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Signed:

INVESTIGATIONS ON THE INNERVATION OF THE DIAPHRAGM IN CATS AND RODENTS WITH SPECIAL REFERENCE TO THE PHRENIC NERVE IN CATS.

by

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- being a thesis submitted in fulfillment of the requirements for the degree of Doctor of Medicine in the University of Glasgow.

SUMMARY.

This thesis is a morphological study of the innervation of the diaphragm in cats and rodents. The observations are based on preparations of normal material as well as material taken at different periods following selective partial denervation of the diaphragm.

At the outset it was considered desirable to carry out quantitative investigations on the fibre components of the phrenic nerves in normal animals so that criteria could be established for subsequent comparison of neurohistological observations. Myelinated fibres within the nerve were clearly demonstrated in osmium preparations and quantitative studies, carried out at three separate levels, indicated a distinct increase in fibres of larger diameter (12-18μ) in the peripheral third of the intrathoracic part of the nerve trunk. This observation raised the possibility that the phrenic nerve receives an accession of fibres from an extra-phrenic source close to its termination within the thorax. No correspondingly reliable method could be found for quantitative studies on the smallest neural components which are commonly called unmyelinated fibres. It was necessary, therefore, to devise a more reliable method before any systematic investigation could be undertaken.

A new ultrastructural technique for demonstrating the components within a transverse section of the phrenic nerve in cats was developed and its mode of employment is described. Its evaluation and characteristics are discussed in the light of previous histological findings. The new method can be used to determine the absolute numbers of nerve fibres and vascular components present at one particular level within the phrenic nerve.

The general arrangements of the diaphragmatic plexus, demonstrated by silver impregnation of whole preparations in mice and rats were compared with the findings in frozen sections of the diaphragm in cats. Detailed studies on the participation of various peripheral nerves in the innervation of the diaphragm can be demonstrated only after selective denervation. It was necessary, therefore, to establish the time course of the changes following these operative procedures and to determine the optimum survival period applicable to this method of investigation.

After unilateral intrathoracic transection of the phrenic nerve in cats, a small number of axis cylinders persist within the otherwise degenerating portion of the nerve peripheral to the level of the lesion and within the area of the diaphragm which is normally supplied by the homolateral phrenic nerve. This is in general agreement with the peripheral increase in numbers of myelinated fibres found within the phrenic nerves of normal animals and further experimental procedures were designed to investigate the possible existence of such an accession and to define its source. While unilateral extirpation of the coeliac ganglion induced very limited changes within the diaphragmatic nerve plexus, ligation of the dorsal roots of the caudal six thoracic segmental nerves central to the spinal ganglion indicated widespread participation of these nerves in the innervation of the diaphragm.

Finally, following separation of the peripheral third of the intrathoracic part of the intact phrenic nerve from its subjacent connective tissue, degenerative changes were observed in the nerve fibres within the homolateral diaphragmatic plexus. In view of these findings it seems reasonable to suggest that the phrenic nerve receives an accession of nerve fibres at this level and that they may arise from the spinal cord in the region of origin of the caudal six thoracic segmental nerves.

ACKNOWLEDGMENTS.

It is a pleasure to record my indebtedness to the members of the Department of Anatomy in Monash University who have assisted in the preparation of this thesis. To Professor Graeme C. Schofield my special thanks are due for his guidance and helpful criticism, during the course of the investigation, which proved to be of great value.

I am most grateful to Professor R. E. Coupland and his colleagues for the hospitality extended to me in the Department of Anatomy at Queen's College, Dundee, during my sabbatical year. It was of inestimable value to discuss with them, histochemical methods which have recently been developed for the investigation of the peripheral nervous system.

I am also indebted to Dr. Jane Wilson for her invaluable assistance in the final compilation of written material and to Mrs. J. Baillie for her generous help with the bibliography.

To Mr. J. S. Simmons, Miss S. Flett and Miss W. Kemp I am indebted for technical assistance at various stages in the preparation of this thesis. For technical help in ultrastructural studies, I am grateful to Mr. J. Preston and Dr. D. G. Silva.

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INTRODUCTION.

The structural and functional characteristics of the peripheral nerves which ramify in the substance of the diaphragm have been the subject of many investigations for more than a hundred years. Two major factors have been responsible for maintaining interest in this problem; the importance of the diaphragm in the essential function of respiration and the complexity of its embryological development. Problems concerning the nerve supply of the diaphragm, which were investigated by physiologists and anatomists during the earlier part of this century have been reinvestigated frequently in the intervening period but, from the inconclusive and frequently contradictory statements which have been made, it is apparent that many of these problems remain unsolved.

One of the main reasons for divergent opinions between authors appears to be the large variety of methods used to study the innervation of the diaphragm. While some investigators restricted their studies to macroscopic observations on material obtained from normal individuals at autopsy, others considered only such circumscribed facets as the afferent fibres of the phrenic nerve. Very few of the existing accounts of morphological investigations can therefore be regarded as comprehensive studies on the innervation of the diaphragm and many of the conclusions are unsupported by photographic evidence.

Those histologists who have investigated the neural components of the diaphragm have employed such agents as methylene blue, and the salts of heavy metals such as silver and gold. The unreliable nature of the results obtained from the application of these methods to tissues from normal animals led to disagreement between the investigators and may have contributed to their seemingly general reluctance to apply these histological methods to experimental investigations. It is evident, therefore, at the commencement of this study, that methods for the demonstration of neural elements within the diaphragm would require to be based on previous successful investigations on the nerve fibres present within tissues other than the diaphragm.

Schofield (1960), in his investigation of the innervation of the alimentary tract in rats, besides evaluating the various techniques available for the demonstration of neural structures, evolved a method of silver impregnation which was applicable to studies on tissue from both normal animals and animals in which degenerative changes had been evoked by interruption of nerve trunks. This method offered several advantages in the present study. It was found to stain neural tissue selectively and consistently and it could be applied with ease to thick sections of tissue. The general relationships between nerve fibres and other components of the diaphragm could thus be observed more easily and their source more accurately defined. With minor modifications it was possible to apply this technique to the study of whole preparations of the diaphragm in small mammals.

As morphological studies on the phrenic nerves formed an important part of this investigation, the various techniques available for the demonstration of fibre components of peripheral nerves were examined. It was found that, while myelinated fibres could be studied satisfactorily both qualitatively and quantitatively in osmium preparations there was no corresponding reliable method for the demonstration of unmyelinated fibres. Each of the silver methods employed for the demonstration of axis cylinders was found to enable qualitative observations to be made but consistent quantitative estimates of unmyelinated fibres could not be obtained. The inconsistency of these findings was considered to be due to the size of the unmyelinated fibres many of which are beyond the resolving power of the light microscope. The possibility of studying these smaller fibre components with the electron microscope was examined and it resulted in the evolution of a method which enabled accurate quantitative observations to be made on the nerve fibres and vascular structures at a particular level within the trunk of the phrenic nerve.

The objectives of the present investigation were to study those portions of the peripheral nervous system which are related to the diaphragm and to carry out comprehensive observations on their ramifications within its substance.

The investigations which have been undertaken in normal and partially denervated animals can be summarised as follows:-

i) The structural features of the components of the peripheral nervous system which contribute to the innervation of the diaphragm, including their intra-diaphragmatic ramifications.

ii) The distribution, within the diaphragm, of nerve fibres derived from each of the contributing components of the peripheral nervous system.

Certain features of the innervation of the diaphragm have been demonstrated with the aid of silver impregnation and the findings were confirmed, wherever possible, by employing other specific techniques such as cholinesterase methods. In detailed studies on the phrenic nerve the newly developed ultrastructural technique provided much valuable information on the quantitative aspects of the phrenic nerve. It is apparent, however, that this investigation has given rise to other problems which, it seems likely, can only be solved by wider application of ultrastructural methods to the study of the peripheral nervous system.

REVIEW OF LITERATURE.

Earlier Studies on the Innervation of the Diaphragm.

The history of the study of the phrenic nerve and diaphragm, appears to extend at least as far as the time of Hippocrates, (400 B.C.), who, according to Geneau de Mussy (1853), associated subdiaphragmatic pain with pain in the clavicular region.

Aristotle (360 B.C.) also examined the relationship between the heart and the septum transversum or diaphragm. In his "Historia Animalum" translated by Thompson (1910) he commented on the presence of a heart in all animals that possess a septum transversum.

In the works of Galen (200 A.D.), several references are made to the diaphragm and the phrenic nerves. These facts are recorded by Duckworth (1948) in "Galen on Anatomical Procedures". Galen examined the origin of the phrenic nerve, and commented on its connections with the cervical region of the spinal cord. In addition, he studied the functions of the diaphragm, which he knew to be associated with both respiration and the production of speech. Geneau de Mussy stated that Galen had clinical interest in the phrenic nerve and its ramifications for he associated certain forms of pain in the cervical region, with maladies of the liver.

As physician to the gladiators, Galen observed, at first hand, the effects of injury to the spinal cord in the cervical region. He noted the clinical signs produced by such injuries at different levels and derived conclusions regarding the spinal level of origin of the motor control of the diaphragm. These observations he later confirmed by experiment in animals. Furthermore, he appreciated that the phrenic nerve acted, not only as an efferent pathway, but also that it contained afferent fibres. This fact was debated over succeeding centuries until it was conclusively proved by Sherrington (1894).

From the time of Galen until 1050 A.D. there was apparently no major advance in the study of respiratory mechanisms, although, in the historical review by Fenn and Rahn (1965), five investigators who perpetuated the study of medical science are referred to. Of these, Oribasios of Byzantium (325-403), who preserved the works of Galen in an Encyclopedia of Medicine, and Alexander of Tralles (525-605), who presented a classical description of pleurisy, appear to have been outstanding figures; little is known, however, of the work of the remaining two investigators apart from their names which are Rhazes (860-1037) and Hali Abbas (died 994).

The name of Mundinus (1270-1326), is associated with the earliest organised dissection of human remains. He was possibly the first anatomist to perform dissections himself, previous workers having depended on assistants to do this task. Among the writings of Mundinus are his "Anathomia", in which most of the views expressed are attributable to Galen. No major advances in the study of respiration were presented.

With the advent of the Renaissance, the study of anatomy was influenced by pictorial representation of human remains. Artists responsible for the renewal of interest in anatomy included Verrochio, Leonardo da Vinci, Michelangelo and Rafael. Outstanding among these is Leonardo da Vinci (1452-1519), whose descriptions of human anatomy have recently been translated by O'Malley and Saunders (1952).

On the subject of the respiratory apparatus, da Vinci expressed the view that the diaphragm performs four functions:-

(1) It is the main inspiratory muscle.

(2) It acts as a 'compressor' on the stomach, aiding the expulsion of food from the stomach into the intestine.

(3) It acts, in conjunction with the abdominal muscles, in the process of defaecation.

(4) It separates the thoracic from the abdominal contents.

In these views, da Vinci is said to have been greatly influenced by the teachings of Mundinus.

Berengario da Carpi (1500) wrote "A Short Introduction to Anatomy", in which reference was made to the diaphragm. In this work, the structure of the diaphragm was investigated in a detailed fashion and its association with respiratory function was explained. The diaphragm was described as a 'panniculus' which separates the thorax and abdomen but permits the passage of various structures between those two cavities. He noted that there were arteries and veins associated with the diaphragm and stated that it was connected to the brain by three pairs of nerves; thus "two pairs come from the nape of the neck" (apparently the sympathetic trunks and phrenic nerves respectively) and "one from the brain" (the vagus nerves).

Vesalius (1514-1564) understood the structure of the diaphragm and other respiratory muscles. In the seventh of his "Tabulae" showing the body musculature, there is a small inset illustrating a human diaphragm in which several areas are clearly labelled. The two crura depicted are of the same length, both in the inset and in the figure accompanying it. According to O'Malley (1964), written descriptions accompanied each of the illustrations in the tabulae. To date, however, no legend on the diaphragmatic illustrations, has been discovered. Specific reference to the diaphragm is made by Vesalius in the dissection instructions given to students investigating the extent of the parietal peritoneum. A brief paragraph on the nerves of the diaphragm is also recorded in the study of the organs of respiration. The description of the procedure used in displaying these nerves, leaves no doubt that they are, in fact, the phrenic nerves. However, the use of the term 'phrenic' attributed to Bauhin, was not introduced until approximately one century later. Vesalius also studied the function of the diaphragm by exposing the costal pleura and, in 1543, he described the rapid collapse of the lung on opening the thorax.

Harvey (1578-1657) studied the diaphragm and defined three degrees of respiratory movement as follows:-

(a) Free respiration, in which the diaphragm and 'underlying' parts are used.
(b) Violent respiration, in which the intercostal muscles are active.
(c) Very violent respiration, in which the muscles of the abdominal wall and scapular regions are used.

Fallopius (1523-1563) and Fabricius (1533-1619) also described the respiratory apparatus in their works but made no substantial changes in the views put forward by Vesalius.

Bauhin (1600) introduced the term 'phrenic' to describe the principal nerves supplying the diaphragm.

Sylvius (1660) was first to express the principle of 'passivity' of the lungs in respiration implying that active respiratory movements were attributable to agencies other than the lungs themselves.

Gibson (1697) in his "Anatomy of Humane Bodies Epitomised" included a valuable account of the anatomy of the diaphragm. He referred to it as a 'digastric muscle', consisting of sterno-costal and vertebral parts and he discussed the structure of the muscle in the region of the oesophageal hiatus. He concurred with the views of Bartholin (1668) with respect to the vascular supply including the importance of the upper lumbar arteries.

The essential nature of respiratory function became more fully understood following observations by Malphigii (1628-1694) who discovered the structure of the lung alveoli, by Stephen Hales (1733) who recognised the significance of capillary vessels, and by Hooke (1667) who demonstrated the principle of artificial respiration. Indeed, Haller (1776) believed that, with the exception of the heart, the diaphragm is the most important muscle in the body.

Investigations on the central control of respiration followed on advances in the knowledge of the functions of the more peripheral portions of the respiratory apparatus. Thus, Lorry (1760) repeated the experimental work of Galen in an attempt to locate a "respiratory centre"; similar methods of study were also applied by Cruickshank (1797). Legallois (1811) made a series of transections in the medullo-pontine region of the brain and concluded that a respiratory 'centre' was located within the brain stem just caudal to the medullo-pontine angle. Flourens (1837) localised an area in the hind brain, the integrity of which was essential for the control of respiration; he referred to this region as the 'vital node'. It was situated at the apex of the calamus scriptorius and was less than one millimetre in diameter.

Gad and Marinesco (1892) and Arnheim (1894) also established the importance of the neural tissue in the floor of the fourth ventricle in the control of respiration.

Bell (1802) postulated that the portion of the spinal cord which lies between the roots of the spinal nerves (the middle column) might have a special governing influence over respiratory movements. Indisputable anatomical evidence for this view was subsequently found to be lacking and it was consequently abandoned. In Todd's Encyclopedia (1847), page 721R, it is stated that - "Among the nerves which Sir C. Bell had classed together as nerves of respiration, were some which had no necessary connection with that function. Of these, the portio dura and glossopharyngeal are examples".

Cruveilhier (1841) studied the diaphragm from the structural and functional point of view and reported his observations in his "Traité d'Anatomie Déscriptive" (page 503). Cruveilhier described the muscle sheet of the diaphragm as forming, with transversus abdominis, a continuous muscular sac which is interrupted only by the costal attachment. In discussing the effect of diaphragmatic contraction on the lower ribs, he stated that the ribs were drawn inwards when the diaphragm contracts; a statement subsequently debated by many authors (see below). The phrenic nerve, according to Cruveilhier, has no communication with the coeliac plexus nor does it give branches to the oesophagus. However, he observed a terminal branch of the right phrenic nerve which passed behind the inferior vena cava and communicated with the transverse branches of the left phrenic nerve before entering the muscle fibres of the right crus. According to Cruveilhier the terminal branches of the phrenic nerves may pierce either the thoracic or abdominal surface of the diaphragm. He also traced terminal twigs of the phrenic nerve within the diaphragm as far as the peripheral attachments of the muscle fibres. Cruveilhier made a particular study of the pericardium but was unable to identify nerves within it; however, the possibility of their existence within it was admitted.

Cruveilhier was not alone in observing partial peripheral decussation of fibres of the phrenic nerves. Instances are given of similar observations by Hirschfeld (1853) and Bourgery (1864), who both found subdiaphragmatic communications between left and right phrenic nerves in man.

Testut (1891) also found that the phrenic nerves within the thorax occasionally undergo partial decussation, the exchange of a few nerve fibres between right and left sides occurring in the anterior mediastinum.

Rouget (1851) believed that the phrenic nerve neither gives nor receives communications in the chest but his categorical statements in this respect are not supported by experimental evidence. He recorded the presence of communications between the right greater splanchnic nerve and the right phrenic nerve in the region of the right crus of the diaphragm. Unlike Valentin (1839) he was unable to identify communications between the left phrenic and left vagus nerves.

Studies by Luschka (1853-1863) confirmed many of the observations of the earlier anatomists and revived interest in the study of the innervation of the diaphragm. His work included a survey of the afferent nerve fibres supplying serosal coverings of the diaphragm; the subdiaphragmatic ramifications of the phrenic nerve were also studied. Luschka's findings can be summarised as follows:-

In addition to supplying diaphragmatic musculature, both nerves are distributed to the pleural and peritoneal coverings. The right phrenic nerve supplies branches to the right atrium, inferior vena cava and liver. Small, thread-like branches were traced from the abdominal surface of the diaphragm into the coeliac plexus and thence to the local branches of the abdominal aorta with which the nerve fasciculi were distributed. A definite group of such nerve fibres were traced to the suprarenal gland on the right and left sides, the right component being larger than the left. The left and right sides showed variation in the extent of their suprarenal branches from the phrenic nerve. He also observed asymmetry in the distribution of the phrenic nerves, to the left and right sides of the diaphragm.

In 1863, Luschka confirmed an earlier finding of peritoneal branches arising from both right and left phrenic nerves and, on the right side only, a branch to the inferior vena cava and to the right atrium. His investigations included topographical studies in animals and man and experimental section of the roots of the phrenic nerve in small mammals. Luschka was not satisfied that only the phrenic nerve supplied the diaphragm, for his observations indicated the possibility that the lower intercostal nerves, particularly the lowest pair, participate in its sensory and motor innervation.

Pansini (1888) investigated two very important aspects of the innervation of the diaphragm, namely, the intrathoracic communications of the phrenic nerves and the morphology of the terminal branches of these nerves within the substance of the diaphragm. In this study the experimental animals used were guinea pigs, rabbits, and foetal dogs and cats. Observations on the intrathoracic part of the phrenic nerve were made on formalin-fixed specimens and the finer details were studied by dissection on material impregnated with osmic acid (1%). Regarding intrathoracic communications, Pansini stated that the phrenic nerve is connected to the autonomic nervous system by fine nerve fibres arising from the inferior cervical and first thoracic sympathetic ganglia, from the periarterial plexus of the internal thoracic artery and from the cardiac and pulmonary plexuses. In this respect he was in agreement with earlier studies by Sappey (1840), Beaunis (1872) and Bouchard (1873). Connections between the phrenic nerve and the ansa hypoglossi, previously observed by Hirschfeld (1820) and Valentin (1840), were also demonstrated. Pansini's finding that communications exist between the phrenic and accessory nerves was in agreement with the views of Sappey; he also agreed with the latter author on the presence of communications between the vagus and phrenic nerves. His observation, that communications exist between the phrenic nerve and the nerve to subclavius muscle, was also in accord with the findings of Valentin and Hirschfeld. In the light of all the above observations, Rouget's (1851) contention that the phrenic nerve had no intrathoracic branches was clearly untenable.

Pansini used two principal methods for demonstrating the intradiaphragmatic terminals of the phrenic nerve. Fixation in formic acid (2%) followed by immersion in arsenious acid (1%) until the tissue became almost transparent was used to study the diaphragm in young rabbits. Microscopic examination was then performed with the diaphragm mounted on a slide of blue glass. The use of Löwit's gold chloride method (1875) to outline the neural components was applied to the diaphragm in guinea pigs. Irrespective of the species or of the method of study employed, the general arrangements of the diaphragmatic plexus were comparable.

According to Pansini, each phrenic nerve, on reaching the thoracic surface of the diaphragm, divides into three branches which are distributed to the following areas; an anterior or sterno-costal area, a lateral or costal area and a posterior or lumbo-vertebral area. Communicating fibres were identified between the terminal branches and it was inferred that these were functionally significant with respect to local co-ordination of muscular contraction.

In gold impregnated specimens, Pansini demonstrated small, irregular gangliform swellings in relation to the diaphragmatic terminals, 'presque jusqu'à leur terminasion'. He postulated that these form centres of local reflex control of diaphragmatic contraction and named them 'vrais ganglions intrinsiques'. In addition, he observed small 'collarettes' around the nerve fibres, these being associated with neurilemma.

From these observations Pansini derived the following conclusions:-

(1) The trunk of the phrenic nerve, like other nerves, is not formed by fasciculi which run parallel to one another but by bundles of nerve fibres which intermingle with each other in a more or less complicated fashion.

(2) The phrenic nerve, in its intradiaphragmatic distribution forms a very complex network of nerve fibres in which the three lower intercostal nerves participate (the division of the diaphragm into three regions of phrenic nerve distribution has been described above).

(3) The diaphragmatic plexus, particularly in its

> posterior part, possesses intrinsic gangliform structures which are related, functionally, to local control and coordination of muscle contraction.

Pansini was unable to determine whether the intrathoracic communications of the phrenic nerves were branches arising from the phrenic nerve itself, or whether they were accessions to it from other peripheral nerves. It was clearly stated, however, that he observed a recurrent branch of the right phrenic nerve which arose from the terminal part of the nerve trunk and ascended on the surface of the inferior vena cava terminating in the region of the right atrium.

INTERCOMMUNICATIONS OF THE PHRENIC NERVE WITH ADJACENT PERIPHERAL NERVES.

Many anatomists have observed communications between the phrenic and adjacent peripheral nerves and it is convenient to include, here a resumé of the relevant investigations.

In man, communications with the hypoglossal nerve via the ansa hypoglossi have been recorded by Haller (1747), Wrisberg (1780), Rüdinger (1870), Henle (1879), Schwalbe (1881) and Turner (1893). Luschka (1853), and Henle, believed it unlikely that the phrenic nerve receives fibres of cranial origin by way of this communication. It was thought to be more likely that the communicating fibres from the hypoglossal nerve are of spinal origin. The level at which these fibres enter the phrenic nerve varies between the posterior triangle of the neck and the lung root. Henle (1879) reported one instance of communication between the accessory (eleventh cranial) and phrenic nerves. The vagus nerve, studied by Wrisberg (1780) was seen to communicate with the phrenic nerve in one case out of thirty-seven nerves studied.

The presence of communications between the nerve to subclavius muscle and the phrenic nerve has been described by many investigators, such as Hirschfeld and Leveillé (1853), Henle (1879), Walsham (1881), Spalteholtz (1896), Merkel (1918) and Rauber-Kopsch (1920). In a series of 17 nerves, Felix (1922) observed three communications of this type. This communication has been observed so frequently in subsequent studies, that it has been named the 'accessory phrenic nerve'. There is some disagreement as to its incidence in humans, but Hovelacque (1936) commented that it is more common than is generally believed. Yano (1925) surveyed the incidence of accessory phrenic nerves and found that it occurred in 47 of 120 phrenic nerves examined.

It is significant that only one of these communications, the classical 'accessory phrenic', has been investigated histologically. (Yano 1928). There is no evidence, in the literature, to show whether these communications are branches of the phrenic nerve contributing fibres to other parts or if they are accessions to the phrenic nerve from extra-phrenic sources.

Anomalies of the Phrenic Nerve.

Perhaps the most unusual of all anomalies in the course of the phrenic nerve is that described by Virchow (1872) and Zeren (1952), who found the nerve passing through a defect in the subclavian vein. Hovelacque (1936), commenting on abnormal positions of the phrenic nerve, stated that the nerve, if not in its usual position, should be sought in a more lateral plane. Its medial displacement is limited by local vessels such as the transverse cervical artery.

COMPARATIVE ANATOMY OF THE DIAPHRAGM.

It is generally agreed that the fully developed diaphragm is a structure peculiar to mammalian anatomy, but controversy still exists regarding its evolutional development. Repeated attempts have been made, throughout the last hundred years to establish the point in evolutional development at which the diaphragm first made its appearance. Some investigators such as Aristotle (360 B.C.) have made observations relevant to this problem in the course of more general studies while others such as Sappey (1847) have considered the evolutional development of the diaphragm and its innervation as a separate entity.

Sibson (1846), in his studies on the mechanics of respiration, considered the importance of the ribs. He recorded that, in snakes, the simplest form of ribs are seen. These are attached to the vertebrae dorsally, but have no osseous ventral connection. In birds there is a group of sternal ribs, which move in the direction opposite to those of the snake in response to the phasic contraction of the intercostal muscles. He observed that both types are present in man, and suggested the name of 'diaphragmatic' ribs for the lower group.

Sappey (1847) compared the innervation of the diaphragm in birds and humans. He found that, in man, the nerve fibres which reach the diaphragm are derived from two sources, the cervical spinal cord, (phrenic nerve) and the solar plexus, the fibres of which reach the phrenic nerve distribution, by way of the inferior phrenic arteries. No mention was made of the possible participation of the intercostal nerves in the innervation of the diaphragm. In Sappey's opinion, two separate sources of diaphragmatic innervation might also be expected to exist in birds, but this was not supported by his own findings. The intercostal nerves supply the diaphragm, and the apparent second source of nerve fibres, the sympathetic chain, simply provides a convenient pathway for the conduction of somatic segmental nerve fibres to their destination in the diaphragm.

Cavalié (1896), carried out comparative studies on the innervation of the diaphragm in birds, dogs, rabbits, guinea-pigs, and rats. His findings concurred with Sappey's that, birds possess no phrenic nerve and that the muscle fibres of the diaphragm are controlled by the third, fourth, fifth, and sixth thoracic nerves. Cavalié also found that electrical stimulation of the intercostal nerves, caused recordable contractions of the diaphragmatic musculature in dogs, rabbits, guinea-pigs, and rats. The pathway of these contributions from local segmental nerves, was also considered, and it was concluded that the diaphragmatic branches of the segmental nerves could be:-

(a) Direct terminal arborisations, and

(b) Indirect - passing through the autonomic ganglia.

This hypothesis, regarding the mingling of nerve fibres of autonomic and cerebro-spinal components of the nervous system, was inferred by Sappey (1847) in his studies on the respiratory apparatus in birds. Subsequent investigations by Ranson and Billingsley (1916) particularly related to the autonomic nervous system, have produced evidence for the existence of a close association between the peripheral fibres of these two divisions of the nervous system. In his conclusions, Cavalié supported the theory that the intercostal nerves contribute motor fibres to the diaphragm in mammals and perhaps also in man.

Robinson (1897) stated that, "amphibia and animals below them in scale of structure, have no diaphragm. The first distinct trace of it may be found in crocodiles and birds where the muscle fibres concerned in its formation arise from the ribs". It is not stated in the record of his investigation, exactly how these observations were made, and there is no illustration presented in support of them.

Keith (1905), believed that there are three different stages in the evolutional development of the diaphragm.

(a) In fish, the septum transversum divides the coelom into two parts - cardiac and abdominal, in the latter of which lie the lungs.

(b) In birds, appears an intermediate stage, where the greater part of the lung is extruded from the abdomen into the cardiac cavity.

(c) In mammals the lungs have been entirely extruded from the abdominal cavity.

The author considered this extrusion from the abdominal cavity to be similar to the descent of the testes.

Van Bambeke (1906) believed that the diaphragm is developed from two distinct halves, an anterior and a posterior, to which he gave the names ventral and dorsal diaphragms respectively. He considered that the ventral diaphragm is present in all vertebrates, and that it has the same morphological significance in each species. He observed, however, that the dorsal diaphragm is present, in the fully developed state, only in mammals and that it is closely linked with the development of the pleuro-peritoneal membranes and body cavities. Fish (Selachii) and humans were compared, since they represent the extreme degrees of diaphragmatic development. Embryological studies revealed that the liver developed in an exactly opposite direction in the two species. It was therefore concluded that the development of the liver is closely related to the development of the diaphragm and that there are three evolutional types of diaphragm:-

(i) Selachii
(ii) Reptiles
(iii) Mammals.

It will be noted that although Van Bambeke and Keith differed with regard to the intermediate stage of evolution, they were in agreement about the two extremes.

Dally (1908) also believed that the innervation of the diaphragm could be considered in three evolutional stages. He quoted Cavalié, who held the view that in vertebrates, which precede birds, the diaphragm is supplied by one set of nerves, the intercostals. Birds represent the intermediate stage of evolution in this respect and here, the diaphragm is supplied by two sets of nerves, the intercostal nerves providing for the costal portion. The remainder of the muscle, the dorsal diaphragm of Van Bambeke, is supplied by nerve fibres derived from the sympathetic system, which also extend into the area supplied by the intercostal nerves. These views are at variance with the findings of Sappey. In the final stage of evolution, and particularly in man, a third source of diaphragmatic nerve fibres, the phrenic nerve becomes predominant.

Turner (1913), compared the origins of the phrenic nerves in rabbits, cats, and dogs. In rabbits, the phrenic nerve was difficult to locate in the neck. Thirty-three dissections were performed, and showed, with minor variations, a tri-partite origin from cervical segments, 4, 5, and 6. In a series of seven cats, each phrenic nerve had two roots, one originating in the fifth and one in the sixth cervical segment. The author observed that the cervical portion of the phrenic nerve was sufficiently long to facilitate the attachment of electrodes in this region. He also observed great variation in the level of union of the component roots which form the trunk of the phrenic nerve. Similar investigations were made in two dogs and, here the roots forming the phrenic nerve were identified as coming from the fifth, sixth and seventh cervical segments. From his observations, the author concluded that experimental operations on the phrenic nerve, should be performed in the thoracic cavity and not in the neck.

Lutnicki (1950) studied the mode of origin of the phrenic nerves in sheep by dissection in forty-five animals and found that 84% of all nerves were formed by union of the roots of C5, 6, and 7, on the ventral surface of scalenus anterior.

In 'Evolution Emerging' (1951), Gregory stated that, in the fossil remains of a very primitive type of mammal, the cynodont, a structure suggestive of a precursor of the diaphragm, is present in the region of the caudal thoracic segments. It is inferred that, in this animal, the diaphragm may have been partly of an osseous or cartilagenous nature.

The participation of bony elements in the formation of the diaphragm, is not perpetuated in the process of evolution. However, Maskar (1957) in his anatomical observations in camels included an account of a diaphragmatic bone situated in the region of the hiatus for the inferior vena cava. This is present in all camels, but it is not seen, as a normal feature, in the diaphragm of other mammalian species.

Walker and Attwood (1960) investigated the structure of the hiatus for the inferior vena cava in the diaphragm. A total of forty-four mammalian diaphragms were studied by dissection in dogs, cats, sheep, pigs, rabbits, cows, seals (phoca vitulina) and humans. It was concluded that the anatomical structure of the inferior vena caval opening in the diaphragm was comparable in all the species examined except the seal. The most striking feature in the majority of species was a distinct crossover of connective tissue fibres at the right lateral margin where a small fat-containing space was thus produced separating the two bundles of fibres. This space enclosed the right phrenic vessels. The margin of the hiatus was invariably pear shaped as shown in the illustrations in Haller's Opera Minora (1763) the broader portion being directed medially. This conflicts with the concept of the term "foramen quadratum" used by many authorities such as Lanza (1958) to describe the hiatus for the inferior vena cava.

In the seal, the hiatus was almost circular and it was formed by a thick ring of muscular and tendinous tissue from which the cuff-like sub-diaphragmatic muscular sphincter took origin. This structure, according to the authors indicates structuro-functional adaptation of the diaphragm to the diving habits of the seal.

None of the investigators of the comparative anatomy of the diaphragm, claim to have established the precise nature of its evolutional development. There exists, however, a measure of agreement between the statements of Robinson (1897) and Gregory (1951) regarding the presence of a diaphragm in the higher reptilian forms. The cynodont, whose fossil remains are described by Gregory was an organism intermediate between reptile and mammal and, in general, resembled the crocodile.

Direct comparisons of the mode of origin of the phrenic nerve in different mammals indicate that this feature shows a distinct species difference. According to Turner (1913), whose work is described above, the cervical nerves which give rise to the phrenic nerve are, in dogs, 5, 6, and 7; in cats 5 and 6 and in rabbits 4, 5 and 6. The findings of Lutnicki (1950) indicated that, in this respect, sheep resemble dogs. In the macaque, according to Warwick and Mitchell (1956) the two main contributory nerve roots are the fourth and fifth cervical whereas in man the most frequently quoted roots of origin are the third, fourth and fifth cervical nerves. It appears, therefore, that the origin of the phrenic nerve reaches its highest level in the primates.

DEVELOPMENTAL ANATOMY OF THE DIAPHRAGM.

One of the earliest authorities on this topic was Von Baer (1827), who stated that, the diaphragm starts to develop from the septum transversum at a time when the occipital and cervical somites together make up about three-quarters of the crown-rump length of the human embryo.

Mall (1901) stated that His, (1880), was first to propose the use of the term, septum transversum, to describe the musculo-aponeurotic sheet that separates the thoracic from the abdominal cavity.

Uskow (1883), placed great emphasis on the influence of development of great veins upon the development of the diaphragm. According to Uskow, the 'dorsal diaphragm' (dorsal component) arises from the body wall in a position rostral to its definitive position, is shaped like the letter 'V' and is in continuity with the dorsal mesocardium dorsally, and with the Wolffian body caudally.

Comparisons were made by Lockwood (1888) of the early development of the diaphragm in rabbits and humans. He agreed with the conclusion of Uskow.

Debeyre (1901), observed a developmental association between the crura of the diaphragm and the Wolffian bodies (mesonephros) to the rostral pole of which, the developing crura are attached. Direct continuity between these two structures was observed in Tarsius which was used here as an experimental animal. In rats and humans, comparable developmental relationships between these structures was also found, but in a modified form. In man the internal surface of the Wolffian body is involved in the formation of the crus of the diaphragm and the junctional zone between these primitive precursors contains the posterior cardinal vein.

Lack of quantitative data from nineteenth century literature on the development of the diaphragm reduces the value of observations such as those quoted above. In addition, many of the conclusions drawn were based on evidence, the accuracy of which was governed by the dissection methods then in use.

Dally (1908) summarised current views on the embryology of the diaphragm in man. The septum transversum, originally formed rostral to the developing protocardiac area, migrates caudally under the influence of the formation of the head-fold of the embryo. The mesodermal components are innervated as they descend, by the mid-cervical segments of the spinal cord, the segmental nerves involved depending on the species under consideration. Dally believed that the septum transversum is composed of two parts - dorsal and ventral - the dorsal part being formed by the fusion of a portion of the cervical aponeurosis with the dorsal wall of the pericardium. The ventral part represents the primitive septum transversum seen in fish and amphibians.

Five developmental parts are represented in the fully developed diaphragm; one mesial, two ventro-lateral, and two dorso-lateral. According to Dally, the mesial and ventro-lateral components together form four fifths of the total definitive structure. The pleuro-peritoneal membranes, now known to contribute to the structure of the diaphragm (Frazer 1940), are not described by Dally.

The current view of the development of the human diaphragm, is that it has six distinct embryological components. According to Wells (1954), those arise from mesodermal masses of which, the septum transversum, is the largest and the pleuro-peritoneal membrane is the smallest. There is a peripheral contribution from the developing chest wall and three smaller central contributions from the mesoderm dorsal to the liver, from the primitive mesogastrium and from the peri-aortic mesoderm. These views agree in general, with the findings of Patten (1946).

More Recent Studies on the Innervation of the Diaphragm.

In the decade 1853-1863, Luschka made a comprehensive study of the afferent and efferent nerve fibres which innervate the diaphragm. His findings, together with his views on the subdiaphragmatic ramifications of the phrenic nerves are described above. In contrast to this, the vast majority of more recent investigations of this problem have been considerably more restricted. For example, various groups of workers have confined their investigations to topics such as, the central nervous control of respiratory muscles, the afferent fibres of the phrenic nerve and the sensory receptors in the diaphragm.

Investigations on the central nervous control of respiratory muscles.

Legallois (1811) and Flourens (1837) whose experimental investigations are described above found evidence for the existence of a medullary 'centre' which controls phasic contraction of respiratory muscles. Schiff (1837) demonstrated that, after total destruction of the medulla oblongata in small mammals, the diaphragm continued to contract reflexly on physical stimulation of the abdominal skin. This finding suggests that, in addition to medullary control, there is a spinal centre which influences the function of the diaphragm. Schiff also observed that efferent respiratory impulses from the medulla descend through the white matter of the spinal cord in the lateral columns, the integrity of which is essential to normal contraction of the diaphragm.

It has been reported by Dally (1908) that Brown-Séquard (1855) demonstrated continued phasic contraction of the diaphragm after 'blocking' the phrenic nerves and that, after death, diaphragmatic movement continued for a considerable time after all other striated muscles apart from the myocardium had ceased to contract. Extensive search of the appropriate literature, however, has failed to confirm Dally's report.

Marey (1863) observed that, in obstructed respiration, the diaphragm enters into action before the thoracic muscles and that it relaxes while the latter group continues to contract.

Bert (1870) carried out experimental studies in small mammals and observed that the following procedures did not interfere with diaphragmatic contraction:-

(1) Longitudinal bisection of the spinal cord from C1-C4 segments.

(2) Complete transection of the spinal cord above the first pair of spinal nerves.

(3) Complete transection of the spinal cord below the second pair of spinal nerves.

Section, caudal to the third pair of spinal nerves, caused cessation of all movement of the diaphragm.

Rokitansky (1874) pointed out that when the medulla is ablated in young rabbits which have previously been treated with strychnine, the animals can still carry out respiratory movements. In a series of experimental investigations, Langendorff and Nitschmann (1880) discovered spinal centres apart from the medulla, capable of reflex control of respiratory movement.

Mosso (1903) investigated the timing of respiratory movements in man and concluded that contractions of the muscles of the face, thorax, diaphragm, and abdomen are independent of one another. Evidence was presented, indicating alternation between abdominal and thoracic respiration, reduction in the strength of diaphragmatic contraction being associated normally with increase in strength of contraction of intercostal muscles. During intermissions in diaphragmatic contraction Mosso observed that the phasic contractions of the intercostal muscles continued unchanged and that spasmodic contractions of the diaphragm can occur in hiccough without participation of the intercostal muscles.

It may be concluded from these findings, that there is a centre for the control of respiratory movement in the medulla oblongata and that there is at least one additional control centre situated in the spinal cord. The possibility of the existence of more than one "spinal centre" is suggested by the findings of Langendorff and Nitschmann (1880) whose views, described above, were supported by the observations of Mosso (1903).

THE PHRENIC NUCLEUS.

The phrenic nucleus is situated at the ventral extremity of the grey matter of the spinal cord and consists of a discrete column of neurones the peripheral processes of which descend towards the diaphragm in the trunk of the phrenic nerve.

The views of Bell (1802) regarding the nervous control of respiration are reported in Todd's Encyclopedia (1847). Although some of Bell's findings, particularly on the participation of the hypoglossal nerve in the control of respiration, were subsequently disproved his investigations promoted interest in the precise cellular origin of nerves which control respiratory movement.

Kohnstamm (1898) attempted to locate the phrenic nucleus in rabbits by section of the cervical roots contributing to the phrenic nerve and subsequent examination of the spinal cord for degenerative changes. This method of investigation was repeated by Marinesco and Sano (1892) in a variety of experimental animals including dogs, rats and cats and by Hirako (1928) in albino rats. General agreement is to be found in the conclusions derived by these investigators; They all located the origin of the phrenic nerve in the mid-cervical region of the spinal cord. The reports of the above investigations do not include exact details of the numbers of experiments performed. In the absence of these quantitative data, the validity of the conclusions is in doubt.

Accurate identification and subsequent transection of spinal nerve roots necessitates mobilisation of the spinal cord with concomitant danger of damage to neighbouring nerve roots. In view of these sources of error, the observations of Kohnstamm, Marinesco and Sano, and Hirako, at best may be regarded as an approximate guide to the location of the phrenic nucleus.

Keswani (1954) induced chromatolysis in the anterior horn cells of the cervical spinal cord by section of the motor roots of the phrenic nerve and located the phrenic nucleus accurately. He observed that in cats, it is situated near the ventral extremity of the grey matter and extends from the rostral extremity of the fifth cervical to the caudal extremity of the subjacent segment.

Samuel and Warwick (1955) investigated the topography of the roots of the phrenic nerve in macaque monkeys. Twenty-three phrenic nerves were studied and it was concluded that:-

(a) As far as dissection can show, the phrenic nerve may receive fibres from C3, C4, C5, and C6.

(b) The nerve to Subclavius gave a communicating branch to just over half of the nerves studied in this series.

(c) The relationship of the roots of the nerves to Scalenus anterior brevis revealed gross variability.

Warwick and Mitchell (1956) investigated the location of the phrenic nucleus in the same species. Twenty-three monkeys were used and the standard procedure was unilateral division of the phrenic nerve at various levels with observation of resultant degenerative changes in the spinal cord after survival periods ranging from 10-14 days. The results of twenty-one of the experiments were presented and the following conclusions were derived:-

(1) "The phrenic motor pool is a topographical entity. Its constituent cells always appear in transverse sections as a well defined group at an intermediate position in the most ventral part of the ventral grey column". The extent of this cell group was then described and there was an additional comment that "there is no evidence of central decussation of phrenic axons in monkeys".

In a final comment however, it was stated that "although the majority of the axons from each phrenic nucleus are distributed in the ipselateral nerve, we found evidence, in two cases, that a considerable number cross from the left to the right nerve at the level of the pericardium. We conclude that in the macaque, at least, the right phrenic trunk, as it enters the diaphragm, contains fibres from both phrenic nuclei".

The afferent fibres of the phrenic nerve.

Ferguson (1891) attempted to establish, on a clinico-pathological basis, the fact that the phrenic nerves contain sensory as well as motor nerve fibres. His preliminary observations were made on one patient who had suffered from progressive muscular atrophy which involved the diaphragm, and, at autopsy, the phrenic nerve, examined histologically, contained an unspecified number of normal nerve fibres. It was deduced by the author that these surviving fibres must be sensory in nature.

These observations led Ferguson to attempt further experimental procedures in cats, the first involving division of the right phrenic nerve. Following a post-operative interval of three weeks, "diaphragmatic sensation was tested" and found to be reduced on the right side. Examination of the divided nerve showed atrophy of all its fibres. Ferguson thus concluded "Here, then, what had escaped in the case of progressive muscular atrophy had been included in the case of a transverse lesion".

In a second cat Ferguson exposed the third, fourth, fifth and sixth cervical spinal nerves on one side and divided the corresponding posterior roots just peripheral to the ganglia. Ten days later, the animal was killed and the phrenic nerve on the operated side was examined histologically. There was "degeneration of approximately one-third of all its fibres". According to Ferguson, these degenerated fibres represented the sensory component of the phrenic nerve.

These observations are in no sense conclusive but represent an attempt to establish the mixed nature of the fibre content of the phrenic nerve.

It is not recorded by what means the author diagnosed atrophy of the diaphragm in his clinico-pathological investigation nor is it indicated in the experimental studies, how sensation in the diaphragm or degeneration of nerve fibres in the phrenic nerve was determined.

Sherrington (1894) made a study of the anatomical constitution of nerves of skeletal muscles in cats and monkeys. The four main methods used were described by the author as follows:-

1. Comparison of the fibres of certain spinal roots with those of the corresponding peripheral nerve trunks entering the muscle and the skin respectively.
2. The alteration of composition of the nerve trunks induced by degeneration of spinal roots contributing to it.
3. The examination of the nerve trunks of muscles where the spinal cord has been arrested early in its development.
4. The search for end organs within the muscular tissue.

In his conclusions (p. 255) Sherrington made the following statements:-

1. In a muscular nerve trunk from one third to one half of the myelinated fibres are from cells of the spinal root ganglion.
2. These fibres range in size (in fresh preparations) from 1.5 to 20μ in diameter.
3. The largest fibres arising from spinal ganglion neurons are not the largest fibres in the muscular nerve trunk; the largest in the nerve trunk come from the ventral

(motor) spinal root.

4. The largest fibres arising from spinal ganglion neurons present in the muscular nerve are larger than any fibres in the cutaneous nerves of the limb and than any articular nerves examined.
5. The smallest myelinated fibres in the muscular nerve are for the most part, perhaps entirely, root ganglion fibres.
6. "Microscopic nerve trunks are, as regards their myelinated fibres in no case purely motor, all are sensory-motor or purely sensory. Such nerves as phrenic, hypoglossal, recurrent laryngeal, posterior interosseous, contain abundance of fibres from sensory ganglia".

Sherrington's conclusions constituted a major advance in the knowledge of peripheral nerves and, besides confirming the earlier investigations of Ferguson, they formed a basis for future study of the peripheral nervous system in general and of specific components such as the phrenic nerve.

Kidd (1911) surveyed the literature relevant to the sensory functions of the phrenic nerve and referred to Luschka (1853), Pansini (1888), Ferguson (1891) and Sherrington (1894) whose work, described above, established the fact that the phrenic nerves contain afferent fibres. The findings of Sano (1898), that the sensory fibres in the phrenic nerve originate in the third to the sixth spinal ganglia, are recorded in Quain's Anatomy (1908). On this basis, Kidd commenced his consideration of the sensory components of the phrenic nerve with the following statement - "We can hardly doubt that these (the sensory fibres of the phrenic nerve) are of two types:-

1) Muscle sensory.
2) Postural."

No reasons were offered in support of this statement. He considered briefly, the controversial issue of the origin of splanchnic afferent nerve fibres and stated the views of Kölliker (1894) and Langley (1903) who supported the theory that these fibres arise from spinal ganglia. Opposing this school of thought, Dogiel (1896) maintained that visceral afferent nerve fibres must have their origin in the ganglia or plexuses of the sympathetic nervous system. With particular reference to the splanchnic afferent nerve fibres of the phrenic nerve, Kidd described the multiple communications which exist between the phrenic nerves and components of the autonomic nervous system. He stated "It is difficult to see what can be the meaning of these phrenic communications if not to transmit splanchnic afferent fibres to the organs it (the phrenic nerve) supplies". There is no evidence that Kidd attempted to investigate this question.

He also defined two more defects in current knowledge of the phrenic nerve; the absence of a thorough histological analysis of the nerve trunk and the unsatisfactory state of knowledge of the development of the phrenic nerve.

Up to this point Kidd's contribution was limited to defining problems but the final and most important part of his work was devoted to a survey of the "clinical importance of the sensory phrenic", an account of which appears below. (p. 95).

Mathieson (1912) investigated the sensory fibres of the phrenic nerve using experimental physiological techniques in dogs. In preliminary experiments he demonstrated the flow of impulses in a centripetal direction along the phrenic nerves. Having established the normal topography of the phrenic nerve, its roots of origin, and its intrathoracic communications, Mathieson found that his observations were in general agreement with the findings of Lim Boon Keng (1893) and Ellenberger and Baum (1891) particularly regarding communications with the inferior cervical sympathetic ganglion. Histological examination of the most constant of these communications showed a preponderance of unmyelinated nerve fibres, some myelinated fibres of large diameter (18-20µ) and a few myelinated fibres of small diameter (4µ). Pericardial branches corresponding closely to those described by Luschka (1863) were observed in dogs by Mathieson. On the right side these branches arose from the phrenic nerve close to the thoracic surface of the diaphragm, and ascended in two strands, along the inferior vena cava to be distributed to the pericardium, one branch going to the atrial portion. From the left phrenic nerve, two branches pass dorso-lateral to the heart to ramify in the pericardium. Histological studies showed that these branches contain a very small number of myelinated fibres of medium diameter and a corresponding number of unmyelinated fibres.

The experimental studies carried out by Mathieson are not supported by photographic evidence.

Felix (1922) concluded, from dissections of human foetuses, that the phrenic nerve gives no branches to the pericardium, nor to the costal or mediastinal pleura. His views regarding the pericardial branches of the phrenic nerve are supported only by Rouget (1851) and are opposed by the findings of Luschka (1853) and Pansini (1888). According to Felix, sensory innervation of the diaphragmatic pleura and peritoneum by branches of the phrenic nerve, is limited to the central and lumbar zones of the diaphragm, the remainder of the serosal surfaces being supplied by the lower intercostal nerves.

AFFERENT ENDINGS OF PHRENIC NERVE FIBRES.

a) Within the diaphragm.

Dogiel and Timofejew (1902) were among the earliest investigators to examine afferent nerve endings in the diaphragm. In small mammals they found diaphragmatic nerve endings which resembled those in muscle spindles of skeletal muscles.

Lanza (1958), using gold chloride techniques, investigated the neural elements within the central tendon of the diaphragm in cats and he observed tendon organs comparable to the stretch receptors present in the tendons of other skeletal muscles.

Winckler (1962) described the structure of tendon organs in the central tendon of the diaphragm in man. They resembled corresponding structures found in the tendons of skeletal muscles.

b) Within the pericardium.

Ruhemann (1926) investigated the innervation of the pericardium in rats and infant humans by silver impregnation of the whole pericardium. Myelinated and unmyelinated nerve fibres were observed in each specimen examined. No intensive search was made to determine the source of these nerve fibres.

Bonivento (1940) studied the innervation of the pericardium in mammals with specific reference to fibres arising from the phrenic nerves. The Bielschowsky silver impregnation method was used and the observations regarding the types of nerve fibres present were in agreement with Ruhemann's findings. Bonivento observed that the myelinated fibres possess end organs while the unmyelinated fibres end in a diffuse network of terminal branches. The source of nerve fibres supplying the pericardium was studied experimentally in small mammals by observing the changes in the pericardial neural elements following section of the vagus or phrenic nerves respectively. No changes were observed in the pericardium following section of the vagus in the upper third of the thoracic cavity but, following section of the phrenic nerve at the same level, all the neural elements of the pericardium underwent degeneration.

In conclusion, Bonivento stated that the myelinated and unmyelinated nerve fibres present in the pericardial plexus originate in the phrenic nerve.

The efferent fibres of the phrenic nerve.

With regard to the participation of the lower intercostal nerves in the efferent innervation of the diaphragm, two opposing schools of thought have existed for many years. One supports the view that the phrenic nerve is the only source supplying efferent nerve fibres to the musculature of the diaphragm while the other, admits the possibility that the lower two or three intercostal nerves may participate in this function.

The following investigations of the diaphragmatic plexus in normal mammals such as rats, mice and guinea-pigs are relevant to the problem.

Eisler (1901) concluded from histological observations that the fasciculi of nerve fibres which arise from the intercostal nerves and the coeliac ganglia and ramify within the diaphragm are devoid of 'muscle bundles'; none of the individual nerve fibres have a diameter compatible with motor function.

Gössnitz (1901) concurred with Eisler and stated categorically that contributions from the intercostal nerves, because of their small diameter, could only be classified as afferent fibres. In the same histological study, he traced nerve fibres of phrenic origin centrifugally and found that terminal twigs were present to within a few millimetres of the peripheral attachments of the diaphragmatic muscle fibres. From this observation he concluded that it was not necessary to postulate any other source of motor innervation.

Ramström (1906) examined the neural elements of the diaphragm and, regarding the general arrangements of the plexus, he agreed with Eisler and Gössnitz. Ramström emphasised that nowhere did he find physical contact between the phrenic and intercostal neural elements. In the absence of experimental evidence from these studies and because of the complete lack of quantitative data and photographic illustrations, it is not easy to accept completely the views of the latter three authors.

The following investigators held the opposing view, that the intercostal nerves do supply motor fibres to the diaphragm.

Timofejew (1902) who studied the problem histologically in cats, young dogs and guinea-pigs using the methylene blue technique described by Ehrlich (1886) concluded that the lowest pair of intercostal nerves participate in the motor innervation of the diaphragm. He also observed that motor terminals in the diaphragm have the same morphological characteristics as those present in other skeletal muscles.

Ellenberger and Baum (1891) studied the topographical anatomy of dogs and concluded that, in these animals at least, the intercostal nerves (8-10) may participate in the motor innervation of the diaphragm.

Oppenheim (1902), Oehlecker (1913) and Kappis (1920) considered the problem from the clinical point of view in patients previously subjected to section of the phrenic nerve and concluded that there was a possibility of the lower intercostal nerves contributing to the motor supply of the diaphragm.

Felix (1922), in his studies on human foetuses, using the same histological techniques as Ramström (1906), observed the terminal fibres involved in the formation of the diaphragmatic plexus. The conclusions of Felix, represented diagrammatically, clearly showed that the muscle fibres of the diaphragm arising from the lumbar vertebrae and medial arcuate ligament receive motor nerve fibres from the lowest pair of thoracic nerves.

The significance of the accessory phrenic nerve and the possible abnormality of the course of the phrenic nerve itself, was considered by Felix, from the practical point of view. He pointed out that, apart from difficulty in locating an aberrant phrenic nerve, selective transection of the main trunk, may not produce the desired effect of paralysis of the corresponding hemi-diaphragm. The work of Felix remains the most important anatomical contribution to the present day knowledge of the innervation of the diaphragm.

Yano (1928), following his topographical study of the phrenic and accessory phrenic nerves, examined their histological structure. His conclusions, though mainly of a qualitative nature, indicated the manner in which unmyelinated fibres of the phrenic nerve are distributed within the transverse section of the nerve trunk. He observed that the unmyelinated fibres increase in number towards the distal end of the phrenic nerve. From the histological illustrations in his records, it is not clear how he arrived at any accurate quantitative conclusion. His results, however, are quoted by contempoary authorities such as Fenn and Rahn (1964).

Kiss and Ballon (1929) examined the histological structure of the phrenic nerve in man and their illustrations of myelinated nerve fibres are of good quality. However, their conclusions were qualitative in nature and were not comprehensive. Their concurrent experimental work was performed on humans, in whom the abdominal surface of the diaphragm, exposed for surgical operations, was stimulated at various points while the patient was still partly conscious. They relied on the subject to indicate the site where he felt pain. This attempt to define the distribution of afferent nerves in the serosa, was fraught with great inaccuracy. The most useful function of their investigation was to renew interest in the subdiaphragmatic plexus of fine nerve fibres which link the phrenic nerve to the coeliac plexus.

Strauss (1933), studied the innervation of the diaphragm by dissection in normal rats and dogs including a brief comparison between those animals and man. The observations made are presented in diagrammatic form and there are no supporting photographic records of his observations.

Strauss's findings do not agree with those of Ramström (1906) who, in his histological studies, found a 'nerve rich zone' close to the equator of the muscle sheet of the diaphragm. Strauss found a more diffuse distribution of nerve fibres with no definable areas of concentration.

Masumoto (1934), using a modification of Cajal's technique for the demonstration of nerve fibres, studied the neural elements present within the diaphragm in normal mammals. Thus eleven different species were examined and it was found that, in general, the nerve fibres and nerve endings present, resembled corresponding structures in other skeletal muscles. Motor and sensory nerve fibres were identified according to their association with recognisable end-organs such as motor end-plates and muscle spindles. Other neural components such as unmyelinated fibres and 'ganglion cells' were identified but were not described in detail. No attempt was made to present the findings on a quantitative basis.

Masumoto then applied the same histological method to the study of the effects produced in the diaphragmatic plexus by experimental procedures such as the administration of neurotoxins and surgical interference with various sources of nerve fibres which reach the diaphragm.

From the pharmacological investigations, Masumoto concluded that each neurotoxin studied had a specific effect upon the components of the diaphragmatic plexus. Curare, especially, produced changes in the terminal apparatus of motor nerve fibres and the neural components of muscle spindles showed distinct swelling. Tetanus toxin produced shrinkage of the cytoplasm of 'ganglion cells' but there was no associated effect on the nuclei of these cells. Strychnine, like curare, had an apparent predilection for progressive damage to motor terminals on repeated injection of the drug. The unmyelinated fibres were also affected by strychnine to a lesser extent. The effects of massive doses of adrenaline were well tolerated by dogs and the changes in nerve fibres were limited to swelling and loss of argentophilic properties. The effects of lethal doses of atropine on the nerve fibres were limited to slight swelling and increased tortuosity in both motor and sensory components. Corresponding changes were observed on the administration of pilocarpine but there were also concomitant structural changes in ganglion cells. The administration of nicotine led to irregular and patchy silver impregnation in the paravascular unmyelinated fibres.

The details of this investigation have been described at some length because of their unusual nature. So far no comparable study has been found in the literature concerning the diaphragm.

Studies by Masumoto involving experimental surgery included transection of the trunks of the following nerves - one or both phrenic nerves in the neck or within the thoracic cavity; the seventh to the eleventh intercostal nerves inclusive, close to their emergence from the inter-vertebral foramina; the vagus and greater and lesser splanchnic nerves (level unspecified). The effects of extirpation of the coeliac ganglia were also observed.

Masumoto's first conclusion from these studies was that the changes seen in the nerve fibres of the diaphragmatic plexus depend, largely, on the level of transection of the nerve source under investigation. If the main trunk of one phrenic nerve is severed in the cervical region, the changes in the diaphragmatic nerve endings are typified by increased tortuosity and broadening of the motor fibres associated with loss of affinity for silver salts. The sensory nerve fibres show corresponding changes. Unmyelinated fibres are, however, unchanged in structure; some motor fibres also show normal structure. Unilateral transection of the phrenic nerve in the thoracic cavity produced changes which were progressive with prolonged survival periods. A small proportion of unmyelinated and motor fibres, however, retain their normal structure.

Bilateral transection of the phrenic nerves (level unspecified), although it produces obvious changes in the musculature of the diaphragm, does not produce total degeneration of all nerve fibres in the muscle. There still remains a proportion (magnitude unspecified) of normal motor and unmyelinated nerve fibres.

By dividing the intercostal nerves as close as possible to their origin, Masumoto demonstrated that a small number of nerve fibres identified as motor fibres showed changes suggestive of degeneration. No changes were observed in the diaphragmatic plexus following transection of the vagus, or greater or lesser splanchnic nerves or following extirpation of the coeliac ganglion.

The records of this work indicate that little attention was given to the quantitative aspects of the subject. Details regarding the length of the survival periods following surgical manipulations in experimental animals are not presented, nor are there records of the numbers of persisting diaphragmatic nerve fibres. These omissions detract from the value of the conclusions drawn. Nevertheless, there is evidence of originality in the experimental methods used in the pharmacological part of the investigations.

The conclusions formed by Masumoto, although not enunciated, indicate agreement with the supporters of 'extra-phrenic' motor innervation of the diaphragm, such as Timofejew (1902) and Felix (1922).

Botha (1956) studied the innervation of the diaphragm by dissection in fifty humans, ranging in age from premature infancy to late senescence (85 years). His findings in human material were then compared with similar studies on rats, ferrets, dogs, cats, rabbits and monkeys, a further eleven species being examined in less detail.

He observed that the general arrangements of the diaphragmatic plexus were comparable in all of the species examined. The principal findings were as follows:
At its distal end the left phrenic nerve occupies a more ventral position than the right; both nerves divide into a variable number of branches, the range being 2-7, before they penetrate the thoracic surface of the diaphragm; the postero-medial branch, which is usually the largest, is "absolutely constant" and always runs in the same direction, towards the crural region of the corresponding side. No macroscopic communication between the right and left phrenic nerves was found. Botha also observed thread-like filaments, which pass from the coeliac ganglion and splanchnic nerves to the substance of the diaphragm.

In order to confirm these findings, he carried out studies involving electrical stimulation of the various possible sources of diaphragmatic nerve fibres. With the diaphragm under constant surveillance, stimuli were applied to the phrenic, vagus, and splanchnic nerves, and to the sympathetic trunk. The conclusions made from these studies confirmed the anatomical finding that only the phrenic nerve is significant in the motor innervation of the diaphragm. No contraction of muscle fibres occurred, unless the phrenic nerves were stimulated, and when this was done, the muscle response was unilateral. In this respect, Botha's findings were in agreement with those of Schlaepfer (1923). Details of the numbers of animals studied are not presented.

As a final experiment, Botha applied the principles of nerve degeneration to the problem. The left phrenic nerve of one fully grown rabbit was sectioned three centimetres proximal to the diaphragm, and following a period of five months, radiological inspection was made. It indicated no contraction of the left hemidiaphragm, which was two centimetres higher than the right one. Naked eye inspection of the diaphragm at laparotomy confirmed complete inactivity of the left hemidiaphragm, which was 'flabby and thin'. The histological appearances of the muscle fibres were atrophic.

In his discussion of the findings, Botha now stated that although the phrenic nerve is the principal motor supply to the diaphragmatic muscle, motor innervation from other sources cannot be excluded. In this respect, he agreed with the views of Luschka (1853), Cavalié (1896) and Felix (1922). He also quoted the views of Last (1954) that, "the crura, derivatives of the prevertebral rectus (q.v.) are supplied by the lower intercostal nerves and not by the phrenic". Then, surprisingly, in his conclusions he stated, "In man as well as in the animal species investigated, the phrenic nerve is the sole motor innervation of the diaphragm".

In summarising the result of the investigation, he stated, "Anatomical dissections in man and animal species strongly suggested the phrenic nerve as the main motor supply to the diaphragm; nerve stimulation and degeneration experiments proved that it is the sole innervator".

It is, thus, obvious from the surprisingly different views expressed in his discussion, conclusions and summary, that Botha was undecided regarding the question of "extra-phrenic" motor innervation of the diaphragm. It is also significant that only one animal was studied by experimental section of the phrenic nerve and that the survival period was of five months duration. The main contribution made by this investigation lies in the comparative studies carried out by Botha, although, even in this part of the work, the findings are almost purely qualitative.

Prioton and Thevenet (1958) studied the distribution of the terminal branches of the phrenic nerve in humans, with special reference to the region of the oesophageal hiatus. In all, forty human cadavers were dissected, eight of them being foetuses of between four and seven months gestation. Three conclusions were made from this purely topographical study.

(1) Three terminal branches are present in the right and left phrenic nerves; they are anterior, lateral, and posterior, and are distributed to the corresponding areas of the diaphragm.

(2) The terminal branches of the right and left phrenic

nerves are distributed in exactly the same fashion within the corresponding hemi-diaphragm. Any difference that exists is explained by the more anterior and more lateral placing of the left phrenic nerve.

(3) With regard to the muscle fibres of the oesophageal hiatus, these authors stated that the right and left crura are supplied by the corresponding phrenic nerve. Where this normal arrangement is disturbed, it can be explained by minor aberrations of the muscle fibres of the crural region.

The right phrenic nerve in man, was investigated in detail by Delaloye (1958). Histological examination of the nerve trunk was purely qualitative, although an attempt was made to estimate the numbers and sizes of the nerve fibres within it. From this study, it was concluded that there is a preponderance of myelinated fibres of large diameter; several myelinated fibres of medium to small diameter, and several unmyelinated fibres.

A small gangliform structure was observed lying on the abdominal surface of the right crus of the diaphragm. From this structure, thread-like filaments radiated medially to the right coeliac ganglion and laterally to penetrate the muscle fibres of the diaphragm. This gangliform structure had been described previously by Schwalbe (1881) and Kiss and Ballon (1929) but its diaphragmatic connections were not studied in detail. Delaloye traced these nerve fibres upwards through the diaphragm to their termination in one or other of the terminal branches of the right phrenic nerve, and he suggested that this structure warranted the name of "inferior diaphragmatic nerve". Histological studies on the fibre content of this nerve showed that whilst it comprised mainly unmyelinated fibres, a number of myelinated nerve fibres were also present.

Ogawa (1958) and his collaborators, having observed subtotal degeneration of the appropriate hemi-diaphragm following section of the phrenic nerve in dogs, investigated the possibility that the phrenic nerves undergo partial peripheral decussation. By electrical stimulation of the phrenic nerve they were able to elicit contractions in the muscle fibres of the contra-lateral hemi-diaphragm in thirteen out of forty-eight dogs. This was followed by dissection of the diaphragm in an attempt to locate the nerve fibres which were responsible for this unexpected muscle contraction. Thus, Ogawa and his colleagues concluded that some fibres of the left phrenic nerve may cross over to supply the right side of the diaphragm, but there was no evidence that exchange of nerve fibres occurred in the opposite direction.

To explain this, it was postulated, in concurrence with Collis (1954) and his collaborators (see below), that the arrangement of muscle fibres of the crural region of the diaphragm is more easily understood, if the central tendon is considered as the site of origin of the muscle fibres. Accordingly, Ogawa and his collaborators concluded that all muscle fibres to the right side of the oesophageal hiatus are innervated by the right phrenic nerve, while those to the left of the hiatus are supplied by the left phrenic nerve, irrespective of their vertebral attachments.

In a series of experiments in dogs, Matochkin (1959) studied the zones of distribution of the phrenic nerve within the diaphragm. He observed that all regions of the musculature possess a large number of myelinated nerve fibres. Following repeated branching, they terminate at motor end-plates. Some unmyelinated nerve fibres terminate close to the walls of blood vessels, ramifying in the tunica media.

Following section of the nerve trunk, the effects are most marked in the motor terminals, which undergo fragmentation. It was noted also, that nerve fibres entering the diaphragm from intercostal nerves remained structurally normal, but details of their distribution were not given.

Autonomic fibres of the phrenic nerve.

Anatomists, since the time of Luschka (1853), have been aware of the connections which exist between the phrenic nerve and adjacent components of the autonomic nervous system. Physiological evidence suggesting autonomic function in the phrenic nerve was obtained by Kowalesky and Adamuk (1868) in a series of experimental studies in which they observed elevation of the blood pressure coinciding with stimulation of the phrenic nerve.

On the subject of phrenico-sympathetic communications, most of the earlier authorities considered two principal regions, the neck and the diaphragm. In the first of these regions, the findings of Luschka (1853), Henle (1879), Schwalbe (1881), and Rauber-Kopsch (1908) were comparable. Each of them observed multiple and diffuse communications, the supra-pleural plexus, associated with the stellate ganglion, being the principal region of contact with the sympathetic nervous system.

Pansini (1888) and Timofejew (1902) observed a small gangliform structure linking the right coeliac ganglion with the adjacent musculature of the diaphragm, while Rauber-Kopsch (1908) drew attention to another possible pathway of phrenico-sympathetic communication along the inferior phrenic vessels.

Felix (1922) also agreed with Luschka, Pansini and Timofejew on the anatomy of peripheral phrenico-sympathetic communications. Sub-diaphragmatic twigs arising from the right phrenic nerve entered a small gangliform structure, which, in turn, communicated with the right coeliac ganglion. On the left side, according to Luschka, there was no corresponding gangliform structure or surrounding plexus of nerve fibres. Felix, however, observed several fine neural fasciculi which communicated directly between the abdominal surface of the diaphragm and the left coeliac ganglion.

Delmas and Cabanac (1934) observed, in man, the effects of therapeutic phrenectomy and concluded that some of the salutory consequences of this procedure were attributable to coincidental division of the autonomic fibres present within the phrenic nerve; involvement of the diaphragm in involuntary activities such as laughing, crying, hiccough and vomiting was curtailed by interruption of the autonomic fibres.

Sergent (1935), concluded from experimental studies that stimulation of the nerve fibres of the phrenic nerve, produced a vasomotor response in the lung. Ungar (1936), confirmed these findings by performing experiments on the pulmonary circulation in dogs. Electrical stimulation of the central stump of the severed phrenic nerve for two minutes, produced a hypotensive response in the pulmonary vessels and 'lesions', the nature of which is not recorded, were found in all regions of both lungs. The pathway followed by the nerve impulse was thought to be by way of the stellate ganglion because the vasomotor effects could not be produced on stimulating the distal stump of the phrenic nerve.

Following this investigation, histamine hydrochloride was injected into the internal jugular vein in normal animals and subsequent examination of the lungs revealed 'lesions' which resembled those found in the first series of experiments. These investigations raised the possibility that the phrenic nerve influences the control of blood supply to the lungs.

Ogawa (1959) studied the phrenic nerve and the diaphragm in dogs, with particular reference to the trophic maintenance of the hemi-diaphragm after section of the corresponding phrenic nerve.

It had been assumed by Aoyagi (1913), Kuré (1928), Rossi (1928) and Kiss and Ballon (1929) that each of the sources providing autonomic nerve fibres for the diaphragm plays a part in the trophic maintenance of the diaphragmatic musculature. Ogawa held the opposing view, as stated by Schlaepfer (1923), Strauss (1933) and Masumoto (1934), that the nerve of importance, in this regard, is the phrenic nerve. The general statement by Tower (1939), that the degeneration of skeletal muscle follows only motor denervation is used by Ogawa in support of his conclusions.

Ogawa also considered the question of the rapid return of motor function to the paralysed hemi-diaphragm, following primary suture of a divided phrenic nerve. This effect he ascribed speculatively to rapid re-establishment of trophic maintenance by regenerating efferent nerve fibres, as yet unable to transmit effective efferent impulses.

Investigations on the structure of diaphragmatic muscle fibres.

Schiefferdecker (1911) studied the detailed structure of human diaphragm at various stages of development including embryos and adults. He observed that the muscle fibres vary in size, the larger ones staining more darkly with a variety of reagents. From foetal to pre-pubertal age groups, two types of muscle can be distinguished on the basis of nuclear morphology:

i) A type in which the nucleus is long and ovoid.

ii) A type which possesses a rod-shaped nucleus.

He also made the general statement that the muscle fibres of the diaphragm are 'inordinately rich' in nuclei.

These conclusions have been confirmed in the more recent studies of Günther (1952) who also observed two distinct types of muscle fibres in the diaphragm on histological examination. He attempted to relate this difference in morphological appearances, to diaphragmatic function. Sittaramayya and Bluhm (1950) studied the ultrastructural characteristics of the muscle fibres of the diaphragm in rats and showed that the fundamental structural difference between these two types of muscle fibres was the variation in length between sarcomeres.

Von Hayek (1953) studied the muscle fibres of the diaphragm in small mammals with the light microscope, and concluded that here, as elsewhere, the muscle tissue is composed of red and white muscle fibres. He investigated the regional distribution of these two types of fibres within the diaphragm and found that the lumbar and costal regions possess mainly red muscle fibres, while, in the sternal region, the fibres are predominantly of the white variety. He made two additional observations; that there is a distinct species difference - with respect to the proportions of red and white fibres present and that the red muscle fibres possess more nuclei than the white.

His conclusions, therefore, agreed in principle with those made in previous investigations, that there are two distinct types of muscle fibres present. He did not, however, indicate that this was a point of special interest, as previous workers, such as Günther (1952) and Sitaramayya and Bluhm (1950) had done. Von Hayek, in agreement with Timofejew (1902), regarded the histological features of diaphragmatic motor end-plates as no different from corresponding structures in skeletal muscles elsewhere.

Experimental Studies on the Innervation of the Diaphragm.

The majority of experimental investigations on the detailed anatomy of the innervation of respiratory muscles is to be found in the literature of the last hundred years.

Legallois (1811) studied the respiratory movements in young rabbits and concluded that there were four constant features:

(1) 'Un baillement' (gaping action of the mouth).
(2) Opening of the glottis.
(3) Elevation of the ribs.
(4) Contraction of the diaphragm.

No attempt was made to put these events in order of occurrence.

On removal of the spinal cord from these animals, Legallois observed complete cessation of all respiratory movements. In subsequent studies it was found that removal of the first three segments of the spinal cord produced no obvious effects on respiratory movements but removal of the fourth caused cessation of all respiratory movement. Transection of the spinal cord caudal to the origin of the phrenic nerve, caused paralysis of intercostal muscles, but the diaphragm continued to contract rhythmically. He concluded that the segment responsible for control of the diaphragm lies within the mid-cervical region of the spinal cord.

Beau and Maissiat (1824) investigated respiratory movements in dogs, and concluded, from a series of studies involving selective removal of respiratory muscles, that the diaphragm elevates the lower ribs when it contracts. This conclusion concurred with the findings of earlier investigators such as Galen (200 A.D.) and Magendie (1811) but disagreed with the views of Vesalius (1543), who believed that the lower ribs are depressed by contraction of the diaphragm.

Hénocque and Eloy (1882) studied the innervation of the diaphragm by transection of all the cervical nerve roots contributing to the phrenic nerve in cats, dogs, guinea-pigs, and monkeys. A daily commentary is given on the animal's progress following operation, with particular reference to respiratory movements. The animals were subsequently killed and the effects were studied topographically and histologically. It was observed, on the ninth day after operation, that the sixth, seventh, eighth and ninth intercostal nerves had undergone hypertrophy but the details of this phenomenon are not described.

Having observed that, in these animals, the phrenic nerve usually springs from two principal roots, Hénocque and Eloy investigated the effects of section of each of these in turn. It had been suggested by previous workers such as Beau and Maissiat that the upper root of the phrenic nerve supplied the sterno-costal portion of the diaphragm, while the lower root supplied mainly the crural fibres. The findings, in this investigation, confirmed that the two roots are different in function but that the exact nature of these differences is obscure. The effects of section were assessed by examination of the contractions of the exposed diaphragm and no histological survey was made.

Billard and Cavalié (1898) carried out experiments on the phrenic nerve in dogs, the nerve trunks being divided in the neck. It was found that, while the operation was compatible with survival, the dogs became progressively emaciated in the post-operative period. Billard and Cavalié observed that, in the immediate post-operative stage, the animals showed acute respiratory distress and made maximum use of all the accessory muscles of respiration, such as neck and abdominal musculature. Subsequently, there was gradual diminution of distress and progressive adaptation to the new mode of respiration, which relied mainly on the use of intercostal muscles. No attempt was made to study the histological changes produced in the diaphragm by these surgical procedures.

Investigating the effects of experimental anastomosis of nerves Langley and Anderson (1904) concluded that 'the cervical sympathetic can unite with the phrenic nerve and cause contraction of the diaphragm'. It was added, however, that artificial stimulation of the proximal component was required to produce contraction of the diaphragmatic musculature.

Lee, Guenther, and Meleney (1916) compared the diaphragm with Extensor longus digitorum on a clinico-physiological basis. These workers considered the diaphragm to be an almost perfect muscular apparatus, because of its greater resistance to fatigue and its relative invulnerability to paralysis by curare, when compared with other muscles such as Extensor longus digitorum and Sartorius. The enzyme systems present in the diaphragmatic muscle fibres - for example the cytochrome oxidase group, are said to be more efficient than their counterparts in other muscles.

Schlaepfer (1923), while investigating the effects of ligation of the pulmonary artery, also resected a portion of the phrenic nerve 2 cms long at the level where it reaches the pericardium. The procedure, carried out in seven dogs, led to the same effects on the diaphragmatic muscle in each animal. These changes were interpreted as indicating total paralysis of the appropriate hemi-diaphragm - the line of demarkation being distinct, and running exactly in the mid-sagittal plane. The structural evidence was thus in favour of the theory that, the phrenic nerve is the only source of motor innervation of the corresponding hemidiaphragm.

In support of his findings Schlaepfer quoted Russel (1897), who stimulated the roots of the phrenic nerve in dogs, electrically, and observed only unilateral muscular contraction of the diaphragm. A further experiment, (the investigator's name is not given), was conducted at Johns Hopkins Hospital, involving thoracotomy in a dog with subsequent stimulation of the various possible sources of diaphragmatic nerve fibres. The exposed diaphragm was inspected for signs of muscle contraction on stimulation of the lower intercostal nerves, (11, 12 and 13) the vagus nerve, and finally, the roots of the lower thoracic and upper lumbar nerves. No contraction of any part of the diaphragm was said to have occurred but it must be borne in mind that the conclusions made from this study are subject to the accuracy of the observation of the diaphragm with the naked eye.

Colledge and Ballance (1927) examined the possibility of restoring the paralysed diaphragm to active contraction and concluded that, in the neck, several nerves such as the vagus and recurrent laryngeal nerves and the descendens hypoglossi, could conveniently be used as the proximal component for union with the distal portion of the phrenic nerve. It is not clear from the record of this investigation whether the level of transection of the phrenic nerve was considered as an important factor. If the operative procedure was entirely confined to the cervical region, then the continued function of the diaphragm could possibly be attributed to an intact accessory phrenic nerve which joined the main trunk peripheral to the level of the lesion.

Regarding the nerve supply to the diaphragm, Lemon (1930) raised the question of a second source of motor innervation, in addition to the phrenic nerve. He did not indicate clearly his conclusions on this subject, although he quoted the views of several authorities, such as Piersol (1923), Cunningham (1923) and Schlaepfer (1923) who regarded the phrenic nerve as the only source of motor innervation of the diaphragm. Morris (1923) is quoted as supporting the opposite view. In the manner of Leonardo da Vinci, Lemon attributed several non-respiratory functions to the diaphragm and these can be summarised as a group of functional properties which influence the balance between intra-abdominal and intrathoracic pressure. In addition, he mentioned specifically, the secondary influence of these functions upon the control of blood pressure and upon the movements of the alimentary tract. Personal investigations conducted by Lemon in experimental animals, included the following procedures in adult dogs:-

Experiment (1). Unilateral avulsion of the phrenic nerve followed by transection of the spinal cord, at the level of the sixth cervical segment. The animal withstood the first procedure very well, and showed quick adaptation to the new demands on the remaining hemidiaphragm. However, following transection of the spinal cord at the level of the sixth cervical vertebra, great respiratory difficulty developed. From this experiment, he concluded that influences other than those exerted by the phrenic nerve, contributed to the respiratory function of the animal with one intact hemidiaphragm.

Experiment (2). One phrenic nerve was removed by avulsion and this was followed by a recovery period of one week; the other phrenic nerve was then avulsed and, following the second operation, the animal recovered within a few days. The next stage of the experiment involved detachment of the diaphragm from its costal attachments and this produced marked respiratory embarrassment. Adaptation to this new condition of the respiratory apparatus, was slower, but nevertheless effective. Further damage to the diaphragm e.g., resection of one hemidiaphragm was then performed and again adaptation occurred. One such animal was still surviving, although in a less active state, three years after operation. Lemon concluded inter alia, that damage to both phrenic nerves, was entirely compatible with life.

Brown and Satinsky (1951) carried out experimental vago-phrenic anastomosis in dogs and concluded from fluoroscopic evidence, that the diaphragm contracted normally following this procedure. The criteria which they used in support of normal diaphragmatic contraction were considered inadequate by Duncan and Eanes (1952) whose criticism was that the influence of the normal hemidiaphragm plus the effect of accessory respiratory muscles, had not been taken into account. The latter workers (Duncan and Eanes), from comparable investigations in which local nerves were used as the proximal component, agreed that contraction of the diaphragm could be elicited by artificial stimulation, proximal to the site of union. They observed, however, that the newly constituted nervous pathway was inadequate for normal diaphragmatic function. This, they attributed to a central, rather than a peripheral defect in nervous control of the diaphragm.

Duncan, Holtmann and Barnett (1953) made a study of the survival of dogs, with bilateral phrenectomy and extensive ablation of the intercostal nerves. Seven animals were maintained in good health for periods of up to sixty-seven days following this extensive procedure.

Two possible conclusions may be drawn from this evidence:-

(a) That the diaphragm is totally paralysed, and that the auxiliary respiratory muscles have taken over its function.

(b) That the diaphragm and intercostal muscles may possess sufficient residual motor supply to support respiration by their own modified contraction.

The second of these two possibilities is strengthened in that the intercostal nerve section was performed in the mid-axillary line. It is therefore possible that the segmental nerves contribute some of their branches to respiratory musculature, central to the site of the lesion. The presence of more than one neuro-muscular system for the control of respiratory function, is strongly suggested by this series of experiments.

Thomson (1955) studying the reaction of the rat diaphragm to denervation, concluded that, there is an increase in the weight of the diaphragm in the first few days following transection of the phrenic nerve. He found that the right and left halves of the diaphragm did not react symmetrically and, by way of explanation, stated the views of Greene (1935) who had described an asymmetrical diaphragmatic blood supply. Greene found that, in the rat, the left hemidiaphragm receives its arterial blood from the left phrenic artery, which arises from the aorta whereas the right hemidiaphragm is supplied by the right phrenic artery, which is, itself, a branch of the right superior adrenal artery. The venous outflow is to the left superior adrenal vein on the left side, and directly to the inferior vena cava, on the right. Quantitative data are entirely lacking from the records of Thomson and Greene.

Stewart and Martin (1956), studying the same problem experimentally provided quantitative data, such as the length of the survival period, which in their experiments ranged from 5-10 days. They recorded the following gains in the weight of the diaphragm (measured wet); mouse 24-57% in 5-8 days, rabbit (only one experiment) 29.5% in 10 days. In contrast to the findings of Thomson, they observed that the right and left hemidiaphragm reacted almost equally and that the dry weight showed corresponding changes. The explanation of these results is obscure but Stewart and Martin believed the effect to be due to a substance which is released by degenerating nerve fibres.

Guth and Frank (1959), also investigated the possibility of restoration of the paralysed diaphragm in rats by vago-phrenic anastomosis. Their assessment of the efficiency of this procedure involved electro-physiological recording from the 'restored' phrenic nerve. They also exposed the re-innervated diaphragm and made permanent records of their observations using a cine-camera. They claimed good functional restoration in three out of four animals so treated. According to these authors, the essence of successful restoration of a paralysed diaphragm is the selection of a nerve with the same electro-physiological properties as the phrenic nerve. A significant incidental finding, was made in the histological survey of the regenerating phrenic nerve. Guth and Frank (1959) found, lying alongside the regenerated fibres originating from the vagus nerve, some persisting fibres of the phrenic nerve. Further histological evidence of satisfactory restoration was denoted by the presence of normal motor end-plates in the diaphragmatic muscle. The main conclusion from this study was that the peripheral nerve most suitable for restoration of the paralysed diaphragm, would be the recurrent laryngeal nerve. This procedure was, therefore, carried out in the next series of experiments by Guth and his collaborators (1960). Six out of eight rats had satisfactory restoration of diaphragmatic contraction but only one monkey out of three subjected to the procedure showed this measure of success. It was again recorded that a small number of undegenerated fibres were present in the distal stump of the reconstituted nerve; this was attributed to the presence of an accessory phrenic nerve which had passed unnoticed at operation.

The authors concluded that the presence of this accession to the phrenic nerve must be taken into account in designing future experiments.

The significance of the findings of these workers is not only of a theoretical nature. The suggestion, following their first series of vago-phrenic anastomoses, that the procedure might be applicable to the restoration of the paralysed diaphragm in victims of 'bulbar' poliomyelitis, is of some practical value. It is also of interest that, in discussing the results of recurrent laryngeal-phrenic nerve anastomosis, they made the following statement: "As hypothesis, we suggest that the ipselateral hemidiaphragm (on the side of the experimental anastomosis) may have been innervated as well by accessory phrenic fibres joining the phrenic nerve distal to the site of anastomosis or by collateral sprouts from intercostal nerves".

Experimental investigations of this topic by Jefferson and his collaborators (1949), led to the conclusion that the phrenic nerve is the only source of motor nerve fibres supplying the diaphragm. In order to determine the length of time required for regeneration following total transection of the phrenic nerve in the thorax, they performed, in 1950, phreno-phrenic anastomosis in dogs. In the series of animals studied, function returned to the paralysed hemidiaphragm in three to six months. Subsequent removal of the diaphragm was followed by histological examination. It showed that the muscle fibres of the diaphragm were morphologically normal and this raised the question of trophic maintenance of the musculature during the period of paralysis. The authors suggested that the normal contra-lateral hemidiaphragm has a trophic influence on the denervated part. This is in opposition to the work of Gutmann (1962), who suggested that the trophic maintenance of a muscle depended, not at all on the surrounding musculature.

In 1958, Ogawa and his colleagues studied the phrenic nerves and diaphragm in dogs. An initial survey was made of decussation of fibres between the left and right phrenic nerves. They concluded that the phrenic nerves constitute the 'sole motor pathway' to the diaphragm and attributed any conclusions contrary to this to faulty technique. As a second conclusion, they suggested that the sympathetic nerves reaching the diaphragm, may exert a trophic influence on the musculature. In 1959 the same group of workers introduced a new experimental procedure, namely, anastomosis of the transected phrenic nerve (distal component) to an intercostal nerve. Trophic changes in the denervated hemidiaphragm were minimal.

One significant conclusion, derived from these studies, is that the trophic maintenance of the diaphragmatic musculature continues in the absence of demonstrable functional continuity across the site of anastomosis. This effect appears to be independent of the nerve used to form the proximal component of the reconstituted nerve trunk. Ogawa and his collaborators observed the same trophic maintenance of the diaphragm whether the phrenic, vagus or intercostal nerve was used as the proximal component. It was also concluded that the phrenic nerve is not unique in its ability to maintain trophic influences on the diaphragm.

Hess (1906) studied the diaphragm in humans using radiological methods. He described a diaphragmatic reflex produced by percussion of the lower ribs and from the radiological form of the ensuing wave of contraction, he deduced that the innervation of the diaphragm is divided into an anterior and posterior part. This type of radiological investigation is now largely discredited for the reason that it is difficult to differentiate active movement of the diaphragm from passive movement induced by contraction of auxiliary muscles of respiration (Duncan and Eanes 1952).

Physiological Studies Related to the Innervation of the Diaphragm.

In his Elementae Physiologiae, Haller (1759) commences his description of respiration by considering the functions of the intercostal muscles and the accessory muscles of respiration such as Sterno-mastoid, Trapezius, and Levator scapulae. He attributes the introduction of the term 'diaphragm', to Plato, and the alternative name 'septum transversum', to Celsus (p. 74). The description of the diaphragm includes an account of its attachments, its division into fleshy and tendinous parts and an account of the structure of the oesophageal hiatus. The various structures passing through the diaphragm are also enumerated (p. 88) along with an account of the 'nervi diaphragmatis' which are easily recognisable from the description as the phrenic nerves. The course of the phrenic nerve is described along with its branches, some of which are said to be directed towards the thoracic inlet. Experimental procedures carried out by Haller in dogs, (p. 92) included the application of pressure or irritants to the nerve trunk or resection of a portion of the phrenic nerve. Besides these techniques, he also observed the effects of transection of the spinal cord at various levels, and concluded that there is, in the cervical region of the spinal cord, a centre controlling respiration. These observations, made by Haller, were subsequently confirmed by numerous investigators.

Many of the early investigators such as Séguin and Lavoisier

(1790) were mainly concerned with the general effects of respiration. There are no detailed descriptions of structural observations in their records. Bichat (1823) made comparisons between living and dead animals and pointed out the vital nature of respiratory movements.

Duchenne (1823), in his 'Physiology of Motion', described the movements of respiratory muscles including the diaphragm (p. 443). His observations on the movements of the lower ribs concurred with those of Galen who concluded that the lowest ribs are elevated and displaced outwards by contraction of the diaphragm. This same facet of the subject was studied by Vesalius (1543) who concluded that the diaphragm ascends on inspiration thus producing elevation of the lower ribs. Riolan (1602) concluded that the ribs were depressed 'en masse' by inspiration. Borelli (1681) believed that the synergic action of the intercostal muscles was important in inspiratory movement and that this influenced the motion of the ribs. He did not commit himself to one or other opinion on the direction of rib movement. Magendie (1811) restated and confirmed the findings of Galen and added the view that the contracting diaphragm as it descends, makes use of the abdominal viscera as a fulcrum.

The electro-physiological experiments carried out in normal animals by Duchenne included stimulation of the exposed phrenic nerve in the cervical region, thus producing contraction of the diaphragm and elevation of the ribs (p 449). When the abdominal viscera had been removed the effect of diaphragmatic contraction was depression of the lower ribs; this confirmed the findings of Magendie. Duchenne added the comment that he disagreed with Haller who stated that the diaphragm is convex downwards in the terminal stages of deep inspiration. In his "Leçons sur la Physiologie Comparée", Bert (1870) considered in detail, certain aspects of respiratory movement and their control. His views on the controversial topic of movements of the lower ribs, were that coincident with diaphragmatic contraction, the lower ribs are elevated. Bert described the experiments which he used in investigating the innervation of the diaphragm in small mammals (p. 347). His modification of previous cord transection experiments included longitudinal bisection of the spinal cord. Thus, he observed to what extent the control of respiratory movement is unilateral. Following longitudinal bisection of the spinal cord, the exposed diaphragm could be seen to function normally. Transection of the bisected spinal cord at the level of the third pair of segmental nerves, produced paralysis of the homo-lateral hemidiaphragm. The contra-lateral hemidiaphragm continued to contract, but in a modified fashion. The reason for this contra-lateral effect of hemisection of the spinal cord is not given, but it is inferred that there is some contra-lateral control of each hemidiaphragm.

Regarding the distribution of nerve fibres arising from the individual roots of the phrenic nerve, Bert concluded, from experimental rhizotomy, that the upper root has functional control over the entire hemidiaphragm, but especially upon the muscle fibres of sternocostal origin. The lower root also has a general influence over the whole hemidiaphragm but especially upon the dorsal portion of the musculature. Bert concluded from experiments involving paralysis of the whole diaphragm, that this condition is not incompatible with life.

General studies by Sherrington (1898) on the peripheral distribution of the posterior roots of spinal nerves, included investigations of the motor roots of the fourth and fifth cervical nerves in monkeys. This provided a good opportunity for comparison with the findings of Bert, described above. Sherrington found, in three experiments, that section of the motor root of the fourth cervical nerve, produced loss of function, mainly in the ventral portion of the diaphragm while, in four subsequent experiments on the fifth cervical nerve, he defined the affected area, as the sternal and vertebral portion of the diaphragm. In previous studies on the nerves of skeletal muscles, Sherrington (1894) performed nerve transection on experimental animals, and after a survival period of fourteen days, tested the effects of faradic stimulation on the preparation. Although not directly concerning the innervation of the diaphragm, this investigation included consideration of several relevant factors. For example, the general rule, that paired nerves contain equal numbers of fasciculi and nerve fibres, was disputed by Sherrington, who stated that fascicular asymmetry and approximate equality of fibre content had been the rule in his observations on paired peripheral nerves. The question of intra-neural branching of axis cylinders was also considered by Sherrington in the light of the observations made by Schwalbe (1881) and Kölliker (1892). Sherrington stated that he could not record personal observations of this phenomenon. His opinion on the diameters of myelinated nerve fibres was that the largest ones are motor in function, but that the afferent fibres from muscle spindles, usually measure about 20µ in external diameter. In several experiments he observed 'surviving' fibres in the distal stump of a transected nerve and he regarded them as fibres originating in the spinal ganglia. He did not believe that they arise from the local components of the autonomic nervous system.

This observation is interesting when compared with the findings of Lloyd (1959) described above, who, with his collaborators, found surviving fibres in the distal stump of the transected phrenic nerve.

Head (1889) introduced the diaphragm-phrenic nerve preparation in rabbits, for the investigation of neuromuscular physiology. This technique was widely used by Head himself and, in the hands of others such as Walther (1962), Sant' Ambrogio (1963) and Bowman and Raper (1964), it has proved of great value for the study of physio-pharmacological features of nerve-muscle relationships.

The complexity of the neural pathways involved in respiration, was demonstrated by Porter (1895), who observed the 'crossed phrenic phenomenon'. Briefly, his findings were that, following hemitransection of the spinal cord close to the spino-medullary junction, the homolateral hemidiaphragm shows only minimal electrical activity and ineffectual contraction of muscle fibres. Subsequent blocking of the contra-lateral phrenic nerve, restores contractility to the previously paralysed hemidiaphragm and produces total paralysis of the hemidiaphragm on the side where the 'block' has been produced. Porter concluded that this phenomenon could only be explained on the grounds that there are connections between the left and right phrenic nuclei in the cervical region of the spinal cord.

Subsequent anatomical studies, such as those of Warwick and Mitchell (1956), described above, have failed to confirm Porter's conclusions and in order to explain the phenomenon, these workers suggested that there may be a partial decussation of nerve fibres between the right and left phrenic nerves in the intrathoracic part of their course. The investigators of the 'crossed phrenic' phenomenon stimulated interest, not only in the control of respiration but also in the neuro-physiological processes involved. Such a stimulus led to the use of this type of preparation by Grundfest (1963) in his investigation of the functions of the neurone. (see below).

Physiological properties of the nerve fibres of the phrenic nerve.

As in morphological studies, most of the physiological investigations have been restricted to circumscribed features of the innervation of the diaphragm.

AFFERENT FIBRES.

Anatomical and clinical evidence for the presence of afferent fibres in the phrenic nerve has been presented in accounts of earlier studies on the innervation of the diaphragm. (pp. 32-42). It was not until the latter part of the 19th century that corresponding physiological evidence was found.

Schreiber (1883) was among the first to observe an increase in the blood pressure due to stimulation of the central end of the severed phrenic nerve in dogs. This effect had been observed by Kowalewsky and Adamuk (1868) and it was attributed to the autonomic fibres present within the trunk of the phrenic nerve.

Thornton (1937) found that traction upon the phrenic nerve in dogs, produced sudden broncho-dilatation in the intact animal, the effect lasting only for a few minutes. He claimed clinical support for this finding in the work of Livingston and Gillespie (1935) who advocated the use of deep breathing exercises as a therapeutic measure in asthma.

In his investigation of the innervation of skeletal muscle, Hinsey (1934) considered at length the functions of muscle spindles and, in 1939, he studied the sensory components of the phrenic nerve. Histological studies on the size-distribution of myelinated nerve fibres in cats were presented in the form of histograms, representing both normal nerves and phrenic nerves whose motor nerve roots had previously been divided. He found that, after section of the motor nerve roots, approximately 100 myelinated nerve fibres remained following survival periods which ranged from 16-21 days. Corresponding quantitative studies on normal phrenic nerves, gave total counts of approximately 1,000 myelinated nerve fibres. He concluded that the ratio of the number of sensory to the number of motor fibres is very low in comparison with other mixed nerves, such as a motor branch of the femoral nerve. His concurrent histological studies on the unmyelinated fibres of the phrenic nerve led him to believe that the number of these fibres present in the normal nerve is approximately three times the number of myelinated fibres. There is no illustration of the histological preparations used in these quantitative studies. The number of unmyelinated fibres found in the phrenic nerve, compared with the corresponding number found in a muscle branch of the femoral nerve by Ranson and Davenport (1931) shows that Hinsey's figures are proportionately higher in relation to the total count of myelinated nerve fibres.

A mass of evidence has been accumulating, in the past thirty years to suggest that unmyelinated nerve fibres within the trunks of peripheral nerves may function as afferent pathways.

For example, in considering this question, Clark, Hughes and Gasser (1936) quoted the findings of Ranson and Billingsley (1916), Ranson and Davenport (1931), Erlanger and Gasser (1930), Adrian (1933), Tower (1939) and Hogg (1935). Those investigators were all agreed that unmyelinated fibres might well provide pathways for afferent impulses. Clark and his colleagues (1935) blocked conduction in the myelinated fibres within a peripheral nerve selectively and found residual reflex activity when the nerves were subjected to compression or anoxia.

At the same time Bishop and Heinbecker (1935) conducted a series of experiments on anaesthetised animals. They produced a reversible selective block to conduction in myelinated fibres by using alternating current and observed residual function in the nerve trunk so treated.

In item 5 of their summary they state: "The reflex responses to nonmyelinated fibre stimulation in the saphenous nerve, in the nerve to the medial head of Gastrocnemius, in the sympathetic trunk and in the vagus nerve, are similar with regard to respiration, blood pressure and pupillary dilatation, and, except for localisation, with respect to muscle responses. They are undoubtedly capable of producing pain in all these nerves, the degree depending chiefly on the number of fibres available".

The measurement of the conduction velocity of nerve fibres has proved a useful means of studying the fibre spectrum of peripheral nerves. It relies on the findings of Gasser and Grundfest (1939) that the conduction velocity of a nerve fibre is directly proportional to its external diameter. The relationship between these properties has been used by various investigators, such as Hunt and MacIntyre (1960) in electro-physiological investigations in the peripheral nervous system. This method of study provides a valuable means of confirming findings of morphological studies on the fibre content of peripheral nerves.

Gernandt (1946) stated that, the normal impulses attributable to conduction in myelinated fibres of the phrenic nerve, produce large spikes on the recording apparatus. In addition to these, a second type of spike can be produced by dropping acetic acid onto the diaphragm. His experiments in cats and one dog indicated that the impulses arising from this noxious stimulus were transmitted in the small myelinated (A) fibres and unmyelinated (C) fibres.

Investigating afferent impulses of pericardial origin Ruckebusch (1961) found evidence to suggest that these impulses may be transmitted from the pericardium by way of fine myelinated fibres which terminate in mechano-receptors in the parietal pericardium. They tend to transmit in rhythm with the heart beat.

Cuénod (1961) studied the proprioceptive reflexes of the diaphragm in rabbits, using the same methods as Fleisch (1928) which were based on simultaneous recording of action potentials in the phrenic nerve and in the diaphragm. It was concluded that the adjustment of the inspiratory inflow to sudden increase in normal resistance, is the result of three events:-

(a) A quick mechanical reaction, attributable to the elastic properties of the lung-thorax mechanism.

(b) A decrease in vagal inhibition due to delay in lung inflation by resistance to inspiration.

(c) A proprioceptive muscular reflex stimulated by the increased tension in the diaphragm.

In the same year, Yasargil (1961) studied the proprioceptive afferent fibres of the phrenic nerves in cats using electrophysiological methods based on action current recording by means of double bipolar leads. He placed the leads on the intact phrenic nerve and found that it was possible to differentiate the afferent from the efferent impulses passing along the nerve by their 'spike polarity'.

From concurrent measurement of the impulse velocity in the afferent fibres and, from the type of discharge of corresponding receptors, Yasargil concluded that the proprioceptive innervation of the diaphragm resembles that in other skeletal muscles but that proprioceptive function is less obvious in the phrenic nerve than in the motor nerves of limb muscles.

The conduction velocity of the efferent fibres of the phrenic nerve, measured in the same way, strongly suggests that the motor innervation of the diaphragm resembles that of postural muscles of the lower limb.

EFFERENT FIBRES.

Gasser and Newcomer (1921) in their study of action currents in the phrenic nerve, found that inspiratory contraction of the diaphragm is brought about by the discharge along the phrenic nerve, of 78-178 volleys of impulses at a rate which ranges from 71-106 metres per second. The waves of the recording vary in size, suggesting a variation in the number of nerve fibres involved in each one. The average size of the wave is larger towards the end of the inspiratory phase. Simultaneous recording of phrenic nerve impulses and diaphragmatic contraction, showed a 'cause and effect' relationship. Similarity in the spacing and size of the volleys on the left and right phrenic nerves suggested that the discharges from the spinal cord onto the phrenic nerves are controlled from some common source.

In 1930 Coombs and Pike compared the nervous control of respiration in kittens and in fully grown cats. They concluded that, in the young, the pathway of nerve impulses is very simple - the vagus nerve providing the afferent component and the phrenic nerves, the efferent. In older animals, however, they found that the conducting pathways are more complex and that the higher centre controlling respiration, is in the region of the quadrigeminal bodies. In a previous study, Pike (1916) had found that the movements of the heart produced an effect on the action current of the phrenic nerve, his opinion being that this effect was due to phasic changes in the blood supply of the nerve trunk.

According to Lee, Guenther and Meleney (1916) whose investigations of diaphragmatic functions have been described above, Fabricius (1603) and Haller (1733) were aware that the diaphragm possesses physiological characteristics which differentiate it from other skeletal muscles. The findings of Lee and his collaborators were followed by attempts to correlate these exceptional functional characteristics with the morphology of the diaphragmatic muscle fibres and the relevant investigations of several workers such as Sittaramayya and Bluhm (1950) and von Hayek (1953) have been described above.

In a search for further evidence to explain the exceptional resistance of the diaphragm to fatigue and paralysant drugs, Krnjević and Miledi (1958) studied the motor units of the diaphragm in rats using the 'diaphragm-phrenic nerve' preparation. This investigation was based upon the earlier general studies of Sherrington (1925) who defined a motor unit as 'an individual motor nerve fibre together with the bunch of muscle fibres which it innervates'. This concept was used by Clark (1931) to compare innervation ratios (i.e. the ratio of the number of motor nerve fibres in a muscle nerve to the number of muscle fibres innervated by that nerve) in different muscles such as Soleus and Extensor longus digitorum in cats. The values for these two muscles were 120 and 165 muscle fibres per motor nerve fibre, respectively. Clark's electrophysiological studies were carried out on animals in which the appropriate spinal ganglia had been removed previously, thus producing a pure 'motor preparation'.

Krnjević and Miledi isolated a single efferent nerve fibre within the trunk of the phrenic nerve and subjected it to electrical stimulation while still in continuity with the diaphragm. The resulting contraction of the muscle fibres was dispersed throughout the corresponding hemidiaphragm, and, on one occasion, it involved the entire diaphragm. This observation was at variance with the finding of Coërs (1953) who concluded that only a narrow strip of muscle is involved in any one motor unit. Krnjević and Miledi placed recording electrodes in individual muscle fibres of the diaphragm to observe the effects of single-fibre stimulation and confirmed their previous findings. Thus, they stated that single active muscle fibres are present throughout the musculature, that the active fibres are not all at the same distance from the surface of the diaphragm and that there are 5-10 muscle fibres in each motor unit. These findings are in keeping with the morphology of the diaphragmatic plexus as seen by Pansini (1888).

Krnjević and Miledi measured the thickness of the diaphragm and found that it ranges from 0.31 to 0.78 mms, the number of layers of muscle fibres being 12-24. The diameters of muscle fibres varied within the range of 25-50µ and the total number of muscle fibres present was estimated at 10,000. The total count of myelinated nerve fibres present in the phrenic nerve was given as 400 and the innervation ratio was, therefore, 25:1. This value must be regarded as approximate for several reasons. The criteria for identification of 400 myelinated nerve fibres in the phrenic nerve as efferent in function are not clearly stated, and it is therefore likely that this figure is inaccurate. The possibility of intraneural branching of the 400 myelinated fibres was not mentioned by these investigators and this would detract further from the accuracy of their estimation of the innervation ratio.

GENERAL STUDIES ON THE INNERVATION OF THE DIAPHRAGM.

Studies on the innervation of the diaphragm in dogs were conducted by Landau, Akert and Roberts (1962), who destroyed the spinal ganglia which give rise to the sensory fibres of one phrenic nerve and constructed histograms of the resulting spectrum of myelinated fibres in the main trunk. These were compared with histograms constructed to represent the distribution of myelinated fibres within the contra-lateral normal phrenic nerve. Acknowledging the fact that the number of fibres in the left and right phrenic nerves may not be equal, these investigators presented their results as percentages. It was found that 80-85% of the myelinated fibres in the intact nerve are within the size range of 9-12µ (diameter) and that 8-10% lie within the size range of 2-4µ. In the de-afferented phrenic nerve, there is a single 'peak' in the histogram in the area representing fibres of diameter

9-12µ which constitutes 80-85% of the residual fibres. The areas representing fibres of smaller diameter become progressively smaller with decrease in diameter of fibres. The efferent component comprises 55-56% of all fibres.

These figures may be compared with the corresponding values obtained by Hinsey (1939) who found that the efferent fibres constitute 90% of all nerve fibres within the phrenic nerve. However, Hinsey stated that this was an unexpectedly high result when comparing the results of studies in the phrenic nerve with the findings obtained from similar studies in a motor branch of the femoral nerve.

Landau and her collaborators (1962) also performed electrophysiological studies on the peripheral distribution of the three roots of the phrenic nerve in dogs, and were in general agreement with the work of previous authors such as Bert (1870) and Sherrington (1894). They observed that there is a medial to dorso-lateral arrangement of the myotomes corresponding to the spinal roots of the fifth, sixth and seventh cervical nerves. The fifth cervical nerve supplies the pars sternalis and the medial part of pars lumbalis, the sixth cervical nerve supplies the entire diaphragm except for pars costalis and the seventh cervical nerve supplies a relatively small dorso-lateral area around the posterior extremity of the central tendon. They found no evidence of contralateral innervation of the diaphragm.

Massion and Colle (1960) having found, in a previous study, that in decerebrate rabbits, the intercostal muscles have an important influence on posture, proceeded, in the same year, to study the reflex pathways involved in this mechanism. In the knowledge that certain impulses, of vagal origin exert an influence upon the tonus of the diaphragm (Hess 1938), Massion and Colle compared the responses of the diaphragm and intercostal muscles to stimulation of the central stump of the severed vagus nerve. They found that the inspiratory group of intercostal muscles reacted similarly to the diaphragm, in that tonus was increased, but that the expiratory group of intercostal muscles reacted by becoming hypotonic. Stimulation of the central stump of the vagus nerve in decerebrate animals did not affect the postural functions of intercostal muscles. These observations tend to eliminate the vagus nerve as a possible pathway of afferent impulses controlling postural function in the intercostal muscles of decerebrate animals. On the other hand, they indicate a distinct functional relationship between the diaphragm and the intercostal muscles concerned in inspiration.

Glebovskii (1961) investigated the physiological properties of motor fibres in the phrenic and intercostal nerves in newborn and adult cats. He described the work of Luriya (1902) who is said to have observed three types of nerve fibres present within the phrenic nerve, motor, sensory and autonomic. This is in agreement with the subsequent observations of several workers, such as Hinsey and his collaborators (1939) and Delaloye (1957). Glebovskii stated the following conclusions. 'The phrenic and intercostal nerves, supplying the main respiratory muscles, contain various kinds of motor fibres, differing in excitability and rate of conduction. In addition to the main group of myelinated fibres of large diameter (A, alpha fibres), these nerves contain beta, gamma, and B fibres. Contraction of the diaphragm is produced by the excitation of alpha and possibly beta fibres. Stimulation of gamma fibres, does not intensify contractions; it is possible that they innervate muscle spindles'. Regarding the comparison of newborn with adult animals he concluded that conduction velocity increases with age.

Rosenblueth, Alanis, and Pilar (1961), investigated the motor innervation of the diaphragm by electro-physiological methods and produced evidence in support of the hypothesis that the diaphragm possesses an 'accessory motor innervation'.

The following three statements summarise the investigation:

1. Direct observations, followed by confirmatory electromyographic studies, indicate that, following phrenectomy, the muscle fibres of the diaphragm continue to contract in a modified fashion.
2. Possible sources of motor nerve fibres, supplying the diaphragm, were subjected to stimulation while simultaneous recordings were made from an electrode placed in the muscle fibres of the diaphragm.

Contraction of muscle fibres was found to occur on electrical stimulation of the following sources:-

(a) From the 4th to the 7th cervical segment (phrenic nerve).

(b) From the 5th to the 13th thoracic segment (both of these sources (a) and (b) are controlled by the respiratory centre.

(c) From the 4th to the 9th thoracic segment (this component is not under the control of the respiratory centre).

This careful investigation, which set out to prove that the entire motor innervation of the diaphragm is controlled by the respiratory centre, failed in this main objective but produced physiological evidence regarding the existence of an "accessory motor innervation" of the diaphragm. The investigation was enlarged subsequently to include a number of different mammalian species in which the above findings were confirmed. The conclusions made from this study suggest that there is a wide spread representation of the motor control of the diaphragm within the spinal cord.

Sant' Ambrogio and his collaborators (1963), who studied the motor innervation and pattern of muscular contraction in the diaphragm in cats, used electro-myographic techniques which resembled those used by Rosenblueth and his colleagues (1961). They carried out transection of cervical segmental nerves (4,5, and 6) in serial fashion and, following section of all three roots on the right side, they found no residual activity in the homolateral hemidiaphragm. When this procedure was repeated on the left side, they found that there was residual, recordable electrical activity, in the homo-lateral hemidiaphragm. This residual activity was abolished by transection of the right phrenic nerve. These conclusions of Sant' Ambrogio and his colleagues are directly opposed to the views of Ogawa (1958) who observed, in dogs, decussation of phrenic nerve fibres from left to right only.

These conflicting views may be explained on the basis of species difference and it will be recalled that Warwick and Mitchell (1956) concluded from experimental studies on monkeys, that some decussation of phrenic nerve fibres also occurred in those animals. The direction of decussation in this species was from left to right.

Clinical Aspects of the Diaphragm and its Innervation.

Progress in the study of the diaphragm and its innervation has been influenced significantly by phasic changes in the character of clinical medicine. For example, from the time of Hippocrates until the inception of modern scientific surgery, studies on the diaphragm and its innervation were more frequently medical than surgical. Thus, Kidd (1911) in his review of the literature concerning the afferent fibres of the phrenic nerve, reported the findings of Bouillaud (1835), Geneau de Mussy (1853) and Peter (1871).

Bouillaud (1835) concluded, from his observations on patients suffering from 'cardiac neuralgias', that 'they (the pains) seem to reside in the phrenic and intercostal nerves'. Geneau de Mussy (1853), in a study of diaphragmatic pleurisy, considered the effects of this condition on the phrenic nerve. He found tenderness on pressure over the lower part of the Sterno-mastoid muscle in relation to the course of the phrenic nerve. He called these pains 'reflex' and referred to similar spontaneous pains, found in this region, in association with pleural affections, pericarditis and hepatitis. He accounted for 'reflex pain' as follows: 'it is phenomenon of irritation by continuity and whereas irritation is usually propagated in a centrifugal manner, here, on the contrary, it travels from the periphery of the phrenic nerve to its origin. The vomitings that are frequently seen in diaphragmatic pleurisy are the result of reflex action'.

Peter (1871), describing phrenic neuralgia, referred to the work of Bouillaud and Geneau de Mussy mentioned above. He stated that diaphragmatic or phrenic neuralgia was common and that 'it is perhaps associated with dorso-intercostal neuralgia, angina pectoris, certain affections of the heart and aorta, spleen or liver'.

Ross (1888) was greatly influenced by the preceding worker, Peter, and his clinical observations made only a minor advance in the knowledge of this topic. The conditions detailed by Ross, in which there was associated pain in the clavicular region, were pleurisy, pericarditis, peritonitis, passage of gall stones, angina pectoris, liver abscess and hepatitis.

McKenzie (1893) wrote that in several cases of basal pneumonia and pleurisy limited to the lower margin of the lungs, he found, not only shoulder pain, but also a distinct field of hyperaesthesia in the shoulder region. He attributed this to diaphragmatic pleurisy but added that it 'may be due to the vagus terminations being involved by inflammation of the lungs'. Clearly, McKenzie believed that the phrenic nerve was not the only pathway along which this type of pain might be projected.

Ramsay (1899) studied sixty-seven patients suffering from suprarenal tumour. In twenty-five there was a history of pain in the following situations:-

1. One or other renal region.
2. Over the whole back extending upwards into the shoulders

or downwards into the thighs.

3. Epigastrium or hypochondriac regions.

Robson (1899) published reports on three such cases and shoulder tip pain was present in each one. These clinical observations are supported by the findings of anatomists regarding the terminal distribution of subdiaphragmatic branches of the phrenic nerve.

Lenormant (1903) wrote an account of the surgical aspects of injuries to the diaphragm. He advocated prompt surgical intervention in every case where injury to the diaphragm was suspected. This was one of the earliest references to the diaphragm in a surgical context and it indicated the gradual entrance of the diaphragm into the field of clinical surgery.

PULMONARY TUBERCULOSIS.

Surgical interest in the diaphragm and, more particularly, in its innervation was very great in the early part of this century because of its relation to the treatment of pulmonary tuberculosis. Stuertz (1911) was one of the first clinicians to advocate elective paralysis of one hemidiaphragm in the treatment of this condition by section of the phrenic nerve in the neck. Sauerbruch (1913) supported the view of Stuertz and, rapidly, this procedure became one of the most common therapeutic measures used in the treatment of pulmonary tuberculosis.

There were, however, certain unpredictable results associated with this procedure, the effects of which were observed by many clinicians. Failure to obtain paralysis of the hemidiaphragm was noticed in many instances. The most frequently quoted reason for this, was the existence of an 'accessory' phrenic nerve, which had not been identified during the operation on the main trunk. While this communication between the nerve to Subclavius and the phrenic nerve had been reported by current authorities on human anatomy, such as Testut (1897), its clinical significance, was only now becoming apparent. Stimulated by this new surgical interest, anatomists began to take note of the incidence of 'accessory phrenic' nerves and the observations of several workers including Spalteholz (1896), Rauber-Kopsch (1920) and Felix have been mentioned above. It will be recalled that Yano (1928) conducted a topographical survey of human cadavers and confirmed that the incidence of 'accessory phrenic' nerves, was significantly high.

In the light of this knowledge, Anderson (1934) advocated the more radical procedure of 'phrenic avulsion' for routine use in elective paralysis of one hemidiaphragm. This new method was more reliable for the production of paralysis of the hemidiaphragm but it introduced the theoretical hazard of accidental damage to the subclavian vein in cases where the accessory phrenic nerve passes in front of the subclavian vein. Fortunately, despite frequent use of this procedure, the incidence of fatal haemorrhage from the subclavian vein has been negligable.

Stenson (1940) reviewed 'the pitfalls of phrenic nerve crush' and emphasised the unsatisfactory nature of this, the most conservative of all procedures for the elective paralysis of one hemidiaphragm. This work was produced rather late to be of any real use in the treatment of pulmonary tuberculosis as new methods, involving chemo-therapy were in the ascendancy at that time. However, the theoretical considerations were of some value when clinicians began to use 'phrenic crush' techniques in the treatment of other conditions such as diaphragmatic spasm.

PHRENIC NERVE INJURY IN THE NEWBORN.

Throughout the clinical literature on the phrenic nerve and diaphragm, there are reports of unilateral paralysis of the diaphragm in newborn infants. This can be attributed, in most cases, to birth trauma, and is frequently associated with damage to the brachial plexus, such as Erb's palsy. Among the earliest records of this condition are those of Weigert (1920), Kofferath (1921), Ehrenfest (1922) and Finkelstein (1924). In a review of the literature on the condition, Friedman and Chamberlain (1926) concluded that phrenic nerve paralysis is not a rare type of birth injury and advised that its presence should be excluded in every case of neonatal brachial palsy.

Subsequent reports by Tyson (1933), Turner and Bakst (1949) and by Schifrin (1951) further emphasise the importance of early diagnosis of this condition. The last author, Schifrin, reviewed the relevant literature and presented, in tabular form, the clinical data of each reported case from 1902. He concluded that most of these cases of diaphragmatic paralysis followed delivery from the breech position. Maintenance of adequate oxygenation formed the basis of treatment and, in one case, all evidence of paralysis had vanished by the 19th day, while in two others, there was normal diaphragmatic function, when the child had reached the age of six months.

DIAPHRAGMATIC HERNIA.

Discovery of a para-oesophageal recess in the diaphragm of a female cadaver in the dissecting room prompted Fraser (1934) to examine the morphology of this abnormality in detail. From a review of the literature on diaphragmatic anomalies, he suggested that the current classification of these conditions should be changed to conform to the Gruber classification (1927) which Fraser then described (p.122).

He considered the mode of production of diaphragmatic anomalies and postulated two main types of defect:-

1. Primary defects, such as the absence of the septum transversum, persistence of the pleuro-peritoneal canals and anomalous muscle invasion.
2. Secondary defects, due to extrinsic factors which can be more or less clearly defined.

Predominant among these, are disturbances of pressure relationships between the two surfaces of the diaphragm, e.g., the normal herniation of the midgut into the umbilical coelom. It was noted by Fraser that, in several instances, diaphragmatic hernia has been associated with mal-rotation of the gut.

Benjamin (1963) studied the structure of the diaphragm in 900 patients undergoing laparatomy. In this series it was found that eight out of ten people had diaphragmatic defects which were large enough to admit two to four fingers. The condition was more common in males and occurred more frequently on the left side than on the right. It was concluded that most of these defects were due to developmental abnormalities in the pleuro-peritoneal membrane.

Further evidence of the relatively high incidence of this condition was described by Hartzell (1940) who, in his review of diaphragmatic hernia in children, presented the data of other surveys. The work of Hedblom (1934) is mentioned, because of the large number of individual cases studied by this investigator who observed the condition in 2,137 children. Hartzell's personal series of sixty-eight cases is also described.

In order to establish the nature of the muscle architecture in the region of the oesophageal hiatus, Listerud and Harkins (1959) studied 204 human cadavers. They found eleven recognisable types of hiatal musculature but the three most common arrangements accounted for 90% of the 204 subjects studied. In the majority of these, the muscle fibres surrounding the hiatus arose from the right crus. The significance of this investigation goes beyond the mere establishment of a concept of the normal muscular architecture of the para-oesophageal region of the diaphragm. It suggests that the nerve supply to the crural region might also be variable to the same extent.

Throughout the literature, there is a considerable amount of evidence which suggests that the nerve supply to the diaphragm is less consistent in the crural region than elsewhere. From their dissections of the crural region of the diaphragm in 14 human cadavers, Collis, Satchwell and Abrams (1954) concluded that all the muscle fibres arising from the left of the mid-line, are supplied by the left phrenic nerve while the remainder of the muscle fibres are supplied by the right phrenic nerve. Those fibres of the right crus which cross the mid-line are supplied by the left phrenic nerve.

An investigation of this region in 25 human cadavers was performed by Sery and Králik (1955), whose results were entirely in agreement with those of Collis and his colleagues (1954). The contributions of Collis and his colleagues and Sery and Králik would have been greatly improved, had they been supported by confirmatory studies in experimental animals. It is also noteworthy that neither of these two groups of workers studied the subject histologically, thus leaving room for doubt regarding accurate observations of small groups of nerve fibres invisible to the naked eye.

Merendino and his collaborators (1956) investigated the topographical distribution of the diaphragmatic terminals of the phrenic nerve in dogs. Their objective was to determine the optimum positions for diaphragmatic incisions in combined thoraco-abdominal operations. Their conclusions, like those of Ramström (1906), were that the main distributive network of the phrenic nerve runs parallel to the peripheral border of the central tendon and lies about half-way between that structure and the peripheral attachment of the diaphragm. They also observed the possibility of paralysing selected small regions of the diaphragm by local surgical interruption of the diaphragmatic plexus. In a subsequent study of this subject, Sarra (1959) was able to confirm the findings of Merendino and his colleagues.

Aufses (1940), reviewing the surgical aspects of this subject described briefly the history of selective paralysis of the diaphragm, stating that it was widely used from 1925-1935, as a therapeutic measure in the treatment of pulmonary tuberculosis. His table, presenting indications for the use of this procedure, includes the following main points:-

1. Treatment of pulmonary tuberculosis as an independent procedure and as an adjunct to other forms of therapy.
2. Treatment of non-tuberculous conditions, such as the following:-
    (a) Thoracic surgery, including pneumonectomy, lobectomy and diaphragmatic lesions.

(b) Spasm of tetanus or intractable hiccough.

(c) Diaphragmatic hernia.

(d) Haemoptysis.

(e) To increase the volume of the abdominal cavity before surgical reduction of large hernias.

In discussing the anatomy of the phrenic nerve, he points out the commoner anomalies of the phrenic nerve and mentions the importance of cutting all contributing sources, where total hemi-diaphragmatic paralysis is desired. Where total, permanent paralysis is indicated, he advocates the use of 'avulsion' of the phrenic nerve. It is of interest that in the records of recent investigators many of the uses of operations on the phrenic nerve, recommended by Aufses, are represented. Thus, Cohen (1939) reported the successful use of phrenectomy for the relief of symptoms in two cases of para-oesophageal hernia. Greene (1932) employed bilateral division of the phrenic nerve for the relief of diaphragmatic spasm following encephalitis in one patient. The result was excellent and about six weeks after operation the patient reported that her breathing was almost normal. This proved, clinically, the theory of several anatomists, including Duncan and Eanes (1952), that the division of both phrenic nerves is not incompatible with life. Greene made no claim to be the first clinician to use this procedure, quoting in his report the work of Dowman (1927) and Skillern (1931) which also involved this seemingly hazardous procedure.

Clinicians performing surgical interruptions of the phrenic nerve, provided material for physiological study of the effects of elective diaphragmatic paralysis. One such investigation was carried out by Werner (1930) who studied the changes in respiratory function and reached the following conclusions:-

1. The vital capacity was reduced in all patients.
2. In the vast majority of patients, the tidal air was reduced.
3. The oxygen consumption remained unaltered.

Banyai (1938) studied the radiological features of respiratory movement and concluded that fluoroscopy, when used alone, could be misleading with respect to diaphragmatic mobility.

A comparatively new term appearing in present day clinical literature is 'diaphragmatic flutter'. This term is best defined in the words of an article in the Lancet (1962). It consists of paroxysmal contractions of either, or both leaflets of the diaphragm at a rate of 35-480 per minute, lasting from a few seconds to several months. It has been seen classically, in association with epidemic encephalitis and in conditions involving cardiac enlargement. Its relationship to hiccough, is not clear, and there may well be no connection between the two conditions apart from the involuntary nature of both.

Several points of interest arise in the consideration of this condition, the first being that, the aetiology is quite unknown.

It is not possible to state whether the cause of the disease is centrally or peripherally located on the grounds of present knowledge. It is quite clear, however, that the condition can, almost invariably, be relieved by section of the appropriate phrenic nerve in cases where one side is involved. It has been found, however, by Dressler and his colleagues (1954) that the right sided condition is unchanged by 'blocking' of the right phrenic nerve. No reason is given for this failure but it is inferred that this is due to the bilateral derivation of the nerve supply of the right hemidiaphragm. These authors also indicate a close connection between abnormal contraction of the diaphragm and concurrent incoordination of the intercostal musculature.

Although a comparatively new term, spasms of the diaphragm were encountered personally by Leuwenhock (1723), himself a sufferer from the condition. It has also been reported that this condition is occasionally accompanied by pain, simulating 'angina pectoris' and, in some instances, this symptom has led to the erroneous diagnosis of heart disease. Attempts are made by several authorities, to explain the aetiology of the condition, and the consensus of opinion is that increased excitability of the phrenic nerve, is the most important factor. The condition is not common, but may assist the understanding of normal function of the diaphragm and phrenic nerve.

MATERIALS AND METHODS.

Investigations on the nerves reaching the diaphragm were carried out in two parts:-

A. Studies in normal animals.

B. Studies in experimental animals.

Selection of the animals for both normal and experimental studies was at random, and without reference to age, sex, litters, and feeding habits. In cats, however, only those animals which were physically normal, as assessed by general inspection and auscultation of the thoracic cavity, were selected. The body weight of the cats used, ranged from 2.5 to 4Kg.

STUDIES IN NORMAL ANIMALS.

These studies were carried out in fully grown animals comprising 32 cats, 14 mice, 6 rats and 2 guinea-pigs.

I. Topographical Studies on Nerves Reaching the Diaphragm.

The animals were deeply anaesthetised by intraperitoneal injection of an aqueous solution of sodium pentobarbitone 60mg/Kg body weight. The thoracic and abdominal cavities were opened widely, conserving the integrity of the diaphragm and the cadaver was immersed in 10% formalin (analytical reagent), which had been stored over marble chips for at least four weeks.

The nerves studied in these preparations were:-

a) The right and left phrenic nerves.

b) The lower six right and left intercostal nerves.

c) The ascending branches of the coeliac ganglia.

This preliminary investigation was conducted in four adult cats, and it included a complete survey of the nerves which reach the diaphragm. A Zeiss dissecting microscope, giving magnifications of 10x to 40x, was used to study finer details. Nerve fibres were found reaching the diaphragm from the sources enumerated above, and they approached from above, from the region of the peripheral attachments of muscle fibres and from below respectively. Details of origin, course and distribution of each nerve source, were examined and the main topographical features were photographed for record purposes.

II. Histological Studies on the Phrenic Nerves.

These investigations were carried out in three adult cats. The fascicular pattern and fibre spectrum of individual phrenic nerves were studied by both light and electron microscopy and histograms were prepared.

Each animal studied, was deeply anaesthetised by intraperitoneal injection of sodium pentobarbitone, the dosage being 40mg/Kg body weight. The thoracic cavity was opened by two longitudinal incisions, one in each anterior axillary line. The caudal extremity of these two incisions were joined by a transverse incision passing through the meso-metasternal joint.

The flap of chest wall, so produced, was hinged at the root of the neck and its upward displacement afforded good exposure of the undisturbed phrenic nerves which were removed in the following manner. Commencing at the diaphragmatic extremity, the nerve was removed in lengths of approximately one centimetre, each length being placed on a small cardboard frame, which was labelled so that the proximal and distal ends could be identified. An interval of no more than three seconds was allowed for the nerve portion to adhere to the card. In addition, each card was numbered so that the relation of each portion to the intact nerve, was clearly evident. The first, third, and fifth portions, so removed, were immersed in Flemming's fixative, the second, fourth, and sixth were fixed in formalin 10% (analytical reagent) made up with tap water.

A. QUANTITATIVE STUDIES ON MYELINATED FIBRES AND FASCICULAR PATTERNS.

(i) Fixation of the nerve portions was of twelve hours duration in Flemming's fixative. Excess fixative was then removed by washing in running water for 24 hours. Dehydration of the specimens was then carried out over a period of three hours in ascending concentrations of alcohol. Clearing was effected with cedarwood oil, to which the sections were exposed for 12 hours, after which the specimens were embedded in paraffin wax.

(ii) Transverse sections were cut at 5μ and mounted on slides with gelatin. A preliminary examination of the sections at this stage enabled those which were cut obliquely, to be discarded.

(iii) Counterstaining was performed using an alcoholic solution of lithium carbonate and haematoxylin, and the sections washed in running water.

(iv) Differentiation was then effected in three stages:-

- (a) Immersion in a dilute solution of potassium permanganate.
- (b) Rinsing in water.
- (c) Immersion in a solution containing oxalic acid 1% and sodium sulphite 1%.

This process was carried out under microscopic control and differentiation was considered complete, when the myelin sheaths were intensely black and could be clearly distinguished from the interstitial material around them.

In order to study the myelinated fibres in the phrenic nerves and to make comparisons at different levels along the length of the nerves, the osmium preparations were photographed and processed in the following way.

Using a Leitz microscope, photomicrographs were taken using Agfa I.F.F. 35mm film; the initial magnification being 40x. Subsequent enlargement resulted in prints in which the total magnification was 1,000x.

Measurements were made using a plastic scale 3mm thick with a series of circular holes, varying from 4mm to 20mm in diameter. Since a multiplication factor of 1,000 was common to both the scale and the prints, it was possible to record the measurements in microns. For measurement of the diameters of the myelinated fibres, the criteria, recommended by Rexed (1944) were used. The fibres were classified within the following ranges 2-4μ; 4-6μ; 6-8μ; and so on up to 18-20μ. As the diameter of each myelinated fibre was estimated, it was punctured by a mechanical counting device; to eliminate the possibility of recounting the same fibres.

The sum of myelinated fibres in each range of diameter was expressed as a percentage of the total number of fibres in the nerve as a whole. From these data, histograms were constructed, representing the fibre spectrum at three distinct levels in each nerve. Eighteen histograms were prepared, representing the results of examinations of the right and left phrenic nerves from three animals.

B. STUDIES ON AXIS CYLINDERS.

Light Microscope Studies.

The portions of phrenic nerve fixed in formalin, were subjected to silver impregnation techniques, so that the numbers of unmyelinated fibres present at various levels in the phrenic nerve, could be estimated. The methods used were those of Bodian (1936), Bielschowsky (1940), and Romanes (1950). The variant of Ranson's method described by Jones (1936) was also used to demonstrate axis cylinders in portions of phrenic nerve previously fixed in ammoniated alcohol.

C. STUDIES ON THE RELATIONSHIP BETWEEN THE PHRENIC NERVES AND THE PERICARDIUM.

In order to study the finer details of the pericardial branches of the phrenic nerves, the whole pericardium was studied with the phrenic nerve in situ:-

(i) After intravital staining with methylene blue.

Attempts were made to demonstrate the fibres of the phrenic nerve which ramify within the pericardium. The technique used by Weddell et al. (1954) was applied to three cats including initial injection of spreading factor, hyaluronidase. Preliminary examination of the pericardium showed good differentiation of neurovascular bundles, on more detailed study with the light microscope after clearing and mounting of the specimens, individual nerve fibres could not be defined clearly and consistently. The method was therefore abandoned.

(ii) After silver impregnation.

For this investigation, three cats and four mice, from which the diaphragm had been removed previously for normal studies, were used. Formalin fixation of the pericardium for a period of three weeks was followed by silver impregnation in the manner described by Schofield (1960).

III. Electron Microscope Studies.

Method (i).

(a) Fixation. Portions of phrenic nerve, approximately 5mm long, were removed in the manner previously referred to, transferred to small cardboard frames, and immediately immersed in ice cold 1% buffered osmium tetroxide (see appendix 1). The temperature of the solutions was maintained between 0°C and 4°C for 3-4 hours.

(b) Dehydration, using acetone in increasing concentrations from 10-100% by 10% increments, was then carried out, specimens being kept in each solution for at least 15 minutes.

(c) Embedding. The specimens were immersed in araldite-acetone mixture (8:1 ratio) overnight at 4°C, after which they were placed in a tube containing the same mixture and rotated slowly in a centrifuge for one hour. Pure araldite was used for final embedding.

(d) Cutting of sections. Using an L.K.B. microtome, sections were cut at approximately 500Å, the factor of variation being 200Å. The thickness of the section was judged by its colour, which, optimally, was gold-purple.

(e) The sections were stained with 1% lead hydroxide (Karnowsky 1961) for 30 minutes, placed on a 200-mesh copper grid and examined in a Siemen's Elmiskop at magnifications which ranged from 2,500x to 33,000x.

Adaptations of the ultrastructural technique mentioned above, were found to be necessary for the satisfactory study of the unmyelinated nerve fibres.

Method (ii).

The first three steps in the above procedure were repeated and modifications were made to the later steps which, for clarity, are given corresponding letters.

(d) Sections were cut as before, but at a greater thickness, approximately 600Å, the factor of variation being again ± 200Å.

(e) The staining technique was the same as before, but the exposure to lead hydroxide was prolonged to 45 minutes.

The stained section was then placed on a copper specimen grid, which, instead of having a supporting meshwork, had a single aperture with a diameter of 750 microns.

The object of these modifications of the previous method, was to examine, on one grid, the entire cross-section of the nerve trunk. To give extra support to the specimen, the grid was first coated with carbon and a film of "Parlodian" of uniform thickness was applied.

Exposure of the specimen to the electron beam in the microscope was carefully controlled to avoid overheating and damage. The electron microscope was adjusted to give a magnification of 2,500x on the fluorescent screen. The image, so produced, was photographed using Adox 35mm film, and allowing appropriate overlap between contiguous areas. From the photographic prints (numbering approximately 500) a montage was constructed and the entire transverse section of the phrenic nerve, was represented on a large composite photograph, at a total magnification of approximately 4,500x.

STUDIES ON THE STRUCTURE OF THE SPINAL MENINGES.

In one adult cat, the entire bundle of filaments comprising the dorsal nerve root, together with its meningeal coverings, was taken from the fifth cervical segment. (fig. 1). Following preparation by the same techniques as those used for the study of the phrenic nerve, transverse sections were examined by light and electron microscopy.

Those sections which were prepared for ultrastructural study, were mounted on standard 200-mesh copper grids and examined at magnifications ranging from 2,000x to 30,000x.

Studies on Intercostal Nerves.

TOPOGRAPHICAL INVESTIGATION.

The intercostal nerves were traced from the intercostal spaces into the region at which the muscle fibres of the diaphragm are attached to the inner aspect of the thoracic wall. The thread-like diaphragmatic branches arising from the lower six intercostal nerves, were barely visible at this point, and, in order to trace them into the diaphragm, the dissecting microscope was used. Three normal adult cats were used in this investigation.

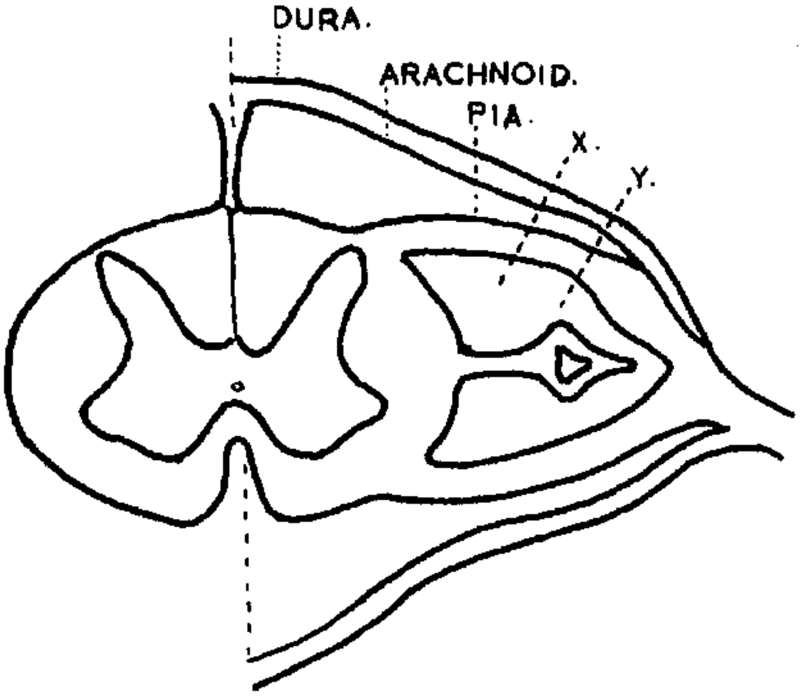


Fig. 1

Illustrating the portion of tissue, between line X and line Y, which was removed for studies on the spinal meninges.

HISTOLOGICAL INVESTIGATIONS.

The terminal arborisations of intercostal and phrenic nerves within the substance of the diaphragm, referred to collectively, as the diaphragmatic plexus, were studied by two main methods which were applied to three cats, six mice, two rats, and two guinea-pigs, all of the animals being fully grown:-

(i) Frozen sections were cut at a thickness of 50µ from portions of muscle removed from representative areas of the diaphragm in cats (fig. 2).

(ii) "Whole preparation" techniques were applied to the study of the diaphragmatic plexus in mice, rats and guinea-pigs.
Two staining procedures were used:-

(a) A modification of the Bielschowsky silver impregnation technique was applied to the "whole preparations" and to the frozen sections cut at 50µ (Schofield 1960).

(b) A cholinesterase technique (Coupland 1957) was applied to whole preparations of the diaphragm in mice, rats, and guinea-pigs.

It is emphasised that, by using these methods, it is possible to study not only the intercostal, but also the phrenic nerve components of the diaphragmatic plexus, and, accordingly, these two features were studied together.

The Diaphragmatic Plexus.

Initially, attempts were made, in cats, to study the diaphragmatic branches of intercostal nerves in silver-impregnated

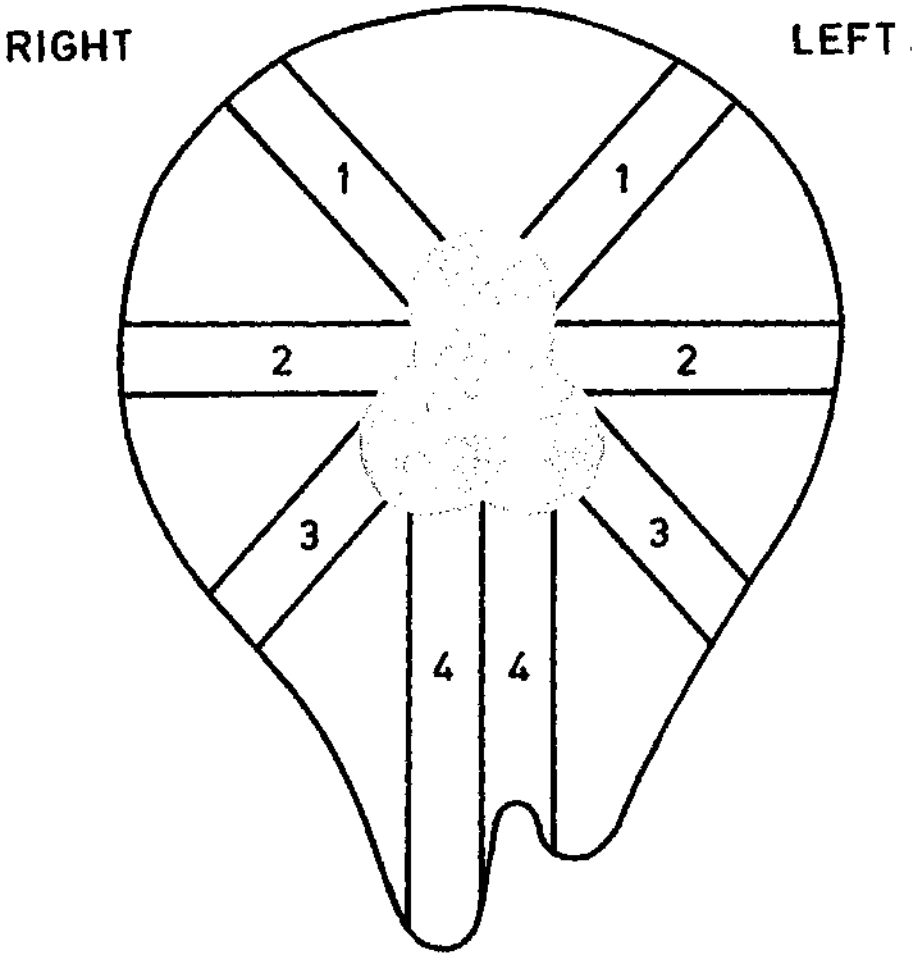


Fig. 2

Illustrating the zones of the diaphragm which were removed for histological study in normal and operated animals.

frozen sections of the diaphragm cut at 50µ. (fig. 2). Although nerve bundles and individual nerve fibres were defined satisfactorily, reconstruction of the plexiform arrangement of the diaphragmatic nerves proved to be difficult in cats and a method using whole preparations was therefore applied to the study of the diaphragm in smaller animals.

In mice, anaesthetised with ether, normal saline was injected intraperitoneally, to produce abdominal distension and stretching of the diaphragm. The thoracic cavity was then opened, and pledgets of cotton wool, soaked with 10% formalin, were placed against the thoracic surface of the diaphragm. After preliminary fixation for 10 minutes, the diaphragm, along with adjacent parts of the body wall, were removed in one piece, and immersed in 10% formalin for at least three weeks. Fragments of ribs and costal cartilages were then removed from the specimen, leaving a fringe of intercostal and abdominal wall musculature attached to the periphery of the diaphragm. The diaphragm was dissected free from its vertebral attachments, the whole specimen was impregnated with silver, and then cleared, mounted and examined as a whole preparation.

This procedure was also carried out in two young rats in which the diaphragm was sufficiently thin to enable nerve trunks and even individual fibres to be identified clearly.

Whole preparations of the hemidiaphragm from three mice, two rats, and two guinea-pigs were used for the demonstration of neuromuscular junctions in the following manner:-

The animal was anaesthetised with ether and the skin was removed from the thorax and abdomen. By means of two complete transverse sections, one in the mid-thoracic region, and one at the level of the iliac crests, the part of the trunk containing the diaphragm was isolated. Following complete removal of thoracic and abdominal viscera, the peripheral attachments of the diaphragm were divided as close to the body wall as possible.

The diaphragm was then divided into two, by an incision in the median sagittal plane and the two portions were treated according to the cholinesterase technique described by Coupland (1957).

STUDIES IN EXPERIMENTAL ANIMALS.

Experimental procedures were attempted in rodents, but proved to be unsuccessful due to difficulties with artificial respiration, an essential concomitant of techniques involving thoracotomy. Cats were used in this part of the investigation because of their availability, size, and suitability for surgical manipulation. In thirty animals, one or other of the following surgical procedures was carried out.

Ligation or transection of nerve trunks was performed, either unilaterally or bilaterally, at selected situations as follows:-

(i) The right or left phrenic nerve.
(ii) The roots of cervical or thoracic segmental nerves.
(iii) The right or left coeliac ganglion.

Following survival periods which ranged from 1-30 days, the animals were anaesthetised and the effects of the above procedures on the phrenic nerves and on the diaphragm were studied.

Transection of the phrenic nerve in the posterior triangle of the neck was attempted in two cats, but it was not used as a standard procedure because of the difficulty of ensuring that all of its roots had been divided.

Pre-operative Procedure.

The animals were anaesthetised by intraperitoneal injection of sodium pentobarbitone. (35mg/Kg body weight). At the same time, Pethilorfan (50mg) and atropine sulphate solution 0.66mg were given by intramuscular injection in the gluteal region. When the animals were deeply unconscious the appropriate regions were shaved, washed with cetavalon and soaked with alcohol (70%). The area was then isolated with sterile towels and the appropriate incision was made. Throughout the entire procedure sterility precautions were observed by the operator and assistant.

Unilateral Operative Procedures.

(A) DIVISION OF THE CERVICAL NERVE ROOTS:-

Four adult cats were used in this procedure. The skin incision extended from the occipital region to the spine of the seventh cervical vertebra in the midline, dorsally. Vertebral levels were located by identifying the first cervical spine and proceeding caudally. The spinal dura was exposed by the removal of the laminae of the fourth, fifth, sixth and seventh cervical vertebrae. The nerve roots of one side of the cord were picked up on a nerve hook and severed, peripheral to the corresponding spinal ganglion.

(B) DIVISION OF THE PHRENIC NERVE IN THE THORAX.

This procedure involved thoracotomy and it was, therefore, necessary to maintain artificial respiration with the prior insertion of an intratracheal tube. A McGill's 'neonatal' tube was used where possible but, if this was impracticable, a noncuffed rubber tube was passed, the cavity of the oral pharynx being loosely packed with moistened gauze. In either case the tube was then connected to an automatic respirator with the side valve in open position to avoid overdistension of the lungs.

The skin incision was made in the fifth intercostal space and the blade discarded. The skin was retracted and the pectoral and intercostal muscles were incised down to the parietal pleura. Haemostasis was secured by ligation or diathermy before the pleural cavity was opened. When the pleural cavity was opened, the lung collapsed, and this was followed by a few moments of violent respiratory effort on the part of the animal. During this time, the side valve of the automatic respirator was partially closed and the respirator switched on. When the respiratory effort had abated, the side valve was adjusted to produce adequate ventilation. The heart, now under direct vision, could be watched, and changes in its rate of beating were observed. The ribs were retracted to aid exposure. The phrenic nerve was now located, mobilised from its pleural covering, and interrupted either by transection or ligation with 00 silk, at the level of the lung hilum.

To close the chest wall, three or four sutures were inserted embracing the rib above and the rib below the incision; the skin was not included in these. The two sutures at the extremes of the incision were tightened and tied in two successive phases of inspiration. The middle one (or two) sutures were tightened with the lung in the state of maximum inflation which was achieved by switching off the respirator and blowing down the open side valve of the connecting tube. The respirator was restarted in the expiratory phase and the skin was closed with silk sutures. Artificial respiration was usually required for approximately 20 minutes and the side tube was opened gradually until the animal was maintaining adequate oxygenation as assessed by the colour of the mucous membranes and by the regularity of the heart rate as estimated by auscultation of the chest.

(C) LIGATION OF THE PHRENIC NERVE IN THE THORAX.

Surgical interruption of the phrenic nerve by occlusive ligation at the level of the lung hilum was carried out in 4 cats. The operative procedure was the same as that used in transection of the nerve trunk.

(D) MOBILISATION OF THE CAUDAL PORTION OF THE PHRENIC NERVE.

In one cat, the procedure for exposure of the phrenic nerve was the same as that used in (B) and (C). Through a needle puncture in the mediastinal pleura, approximately 0.5cm from the nerve, a blunt nerve hook was insinuated between the subjacent fibrous pericardium and the phrenic nerve. With minimal traction on the nerve hook, the pleura was incised parallel to the phrenic nerve which was thus mobilised in its entirety from the level of the lung hilum to its terminal intrathoracic arborisation at the level of the diaphragm. Repair of the thoracic wall was carried out in the manner described for procedures (B) and (C).

(E) EXCISION OF THE COELIAC GANGLION.

One adult animal was used in this part of the study. The abdominal cavity was opened by a left subcostal incision extending from the midline in front to the posterior axillary line. The coeliac ganglion was located on the left crus of the diaphragm and was removed intact by severing all of its connections. The abdomen was closed in layers.

Bilateral Operative Procedures.

LIGATION OF LOWER THORACIC SEGMENTAL NERVE ROOTS.

Two adult cats were used in this procedure. Following the pre-operative procedure described above, a longitudinal midline incision was made overlying the appropriate region of the vertebral column. The spinous processes were located using the lowest rib as a landmark and laminectomy was performed, the filaments of the appropriate nerve roots being found and ligated, central to the spinal ganglia.

The neural components ligated in these animals comprised the filaments of the eighth to the thirteenth thoracic spinal nerves inclusive. The animals were killed after survival periods of 24 and 48 hours respectively.

General Post-operative Procedure.

Penicillin, 500,000 units, was administered by intramuscular injection immediately after the operation and the animal was transferred to the recovery room. Post-operative maintenance depended on the time taken for recovery from the anaesthetic, the average being about 4 hours. Animals which took longer than 4 hours to recover, were given intraperitoneal fluids in the form of 5% dextrose in normal saline. Those which recovered quickly, were given oral fliuds by means of a dropper bottle. In animals where unconsciousness was prolonged, the dosage of penicillin was repeated daily for three days to avoid infection. When the animal was able to feed itself, it was returned to the animal house.

Histological Examination of Tissues from Experimental Animals.

The animals used for experimental study were maintained for periods ranging from 1 to 30 days. They were then killed under sodium pentobarbitone anaesthesia and the diaphragm and phrenic nerves were removed for histological examination.

(A) THE RIGHT AND LEFT PHRENIC NERVES.

The techniques employed in this study were the same as those used in the study of normal phrenic nerves and involved the use of osmic acid, silver impregnation, and ultrastructural techniques.

(B) THE NEURAL PLEXUS OF THE DIAPHRAGM.

The method used for this purpose was as follows:-
The diaphragm was divided into zones as shown in figure 2. To

facilitate sectioning and mounting of these specimens, each strip of muscle was divided into peripheral and central portions. Frozen sections were cut at 50µ and stained by the Bielschowsky silver impregnation technique. (Schofield 1960).

OBSERVATIONS.

STUDIES IN NORMAL ANIMALS.

Topographical Studies.

THE PHRENIC NERVE IN CATS.

In four adult animals it was observed that the phrenic nerve is formed on the ventral aspect of Scalenus anterior muscle, mainly by the union of two roots which issue from the cervical plexus as is the case in man, but the segments involved in cats are the fifth and sixth cervical. The nerve then passes downwards almost vertically on the surface of Scalenus anterior deep to the prevertebral fascia and, on leaving the surface of that muscle, it passes between the subclavian artery and the subclavian vein. At the point where it enters the thoracic cavity, the phrenic nerve is immediately ventral to the internal thoracic artery. Within the thorax it descends between the fibrous pericardium and the mediastinal pleura passing ventral to the lung hilum to reach the diaphragm slightly in front of the mid-coronal plane. The nerve is accompanied in its lower two thirds by the pericardiaco-phrenic vessels. The relationships of the phrenic nerves to mediastinal structures are almost identical to those found in man and, as the nerves leave the surface of the heart, they remain adherent to the mediastinal pleura until they reach the upper surface of the diaphragm. Throughout their course in the thorax, both the nerves and their companion vessels are partly embedded within a narrow strip of fatty-areolar tissue. In addition to the evidence derived from studies on the four animals used specifically for topographical investigations, supplementary macroscopic observations were made on a further eleven cats which had been sacrificed for other reasons.

As experimental procedures were envisaged it was found convenient to carry out detailed topographical studies by dividing the nerve, arbitrarily into three portions so that the optimum position for surgical interference could be determined. These three portions were:-

(a) A proximal part, extending from the spinal cord at the level of the fifth and sixth cervical nerve roots to the thoracic inlet.

(b) A middle part, extending from the thoracic inlet to the lung hilum and

(c) A distal part, extending from the lung hilum to the diaphragm.

The detailed topography of these parts is as follows:-

(A) THE PROXIMAL PART OF THE PHRENIC NERVE.

The spinal nerve roots contributing to the phrenic nerve arose from the fifth and sixth cervical segments. These components were approximately equal in size in more than half of the animals examined. The trunk of the nerve was formed by the union of these nerve roots on the ventral aspect of Scalenus anterior muscle, usually at about the level of the upper trunk of the brachial plexus. The close proximity of the newly formed trunk of the phrenic nerve to the branches of the other components of the brachial plexus, for example the nerve to Subclavius muscle, made positive identification particularly difficult in living animals. This difficulty was augmented by the close ventro-medial relationship of the vagus nerve and the cervical portion of the sympathetic trunk. Extremely slender connections with the inferior cervical ganglion were observed in two animals in which the phrenic nerve, at this level, was studied in detail with the dissecting microscope. Apart from these thread-like communications, the majority of nerves studied had no other visible connections at this level. In approximately one third of all the nerves examined, an accessory phrenic nerve was present. Further observations on this topic are described below (p. 131).

(B) THE MIDDLE PART OF THE PHRENIC NERVE.

In this part of its course the nerve was conspicuous and easily defined on the surface of the pericardium which clothes the right atrium. In most of the animals, studied by dissection, slender branches numbering from two to six, arose from the phrenic nerve and ran ventro-medially to arborise on the pericardium.

An interesting feature of the phrenic nerves on both sides in most of the animals studied, was the presence of branches which left the main trunk and recurred on the pericardium. These branches varied in number from one to four in different animals and they emerged obliquely from the nerve trunk. They were traced macroscopically and by means of the dissecting microscope superiorly as far as the level of origin of the aorta and pulmonary artery where their size diminished so markedly due to arborisation that they could be followed no further.

The remainder of the pericardial branches of the phrenic nerve, comprising about two thirds of the total number ran caudally from the main trunk and were traced to the level of the apex of the heart and to the terminal portion of the inferior vena cava. Both groups of branches, whether recurrent or otherwise, were traced to the midline, ventrally, where they formed a network of terminals intermingling with corresponding branches from the contra-lateral phrenic nerve.

Detailed examinations failed to demonstrate the presence of corresponding branches entering the posterior mediastinum. Connections were identified passing between the phrenic nerve and mediastinal components of the autonomic nervous system. Thus, in three of the nerves studied, a slender, thread-like bundle of nerve fibres connected the phrenic nerve with the paravascular plexus accompanying the internal thoracic vessels. No macroscopic connections were found between the phrenic and vagus nerves in the thorax, even although, at the upper limit of the lung hilum, these two nerve trunks are no more than 1-2mm apart.

(C) THE DISTAL PART OF THE PHRENIC NERVE.

In this region, the nerve followed a direct course to the surface of the diaphragm in about three quarters of the total number of phrenic nerves examined; no macroscopic connections with adjacent structures were identified in these, apart from the terminal branches entering the diaphragm. In the remainder, about one quarter of the total number of animals studied, the phrenic nerve and its companion vessels were joined by a vascular bundle, distal to the level of the lung hilum. Although the presence of a vessel within the leash, was clearly evident, there was no macroscopic evidence of nerve fibres (fig. 3). The bundle (N-V B.) is, like the phrenic nerve itself, partly enveloped in fatty areolar tissue. Distally, it merges with the pedicle containing the right phrenic nerve and traced proximally, the bundle (N-V B.) was found to have a corresponding relation to the left phrenic nerve.

In all of the animals examined, the terminal branching of the phrenic nerves occurred before penetration of the diaphragm. About two thirds of all the nerves had three major terminal branches, one being directed ventrally, the second laterally, and the third dorsally. One sixth of all the nerves examined had four or five terminal branches and the remainder had two. The majority of nerves examined were ribbon-like in the distal part, in contrast to the proximal part which was more cylindrical in shape (fig. 4).

Fig. 3

A right lateral view of the mediastinum in an adult cat. The chest wall and right lung have been removed to show the diaphragm, the heart, and inferior vena cava. The right phrenic nerve which is, here, more ventrally placed and less conspicuous than normal, is joined by a neurovascular bundle (N-V B.) which approaches from the left. Magnification = 1.5x.

Fig. 4

A left dorso-lateral view of the diaphragm and phrenic nerves of an adult cat. Subtotal removal of the chest wall and mediastinal viscera has been carried out to highlight the intrathoracic terminations of the left and right phrenic nerves.

(Approximately life-size.)

ACCESSORY PHRENIC NERVE.

In 15 cats, the arrangements and variations in incidence of this nerve, were comparable to the descriptions of the arrangements observed in man. It originated from the nerve to Subclavius muscle and approached the phrenic nerve from the lateral aspect, passing most frequently, in front of the subclavian vein. The level of junction of the accessory phrenic and phrenic nerves was variable, but was always within the upper third of the thoracic cavity, the most caudal level of this junction recorded in the present series being at the upper level of the lung hilum.

The incidence of accessory phrenic nerves in this series of 15 animals is shown in Table I.

TOPOGRAPHICAL STUDIES ON THE INTERCOSTAL NERVES IN CATS.

The diaphragmatic branches of the lower six intercostal nerves were threadlike in size and topographical investigations were, therefore, strictly limited. Using a dissecting microscope, the point of emergence of the diaphragmatic branches was found to be no more than 5mm from the fibrous arches spanning intercostal spaces and giving attachment to the costal fibres of the diaphragmatic musculature. Each nerve was accompanied by small vessels derived from the corresponding intercostal artery and vein. These small neurovascular bundles were situated radially in relation to the periphery of the diaphragm into which they could be traced, with the aid of a dissecting microscope, for a distance of approximately

TABLE 1.

Accessory phrenic nerves, identified in a random series of fifteen adult cats, are denoted by X in the appropriate columns.

Cat Number	Right Side	Left Side
1	X	
2		
3	X	
4		
5	X	X
6		
7	X	
8	X	X
9		
10	X	
11		
12		X
13	X	
14		
15		

1cm. Although a small number of branches ramified on the thoracic surface of the pleura, the great majority were situated in the loose connective tissue separating the pleura from the muscle fibres and they were directed towards the central tendon. It was not possible to determine whether these branches established contact with the centrifugal branches of the phrenic nerve by this method.

TOPOGRAPHICAL STUDIES ON THE COELIAC PLEXUS IN CATS.

In all of the animals examined, the coeliac plexus gave communications, including some directly from the left and right coeliac ganglia, to a small plexus of nerves lying on the abdominal surface of each crus of the diaphragm. The branches and the 'crural plexus' were more prominent on the right side than on the left. From this ensemble, at least one nerve bundle in each animal could be traced into the corresponding crus of the diaphragm.

The general relationships of the left coeliac ganglion are illustrated. (fig. 5). Here are depicted, the right and left crura of the diaphragm with the abdominal aorta emerging from the intercrural space. The medial and dorsal connections of the left coeliac ganglion have been displayed by raising the ganglion from its normal position. It can be seen that the uppermost branch penetrates the diaphragmatic musculature in the upper portion of the left crus.

Fig. 5

A right ventro-lateral view of the crural region of the diaphragm in an adult cat. The aortic hiatus has been exposed by removal of the abdominal viscera and the left coeliac ganglion has been raised from its normal position to show the general relationships of its medial and dorsal connections. Magnification = 4.5x.

THE DIAPHRAGM IN NORMAL CATS.

Since frequent reference will be made, in the course of this work, to the various parts of the diaphragm, it is convenient here, to review, briefly, its chief structural characteristics.

The muscle fibres which form more than half of the entire structure, are attached peripherally to the metasternum, the lower six ribs, and the upper three lumbar vertebrae. In this respect, the arrangements in cats are comparable to those in man except that the cat has thirteen ribs. While the lower six ribs form the basis of the costal attachments of the muscle fibres, a large proportion of the area required is provided by fibrous arches which span the intercostal spaces. Whereas the muscle fibres of sterno-costal origin converge directly upon the central tendon, those of vertebral origin describe a curve which is almost rectangular before reaching their central attachment. Fibres arising from the medial and lateral arcuate ligaments and adjacent parts of the thoracic cage also form an arch as they course towards the central tendon. The muscle fibres of the right crus which arise from the upper three or four lumbar vertebrae and intervening intervertebral discs, can be divided into two groups:-

(a) Those which are superficial, as seen from the abdominal aspect, and form the annular fibres surrounding the oesophageal hiatus.

(b) Those which are deep to the above group and, remaining on the right side of the midline, are inserted into the central tendon. The muscle fibres of the left crus, do not cross the midline but are attached directly to the central tendon on the left side.

The central tendon is trefoil in shape, each of the leaflets having a rounded peripheral margin.

Structures passing between the thorax and abdomen such as the aorta, oesophagus, inferior vena cava and splanchnic nerves, do so in a manner which is comparable to that seen in man.

Light Microscope Studies on the Phrenic Nerve.

HISTOLOGICAL STUDIES IN CATS.

Only the intrathoracic portions of the phrenic nerves were studied in this part of the investigation as previous topographical studies indicated that the nerves were not regularly, fully constituted in the neck. For the purpose of this study, 10 adult cats were used and, irrespective of the presence or absence of an accessory phrenic nerve, the phrenic nerves were studied in three portions:-

(a) A proximal portion - proximal to the level of the lung hilum.

(b) A middle portion - at the level of the lung hilum.

(c) A distal portion - distal to the level of the lung hilum (fig. 6).

Two main structural features were studied in osmium-treated

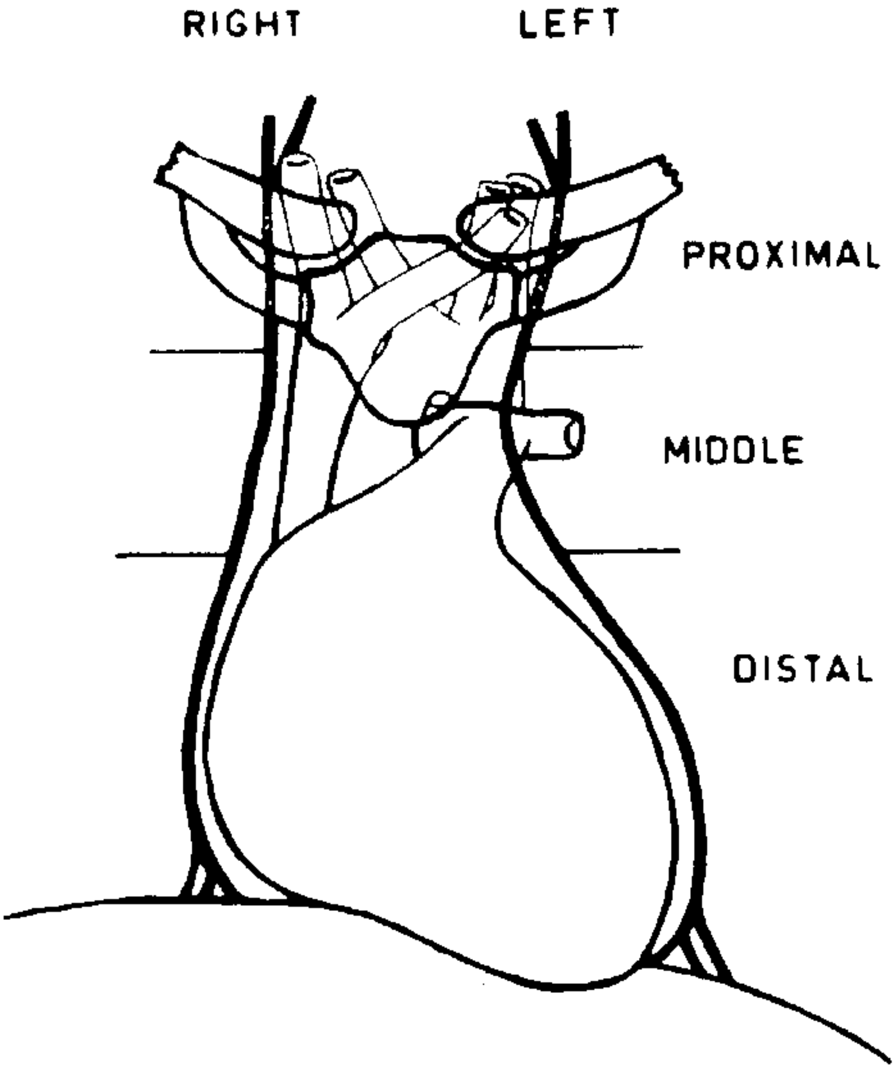


Fig. 6

Illustrating the arbitrary portions, proximal, middle and distal, into which each phrenic nerve was subdivided for histological study.

portions of the nerve. (fig. 7).

(i) The fascicular pattern.

(ii) The myelinated nerve fibres.

(i) FASCICULAR PATTERN OF THE PHRENIC NERVE.

In the osmium-treated sections taken from proximal, middle and distal portions of the right and left phrenic nerves, the size and number of fasciculi were examined. The number of fasciculi present in the right and left phrenic nerves in seven cats and in one phrenic nerve, in each of three other cats selected at random without reference to side, are shown in Table 2.

There is considerable variation between the numbers of fasciculi present at corresponding levels in the phrenic nerves of different animals; the range of variation is as follows:-

(i)	Proximal	1-5.
(ii)	Middle	1-9.
(iii)	Distal	1-10.

In those nerves which show a proximo-distal increase in numbers of fasciculi, there is a reduction in the diameter of the individual fasciculi present.

With regard to individual animals, the same relationship obtains between the number of fasciculi present and the fascicular diameter. Only in two of the seventeen nerves studied, did the number of fasciculi remain constant throughout the length of the phrenic nerve. In the remaining fifteen nerves, considering only

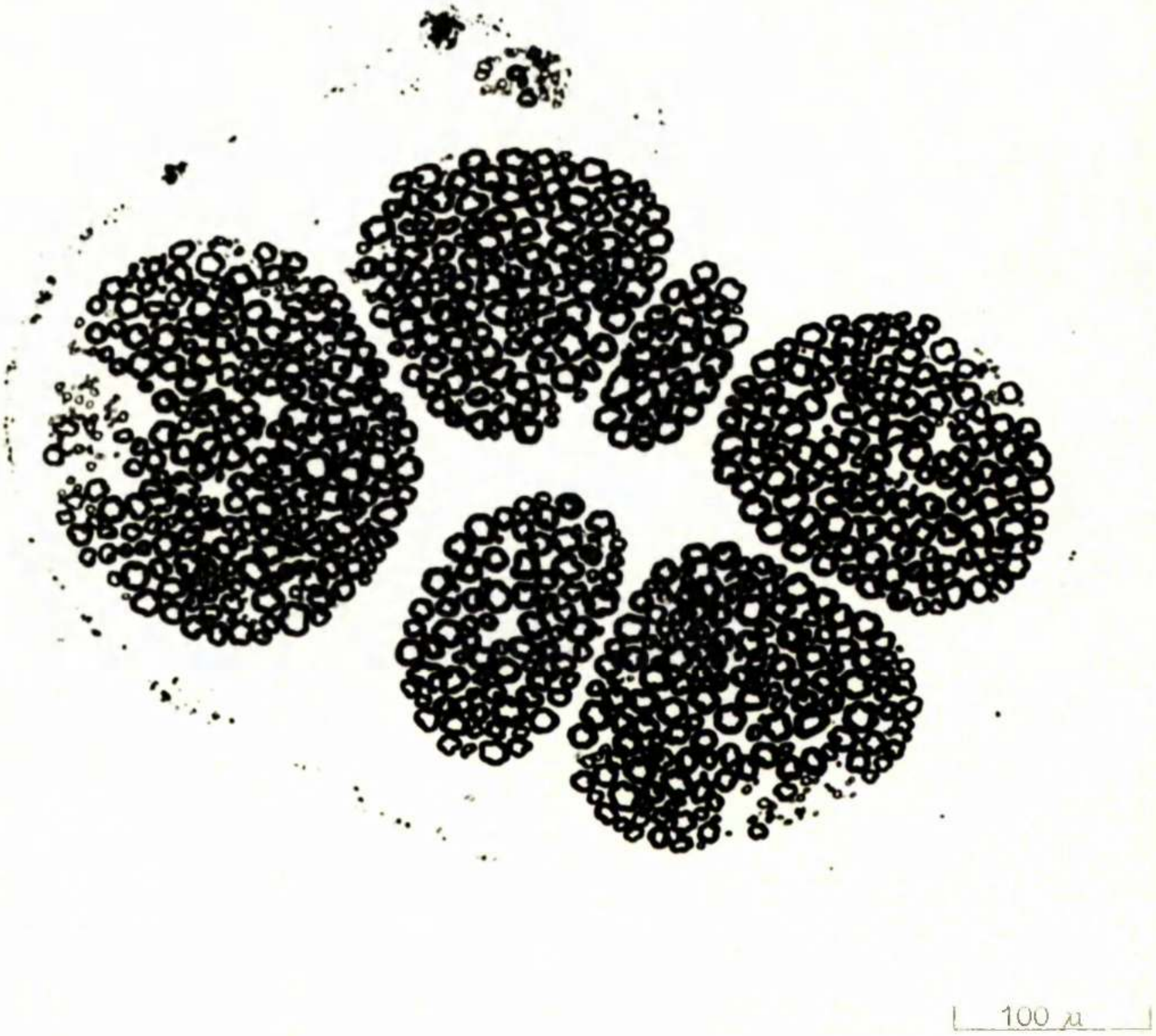


Fig. 7

A complete transverse section of the phrenic nerve of a cat. This type of osmium preparation was used to study the fascicular pattern and, after magnification to 1,000x, for investigation of the size distribution of myelinated fibres at separate levels within the nerve trunk.

TABLE 2.

The number of fasciculi present at three distinct levels within 17 phrenic nerves demonstrates the variations which may occur.

Cat	Side	Proximal	Middle	Distal
	Right	1	2	2
1	Left	1	4	4
	Right	1	7	2
2	Left	4	9	10
	Right	2	7	4
3	Left	2	7	7
	Right	2	1	3
4	Left	1	2	1
	Right	3	8	3
5	Left	3	5	2
	Right	2	1	1
6	Left	1	1	1
	Right	4	1	1
7	Left	2	2	2
8		1	2	7
9		4	6	6
10		5	5	3

proximal and distal levels, the numbers of fasciculi showed the following variations:-

1. (a) Increase in numbers from proximal to distal - 9 nerves.
   (b) Equality in numbers from proximal to distal - 2 nerves.
   (c) Decrease in numbers from proximal to distal - 4 nerves.

Corresponding studies at the proximal and middle levels gave the following results:-

2. (a) Increase from proximal to middle - 11 nerves.
   (b) Equality from proximal to middle - 1 nerve.
   (c) Decrease from proximal to middle - 3 nerves.

The results of comparisons at middle and distal levels were as follows:-

3. (a) Increase from middle to distal - 3 nerves.
   (b) Equality from middle to distal - 6 nerves.
   (c) Decrease from middle to distal - 6 nerves.

(ii) QUANTITATIVE STUDIES ON THE MYELINATED FIBRES IN THE PHRENIC NERVE.

(1) General Observations.

Counts of the total numbers of myelinated fibres were made in three cats, and they showed only minor variations at proximal, middle and distal levels in any one phrenic nerve. The results obtained in one animal, studied in detail, are shown in Table 3.

It will be seen that:-

(i) Approximately 80% of the fibres range in diameter from 8-16µ, that 15% are less than 8µ in diameter and that 5% are greater than 16µ.

(ii) There is a marked increase in the number of fibres of large diameter particularly between middle and distal levels in the phrenic nerves (represented in Table 3). These features are illustrated in more graphic form in figure 8.

In the left phrenic nerve represented in Table 3, there is a net decrease of approximately 3% in the total count of fibres between proximal and middle levels and a further decrease of approximately 10% between middle and distal levels. On the other hand, the right phrenic nerve shows a marked increase in the total count of fibres between proximal and middle levels. In this animal, an accessory phrenic nerve was present and it joined the main trunk of the right phrenic nerve about 1.5cm proximal to the lung hilum. The net decrease in the total count between middle and distal levels in this nerve, is identical to that in the left phrenic nerve.

The numerical values obtained in comparable quantitative studies on another phrenic nerve selected at random are presented in Table 4.

TABLE 3.

Quantitative studies on the myelinated fibres of the right and left phrenic nerves in one adult cat.

	Right Phrenic Nerve.			Left Phrenic Nerve.		
Fibre Diameter in Microns	Proximal	Middle	Distal	Proximal	Middle	Distal
4-6	2	1	15	36	82	22
6-8	26	3	63	85	207	59
8-10	58	46	143	291	203	173
10-12	128	209	192	219	231	208
12-14	152	219	152	184	113	180
14-16	192	201	99	63	25	110
16-18	137	102	42	20	7	26
18-20	40	58	48	3	0	5
Total Number of Fibres	735	839	754	901	868	783

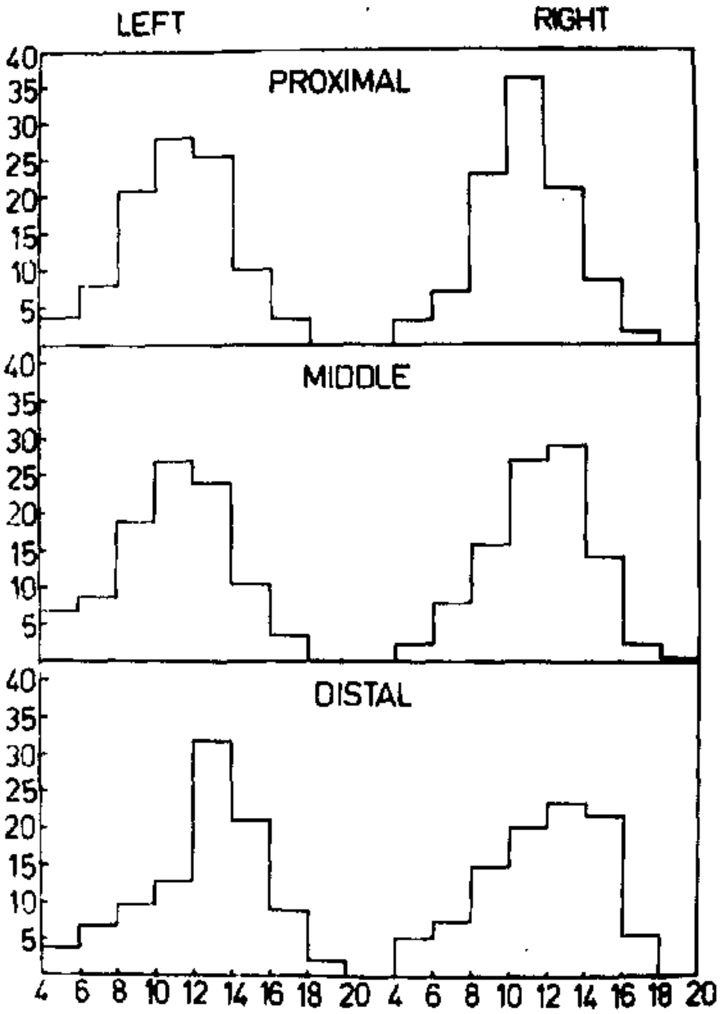


Fig. 8

Histograms illustrating the size-distribution of myelinated fibres at three different levels within the left and right phrenic nerves in an adult cat.

Ordinate. Numbers of myelinated fibres expressed as percentages of the total count at corresponding levels.

Abscissa. Diameter of nerve fibres expressed in microns.

TABLE 4.

Quantitative studies on the myelinated fibres of a phrenic nerve selected at random without regard to side.

Fibre Diameter in Microns	Proximal	Middle	Distal
4–6	28	52	30
6–8	59	66	58
8–10	151	144	79
10–12	204	203	103
12–14	186	180	256
14–16	72	79	167
16–18	25	26	75
18–20	1	0	17
Total Number of Fibres	726	750	785

(b) Examination of Histograms.

The values included in Table 3 have been used to construct a histogram. (fig. 8). It can be seen that the outline representing size distribution, has one single peak which lies in the zone in which fibres of intermediate diameter are represented. The fibres with a diameter of less than 10µ were more numerous at the proximal level than at the distal level. On the other hand, the number of fibres whose diameter ranged between 10 and 12µ, was approximately equal at proximal and distal levels, but showed an increase of about 10% at the level of the lung hilum.

A surprising observation was, that fibres ranging from 12-20µ in external diameter, were more numerous distally than proximally. These observations were confirmed in similar histograms from a further four animals. (fig. 9).

Unmyelinated Fibres in the Phrenic Nerve.

Repeated attempts were made initially, to estimate the numbers of unmyelinated fibres in formalin fixed portions of the phrenic nerves from which 5µ transverse sections impregnated with silver, were examined with the light microscope. Although it was possible to make observations of a general, qualitative nature on the axis cylinders present in a given transverse section of the phrenic nerve, inconsistencies were found in the results of quantitative studies performed at different levels in individual phrenic nerves. Results using the silver impregnation method described by Bodian

Fig. 9

Histograms illustrating the size-distribution of myelinated fibre at three different levels within the left and right phrenic nerves of 4 adult cats. The parameters here, correspond exactly to those used in figure 8.

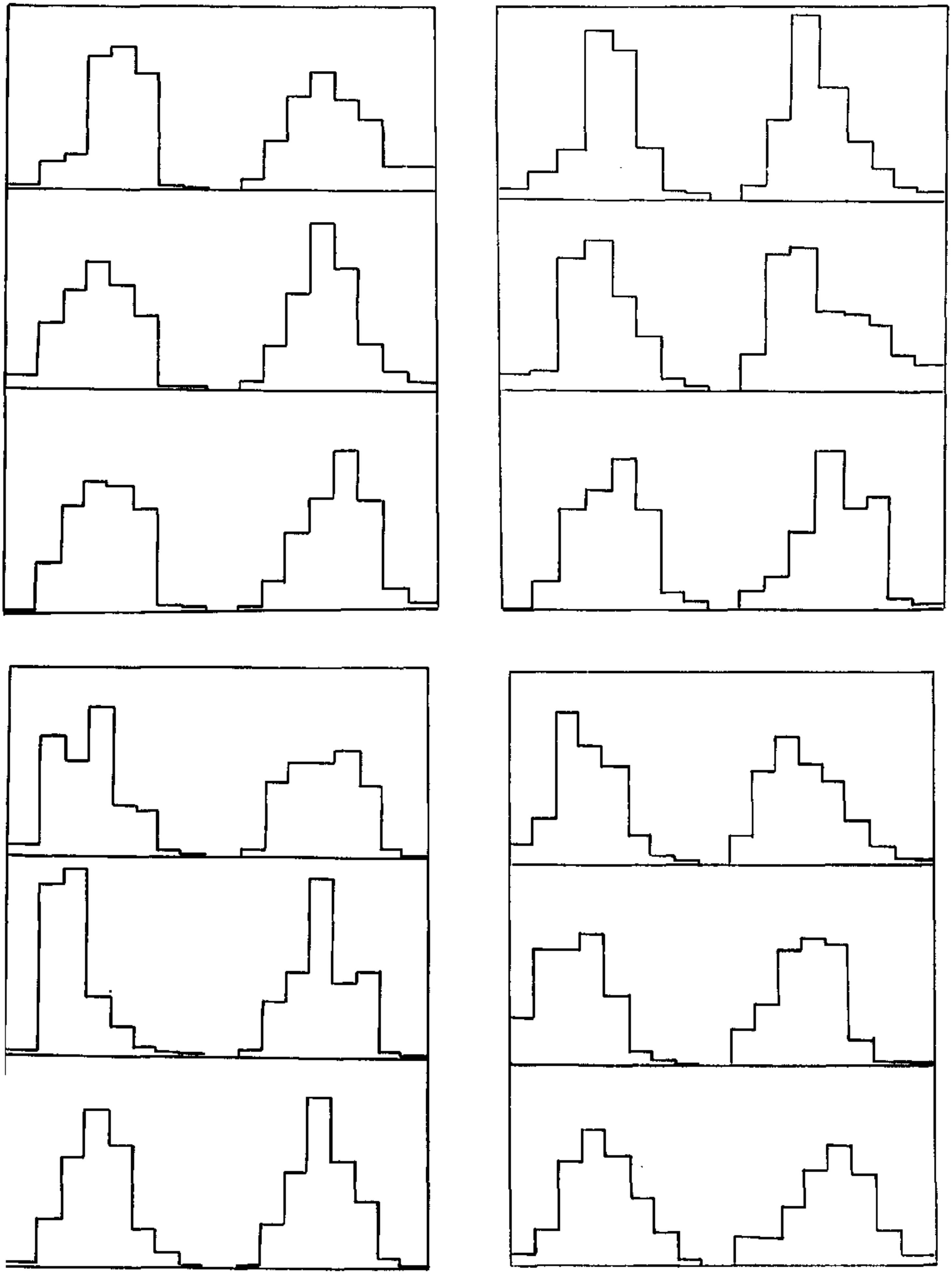


Fig. 9

(1936) are presented in Table 5.

The values obtained by this method, for total counts of axis cylinders, are surprisingly low in comparison to the corresponding counts of myelinated fibres as shown in Tables 3 and 4. It can be seen that the total numbers of axis cylinders present at comparable levels within the right and left phrenic nerves are variable.

A corresponding degree of inconsistency was found in quantitative studies where the methods described by Bielschowsky (1940) and Romanes (1950) were used.

A modification of Ranson's silver-pyridine method described by Jones (1936) was applied to the middle portion of the left phrenic nerve and it was found that the total number of axis cylinders present at this level was 1,123; this was the highest value obtained by light microscopy.

The results of counts such as those illustrated in Table 5 are considered unreliable because of the wide numerical variation within single nerve trunks. The failure of this method to give consistent results was attributed, inter alia, to the limited resolving power of the light microscope and for this reason it was decided to examine the possibility of estimating the numbers of unmyelinated fibres by means of ultrastructural methods.

TABLE 5.

Total counts of axis cylinders present within the trunk of the right and left phrenic nerves in one adult cat.

Level	Right Phrenic Nerve	Left Phrenic Nerve
Proximal	640	696
Middle	574	750
Distal	964	700

Histological Studies on the Intrathoracic Connections of the Phrenic Nerve.

In 3 cats and 4 mice, the pericardium, with the phrenic nerve in situ, was impregnated with silver and studied with the light microscope. Branches arising from the phrenic nerve, previously observed in cats with the dissecting microscope, were seen to comprise a small number of myelinated fibres of small diameter and a greater number of fasciculi of unmyelinated fibres. No specialised nerve endings were seen in relation to the terminations of myelinated fibres and the fasciculi of unmyelinated fibres could be traced to the midline ventrally where interdigitation occurred with the corresponding nerve fibres from the opposite side. The recurrent pericardial branches of the phrenic nerve were traced rostrally to the region of the roots of the great vessels where they participated in the formation of a dense nerve network. It was not possible to trace individual fasciculi beyond this region.

In one cat, in which the topographical features closely resembled those illustrated in figure 3, the vascular pedicle which converged with the distal portion of the right phrenic nerve was removed 'en bloc' and impregnated with silver. It was examined histologically as a whole preparation and it was found to contain a bundle of nerve fibres. Approximately 10 of these were myelinated fibres of medium diameter and there were several fasciculi of unmyelinated fibres present. The trunk of the right phrenic nerve, which here occupied a more ventral plane than normally, possessed a fibre-spectrum which was comparable to all other nerves examined. The total count of nerve fibres at proximal, middle and distal levels showed variations of the same order as the nerves studied previously.

Electron Microscope Studies on the Phrenic Nerve.

I. "SAMPLING" METHOD.

In one adult cat, the left phrenic nerve, at the level of the lung hilum was appropriately prepared, sectioned transversely, and mounted on a standard 200 mesh copper grid. Six areas, equal in size, of the total cross section of the nerve at this level, were photographed.

Initial qualitative observations made on enlarged photographic prints, indicated the mode of distribution of unmyelinated fibres throughout the transverse section and this influenced the selection of photographs used for quantitative studies. (see fig. 10). Counts were made of the numbers of myelinated fibres, unmyelinated fibres, Schwann cells, and vascular structures with the intention of estimating the total numbers of unmyelinated fibres present at this level within the trunk of the phrenic nerve.

The figures presented in Table 6 represent the values obtained in this investigation.

From this study the following observations were made:-

1. There is some variation in the numbers of myelinated

Fig. 10

An area within the transverse section of the phrenic nerve of a normal cat. Myelinated fibres, unmyelinated fibres, nuclei of Schwann cells and vascular structures can be identified.

TABLE 6.

Quantitative findings in six selected areas of the transverse section of one phrenic nerve.

Area No.	Myelinated Nerve Fibres	Schwann Cell Nuclei	Unmyelinated Nerve Fibres	Vessels
1	60	8	112	3
2	56	7	171	2
3	73	9	385	1
4	62	9	194	1
5	68	8	135	2
6	49	7	299	3
Total	368	48	1296	12

fibres present in the six equal areas studied. The minimum is 49 and the maximum is 73.

2. An even greater variation is seen in the numbers of unmyelinated fibres present, the minimum being 112 and the maximum 385.

Therefore, the value of this method of sampling, which in any case, was not at random, is limited for accurate estimates of:-

(a) Total numbers and categories of fibres present at a particular level within the phrenic nerve.

(b) Proportional representation of myelinated and unmyelinated fibres within the nerve trunk.

The main feature emerging, therefore, is that the unmyelinated fibres are not evenly and regularly distributed throughout the phrenic nerve, so that 'sampling', as used here, is an unsatisfactory technique. Nevertheless, interesting observations were made from the results recorded in Table 6.

For example:-

1. Observations on the numbers of unmyelinated fibres using the light microscope, are unreliable. (see Table 5).
2. Taking total counts of the six areas into consideration, the ratio of myelinated to unmyelinated fibres is of the order of 1:4 and the ratio of Schwann cell nuclei to myelinated and unmyelinated fibres is 1:7 and 1:27, respectively.
3. It led to consideration of the possibility of studying the whole transverse section of the phrenic nerve by

means of the electron microscope in order to obtain absolute values for the components of the nerve trunk.

It is emphasised, that the above attempts at sampling were unsatisfactory because representative areas were sought initially, and it was decided, therefore, to proceed with attempts to study a complete cross-section of the nerve.

2. MONTAGE METHOD.

The essential features of the technique employed in this part of the investigation were, firstly, that a grid with an aperture was used in order to afford an unobstructed view of the whole cross-section of the nerve. Secondly, the section, cut at a slightly greater thickness than normally, was supported on a carbon coated aperture grid on both sides by a thin film of a clear plastic material, "Parlodian".

The image, projected on the fluorescent screen, was photographed in serial fashion, as described on page 114, and, from photographic enlargements, the entire cross-section was reconstructed to form a montage (mag. approximately 4,500). The maximum diameter of the montage was approximately 2.65 metres and it was at this magnification that the following studies were made. A photographic reduction of the original montage shows the main features of this preparation. (fig. 11).

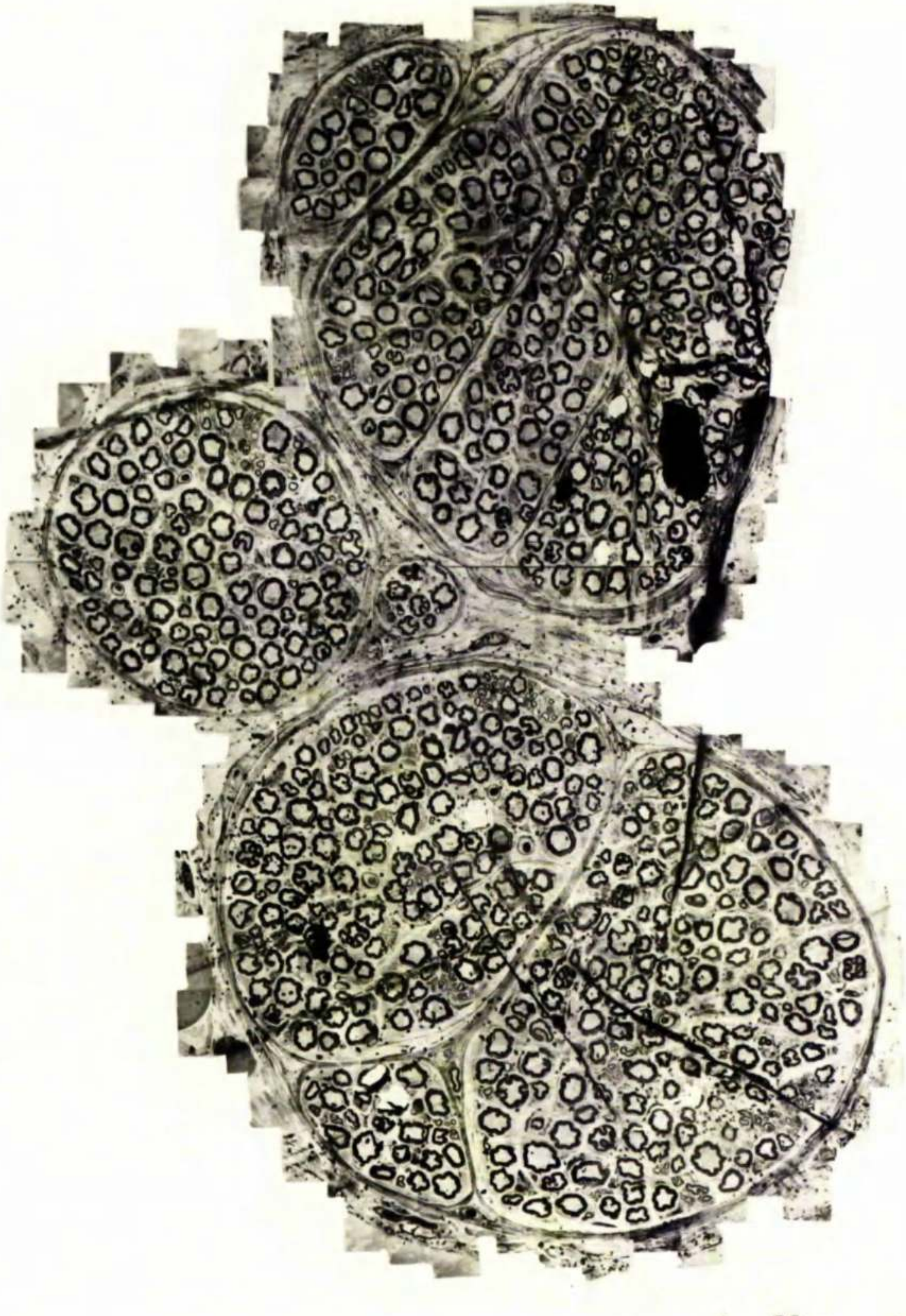


Fig. 11

A transverse section of the left phrenic nerve at the level of the lung hilum in an adult cat. This was prepared by photographic reduction of the original montage.

Quantitative Studies on the Left Phrenic Nerve at the Level of the Lung Hilum in an Adult Cat.

The phrenic nerve used in this study was topographically normal, as far as could be judged, at the time of removal. There was no accessory phrenic nerve present, and subsequent post-mortem dissection showed that its roots of origin came from the fifth and sixth cervical segments of the spinal cord. It was normal with regard to its terminal branching.

a) Fasciculi and extra-fascicular tissue. The phrenic nerve, at this level, consists of four fasciculi, of which the two larger ones are further subdivided into portions referred to here as subfasciculi by extensions of the nerve sheath. The general arrangements of the fasciculi (A,B,C, and D) and their further subdivision into A(I-VI) and C(I-III) are illustrated (fig. 12). The boundaries between contiguous fasciculi and subfasciculi formed by extensions of the nerve sheath, are referred to here as the perineurium, the structure of which is described below (p. 150). Besides forming a multi-layered boundary between contiguous fasciculi and subfasciculi, the innermost layer of the perineurium forms extensions which penetrate the substance of the nerve fasciculi. One example of this is seen in the upper part of subfasciculus CIII, where two small groups of nerve fibres are separated from the other components of the fasciculus by a perineurial extension. In subfasciculus CI, a similar extension of the perineurium courses among the nerve fibres but does not form an investment for a bundle

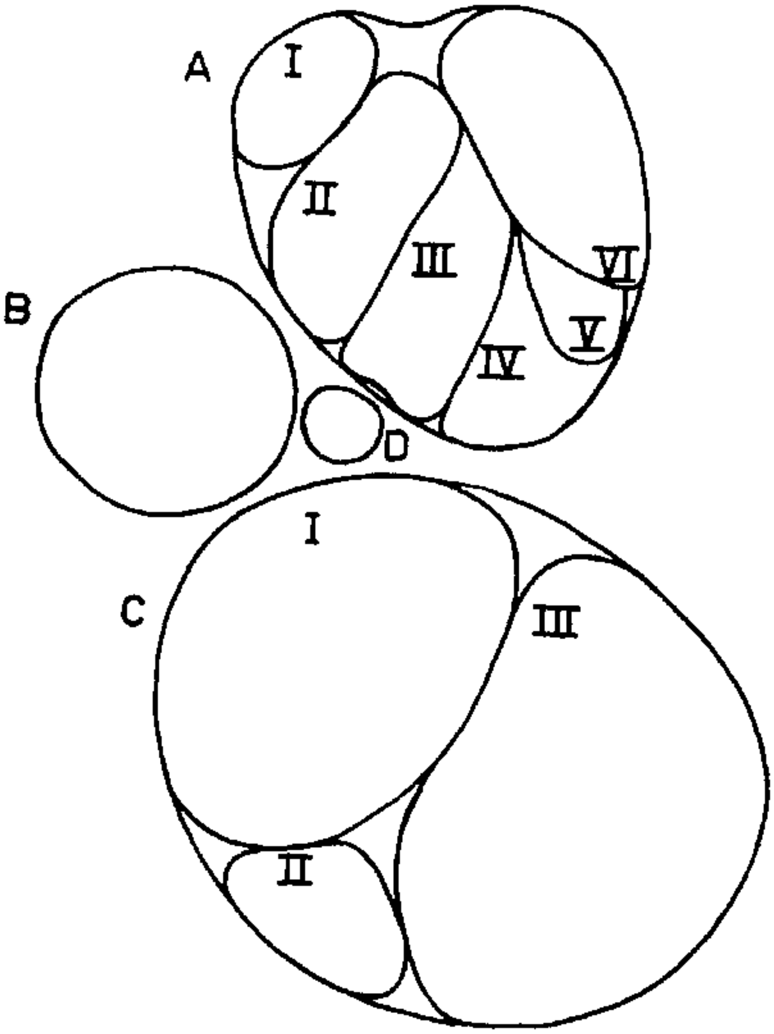


Fig. 12

Illustrates, in outline, the fascicular pattern of the transverse section of the phrenic nerve represented in figure 11. Each subdivision was considered separately in the quantitative studies which are presented in Table 7.

of nerve fibres. Along the length of each of these perineurial extensions, small ovoid spaces which contain a granular substance can be seen. In subfasciculus CI two vessels lie subjacent to the perineurial extension but there is no continuity between the latter and the connective tissue surrounding the vessels.

The well-organised components within the nerve fasciculi stand in sharp contrast to the extrafascicular tissue which consists of irregular clumps of collagen fibres with a few interspersed cells identified as fibroblasts. Five vascular structures are present in the extrafascicular tissue immediately outside the perineurium.

(b) Myelinated nerve fibres.

While the higher magnifications used in ultrastructural studies are an aid to accurate counts of nerve fibres, they are of no great advantage in measurement of the diameter of individual myelinated fibres whose contours show varying degrees of irregularity (c.f. figs. 7 and 11). The membrane which forms the external limit of the myelin sheath, however, remains independent of the sheath itself and can be used for approximate estimation of the external diameter of the myelinated fibre which it ensheaths. On this premise the majority of myelinated fibres present at the level studied were found to lie within the size range 4-20μ while the remainder ranged from 1-4μ in external diameter. There is an apparently random distribution of these fibres throughout the nerve trunk and the total number present in each fasciculus is recorded in

Table 7.

In the space between subfasciculi CII and CIII, there is one single myelinated fibre accompanied by a small group of unmyelinated fibres, which, for the purpose of this record, were included with the fibres of CII. Throughout the phrenic nerve (in both central and peripheral portions within each fasciculus), nerve fibres were seen, whose myelin sheath showed two distinct layers of electron-dense material separated by a clear interval 0.5-1µ in thickness. In a few other nerve fibres the lamination of the myelin sheath was more pronounced and consisted of up to four layers. This type of layering was distinct from the lamination seen in the myelin sheaths at higher magnifications (fig. 13), but was not studied further in the present investigation. It will be observed that the count of the total number of myelinated fibres using the ultrastructural method, compares favourably with that found in light microscope investigations. By both methods, the count of fibres ranging from 4-18µ in diameter, is approximately 800.

(c) Unmyelinated nerve fibres.

A profusion of these fibres was found at this level in the phrenic nerve; they number 2,094. Although actual measurement of all unmyelinated fibres was not performed, it was estimated that at least half are less than 0.5µ in diameter, while some are as large as the smallest myelinated fibres present in the section. The unmyelinated fibres are aggregated in groups throughout the

TABLE 7.

Quantitative findings obtained from studies on a complete transverse section of the left phrenic nerve of a cat.

F	SF	M.F's	Groups of U.M.F's	U.M.F's	Vessels
A	I	34	4	92	1
	II	67	8	147	2
	III	48	5	103	2
	IV	38	4	62	1
	V	20	4	10	1
	VI	100	13	183	3
B		122	13	338	
C	I	158	31	562	4
	II	29	5	50	2
	III	174	31	522	5
D		11	2	25	
Total 4	11	801	120	2,094	21

Key to abbreviations used in Table 7.

F---- Fasciculus

SF--- Subfasciculus

M.F's--- Myelinated Fibres

U.M.F's--- Unmyelinated Fibres

Fig. 13

A portion of a myelinated fibre within a transverse section of the phrenic nerve. The electron-dense sheath, in which regular layering is evident, shows an indentation which is limited peripherally by the outer limiting membrane of the sheath.

fasciculi. The larger groups, containing 20-200 fibres, were seen to be situated near the periphery of each fasciculus, while the smaller groups, containing 2-20 fibres, were nearer the centre. The largest groups were found immediately subjacent to the perineurial sheath and they contained a small number of small myelinated fibres whose diameter ranged from 1-4µ.

The close association between Schwann cells and unmyelinated fibres, observed at higher magnifications, was comparable to that which obtains in other situations. Thus the unmyelinated fibres are seen to be smooth and regular in contour, and they possess mesaxons by which they are suspended within the cytoplasm of the Schwann cell. (fig. 14).

From quantitative studies it is evident that the ratio of the number of groups of unmyelinated fibres to the number of myelinated fibres is approximately 1:8 in each of the subfasciculi of fasciculus A (discounting subfasciculus V); the corresponding ratio in fasciculus C is approximately 1:5. (see Table 7).

It is clear from these findings, that counts of the unmyelinated fibres made with the light microscope are very much lower than those observed in ultrastructural studies. (cf. Tables 5 and 7).

(d) Blood and lymphatic vessels.

Surprisingly few blood and lymphatic vessels were present

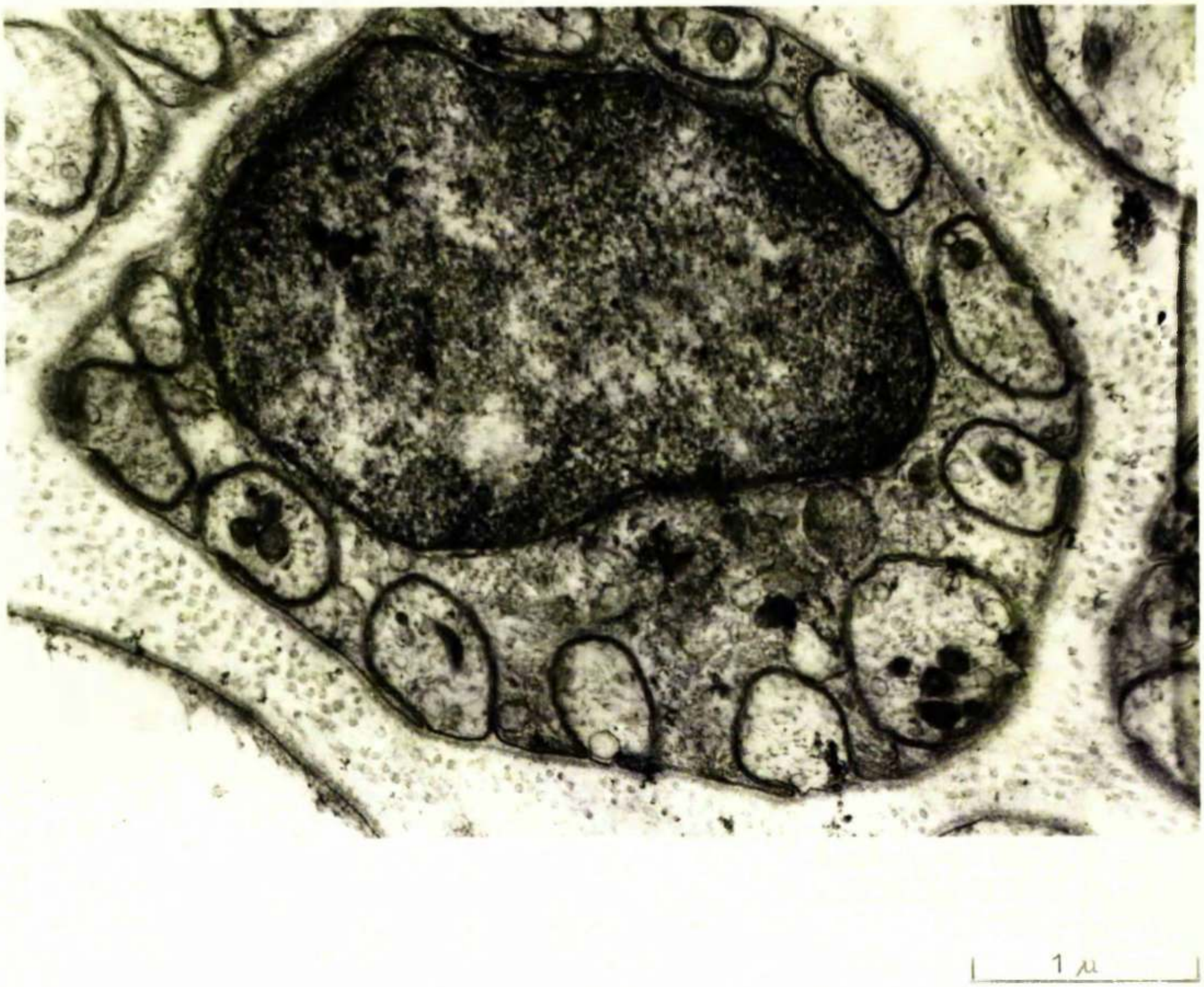


Fig. 14

A Schwann cell within a transverse section of the phrenic nerve. The granular nucleus is of greater electron density than the surrounding cytoplasm which contains 12 unmyelinated nerve fibres. Interstitial collagen fibres, cut transversely, are present around the periphery of the Schwann cell.

at the level studied. Within the fasciculi, there were 23 such vessels, which were well defined and therefore measurable. None exceeded 10µ in diameter. In the extrafascicular tissue, 5 vessels of similar size to those just described were seen immediately outside the perineurium. Two of the latter were in a collapsed state, and accurate measurement was not possible. The estimated diameters of these, are of the same order as those of the intrafascicular vessels because of the presence of blood cells within the lumen. In the remainder identification was uncertain.

The distribution of the vessels within the fasciculi indicates that each subfasciculus and fasciculus possesses at least one vessel, and that the maximum number of vessels in any one of these, is five. In transverse section the intrafascicular vessels resemble capillaries; they are formed by endothelial cells surrounded usually by one cell identified as a pericyte. (fig. 15).

No smooth muscle cells were seen in relation to the walls of any of these vessels.

(e) Schwann cells and intrafascicular tissue.

The relationship of unmyelinated fibres to the cytoplasm of Schwann cells, has been referred to briefly above (fig. 14). Several Schwann cell nuclei are present at the level illustrated in the montage, (fig. 11) and they are situated mainly in the peripheral portions of the nerve fasciculi and subfasciculi.

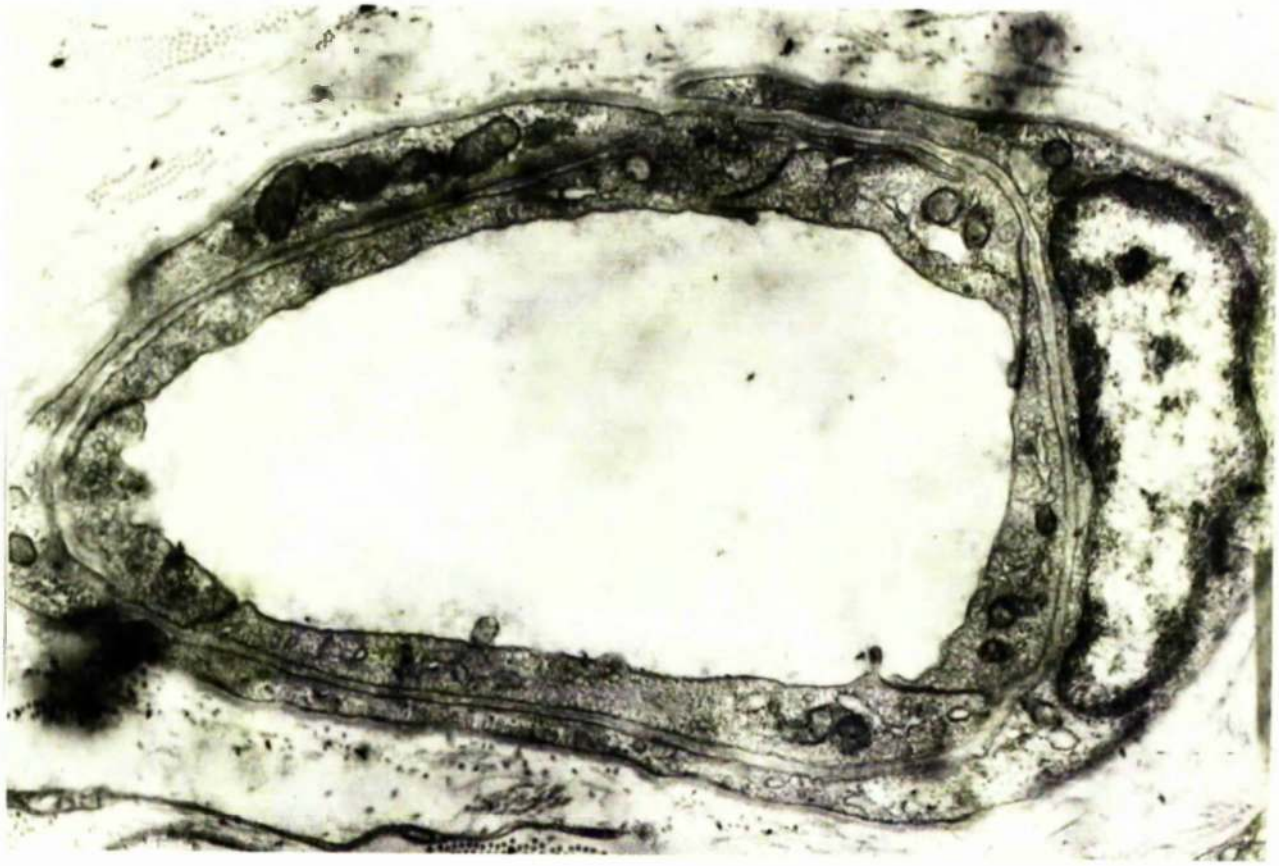


Fig. 15

An intrafascicular vessel within a transverse section of the phrenic nerve. The inner endothelial lining is embraced by cytoplasmic processes which extend from a cell resembling a pericyte. Five regions of intercellular contact are seen within the endothelial lining.

Attention is drawn to the smoothly undulating nuclear membrane of the Schwann cell (fig. 14) and to the variable electron density of the nucleoplasm which, overall, is darker than the cytoplasm. The latter has a heterogeneous appearance in this particular illustration and it contains circular endoplasmic inclusions, but no mitochondria or other specific organelles, are seen.

Immediately surrounding the Schwann cell membrane, collagen fibres are seen cut in transverse section (fig. 14). This is a typical finding throughout the portions of nerve studied, and collagen fibres regularly occupy the interstices between the other components of the nerve trunk. The orientation of the collagen fibres within the transverse section of the nerve trunk is variable, however, and on the basis of this study, at least two groups of fibres approximately equal in numbers can be identified.

(1) A group which follows the direction of adjacent nerve fibres and which, therefore, is cut transversely. (fig. 14).

(2) A group which has an oblique direction and thus appears obliquely cut in transverse sections of the nerve. (fig. 16). No truly circular group of intrafascicular collagen fibres was seen in any region of the transverse section of the nerve studied.

Cells identified as fibroblasts, were present in small numbers in the spaces occupied by larger aggregates of collagen fibres. No other cell types were identified.

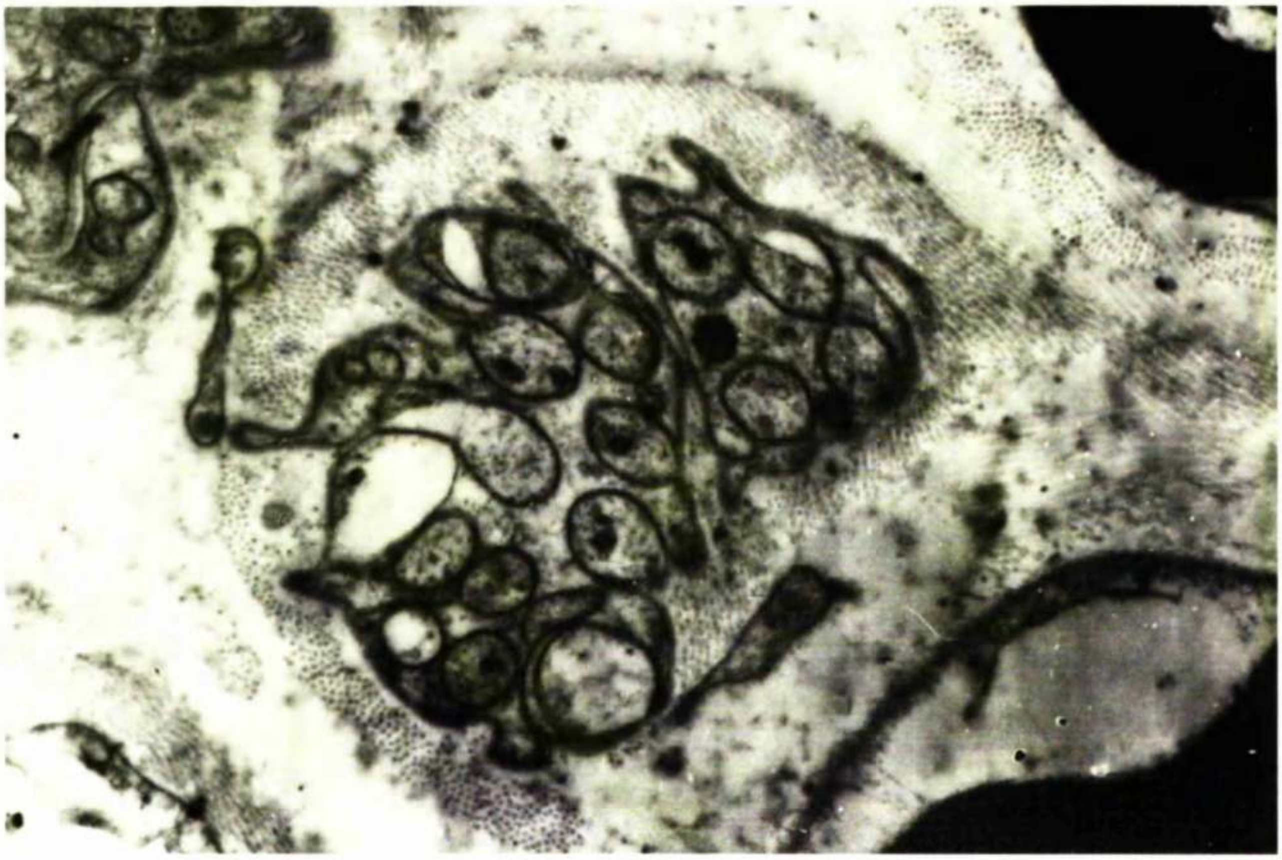


Fig. 16

A fasciculus of 16 unmyelinated fibres within a transverse section of the phrenic nerve. Interstitial collagen fibres are profuse and although the majority are cut transversely some appear to be obliquely orientated.

(f) Perineurium.

It will be recalled that the perineurium was defined previously as the multilayered sheath which surrounds the fasciculi and subfasciculi. (fig. 11 and 12). It consists of alternate layers of cells and collagen fibres, and the number of layers present is directly proportional to the size of the fasciculus or subfasciculus contained. The perineurium forming the sheaths of fasciculi A and C, is about six layers thick, while the corresponding structures in fasciculi B and D, possess four and two layers respectively. The number of laminae is also variable in the perineurium surrounding the subfasciculi, the larger of these, CI, II, and III possessing, in most parts of their circumference, three layers, while the smaller ones, AI-VI, have two layers. Only the inner layers of the perineurium extend inwards to form the boundaries which separate the subfasciculi, while the outer layers form the basis of the fascicular sheaths.

The cells of the perineurium are characterised by their great length, most of them encompassing approximately one third of the entire circumference of the subjacent nerve bundle. The nuclei of these cells are seen to be 'spindle shaped' and of greater electron density than the surrounding cytoplasm. (fig. 17 and 18). The cytoplasmic processes branch frequently, and subsequently rejoin to enclose ovoid spaces which are occupied by collagen fibres. The sizes of these spaces vary within a wide range, the majority being twice to three times the size of the one illustrated. (fig. 19).

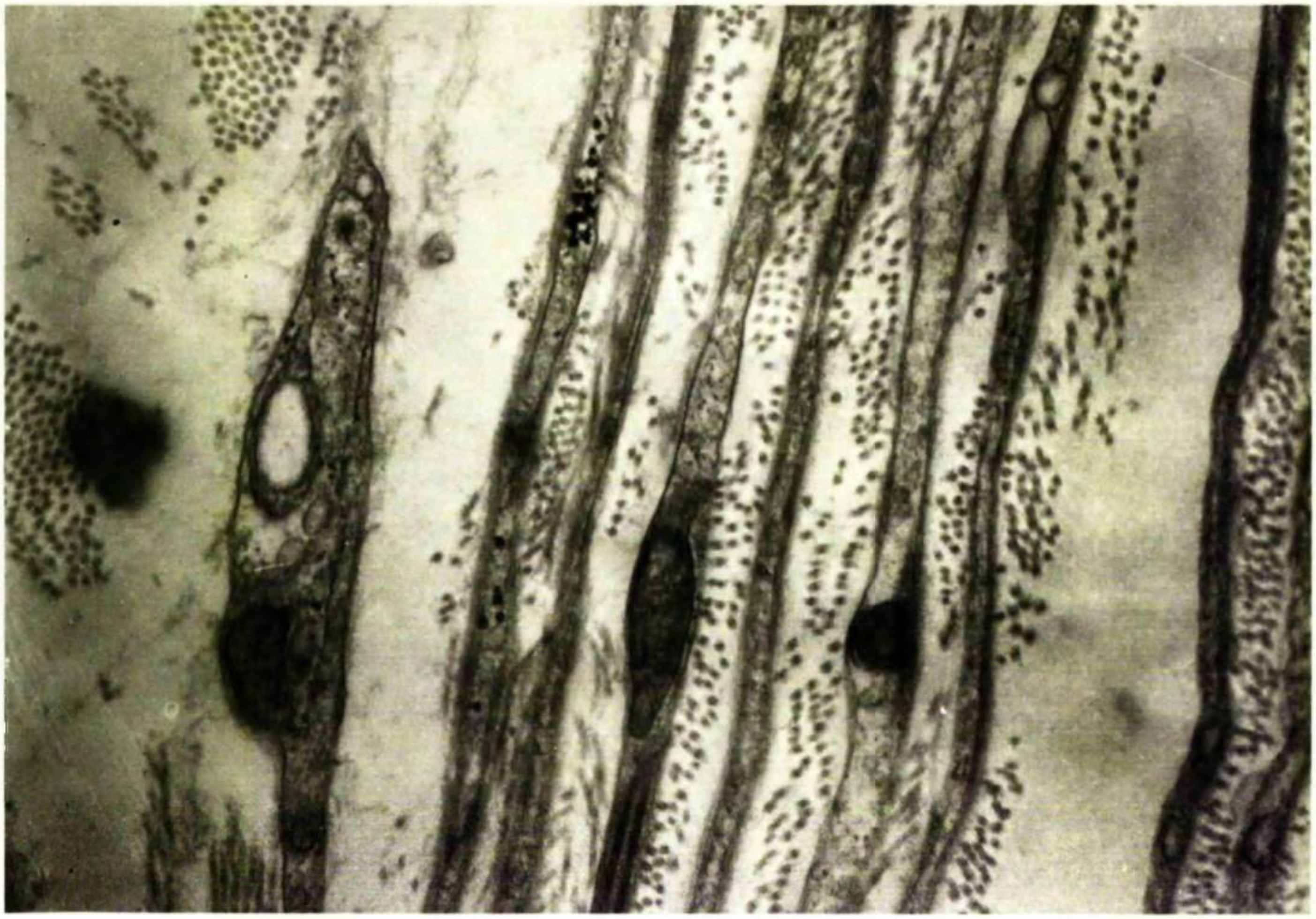


Fig. 17

The components of the perineurium in a transverse section of the phrenic nerve. Alternating layers of perineurial cells and collagen fibres can be clearly defined.

Fig. 18

A higher magnification of perineurial components counterstained with phosphotungstic acid to show the 640 Å 'banding' of interstitial collagen fibres.

Fig. 19

From the same preparation as figure 18. The ovoid space which contains collagen fibres is bounded by processes of perineurial cells, which show local variations in their limiting membranes particularly in the regions adjacent to interstitial collagen.

Irrespective of their size or shape, all of these spaces contain collagen fibres. It will be observed that the cell membrane is indistinct in one part of the space and that, near this hiatus, a structure resembling a collagen fibre appears to lie within the cytoplasm of the adjacent cell. Close association of the cellular components with collagen fibres is seen throughout the perineurium. (fig. 19). Another field of perineurium in a specimen counter-stained with phospho-tungstic acid illustrates the cross-banding typical of collagen where a bundle of these fibres has been cut obliquely. (fig. 18). Sites of contact between adjacent perineurial cells, can be identified and, in the example shown here, some interdigitation is evident. (fig. 20). In regions where the perineurial sheaths of adjacent subfasciculi are confluent, the intercellular spaces, as elsewhere in the sheath, are partly occupied by collagen fibres. In two of these 'junctional zones' viz. where AI and AII are contiguous and where CII and CIII are contiguous peripherally (fig. 11) a vessel is present within the local meshwork of the processes of perineurial cells.

Comparison of the perineurium with the spinal meninges.

In view of the findings of Shanthaveerappa and his colleagues (1963) that the perineurium is continuous with the meninges of the spinal cord and that there are extensions of the subarachnoid space along the length of the peripheral nerves, it was decided to compare the perineurium of the phrenic nerve with the meninges by ultrastructural studies in cats. Accordingly, filaments of the

0.5 µ

Fig. 20

Perineurial cells and interstitial collagen fibres prepared without special counterstaining. In this field the lowermost cell is seen to form an interlocking junction with a contiguous cell.

dorsal nerve root together with their meningeal coverings were taken from the fifth cervical segment and following preparation by the same techniques as those used for the study of the perineurium, transverse sections of the specimens were examined, both by light microscopy and by electron microscopy. In the specimen taken from the dorsal nerve root, central to the spinal ganglion, the three meningeal layers were identified along with the spaces separating them. (fig. 1). It was found that the investing layer of the pia mater consists of a single layer of elongated cells which differ morphologically from the cells of the outer two layers. Compared with cells in the two outer layers of the meninges and in the perineurium, the pial cells show greater electron density both in the nucleus and in the cytoplasm, the elongated processes of which are perceptibly narrower than those of the dura, arachnoid or perineurial cells. (c.f. fig. 17 and 21). The most significant finding, however, is that the pial cells, like the cells of the arachnoid, generally have very few collagen fibres lying on the surface which bounds the subarachnoid space. (fig. 21). This is in marked contrast to the layers of the perineurial cells which are invariably separated by numerous collagen fibres which occupy the intercellular spaces. (see figs. 17-20).

Fig. 21

A small portion of a transverse section of the dorsal root of a cervical spinal nerve central to the spinal ganglion in a cat. A cell of the pia mater forms the external boundary of the bundle of nerve filaments. Circular and longitudinal collagen fibres are identifiable on the inner aspect of the pia but none are present on the surface which forms the boundary of the subarachnoid space.

Histological Studies on the Diaphragmatic Plexus.

A) IN CATS.

Preceding studies in cats, prompted the use of the same species for preliminary investigation of the diaphragmatic plexus. Four representative areas were selected in each hemidiaphragm, and these were further subdivided into peripheral and central portions (fig. 2). From these areas, 50μ frozen, silver impregnated sections, prepared according to Schofield (1960) were examined by light microscopy.

Comparatively few tissue neural elements were present in the samples taken from the periphery of the diaphragm. Nerve fibres and their terminations were found to be concentrated in a narrow band parallel to the outer border of the central tendon and situated about midway between that structure and the peripheral attachments of the diaphragmatic musculature. This region, referred to here as the 'nerve rich' zone, partly encircles the central tendon. In order to display the 'nerve rich' zone, the diaphragm of a half grown cat was impregnated with silver as a whole preparation and studied histologically. Although it was possible to demonstrate the main fasciculi of the plexus by this method, the thickness of the preparation precluded more detailed studies at higher magnifications. It was therefore decided to apply whole preparation techniques to the study of the diaphragm in smaller animals.

B) IN RODENTS.

In 6 mice and 2 rats, the diaphragm was impregnated with silver by the method mentioned above and the diaphragmatic plexus was examined. Almost all of the neural tissue in the diaphragmatic musculature stemmed from a conspicuous 'nerve rich' zone comparable to that observed in cats. Mice were particularly useful for examination of these general features of the diaphragmatic nerve plexus, and it was possible to trace the majority of the constituent fasciculi to their source in either the phrenic or intercostal nerves. In all of the animals studied, the radially arranged diaphragmatic branches arising from the lower six intercostal nerves, comparable to those previously traced centrally in cats, were found to participate in the formation of the plexus.

CONTRIBUTIONS TO THE DIAPHRAGMATIC PLEXUS FROM THE LOWER INTERCOSTAL NERVES.

These contributions were most conveniently studied by locating a main intercostal trunk in the peripheral fringe of the specimen and tracing its diaphragmatic branches towards the central tendon of the diaphragm. (It will be recalled that the whole preparations of the diaphragm included a narrow fringe of body wall musculature). The general arrangements of the diaphragmatic plexus can be seen in specimens before and after mounting (figs 22 and 23 respectively). In general, The diaphragmatic branches of the intercostal nerves comprise three categories, all of which

Fig. 22

The diaphragm and adjacent skeletal components of a mouse. This unmounted specimen was prepared by silver impregnation as a whole preparation. The translucency of the diaphragmatic tissue permits histological examination of the diaphragmatic plexus the general arrangements of which are clearly evident.

Magnification = 5x.

Fig. 22

Fig. 23

This diaphragm, also from a mouse, was prepared by the same technique as that shown in figure 22. The entire diaphragm is present along with portions of intercostal musculature peripherally. Magnification = 4x.

terminate centrally where they arborise in company with terminals of the phrenic nerve.

(1) Paravascular Fasciculi.

By far the most common of the intercostal contributions are bundles of unmyelinated nerve fibres which occupy a paravascular position, and give rise, at irregular intervals, to branches which ramify upon the wall of the adjacent vessel. The axis cylinders of these unmyelinated fibres, are regular in outline and remain discrete when traced centrally; no evidence of terminal reticulum (Stöhr 1952) was found. Near to their termination the vast majority of these fasciculi become closely approximated to corresponding paravascular components of the phrenic nerve. Of all the intercostal components which ramify in the diaphragm, these fibres are of the smallest diameter. (fig. 24).

(2) Serosal Fasciculi.

These are the next most common of the nerve fibres which arise from the intercostal nerves. They are seen as tortuous dark brown lines on a focal plane which is distinctly different from the focal plane of the muscle fibres of the diaphragm. In addition to the tortuosity mentioned above, these fibres describe frequent right-angled bends throughout their course. In size, they are intermediate between the preceding and succeeding groups of fibres and they ramify in the peripheral regions of the diaphragmatic pleura and peritoneum.(fig. 25). Only a few of these

100 µ

Fig. 24

From the peripheral region of the diaphragm of a mouse. The profile of a vessel is represented by parallel curved lines which traverse the field diagonally. A fasciculus of paravascular nerve fibres is present in the concavity of the vessel profile. Note the clarity with which the transverse striations of the diaphragmatic muscle fibres can be discerned.

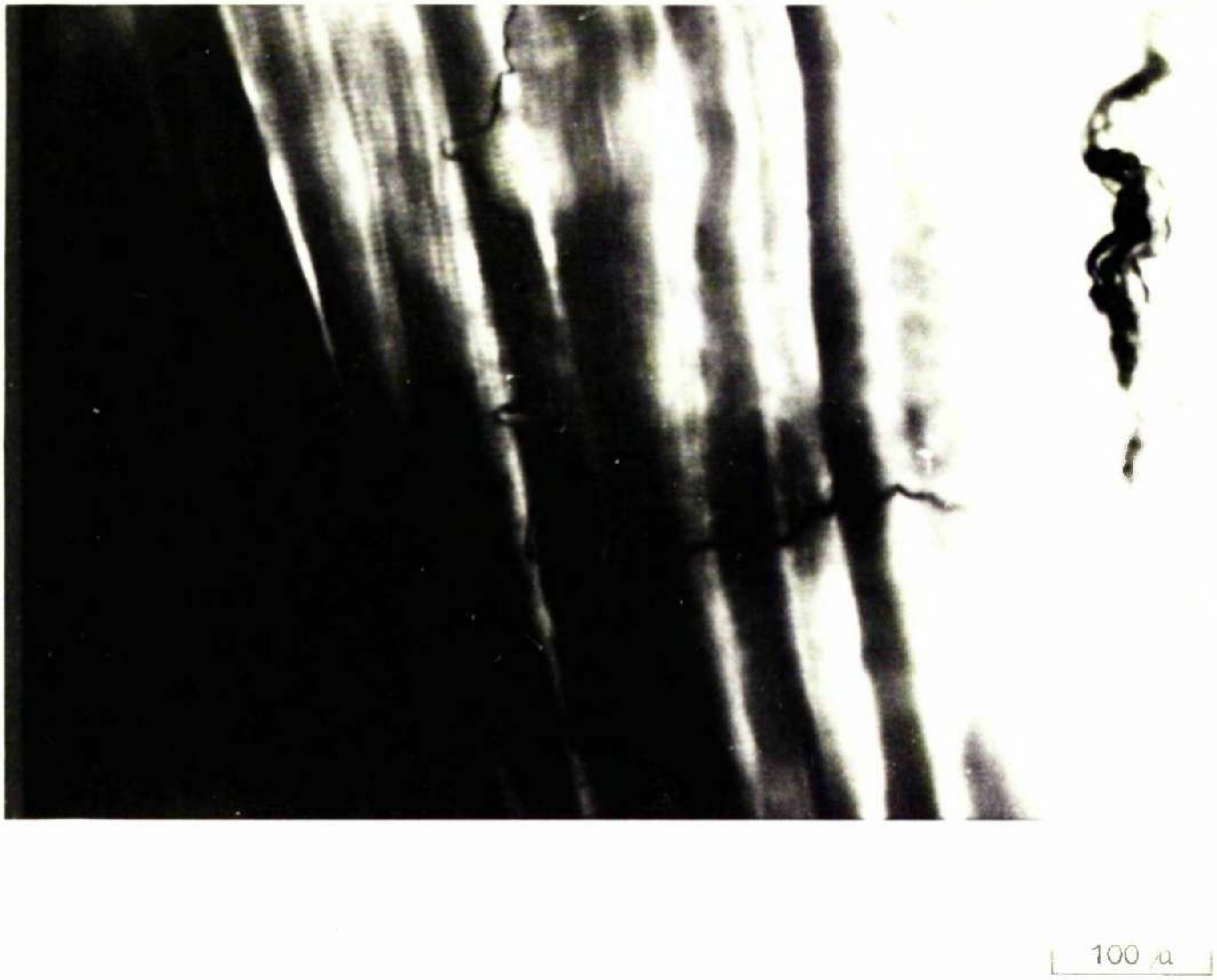


Fig. 25

From the same region of the diaphragm depicted in the previous two figures. A serosal nerve fibre, arising from an intercostal nerve, describes a tortuous course on the diaphragm. Note that the focal plane of the muscle fibres does not coincide with that of the nerve.

fibres reach as far centrally as the corresponding serosal fasciculi which arise from the phrenic nerve.

(3) Intramuscular Fasciculi.

These fasciculi, composed in the main, of myelinated fibres which ranged from 8-12μ in diameter, were seen to pass through the connective tissue which joins the diaphragm to the chest wall; they then enter and merge with the most peripheral parts of the diaphragmatic musculature. (fig. 26). In this position the nerve fibres of these fasciculi and the muscle fibres of the diaphragm are co-planar. The majority of these neural elements could be followed no further than the peripheral extremity of the muscle fibres. Eight such fasciculi, however, were traced centripetally to regions of contact between intercostal and phrenic nerve components within the substance of the diaphragm. The association of the two is so close that it suggests actual physical contact between them. (fig. 27).

CONTRIBUTIONS TO THE DIAPHRAGMATIC PLEXUS FROM THE PHRENIC NERVES.

From topographical studies in mice, just as in cats, it was noted that the phrenic nerves form branches before penetrating the diaphragm. As these diverge, to run ventrally, laterally and dorsally, they also give rise to a series of intercommunications which lie on the diaphragmatic musculature. Thus, on each hemidiaphragm, there are multiple neural arcades comprising nerve fasciculi which are situated close to the point at which the phrenic

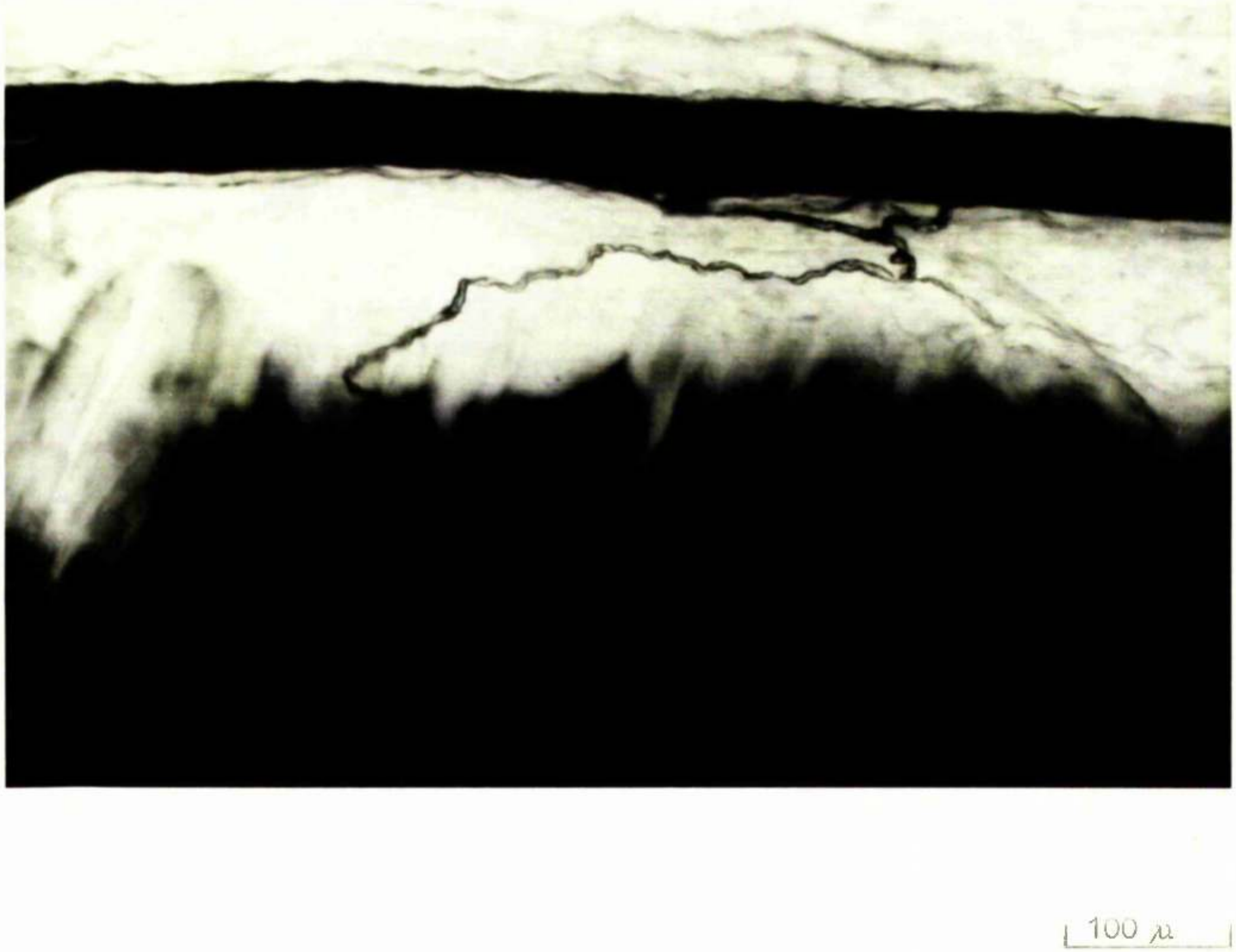


Fig. 26

From the same specimen as figure 25. A small fasciculus of nerve fibres can be seen arising from an intercostal nerve and, approaching the periphery of the diaphragm, they merge with the muscle fibres.

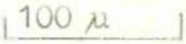


Fig. 27

From a silver-impregnated whole preparation of the diaphragm of a mouse. A centrifugal fasciculus containing about six nerve fibres approaches closely a centripetal fasciculus containing two nerve fibres. Note that the individual axis cylinders of the two fasciculi are approximately equal in calibre.

nerve terminals reach the diaphragm. From these arcades the three main fasciculi arborise to participate in the formation of the diaphragmatic plexus.

Each of the three main fasciculi follows a course which is parallel to the peripheral edge of the central tendon, thus, forming the "nerve rich" zone. The nerve fasciculi which run towards the metasternal attachments of the diaphragm from the right and left phrenic nerves terminate within a few millimetres of each other at their ventral extremities. The lateral terminal branches of the phrenic nerve are multiple and diverge from one another as they proceed centrifugally. The dorsal fasciculi proceed towards the corresponding crus of the diaphragm and terminate within its substance ramifying among the crural muscle fibres.

As these branches approach their termination, they give rise to smaller bundles of nerve fibres which leave the main fasciculi at approximately right angles. These small bundles are surprisingly uniform in length and, in the mouse, they undergo further branching at a distance of 1.5-2mms from the parent fasciculus. The same uniformity of terminal branching was seen in the rat, cat, and guinea-pig.

In order to confirm that the nerve terminals of the diaphragm are confined to a "nerve rich" zone, a cholinesterase technique was used to demonstrate the positions of myoneural junctions in guinea-pigs and mice.

It can be seen that diaphragmatic myoneural junctions as indicated by deposits of cholinesterase, are confined to a region which is coincident with the "nerve rich" zone defined by silver impregnation. (figs. 28 and 29). The most darkly stained regions within both of these illustrations are seen to be composed of many units which can be described as discrete black dots. These units are more easily distinguished in the diaphragm of the guinea-pig (fig. 28) because they are more widely spaced in this animal than in the mouse. (fig. 29). In regions where they are widely spaced, all of these dots, representing myoneural junctions, are approximately equal in size.

Characteristics of the Intradiaphragmatic Fasciculi of the Phrenic Nerve.

In four mice and two rats, whole preparations of the diaphragm, impregnated with silver were studied with the light microscope and the morphological features of the intradiaphragmatic branches of the phrenic nerve were observed.

It will be recalled that the main diaphragmatic branches of the phrenic nerve form neural arcades and then participate in the formation of the "nerve rich" zone which is parallel to the central tendon. (p. 150). This network of nerve fibres, referred to here as the 'diaphragmatic plexus' is equally visible, under the light microscope, from both the superior and inferior aspects of the

Fig. 28

The "nerve-rich" zone of the diaphragm of a guinea-pig. In this specimen the positions of neuro-muscular junctions have been indicated by a cholinesterase method. Magnification = 3x.

Fig. 29

The "nerve-rich" zone of the diaphragm of a mouse. The method of preparation was the same in this case as in figure 28.

Magnification = 4x.

diaphragm in rats and mice.

The main fasciculi, traced from the region of the neural arcades are composed, mainly, of conspicuous myelinated fibres whose diameters were estimated as ranging from 4-16μ, approximately. These myelinated fibres are accompanied throughout the entire diaphragmatic plexus by fasciculi of unmyelinated fibres. The numbers of fibres in fasciculi of phrenic origin, could not always be accurately determined. However, although some fasciculi contained as many as 40 nerve fibres and others as few as 2, most fasciculi in which individual fibres could be distinguished, contained between 20 and 30 nerve fibres. These estimates of fibre numbers although only approximate, enabled comparisons to be made between the various branches of the phrenic nerve which give rise to intra-diaphragmatic fasciculi.

The diaphragmatic plexus, including the components of the phrenic nerve, was seen to branch profusely, particularly in those parts which extended ventrally towards the metasternal attachments and dorsally towards the crural attachments of the diaphragm. At its ventral and dorsal extremities, the diaphragmatic plexus was reduced practically to a single nerve fibre due to continuous branching. The nerve fibres of the lateral diaphragmatic branch of the phrenic nerve were seen to be almost entirely deployed in the formation of the neural arcades. (fig. 22). Many of these lateral components then became associated with fasciculi which were directed ventrally and dorsally. A few of the fasciculi of the lateral branch, gave rise to nerve fibres which arborised near to their parent trunk.

The distribution of the branches of the diaphragmatic plexus, occurs mainly in the centrifugal direction (fig. 22). In this illustration, the internal aspect of the diaphragmatic plexus is seen to be smoother than the external aspect, indicating that the majority of branches originate from the external aspect. The posterior branches, seen in this illustration, cross the junction between the posterior leaflet and the central region of the central tendon and, in this position, they give rise to local branches which ramify in the central tendon.

By far the greatest majority of the fasciculi originating in the phrenic nerve, ramify among the muscle fibres of the diaphragm. A profusion of branches arise from the ventral and lateral components of the plexus almost at right angles to their parent fasciculi and they extend centrifugally for approximately 1.5 mm before terminating. Very few such branches could be traced in a centripetal direction and, when present, they were seen to consist of fine myelinated and unmyelinated fibres which ramify within the central tendon. The dorsal component of the diaphragmatic plexus pursued an uninterrupted course towards the crural attachments of the diaphragm giving rise to a few fine myelinated fibres which ramified upon the surface of the dorsal leaflet of the central tendon. On reaching the crural region, these fasciculi terminate in a fashion comparable to the ventral and lateral fasciculi.

The Morphology of the Terminal Branches of the Diaphragmatic Plexus.

In all of the animals studied, the majority of myelinated nerve fibres present within the terminal branches of the diaphragmatic plexus were seen to range in diameter from 4-16µ approximately; in this respect, they correspond to the myelinated fibres present within the trunk of the phrenic nerve. Individual unmyelinated fibres could not be demonstrated, due mainly to the thickness of the preparation, but small bundles of these fibres could be identified in most of the fasciculi.

(i) More than half of the myelinated fibres in the diaphragmatic plexus have an estimated diameter of 10-14µ, and they are uniformly cylindrical, close to their parent fasciculus. Traced centrifugally, however, these fibres become suddenly enlarged close to their point of arborisation. (fig. 30). These preterminal enlargements are single in some nerve fibres and multiple in others; there is great individual variation in their shape. In 'whole preparations' it was not possible to trace these nerve fibres beyond the site of local enlargement due, mainly, to the thickness of the preparation but, in subsequent studies, where 50µ frozen sections were examined, it was possible to trace them to end formations which had many of the features of motor end plates. (fig. 31). Thus, sole-plate nuclei and terminal arborisations, both of which are recognised features of this type of motor end formation, were

Fig. 30

A silver preparation of the diaphragm in a mouse. The complexity of the diaphragmatic plexus is conspicuous in this zone which is close to the point of terminal branching of the phrenic nerve. Note the profusion of interfascicular communications and the irregularity of the outlines of fibre terminations.

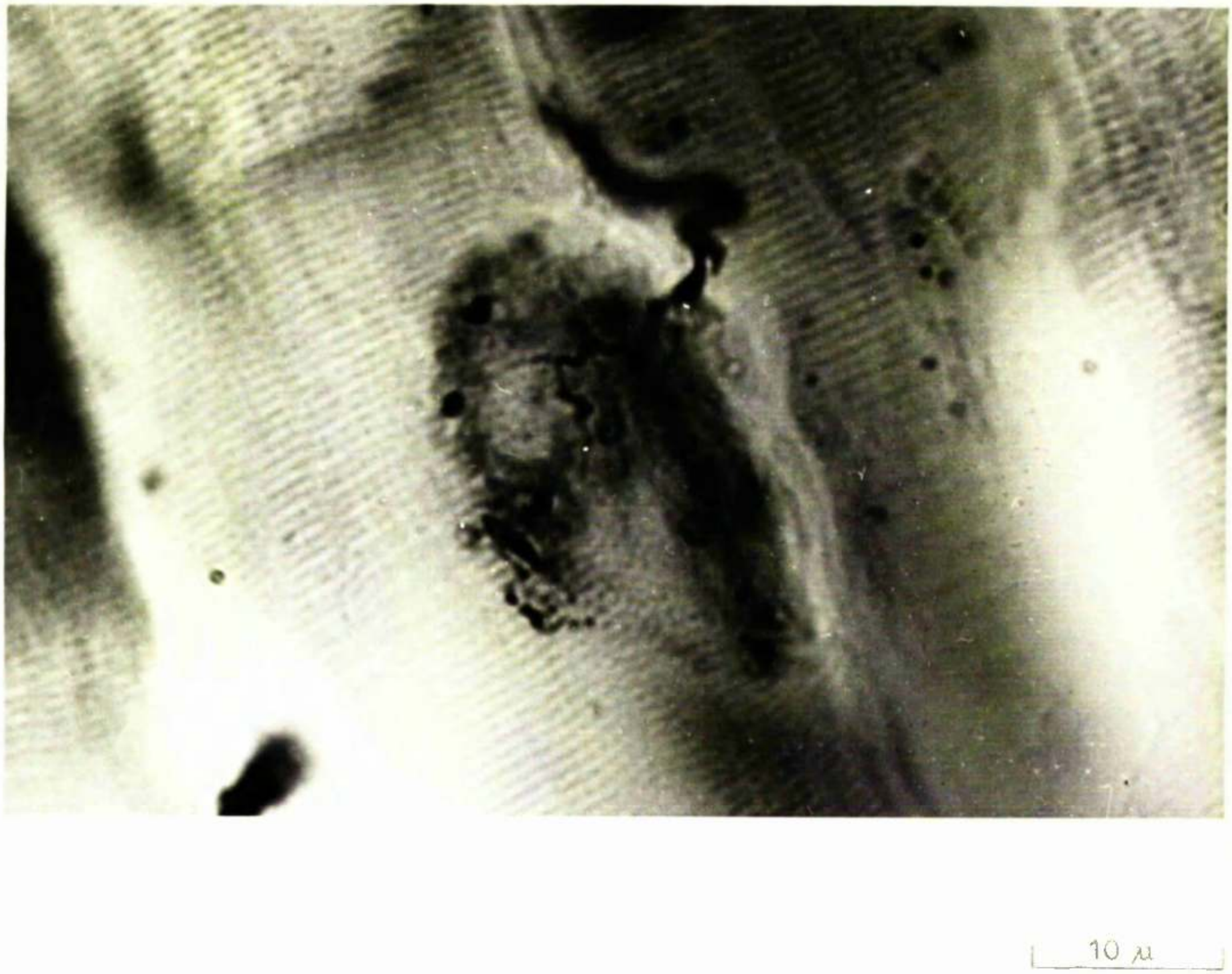


Fig. 31

A nerve ending resembling a motor end-plate in a frozen section of the diaphragm in a cat. Note that the argyrophilic ending is in continuity with an axis-cylinder which shows preterminal irregularity of shape.

present. In their preterminal and terminal regions these fibres were in the same focal plane as the muscle fibres.

(ii) Within the fasciculi of the diaphragmatic plexus there are myelinated fibres in which the estimated diameters range from 6-12µ approximately. These are fewer in number than the nerve fibres of the previous group and they show the following structural characteristics. Traced centrifugally, they were seen to lie in a focal plane different from that of the muscle fibres, a few of them being coplanar with the serosal covering of the diaphragm, while the majority were situated in the plane between the muscle fibres and the serosa. In both of these situations the fasciculi branched repeatedly to form communications with adjacent nerve fibres and they became very much reduced in size about half way between the "nerve rich" zone and the peripheral attachment of the diaphragm. No identifiable end-formations were seen in relation to individual fibres of this group. (fig. 32).

(iii) The smallest nerve fibres present within the fasciculi of the diaphragmatic plexus, have an estimated diameter which ranges from 2-6µ approximately. Although myelin sheaths of individual fibres were clearly seen in the more central part of their course, careful examination of their more peripheral parts with the highest magnification of the light microscope, failed to determine clearly, the presence or absence of a myelin sheath. Infrequent branching was seen to occur, and the branches, so formed, arborised on the wall of an adjacent vessel. It was also noticed that bulbous swellings, identified as Schwann cells, were

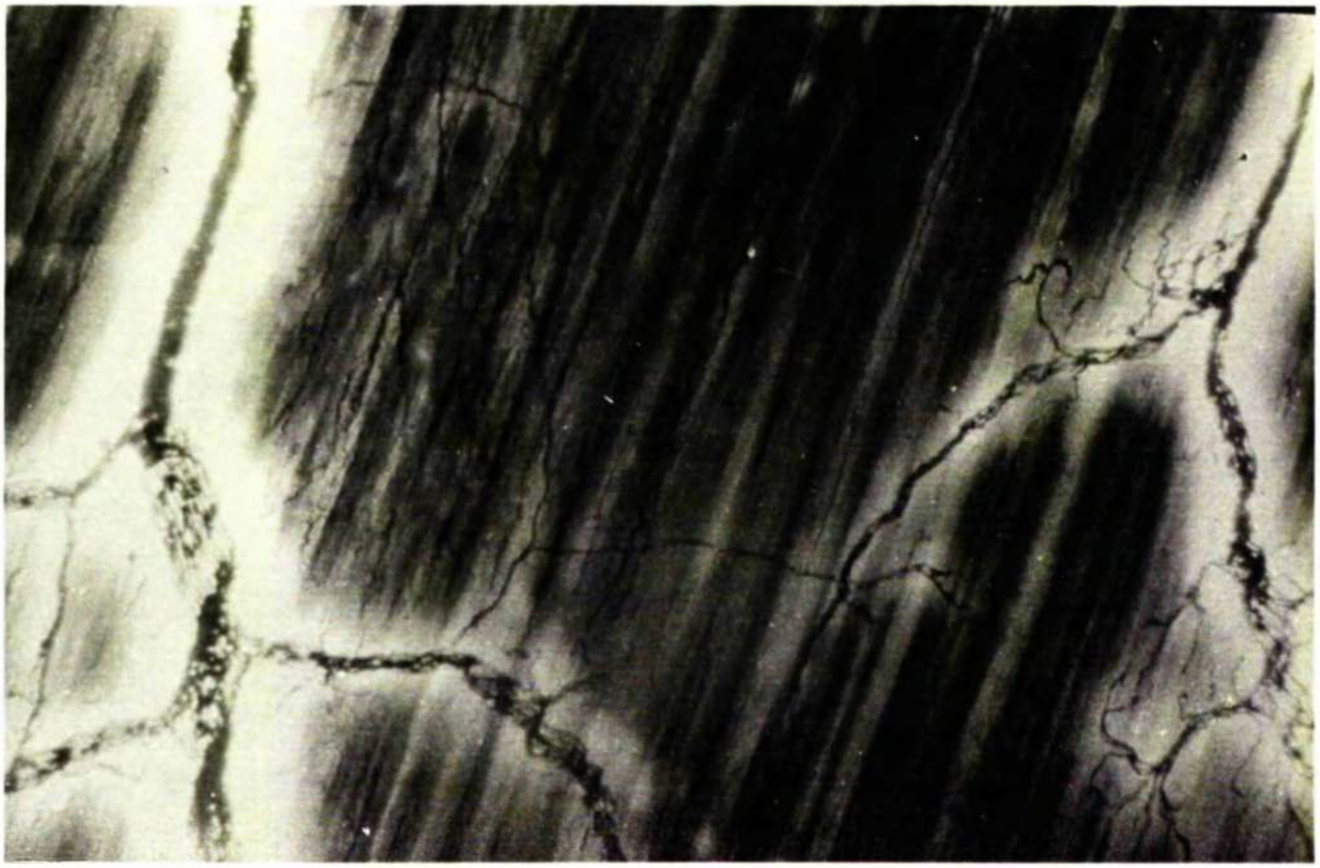


Fig. 32

This region of the diaphragm in a mouse shows nerve fibres of medium diameter which form interfascicular connections. Tortuosity is a prominent feature in these components of the plexus.

present at irregular intervals along the length of these nerve fibres.

(iv) Approximately one third of all the preterminal enlargements situated on nerve fibres associated with motor end-plates, possess a centrifugal extension in the form of an axis cylinder of small diameter which could be traced to the point where it merged with other branches of the diaphragmatic plexus. (fig. 33). In one instance, this type of fibre was traced beyond the "nerve rich" zone to the vicinity of a structure identified as a muscle spindle, although actual physical contact between these two neural elements was indefinite.

In spite of repeated attempts to demonstrate muscle spindles only one was found in a 50μ frozen section taken from the ventral region of the diaphragm in a cat.

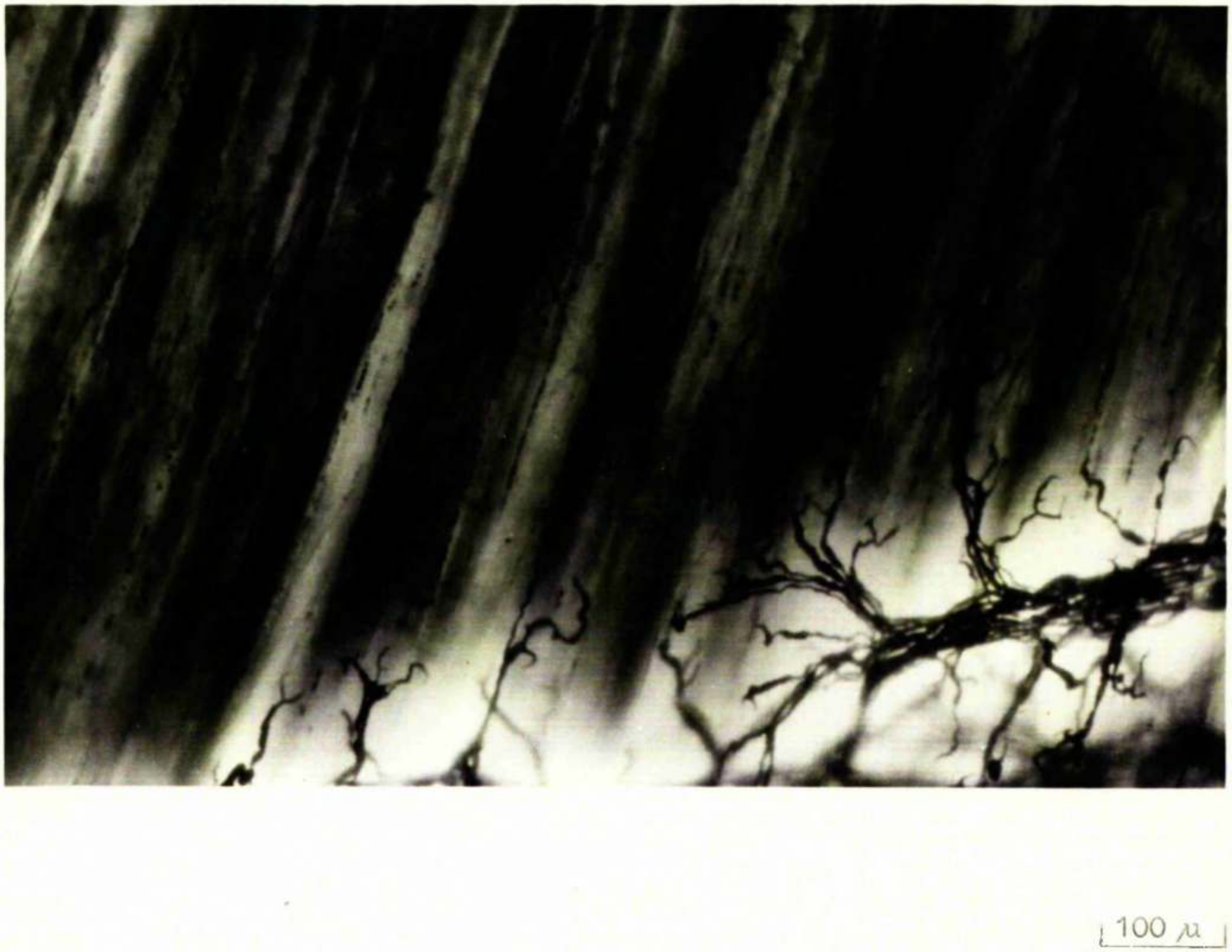


Fig. 33

From the same specimen as figure 32. An argyrophilic filament resembling an axis-cylinder of small diameter projects centrifugally from a short, stubby terminal of the diaphragmatic plexus.

EXPERIMENTAL OBSERVATIONS.

The experimental procedures carried out in the present study involved interruption of individual groups of nerve fibres supplying the diaphragm. Thus, each experimental animal of the series was subjected to one or other of the procedures described above and the diaphragmatic plexus and phrenic nerves were examined histologically following survival periods which ranged from 1-30 days. Ultrastructural changes in the phrenic nerve were also explored in 4 animals following ligation of the phrenic nerve.

A. Unilateral Division of the Phrenic Nerve at the Level of the Lung Hilum.

In two adult cats, attempts were made to locate the left and right phrenic nerves respectively, by exploration of the posterior triangle of the neck. The observations confirmed previous topographical findings that precise identification of the roots of the phrenic nerve was difficult to achieve and since it was clearly impossible to be certain that all fibres reaching the phrenic nerve were severed at operation, this procedure was abandoned. All subsequent attempts to interrupt the phrenic nerve were, therefore, confined to the intrathoracic portion of the nerve trunk or to its cervical roots of origin.

Successful interruption of the intrathoracic part of the phrenic nerve depended on satisfactory maintenance of respiration for the duration of the operation. Initially, this was achieved by means of tracheostomy, the airway being connected to a mechanical pump by means of a glass cannula tied into the trachea. In subsequent experiments, however, an intratracheal tube was used in fully grown animals and it was found that the mortality was reduced from 50% in tracheostomy, to 10% in intratracheal intubation. Fourteen adult cats recovered following intrathoracic transection of the phrenic nerve and the survival period of these animals ranged from 2-30 days.

"In vivo" observations in normal animals confirmed that intrathoracic section of the phrenic nerve was a less hazardous procedure on the left side than on the right because the left nerve is more accessible and is related to less vulnerable vascular structures than the right nerve. In 10 of the 14 animals surviving the operation, the left phrenic nerve was severed and in 4, the right was severed.

Macroscopic observations.

(a) During approach to the phrenic nerve.

On opening one side of the thorax, the subjacent lung collapsed within two or three seconds, while the expansion of the other lung became reduced. Further observations on the lungs were precluded by the necessity of maintaining oxygenation, using the automatic respirator. The animal continued to make respiratory efforts during this stage and it was found advantageous to synchronise the inspiratory and expiratory phases of the respirator with those of the animal. If the two were out of phase, peripheral cyanosis became established within one minute as evidenced by the blue discolouration of the cat's tongue. The heart beat was also adversely affected, becoming very rapid and weak after exposure to this type of asynchrony for about ten seconds. In about two-thirds of all animals subjected to thoracotomy, respiratory effort ceased when the mechanical respirator had assumed control. In the remainder of the animals respiratory effort was maintained until the end of the operation and spontaneous recovery of natural respiration was correspondingly quicker.

(b) During localisation and transection of the phrenic nerve.

Previous topographical observations on the intrathoracic portion of the phrenic nerve were confirmed and attention was given to the dynamic state of neighbouring structures. The nerve, as observed at thoracotomy, was never completely at rest being affected by cardiac and pulmonary movements. During artificial respiration the lung movements produced approximately equal displacement of the left and right phrenic nerves. The effects of cardiac contraction were, however, asymmetrical and displaced the left nerve more than the right one. This dynamic asymmetry was, to some extent, diminished by the slightly greater length of the left phrenic nerve but the difference in movement of the two nerves, remained obvious. With the thoracic cavity open to the atmosphere, movements of the diaphragm became progressively reduced in amplitude and irregular in rhythm, producing only minor displacement of the phrenic nerves. When the nerve trunk was stimulated unilaterally by squeezing with artery forceps, the appropriate hemidiaphragm contracted vigorously and there was considerable traction exerted upon the mediastinal viscera including the phrenic nerve itself. Alteration in the relative positions of the heart and phrenic nerves was seen to occur in a dorso-ventral direction, indicating that the nerves are not completely bound down by surrounding connective tissue. During right atrial contraction, the resultant pulse wave, propagated along the inferior and superior vena cava, caused a slight ripple of movement along the length of the right phrenic nerve. The corresponding movement, due to ventricular contraction, on the left side involved a shorter length of the left phrenic nerve.

Transection of the nerve at the lung hilum, produced immediate cessation of all rhythmic movement of the diaphragm on the side of the lesion.

(c) During closure of the thorax following transection of the phrenic nerve.

The lungs, which had been aerated so as to produce the least excursion compatible with adequate oxygenation, were now inflated more vigorously for five minutes by appropriate adjustment of the respirator. In the phase of maximum inflation, the wall of the thorax was closed, respiratory assistance being reduced gradually during repair of the skin wound. Signs of returning spontaneous respiration were usually observed when slight cyanosis and tachycardia were becoming apparent. Irregular, spasmodic contraction of the upper abdominal musculature was invariably the first manifestation of the onset of respiration and it was best observed with the animal lying in the supine position. In order to increase the phasic inflation of the lung, the animal was placed in the semi-prone position with the unoperated side uppermost and the automatic respirator was stopped but not disconnected. The animal was not removed from this position until the respiratory rate had reached almost twice its pre-operative value. The time taken for this to occur was longer in older animals and in animals in which anaesthesia was prolonged. The time taken for the animal to become ambulant was seldom less than 48 hours after operation and, during this recovery period, normal saline solution containing 5% dextrose was administered by intraperitoneal injection in doses of 20ml three times each day. Careful assessment of the respiratory movement, two to three days after operation indicated asymmetry of movement much less pronounced than in the immediate post-operative state.

Preliminary histological observations on the effects of transection of the phrenic nerve indicated that the structural changes in axis cylinders were more conspicuous in the diaphragmatic plexus than in the nerve trunk itself. Thus it was decided to examine the effects of transection in cats, first in the diaphragmatic plexus and then in the trunk of the phrenic nerve following survival periods which ranged from 48 hours to 30 days. The maximum survival period of 30 days was selected in order to obviate the possible complication of regeneration of axis cylinders.

Microscopic observations.

CHANGES IN THE DIAPHRAGMATIC PLEXUS.

The structural features of the diaphragmatic plexus in normal animals have been described above (p 153 to p 163) and they form a basis for comparison with the experimental observations described below, since comparable methods of tissue preparation were used in each case.

The morphological changes in the plexus were examined in 8 cats in which the survival period ranged from 48 hours to 30 days. While, in all of the animals examined, the majority of diaphragmatic nerve fibres on the operated side showed structural changes indicative of degeneration, there were some axis cylinders which were normal in appearance. Although most of these persisting fibres were present in sections taken from the periphery of the diaphragm and were identified as branches of intercostal nerves, a small proportion of them occupied more central positions in the region which is normally supplied by the phrenic nerve; their distribution is discussed below. (p 176).

At 48-84 hours.

The changes in individual nerve fibres of the diaphragmatic plexus on the side of the lesion were characterised by varicosities of axis cylinders, in the case of fibres of medium to large diameter and fragmentation of axis cylinders in the case of an increasing proportion of fibres of small diameter. (fig. 34).

At 4-13 days.

Comparatively few normal fibres could be distinguished. Most fibres were either markedly irregular in outline or were represented by argyrophilic debris arranged in series and confined within endoneurial sheaths. (fig. 35). There was an obvious reduction in the number of intact nerve fibres in specimens taken at more prolonged post-operative intervals. There was, however, a corresponding increase in nucleated Schwann bands many of which contained argyrophilic debris which was seldom regularly arranged in linear aggregates at 8 days or longer after operation.

At 18-30 days.

The argyrophilic remnants of degenerated nerve fibres disappeared progressively until only empty Schwann bands remained within the fasciculi. (fig. 36).

Detailed observations after specified post-operative intervals follow.

At 48 hours.

The vast majority of the fasciculi of the diaphragmatic

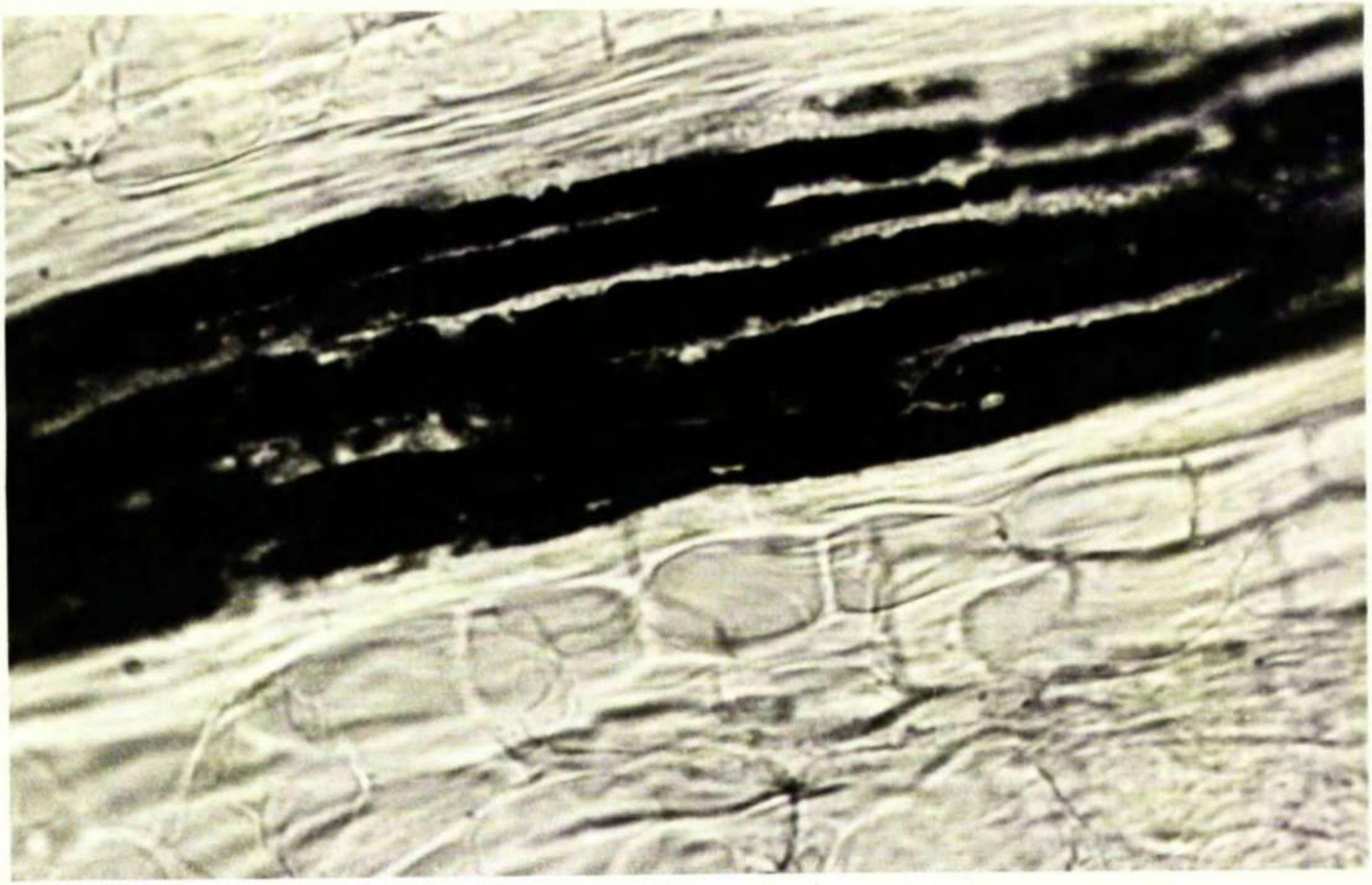


Fig. 34

A fasciculus of nerve fibres within the right hemi-diaphragm of a cat in which the right phrenic nerve had been transected $3\frac{1}{2}$ days previously. The space which is normally present between individual fibres has, apparently been reduced in size by swelling of axis cylinders.

Fig. 35

A large fasciculus of nerve fibres within the left hemi-diaphragm of a cat in which the left phrenic nerve had been interrupted 4 days previously. Fragmentation of axis cylinders is obvious and, in some fibres, the argyrophilic material has a varicose appearance. Note the presence of argyrophilic debris in the extrafascicular tissues.

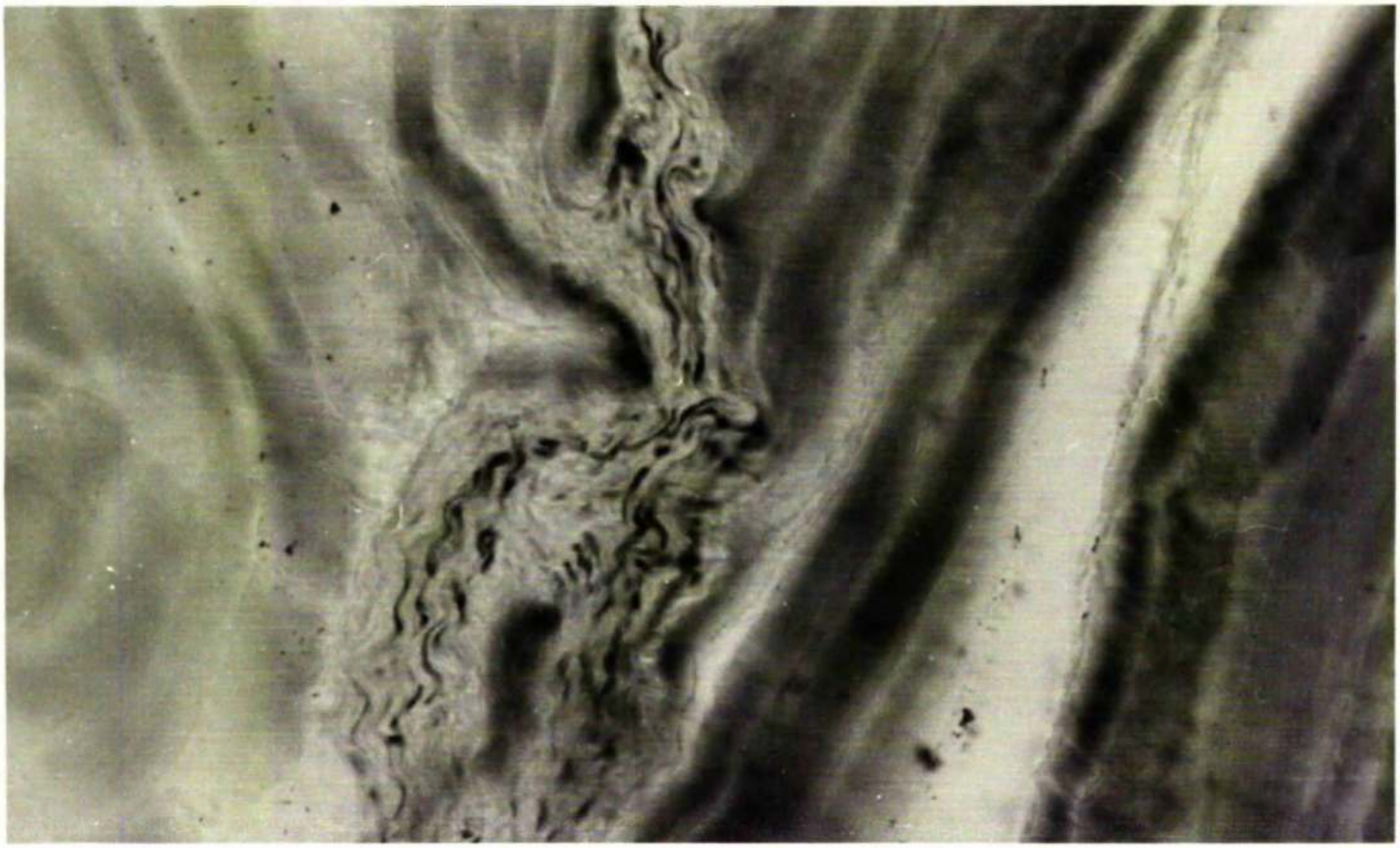


Fig. 36

Remnants of a fasciculus of nerve fibres within the right hemi-diaphragm of a cat in which the right phrenic nerve had been transected 30 days previously. Argyrophilic material has been reduced to small, discontinuous spindle-shaped fragments and the majority of nerve fibres are represented by empty Schwann bands.

plexus on the side of the lesion, possessed nerve fibres which were apparently swollen, for the clear spaces seen separating adjacent axis cylinders in normal animals were either reduced in width or completely obscured. (fig. 37).

At 84 hours.

Most of the axis cylinders of medium to large diameter showed early degenerative changes. (fig. 34). Traced peripherally, this fasciculus was seen to contain one axis cylinder of medium diameter in which the argyrophilic material was 'beaded' and two axis cylinders of small diameter in which there was evidence of fragmentation. (fig. 38).

At 4 days.

Although the survival period was only twelve hours longer, the degenerative changes were much more pronounced and the argyrophilic material showed marked fragmentation. Within the fasciculi, spindle-shaped cells were present, and in the nuclear zone these were approximately twice the diameter of the largest axis cylinders. (fig. 39). In the perifascicular zone there was a profusion of argyrophilic droplets.

At 10 days.

Fragmentation of argyrophilic material within the fasciculi was further advanced but was not present to the same extent in all axis cylinders. (fig. 40). Changes in nerve fibres of large

Fig. 37

A small fasciculus of nerve fibres within the right hemi-diaphragm of a cat in which the right phrenic nerve had been transected 2 days previously. The only evidence of change, in this case, is narrowing of the space which lies between adjacent nerve fibres. The general increase in intensity of staining was seen in all regions of this diaphragm.

Fig. 38

A small fasciculus of fibres in the left hemi-diaphragm of a cat in which the left phrenic nerve was transected 3½ days previously. The prominent changes in axons, here, are 'beading' and fragmentation of argyrophilic material.

Fig. 39

A fasciculus within the left hemi-diaphragm of a cat in which the left phrenic nerve had been interrupted 4 days previously. Some fragmentation of axis cylinders is present in fibres of smaller diameter and the outlines of the majority of the fibres appear to be more irregular than normal. Note the presence of spindle-shaped, intrafascicular cells with prominent pale-staining nuclear zones.

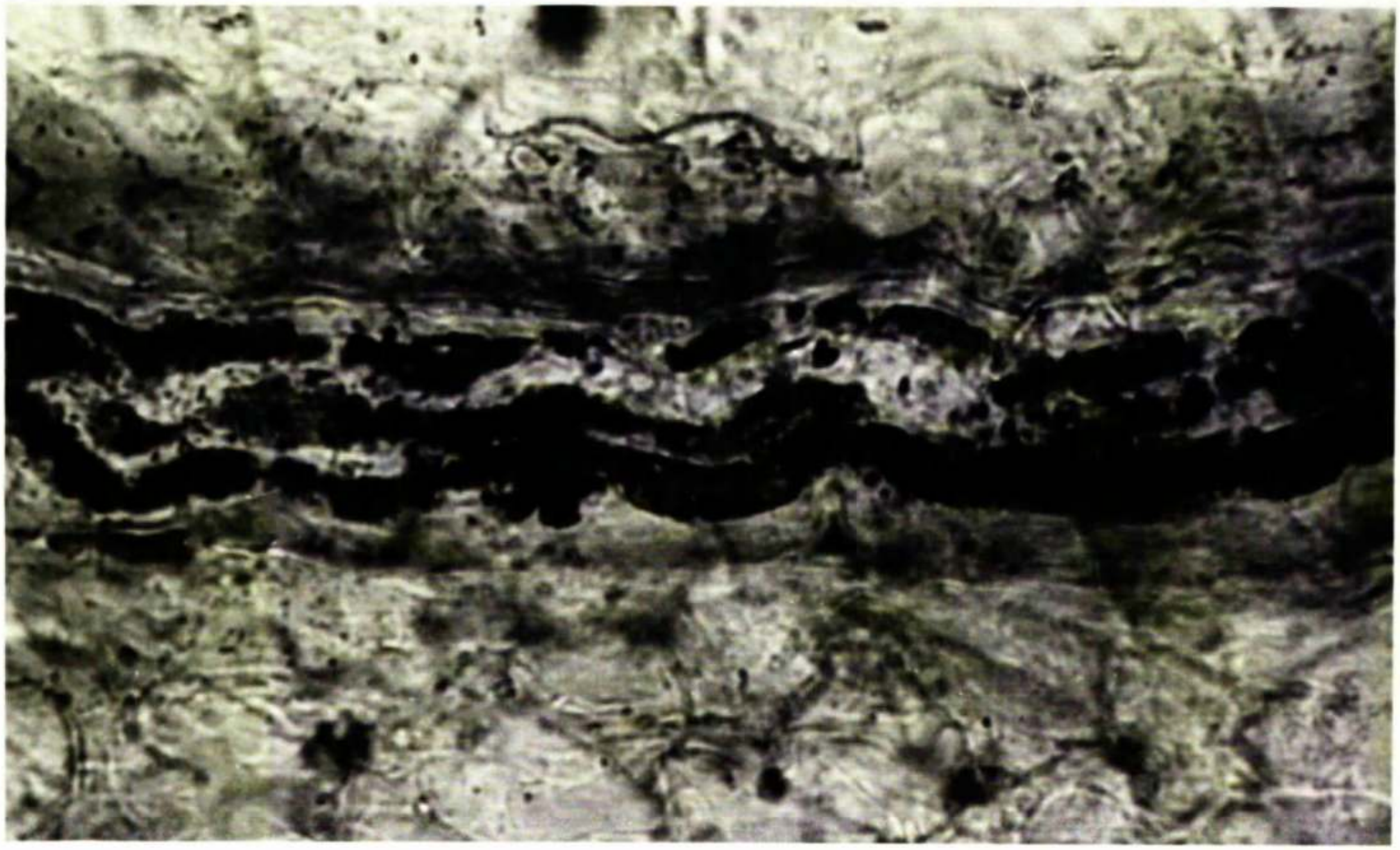


Fig. 40

A small fasciculus of fibres within the left hemi-diaphragm of a cat in which the left phrenic nerve had been interrupted 10 days previously. Fragmentation of two axis cylinders is clearly evident, the remaining fibre of larger diameter showing distinct irregularity in outline but no disruption of continuity. Note the abundance of extrafascicular argyrophilic debris.

diameter were less obvious than those in fibres of smaller diameter in which argyrophilic material was reduced to the form of discontinuous droplets. Perifascicular argyrophilic debris was also observed within a few millimetres of the fasciculi of the diaphragmatic plexus.

At 13 days.

The majority of axis cylinders within the fasciculi showed further fragmentation and the discontinuous, irregularly shaped particles were intensely argyrophilic. (fig. 41). In those fibres where the axoplasmic continuity was preserved for distances of a few millimetres, the argyrophilic material was varicose.

At 18 days.

The nerve fibres of the diaphragmatic plexus were, in general, less intensely argyrophilic than those seen in any of the animals described above. (fig. 42). Perifascicular argyrophilic debris was a prominent feature suggesting that a process of rarifaction of axis cylinders had occurred.

The changes observed were not identical in all regions of the diaphragmatic plexus for, in the larger fasciculi of the "nerve rich" zone, particulate argyrophilic material was present within the individual axis cylinders.

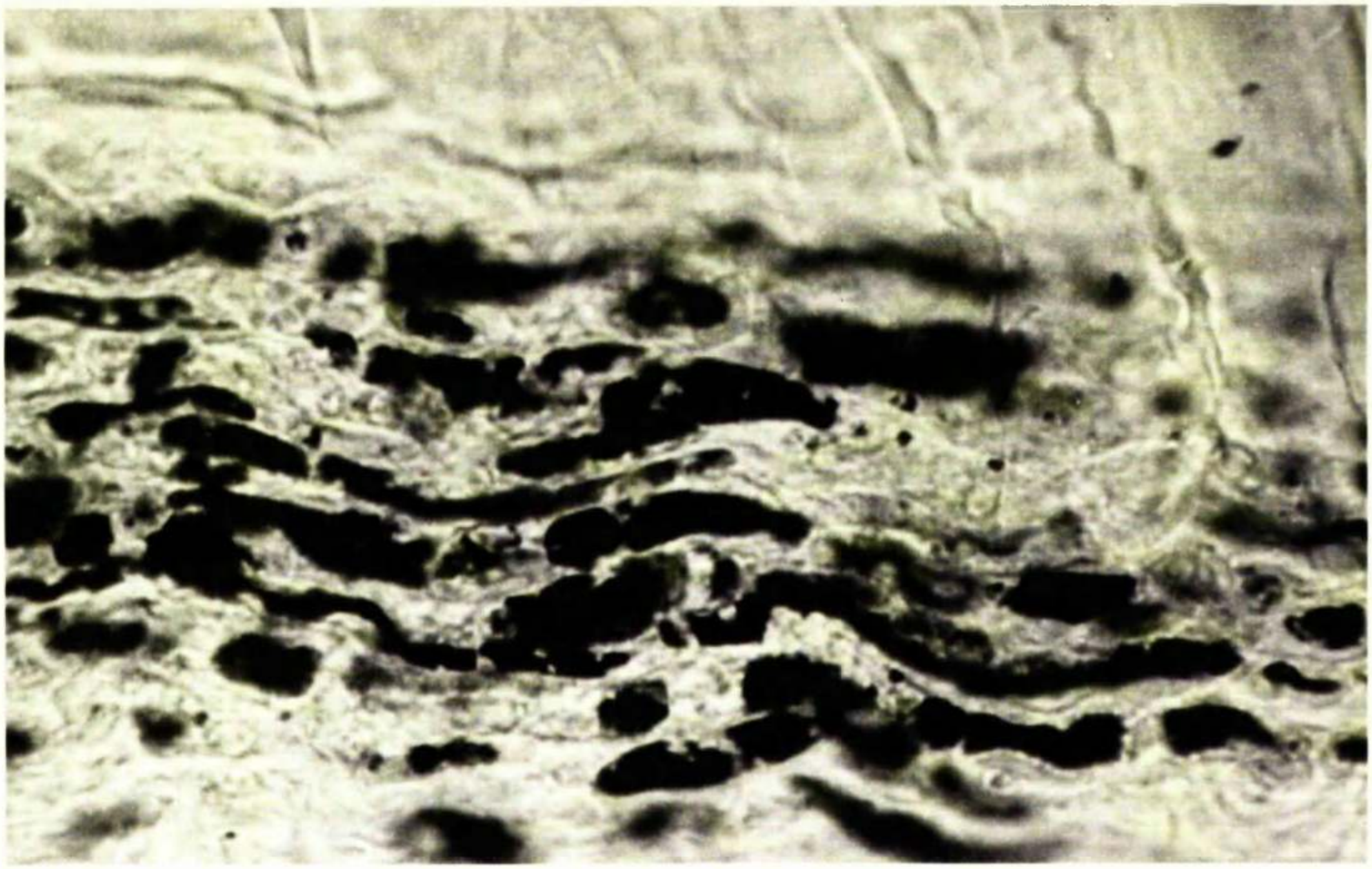


Fig. 41

A portion of a large fasciculus within the left hemi-diaphragm of a cat in which the left phrenic nerve had been interrupted 13 days previously. Fragmentation and varicosity of all axis cylinders are clearly seen.

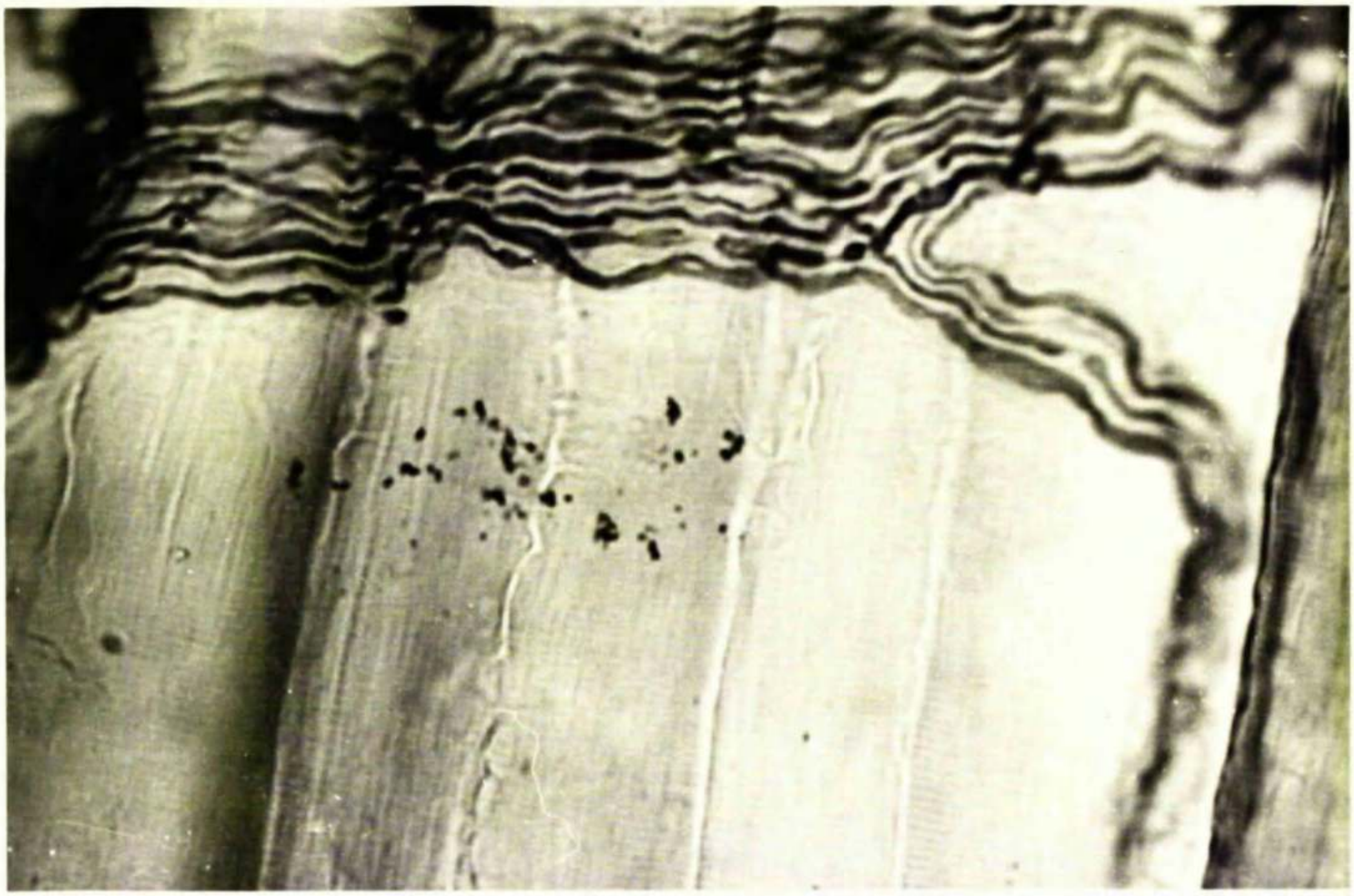


Fig. 42

A portion of a large fasciculus within the left hemi-diaphragm of a cat in which the left phrenic nerve had been transected 18 days previously. The axis cylinders are distinctly less argyrophilic than those of other animals of the series. Argyrophilic droplets are clearly seen in the muscle tissue which surrounds the fasciculus. Note that the transverse striations of the muscle fibres appear virtually normal.

At 21 days.

Identification of individual nerve fibres and even of fasciculi within the diaphragmatic plexus was extremely difficult to achieve. Particles of argyrophilic material within the axis cylinders were so sparse that they could not be relied upon to indicate the position of fasciculi. It was possible, however, to identify very short fragments of Schwann sheaths because the remnants of fasciculi contained large cellular components resembling those described in the four day study. (fig. 43).

At 30 days.

The degenerative changes had advanced to a stage at which all vestiges of intrafascicular argyrophilic material were completely absent, only empty Schwann bands remaining. (fig. 36).

Topographical Distribution of Degenerated Diaphragmatic Nerve Fibres.

In four animals, in which the survival periods were $3\frac{1}{2}$, 10, 13 and 30 days respectively, the diaphragmatic plexus in both hemidiaphragms was examined in serial frozen sections through the entire thickness of the muscle in selected areas. In one additional animal, in which the phrenic nerve had been ligated instead of transected at the level of the lung hilum 84 hours previously, the diaphragmatic plexus was similarly examined in detail so that comparisons could be made between the effects of the two

Fig. 43

Remnants of nerve fibres within a degenerated fasciculus of the left hemi-diaphragm in a cat in which the left phrenic nerve had been transected 21 days previously. The small amount of intrafascicular argyrophilic material which remains is in the form of small, discontinuous droplets. The conspicuous ovoid profile within the fasciculus resembles the intrafascicular cell seen in the 4 day study.

procedures after equal post-operative intervals.

The anterior, anterolateral, posterolateral and posterior areas of the diaphragm from which samples were taken will be referred to as zones 1, 2, 3 and 4 respectively. (fig. 2).

At $3\frac{1}{2}$ days after transection or ligation of the phrenic nerve.

On the side of the lesion the axis cylinders within all zones of the diaphragmatic plexus were represented as continuous bands of argyrophilic material and no indisputable evidence of degeneration was seen. The majority of the fasciculi within the central region of the diaphragm, however, showed structural changes comparable to those described above. (fig. 34).

On the side opposite the lesion in zones 1, 2, and 3 the nerve fasciculi were entirely normal but, in zone 4, a few bundles of nerve fibres were seen to have undergone structural changes resembling those observed in the diaphragmatic plexus on the side of the lesion.

At 10 days after transection of the left phrenic nerve.

On the side of the lesion the vast majority of fasciculi in zones 1, 2 and 3 of the diaphragm showed degeneration of axis cylinders although fragmentation was seen to be further advanced in fibres of small diameter than in those of large diameter. (fig. 40).

On the side opposite the lesion the nerve fasciculi in zones 2, and 3 were normal in appearance while those in zones 1 and 4 showed indisputable degenerative changes in axis cylinders in which the estimated diameter ranged from 2-14μ.

At 13 days after transection of the left phrenic nerve.

On the side of the lesion no normal fibres were seen in zones 1, 2 and 3, all of the axis cylinders being represented by discontinuous clumps of argyrophilic material. (fig. 41).

On the side opposite the lesion all of the axis cylinders appeared normal in zones 1, 2 and 3 but in zone 4, degenerated axis cylinders were present, either singly or within fasciculi which also contained normal axis cylinders.

At 26 days after transection of the right phrenic nerve.

In this animal the level of transection was somewhat more caudal, being 1.5cm closer to the diaphragm.

On the side of the lesion the components of the diaphragmatic plexus were difficult to define, most fibres, in all but a few fasciculi having undergone marked degenerative changes which were almost identical to those observed at 30 days. (fig. 36).

On the side opposite the lesion, although repeated attempts were made to identify degenerated nerve fibres, none were found.

Topographical Distribution of Persisting Diaphragmatic Nerve Fibres.

In all animals subjected to unilateral division of the phrenic nerve, some of the fasciculi within the diaphragmatic plexus on the side of the lesion showed no evidence of degeneration. These persisting fibres were observed in greatest numbers in the peripheral regions of the diaphragm and they appeared to be directed radially towards the central tendon. The estimated diameters of their individual axis cylinders ranged from 4-16µ and the largest of them could be traced to the vicinity of the "nerve rich" zone which has been referred to in normal studies. They were identified as branches of intercostal nerves because the number of axis cylinders within them diminished from the periphery inwards. Furthermore, they resembled those peripheral components of the diaphragmatic plexus which were identified as intercostal contributions in normal animals. All of these fasciculi contained bundles of fibres of small diameter which appeared to be unmyelinated and which, on emerging from the parent fasciculus, occupied paravascular positions.

In addition to those present in the region of the costal attachment of the diaphragm, small fasciculi of persisting fibres were also observed in the zone of distribution of the phrenic nerve. The majority of these fasciculi could not be traced to intercostal origins nor could their direction be clearly defined as radial with respect to the diaphragm. The individual nerve fibres within them resembled the persisting fibres observed at the periphery with regard to size and myelination.

In those animals in which the survival period was less than 4 days it was difficult to distinguish clearly between normal nerve fibres and those affected by the experimental lesion. In two animals, however, in both of which the survival period was 3½ days, a few of the nerve fasciculi in zones 3 and 4 of the hemidiaphragm on the side of the lesion were apparently normal. Following survival periods of 10, 13 and 26 days, the structural differences between degenerated and persisting nerve fibres were much more distinct and their distribution was more easily defined.

At 10 days after transection of the left phrenic nerve.

Persisting nerve fibres were observed in zone 3 of the hemidiaphragm on the same side as the lesion. It was estimated that the number of individual fibres was no greater than one-third of the number present in the corresponding area in normal animals. Although a few of the persisting fibres were of small diameter and occupied paravascular positions, some of them were intermediate in size, having estimated diameters of 10-14µ.

At 13 days after transection of the left phrenic nerve.

Persisting fibres were present in zone 4 of the left hemidiaphragm. In this animal, some of the persisting fibres were contained within fasciculi, the other fibres of which showed degenerative changes. Qualitative and quantitative assessment of these fibres corresponded closely to the findings in the 10 day study.

At 26 days after transection of the right phrenic nerve.

Zones 3 and 4 of the right hemidiaphragm contained persisting nerve fibres, some of which occupied paravascular positions. (fig. 44). In addition to these, fasciculi containing axis cylinders which ranged from 8-14µ in diameter were identified within the musculature in the region of the lumbar attachment of the diaphragm. (fig. 45). The number of individual fibres within these fasciculi was seldom more than five and the total number present was estimated as one quarter of the corresponding value in normal animals.

The results of these studies on the distribution of degenerated and persisting nerve fibres within the diaphragm are presented in more graphic form below. (fig. 46).

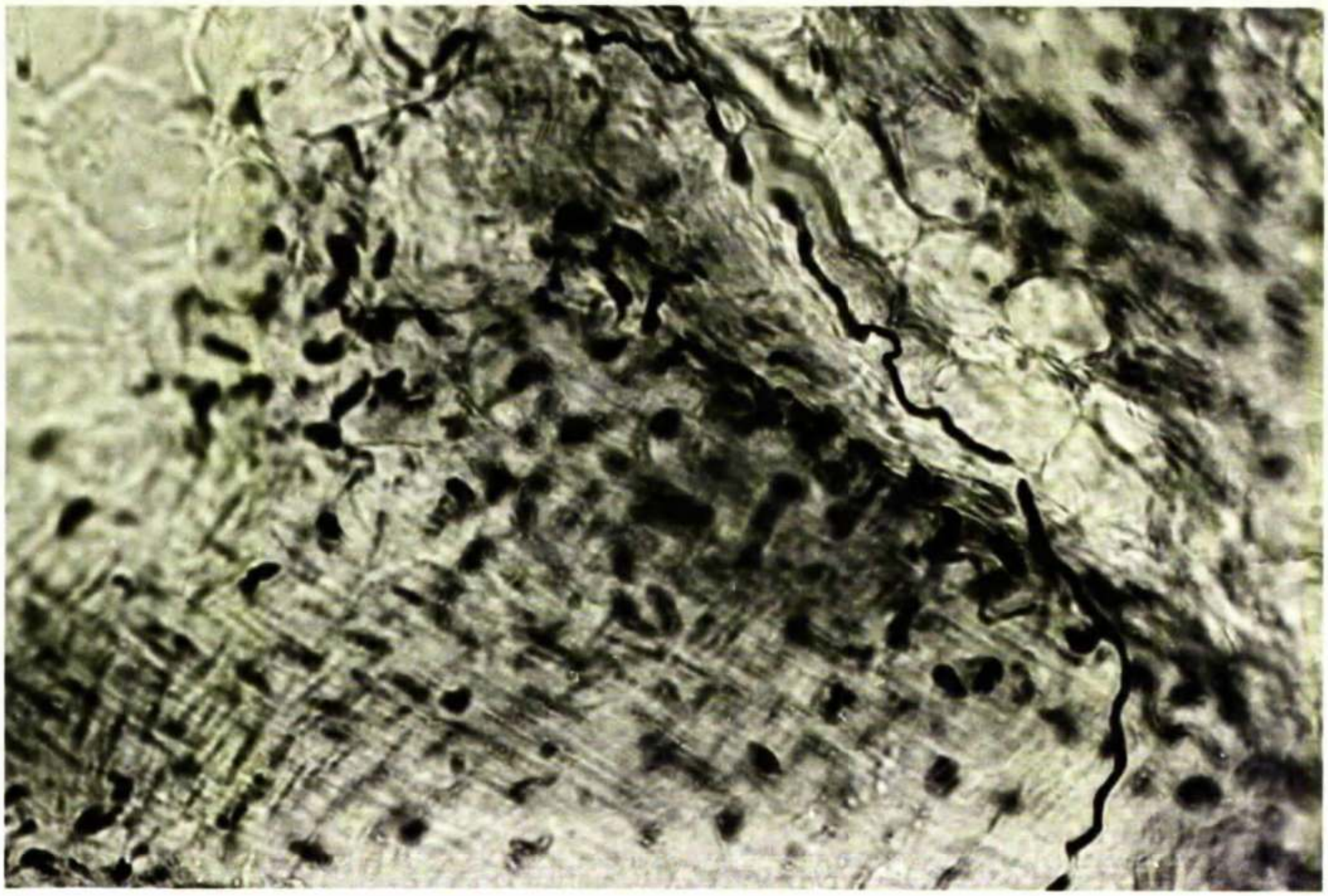


Fig. 44

A nerve fibre of small diameter within the postero-lateral zone of the right hemi-diaphragm in a cat in which the right phrenic nerve had been transected 26 days previously. The continuity of this axis cylinder suggests that the nerve fibre is structurally intact.

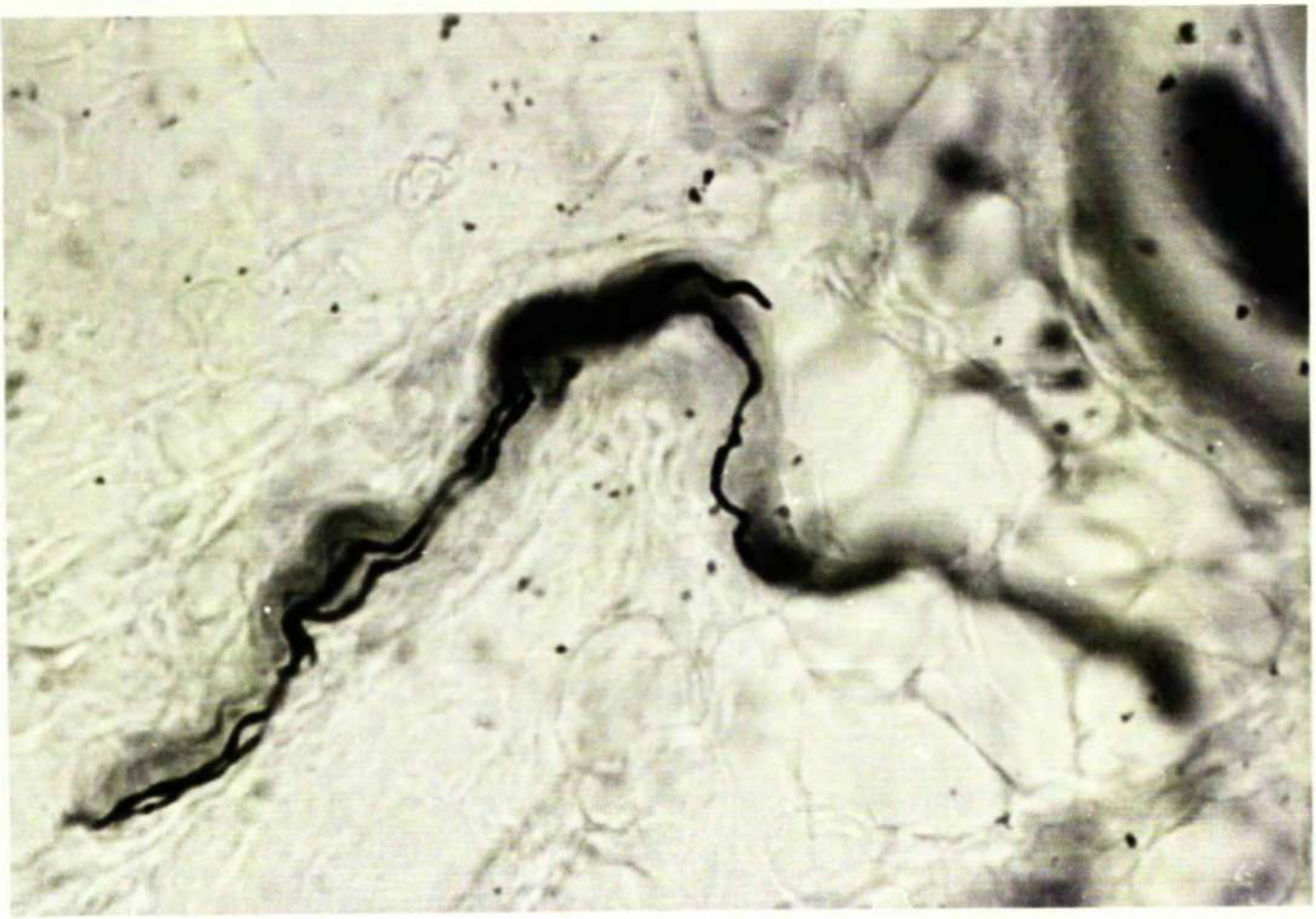


Fig. 45

From the same specimen as figure 44. A small fasciculus within the posterior zone of the right hemi-diaphragm. Small irregularities in the outlines of individual fibres are compatible with normal structure. There is no apparent break in the continuity of the axis cylinders.

Fig. 46

Illustrating the results of studies on the topographical distribution of degenerated and persisting fibres within the diaphragm following unilateral interruption of the phrenic nerve. The broken line indicates the inner limit of the peripheral zone in which persisting branches of the intercostal nerves were consistently present. The grey-shaded zone indicates the side on which the phrenic nerve was interrupted. X denotes regions in which degenerated fibres were present in the side opposite the experimental lesion. O denotes regions in which persisting fibres were found on the same side as the experimental lesion.

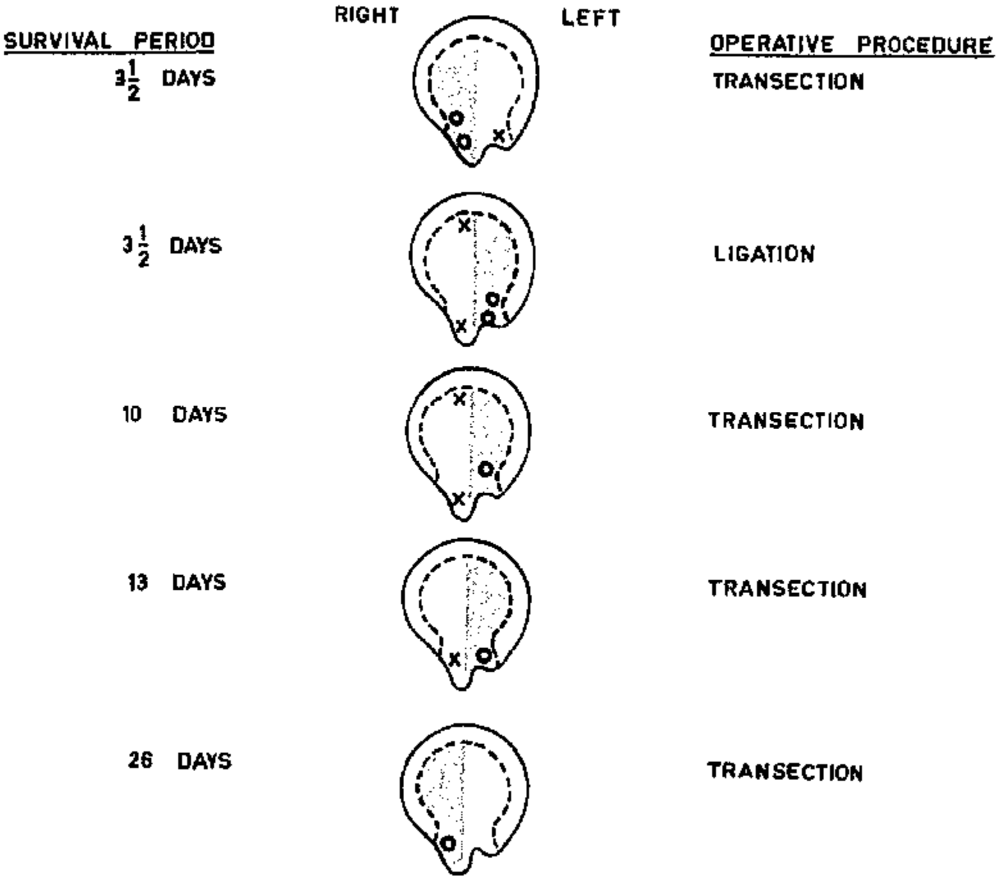

RIGHT
LEFT
SURVIVAL PERIOD
OPERATIVE PROCEDURE
3½ DAYS
TRANSECTION
3½ DAYS
LIGATION
10 DAYS
TRANSECTION
13 DAYS
TRANSECTION
26 DAYS
TRANSECTION

Fig. 46

CHANGES IN THE PHRENIC NERVE.

In 14 adult animals, unilateral transection of the phrenic nerve was carried out and, following survival periods which ranged from 1-30 days, the fibres within the nerve trunk were examined with the light microscope. Portions of the nerve approximately 1cm in length were taken from levels central and peripheral to the experimental lesion and they were immersed in Flemming's fixative and neutral formalin alternately. In the osmium-fixed portions the changes in myelinated fibres were studied while those portions which were fixed in formalin were impregnated with silver to demonstrate changes in the axis cylinders. The outline of the nerve trunk and its myelinated fibres were well defined in transverse and longitudinal sections of the osmium-fixed portions but a considerable degree of shrinkage and distortion of the tissue was evident in the corresponding formalin-fixed preparations. While it was considered reasonable to attempt quantitative studies on the myelinated nerve fibres, it was decided to restrict studies on the axis cylinders to qualitative observations.

(1) Changes in Axis Cylinders.

In six cats in which unilateral transection of the phrenic nerve had been carried out at the level of the lung hilum the changes in axis cylinders were studied in detail at levels central and peripheral to the level of the lesion, after survival periods which ranged from 3-25 days. The findings in these animals were compared directly with observations made on corresponding preparations in normal animals, in which the axis cylinders were represented as ribbons of argyrophilic material which, apart from symmetrical constriction at nodes of Ranvier, were regular in outline. (fig. 47).

a) Peripheral to the level of the lesion.

The axis cylinders in this situation were studied in serial longitudinal sections of the nerve trunk and the structural changes were seen to correspond to those observed in the diaphragmatic plexus. After a survival period of 3 days the axis cylinders showed widening, at 5, 8 and 14 days fragmentation was evident and at 21 and 25 days, all argyrophilic material had gone from the axis cylinders only empty Schwann bands remaining. In three animals where the survival periods were 5, 14 and 21 days respectively, all of the axis cylinders within this portion of the nerve showed degenerative changes. In the remaining 3 animals, however, in which the survival periods were 3, 8 and 25 days respectively, a small minority of the axis cylinders showed persistence of axoplasmic continuity and were identified as persisting nerve fibres.

Changes at 3 days. The vast majority of nerve fibres showed marked widening of the axoplasm with localised varicosity and the spaces between adjacent fibres were correspondingly reduced in width. (fig. 48). In a small group of fibres immediately subjacent

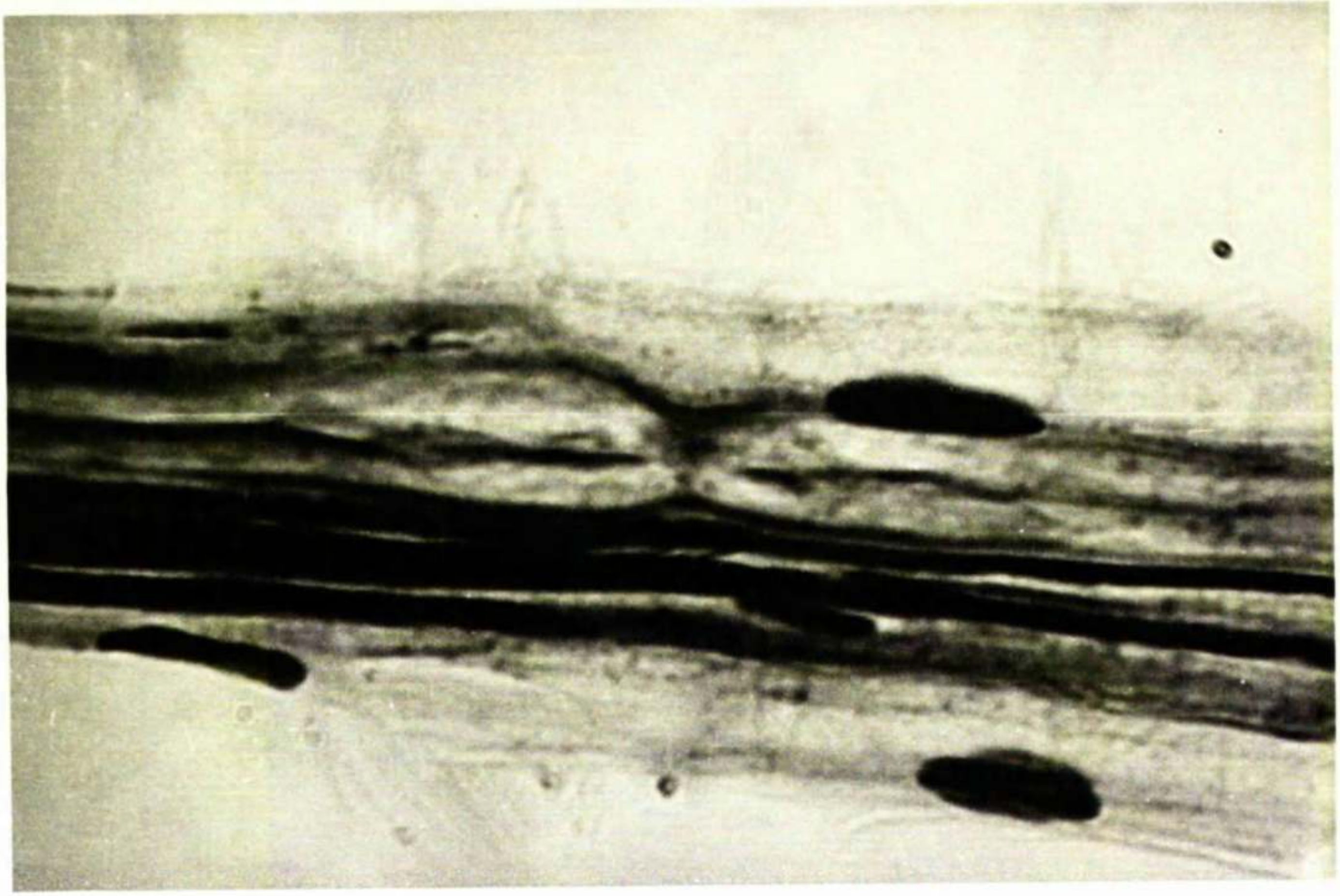


Fig. 47

A silver-impregnated portion of a fasciculus within the phrenic nerve of a normal cat. Three prominent axis cylinders, with distinct regular outlines are present along with three ovoid profiles of Schwann cell nuclei. The outline of a myelin sheath, in the region of a node of Ranvier is evident in the upper part of the illustration.

Fig. 48

Axis cylinders within the peripheral stump of a phrenic nerve which had been transected 3 days previously. The vast majority of the nerve fibres are irregular in outline and the space between them appears to be reduced. In the majority of the axis cylinders continuity has persisted.

to the perineurium in the most distal portion of the nerve trunk these morphological changes were absent. (fig. 49).

Changes at 8 days. In this animal the vast majority of nerve fibres showed marked fragmentation of the axoplasm. At its distal extremity, however, the nerve trunk was seen to contain a bundle of fibres in which the continuity of argyrophilic material remained obvious. (fig. 50). These axis cylinders, though not entirely regular in outline, were thought to represent a group of persisting nerve fibres.

Changes at 25 days. Here, the degenerative changes affecting the majority of fibres had advanced to a stage where the axis cylinders were represented by empty Schwann bands. (fig. 51). A small group of intact nerve fibres, rendered conspicuous by the surrounding changes of degeneration, was observed within the trunk at a distance of approximately 0.5cm from the diaphragm. (fig. 52)

b) Central to the level of the lesion.

In portions of the nerve trunk, within 0.5cm of the level of transection, the axis cylinders showed changes corresponding to those observed within the nerve trunk peripheral to the level of the lesion. With survival periods ranging from 3-21 days, these axonic changes did not ascend the nerve trunk beyond the first half centimetre. In the animal, in which the survival period was 25 days, a group of approximately 20 fibres showed axoplasmic widening, at a level which was 1.5cm central to the level of the lesion.

Fig. 49

From the same specimen as figure 48. The axis cylinders appear to have retained their continuity and, in most cases, their regular outlines. The morphological features of these fibres are compatible with normal appearances.

Fig. 50

A small fasciculus of nerve fibres within the peripheral stump of a phrenic nerve which had been transected 8 days previously. The argyrophilic axis cylinders are irregular in outline but they appear to have retained their continuity.

25 µ

Fig. 51

Degenerated fibres within the peripheral stump of a phrenic nerve which had been transected 25 days previously. The argyrophilic material is in the form of discontinuous droplets in some axis cylinders whereas other nerve fibres are represented by empty Schwann bands.

Fig. 52

From the same specimen as figure 51. The individual axis cylinders of this small fasciculus are regular in outline and show no evidence of degeneration.

(2) Changes in Myelin Sheaths.

In 8 cats in which unilateral transection of the phrenic nerve was followed by survival periods ranging from 5-30 days, the myelinated fibres were examined in osmium preparations of the nerve trunk at levels central and peripheral to the experimental lesion. The sheaths were distinguished from other osmiophilic droplets within the nerve trunk by estimation of their external diameters.

a) Peripheral to the level of the lesion.

There was invariably a marked reduction in the total number of myelin sheaths in this portion of the transected nerve, the total count being less than 300 in every case. This number was less than half of the corresponding value found in normal animals, and the fibres were interspersed with osmiophilic debris. Whether this material occupied an extracellular position in the endoneurium or was enclosed within cells of the macrophage series was not determined in the present study.

At 5-13 days. The myelin sheaths, which, in normal animals were circular, having internal and external concentric borders, showed irregular inward projections which encroached on the axoplasmic space. In an appreciable number of these sheaths the projection of osmiophilic material obliterated the axoplasmic space entirely. (fig. 53). In the majority of sheaths, however, the external border retained its distinct circularity and, with successively prolonged survival periods, the total number of these

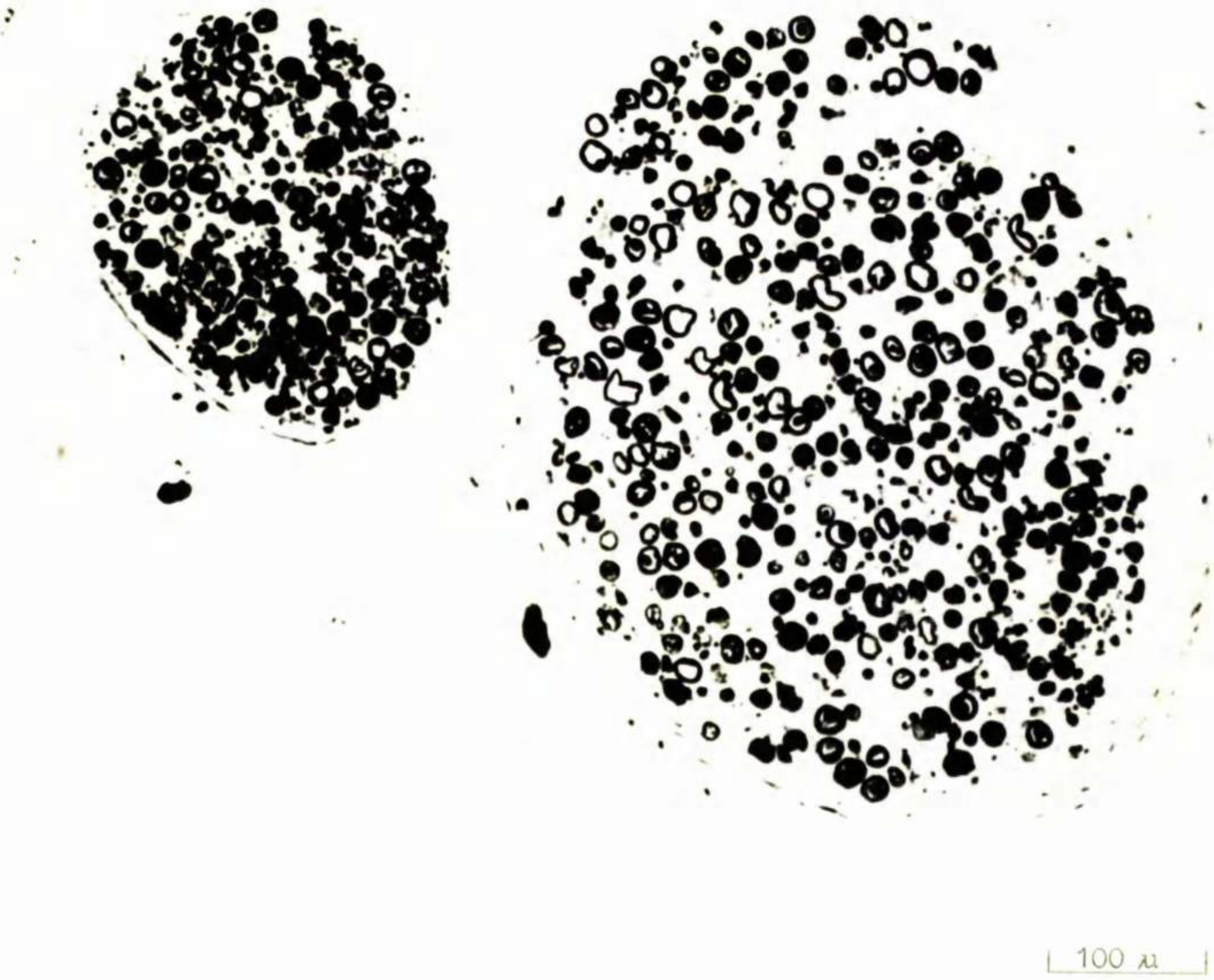


Fig. 53

An osmium-treated complete transverse section of the peripheral stump of a phrenic nerve which had been transected 5 days previously. There is an obvious reduction in the total number of myelinated fibres and, of those which remain, many appear to have osmiophilic material in the axoplasmic space. A small number of the profiles which represent myelin sheaths are normal in appearance.

structures became progressively fewer, two categories of sheaths being recognisable in every animal of this group.

(i) In this category the concentric internal and external margins of the sheaths were recognisable and centripetal extension of osmiophilic material was minimal.

(ii) In this category only the external margin of the sheath was identifiable, the central space which is normally occupied by the axis cylinder having been obliterated by osmiophilic material. For purposes of description, these two categories of persisting sheaths have been named 'type 1' and 'type 2' respectively.

At 13-26 days. No persisting sheaths of type 1 were observed in transverse sections of this portion of the nerve but sheaths of type 2 were present even after a survival period of 26 days.

The quantitative observations made in 8 animals are presented in table 8.

In the photomicrographs which correspond to the quantitative data presented, it can be seen that some of the osmiophilic structures within the nerve trunk are clearly in the form of debris. These were distinguished from persisting sheaths because of their irregularity of shape and were excluded from the count. A small number of persisting sheaths could not be classified with certainty as belonging to type 1 or type 2 and on account of these intermediate forms, the quantitative results obtained constitute

TABLE 8.

Quantitative studies on the myelinated fibres of the transected phrenic nerve peripheral to the level of the lesion.

Survival Period in Days.	Numbers of Persisting Sheaths.		
	Type I	Type II	Figure
5	50	200	53
10	40	200	54
11	40	150	-
13	30	70	55
20	0	100	56
20	0	100	-
26	0	120	57
30	0	120	58

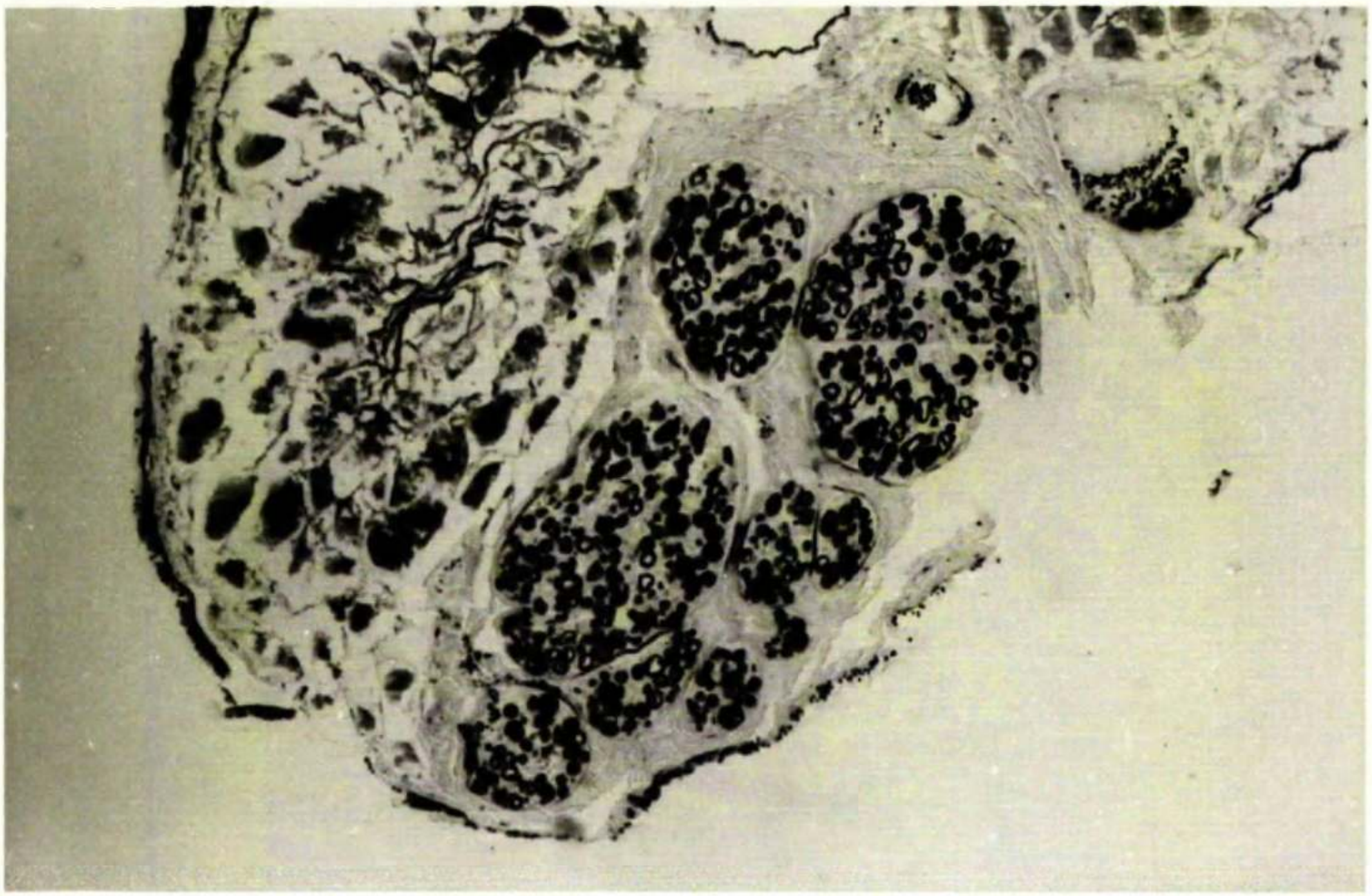


Fig. 54

From a preparation comparable to that shown in figure 53. In this case the survival period, following phrenic transection, was 10 days.

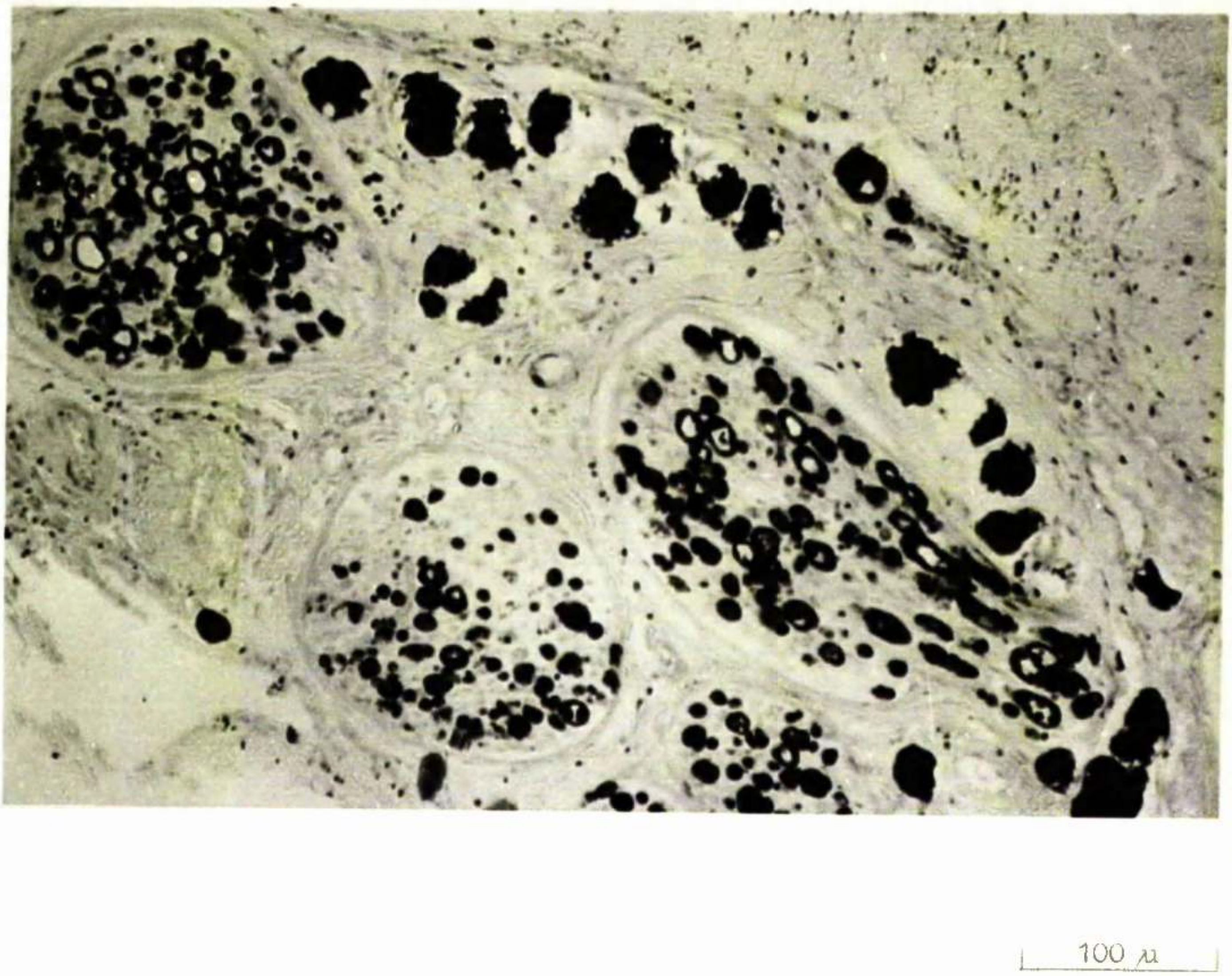
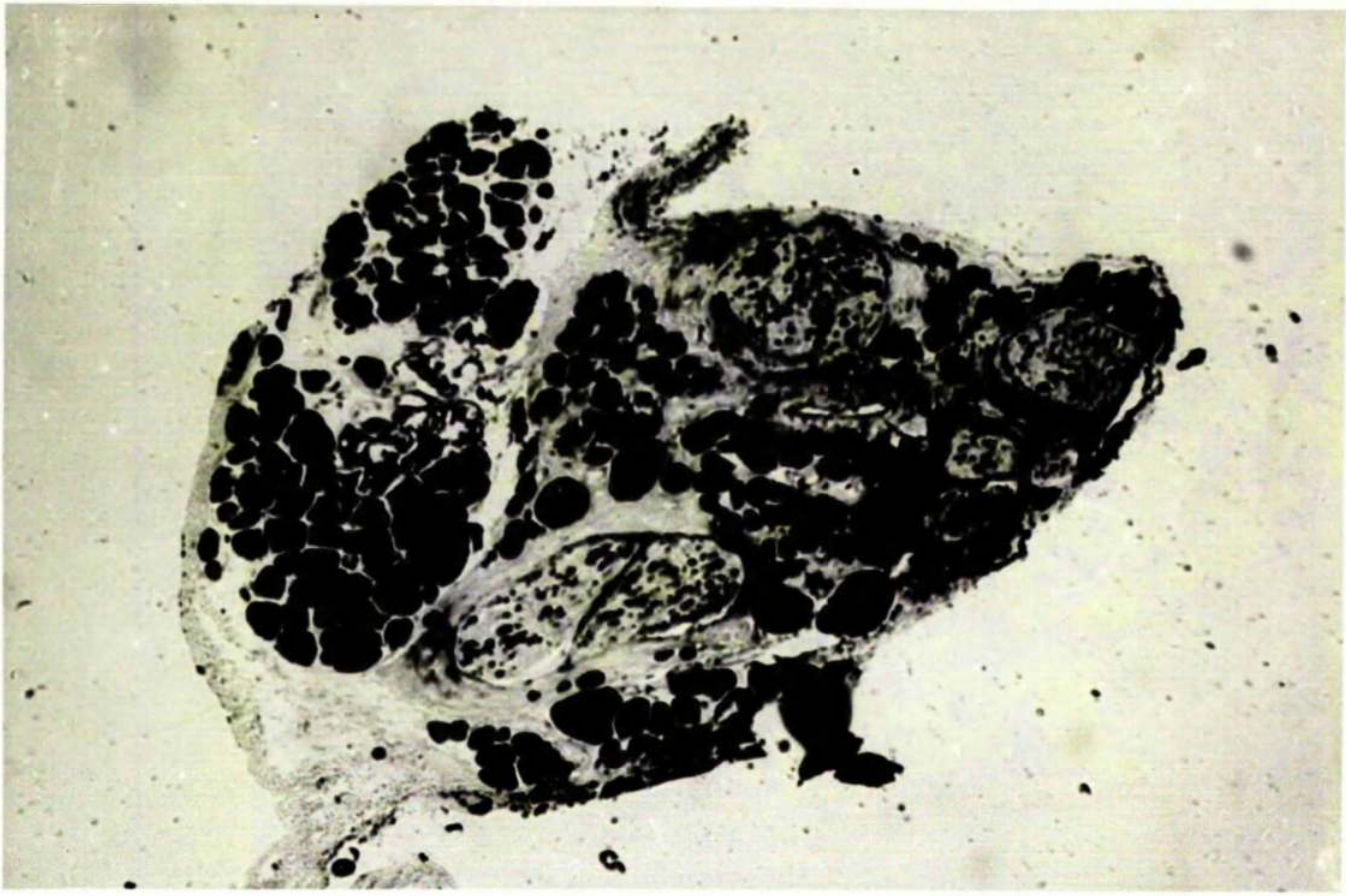


Fig. 55

From a preparation comparable to that shown in figure 53. In this case the survival period following transection was 13 days.



200 μ

Fig. 56

From a preparation comparable to that shown in figure 53. In this case the survival period following transection was 20 days.

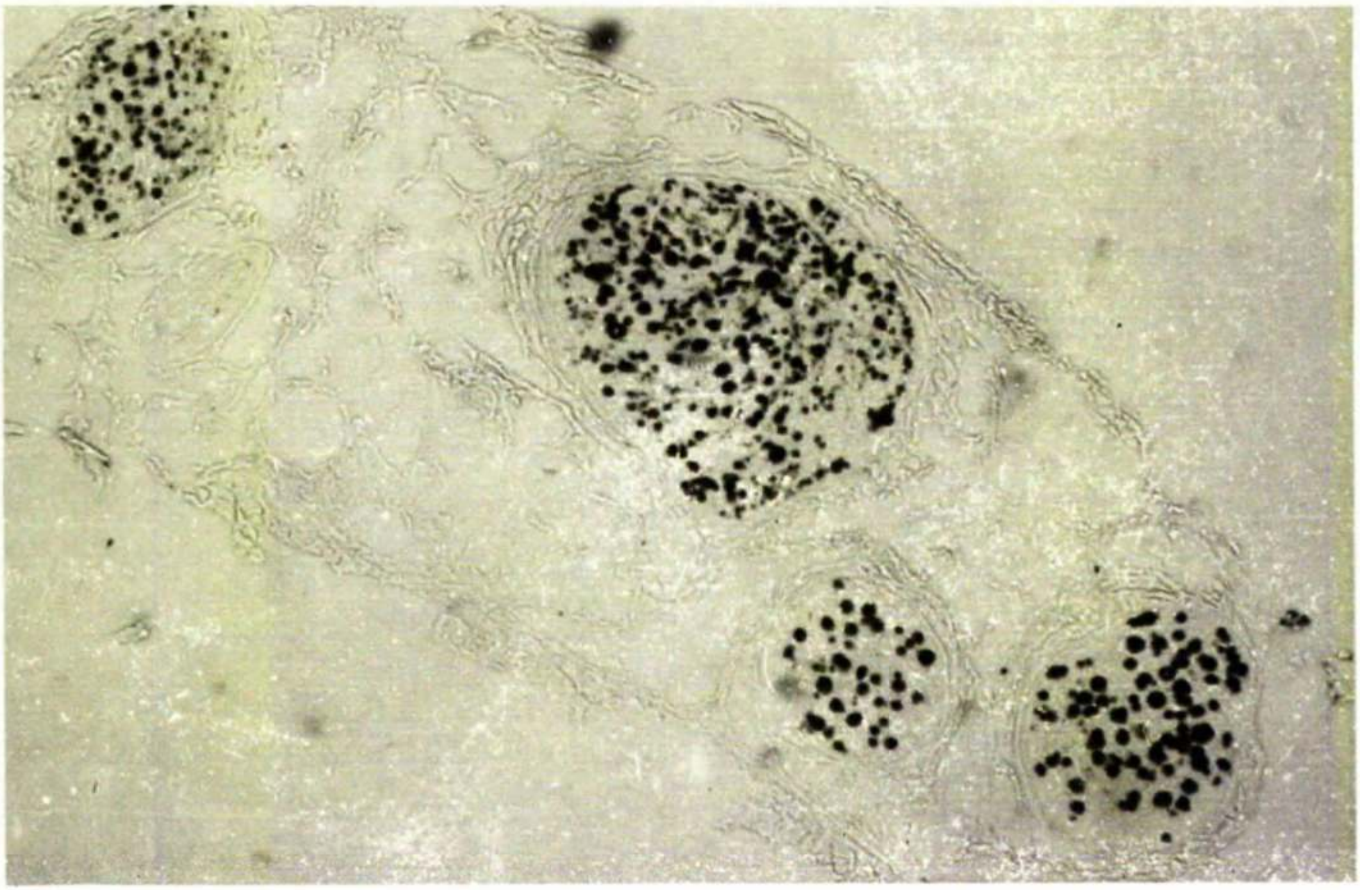
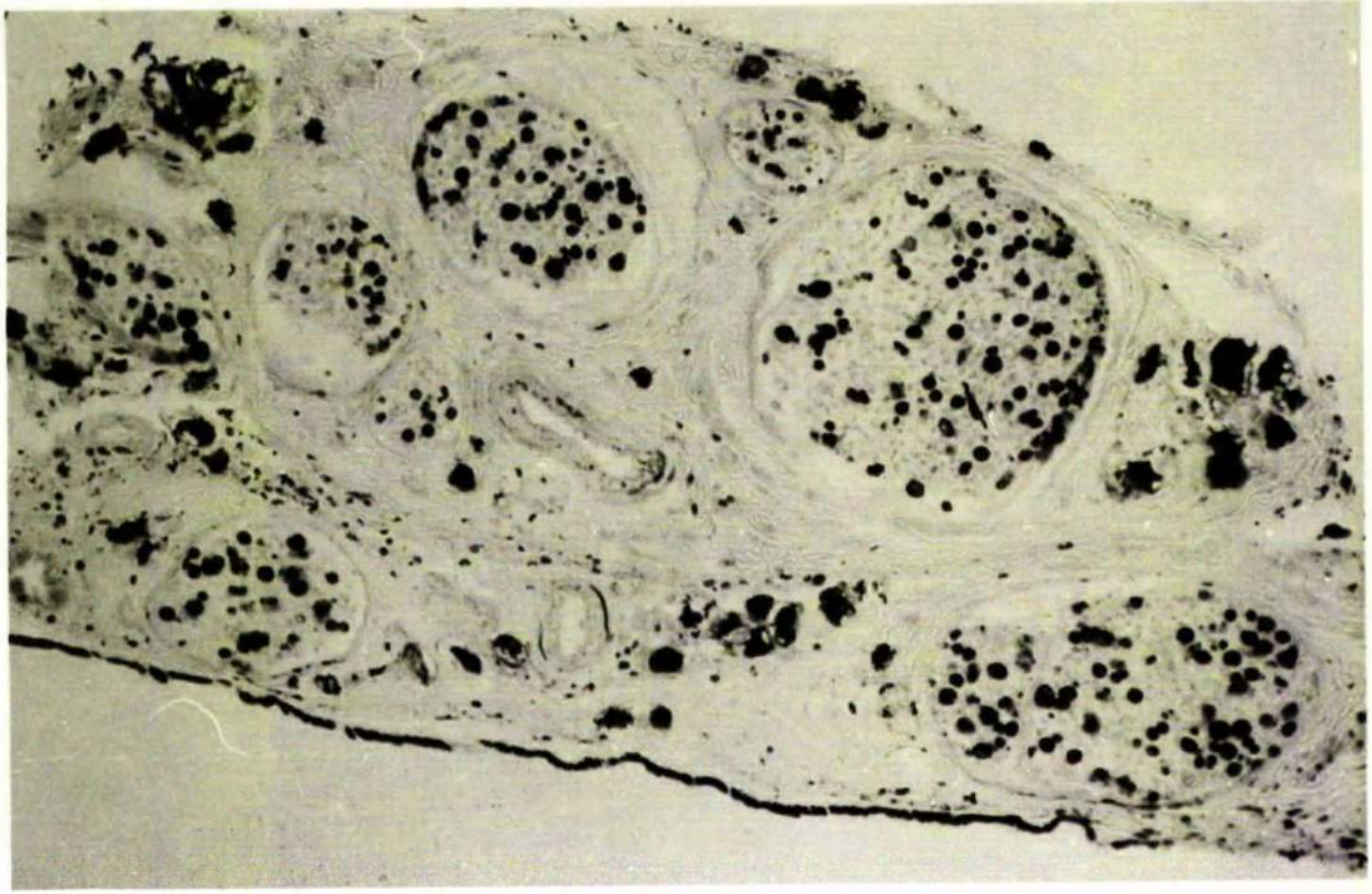


Fig. 57

From a preparation comparable to that shown in figure 53. In this case the survival period was 26 days.



200 u

Fig. 58

From a preparation comparable to that shown in figure 53. In this case the survival period was 30 days. Note that circular osmiophilic profiles are present within all fasciculi and that their diameter is comparable to that found in normal myelinated fibres.

an estimate rather than an accurate count.

b) Central to the level of the lesion.

These studies were carried out in two portions of the nerve trunk, one between 0 and 2cm, and the other between 4 and 6cm from the level of transection.

(i) Within 2 cm of the lesion. The structural changes observed in the myelinated fibres in this portion of the nerve trunk resembled those found peripheral to the level of the lesion, two types of persisting sheaths being present. The quantitative values obtained from 4 animals of the series are presented in table 9.

It is immediately apparent, that in this portion of the nerve, persisting sheaths of type 1 are represented only in the 5 day study and that with prolongation of the survival period there is a progressive reduction in the number of persisting sheaths of type 2.

(ii) At 4 cm from the lesion. In the transected phrenic nerve, no changes were seen in the more central portions of the nerve trunk after survival periods of up to 26 days. However, in the animal in which the survival period was 30 days, there were changes in the myelinated fibres within the phrenic nerve at approximately 4cm central to the lesion Thus, approximately 30 myelinated fibres, situated in this portion of the nerve, were seen to have osmiophilic material in the axoplasmic space. (fig. 62). The vast

TABLE 9.

Quantitative studies on the myelinated fibres of the transected phrenic nerve central to the level of the lesion.

Survival Period in Days.	Numbers of Persisting Sheaths.		
	Type I	Type II	Figure
5	15	80	59
10	0	160	60
11	0	140	-
26	0	35	61

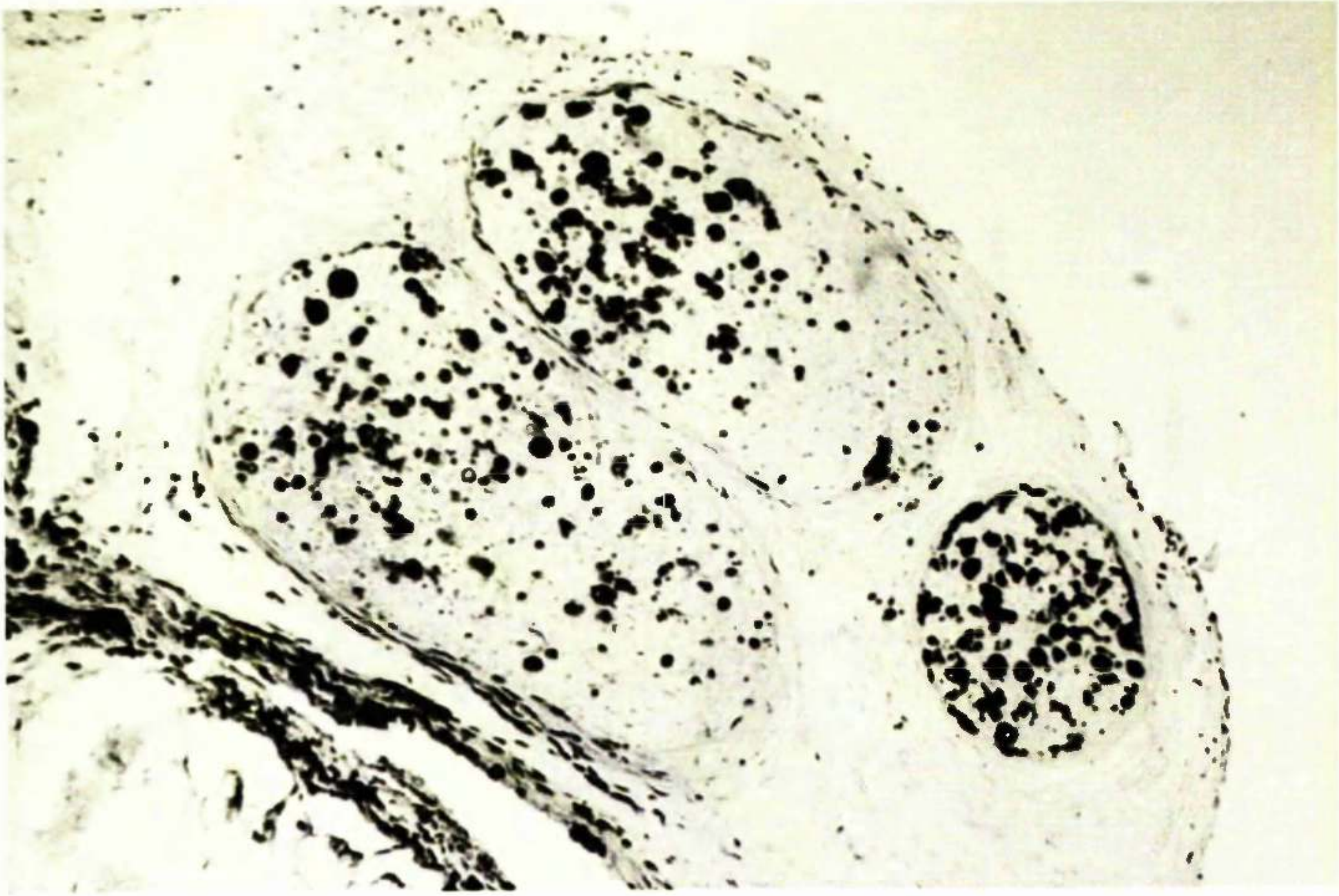


Fig. 59

An osmium-treated complete transverse section of a transected phrenic nerve. The specimen was taken from the trunk of the nerve approximately 2cm central to the level of the lesion after a survival period of 5 days. No normal myelin sheaths are present.

Fig. 60

From a preparation comparable to that shown in figure 59. In this case the survival period was 10 days. No normal myelin sheaths are present and those which show axoplasmic osmiophilia are greatly reduced in number.

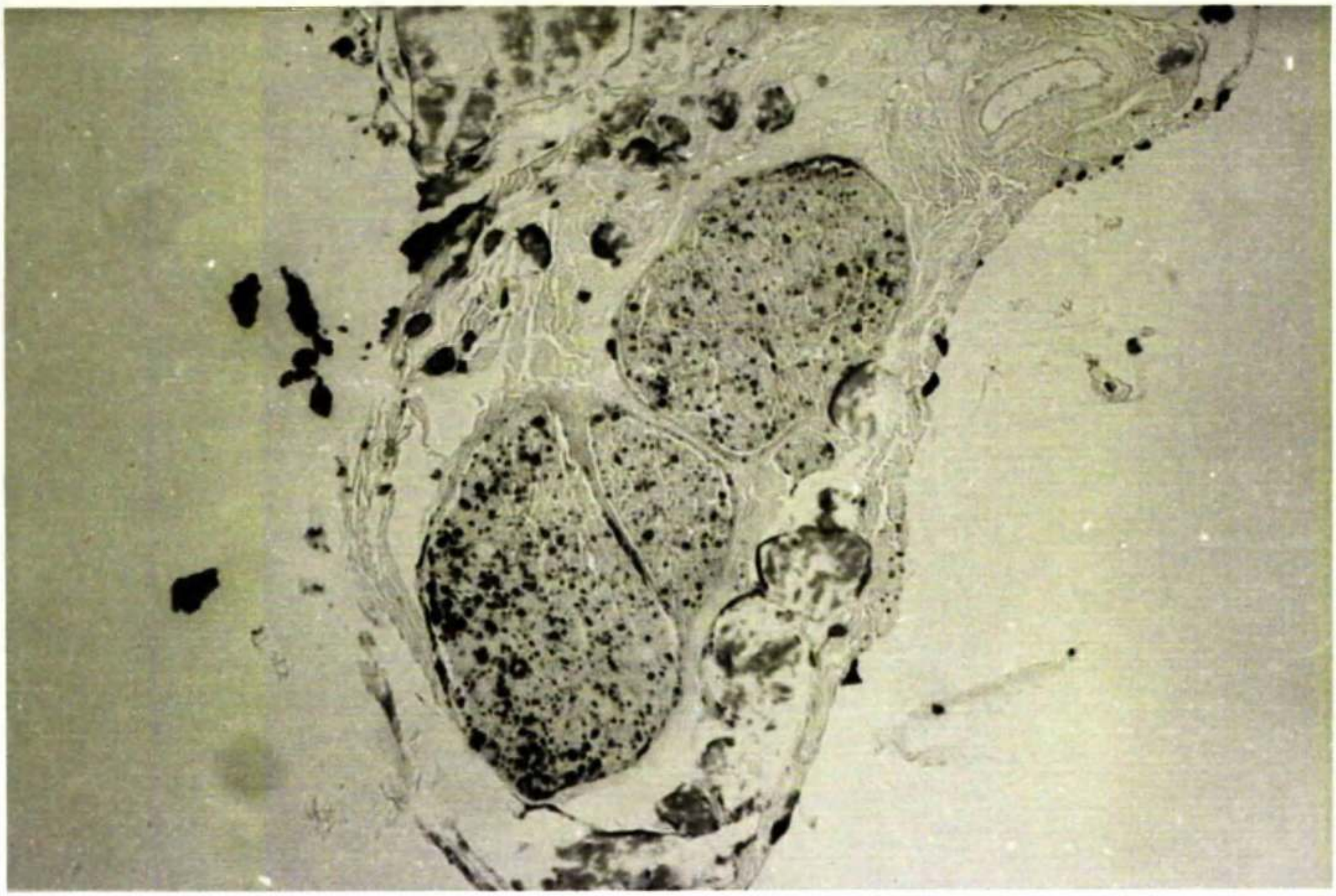


Fig. 61

From a preparation comparable to that shown in figure 59. The survival period in this case was 26 days. Very few osmiophilic structures are present within the section.

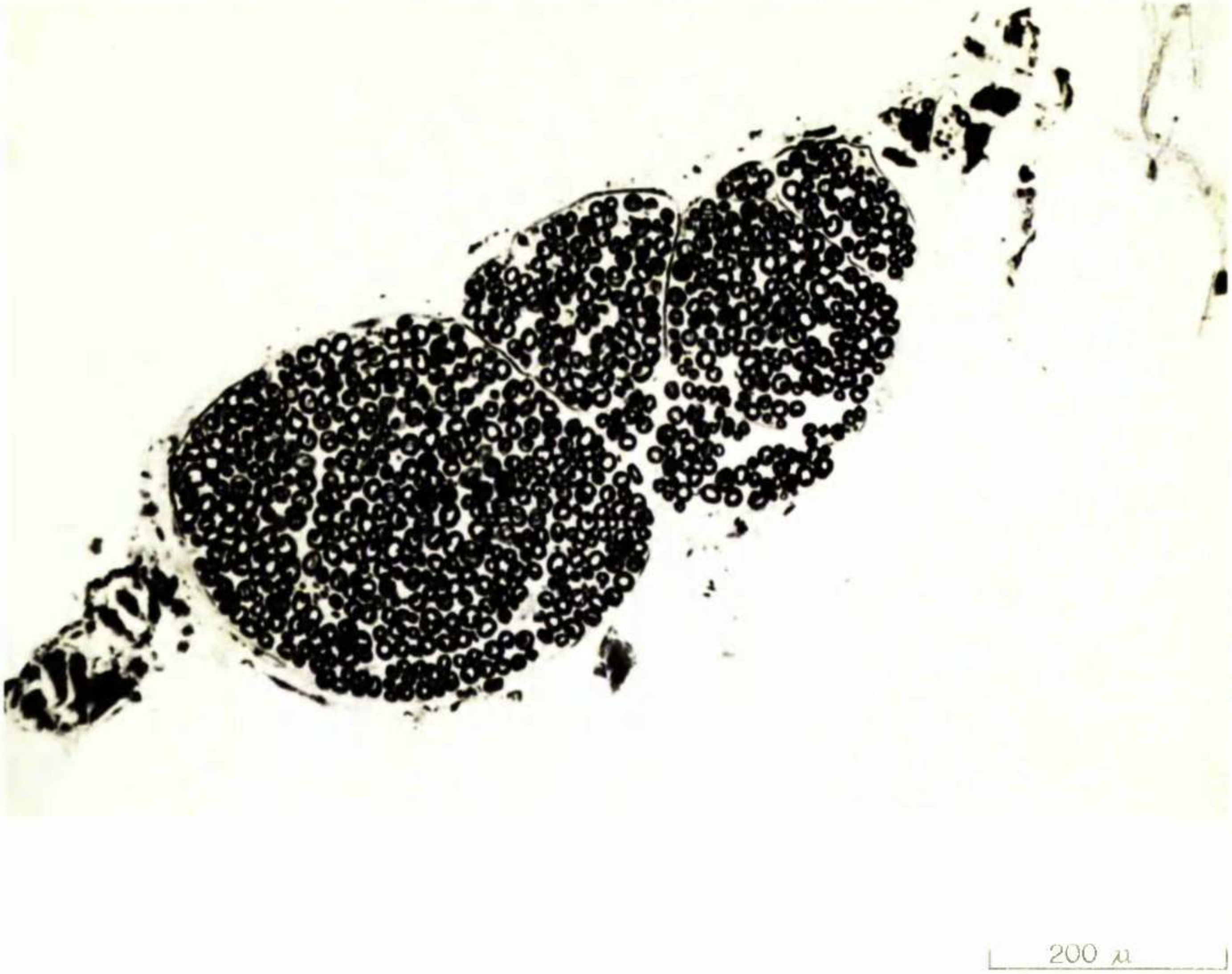


Fig. 62

An osmium-treated transverse section of a transected phrenic nerve The specimen was taken from the nerve trunk approximately 4cm central to the level of the lesion following a survival period of 30 days. Note the presence of approximately 30 nerve fibres which show axoplasmic osmiophilia.

majority of these fibres are confined to one (the largest) fasciculus.

c) Changes in longitudinal sections at the site of transection.

In one animal in which the survival period was 26 days the site of the transection was removed in continuity with portions of the central and peripheral stumps of the nerve, the total length of the specimen being approximately 1.5cm. In longitudinal sections the site of the lesion was conspicuous due to the absence of myelinated fibres from this portion of the nerve while the contiguous ends of the transected nerve showed localised widening. This feature which appeared to be due to aggregation of osmiophilic material was more prominent in the central than in the peripheral portion of the transected nerve. (fig. 63a and fig. 63b respectively). Although a relatively large proportion of the osmiophilic material within the peripheral portion of the nerve is present in droplet form, there are nevertheless, a number of continuous osmiophilic bands coursing through the nerve for considerable distances. These resemble myelin sheaths as seen in comparable studies in normal animals and it was considered that they correspond to the persisting sheaths observed in transverse sections of the peripheral portion of the transected nerve. (fig. 64).

d) Changes in the contralateral phrenic nerve.

In every animal subjected to unilateral transection of the

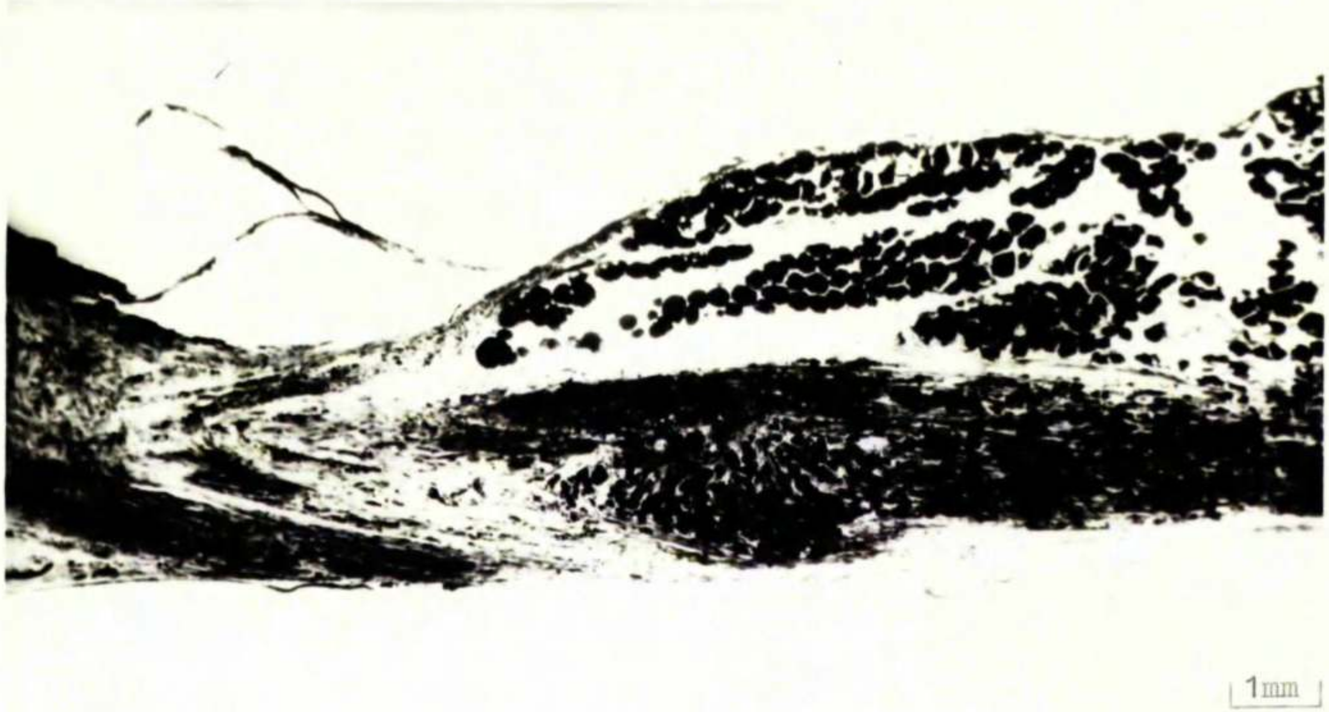


Fig. 63(a)

A longitudinal section of the phrenic nerve at the level of transection in an animal in which the survival period was 26 days. A portion of the nerve trunk central to the level of the lesion has been included. Note the presence of an osmiophilic swelling in the central portion adjacent to the level of the lesion.

Fig. 63(b)

From the same specimen as figure 63(a). The longer portion of the nerve trunk, here, is peripheral to the level of the lesion. Osmiophilic bands resembling those seen in normal nerves extend peripherally in the nerve trunk at least as far as the limit of the section.

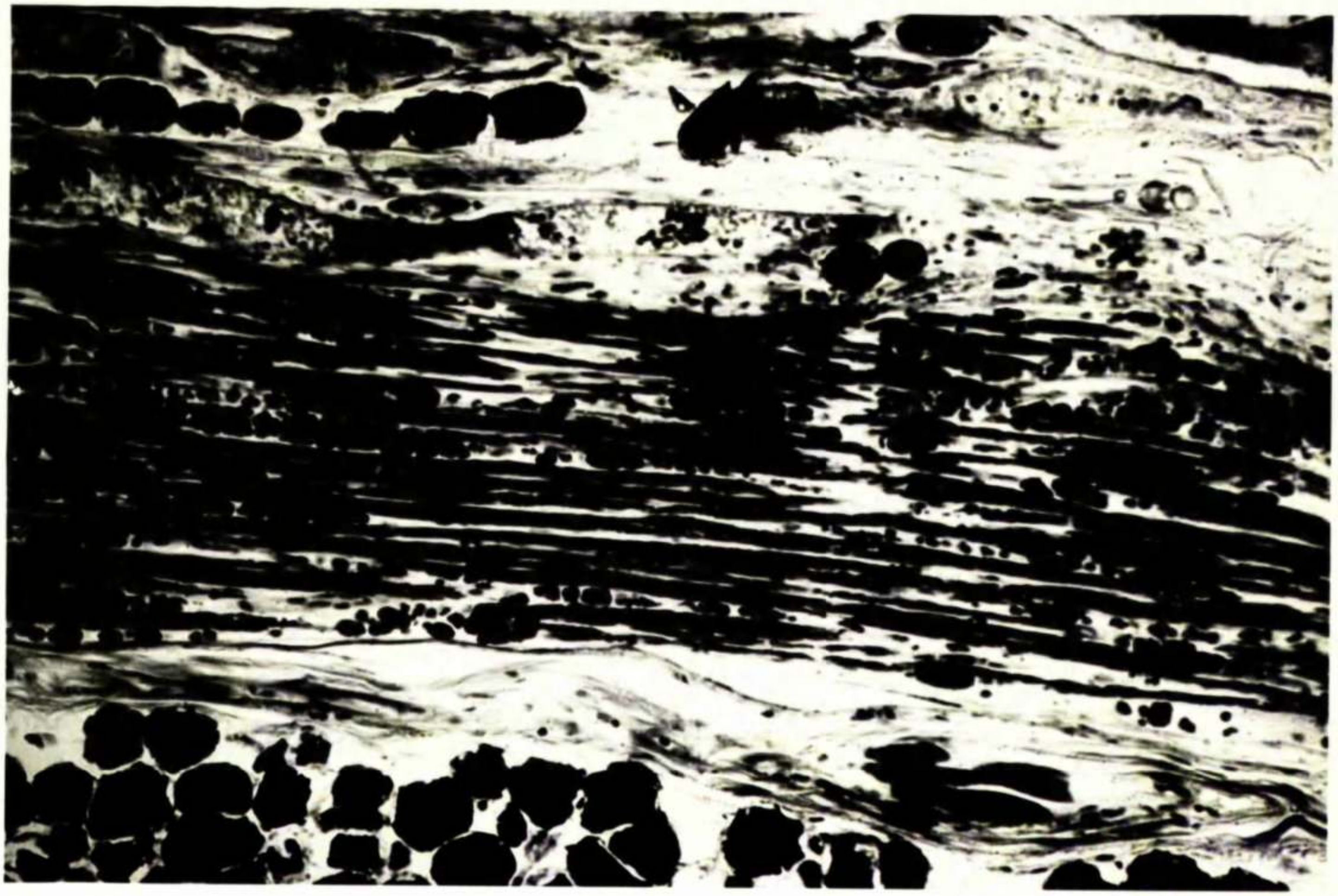


Fig. 64

From the same specimen as figure 63(b). The magnification has been increased to show more clearly the profiles of myelin sheaths within the peripheral portion of the nerve trunk.

phrenic nerve, the unoperated phrenic nerve was examined and the myelinated nerve fibres were seen to be normal in structure.

B. Unilateral Ligation of the Phrenic Nerve at the Level of the Lung Hilum.

In 3 adult cats the phrenic nerve was ligated firmly at the level of the lung hilum so that retraction of the nerve trunk, a natural consequence of transection, could be avoided, thus facilitating the preparation of transverse sections for ultra-structural studies. Following survival periods of 72, 84 and 96 hours respectively, portions of the ligated nerve, 2-3cm central and 0-1cm peripheral to the level of the lesion were removed and prepared by the same method as was used in normal studies. (fig. 65).

Ultrastructural observations on the phrenic nerve.

CHANGES AFTER A SURVIVAL PERIOD OF 72 HOURS.

a) Peripheral to the Level of Ligation.

In transverse sections the structural components of the nerve trunk, were examined in detail.

(1) Myelinated nerve fibres.

Whereas in normal studies, the axoplasm of myelinated fibres filled the entire space enclosed by the myelin sheath following ligation of the nerve trunk, the axoplasm and the inner boundary of the myelin sheath were separated by a distinct gap which was

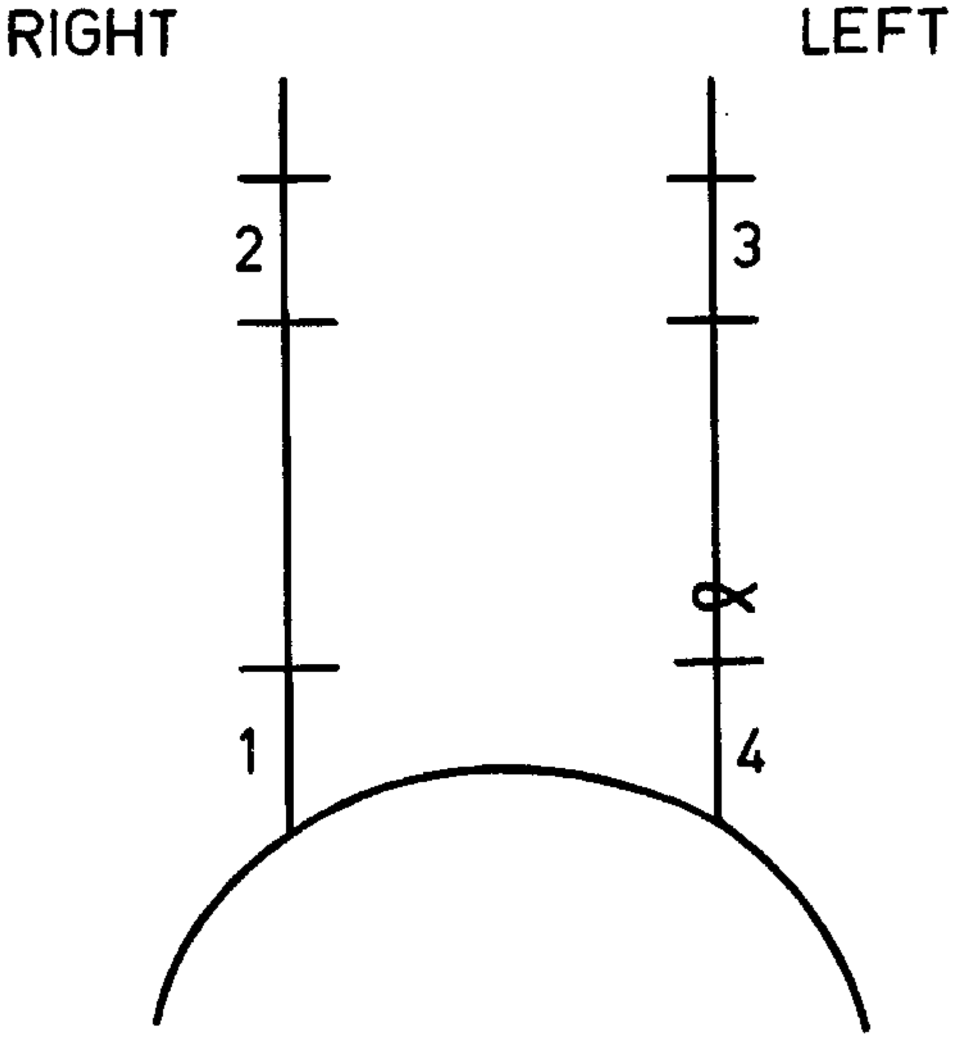


Fig. 65

Illustrating the regions 1,2,3 and 4, from which portions of the phrenic nerves were removed for ultrastructural investigation following ligation at the level indicated.

irregular in width. (fig. 66). For purposes of description this structural change is referred to here as axoplasmic retraction. In the axoplasm there was an apparent increase in the number of electron-dense particles present and the myelin sheath, while it showed the lamination typical of normal myelinated nerve fibres, also showed intralaminar cleft formation in all parts of its thickness. (fig. 66). A small number of myelinated fibres in each field examined were structurally normal. (fig. 67).

(2) Schwann cells.

The most marked change in these cells was seen in the nuclei which had lost their normal ovoid contour and had become multi-angular. The nucleoplasm was less electron dense than in the nuclei of Schwann cells seen in comparable preparations of normal phrenic nerves. (fig. 68).

(3) Unmyelinated fibres.

In normal nerves the outlines of these were seen to be regular and in the main, ovoid, but in the distal portion of the ligated nerves, the axis cylinders were multi-angular in outline as seen in transverse section. The cross-sectional area of most of the unmyelinated fibres appeared to have undergone an increase, but a small minority did not show changes in contour nor in size. Vesicular structures were observed within the cytoplasm of the unmyelinated fibres, and their mesaxons were seen to be tortuous in contrast to the smooth, regular outline of those seen in transverse

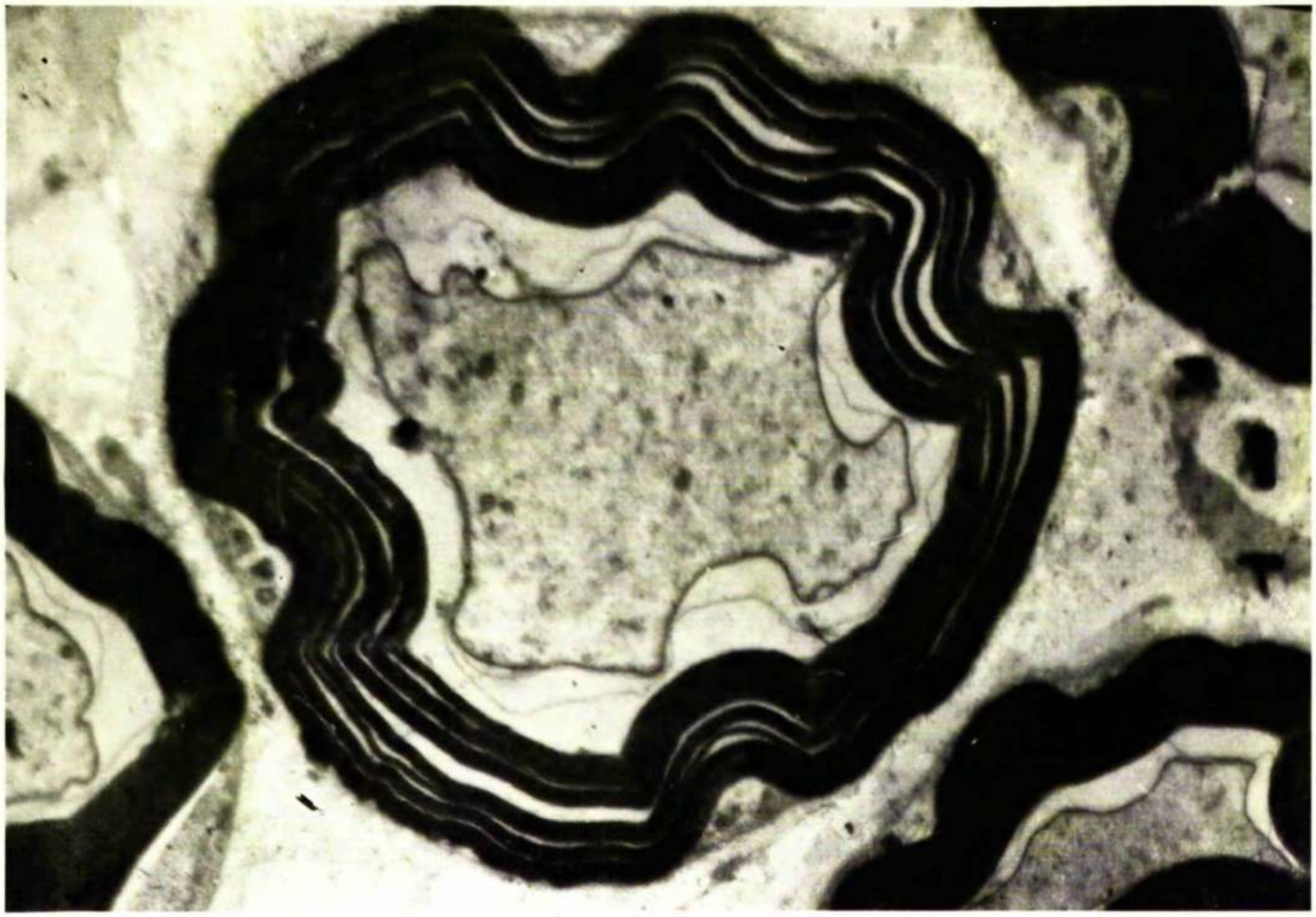


Fig. 66

A myelinated fibre within the peripheral portion of a phrenic nerve which had been ligated 3 days previously. A small gap of irregular width is seen separating the axoplasm from the sheath which surrounds it.

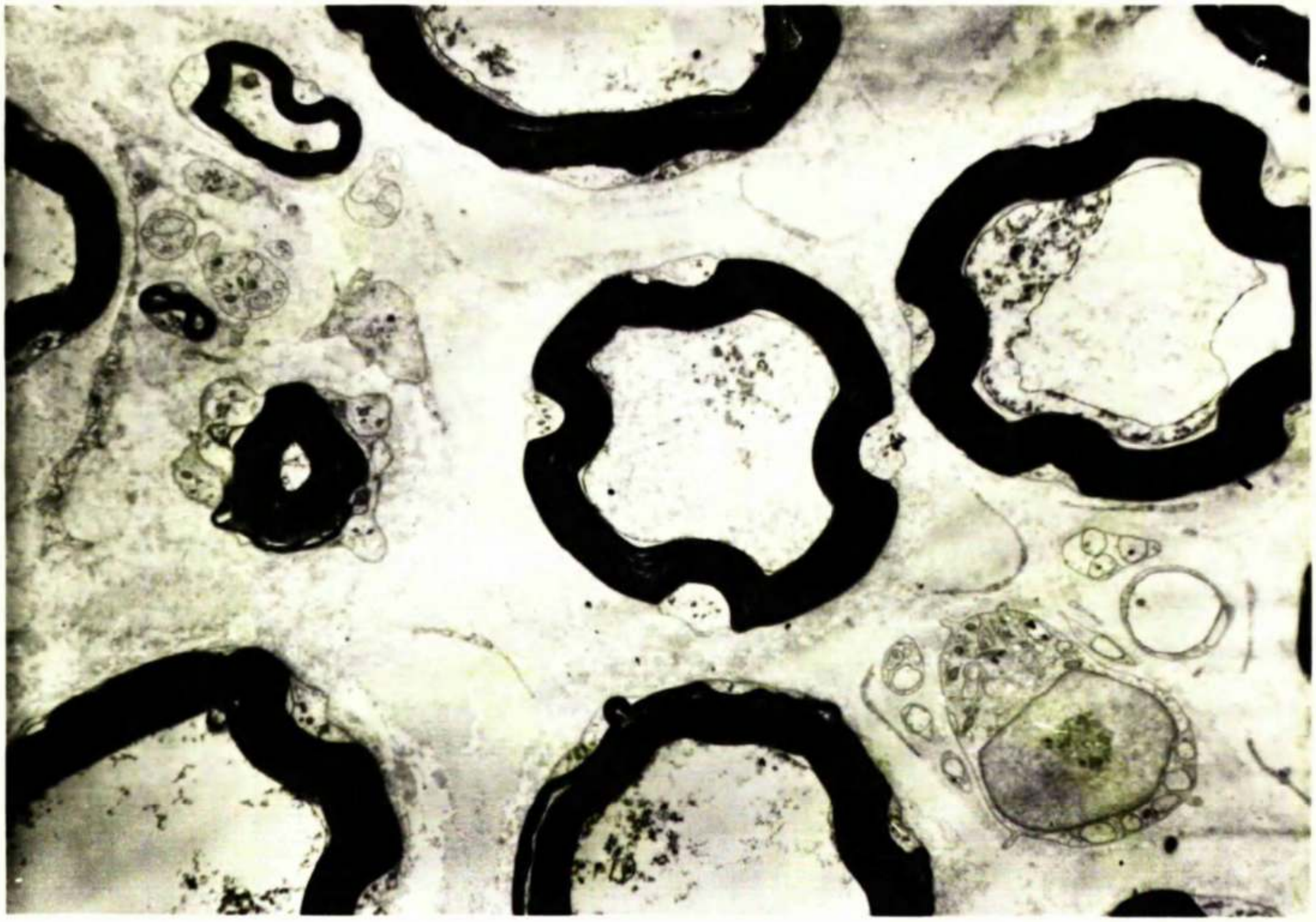


Fig. 67

A group of myelinated fibres within the peripheral portion of a phrenic nerve which had been ligated 3½ days previously. Note that only one myelinated fibre of this group shows axoplasmic retraction. The unmyelinated fibres within this section are normal in appearance.

Fig. 68

Schwann cells within the peripheral portion of a phrenic nerve which had been ligated 3 days previously. The two nuclear masses appear less regular in outline than corresponding structures within normal nerve trunks. The cytoplasm surrounding these nuclei is less homogeneous and appears to contain more organelles than normally.

sections of normal nerves. (fig. 69).

(4) The perineurium and interstitial collagen fibres.

These possessed the same morphological characteristics as the corresponding structures seen in transverse sections of normal nerves. The amount of collagen present, however, appeared to have increased.

b) Central to the Level of the Lesion.

(1) Myelinated fibres.

In this portion of the nerve trunk, axoplasmic retraction, as seen in the peripheral portions of the nerve was less generalised, affecting fewer myelinated nerve fibres. As an exception to this rule, however, a small number of myelinated fibres showed this change to a marked extent. (fig. 70).

(2) Schwann cells.

The majority of Schwann cells present in this portion of the nerve were normal in appearance but a small number showed changes which resembled those seen in the peripheral portion of the nerve. Intracytoplasmic vesicles were more prominent in the Schwann cells here than in the corresponding sections of the peripheral stump. (fig. 70).

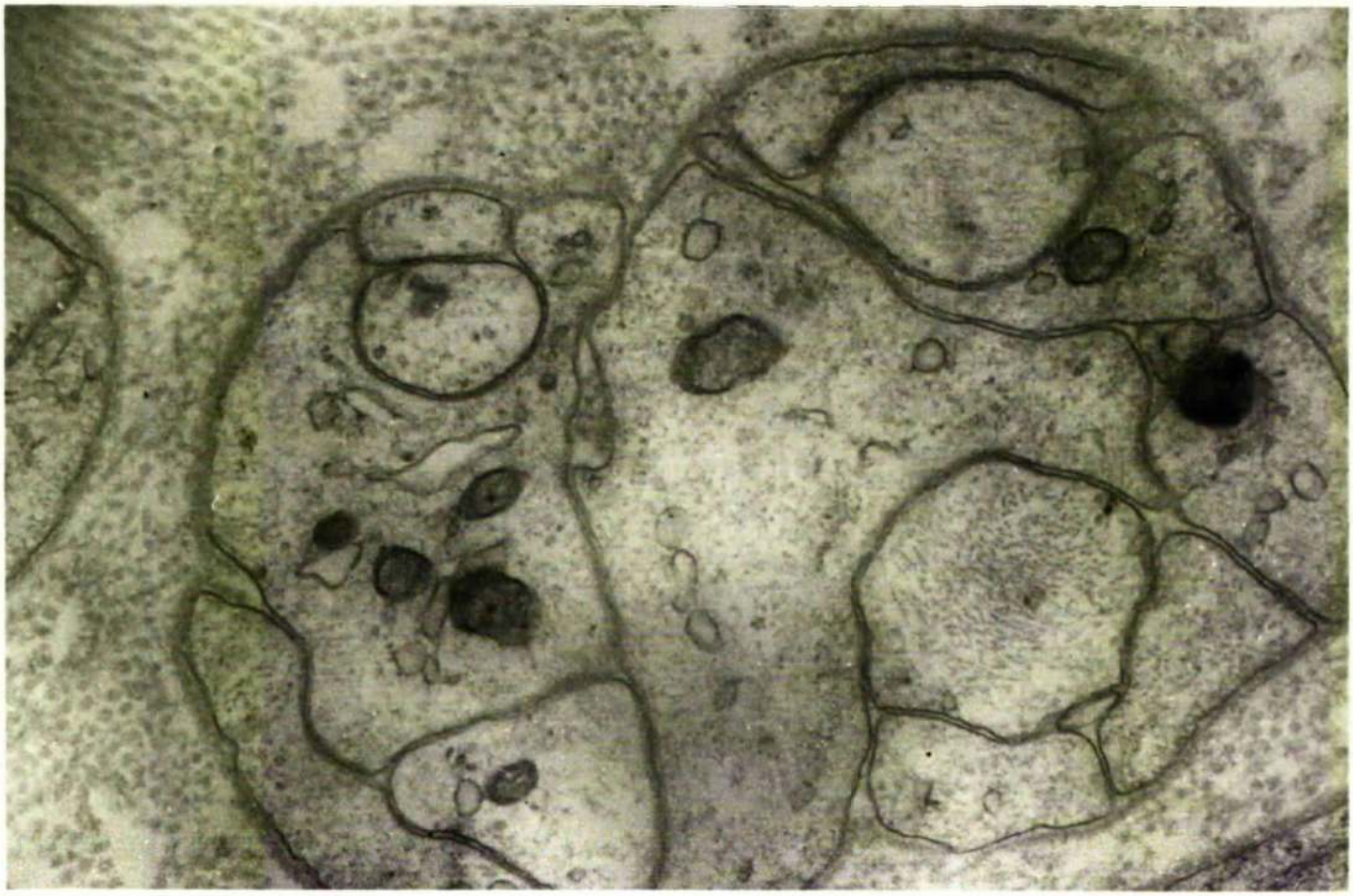


Fig. 69

A group of unmyelinated fibres within the peripheral portion of a phrenic nerve which had been ligated 3½ days previously. The majority of the axis cylinders are multi-angular in outline but a few remain ovoid. Note that there is an apparent increase in the quantity of interstitial collagen.

Fig. 70

A myelinated fibre within the central portion of a phrenic nerve which had been ligated 3½ days previously. There is obvious retraction of the axoplasm from its surrounding sheath. The contiguous Schwann cell shows marked vacuolation within its cytoplasm.

(3) Unmyelinated fibres.

The change most frequently seen in unmyelinated fibres was angularity of the axoplasmic membrane. In approximately one-third of the groups of unmyelinated fibres this change was observed in most of the axis cylinders. (fig. 71). In the remaining two-thirds of the groups examined, only a few of the individual axis cylinders within a particular group showed this feature. Changes in the electron density of the axoplasm caused the axis cylinders to have a stippled appearance when examined at high magnifications. In a few isolated instances the axoplasmic membrane showed localised thinning and on one occasion, a breach in continuity of this structure was observed.

(4) The perineurium and interstitial collagen fibres.

The nerve sheath showed no changes whatsoever, but the amount of intrafascicular collagen was appreciably greater in this portion of the ligated nerve than in portions of normal phrenic nerve. (fig. 69).

CHANGES AFTER SURVIVAL PERIODS OF 84 AND 96 HOURS.

It has been considered unnecessary to describe the observations in these animals singly because of the resemblance they bear to each other and to the findings in the preceding study. Changes in axoplasmic membranes became progressively more prominent with increases in the survival period. The distribution

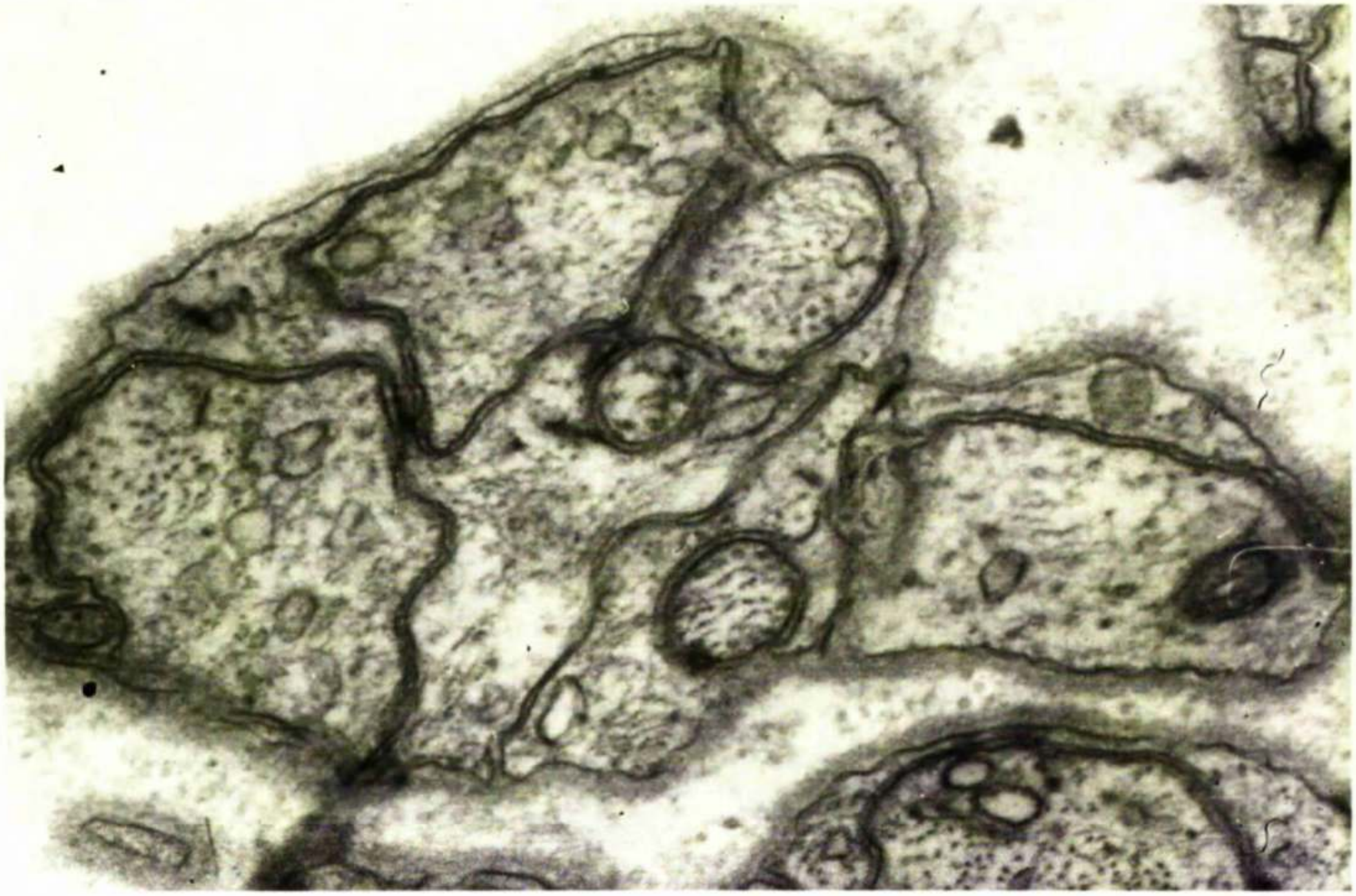


Fig. 71

A group of unmyelinated fibres within the central portion of a phrenic nerve which had been ligated 3½ days previously. Irregularity in the membranes surrounding individual fibres is obvious and there is a generalised increase in the electron-density of the axoplasm. Note that two fibres have retained their normal ovoid outline.

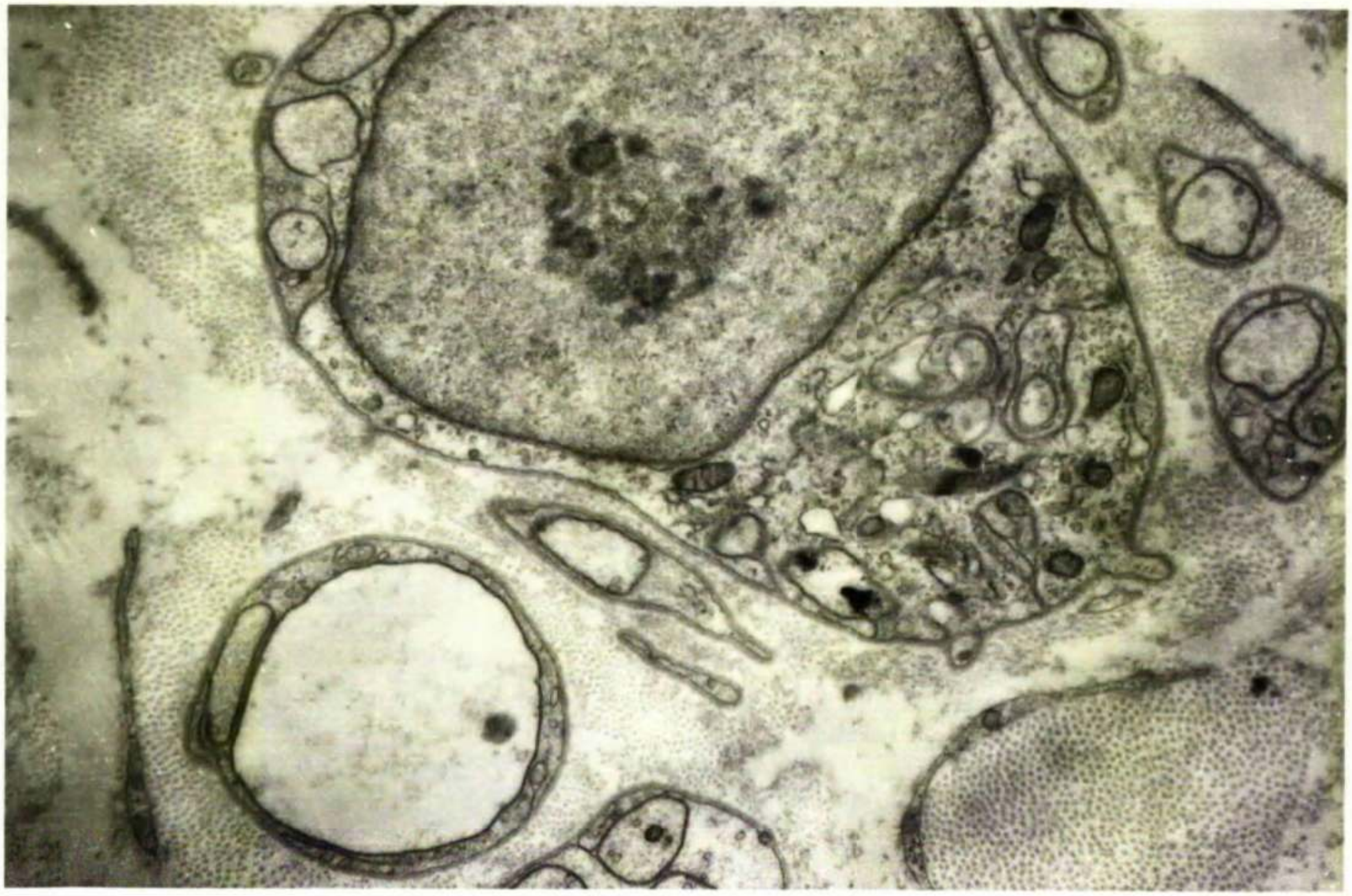


Fig. 72

A Schwann cell within the proximal portion of a phrenic nerve which had been ligated 3½ days previously. Note that at one pole of the cell there are several unmyelinated nerve fibres while, at the opposite pole there are features which suggest phagocytic activity.

of these changes within the nerve trunk, however, showed no appreciable differences as far as could be ascertained in this series of short term studies. The observation of normal myelinated fibres within each one of the nerves, peripheral to the level of ligation, was particularly consistent. Attempts were made initially to study these persisting fibres quantitatively but the friability of the nerve trunk following its ligation precluded the preparation of an entire transverse section for ultrastructural observation.

Striking changes were observed in the Schwann cells of the nerve trunk peripheral to the ligature. The contours, both of the nucleus and the cytoplasm, were less regular than normal, the cell membranes showing occasional short protrusions, resembling pseudopodia. (fig. 72). In this particular cell the cytoplasm is clearly divisible into two zones, which have entirely different morphological features. At the broader end of the profile the normal constituents of a Schwann cell are present; three unmyelinated fibres with recognisable mesaxons can be seen. At the narrower end of the cell, many cytoplasmic inclusions are present comprising five large and two small mitochondria, five vesicular components and four structures resembling remnants of unmyelinated nerve fibres. Approximately halfway between the two pseudopodial projections, there is a distinct chain of microvesicles within the cell. The nucleus, besides the change in shape already mentioned, shows a central region of increased electron density.

In contrast to the changes observed in this cell it may also be observed that the unmyelinated fibres grouped around it are normal in structure. The structure, shaped like a 'signet ring', which lies subjacent to this cell, may be seen to have structures resembling unmyelinated nerve fibres suspended within its cytoplasm. Three cytoplasmic extensions of fibroblasts, recognised by their association with collagen fibres, are also present within this region.

The unoperated phrenic nerve in each of these animals was invariably normal in structure.

C. Interruption of the Roots of Segmental Nerves.

Operations on the roots of spinal nerves were carried out with difficulty and, it was necessary to make frequent use of the dissecting microscope during the procedure.

In the region of cervical segments 5 and 6, even after total removal of the vertebral laminae, it was not possible to obtain adequate exposure of the filaments of ventral roots without considerable displacement of the spinal cord. This difficulty was increased by the presence, at this level, of the cervical enlargement of the spinal cord and a rich plexus of spinal vessels which gave rise to troublesome haemorrhage.

In the region of thoracic segments 8-13, less difficulty was experienced because, here, the operation site was rostral to the region of the lumbar enlargement, the spinal cord being more mobile as a result. In this situation, exposure and identification of the ventral roots of the spinal nerves was difficult to achieve and surgical procedures were confined to the dorsal roots.

Of the 4 animals which were subjected to posterior rhizotomy in the cervical region, 3 died without recovering from the anaesthetic while the remaining one lived for 48 hours. The 2 animals, in which the dorsal roots of the lower thoracic spinal nerves were ligated were sacrificed 48 hours after operation.

Changes in the diaphragmatic plexus following bilateral ligation of the dorsal nerve roots of the lower thoracic spinal nerves.

In two adult cats the dorsal roots of the thoracic spinal nerves, from the eighth to the thirteenth, inclusive, were exposed and tight ligatures were applied central to the corresponding spinal ganglia. Following a survival period of 48 hours the animals were sacrificed and the diaphragmatic plexus was examined in silver impregnated frozen sections, selected and prepared as in previous studies.

GENERAL OBSERVATIONS.

In both animals, axis cylinders, showing preterminal widening, were observed in all zones of the diaphragmatic plexus. (fig. 73). The nerve fibres in which this change was present appeared to be more tortuous than the nerve fibres of the plexus in

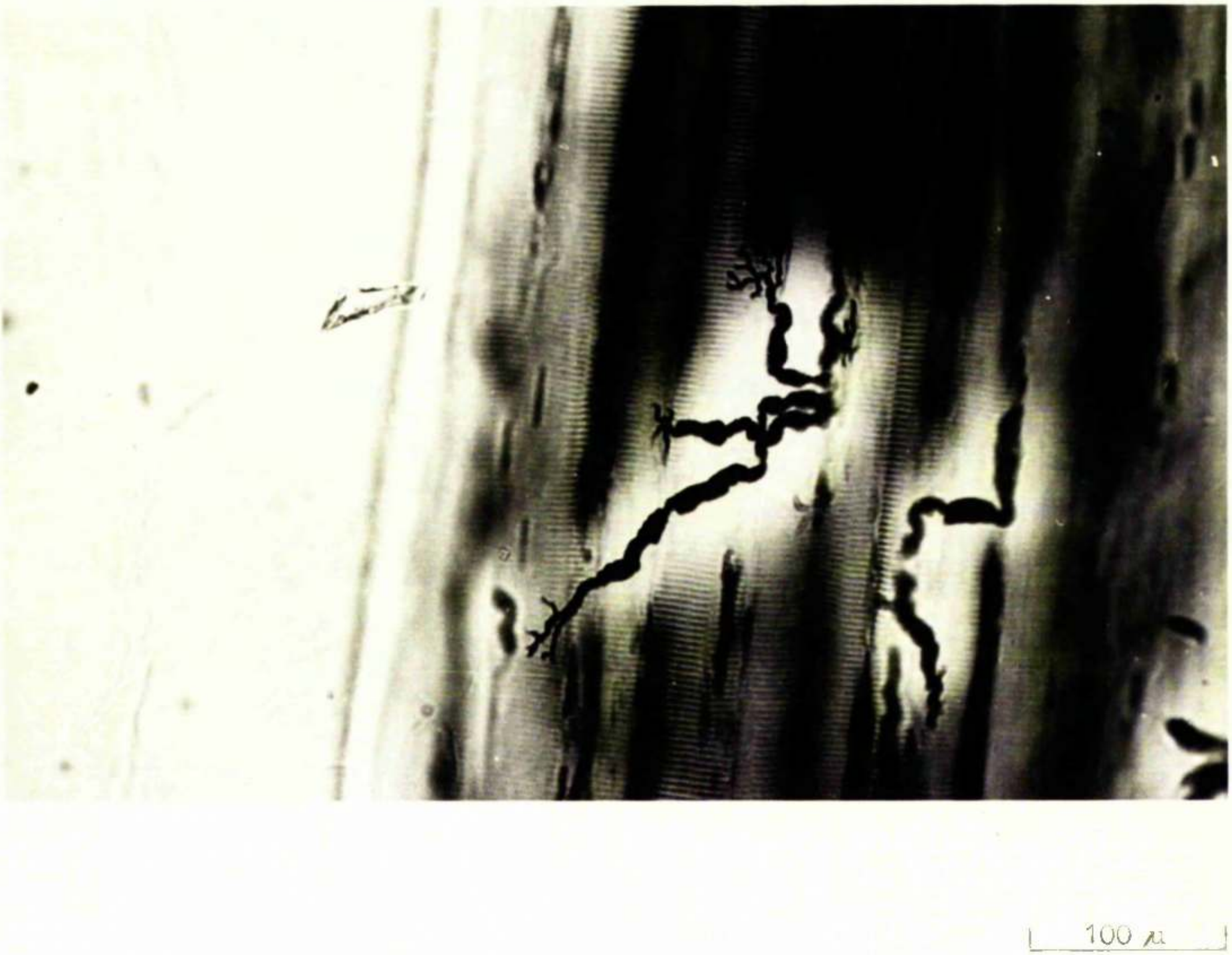


Fig. 73

Nerve fibres within the ventro-lateral zone of the diaphragm of an animal in which the dorsal roots of the lower six thoracic nerves had been ligated central to the spinal ganglion. Note that all of the fibres present show considerable preterminal distension.

normal animals. On tracing these fibres peripherally, their terminals were seen to be less argyrophilic than their more proximal portions. Small circular zones, completely devoid of argyrophilic material were observed at their extremities. (fig. 74).

Detailed Observations on the Distribution of Changes.

Although preterminal axonal swelling was seen in a small proportion of fibres in every zone of the diaphragm, the majority of nerve fibres remained morphologically normal. In the peripheral portions of the diaphragm all of the individual axons within the majority of fasciculi showed these structural changes. (fig. 73). In the more central parts of zones 1-4, however, the fasciculi of the diaphragmatic plexus were commonly seen to contain one or two nerve fibres with preterminal swelling. (fig. 75). In the more central zones of the diaphragm where fibre changes were minimal the argyrophilia of nerve terminals was increased to such an extent that nerve endings could be examined with more ease than in normal animals. One such ending, suggestive of a muscle spindle was observed. (fig. 76).

D. Extirpation of the Left Coeliac Ganglion.

The posterior abdominal wall was exposed through a left subcostal incision which extended from the midline ventrally to the posterior axillary line. The left coeliac ganglion was located by identifying the adjacent suprarenal gland which lies immediately lateral to the crus of the diaphragm on the ventral aspect of which

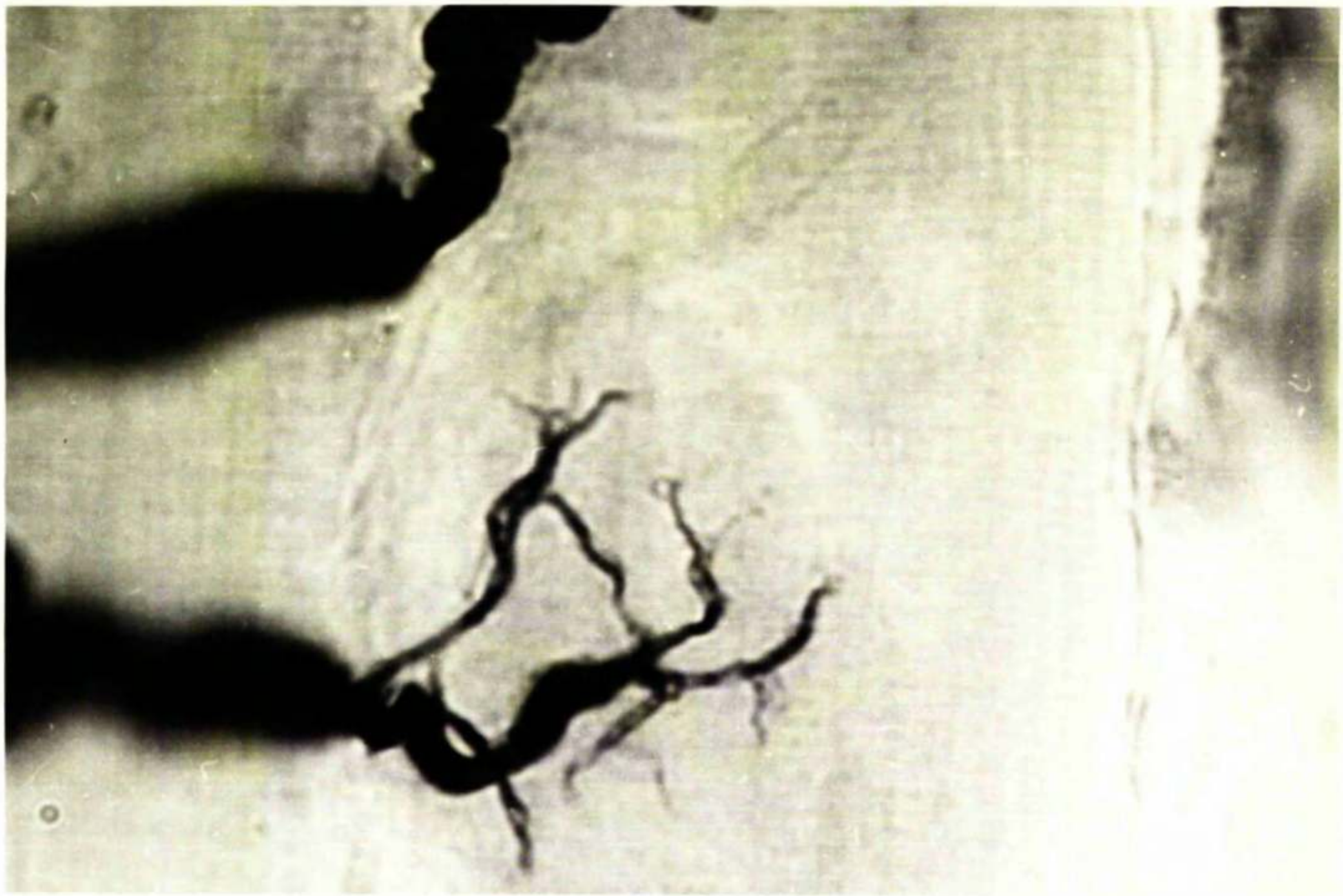


Fig. 74

From the same specimen as figure 73. Tortuosity and axonic distension are the most obvious features but there are also small circular zones at the periphery of the axis cylinders which have not been impregnated with silver.

Fig. 75

From a specimen prepared in the same way as for figure 74. A fasciculus of nerve fibres within the more central zone of the diaphragm. One axis cylinder shows distinct axonic distension while the rest of the fibres appear to be normal.

Fig. 76

From the same specimen as figure 75. A structure resembling a muscle spindle within the ventro-lateral portion of the diaphragm.

the coeliac ganglion is situated. Careful dissection revealed that, in every case, the ganglionic connections with adjecent nerves and vessels are profuse. It was also confirmed that fasciculi of nerve fibres stemming from the ganglion, penetrate the muscle fibres of the diaphragmatic crus.

Changes in the diaphragmatic plexus.

In this animal the only changes in the diaphragmatic plexus were seen in paravascular fibres of the left hemi-diaphragm after a survival period of 5 days. These changes were confined to zones 3 and 4 and were characterised by fragmentation of axis cylinders.

E. Mobilisation of the Distal Third of the Phrenic Nerve.

Through the standard thoracotomy incision, the left phrenic nerve was located in its lower third. The nerve was mobilised by incising the mediastinal pleura leaving a margin of safety to avoid damage to the nerve trunk. Throughout the procedure, no ligatures or pressure forceps were used in the vicinity of the nerve.

Changes in the diaphragmatic plexus.

Following a survival period of ten days, the diaphragm was removed and subsequent histological examination revealed a diaphragmatic plexus which was normal in most areas. However, in the anterior area of the left hemi-diaphragm there was a fasciculus comprising fifteen to twenty nerve fibres which showed changes comparable to those seen in the previous 10 day study following transection of the phrenic nerve. A fasciculus showing these changes, also appeared in the anterolateral area of the left hemidiaphragm.

DISCUSSION.

The importance of the diaphragm in respiration and in other physiological functions which require adjustment of intra-abdominal pressure has been recognised for many centuries. Since the end of last century, embryologists have been aware that the mesodermal precursors which participate in the formation of the diaphragm include the septum transversum, the somatopleure, the dorsal mesentery of the foregut and the pleuro-peritoneal membranes. An embryological history as complex as this, is difficult to reconcile with the generally accepted descriptions of the innervation of the diaphragm in man. (Hollinshead, 1956). With very few exceptions, previous authors have restricted their investigations to circumscribed facets of the problem and, since this appears to have been largely responsiblé for many controversies, the present studies were directed towards diaphragmatic innervation as a whole. It was clearly desirable, however, to examine in detail, the phrenic nerves from which most of the fibres of the diaphragmatic plexus arise and to correlate their topographical, histological and ultrastructural features.

It was intended that the results of these studies should form a comprehensive morphological basis for comparison with the findings derived from previous anatomical, physiological and clinical investigations.

Selection of Animals.

The selection of animals for the purposes of this investigation, was, of necessity, restricted to the mammalian species, since those animals which precede mammals on the evolutional scale do not possess a diaphragm such as is found in man. Since many studies, relevant to the innervation of the diaphragm, have been carried out in humans, it was considered reasonable to select mammals in which the form of the diaphragm and its innervation resemble the arrangements found in man. Although, on these grounds, primates would have been the obvious choice, fully grown cats were selected because of their greater availability and because of the convenient size of the peripheral nervous structures which required to be examined. With regard to the surgical procedures envisaged, cats were considered to offer definite advantages over smaller mammals which presented difficulties in the controlled administration of anaesthetics. Endotracheal intubation, an essential adjunct to endothoracic operations, could be successfully carried out in cats with the aid of a laryngoscope designed for use in neonatal humans, but this procedure was found to be impossible in mammals smaller than cats with the equipment which was currently available. The relative rapidity with which cats recover from major surgical procedures was regarded as an important advantage. Attempts to demonstrate the diaphragmatic neural tissues by silver impregnation of whole preparations of the diaphragm were unsuccessful in cats because of the thickness of the musculature, but, when this method was applied to rats, mice and guinea-pigs, it provided a clear picture of the general structure of the diaphragmatic plexus. Direct comparison of the neural elements observed in "whole preparations" with those seen in frozen sections taken from selected portions of the diaphragm in cats indicated that, in all of the species examined, the components of the diaphragmatic plexus possessed many structural similarities.

Apart from these observations in normal animals, the details of which are discussed below, rats, mice and guinea-pigs were not used to any great extent in the present investigation, most of the experimental observations having been carried out in cats.

The advantages of carrying out these studies in cats were further increased by the fact that this species has been widely used in relevant physiological and pharmacological investigations.

Techniques Used in the Present Investigation.

a) HISTOLOGICAL.

The value of osmium preparations for studies on the myelinated fibres of peripheral nerves, has been recognised by histologists for more than a hundred years. Ranvier (1875) provided comprehensive qualitative descriptions of these components of peripheral nerves. Rexed and Therman (1948) carried out quantitative studies on osmium-treated transverse sections of peripheral nerves and established criteria for the measurement of the external diameters of myelinated fibres so that they could be placed in categories according to size. These findings provided a morphological basis for comparison with the results of electrophysiological studies on the conduction velocities of individual nerve fibres. (Gasser and Grundfest, 1939).

In applying osmium techniques to the present studies on the phrenic nerve, it was found advantageous to follow fixation in Flemming's solution (see appendix 1) with the Weigert-Pal differentiation technique. Following this treatment, the myelin sheaths were found to stain more intensely black and they retained their intense blackness even after photographic magnification up to 1,000x. It was of no advantage to carry out the additional procedure of counterstaining as the fascicular pattern was sufficiently conspicuous without it. One possible explanation of the advantage of using the Weigert-Pal manoeuvre is that only partial staining of the components of the myelin sheath is obtained with osmium, while the remaining non-osmiophilic material takes up the haematoxylin which is used in the differentiation procedure. The findings derived from quantitative studies were in agreement with the observations of previous authors who found that the histogram of the myelinated fibres of the phrenic nerve is unimodal and resembles the constitution of the motor nerves to postural muscles. (Sherrington 1894).

While the magnifications used for light microphotography were commonly 40x in osmium treated transverse sections, observations made with the oil immersion lens showed that, even in normal animals, approximately half of the myelin sheaths of the phrenic nerve did not have concentric inner and outer margins. In a small number of the sheaths examined, the black circular profiles, representing the myelin sheaths, were divided into inner and outer concentric osmiophilic circles by a white line. These findings are in keeping with the descriptions of Ranvier (1875), who attributed these two apparent irregularities to the proximity of a node and to "renflements biconiques" respectively.

In order to demonstrate the axis cylinders within the nerve trunk, silver impregnation methods described by Bodian (1936) and Romanes (1950) were applied repeatedly but although these were successful from the point of view of defining nerve fibres of external diameter 4-18µ, they gave inconsistent results in quantitative studies on fibres of smaller diameter (1.5-4µ). The more elaborate silver method described by Jones (1936) was applied with a greater measure of success but the results remained inconsistent. The failure of these histological methods to give satisfactory quantitative results was attributed to the following factors. The techniques of Bodian and Romanes resulted, invariably, in distortion and irregular shrinkage of the tissue during preparation. Attempts were made to prevent this by placing the nerve portions on cardboard frames during fixation but this measure produced no improvement. None of the nerve fasciculi within the transverse sections obtained was truly circular in outline and the individual axis cylinders appeared to have undergone corresponding distortion. To assess how much of this effect was due to fixation in formalin, portions of nerve were treated with Bouin's fixative and the nerve was found to retain its circular outline. Subsequent silver impregnation of this tissue, however, was found to produce inconsistent staining of axis cylinders. Jones' method, though technically more cumbersome than Bodian's and Romanes', had the advantage that the transverse sections obtained were circular in outline and showed only slight distortion of nerve bundles and individual nerve fibres.

All of the histological methods used had one defect in common; it was not possible to establish criteria for the positive identification of unmyelinated nerve fibres in transverse sections of the phrenic nerve. Inconsistent quantitative results were therefore inevitable as it was impossible to differentiate between axis cylinders of small diameter and other components of the nerve such as the nuclei of Schwann cells, fibroblasts and other intrafascicular connective tissue components. The possibility of solving this problem histologically appeared to be remote because of the incompatibility of the size of unmyelinated fibres with the present limits of resolving power of the light microscope. The possibility of using an ultrastructural method for quantitative estimation of unmyelinated fibres was therefore examined and the development of this method is discussed below. (p. 204-206).

Although silver impregnation was found to be unreliable for quantitative studies on the axis cylinders of the phrenic nerve, it was found that nerve fibres could be studied qualitatively in longitudinal sections of the nerve trunk by employing the method of Bielschowsky (1940).

In order to demonstrate clearly the components of the diaphragmatic plexus in normal animals, the most reliable histological method was found to be a modification of the silver technique employed by Schofield (1960) in his studies on the innervation of the alimentary tract. Methods of tissue fixation and clearing corresponded exactly to those described by Schofield, but reduction of the strength of the silver nitrate to 10% was found to be advantageous in the initial impregnation, in order to avoid intense staining of diaphragmatic muscle fibres. The critical stage of the procedure appeared to be the length of time of immersion in ammoniacal silver nitrate; if this exceeded 10 seconds the tissue became uniformly blackened. The most suitable time, as used in this study, was three seconds. At times shorter than three seconds, subsequent immersion in formalin resulted in patchy brown staining of the muscle fibres and corresponding variations in the intensity of axoplasmic argyrophilia. This method was particularly valuable for the demonstration of the general arrangements of the diaphragmatic plexus and it enabled observations to be made on nerve fibres as far peripherally as their preterminal arborisations. Due to the thickness of the sections only a few of the actual terminals were identifiable. Studies on the diaphragm in cats were carried out, initially, on 50µ frozen sections of the musculature, but, since this method was unsatisfactory for producing an overall picture of the plexus, attempts were made to demonstrate it in silver preparations of the whole diaphragm in rats, mice and guinea-pigs. In these preparations, it was possible to trace the intrinsic nerve fibres of the diaphragm to their origin in the phrenic or intercostal nerves and to make comparisons between the arrangements in the species studied. The form of the fibres was clearly discernible and their sizes were compared in an attempt to distinguish between afferent and efferent components.

The general arrangement of the diaphragmatic plexus, as seen in silver studies, was further investigated in whole preparations of the diaphragm in normal rats, mice and guinea-pigs by a histochemical method described by Coupland and Holmes (1957). This procedure, referred to previously as a 'cholinesterase method', is said to result in the deposition of acetylthiocholine iodide at sites where the enzyme esterase is present. In order to increase the specificity of this method for acetyl cholinesterase, the tissue was subsequently immersed in a dilute solution of diisopropyl fluorophosphate (D.F.P.). When this method was applied to the diaphragm in rats, mice and guinea-pigs, the distribution of opaque deposits corresponded closely to the 'nerve-rich' zone observed in silver preparations. In view of the findings of previous authors, the regions which showed intense staining, were thought to indicate the position of neuro-muscular junctions such as are found at motor-end plates. These have been demonstrated in the diaphragm in rats, cats and dogs by this method (Gerebtzoff, Philippot and Dallemagne, 1954). The precise nature of the reaction which causes localised deposition of acetylthiocholine in animal tissues is not fully understood but it is generally believed that, by a hydrolytic process, acetylthiocholine reacts with acetyl cholinesterase to form a mercaptide which, in the presence of copper ions and ammonium sulphide, gives rise to an opaque deposit.

b) ULTRASTRUCTURAL.

In applying ultrastructural methods to quantitative studies on the unmyelinated fibres of the phrenic nerve, it was found necessary to consider two alternative approaches to the problem. The most satisfactory approach would have been to simulate histological methods in which entire transverse sections of the nerve trunk were examined. This presented technical problems of obtaining complete transverse sections of suitable thickness for examination with the electron microscope and of maintaining the integrity of the specimen for a time sufficiently long to enable the entire image to be photographed. In order to eliminate these difficulties, attempts were made to estimate the total numbers of unmyelinated fibres within the phrenic nerve by counting the numbers present in selected areas of transverse sections.

This procedure, referred to previously as the "sampling method", was unsatisfactory as conducted here but it provided valuable information on the qualitative features of nerve fibres and connective tissue components of the phrenic nerve in normal cats. It aided in the selection of the optimum magnification for implementation of the "montage method" and it indicated clearly the degree of error in previous attempts to study the unmyelinated fibres with the aid of the light microscope.

In the montage method, the difficulties mentioned above were overcome by using transverse sections which were approximately 100 Ångstrom units thicker than the specimens used for qualitative investigations. This innovation was adopted in order to afford the specimen greater resistance to mechanical stress and to the thermal effects of exposure to the electron beam. It was further supported by the application of a film of clear plastic material to the specimen grid. Mechanical support was essential because the specimen required to be mounted on an aperture grid, so that an unobstructed view of the entire image could be obtained. When the specimen was placed in the column of the electron microscope, the intensity of the electron beam was increased very slowly so that the inevitable thermal effects could be reduced to a minimum. Each field photographed overlapped its contiguous fields by a margin of one sixth of the length of the appropriate side of the rectangle and, in this way, a total of approximately 500 photographic prints were produced. Care was taken to adjust the time of exposure so that individual photographic prints showed uniform intensities of black and white. The resulting montage was 2,67 metres in its largest diameter while the corresponding dimension of the specimen was 0.6mm approximately. The size of unmyelinated fibres at this magnification was such that criteria for identification could be clearly established. The form of these fibres is discussed below, along with myelinated fibres and other components of the phrenic nerve (p. 225-226).

c) SURGICAL.

In determining the optimum position for surgical interruption of the peripheral nerves which supply the diaphragm, the principal factor considered was the site at which each of these nerves could be identified with greatest accuracy and divided or ligated with minimum risk of damage to adjacent structures. Operations on the phrenic nerve in the neck were, thus, avoided, because of the concomitant danger to the brachial plexus which, in cats, is intimately related to the origin of the nerve trunk. Furthermore, it has been established by previous workers such as Yano (1928) and confirmed by observations on cats in this investigation, that the incidence of 'accessory phrenic' nerves is significantly high. The possibility that an accession such as this might pass unrecognised at operation was a further deterrent to the use of the cervical approach. In order to be reasonably certain of dividing all of the nerve fibres which originate in the phrenic nucleus, therefore, it was decided to confine surgical procedures either to the intrathoracic portion of the phrenic nerve or to the roots of the spinal nerves from which it arises.

In operations on the roots of the fifth and sixth cervical segmental nerves, the spinal cord was exposed by dorsal laminectomy of the appropriate vertebrae. The dorsal roots were clearly seen after opening the dura mater but attempts to expose the ventral roots were unrewarding because they required considerable displacement of the spinal cord which, at that level, filled the spinal canal almost completely and was surrounded by a network of blood vessels. These practical difficulties, coupled with the possibility that neighbouring nerve roots might be damaged accidently by traction on the spinal cord, led to the exclusion of this method as a standard procedure. Both of the animals in which the operation was carried out unilaterally, died within 48 hours of operation.

The intrathoracic portion of the phrenic nerve, particularly where it lies between the mediastinal pleura and the pericardium, is conspicuous and easily defined. This was, therefore, selected as the most suitable region in which to transect the nerve trunk. In determining the optimum level for division, the main deciding factor was the variability of the level of the junction between the main trunk and the accessory phrenic nerve, which, when present, descended as a separate trunk as far as the rostral limit of the lung hilum. On these grounds, it was decided to carry out operations on the phrenic nerve as close to the caudal extremity of the lung hilum as possible and to make the approach through the fifth intercostal space so that the trunks of the lower six intercostal nerves which give branches to the diaphragm, might remain intact.

Masumoto (1934) inferred that the lower intercostal nerves may give rise to diaphragmatic branches almost immediately on emerging from the intervertebral foramina and this prompted the selection of the optimum position for experimental lesions in these nerves. Thus it was decided to perform dorsal laminectomy of the appropriate vertebrae and to ligate the dorsal roots of the eighth to the thirteenth thoracic segmental nerves inclusive. These procedures were confined to the dorsal roots because of the technical difficulties involved in operating on the ventral roots. The aim of this procedure differed from that of the transection experiments in that it was not intended to produce axonal degeneration but simply to evoke identifiable preterminal widening such as that described by Schofield (1960) so that the intra-diaphragmatic distribution of these nerve fibres might be delineated.

The likelihood of diaphragmatic nerve fibres arising either directly or indirectly from the coeliac ganglia has been recognised since the time of Schwalbe (1881) and an investigation of this feature has been conducted by Delaloye (1958). There is, however, very little experimental evidence to confirm these findings and, it was considered worthwhile to study, in cats, the effects of unilateral coeliac ganglionectomy on the components of the diaphragmatic plexus. For the purposes of these studies it was found convenient to extirpate the coeliac ganglion through an oblique subcostal incision which extended from the lateral border of rectus abdominis to the mid-axillary line.

This method possessed the advantage that the ganglion could be identified clearly, elevated from its retroperitoneal position and its connections severed one by one.

The last of the surgical procedures cited in this investigation was evolved as a means of determining whether the phrenic nerve receives an accession of fibres in its distal portion. The operation has been referred to previously in the text as "mobilisation of the caudal portion of the phrenic nerve" and the technique has been described (p. 122). The aim of this study was to evoke degeneration of nerve fibres which enter the phrenic nerve in the distal part of its intrathoracic course and ramify within the diaphragmatic plexus.

THE PHRENIC AND INTERCOSTAL NERVES.

ORIGIN.

Many references to the central control of respiration and to the spinal origin of the phrenic nerves are to be found in the literature, particularly in the records of the earlier physiological investigators. The precise nature of the cellular origin of the nerve processes which contribute to the phrenic nerves has been investigated frequently in many species, the most recent account being that of Warwick and Mitchell (1956) who defined clearly the segments of the cervical spinal cord which give origin to the phrenic nerves in primates (macaque).

The spinal nerves which are responsible for the formation of the phrenic nerves have also been investigated widely in many different species and it has been shown that the highest levels of phrenic origin are to be found in man. The incidence of 'accessory' phrenic nerve in the present investigation compared favourably with the observations of previous authors.

The comparative wealth of information obtainable on the central connections of the phrenic nerve stands in sharp contrast to the paucity of corresponding factual data on the intercostal nerves. Recent investigations of this problem have been, in the main, physiological. For example, Rosenblueth (1961) and his collaborators defined in cats, spinal centres which extended from the fifth to the thirteenth and from the fourth to the ninth thoracic segments respectively. The latter was considered to be outwith the control of the medullary respiratory centre. This investigation confirmed that of the intercostal nerves which give branches to the diaphragm, some arise from the seventh to the thirteenth thoracic spinal segments in cats.

DISTRIBUTION.

In this, and previous studies, there has been frequent and convincing confirmation of the fact that the main distribution of the phrenic nerves is within the substance of the diaphragm. The developmental history of the diaphragm, however, suggests that the phrenic nerves might be expected to extend their influence to other structures which develop in relation to the mesoderm of the septum transversum, for example, the pericardium and the liver. It has been shown by Ruhemann (1926) in topographical and experimental studies that the phrenic nerves give rise to pericardial branches, the presence of which was confirmed in cats and mice, in this investigation. Luschka, whose findings have been summarised above (p. 13), observed that the phrenic nerves had a significant number of abdominal branches and considered it likely that the liver and the adrenal glands may derive part of their nerve supply from this source. The present investigation, however, was not specifically orientated towards these features of the problem but was principally concerned with the intra-diaphragmatic distribution. The manner in which the nerve fibres descend in the phrenic nerves to reach the diaphragm is somewhat more complex than many textbooks suggest. For example, it is considered likely by authorities such as Cruveilhier (1845) and Testut (1891) that the left and right phrenic nerves may exchange a few fibres either in the pre-pericardial or in the infra-diaphragmatic position, thus providing a pathway for contralateral innervation. This possibility has been acknowledged by physiologists such as Langendorff (1887) who regarded it as a reasonable explanation for the "crossed-phrenic phenomenon" which was described above. (p. 80).

The findings derived from topographical and experimental studies in this investigation strongly support the possibility that some nerve fibres may cross from one phrenic nerve to the other in the lower portion of their intrathoracic course. Thus, in addition to the intermingling of nerve fibres which may occur within the diaphragmatic plexus, provision is made for extra-diaphragmatic communications between the left and right phrenic nerves. The possible functional significance of this fact may well be in the provision of a peripheral pathway for coordination of the left and right halves of the diaphragm.

At a distance of a few millimetres rostral to the thoracic surface of the diaphragm the left and right phrenic nerves both divide to form their terminal branches. This finding has been consistent both in the present study and in the records of other investigators. (Botha, 1956). In cats, most of the terminal branches of the left phrenic nerve penetrate the muscular part of the diaphragm to ramify on its abdominal aspect and, as in man, the corresponding branches of the right nerve pass through the right leaf of the central tendon. The shape of the hiatus for the inferior vena cava, through which some of these branches pass, resembled more closely the pear-shape described by Haller (1763) and Walker (1960) than the foramen quadratum described by other authorities such as Lanza (1958). There exist only minor differences of opinion regarding the actual numbers of terminal branches in different mammalian species, the general pattern of, one lateral, one anterior, and one posterior branch being surprisingly uniform throughout.

The deployment of the branches of the phrenic nerve within the substance of the diaphragm in all of the species examined is also very consistent, a 'nerve-rich zone' being formed by the nerve fasciculi approximately halfway between the peripheral and central attachments of the muscle fibres. This pattern of innervation has been recorded previously both for the diaphragm and for other skeletal muscles such as the extrinsic muscles of the eye in which the muscle fibres are supplied near to their equatorial region. (Merrillees, Sunderland and Hayhow, 1950).

With regard to the diaphragmatic branches of the intercostal nerves, very few comprehensive descriptions appear in the literature. Topographical accounts indicate that the portal of entry of these neural elements into the diaphragm is closely related to the interdigitations of Transversus abdominis and the diaphragm. Diversity of opinion regarding the structure and function of these branches is a conspicuous feature of the literature; clearly illustrated records of morphological studies are almost entirely absent. In this investigation, preliminary topographical studies on the intercostal nerves in cats led to the conclusion that little could be learned about diaphragmatic branches without the aid of the light microscope. Even at relatively low magnifications it was possible to identify and categorise these branches morphologically and to establish their mode of distribution. This part of the investigation was simplified considerably by the use of 'whole preparation' techniques in rats, mice and guinea-pigs. It became evident that the direct diaphragmatic branches of intercostal nerves are not only distributed locally at the periphery of the diaphragm but also communicate, through a few fibres with intra-diaphragmatic fasciculi which originate in the phrenic nerve. The possibility that branches of the caudal six intercostal nerves reach the diaphragm by routes other then the direct one just described has been suggested by previous authors such as Masumoto (1934). There is some evidence in the present study which supports this theory and it is discussed in more detail below.

Fibre Components of the Phrenic Nerve.

1) WITHIN THE NERVE TRUNK.

At the outset it was clearly desirable to examine the fascicular pattern and fibre content of phrenic nerves in normal animals at three different levels to provide a structural basis for comparisons with the appearances in animals subjected to experimental procedures.

A) <u>In Normal Animals.</u>

No uniformity of fascicular pattern was recognisable and there was no distinct quantitative relationship either between the number of contributing roots and fasciculi centrally or between the number of terminal branches and fasciculi peripherally. These results are in agreement with the views of Pansini (1888), who concluded that the phrenic nerve is composed of a number of fasciculi which follow a serpentine course along the nerve trunk. The technical difficulty of obtaining a true transverse section of the nerve trunk in which all fasciculi and fibres are cut transversely, supports this finding. Asymmetry of fascicular patterns at comparable levels in the left and right phrenic nerve in the same animal was a consistent finding. This is in agreement with one of the conclusions of Sherrington (1894) who stated that asymmetry of fascicular patterns was the rule in paired peripheral nerves. The quantitative findings in this investigation show that the number of fasciculi within the nerve trunk increases towards the peripheral end of the phrenic nerve. These changes in the numbers of fasciculi may have the double significance that bundles of fibres which originate from neurones in the cervical spinal cord are being apportioned and that the nerve trunk is receiving accessions as it courses through the thoracic cavity.

Myelinated fibres.

In preliminary histological studies on osmium-treated transverse and longitudinal sections, these components of the phrenic nerve resembled closely myelinated fibres in other peripheral nerves. It was observed that the profiles of the myelin sheaths, although approximately circular, frequently had non-circular inner margins and that the thickness of the osmiophilic material was occasionally interrupted by an unstained cleft which encircled the axoplasmic space. Ranvier (1875) described these apparent irregularities in the sheaths of myelinated fibres and put forward the following explanations.

1) The myelin sheath is specially adapted to the axis cylinder in the region of a node thus conferring on the transverse section of the sheath an irregularly grooved or fluted outline.

The fact that the internodal segment of the myelin sheath is not uniformly cylindrical is supported by the views of Thompson (1959) who, in his studies on growth and form, considered the growth of a cylindrical unicellular organism. He concluded that, at its extremities, the cylinder must develop ridges and grooves to allow for the expansion of cellular contents. Extrapolating from this premise, it seems reasonable to assume that the cylindrical myelin sheath, in the region of the paranodal constriction, may have an irregular form and may even appear cruciform in transverse section.

Support is also found for this viewpoint in the more recent ultrastructural studies of Williams and Landon (1963) who regarded local irregularities in the shape of the sheath as one of the features of the paranodal apparatus.

2) The term 'renflements biconiques' used by Ranvier to describe the apparent duplication of the myelin sheath, suggests that there is a region in the internodal segment where a process resembling intussusception has occurred in the myelin sheath.

Whatever the explanation of these structural characteristics may be, there was no doubt about their frequent presence in this investigation both in histological and ultrastructural studies.

Sherrington's analyses of the fibre content of peripheral nerves revealed that, in paired nerves, the numbers of fibres present were approximately equal. In this investigation, the myelinated fibres of the left and right phrenic nerves of each cat studied showed this quantitative characteristic. In one animal in which there was an accessory phrenic nerve present unilaterally, the total numbers of fibres present at corresponding levels peripheral to the junction of the accessory phrenic nerve with the main nerve trunk, were approximately equal. Variation in the total numbers of myelinated fibres from level to level within one phrenic nerve was not very marked and this was surprising in view of the numbers of fibres distributed to the pericardium and other thoracic contents such as the pleura. If it is true that the nerve trunk is fully formed centrally by the union of the fifth and sixth cervical nerve roots, then the total number of fibres within the phrenic nerve might be expected to diminish steadily as the nerve proceeds peripherally. The observation that this was not so in the present study, led to more detailed examination of the histograms in which myelinated fibres were represented according to measurement of their external diameter.

The diminution in numbers of fibres which are represented on the left side of the histograms was explicable by the observations of Luschka (1865), Ruhemann (1925) and Bonivento (1940), who observed that a small number of the fibres of the phrenic nerve were distributed to the pericardium. Confirmation of this observation was obtained in the present study and a large proportion of these nerve fibres were myelinated.

On the other hand, the nerve fibres of medium to large diameter, represented on the right side of the histogram, generally show an increase in numbers at more peripheral levels of the nerve trunk. No immediate explanation could be found for this observation although Greenman (1913), Bors (1925) and Häggqvist (1938) had all observed an increase in the numbers of fibres within peripheral nerves close to their peripheral extremity. These workers did not comment on the sizes of nerve fibres affected by this increase.

It would seem that two rational explanations are possible;

1) That the myelinated fibres within the phrenic nerve undergo intraneural branching.
2) That the phrenic nerve receives an accession of myelinated fibres from an extra-phrenic source close to its termination.

If the first of these possibilities is to be accepted, then the implications of intra-neural branching must be examined closely. Records of previous morphological studies on the branching of nerve fibres, state that when axis cylinders branch they do so at a node of Ranvier and that the diameter of the newly formed processes may be less than or equal to the diameter of the parent stem. These views have been clearly expressed by Sunderland and Lavarack (1953) who reviewed the findings of Langley (1902) and Cooper and Sherrington (1929).

The concensus of opinion is that intraneural branches of nerve fibres, although occasionally equal in diameter to the parent fibre, generally show an immediate reduction in size at their point of formation.

On these grounds, the increase in numbers of myelinated fibres of diameter 16-20µ within the most peripheral portion of the phrenic nerve can only be produced by branching of intrinsic myelinated fibres represented at the extreme right in the histograms. While this might be a satisfactory explanation in the case of nerves where the peripheral increase is small, it becomes less acceptable in cases where these nerve fibres show a numerical increase of approximately fifty per cent. Although it is recognised that intra-neural branching of fibres may influence the fibre-spectrum of the nerve, it was not pursued further in the present study.

The second possibility, that the phrenic nerve is joined, in its most peripheral portion, by a small number of myelinated nerve fibres which arise from an extra-phrenic source, is of more fundamental significance than the first explanation. The presence of such an accession would imply the existence of a group of neurones, other than those of the phrenic nucleus, the processes of which are distributed with the phrenic nerve and are thus capable of influencing the nervous control of diaphragmatic function. At least four significant portions of evidence, derived from the relevant literature, support this hypothesis.

The records of previous authors who have investigated diaphragmatic innervation have frequently been diametrically opposed to one another, the most controversial topic being the manner in which intercostal nerves participate. Those studies in which the findings support the possibility of motor function of the diaphragmatic branches of intercostal nerves are almost equal in number and significance to those which support the opposing view. The equivocal nature of the available evidence thus indicates, at least, the necessity for considering hypotheses such as that expressed above. Pansini (1888) inferred that some of the connections between the phrenic nerve and the pericardium might be accessions to the phrenic nerve rather than branches arising from it. In the era of elective paralysis of the diaphragm for the treatment of pulmonary tuberculosis, clinicians observed frequently that crushing or dividing the phrenic nerve in the cervical region failed to produce the desired effect. Although this was, almost invariably attributed to the co-existence of an unidentified accessory phrenic nerve which arose from the nerve to Subclavius, it was also possible that the accessory fibres were not entirely confined to the cervical region but could have originated in an adjacent nerve trunk to unite with the phrenic nerve at any point within the thoracic cavity. The electrophysiological studies carried out by Rosenblueth and his collaborators (1961) provided evidence in support of the existence of an accessory motor innervation of the diaphragm in cats.

The findings in normal animals in this investigation combined with the conclusions of previous authors indicate that the possibility of the existence of an accession of nerve fibres to the phrenic nerve in its most peripheral portion cannot be denied. On this premise, the second of the two explanations offered above, influenced the further extension of these studies.

Although the results of quantitative studies on myelinated fibres were suggestive of the existence of an accession of fibres to the phrenic nerve in its most peripheral portion, no indisputable direct evidence of this communication was observed. In about one quarter of all the animals examined, however, there was a thread-like neuro-vascular bundle connecting the left with the right phrenic nerve. The presence of this structure indicates that, in a small number of cats, there is a commisural pathway from the left to the right phrenic nerve.

Reference has been made above to intrathoracic communications between the two phrenic nerves and the records of investigators such as Hovelacque (1927), support the findings made in this study. This observation is also in agreement with the findings of Collis (1954) and his collaborators, who concluded that the left and right phrenic nerves supply the entire left and right hemidiaphragms respectively. The muscle fibres of the right crus which cross the midline to encircle the oesophageal hiatus, according to them, are supplied by the left phrenic nerve. This infers that a small fasciculus of nerve fibres must also cross the midline. It is not surprising, therefore, that in some animals, these nerve fibres cross from left to right within the thoracic cavity. In animals which lack this communication there may well be another less conspicuous commisural pathway associated with the coeliac plexus or the connection may occur within the diaphragmatic plexus itself.

The decision to examine the trunk of the phrenic nerve with the aid of the electron microscope was prompted by the failure of existing histological methods to demonstrate consistently the unmyelinated fibres which are present within the nerve trunk.

Besides enabling quantitative studies on these smaller components to be carried out, the 'montage' method provided an opportunity to examine in greater detail the other components, including the myelinated fibres of the nerve trunk. Both qualitatively and quantitatively, the histological and ultrastructural observations on these fibres had several comparable features. The division of the nerve trunk into fasciculi was obvious in both studies but the electron microscope provided a more detailed picture of the subfasciculi which were inconspicuous in previous histological studies.

The myelinated fibres, as seen in transverse sections of the phrenic nerve, were much less regular in outline in ultrastructural preparations than in corresponding histological studies. In the montage, which represents the transverse section of the left phrenic nerve at the level of the lung hilum of a cat, the myelinated fibres are represented by non-circular profiles, many of them showing marked irregularity in outline while some sheaths are distinctly cruciate. A satisfactory explanation of this is lacking and although some of the irregularity may be due to methods of preparation, it is possible that, to some extent, these appearances are genuine and represent the irregularities observed in corresponding histological studies discussed above. In any case, the main purpose of this portion of the study was to derive quantitative data and it was not unduly influenced by this finding. Other ultrastructural characteristics of myelinated fibres, such as the contiguity of the axoplasm and the sheath, and the approximately circular outer limiting membrane of the sheath were easily distinguishable at magnifications of 2,000x and at magnifications in excess of 30,000x, the regular layering of the myelin could be identified clearly. These morphological features which were observed in each portion of nerve examined, were regarded as significant in establishing the criteria for identification of normal myelinated nerve fibres.

Quantitative studies on myelinated fibres, carried out by ultrastructural methods gave total counts of a somewhat higher value than those obtained from histological investigations. This was attributed to the presence of groups of myelinated fibres of small diameter which are present in peripheral parts of the fasciculi and are associated with aggregates of unmyelinated fibres. These myelinated fibres of small diameter are inconspicuous and may pass unnoticed in histological preparations. Their association with unmyelinated fibres was thought to indicate some form of functional grouping since they were all of small diameter and might be expected to conduct at low velocities. No other evidence of grouping of myelinated fibres within the nerve trunk was found, fibres of larger diameter being distributed among fibres of smaller diameter in an apparently random fashion throughout the entire transverse section of the nerve. The absence of an identifiable pattern of distribution of these fibres suggests that their myelin sheaths, coupled with the interstitial collagen fibres which surround them, afford sufficient insulation to obviate functional interference of fibres of high conduction velocity with neighbouring fibres of low conduction velocity. Because of the generalised irregularity of the outer margins of the osmiophilic myelin sheath, estimates of external diameters were made by using the circular outer limiting membrane of the sheath as the line of reference. In this way, the results obtained by electron microscopy confirmed that the vast majority of myelinated fibres range in diameter from 4-18μ and they established the fact that each fasciculus contains a small number of fibres of smaller diameter (1-4μ) near to its periphery.

Unmyelinated fibres.

While it is recognised that the term 'unmyelinated fibre' is misleading in that it suggests the absence of a sheath from these neural components, it has been used here to describe those nerve fibres of small diameter in which the sheath cannot be demonstrated by light microscopy. Despite their relatively small diameter, these fibres have been recognised as components of peripheral nerves since the time of Remak (1839) whose association with their early history earned them the title 'fibres of Remak'. The presence of these components within the phrenic nerve has been noted by Felix (1922) and, in the studies of Yano (1928) reference is made to the general distribution of these fibres at selected levels within the nerve trunk. The microphotographs presented by Yano are of a standard which casts doubt on the validity of his quantitative findings, but his general conclusions regarding the distribution of unmyelinated fibres within the phrenic nerve are in agreement with the findings obtained in this investigation.

It will be recalled that the failure of existing histological methods to give consistent quantitative results in this study indicated the need for ultrastructural investigation of these fibres. The sampling method which relied on statistical methods, although it provided valuable general information on the components of the nerve trunk, was not entirely satisfactory for accurate quantitative studies on unmyelinated fibres. The advantages of the montage method are that absolute numbers of nerve fibres, including the unmyelinated variety, can be obtained. Before any attempt was made to count these fibres, three main criteria for their identification were established.

1) They should have a circular or ovoid profile with diameters of approximately 1 micron or less.
2) There should be clear evidence of their association with an adjacent Schwann cell in the form of an identifiable mesaxon.
3) The axoplasm should contain axoplasmic organelles such as mitochondria, vesicles or electron-dense granular material.

In addition to these salient features, identification was facilitated by their almost invariable aggregation into groups which were larger at the periphery than in the central zones of each fasciculus. More reliance was placed on the first and second criteria than on the third which was found to be very variable. It was unusual to find evidence of all three types of axoplasmic organelles mentioned in a single transverse section of one unmyelinated fibre.

The apparent predilection of unmyelinated fibres for the periphery of fasciculi could be attributed to the fact that these components are destined for innervation of smooth muscle which, if present at all, is very sparse in the trunk of the phrenic nerve. The appearances are consistent with these fibres making use of the trunk of the phrenic nerve as a convenient means of reaching an extra-phrenic destination in structures such as the walls of blood vessels in the pleura, pericardium and diaphragm. Their cell bodies are most likely to be situated in peripheral components of the autonomic nervous system such as the inferior cervical ganglion which has connections with the phrenic nerve. Nerve fasciculi which connect the perivascular plexus of the internal thoracic artery with the phrenic nerve also constitute a possible pathway for the exchange of post ganglionic autonomic nerve fibres. These connections have been observed in this study and their identification confirms the findings of Luschka (1853), Henle (1863) and Schwalbe (1881) who studied the communications between the phrenic nerve and the autonomic system. The multiple nature of these connections is compatible with the numerical value obtained for the total count of unmyelinated fibres present within the phrenic nerve. Furthermore it has been observed by Delaloye (1958) that the right phrenic nerve is connected, in the most peripheral part of its course, to a bundle of nerve fibres which originate in a ganglionic structure situated on the abdominal surface of the right crus of the diaphragm. This accession, where present, constitutes another source of unmyelinated fibres which enter the trunk of the phrenic nerve and pass along it. It is interesting to speculate as to the situation of the cell bodies of these nerve fibres and to consider their possible functional significance. The two most likely sources are the coeliac ganglion and the subdiaphragmatic ganglion described by Delaloye (1958). Originating in either of these ganglia, these fibres would be in a position to communicate with the corresponding structure of the opposite side by way of the coeliac plexus thus establishing a pathway for coordination of autonomic activity within the two halves of the diaphragm. Apart from this possibility these communications may be part of the nervous apparatus responsible for the innervation of subdiaphragmatic viscera such as the liver. These abdominal branches of the phrenic nerve were described in detail by Luschka (1865) but were not studied intensively in this investigation. If these subdiaphragmatic communications act as a peripheral commisural pathway they could be regarded as supplementary to the central coordination of respiration, which occurs at the level of the medulla or in the nuclei of the hypothalamus.

The pericardial branches of the phrenic nerve, as described above, contain a large number of unmyelinated fibres which are distributed along the walls of adjacent vessels. A small proportion of these branches, however, do not come into close relationship with vascular structures and, since they are not closely related to identifiable contractile elements, it seems unlikely that they are directly concerned in vasomotor control. It may be deduced, therefore, that these fibres subserve an afferent function and, because they are of small diameter, that they have a low conduction velocity compatible with the transmission of pain from the pericardium. The extension of unmyelinated fibres from the phrenic nerve on to the pericardium which overlies the origins of the great vessels has also been observed and in this situation many fasciculi ramify within the adjacent tunica adventitia. Although these findings are based on observations in cats, rats and mice, it is interesting to speculate on the possible functional significance of the corresponding fibres, should such exist in humans. Their topographical distribution coincides, approximately, with the region in which the pain of myocardial ischaemia is commonly felt. Furthermore, the precordial pain which accompanies diaphragmatic flutter, because of its distribution and severity, has occasionally been mistaken for angina pectoris but, so far, no satisfactory explanation has been discovered for this distressing symptom. The possibility that the pain associated with these two clinical conditions is mediated by the pericardial branches of the phrenic nerve seems worthy of further investigation.

B. In Animals Subjected to Experimental Procedures.

The observations of Waller (1852), that interruption of a process of a neurone results in degeneration of that portion which is separated from the cell body, formed the basic premise of these experimental studies. It was confirmed initially, following transection of the phrenic nerve, that Wallerian degeneration occurs in the fibres of that portion of the nerve which is peripheral to the lesion. The morphological features of nerve fibres were studied with the aid of the light microscope to establish criteria for the identification of degeneration in axis cylinders and myelin sheaths after survival periods which ranged from 24 hours to 30 days.

Changes in axis cylinders.

The appearance of axis cylinders in longitudinal sections of the phrenic nerve in normal animals was established by the silver-impregnation method described above and the predominant features of the argyrophilic profiles was their parallel outlines. Irregular widening of the axis cylinders which is the most obvious structural change after survival periods of 24-84 hours, suggests that these aixs cylinders, divorced from their cell bodies, have imbibed extra fluids from the surrounding intrafascicular tissues. The somewhat indefinite border which separates the argyrophilic axoplasm and the myelin sheath is compatible with damage to limiting membranes, the integrity of which is essential to the control of the fluid content of the axoplasm. The progressive nature of these changes is not very striking within the range of survival periods mentioned above, except that the fibres of small diameter show greater susceptibility to degenerative changes than those of large diameter. In these relatively short-term studies, no irrefutable evidence of degeneration was observed, the axis cylinders having retained their continuity.

The rate of development of structural changes in the peripheral portion of the nerve was much faster at survival periods in excess of 84 hours. Marked irregular widening of axis cylinders, was seen at 4 days and this was accompanied by the appearance of intrafascicular cells, the morphology of which was compatible with that of enlarged Schwann cells. In studies where the survival period was greater than 4 days, the boundaries between the argyrophilic axoplasm and neighbouring components became progressively less distinct, suggesting that damage to the limiting membranes was continuing. Although each of these structural changes might be regarded as presumptive evidence of degeneration within nerve fibres, discontinuity of the argyrophilic outlines which represent axis cylinders did not become generalised until survival periods were of 10 days and over. Loss of continuity of the axoplasm was regarded as a most important criterion in the definite identification of degeneration. At survival periods of less than 10 days, duration, some of the axis cylinders of large diameter retained their continuity but in animals where the survival periods exceeded 10 days, no normal axis cylinders could be identified in degenerated fasciculi. In animals in which the survival periods ranged from 10-30 days, the argyrophilic material became progressively more fragmented and clumps of densely staining material were present throughout degenerated fasciculi. These clumps were very conspicuous in short term studies of this group, gradually becoming less marked towards 20 days. At 30 days, all argyrophilic material had gone from the fasciculi, only empty Schwann bands remaining.

From this series of studies, it would appear that four stages of degeneration can be recognised in the axis cylinders of the transected phrenic nerve. The initial increase in width of individual fibres may be attributed to damage to cell membranes such as that which separates the axis cylinder from the myelin sheath but, even at 84 hours after transection, the continuity of nerve fibres within the nerve trunk peripheral to the lesion is maintained. The presence of intra-neural spindle shaped cells with prominent nuclei suggests a considerable cellular reaction to the experimental procedure. The reason for the comparatively sudden structural change which occurs between 3½ and 4 days after operation is obscure but the appearances suggest that a process of decongestion has supervened within the peripheral portion of the transected nerve. The damage to membranes which has been presumed to influence the initial changes may also be responsible, to some extent, for the progressive fragmentation of the axoplasm which becomes obvious after 10 days.

Removal of debris from the peripheral stump by phagocytosis may contribute appreciably to the shrinkage of the nerve in which axoplasmic retraction in the long axis of the nerve fibres is observed. The gradual disappearance of all argyrophilic material from the peripheral stump was undoubtedly due to phagocytosis in those animals in which survival periods ranged from 10-30 days. From the twentieth day after transection, the axis cylinders of the phrenic nerve peripheral to the level of the lesion contained progressively less argyrophilic material and were not easily defined. The optimum survival period for identification of degenerating nerve fibres in the nerve trunk was from the tenth to the thirteenth day as the fragmented argyrophilic material was most conspicuous at that time.

Although the vast majority of axis cylinders within the peripheral portion of the transected phrenic nerve conformed to the pattern of degeneration described above, a significant number of nerve fibres did not show these degenerative changes. The persistence of a small number of axis cylinders, which were observed in three of the six animals studied in detail, thus supports the quantitative observations in normal animals. This evidence suggests that, within the peripheral portion of the phrenic nerve, there are fibres, the cell bodies of which are not situated in the phrenic nucleus.

Persisting fibres in the peripheral portion of transected nerves have been observed by investigators such as Erhart (1965), who studied the effects of experimental interruption of branches of the brachial plexus in dogs. The origin of these accessory fibres, however, remains unexplained perhaps because of the relative paucity of possible local sources of neurones in the peripheral region of the upper limb. In the case of the phrenic nerve, however, several peripheral nerves are in a position to provide accessory contributions of fibres to the main nerve trunk. The proximity of the phrenic to the intercostal nerves, for example, is entirely compatible with exchange of fibres between these two structures. If this accession to the phrenic nerve exists, and the possibility is strengthened by the findings of Rosenblueth (1961), it is not of a macroscopic size because it was not identifiable by ordinary dissection in any of the animals studied. Such an accession, however, might originate from multiple sources such as the lower intercostal nerves and communicate with the phrenic nerve by means of very small fasciculi which are visible only with the aid of the light microscope.

Changes in myelinated fibres.

Current views on the effects of transection of the trunk of a peripheral nerve, suggest that, all the fibres present within the peripheral portion will ultimately degenerate. The incomplete nature of this process was very striking in the present investigation for, in animals in which the survival period ranged from 5-30 days, osmiophilic material persisted in that portion of the nerve which was peripheral to the lesion. The degenerative changes observed at 5 days after operation were in agreement with the findings of Causey and Palmer (1951), who, in short term studies on the effects of nerve crush, observed that mild osmiophilia was present in the axoplasmic space. In identifying this as centripetal migration of myelin, these workers claim support from the findings of Young (1949) who observed corresponding centrifugal migration of argyrophilic material under comparable experimental conditions. Both effects could be attributed to damage to the limiting membranes and the findings from the present study strengthen this possibility. It is significant that Causey and Palmer (1951) considered nerve fibres which showed this change to be capable of functional recovery.

In the peripheral portion of the transected phrenic nerve, while most of the myelinated fibres degenerated leaving no visible trace of their presence, approximately 100 of the myelin sheaths, having reached the stage of axoplasmic osmiophilia, appeared to degenerate no further. In transverse sections two observations favoured the identification of these structures as persisting sheaths:-

1) Their circular outer boundaries.
2) Their diameters which were comparable with the myelin sheaths of normal nerves.

Longitudinal sections of this portion of nerve also showed that although the vast majority of myelin sheaths were reduced to osmiophilic droplets, some of them retained their normal histological appearance. The persistence of a number of myelin sheaths after survival periods of up to 30 days, suggests that a considerable number of myelinated fibres do not conform to the general pattern of degeneration. It is tempting to regard them as equivalent to the fibres which give rise to the increased numbers of myelinated fibres which are found in the peripheral portion of normal phrenic nerves and to equate the persisting myelin sheaths with the persisting axis cylinders described above (p. 180). In the present study, however, the axis cylinders which remained intact within this part of the nerve could not be counted accurately and so numerical comparisons were essentially of an approximate nature.

Structural changes in the fibres of transected nerves at levels central to the level of the lesion were also described by Waller (1852) whose observations suggested that degenerative changes extend centripetally to the nearest node of Ranvier. Cajal (1928) expressed the view that Waller might have observed more extensive centripetal degeneration if a longer interval had elapsed between operation and histological examination of the components of the nerve trunk. The present studies on myelinated nerve fibres within the transected phrenic nerve in one animal, support the views of Cajal in that axoplasmic osmiophilia was present in a significant number of fibres at a distance of 4cm central to the level of the lesion. The survival period was 30 days and the changes in fibres resembled those observed in some fibres within the peripheral portion of the nerve after a survival period of 5 days.

In every animal examined, the myelinated fibres of the central portion of the transected phrenic nerve, within 1cm of the lesion showed degenerative changes which were much more striking than those observed at more central levels. The myelin sheaths and the axis cylinders adjacent to the lesion showed almost complete disintegration. Quantitative studies on persisting sheaths, within the nerve trunk, indicated that a greater number was present at 1cm peripheral to the lesion than at 1cm central to the lesion. It seems improbable that the severity of the inflammatory response of the tissue to the experimental procedure could differ greatly in these two closely related portions of the nerve and it is worthwhile considering the possible reasons for the persistence of greater numbers of myelin sheaths within the peripheral portion.

If one regards the perineurium as an almost inelastic sheath and accepts the views of Weiss (1941), that transport of metabolites is continuing in a centrifugal flow from the neurone along the axis cylinder, then increased intraneural pressure within the central portion of the nerve may be expected to occur, the severed end of the nerve being plugged by a connective tissue proliferation. Under these conditions, the contained nerve fibres would be subject to compression and might therefore be expected to degenerate more readily than the corresponding components of the peripheral stump.

While this mechanism may be partially responsible, it is possible that the larger numbers of persisting sheaths in the peripheral portion of the nerve may be due to an actual accession of myelinated fibres to the phrenic nerve at this level.

Ultrastructural changes in the components of the phrenic nerve.

It has been stated previously (p. 186), and it is further emphasised here, that the experimental procedure carried out in these studies was ligation instead of transection of the phrenic nerve but the level was the same in both cases. For this reason, and because the survival periods in these animals ranged from 72-96 hours, the results are not directly comparable with the previous histological findings. The advantages of this method were, that it was technically suitable for preparation of transverse sections of the nerve trunk, the continuity of which had been preserved. Furthermore, since damage to the nerve was less than in transection experiments and since the duration of the survival periods was shorter, a picture, more closely resembling the normal appearance, was obtained. In this way, the possibility of errors of identification were reduced. This method provided an opportunity of studying, not only the fibre components of the phrenic nerve but also the Schwann cells and connective tissues present within the nerve trunk, both central and peripheral to the level of the lesion.

The findings peripheral to the level of the lesion were of particular interest because the morphological changes in the nerve fibres were not entirely uniform. It might be argued that this was due to the fact that the ligature had not affected all fibres equally but it has been shown, histologically at least, that the effects of transection and ligation correspond closely to one another after a survival period of 3½ days. The ligature, inspected with the dissecting microscope both at operation and when the animal was sacrificed, appeared to interrupt the nerve completely. While the vast majority of myelinated fibres showed axoplasmic retraction, some remained apparently intact and the distribution of these changes within the transverse section did not appear to bear any relationship either to the position of the fibres within the nerve or to the magnitude of their external diameter. One possible explanation of axoplasmic retraction is that the isolated portiom of axis cylinder, deprived of its central source of nutrition, undergoes atrophy. This is in keeping with histological observations. The absence of this ultrastructural change in a small number of myelinated fibres provides evidence that the individual fibres of this persistent group are still in continuity with their cell bodies which must, therefore, be situated in an extra phrenic position. In the sheaths of myelinated fibres which show axoplasmic retraction there are electron-lucid clefts which separate the layers of the regularly laminated myelin. This may be due to the presence of excess fluid which has been produced as part of the degenerative process in the peripheral portion of the nerve. Within the axoplasm and in the small rim of Schwann cell cytoplasm which surrounds the osmiophilic myelin, there is an apparent increase in the numbers of electron-dense particles.

The distribution of changes in the unmyelinated fibres resemble those just described for myelinated fibres, some groups showing evidence of damage to membranes while others appear to remain intact. This is not surprising because the phrenic nerve has multiple communications with the autonomic nervous system within the thoracic cavity and may receive accessions of unmyelinated fibres which ascend from the autonomic plexuses of the upper abdomen. It is unlikely, therefore, that all of the intraneural unmyelinated fibres would be damaged by a single lesion at the level of the lung hilum. These ultrastructural observations support the histological evidence which suggests that the membranes of nerve fibres are among the first components to be affected by damage to the nerve trunk. This response to ligation of the nerve was seen as an increase in the tortuosity both of the limiting membrane of the axis cylinder and of the contiguous membrane of the Schwann cell which forms the mesaxon. In the present study it was not determined whether the tortuosity arose 'de novo' or if it was secondary to changes in the underlying cytoplasm.

The Schwann cells also showed changes in their limiting outlines, their undulating ovoid membranes having become distinctly more tortuous. Their nuclei were generally more electron-dense and nucleoli which were seldom seen in Schwann cells of normal animals had become clearly demarcated. Changes in the cytoplasm were not dramatic even after a survival period of 72 hours but the general impression was one of enlargement and increased activity of the Schwann cells.

When the survival period was prolonged to 96 hours, there was an obvious accentuation of each of the features described although their distribution within the transverse section of the nerve remained about the same. The axoplasm of myelinated fibres became progressively more retracted, the myelin sheaths developed larger lucid clefts and the increase in electron-dense particles became more conspicuous both in the axoplasm and in the peripheral cytoplasm of the Schwann cell. Unmyelinated fibres became more irregular in outline and appeared in a variety of bizarre shapes. In some, there was evidence of complete rupture of limiting membranes with extrusion of cytoplasm. The most dramatic change, at 96 hours was in the Schwann cells which enfold unmyelinated fibres. Several of these within one transverse section possessed the characteristics of Schwann cells at one pole and those of a macrophage at the other. This was accompanied by a conspicuous increase in the numbers and sizes of intracellular organelles, a prominent nucleolar formation and an apparently general increase in size. These characteristics suggest that the Schwann cell is capable of adopting, in part at least, the functions of a macrophage cell and that this is accompanied by a corresponding process of metamorphosis. This observation is in agreement with the findings of Blumcke (1963). The quantity of intrafascicular collagen surrounding nerve fibres appeared to have undergone an increase.

In the portion of the nerve trunk central to the level of the lesion, ultrastructural changes were generally less marked but a small minority of myelinated fibres showed axoplasmic retraction equal in extent, to that present in the peripheral portion of the nerve. In accounting for this structural change consideration was given to the level from which the sample was taken. Close to the experimental lesion the inflammatory response of the nerve to ligation is highly significant but, with increasing distance from the lesion this factor diminishes in importance. It is likely, therefore, that in the present study the influence of the tissue inflammatory response was not a major factor since the portion of nerve was approximately 2cm central to the ligature. Centripetal degeneration can be identified with the aid of the light microscope only after survival periods in excess of 10 days. The electron microscope, however, reveals axoplasmic retraction in myelinated fibres at a distance of 2cm central to the lesion after survival periods of 96 hours. There is another interesting possibility, that these myelinated fibres represent the centripetal processes of neurones which are situated peripherally but no indisputable evidence of the existence of such components was obtained in this investigation.

The unmyelinated fibres within the central portion of the nerve showed structural changes which corresponded to those observed in the peripheral portion but estimates showed that fewer fibres were involved centrally than peripherally. If the unmyelinated fibres are considered as centrifugal and centripetal in direction with reference to the phrenic nerve the findings suggest that the former exceed the latter in numbers.

2. WITHIN THE DIAPHRAGMATIC PLEXUS.

The types of nerve fibres which originate from the phrenic nerve and ramify within the diaphragmatic plexus showed very little morphological variation in cats, rats, mice and guinea-pigs. The general arrangements of the diaphragmatic plexus were demonstrated most clearly in the smaller animals of the series by the 'whole preparation' technique, but it was possible, in cats, to identify the corresponding neural components in frozen sections of the diaphragm cut at a thickness of 50µ and from this, to reconstruct the whole picture. These observations formed a basis for comparison with the diaphragmatic plexus in cats which were subjected to experimental procedures, the same preparatory techniques being employed throughout the entire series.

A. In Normal Animals.

In the general description of the distribution of the phrenic nerve (p. 211), the pattern of its terminal branching was described and reference was made to the 'nerve rich' zone which encircles the central tendon. This was the most conspicuous structural feature of the innervation of the diaphragm in all of the species examined and it has been observed by many investigators such as Ramström (1906), Waser (1963) and Aitken (1950).

Three main types of fibres arise from the fasciculi of the nerve rich zone. It was estimated that approximately two thirds of the axis cylinders are of the first type and they extend for very short distances in a centrifugal direction to terminate in the region of the mid point of the radially directed muscle fibres. The individual axis cylinders of this type are of larger diameter than any other diaphragmatic nerve fibres and, at magnifications of 20x, they appear to terminate in irregularly-shaped expansions which are argyrophilic. At magnifications in excess of 40x, these expansions were seen to be in continuity with fine filamentous axis cylinders which terminated as motor end-plates in relation to muscle fibres. It was, therefore, concluded that these neural components could be identified as efferent in function. The presence of local preterminal expansions on these fibres is difficult to explain but there is little doubt that they represent the structures described by Pansini (1888), as 'vraies ganglions intrinsiques'. In this investigation it was not possible to confirm Pansini's views and it was considered more likely that they represent preterminal axonal expansions produced by a local accumulation of neurosecretions or their precursors. The very fine axis cylinders which extend into motor-end-plate formations were comparatively short but it was observed that, in some cases, there was an elongated argyrophilic process of small diameter which extended from the preterminal expansion, for a distance of a few millimetres. There appeared to be no structural association between this component and motor end-plates but, in one specimen, the process terminated in the region of an argyrophilic structure which resembled a muscle spindle. This finding, while far from conclusive, suggested that this filamentous extension from motor components of the diaphragmatic plexus might be the effector part of the mechanism for maintenance of tone within the diaphragmatic musculature.

The second type of nerve fibre in which the diameter of the axis cylinder was generally smaller, ramified freely both in the muscular and in the tendinous part of the diaphragm. The majority of these, because they ramified as free nerve endings in the plane which separates the serosal covering from the muscle fibres, were identified as general afferent components. The remaining axis cylinders of this group, however, were of a diameter equal to that of the efferent components and they were co-planar with the muscle fibres. These were identified as the centripetal processes of muscle spindles since, according to Sherrington (1895), the fibres which subserve this function are of larger diameter than other afferent fibres.

These two groups together represent the peripheral moieties of myelinated fibres which were observed more centrally in the trunk of the phrenic nerve and, although the myelin sheaths were not distinctly visible within the diaphragmatic plexus, efficient silver impregnation made it possible to estimate the position and thickness of the myelin sheath.

The third type of nerve fibre because it was of small diameter was not observed as an individual axis cylinder but was ubiquitous in the fasciculi of the diaphragmatic plexus. These fibres were regarded as the peripheral processes of unmyelinated nerves which originate in the phrenic and intercostal nerves and in the coeliac ganglia. Fasciculi of these nerves were plainly visible, with an oil immersion lens and many of them occupied paravascular positions. They were seen to ramify also in the serosal covering of the diaphragm. Those which were situated along the walls of vessels arborised at regular intervals and the branches penetrated the adventitious coat of the vessel. The nerve fibres of the serosa ramified freely in all directions within the pleural or peritoneal membrane. No identifiable end formations were seen in relation to these fibres.

The ratio of numbers of somatic afferents to somatic efferent myelinated fibres within the diaphragmatic plexus was estimated as 1:2, which corresponds to the currently accepted corresponding ratio within the phrenic nerve. (Last, 1954). It was not possible to make an accurate quantitative study of the intrinsic unmyelinated fibres of the diaphragm by the histological methods used in this study. The profusion of these fibres present within the diaphragmatic plexus, however, was entirely compatible with the quantitative studies in the phrenic nerve.

The functional significance of these small but numerous neural components is a matter for speculation although it may be inferred that they are largely concerned in the vasomotor control of diaphragmatic blood vessels. While this explanation may be apt for those unmyelinated fibres which accompany vessels there is no convenient means of accounting for the function of those which branch freely within the serosal coverings of the diaphragm. Whatever their function, these components resemble morphologically, the unmyelinated fibres which arise from the phrenic nerve and ramify within the pericardium. It may be that the morphological similarity of the two groups of fibres denotes a common functional significance.

The relationships which exist between nerve fibres of the diaphragmatic plexus in normal animals indicate no orderly grouping of morphological types. Axis cylinders of large diameter are distributed at random along with those of small diameter within the fasciculi even in the most peripheral parts of the diaphragm. A very prominent feature of the diaphragmatic plexus is the number and variety of communications which exist between adjacent nerve fasciculi. At the point of entry of the main branches of the phrenic nerve these communications take the form of neural 'arcades', while, more peripherally, they appear as collateral axis cylinders which accompany the main fasciculi of the plexus. In addition to these relatively short communications between fasciculi there are structural connections between more widely separated components of the diaphragmatic plexus. Some of these appear to pass, by tortuous pathways, from one side of the diaphragmatic plexus to the other. The individual axis cylinders which take part in the formation of these communications arise as branches of one fasciculus and merge with the nerve fibres of another. No bundle of nerve fibres within the plexus can be regarded as entirely separate from the other fasciculi which are present within the diaphragm. Thus, it may be deduced that there is structural provision for the coordination of diaphragmatic function both within each hemidiaphragm and throughout the diaphragm as a whole. This arrangement is not surprising since synchronisation of the phasic contraction of the diaphragm is essential to symmetrical function both in respiratory and other functions of this muscle.

B. In Animals Subjected to Experimental Procedures.

Studies on experimental animals in which the phrenic nerve had been transected indicated that degeneration of axis cylinders of the diaphragmatic plexus was comparable with the changes observed in the axis cylinders of the transected nerve trunk peripheral to the lesion. Thus, after survival periods which ranged from 1-3½ days, individual axis cylinders of the plexus showed considerable widening but remained in continuity. After survival periods of 4-10 days, the argyrophilic axis cylinders became increasingly irregular in outline. Although some evidence of commencing fragmentation was observed in fibres of small diameter after survival periods of less than 10 days, this characteristic feature of degeneration was not general within the plexus until the tenth day after transection.

Throughout this study no nerve fibre was considered as having degenerated on morphological grounds unless there was clear evidence that axoplasmic continuity had been lost. It is clear, therefore, that transection of the phrenic nerve does not produce widespread degeneration of the fibres within the diaphragmatic plexus until at least 10 days after the operation.

After survival periods of 10-30 days the axoplasm of nerve fibres became progressively more fragmented and the size of argyrophilic droplets gradually decreased. A concurrent process of rarefaction of the axis cylinders appeared to be in operation for the argyrophilic material became progressively scarcer within the Schwann sheath. At 30 days after transection, which was the longest survival period allowed in this investigation, only empty Schwann sheaths remained. The presence of argyrophilic debris in the perineurial region after survival periods of 10 days or more, provided further evidence that axoplasm was being displaced either actively, by cell transport, or by passive extrusion from the degenerating axis cylinders. Active removal of axoplasmic debris by transport within cells, presumes the participation of phagocytes and, therefore, the presence of cells of the macrophage series within the fasciculi. Large ovoid intrafascicular cells have been observed in close association with degenerating axons and it seems likely that these correspond to the Schwann cells observed in ultrastructural studies on the peripheral portion of the transected phrenic nerve. It will be recalled that the Schwann cells, according to Blumcke (1963) are capable of undertaking phagocytic functions within degenerating nerves and corresponding observations made in this investigation strongly support his views.

Although the vast majority of nerve fibres which arise in the phrenic nerve and ramify within the substance of the diaphragm show degenerative changes after transection of the nerve trunk, there are, invariably, some nerve fibres present in areas normally supplied by the phrenic nerve in which these changes are absent. At survival periods of less than 10 days, it is difficult to distinguish intact axis cylinders from degenerating ones and so, firm conclusions regarding persistence of nerve fibres cannot be made in these animals. However, when persisting nerve fibres were seen after survival periods of more than 10 days, it seemed reasonable to conclude that these fibres arose from some source other than the homolateral phrenic nerve.

It has been shown that, in a small proportion of cats a connection exists between the left and right phrenic nerves within the thoracic cavity. This fact suggests one possible explanation for the existence of persisting nerve fibres within the right hemidiaphragm when the right phrenic nerve is severed, provided that the commisural fibres enter the right nerve peripheral to the level of transection. The crossing of fibres from left to right was verified by histological examination of a neuro-vascular bundle such as that discussed above. Although the communication was sufficiently large to be observed macroscopically, only in a few animals, there was histological evidence of its existence in every animal examined. Thus, transection of the right phrenic nerve was followed by degeneration in most of the fibres of the diaphragmatic plexus on the same side as the lesion but a few axis cylinders within the right crural region remained intact. Furthermore, although there was no macroscopic evidence of a corresponding communication from the right to the left phrenic nerve, persisting fibres were found in the left crural region after transection of the left phrenic nerve. In support of the possibility that there is an exchange of fibres between the phrenic nerves, it was observed that transection of one or other of the phrenic nerves was followed by the degeneration of a small number of axis cylinders in the contralateral crural region. It seems likely that this peripheral communication between left and right phrenic nerves exists to provide a pathway for the nervous coordination of the right and left halves of the diaphragm.

The methods employed for identification of persisting fibres within the diaphragmatic plexus were not suitable for accurate quantitative study but it was considered improbable that commisural fibres alone could account for the entire increase in numbers of myelinated fibres in the peripheral portion of the phrenic nerve in normal animals. Neither did it seem likely that the numbers of persisting fibres observed within the diaphragmatic plexus could be attributed to the comparatively small numbers of fibres which are exchanged between the two phrenic nerves. Accordingly three additional experimental procedures were carried out to elucidate the problem and attention was directed to the intercostal nerves and the coeliac ganglia which are in a position to provide accessory fibres for incorporation in the trunk of the phrenic nerve. Before proceeding to consider these sources of nerve fibres, however, it is convenient to consider the remaining intrinsic components of the phrenic nerve.

The Vascular Components and Connective Tissues of the Phrenic Nerve.

Adams (1942) provided an extensive review of the literature relevant to the blood supply of nerves and pointed out clearly the importance of these vessels. None of the histological techniques used in this investigation was entirely satisfactory for quantitative assessments of the vascular components of the nerve trunk. In osmium studies, the profiles of vessels were distinct while, in silver studies, transverse sections of the nerve trunk were so distorted that identification of vascular structures presented considerable difficulties. Thus, the only possible conclusion from observations with the light microscope was that there are vessels within the fasciculi of the phrenic nerve.

In ultrastructural studies, however, the vessels present within individual fasciculi and subfasciculi were clearly demonstrated. It was not possible to classify the vascular structures as arteries, veins or lymphatics but the importance of this distinction was somewhat diminished by the fact that all of the intrafascicular vessels were of the size and structure of capillaries. The best demonstration of these vascular structures and their intraneural relationships was obtained in a transverse section of the left phrenic nerve prepared by the montage method. As with the nerve fibres, absolute numbers of these structures were obtained at one level within the nerve trunk. The total of 21 vessels appears to be very small in relation to the number of nerve fibres present. It will be recalled that, at this level, there were 802 myelinated and 2,094 unmyelinated nerve fibres. The fact that this number of fibres is provided with 21 capillaries for purposes of respiration, nutrition and excretion is in agreement with the views of Weiss (1941) that the main metabolic functions of nerve fibres are carried out at the cell body rather than in relation to its processes within the nerve trunk. In view of this surprisingly low capillary density, these values were compared with the figures obtained by Krogh (1929) for other tissues such as the cerebral cortex in rats in which there were 1,470 capillaries per square millimetre. Making use of the fact that the capillary density of a tissue is directly proportional to the basal metabolic rate of that tissue, the following equation, applicable to rats, was constructed.

$$\frac{\text{Capillary density in peripheral nerve}}{\text{Capillary density in cerebral cortex}} = \frac{-QO_2 \text{ in peripheral nerve}}{-QO_2 \text{ in cerebral cortex}}$$

where $-QO_2$ stands for rate of oxygen uptake per hour, measured, in each case, by the same method.

Substituting the values obtained by Krogh for the cerebral cortex in rats and the $-QO_2$ values as expressed in the Biochemist's Handbook (1961), it was possible to arrive at an approximate estimate of the capillary density within peripheral nerves in rats. If X = Capillary density in peripheral nerves in rats, then

$$\frac{X}{1,470} \qquad \frac{1.1}{8.9}$$

Thus, the capillary density in peripheral nerve in rats is 182 per sq. mm approximately.

Since the transverse section of the phrenic nerve was approximately circular, having a radius of 0.3mm, it was possible to calculate the cross sectional area by applying the formula:-

The area of a circle = Πr^2 where $\Pi = 3.143$ approximately

and r = radius of the circle.

Thus the area of the transverse section of the left phrenic nerve at the level of the lung hilum in this particular animal was 3.143x 0.09 sq. mm which gives a result of 0.28 sq. mm. Since this area contained 21 capillaries, the capillary density of the phrenic nerve at that level is approximately 75 per sq. mm.

The difference between the values, 182 for peripheral nerve in rats and 75 for phrenic nerve in cats, may be partly explained by the higher basal metabolic rate in the smaller mammal but there may well be other significances as yet unknown. In order to elucidate this problem, total counts of the vascular structures present at several levels within peripheral nerves would require to be known.

Histological studies on transverse sections of the phrenic nerve in normal cats were useful only for defining the general arrangements of the connective tissue components of the nerve. In osmium studies it was possible to make quantitative observations on the fascicular pattern at different levels within the nerve trunk but it was not possible to examine the finer details of subdivisions within fasciculi.

A much more detailed picture was obtained from ultrastructural studies, particularly in preparations such as the montage which has been fully described. The epineurium was seen to be composed of fibroblasts and densely interwoven bundles of collagen fibres while the perineurial sheath was composed of alternating layers of fibroblasts and collagen fibres, The intrafascicular connective tissue was less dense than the epineurium and perineurium and it consisted of occasional fibroblasts interspersed with collagen fibres. While the epineurium and endoneurium may be simply described as dense and loose connective tissue respectively, the perineurium showed several structural features of particular interest.

The stratified nature of the perineurium is its most distinctive feature and the elongated cytoplasmic process of its cells were seen to extend for approximately one third of the underlying fasciculus. The cells were identified as fibroblasts in view of the long attenuated processes which they possess and because of their intimate relationships with collagen fibres. Porter and Pappas (1959) have described corresponding relationships between fibroblasts and collagen fibres in tissues other than the perineurium while the observations of Wessel (1959) led to the conclusion that collagen production occurred in the juxta-cellular position in the presence of fibroblasts. Furthermore, the identification of these cells as fibroblasts is supported by the presence of multiple branching in all layers of the perineurium. While the branching and rejoining of cell processes was seen most frequently in the intermediate layers of the perineurium, particularly where the sheaths of two contiguous fasciculi became confluent, the innermost layer of cells also formed branches at infrequent intervals. The centripetal processes of the innermost perineurial cells entered the subjacent fasciculi and formed connective tissue boundaries between bundles of nerve fibres thus forming separate small subfasciculi. This structural characteristic suggested that these small bundles of nerve fibres had either just joined the main nerve trunk or that they were about to leave it. One exceptional centripetal process of the perineurium penetrated deeply into a major fasciculus and terminated without further branching. The significance of this structure is obscure but it seems possible that it may unite with a centripetal process of another perineurial cell at an adjacent level within the nerve trunk and form a subfasciculus. At irregular intervals along this process there were small intracellular vacuoles which contained granular material of greater electron-density than the surrounding cytoplasm.

They resembled corresponding structures which were observed in other connective tissue cells and it was considered likely that their contents represented intracellular metabolites concerned in the fibroblastic function of the perineurial cells. Specialised orientation and shape are the only obvious morphological features which distinguish perineurial cells from the fibroblasts of other tissues and these differences are attributable to the function which the perineurium performs.

Peele (1961) described the perineurium as a connective tissue sheath composed of fibroblasts and collageneous fibres but Shanthaveerappa (1964) and his collaborators were of the opinion that the perineurium is partly formed by continuation of spinal meninges along peripheral nerves. In a direct comparison between the ultrastructure of spinal meninges and the perineurium it was observed, in this investigation, that the meningocytes of both the pia mater and arachnoid, in the region of spinal nerve roots, generally form a limiting membrane to the subarachnoid space and are only rarely separated from it by tissue containing collagen fibres (Wilson and Silva, 1965). It was, therefore, concluded that the perineurium is composed of fibroblasts and collagen fibres. No support was found for the existence of extensions of subarachnoid space along peripheral nerves. The recent findings of Gamble and Breathnach (1965) suggest that the perineurium is composed of 'fibroblast-like' cells and collagen fibres, thus providing support for the observations made in this investigation.

CONTRIBUTIONS OF INTERCOSTAL NERVES TO THE DIAPHRAGMATIC PLEXUS.

(A) In Normal Animals.

In mice, whole preparations of the diaphragm impregnated with silver provided a comprehensive picture of the entire diaphragmatic plexus and fibres arising from the phrenic and intercostal nerves could be easily distinguished. In the branches which arose from the intercostal nerves and entered the diaphragm at its periphery, three distinct categories of nerve fibres were identified. These have been described previously as serosal, paravascular and muscular fibres. The first two of these categories can be associated with specific functions, the serosal fibres being afferents from the pleura and peritoneum, while the paravascular fibres are concerned in vasomotor control of the diaphragmatic blood vessels. The third group consists of fasciculi in which the nerve fibres are few in number, their diameters ranging from 10-16µ approximately. In size, therefore, they resemble those fibres which were identified as efferent components of the phrenic nerve. Their immediate penetration into the muscle fibres of the diaphragm at the periphery prevented accurate tracing of the majority of these fasciculi. Those which could be traced, however, were seen to come into close relationship with the fasciculi which arose from the phrenic nerve and took part in the formation of the diaphragmatic plexus (Wilson 1963). This region of intermingling of fibres which originate in the phrenic and intercostal nerves,respectively, provides a peripheral means of communication for the coordination of diaphragmatic functions. It would appear to have the same functional significance as the connections which exist between the left and right phrenic nerves. Although no motor-end-plates were clearly identified in relation to these contributions, some of the fibres possessed irregular preterminal expansions which were observed in association with motor-end-plates in the efferent fibres which arose from the phrenic nerve. Thus, although no conclusive morphological evidence of motor-end-plates was obtained, it is possible that these muscular fibres represent the group which, in the experiments of Rosenblueth (1961) and his collaborators, gave rise to recordable contractions within the musculature of the diaphragm, when the lower intercostal nerves were stimulated.

Since, according to Wells (1954) the peripheral part of the diaphragm is developed in mesoderm which is continuous with the body wall, there is a possibility of extension of nerve fibres from the body wall into the substance of the diaphragm. Many embryologists such as Hamilton (1947) have inferred that this is so in respect of afferent fibres but there is no general agreement among anatomists that it also applies to efferent fibres. Dally (1909) attempted to summarise this viewpoint by stating that, "being polymeric, the diaphragm is essentially heteroneural" but Dally's own observations, like those of many other investigators, proved to be inconclusive regarding the participation of nerves, other than the phrenic in the efferent innervation of the diaphragm.

(B) In Animals Subjected to Experimental Procedures.

In a further attempt to define the relationships of the intercostal nerves to the innervation of the diaphragm, the effects of various experimental procedures were observed within the fasciculi of the diaphragmatic plexus in cats.

1. After unilateral transection of the phrenic nerve.

In every animal in which this experimental procedure was carried out, persistence of the diaphragmatic branches of intercostal nerves was clearly observed. The radial direction of these fibres was confirmed and it was clear that a number of them reached the region of the main fasciculi of the diaphragmatic plexus. These persisting nerve fibres were most conspicuous in animals in which the survival period was longer than 10 days and many of the preparations showed degenerated phrenic and persisting intercostal fibres within the same microscopic field. Their prominent argyrophilia in relation to the degenerated fibres of the phrenic nerve made it possible to reassess the diameter and shape of individual axis cylinders and, in all respects they confirmed the corresponding findings in normal animals.

Not all of the persisting nerve fibres within the hemidiaphragm on the same side as the lesion could be identified clearly as direct contributions from intercostal nerves. The following criteria were established in order to differentiate between those persisting axis cylinders which arose from intercostal and phrenic nerves respectively. If the axis cylinders were intact from the periphery of the specimen and formed branches when traced centripetally, they were regarded as branches of intercostal nerves. If the converse were true, the persisting fibres were identified as branches of the phrenic nerve. Although accurate quantitative studies were not possible because of the methods used in this investigation, the persisting fibres of the phrenic nerve appeared to outnumber those of the intercostal nerves in a ratio of approximately 2:1.

2. After bilateral ligation of the lower thoracic spinal nerve roots.

By ligating the centripetal fasciculi of mesenteric nerves close to their point of origin in autonomic ganglia, Schofield (1960) demonstrated preterminal widening of the corresponding centrifugal axis cylinders. This experimental technique was applied, in this investigation, to those spinal nerves which are known to contribute to the lower six intercostal nerves, namely the eighth to the thirteenth thoracic nerves in cats. After survival periods of 24 and 48 hours respectively, in two cats, preterminal widening was observed in a surprisingly large number of the axis cylinders in all regions of the diaphragmatic plexus.

It was necessary, at the outset, to distinguish between this abnormality in axis cylinders and the preterminal dilatations which were present in relation to nerve fibres within the diaphragmatic plexus in normal animals. In those animals which had been subjected to ligation of the lower six thoracic nerves, central to the spinal ganglia, preterminal widening of axis cylinders extended centrally for a distance of approximately 0.2mm from the zone of terminal arborisation. The swelling affected the individual axis cylinders in a symmetrical manner, the smooth outer margin of the axoplasm being maintained. The preterminal dilatations seen in normal animals were irregular in outline, presenting a variety of bizarre shapes and they seldom extended along nerve fibres for distances greater than 20 microns. Using these criteria, the ramifications of afferent fibres, which originate from the lower six thoracic segmental nerves were defined within the diaphragmatic plexus. The large numbers of fibres identified in this way and their distribution within the more central zones of the musculature in all regions of the diaphragm are both incompatible with the findings in the direct branches of intercostal nerves in normal animals.

These discrepancies can be considered together. If the direct diaphragmatic branches of intercostal nerves in normal animals are too few in numbers to account for all of the axis cylinders observed in this experimental study, it seems reasonable to assume that some branches of the local segmental nerves enter the diaphragm by an indirect route. In this investigation there is some support for this possible explanation. It may be that these indirect branches from the lower six thoracic nerves, originate close to the origin of the intercostal nerves and that they traverse the pericardium to reach the homolateral phrenic nerve. This arrangement would account, not only for the additional fibres identified experimentally but also for their distribution along with components of the phrenic nerve, within the diaphragmatic plexus. Masumoto (1934), found that division of the lower intercostal nerves near to their point of origin gave rise to degeneration of some fibres within the diaphragmatic plexus. Furthermore, if such fasciculi of nerve fibres were to join the phrenic nerve in the peripheral part of its intrathoracic course, they would explain the presence of increased numbers of nerve fibres within the nerve trunk at this level. Evidence in support of this possibility has been presented above in consideration of normal animals. The striking results of studies of the afferent components of branches arising from thoracic segmental nerves, suggested the possibility of carrying out corresponding studies on the efferent nerve fibres. These were not included in the present investigation, however, because of the unsatisfactory nature of available experimental methods. Selective transection of motor roots of segmental nerves is complicated in experimental animals such as cats by the necessity of mobilising the spinal cord. In order to produce satisfactory exposure of the motor roots, it is necessary, in the posterior approach, to remove the laminae of the vertebrae which overlie the appropriate segments plus the laminae of the two adjacent vertebrae rostral and caudal to these. Having, thus, achieved adequate exposure, there remains the danger of inflicting unwanted damage on adjacent nerve roots. A method of overcoming this technical difficulty is, at present, under consideration but it requires further adaptation before being applied to investigations such as this.

OTHER CONTRIBUTIONS TO THE DIAPHRAGMATIC PLEXUS.

A. In Normal Animals.

In topographical studies on normal cats the posterior region of the diaphragm was seen to receive two or three thread-like fasciculi of nerve fibres from each of the coeliac ganglia. In histological preparations the diaphragmatic branches of these nerve fibres could not be distinguished from those which arose from the phrenic and intercostal nerves. It was clear, however, that the nerve fibres of large diameter which are present in this region descend in a direction which is parallel with the muscle fibres of the crus. Their orientation is compatible with that of the posterior branch of the phrenic nerve which reaches the crural region by traversing the lateral leaf of the central tendon to descend towards the vertebral attachment of the muscle fibres of the diaphragm. The only fasciculi which could be identified as ascending from the region of the coeliac ganglion were of the paravascular variety.

It seems appropriate to mention, here, that each of the main sources of diaphragmatic nerve fibres contains a profusion of unmyelinated fibres which ramify within the diaphragmatic plexus and appear to communicate freely with one another in paravascular situations. Whether these fibres are deployed entirely in the innervation of smooth muscle in vessel walls or take part in some other autonomic function within the diaphragm remains doubtful. While most of these nerve fibres can be traced to arborisations in relation to the walls of vessels, a considerable proportion of them diverge from their paravascular positions to enter the surrounding muscular tissue. The functional significance of these non-vascular fibres of small diameter was not fully considered in the present study as their morphological characteristics could not be demonstrated with the aid of the light microscope. For satisfactory understanding of the structure and function of these neural components, extensive ultrastructural investigations beyond the scope of the present study would be essential.

B) In Animals Subjected to Experimental Procedures.

1) After left coeliac ganglionectomy.

In the animal in which the left coeliac ganglion was extirpated, the posterior portion of the diaphragmatic plexus on the same side as the lesion showed degeneration of paravascular fibres after a survival period of 5 days. The fibres of larger diameter which were present in the posterior part of the diaphragm were structurally unchanged by this experimental procedure. The findings, in this investigation, therefore, indicate that the left coeliac ganglion in cats, contributes only fibres of small diameter to the innervation of the diaphragm.

2) After mobilisation of the phrenic nerve.

The experimental procedure, referred to above as 'mobilisation' of the phrenic nerve has been described in detail.

In this animal the survival period was 10 days because previous studies indicated that, after this interval, degeneration of axis cylinders was most easily defined. On the same side as the lesion one large fasciculus of nerve fibres within the diaphragmatic plexus showed degeneration of all axis cylinders. This was regarded as an indication that, in this animal, the phrenic nerve received an accession of fibres in the peripheral third of its intrathoracic course and that these fibres, damaged by separating the nerve trunk from its surrounding connective tissue in the thorax, accompanied the fibres of the homolateral phrenic nerve to their distribution within the diaphragm. Post-mortem examination of the thorax produced no evidence of a macroscopic nature to confirm the presence of this accession but subsequent histological studies on the pericardium showed that the phrenic nerve was joined by several nerve fibres at intervals along its length, in the lower part of the thorax. There was no evidence of prepericardial commisural fibres from the other phrenic nerve and dissection at the thoracic inlet showed that there was no accession to the nerve in that region.

GENERAL CONCLUSIONS.

Quantitative studies on the myelinated fibres within the phrenic nerve in normal adult cats suggest that the nerve trunk may receive an accession of fibres in the peripheral third of its intrathoracic course. Qualitative findings in support of this possibility were obtained from histological observations on the myelin sheaths and axis cylinders which were identified in transected phrenic nerves peripheral to the level of the lesion. Studies on the diaphragmatic plexus in those animals which had been subjected to unilateral transection of the phrenic nerve indicated that the phrenic nerves each contribute a few fibres to the contralateral hemidiaphragm. In a small proportion of the animals studied there was clear topographical evidence of these commisural fibres which were, however, too few in number to account adequately for appreciable increase in the numbers of myelinated fibres in the peripheral third of the phrenic nerve. Although indisputable proof of the existence of peripheral accessory fibres was not obtained, the results of experimental studies on the lower thoracic nerves are almost impossible to explain in the absence of such a communication. Accessory fibres, such as those which may arise from the lower intercostal nerves and enter the diaphragm by way of the phrenic nerve would explain some of the conflicting conclusions derived from previous investigations on the innervation of the diaphragm. Furthermore, such an arrangement would provide a peripheral pathway for the coordination of function of the two main muscular masses which are responsible for respiratory movements, namely the diaphragm and intercostal muscles. When the right phrenic nerve was separated from its surrounding connective tissue in the peripheral third of its course, the degeneration of a large homolateral intradiaphragmatic fasciculus of nerve fibres provided further evidence that such an accession exists.

Ultrastructural studies on the phrenic nerve in normal cats resulted in the finding of absolute numbers of nerve fibres present within the nerve trunk at one particular level. Moreover, it facilitated the counting of unmyelinated fibres which could not be demonstrated consistently by the histological methods used in this investigation. Much additional quantitative information was obtained in respect of the vascular components and connective tissues present within the nerve trunk and it was found that, the 'montage' method provided a comprehensive picture of the perineurium. In animals which were subjected to unilateral ligation of the phrenic nerve, the effects of the procedure on the fibres within the nerve peripheral to the level of the lesion were comparable to those observed in histological studies. Thus, it was found, that the ultrastructural characteristics of a small minority of myelinated nerve fibres remained unchanged although the vast majority appeared to be undergoing degeneration after survival times of 72 hours. The significance of these experimental observations on the ultrastructural characteristics of ligated phrenic nerves is not entirely certain, since the nerve trunk remained in continuity at the site of the lesion. In order to confirm these observations further ultrastructural studies would require to be carried out on transected phrenic nerves.

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APPENDIX I.

1. Silver techniques for nerve fibres.

A. Bodian (1936)

1) Portions of phrenic nerve 1cm long are fixed in 10% neutral formalin. Paraffin sections, cut at a thickness of 5µ, are placed on slides, dewaxed, coated with nitro-cellulose to prevent detachment of the section and hydrated.

2) The sections are immersed overnight at 37°C in Protargol solution, the composition of which is described below.

3) Rinsing of the preparations is then carried out in running tap water for 2 minutes and in two changes of distilled water for a total of 2 minutes.

4) Reduction is then effected by immersion of the sections in a solution which contains 1gm hydroquinone and 5gm sodium sulphite per 100ml distilled water. The sections are then washed in running tap water for 10 minutes followed by several changes of distilled water for 10 minutes.

5) The preparations are then placed in a chilled 0.5% solution of gold chloride for 10 minutes and then in several changes of distilled water in which they are rinsed gently.

6) Blackening of nerve fibres is then induced under microscopic control in 0.25% oxalic acid, this being achieved usually

after about 3 minutes. This is followed by a rinse in tap water and immersion in 5% sodium thiosulphite for 5 minutes. (As a final step the sections may be counterstained but this was omitted from the present study).

PROTARGOL SOLUTION.

This is prepared from protargol (Winthrop-Stearns Inc.) in the following way. 0.5gm protargol is sifted gently into distilled water (50ml) and allowed to dissolve at room temperature for approximately 2 hours. The resulting solution is placed in an upright Choplin jar containing 5gm of clean copper wire in convenient lengths.

Acid clean glassware is used throughout the procedure.

B. Romanes (1950).

1) Fixation of nerve portions is produced by immersion in 10% neutral formalin and paraffin sections, cut at a thickness of 5µ are mounted on slides with dichromate gelatin solution as described in method 2 (below). The sections are then dewaxed and hydrated.

2) The sections are placed in solution A for 16 hours in total darkness at 58^{0}C, after which they are immersed directly in solution B for reduction which takes about 5 minutes. (The composition of solutions A and B are described below). This is followed by thorough washing, first in tap water and

then in distilled water.

3) The sections are then immersed in 0.5% gold chloride for 10 minutes and washed again in distilled water.

4) Immersion in 2% oxalic acid is then carried out, the optimum time for this procedure being about 1½ hours after which the sections are thoroughly rinsed and placed in 5% sodium hyposulphite.

5) After a thorough wash the tissues are dehydrated, cleared and mounted.

SOLUTION (a).

0.1% silver nitrate	3ml
0.1% sodium chloride (A.R.)	1ml
Distilled water	100ml

These substances are thoroughly mixed just before use and the pH of the solution is adjusted to 7.8 by the addition of a very dilute ammoniacal solution.

SOLUTION (b).

Hydroquinone	1gm
Anhydrous sodium sulphite (A.R.)	10gm
Distilled water	100ml

C. Jones (1936)

1) A portion of spinal cord 4-5cm long is removed from a cat and placed on cotton wool soaked in 1% ammoniated alcohol. A large darning needle is passed up through the spinal cord parellel with the long axis and left 'in situ', both ends protruding from the cut surfaces. A slightly longer portion of the phrenic nerve (almost its entire intrathoracic part) is removed from the animal, threaded through the cord and secured firmly at one end. The cord is then stripped of its outer meningeal coverings and suspended in a vertical tube which is held upright within a measuring cylinder. A weight 12-14gm, is tied to the protruding lower end of the nerve and the whole preparation is immersed in a solution of 1% ammoniated alcohol for 48 hours.

2) The tissues are washed for 1-3 minutes in distilled water and then immersed in pyridine for 24 hours after which they are washed again in many changes of distilled water for 24 hours.

3) Tissues are immersed in 2% silver nitrate for 3-5 days at $37^{O}C$ and then rinsed with distilled water for 30 seconds.

4) Reduction is achieved by immersion in the following solution for 24 hours:-

4gm pyrogallol

100ml 5% formalin

and further washing is carried out for 24 hours in running tap water.

5) Dehydration is effected by immersion for 30 minutes in each of the following concentrations of alcohol, 50%, 70%, 80%, 90% and 100% (bis).

6) Sections are cut at a thickness of 3μ and are mounted on slides without further counterstaining or toning.

2. Osmium technique for myelinated fibres of peripheral nerves.

The method used in this study incorporated procedures described by Flemming, Loyez and Pal.

Portions of the phrenic nerve, removed with minimal delay (5-10 seconds) from live animals are placed on cardboard frames to reduce longitudinal shrinkage during fixation.

1) The tissue is placed in Flemming's solution, the composition of which is described below. After 5 hours it is removed and washed in running tap water overnight.

2) The nerve portions are removed from the frames and dehydrated by immersion, for 30 minutes, in each of the following concentrations of alcohol:- 50%, 70%, 80%, 90% and 100%. The specimens are then cleared in cedarwood oil for 24 hours and embedded in paraffin wax.

3) Sections, cut at a thickness of 6μ, are mounted on slides with specially prepared adhesive which consists of:-

Potassium dichromate	200gm
Gelatin	200gm
Water	1 litre

(Preliminary examination of the sections at this stage permits selection of those which are complete and transversely orientated, with reference to the nerve trunk, for further processing.)

4) The sections are then incubated overnight at 37°C in concentrated formalin vapour. This is followed immediately by dewaxing and hydration.

5) Intensification of staining of the myelin sheath is effected by immersion in the following solution for 24 hours at 37°C:-

10% haematoxylin in absolute alcohol	10ml (ripened solution)
Saturated lithium carbonate	1-2ml
Distilled water	90ml

This is followed by rinsing in tap water.

6) Differentiation of myelin sheaths from surrounding tissue components is carried out in the following two solutions:-

a) 0.25% aqueous potassium permanganate.

b) (Pal's solution)

1% aqueous oxalic acid	50ml
1% sodium sulphite	50ml

The sections are immersed in solution (a) for ½-3 minutes, rinsed in distilled water and transferred to solution (b). After a quick rinse in distilled water the section is examined to assess the specificity and intensity of staining in the myelin sheaths. If further differentiation is required, the procedure is repeated using shorter times in solutions (a) and (b) respectively. Differentiation is regarded as complete when the sheaths are uniformly black and the axoplasmic space is clear.

FLEMMING'S FLUID.

Solution A	2% osmium tetroxide
Solution B	1% chromium trioxide (freshly prepared)
Solution C	glacial acetic acid

The solution is prepared by mixing solutions A, B and C, in the proportion of 4:15:1.

3. Preparation of portions of the phrenic nerve for ultrastructural studies.

Portions of the phrenic nerve approximately 5mm long are placed on small cardboard frames and immersed for 2-4 hours in freshly prepared, ice-cold fluid fixative prepared according to the method of Dalton. (1955).

The solution has the following composition.

Solution D. 4% potassium dichromate in water neutralised with normal potassium hydroxide.

Solution E. 3.4% sodium chloride

One part of solution D is mixed with one part of solution E and added to two parts of 5% osmium tetroxide. The temperature of the fixative is maintained at 0-5°C during fixation. Subsequent dehydration and embedding and sectioning of the nerve have been described above (p. 113).