In the third sub-clinical case, the cricothyroid muscles were not measured as the sections were considered unsatisfactory. In the first clinical case (49589) measurements were made of the left C.A.D. and C.A.L. only, as the other muscles had evidence of post mortem change. In the second case (47988) only the right C.A.D. and C.A.L. were measured, the contralateral muscles not being available for cryostat sections. The right C.T. was thought to be unremarkable and was not measured due to considerable ice artefact. In the normal pony only the left and right C.A.D. and C.A.L. muscles were measured as controls.

The method involved back projection of an image through a microscope onto a glass plate. The selected slide was placed on the platform of the microscope and a strong light source (Zeiss - Tungsten filament lamp) shone onto the microscope's mirror. The image of the slide was focussed onto a piece of semi-transparent paper on top of a glass screen. In order to obtain reasonable illumination of the image, this was performed in a darkened room.

The area of the slide occupied by the section was then determined using the vernier scale on the microscope's platform. In order to sample the muscle randomly, numbers from the table of random numbers in the Cambridge Elementary Statistical Tables which were included within the known area of the section, were selected. These pairs of numbers, were then chosen as co-ordinates on the vernier scale, and in order to make the selection of an area more random, the area was selected after adjustment of/



of the vernier scales, with the slide out of focus (Bell and Conen 1967). The fascicle within the centre of the field, and its constituent muscle fibres were drawn out carefully. Great care was taken to see that only fascicles were drawn in which the muscle fibres were cut in T.S., any which had evidence of obliquity being rejected.

Four to six fascicles randomly selected, were then drawn out. The number of fascicles varied as an attempt was being made to limit the muscle fibres measured to two to three hundred.

It was felt that this method of selection was indeed truly random in normal muscles, where perhaps the need for such caution is not as great as in diseased muscles. However, in some of the affected laryngeal muscles a small degree of observer selection was necessary as our method of selection occasionally was found to have missed the affected areas. To overcome this, one could either measure more fascicles or in fact select one or two fascicles at the end of such measurements for inclusion, to cover the range of changes. This was only done where absolutely necessary but it is agreed, that when it was done, then the random selection method was affected if not negated.

Each of the fibres was then measured using a compensating planimeter (HAFF 315), repetition of each measurement three times at the beginning of the study, indicating that the one measurement was accurately reproducible. Calibration of a known area enabled the transverse sectional area of each of the individual fibres to be measured.

The results were then subjected to statistical analysis, in particular to :

- a) to fit a model to the data
- b) compare the variance of muscle fibre area
- c)/

c) compared mean muscle fibre area

A programme was written for the Glasgow University K.D.F.9. computer and the mean, variance, standard deviation, standard error, and sum of the squares was computed for each muscle.

### Results

Sample sizes, mean muscle fibre area and variance, are shown in tables 8 - 10 respectively. Histograms of muscle fibre frequency (%) against fibre area were drawn and are illustrated in figs. 44-48.

In addition, in order to compare the histograms with each other several were superimposed (fig. 49).

Statistical Analysis

a) Fitting a model to the data

On the basis of histogram inspection, a normal model (or a variant of it) seemed to be appropriate. In order to test this hypothesis a programme was written to perform a  $\chi^2$  - Goodness of Fit test.

This involved:-

- i) arranging the data in intervals
- ii) ensuring that the expected number of observations in any interval was at least 5 and combining intervals until this was the case.

Comparing the observed number of observations with the expected number in each interval under the null hypothesis using the formula

$$\chi^2 = \sum_{i=1}^S \frac{(O_i - E_i)^2}{E_i} = \sum_{i=1}^S \frac{O_i^2}{E_i} - n$$

(where S is the number of intervals, n is the number of observations,  $O_i$  is the observed number in the  $i^{\text{th}}$  interval,  $E_i$  is the expected number in the  $i^{\text{th}}$  interval).

This is distributed as  $\chi^2$  with  $S - \gamma - 1$  degrees of freedom, (where  $\gamma$  is the number of estimated parameters).

The following hypotheses were used -

- i) the data was normal.
- ii) the logarithm of the data was normal.
- iii) the square root of the data was normal.

The fits obtained using this programme are shown in table 11.

b) Comparison of the variance of muscle fibre area

Because both atrophy and hypertrophy occur in the diseased laryngeal muscle it was decided to measure the variance of fibre area in each muscle and compare these with the variance of ipsilateral and contralateral muscles./

muscles.

It was thought that in some cases the variance in one muscle was greater than in another for example

$$\sigma^2 \text{ L CAD} > \sigma^2 \text{ R CAD}$$

Suppose that  $\sigma_1^2$  and  $\sigma_2^2$  are two unknown variances, but we suspect as above that  $\sigma_1 > \sigma_2$ . Let  $S_1^2$  and  $S_2^2$  be estimates of  $\sigma_1^2$  and  $\sigma_2^2$ , and

$$F = S_1^2 / S_2^2$$

Clearly the larger  $S_1^2$  is over  $S_2^2$ , the larger will be the value of  $F$ , and the more confident we are that  $\sigma_1 > \sigma_2$ . Hence if  $F$  exceeds some upper limit we should conclude that in fact  $\sigma_1^2 > \sigma_2^2$ .

Table 7 of the Cambridge Elementary Statistical Tables (Lindley and Miller 1968) provides such critical limits and when  $F$  exceeds the appropriate limit, it becomes significant.

The technique is known as a test of significance and conventionally is regarded as a test for deciding between two hypotheses, for example

$$H_0: \sigma_1 < \sigma_2 \quad H_1: \sigma_1 > \sigma_2$$

As we have expressed these,  $H_1$  is the hypothesis in the above example which we hope to establish as the truth,  $H_0$  is its opposite.

The test treats these two unequally in that there requires to be strong evidence in the data in favour of  $H_1$  before a significant  $F$  value is realised. This inequality is reflected in the standard terminology;  $H_0$  is known as the null hypothesis,  $H_1$  as the alternative hypothesis. This will be the standard test used. In all of the cases we take as the alternative hypothesis the hypothesis that we hope to establish as the truth.

In particular we shall consider  $F$  tests where  $H_0$  and  $H_1$  are

$$H_0: \sigma_1 = \sigma_2 \quad H_1: \sigma_1 \neq \sigma_2$$

i.e./

i.e. we suspect two variances to differ but we have no strong feeling about which is the larger. Such a test is known as a 2 sided test, the example given above being a one sided test. Clearly in this case we will suspect  $H_1$  to be true if  $F$  is either large or small. Hence we will conclude that  $H_1$  is true if  $F$  exceeds an appropriate upper limit (different from the upper limit in the above one sided test) or falls below a corresponding lower limit. Again if this happens we say that  $F$  is significant.

If a significant  $F$  value is obtained a re-examination of will indicate which of  $\sigma_1$  and  $\sigma_2$  is the larger. Further comparisons between  $\sigma_1$  and  $\sigma_2$ , given new data might then use an appropriate one sided  $F$ -test.

The following comparisons were therefore made using the  $F$  test.

- |   |   |   |
|---|---|---|
| i) $H_0: \sigma^2_{L\ CAD} < \sigma^2_{R\ CAD}$   | V | $H_1: \sigma^2_{L\ CAD} > \sigma^2_{R\ CAD}$    |
| ii) $H_0: \sigma^2_{L\ CAL} < \sigma^2_{R\ CAL}$  | V | $H_1: \sigma^2_{L\ CAL} > \sigma^2_{R\ CAL}$    |
| iii) $H_0: \sigma^2_{L\ CAL} < \sigma^2_{L\ CAD}$ | V | $H_1: \sigma^2_{L\ CAL} > \sigma^2_{L\ CAD}$    |
| iv) $H_0: \sigma^2_{R\ CAL} = \sigma^2_{R\ CAD}$  | V | $H_1: \sigma^2_{R\ CAL} \neq \sigma^2_{R\ CAD}$ |
| v) $H_0: \sigma^2_{R\ CT} = \sigma^2_{L\ CT}$     | V | $H_1: \sigma^2_{R\ CT} \neq \sigma^2_{L\ CT}$   |

RESULTS (all at the 5% significance level)

i) Null Ho:  $\sigma^2$  L CAD <  $\sigma^2$  R CAD    V    Hi:  $\sigma^2$  L CAD >  $\sigma^2$  R CAD

<u>Horse</u>	<u><math>\sigma^2</math> L CAD</u>	<u><math>\sigma^2</math> R CAD</u>	<u>F Value</u>	<u>Results</u>
49696	2894711.	417413.	6.935	Significant
26443	3094477.	569591.	5.433	"
50316	1093702.	539398.	2.028	"
Pony 2	37968.	60799.	0.6245	"

Comment

Comparing the actual variances of the first three cases, all of which were sub-clinical cases, it can be seen that as expected, the variance on the left side is greater than on the right. There was also a significant difference between the muscles in pony 2, but with the variance on the right being greater than the variance on the left. However these variances were much less than in the sub-clinical cases.

ii) Null Ho:  $\sigma^2$  L CAL <  $\sigma^2$  R CAL    V    Hi:  $\sigma^2$  L CAL >  $\sigma^2$  R CAL

<u>Horse</u>	<u><math>\sigma^2</math> L CAL</u>	<u><math>\sigma^2</math> R CAL</u>	<u>F Value</u>	<u>Result</u>
26443	4111571.	1206532.	3.797	Significant
49696	5054894.	1331173.	3.408	"
50316	3119254.	1288051.	2.420	"
Pony 2	81807.	80365.	1.018	not significant

Comment

As in the first comparison i) these results are to be expected.

iii) Null Ho:  $\sigma^2$  L CAL <  $\sigma^2$  L CAD    V    Hi:  $\sigma^2$  L CAL >  $\sigma^2$  L CAD

<u>Horse</u>	<u><math>\sigma^2</math> L CAL</u>	<u><math>\sigma^2</math> L CAD</u>	<u>F Value</u>	<u>Result</u>
49696	41117571.	2894711.	1.420	Significant
26443	5054894.	3094477.	1.633	"

50316/

<u>Horse</u>	<u><math>\sigma^2</math> L CAL</u>	<u><math>\sigma^2</math> L CAD</u>	<u>F Value</u>	<u>Result</u>
50316	3119254.	1093702.	2.825	significant
49589	1252000.	3174084.	2.535	"
Pony 2	81807.	37968.	2.155	"

Comment

These results confirm the qualitative histological evaluation that the degree of atrophy and hypertrophy (i.e. the variance) was greater in the CAL than in the CAD muscles of the sub-clinical cases. However, in the one clinical case examined (49589), the calculated value for the variance was greater in the CAD than in the CAL, and this will be discussed later.

iv) Null Ho:  $\sigma^2$  R CAL =  $\sigma^2$  R CAD    V    H<sub>1</sub>:  $\sigma^2$  R CAL  $\neq$   $\sigma^2$  R CAD

<u>Horse</u>	<u><math>\sigma^2</math> R CAL</u>	<u><math>\sigma^2</math> R CAD</u>	<u>F Value</u>	<u>Result</u>
49696	1206532.	417413.	2.890	Significant
26443	1331173.	569591.	2.337	"
50316	1288851.	539398.	2.389	"
47988	672771.	331857.	2.027	"
Pony 2	80365.	60799	1.322	"

Comment

Despite the fact that a 2-sided test has been used here the results are again all significant and it may be that such future comparisons between these two muscles could be compared on a one-sided basis with some confidence i.e.  $\sigma^2$  R CAL  $\gg$   $\sigma^2$  R CAD. These results also confirm the qualitative histological study as in all cases where right sided involvement was found, the CAL was affected to a greater degree than the CAD.

v)/

Horse	$\sigma^2_{R CT}$	$\sigma^2_{L CT}$	F Value	Result
49696	1019033.	937071.	1.087	non-significant
26443	336994.	841357.	2.497	significant

Comment

In the first case the variance in the right CT is actually greater than in the left CT but the result was not significant. In the second case however there was a significant difference and this may have been due to the hypertrophy that was noted in this muscle.

c) Comparison of mean muscle fibre area

If we now consider the Welsh test for comparing two means from two populations of sizes  $n_1$  and  $n_2$  with means  $\mu_1$  and  $\mu_2$  variances  $\sigma_1^2$  and  $\sigma_2^2$ . Let the estimates of these, obtained from the 2 samples, be

$\bar{x}_1, \bar{x}_2$   $s_1^2$  and  $s_2^2$  respectively.

$$\text{Let } V = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s_1^2/n_1 + s_2^2/n_2}}$$

Consider

$$H_0: \mu_1 = \mu_2 \quad V \quad H_1: \mu_1 \neq \mu_2$$

ie we suspect  $\mu_1$  and  $\mu_2$  to differ but we do not know which is larger.

Under  $H_1$ , we expect the numerical difference between  $\bar{x}_1$  and  $\bar{x}_2$  to be large, and hence (V) to be large. Hence if (V) exceeds a critical limit we conclude that  $H_1$  is true and say that (V) is significant. Again as with the F statistic, appropriate critical values of (V) are available.

This test is known as the Welsh test for comparing two means, and differs from the standard 2 sample t test in that it does not assume the two variances  $\sigma_1^2$  and  $\sigma_2^2$  to be equal.

The following comparisons were made.

i)/



- i) Ho:  $\mu L CAD = \mu L CAL$  V H1:  $\mu L CAD \neq \mu L CAL$
- ii) Ho:  $\mu L CAD = \mu R CAD$  V H1:  $\mu L CAD \neq \mu R CAD$
- iii) Ho:  $\mu L CT = \mu R CT$  V H1:  $\mu L CT \neq \mu R CT$
- iv) Ho:  $\mu L CAL = \mu R CAL$  V H1:  $\mu L CAL \neq \mu R CAL$
- v) Ho:  $\mu R CAL = \mu R CAD$  V H1:  $\mu R CAL \neq \mu R CAD$

Results (all at the 5% significance level)

i) Null Ho  $\mu L CAD = \mu L CAL$  V H1  $\mu L CAD \neq \mu L CAL$

<u>Horse</u>	<u><math>\mu L CAD</math></u>	<u><math>\mu L CAL</math></u>	<u>V</u>	<u>Result</u>
49696	3711.23	2581.22	6.975	significant
26443	1899.91	1669.23	1.258	non-significant
50316	2548.83	3424.97	-6.967	significant
49589	1274.48	543.49	6.840	"
Pony 2	745.36	1060.15	-16.344	"

Comment

In the more advanced sub-clinical cases (49696 and 26443) it can be seen that the means are less in the CAL than in the CAD and in the clinical case (49589) this difference is marked. The two negative results, while still significant indicate that the means are greater in the CAL than CAD. The larger mean in the case of Pony 2 along with the larger mean of the same contralateral muscle may indicate that it is 'normal' for this muscle to have a larger mean fibre area.

ii) Null Ho  $\mu L CAD = \mu R CAD$  V H1  $\mu L CAD \neq \mu R CAD$

<u>Horse</u>	<u><math>\mu L CAD</math></u>	<u><math>\mu R CAD</math></u>	<u>V</u>	<u>Result</u>
49696	3711.23	2558.24	9.945	significant
26443	1899.91	1974.66	0.580	non-significant
50316/				

<u>Horse</u>	<u><math>\mu</math> L CAD</u>	<u><math>\mu</math> R CAD</u>	<u>V</u>	<u>Result</u>
50316	2548.83	2173.45	5.685	Significant
Pony 2	745.36	886.6	-8.537	"

Comment

In two of the sub-clinical cases (49696 and 50316) the mean fibre area is larger in the left CAD than the right CAD, probably due to the greater degree of hypertrophy than atrophy. In the more advanced sub-clinical case (26443) the mean of the left CAD is slightly smaller than the right CAD presumably to the combined effects of greater atrophy and less hypertrophy. The means of the left and right CAD of pony 2 are very similar despite the large value of V.

iii) Null  $H_0$   $\mu$  L CT =  $\mu$  R CT    V     $H_1$   $\mu$  L CT  $\neq$   $\mu$  R CT

<u>Horse</u>	<u><math>\mu</math> L CT</u>	<u><math>\mu</math> R CT</u>	<u>V</u>	<u>Result</u>
49696	2714.59	2589.88	-1.464	non-significant
26443	1905.09	1266.23	-9.106	significant

Comment

As in the comparison of the variance, the greater mean value for the left CT may be due to hypertrophy and this will be discussed later.

iv) Null  $H_0$   $\mu$  L CAL =  $\mu$  R CAL    V     $H_1$   $\mu$  L CAL  $\neq$   $\mu$  R CAL

<u>Horse</u>	<u><math>\mu</math> L CAL</u>	<u><math>\mu</math> R CAL</u>	<u>V</u>	<u>Result</u>
49696	2581.22	2982.10	-2.842	Significant
26443	1669.23	2103.59	-2.683	"
50316	3424.97	2722.38	5.487	"
Pony 2	1060.15	978.14	3.722	"

Comment

In only one of the sub-clinical cases (50316) was the mean in left/

left CAL greater than in the right CAL and in the other two (49696 and 26443) the result was reversed. This is probably due to the more severe nature of the lesion in the latter two, where much more atrophy was noted. Despite the fact that the means in Pony 2 are very similar there is still a significant difference between them.

<u>Horse</u>	<u><math>\mu</math> R CAL</u>	<u><math>\mu</math> R CAD</u>	<u>V</u>	<u>Result</u>
49696	2982.10	2558.24	-5.042	Significant
26443	2103.59	1974.66	-1.342	Non-significant
50316	2722.38	2173.45	-7.810	Significant
47988	1506.68	1765.30	4.832	"
Pony 2	978.14	886.60	-4.724	"

#### Comment

In only one case (47988) was the mean right CAD greater than the right CAL and this was thought to be due to the atrophy which was qualitatively noted in the right CAL. Although atrophy was also noted in the histological sections in the right CAL of one sub-clinical case (26443) there was no significant difference between the means.

#### Summary of Statistical Conclusions

- 1) The variance in the L CAL muscle was greater than in the L CAD muscle in all the sub-clinical cases but was less in the one clinical case.
- 2) The variance in the L CAD muscle in the sub-clinical cases was greater than in the R CAD muscle.
- 3) The variance in the L CAL muscle in the sub-clinical cases was greater than in the R CAL muscle.
- 4) The variance in the R CAL muscle was significantly greater in the sub-clinical/

clinical cases and the one clinical case, than in the R CAD muscle.

- 5) The mean muscle fibre area of the L CAD muscle was significantly greater than in the R CAD muscle in two of the sub-clinical cases (49696 and 50316).
- 6) The mean muscle fibre area of the R CAL muscle is greater than that of the R CAD muscle in all the sub-clinical cases and the normal pony, but the reverse was true in the one clinical case measured.
- 7) The mean muscle fibre area of the L CAL muscle was significantly less than of the L CAD, in the one clinical case measured.

### Discussion

The first object of this quantitative study of laryngeal muscle fibre area was to obtain accurate values for fibre size in health and disease. Whether or not this study has produced accurate values for normal laryngeal muscle fibre size is perhaps open to question, as too few normal larynges were collected from various ages of animal. The premise that the right sided muscles would act as normal controls is perhaps in doubt due to the histological findings. However, it is not unreasonable to examine the right sided muscles from three age groups (1, 10 and 20 years) in order to examine the effect of age on muscle size, an effect which if present may influence the diseased left side. Moore et al (1971) showed that in man there was a progressive increase in muscle fibre size until middle age, and from then on, a decrease. A comparison of the mean fibre area of the right CAD muscle from cases which might correspond to the human periods mentioned, (pony 2, 1 year; 50316, 10 years; 26443, 20 years) shows that there is this increase in fibre area to middle age followed by a decrease ( $886.6 \rightarrow 2173.4 \rightarrow 197.4 \mu^2$ ). A similar result is seen if the right CAL muscle from these three cases is examined. Also if all the mean fibre areas of the 20 year old subject (26443) are compared with the mean fibre areas of the other two sub-clinical cases, they are seen to be smaller, including those of the control muscle, the cricothyroids. This comparison however has not taken into account the possibility of breed differences being present, and to be certain of this trend a yearling thoroughbred might have been a better example than the case chosen (pony 2). Future work on the effect of ageing on laryngeal muscle fibre size would be best performed in the same breed of horse and might usefully be compared with similar/

similar effects on limb skeletal muscle.

The effect of neurogenic atrophy on mean fibre size is entirely dependent on the stage of the disease, that is whether atrophy or hypertrophy predominate. In some muscles in the present study, hypertrophy had more than compensated for atrophy, in that there was an increase in mean fibre area of such muscles compared with their contralateral controls. This was seen in three muscles ( L CAD 49696, L CAD and L CAL 50316). Such a finding was also noted by Johnson et al (1974) who found 8 muscles undergoing neurogenic atrophy in which there was a shift of mean fibre diameter, only one of these showing a decrease. In the sub-clinical cases of the present study, only three diseased muscles showed a decrease in mean muscle fibre area compared with their contralateral controls. This presumably reflects a stage in the disease when the effects of atrophy and hypertrophy have cancelled each other out and further atrophy leads to a decrease in mean fibre size.

The distribution of muscle fibre area is complex but in nearly every case where the muscle was histologically normal, a normal distribution was present (table 11). The distribution of muscle fibre area has been a contentious one. Sisson (1965) in his careful study on normal muscle showed that frequency-distribution histograms for fibre area were unimodal but were skewed slightly to the right, whilst the same data converted to diameters resulted in a normal distribution. When our results were converted either to the square root or logarithm, no more normal distributions were found. Other authors however have found a normal distribution for muscle fibre area both in infants, children and adolescents (Aherne et al 1971) and in young adults (Reske-Nielsen et al 1970). Both of these authors found that muscle fibre/

fibre area had a unimodal frequency distribution and they failed to find evidence for bimodal curves such as those described in infant muscle by Wolfhart (1937) or by Goldspink and Rowe (1968) in the normal mouse. Reniers et al (1970) in a detailed study of type I and II fibres in normal and pathological human muscle found that in normal muscle the distribution curve was normal but when taken separately were slightly negatively and positively skewed respectively (as judged by the myosin ATP ase reaction).

In all the histologically normal muscles in this study a normal frequency distribution was seen. However in three muscles in which there was evidence of histological abnormality a normal distribution was also found on the basis of the  $\chi^2$  Goodness of Fit test (50316, left CAD and CAL and 49696, left CAD). In all the other diseased muscles from the sub-clinical cases, abnormal distributions were found. As in other studies on the muscle fibre size in neurogenic atrophies, marked evidence of both atrophy and hypertrophy were found (Reniers et al 1970, Johnson et al 1974). There was little evidence in the current study of bimodal distribution despite the fact that the authors of the sub-committee report on muscle quantitation (1968) agreed that this was the case in denervation. In the study of Reniers et al (1970) however, there was no evidence in both the Werdnig-Hoffman and Kugelberg-Welander forms of spinal muscular atrophy of a bimodal distribution, but occasionally evidence for a plurimodal distribution. Johnson et al (1974) also could find no evidence for a bimodal distribution in neurogenic atrophy and suggested that this might only be seen in acute denervating processes such as Werdnig-Hoffman disease where atrophy of individual motor units in an otherwise normal fibre population is occurring. The reason for the absence/

absence of such findings in Reniers et al (1970) case of Werdnig-Hoffman disease, was possible because the biopsy was taken 30 months after the onset of clinical symptoms. In these circumstances considerable atrophy will have taken place with little compensatory hypertrophy remaining and hence a bimodal distribution is unlikely. It would seem from current results that in a chronic neurogenic disease a number of different distributions can be found depending on the stage of the disease. In the early stages where both atrophy and hypertrophy are present there is a widening of the distribution with a possibility of plurimodality (fig. 46) In the later stages of the disease further atrophy leads to a skew to the left and a return to a unimodal distribution. In contrast of this move to the left a possible example of a skew to the right is seen in the left CT of a sub-clinical case (26643) where hypertrophy in a muscle without atrophy might have been due to a compensatory mechanism.

Analysis and interpretation of the histograms may be useful in the understanding of the disease process and any obvious differences between contralateral or ipsilateral muscles. As in the subjective scoring system of the pathological changes in the muscles, the histograms show that there is an apparent variation in the effect of the disease in the affected muscles, namely the dorsal and lateral cricoarytenoid muscles. This was also found in the analysis of the variance of and the mean of muscle fibre size and will be discussed later. In all the histograms from the sub-clinical cases however it is obvious that the adductor muscle (CAL) is affected more severely than the abductor (CAD). In the former, both atrophy and hypertrophy are more obvious. This confirms/



confirms the evidence of the histological scoring system in which in all the sub-clinical cases examined, the range of change was thought to be more severe in the CAL muscles than in the CAD muscles examined. The histograms from the two clinical cases were also interesting, but in only one of these was it possible to measure the muscles from the left side. In this case (49589) it was obvious from the histograms that atrophy in the left CAL muscle was more marked than in the CAD but that hypertrophy was slightly greater in the CAD. In the second case (47988) it was obvious that atrophy was greater in the CAL than in the CAD but that hypertrophy was not obvious in either.

These findings would seem to contradict those of Cole (1946) who found that the left CAD muscle was always more severely atrophied than left CAL in his series of equine larynges. Indeed it would also seem to contradict an old law of laryngology - Semon's Law, which states that in a partial lesion of the recurrent laryngeal nerve the abductor fibres are more susceptible to injury than the adductor fibres. While this law has been the subject of much discussion, no attempts have been made to systematically examine human laryngeal muscles following such lesions. In all the horses examined, it would seem from the histograms, especially where they are compared by superimposition that the reverse of Semon's Law is the case i.e. the disease is more apparent in the adductor (CAL) than the abductor (CAD) muscle (fig. 49).

However, this apparent disparity between the two groups of muscles is most obvious in the sub-clinical case and becomes less obvious when the clinical disease develops.

Confirmation that the disease results in differing degrees of atrophy and hypertrophy of the adductor and abductor group of muscles at/

at various stages of the disease process are found in the results of analysis of the variance of fibre area and of mean fibre area. The variance which was found in the normal pony (pony 2) was perhaps surprising but it was not thought to be significant in comparison with the large variance that was seen in the sub-clinical cases. These results have been summarised earlier but some comment on them is necessary. The variance in the left CAL muscle of all the sub-clinical cases was statistically greater than in the left CAD muscles and the same was true in the contralateral muscles in the sub-clinical cases and in one clinical case. Whilst there may be a normal biological variation in these muscles (of pony 2), this variance was considered outside normal limits. In the one clinical case in which the left sided muscles were measured the variance in the left CAL muscle was significantly less than that in the left CAD, as was the mean muscle fibre size. Mean muscle fibre size in the left CAL was significantly different to the mean size in the left CAD in three sub-clinical cases. Finally, mean muscle fibre size in the left CT muscle was significantly greater than in the right CT in one sub-clinical case.

It is thought that all of these observations may be important in the pathogenesis of the muscle lesion. It is apparent that out of the three muscles of the larynx examined (CAD, CAL and CT on each side), the lesion is most obvious firstly in the CAL, both on the left and right sides of the larynx. This is seen in the histograms where firstly atrophy, and then hypertrophy result in a large standard deviation and hence variance. With increasing severity of the lesion both atrophy and hypertrophy are found in the left CAL and CAD and the difference between the two becomes less as the disease progresses from the sub-clinical to clinical/

clinical stages. With chronic sub-clinical or clinical disease, there is an increased chance of right sided involvement. It is also possible that in the chronic case which is either clinical or sub-clinical (e.g. 26443), there is compensatory hypertrophy of the left CT following chronic atrophy of the other left sided adductor muscles. It would be necessary however to look for this possibility in further cases.

Lastly it must be considered whether the sampling technique used was a valid method of examining muscles affected by a neurogenic disorder. Unlike myopathic change where the effect is purely random, neurogenic atrophy can affect fibres in close proximity giving rise to small, grouped atrophy, or finally may result in fascicular atrophy due to denervation following reinnervation. Two methods are therefore available to quantitate muscle fibre size in such cases a) measure a very large number of fibres and hope to include a satisfactory random sample (e.g. Reniers et al 1970 - measured up to 1500 muscle fibres in a diseased muscle and b) completely random selection of a number of areas measuring a small number of fibres in each area. We chose to attempt the latter but decided to measure all the fibres in each fascicle which lay within the randomly selected area. This involves an assumption that some of the fibres within that fascicle are independent i.e. have different innervation. This assumption is feasible in most cases as muscle fibres within a single motor unit are scattered and may lie in different fascicles although tending to be reasonably adjacent. However, following denervation and reinnervation it is possible that all the fibres within one fascicle belong to the same motor unit and hence are not independent. Denervation of such fascicles results in fascicular atrophy. However, it is felt that very few of these fascicles were measured and hence would make little difference/

difference to the analysis. Another possible difficulty of this method in muscles in which there was either minimal pathology, or in which there was marked atrophy but with little hypertrophy, was to sample all such areas. In two cases it was found necessary to trace additional fascicles to include such areas. However, it was felt that sufficient areas were selected to ensure the accuracy of the fibre measurements. Indeed Sisson (1965) showed that there was a significant difference between the area of fibres, when a few areas containing a large number of fibres were compared with a large number of areas with few fibres. In this study up to 6 or 7 fascicles were drawn from widely dispersed areas within the muscle sample.

A drawback of this method of quantitation was undoubtedly the necessity to measure only those fibres which were completely transverse in section. This excluded many near perfect samples from inclusion. Measurement of the lesser diameter of the muscle fibre is not excluded where slight obliquity is present and may be a more useful parameter than fibre area. Brooke and Engel (1969a) devised a method whereby the measurement of lesser diameter of a number of randomly selected muscle fibres could be used to calculate 'Atrophy - Hypertrophy factors'. This involves knowledge of the range of normal diameters for each muscle; the number of fibres below and above this range are calculated. Atrophy and hypertrophy factors are then calculated by counting the total number of fibres above and below this range and their degree of deviation from the norm. However, such a method does not allow further statistical analysis of the data as was performed in the current study but could be a quick and useful method in future work.

<u>Case No.</u>	<u>Age</u>	<u>Clinical Status</u>	<u>Muscle Measured</u>					
			<u>Left</u>			<u>Right</u>		
			<u>CAD</u>	<u>CAL</u>	<u>CT</u>	<u>CAD</u>	<u>CAL</u>	<u>CT</u>
26443	20 yrs.	sub-clinical	*	*	*	*	*	*
49696	10 yrs.	"	*	*	*	*	*	*
50316	8 yrs.	"	*	*	-	*	*	-
49589	9 yrs.	clinical	*	*	-	-	-	-
47988	12 yrs.	"	-	-	-	*	*	-
Pony 2	1 yr.	normal	*	*	-	*	*	-

\* = muscle measured  
 - = no sample available

TABLE 7 Details of muscles measured.

<u>Horse</u>	R CAD	L CAD	R CAL	L CAL	R CT	L CT
49696	245	247	225	283	296	246
26443	219	221	201	258	260	232
50513	467	268	283	349	286	316
50316	440	349	347	246	-	-
47988	393	-	333	-	-	-
49589	-	374	-	427	-	-
Pony 2	380	334	373	318	-	-

TABLE 8 Total number of muscle fibres measured in each case.

<u>Horse</u>	R CAD	L CAD	R CAL	L CAL	R CT	L CT
49696	2558.24	3711.23	2982.10	2581.22	2589.88	2714.59
26443	1974.66	1899.91	2103.59	1669.23	1266.23	1905.09
50513	2157.46	2873.50	2030.42	2043.55	2069.11	2156.80
50316	2173.45	2548.83	2722.38	3424.97	-	-
47988	1765.30	-	1506.68	-	-	-
49589	-	1274.48	-	543.49	-	-
Pony 2	886.60	745.36	978.14	1060.15	-	-

TABLE 9 Arithmetic means of muscle fibre areas

<u>Horse</u>	R CAD	L CAD	R CAL	L CAL	R CT	L CT
49696	417413.575	2894711.837	1206532.446	4111571.297	1019033.280	937071.973
26443	569591.069	3094477.520	1331173.119	5054894.495	336994.600	841357.422
50513	1099389.017	1931626.082	864890.670	796627.001	1013290.654	783377.796
50316	539398.750	1093702.591	1288851.254	3119254.962	-	-
47988	331857.189	-	672771.417	-	-	-
49589	-	3174084.958	-	1252000.692	-	-
Pony 2	60799.850	37968.869	80365.710	81807.725	-	-

TABLE 10 Variances



Case No.	L CAD	R CAD	L CAL	R CAL	L CT	R CT
26443	/	N	/	/	/	$\sqrt{N}$
49696	N	N	/	N	$\sqrt{N}$	$\sqrt{N}$
50316	N	/	N	N	-	-
47988	N	/	-	-	-	-
49589	/	-	/	-	-	-
Pony 2	N	N	N	/	-	-

- = no data

N = Normal fit

/ = no fit obtained

N = Square root of normal fit

TABLE 11 Results of fitting a model to the data.

Figs. (44) to (48): Histograms of muscle fibre area  
(X axis,  $\mu^2$ ) against fibre frequency (Y axis, %)

Fig. (44): Muscle fibre area of two clinical cases. Note  
that the C.A.L. muscle is more severely atrophied in each  
case and that in the second case (49589) there are more  
normal sized or hypertrophic fibres in the C.A.D. than  
the C.A.L.

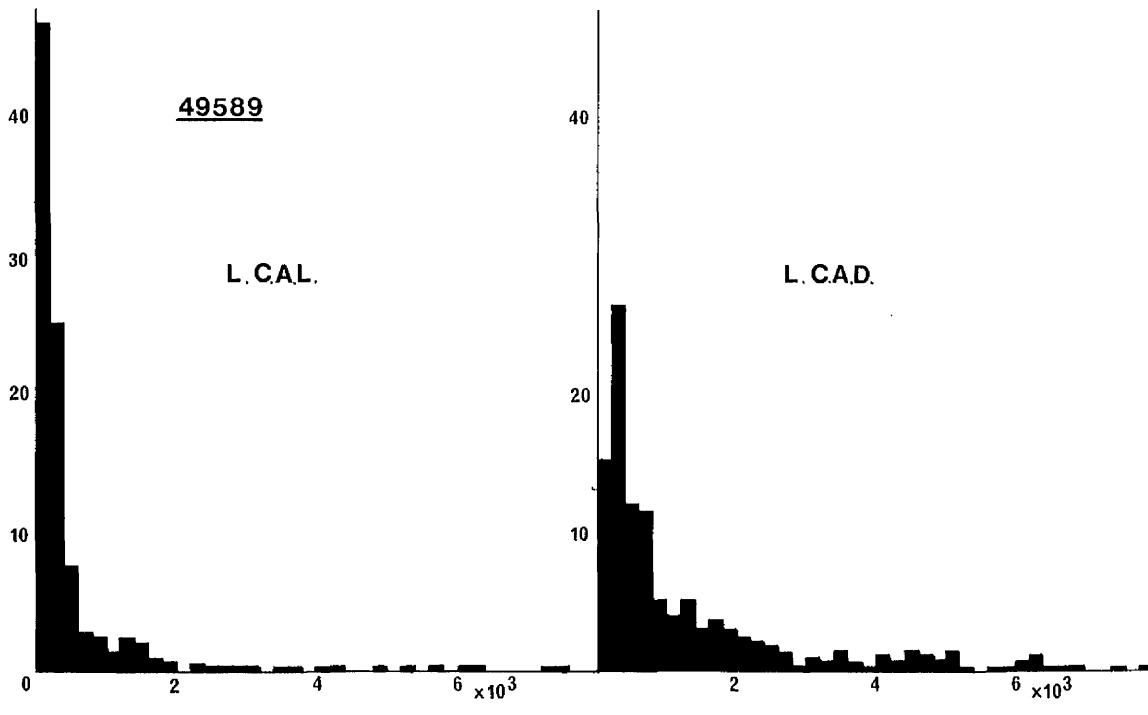
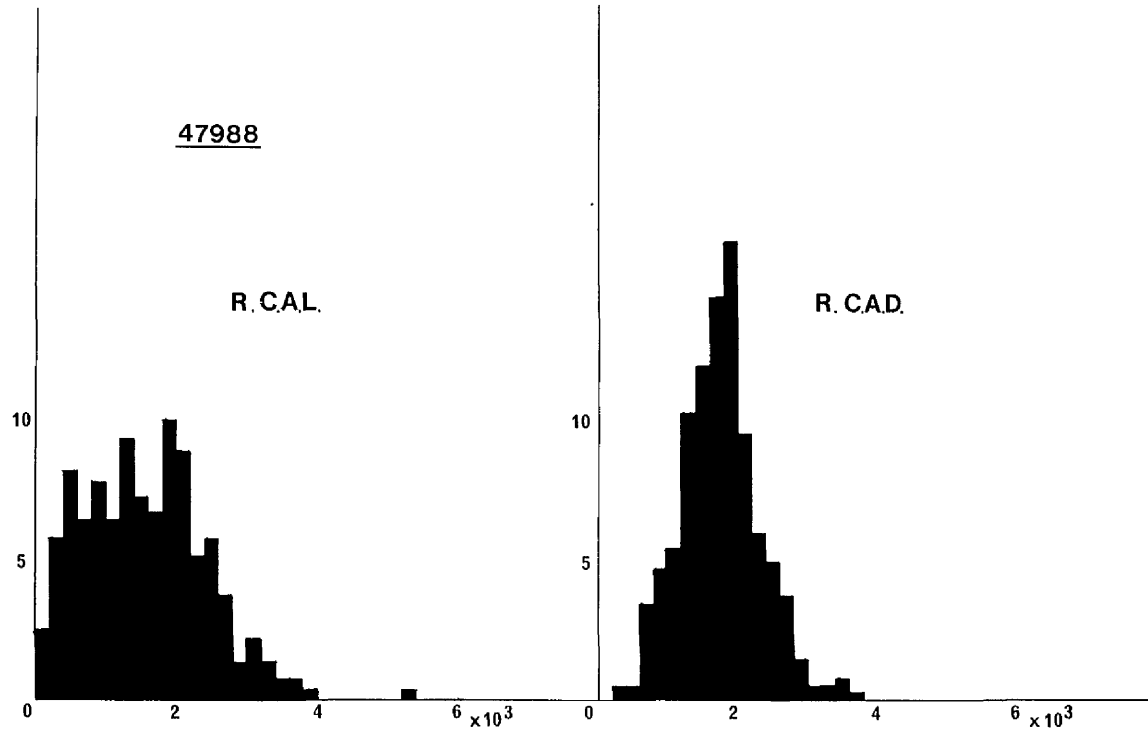


Fig. (45): Muscle fibre area of a sub-clinical case.

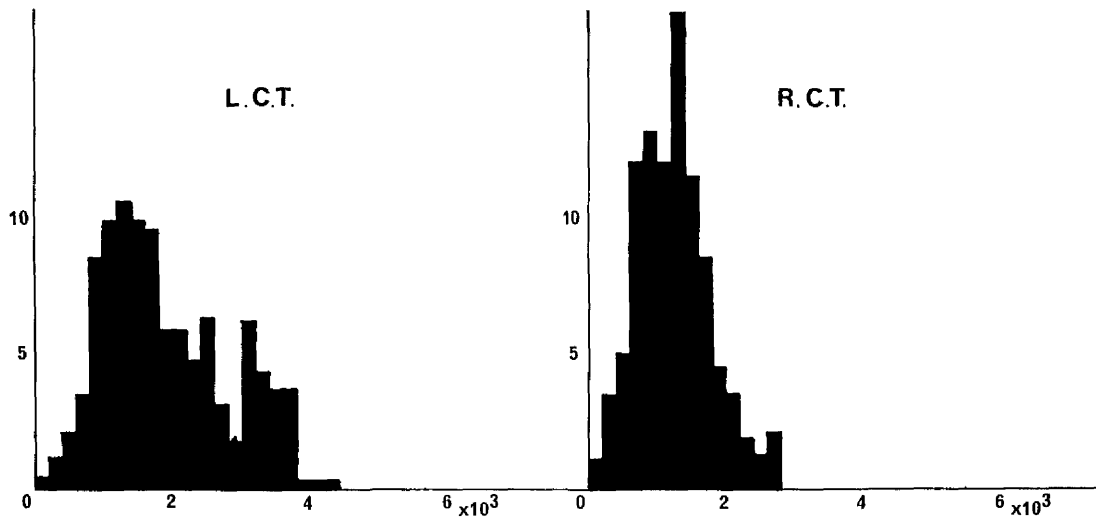
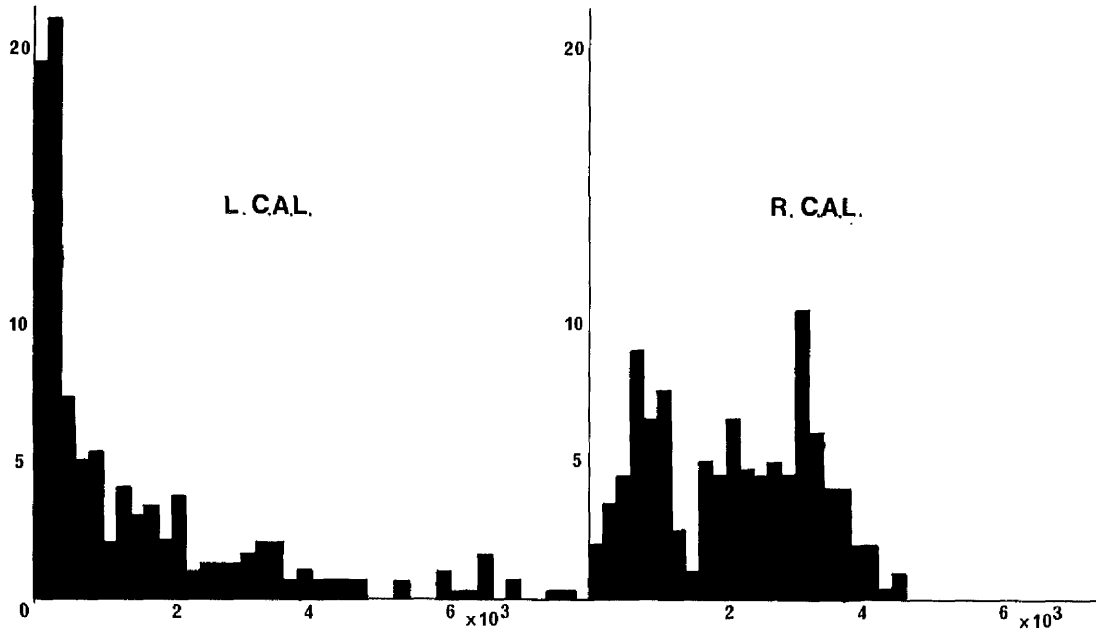
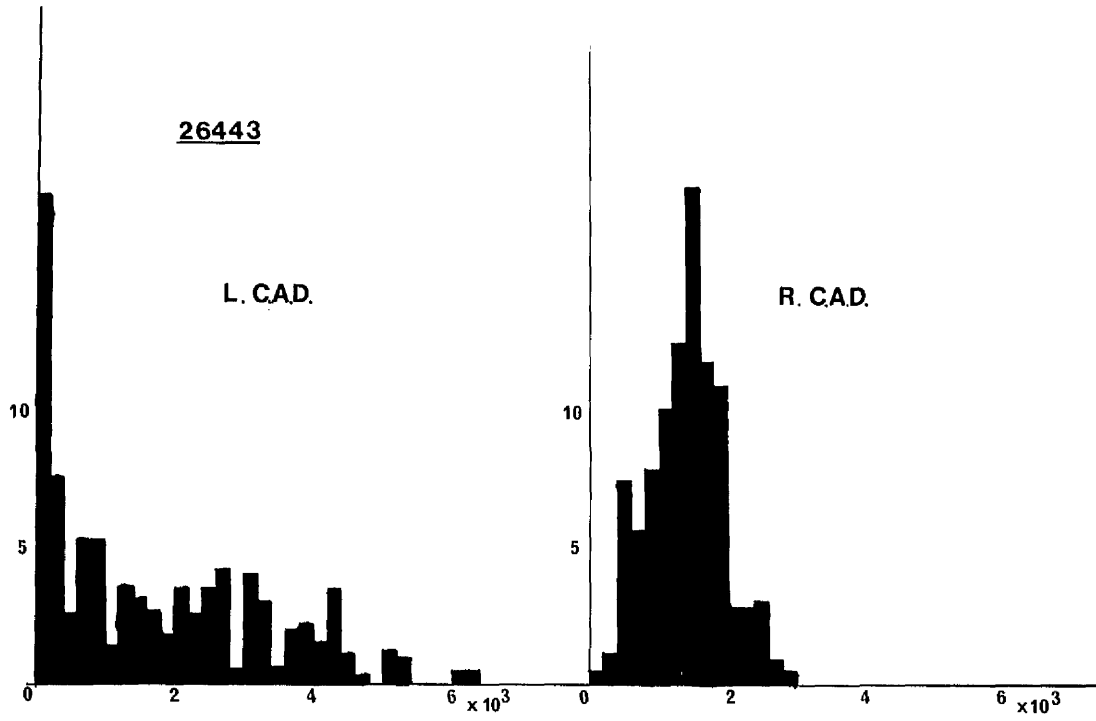
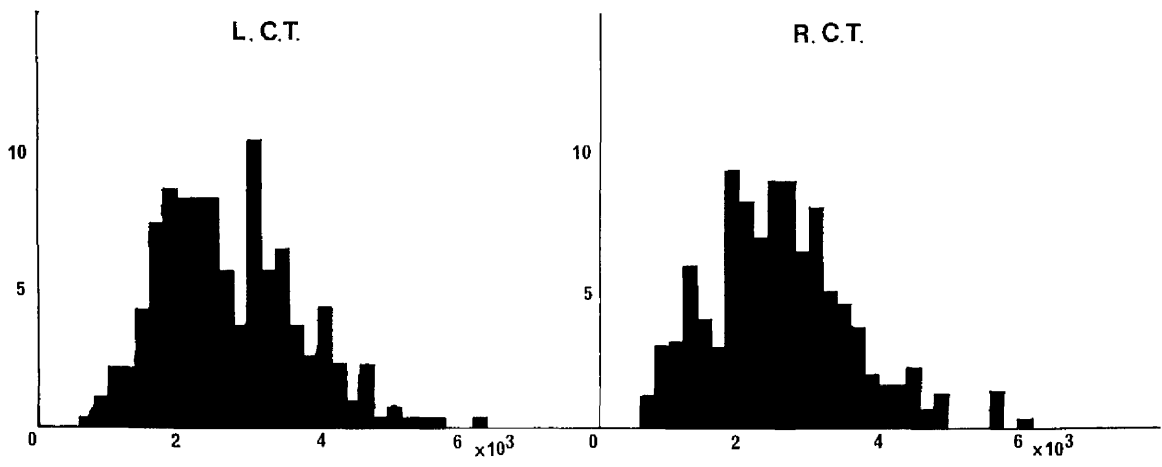
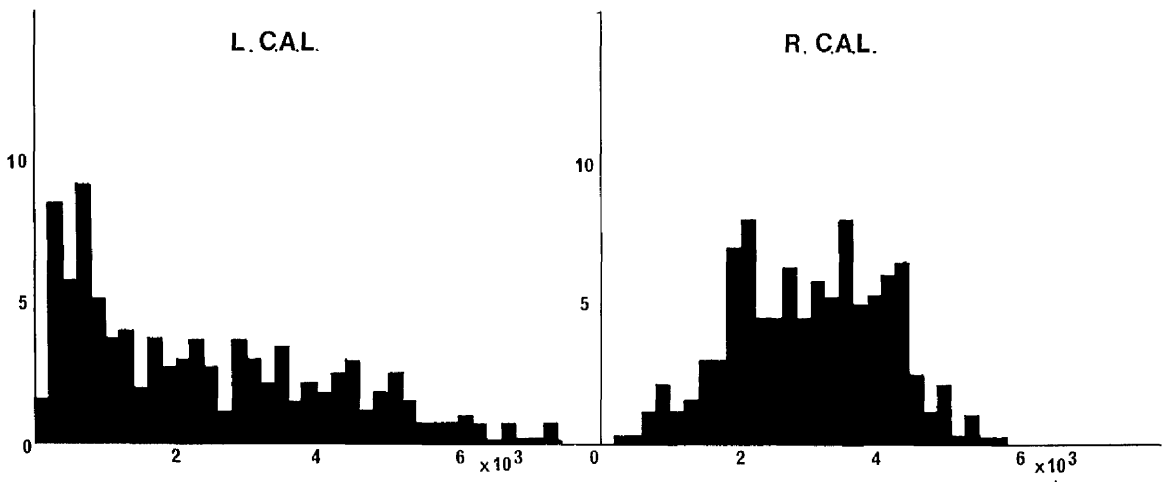
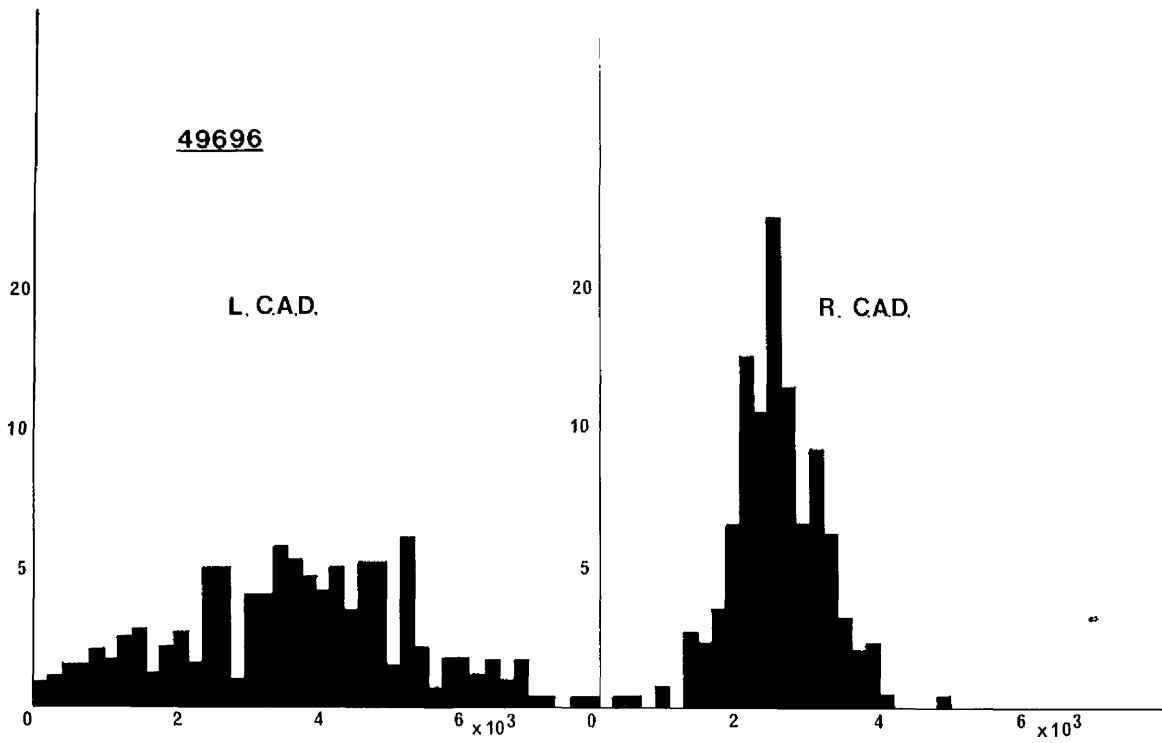
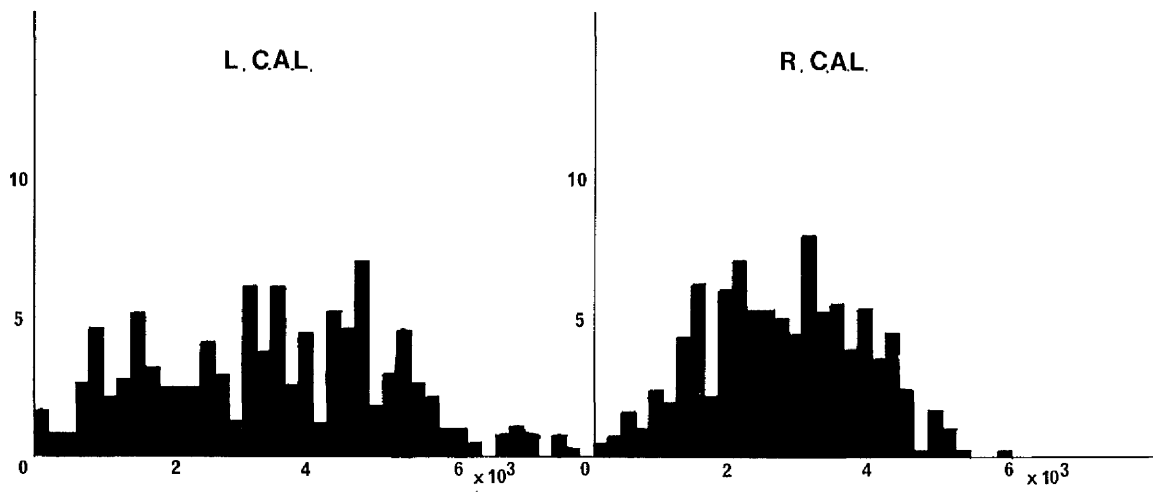
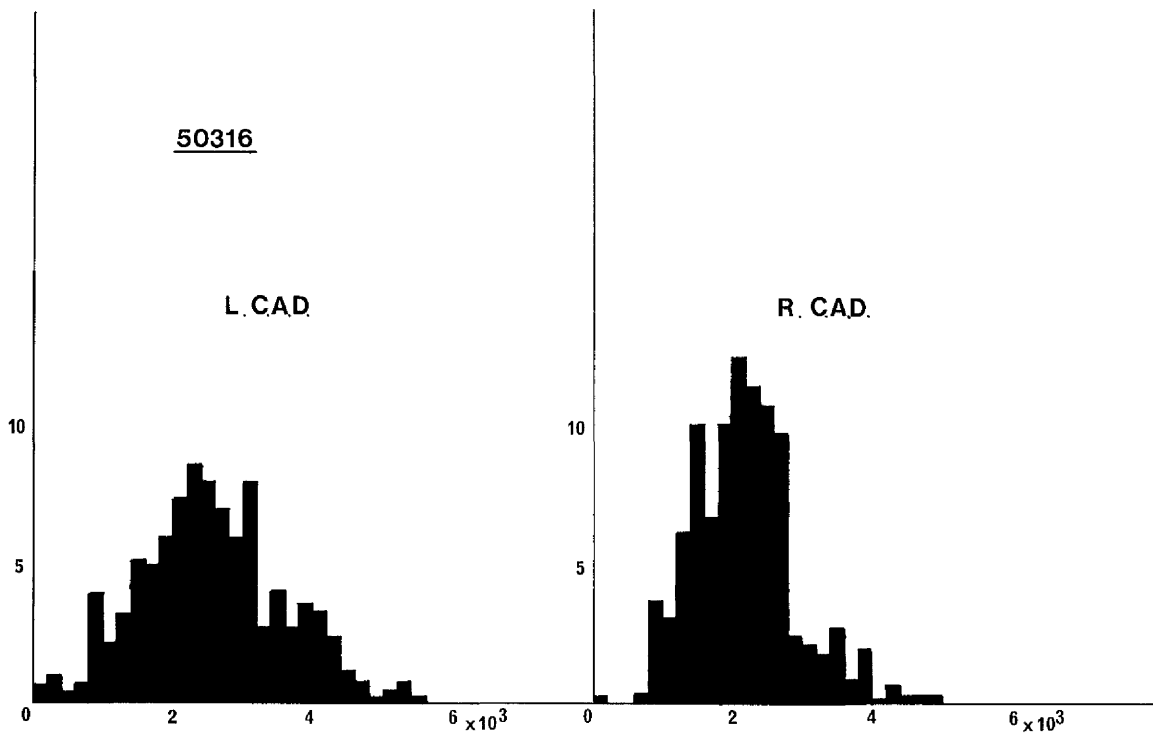


Fig. (46): Muscle fibre area of a sub-clinical case.







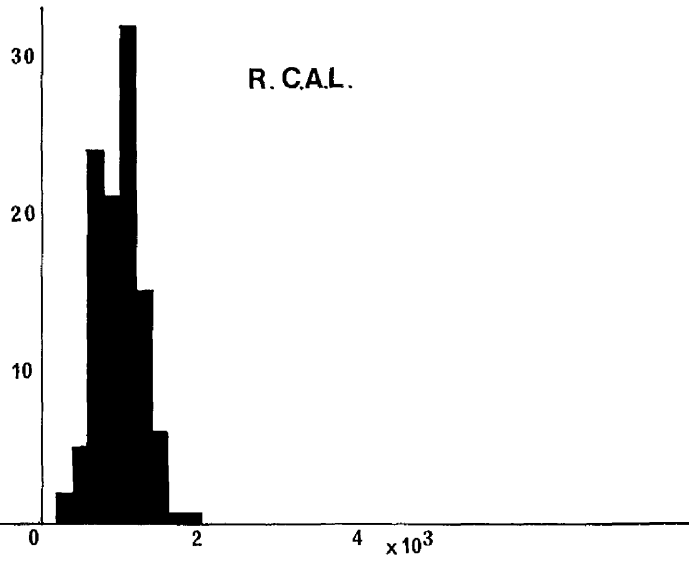
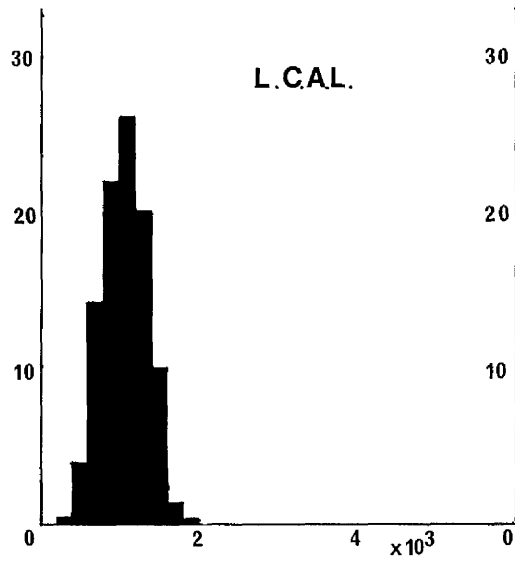
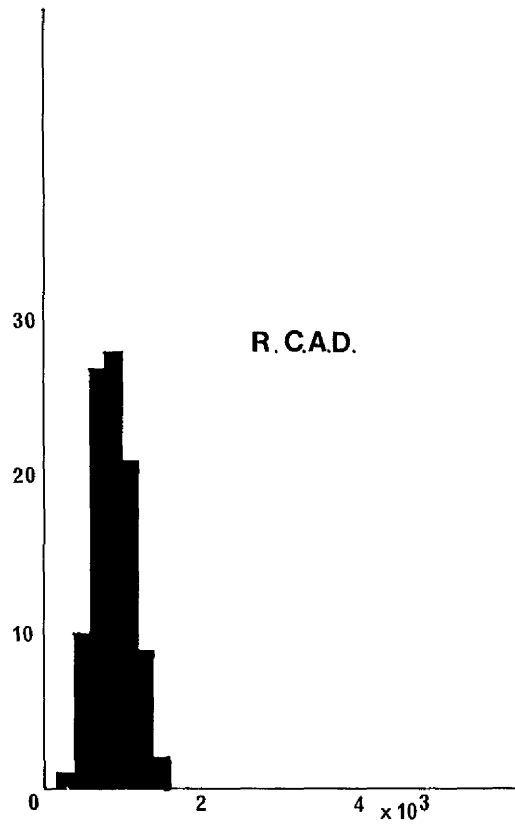
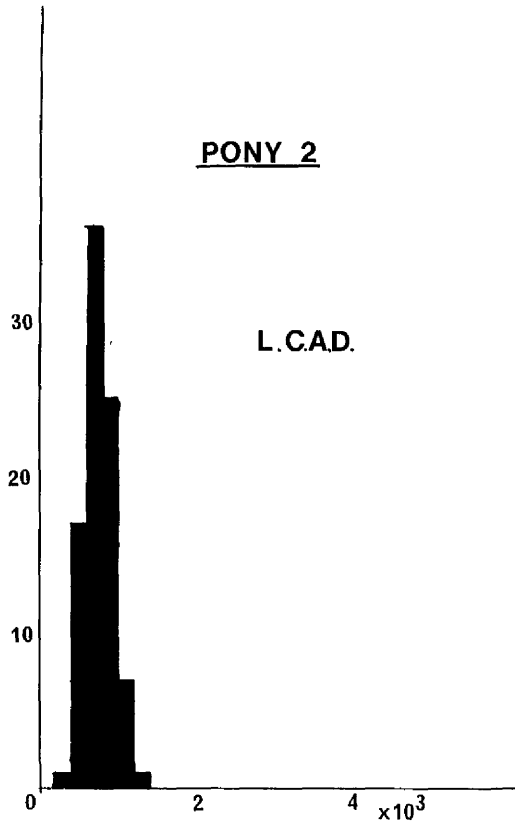
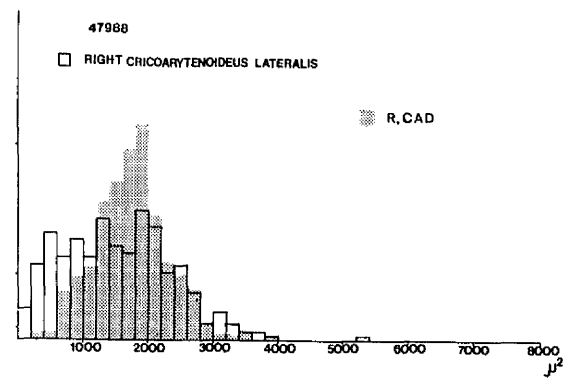
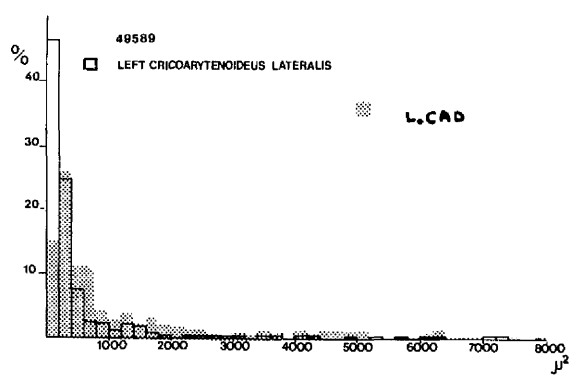
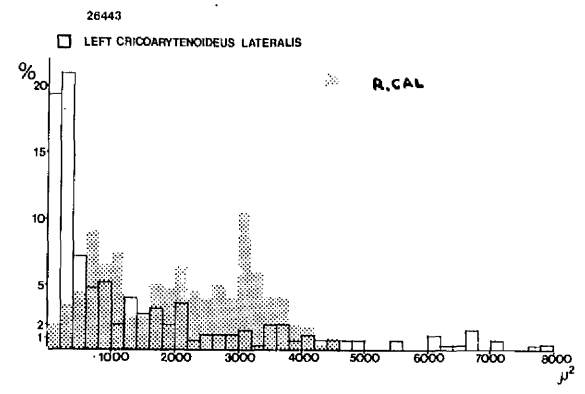
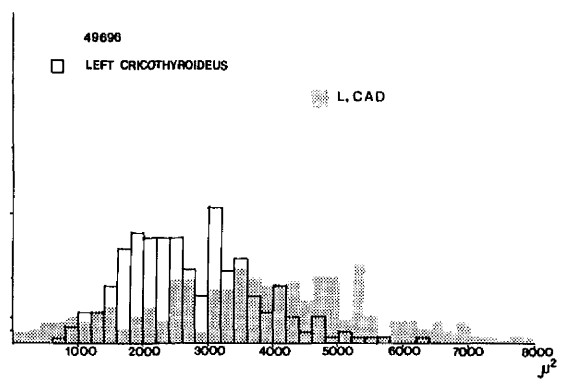
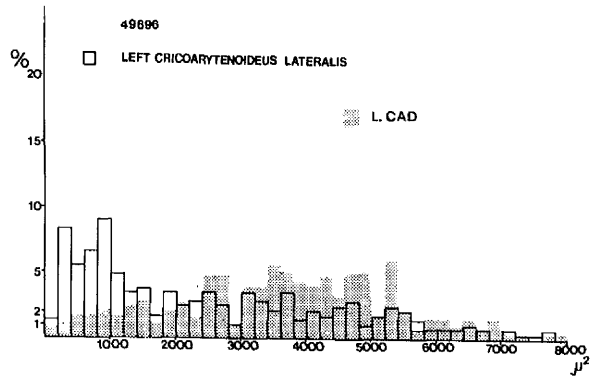


Fig. (49): Superimposition of muscle fibre area histograms  
from clinical and sub-clinical cases to stress the  
difference between C.A.D. and C.A.L. muscles.



SECTION 4

NERVE PATHOLOGY

INTRODUCTION

The pathology of the recurrent laryngeal neuropathy in equine laryngeal paralysis is not well documented. This is especially so in the light of the new techniques such as single fibre teasing and electron microscopy which have been used to investigate many human clinical, and experimentally induced neuropathies. However, one important fact has been described, namely that the part of the left recurrent nerve nearest to the larynx is most severely affected (Thomassen 1901 and Cole 1946) whilst there is little evidence of loss of nerve fibres or degeneration in the more proximal portions of the nerve. Cole (1946) also described fragmentation of axis cylinders with eventual endoneurial fibrosis, and stated that smaller diameter fibres were preferentially affected.

Mason (1973) reintroduced an interesting finding into the discussion of the recurrent laryngeal neuropathy, that of the anomalous course of the left recurrent nerve in some horses (Haslam's anomaly 1893). This anomaly is present when the left recurrent nerve lies lateral to the trachea and between it and aortic arch, as the left nerve emerges from behind the aorta. Normally the left nerve is ventral to the trachea at this point. However, he could find no correlation between this anomaly and the clinical disease of laryngeal hemiplegia and pointed out that both Haslam (1893) and Argyle (1932) had found the anomaly in normal horses. In the five cases described by Mason (1973) which showed the anomaly, at the point of compression between the trachea and oesophagus the left recurrent nerve was seen to be flattened and "ribbon-like". Histologically there was a gross displacement of the fascicles, which are found to lie along the line of flattening. The perineurium was thickened, in some cases up to/

to eight times the normal thickness, and such changes may be seen distal to the point of flattening. In addition Mason described Renault bodies at this point.

The significance of this latter finding is unknown. Renault bodies were well known to the histologists of the late nineteenth century but it would appear that they have been forgotten, with a few exceptions, in the modern literature. Asbury (1973) renewed the interest in these structures in a paper entitled "Renault bodies; A forgotten endoneurial structure". These structures, which are found in the subperineurial space are 'cylindrical, hyaline appearing, loosely textured whorled and cell sparse'. They were first described by Renault (1881) in the peripheral nerves of man and animals although they were particularly well developed in the horse and ass. Histochemically these structures are negative except when stained by alcian blue.

Their possible link with chronic compression and injury as suggested by Mason (1973) was confirmed by Asbury (1973) and by a recent publication on the pathology of human ulnar nerve compression (Neary and Eames 1975). However, Asbury (1973) emphasises that Renault bodies are probably non-specific as they can be found in normal nerves, hypothyroid neuropathy, cretinism and, necrotising angiopathic neuropathy (Dyck et al 1972) as well as in association with compression.

In order that the laryngeal neuropathy can be considered in more detail in modern terms it is necessary to discuss the recent advances that have been made into the investigation of peripheral neuropathies.

Two main types of nerve fibre degeneration are now recognised, 1) axonal degeneration 2) demyelination. In the former the primary change/

change is in the axon with secondary degeneration of the myelin sheath. Originally termed Wallerian degeneration, it is more accurate to describe such changes as Wallerian type degeneration, since these changes were first described by Waller (1850) distal to nerve section. Today, the term is used to describe any lesion in association with focal trauma, and as Bradley (1974) indicates, the term axonal neuropathy is perhaps a better description for diseases in which axonal degeneration is not secondary to trauma.

Demyelination describes the change where the Schwann cell and the myelin sheath degenerate but where the axon remains intact. This degeneration is often haphazard, affecting some internodes and not others, and is often termed segmental demyelination. Paranodal demyelination described the loss of myelin from only the paranodal area.

While most human neuropathies are of the mixed variety (that is with both axonal degeneration and demyelination), some are predominantly of the axonal type, for example that of acute porphyria, while others are primarily demyelinating e.g. diphtheria. (Jacobs et al 1967). However, even in local diphtheritic neuropathy, in which the primary change is demyelination, there can be evidence of degeneration if the lesion is severe (Bradley and Jennekens 1971).

The distribution of degeneration is often found to be predominantly distal, a fact first described by Gowers (1886) in his description of the central changes in amyotrophic lateral sclerosis (A.L.S.). Such a process, where degeneration is first seen distally with progression towards the nerve cell body has been called the "Dying-back" process. To date this "Dying-back" process has been described in many conditions, in particular in Friedreich's ataxia, A.L.S., where the changes can be seen/

seen both in C.N.S. and peripheral nervous systems, and in the neuropathies occurring in association with thiamine deficiency and acrylamide and tri-orthocresyl phosphate (TOCP) intoxication. Cavanagh (1964) has reviewed both the experimental and clinical aspects of the "Dying-back" process.

The description of "distal degeneration" in the acrylamide neuropathy (Hopkins 1970) is one of the few studies to-date which rigorously prove the "Dying-back" hypothesis (Bradley 1974). In this study (Hopkins 1970) the left recurrent laryngeal (a non-branching motor nerve) was found to be normal at the level of the aortic arch but to be almost totally degenerate distally. In addition, a few single teased fibres were seen to be intact proximally, but degenerate distally. By comparison the right recurrent laryngeal nerve was found to be comparatively normal throughout (Hopkins 1975 personal communication). This involvement of the laryngeal nerves has been described in other toxic neuropathies. Cavanagh (1964) found evidence of laryngeal involvement in cats poisoned with TOCP after noting a change of voice, and commented on similar problems in an out-break of trixylyl phosphate poisoning in cattle.

Hern (1971), in a detailed study on the physiology and pathology of TOCP poisoning in the baboon, showed unequivocally that degeneration was most severe in the distal portions of the left recurrent nerve while normal at the aortic arch, and distally on the right side.

Not only are the longest nerve fibres preferentially attacked by a "Dying-back" process but also the largest diameter nerve fibres appear to degenerate first. It has been suggested that the nutritional requirements of the longest and largest fibres may impose a greater strain on/



on the perikarya than in shorter smaller fibres. It has been shown that protein synthesis is decreased in the perikarya prior to the appearance of distal degeneration in a toxic neuropathy (Cavanagh and Chen 1971). Such a decrease may inhibit axoplasmic-flow in affected fibres and this may be most severe in longest, largest fibres.

Axoplasmic flow has become a major corner stone of research into peripheral nerve disorders. Flow of axoplasm in an orthograde direction was first shown by Weiss and Hiscoe (1948) who demonstrated swelling of the axon proximal to ligatures tied round regenerating nerves. Since then, retrograde flow has also been demonstrated (Lubinska et al 1963), although this is probably smaller in amount than orthograde flow. The function of material which is transferred along the axon distally is uncertain but has been suggested to include transmitter substance (Ach) or its precursors, nutrients for the nerve terminals, trophic factors with effects either at the neuromuscular junction or in the Schwann cell (Bradley 1974). This flow of material has been shown to travel at both a fast and a slow rate (Lasek 1968).

Impairment of such flow would seem an obvious explanation for many of the distal neuropathies and Pleasure et al (1969) showed that there was a reduction in the slow rate of flow in acrylamide neuropathy. However, Bradley and Williams (1973) could not confirm this finding but found a decrease in the velocity of the crest of fast axoplasmic flow, but thought this unlikely to be responsible for the resultant distal neuropathy. There was no significant decrease in flow in cats with TOCP or vincristine neuropathy (Bradley and Williams 1973) nor in mice with inherited motor neuron disease (Bradley and Jaros 1973). The possibility of direct local damage by acrylamide has been suggested (Schaumburg et al 1974)/

1974) but remains to be proven. However, the role of axoplasmic flow in the "Dying-back" neuropathies will be studied further to determine whether impairment of a small subfraction of total protein may be sufficient to lead to distal degeneration (Bradley 1974).

Despite the fact that the primary lesion of the "Dying-back" process may be in the nerve cell body, these are often normal or show only temporary chromatolytic changes (Cavanagh 1964). It may therefore be impossible to find changes in the affected nucleus or indeed distinguish a "Dying-back" process from a "longest fibre" disease (Bradley and Thomas 1973). In the latter there is total loss of the perikarya and axons of the longest, largest fibres. Although the nerve cell bodies are lost this may be difficult to determine as the nuclei lie scattered in a large population, and any proximal axonal changes will be difficult to find due to their small number.

Recognition of the two main types of degeneration within the peripheral nervous system has been aided by the development of new histological and quantitative techniques (Thomas 1970, Bradley 1974). The main advances that have been made are (1) the examination of isolated single nerves fibres (2) the quantitation of myelinated and unmyelinated nerve fibres (3) electron microscopy. The former two have been investigated in this study. It was Gombault in 1880 who published the first account of teased fibre preparations in lead neuropathy, in which there was clear evidence of segmental demyelination. As a method of investigation, teased fibre preparations were then largely forgotten by histologists who relied on the more conventional histological methods for examination of peripheral nerves. While these may demonstrate axonal degeneration they do not demonstrate demyelination adequately and it/

it is necessary to examine single fibre preparations especially if segmental demyelination or remyelination is present. Using this technique, segmental demyelination has been described in a number of different neuropathies for example, diabetic neuropathy (Thomas and Lascelles 1966) liver disease (Dayan and Williams 1967) diphtheria (Cavanagh and Jacobs 1964), peroneal muscular atrophy (Gutrecht and Dyck 1966) and myxoedema (Dyck and Lambert 1970). In other cases, segmental demyelination has been thought to be secondary to axonal change, as in uraemic neuropathy (Jennekens et al 1969, Dyck et al 1971). It is possible by measuring the internode distance/diameter to obtain quantitative information on the neuropathy in question. It is now well accepted that internode length increases linearly with internode diameter, in other words the largest diameter fibres have the longest internodes and the shortest diameter have correspondingly short internodes.

This relationship is however affected by age, humans over 65 showing evidence of alteration of this linear relationship due to demyelination and remyelination and degeneration and regeneration (Lascelles and Thomas 1966).

The loss of linear relationship can be identified in the single fibre graphs as described by Fullerton et al (1965). In these the internodal lengths of single fibres are plotted against their maximum diameter. In normal fibres there should be little variation in internode length but in those affected by segmental demyelination and remyelination (such as in the work of Lascelles and Thomas 1966) there is an abnormal variation in internode length. These single fibre graphs are also able to clearly illustrate fibres which have regenerated after axonal degeneration. It has been shown that the internode lengths of regenerated fibres/

fibres of all diameters are uniformly reduced in length (Hiscoe 1947, Vizoso and Young 1948), and as such are easily recognisable on the single fibre graphs.

The importance of being able to define the normal appearance, both qualitative and quantitative, of teased fibres from different nerves and different ages has been reviewed (Arnold and Harriman 1970, Stevens et al 1973). While differing in their classification and interpretation of changes seen, they both illustrate that degeneration and demyelination and remyelination can be seen in "normal" subjects. Both papers also note the presence of "intercalated" internodes, a finding that was discussed in an earlier publication by Dyck and his colleagues at the Mayo Clinic (1968). These intercalated internodes were first described in the median and sciatic nerves of asses and horses by Renaut (1881), who took them to be evidence of distal nerve growth. They are now known to result from remyelination following paranodal demyelination. Such segments have also been described in peripheral nerves and nerve roots of normal cats by Lubinska (1958). Intercalated segments were found more frequently in older cats but were only found to occur in one out of every thousand nodes examined. In a later study on nerve roots in dogs (Griffiths et al 1975), intercalated segments were found more frequently than in Lubinska's study. It would appear that intercalated segments are common features of a number of neuropathies and can be found in both the primary axonal neuropathies e.g. TOCP intoxication (Hern 1971) and in those where the primary damage is thought to be in the Schwann cell e.g. ischaemia (Eames and Lange 1967).

Quantitation of the myelinated nerve population can be calculated from photographic enlargements of transverse sections (Thomas 1970).

Enlargements/

Enlargements at 1000 times can be used to calculate the total number of myelinated nerve fibres and also their diameters. Although it is possible to measure fibre density rather than count the total number of fibres, Swallow (1966) suggested that there was no advantage in expressing results as densities when the nerve was small enough to allow total counts to be made.

Different methods of fixation and processing result in different degrees of shrinkage and therefore it is important to compare results only from material produced in a standardised manner. A common method used for such quantitative studies of myelinated fibre population is fixation in Fleming's fluid followed by Weigert-Pal or Kultschitzky staining. It is also important to realise that nerve fibres branch and taper, in particular if a nerve is sampled near to the muscle it is innervating considerable branching will probably have occurred. (Eccles and Sherrington 1930). Similar sites on the nerve should therefore be chosen for comparison.

In most normal human nerves, the fibre distribution has been shown to be bimodal (Thomas 1970). However, some nerves are thought to be exceptional in having a unimodal distribution (see section on normal larynx), the recurrent laryngeal nerve being described as having this distribution. The main significance of nerve fibre size spectra when investigating peripheral neuropathies is that it is well known that some diseases selectively affect particular sizes of fibres. Large diameter fibres seem to be most susceptible and selective loss of this spectra has been described in motor neurone disease (Wolfhart and Swank 1941), Friedreich's ataxia (Dyck et al 1968), experimental TDCP poisoning (Hern 1971), ischaemic neuropathy (Garven et al 1962), nerve compression/

compression in the guinea pig (Anderson et al 1970) and human ulnar nerve compression (Neary and Eames 1975). Selective loss of small diameter fibres has been reported in amyloid neuropathy (Dyck and Lambert 1969) and Fabry's disease (Kocen and Thomas 1970).

Despite the fact that it might seem that both qualitative and quantitative studies of teased fibres, and of myelinated fibres on transverse section, would provide unequivocal evidence of the type of disease and its pathology, Bradley (1974) has discussed some drawbacks which may occur. These are that -

- a) fibres which have undergone axonal degeneration may appear normal on transverse section
- b) if totally degenerate, fibres leave no mark and may not produce a sufficient effect on the total number of fibres to be noticed on transverse section.
- c) fibres which have demyelinated areas remain and hence may accentuate the proportion of demyelination in the teased fibre preparations.
- d) if demyelinated segments are numerous, they may not be apparent on transverse section and hence produce an erroneously low total fibre count on transverse section.

However, as Bradley points out, these methods of investigation can still produce much worthwhile information provided these facts are kept in mind.

Electron microscopy has been widely used to study both experimental and human neuropathies but has not been employed in this study to-date. However, light microscopy on  $1\mu$  sections has been carried out in some cases.

From the preceding discussion it is apparent that basic disease processes in peripheral nerves and their methods of investigation can enable/

enable one to classify many of the peripheral neuropathies on aetiological or pathological grounds. It may therefore be possible by examination of the recurrent laryngeal neuropathy, using such techniques as have been described, to compare it with other known conditions in an attempt to classify and perhaps discover its aetiology. As with the pathology of muscle however, many of the pathological features of peripheral nerve disease are non-specific and not pathognomonic of any one disease. However, it is perhaps worthwhile to examine some of the suggested causes of the laryngeal neuropathy and discuss the known features that such agents produce in other circumstances. It will therefore be possible to compare the findings of this study with the suggested causes of laryngeal paralysis.

One of the very first suggestions of the cause of "roaring" was thyroid dysfunction (Vermeulen 1914/15), this enabling bacteria and toxins to circulate in the blood causing nervous tissue damage. Several authors to-date have shown that thyroid disease particularly myxoedema can give rise to peripheral neuropathies. Dyck and Lambert (1970) showed physiological and histological evidence of segmental demyelination and remyelination in myxoedematous neuropathy.

It was commonly suggested that the disease was due to compression of the left recurrent nerve as it passed around the aorta, and indeed Haslam (1893) showed that some gross flattening could occasionally be found in the nerve as it passed between aorta and trachea. However, due to inadequate proof, such a suggestion has been more or less forgotten. Compression or entrapment neuropathies are now in vogue in human and in experimental neurology as much has been discovered about them in recent years. Entrapment neuropathies have been reported in man in many anatomical/

anatomical sites, by Thomas and Fullerton (1963), Kopell and Thompson (1963) Neary and Eames (1975) and Neary et al (1975). In addition, peripheral nerve entrapment has been described in association both with hereditary and infectious neuropathies (Earl et al 1964, Hopkins and Morgan-Hughes 1969, Behae et al 1972, and Karpati et al 1974).

The pathogenesis of such lesions and the effect of pressure on peripheral nerves has raised considerable interest in the past and stimulated much research. Erlanger and Gasser (1929) were the first to show that larger diameter fibres were preferentially affected by pressure. Denny-Brown and Brenner (1944) using a sphygmomanometer cuff to produce acute pressure suggested that ischaemia was responsible for the conduction block and degeneration found. Ochoa et al (1972) however disagreed with this hypothesis, giving evidence that acute pressure could produce direct damage to the nerve fibres which was not secondary to ischaemia. Using a pneumatic cuff placed around the leg of a baboon, they found evidence of mechanical damage to fibres in the medial popliteal nerve, just below the edges of the cuff. Single teased fibres from this area were found to have displaced nodes of Ranvier in which there was invagination of the paranodal myelin. Invagination of one paranode produced stretching of the paranodal myelin at the opposite end of the internode. These lesions were always polarised so that the invagination areas were always distal to the pressure point.

Aguayo et al (1971) experimentally produced entrapment in young rabbits by fitting a silastic tube round the medial popliteal nerve. Subsequent growth produced compression resulting in segmental demyelination at the level of entrapment with the outer fibres being more affected than the inner ones.



A natural animal model of entrapment neuropathy was first described by Fullerton and Gilliatt (1967) in guinea pigs. As they grow older, guinea pigs, if kept in wire cages develop a compressive neuropathy of the median nerve at the wrist. Anderson et al (1970) first noticed that internodes in teased fibre preparations from such cases were asymmetrical with bulbous swellings at one end and tapering at the other. Ochoa and Marotte (1973), in a beautifully illustrated study, showed that all such internodes lay in one direction in relation to the entrapment site and that the polarity of the swellings and taperings was reversed on the opposite side. Single fibre E.M. preparations showed that there was slippage of the inner myelin lamellae relative to the axon, away from the site of compression. A similar process has now been shown to occur in ulnar nerve compression in man (Neary and Eames 1975).

While the theory of aortic arch compression was thought unlikely, others thought that the peculiar anatomical course of the left recurrent nerve was still responsible for the asymmetry of the clinical disease. It was suggested by two authors that due to the longer course of the left recurrent nerve, and its different geometric axis compared with the right nerve, greater tension is placed on the left nerve on extension or movement of the head to right than on the right nerve on an opposite manoeuvre, (Marks et al 1970, Rooney and Delaney 1970). The former authors suggested that the pathological changes in the laryngeal nerves were identical to those seen in experimentally produced stretch lesions in the peripheral nerves in dogs (Highett and Sanders 1943). Their method was to excise a portion of nerve, then suture together the viable remnants. It was thus sudden and traumatic and not comparable with the apparent chronic, possibly "atraumatic" lesions of the R.L.N. lesion. Stress-strain phenomena/

phenomena in peripheral nerves have been carefully investigated by Sunderland and Bradley (1961) who remarked that there was considerable clinical evidence to suggest that slow stretching of a nerve over some years leads to a remarkable increase in length of the nerve without any disturbance in function. Highett and Sanders (1943) however found that nerves kept under a constant tension do not remain permanently elongated. More recently Haftek (1970) studied the effect of both sudden and gradual stretch on rabbit tibial nerve in vitro. Gradually increasing stretch, initially produces rupture of the epineurium and then damage to the myelin sheath with concomitant axonal compression due to pressure by the elongated perineurium, which itself eventually ruptures. Finally complete division of the nerve fibres results.

Rooney and Delaney (1970) however thought it more likely that such a chronic stretching of the left recurrent nerve would be more likely to cause ischaemic damage rather than direct mechanical damage. Ischaemic nerve changes have been the subject of numerous experimental studies as it is well known in man that ischaemic neuropathies due to changes in the vasa nervorum are common and may be seen in arteriosclerosis and thromboangitis obliterans (Richards 1951). While neither of these diseases has been described in the horse, it may be important to remember that age changes have been described in the vasa nervorum of man (Cottrell 1940) and could give rise to ischaemic change.

It has been shown by Roberts (1948) in acute experiments, that the vasa nervorum can be obliterated by stretching, and Lundborg (1973) also showed that the stretching could affect the microcirculation of nerves. He showed that the "upper stretching limit" when all microcirculation ceases, is between 11% and 18% of the nerve's length. If this ischaemia was/

was maintained over long periods, definite damage could be expected despite the axon's remarkable resistance to ischaemia (Lundborg 1970). It should be borne in mind that while nutrient artery supply to nerve is important, the epineurial plexus is so extensive that the rabbit sciatic nerve can survive total exclusion of its nutrient vessels (Adams 1943).

The pathology of ischaemic neuropathy in man has been investigated in detail (Garven et al 1962, Eames and Lange, 1967, Chopra and Hurwitz, 1967). It is apparent from their work that ischaemia preferentially affects the larger diameter nerve fibres and that segmental demyelination and remyelination are the primary changes. Axonal degeneration and regeneration are also seen. As yet however no quantitative studies on equine peripheral nerve have been carried out.

Finally, the most recent suggestion as to the cause of equine laryngeal paralysis was proposed by Loew (1973). He suggested that chronic thiamine deficiency was the cause, basing his argument on the fact that the laryngeal nerves may be involved in human thiamine deficiency (beriberi) and that cattle with thiamine deficiency are said to "roar". Cavanagh (1964) discussed the neuropathy of thiamine deficiency in beriberi and in experimentally produced deficiencies in animals. He found that there was evidence of distal degeneration in the long peripheral nerves with minimal changes in the anterior horn cells themselves, which is suggestive of a "Dying-back" phenomenon.

In a review article on the nutritional neuropathies, Erbslöh and Abel (1970) state that in the polyneuropathy of beriberi, the vagal branches especially the cardiac and recurrent laryngeal nerves are particularly affected. However they also note that facial nerve (a short motor nerve) involvement/

involvement has been described. Older reports of the neuropathology suggest that demyelination may precede axonal degeneration (Erbslöh and Abel 1970).

The possibility of a "Dying-back" phenomenon being involved in the equine laryngeal neuropathy will be discussed later as will the possibility of thiamine deficiency, ischaemia, compression or one of the other possible aetiologies, being a possible or contributing cause will be discussed .

## RESULTS

### a) Gross findings

Laryngeal and other peripheral nerves were only available in three out of the four clinical cases examined. In two of the cases (47988 and 52838) the distal portion of the left nerve was considerably thinner than the right nerve. The only other gross finding was a slight flattening of the left recurrent nerve in one case (40733), as it passed around the aorta.

### b) Routine light microscopical findings

#### i) Clinical cases -

In each of the three cases the changes were similar, varying only in degree. In each case there was a progressive loss in a distal direction of myelinated nerve fibres in the left recurrent nerve with only a few fibres being present at the level of the larynx (fig. 50). In comparison the right recurrent nerve at similar levels was normal (fig. 51). Silver stains for axons showed that few were present. This distal loss of nerve fibres was particularly obvious when the left recurrent nerve at the level of the larynx and the aortic arch were compared (fig. 52), and the difference between both left and right nerves at the same level was equally clear (figs. 50 & 51). It was however also possible to discern a slight drop out of myelinated nerve fibres in the right recurrent nerve of one clinical case (47988).

In close association with this loss of fibres there was an increase in both endoneurial connective tissue and nuclei. As described in the chapter on muscle pathology, this endoneurial fibrosis was a marked feature of the intramuscular nerve bundles. Perineurial fibrosis with marked decrease in the fascicular area was prominent in one case (52838) in the most distal portions of the left nerve. There was little evidence of active axonal degeneration and demyelination was not easily/

easily observed in the routine sections. In no case was there any evidence of changes in the vasa nervorum.

In two cases (52838 and 40733), other peripheral and cranial nerves were examined but no obvious lesions were seen, and there was no obvious 'drop-out' of myelinated fibres. The nerves sampled included the facial, phrenic, vagus, ulnar, tibial and digital nerves (fig. 53)

ii) Sub-clinical cases and adductor paralysis cases -

In general the changes in the left nerve in these cases were similar to those described in the clinical cases but much less severe. Indeed in a few of the least affected larynges, it was difficult to observe any decrease in myelinated fibre population in their nerve supply (fig. 54). In one of the cases with adductor paralysis and in one sub-clinical case however, this was obvious and the drop out of fibres was most marked in the distal reaches of the nerve (figs. 55 & 56). It was apparent in figs. 55 & 56 that the decrease in fibre number was due to the loss of the large myelinated fibres. In the second case of adductor paralysis, examination of the adductor branch of the LRN showed that there was a marked drop out of nerve fibres (fig. 59). Examination of the adductor/abductor branches in one sub-clinical case again showed that the decrease in fibres was due to the loss of the larger diameter fibres, (this being obvious when compared with the same branches on the right side) (figs. 57 & 58).

Occasional thinly myelinated fibres were seen (fig. 60), as were clusters of small fibres, which were also seen in one clinical case (fig. 61). In cases where there was obvious decrease in the laryngeal myelinated fibre population, the fascicles containing sensory fibres from trachea and oesophagus, appeared normal (fig. 62).

There was no evidence of an active lesion in any of the other routine preparations, /

preparations, but an increase in endoneurial connective tissue, was seen (fig. 56). All other peripheral nerves which were examined, were normal.

### iii) Renaut bodies -

Description of these was separated from the previous results, as interest in these structures and their significance has increased in the last few years.

Renaut bodies were common findings in both the right and left recurrent laryngeal nerves of both clinical and sub-clinical cases (Figs. 51 & 56). They were found to lie beneath the perineurium and were acellular, whorled structures. They were found at all levels of the laryngeal nerves examined. However, they were not a prominent feature of any of the other peripheral nerves examined.

### c) Teased fibre results.

The results for both the clinical and sub-clinical cases will be described together as the findings were similar.

The main abnormalities seen in the single fibre preparations were intercalated segments, and segmental demyelination. Intercalated segments were frequent findings in both left and right nerves. These short segments were often at varied stages of development, from the very earliest stages of remyelination, where Schmidt-Lanterman incisures were prominent, to later stages where thinly remyelinated short segments were seen (fig. 63). Paranodal demyelination was frequently noted and occasional areas of paranodal or segmental demyelination, where the old myelin sheath had not been completely digested were seen. Segmental demyelination however was not a common finding. Many intercalated segments could be found in the same fibre nodes (fig. 64) and up to six of these were seen in one fibre at consecutive nodes. Occasionally two short intercalated segments were found/

found adjacent, each being short but the same length. Little evidence was seen of axonal degeneration (fig. 63).

With material fixed in formalin/post fixed in osmic acid, artefactual staining was commonly seen, with "dotting" of the myelin sheath. Paranodal oedema was also seen infrequently and was regarded as non-significant.

When a number of successive internodes were photographed and arranged in order, it was apparent in many that correlation between internode length/diameter in the normal nerve (fig. 65 ) was lost. A number of these have been included to illustrate such changes. There was a marked variation of internode length and diameter in some teased fibres, some internodes of which had fully remyelinated and some of which had short remyelinating segments with other demyelinated nodes (fig. 66) Some fibres were seen which had obviously completely remyelinated as there was variation in internode length but internode diameter was uniform (fig. 67). Occasional fibres were seen in which part of the myelin sheath at the node was still present after the remainder of the node had disappeared (fig. 68). Figure 69 illustrates a bizarre fibre in which there was variation in internode length and diameter, with paranodal demyelination and remyelination. Very occasional regenerating fibres, that is those with consecutively short internodes (for that diameter of fibre) were seen (fig. 70). Finally one interesting finding was noted in the most recent clinical case (40733). Fibres teased from the left nerve 50 cms from the larynx, showed evidence of tapering and swelling of the opposite end of internodes. All of these swollen/tapered internodes were found to be polarised in the same direction although no proximal/distal markers had been placed on this nerve before processing. The large and medium sized diameter/



diameter fibres were principally affected. The tapering of one end of the internode was perhaps more obvious than a swelling of the other end, but occasional fibres were seen in which several internodes were affected (fig. 71).

In the other peripheral nerves examined, the range of changes were much as described for the laryngeal nerves but were less frequently seen. Ageing, however, possibly had an affect on both the laryngeal and other peripheral nerves examined and this will be discussed later.

Scatter graphs and single fibre graphs were useful in illustrating many of the changes described above. In the laryngeal nerves of the sub-clinical cases it can be seen in the single fibre graphs that there is a wide variation of internode length indicating segmental demyelination and remyelination (figs. 72,73,74). Intercalated segments can be recognised on these graphs as points on large diameter fibres with short internode length (e.g. 54788 RRN distal). Both right and left recurrent nerves of the sub-clinical and clinical cases reflected these changes, which were seen mainly in the larger and medium sized fibres, although they could also be noted in the smaller fibres.

The scatter graphs of these cases showed much the same features i.e. wide scatter of points from the regression line, but flattening of the regression line was not obvious. This was probably due to the fact that many of the short intercalated segments had not completely remyelinated and had not regained their original diameter. This led on occasions to a clustering of points in the area containing short, thin internodes (fig. 72).

Graphs were plotted of other peripheral nerves in one sub-clinical case and one case of adductor paralysis. In the first case (57510), there was some/

some scatter of points on the scatter graph and two medium/small diameter fibres have variation in internode length. In the second case (57769) there was possibly a greater variation in internode length in the ulnar nerve than in the previous case, and the digital nerve in this case showed a greater scatter than the ulnar nerve of the same case (fig. 74).

As the right recurrent laryngeal nerve could not be used as a control, two ponies in which the laryngeal muscles and their nerve supply (on transverse section) were normal, were used as comparisons, one aged 5 years (53273) and the other 25 years (55028). The latter was selected to test any variation that might occur with ageing. The former case (53273) was almost entirely normal, but one abnormal fibre can be seen in the single fibre graph (fig. 75). No more nerves were teased in this case as it was thought that the left recurrent nerve was a sufficient control. In the older pony (55028), however, many more abnormalities were seen, and in this case both laryngeal nerves and the ulnar and tibial nerves were measured (fig. 76). In the left recurrent nerve there is evidence of a wide scatter of points on the scatter graph. This was due to demyelination and remyelination, but unlike many of the sub-clinical cases some of the short internodes had fully remyelinated. On the single fibre graph, as well as variation of internode length, two fibres with almost uniformly short internodes were seen (fig. 75). These were thought to be regenerating fibres. In the right recurrent nerve and the ulnar and tibial nerves there was variation and scatter of points, with noticeable flattening of the regression lines in the latter two cases (fig. 75). The regression coefficient ( $b$ ) which indicates the slope of the line in the scatter graphs also indicated this flattening (table 12).

In order to compare the incidence of changes found in the teased nerve fibres in different nerves and at different levels of the laryngeal nerves, the combined number of demyelinated/remyelinated and normal fibres examined/

examined (table 13) were compared using a  $\chi^2$  ( $X^2$ ) test. The significant results are noted below.

- i) In cases 57769 and 54788 the incidence of demyelination/remyelination was significantly greater in LRN distal than RRN distal ( $p < 0.001$ ).
- ii) In both these cases the incidence of demyelination/remyelination was significantly greater in the LRN distal than RRN distal of the normal pony, 53273 ( $p < 0.001$ ).
- iii) In the one clinical case (47988) the incidence of demyelination/remyelination was significantly greater in the LRN proximal than in the RRN proximal ( $p < 0.001$ ).
- iv) In two cases (57510 and 57769) the incidence of demyelination/remyelination was significantly greater in the LRN distal than in the ulnar nerve ( $p < 0.001$  and  $p < 0.02$  respectively).

d) Myelinated nerve fibre population.

i) Clinical cases -

In three of these cases the total myelinated nerve fibre population was counted at three levels of the nerve. The results are illustrated in figure 77. It can be seen that there is a progressive reduction in the number of nerve fibres in a distal direction. In two of these cases fibre density was also calculated and was also found to decrease markedly towards the larynx (fig. 78).

ii) Sub-clinical cases -

Total fibre counts were done in two cases, in one comparing left and right recurrent nerves and in the other, comparing left and right abductor/adductor branches of the recurrent nerve (table 14). In this latter case the fibre diameter spectra were also measured (fig. 57 & 58). In order to compare the effect of ageing on fibre diameter, the left abductor/adductor branches from/

from one aged pony (20 years) were measured and compared with the same branches on the right side of a 15 year old pony (figs. 79 & 80). In both cases the muscles supplied by these nerves were normal. The contralateral abductor/adductor branches in each of these cases were not available.

In the first case (57510) there is a noticeable decrease in total number of fibres in left nerve compared with the right, and also in fibre density (table 14). In the second case there is an obvious decrease in fibres in both the adductor and abductor branches (table 14) and a marked loss of the larger diameter fibres on the left side (figs. 57 & 58). In the two normal ponies, the older pony (fig. 79) had fewer larger diameter fibres than the slightly younger animal (fig. 80).

e) Terminal innervation

Extrafusal motor end-plates in the laryngeal muscles varied from simple "en plaque" type endings (fig. 81 a,b,c) to extremely complex endings, one of which was seen to contain four separate endings all branching within the same area (fig. 81d). Many different types of ending however were seen in normal muscle (figs. 82, 83). The variety of types of end-plates that could be found together is illustrated in fig. 84. The sub-terminal axon in the centre has split to supply two separate end-plates (fig. 84). In fact many sub-terminal nerve fibres were noted to have split to supply separate end-plates (double end-plates). These end-plates were either simple or "en plaque" in structure (fig. 85 a,b) or resembled a tree which was bearing fruit (fig. 85 c). This degree of division of the sub-terminal fibre varied from the fibre which had split, but in which the divisions were still running to a single motor-end plate (fig 85 a,b), to cases where there was a strong possibility that each end plate was separate and was supplying a different muscle fibre (fig. 84). Confirmation/

Confirmation of this was sometimes difficult as the muscle fibre was blurred due to superimposition. End-plate morphology often became complex and bizzare in the sub-clinical cases (fig. 86).

Collateral sprouting was a marked feature in some of the sub-clinical cases, in one case six end-plates were seen to arise from one sub-terminal nerve fibre which had already branched (fig. 87 a). Figure 87 b illustrates the often apparent random innervation that was commonly seen. In one sub-clinical case, one sub-terminal had given rise to three separate end-plates two of which were extremely complex and had split to form double end-plates (fig. 88). Ultra-terminal sprouts were also occasionally seen (fig. 86). Correlation between collateral sprouting and muscle histological fibre type grouping was noted in one clinical case in which there was evidence of both of these processes on the right side of the larynx (fig. 89).

### DISCUSSION

As in most peripheral neuropathies little information can be gained from routine histological techniques and stains of affected nerves. It is necessary to resort to special staining techniques, single fibre and quantitative studies in order to elucidate the nature of the disease. Electron microscopy (EM) may finally be necessary to produce a complete understanding of the disease process. In this study all of these techniques with the exception of EM have been applied to the recurrent laryngeal and other peripheral nerves.

Routine histology showed that there was a loss of fibres in distal part of the left recurrent nerve in both clinical and sub-clinical cases, which differed in severity, the clinical cases having fewer intact fibres. Stains for myelinated fibres also demonstrated this loss in the right nerve in one clinical and one sub-clinical case. As well as demonstrating this loss, the routine histological techniques were able to demonstrate a resultant endoneurial fibrosis which occurred *pari passu* with a decrease in nerve fibres, with a marked increase in Schwann cell nuclei.

Apart from the obvious loss of fibres and endoneurial fibrosis the other main histological finding was the presence of Renaut bodies in the laryngeal nerves. These were described in the introduction and it was pointed out that they are found in association with chronic compression and are more highly developed in species such as the horse than in man (Asbury 1973). However, they are thought to be non-specific and may in fact be found in some normal nerves. Their incidence in the laryngeal nerves is extremely high and the question must be raised as to whether or not they have any significance in these nerves. They are found in both the left and right recurrent nerves of clinical and sub-clinical cases/

cases (figs. 51 & 54) and when found on the right, were often in association with otherwise normal nerves. Other peripheral nerves examined contained few or no Renault bodies. Their finding at all levels of both recurrent nerves might exclude the possibility of their association with compression, as such a lesion has only been suggested to arise as the left recurrent nerve passes around the aorta. In human ulnar nerve compression many Renault bodies can be found at the site of entrapment (Neary 1975, personal communication), and therefore it might be expected to find them in the left nerve at the area of the aortic arch. In only two or three cases has the nerve been available for study at this level and Renault bodies were not an outstanding feature, although Mason (1973) described them as occurring in the nerve in cases in which there was an anomalous course of the left nerve with consequent compression. The significance of Renault bodies in the laryngeal neuropathy remains uncertain but it may be that they play a protective cushioning role in nerves subjected to chronic trauma. In view of their apparent greater frequency of occurrence in the laryngeal nerves compared with other peripheral nerves examined a quantitative study of the incidence of such findings is indicated.

It is obvious both from the present qualitative and quantitative studies on the left RLN of three of the clinical cases that there is a loss of nerve fibres which is progressively more severe in a distal direction. As the recurrent laryngeal nerve is a non-branching motor nerve (as discussed in section 1), the hypothesis of a "Dying-back" phenomenon being involved in the disease process would seem to be proven. In two toxic neuropathies, (acrylamide and TOCP intoxication in the baboon) which are thought to be classical experimental models of "Dying-back", the finding of a distal loss of myelinated fibres in/

in the left recurrent laryngeal nerve was thought to be of prime significance (Bradley 1974).

It is not known from the quantitative data whether or not there is an equal loss of both sensory fibres to the laryngeal muscle receptors and motor fibres to extrafusal fibres. Equally, the effect of the disease on non-myelinated fibres is not known. However, it is felt that the quantitative studies on total number of myelinated nerve fibres have shown a significant difference in the numbers of myelinated fibres at different levels of the laryngeal nerves of the clinical cases. Despite the apparently significant number of demyelinated segments as seen in the teased fibre preparations, silver stains showed that there was an axonal loss as well as the apparent loss of myelinated fibres as seen in the Kultschitzky stained sections. EM counts of myelinated and unmyelinated fibres would be conclusive but time-consuming, and has not been included. The sub-clinical cases in which total fibre counts and densities were performed, also showed a decrease in myelinated nerve fibres. In one case there was a decrease in both myelinated nerve fibres and fibre density (table 14, case No. 51510) in left nerve near the larynx, and in the other case (table 14, case No. 59190) there was a decrease in total number of fibres in the left abductor/adductor branches, compared with the right nerve at similar levels.

Fibre density also showed a progressive decrease distally in the LRN of the clinical cases, even where extensive perineurial fibrosis and fascicular shrinkage might have produced normal fibre densities (Stevens et al 1973). Mean fascicular area might decrease with age and so invalidate such methods of quantitation in older cases. However, it is known that this does not occur (Swallow 1966, O'Sullivan and Swallow 1968) and/



and so fibre density might be a useful index of disease. However Swallow (1966), in a study on the effect of age of the nerve fibre population of the anterior tibial nerve, showed that there was a greater significant correlation between age and total fibre count than between age and fibre density. He therefore suggested that in nerves which had a small number of fibres, no advantage would be gained from measuring fibre density. Indeed this is probably the case in the recurrent laryngeal nerve where the total number of fibres is small (600-900) and the exact fascicular area can be difficult to judge due to Renaut body formation.

Quantitative studies raise another important consideration, namely, is one particular diameter range of fibres affected in this disease? As mentioned in the introduction, a number of diseases preferentially affect large or small diameter fibres, although there is predominance of those affecting the former. From the qualitative histological studies of the sub-clinical cases it was apparent that there was a decrease in the larger diameter fibres (fig. 56). Early quantitative studies on the abductor/adductor branches of the left and right recurrent nerves (figs. 57 & 58) have also shown this to be the case.

In addition there may in fact be a loss of larger diameter fibres with age (see figs. 79 & 80). Figure 79 is the fibre spectra of the left adductor/abductor branch of a normal pony of over 20 years, and figure 80, gives similar results from the right side of a sub-clinical 15 year old pony. Such comparisons are possibly unjustified as little normal data is available on the difference between right and left nerves at this point.

Swallow (1966) showed that there was a loss of larger diameter fibres with ageing in man and this may be worth examining in the older horse.

However/

However, the results of similar quantitative studies in these previous cases (figs. 79 & 80) when compared with fibre diameter spectra of a young normal pony (fig. 11) are at variance, in particular with regard to the almost total lack of fibres over 15  $\mu$  in the normal case. Such conflicting evidence will need clarification, but it is felt that when the diameter spectra in left and right nerves at identical positions are compared, then any obvious differences, such as shown in figures 57 & 58 is significant.

Any apparent increase in the percentage of smaller diameter fibres may of course be relative, but could also be due to the presence of small regenerating fibres. Such fibres are often found in clusters, which are found in neuropathies in which axonal degeneration and regeneration occur (Thomas 1968). However, clusters of regenerating fibres were not often seen on histological examination and only occasional examples were noted (fig. 61). Due to the predominantly medium to large diameter fibre composition of most of the fascicles of the recurrent nerves, such clusters should be obvious if regeneration was occurring. It would therefore seem that in the cases examined so far, there was a relative and not absolute increase in small diameter fibres.

The findings from the motor end point studies are difficult to comment on, due to the difficulty in defining the norm. It is well known that laryngeal extrafusal motor end plates of man and other species, are complex in nature (Rossi and Cortesina 1965, Feindel et al 1952, Doyle 1972, personal communication). It was not the purpose of this study to define or describe in detail the morphology of laryngeal motor end plates but to look for abnormalities such as collateral sprouting or distal degenerative which might be associated with a "Dying-back" phenomenon. However, a considerable amount/

amount of material was gathered and it may be worth comment and comparison with previous work. Rossi and Cortesina (1965) showed that laryngeal muscle fibres in man could have up to five motor end-plates per fibre, and they called such structures, multi-motor end-plates. Many fibres had double end-plates as have been described in the rabbit's cricothyroid muscle (Feindel et al 1952). These double end-plates however were always seen to be supplied by a common axon. Feindel et al also described similar findings in the rabbit extraocular muscles, and using the methylene blue technique a large range of types of double end-plates have been found in the equine laryngeal muscle. These vary from division of sub-terminal nerve fibre with the simple terminal expansions supplying one muscle fibre (fig. 85), to distant splitting of the sub-terminal fibre with complex expansions which may be supplying more than one muscle fibre (fig. 84). Many of the end-plates are extremely complex in nature and it seems that the larger they become, the more likely they are to be split into separate end-plates (fig. 88). These morphological complexities may change the physiological characteristics of the muscle fibres so innervated.

The morphology of individual equine laryngeal motor end-plates is complex and their normality or abnormality unknown. The morphology of developing motor end-plates and their physiological role has been discussed by Nystrom (1968). He also discussed the division of motor end-plates into the "en grappe" and "en plaque" type. Woolf and Till (1955) describe in the cat, the "en grappe" endings as being "glomerular" like and the "en plaque" endings as being "cherry-like".

In the horse laryngeal muscle it appears that some end-plates fall into the category of "en grappe" or "en plaque" but that many are either simpler/

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simpler in nature or much more complex. Woolf and Till (1955) note that in the rabbit, terminations which resembled a tree, bearing fruit, could be seen and many of the equine laryngeal end-plates could be similarly described. Woolf and Till also note that in the rat, terminal expansions could be seen which had a plexiform arrangement. There are obviously marked species differences in the morphology of the motor end-plates.

The corresponding size of motor end-plate with the muscle fibre it innervates has been the subject of much controversy (Swatland and Cassens 1972). It has been shown by a number of authors that end-plate diameters were larger in red parts of a muscle (mainly oxidative fibres) whereas mean muscle fibre diameter tends to be larger in the white parts of muscle - mainly anaerobic fibres, see Swatland and Cassens (1972). These authors, in an investigation of the peripheral innervation of stress susceptible pigs, showed that end-plates were proportional in size to the innervated muscle fibre regardless of histochemical differences. They also found a large number of double end-plates and suggested that these might be directly due to hypertrophy of the muscle fibre. It was apparent that single end-plates in the pig could be large and complex and show early evidence of axonal bifurcation, a process which ultimately led to the formation of a double end-plate, each of which tended to be smaller than the previous single ending. In a later study on an inherited bovine condition known as double muscleing, Swatland (1973) showed that hypertrophy of muscle fibres was not the complete explanation of the increase in size of the motor end-plates or of their complexity. He showed that full sized motor-end plates could be seen on immature muscle fibres but he suggested that the large size of the end-plate may be due to/

to the rate of growth (or hypertrophy) rather than hypertrophy alone.

In a study on the terminal innervation in dystrophica myotonica (Allen et al 1969), a disease in which muscle hypertrophy as well as atrophy is found, there was a high incidence of double end-plates and an increase in end-plate diameter. The equine laryngeal end-plates demonstrated in this study could therefore be showing a similar response, in that muscle fibre hypertrophy is a common sequel to atrophy.

No attempt was made to quantitate the innervation ratios (Coërs and Woolf 1959) of the laryngeal muscles, due to the enormous complexity of the terminal innervation. However it was felt that there were sufficient abnormalities to be significant. It is well known that axonal sprouting follows denervation (Hoffman 1950, Edds, 1950) and that this collateral sprouting results in reinnervation and histochemical fibre type grouping (Morris 1969). In the equine laryngeal muscles, collateral sprouting was more frequent in muscles which showed evidence of fibre type grouping (fig. 87). Collateral sprouting is of course an earlier index of reinnervation than fibre type grouping as they occur in that sequence.

In many respects the finding of little or no change in the brains of the three cases examined, was not surprising. To date, there have been conflicting reports on whether or not the nucleus ambiguus was affected (see discussion). Changes which one might expect to see, such as chromatolysis or neuronal drop out with gliosis were not found. If one was to assume that a "Dying-back" process was involved then it is not surprising that no changes are seen as Cavanagh (1964) pointed out that changes in the central nervous system in experimental "Dying-back" diseases were often temporary or absent. In experimental thiamine deficiency for example, /

example, the nerve cell bodies in both spinal cord and dorsal ganglia were either normal or showed only chromatolytic changes.

The teased nerve fibre findings were useful in elucidating some of the changes that are found to affect individual fibres. In general, nearly all the laryngeal nerves, both from the left and right sides were found to be abnormal, with one exception, the young control pony (53273) which had little evidence of abnormality. In both the sub-clinical and clinical cases, demyelination and remyelination were prominent features with little evidence of axonal degeneration.

While Renault (1881) originally described the presence of intercalated internodes in the sciatic and median nerves of horses it is not known how frequent these were. In the laryngeal nerves of the current study, paranodal demyelination with subsequent remyelination was a common feature (see table 13), with many fibres being found in which up to six successive nodes were affected. A large proportion of the fibres of both medium and large diameter, and occasional smaller size were affected. Two intercalated segments, each of the same length were occasionally found adjacently, and infrequently demyelination was seen to spread further than the paranodal area. More commonly, however fibres at all stages of remyelination and some with demyelination as well were found. These gave rise to the characteristic variation of internode length as seen on the individual fibre graphs.

It was thought that some of the internodes in apparently healthy fibres were longer than normal for that particular diameter of fibre. It has been suggested by Vizoso (1950) that internode length is proportional to the length a nerve has to grow from the time of myelination. He compared the ulnar, tibial and facial nerves in an 18 year old girl, assuming/

assuming that the facial nerve had a much shorter length to achieve than the other limb nerves. He found that the large diameter fibres in the facial nerve had shorter internodes than comparable fibres in the limb nerves, but that small diameter fibres had a similar length. Stevens et al (1973) however disagreed with this concept, stating that they had found adult values of internode length before cessation of growth. This present study does not resolve this point, but peripheral nerves of the horse possibly provide a possible means of testing this hypothesis. The recurrent laryngeal nerve is longer than most limb nerves and therefore it might be expected that internode length would be correspondingly longer. Internode length is reflected by the slope of the line on the scatter graph, which is expressed mathematically in the regression coefficient (b). In the two sub-clinical cases where the ulnar nerve and left RLN were measured it can be seen that the slope is greater in the latter in both cases. No firm conclusions can be drawn from this however as neither case was normal, but it would seem an obvious and simple exercise, to compare the facial, ulnar and left RLN of a normal pony.

The single fibre graphs while illustrating the wide variation in internode length with very short intercalated segments, also showed that excessively long segments could be seen occasionally (fig.72, 54788 right RLN). Vizoso (1950) also found evidence of these long segments in older human subjects. He suggested that this was due to a neighbouring Schwann cell extending its territory following local or paranodal demyelination. This wide variation of internode length of a single fibre is taken to be positive evidence of segmental demyelination and remyelination. The intercalated segments perhaps however exaggerate this difference, but their affect on the scatter graphs of the sub-clinical/

clinical cases was not as might have been expected. It has been shown by numerous investigators that such segments cause a reduction in the slope of the regression line. However, in the laryngeal nerves most of the intercalated segments were at a very early stage of remyelination and hence were still of thin diameter. These segments in fact in some cases, caused a clustering of points in the lower left hand corner of the scatter graphs (fig. 72, 54788 LRN distal).

However, the single fibre graphs from the laryngeal nerves did not show one feature which might have been expected, that is regenerating fibres, and indeed few or none of these were found in the laryngeal nerves.

Before going on to consider whether or not demyelination or degeneration are the primary changes in the laryngeal nerves it is advisable to consider two other points first, a) are other peripheral nerves similarly affected in the horse?, and b) does age have an effect on the laryngeal nerves and other peripheral nerves? In one case with adductor paralysis, one sub-clinical case and one normal old pony, such comparisons were possible. In the former two cases (figs. 74 & 76) it can be seen that there was evidence of variation of internode length of some fibres of limb nerves, but they were considerably less abnormal than their laryngeal counterparts.

The possibility of an ageing factor is interesting. If the two normal ponies are compared (i.e. 53273 - 5 years and 55028 - 20+ years) a marked difference is noted between both single fibre and scatter graphs (figs. 75 & 76). While there was still one abnormal fibre in the left RLN of the young pony, the older pony showed much more evidence of abnormality in all the nerves examined. These changes were similar to those/



those described by Lascelles and Thomas (1966) in age related changes in man. In the left RLN of 55028 as well as evidence of segmental demyelination, fibres with regularly short internodes of large diameter were seen indicating probable regeneration. Intercalated segments were found in this nerve, and the right RLN, tibial and ulnar nerves, but in comparison to previous findings in the sub-clinical cases, these segments had completely remyelinated and had achieved their normal diameter. This led to the flattening of the slope that was noted in all scatter graphs of 55028, in direct contrast to the sub-clinical cases. As has already been noted in the latter, intercalated segments were nearly always thin, and led to a clustering of points in the lower left hand corner of the scatter graph. Direct comparison of the regression coefficients of the nerves measured in 55028 with the control pony (53273) and other nerves demonstrates the flattening of the slopes in the former.

As Bradley (1974) explained, single fibre preparations tend to exaggerate the incidence of demyelination compared to degeneration. Despite this fact, the incidence of demyelination in the laryngeal nerves of both the clinical and sub-clinical cases leads one to consider where the primary lesion lies i.e. is the Schwann cell or the axon affected first, and if the primary lesion is in the axon, is the demyelination which is seen, secondary to axonal degeneration? The incidence of demyelination and remyelination in a number of cases has been tabulated and the results statistically compared (see results). In summary it was noted that the degree of demyelination/remyelination was significantly greater in the left RLN than the right RLN in the sub-clinical and normal cases (no muscle pathology) and also greater than in the other peripheral nerves. It was also noted that this incidence was greater in/

in the more proximal portions of the left RLN compared with the right RLN in one clinical case. Are these therefore the primary changes or are they secondary to axonal degeneration?

Bradley and Jennekens (1971) discuss informatively the mixed nature of many neuropathies but demonstrate that axonal degeneration can occur in the primary demyelinating, diphtheritic neuropathy. In contrast they note that segmental demyelination can be seen in conditions such as Friedreich's ataxia and motor neuron disease where the condition is thought to be entirely due to axonal degeneration. It has been statistically demonstrated by Dyck et al (1971) that the segmental demyelination found in uraemic neuropathy, is secondary to axonal degeneration. This was suspected by them, as they noted that the demyelination seen was not random, but was found in individual fibres which had many consecutive affected nodes. This was previously described by Jennekens et al (1969). By counting the number of consecutive normal nodes in 100 fibres arranged in order they were able to show that the demyelination was not random but was affecting fibres which were undergoing degeneration; such fibres were later shown to have axonal atrophy. It therefore remains to perform such an exercise on the equine laryngeal nerves to determine where the primary change lies.

In the light of this discussion on the changes seen in the laryngeal nerves it might be possible to suggest or exclude some of the possible suggestions as to the cause of the neuropathy.

If a "Dying-back" phenomenon is involved in the pathogenesis of the neuropathy then what are the implications? As mentioned in the discussion, the left recurrent nerve has been used to illustrate evidence for Dying-back in two toxic neuropathies, acrylamide (Hopkins 1970) and TOCP/

TOCP (Hern 1971). Although they found that the right recurrent nerve was normal near to the larynx in contrast to the left, they provided no explanation for this. One possible explanation may be the difference in length of the two nerves. This could explain the asymmetry of the lesion in the horse where the left nerve can be as much as 20-25 cms longer than the right (see section 1 ). In fact the enormous length of both equine recurrent nerves, especially the left nerve compared with other long limb nerves, (table 5 ) might be a major factor in the causation of the disease. The preferential susceptibility of large diameter, long nerve fibres are the definitive features of a "Dying-back" disease. It could be asked if a "toxin" is affecting the nerve cell bodies of the left nerve, then why is the anterior laryngeal nerve and hence the cricothyroid muscle not affected?. If this disease is simply a case of the "Dying-back" of the longest neurones, then the anterior laryngeal nerve, being shorter in length, would probably remain unaffected. Length however may not completely account for the apparent preferential susceptibility of the left recurrent nerve, despite the apparent difference in length between left and right. This difference may not be great enough to account for the infrequent and minimal pathology seen in the right recurrent nerve. If length were totally responsible then it could be hypothesised that a normal number of fibres should be found in the left nerve 20-30 cms from the larynx (i.e. this is the extra distance the left nerve has to travel compared with the right). If the first two clinical cases quantitated (47988 and 52838) are compared then this is not the case (fig. 77). In these two cases, especially 47988, a 'drop-out' of fibres was also noted in the distal portion of the right nerve. In other words quantitation of both left and right nerves in the clinical cases/

cases at accurately known distances from the larynx may solve this point.

It is still possible that chronic thiamine deficiency as suggested by Loew (1973), could be responsible for the laryngeal neuropathy. It was noted in the introduction that thiamine deficiency is responsible for a "Dying-back" neuropathy in which there has been laryngeal involvement (Cavanagh 1964). Too little detail is known about the pathology of the naturally occurring polyneuropathy in beriberi. to make accurate comparison, but on current evidence the similarity between it, and the equine laryngeal neuropathy, cannot be overlooked. Evidence against thiamine deficiency, may be the finding of the disease in one very young horse (7 months) and the fact that there was never any evidence of generalised neuropathy which may have been expected in some thiamine deficient cases. Coërs and Woolf (1959) also stated that collateral sprouting of terminal intramuscular nerves was not as prominent a feature in nutritional neuropathies compared with other peripheral nerve diseases. This is not the case in the equine larynx where evidence of reinnervation from both the methylene blue preparations and the muscle enzyme histochemistry was obvious.

Are there therefore any other outstanding features of the nerve pathology which might point towards the causative factor? In both the neuropathies where axonal degeneration or segmental demyelination are prominent features, efforts at repair, or further episodes of the disease can lead to characteristic pathological features. If axonal degeneration and regeneration are continuous, then it is common to find clusters of small myelinated and unmyelinated nerve fibres as evidence of regeneration, in affected nerves (Thomas 1968). Other evidence of regeneration can be found in teased nerve fibres as mentioned earlier, but in none of the teased/

teased laryngeal nerves examined were regenerating fibres a noticeable feature, and in the transverse sections only a few clusters were seen. Many human and experimental axonal neuropathies or mixed neuropathies show evidence of regeneration including the neuropathy of chronic occlusive vascular disease (Garven et al 1962). This specific example is mentioned as a suggestion has been made that chronic ischaemia is the cause of the laryngeal neuropathy (Rooney and Delaney 1970). Electron microscopy however would be needed to confirm the presence or absence of regenerating fibres, but they are certainly not an obvious feature in this disease.

Electron microscopy would also be of value in interpreting the role of segmental demyelination and its significance. It is well known that repeated demyelination and remyelination results in the formation of 'onion-bulbs', structures which consist of central axons with concentric Schwann cell processes proliferating around a central core (Bradley 1974). The role of the Schwann cell in this process is clear (Webster et al 1967), these structures not being found in conditions where axonal degeneration and regeneration occur. Probably the best known human clinical neuropathy where 'onion-bulbs' are found is hypertrophic neuropathy. If therefore, demyelination and remyelination is occurring frequently in the laryngeal nerves it would seem likely that these structures would be seen. Like regeneration clusters they are not apparent, but E.M. examination would be necessary to confirm this. Repair following demyelination does however occur as the evidence of the teased fibres have shown, but it may be that the process is simply not repetitive enough to give rise to 'onion-bulbs'.

It may be that the frequent occurrence of paranodal demyelination and/

and subsequent formation of intercalated internodes is of significance. It has been suggested that the metabolic requirements of the Schwann cell cytoplasm are extremely high in the paranodal area, judging by the increased numbers of mitochondria found in that area (Williams and Langdon 1963). Loss of myelin in these areas is known to occur both in early segmental demyelination and in cases in which the axon is primarily affected. Indeed, paranodal demyelination with resultant intercalation has been seen in teased fibres proximal to regeneration in both acrylamide and TOCP poisoning (Hopkins 1970 and Hern 1971). The finding of so many intercalated internodes in the laryngeal nerves with many of them on the same fibre, may indicate that demyelination is not random and is affecting only fibres which are undergoing degeneration (Dyck et al 1971).

The last morphological abnormality which may have specific significance in the causation of the disease, is the recent finding of tapered and swollen fibres in the left recurrent nerve of a clinical case. There is no doubt that such a finding will require further investigation and that its significance may be in doubt as the lesions were found a long distance (almost 50 cms) from the site of possible compression. In the case of ulnar nerve compression in man affected fibres were only found within a few centimeters of the entrapment site (Neary and Eames 1975), but the chronicity of the disease in the horse may mean that the mechanical effects produced, result in myelin loop slippage a long way from the compression. It is possible that compression is increasing the affect of some other neuropathy for example a "Dying-back" phenomenon affecting the left recurrent nerve due to its length, in a similar way to the examples quoted in the introduction. While doubt may/

may remain about the significance of such an isolated finding, the fact that some fibres (mainly medium and large diameter fibres) were tapered at one end with swollen, bulbous ends at the other, and that all these are polarised in the one direction, (Ochoa 1975, personal communication), requires explanation.

In summary therefore the changes found in the equine laryngeal nerves, other nerves, and brains examined in this study and their possible significance are -

- 1) there is a progressive distal loss of myelinated nerve fibres in the left recurrent laryngeal nerve of clinical cases, and to a much lesser extent in the sub-clinical cases and the right recurrent nerve in a few cases. Large diameter fibres may be affected preferentially.
- 2) such findings may indicate the presence of a "Dying-back" phenomenon.
- 3) it would appear unlikely that the laryngeal neuropathy is part of a more generalised peripheral neuropathy.
- 4) paranodal and segmental demyelination, with remyelination and intercalation are commonly found, but are more frequent in the left recurrent nerve than in the right recurrent nerve or other peripheral nerves. Such findings are not necessarily related to ageing and may well be secondary to axonal degeneration.
- 5) no significant findings were found in the nucleus ambiguus of three clinical cases.

Case No.	Nerve	n	b	r
54788	LRN dis	157	138.3	0.9
"	RRN dis	116	112.9	0.85
57769	LRN dis	98	122.0	0.83
"	RRN dis	87	159.2	0.9
"	ulnar	79	118.9	0.9
"	digital	92	90.0	0.79
57510	LRN dis	129	131.1	0.7
"	ulnar	119	112.0	0.9
36820	LRN dis	197	93.0	0.7
"	RRN dis	50	107.2	0.91
47988	RRN dis	138	115.9	0.7
53273	LRN dis	129	121.5	0.9
55028	LRN dis	88	73.0	0.63
"	RRN dis	41	95.4	0.88
"	ulnar	122	74.1	0.9
"	tibial	138	59.8	0.91

TABLE 12 Number of internodes examined, regression coefficient (b) and correlation coefficient (r).



	<u>Normal</u>	<u>Demyelinated</u>	<u>Remyelinated</u>	<u>Status</u>
<u>53273</u>				
LRN dis	60	2	3	normal
<u>55028</u>				
LRN dis	21	0	7	"
RRN dis	10	0	3	
<u>57769</u>				
LRN dis	29	6	16	
RRN dis	38	0	4	
Ulnar n.	23	0	10	sub-clinical
Digital n.	30	2	4	
<u>54788</u>				
LRN dis	66	20	38	"
RRN dis	38	4	9	"
<u>57510</u>				
LRN dis	43	8	23	"
RRN dis	40	2	1	
Ulnar n.	36	1	3	
<u>47988</u>				
LRN prox.	20	14	25	clinical
RRN prox.	28	4	9	

TABLE 13 The number of normal fibres and those with evidence of demyelinated or remyelinated segments in normal,

sub-clinical and clinical cases.

LRN = left recurrent laryngeal nerve

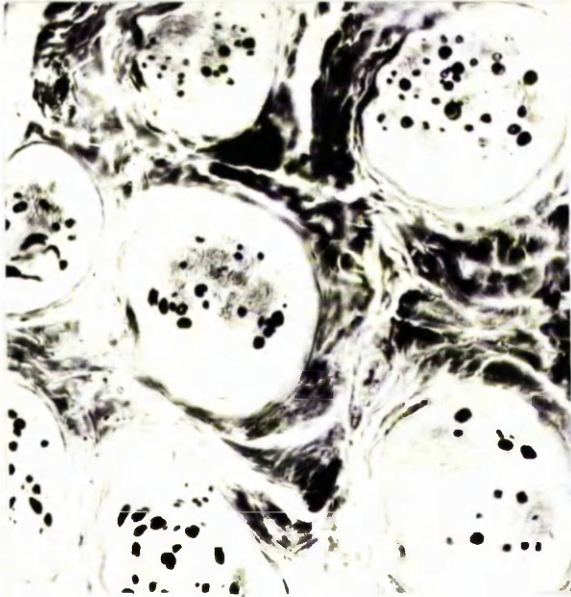
RRN = right recurrent laryngeal nerve

<u>CASE NO</u>	<u>NERVE</u>	<u>TOTAL NO. OF FIBRES</u>	<u>FIBRE DENSITY/mm<sup>2</sup></u>
59190	Left RLN - adductor branch	238	-
	Left RLN - abductor branch	163	-
	Right RLN - adductor branch	375	-
	Right RLN - abductor branch	377	-
57510	Left RLN - distal	461	2,500
	Right RLN - distal	601	3,300

TABLE 14

Fig. (50): Sections of the left R.L.N. of a clinical case (40733) at 4 levels, (a) 10 cms from larynx, (b) 20 cms, (c) 30 cms and (d) 40 cms. Note the progressive distal loss of myelinated fibres. Compare with fig. 51.

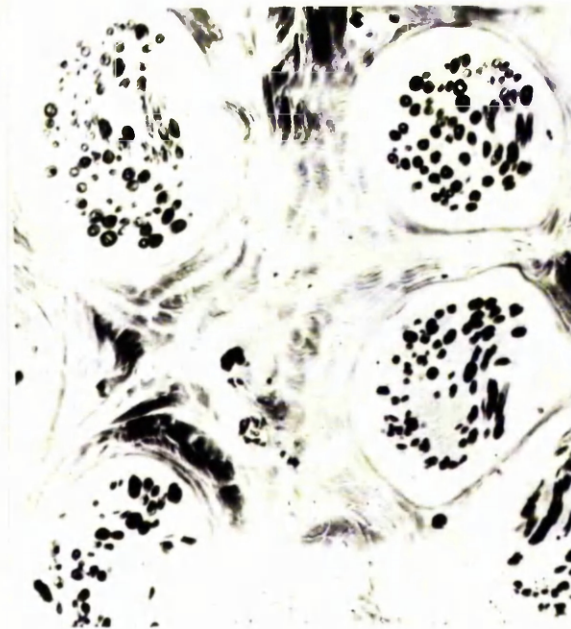
(Kult. X 120)



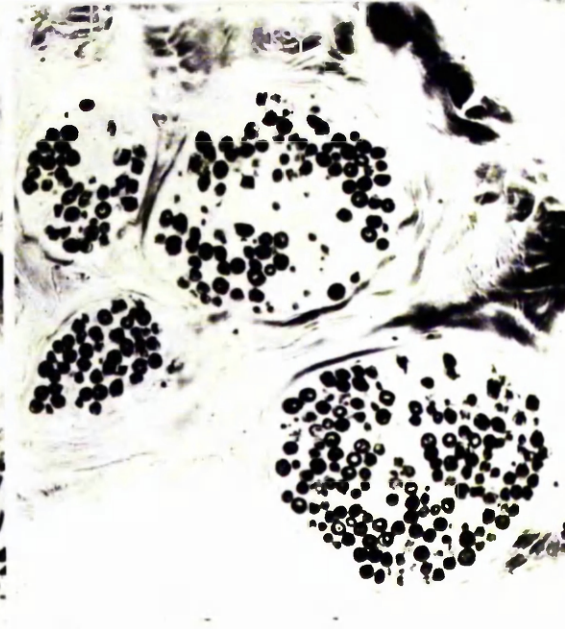
**a**



**b**



**c**



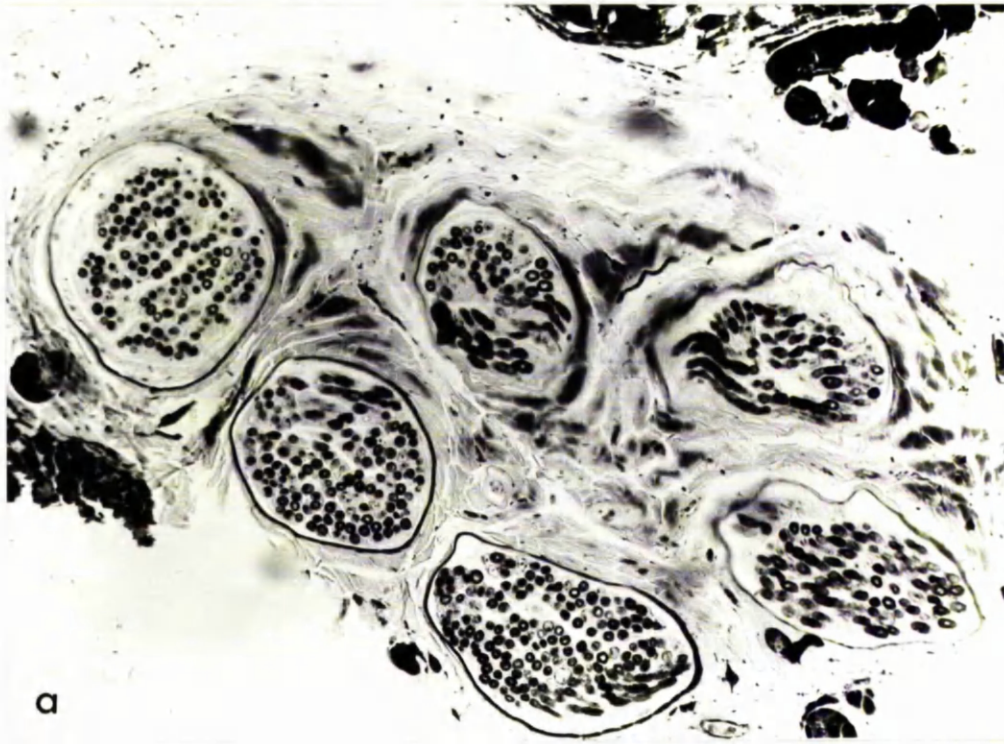
**d**



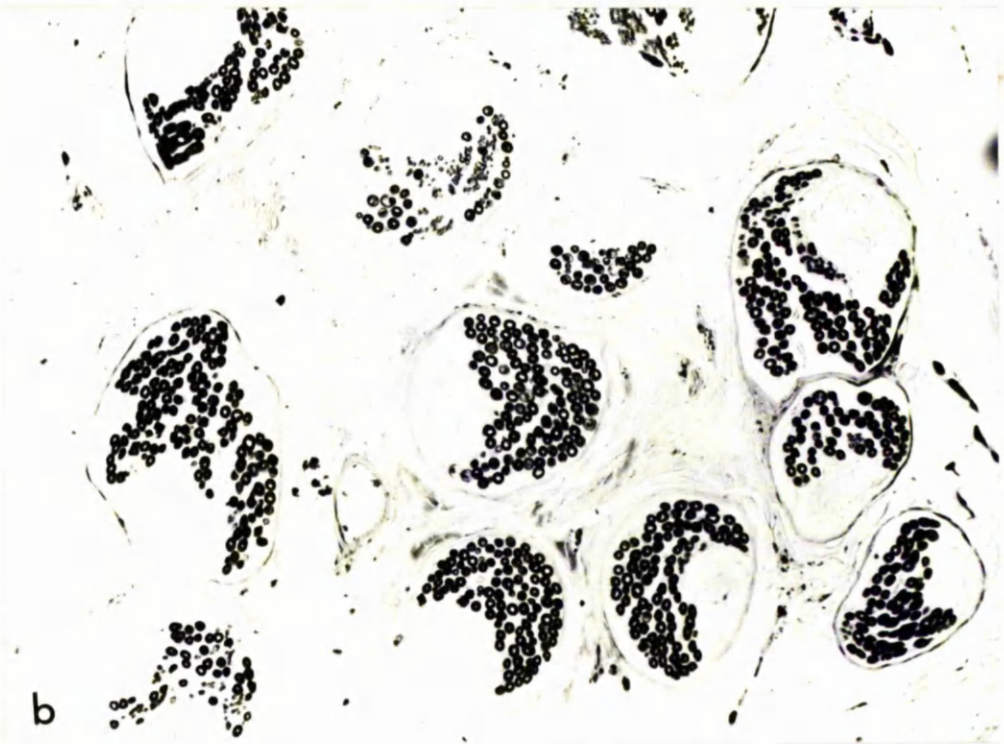
Fig. (51): Sections of the right R.L.N. of a clinical case  
(40733), at 2 levels,(a) 10 cms from larynx and(b) 20 cms.

Note the Renaut body formation in (b.)

(Kult. X 120)



a



b

Fig. (52): Sections of the left R.L.N. of a clinical case (47988) at two levels (a), at the level of the larynx and (b), in the thorax near the aortic arch. Few or no myelinated fibres are present in (a).

(Kult. X 120)



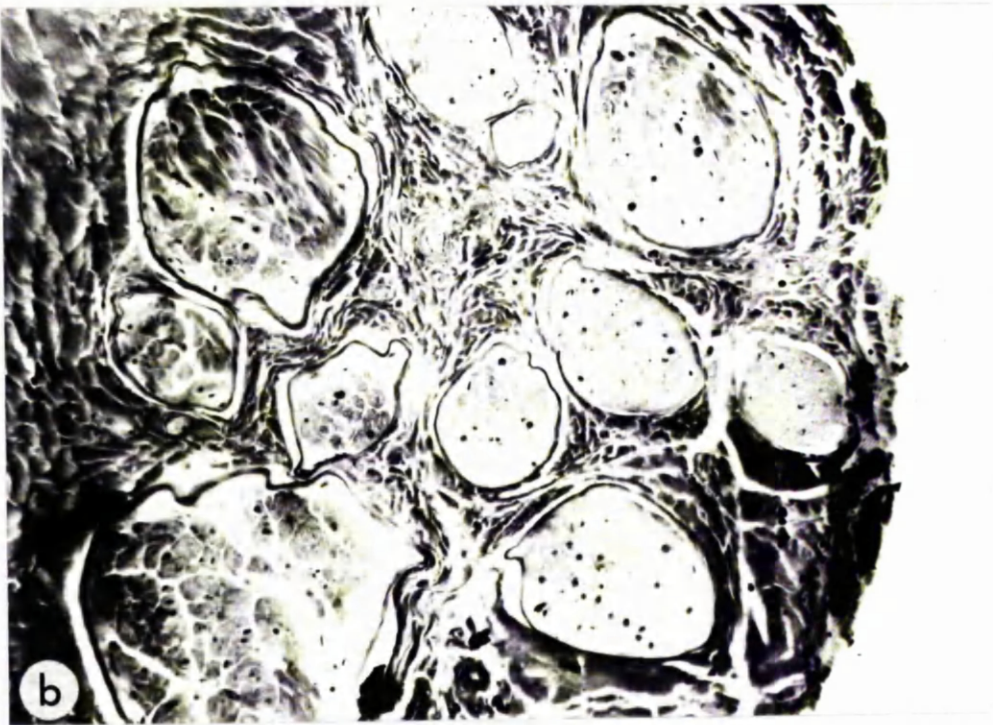


Fig. (53): Three other long peripheral nerves from a clinical case (40733), (a) tibial nerve, (b) phrenic nerve, (c) digital nerve. All appear to be normal, in marked contrast to the left R.L.N. (fig. 50).

(Kult. X 120)

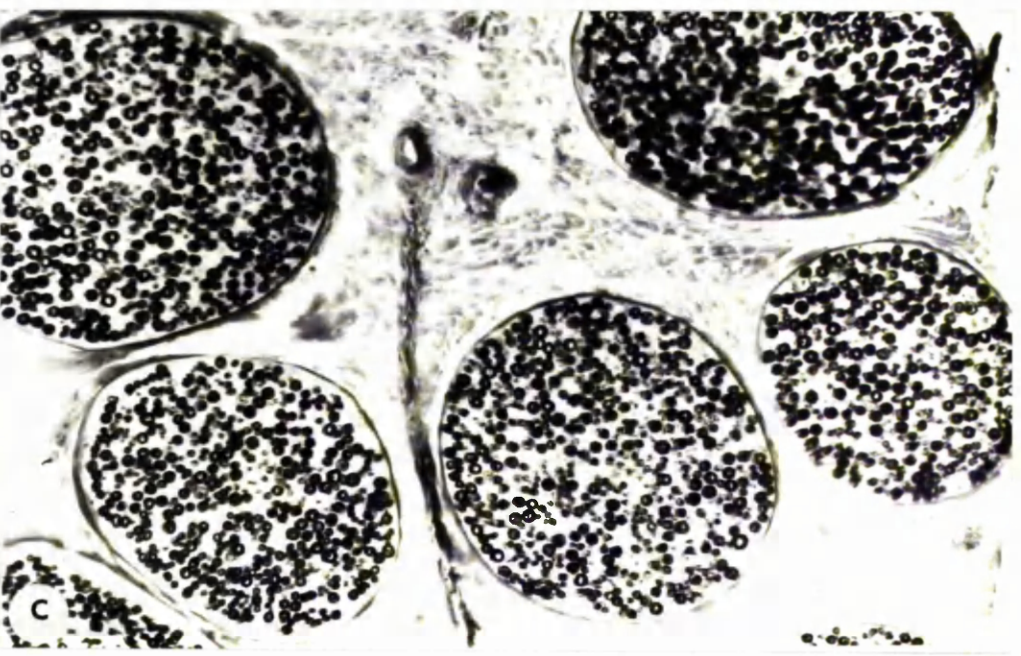
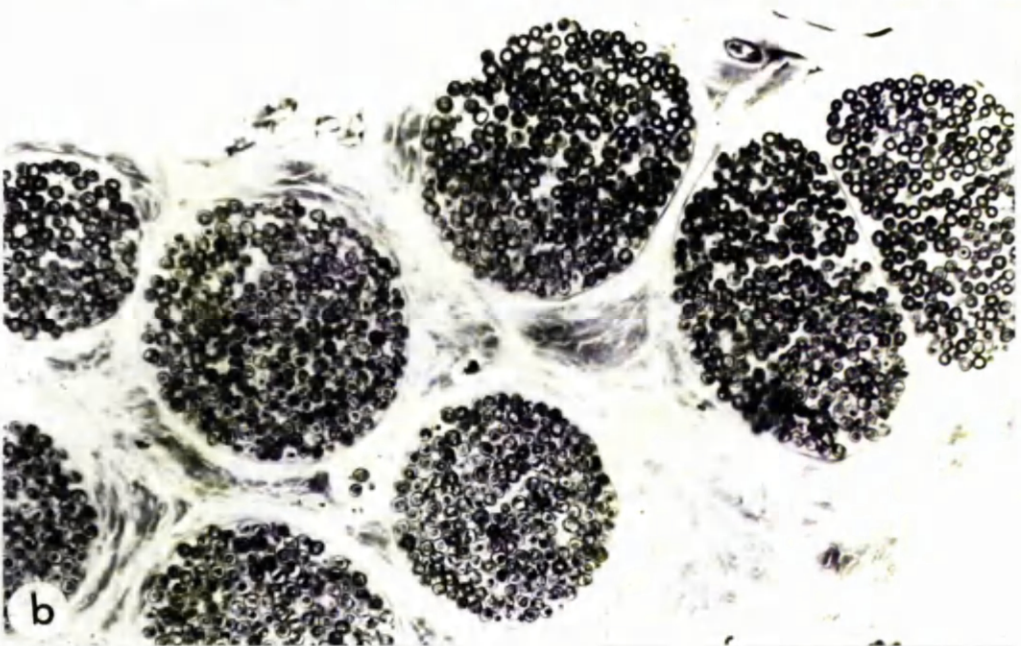
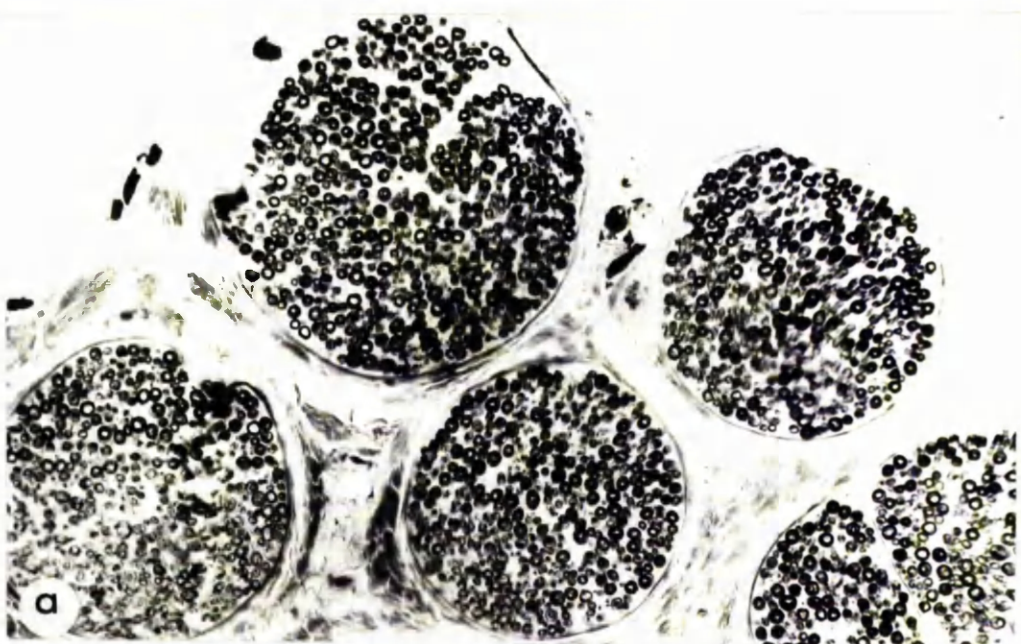


Fig. (54): The left (a) and right (b) R.L.N.'s of a sub-clinical case 10 cms from the larynx (54646). There is no obvious decrease in myelinated nerve fibres but there is marked Renaut body formation.

(Kult. X 120)

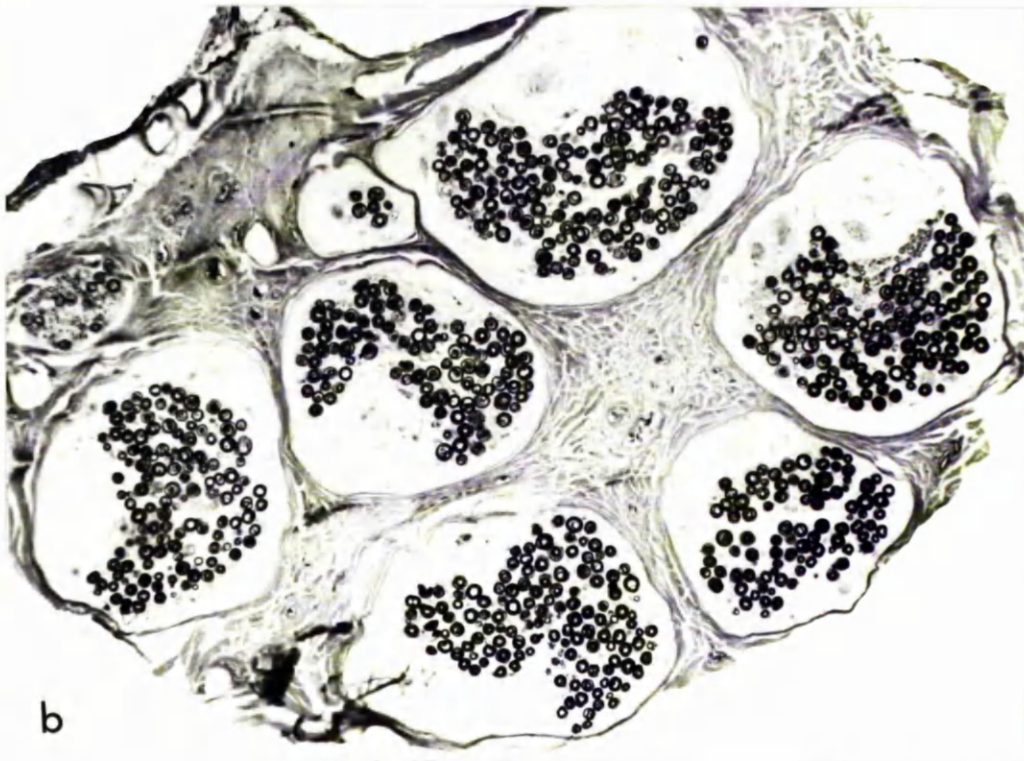
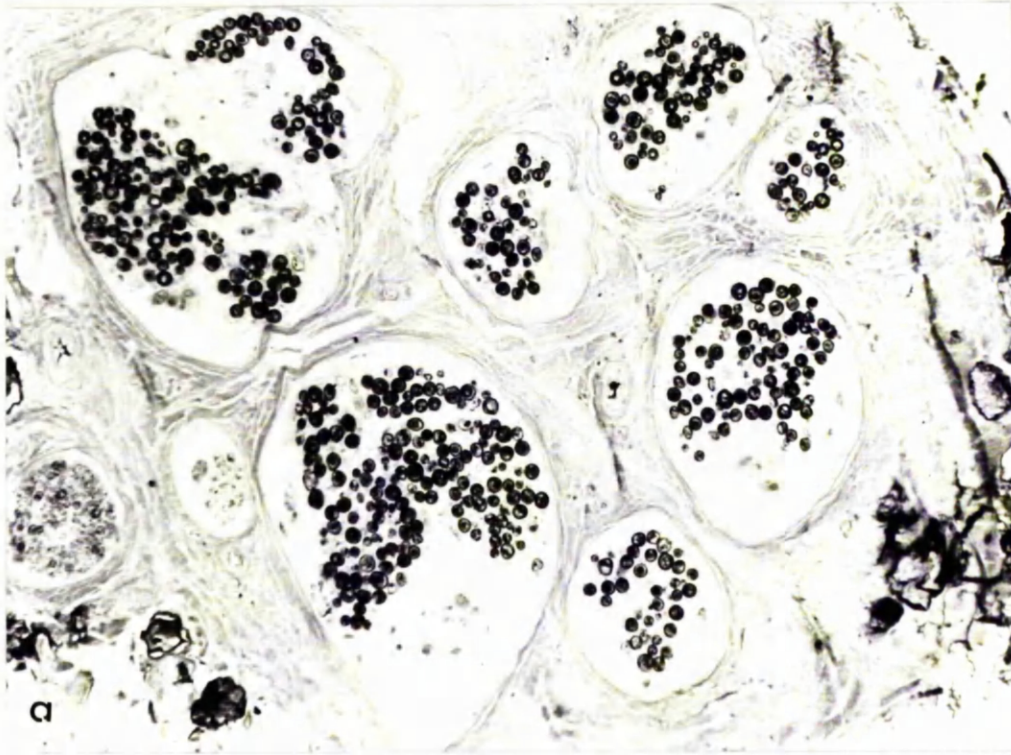




Fig. (55): The left (a) and right (b) R.L.N.'s near the larynx, of a case with adductor paralysis (57769). There is a decrease in fibre content in (a) with few medium/large diameter fibres present. Note the Renault bodies in (b).

(Tol. blue X 120)

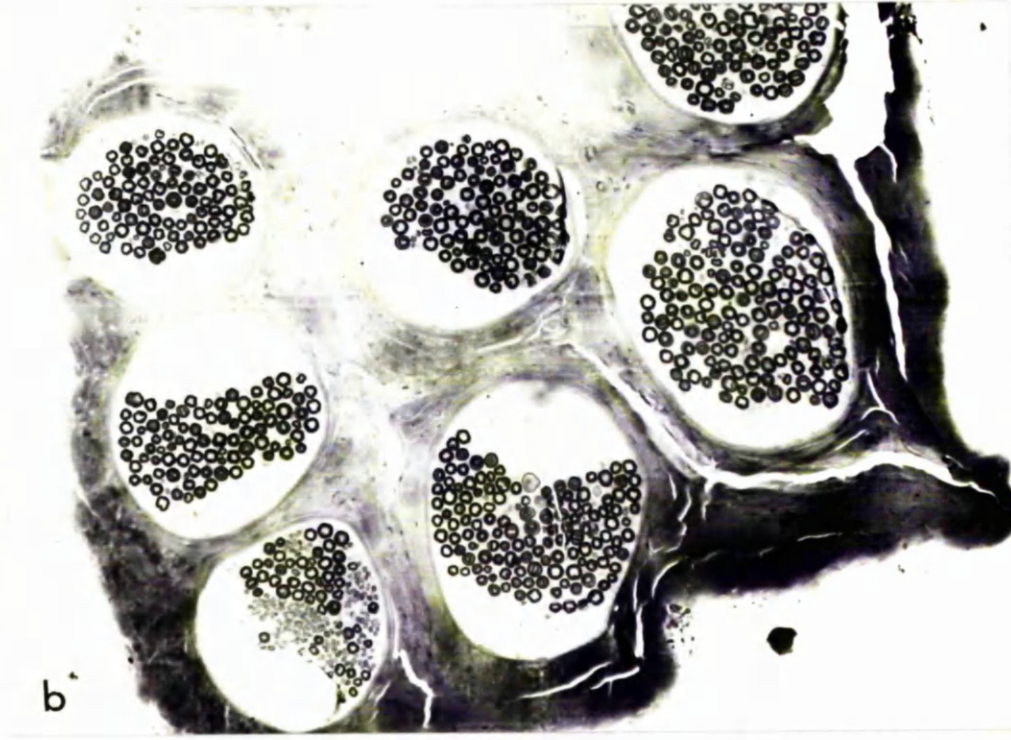
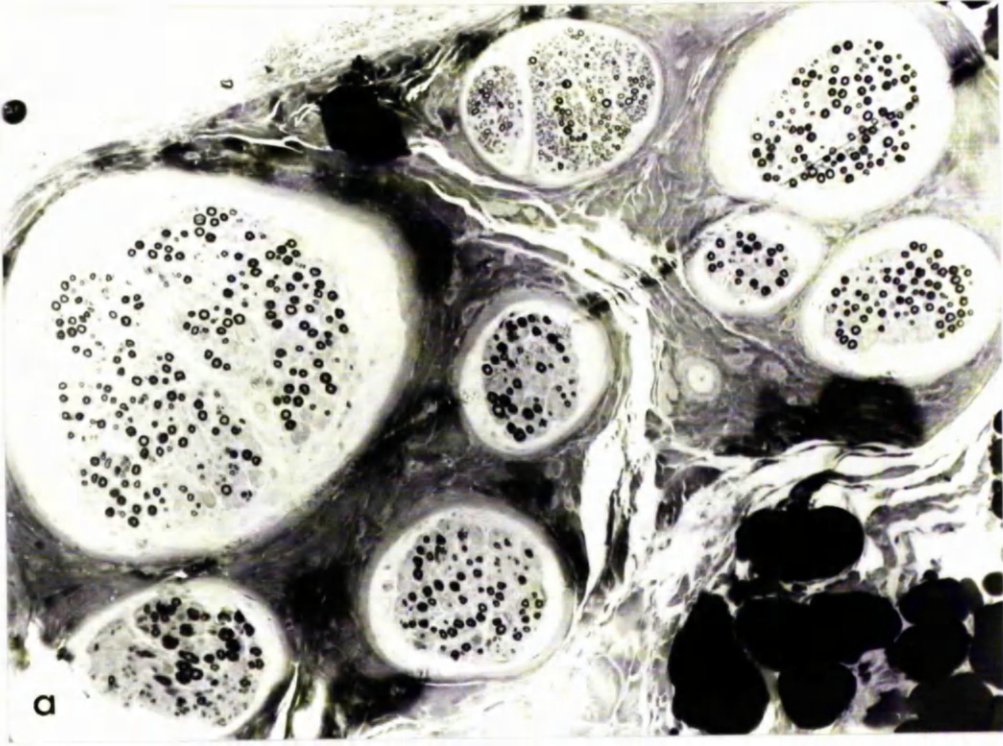




Fig. (56): The left (a) and right (b) R.L.N.'s of a sub-clinical case 5 cms from the larynx (57510). As in fig. (55), there is a decrease in the larger myelinated fibres in (a). Renaut bodies can be seen in (a+b). Note also in (a) that two of the fascicles are divided by connective tissue septae.

(Tol. blue X 120)

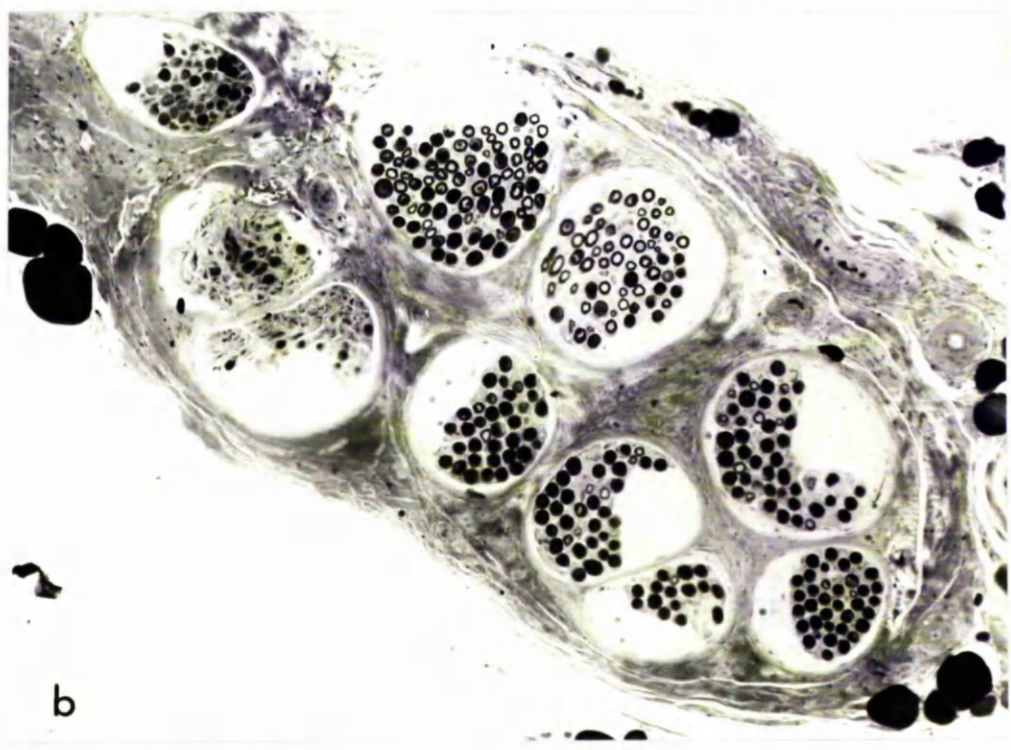
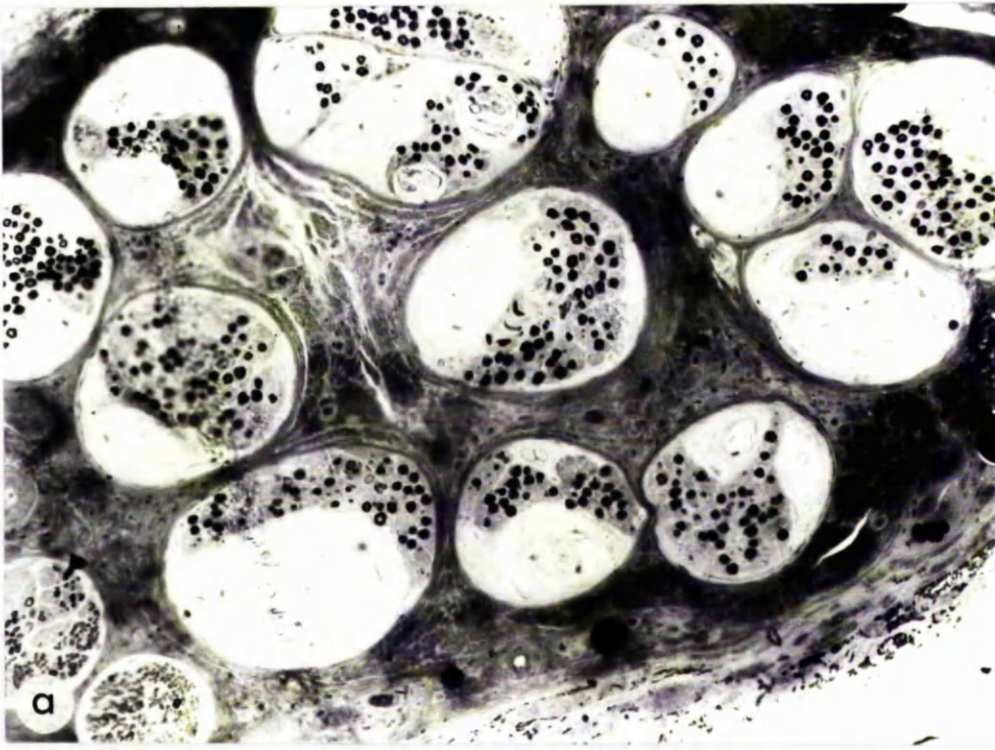


Fig. (57): Part of the left abductor (a) and right abductor  
(b) branches of the R.L.N.'s of a sub-clinical case  
(59190). Below is the histogram of the fibre diameter  
spectra of each branch.

(Kult. X 240)

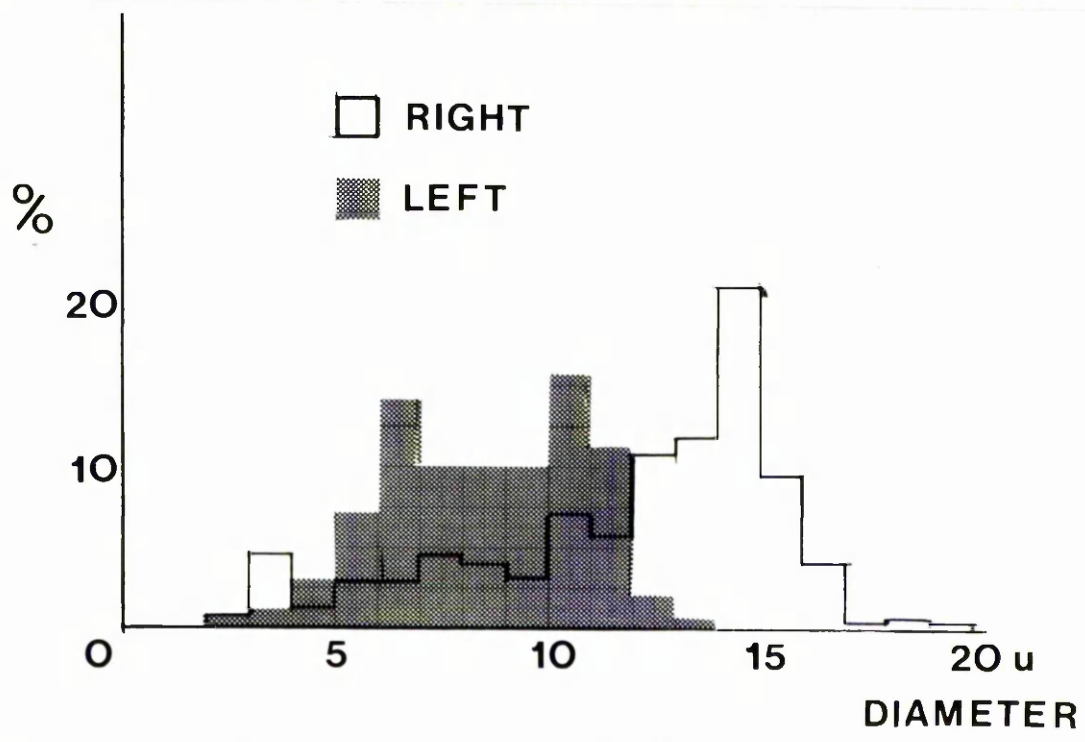
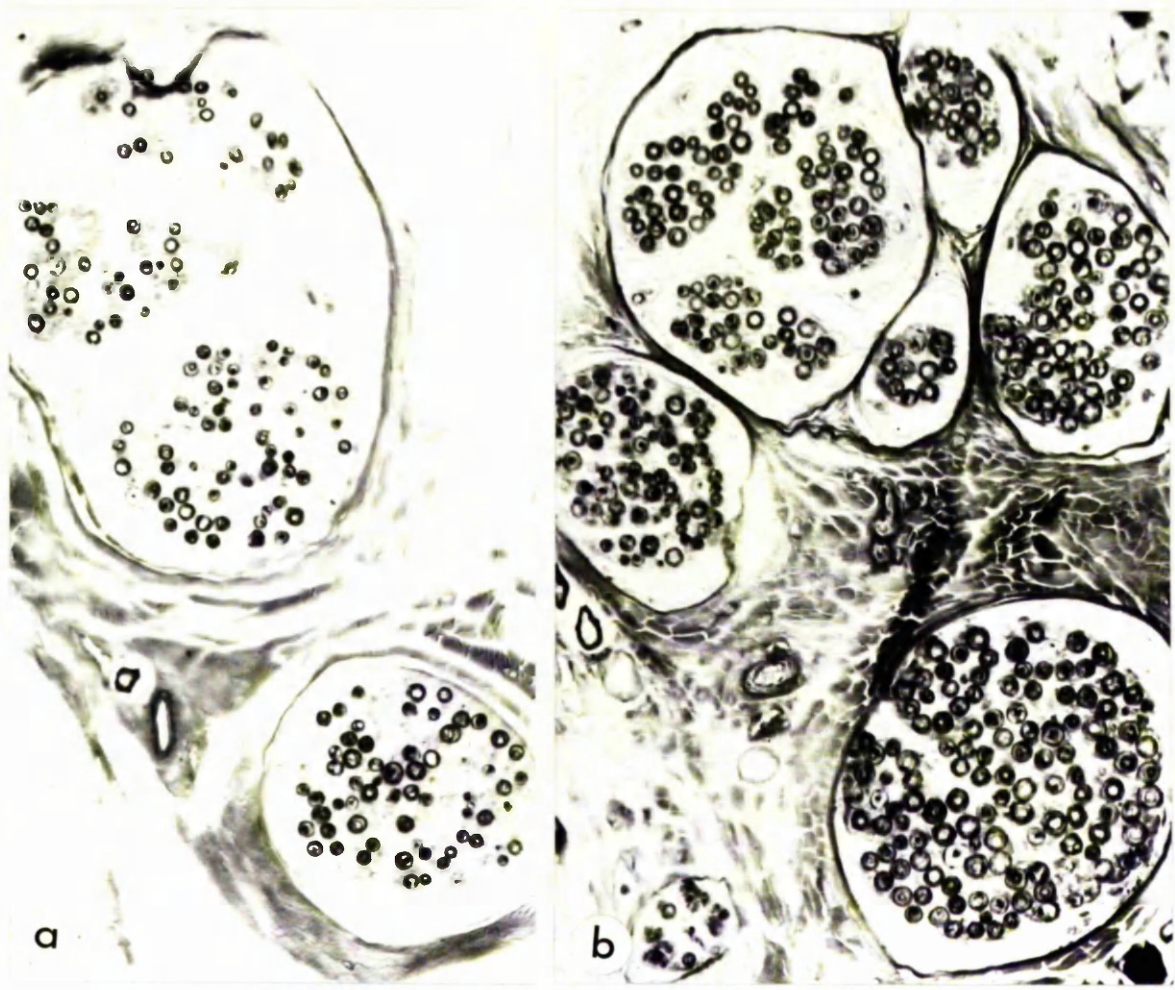


Fig. (58): Part of the left adductor (a) and right adductor  
branches of the R.L.N.'s of a sub-clinical case (59190).  
Below is the fibre diameter histogram of each branch.

(Kult. X 240)

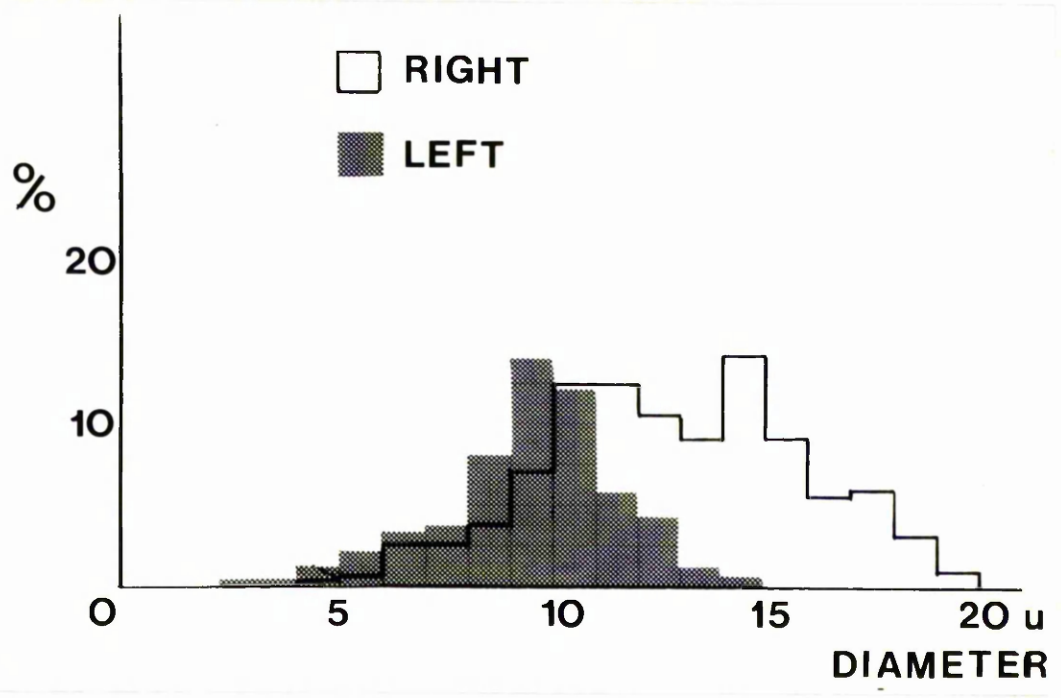
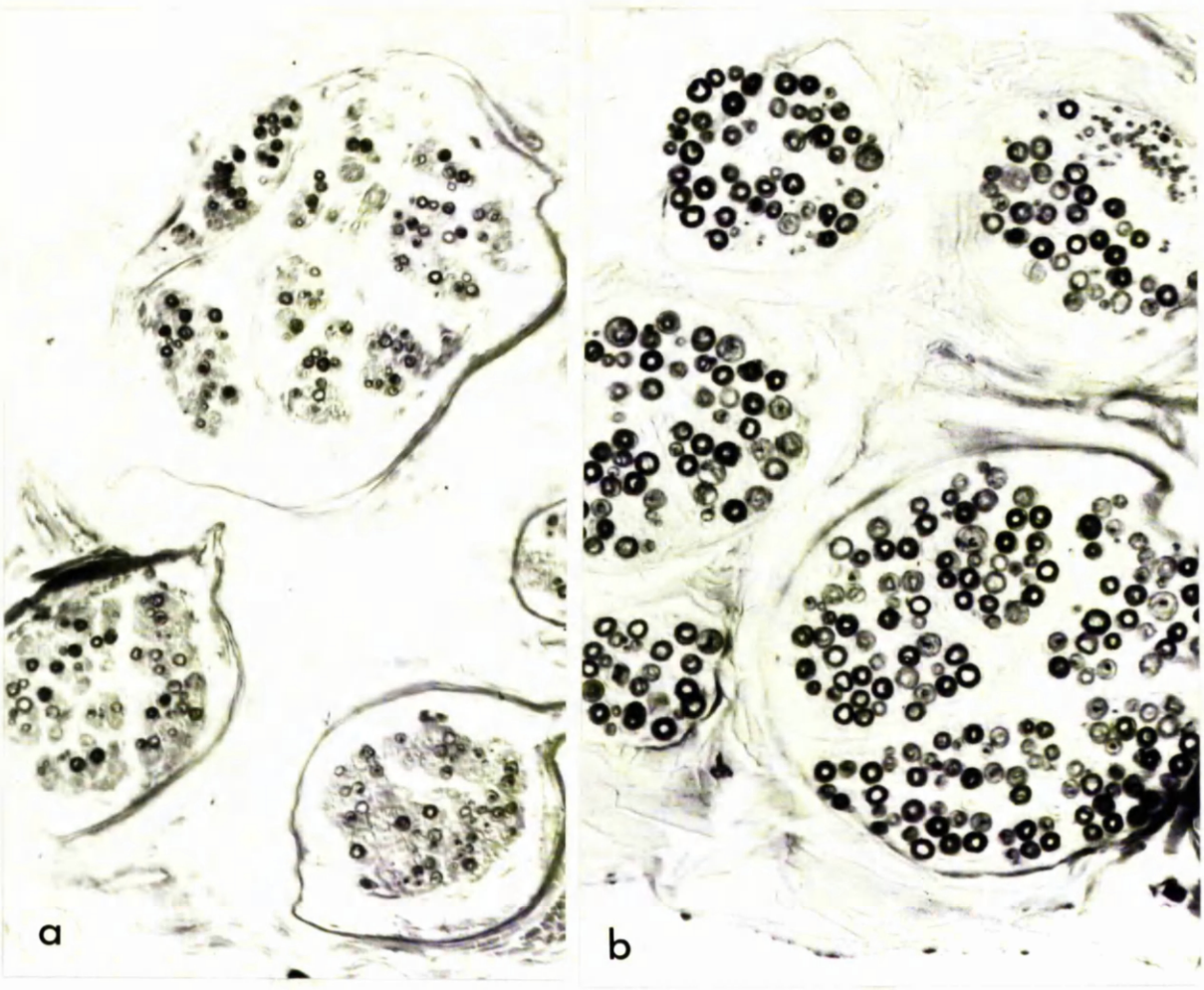


Fig. (59): The adductor branch of the left R.L.N. of a sub-clinical case (58877). There is a marked decrease in myelinated nerve fibres.

(Kult. X 120)

Fig. (60): Part of the left R.L.N. of a sub-clinical case near the larynx. There is a decrease in nerve fibres but some larger fibres have relatively thin myelin sheaths (arrows) indicating remyelination. Fibres which appear completely black have been poorly fixed.

(Tol. blue X 400)

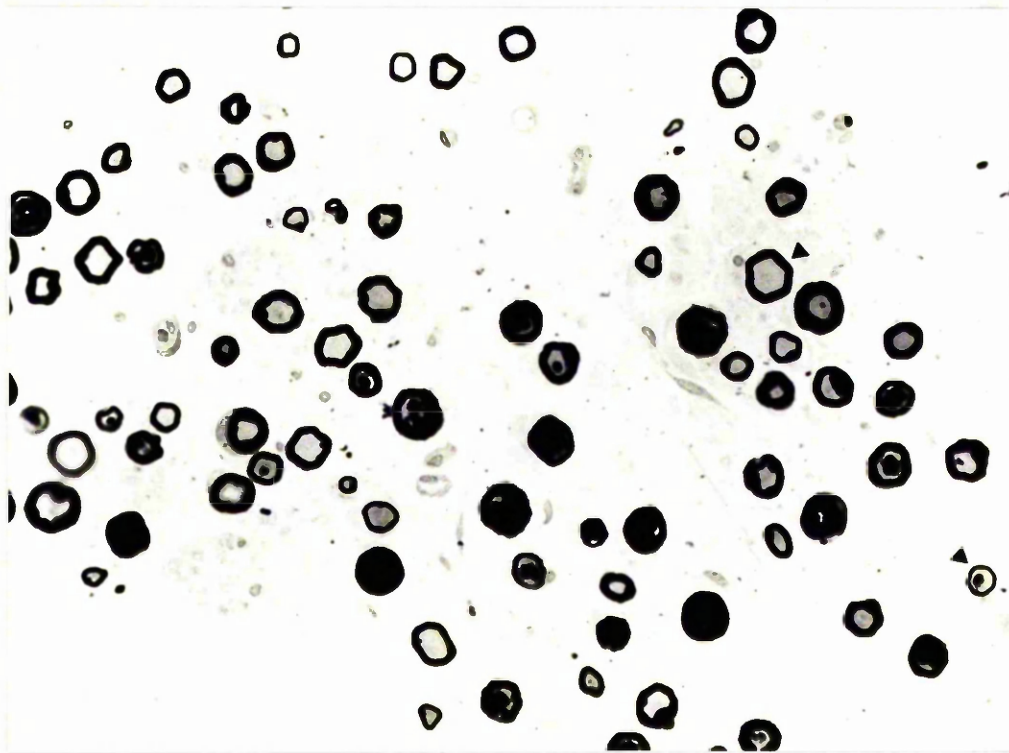
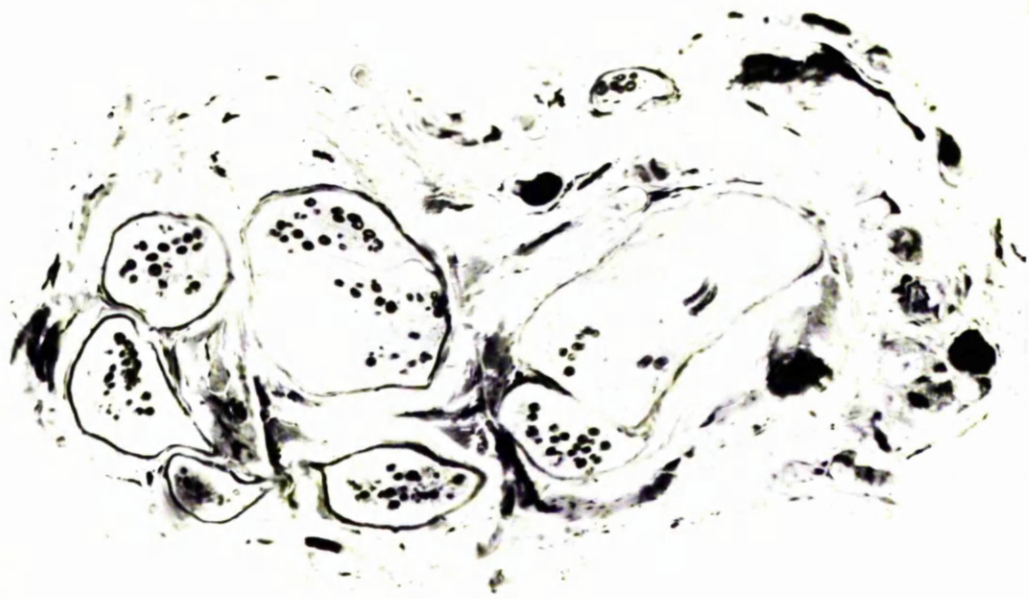




Fig. (61): A cluster of regenerating fibres (arrow) in a fascicle of the left R.L.N. of a clinical case.

(Tol. blue X 250)

Fig. (62): A fascicle from the left R.L.N. of a case with adductor paralysis (57769), containing mainly small diameter fibres which are thought to be sensory fibres from trachea and oesophagus. There appears to be a normal content of fibres in the fascicle compared with the muscular branches in the same nerve (fig. 55).

(Tol. blue X 120)

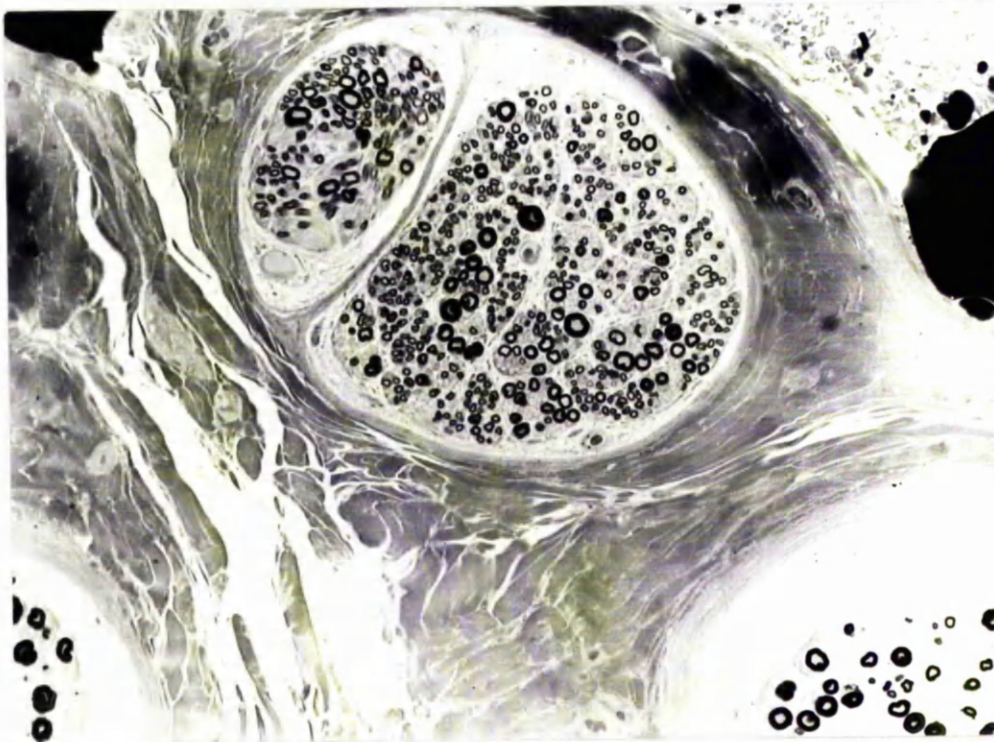
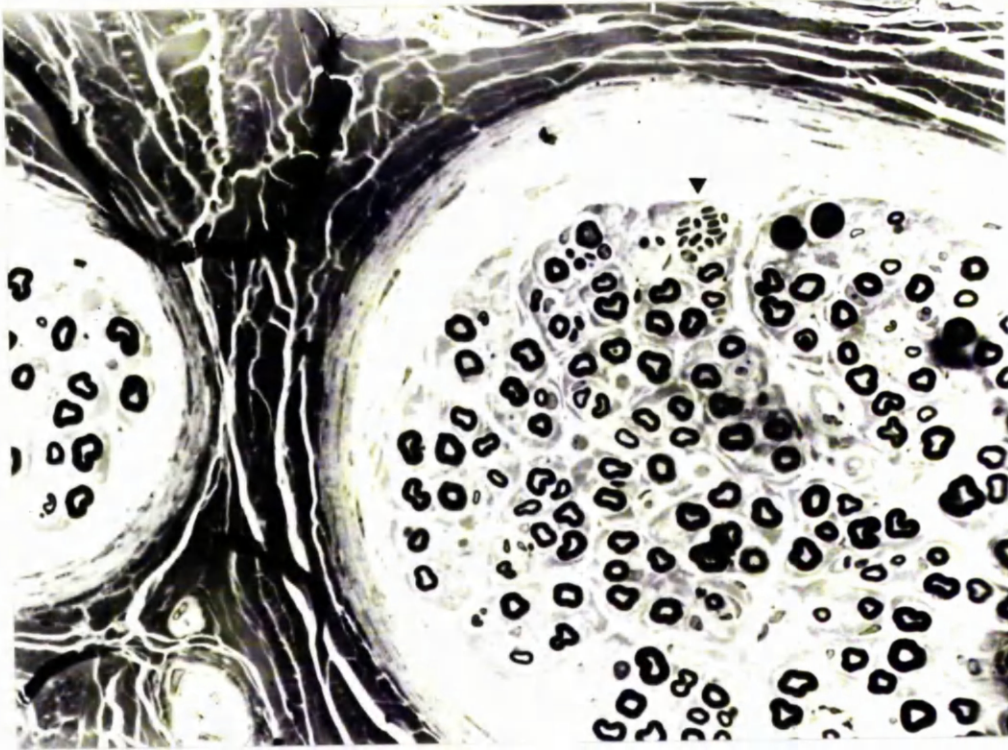
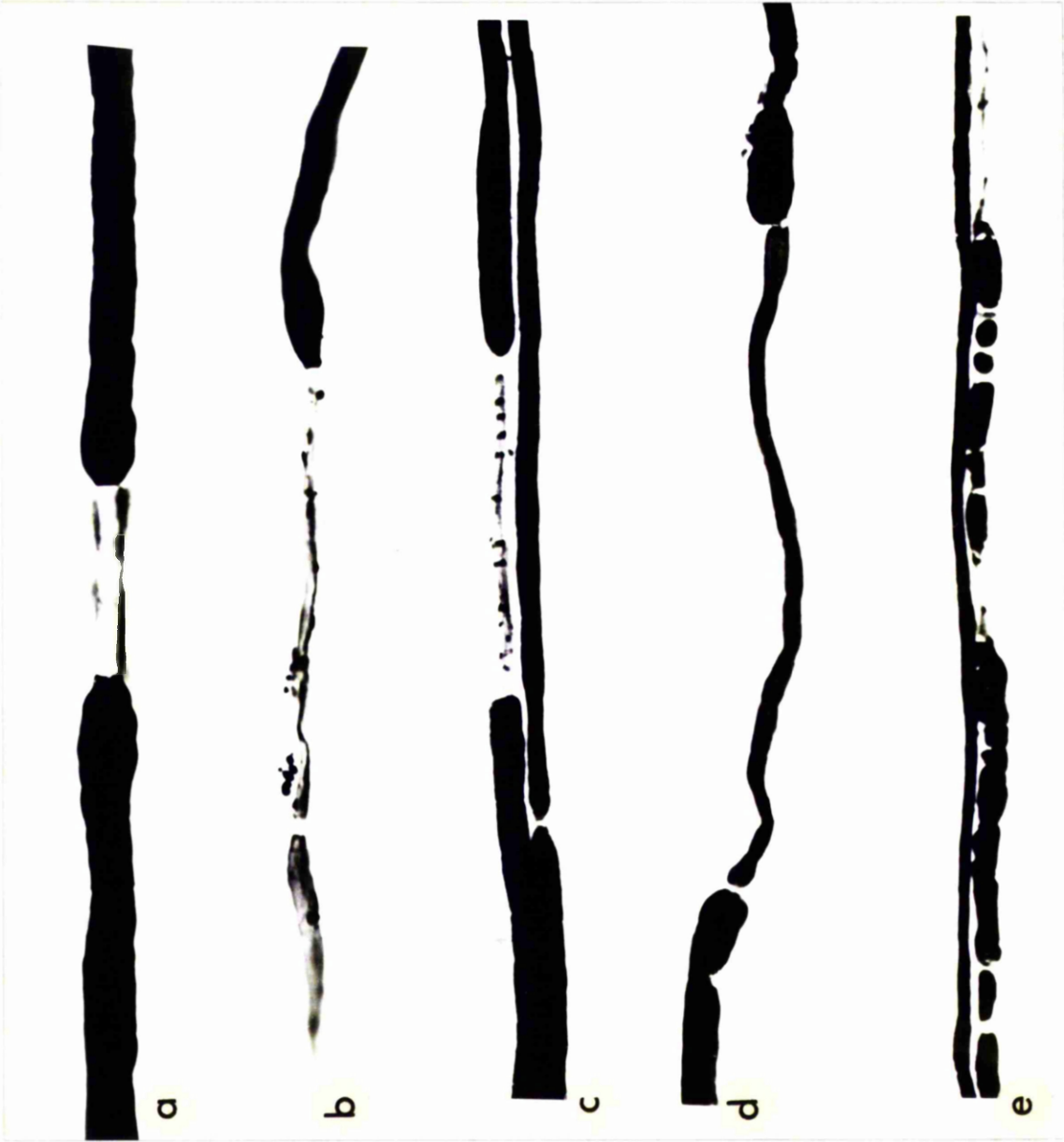


Fig. (63): Range of changes seen in the teased nerve fibre preparations from the laryngeal nerves.

- a) Short intercalated segment, as a result of paranodal demyelination.
- b) Short demyelinated segment in which remnants of the myelin sheath are still present. The internode on the left is also demyelinated.
- c) Short remyelinating intercalated segment with prominent Schmidt - Lanterman incisures.
- d) Short, remyelinated intercalated segment.
- e) Axonal degeneration.

(X 500)

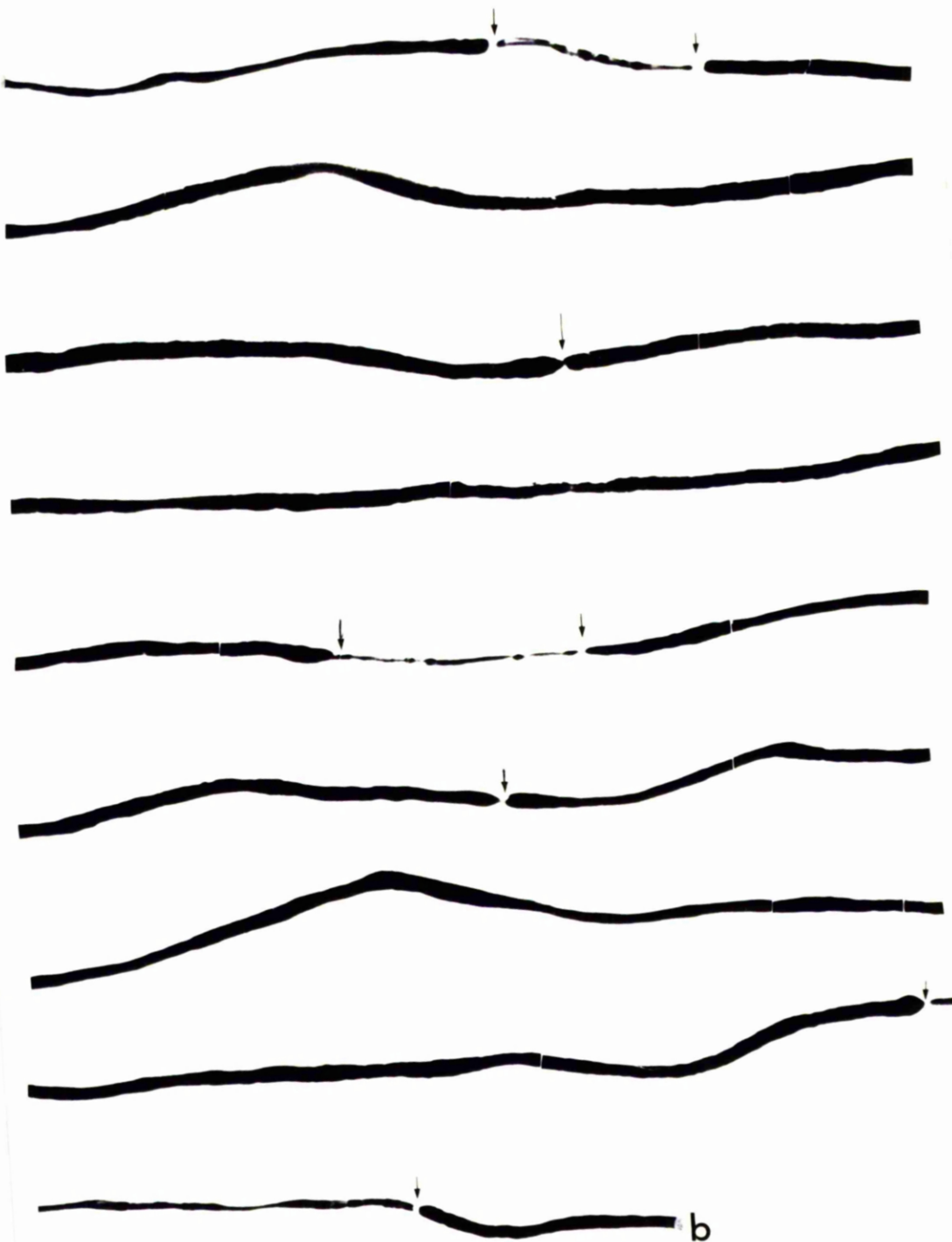


Figs. (64 - 72): All are teased, individual fibres continuous  
from a to b going from left to right.

Fig. (64): Single fibre with three intercalated segments  
see text (p.

(X 400)

a



b

Fig. (65): Normal single fibre. Note the equal distances between nodes of Ranvier and the almost constant internode diameter.

(X 400)

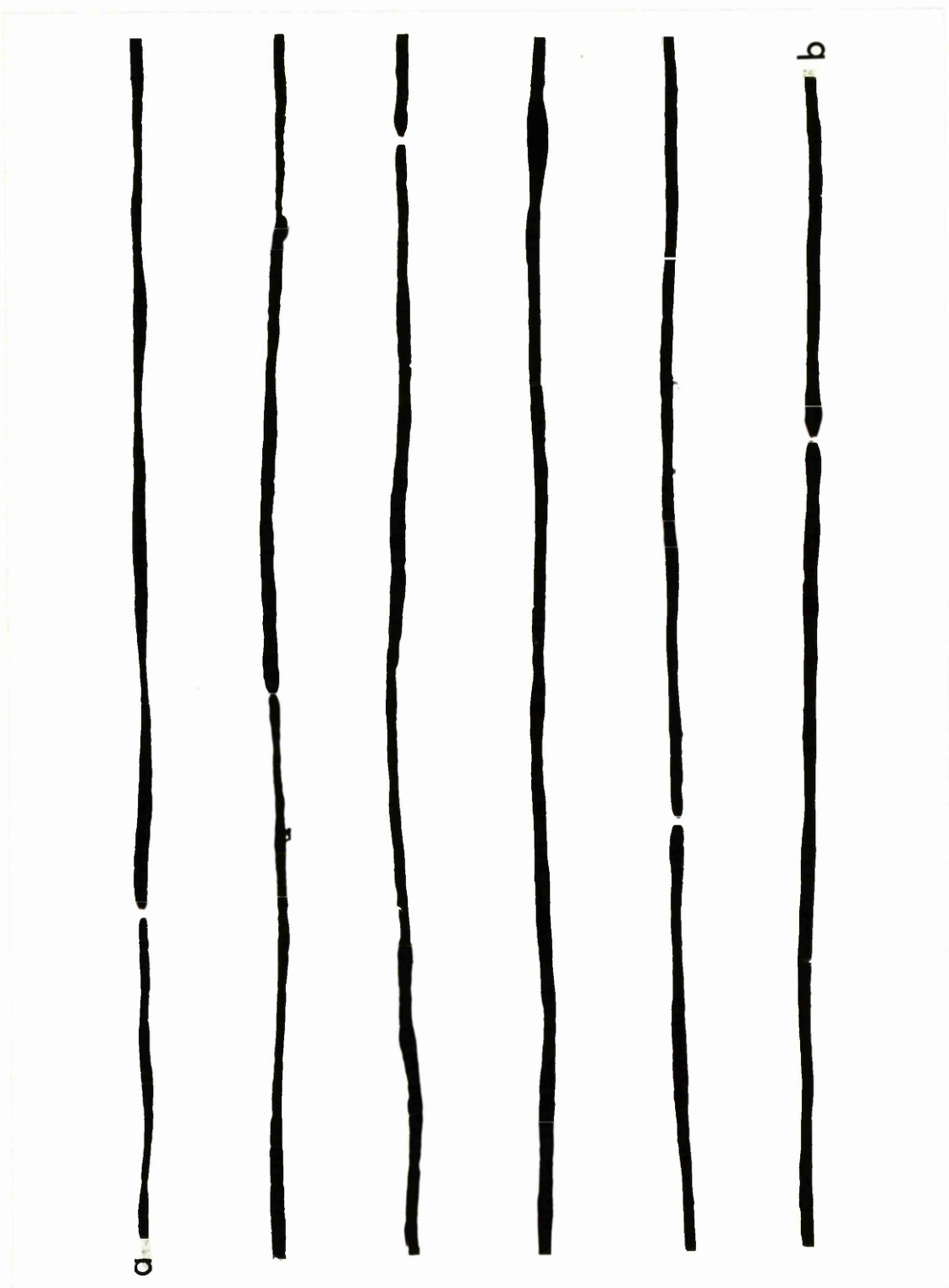




Fig (66): Abnormal single fibre. See text (p 176)

(X 400)

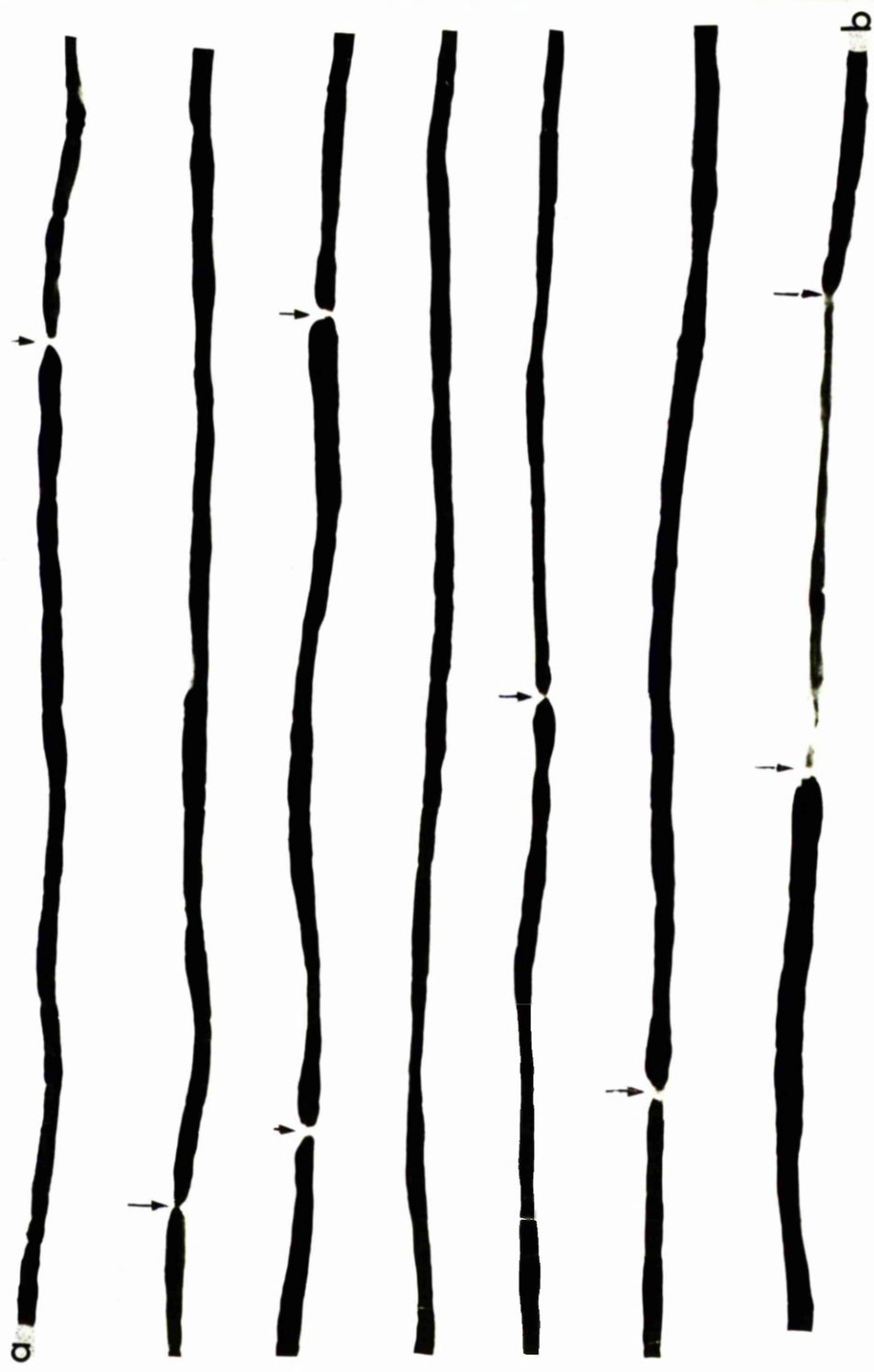
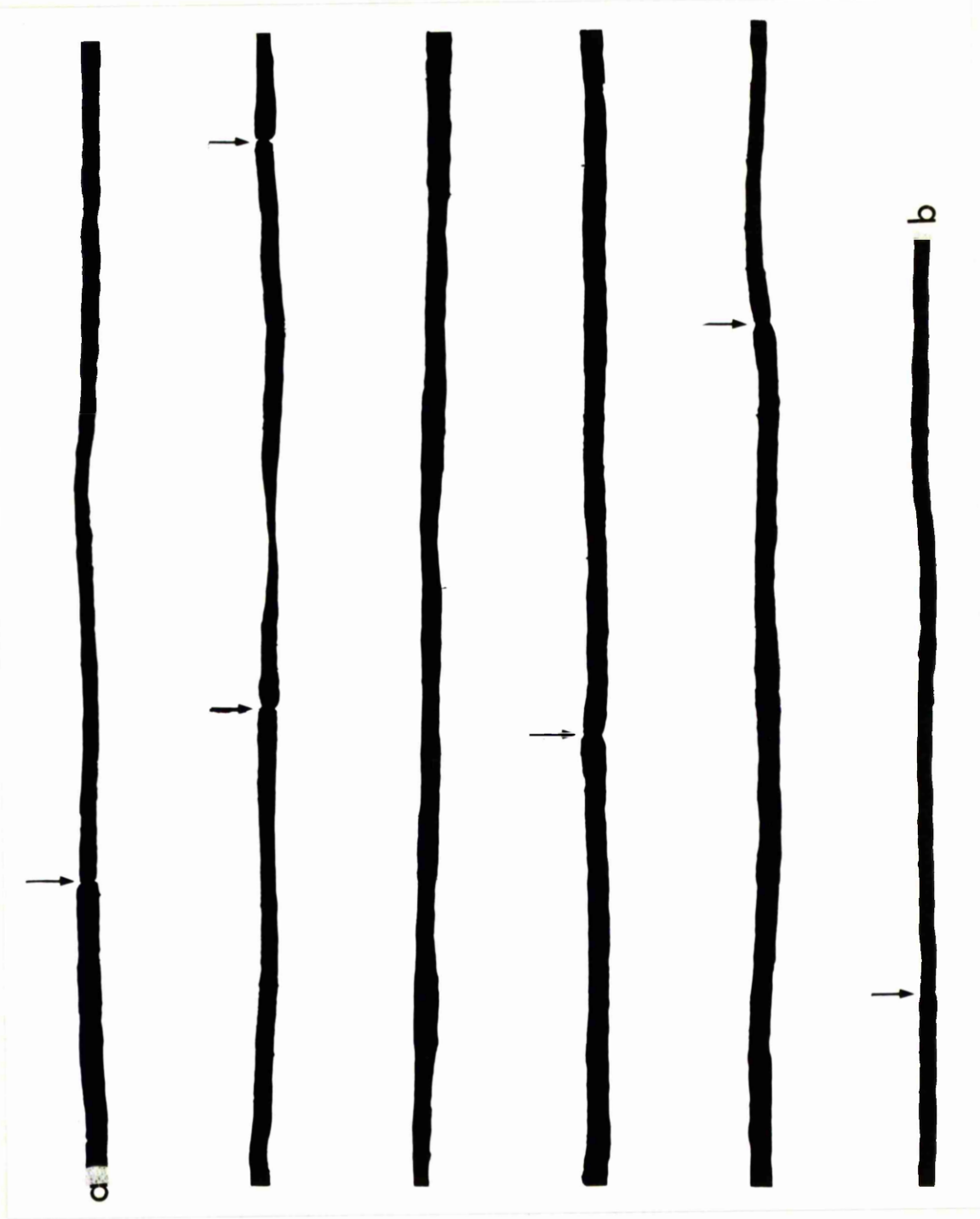


Fig. (67): Remyelinated fibre following segmental demyelination.

(X 400)



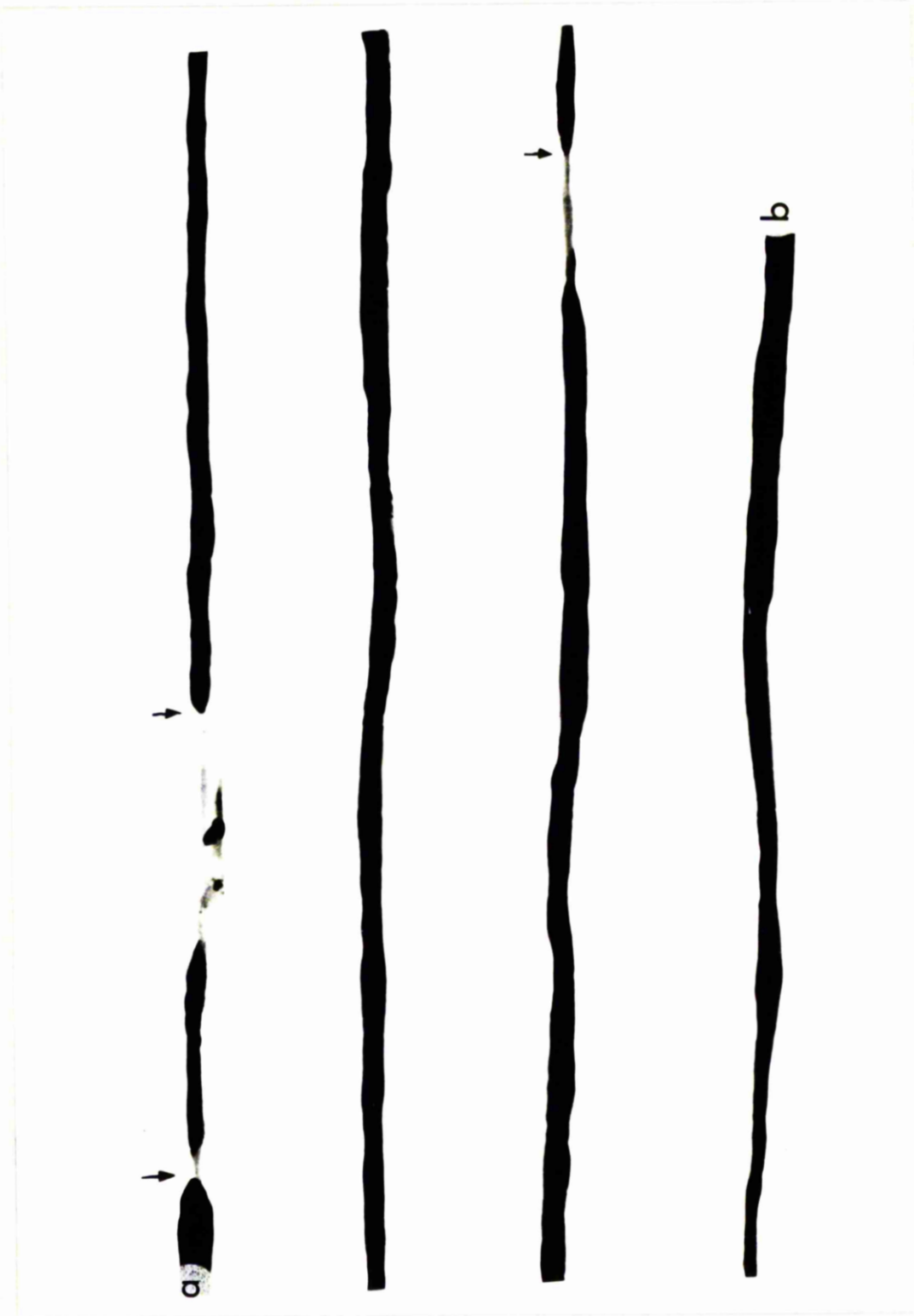
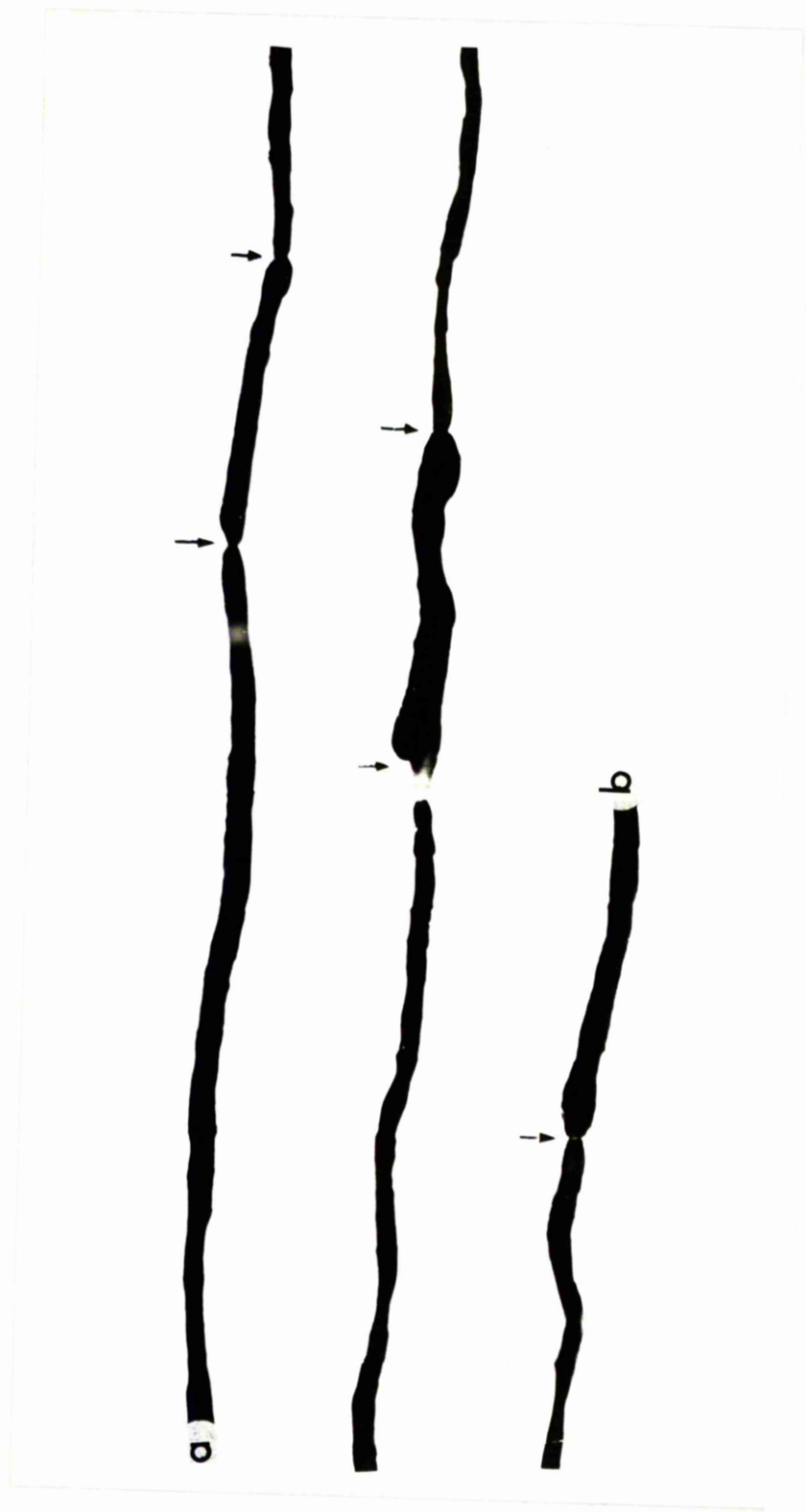


Fig.(69): Abnormal fibre. There is marked variation of internode length and diameter.

(X 400)



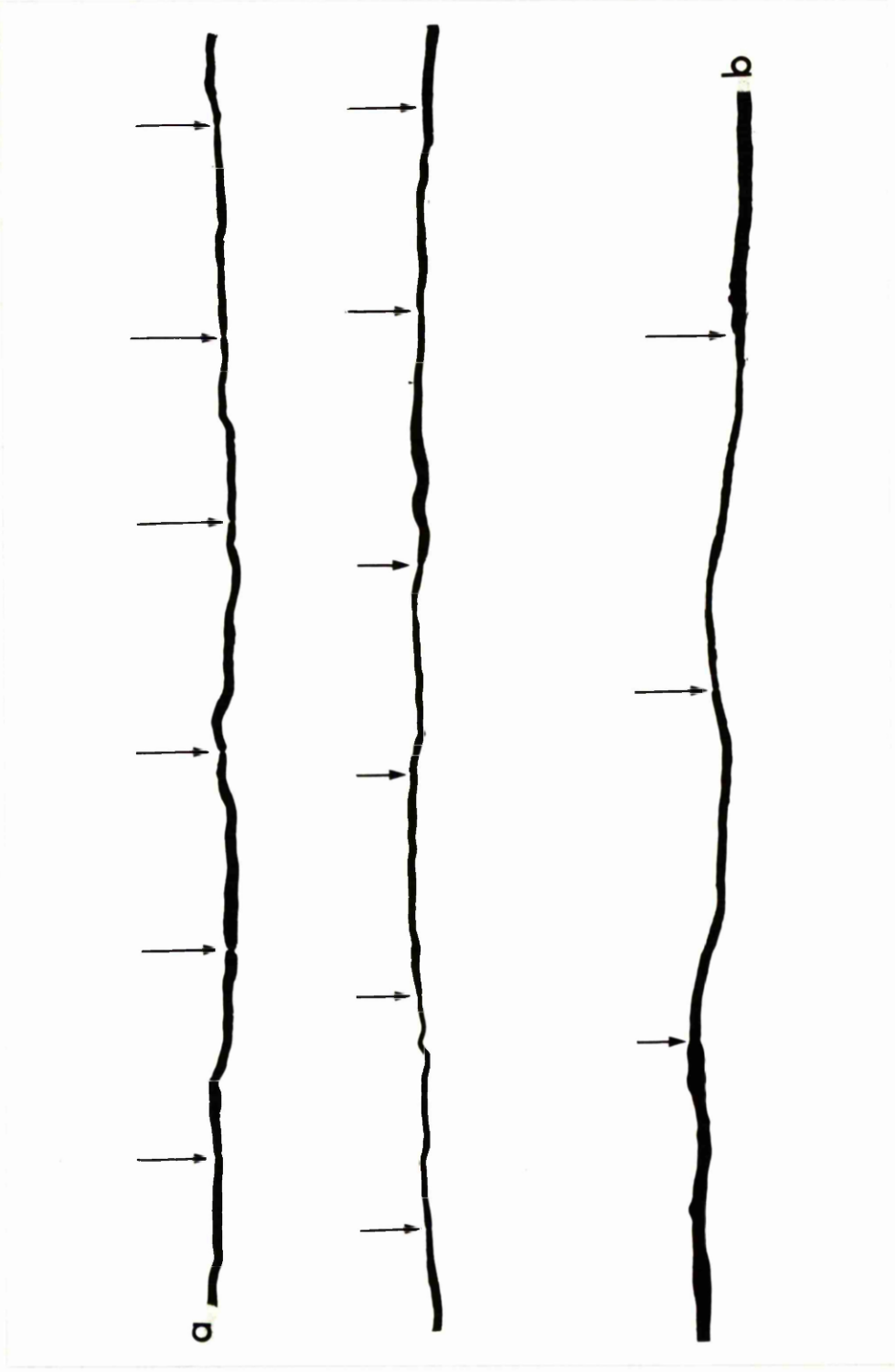
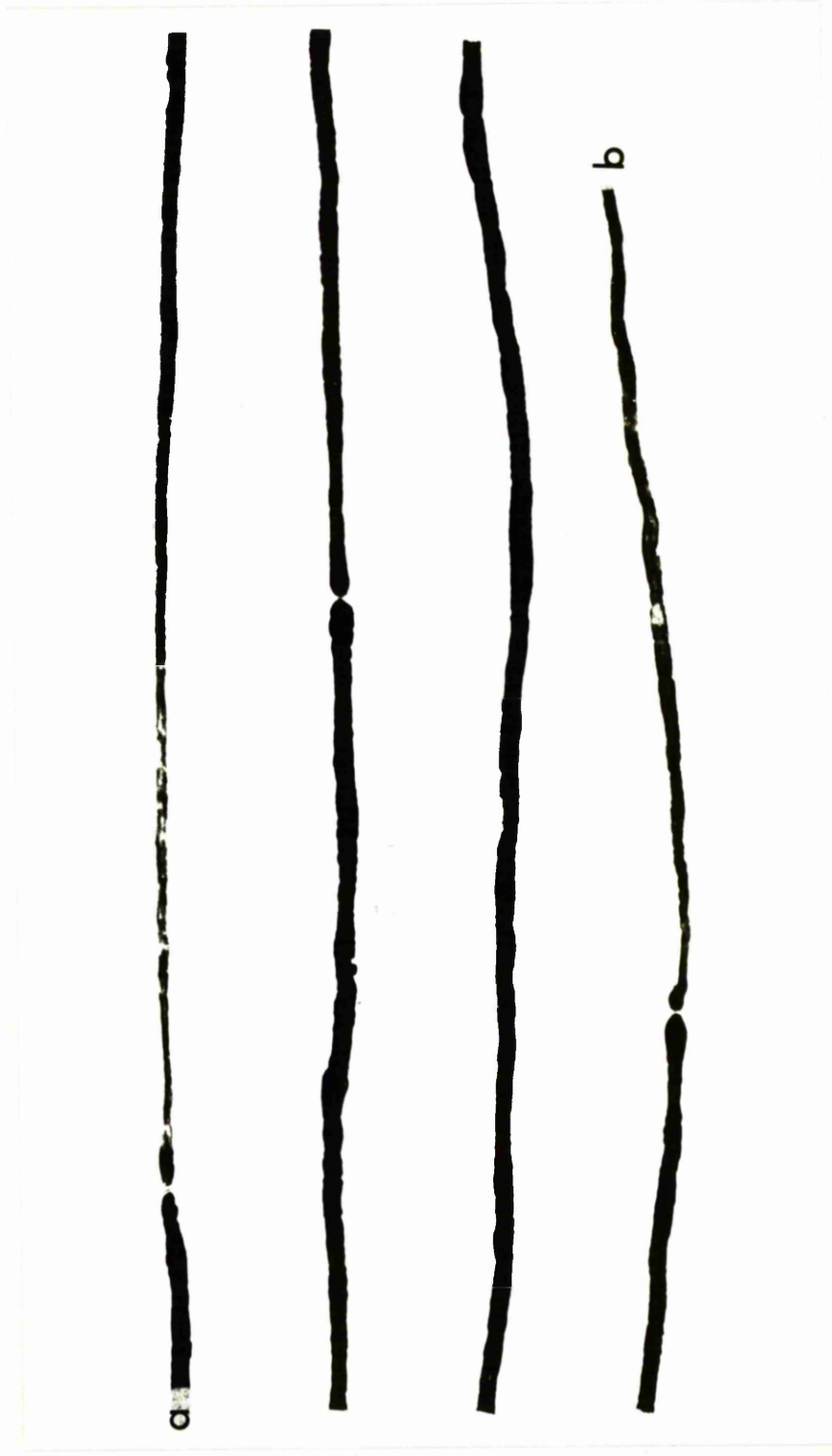




Fig. (71): A single fibre from the left RLN of a clinical case 50 cms from the larynx. Note the marked tapering of two internodes (arrows) both in the same direction.

(X 400)



a

b

Figs. (72 - 76): Single fibre graphs (after the method of Fullerton et al 1965) and scatter graphs of teased nerve fibre preparations. Internode length is plotted against internode diameter.

Fig. (72): Single fibre and scatter graphs of a sub-clinical case (54788) and the right RLN of a clinical case (47988). Note the clustering of points in the scatter graph of 54788, left RLN.

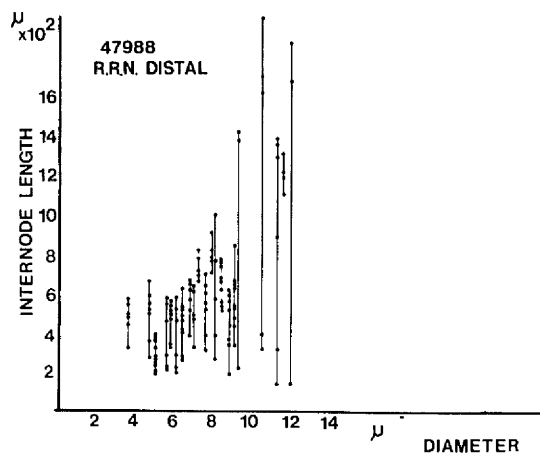
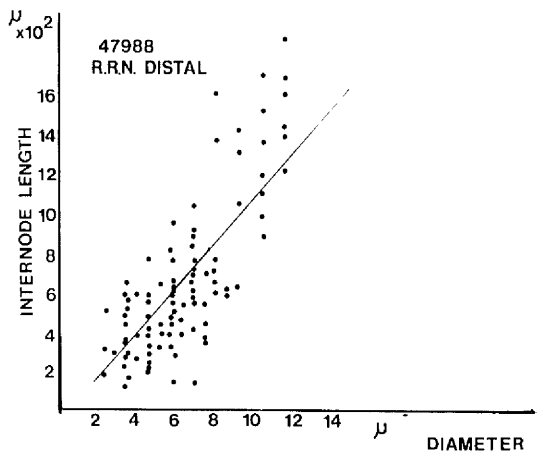
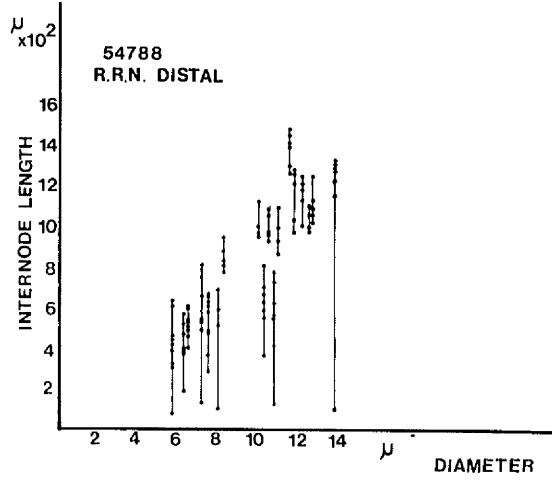
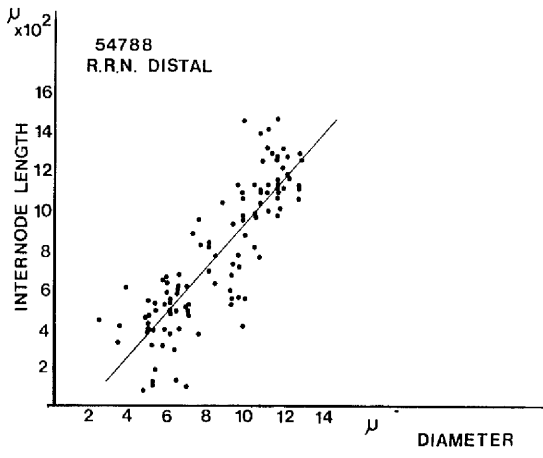
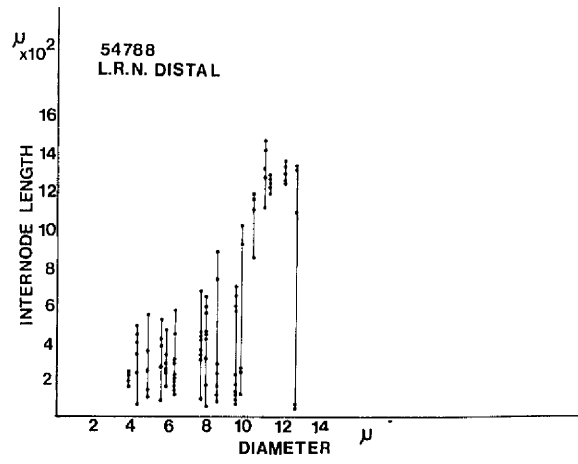
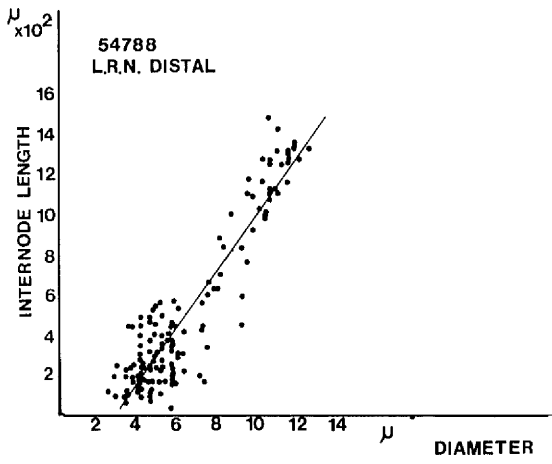


Fig. (73): Single fibre and scatter graphs of two sub-clinical cases.

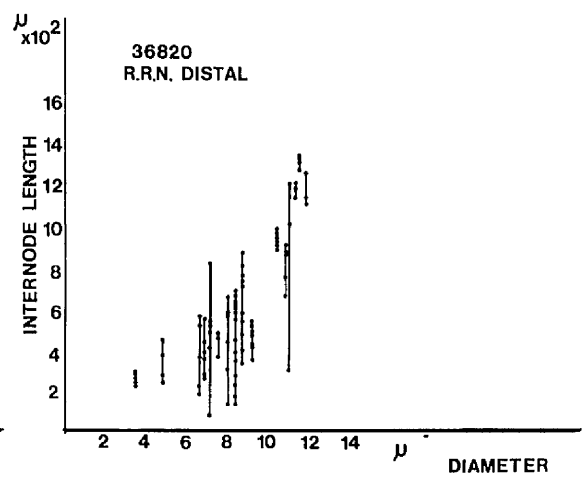
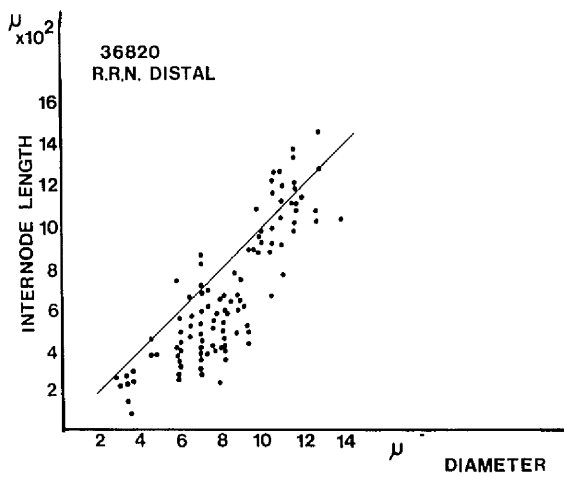
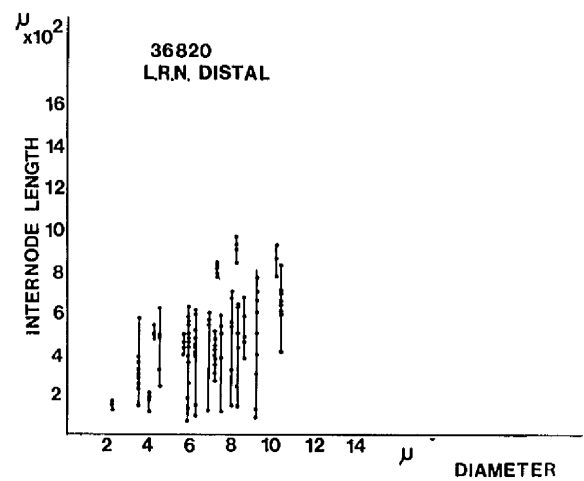
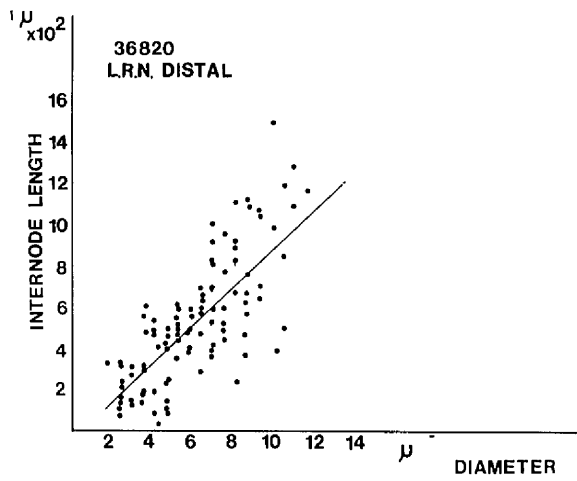
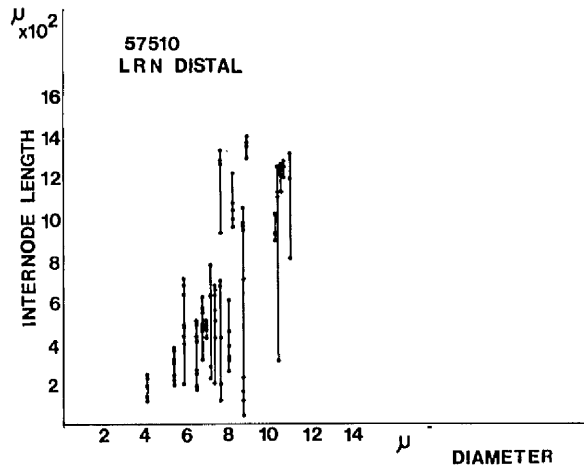
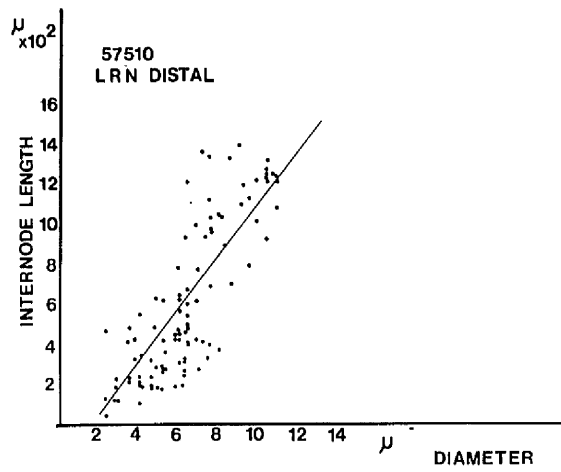


Fig. (74): Scatter graphs of the laryngeal nerves and single fibre and scatter graphs of other peripheral nerves in a case of adductor paralysis (57769).

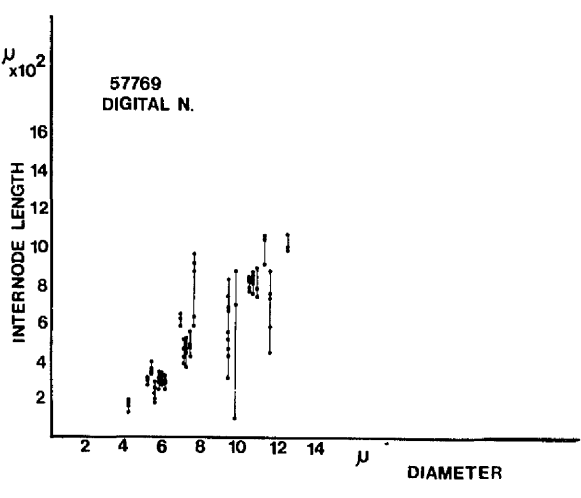
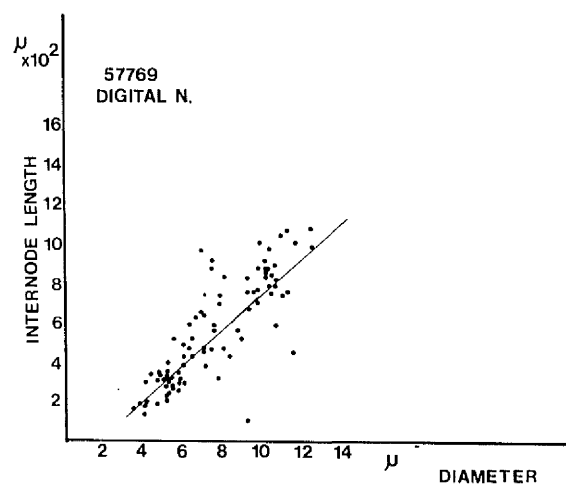
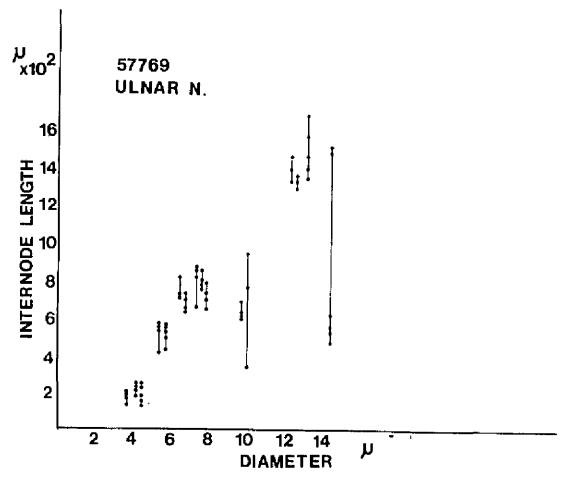
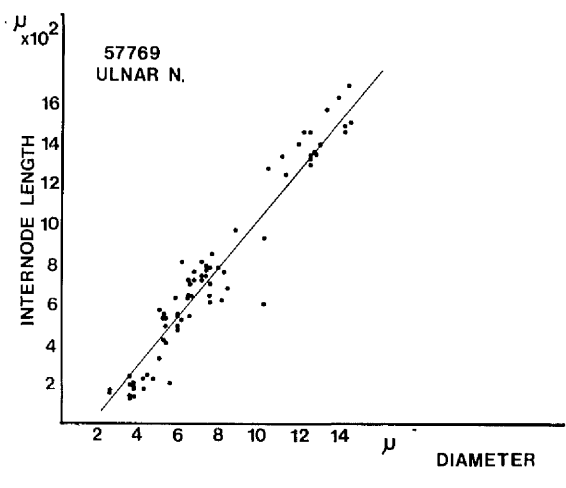
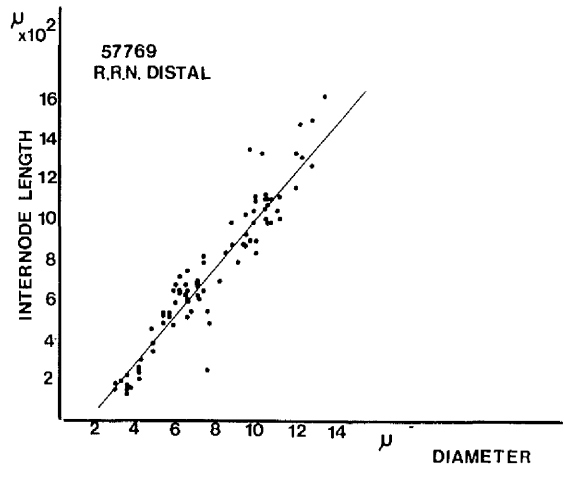
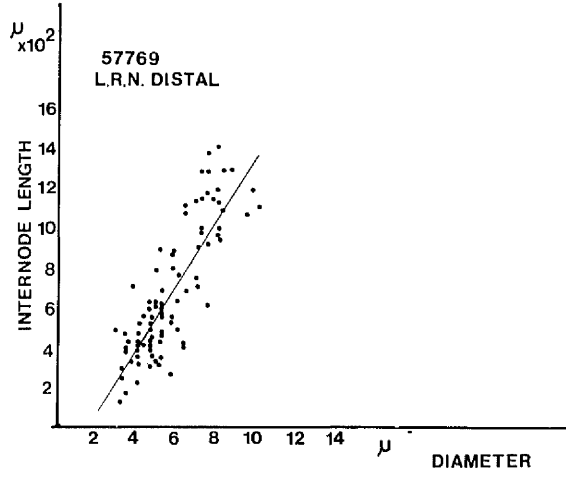




Fig. (75): Single fibre and scatter graphs of the left R.L.N. of a normal, young pony (53273). Single fibre and scatter graphs of the laryngeal nerves of an old, normal pony (55028). The two fibres in single fibre graph of the left R.L.N. of 55028 marked(a), are thought to have regenerated.

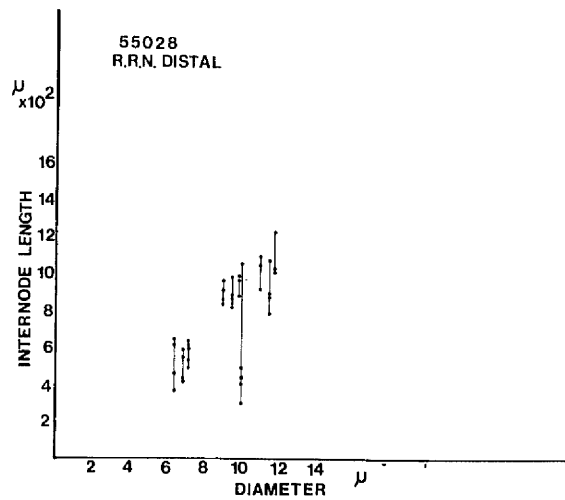
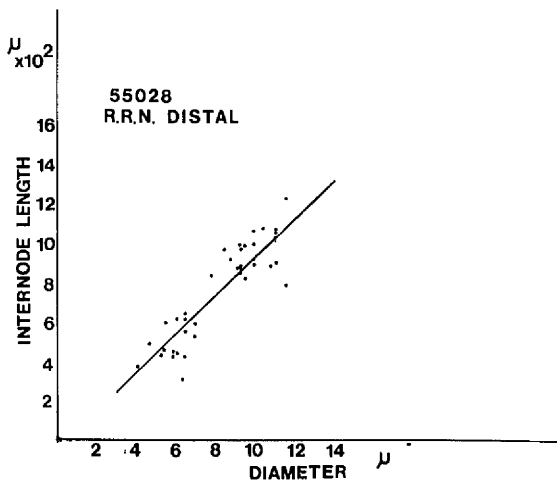
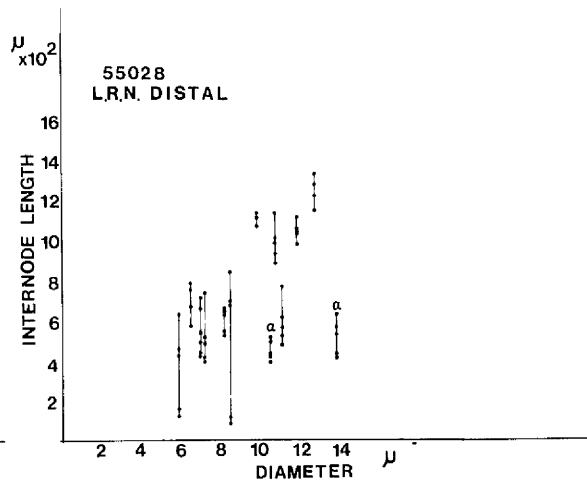
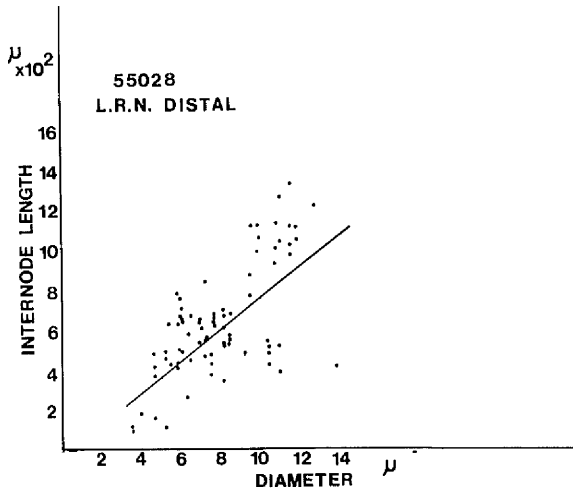
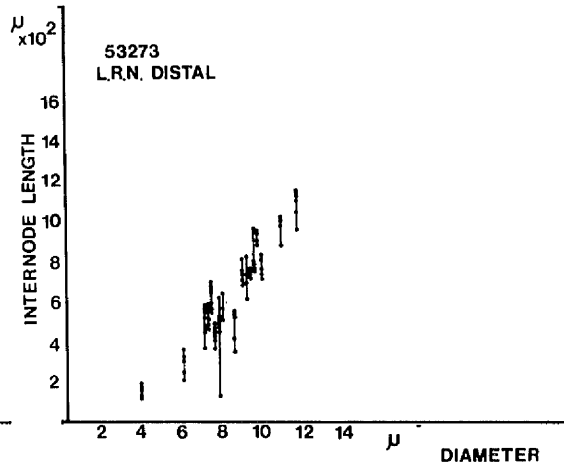
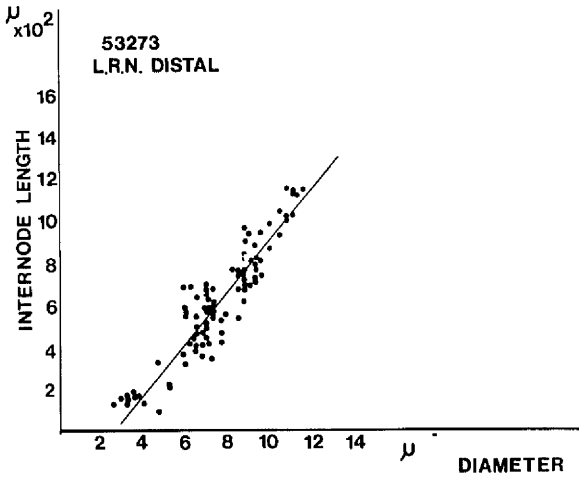


Fig. (76): Single fibre and scatter graphs of limb peripheral nerves of a normal, old pony (55028) and a sub-clinical case (57510).

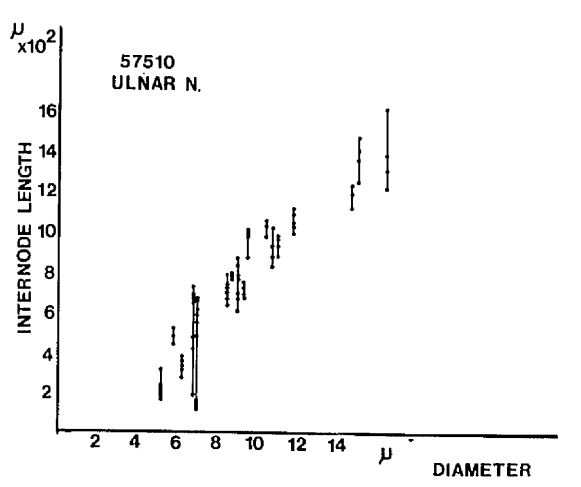
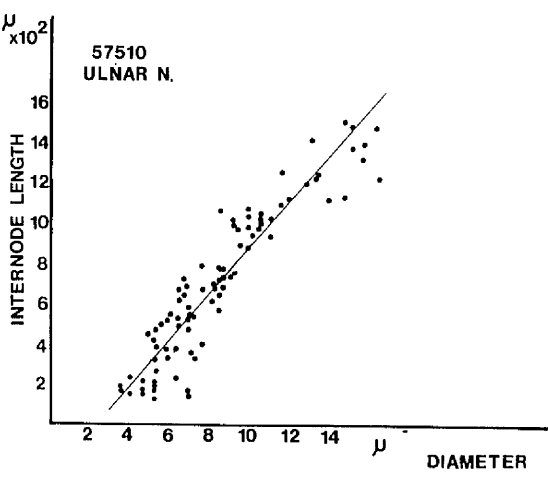
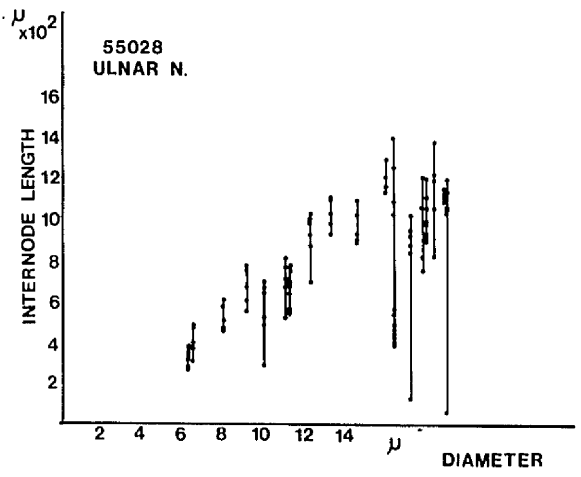
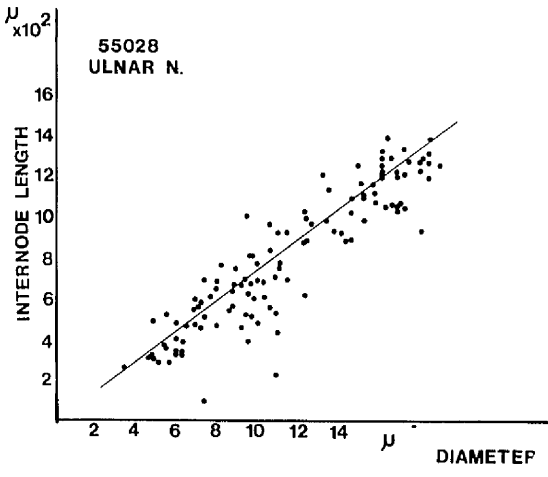
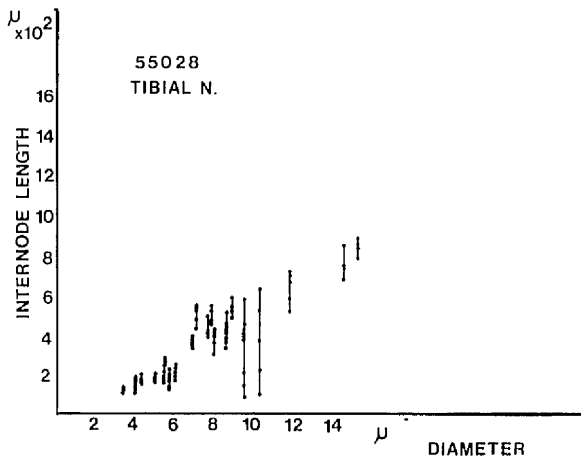
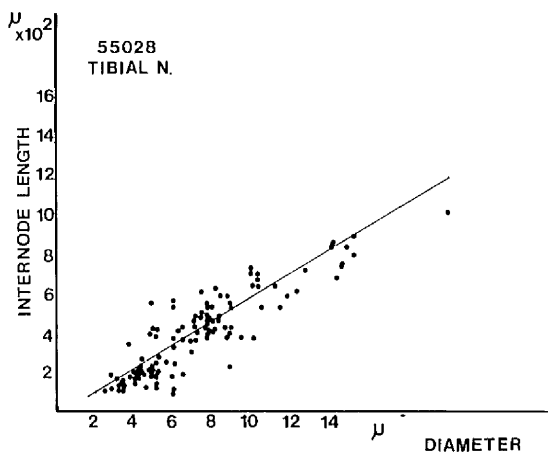


Fig. (77): Total number of fibres in the laryngeal branches  
at different levels of the left R.L.N. of three clinical  
cases.

Fig. (78): Myelinated nerve fibre density of the left R.L.N.  
at different levels in two clinical cases

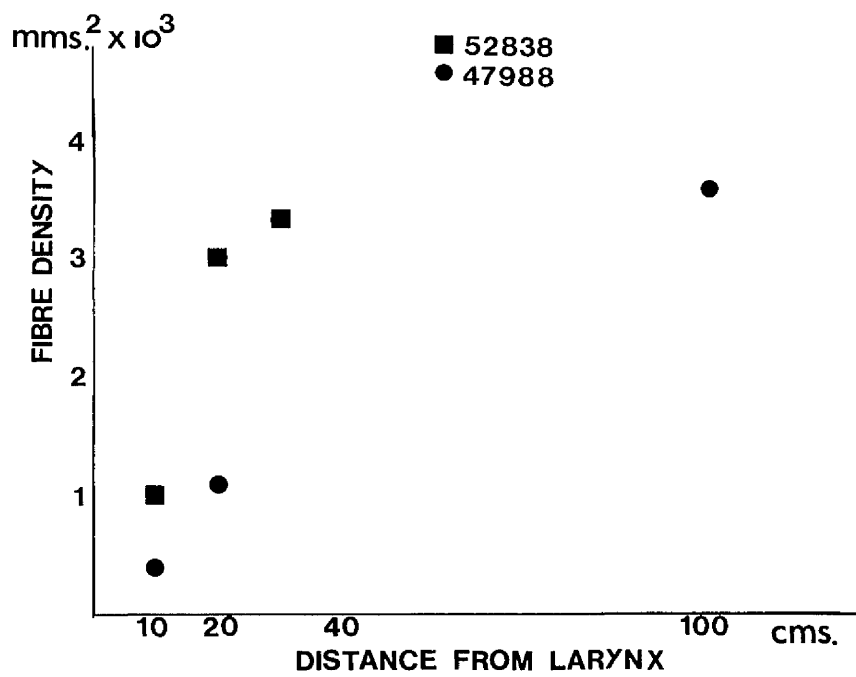
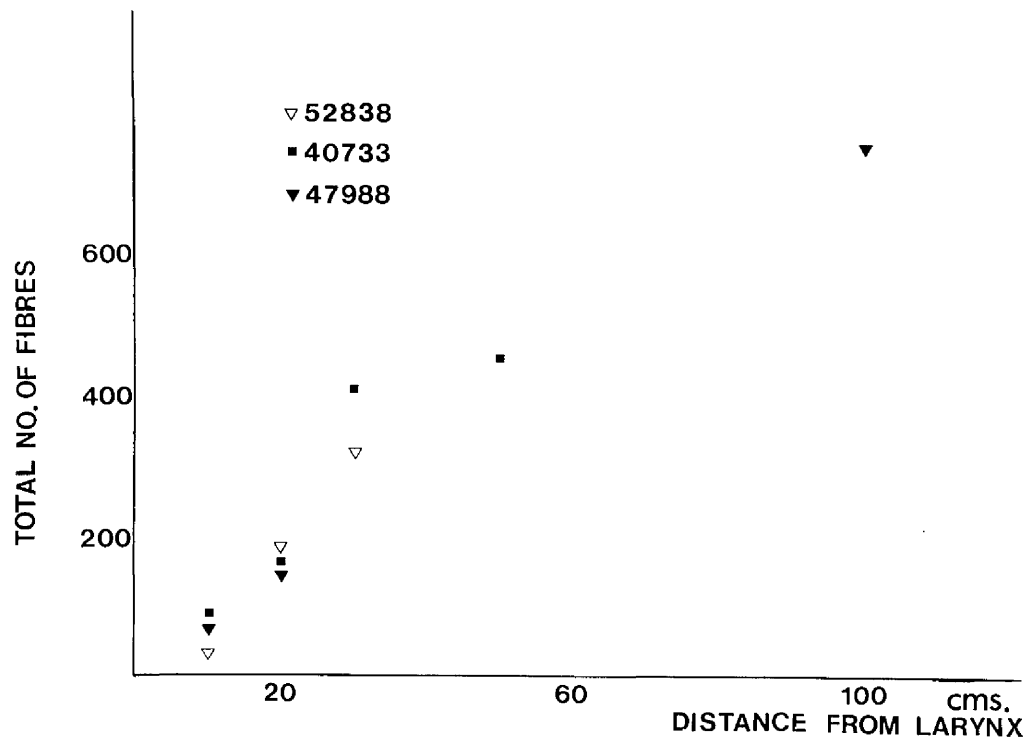
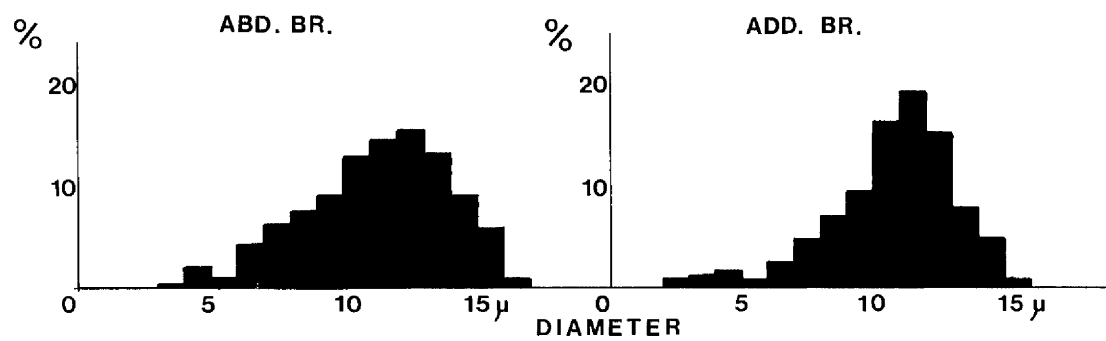


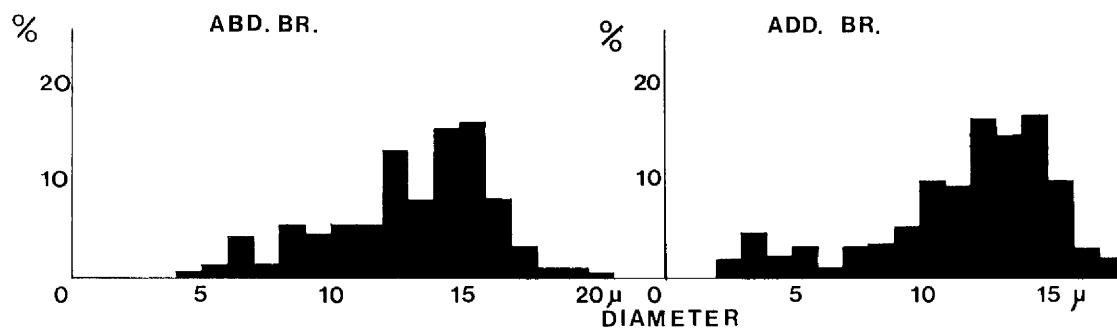
Fig. (79): Nerve fibre diameter spectra of the abductor/  
adductor branches of the left R.L.N. of aged pony.

Fig (80): Nerve fibre diameter spectra of the abductor/  
adductor branches of the right R.L.N. of a 15 year old pony.  
Note that there are more large diameter fibres than in fig.  
(79).

59186



59170





Figs. (81 - 89): All are supravital methylene blue preparations

Some are montage preparations.

Fig. (81): Range of extrafusal end plates seen in normal laryngeal muscle (a, b and c) and the abnormal muscle of clinical and sub-clinical cases (d). Note the extreme complexity of (d).

(Me blue a, b & c X 300  
d X 500)

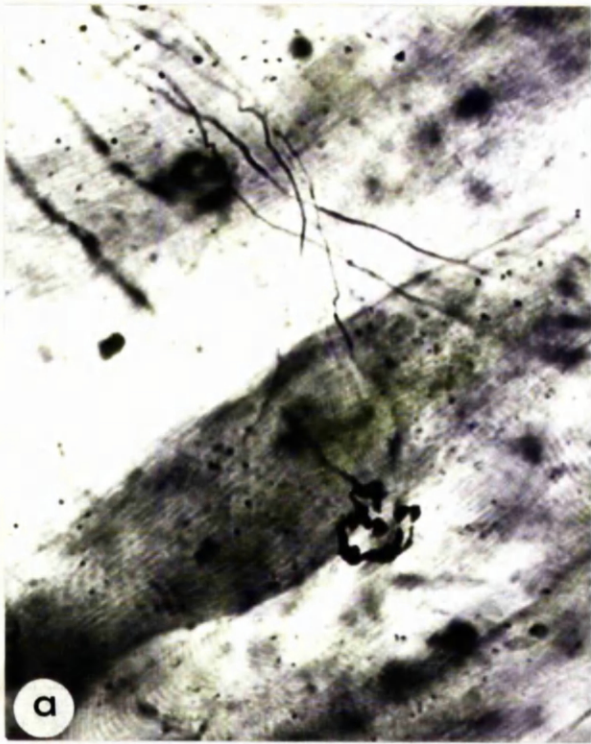


Fig. (82): a) and b)

These two end-plates vary somewhat in structure  
b) having finer terminal expansions with much slimmer  
terminal axons than a).

(a, b X 400)

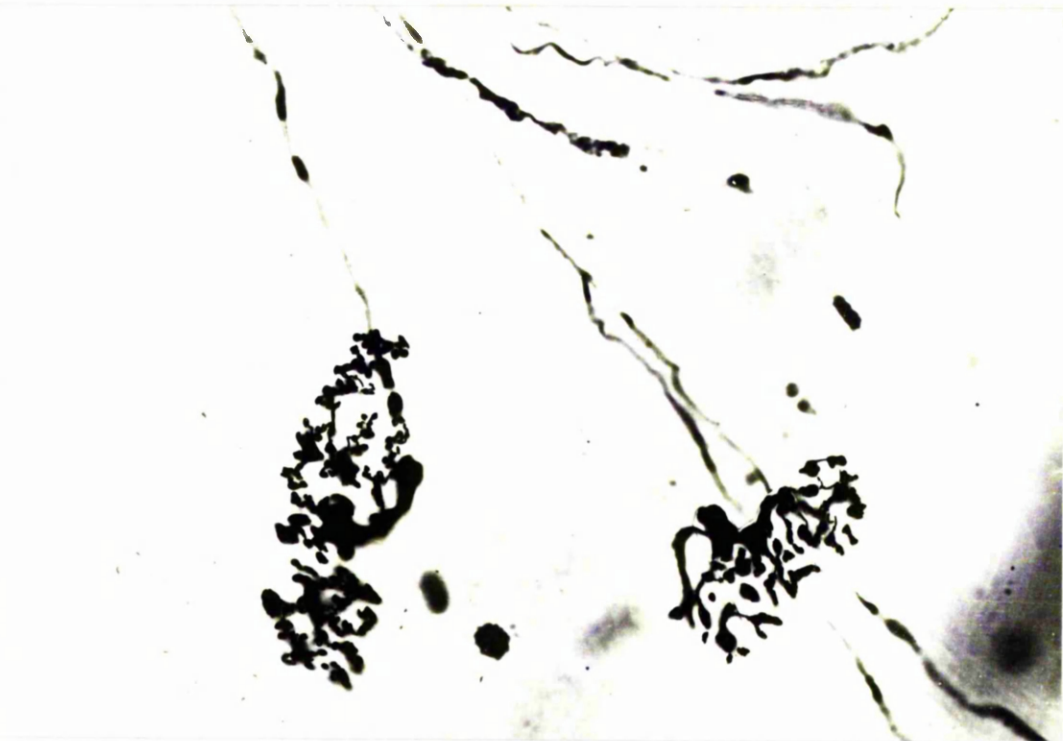
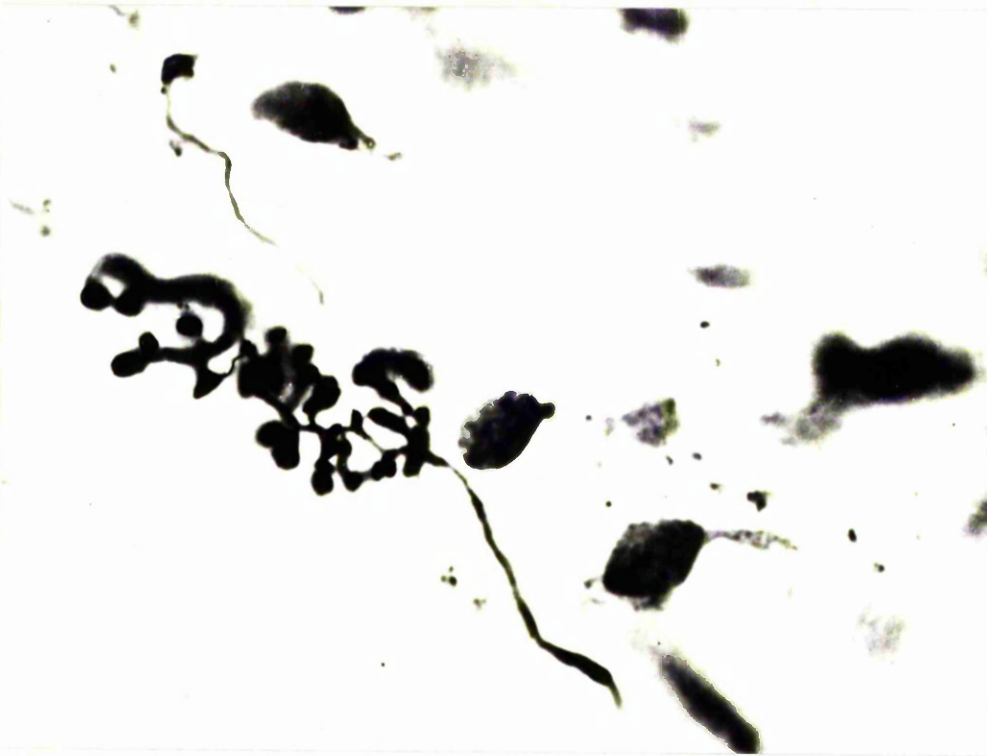


Fig. (83): An end-plate in which all the terminal expansions appear to lie on one side of the sub-terminal or terminal nerve fibre.

(X 500)

Fig. (84): Within this field, three types of end-plates can be seen. The arrow indicates an end-plate which has split into two, each end-plate possibly supplying a different muscle fibre.

(X 400)

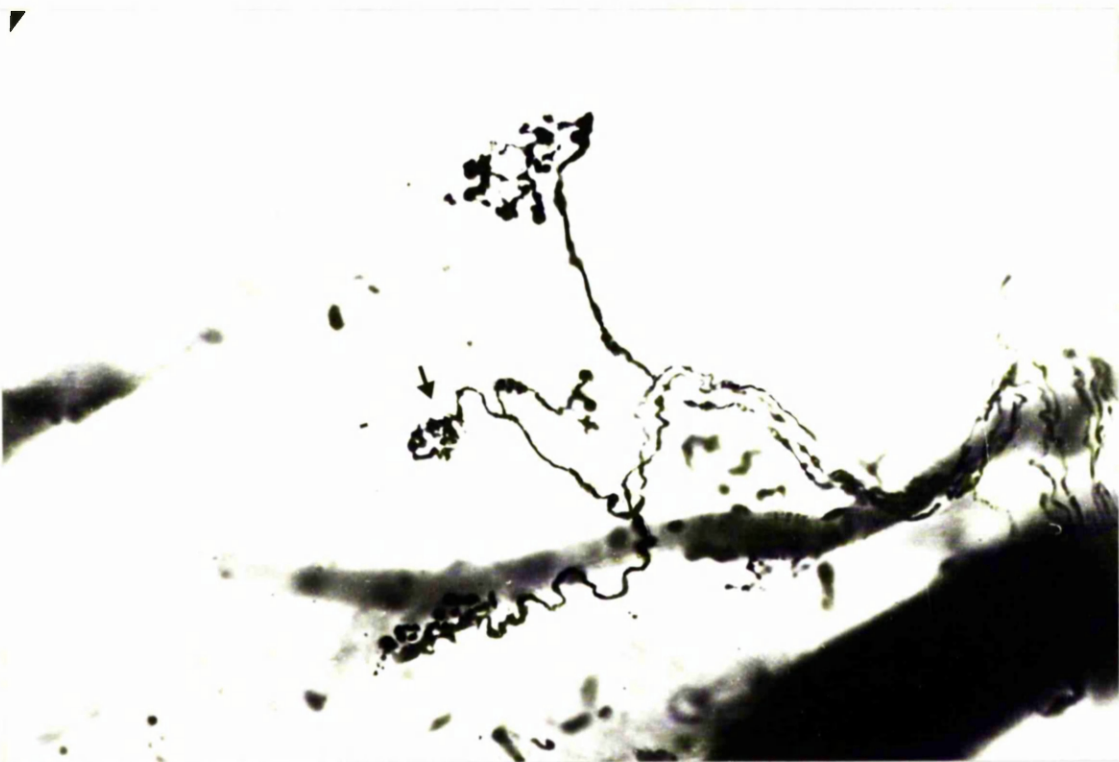


Fig. (85): Different types of double end-plates. Type c were commonly found in both normal and sub-clinical cases.

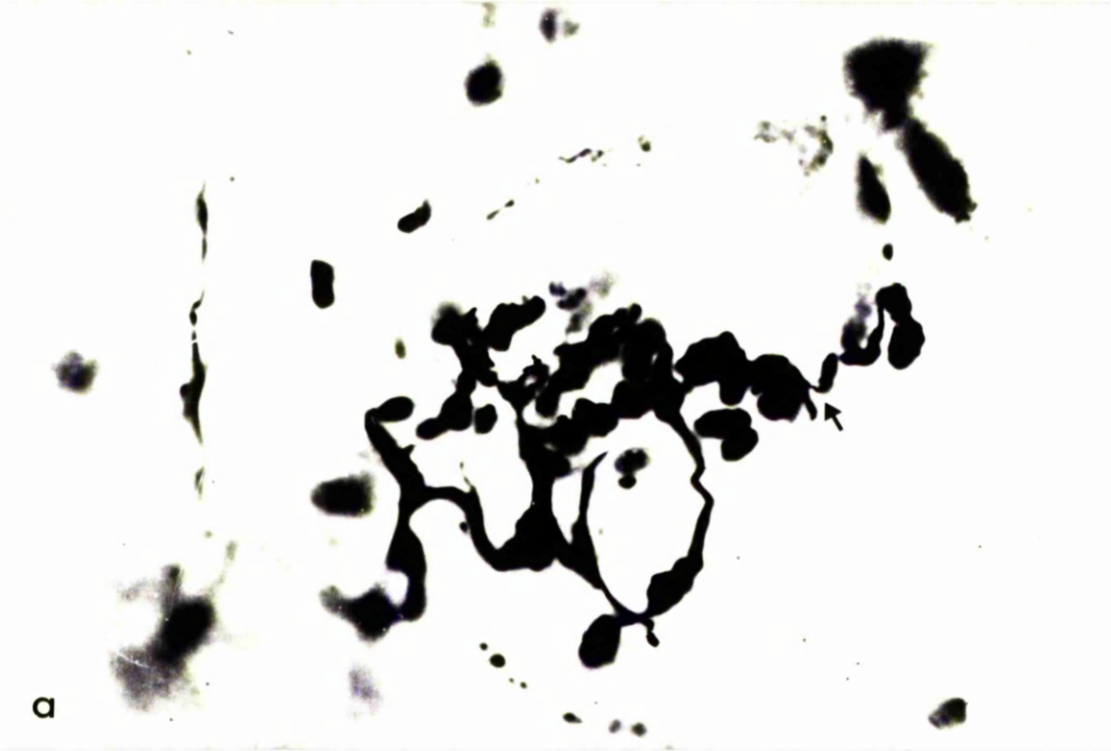
(a, b X 450, c X 400)



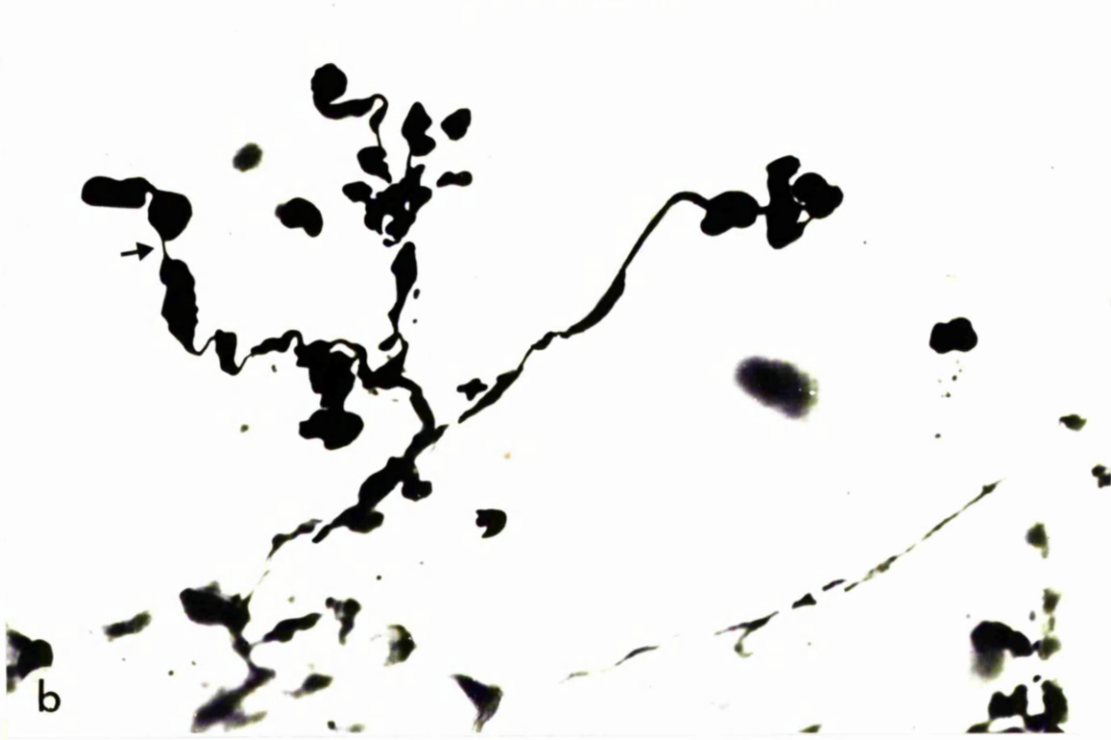


Fig. (86): Bizzare endings from sub-clinical cases in which there was flattening of the terminal axon. In (b) one of the end-plates has split to form a double end-plate. The arrows mark possible ultra-terminal sprouts.

(a, b X 500)



a



b

Fig. (87): a) Camera lucida drawing of a sprouting complex from a sub-clinical case in which there was marked evidence of fibre type-grouping.

(X 120)

b) A sprouting complex from a sub-clinical case. There is a variation in morphology of the endings; in one end-plate, the terminal expansions are at the end of very fine 'twig-like' terminal axons (arrow).

(X 400)

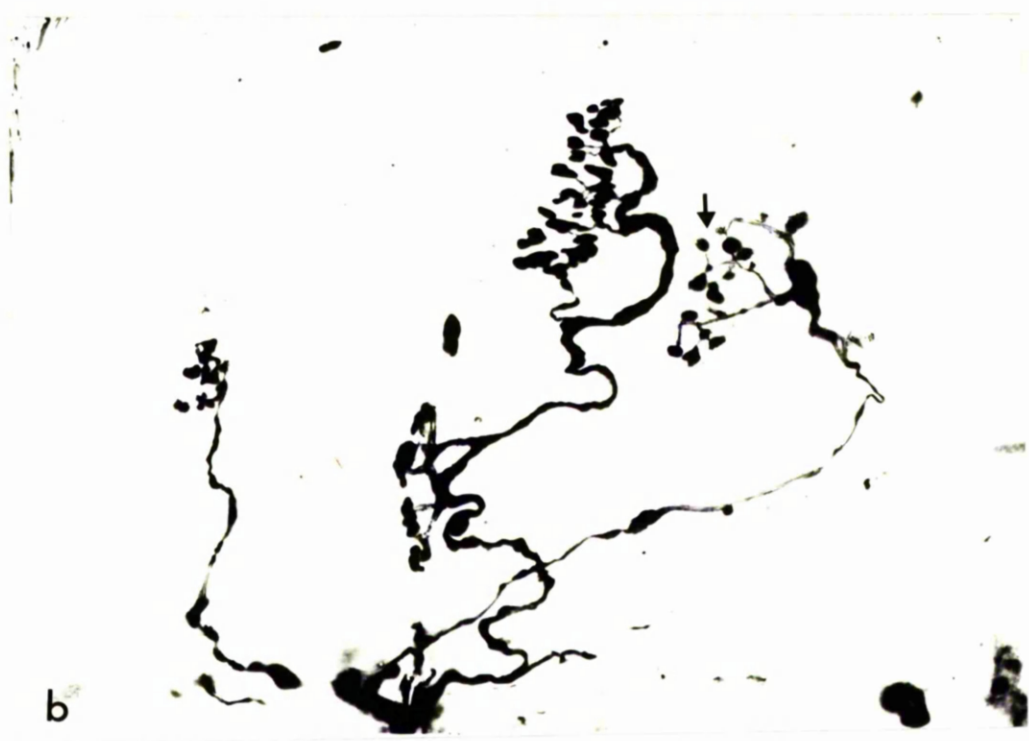
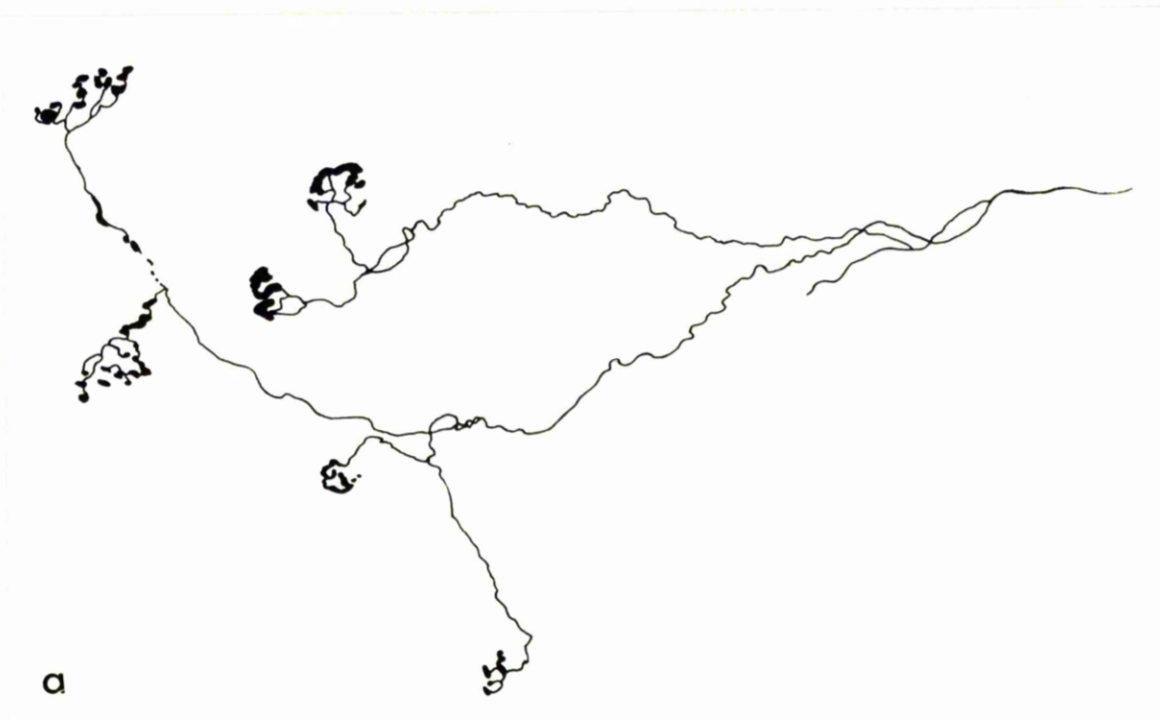
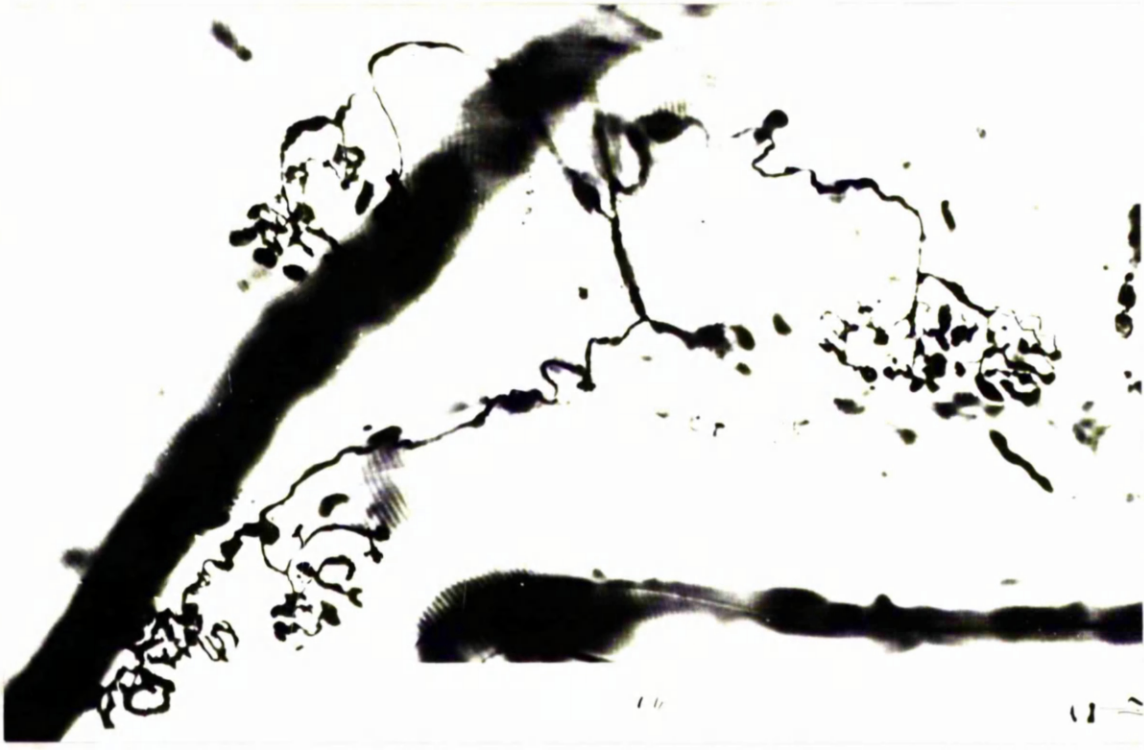


Fig. (88): A sprouting complex for a sub-clinical case. Each of the endings appears to have split into double if not triple end plates.

(X 400)

Fig. (89): A sprouting complex from the right side of a clinical case in which there was evidence of fibre type-grouping.

(X 400)



GENERAL DISCUSSION

It is apparent from the current study that a large number of clinically normal horses have histological evidence of neurogenic atrophy of the muscles of the left side of the larynx, and such cases have been classified in this study as suffering from sub-clinical disease. The possibility of isolated adductor paralysis as judged by the endoscopic findings in two cases must also be considered. It seems possible from both clinical and pathological studies that three stages of the disease may be recognised 1) sub-clinical 2) adductor paralysis 3) total paralysis of the left vocal cord. It is not certain whether all horses which develop total unilateral paralysis, will always have suffered previous loss of adduction alone, but it certainly appears that this is so in a number of cases. It is also interesting to speculate whether or not some or all of the sub-clinical cases would have progressed to exhibit clinical evidence of disease had they lived sufficiently long. This is not known, but it may well be that ageing can also be a primary cause of the disease as two old ponies under 14.2 hands (one was slaughtered recently and was not included in the survey figures) were found to be early sub-clinical cases. This was unusual as all the other small ponies in the survey were normal, and past reports have always denied the existence of the disease in equines less than 15 hands high.

The age range in this study of both clinical and sub-clinical disease was from 6 months to 20+ years. The finding of disease in two young thoroughbred foals could suggest the possibility of the disease being congenital in origin, becoming progressively worse with age. However, the finding in the two old ponies, of early left sided neurogenic atrophy, would suggest that not all cases are congenital and that ageing alone may produce a similar lesion.

Clinical/



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Clinical evidence of laryngeal hemiplegia is most frequently seen in the young horse (3-5 years) and thus it appears likely that the first evidence of neurogenic atrophy in the clinical, and possibly sub-clinical cases normally begins at an early age. This age group (3-5 years) coincides with the period during which the horse is most likely to be under maximum stress and any inspiratory dyspnoea will be obvious. Most authors would deny that clinical signs of the disease are diagnosed for the first time in later life, but this may be due to less severe exercise with age so that any inspiratory dyspnoea may not be noticed, or may even be mis-diagnosed.

As has been discussed earlier (section 2), there is abundant evidence from the histopathology of the laryngeal muscles, that the muscle lesion is due to repetitive cycles of denervation and reinnervation. Paralysis of the muscle involved only occurs when reinnervation fails to compensate for denervation. The finding of focal secondary myopathic change in affected muscles suggests that the process is of marked chronicity. Evidence of this process of denervation/reinnervation was also found in the motor end-plate studies in which collateral sprouting was a feature. Such sprouting increases the size of the motor units in affected muscles, and the number of their adjacent histochemically homogeneous component muscle fibres (fibre type grouping).

The previous suggestion of the possibility of isolated clinical adductor paralysis was derived from the qualitative and quantitative muscle fibre studies. In the qualitative studies where a scoring system was used to classify the muscle changes, it was always noted that changes were more severe, and appeared earlier, in the adductor muscle studied than in the abductor. This was also the case where a lesion was found on/

on the right side. In early clinical disease this was also thought to be the case, but eventually it was found that the degree of atrophy in both was almost equal. The quantitative studies have also shown that preferential atrophy of the adductor muscles occurs and that both atrophy and hypertrophy (as measured by the variance of muscle fibre area) are more marked in adductor than abductor muscles. The histograms of muscle fibre area demonstrate the various stages of the disease both where atrophy and hypertrophy are found (sub-clinical disease) and where atrophy predominates (clinical disease). This is in direct contradiction to Semon's Law and to previous work in the horse.

Studies on the mean muscle fibre area have shown that it was possible for fibre area to be larger in the diseased muscle than in the control, due to hypertrophy in the former. In the case of chronic left sided disease there may be hypertrophy of the normal left cricothyroid muscle. As there was no atrophy in this muscle, this hypertrophy could be due to a physiological compensatory response to atrophy in the other adductor muscles.

The neurogenic origin of the muscle lesion was confirmed in the histological studies of the recurrent laryngeal nerves, in both the clinical and sub-clinical cases. The main findings on routine light microscopy were a decrease in myelinated fibres with concurrent endoneurial fibrosis. Renault bodies were found in the laryngeal nerves but their significance is not known. All other peripheral nerves examined were normal. Studies on single fibre preparations showed that demyelination and remyelination were commonly found in the laryngeal nerves but little or no active axonal degeneration was seen. It is possible that demyelination was secondary to axonal degeneration and this/

this possibility has been discussed in detail (section 4). These changes were found in both laryngeal nerves and in other peripheral nerves, but were proved statistically to be more prevalent in the left recurrent nerve. Demyelination and remyelination may also become more frequent with age.

Quantitative studies on the myelinated nerve fibre population have shown that in both the clinical and sub-clinical case, there is a progressive, distal decrease in total number of myelinated fibres, this being strong evidence of the occurrence of a "Dying-back" phenomenon. The left recurrent laryngeal nerve is the longest motor nerve in the horse, it is an unbranching motor nerve and the disease process preferentially affects large diameter fibres. All of these add weight to the "Dying-back" hypothesis.

It may well be that some of the qualitative or quantitative findings in this study, indicate the possible aetiology of the disease. Both the muscle and nerve pathology show that this disease is a chronic peripheral neuropathy in which a number of features are outstanding.

- 1) The neurogenic atrophy occurs primarily, and more severely, in the adductor muscles of the left side of the larynx.
- 2) There is strong evidence of a "Dying-back" nature of the disease in the left recurrent nerve.
- 3) The right side of the larynx may also be involved.

In addition three other facts may be of great significance -

- 1) Very young thoroughbreds can be sub-clinically affected.
- 2) The left recurrent nerve is the longest equine motor nerve.
- 3) No evidence of a generalised peripheral neuropathy has been found.

If the disease is due solely to the length of the nerve, this could explain/

explain the early susceptibility of the adductor muscles as the nerve supplying this group of muscles has 2-3 cms further to travel than to the abductor. One other possible explanation for this finding is that the motor units in the adductor are larger than in the abductor, but this would not explain the apparent difference in timing of onset, but could account for an apparent difference in severity. Lastly, it may be that entrapment or compression of the adductor branch occurs as it passes between the crico-pharyngeus and dorsal cricoarytenoid muscles.

The presence of early neurogenic atrophy on the right side of the larynx adds a new dimension to the problem, yet may make it easier to understand. If such a disease were purely unilateral then anatomical factors would probably form the only explanation but the present known facts do not fit this case. However, the possibility of aortic arch compression must still be considered, as must the combination of this with some other type of neuropathy. This could be a hereditary neuropathy of the "Dying-back" type, but if the former is true then on the evidence from the cases seen in this study, the inherited defect is widespread if not endemic in larger equines. There are many other causes of neuropathies of the "Dying-back" type. Among them is thiamine deficiency, and at present it cannot be ruled out that this may play a part in the condition.

It is inevitable that a study such as this produces more problems than answers. Nevertheless, it seems that the work has indicated problems hitherto unknown and the possible methods of solving some of these. If some such answers were forthcoming undoubtedly more would be known about the pathogenesis of equine laryngeal hemiplegia and might lead to identification of its aetiology.

It/

It is suggested therefore that future investigation should follow certain lines -

1) Statistical quantitative teased fibre methods are available to determine whether or not the demyelination and remyelination seen in the laryngeal nerves is secondary to axonal change.

2) Electrophysiology to demonstrate whether or not there is any slowing of motor nerve conduction velocity with disease of the recurrent laryngeal nerves and any difference in terminal latencies between the groups of muscle.

3) Further gross studies to confirm or refute compression of the left recurrent nerve at the aortic arch, and evidence of a reversal of the bulbs and tapers in simple teased fibre preparations on the opposite side of the aorta. Similar compression may be taking place at the larynx and any proof of this on single fibre examination would explain the disparity of the effect of the disease on the abductor/adductor groups of muscles.

4) Electron microscope studies to look for regeneration or "onion-bulb" formation in the laryngeal nerves at any level, and E.M. studies of single teased fibres in which bulbs and tapering segments were seen.

5) Demonstration of the innervation ratios of the laryngeal muscles in both normal and affected muscles using methylene blue preparations.

6) Use of the 'enclosed fibre' method to demonstrate the number of histochemically similar fibres which can be found adjacent to each other within a normal laryngeal muscle. It may also be useful in the detection of early sub-clinical cases, where minimal fibre type grouping (without atrophy) is suspected.

7) To establish the normal myelinated nerve fibre spectra of laryngeal nerves and show how they are modified by age.

These/

These seven areas for study have occurred to the author as the work progressed and were not apparent at the onset. It seems more than possible that attempts to investigate those seven aspects will in turn pose even more questions and thus provide a continuum of investigation.

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APPENDIX

Enzyme Histochemistry MethodsA. Succinic dehydrogenase (after the method of Nachlas et al 1957)

- 1) Incubate fresh sections for 20 mins. at 37°C in air.
- 2) Wash in saline and allow to dry for 1 hour.
- 3) Fix in 4% BNF for 10 mins.
- 4) Rinse in 15% alcohol for 5 mins.
- 5) Mount in glycerine jelly or hydramount.

Substrate solution

Stock buffered succinate: Combine equal volumes of 0.27 phosphate  
pH 7.6: 0.27 Sodium succinate.

Incubating medium

add 10 ml of stock succinate solution to 10 ml aqueous solution  
of nitro BT (1 mg/ml)

B. N.A.D.H. diaphorase (after the method of Scarpelli et al 1968)

- 1) Incubate sections in medium for 20 mins. at 37°C.
- 2) Fix in 4% BNF for 15 mins.
- 3) Rinse in distilled water.
- 4) Counterstain for a few minutes in Mayers haematoxylin.
- 5) Wash.
- 6) Mount in glycerine jelly.

Stock solution

MTT (1 mg/ml)	2.5 ml
Cobaltous chloride 0.5M	0.3 ml
0.2M Tris buffer (pH 8.2)	2.5 ml
Distilled water	4.1 ml
7% Polyvinyl pyrrolidone	0.75 ml

adjust pH of final solution to 7.2 with Tris buffer.

Incubating/

### Incubating medium

To 1 ml of stock solution add 6 mg of reduced diphosphopyridine nucleotide.

### C. Phosphorylase (after the method of Takeuchi 1955)

- 1) Incubate sections in medium for  $1\frac{1}{2}$  hours at  $37^{\circ}\text{C}$ .
- 2) Rinse quickly in water and dry at  $37^{\circ}\text{C}$  for a few minutes.
- 3) Fix sections for 2 mins. in absolute alcohol and dry in air.
- 4) Immerse in dilute gram's iodine - 1:10 with distilled water until blue colour appears (usually 1-2 minutes).
- 5) Drain and mount wet sections in iodine glycerol - 1:1. Seal with Glyceel.

The phosphorylase reaction fades totally after a period, but reimmersion of the sections in dilute gram's iodine will give the same result as before.

### Incubating medium

Glucose - 1 - phosphate (potassium salt)	16.7 mgs.
Adenosine - 5 - phosphate	3.3 mgs.
Glycogen	0.6 mgs.
Distilled water	5 mls.
0.1 M Potassium acetate buffer pH 5.8	3.3 mls.
Insulin (20 units/ml)	1 drop
Reagent ethanol	1.7 mls.

Check and adjust pH to 5.8 to 6.0 with 0.1M acetic acid.

### D. Myosin ATP-ase (modification of several standard methods)

- 1) Incubate sections at pH 9.5 for 20-30 mins at room temperature.
- 2) Wash in 1% calcium chloride solution, 3 changes, 2 mins. each.
- 3) Treat with 2% cobaltous chloride for 3 mins.
- 4) Wash well in distilled water.
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- 5) develop for 1 - 2 mins in fresh 1% ammonium sulphide .
- 6) Wash in tap water.
- 7) Dehydrate, clear and mount in Permount (reduces the fading which occurs due to sections being unfixed).

In addition, sections can be further pre-incubated at an acid pH (4.3 and 4.6) in order to further classify the type II fibres.

- 1) Preincubate sections at pH 4.3 or 4.6 or 4.1 for 5 mins.
- 2) Wash in tap water, then proceed as above.

#### Acid preincubation solution

Walpoles acetate buffer pH 4.6, 4.3 and 3.9, 0.1M.

#### Substrate solution

0.2M Ca Cl<sub>2</sub> in 0.2M Tris buffer - 10 mls.

ATP (Sigma) - 20 mgs.

Adjust final pH of substrate to 9.5 with 0.1M Na OH. Substrate made fresh before use.

#### Tris containing Ca Cl<sub>2</sub>

100 ml Tris buffer pH 9.10 (0.2M). Add 4.381 gms Ca Cl<sub>2</sub>

It is vital in this reaction that the pH of the incubation medium is 9.5 and not above or below. It is also vital that the ammonium sulphide is fresh and contains no sediment.

#### Modification-

Two modifications to the above method were added to this method late in the study.

- 1) All sections were preincubated in the following solution -  
0.2M Calcium chloride (Analar) in 0.05M Tris adjusted to pH 9.5 with 0.1 NHCL before use. This was found to clear a "deposit" of unknown origin which often occurred in the sections, especially in the non-preincubated/



preincubated sections.

2) After treatment with 2% Co Cl<sub>2</sub>, the sections were washed in alkaline distilled water (65 mls H<sub>2</sub>O + 15 mls 0.17 Sodium barbiturate).

Peripheral Nerve Stains

A. Kultschitzky's method for myelin in nerve fibres.

- 1) Plate immediately into Flemings solution at R.T. for 24-48 hours.  
(cut nerve T.S. after being in fixative for a few hours).
- 2) Wash in running water overnight before embedding in paraffin.
- 3) Cut 5  $\mu$  sections and stain 12-24 hours in Kultschitzky's haematoxylin.
- 4) Wash and differentiate 20-30 secs. in .25%  $KMnO_4$  (or until white matter is distinguishable from grey).
- 5) Transfer to Pals bleach for 30 secs to 3 mins.
- 6) Wash well in running water, dehydrate, clear and mount in D.P.X.

Results - Myelin sheaths stain black.

Solution - Kultschitzky's haematoxylin.

10% alcoholic haematoxylin (ripened)	10 ml
Glacial acetic acid	2 ml
Distilled water to make	100 ml

B. Methylene blue technique for end-plates.

- 1) The oesophagus was reflected off the dorsal aspect of the larynx. The motor point of the CAD was located using a Devices Isolated Stimulator (2533), using the method of Coers Woolf (1959). End-plates from the other laryngeal muscles were located similarly.
- 2) A strip of muscle from the motor point was removed and immediately impregnated with methylene blue (concentration 0.3 gms/litre) until uniformly blue. This must be done in less than 5 minutes.
- 3) The muscle is oxygenated between 2 swabs soaked in saline at a flow rate of approximately 4 litres/minute for 1 hour.
- 4) The surface of the muscle is brushed with 50% alcohol to remove any debris.
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- 5) Fix overnight in 8% ammonium molybdate (filtered and cooled to 4°C before use) overnight at 4°C.
  - 6) Wash for  $\frac{1}{2}$  hour in distilled water - at least 4 changes.
  - 7) Cut the muscle with small, sharp pointed dissecting scissors into strips 1 mm wide and place on clear microscope slides and squash the preparation firmly.
  - 8) Examine the slides under the microscope to locate nerves and end-plates. Mark the area on the slides.
  - 9) Squash slides again for 1 hour under a heavy weight.
  - 10) Separate the slides, and cut out the required piece of muscle using a very sharp blade, keeping the specimen as flat as possible.
  - 11) Dehydrate the specimens through 4 changes of absolute alcohol - 15 minutes each. Clear and mount in D.P.X.

ABREVIATIONS

- CAD = Cricoarytenoideus dorsalis muscle
- CAL = Cricoarytenoideus lateralis muscle
- CT = Cricothyroid muscle
- RLN = Recurrent laryngeal nerve

