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THE INNERVATION OF THE AIR PASSAGES OF THE AVIAN LUNG AND OBSERVATIONS ON AFFERENT VAGAL PATHWAYS CONCERNED IN THE REGULATION OF BREATHING

by

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I. INTRODUCTION.

The purpose of this thesis is to contribute new knowledge on the peripheral nerve pathways involved in the regulation of breathing in birds using Gallus domesticus as the subject. Such a project was undertaken for three main reasons. Firstly, very little is known about this aspect of respiratory physiology in birds. Secondly, any information on the regulation of breathing in birds is likely to be helpful in our understanding of the control mechanisms involved in the regulation of breathing in other classes of vertebrates. A final important consideration is the same as that motivating all the work of the Unit of Avian Anatomy of which this study is part, and is the very important position held by the poultry industry in the agricultural economy of the United Kingdom. The viability of the industry inevitably rests on informed preventive medicine and husbandry which in turn are based on a firm understanding of the anatomy and physiology of birds.

Basically the investigation tries to find the answers to three questions:- What is the distribution of the nervous tissue in the intrapulmonary air passages? What afferent nerve pathways in the vagi arise in these passages? What is the role of the afferent vagal pathways in the control of breathing? The distribution of the nervous tissue was studied by gross dissection and light microscopy. The choice of histological techniques was strongly influenced by the special interest of the investigation in the afferent

control of breathing with the result that most of the techniques that were used are generally believed capable of demonstrating the endings of the pathways. For further evidence of the pathways it was necessary to turn to the range of experimental techniques that have formed the basis of extensive studies in other vertebrates. Obviously the most useful sort of information on any afferent pathway includes evidence of its function. The function of an afferent pathway, however, can only be precisely known by demonstrating the reflex of which the pathway is part. To do this, the response to a specific stimulus must be first established and then abolished by interrupting the presumed afferent pathway. However, previous investigations in birds using this approach have met with very mixed results. The only really successful line of study seems to have been that of Peterson & Fedde (1968), Fedde & Peterson (1970) and Peterson (1970) which established the existence of a CO_2-modulated vagal chemoreflex with sensory endings in the walls of the intrapulmonary airways. In this reflex a physiologically low level of CO_2 in the airways results in shallower breathing; a physiologically high level of CO_2 causes hyperventilation. This chemoreflex is now also thought to be responsible for the apnoea described in many accounts of the regulation of breathing which used a unidirectional artificial ventilation technique with ventilating gases low in CO_2 (Bordoni, 1888; Treves, 1905; Foà, 1911; Van Matre, 1957; Burger & Lorenz, 1960).

Direct evidence like this for other pulmonary reflexes is minimal. Most investigations of them have been primarily concerned with identifying an inflation stretch reflex similar to the vagal reflex first described in mammals by Hering (1868) and Breuer (1868) in which sustained distension of the lungs subsequently inhibits the inspiratory effort. Evidence for such a reflex in birds was claimed by Langendorff (1893), Bourgeois (1896), Siefert (1896), Grober (1899), Graham (1940), Hiestand & Randall (1942), Blankart (1960) and Richards (1968, 1969); in general, these workers claimed to have demonstrated a relatively weak apnoeic response after distension of the lungs by inflation or by clamping the trachea in the inspiratory phase of breathing, the apnoeic tendency being prevented by bilateral cervical vagotomy. Fedde (1970), however, showed that any tendency to apnoea here is in fact mainly due to the CO_2-modulated chemoreflex. He argued that inflation washes CO_2 out of the intrapulmonary air passages and into the air sacs, the physiologically low concentration of CO_2 in the airways then exciting chemoreceptors in the air passages to cause apnoea. However, although Fedde found that 6 to 8% CO_2 in the inflating gas did prevent apnoea, there was still a slight prolongation of expiration which was related to the inflation pressure. This observation of Fedde then seems to be the only really direct evidence that there is for a stretch reflex in birds for which no alternative explanation is available. Burger (1968) also felt that the apnoeic response supported the presence of a stretch reflex

but proposed that the low level of CO_2 in the airways acts directly on mechanoreceptors. Fedde (1970) concedes the possibility that one type of afferent nerve ending may lie in the air passages which is modulated by two different sorts of stimuli, stretch and $\overset{\text{low}}{\wedge}\ CO_2$. This question is at present under intensive investigation in our laboratories by V. Molony.

There is no doubt then, that apart from the CO_2-modulated chemoreflex great difficulties exist in birds in demonstrating pulmonary reflexes and therefore establishing precisely the function of the afferent pathways in the vagi. Consequently it was felt that the present investigation was unlikely to obtain any new evidence for the afferent pathways by using this approach. Instead, three other experimental techniques were chosen to investigate the pathways which it was believed might produce fresh evidence. Two of these methods, vagotomy and electrical stimulation of the central stump of the vagus, have been frequently used already in birds although there seems to be little agreement on their effect on breathing. The third technique, the electrophysiological recording of afferent unit activity in the peripheral stump of the vagus, had not been reported before in birds.

II. NOMENCLATURE.

A widely varied terminology exists for the parts of the avian lung. The nomenclature used in this account is based on that suggested by King (1966a).

(1) Primary Bronchus.

The primary bronchus begins at the syrinx and extends caudally through the lung to end at its connexion with the abdominal air sac (fig. 1).

(2) Secondary Bronchi.

The secondary bronchi are named according to their origin from the primary bronchus. In Gallus domesticus there are typically four craniomedial bronchi, eight caudodorsal bronchi, eight caudoventral bronchi and twenty-five to thirty caudolateral bronchi (fig. 1).

(3) Tertiary Bronchi.

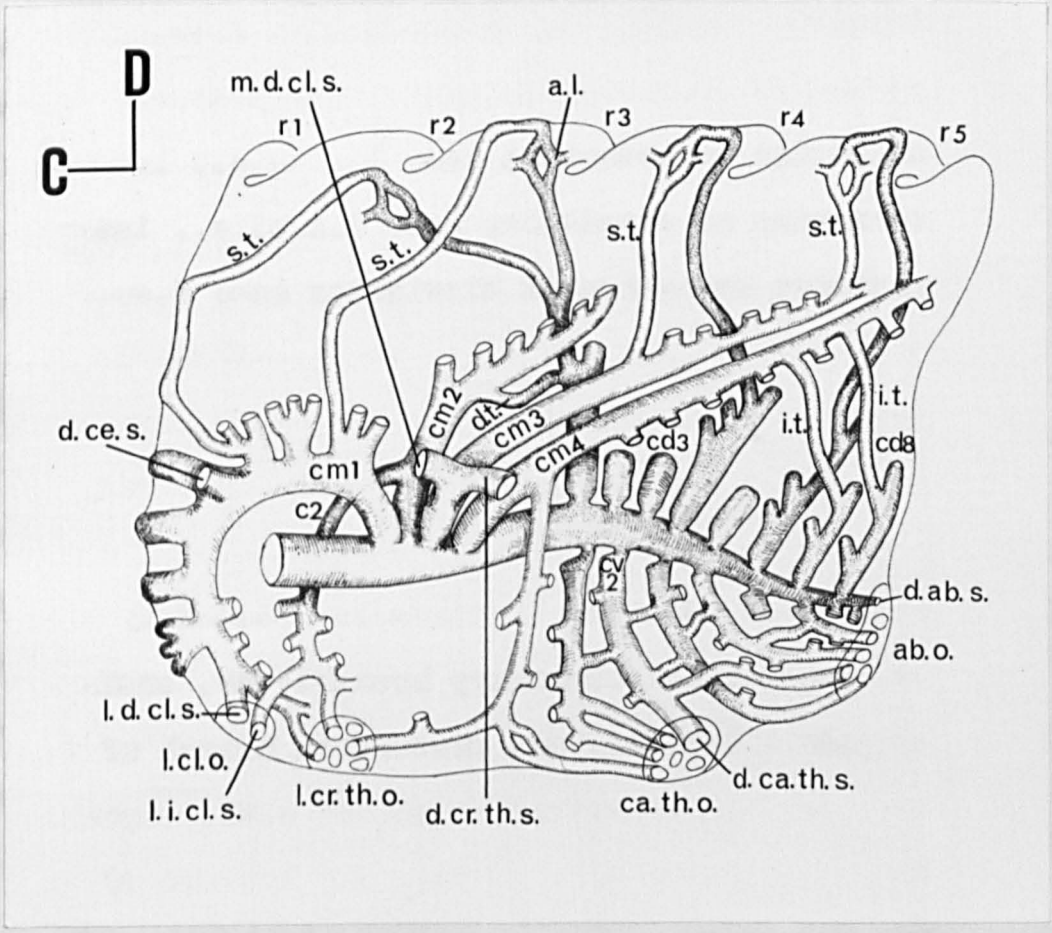
The several hundred tertiary bronchi originate from the secondary bronchi and anastomose with each other (fig. 1). In the walls of the tertiary bronchi are many openings which lead into roughly spherical chambers called atria (figs. 28 and 34). The atria are separated from each other by connective tissue interatrial septa (figs. 34, 35 and 36) and lead to the gaseous exchange areas of the lung (figs. 28 and 31).

(4) Air Capillaries.

The air capillaries form the gaseous exchange areas of the lung as an anastomosing network of minute air tubes which

Figure 1. Diagram of a medial view of the right lung of Gallus domesticus to show the primary bronchus, main secondary bronchi, and examples of tertiary bronchi. Caudolateral secondary bronchi are not visible.

C,cranial; D,dorsal; m.d.cl.s., medial direct connexion to clavicular sac; d.cr.th.s., direct connexion of cranial thoracic sac; d.ca.th.s., direct connexion of caudal thoracic sac; d.ab.s., direct connexion of abdominal sac; l.d.cl.s., lateral direct connexion of clavicular sac; l.i.cl.s., lateral indirect connexion of clavicular sac; d.ce.s., direct connexion of cervical sac; ab.o., ostium of abdominal sac; ca.th.o., ostium of caudal thoracic sac; l.cr.th.o., lateral ostium of cranial thoracic sac, comprising indirect connexion only; l.cl.o., lateral ostium of clavicular sac; cm, craniomedial secondary bronchi; cd, caudodorsal secondary bronchi; cv, caudoventral secondary bronchi; c2, circumflex branch of second craniomedial secondary bronchus; s.t., superficial tertiary bronchi; i.t., tertiary bronchi of intermediate depth; d.t., a deep tertiary bronchus; a.l, anastomotic line; r1 to r5, five impressions made by ribs (in G. domesticus by ribs 2 to 6, inclusive). (From King, 1966a, by permission of the Academic Press).


D
C
m.d.cl.s.
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r1
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s.t.
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l.cr.th.o.
d.cr.th.s.
ca.th.o.
d.ca.th.s.

communicate by the atria with the tertiary bronchi (figs. 28 and 31). An extensive network of blood capillaries lies between the air capillaries.

(5) Air Sacs.

The air sacs are extrapulmonary extensions of the primary and secondary bronchi. In G. domesticus there are eight definitive sacs; one median cervical, one median clavicular, two cranial thoracic, two caudal thoracic and two abdominal sacs. The sacs make "direct" connexions with the secondary bronchi and "indirect" connexions with the tertiary bronchi at regions of the lungs called ostia (fig. 1).

(6) Lung Lobules.

A lung lobule is a descriptive histological term widely used for a tertiary bronchus and the atria and air capillaries which surround the bronchus and communicate with it. Adjacent lung lobules are usually separated by connective tissue septa (figs. 27 and 31), although sometimes the air capillaries of different lobules anastomose with each other.

III. REVIEW OF THE LITERATURE.

A. The Innervation of the Intrapulmonary Air Passages.

(1) The Macroscopic Evidence for Pulmonary Branches of the Vagus in Gallus domesticus.

There is little agreement on the number of branches of the vagus supplying the lung of Gallus domesticus. As many as six of these nerves appear to have been observed by Hsieh (1951, pp. 60-62) and Peterson (1970, pp. 12-13). Further details were provided mainly by Hsieh (1951, pp. 60-62) with some observations by Cords (1904), Kaupp (1918, p. 274), Van Matre (1957, pp. 51-53), Watanabe (1960), Fedde, Burger & Kitchell (1963b) and Peterson (1970, pp. 12-13). Unfortunately, most of these accounts are of very limited value as they do not provide photographic evidence of the innervation and their highly schematic line diagrams fail to demonstrate the true distribution of the nerves.

According to Hsieh (1951) the nerves originate from the vagus at the level of the pulmonary artery and vein although the most rostral branch, the "pulmono-oesophageal" nerve of Fedde et al. (1963b), sometimes arises more rostral to this from the recurrent branch of the vagus. Van Matre (1957), who alone in G. domesticus described pulmonary nerve branches from a collar of the vagus around the pulmonary artery, found more than one pulmonary branch of the recurrent nerve. Hsieh (1951) divided the pulmonary nerves of the vagus into rostral, middle and caudal groups which with

branches of the sympathetic "cardiac" nerve form a pulmonary nerve plexus outside each lung on the lateral surfaces of the pulmonary blood vessels. Fedde et al. (1963b) however, recognised rostral and caudal pulmonary nerve plexuses on each side of the body, but emphasised the variations in the distribution of the nerves here. In most accounts, branches of the pulmonary nerve plexus enter the lung with the pulmonary artery and vein although Hsieh also noted some nerves which formed a network under the visceral pleura on the ventral surface of the lung. Apparently nerves do not enter the lung at the hilus with the primary bronchus.

Obviously further studies are needed to fill in the gaps in our knowledge of this area of avian anatomy. These studies moreover should go some way towards compensating for the inadequacies of most of the existing line diagrams by presenting the photographic evidence which is so essential as a basis for experimental studies on the innervation.

(2) The Histological Evidence for an Innervation of the Intrapulmonary Air Passages.

There are serious problems in assessing the very limited histological investigations of the innervation. Firstly, the widely varied terminology that has been used for the passages makes it difficult to identify them precisely in some of the accounts. Furthermore, several authors (Arimoto & Miyagawa, 1930; Okamura, 1930; Takino, 1933b; Toussaint-Francx & Toussaint-Francx, 1959)

described more or less simultaneously the innervation of the lungs of both birds and other classes of vertebrates without always allowing for differences in the structure of the lungs. In the present account an attempt is made to relate the findings of these authors to the terminology suggested for the air passages by King (1966a) (pp. 5-6). Only the evidence that is definitely known to have been obtained from birds is considered here.

Most investigations have used Gallus domesticus. The relatively few observations in other avian species include the duck, falcon, buzzard, pigeon, canary and sparrow. Nearly all of these studies have employed a silver technique although limited observations were also made with the methylene blue, cholinesterase and fluorescent methods. However, only Okamura (1930), Toussaint-Francx & Toussaint-Francx (1959) and Akester & Mann (1969) used more than one of these techniques. The species and staining methods used to study the innervation are listed in Table 1.

(a) Distribution of Nervous Tissue.

(i) Primary Bronchus.

A nerve plexus in the peribronchial connective tissue was described by Arimoto & Miyagawa (1930), Takino, (1933a,b) and Muratori (1934). The plexus has many myelinated fibres (Arimoto & Miyagawa 1930; Takino 1933b) and is best developed proximally (Muratori, 1934). The ganglia here according to Takino (1933a) are smaller than in other parts of the lung and Muratori (1934) found that they became less numerous in the distal part of the plexus. An innervation

Author	Species	Staining Methods
Eberth (1863)	falcon,pigeon,G. domesticus,buzzard.	unknown
Arimoto and Miyagawa (1930)	pigeon.	silver
Okamura (1930)	G. domesticus.	silver, methylene blue
Takino (1933a,b)	G. domesticus,pigeon.	silver
Muratori (1934)	G. domesticus,sparrow, canary.	silver
Hsieh (1951)	G. domesticus.	silver
Van Campenhaut (1955,1956)	G. domesticus.	silver
Toussaint-Francx and Toussaint-Francx (1959)	G. domesticus,duck.	silver, methylene blue
Akester and Mann (1969)	G. domesticus.	cholinesterase and fluorescent methods
Bennett and Malmfors (1970)	G. domesticus.	fluorescent method

Table 1. The species and staining methods used to study the innervation of the air passages of the avian lung.

of the muscle layer and lamina propria of the primary bronchus was reported by Takino (1933a,b) and Muratori (1934). Some of the nerve fibres in the muscle layer are adrenergic (Bennett & Malmfors, 1970). Intraepithelial fibres were seen in the proximal part of the bronchus by Muratori (1934). Other observations of an epithelial innervation in the avian lung by Takino (1933b) and Toussaint-Francx & Toussaint-Francx (1959) are also likely to have been made in the primary bronchus. A few details of the ganglia in the wall of the primary bronchus were given by Takino (1933a,b).

(ii) Secondary Bronchi.

The accounts of Takino (1933a) and Muratori (1934) imply that the innervation of the secondary bronchi is very similar to that of the primary bronchus. However, only Van Campenhaut (1955, 1956) has described the innervation of these secondary bronchi in any detail. He believed them to be the most profusely innervated air passages in the lung. Many of the nerve bundles lay in the lamina propria and formed a plexus which was especially well-developed next to the epithelium but he was unable to determine if fibres from the plexus actually penetrated the epithelium. The ganglia of the secondary bronchi are small with about 2 to 10 cells.

(iii) Tertiary Bronchi and Air Capillaries.

A nerve plexus was described in the interlobular connective tissue septa by Takino (1933a,b) and Muratori

(1934). The ganglia here according to Muratori are small with only about 6 to 10 cells. Fibres from this plexus pass between the air capillaries (Takino, 1933a,b; Hsieh, 1951, p. 81) to be distributed to the muscle of the tertiary bronchi (Takino, 1933a,b). There are very few observations on the innervation of the tertiary bronchi. A cholinergic innervation was described in the muscle by Akester & Mann (1969). Adrenergic fibres have not been found (Akester & Mann, 1969; Bennett & Malmfors, 1970) inspite of pharmacological evidence for their presence (King & Cowie, 1969). Even less is known about the innervation of the air capillaries. Certainly nerve fibres have not been seen to end here. Only a few ganglia are believed to lie in this part of the lung (Takino, 1933a; Muratori, 1934).

(b) Ganglion Cells.

The nerve cells in the lung are generally believed to be predominantly multipolar (Arimoto & Miyagawa, 1930; Takino, 1933a; Muratori, 1934; Van Campenhaut, 1955, 1956) although Takino (1933a,b) found in addition a small number of pear-shaped unipolar and bipolar cells. The smallest cells that were reported had a diameter of 10μm (Muratori, 1934); the largest cells had a diameter of 28μm (Takino, 1933a). Arimoto & Miyagawa (1930) and Van Campenhaut (1955, 1956) described the argyrophilia of their cytoplasm.

In the lamina propria of the secondary bronchi Van Campenhaut (1955, 1956) also identified ovoid or globular nerve cells as being different in a number of ways from

the other nerve cells in the lung. An outstanding feature of these cells apparently was that their cytoplasm was much less argyrophilic than that of the other ganglion cells. He believed that these cells might by sensory and part of a local reflex.

(c) Sensory Nerve Endings.

From the accounts of Cauna (1959), Miller & Kasahara (1964) and Dwinnell (1966) it appears that in vertebrates in general the following two basic types of sensory nerve endings are widely recognised with the light microscope.

(i) Free fibre endings are unencapsulated and can be divided into simple and complex varieties. The simple free fibre type are slightly branched or unbranched terminations of small myelinated or unmyelinated fibres. The complex free fibre type are discrete structures formed by the repeated branching of the terminal fibres (e.g. Ruffini endings).

(ii) Encapsulated endings are characteristically discrete and have a capsule of modified Schwann cells or connective tissue elements (e.g. Meissner's corpuscles).

In addition, there is some evidence that the neurite-receptor cell complexes (e.g. Merkel cells), first classified (on the basis of electron microscopy) as a distinct type of afferent nerve ending by Munger (1965), can also be seen with the light microscope (e.g. Fröhlich, 1949; Cauna, 1959).

The very few observations on the sensory innervation of the air passages of the avian lung with the light microscope have so far revealed only free unencapsulated

intraepithelial endings in the primary bronchus (Takino, 1933b; Muratori, 1934; Toussaint-Francx & Toussaint-Francx, 1959). Fortunately, much more histological evidence exists for pulmonary receptors in other vertebrates which it was felt might be profitably used as a comparison in the present investigation. Consequently, the accounts of the innervation of the lungs of amphibians, reptiles and mammals that are listed in Table 2 were very carefully studied. Unfortunately, it is very difficult to critically appraise a great deal of this evidence for receptors since it is presented in the form of line diagrams with few photographs. A very striking feature of most accounts, however, is the very limited histological evidence on which claims for endings appear to be based. Whilst all the basic types of receptor have been seen in the air passages there is little agreement on their detailed structure. Elftman (1943) believed that this depended upon the precise position of the receptor in the lung and Fisher (1962, pp. 77-78) noted that in general endings were usually better developed at the bifurcation points of the air passages. The method of staining is another factor thought to influence the appearance of the endings. A good example of this is the complex free fibre intramuscular endings of Fisher (1962, p. 70) which with the methylene blue and gold chloride techniques had numerous fibrils with end-knobs, whereas with the silver method the terminal fibres stood out more clearly since fewer end-knobs were visible.

Most accounts of endings appear to conform to descrip-

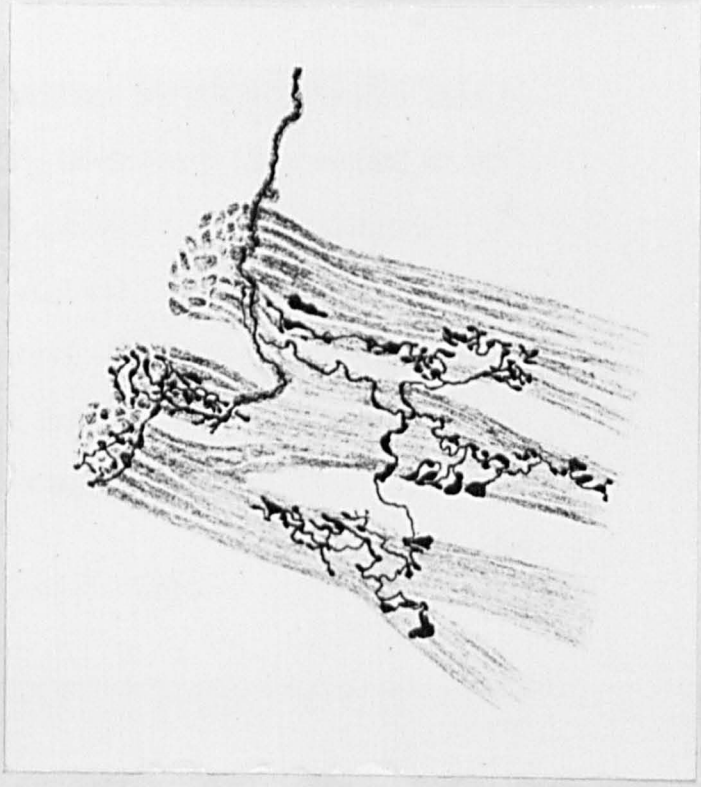
Class of Vertebrate	Authors
Amphibian	Stirling (1876,1882), Egorow (1879), Cuccati (1888,1889), Smirnow (1888), Wolff (1902), Okamura (1930,1937), Bronkhorst & Dijkstra (1940), McLean & Burnstock (1967a,b).
Reptile	Wolff (1902), Jones (1926), Arimoto & Miyagawa (1930), Muratori (1930,1931a,b,c, 1934), Abraham (1941), McLean & Burnstock (1967b).
Mammal	Berkley (1893,1894), Budde (1904), Ponzio (1906), Hill (1907), Müller (1911), Miller (1918), Larsell (1921,1922,1923), Glaser (1927), Arimoto & Miyagawa (1930), Okamura (1930,1937), Terni (1930), Larsell & Dow (1933), Sunder-Plassmann (1933a,b, 1935,1938), Takino (1933b), Gaylor (1934), Hayasi (1937), Takino & Watanabe (1937), Dijkstra (1939), Bronkhorst & Dijkstra (1940), Elftman (1943), Gasparini (1947,1948a,b), Fröhlich (1949), Koelle (1950), Magnenat (1951), Conti & Bariatti (1953), Cookson (1953), Feyrter (1953,1954a,b, 1966), Mitchell (1953), Honjin (1954,1956), Yagita (1954), Mohr (1955), Saito,M. (1955), Numata (1956), Omoto (1958), Toussaint-Francx & Toussaint-Francx (1959), Fukase (1960), v.Hayek (1960), Honma (1960), Saito,R. (1961), Fisher (1962,1963), Spencer & Leof (1964), Dahlström, Fuxe,Hökfelt,& Norberg (1966), Daly & Hebb (1966), Terayama & Soda (1967), Ziemiański, Obrębowski & Kompf (1967), Blümcke (1968), Hebb & Mann (1968), Hirsch,Kaiser,Barner,Cooper & Rams (1968), Blümcke,Dellschau,Nasseri,Eisele, Städtler & Bücherl (1969), Blümcke,Rode,Niedorf, Nasseri,Eisele,Städtler & Bücherl (1969), Blümcke & Schmidt (1969), Lauweryns & Peuskens (1969), Nishimura & Takasu (1969), Fillenz & Woods (1970).

Table 2. The accounts of the innervation of the intrapulmonary air passages of amphibians, reptiles and mammals that were studied in the present investigation.

tions of the simple free fibre type, although on morphological grounds alone there is very little to indicate that these are sensory structures, distinct from the rest of the nervous tissue. The evidence for the complex free fibre type of ending is much more convincing (figs. 2 and 3) even although many of the illustrations of the endings leave much to the imagination. These complex endings are typically described as coming from thick fibres which divide rapidly into several orders of fine branches sometimes with variscosities. Many of the terminal branches appear to end freely, whilst others end in a more complicated way such as by small enlargements or even in fine networks. The evidence for encapsulated endings (fig. 4), which on morphological grounds are presumably the easiest to recognise, is relatively limited. It is probably significant that Fisher (1962, p. 96, 1963) who seems to have demonstrated the most profuse innervation in the mammalian lung was unable to find this type of ending. Neurite-receptor cell complexes seem to have been found in the bronchial epithelium. The receptor cells, compared with other epithelial cells, are more granular (Sunder-Plassmann, 1933a) or more lightly stained (Fröhlich, 1949). However, much of the evidence for an epithelial innervation does not include specialized cells but resembles more the complex free fibre type of ending (fig. 5).

Figure 2. One of the first detailed drawings of a complex free fibre afferent nerve ending in the muscle layer of a mammalian bronchus, from Larsell (1922). According to Larsell, the terminal branches of this "smooth muscle spindle" wrap about the muscle bundles or pass between the muscle fibres. All fibres end in knobs or leaf-like expansions. Methylene blue. Approximately X 430.

Figure 3. Photomicrographs of complex free fibre afferent nerve endings in the muscle layer of a human bronchus stained by silver techniques, on the left from Gaylor (1934), scale unknown; on the right from Spencer & Leof (1964), approximately X 470.



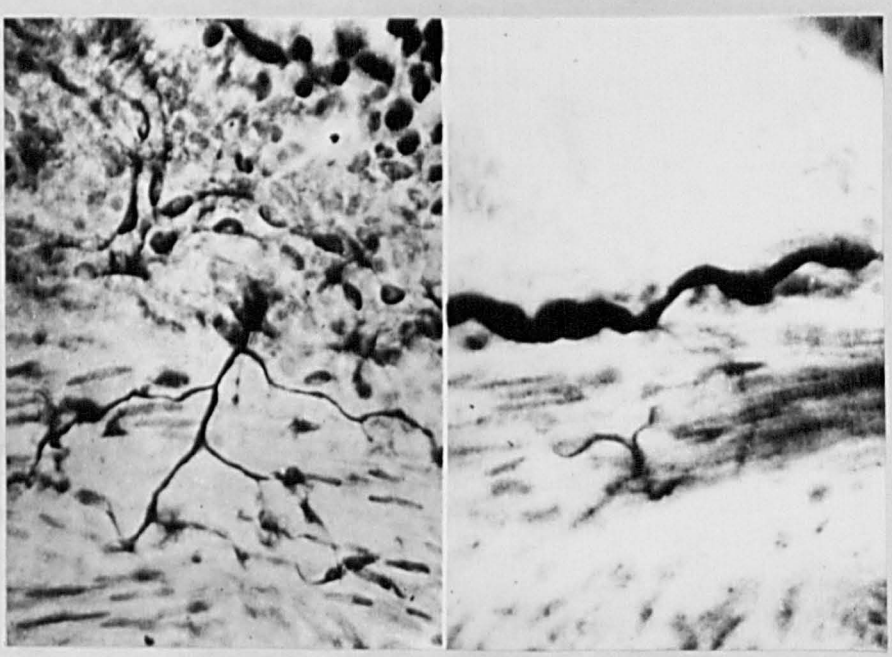
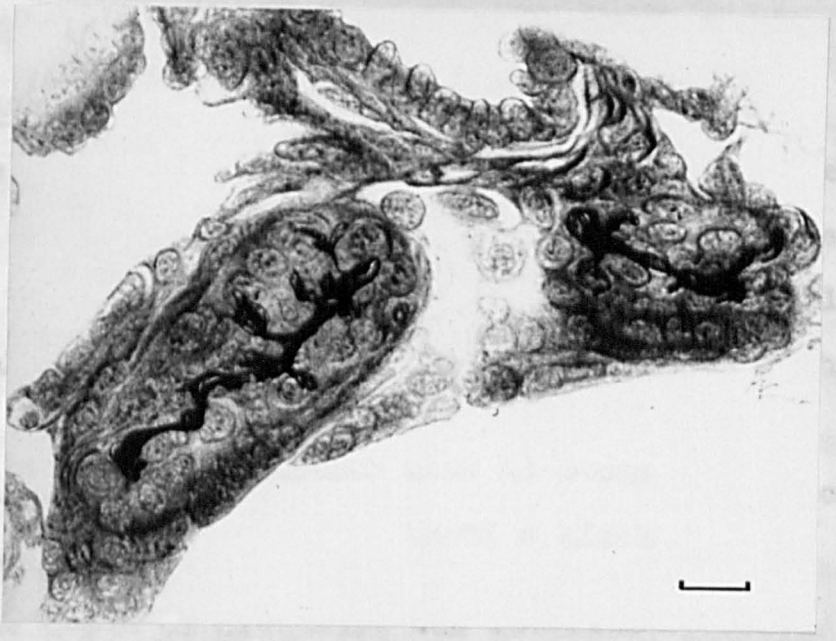
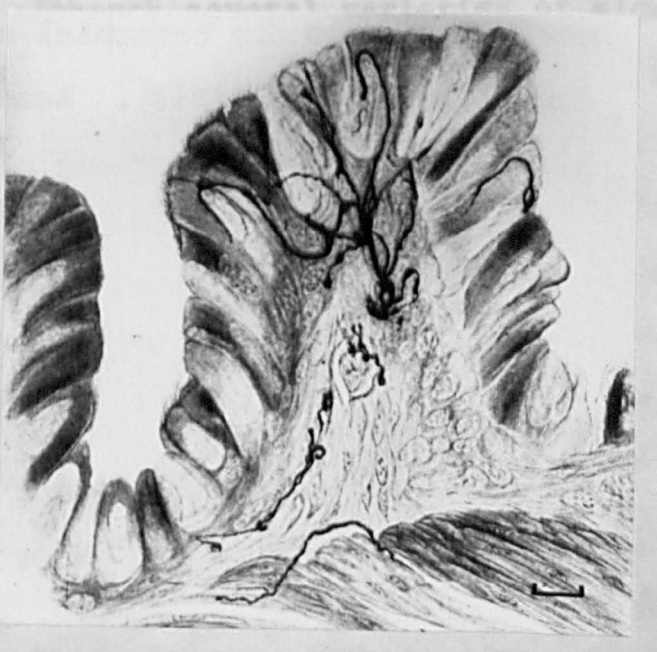


Figure 4. Drawing of an encapsulated afferent nerve ending in the wall of the respiratory bronchiole of a dog, from Elftman (1943). According to Elftman, the two terminal branches of the ending are embedded in elliptical masses of cells in the muscle layer, lamina propria and epithelium of the bronchiole. Several depths of focus are shown in this drawing. Reduced silver method. Scale = 10μm.

Figure 5. Drawing of a complex free fibre type of afferent nerve ending in the bronchial epithelium of a dog, from Elftman (1943). According to Elftman, the numerous terminal fibres pass between the epithelial cells and end near the surface of the epithelium with and without terminal knobs. Some fibres, however, return towards the basement membrane and end in the deep part of the epithelium. One secondary branch of the ending terminates in the lamina propria. Several depths of focus are shown in this drawing. Reduced silver method. Scale = 10μm.





B. Functional Studies on the Afferent Pathways in the Vagus.

(1) The Effects on Breathing of Cervical Vagotomy.

(a) Unilateral Vagotomy.

There are surprisingly few observations on the effects of unilateral cervical vagotomy. Most accounts agree that the breathing becomes slightly slower although both Knoll (1880) and Sinha (1958) observed slowing of up to half the normal rate. The slowing is due to either pauses in expiration (Bert, 1870, p. 447) or to prolonged expirations without pauses (Sinha, 1958; Richards, 1969). A small increase in the depth of breathing was recorded by Bert (1870, p. 447), Grünwald (1904) and Richards (1969). In many pigeons, however, Sinha (1958) found shallower breathing.

(b) Bilateral Vagotomy.

It is generally agreed that the rate of breathing is markedly reduced although several varieties of slowing have been reported. These include pauses in breathing in the extreme expiratory position (Couvreur, 1891, 1892; Siefert, 1896; Grober, 1899; Grünwald, 1904; Richards, 1969), pauses in expiration in the resting position of the thorax (Stübel, 1910), prolonged expirations without pauses (Stübel, 1910; Orr & Watson, 1913; Graham, 1940; Van Matre, 1957 p. 100; Sinha, 1958; Blankart, 1960; Fedde, Burger & Kitchell, 1963a), pauses in inspiration in the resting position of the thorax (Siefert, 1896; Stübel, 1910) and prolonged inspirations without pauses (Stübel, 1910). Observations on the amplitude of breathing are also conflicting. Whilst large increases

in depth were usually recorded, Couvreur (1891, 1892) in the chicken found that the increase lasted only a short time and soon fell below that of normal breathing. Furthermore, some experiments on the pigeon either produced no change in the depth of breathing (Couvreur, 1891, 1892; Sinha, 1958; Richards, 1968) or resulted in shallower breathing (Sinha, 1958; Blankart, 1960).

The observations of Fedde et al. (1963a,b) on vagotomy are especially interesting. They found that in unilateral vagotomized birds, cutting the pulmono-oesophageal and cranial pulmonary branches of the intact vagus changed the breathing so that it resembled that of bilateral vagotomy. To explain some of the different breathing responses reported by previous workers, they studied the effects on the outcome of vagotomy of age and anaesthesia. Whilst breathing in both 16 and 32 week old conscious male chickens became slower and deeper soon after vagotomy, that in the older birds later became progressively faster and shallower. Anaesthesia lessened changes in rate and depth after vagotomy. King (1966b) suggested that posture and species might also influence the effects of vagotomy although Saalfeld (1936) and Richards (1968) both concluded that posture was an unimportant factor. Different responses to vagotomy in different species, however, were shown by Richards (1968) who found markedly deeper breathing in the anaesthetised chicken and quail but frequently no change in the pigeon at presumably the same level of anaesthesia.

Unfortunately most accounts omit details of the birds

including their age and posture and whether they were anaesthetised or not, but it seems likely that the evidence of Fedde et al. (1963a) on age and anaesthesia and Richards (1968) on species differences could account for some of the conflicting results. Other differences, however, remain unexplained, and certainly much more precise information is still required on the acute effect of vagotomy under carefully controlled experimental conditions.

(2) The Effects on Breathing of Electrical Stimulation of the Central Stump of the Cervical Vagus.

As with the accounts of vagotomy, those on stimulation provide few experimental details either of the birds or of the stimulations, including the choice of stimulator and the stimuli. Whilst many of these accounts are therefore of very limited value, they do indicate that there is little agreement on the effects of stimulation and that the respiratory response varies with the characteristics of the stimulus. Thus the conflicting observations of Bert (1870, p. 480), Knoll (1880), Siefert (1896), Couvreur (1891, 1892), Bourgeois (1896), Cavalié (1898), Grober (1899), Grünwald (1904), Stübel (1910), Orr & Watson (1913), Dooley & Koppányi (1929), Saalfeld (1936), Graham (1940) and Ripplinger & Bopelet (1968) include increases and decreases in the rate and amplitude of breathing and arrests of breathing in inspiration, expiration and in the resting position of the thorax. The many factors believed to influence the response include anaesthesia (Couvreur, 1891, 1892; Bourgeois, 1898),

the point on the breathing cycle at which the stimulus is first applied (Bert, 1870, p. 480) and whether it is the right or left vagus that is stimulated (Ripplinger & Bopelet, 1968).

Only Sinha (1958) in pigeons and Richards (1968, 1969) in chickens, however, appear to have used precisely controlled strengths of stimuli. Sinha at low frequencies of stimulation typically induced a shift into inspiration with an increase in the rate of breathing and a reduction in the depth. At high frequencies, the response was expiratory with low voltages and inspiratory with high voltages. Richards also described different responses with different strengths of stimuli, a weak stimulus (1-3V, 5-25 c/s) applied continuously producing faster and shallower breathing whilst stimulation with higher voltages and frequencies caused respiratory inhibition.

It appears therefore, that the effect on breathing of electrical stimulation of the central stump of the vagus in the bird is far from being established. As yet there is little evidence which might suggest reasons for the many different responses that are reported. More precise observations are obviously required to determine the influence of stimulation of the many variables including species, posture, anaesthesia and the characteristics of the stimulus.

(3) Action Potentials in the Peripheral Stump of the Cervical Vagus synchronous with Resting Breathing.

The very first studies of any sort in birds to record

electrophysiological activity in the cervical vagus were those of King, Molony, McLelland, Bowsher, and Mortimer (1968), King, McLelland, Molony, Bowsher, Mortimer and White (1968) and King, McLelland, Molony and Mortimer (1969) who obtained 122 single fibre records in phase with some stages of the eupnoeic breathing cycle. Some of the units were sensitive to CO_2. Details of many of these units are given later in the thesis as new evidence for the afferent vagal pathways.

Since then Jones (1969), Fedde & Peterson (1970) and Peterson (1970, pp. 59-65) have also used this technique to study respiratory reflexes. Jones basically obtained three types of activity which was all in phase with inspiration but which differed in its frequency and pattern of firing. Fedde and Peterson found activity cyclic with one or both phases of resting breathing which was sensitive to the level of CO_2 in the airways. At physiologically low levels of CO_2 the units fired more; at physiologically high levels of CO_2 they fired less. Although the units firing in inspiration responded to inflation of the respiratory system with CO_2-free gas, their firing pattern differed from that of mammalian pulmonary stretch receptors described by Adrian (1933) since they were most active early in the inflation. Another difference of course was that they ceased firing when the inflating gas contained 15% CO_2. Some of the CO_2-sensitive units were identified in pulmonary branches of the vagus. Fedde and Peterson also found other activity in the cervical vagus cyclic with inspiration which was unaffected by 10% CO_2 in the ventilating gas. On

inflation, some of these units increased their rate of firing whilst others ceased firing.

IV. OBJECTIVES.

The detailed objectives were as follows.

(1) To establish the gross anatomy of the pulmonary branches of the vagus in Gallus domesticus.

(2) To investigate with the light microscope the distribution of the nervous tissue in the intrapulmonary air passages of G. domesticus and in particular to find some histological evidence for an afferent innervation.

(3) To investigate afferent respiratory pathways in the vagi of G. domesticus by:-

(a) recording breathing after unilateral and bilateral vagotomy and investigating the effect of anaesthesia on the response.

(b) By recording the respiratory responses to electrical stimulation of the central stump of the vagus and investigating the effects of various factors which might influence the response such as age, posture and anaesthesia.

(c) By obtaining electrophysiological recordings of unit activity in the peripheral stump of the cervical vagus in phase with resting breathing.

(4) To interpret the histological and experimental evidence for the afferent pathways in the vagi in terms of their respiratory function.

V. MATERIALS AND METHODS.

A. The Innervation of the Intrapulmonary Air Passages.

(1) The Macroscopic Study of the Pulmonary Branches of the Vagus.

Five male and fifteen female Rhode Island Red X White Leghorn adult birds were investigated. Three of the males and ten of the females were preserved by intra-arterial perfusion with 10% formalin via the sciatic artery. The remaining seven birds were unfixed.

The vagus lies in the thorax medial to the caudal part of the internal jugular vein and the cranial vena cava and therefore to expose the nerve these veins had to be very gently removed, great care being taken to avoid damaging branches of the nerve. The distribution of all these branches between the levels of the thoracic inlet and the caudal border of the pulmonary vein were investigated with the aid of a dissecting microscope. To demonstrate the distal parts of some of the nerves it was necessary to remove the extrapulmonary part of the pulmonary artery as well as the rostral part of the lung.

(2) The Histological Study of the Innervation of the Intrapulmonary Air Passages.

The histological innervation of the air passages was investigated with the light microscope in male and female Rhode Island Red X White Leghorn, Thornber 404, and Light Sussex birds ranging in age from two months to two years.

The choice of a staining technique was difficult since, as noted by a number of workers including Sutherland (1964) and Gosling (1970), all methods suffer from two main disadvantages. These are firstly their failure sometimes to stain all the nervous elements which are present and secondly the fact that all of them lack complete specificity for nervous tissue. For these reasons, therefore, it was considered unwise to rely on the uncorroborated result of a single technique. Consequently in this study as many as six techniques were used including methylene blue, silver nitrate, osmium tetroxide and gold chloride methods and a cholinesterase technique with and without intensification by silver.

With all methods, blocks of lung tissue were sectioned on a freezing microtome at thicknesses ranging from 10 to 120μm. The number of birds in which each technique was used is shown in brackets.

(a) The Methylene Blue Technique (20 birds).

In many of the birds a combined intravital and supravital method was employed based on the technique described by Drury & Wallington (1967, pp. 287-288). In other birds a supravital method alone was used based on that of Spencer & Leof (1964). With both techniques the lungs were perfused with a freshly made 0.01 - 0.05% solution of methylene blue (Edward Gurr) in normal saline. During

the perfusion in both techniques, the thoracic walls were compressed intermittently to ensure that the solution reached all parts of the lungs. Evidence of adequate filling of the lungs was judged to have taken place when their ventromedial surfaces became light-blue in colour.

(b) The Silver Impregnation Technique (15 birds).

Although the impregnation of nervous tissue with silver has been carried out for over half a century, it is still difficult to choose a silver technique which can be guaranteed to stain the tissue successfully. The development of silver techniques used with frozen sections was reviewed by Fisher (1962, pp. 2-5). The most commonly used of these methods are based on the silver diamine technique of Bielschowsky (1902). A widely used variation of this method is that of Gros-Schultze described by Mallory (1938, pp. 227-228). The empirical nature, however, of this Bielschowsky-Gros technique has often led to disappointing results like those found with the method during pilot investigations to this study. Generally the nervous tissue was inadequately impregnated and at best there was co-impregnation with other tissues. Such shortcomings of the method have inevitably resulted in the faulty interpretation of nervous tissue by many authors, and especially on the position and appearance of the nerve endings.

An important modification of the Bielschowsky-Gros

technique was that devised by Rintoul (1960, pp. 83-92) to stain the nerves in the wall of the intestinal tract. In this modification the tissues are initially fixed for twenty-four hours in ammoniacal formalin before further treatment with neutral formalin. The method has the advantage of being very simple and when used correctly produces selective impregnation of nervous tissue. Furthermore, it was employed very successfully by Fisher (1962) to demonstrate a profuse innervation in the mammalian lung. For these reasons, this modification of Rintoul was chosen for the present investigation.

(c) The Osmic Acid Technique (23 birds).

A modified Champy (1913) method was used. It is well-known that the original technique as described by Champy is extremely capricious. In an attempt to increase the reliability of the method, the modification of Maillet (1959) replaced potassium iodide with zinc iodide. Sutherland (1963) suggested that even better results could be obtained by the use of clean glassware, water which was both deionised and distilled, and thin sections. All these modifications were adopted in the present study.

(d) The Gold Chloride Technique (15 birds).

The modification of Ranvier's (1880) gold chloride technique by Cole (1946) substituted citric acid for lemon juice in an attempt to reduce the capriciousness of the method. Although this modification by Cole was primarily devised to stain motor end plates, it was also used successfully to demonstrate nervous tissue in the lungs of

guinea-pigs and rats by Fisher (1962). Cole (1950), however, pointed out that the optimum concentrations of the staining solutions in fish, amphibians and reptiles were different from those in mammals and that the optimum concentrations also varied between different species of mammals. In the present study, therefore, the concentrations of the staining solutions were varied within the ranges suggested by Cole (1950).

(e) The Cholinesterase Technique (30 birds).

Gomori's (1952, pp. 211-212) modification of the original Koelle and Friedenwald (1949) technique was chosen. The lungs were fixed immediately after death by injection via the pulmonary artery of the formalin-sucrose ammonia mixture of Pearson (1963). To distinguish between acetylcholinesterase and butyrylcholinesterase, the substrates acetylthiocholine iodide and butyrylthiocholine iodide were used in conjunction with specific inhibitors recommended by Bayliss & Todrick (1956). Acetylcholinesterase was selectively inhibited by 1,5 -bis (4-allyl dimethylammoniumphenyl) pentan-3-one dibromide (284C51, Wellcome) at concentrations of 10^{-5}M to 3 X 10^{-4}M. Butyrylcholinesterase was selectively inhibited by ethopropazine methosulphate (Lysivane, May & Baker) at concentrations of 3 X 10^{-5}M to 10^{-4}M, and by tetra-isopropylpyrophosphoramide (iso-OMPA, Koch-Light) at concentrations of 10^{-6}M to 3 X 10^{-6}M.

(f) The Cholinesterase Technique intensified with Silver (25 birds).

In this technique, the relatively pale copper sulphide

deposited on the nerve fibres with the cholinesterase method is converted by immersion of the sections in a solution of silver nitrate to a black precipitate of silver sulphide. According to Henderson (1967) who combined this refinement with the cholinesterase method of Lewis (1961) it has an important advantage when compared with unintensified cholinesterase methods in that the black precipitate of silver sulphide allows fine nerve fibres to be identified after only a short incubation period. The reduction in the time lessens the diffusion of the copper sulphide precipitate and allows better definition of the stained nerve fibres. In the present study, it was considered that a further advantage might be gained from the dense staining of the nerve fibres in that it might permit nerve endings to be seen.

Sections were removed from the copper sulphide stage of Gomori's (1952) cholinesterase method (p. 26) and silvering carried out as described by Henderson (1967). However, Henderson used a 5-10% aqueous solution of silver nitrate for 10-40 seconds with mammalian pancreas and mammary gland but emphasised that both the concentration of the silver nitrate solution and the immersion time might have to be varied according to the organ under examination. With the avian lung and Gomori's (1952) technique a 0.5-1% solution of silver nitrate and an immersion time of approximately five seconds produced satisfactory results.

(g) Control Staining Techniques.

Since the staining techniques used in this study are

not specific for nervous tissue it was possible that connective tissue fibres could be mistaken for nervous tissue. In order, therefore, to establish the distribution and appearance of the connective tissue in the lung, the following techniques were carried out on paraffin sections: haematoxylin and eosin, Weigert and van Gieson's stain, Gomori's stain for reticulin, Gordon and Sweet's stain for reticulin, and Gomori's aldehyde fuchsin stain for elastic tissue. All the techniques used were as described by Culling (1957).

In addition, connective tissue fibres were intentionally demonstrated as suggested by Fisher (1962, p. 61) whenever Rintoul's (1960) silver impregnation technique was used, by allowing excessive formalin to remain on the sections after the blotting stage. This resulted in either co-impregnation of nervous and connective tissues or selective impregnation of connective tissue.

B. Functional Studies on the Afferent Pathways in the Vagus.

(1) The Effects on Breathing of Cervical Vagotomy.

The effects of unilateral and bilateral cervical vagotomy on breathing were observed in five conscious and eight anaesthetised, eight month old, Rhode Island Red X White Leghorn, female birds. Breathing was recorded by a closed circuit apparatus. A short Y-piece from a tracheal cannula led to inspiratory and expiratory fluid valves which contained Fusus oil "A" (Shell-Mex and B.P. Ltd), the oil which Payne (1960, p. 47) found offered the least resistence to breathing. The valves were connected to a float spirometer of about 1000ml. capacity which wrote on a kymograph with an ink pen made of perspex. A flask containing soda-lime (Protosorb) was incorporated into the circuit to absorb CO_2. Before each period of recording, the spirometer was filled with a 95% air/5% CO_2 mixture.

The kymograph paper was calibrated for amplitude of breathing by injecting measured volumes of air into the spirometer and recording the changes in height, and for frequency of breathing by recording from an automatic time marker. The minute volume of breathing was calculated from the observed tidal volumes and frequencies.

The following procedures were carried out on each bird.

(a) Conscious Birds.

(i) Atropine sulphate (2mg/kg body wt.) was injected intramuscularly to reduce the secretion of mucus from the respiratory tract and under local anaesthesia (procaine

hydrochloride) the trachea was cannulated just caudal to its rostral quarter where it is circular in cross-section and has a relatively large diameter (McLelland, 1965).

(ii) With the bird standing and only gently held, breathing was first recorded for five minutes with both vagi intact.

(iii) Under local anaesthesia the right vagus was then cut in the mid-cervical region and breathing recorded for periods of one minute during minutes 1 and 15 after unilateral vagotomy.

(iv) Under local anaesthesia the left vagus was cut in the mid-cervical region and breathing recorded for periods of one minute during minutes 1,10,30,50 and 70 after bilateral vagotomy.

(v) The breathing response to vagotomy was examined for statistical significance with Fisher's "t" test (Paterson, 1939, pp. 17-19).

(b) Anaesthetised Birds.

A 43% (w/v) aqueous solution of ethyl carbamate (urethane, B.D.H. Poole, Dorset) as recommended by King & Biggs (1957) was chosen as an anaesthetic. Light anaesthesia was induced in five birds and deep anaesthesia in three birds by injections via the brachial vein. The depth of anaesthesia was judged according to the criteria selected by Fedde, Burger & Kitchell (1963a). Thus in light anaesthesia there was no response to skin and toe pinches although a response to a comb pinch remained. In deep anaesthesia the

response to a comb pinch was also absent. Basically the experimental procedure was the same as that for conscious birds. However, the birds were held erect by the wings with only the feet touching the surface of the table. Also breathing was only recorded in the lightly anaesthetised birds during minutes 1, 10 and 30 after bilateral vagotomy and in the deeply anaesthetised birds during minutes 1 and 10 after bilateral vagotomy.

(2) The Effects on Breathing of Electrical Stimulation of the Central Stump of the Cervical Vagus.

Seven 9 - 11 month old, female birds and three 2 - 3 month old, male birds were used. The adult birds belonged to the Light Sussex, Rhode Island Red X Black Rock, Thornber 404 and Rhode Island Red X White Leghorn breeds, whilst the immature birds were Sykes Hybrids. Breathing was recorded by one of the following techniques.

(a) In seven erect birds supported by the wings (including the two conscious birds used in the study) breathing was recorded by a float spirometer and kymograph (p. 29).

(b) In three anaesthetised birds firmly supported erect in an iron frame, breathing was recorded by measuring changes in abdominal volume (3 birds), intrarectal pressure (1 bird) and intratracheal pressure (1 bird). The frame consisted of adjustable ear bars, wing supports, a transverse pin for inserting subcutaneously through the coccygeal region, and a tail clamp. Movements in the cervical region were minimized

by inserting into the oesophagus a hollow copper bar closed at one end and through which heated water was circulated to maintain body temperature. Rotation of the ear and oesophageal bars allowed easy access to the vagus from the dorsal aspect.

Abdominal volume was measured by an abdominal stethograph just caudal to the sternum composed of an inflated rubber balloon held firmly to the bird by sutures in the dorsal mid-line. Intrarectal pressure was measured by an inflated rubber balloon inserted via the cloaca into the rectum. Intratracheal pressure was measured by a T-piece attached to a tracheal cannula. Changes in abdominal volume, intrarectal pressure and intratracheal pressure were transmitted to a pressure transducer, amplified and transposed to paper by means of a pen recorder (Devices M2). During inspiration, the increase in abdominal volume was recorded by an upward movement of the pen recorder; the decrease in intrarectal and intratracheal pressures being recorded by a downward movement of the pen recorder.

Stimuli were applied with a bipolar silver electrode having a terminal gap of approximately 5 millimeters. The electrode was either supported by hand or mounted in a three dimensional micromanipulator (Prior). Square wave stimuli of duration 1 - 100 μsec were applied to the vagus from a physiological stimulator designed by Catton, Molyneux & Schofield (1957) (5 birds), from a Tektronix 160 Series pulse generator (5 birds) and from a Grasse pulse generator (1 bird) at 2 to 50 volts and 1 to 1000 cycles per

second. Sine wave stimuli were applied in one bird to the vagus from a Tektronix 160 Series pulse generator at 1 to 50 volts and 2 to 5000 cycles per second. The length of time during which the stimulus was applied to the nerve varied from 3 to 80 seconds.

In most series of stimulations the pulse duration of the stimulus was kept constant and either the voltage or the frequency varied over a selected range. Stimulation was usually begun at the beginning of inspiration although some stimulations were intentionally begun at other points on the breathing cycle. In each bird the following procedures were carried out.

(a) Eight birds were anaesthetised by an intravenous injection of urethane (p. 30). Two birds remained conscious throughout the experiment.

(b) The trachea was cannulated (pp. 29-30) and approximately 1.5cm lengths of one or both vagi dissected out through lateral incisions in the mid-cervical region. A cotton loop was attached around each vagus and the nerve cut peripheral to the loop. In the conscious birds all surgery was carried out under local anaesthesia.

(c) Electrical stimulation of the central stump after unilateral vagotomy was carried out in two conscious birds (37 stimulations) and in eight anaesthetised birds (451 stimulations). Only weak stimuli were applied to the conscious birds and no discomfort of the birds was observed. Except for the stimulations carried out in one anaesthetised bird lying in the supine position (45 stimulations) all the

birds were erect. The left vagus was stimulated in seven birds and the right vagus in three birds. Stimulation of the central stump after bilateral vagotomy was carried out in six, erect, anaesthetised birds (69 stimulations). The left vagus was stimulated in one bird and the right vagus in five birds. In all birds the whole vagus was stimulated with the epineural and perineural sheaths intact but in two birds stimulation was also carried out with the sheaths removed. In one bird, a length of vagus was dissected into five approximately equal parts, each of which was stimulated separately.

(3) Action Potentials in the Peripheral Stump of the Cervical Vagus synchronous with Resting Breathing.

Action potentials in the peripheral stump of the cervical vagus were studied in seventeen male and female Brown Leghorn, Buff Rock, and White Leghorn X Rhode Island Red birds aged between 4 weeks and 18 months. The following procedures were carried out in each bird.

(a) The bird was anaesthetised by an intravenous injection of urethane (p. 30).

(b) The trachea was cannulated by suturing a 2 cm portion of its caudal third inside a close-fitting brass collar. The normal sweeping action of the tracheal cilia was thus preserved and reduced the tendency for mucus to accumulate and block the cannula. The skin incision was closed around the tracheal collar.

(c) The bird was firmly supported erect in the iron

frame described on pages 31-32. Breathing was recorded oscillographically (Tektronix oscilloscope 502A) via a pressure transducer in the form of abdominal volume or intrarectal pressure or intratracheal pressure changes (p. 32).

(d) A longitudinal skin incision was made on the right side of the neck rostral to the level of the tracheal cannula. The skin flaps of the incision were sutured to a copper wire frame to form the sides of a bath in which the right vagus was prepared for recording.

(e) The vagus was dissected free of the jugular vein and a supporting black perspex strip inserted between the nerve and the underlying structures. With the aid of a Zeiss stereoscopic dissecting microscope a 0.5 - 1 cm length of the epineurium was dissected away and the underlying perineurium split longitudinally. The bath was then filled with liquid paraffin at 40°C and saturated with oxygen and avian Ringer's solution.

(f) With the aid of watchmakers forceps and a small triangular fragment of razor blade, short thin nerve filaments were "pulled" from the vagus. Finer nerve strands were then carefully split from the peripheral stumps of each filament and wound around a monopolar silver or platinum electrode held in a three dimensional micromanipulator (Prior). The activity in the strands was passed through a low level pre-amplifier (Tektronix, 127) and displayed on a dual beam oscilloscope (Tektronix, 502A). This activity was photographed by a camera mounted on a "slave" oscilloscope connected to the main oscilloscope.

VI. RESULTS.

A. The Innervation of the Intrapulmonary Air Passages.

(1) Macroscopic Observations on the Pulmonary Branches of the Vagus.

The thoracic part of the vagus lay medial to the jugular vein and the cranial vena cava and crossed the lateral surfaces of the pulmonary blood vessels (fig. 6). At the caudal border of the pulmonary vein it turned medially and went to the ventral surface of the oesophagus. Its thoracic branches originated in the following rostrocaudal order (a) branches to the thyroid, parathyroid and ultimobranchial glands, (b) the rostral cardiac nerves, (c) the recurrent nerve, (d) branches to the pulmonary nerve plexus, and (e) the caudal cardiac nerves.

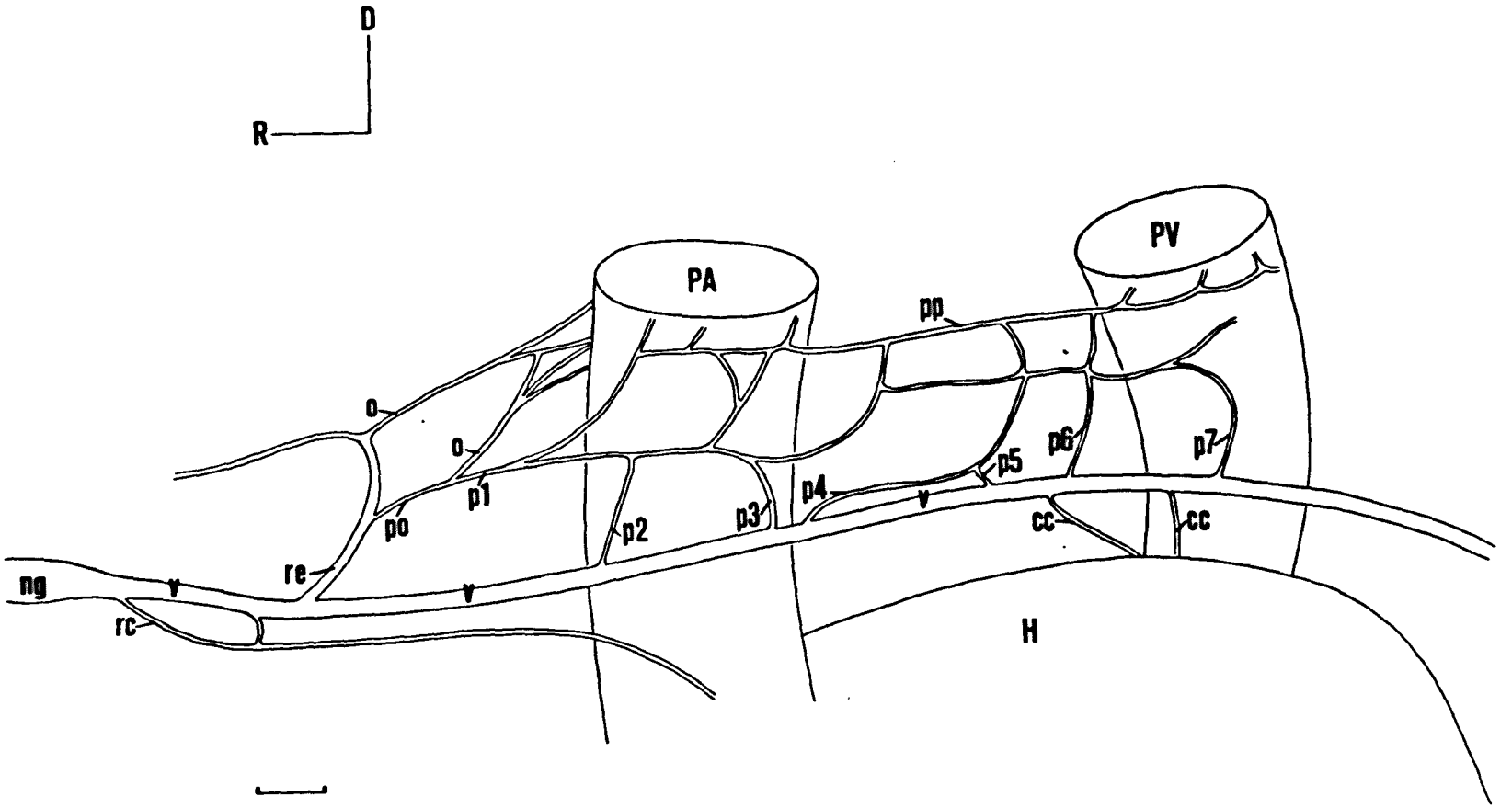
(a) Branches to the Thyroid, Parathyroid and Ultimobranchial Glands.

Several fine, short, anastomotic nerves from the lateral surface of the vagus just caudal to the nodose ganglion appeared to be distributed to these glands.

(b) Rostral Cardiac Nerves (figs. 6 and 8).

Two to three cardiac nerves came from the ventral surface of the vagus between the levels of the nodose ganglion and the recurrent nerve. They joined caudally to form a single nerve which was distributed to the heart, the left nerve travelling on the lateral surface of the pulmonary artery and the right nerve going lateromedially around the concavity of

Figure 6. Semi-diagrammatic representation of some of the branches of the left vagus (v) of G. domesticus. The pulmono-oesophageal nerve (po) originates in this bird from the recurrent nerve (re). Nerves from the dorsal part of the pulmonary nerve plexus (pp) enter the lung close to the pulmonary artery (PA) and the pulmonary vein (PV). The nerves are not drawn accurately to scale. cc = caudal cardiac nerve; D = dorsal; H = heart; ng = nodose ganglion; o = oesophageal nerve; p1 to p7 = pulmonary nerves; R = rostral; rc = rostral cardiac nerve. Scale = 1mm.


D
R
PA
PV
pp
o
o
p1
po
re
p2
p3
p4
p5
p6
p7
cc
cc
ng
rc
H

the aortic arch.

(c) Recurrent Nerve (figs. 6,7,8 and 10).

The thick recurrent nerve originated from the dorsal surface of the vagus several millimetres rostral to the level of the pulmonary artery. The first part of the recurrent nerve descended medially and dorsally for a short distance. This descending part of the nerve was then continued by a much longer ascending part which travelled first lateromedially on the left side of the body around the caudal border of the ligamentum arteriosum, and on the right side of the body around the concavity of the aortic arch, before extending rostrally into the neck. The following fine branches came from the descending part of the nerve.

(i) The pulmono-oesophageal nerve went to the pulmonary nerve plexus and the oesophagus. Further details of the nerve are given below.

(ii) An oesophageal branch came from the descending part of the recurrent nerve distal to the origin of the pulmono-oesophageal nerve (figs. 6,7 and 8). Some of its branches joined the pulmono-oesophageal nerve.

(d) Branches to the Pulmonary Nerve Plexus.

The branches of the vagus to the pulmonary nerve plexus were very fine and frequently hidden by large amounts of fat.

(i) Pulmono-oesophageal Nerve (figs. 6,7,8 and 10).

The pulmono-oesophageal nerve came from either the proximal part of the recurrent nerve (figs. 6 and 8) or the

Figure 7. Semi-diagrammatic representation of part of the left vagus (v) of G. domesticus. The pulmono-oesophageal nerve (po) originates in this bird directly from the vagus. The nerves are not drawn accurately to scale. D = dorsal; o = oesophageal nerve; p1,p2 = pulmonary nerves; PA = pulmonary artery; R = rostral; re = recurrent nerve. Scale = 1 mm.

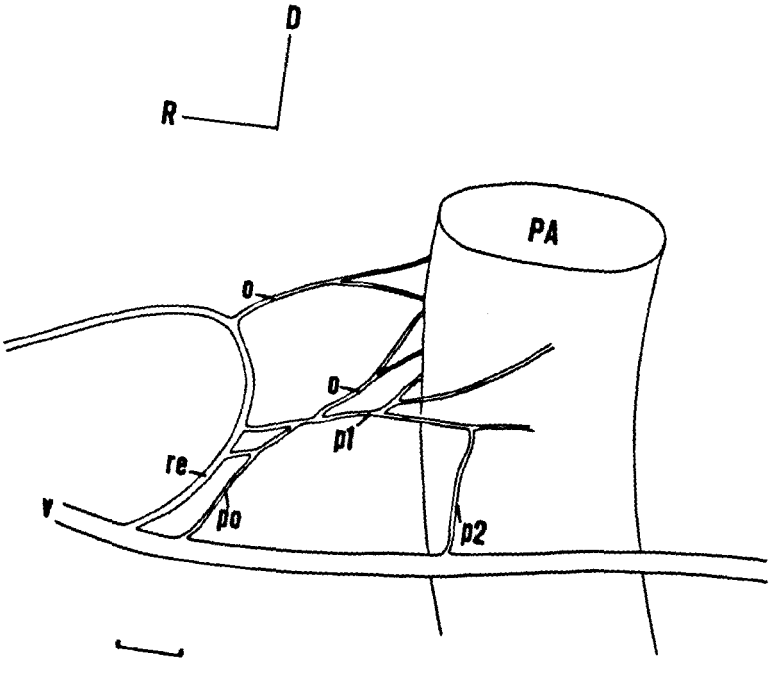
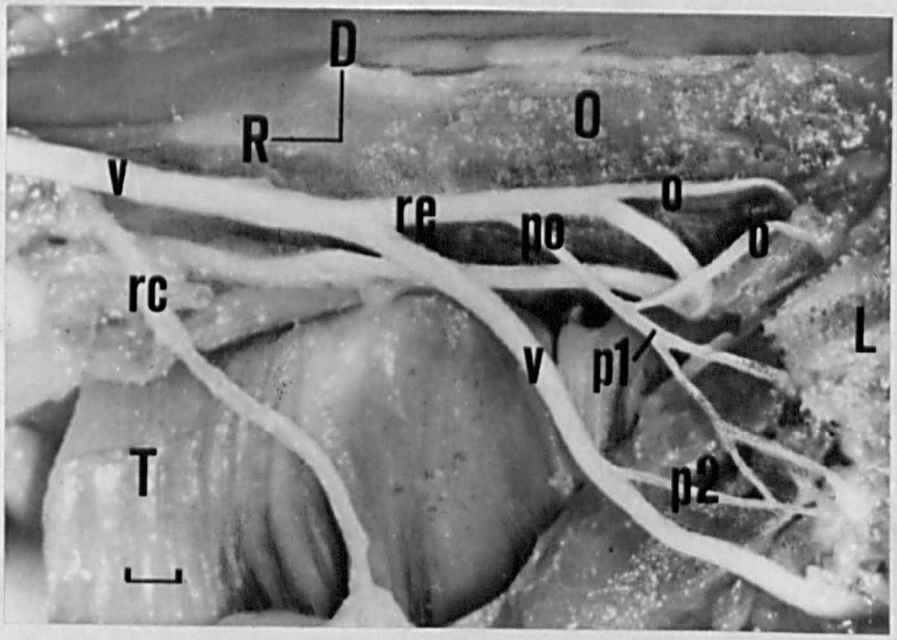

D
R
PA
o
o
p1
re
po
v
p2

Figure 8. Lateral view of the left vagus (v) of an adult female Rhode Island Red X White Leghorn *G. domesticus* to show the recurrent nerve (re) and some of its branches. The pulmonary nerves (p1,p2) anastomose to form the rostral part of the pulmonary nerve plexus. D = dorsal; L = left lung; O = oesophagus; o = oesophageal nerve; po = pulmono-oesophageal nerve; R = rostral; rc = rostral cardiac nerve; T = trachea. Scale = 1mm.


D
R
O
V
re
po
o
o
rc
L
v
p1
T
p2

dorsal surface of the vagus a few millimetres caudal to the recurrent nerve (figs. 7 and 10). Both origins were sometimes seen in the same bird on opposite sides of the body. In one bird, the right nerve appeared to be replaced by separate pulmonary and oesophageal branches of the vagus. The detailed distribution of the nerve varied. Sometimes the proximal part of the nerve had several short branches which travelled rostrodorsally or rostroventrally to join the recurrent nerve (fig. 7). Usually, however, the nerve extended caudodorsally without branching for several millimetres before finally splitting into medial and lateral branches (figs. 6 and 8). The medial branch went caudomedially to ramify in the wall of the oesophagus (figs. 6 and 8). Generally some of its branches anastomosed with the oesophageal branches of the recurrent nerve. The lateral branch (p1) usually divided rostral to the pulmonary artery into dorsal and ventral nerves which went to the rostral part of the pulmonary nerve plexus on the lateral surface of the pulmonary artery (figs. 6,7,8 and 10). Sometimes a branch of this nerve could be followed to the pleura on the ventral surface of the lung.

(ii) Pulmonary Nerves (p2 - p7) (figs. 6,7,8,9 and 10).

In addition to the pulmonary branch (p1) of the pulmonooesophageal nerve, from 2 to 6 other pulmonary nerves (p2 - p7) were found. Most birds appeared to have 5 or 6 of these nerves branching from the dorsal surface of the vagus between the levels of the pulmonary artery and the pulmonary vein.

The most rostral nerve (p2), however, sometimes originated a short distance rostral to the pulmonary artery. The origins of some of the nerves were very close to each other, often less than one millimetre apart. Their lengths varied, the shortest being less than 0.5 millimetres. Typically adjacent nerves travelled in opposite directions to each other before forming the ventral part of the pulmonary nerve plexus (figs. 6 and 9). The most rostral nerve (p2) usually anastomosed with p1 (figs. 6,7 and 8).

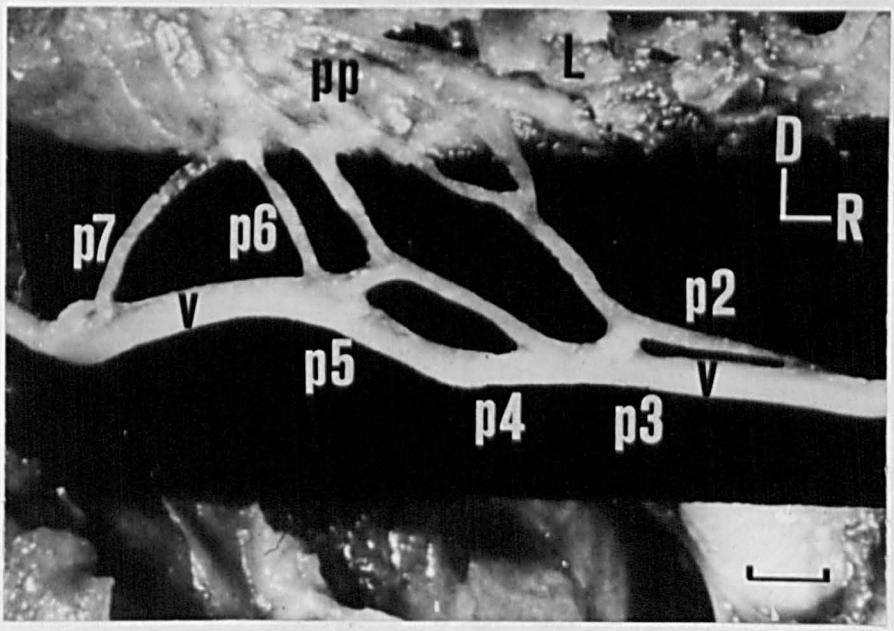
The pulmonary nerve plexus (figs. 6,9 and 10) lay lateral to the pulmonary artery and vein. Macroscopically it appeared as a single layer of very fine anastomotic nerve bundles extending between the vagus ventrally and the hilus of the lung dorsally. Often the plexus was surrounded by a large amount of fat. The distribution of the nerve bundles in the plexus varied and was usually different in the right and left plexuses of the same bird. Nerve bundles from the dorsal part of the plexus entered the lung at the hilus close to the pulmonary artery and vein (fig. 11). Sometimes nerves from the rostral and caudal parts of the plexus were seen to travel to the pleura on the ventral surface of the lung. It was not possible, however, by gross dissection to find out the final destination of these nerves. A nerve plexus was not found close to the primary bronchus and nerves were not seen to enter the lung with the primary bronchus.

(e) Caudal Cardiac Nerves (figs. 6 and 10).

One to three cardiac nerves came from the ventral

Figure 9. Lateral view of the right vagus (v) of an adult female Rhode Island Red X White Leghorn G. domesticus. A strip of black celluloid has been placed beneath the nerve and its pulmonary branches (p2 to p7). D = dorsal; L = right lung; pp = pulmonary nerve plexus; R = rostral. Scale = 1mm.

Figure 10. Lateral view of the left vagus (v) of an adult female Rhode Island Red X White Leghorn G. domesticus. A strip of black celluloid has been placed beneath the nerve and its branches. The pulmono-oesophageal nerve (po) comes directly from the vagus. B = left primary bronchus; cc = caudal cardiac nerve; D = dorsal; L = left lung; p1 to p6 = pulmonary nerves; R = rostral; re = recurrent nerve. Scale = 3mm.


pp
L
D
R
p7
p6
p2
p5
p4
p3

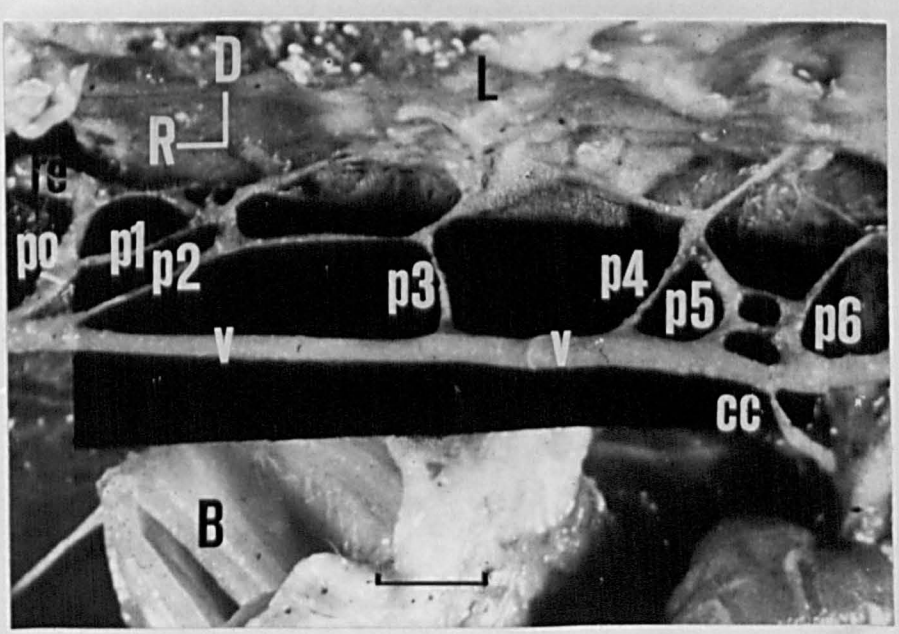
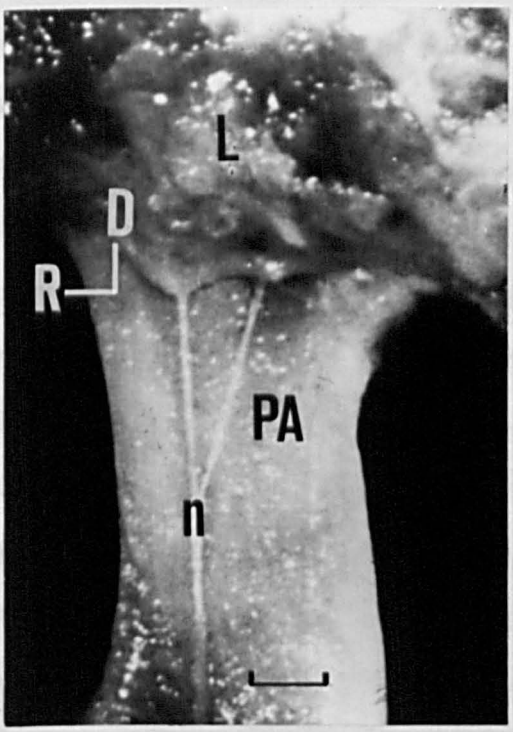

D
L
R
po
p1
p2
p3
p4
p5
p6
cc
B

Figure 11. Lateral view of a nerve (n) in the cranial part of the left pulmonary nerve plexus of an adult, female Rhode Island Red X White Leghorn *G. domesticus*. The nerve enters the lung (L) close to the pulmonary artery (PA). D = dorsal; R = rostral. Scale = 1mm.


L
D
R
PA
n

surface of the vagus close to the pulmonary vein. The most rostral nerve was usually larger than the others and originated close to the rostral border of the vein near the level of p5. Often these cardiac nerves anastomosed with each other on the lateral surface of the pulmonary vein.

(2) Histological Observations on the Innervation of the Intrapulmonary Air Passages.

(A) Appearance of the Nervous Tissue.

A profuse innervation of the lung was demonstrated with all the staining techniques although the methylene blue, silver nitrate, osmium tetroxide and gold chloride methods tended to be capricious and to stain also non-nervous tissues which made the precise identification and interpretation of the innervation sometimes difficult. Despite the disadvantages of these methods, most of them had a special value in the investigation for demonstrating a particular aspect of the innervation like that, for example, of the silver technique which consistently stained a large number of ganglia in the lungs even when there was under-impregnation of the sections and when the surrounding nerve fibres were not stained. The gold chloride technique produced best results with the following concentrations of solutions: 10% citric acid, 0.5% gold chloride, and 10% formic acid, although compared with the other methods that were used this technique was by far the least successful.

The cholinesterase technique at pH6 with an incubation time for the sections in the substrate medium of four hours

always demonstrated a very large amount of nervous tissue. The use of different substrate media and specific inhibitors greatly altered the staining reaction of the nervous tissue, including the amount of tissue which was demonstrated and the intensity with which it was stained. Appropriate inhibitors also greatly increased the contrast in the staining reaction between nervous tissue and the rest of the lung. Thus the intense staining of some of the epithelia and muscle in sections incubated in the acetylthiocholine iodide substrate medium (figs. 15 and 26) did not occur when the sections were exposed to one of the butyrylcholinesterase inhibitors, iso-OMPA or Lysivane (figs. 24 and 35). The staining of nervous tissue in sections incubated in the acetylthiocholine iodide substrate medium and exposed to butyrylcholinesterase inhibitors varied. Usually a profuse innervation was found in all the air passages. However, sometimes the amount of nervous tissue appeared to be reduced. With the acetylcholinesterase inhibitor 284C51, very little nervous tissue was found and the few nerve fibres and ganglion cells that were observed always stained with a greatly reduced intensity. Similarly, there was almost no staining of the nervous tissue in sections incubated in the butyrylthiocholine iodide substrate medium.

When the cholinesterase technique was followed by immersion of the sections in a silver nitrate solution, an even more profuse innervation of the air passages was seen although the basic staining reactions of the tissues still resembled those of sections which had been stained with the

cholinesterase technique alone. However, the staining was always much more intense in silvered sections and consequently finer nerve bundles were observed. Great care had to be taken in interpreting the nervous tissue since immersion in the silver nitrate solution occasionally resulted in the staining of connective tissue fibres which could be confused with nerve fibres.

Most of the nerve fibres in the air passages were less than 1.5μm in width and are described in this account as "fine". The finest had a regular beaded appearance with the methylene blue and osmium tetroxide stains (fig. 17). "Thick" nerve fibres were those which were more than 1.5μm in width (fig. 14), the largest having a width of approximately 4μm. Most of the larger thick fibres with the silver technique had a distinctive vacuolated appearance, which contrasted with the uniform black staining of the other thick fibres.

(B) Distribution of the Nervous Tissue.

Large nerve bundles entered the lung at the hilus close to the pulmonary artery and vein. Many of these bundles supplied the air passages and extended distally from the hilus along the passages dividing and anastomosing to form a plexus. This plexus could be arbitrarily divided into different parts either by its position in the lung or by the relative size of its nerve bundles and its meshwork. In this account nerve plexuses of the primary bronchus, secondary bronchi, tertiary bronchi, interatrial septa and

air capillary regions are distinguished. Further divisions of these plexuses are described later. Nerve fibres were also distributed with many large intrapulmonary blood vessels lying outside the walls of the bronchi but no further observations were made on the innervation of these vessels.

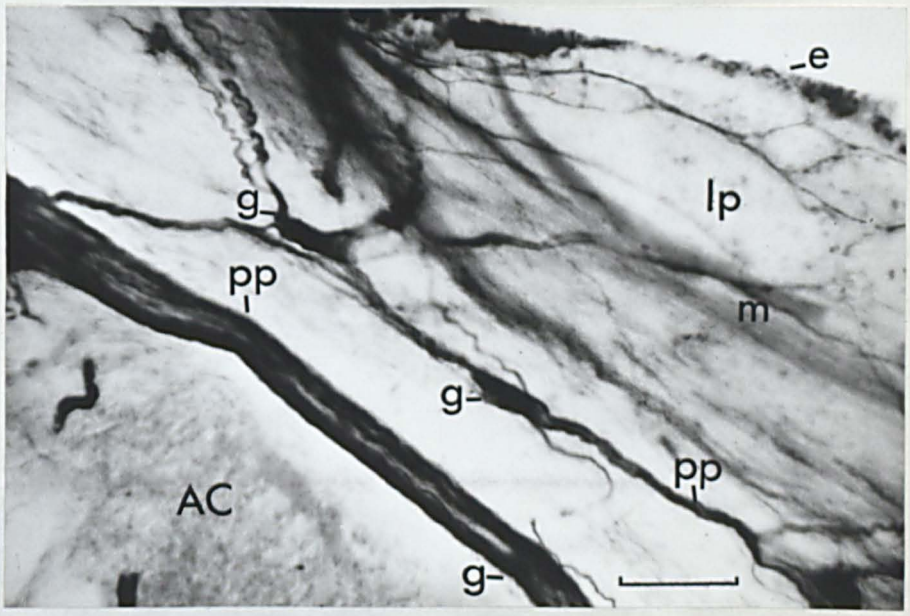
(a) Primary Bronchus.

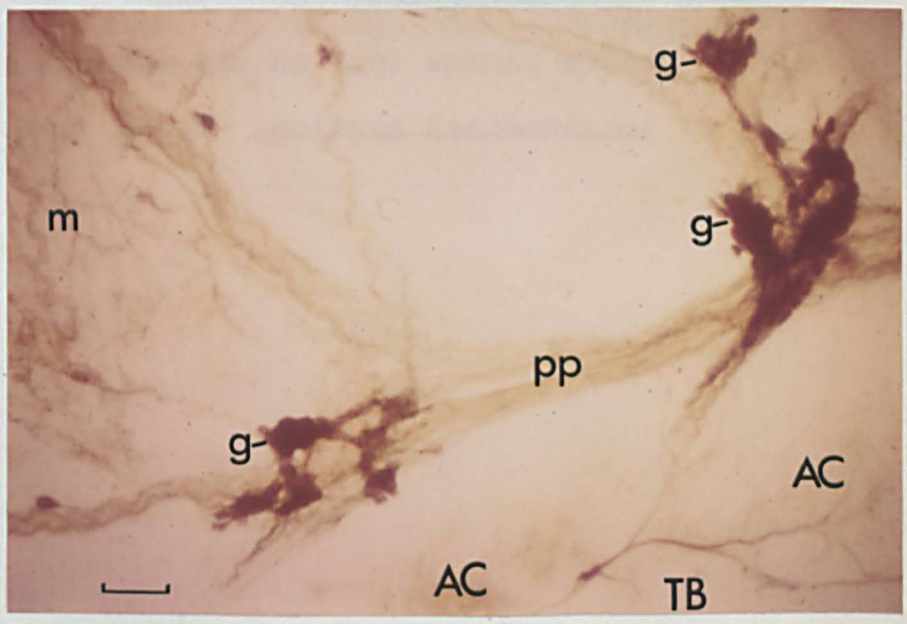
(i) Peribronchial Connective Tissue.

Large branches of the nerve bundles which entered the lung at the hilus with the pulmonary artery and vein travelled to the connective tissue on the outside of the cartilages and the muscle layer of the primary bronchus. Here many of the nerve bundles lay parallel to the bronchus whilst others ran transversely and obliquely around the bronchus, all bundles dividing and anastomosing with each other to form a coarse-meshed peribronchial plexus (figs. 12 and 13). Most of the bundles in the plexus were relatively large, the thickest approximately 300µm in width lying alongside the proximal part of the bronchus. There were many thick fibres (fig. 14) and ganglia were numerous, especially at the points of division of the nerve bundles. The largest ganglia were most often seen at the level of the proximal part of the bronchus and had at least 80 cells. Many branches of this peribronchial plexus were distributed to the muscle layer and lamina propria of the primary bronchus (figs. 12 and 13) whilst others travelled away from the bronchus in the interlobular connective tissue septa or

Figure 12. Low power photomicrograph of the peribronchial nerve plexus (pp) of the primary bronchus in longitudinal section. Nerve fibres from the plexus pass to the muscle layer (m) and lamina propria (lp) of the bronchus. AC = air capillaries; e = epithelium of primary bronchus; g = ganglion. Scale = 100µm. Uninhibited cholinesterase preparation intensified with silver.

Figure 13. Low power photomicrograph of the peribronchial nerve plexus (pp) of the primary bronchus in longitudinal section. Nerve fibres from the plexus pass to the muscle layer (m) of the primary bronchus and to the air capillaries (AC) which surround the tertiary bronchus (TB). The many ganglia (g) of the plexus are intensely stained. Scale = 100µm. Uninhibited cholinesterase preparation.


e
lp
g
pp
m
g
pp
AC
g


g
m
g
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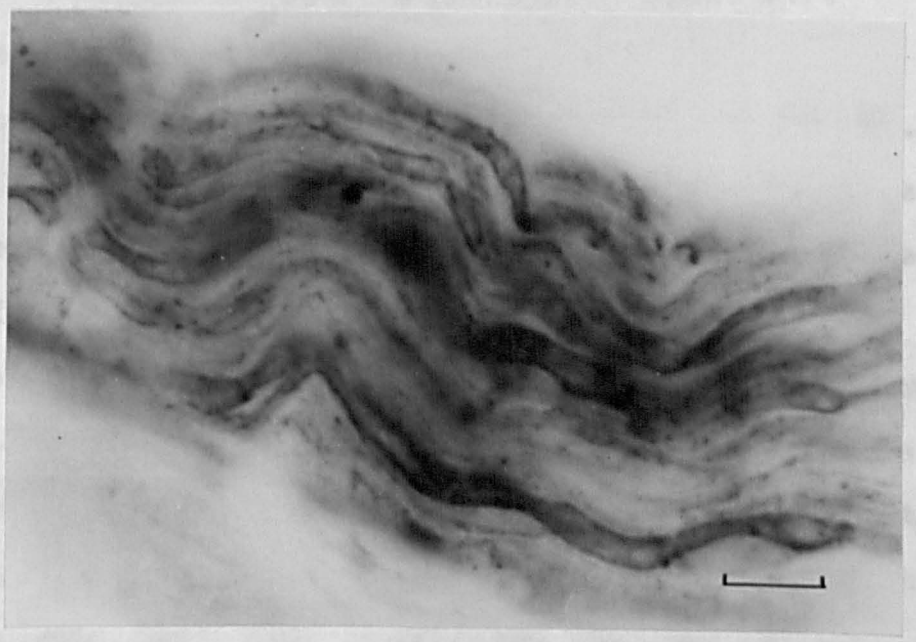
AC
AC
TB

Figure 14. Medium power photomicrograph of "thick" nerve fibres in the peribronchial nerve plexus of the primary bronchus. Scale = 10μm. Osmium tetroxide stain.



in lobules which lay next to the bronchus.(fig. 13).

(ii) Muscle Layer.

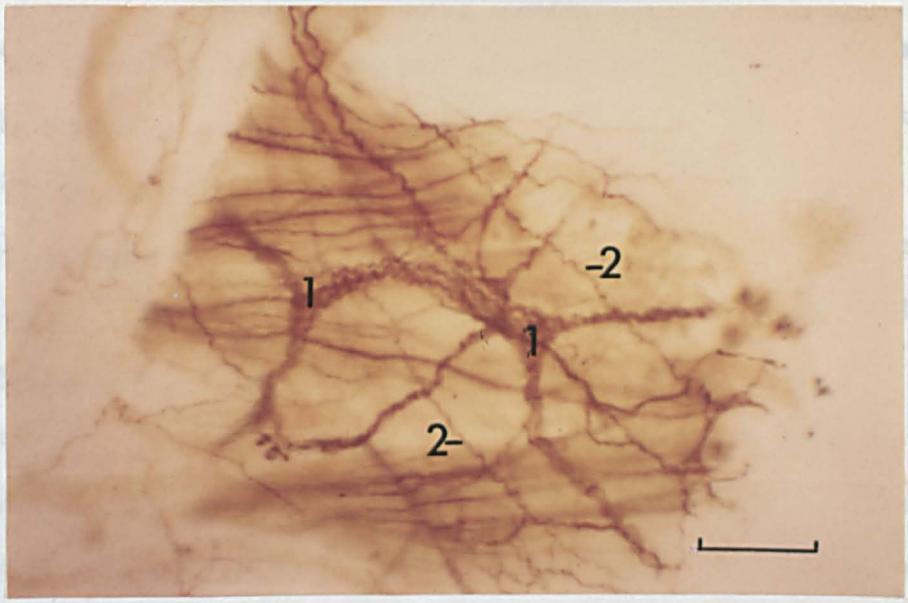
Nerve fibres from the peribronchial plexus formed a plexus in the muscle layer which could be arbitrarily divided into three different parts. The thickest nerve bundles usually anastomosed with each other to form a well-defined "primary" nerve plexus (fig. 15). Similarly the finest nerve bundles usually anastomosed with each other to form a well-defined "tertiary" nerve plexus (fig. 17). Primary and tertiary plexuses were united by a relatively poorly-defined "secondary" nerve plexus (figs. 15 and 16).

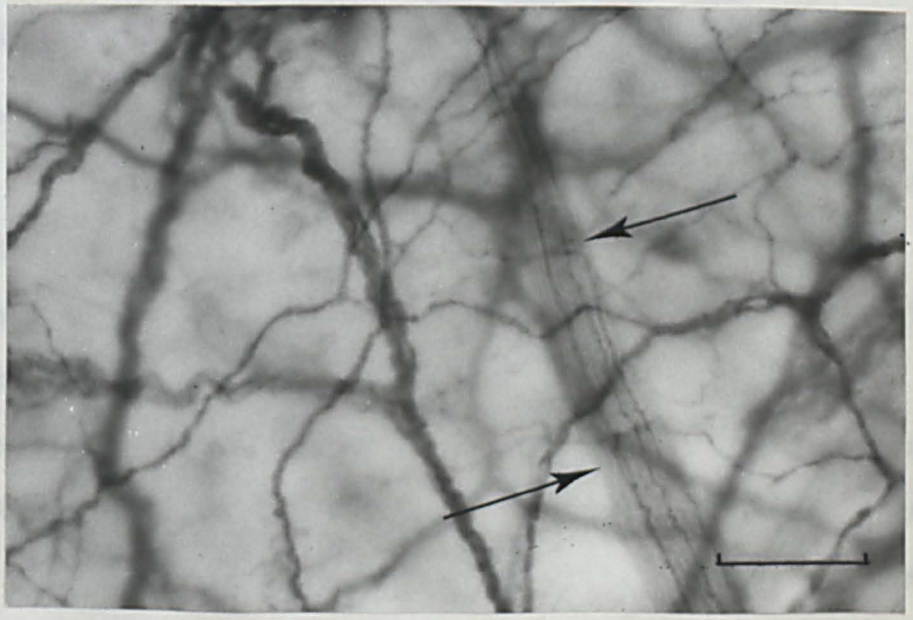
The fibres of the primary nerve plexus (fig. 15) came directly from the peribronchial plexus and formed a coarse-meshwork of thick nerve bundles in the connective tissue between the muscle bundles. The largest nerve bundles were approximately 35μm in width. There were many thick fibres in the plexus, some of which left the plexus and with little or no branching appeared to end abruptly in the connective tissue conforming therefore to previous descriptions of simple free fibre afferent nerve endings. Small ganglia lay at many of the nodal points of the plexus although more nerve cell bodies appeared to be irregularly distributed in the nerve bundles. Branches of the primary plexus gave rise to the secondary and tertiary nerve plexuses in the muscle layer as well as contributing to the innervation of the lamina propria.

The fine secondary nerve plexus (figs. 15 and 16) lay

Figure 15. Low power photomicrograph of nerve plexuses in the muscle layer of the primary bronchus. The largest nerve bundles anastomose with each other to form a coarse-meshed primary plexus (1). A narrower-meshed secondary plexus (2) of finer nerve bundles comes from the primary plexus. Scale = 100μm. Uninhibited cholinesterase preparation.

Figure 16. Medium power photomicrograph of the secondary nerve plexus around a muscle bundle (arrows) in the primary bronchus. Scale = 50 μm. Uninhibited cholinesterase preparation.


1
2
1
2



within the meshwork of the primary plexus. Compared with the primary plexus, its bundles were much finer, more numerous and together formed a narrower mesh. Furthermore, unlike the primary plexus, the secondary plexus appeared to consist only of fine fibres and had no ganglia. Branches of the secondary plexus gave rise to the tertiary nerve plexus in the muscle layer and like the primary plexus also supplied the lamina propria.

The tertiary nerve plexus (fig. 17) came mainly from the secondary plexus with a much smaller contribution from the primary plexus. Most of the nerve bundles in the plexus were less than 1µm in width. Much of the plexus lay parallel and close to the muscle cells and was best demonstrated by the methylene blue and osmium tetroxide techniques which gave its fibres a regular beaded appearance. No ganglia were seen in the plexus.

(iii) Lamina Propria.

Fibres from the primary and secondary nerve plexuses of the muscle layer including some thick fibres formed a plexus in the lamina propria. The bundles of the plexus varied widely in size, the largest being approximately 30µm in width. The meshwork of the plexus in the deep part of the lamina propria was usually very wide (fig. 18). From here bundles travelled towards the epithelium dividing in a Y-shaped fashion just below the basement membrane. The meshwork of the plexus close to the epithelium was usually narrow and therefore this part of the lamina propria generally appeared to be better innervated (figs. 19 and 20). Here many of the

Figure 17. Low power photomicrograph of the fine bundles (arrows) of the tertiary nerve plexus in the thin layer of muscle (m) of the primary bronchus which lies on the outside of the cartilage (C). ct = peribronchial connective tissue. Scale = 15μm. Osmium tetroxide stain.

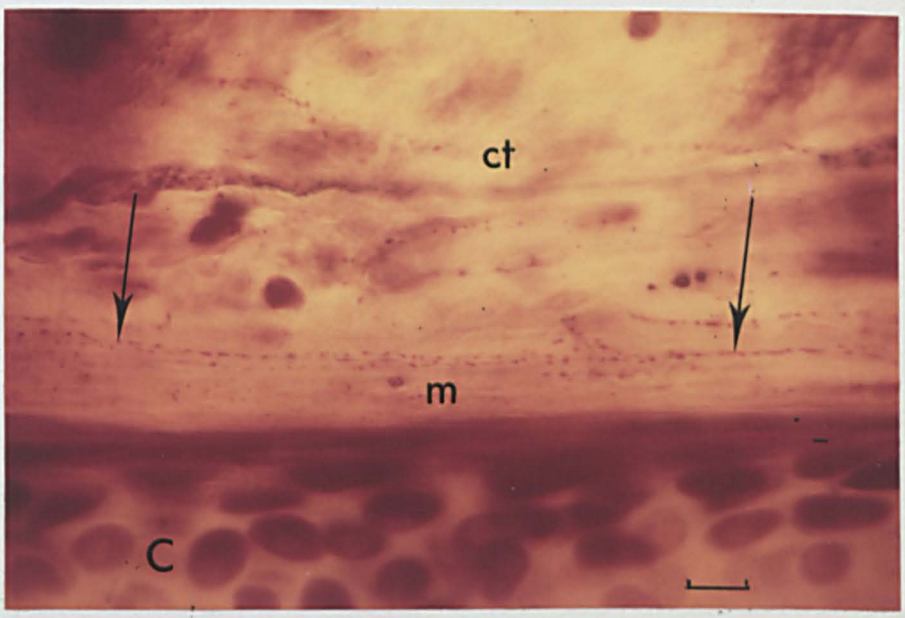

ct
m
C

Figure 18. Medium power photomicrograph of a wide-meshed nerve plexus in the deep part of the lamina propria of the primary bronchus. Scale = 50µm. Inhibited cholinesterase preparation (iso-OMPA, 1.5×10^{-6}M).

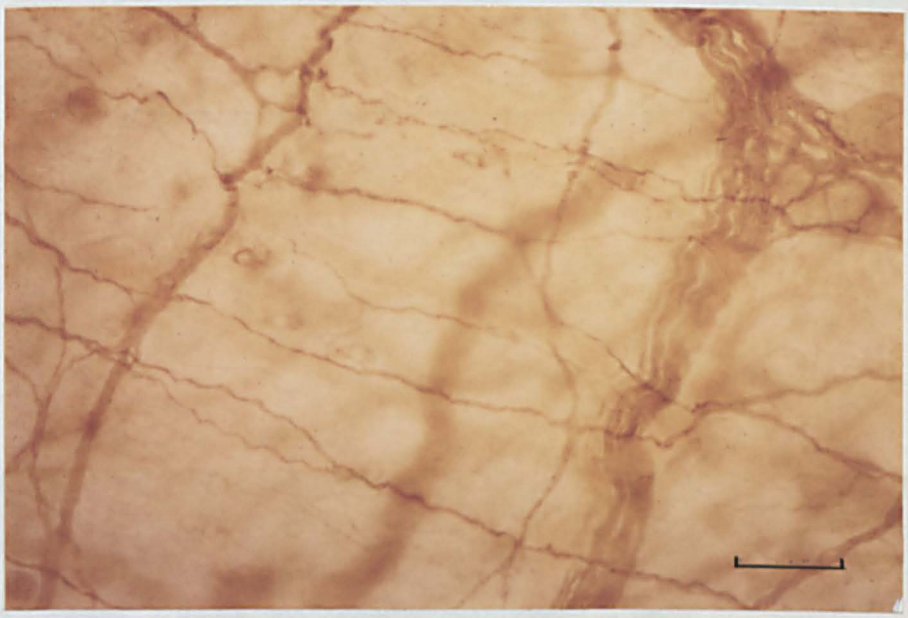
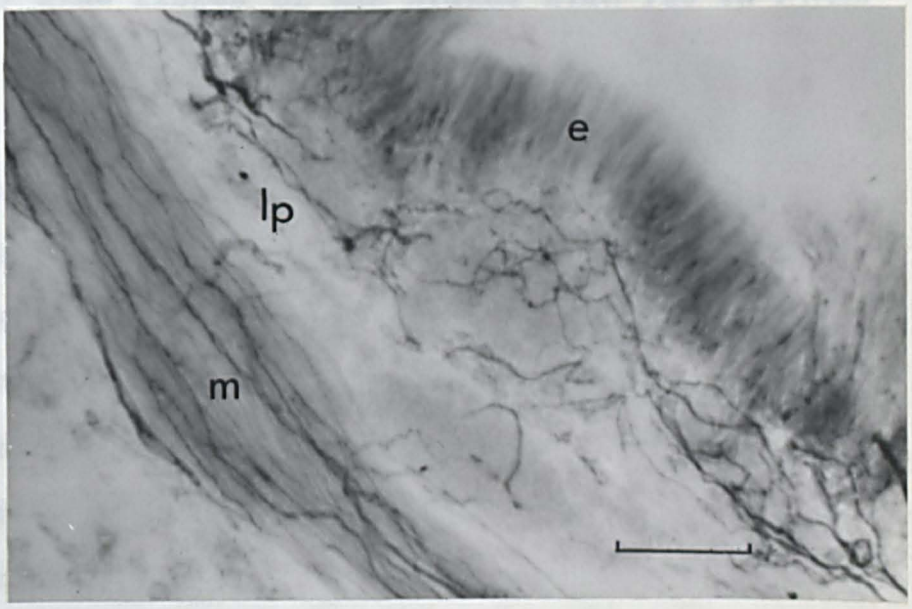


Figure 19. Medium power photomicrograph of nerve plexuses in the muscle layer (m) and lamina propria (lp) of the primary bronchus. The plexus in the lamina propria appears best developed close to the epithelium (e) where there are many small blood vessels. Scale = 50μm. Uninhibited cholinesterase preparation.

Figure 20. Medium power photomicrograph of part of the nerve plexus in the lamina propria of the primary bronchus lying immediately below the epithelium. Most nerve bundles in the plexus lie close to a well-developed plexus of small blood vessels (arrows). Scale = 50μm. Uninhibited cholinesterase preparation.


e
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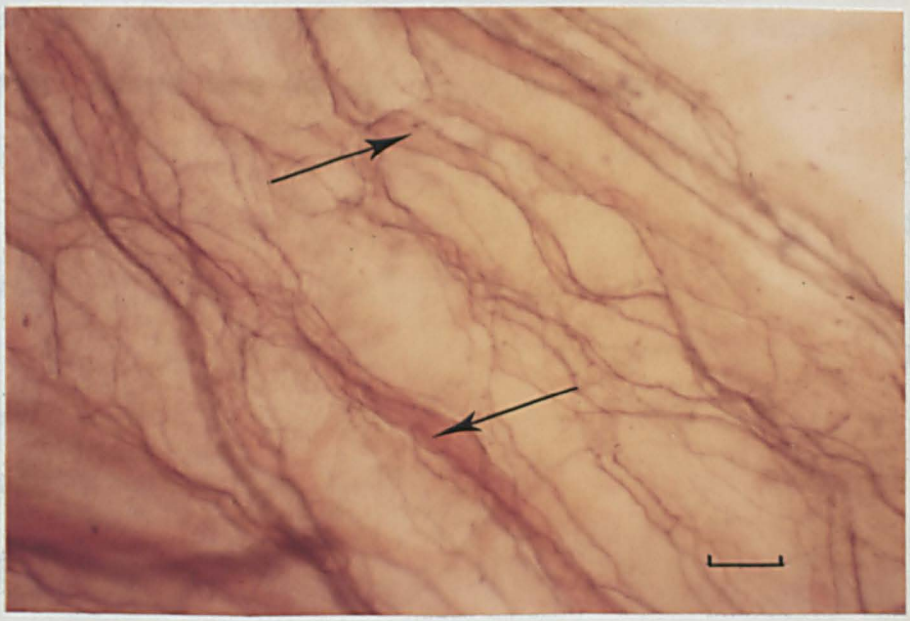


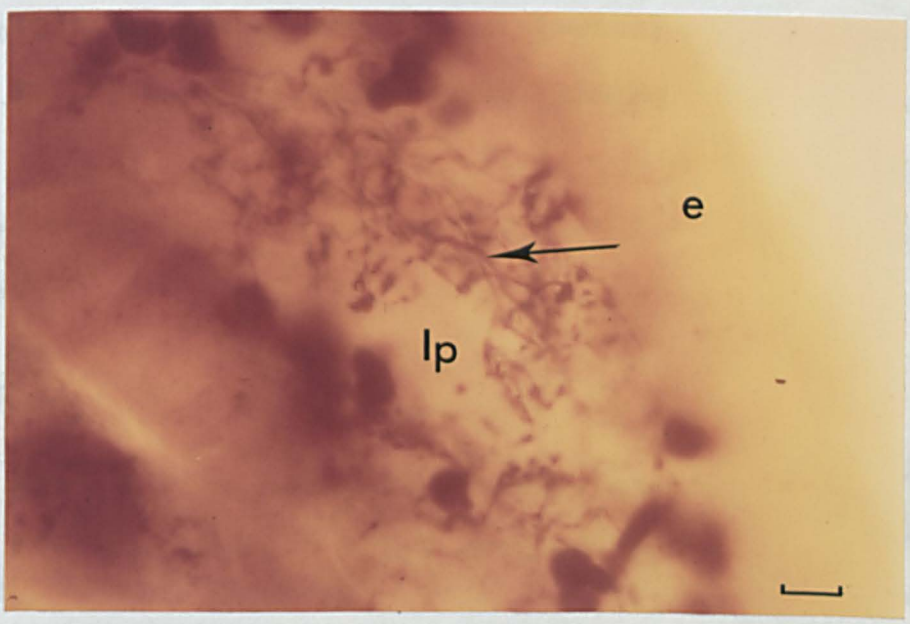
nerve bundles accompanied a well-developed blood capillary plexus. Very fine fibres of the plexus also lay in close contact with the bases of the epithelial cells but did not often appear to enter the epithelium. A perichondrial innervation was not seen.

As in the muscle layer, single thick fibres were distributed separately from the plexus. Sometimes in sections stained by osmium tetroxide these thick fibres were distributed in a way which suggested that they might be complex free fibre sensory endings (fig. 21). Each thick fibre divided rapidly several times into successively finer fibres, the finest branches generally appearing to end in knob-like swellings. A single thick fibre innervated a relatively large area as its many branches usually extended in opposite directions to each other and seemed sometimes even to enter the connective tissue of the muscle layer. It was not possible in a single photograph to demonstrate more than a few branches of one of these thick fibres since they were distributed in several planes. Other more numerous thick fibres in the lamina propria with little or no branching appeared to end abruptly and conformed therefore to previous descriptions of simple free fibre afferent nerve endings. The relatively few ganglia in the lamina propria were small and rarely consisted of more than four cells.

(iv) Epithelium.

There was some evidence for an epithelial innervation in sections stained by the methylene blue, silver nitrate and

Figure 21. Medium power photomicrograph of a nerve fibre (arrow) in the lamina propria (lp) of the primary bronchus which is distributed in a way suggestive of a complex free fibre type of afferent nerve ending. The fibre divides rapidly into successively finer branches many of which appear to end in knob-like swellings. Many other branches of the ending lie outside the plane of the photograph. e = epithelium. Scale = 10μm. Osmium tetroxide stain.


e
lp

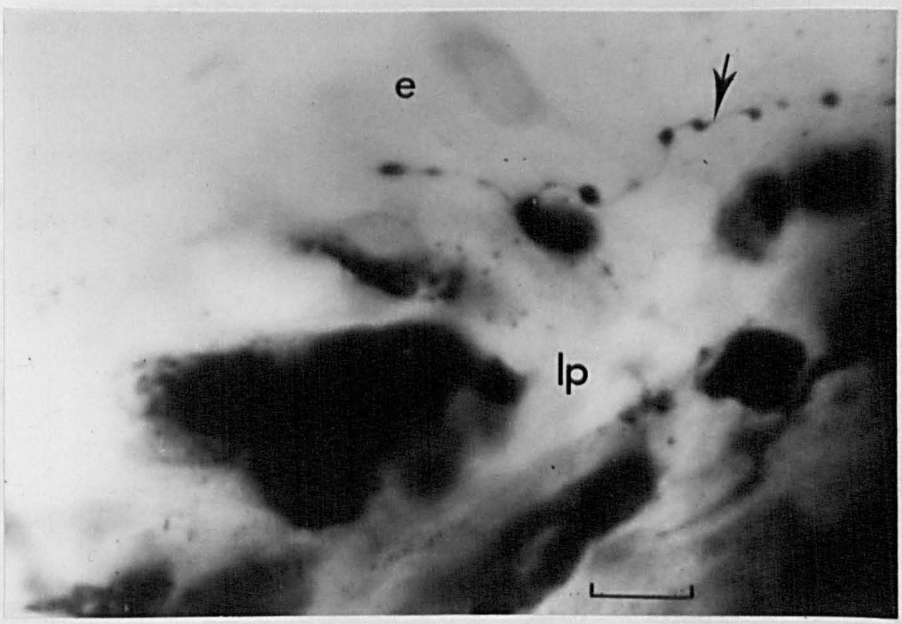
osmium tetroxide techniques (figs. 22 and 23). This seemed to come from two sources. Firstly fine fibres which had usually left the plexus in the lamina propria at some distance from the epithelium, branched dichotomously just below the epithelium and appeared to penetrate between the epithelial cells. Other intraepithelial fibres seemed to branch from the very fine fibres which lay in close contact with the bases of the epithelial cells. All the epithelial fibres ended below the luminal surface of the epithelium without branching or terminal enlargements. The epithelial cells close to the "endings" of the fibres were not stained differently from the other epithelial cells. The innervation was not found to be better developed at any particular level of the bronchus.

(b) Secondary Bronchi.

The innervation of a secondary bronchus was essentially the same as that of the primary bronchus and its peribronchial and intrabronchial nerve plexuses were continuations of those of the primary bronchus (fig. 24). These plexuses in the secondary bronchus were usually best developed proximally (fig. 25) and where the proximal part of a secondary bronchus lay next to another secondary bronchus, the plexuses of the two bronchi were continuous with each other (fig. 26). Further distally, where the wall of the bronchus was much thinner there were fewer nerve fibres and it was impossible to distinguish separate plexuses. The evidence for complex free fibre sensory endings in the secondary bronchi was not

Figure 22. High power photomicrograph of the mucous membrane of the primary bronchus. A beaded nerve fibre (arrow) in the lamina propria (lp) appears to enter the epithelium (e). Scale = 10μm. Osmium tetroxide stain.

Figure 23. Photomicrographs of the mucous membrane of the primary bronchus, on the left stained by the methylene blue technique, scale unknown; on the right stained by a modified Bielschowsky-Gros silver method, scale 10μm. Nerve fibres (arrows) in the lamina propria appears to enter the epithelium (e).


e
lp

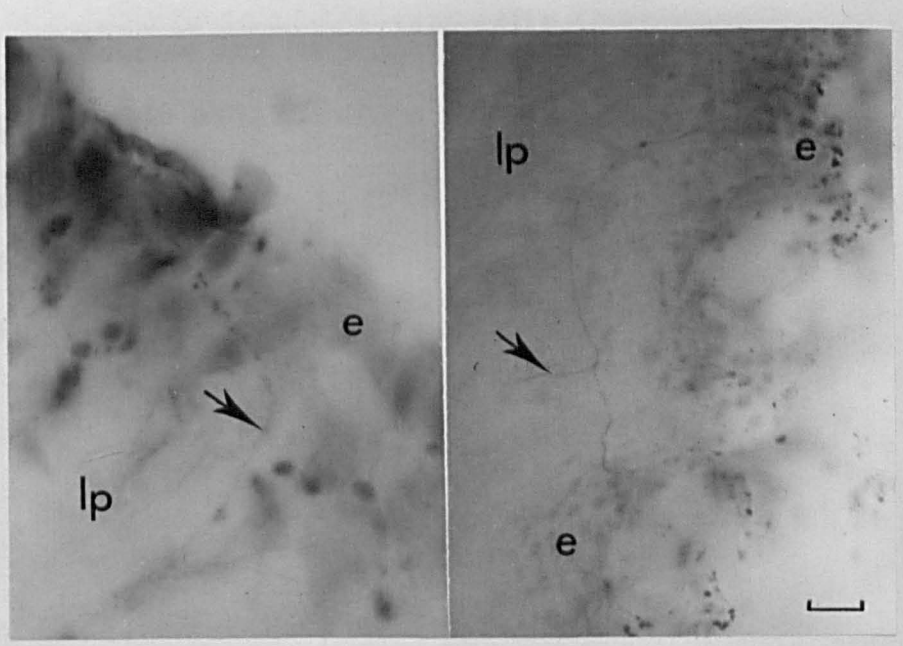
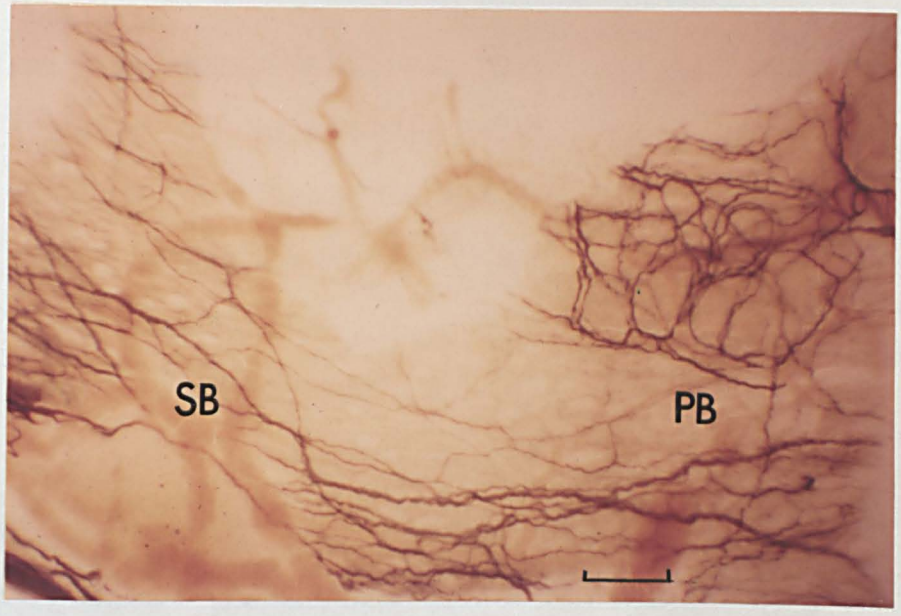

e
lp
lp
e
e

Figure 24. Low power photomicrograph of a section through the lamina propria of the primary bronchus (PB) and the lamina propria of a secondary bronchus (SB) showing that the nerve plexuses of the two bronchi are continuous. Scale = 100μm. Inhibited cholinesterase preparation (iso-OMPA, 1.5×10^{-6}M).

Figure 25. Medium power photomicrograph of nerve fibres in the muscle layer of the proximal part of a caudodorsal secondary bronchus. Scale = 20μm. Uninhibited cholinesterase preparation intensified with silver.


SB
PB

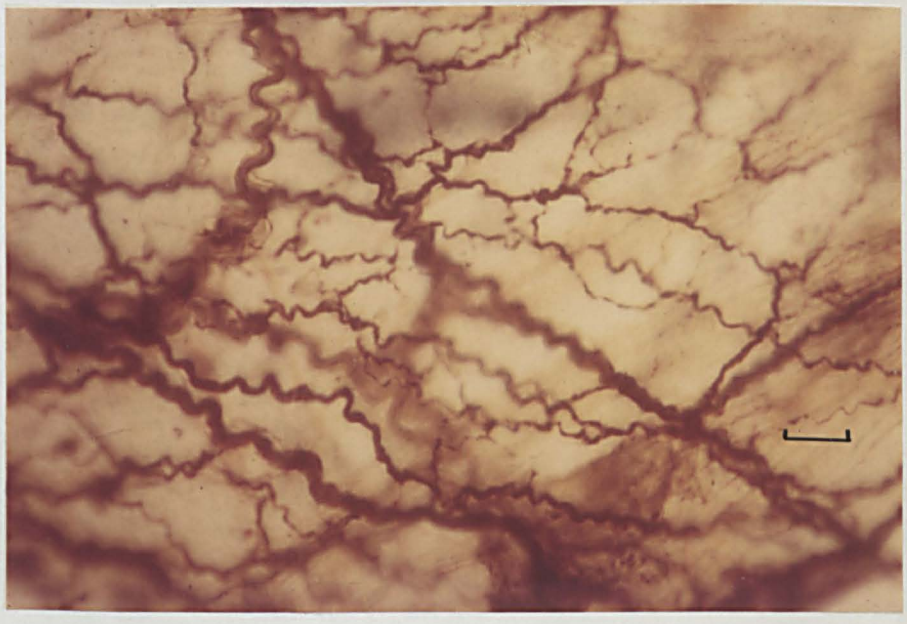
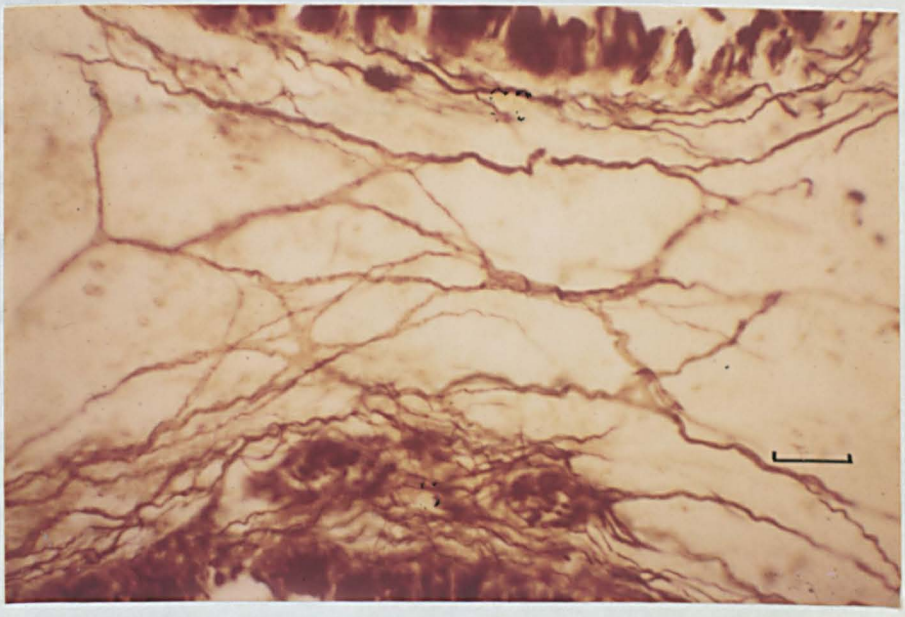


Figure 26. Medium power photomicrograph of the proximal parts of two secondary bronchi in transverse section. The nerve plexuses of the bronchi are continuous with each other. Scale = 50μm. Uninhibited cholinesterase preparation.



nearly so convincing as in the primary bronchus. However, as in the primary bronchus, there were many structures here which resembled simple free fibre endings. Intraepithelial fibres were not seen. The ganglia were larger and more numerous at the level of the proximal part of a bronchus, but even the largest ganglia were smaller than many of the ganglia in the peribronchial plexuses of the primary and tertiary bronchi.

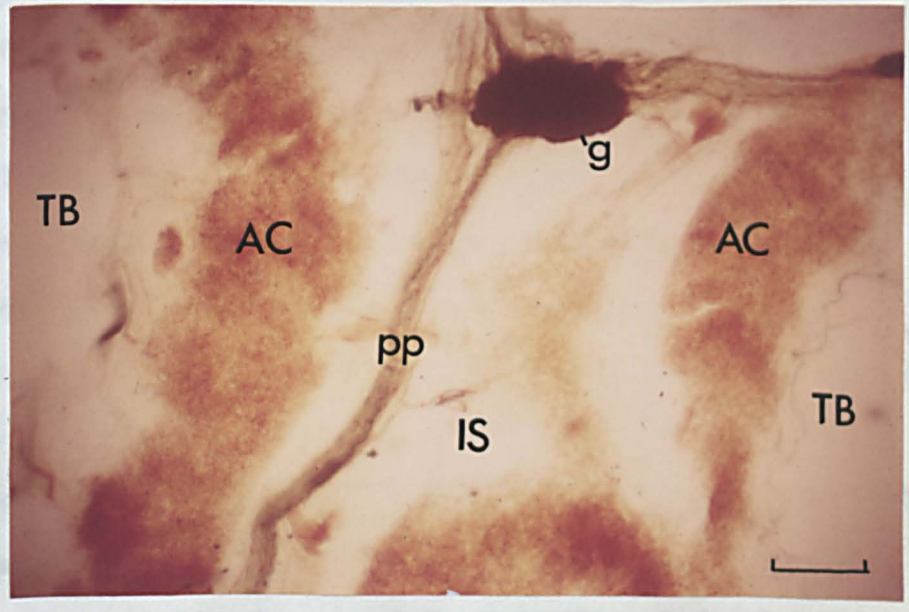
(c) Tertiary Bronchi and Interatrial Septa.

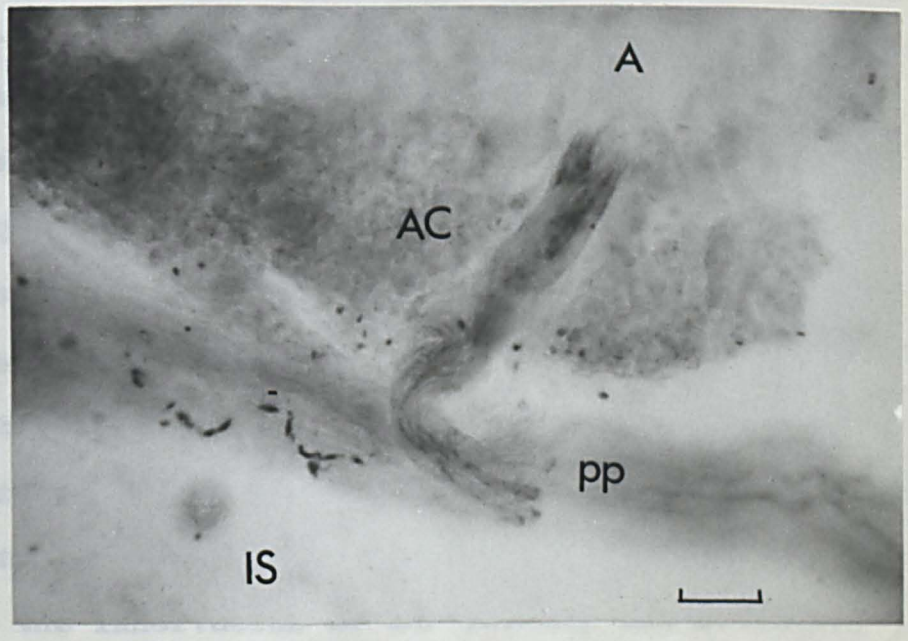
The innervation of the tertiary bronchi could not be distinguished from the innervation of the walls of the atria leading from them, the interatrial septa, and therefore in this account the interatrial septa, for convenience, are considered as part of the tertiary bronchi. The peribronchial area of the tertiary bronchi then consists of the tissues lying peripheral to the atria, that is the air capillary regions and the interlobular connective tissue septa (figs. 28 and 29).

The nervous tissue in the tertiary bronchi was a continuation of plexuses in the secondary bronchi. It also received branches from a coarse-meshed peribronchial nerve plexus (figs. 27,28,29,30 and 31). The tertiary bronchi lying next to the primary bronchus in addition were innervated by branches from the peribronchial plexus of the primary bronchus (fig. 13). The peribronchial nerve plexuses of all the tertiary bronchi were continuous. Those bundles of the plexus which lay in the interlobular septa (figs. 27

Figure 27. Low power photomicrograph of the peribronchial nerve plexus (pp) of the tertiary bronchi (TB). AC = air capillaries; g = ganglion; IS = interlobular septum. Scale = 100μm. Uninhibited cholinesterase preparation.

Figure 28. Low power photomicrograph of the innervation of a tertiary bronchus and interatrial septa. Nerve fibres from the peribronchial plexus (PP) of the tertiary bronchus pass through the air capillaries (AC) to the bronchus and septa. A = atrium; IS = interlobular septum. Scale = 50μm. Methylene blue stain.


TB
AC
g
AC
pp
IS
TB


A
AC
pp
IS

and 28) were relatively large, the thickest being approximately 100μm in width. Those bundles of the plexus which lay in the air capillary regions (figs. 28,29,30 and 31) were usually much smaller than this, the thickest being only about 35μm in width. All the bundles had some thick fibres and many fine fibres. The plexus innervated the tertiary bronchi in two ways. Firstly offshoots from the part of the plexus lying in the interlobular septa travelled with little branching through the air capillaries to the tertiary bronchi (fig. 29). Other branches to the tertiary bronchi came from bundles of the plexus which travelled through the air capillaries to join together interlobular nerve bundles lying on opposite sides of the lobule to each other (figs. 30 and 31). There were no anastomoses between bundles of the plexus lying in the air capillary region. Ganglia were only found in the interlobular part of the plexus (fig. 27). They lay here mainly at the division points of the plexus and were generally relatively large. Some ganglia even appeared to have as many cells as the largest ganglia in the proximal part of the peribronchial nerve plexus of the primary bronchus. The peribronchial nerve plexus of the tertiary bronchi also innervated blood vessels in the interlobular septa (figs. 32 and 33) and in addition contributed to a much narrower-meshed plexus in the air capillary regions which will be described later.

Unlike the innervation of the primary and secondary bronchi, that of a tertiary bronchus could not be divided into separate plexuses, one in the muscle layer and one in

Figure 29. Low power photomicrograph of the innervation of a tertiary bronchus (TB). The nerve bundle of the peribronchial plexus (pp) passes through the air capillaries (AC) to supply the tertiary bronchus and the interatrial septa. A = atrium; IS = interlobular septum. Scale = 50μm. Uninhibited cholinesterase preparation intensified with silver.

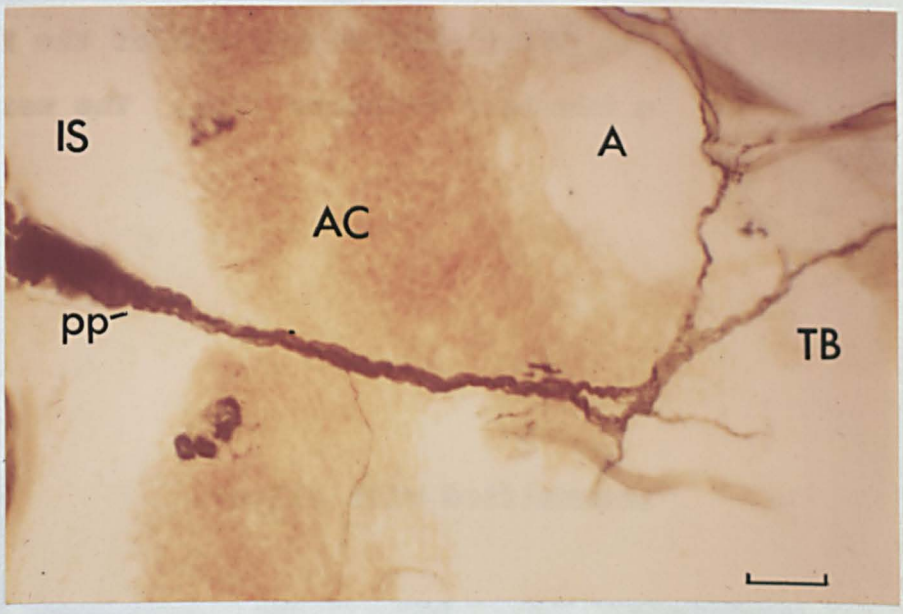
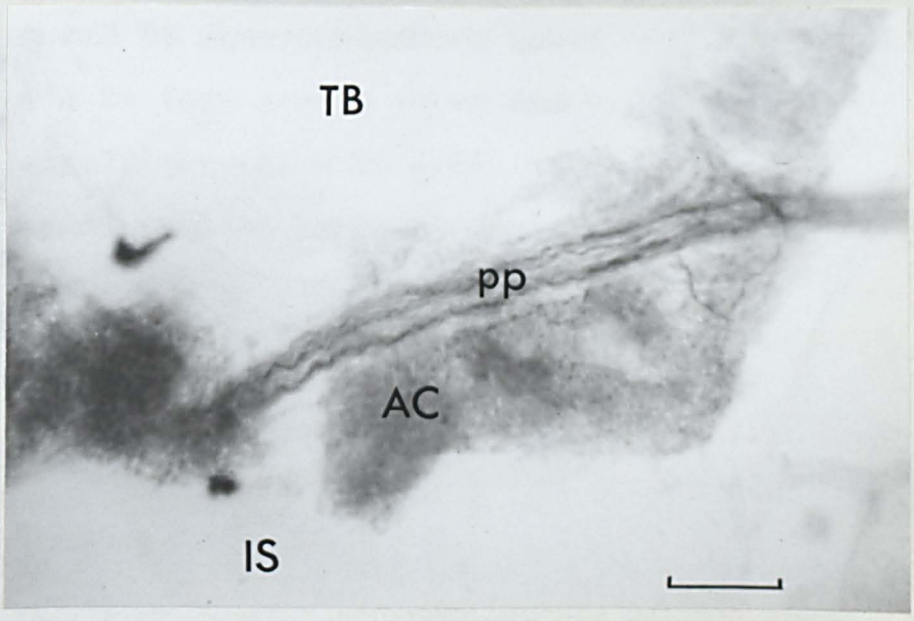

IS
A
AC
pp⁻
TB

Figure 30. Low power photomicrograph of the peribronchial nerve plexus (pp) of a tertiary bronchus (TB). AC = air capillaries; IS interlobular septum. Scale = 100μm. Inhibited cholinesterase preparation (Lysivane, 10^{-4}M).

Figure 31. Low power photomicrograph of the peribronchial nerve plexus (arrows) of a tertiary bronchus (TB) in transverse section. AC = air capillaries; IS = interlobular septum. Scale = 100μm. Modified Bielschowsky-Gros silver technique.


TB
pp
AC
IS

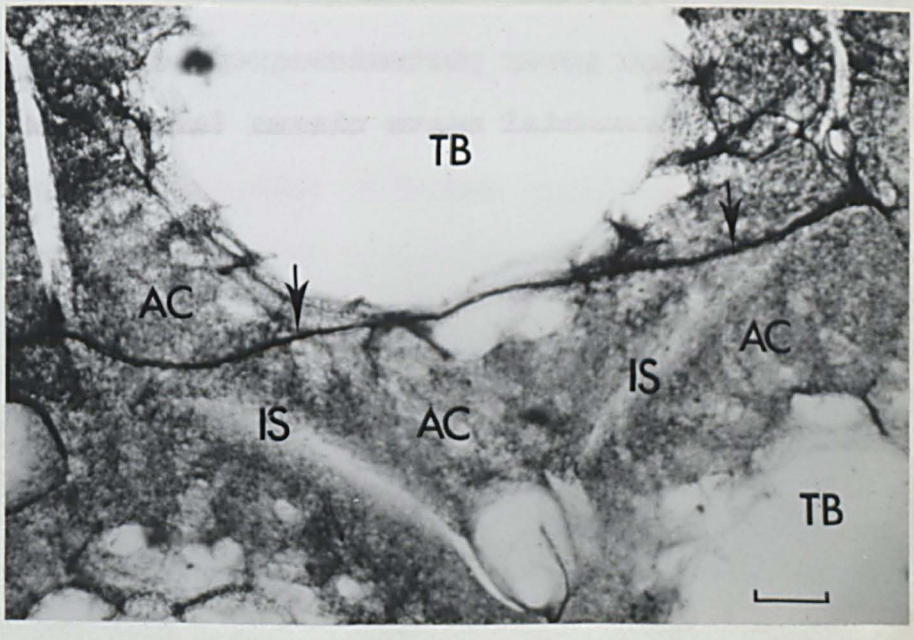
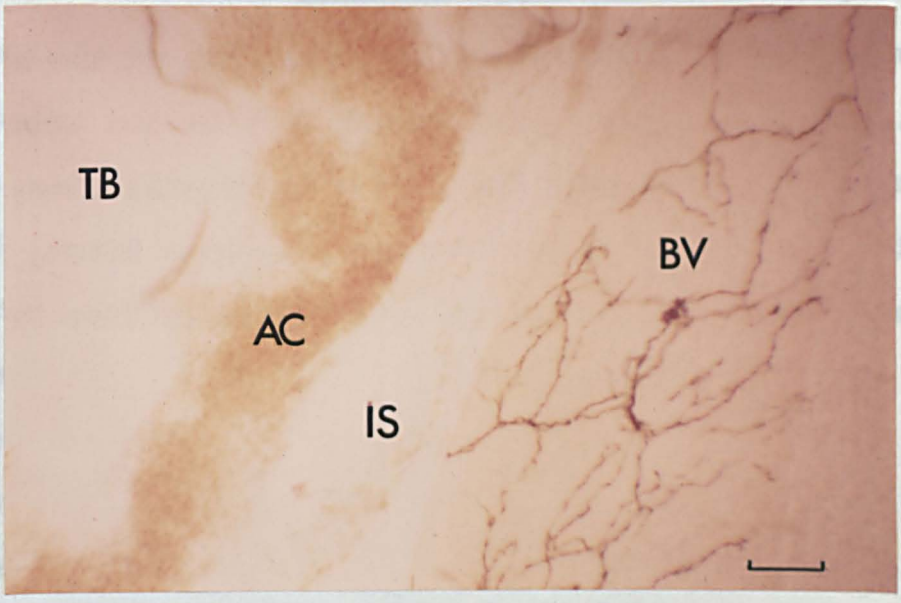
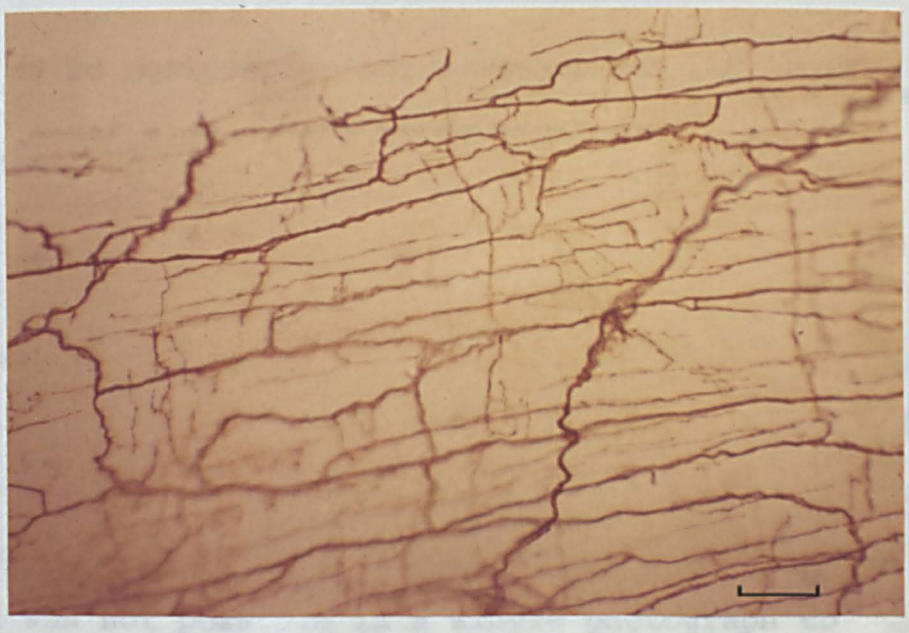

TB
AC
AC
IS
IS
AC
TB

Figure 32. Low power photomicrograph of the nerve plexus in a blood vessel (BV) in the interlobular septum (IS). AC = air capillaries; TB = tertiary bronchus. Scale = 100μm. Inhibited cholinesterase preparation (iso-OMPA, 3 X 10^{-6}M).

Figure 33. Medium power photomicrograph of the well-developed nerve plexus in a large blood vessel in the interlobular septum. Scale = 50μm. Inhibited cholinesterase preparation (iso-OMPA, 3 X 10^{-6}M) intensified with silver.


TB
BV
AC
IS



the lamina propria. Also as already described, the innervation of a tertiary bronchus could not be distinguished from the innervation of the interatrial septa. The larger nerve bundles in the bronchus and septa 2 - 10μm in width, had a small number of thick fibres and many fine fibres and sometimes formed quite a distinct coarse-meshed plexus (fig. 34). However, most of the nerve bundles were smaller than this, had only fine fibres and formed a plexus within the mesh-work of the larger nerve bundles (figs. 35 and 36). The smallest nerve bundles were similar to those of the tertiary nerve plexus in the muscle of the larger bronchi. The muscle tissue of a tertiary bronchus was best innervated close to the origin of the bronchus from a secondary bronchus although its innervation always appeared to be poorer than that of the primary and secondary bronchi.

Some thick nerve fibres in the tertiary bronchi and interatrial septa (figs. 37 and 38) were distributed separately from the plexuses and seemed in sections stained by silver to be essentially similar to the complex free fibres sensory endings observed in the lamina propria of the primary bronchus with the osmium tetroxide stain. However, unlike the endings stained with osmium tetroxide, their finest branches generally appeared to end freely and were only sometimes seen to have end-knobs. As in the primary bronchus, one thick fibre innervated a very large area and it was not possible in a single photograph to demonstrate more than a few of its branches. This type of ending was not seen here with the osmium tetroxide stain.

Figure 34. Medium power photomicrograph of a plexus of relatively large nerve bundles in the darkly stained muscle layer (m) of a tertiary bronchus and in the lightly stained interatrial septa (AS). A = atrium; AC = air capillaries. Scale = 50μm. Uninhibited cholinesterase preparation intensified with silver.

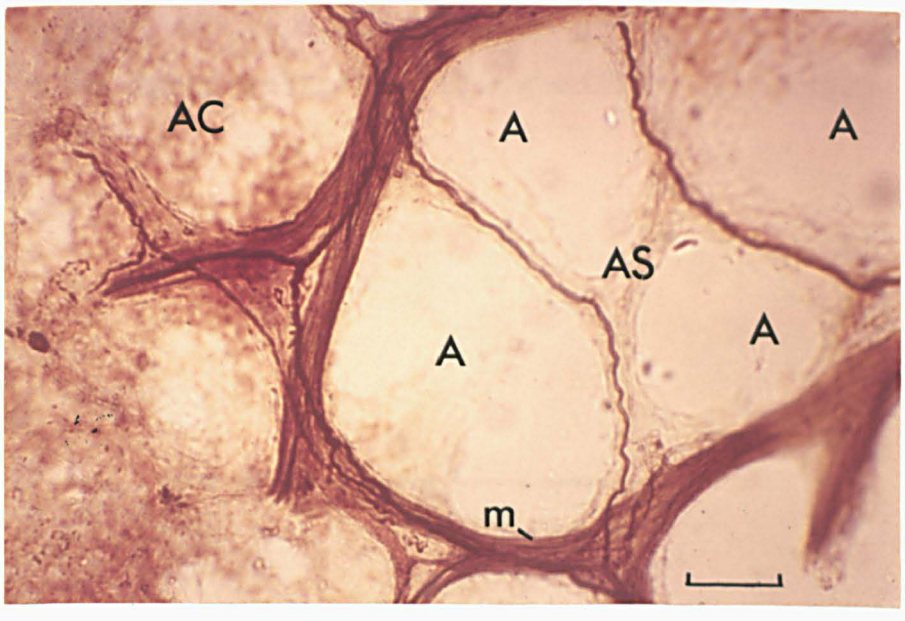
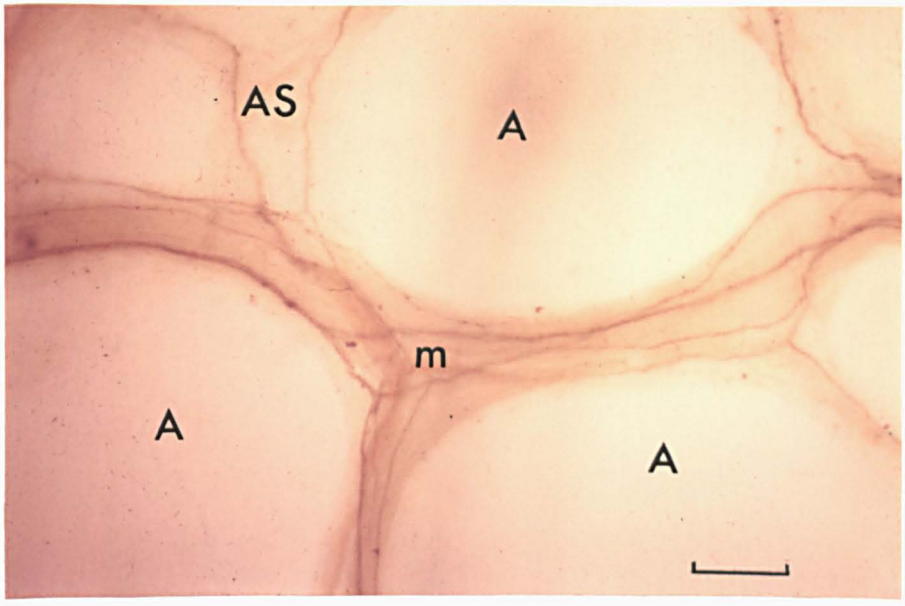

AC
A
A
AS
A
A
m

Figure 35. High power photomicrograph of a plexus of fine nerve bundles in the muscle layer (m) of a tertiary bronchus and in the interatrial septa (AS). A = atrium. Scale = 50μm. Inhibited cholinesterase preparation (Lysivane, 10^{-4}M).

Figure 36. High power photomicrograph of a plexus of fine nerve bundles in the interatrial septa (AS). A = atrium. Scale = 10μm. Modified Bielschowsky-Gros silver technique.


AS
A
m
A
A

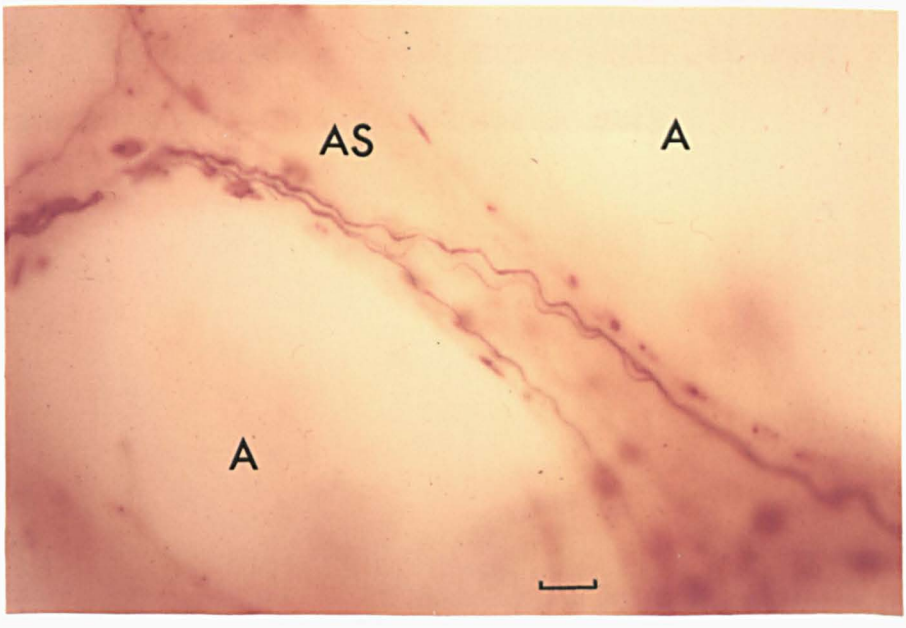
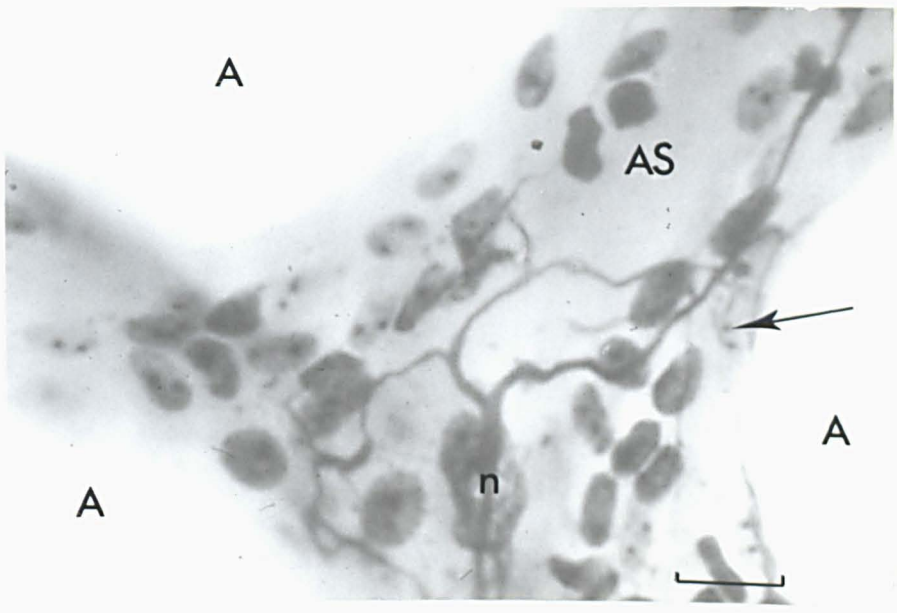
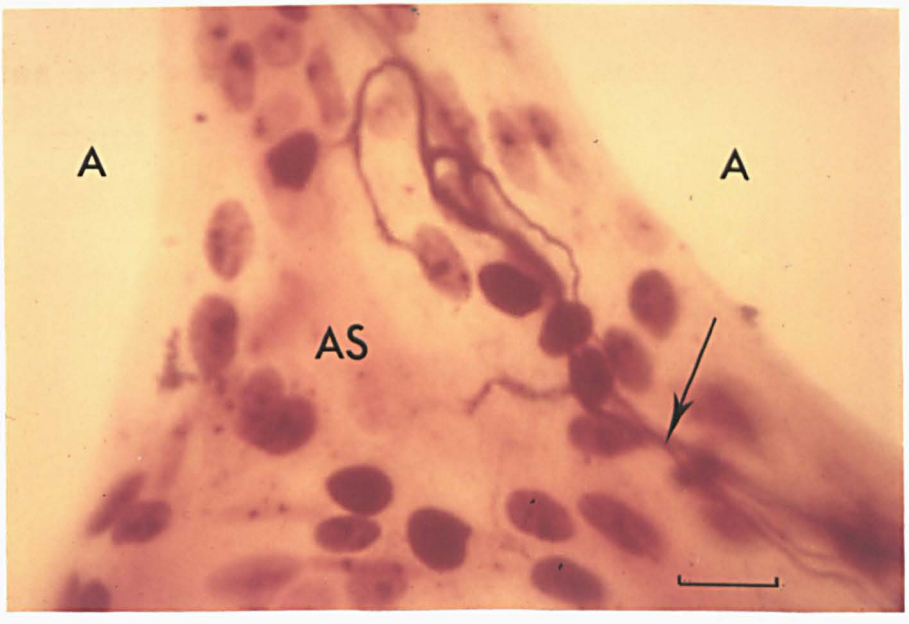

AS
A
A

Figure 37. High power photomicrograph of a nerve fibre (n) in the interatrial septum (AS) which is distributed in a way suggestive of a complex free fibre type of afferent nerve ending. The fibre divides rapidly into progressively finer branches which extend in opposite directions to each other and sometimes appear to end in knob-like swellings (arrow). A = atrium. Scale = 10μm. Modified Bielschowsky-Gros silver technique.

Figure 38. High power photomicrograph of a nerve fibre (arrow) in the interatrial septum (AS) which is distributed in a way suggestive of a complex free fibre type of afferent nerve ending. A = atrium. Scale = 10μm. Modified Bielschowsky-Gros silver technique.

The sensory endings in Figures 37 and 38 innervated relatively large areas of the interatrial septa. However, since the branches of each of these endings were distributed in several planes it was only possible in a single photograph to demonstrate a small part of an ending.


A
AS
A
A
n


A
A
AS

Many other thick fibres in the tertiary bronchi and interatrial septa ended in a way that conformed to the simple free fibre type of afferent ending. No innervation of the simple squamous epithelium of the air passages was observed. Ganglion cells were not found here.

(d) Air Capillaries.

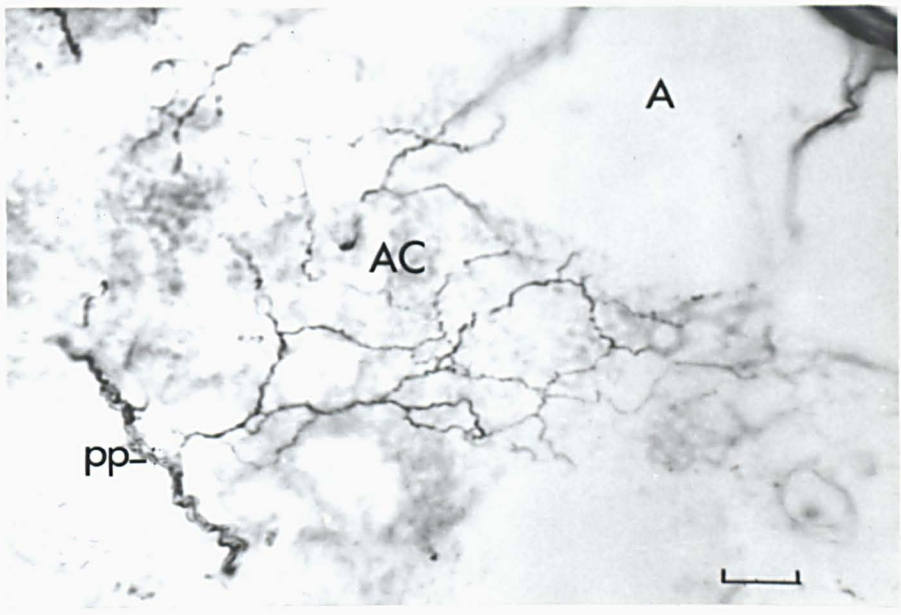
The air capillary region contained many of the large nerve bundles of the coarse peribronchial plexus of the tertiary bronchi which has already been described. Small branches of this peribronchial plexus, less than 2μm in width, were distributed as a separate narrow-meshed plexus of fine fibres in many parts of the air capillary regions (figs. 39 and 40). This narrow-meshed plexus was best demonstrated by the cholinesterase technique intensified by silver. Although much of the plexus appeared to be distributed rather irregularly through the air capillary region, some of the bundles obviously innervated the large blood vessels that lay here (fig. 41). The plexuses around adjacent large blood vessels were joined together in places by small nerve bundles. However, not all large blood vessels appeared to be innervated. Nerve fibres were not seen to end in the air capillaries. No ganglion cells were present in this part of the lung.

(C) Ganglion Cells.

Ganglia were numerous in the peribronchial nerve plexuses of all the bronchi and also in the plexuses in the walls of the primary and secondary bronchi. They were not

Figure 39. Low power photomicrograph of a narrow-meshed nerve plexus in the air capillaries (AC) that is continuous with the large nerve bundle of the peribronchial plexus (pp) of a tertiary bronchus. A = atrium. Scale = 50μm. Uninhibited cholinesterase preparation intensified with silver.

Figure 40. Medium power photomicrograph of the peribronchial nerve plexus (pp) of a tertiary bronchus (TB). Fine branches (arrows) of the plexus extend through the air capillaries (AC) towards the interlobular septum (IS). Scale = 50μm. Inhibited cholinesterase preparation (Lysivane, 10^{-4}M).


A
AC
pp

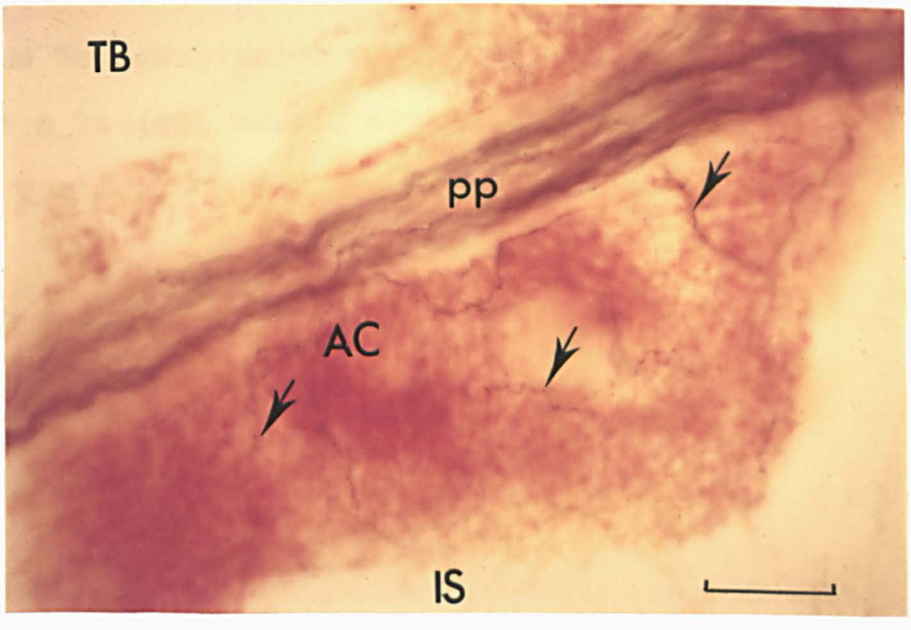
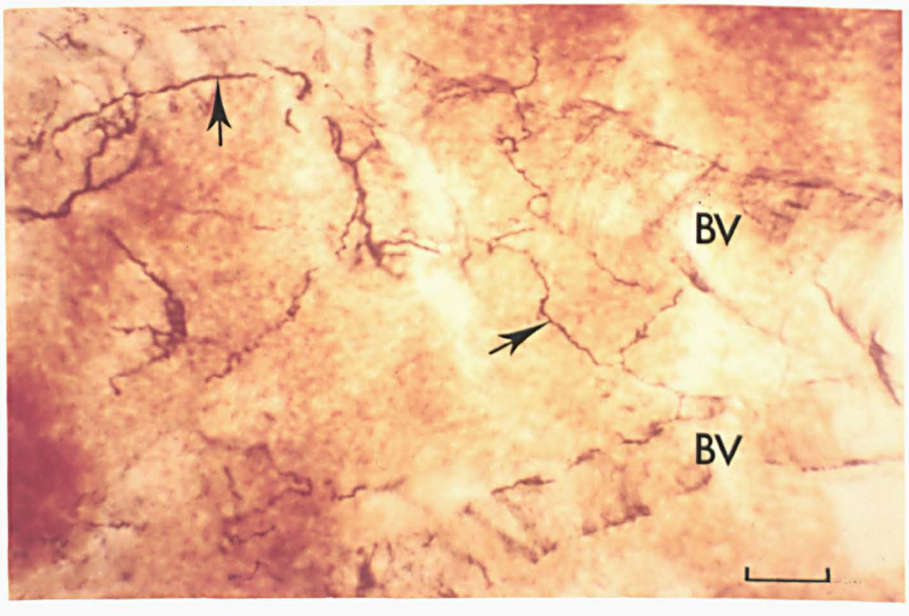

TB
pp
AC
IS

Figure 41. Low power photomicrograph of fine nerve bundles (arrows) near large blood vessels (BV) in the air capillary region. Scale = 50μm. Uninhibited cholinesterase preparation intensified with silver.


BV
BV

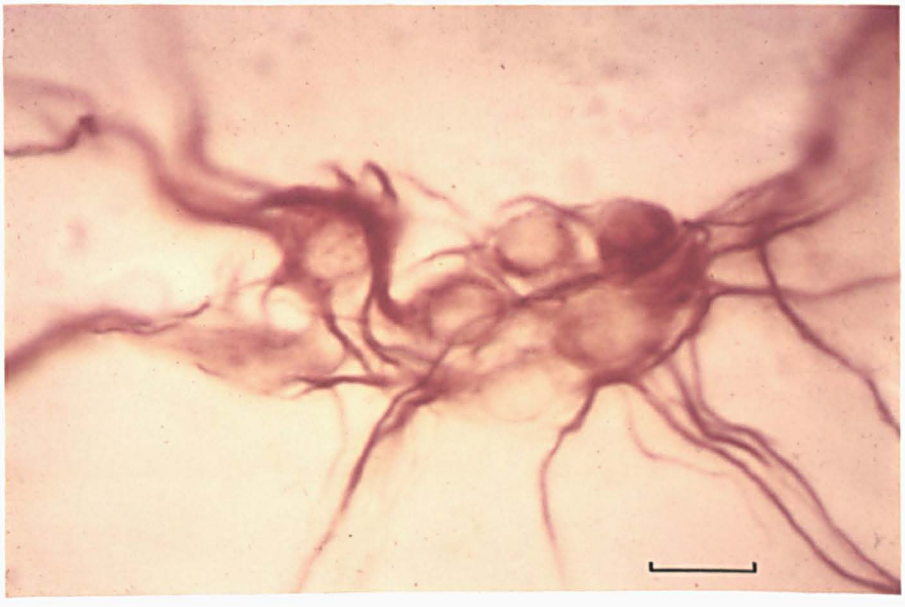
seen in the tertiary bronchi and interatrial septa and in the air capillary regions. Some details of the ganglia have already been given. Further observations on the cells are described below.

A very wide variety of shapes and sizes of ganglion cells were seen (figs. 42,43,44 and 45). The sizes of the round cells appeared to be typical and ranged from approximately 7.5 to 27μm in diameter. The processes of the ganglion cells were only regularly demonstrated by the silver impregnation technique and consequently most observations on them were made with this stain. Most cells were multipolar with between 3 and 9 processes although some cells appeared unipolar and bipolar. The appearance of the processes differed immensely and depended on their thickness and length and whether they were branched or unbranched (figs. 44 and 45). Often the processes of the cells appeared to end or lie in close "association" with other cell bodies or processes of cells in the same ganglion.

When stained by the silver technique most ganglion cells had an argyrophilic cytoplasm and a weakly stained nucleus, the processes then being usually demonstrated (fig. 42). A few cells, however, had an argyrophobic cytoplasm and an intensely stained nucleus, the processes then being usually unstained (fig. 43). Both types of staining reaction were not seen in the same ganglion. Similar staining to this was also found with the methylene blue technique, the nucleus of the ganglion cell being either more heavily stained or

Figure 42. Medium power photomicrograph of a ganglion in the peribronchial nerve plexus of the primary bronchus. All the ganglion cells are multipolar and have argyrophilic cytoplasm and a weakly stained nucleus. Scale = 10μm. Modified Bielschowsky-Gros silver technique.

Figure 43. Medium power photomicrograph of a ganglion in the peribronchial nerve plexus of the primary bronchus. All the ganglion cells have argyrophobic cytoplasm and an intensely stained nucleus (arrow). The processes of the cells are unstained. Scale = 10μm. Modified Bielschowsky-Gros silver technique.



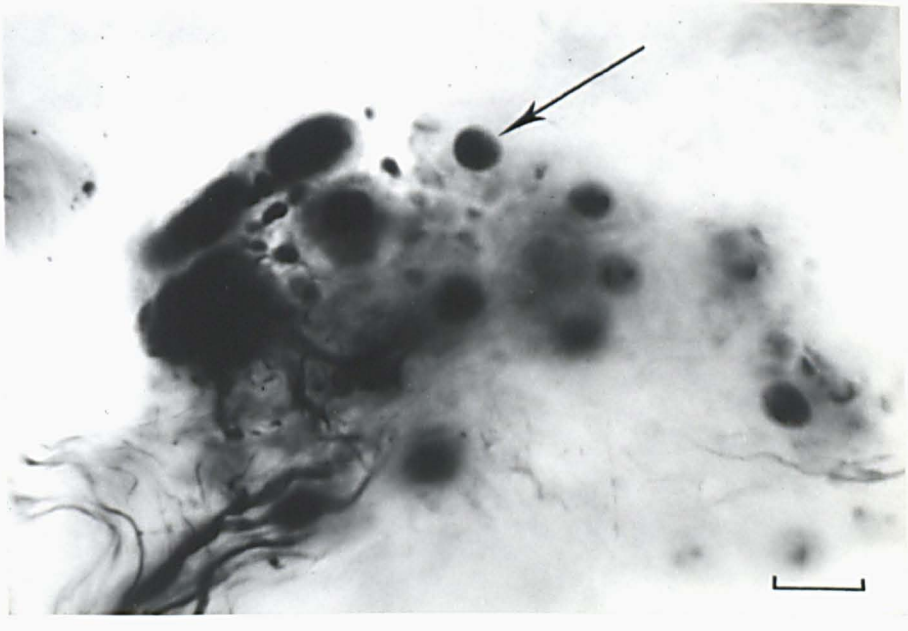
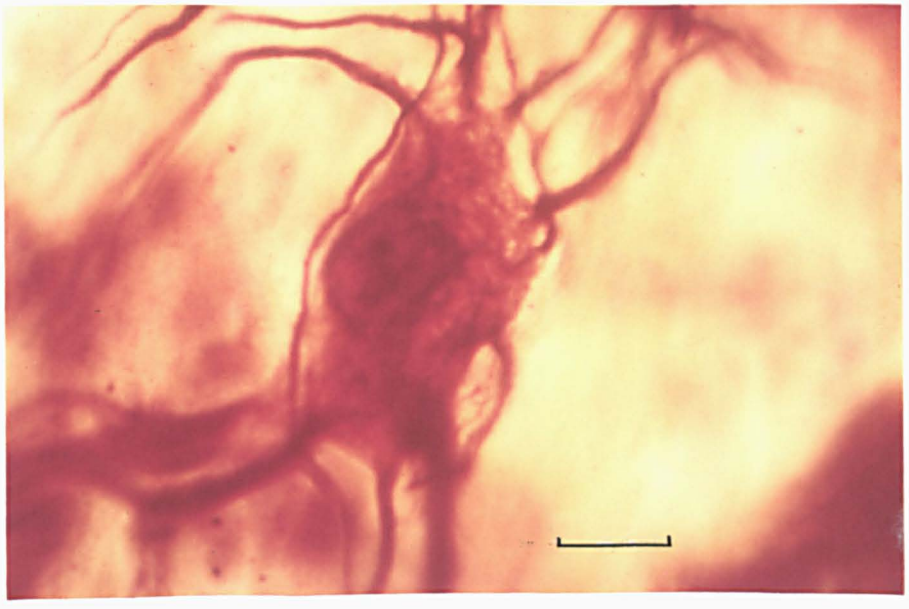
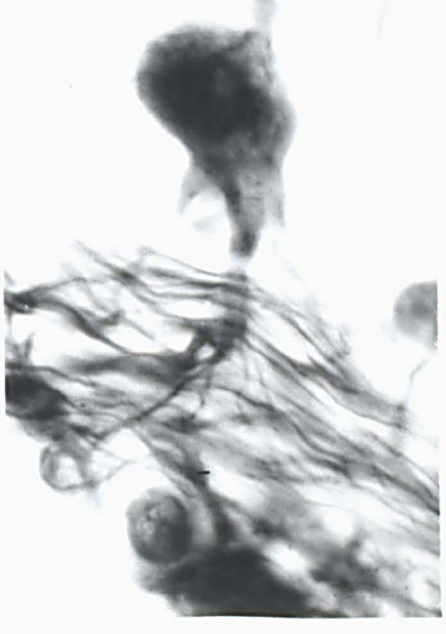
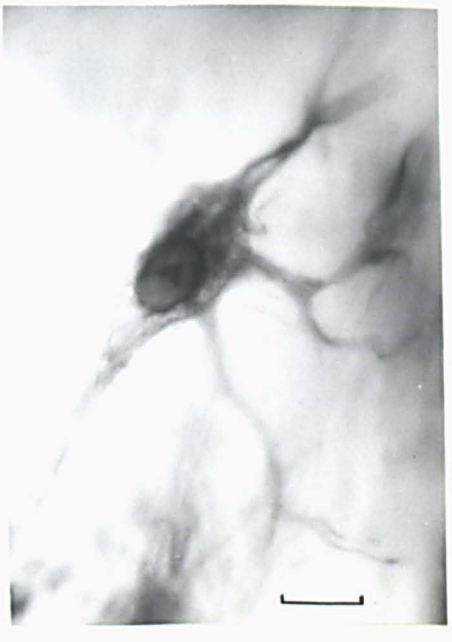


Figure 44. Medium power photomicrograph of a multipolar ganglion cell in the lamina propria of the primary bronchus. Scale = 10μm. Modified Bielschowsky-Gros silver technique.

Figure 45. High power photomicrographs of multipolar ganglion cells, on the left from the interlobular septum; on the right from the lamina propria of the primary bronchus. Scale = 10μm. Modified Bielschowsky-Gros silver technique.







more lightly stained than the cytoplasm. With the cholinesterase technique many of the ganglion cells in sections incubated in an acetylthiocholine iodide substrate medium and exposed to butyrylcholinesterase inhibitors stained at different intensities from each other. The cells in sections incubated in a butyrylthiocholine iodide substrate medium and exposed to acetylcholinesterase inhibitors were either unstained or very weakly stained.

(D) Sensory Nerve Endings.

Details of structures found in the air passages closely resembling previous descriptions of sensory nerve endings have already been given. Some of these corresponded to the so-called complex free fibre endings and were seen in the lamina propria of the primary bronchus with the osmium tetroxide method (fig. 21) and in the walls of the tertiary bronchi and atria with the silver technique (figs. 37 and 38). Many more in all the bronchi with the methylene blue, silver nitrate and osmium tetroxide stains resembled the simple free fibre type of sensory ending in having little or no branching or any other forms of specialization. Evidence for an epithelial innervation was seen in the primary bronchus (figs. 22 and 23) although neurite - receptor cell complexes were not found. No encapsulated sensory endings were demonstrated.

B. Functional Studies on the Afferent Pathways in the Vagus.

(1) The Effects on Breathing of Cervical Vagotomy.

(A) Unilateral Vagotomy.

The effects of unilateral vagotomy on breathing in conscious and anaesthetised birds are summarized in Tables 3,4 and 5, Figures 46,47 and 48 and Graphs 1 and 2. The mean frequencies, tidal volumes and minute volumes were not significantly different from those when both vagi were intact. However, in one lightly anaesthetised bird, the breathing became very irregular with many deep breaths and with pauses in both inspiration and expiration (fig. 49).

(B) Bilateral Vagotomy.

(a) Conscious Birds.

The results are summarized in Table 3, Figure 46 and Graph 1.

(i) Frequency.

The mean frequency of breathing was always significantly less than that when both vagi were intact ($P < 0.01$), the greatest reduction in frequency being 76%, seen ten minutes after vagotomy. This slowing was always due to an increase in the length of expiration.

(ii) Tidal Volume.

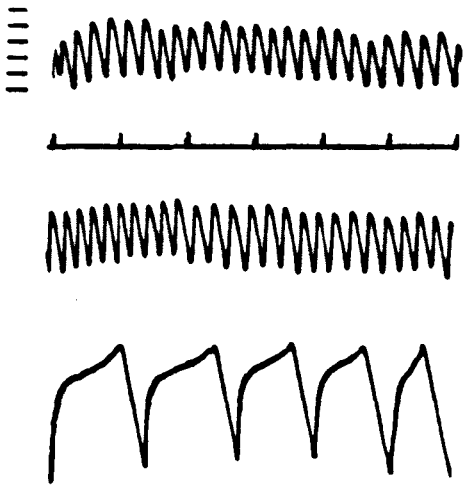
The mean tidal volume was always significantly greater than that when both vagi were intact ($P < 0.01$). The greatest increase was 173%, seen fifty minutes after vagotomy. Usually the depth of breathing was very regular but sometimes

	Time (mins.)	Mean frequency with S.E. and range (breaths/min.)	Mean tidal volume with S.E. and range (ml.)	Mean minute volume with S.E. and range (ml./min.)
I vagi intact	1-5	25.32 ± 2.99 (18.4 - 32)	24.32 ± 4.18 (17.06 - 40.65)	581.18 ± 58 (432.21 - 747.96)
II unilateral vagotomy	6	22 ± 3.13 (15 - 31)	32.05 ± 3.95 (19.12 - 37.33)	724.14 ± 165.53 (344.16 - 1311.61)
	20	18 ± 2.20 (11 - 24)	29.37 ± 4.68 (17.62 - 44.18)	503.53 ± 61.6 (281.92 - 635.88)
III bilateral vagotomy	21	9.80 ± 1.85* (6 - 16)	50.66 ± 3.78* (39.75 - 62.25)	473.9 ± 58.43 (312 - 636)
	30	6 ± 0.32* (5 - 7)	63.50 ± 3.62* (54 - 76.50)	380.38 ± 29.82** (300 - 459)
	50	6.20 ± 0.79* (5 - 9)	61.37 ± 6.55* (47 - 81.60)	372.58 ± 44.71** (243 - 491.96)
	70	6.80 ± 0.48* (6 - 8)	66.45 ± 6.81* (51.16 - 90)	450.16 ± 49.73 (306.96 - 576)
	90	6.80 ± 0.43* (6 - 9)	65.60 ± 8.46* (45.60 - 91.50)	431.08 ± 35.08 (359 - 549)

Values of P for differences between III and I means:
* $P < 0.01$; ** $0.01 < P < 0.02$.

Table 3. Mean values with ranges of frequencies, tidal volumes and minute volumes of the breathing of five conscious adult G. domesticus with both vagi intact (I), one vagus sectioned (II) and both vagi sectioned (III). S.E. is the standard error of the mean. The time at which each observation was made is shown as the number of minutes from the beginning of the experiment.

Figures 46,47 and 48. Kymographic records of breathing of erect, adult, female G. domesticus conscious in Figure 46, lightly anaesthetised in Figure 47, and deeply anaesthetised in Figure 48. In each Figure, the upper record of breathing is with both vagi intact, the middle record with one vagus sectioned and the lower record with both vagi sectioned. Breathing runs from left to right and expiration is ascending. Time trace below upper record in Figure 46 has 10 second intervals. Amplitude trace at left of upper record in Figure 46 has 10 millilitre intervals. Time and amplitude scales in Figures 47 and 48 as for Figure 46.



46

47

48

Graph 1. The breathing of five conscious, erect, adult, female G. domesticus with both vagi intact (minutes 0 to 5), with one vagus sectioned at arrow 1 (minutes 5 to 20), and with the other vagus sectioned at arrow 2 (minutes 20 to 90).

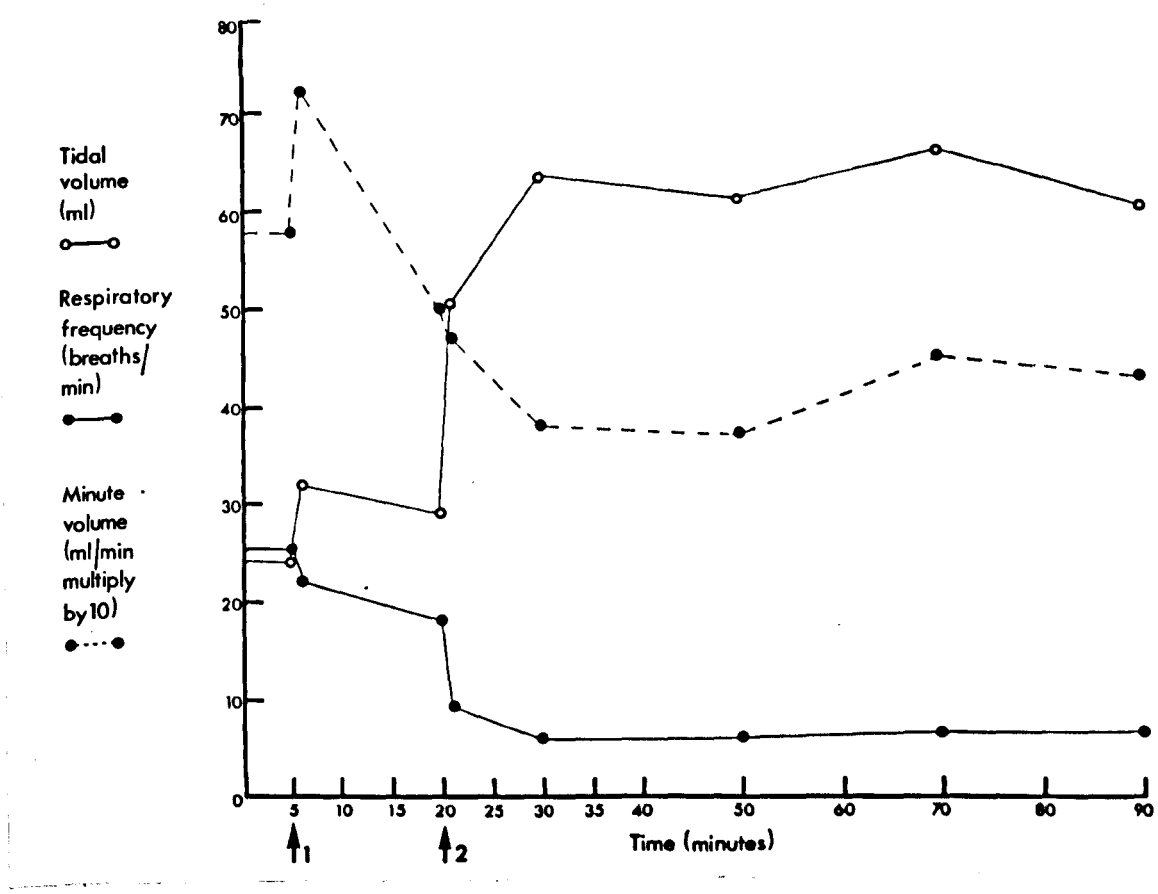
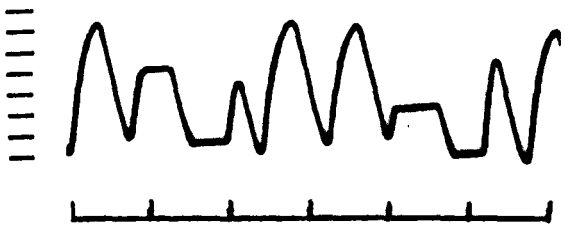

Tidal volume (ml)
Respiratory frequency (breaths/ min)
Minute volume (ml/min multiply by 10)
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
40
50
60
70
80
90
I1
I2
Time (minutes)

Figure 49. Kymographic record of breathing of an erect, lightly anaesthetised adult female *G. domesticus* with one vagus sectioned. Time trace below has 10 second intervals. Amplitude trace on left has 10 millilitre intervals. Breathing runs from left to right and expiration is ascending. The breathing is irregular and has pauses in both inspiration and expiration.



single breaths or short groups of breaths occured which were either much deeper or much shallower than the others. This relatively irregular sort of breathing was seen more frequently in some birds than in others.

(iii) Minute Volume.

The mean minute volume was always less than that when both vagi were intact. However, a significant difference in the minute volume ($0.01 < P < 0.02$) was only seen ten minutes after vagotomy when the greatest reduction was found (36%) and also thirty minutes after vagotomy. The mean minute volumes measured later than this were not significantly different from that when both vagi were intact.

(b) Anaesthetised Birds.

The results are summarized in Tables 4 and 5, Figures 47 and 48 and Graph 2.

(i) Frequency.

The mean frequency was always significantly less than that when both vagi were intact. The greatest reduction in the lightly anaesthetised birds was 69% seen thirty minutes after vagotomy ($P < 0.01$), and in the deeply anaesthetised birds was 39% seen ten minutes after vagotomy ($0.01 < P < 0.02$). This slowing was always due to an increase in the length of expiration.

(ii) Tidal Volume.

The mean tidal volumes were not significantly different from those when both vagi were intact.

(iii) Minute Volume.

The mean minute volumes fell immediately after vagotomy

	Time (mins.)	Mean frequency with S.E. and range (breaths/min.)	Mean tidal volume with S.E. and range (ml.)	Mean minute volume with S.E. and range (ml./min.)
I vagi intact	1-5	16.72 ± 2.4 (11 - 24.8)	33.28 ± 3.58 (25.93 - 43.79)	567.72 ± 131.16 (368.2 - 1085.99)
II unilateral vagotomy	6	14 ± 2.25 (7 - 20)	33.73 ± 3.9 (26.66 - 49)	473.36 ± 95.26 (221.97 - 784)
	20	13 ± 1.99 (7 - 18)	37.22 ± 1.35 (33.66 - 40.71)	484.80 ± 74.37 (240.94 - 624)
III bilateral vagotomy	21	8.80 ± 1.42* (4 - 11)	37.43 ± 2.51 (29.57 - 44.45)	339.80 ± 69.73 (138 - 488.95)
	30	6.6 ± 1.16** (4 - 10)	36.32 ± 4.05 (29.66 - 51.85)	238.08 ± 45.22 (123 - 362.95)
	50	5.2 ± 1.06** (3 - 9)	45 ± 3.88 (38.33 - 60)	235.18 ± 48.03 (141 - 360)

Values of P for differences between III and I means:
*$0.02 < P < 0.05$; **$P < 0.01$.

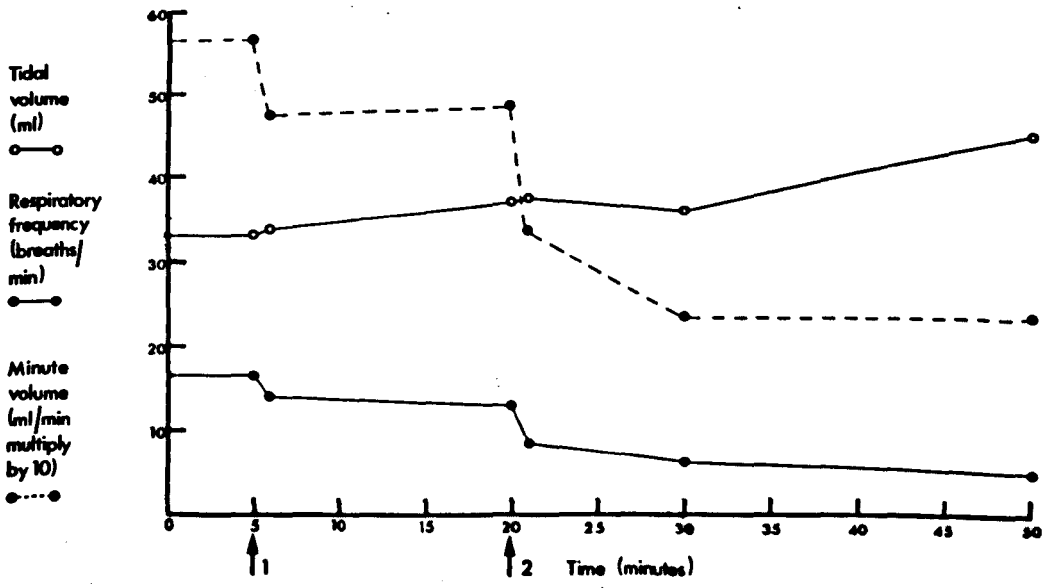
Table 4. Mean values with ranges of frequencies, tidal volumes and minute volumes of the breathing of five lightly anaesthetised adult G. domesticus with both vagi intact (I), one vagus sectioned (II) and both vagi sectioned (III). S.E. is the standard error of the mean. The time at which each observation was made is shown as the number of minutes from the beginning of the experiment.

	Time (mins.)	Mean frequency with S.E. and range (breaths/min.)	Mean tidal volume with S.E. and range (ml.)	Mean minute volume with S.E. and range (ml./min.)
I vagi intact	1-5	15.4 ± 1.17 (13.6 - 17.6)	17.46 ± 1.32 (15.25 - 19.84)	265.93 ± 3.12 (259.65 - 269.82)
II unilateral vagotomy	6	12 ± 0.57 (11 - 13)	19.28 ± 3.09 (13.28 - 23.57)	228.8 ± 31.8 (172.64 - 282.84)
	20	13 ± 0.57 (12 - 14)	20.04 ± 3.05 (14.71 - 25.28)	262.23 ± 58.09 (191.23 - 353.92)
III bilateral vagotomy	21	11 ± 0.57* (10 - 12)	18.42 ± 5.89 (10.71 - 30)	196.23 ± 52.71 (128.52 - 300)
	30	9.33 ± 0.63** (8 - 10)	17.57 ± 1.73 (14.57 - 20.57)	165.96 ± 26.18** (116.56 - 205.7)

Values of P for differences between III and I means:
*$0.02 < P < 0.05$; **$0.01 < P < 0.02$

Table 5. Mean values with ranges of frequencies, tidal volumes and minute volumes of the breathing of three deeply anaesthetised adult G. domesticus with both vagi intact (I), one vagus sectioned (II) and both vagi sectioned (III). S.E. is the standard error of the mean. The time at which each observation was made is shown as the number of minutes from the beginning of the experiment.

Graph 2. The breathing of five lightly anaesthetised, erect, adult, female G. domesticus with both vagi intact (minutes 0 to 5), with one vagus sectioned at arrow 1 (minutes 5 to 20) and with the other vagus sectioned at arrow 2 (minutes 20 to 50).


Tidal volume (ml)
Respiratory frequency (breaths/ min)
Minute volume (ml/min multiply by 10)
60
50
40
30
20
10
0
5
10
15
20
25
30
35
40
45
50
1
2
Time (minutes)

but were not significantly different from those when both vagi were intact until the observations made ten minutes after vagotomy. The greatest reduction in mean minute volume in the lightly anaesthetised birds was 59% seen thirty minutes after vagotomy ($0.02 < P < 0.05$), and in the deeply anaesthetised birds was 38% observed ten minutes after vagotomy ($0.01 < P < 0.02$).

(2) The Effects on Breathing of Electrical Stimulation of the Central Stump of the Cervical Vagus.

(a) Unilateral Vagotomy.

The breathing responses to square and sine wave stimulations of the central stump of the vagus were the same in conscious and anaesthetised birds. Depending on the strength of the stimulus, three types of breathing responses were observed.

(i) At the least effective stimulus there was a slight shift of breathing into the inspiratory phase without any marked changes in the frequency and amplitude (fig. 50).

(ii) At higher strengths of stimulation the shift into inspiration was greater and was nearly always accompanied by an increase in the frequency of breathing and a decrease in the amplitude. These changes in frequency and amplitude became more marked with stronger stimuli (figs. 51 and 52). Increased stimulus frequency was the main factor. Very occasionally a stimulation caused a shift into inspiration with either a decrease in the frequency of breathing or an increase in the amplitude, but the changes in amplitude and frequency were then always very small and

seldom occurred together. Furthermore, unlike the usual response to stimulation they could not be repeated.

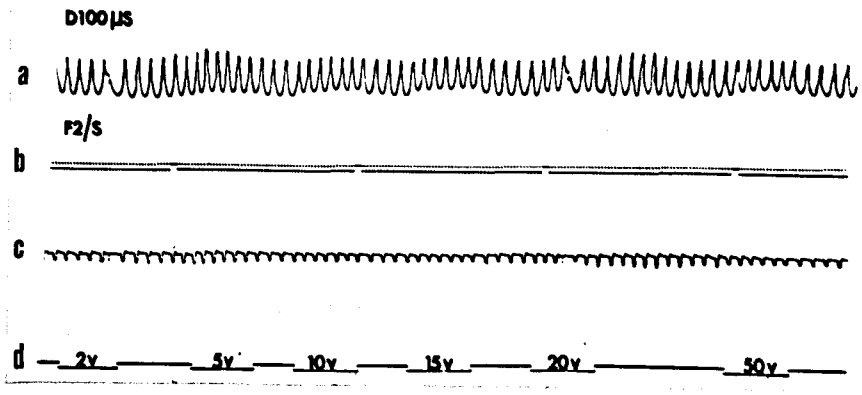
(iii) With the strongest stimuli apneusis nearly always occured (figs. 52 and 53). Only three stimulations caused an arrest of breathing in the middle of inspiration.

When the epineurium of the vagus was removed the response to stimulation was not altered although the least effective strength of stimulus was slightly lowered. Dissection of the vagus and stimulation of the separate filaments resulted in a change in the response to the strongest stimulations in that an arrest of breathing was never produced. Removal of the stimulus always restored the breathing to its prestimulatory level, although the 2 to 3 breaths after stimulation had stopped were usually reduced in amplitude. The response to stimulation was not altered by the age and position of the bird, the type of stimulator that was used and the point on the breathing cycle at which the stimulations were first applied. No differences were observed in the responses to stimulation of the right and left vagi.

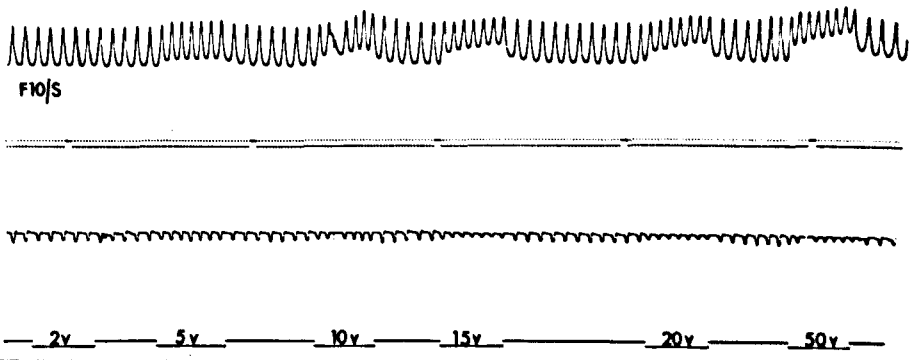
(b) Bilateral Vagotomy.

With both vagi sectioned, square wave stimulation nearly always resulted in a shift into the inspiratory phase and gradual changes in the frequency and amplitude of breathing similar to those seen on stimulation when one vagus was sectioned. Again with the strongest stimuli apneusis resulted. At stimulus strengths below that which resulted in apneusis, the typical slow, deep breathing of

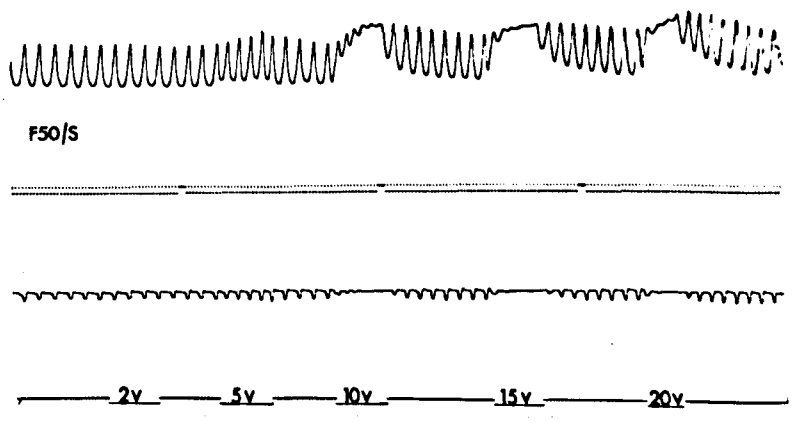
Figures 50,51,52 and 53. Records of breathing of an erect, anaesthetised, immature, male G. domesticus with one vagus sectioned to show the response to square wave stimulation of the central stump of the vagus with increasing stimulus strengths of pulse duration 100μsec, voltages 2 to 50V and frequencies of 2 cycles per second in Figure 50, 10 per second in Figure 51, 50 per second in Figure 52, and 400 per second in Figure 53. In Figure 50 breathing is recorded in trace (a) as abdominal volume changes, and in trace (c) as tracheal pressure changes and runs from left to right. Inspiration in (a) is ascending and in (c) descending. Trace (b): time in minutes. Trace (d): stimulation event marker. Traces in Figures 51, 52 and 53 as in Figure 50. With the lowest frequency of stimulation (fig. 50) there is a shift into inspiration but only small changes in frequency and amplitude. Increasing the strength of stimulus (figs. 51 and 52) resulted in large changes in frequency and amplitude progressing to apneusis (figs. 52 and 53).


D100µS
a
F2/S
b
c
d
2v
5v
10v
15v
20v
50v

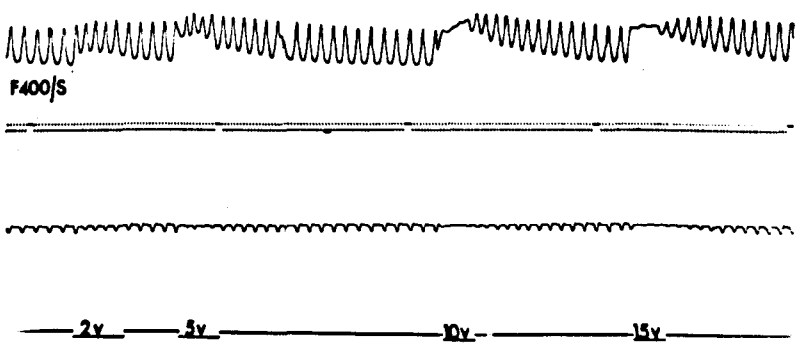
50


F10/S
2v
5v
10v
15v
20v
50v

51


F50/S
2v
5v
10v
15v
20v

52


F400/S
2v
5v
10v
15v

53

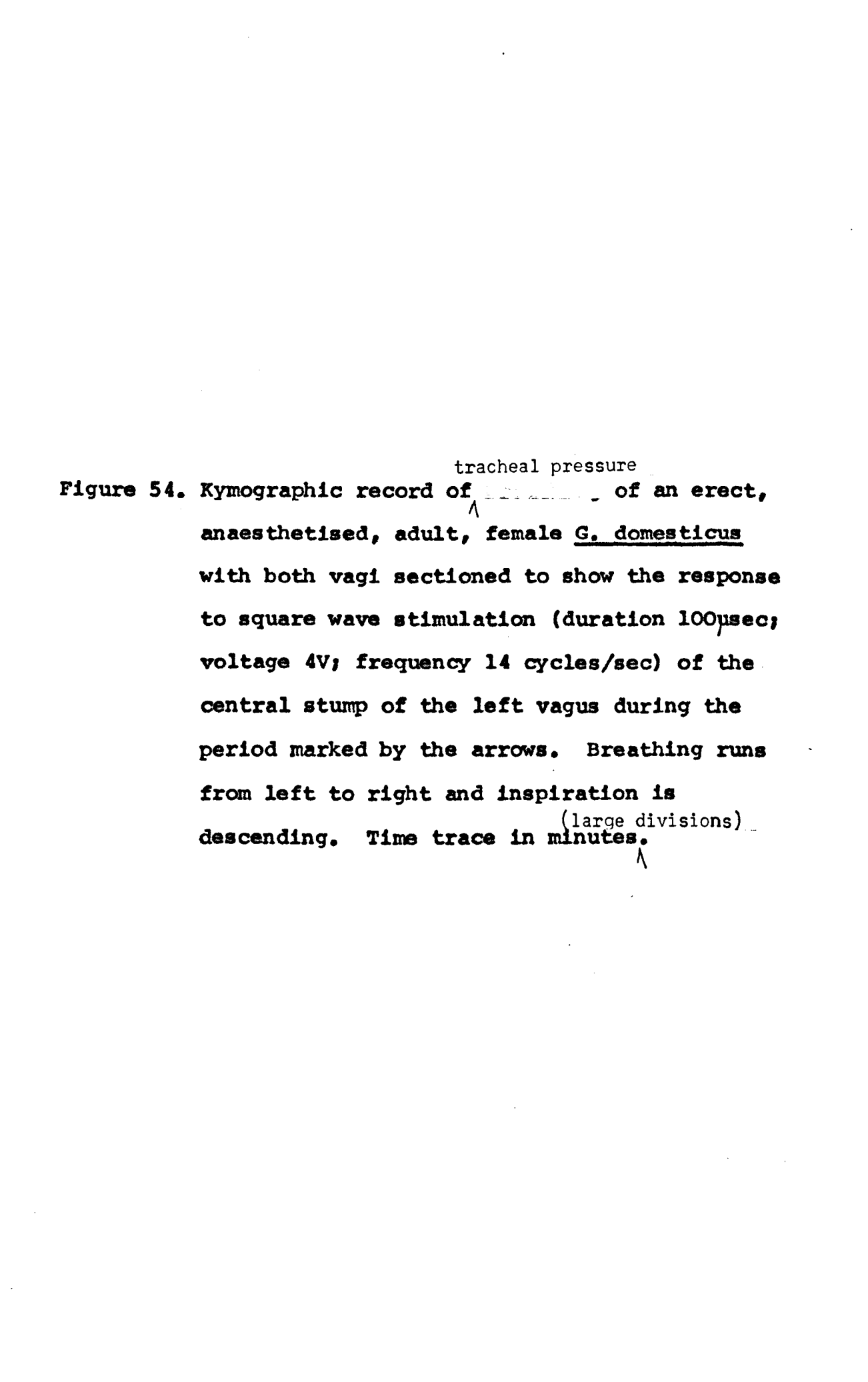
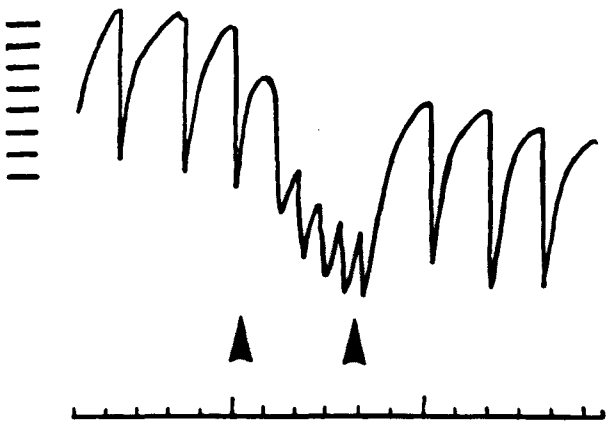


Figure 54. Kymographic record of tracheal pressure of an erect, anaesthetised, adult, female G. domesticus with both vagi sectioned to show the response to square wave stimulation (duration 100μsec; voltage 4V; frequency 14 cycles/sec) of the central stump of the left vagus during the period marked by the arrows. Breathing runs from left to right and inspiration is descending. Time trace in minutes. (large divisions)



the bilaterally vagotomised bird was almost restored to its prevagotomy level by an increase in the frequency of breathing and a decrease in the amplitude (fig. 54).

An expiratory response to stimulation with an arrest of breathing at the peak of expiration was only observed following one stimulation, but could not be repeated.

There was no differences in the breathing responses to stimulation of the right and left vagi and the point on the breathing cycle at which the stimulations were first applied.

(c) Control Stimulations.

Stimulation of the extravagal tissues, including the central stump of the glossopharyngeal nerve, did not result in any change in breathing.

(3) Action Potentials in the Peripheral Stump of the Cervical Vagus synchronous with Resting Breathing.

Single-fibre records have been obtained from the peripheral stump of the cervical vagus of 102 units, all of them showing consistent afferent activity in phase with some stages of the eupnoeic breathing cycle. These afferent units comprised 3 groups: I, units which fired more during inspiration than expiration; II, units which fired more during expiration than inspiration; and III, units which fired at 2 separate points, one in inspiration and one in expiration. 73 units (71%) fired mainly in the inspiratory phase of breathing and 29 units (29%) fired mainly in the expiratory phase of breathing. Some units temporarily changed their eupnoeic firing pattern from one phase to the

other. The units do not include cardiovascular units which fired with a respiratory rhythm. Further details of the units are given below.

(a) Group I Units.

Sixty-three units (62%) fired more during inspiration than expiration. Fifty-two of these units fired only during inspiration (fig. 55, and the tall unit in fig. 58), 41 firing at a constant rate, the others decelerating (10 units) or accelerating (1 unit).

Eleven units fired during both phases of breathing, firing through the end of inspiration and stopping usually in the first half of expiration (fig. 56). Six of these units fired less rapidly during expiration than inspiration, the rest firing at a constant rate.

(b) Group II Units.

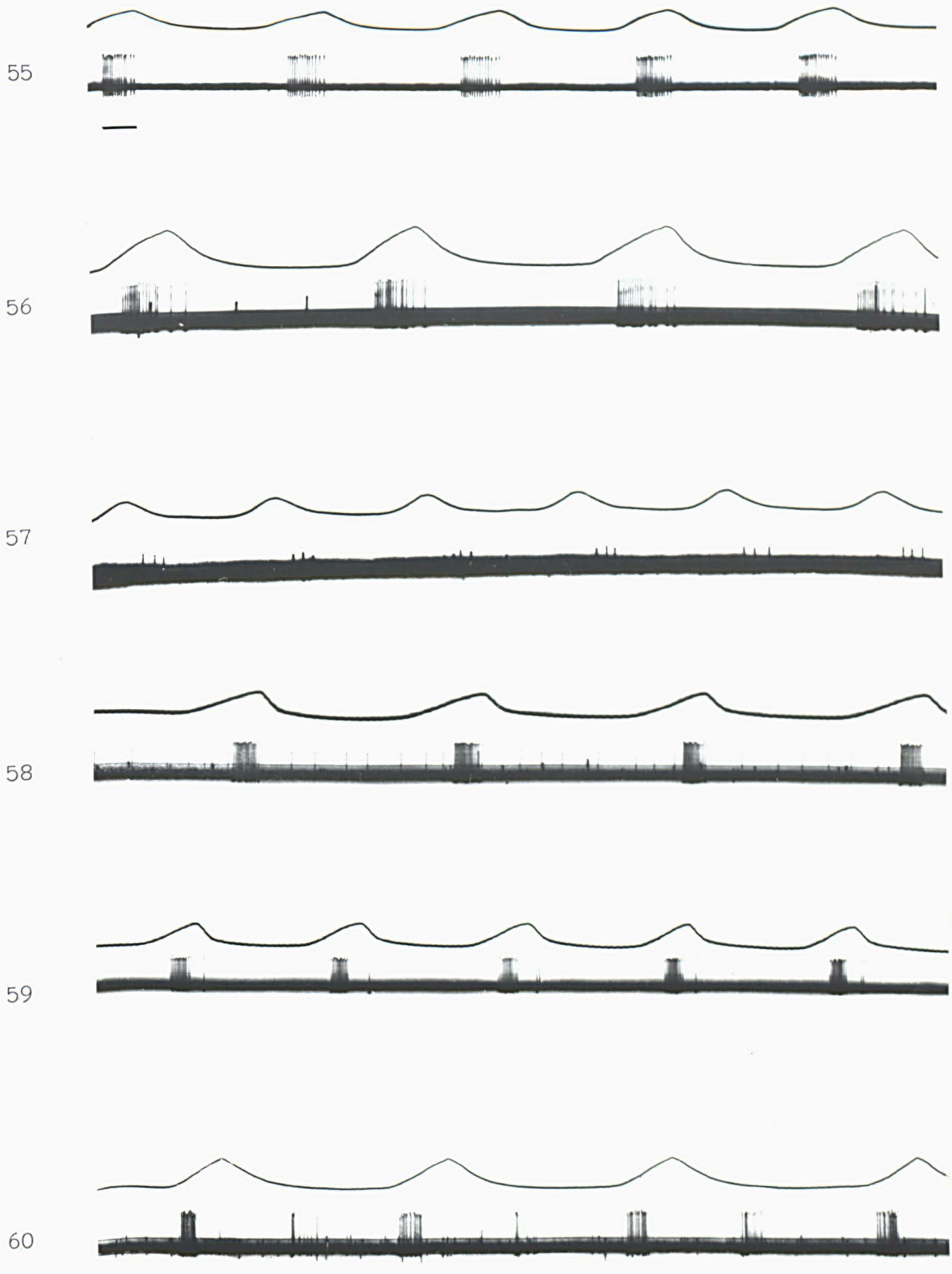
Twenty-eight units (27%) fired more during expiration than inspiration. Most of these units fired relatively slowly. Twenty-one units fired only during expiration (fig. 57), 17 firing at a constant rate, the others decelerating.

Units in group II which fired during both phases of breathing were difficult to identify. However, 7 units appeared to fire in expiration and inspiration, 3 firing at a constant rate through the end of inspiration and 4 firing at a constant rate through the end of expiration (the short unit in fig. 58).

(c) Group III Units.

Eleven units (11%) fired at 2 separate points, one in

Figures 55,56,57,58,59 and 60. In each Figure the upper trace shows eupnoeic breathing in G. domesticus recorded oscillographically be means of an abdominal stethograph and a pressure transducer, inspiration being upwards. The lower trace in each Figure shows afferent nerve impulses in the peripheral stump of the right vagus recorded electrophysiologically, the left vagus being intact. Time scale in Figure 55 is 1 second. Time in Figures 56 - 60 as in Figure 55. In Figure 55 the unit fires only in inspiration. In Figure 56 the unit fires rapidly in inspiration and continues at a slower rate during the beginning of expiration. In Figure 57 the unit fires only in expiration. In Figure 58 the short unit fires mainly in expiration and continues during the beginning of inspiration. The tall unit fires only in inspiration. In Figure 59 the unit fires at two separate points, one in inspiration and one in expiration. The unit fires mainly in inspiration. In Figure 60 the firing of the unit is similar to that in Figure 59 but the firing in expiration is relatively heavier than in Figure 59.


55
56
57
58
59
60

inspiration and one in expiration. Ten of these units fired more in inspiration than expiration (figs. 59 and 60), 9 firing more rapidly in inspiration. Five units fired at a constant rate whilst the others either decelerated in inspiration (1 unit) or decelerated in expiration (3 units).

Only 1 unit in this group fired more in expiration than in inspiration.

The units comprising the three groups are summarised in Table 6.

Group I Units firing more in inspiration than expiration	Units firing only in inspiration	52	63 (62%)
	Units firing in both inspiration and expiration	11	
Group II Units firing more in expiration than inspiration	Units firing only in expiration	21	28 (27%)
	Units firing both in expiration and inspiration	7	
Group III Units firing at two separate points, one in inspiration and one in expiration	Units firing more in inspiration	10	11(11%)
	Units firing more in expiration	1	

Table 6. A summary of the units firing synchronously with resting breathing recorded from the peripheral stump of the cervical vagus of G. domesticus.

VII. DISCUSSION.

A. The Innervation of the Intrapulmonary Air Passages.

(1) Macroscopic Observations on the Pulmonary Branches of the Vagus.

The present investigation of the innervation confirmed the general accuracy of the detailed account of Hsieh (1951, pp. 60-62). Thus in most birds each vagus contributed 6 to 7 branches to a pulmonary nerve plexus on the lateral surfaces of the pulmonary artery and vein. Sometimes, however, fewer branches than this were found and then the innervation resembled more that described by Cords (1904), Kaupp (1918), Watanabe (1960) and Fedde, Burger & Kitchell (1963b). All of these conflicting observations of course strongly suggest that there is a real variation in the number of nerves. However, an equally likely explanation is that parts of the innervation have been overlooked mainly because of the fineness of many of the nerves and because of the large amounts of fat which sometimes surround them.

It was not possible to separate the pulmonary branches of the vagus into rostral, middle and caudal groups of nerves, as claimed by Hsieh (1951, pp. 60-62), although the most rostral nerve (p1) did stand out rather from the other nerves by coming from the pulmono-oesophageal nerve. All the other pulmonary nerves (p2-p7) of course came directly from the vagus but not as reported by Van Matre (1957, pp. 51-52) from a collar of the nerve about the pulmonary artery. The

detailed structure of the pulmonary nerve plexus was not the same from one bird to the next. Despite this, separate rostral and caudal plexuses as described by Fedde et al. (1963b) were never distinguished. Further studies on this plexus are still needed and especially on its sympathetic nerve content already briefly reported by Hsieh (1951, p. 60), and Van Matre (1957, p. 53). Nerves from the plexus were only seen to enter the lung at the hilus with the pulmonary blood vessels which conforms with all other accounts of the innervation. There was certainly no evidence of fibres going into the lung with the primary bronchus. Other nerves from the plexus resembled those of Hsieh (1951, pp. 60 and 62) and were distributed to the pleura on the ventral surface of the lung; unfortunately it was not possible to investigate by dissection Hsieh's claim (p. 81) for an innervation of the lung from this source.

Obviously a macroscopic study like this is of limited value as it omits nerves which are too fine to be identified with the naked eye and cannot establish the existence of fibres which actually go to the lungs in any of the vagal branches. Further information on the distribution of the nerves apparantly going to the lung can be obtained by degeneration studies with the electron microscope as well as from blocking and electrical stimulation experiments on the nerves and electrophysiological recordings of their unit activity. Limited observations with some of these techniques by Fedde et al. (1963b) and Peterson (1970) have already

established pulmonary fibres in some branches of the vagus. Fedde et al. even found that the breathing changes in some birds after sectioning the oesophageal branch of the pulmono-oesophageal nerve which surely emphasises the limitations of purely structural investigations. It is hoped that the present account of the innervation will prove helpful to future studies on the nervous control of breathing by providing such evidence of the innervation as will allow more sophisticated experimental approaches in this field to be made. It is important, however, at the moment that this information is only applied to G. domesticus as evidence is available (Malinovský, 1962) that in the pigeon at least, the anatomy of the pulmonary branches of the vagus are very different.

(2) Histological Observations on the Innervation of the Intrapulmonary Air Passages.

Most previous accounts of the microscopic innervation of the air passages of the avian lung have apparently failed to appreciate that there may be variations in the degree of impregnation of histological sections and that these variations moreover can account for differences in the amount of nervous tissue that is demonstrated in the sections. Consequently, only Akester & Mann (1969) and Bennett & Malmfors (1970) appear to have shown as profuse an innervation as the present study, most other workers basing their observations on histological sections in which the nervous tissue by comparison seems to be understained. Furthermore, no previous investigation has considered the lack of complete specificity of the staining

methods for nervous tissue important enough to warrant checking the identity of the nerves by means of a wide range of histological techniques. Inevitably then, non-nervous tissue has often in the past been mistaken for nervous tissue as for example in the observations by Toussaint-Francx & Toussaint-Francx (1959) on interstitial cells (see Rogers & Burnstock, 1966) and Boeke's "sympathischer Grundplexus" (see Botár, 1966, pp. 48-62). It is hoped therefore that the decision to use a number of staining methods in the present investigation was justified by at least establishing the true identity and distribution of the nervous tissue in the air passages.

(a) Types of Nerve Fibre.

It was not possible to make more than very limited observations on the types of nerve fibre in the lung. No consistent attempt was made to distinguish between myelinated and unmyelinated fibres as electron microscopic studies in general have cast some doubt on the validity of such observations with the light microscope. Most fibres were "fine" and less than 1.5μm in width. This closely conforms to observations on the fibre spectrum of the recurrent and pulmono-oesophageal branches of the vagus by Brown (1970, pp. 53-54). Ultrastructural observations on the innervation by Cook (1970b) suggests that nearly all of these fine fibres were unmyelinated. "Thick" nerve fibres were more than 1.5μm in width. The largest of these were approximately 4μm which is similar to the largest fibres measured by Brown in the pulmono-oesophageal nerve. Again, ultrastructural observations of Cook (1970b)

suggest that most of these thick fibres in the air passages were myelinated. In the bronchial nerves of the cat Agostoni, Chinnock, Daly & Murray (1957) found fibres of up to 14μm in width which are much larger than anything that has so far been found in the vagus and its branches in the bird.

Attempts to identify some nerve fibres histochemically proved inconclusive. Acetylcholinesterase positive fibres were identified in most parts of the air passages by the cholinesterase technique in conjunction with the butyryl-cholinesterase inhibitors iso-OMPA and Lysivane. It is interesting to note here that a non-specific inhibition was sometimes seen with these inhibitors similar to that reported by Sutherland (1964) in the mammalian liver when acetyl-cholinesterase activity as well as butyrylcholinesterase activity appeared to be diminished. Although the cholinesterase technique is generally believed to demonstrate only cholinergic fibres there is some evidence (Jakobowitz & Koelle, 1965; Cook, 1970b) that acetylcholinesterase activity can also be associated with adrenergic fibres. Consequently, it cannot be assumed that the profuse innervation demonstrated with the cholinesterase technique is due entirely to cholinergic fibres. Further histochemical observations therefore with the electron microscope are necessary to determine the true extent of the cholinergic innervation. No method was used which selectively stained adrenergic fibres. Whilst Champy & Hatem (1955) believed that the osmium tetroxide method might be used as a histochemical technique for these fibres, the light and electron microscopic study of Lauweryns & Peuskens (1969) has

now conclusively demonstrated the complete lack of selectivity of this stain.

(b) Distribution of the Nerve Plexuses.

The present account of the innervation in birds in many ways closely resembles that described in the mammalian lung by Fisher (1962). Thus in both classes of vertebrates the innervation is as a whole very well-developed and is distributed in rather similar peribronchial and intrabronchial nerve plexuses. In birds, as in mammals, the number of nerve fibres seemed to decrease distally along the air passages and was not greatest in the secondary bronchi as reported by Van Campenhaut (1955, 1956).

(i) Primary and Secondary Bronchi.

The distribution of the nerve fibres was basically similar in these bronchi. The primary bronchus was best innervated rostrally. Similarly, the secondary bronchi were best innervated close to the primary bronchus and this is almost certainly due to the greater development of the muscle here.

Most of the nerve fibres in these bronchi lay in the muscle layer and in the part of the lamina propria next to the epithelium. The nerve fibres in the muscle layer were distributed in primary, secondary and tertiary plexuses apparently identical to those described by Fisher (1962, 1963) in the bronchial muscle of mammals. Only the plexus with the finest bundles, the tertiary plexus, seemed close to the muscle cells. This plexus almost certainly corresponds to the autonomic ground plexus described in many parts of the body by other workers and also to the small nerve bundles observed

between the muscle cells with the electron microscope by Cook & King (1970) which have axons containing enlargements and both granular and agranular vesicles. It is very likely therefore that this tertiary plexus consists of motor nerve terminations and is the effector nerve plexus of the muscle layer.

The dense innervation of the lamina propria immediately below the epithelium was previously described in the secondary bronchi by Van Campenhaut (1955, 1956). In mammals, an apparently identical subepithelial plexus was seen in the lung by several workers including Hayasi (1937) as well as in various other parts of the body such as the upper urinary tract (Gosling, 1970). However, the functional significance of a plexus in this position is still unknown. Part of it in the avian bronchi is probably concerned with the well-developed blood capillary plexus which also lies here. Part of it may innervate the overlying epithelium although certainly the plexus did not seem better developed around the mucous alveoli. Furthermore, relatively few fibres of the plexus were actually observed to enter the epithelium. Possibly most of the intraepithelial fibres were too fine to be identified with the light microscope. An alternative explanation is that nerve fibres of the plexus mainly innervated the epithelium where the fibres lay close to the bases of the epithelial cells. Such a relationship between epithelial cells and axons has also been seen in the avian lung with the electron microscope by Cook (1970b).

(ii) Tertiary Bronchi.

The present observations agree with Takino (1933a,b) and Hsieh (1951, p. 81) that part of the peribronchial nerve plexus of the tertiary bronchi actually lies in the air capillary regions. The innervation of the tertiary bronchi was greatest close to the origins of the bronchi from the secondary bronchi. At this level of a tertiary bronchus the muscle is especially well-developed and is arranged in relatively long and massive spiral bands several of which surround the orifice of the tertiary bronchus like a sphincter (King & Cowie, 1969). Precise neural control of this muscle would be very important in regulating the relative ventilation of the pulmonary exchange areas (the air capillaries) and the air sacs. However, the innervation of the tertiary bronchi as a whole seemed to be much less developed compared with that of the primary bronchus. This was confirmed by the electron microscopic study of the bronchial muscle by Cook & King (1970). Their detailed observations enabled them to conclude that the neural control of the muscle of the primary bronchus is much more precise that that of the tertiary bronchi and that this may allow non-uniform changes in the calibres of the openings of the secondary bronchi from the primary bronchus. Further observations on the innervation of the tertiary bronchi with the electron microscope might however establish a relatively denser innervation of the tertiary bronchi where the muscle layer is more developed, such as at the junctions between secondary and tertiary bronchi, at the junctions between two tertiary bronchi and possibly also at the junctions between tertiary bronchi and air sacs (King & Cowie, 1969).

(iii) Air Capillaries.

The narrow-meshed plexus of fine nerve bundles in many parts of the air capillary regions does not seem to have been observed before. Small bundles of axons like these, however, were also found here with the electron microscope by Cook & King (1969a). Although some of the plexus lay close to the large blood vessels that passed through the air capillary region most of it was not obviously associated with blood vessels. Whilst in mammals there is physiological evidence for a sensory innervation of the gaseous exchange areas (Paintal, 1970) the histological evidence for such an innervation is still controversial. Thus although nerve fibres have often been described in the alveoli there is still great doubt as to whether these fibres actually terminate in the alveoli or are in fact distributed to other parts of the lung such as the bronchi (Takino, 1933b), the bronchioles (Magnenat, 1951) and the subpleural tissues (Honjin, 1956; Honma, 1960; Watanabe, 1960). Certainly, it is now generally agreed that many claims for nerve endings in the alveoli including those of Ponzio (1906), Hill (1907) and Sunder-Plassmann (1938) were in fact based on connective tissue (Larsell, 1921; Gaylor, 1934; Magnenat, 1951; Cookson, 1953, p. 13; Fisher, 1962, p. 92). Some of the evidence for an alveolar innervation, like that for example of Spencer & Leof (1964), is much more convincing than this and suggests that there are only discrete sensory endings in the alveoli of certain parts of the lungs and certainly never the sort of plexus seen in the bird. An explanation for this innervation

in the bird is consequently difficult to find especially since most of the nerve fibres did not lie near structures which are known to require a motor innervation. Possibly all these fibres in the air capillaries are in fact efferent and join together the plexuses of adjacent blood vessels. However, the close association observed with the electron microscope by Cook & King (1969a) between unmyelinated axons and the epithelial cells of the air capillaries suggests that some fibres of the plexus at least may have a sensory function.

(c) Ganglia.

There are very few accounts of the ganglia in the avian lung. As in other vertebrates the ganglia became less numerous distally along the air passages and were absent from the tertiary bronchi and also contrary to the observations of Takino (1933a) and Muratori (1934), from the air capillaries. The best developed ganglia lay in the peribronchial plexus of the primary and tertiary bronchi and consisted of many more nerve cells than seems to have been previously realised. Rintoul (1960, pp. 142-146) believed that the appearance of the ganglion cells in the intestinal tract of birds and mammals was characteristic of each species. This is unlikely, however, to be so in the avian lung since nearly all the different types of ganglion cells described by Rintoul were observed in G. domesticus. The size of the cells greatly varied although the largest were small when compared with some of the cells observed in the lung with the electron

microscope by Cook & King (1970). These very large cells appear to be close in size to the bipolar ganglion cells in the nodose ganglion (McLelland, unpublished observation) and like them may be sensory. However, since they were not found with the light microscope it is unlikely that they are very numerous. Most ganglion cells appeared with the silver technique to be multipolar and only a few cells seemed to be unipolar and bipolar like those described in the avian lung by Takino (1933a,b) and in the mammalian lung by Berkley (1893, 1894), Okamura (1930), Sunder-Plassmann (1933a), Takino (1933b), Takino & Watanabe (1937), Honjin (1956), Toussaint-Francx & Toussaint-Francx (1959) and Fisher (1962, p. 77). As observed by Okamura (1930) and Fisher (1962, p. 97) however, these unipolar and bipolar cells were probably multipolar and their appearance the result of imperfect or incomplete staining. A simpler explanation for the appearance of these cells is that most of their processes lay outside the planes of the sections.

Fisher (1962, p. 93) identified the ganglion cells in the mammalian lung as the type II cell of the widely accepted classification of the cells suggested by Dogiel (1895, 1899) and later modified by Rintoul (1960). This type II cell has argyrophobic cytoplasm. In contrast, most cells in the avian lung conformed to Rintoul's type I cell which has argyrophilic cytoplasm and this agrees with earlier observations by Arimoto & Miyagawa (1930) and Van Campenhaut (1955, 1956). Only a few cells conformed to Rintoul's type II cell, although unfortunately in this investigation the processes of these

cells were not demonstrated. Rintoul believed that the type I enteric ganglion cell was an association neuron which synapsed with type II cells, the parasympathetic effector neurons. This is unlikely to be so in the avian lung since most cells conformed to the "association" type I cell. Furthermore, the precise associations of the enteric ganglion cells would seem to be in dispute (Gunn, 1968). Possibly, variations in the impregnation of the sections with the silver technique would explain the different staining reactions of the cells. However, the ganglion cells found in the present study did show differences in their cholinesterase activity rather like those reported in ganglia in other parts of the body by Leaming & Cauna (1961), Fisher (1965), Sutherland (1967) and Gunn (1968). Leaming & Cauna believed that such differences in cholinesterase activity supported Dogiel's suggestion for 2 or 3 types of nerve cells. Obviously further observations on the staining reactions of the ganglion cells are needed.

(d) Sensory Nerve Endings.

As stated in the review of the literature (p. 12) the evidence compiled by Cauna (1959), Miller & Kasahara (1964), Munger (1955) and Dwinnell (1966) suggests that there are three classes of afferent nerve endings. These are free fibre endings, encapsulated endings and neurite-receptor cell complexes. This classification was used as the basis for my search for the afferent endings in the air passages. Such a search, however, proved in practice very difficult and seemed to encounter most of the problems of interpretation

described by other workers. The possibility of artefacts, for example, had to be considered at all times and especially those resulting from the impregnation of connective tissue.

There was no evidence for encapsulated endings. Whilst this type of ending has been reported in the mammalian lung by several workers including Larsell (1921), Larsell & Dow (1933), Elftman (1943), Conti & Bariatti (1953) and Hirsch, Kaiser, Barner, Cooper & Rams (1968) most of the evidence for it is not very convincing. Furthermore, as noted previously, Fisher (1962, p. 96, 1963) who probably made the most critical assessment of the innervation of the mammalian lung, did not see this type of ending.

Of the free fibre endings, simple and complex types are usually distinguished according to the degree of branching of the endings. In all the bronchi of the bird many unbranched and slightly branched thick fibres were observed which resembled descriptions of the simple type of ending. However, to interpret such evidence as being sensory must only seem to condone many of the earlier accounts of the innervation of the lungs of vertebrates which claimed the existence of sensory endings on the basis of such limited histological evidence. In many of these earlier studies the supposed simple free fibre endings can very easily be explained by poor impregnation of the sections so that further distribution of the fibres and their branches were simply not visible. Likewise, in my own material, it is more than likely that the apparent observation of simple free fibre endings was also due to a failure to impregnate their subsequent branches.

The only really convincing evidence for free fibre endings was found in the lamina propria and muscle layer of the primary bronchus and in the tertiary bronchi and interatrial septa where relatively thick nerve fibres were distributed in a way that strongly suggested the complex type of ending. Always these thick nerve fibres divided rapidly into successively finer branches which appeared to end either freely or in knob-like swellings. An interesting feature of the endings was the very large area which they seemed to innervate, some in the primary bronchus for example being distributed in both the muscle layer and the lamina propria. Unfortunately, it was not possible to confirm them with different staining techniques since the endings in the primary bronchus were only seen with the osmium tetroxide method whilst those in the tertiary bronchi and interatrial septa were only found with the silver method. In interpreting these structures as sensory therefore it must be remembered that neither of the stains are completely selective for nervous tissue and that the osmium tetroxide technique especially as demonstrated by Lauweryns & Peuskens (1970) can stain connective tissue in such a way that it cannot be distinguished from nerve endings. All these complex free fibre endings in the bird are very like certain endings in the mammalian lung first described in detail by Larsell (1922, 1923) and Larsell & Dow (1933) and later confirmed by a number of workers. Larsell believed that the endings in the muscle layer were very like the neuromuscular spindles of striated muscle and therefore named them "smooth muscle spindles". However, a

few workers including Fisher (1962, p. 37) commented on the marked lack of similarity between Larsell's illustration of the endings and striated muscle spindles. Certainly, none of the endings found in the present study resembled muscle spindles. At the moment it is impossible to match these observations made with the light microscope with the ultrastructural descriptions of the sensory endings by Cook (1970a). It is probably significant, however, that the distribution of the endings found by both methods of study is very similar.

The demonstration of intraepithelial nerve fibres in the primary bronchus appears to confirm the endings described by Takino (1933b), Muratori (1934) and Toussaint-Francx & Toussaint-Francx (1959). Many of these intraepithelial fibres branched and therefore conformed to the complex free fibre type of ending. Some care however, must be taken in interpreting this innervation since as observed by Cauna (1959) and others, nerve fibres in the lamina propria often appear, in thick and obliquely cut sections, to be in the epithelium. Certainly only electronmicroscopic observations can provide conclusive evidence of intraepithelial fibres. Such a study by Cook & King (1969a,b) in fact has established an epithelial innervation of the primary bronchus. They found here specialized cells which resembled certain well-known sensory cells including glomus cells and Merkel cells as well as the specialized cells described in the human bronchial epithelium by Bensch, Gordon & Miller (1965). Like these other cells, the specialized cells in the epithelium of the

bird lay close to unmyelinated axons and with the axons probably formed sensory neurite-receptor cell complexes. There was no evidence, however, in the present study for the "light cells" found with the light microscope by Fröhlich (1949) and others in the bronchial epithelium of mammals and which may be the specialized cells of the neurite-receptor cell complexes. Some of the intraepithelial fibres identified with the light microscope may belong to these complexes; others could correspond to the intraepithelial axons found by Cook (1970a) which do not appear to be associated with specialized epithelial cells. It is interesting to note that the restriction of the intraepithelial fibres to the primary bronchus seen in the present investigation agrees with all the available electron microscopic evidence.

Both Miller & Kasahara (1964) and Dwinnell (1966) emphasised the importance in neurohistology of interpretation and the need therefore for adequate photographic evidence with all descriptive accounts. Consequently, it is hoped that the photomicrographs of the presumed afferent nerve endings described in this account will prove both convincing as evidence of afferent nerve endings and useful as comparisons for other studies on the innervation of the air passages, not only of birds but also of other vertebrates. The functional significance of these endings is discussed later.

(e) Need for further Observations.

Although very precise and detailed information on the innervation of the lungs can only be obtained with the electron

microscope, there is still room for further investigations with the light microscope. Obviously more studies are needed to confirm the innervation of the air passages reported in this account and especially those relatively controversial aspects of the innervation such as the distribution of the nerve fibres in the air capillaries and the possible afferent nerve endings. Again the degeneration technique might be useful here to provide much additional information on the sensory innervation. More detailed investigations are also needed on the sympathetic innervation of the air passages, since at the moment only Akester & Mann (1969) and Bennett & Malmfors (1970) appear to have looked at this aspect of the innervation with the light microscope. Observations on the nerve fibres in other parts of the lungs are also essential. Of particular relevance to the present study is the innervation of the visceral pleura since Hsieh (1951, p. 81) described pulmonary branches of the vagus entering the ventral part of the lung via the pleura. Furthermore, Dwinnell (1966) obtained excellent evidence for afferent nerve endings in the visceral pleura of mammals. The bronchopleural membrane, a two-layered structure formed by the parietal pleura and the walls of the thoracic air sacs, should also be examined. Evidence already exists with the light microscope for a profuse innervation of this membrane (Bennett & Malmfors, 1970; King, 1970) and furthermore Cook (1970b) has some electron microscopic evidence for specialized cells here which appear to be identical to those of the neurite-receptor cell complexes in the epithelium of the primary bronchus. It is likely, therefore, that this

membrane may play a more important part in the control of breathing than has previously been realised, as Bennett & Malmfors (1970) predicted. Finally, little seems to be known about the innervation of the pulmonary blood vessels. Unfortunately constructive studies on this important topic must await investigation on the basic structure of these vessels since at the moment it is impossible to distinguish pulmonary arteries from pulmonary veins in histological sections of the lung. Certainly, the very limited observations on the vascular innervation in the present study indicate the need for a much more extensive investigation.

B. Functional Studies on The Afferent Pathways in the Vagus.

(1) The Effects on Breathing of Cervical Vagotomy.

One of the objects of this study was to establish the effects of cervical vagotomy under controlled experimental conditions since very few accounts on vagotomy are available which provide details of the birds including their age and position and whether they were anaesthetised or not. Whilst in the conscious, erect, adult bird unilateral vagotomy had usually little effect on breathing, bilateral vagotomy was always followed by much slower and deeper breathing, apparently identical to that found in many previous studies on vagotomy. Furthermore, as observed by most other workers, the slower breathing was due to prolonged expirations although an actual pause in expiration as described by Couvreur (1891, 1892), Siefert (1896), Grober (1899), Grünwald (1904), Stübel (1910) and Richards (1969) did not occur. Never was the slower breathing explained by the prolonged inspirations that were sometimes found by Siefert (1896) and Stübel (1910). The response also contrasts very strongly with the very shallow type of breathing observed by Couvreur (1891, 1892), Sinha (1958) and Blankart (1960).

Fedde, Burger & Kitchell (1963a) showed that age and anaesthesia could account for some of the different responses to vagotomy although the precise effect of anaesthesia does not seem to have been fully established. Urethane anaesthesia for example in the present study resembled barbiturate

anaesthesia described by Fedde et al. (1963a) in preventing the changes in the depth of breathing that usually followed vagotomy but unlike barbiturates did not prevent the significant slowing of breathing. It seems that much more work is required on the effects of different anaesthetics on the response to vagotomy. Unfortunately it is not really possible to relate this sort of information with any certainty to those other accounts which omit essential experimental details. Certainly some of the observations of other workers still remain unexplained. Species differences as noted by Richards (1968) must be considered and the present observations add to the growing evidence that the birds in which the effects of vagotomy have been most frequently studied, the pigeon and chicken, do not in fact respond to vagotomy in identical ways.

(2) The Effects on Breathing of Electrical Stimulation of the Central Stump of the Cervical Vagus.

Electrical stimulation of the central stump of the vagus in both unilateral and bilateral vagotomized birds induced an inspiratory reaction and faster, shallower breathing progressing to apneusis with increasing strengths of stimuli. Similar respiratory responses always followed stimulation of a part of the vagus although the response was much weaker than when the whole vagus was stimulated. This seems to indicate that the fibres which are responsible for the change of breathing on stimulation are widely distributed in the vagus. Obviously it is extremely difficult to compare these responses with those found by most other workers since few accounts

includes details of the stimuli. However, it would appear that of the many studies of stimulation only those of Couvreur (1891, 1892), Bourgeois (1896) and Richards (1968, 1969) reported identical respiratory responses to those observed here. Furthermore, the latter are strikingly different from the slower and deeper type of breathing found by Siefert (1868), Grünwald (1904), Stübel (1910), Dooley & Koppányi (1929) and Saalfeld (1936). Arrests of breathing outside inspiration either in expiration or in the resting position of the thorax as reported by Bert (1870, p. 480), Knoll (1888), Siefert (1896), Cavalié (1898), Grober (1899) and Grünwald (1904) were never observed. None of the many factors which could reasonably be expected to influence the outcome of electrical stimulation of the vagus in unilateral vagotomized birds were found to alter the breathing response in any way. These included age, posture and anaesthesia as well as the use of different stimulators, stimulation of the left or right vagus and the precise point on the breathing cycle when the stimulus was first applied. Most interesting was the failure of widely different stimulus frequencies to induce radically different breathing responses like those obtained by Sinha (1958) in the pigeon when low-frequency stimulation resulted in inspiration and high-frequency stimulation induced an expiratory reaction. Similar observations to those of Sinha moreover have also been made in mammals by many workers including Wyss (1954).

One of the objects of this study, like that on the effects of vagotomy, was to establish the respiratory response to electrical stimulation of the vagus under certain precise

experimental conditions. Although it was not possible to reproduce the breathing elicited by most other workers, by no means all the variables were investigated. For example, a very different response might be obtained with other anaesthetics as suggested by the findings of Bourgeois (1896) during stimulation under chloral hydrate anaesthesia. Like the response to vagotomy, species differences must be considered as an explanation, and it is very interesting that most of the investigations on stimulation which have produced results different from those of the present study have mainly used pigeons and ducks.

(3) Action Potentials in the Peripheral Stump of the Cervical Vagus synchronous with Resting Breathing.

The records of afferent discharges in phase with resting breathing obtained in this investigation seem to be the first direct evidence in birds of afferent neural activity capable of continuously informing the central nervous system of the state of the respiratory cycle. The activity was very varied although approximately half the fibres fired only in inspiration. The separation of the fibres into three groups according to their pattern of firing is of value for descriptive convenience rather than for any known functional significance. Whilst much of this activity was also observed by Jones (1969), Fedde & Peterson (1970) and Peterson (1970) they appear to have not much evidence for fibres which fired only in expiration.

This very varied activity in the avian vagus cyclic with eupnoeic breathing contrasts markedly with that during resting

breathing in mammals as described by Paintal (1963) when firing only occurs in Hering-Breuer fibres from slowly adapting broncho-pulmonary stretch endings; in these mammalian fibres the rate of firing is linearly related to the degree of inflation, whereas in the bird the firing rate tends to slow down towards the peak of inspiration as in Figure 55.

C. The Interpretation of the Histological and Experimental Evidence for Afferent Pathways in the Vagi in terms of their Respiratory Function.

The investigation of a chemoreflex modulated by CO_2 by Peterson & Fedde (1968), Fedde & Peterson (1970) and Peterson (1970) established the function of one afferent pathway in the vagus from the air passages. Richards (1968) and Fedde (1970) may have evidence for a weak stretch reflex although the rest of the direct evidence for this type of reflex is equivocal. What additional information on the function of the pathways, therefore, has been obtained from the present investigation? None of the techniques that were used was capable of establishing precisely the function of the pathways. The electrophysiological recording of single units in phase with resting breathing was the most informative experimental technique since it provided direct evidence of afferent neural activity. The intrapulmonary origin of such activity was not established and therefore future studies should try to verify this point. One way of doing this would be to record directly from the pulmonary branches of the vagus. Preliminary observations on this, however, by Brown (1970, pp. 39-41) suggest many problems. It is extremely likely that many of the units that were recorded correspond to the CO_2-sensitive units described by Fedde & Peterson (1970), Molony (1970) and Peterson (1970) which also fired in phase with resting breathing. Others are probably associated with pulmonary reflexes which are still unidentified like the CO_2-

insensitive units recorded by Fedde & Peterson (1970) and Peterson (1970, p. 65).

In comparison with the recording of action potentials, vagotomy and electrical stimulation proved much less rewarding. Many workers in birds who have used these techniques, have been very happy to draw far reaching conclusions from their observations. In contrast, I found it extremely difficult to interpret the results of these procedures because the essential background of information about respiratory reflexes, fibre sizes etc. which is available for mammals is almost non-existent for birds. The significance of observations with both these methods even in mammals has rightly been doubted by Widdicombe (1964) and Comroe (1965, p. 77). They pointed out that the vagi are likely to be the afferent pathways of many reflexes which influence breathing and therefore vagotomy can only provide information on the dominant activity in the nerves. In mammals, this activity is due mainly to fibres of the Hering-Breuer inflation reflex and vagotomy therefore slows and deepens breathing (Widdicombe, 1964). The basic similarity between this response and the breathing following vagotomy in the bird inevitably has suggested to most workers that a Hering-Breuer reflex also exists in birds. However, the equivocal outcome of direct attempts to demonstrate an avian Hering-Breuer reflex would indicate that even if such a reflex did exist it is unlikely to be responsible for the <u>dominant</u> activity in the nerves. Moreover, the response to vagotomy in mammals and birds differs considerably in detail,

the slowing in breathing in mammals being much smaller than in birds and unlike that in birds is often due to prolonged inspirations. Furthermore, the full effect of vagotomy seems to occur much quicker in mammals than in birds. It is much more likely therefore, that the breathing response to vagotomy in birds is associated with a pulmonary reflex which is not directly modulated by volume and pressure changes in the airways.

The main disadvantage of electrical stimulation seems to be that it excites afferent nerve fibres of several reflexes. In mammals, for example, most of the pulmonary stretch fibres belong to the same physiological groups of fibres, Aα and Aβ , as those from rapidly adapting irritant receptors in the airways. Consequently, the fibres of both these reflexes share the lowest threshold to electrical stimulation (Paintal, 1953). Despite this drawback, Wyss (1954) felt able to interpret his observations on electrical stimulation in mammals in terms of the inflation reflex. The frequency of the impulses he believed was critical, impulses at low-frequency augmenting inspiration as in the early stages of the inspiratory phase and impulses at high-frequency causing an expiratory pause as occurs immediately following inspiration. The failure of widely different stimulus frequencies to induce similar breathing responses in G. domesticus suggests that in this species at least the regulation of breathing is very different from that in mammals. Furthermore, the lack of any marked variation in the response to stimulation in G. domesticus is really surprising when one considers the very varied patterns of firing of the units in the vagus of this bird in phase with

resting breathing.

Further evidence for pulmonary reflexes came from the histological observations on the possible afferent receptors in the bronchi. Unfortunately, one can only speculate on the modalities of these structures. Some of the intra-epithelial fibres which were observed in the primary bronchus probably belonged to neurite-receptor cell complexes described with the electron microscope by Cook & King (1969b) and Cook (1970a). The ultrastructure of many of the specialized cells of these complexes resembles that of both glomus cells which have been widely regarded as being chemoreceptors and also Merkel cells which are thought to be mechanoreceptors. The existing experimental evidence in birds for pulmonary reflexes strongly suggests a chemical modality for these intraepithelial endings and as noted by Cook & King (1969b) this would be a very direct mechanism for analyzing the composition of the gas in the airways. On the other hand Cook and King pointed out that the resemblance to Merkel cells suggests that these neurite-receptor cell complexes could just as well be mechanoreceptors. Another possibility, however, is that any intraepithelial fibre could be the same as the "lung irritant receptors" in the bronchial epithelium of mammals which according to all the physiological evidence are relatively unspecialized and have both chemoreceptor and mechanoreceptor activity (Mills, Sellick & Widdicombe, 1970). Then the endings in birds would respond to a wide variety of stimuli including different chemical and physical irritants as well as deformation of the epithelium. Changes in the

shape of the epithelial cells of course could also excite other sensory endings which might lie in the well-developed plexus immediately below the epithelium.

The most convincing sensory endings seen with the light microscope were the supposed complex free endings. These lay at some distance from the epithelium and are more likely therefore to belong to mechanoreceptors. Fedde, Burger & Kitchell (1963a), however, argued that the avian lung was "relatively non-expansive" so that stretch receptors would scarcely be activated. On the other hand, King (1966b) concluded from the experimental evidence of earlier workers including Soum (1896), that the volume changes in the lung are in fact sufficient to excite stretch endings. Presumably as described by King & Molony (1970) the increased diameters of the thoracic cage during inspiration result in a sufficiently low pressure within the coelom and the air sacs surrounding the lungs to cause an outward movement of the lungs. Obviously the parts of the lung with relatively well-developed elastic tissue are likely to be more mobile than other regions. Such parts are the interatrial septa which have a very well-developed network of elastic fibres (King, Ellis & Watts, 1967). King & Molony (1970) believed that this network was an energy-storing system which by opposing the intrinsic tone of the muscle of the tertiary bronchi tended to keep the airways of these bronchi open. Furthermore, they suggested that the elastic atria acted as a spring balancing the tone of the bronchial muscle against the surface tension in the air capillaries. Consequently, it is tempting to believe that

the sensory nerve endings which lie in the atria are related to the great mobility here. It is interesting to note that similar endings to these were also found in the lamina propria of the primary bronchus, another part of the lung in which the elastic tissue is well-developed (King et al., 1967).

Stretch receptors need not lie in areas of great mobility. The juxtapulmonary capillary stretch receptors (type J receptors) described by Paintal (1970) in mammals, for example, are stimulated by the increased interstitial volume which follows the rise in pulmonary capillary pressure during excercise. It is interesting to note that supposed afferent endings were found most frequently in the avian lung in the interatrial septa which are also very well supplied with blood vessels. Interstitial receptors certainly may exist here and in even more vascular parts of the avian lung such as the lamina propria of the primary and secondary bronchi and the air capillary regions. Certainly, a profuse innervation lies in both these parts of the lung which is so far unexplained.

In conclusion, therefore, some new evidence for the afferent vagal pathways from the intrapulmonary air passages has been obtained from the present investigation although all of the experimental approaches that were taken have their limitations. The most convincing evidence for these pathways came from two sources. The first of these was the electrophysiological recordings of unit activity which seemed to belong to receptors monitoring the state of the breathing cycle. The other was the histological demonstration of

possible receptors in the air passages. Both these lines of inquiry suggest that there are many more functionally different pathways in the vagus concerned in the regulation of breathing than is known at present.

VIII. SUMMARY.

(1) The general aim of the thesis was to contribute new knowledge on the peripheral control of breathing in birds using Gallus domesticus as the subject.

(2) All the available evidence was considered for the innervation of the intrapulmonary air passages in birds including experimental observations on afferent pathways in the vagi that originate from the passages.

(3) The detailed OBJECTIVES were as follows.

(a) To establish the gross anatomy of the pulmonary branches of the vagus.

(b) To establish the distribution of the nervous tissue in the intrapulmonary air passages and especially to search for evidence of an afferent innervation.

(c) To investigate afferent pathways in the vagi that come from the intrapulmonary air passages.

(d) To interpret functionally the histological and experimental evidence for the afferent pathways in the vagi.

(4) The macroscopic investigation of the pulmonary branches of the vagus demonstrated in adult male and female birds the following innervation.

(a) Five to six fine pulmonary nerves usually originate from the vagus at the level of the pulmonary artery and vein although in some birds there appears to be a smaller number of these nerves. One pulmonary nerve always comes from the

pulmono-oesophageal nerve, an offshoot of either the vagus or its recurrent branch.

(b) All the pulmonary nerves contribute to a pulmonary nerve plexus lying on the lateral surfaces of the pulmonary artery and vein.

(c) Many branches of the pulmonary nerve plexus enter the lung at the hilus close to the pulmonary blood vessels. A few branches of the plexus go to the pleura on the ventral surface of the lung.

(d) Nerves from the pulmonary plexus do not enter the lung close to the primary bronchus.

(e) The pulmonary nerves and the pulmonary nerve plexus are often hidden by large amounts of fat.

These findings are compared with the observations of other authors. The possibility is considered that the variation that is sometimes described in the number of pulmonary nerves is often due to the nerves being overlooked because they are either too fine or are hidden by large amounts of fat.

(5) The distribution of the nervous tissue in the intra-pulmonary air passages was investigated in male and female, immature and adult birds of a number of breeds with the light microscope and using methylene blue, silver nitrate, osmium tetroxide and gold chloride methods and a cholinesterase technique with and without intensification by silver.

(a) Nerve fibres which enter the lung at the hilus close

to the pulmonary blood vessels are distributed in the bronchi as a plexus which can be arbitrarily divided into different parts according to its position in the lung and the sizes of its nerve bundles and its meshwork.

(b) Most of the nerve fibres in the air passages are "fine" and less than 1.5μm in width. The other fibres are "thick", the largest being approximately 4μm in width.

(c) The innervation of the primary and secondary bronchi is best developed proximally. The distribution of the nervous tissue in both types of bronchi is basically the same although in the distal parts of the secondary bronchi, separate nerve plexuses cannot be distinguished.

(i) Both types of bronchi have a coarse-meshed peribronchial nerve plexus of thick bundles containing both thick and fine fibres and numerous ganglia.

(ii) Branches of the peribronchial plexus innervate the muscle layer. The larger nerve bundles in the muscle layer have many thick and fine fibres and anastomose with each other to form a coarse-meshed primary plexus in the connective tissue between the muscle bundles. The finest nerve bundles in the muscle layer have only fine fibres and form a narrow-meshed tertiary or autonomic ground plexus lying close to the muscle cells. The remaining nerve bundles form a relatively poorly defined secondary nerve plexus joining the primary nerve plexus to the tertiary nerve plexus. Ganglia are only present in the primary plexus.

(iii) Branches of the nerve plexuses in the peribronchial

connective tissue and the muscle layer supply the lamina propria. The lamina propria appears best innervated just below the epithelium. Here many nerve bundles lie close to a blood capillary plexus. Numerous very fine fibres of the plexus appear to be in close contact with the bases of the epithelial cells. A perichondrial innervation was not seen. There are only a few small ganglia in the lamina propria.

(iv) An intraepithelial innervation is only present in the primary bronchus.

(d) The tertiary bronchi and interatrial septa are innervated by a coarse-meshed peribronchial plexus in the interlobular connective tissue septa and air capillary regions formed by large bundles consisting of a small number of thick fibres and many fine fibres. The innervation of the tertiary bronchi cannot be distinguished from that of the interatrial septa outside them. It consists of a small number of thick fibres and many fine fibres. The innervation of the muscle layer is best developed at the origin of the bronchus from a secondary bronchus although the muscle layer of the tertiary bronchus is always less well innervated than that of the primary and secondary bronchi. Ganglia are absent from the tertiary bronchi and interatrial septa.

(e) Branches of the peribronchial nerve plexus of the tertiary bronchi form a narrow-meshed plexus of fine fibres in many parts of the air capillary regions. Some of the narrow-meshed plexus innervates large blood vessels here

although other parts of the plexus are not near blood vessels of this size. Ganglia are absent from the air capillary regions.

(f) The substantial variations in the appearance of the ganglion cells is described. The smallest cells are approximately 7.5μm in diameter and the largest cells are about 27μm in diameter. With the silver technique most cells appear multipolar although there are bipolar and unipolar cells. Variations in the staining reactions of the ganglion cells occur with the methylene blue, silver nitrate and cholinesterase techniques.

(g) Structures which closely resemble the descriptions of the complex free fibre sensory nerve endings of other workers lie in the lamina propria of the primary bronchus and in the walls of the tertiary bronchi and atria. Furthermore, there are other nerve fibres in all the bronchi which resemble the descriptions of the simple free fibre afferent nerve endings of other workers. Although there is an intraepithelial innervation in the primary bronchus, there is no evidence with the light microscope for neurite-receptor cell complexes. Encapsulated sensory nerve endings are not present in the air passages.

These observations are compared with the light and electron microscopic evidence of other workers in the bird. The findings are also discussed in the light of existing knowledge of the innervation of the lungs of other vertebrates.

(6) The afferent pathways in the vagi coming from the intra-

pulmonary air passages were investigated by a number of experimental techniques including vagotomy and electrical stimulation of the central stump of the vagus as well as electrophysiologically recording unit activity in the peripheral stump of the nerve in phase with resting breathing.

(a) In erect, conscious, adult birds bilateral cervical vagotomy resulted in the breathing becoming significantly slower and deeper, the reduction in rate being due to prolonged expirations without pauses. *Urethane anaesthesia* prevented significant changes in the depth of breathing.

These findings are compared with the accounts of vagotomy of other workers. It is concluded that some of the different responses to vagotomy that are reported are probably due to the use of different anaesthetics and different species of birds.

(b) Electrical stimulation of the central stump of the vagus always induced an inspiratory reaction and faster, shallower breathing progressing to apneusis with increasing strengths of stimuli. Increased stimulus frequency was the main factor. The breathing response to stimulation was not affected by age, posture, anaesthesia, the use of unilateral or bilateral vagotomized birds, different stimulators, stimulation of the left or right vagus and the precise point on the breathing cycle at which the stimuli were first applied.

These findings appear to be very different from the responses found by most other workers in both birds and mammals. The possible reasons for this are discussed.

(c) 102 single units which showed activity synchronous with the eupnoeic breathing cycle were recorded from the peripheral stump of the cervical vagus. Group I units (63 units) fired more during inspiration than expiration. Group II units (28 units) fired more during expiration than inspiration. Group III units (11 units) fired at two separate points, one in inspiration and one in expiration.

The units are compared with those recorded from the vagi in birds by other workers. The very varied activity in the avian vagus in phase with eupnoeic breathing contrasts very markedly with the more limited firing that occurs during resting breathing in the vagus of mammals.

(7) The difficulties are discussed of interpreting the histological and experimental evidence for the afferent pathways in terms of their function. However, functions for the pathways are suggested on the basis of existing knowledge of the regulation of breathing in birds and other vertebrates. It seems very likely from the evidence that a much greater variety of pulmonary reflex activity exists in birds than is known at present.

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