A MULTIDISCIPLINARY STUDY INTO CHANGES IN THE VAGUS NERVE FOLLOWING PARTIAL CHRONIC VAGOTOMY

A thesis submitted by

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Dedicated to

my grandmother, Fayomi Ajayi,

the memory of my sister,

Ebun Omowumi

and the glory of God

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SUMMARY

The ferret was used as a model for studying the effect of chronic abdominal partial vagotomy on gastric motility and innervation of the gastric wall by the surviving abdominal vagal trunk. The fibre composition of the vagus nerve trunks was also determined and correlated as much as possible with the results of the two series of experiments above. Gastric motility was measured indirectly by measuring the intragastric pressure that was associated with it in response to stimulation of the cervical vagus nerves.

Acute ventral trunk vagotomy did not significantly reduce the intragastric pressure response to cervical vagal stimulation. Similarly, following chronic ventral trunk vagotomy, intragastric pressure response to cervical vagal stimulation did not differ significantly from the acute and control values until after three months when it increased significantly above acute values probably as a result of re-innervation of the gastric wall by regenerative sprouts from the proximal nerve segment. Acute dorsal trunk vagotomy resulted in a significant reduction of intragastric pressure response to cervical vagal stimulation below control values. Following chronic dorsal trunk vagotomy, intragastric pressure response to cervical vagal stimulation significantly increased above the acute value and reached the control value five days after vagotomy. The intragastric pressure maintained a plateau at this normal value

throughout the three months period of the experiments. Regenerative sprouts were not seen after chronic dorsal trunk vagotomy.

Light microscope autoradiographic study of the normal distribution of each of the abdominal vagal trunks in the gastric wall showed that the ventral trunk was distributed exclusively to the ventral surface of the stomach. The dorsal trunk was distributed to the dorsal surface of the stomach as well as the ventral surface of the pyloric antrum and the pylorus. Three weeks after ventral trunk vagotomy or dorsal trunk vagotomy, there was no change in the pattern of distribution of both abdominal vagal trunks. There was no tendency for compensatory spread of the surviving vagal trunk to the denervated areas of the stomach. Pre-ganglionic vagal efferent fibres reach the submucosal plexus but not the mucosa.

Electron microscope analysis of vagus nerve fibre composition showed that the left and right cervical vagus nerves contain equal numbers of fibres whereas the ventral trunk contains only about 50 percent of the number of fibres in the dorsal trunk. The total number of fibres in both abdominal vagus nerve trunks is only 50 percent of all fibres in the cervical trunks. Thirteen percent of all fibres in the cervical vagus nerves and less than 1 percent of those in the abdominal trunks are myelinated. The vagus nerve is essentially composed of afferent fibres with only 12 percent of cervical and 7 percent of abdominal vagal

trunks being efferent. The size-frequency distribution of the myelinated fibres is unimodal with a distinct peak at 2 to 3 µm diameter. Less than 1 percent of the myelinated fibres have diameters above 8 µm. Myelinated fibres less than 1 um diameter were seen but they form a very small percentage of all myelinated fibres. Because the myelinated fibres of the vagus nerve in the ferret have generally small diameters, and because the nerve is composed mostly of non-myelinated C-fibres, the vagus nerve in this animal is a slowly conducting nerve.

LIST OF ABBREVIATIONS USED IN THIS THESIS

SVE	-	Special Visceral Efferent
GVE	-	General Visceral Efferemt
GSA	-	General Somatic Afferent
SVA	-	Special Visceral Afferent
GVA	-	General Visceral Afferent
DMN	-	Dorsal Motor Nucleus
HCl	-	Hydrochloric Acid
DU	-	Duodenal Ulcer
SV	-	Selective Vagotomy
HSV	-	Highly Selective Vagotomy
PGV	-	Proximal Gastric Vagotomy
PCV	-	Parietal Cell Vagotomy
SPV	-	Selective Proximal Vagotomy
PV	-	Proximal Vagotomy
G-cells	-	Gastrin-secreting cells
DTV	-	Dorsal (Abdominal) Trunk Vagotomy
VTV	-	Ventral (Abdominal) Trunk Vagotomy
H_2O	-	Water
$\|^3H\|$leucine	-	Tritiated leucine
mRNA	-	Messenger Ribonucleic Acid
mCi	-	Millicurie
LCV	-	Left Cervical Vagus
RCV	-	Right Cervical Vagus
DT	-	Dorsal (Vagal) Trunk
VT	-	Ventral (Vagal) Trunk
LSNV	-	Left Supranodose Vagotomy
RSNV	-	Right Supranodose Vagotomy
SNV	-	Supranodose Vagotomy
CO_2	-	Carbondioxide
p	-	circumference
r	-	radius
π	-	pi

Continued

a	-	diameter of internal circumference
b	-	diameter of external circumference
t	-	myelin thickness
A_V	-	Total number of axons in ventral trunk
A_D	-	Total number of axons in dorsal trunk
L_V	-	Total number of axons in ventral trunk after LSNV
L_D	-	Total number of axons in dorsal trunk after LSNV
R_V	-	Total number of axons in ventral trunk after RSNV
R_D	-	Total number of axons in dorsal trunk after RSNV

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1. Time course of recovery of gastric motility in the ferret after chronic vagotomy.

2. Vagal innervation of the gastric wall - an autoradiographic study in the ferret.

CHAPTER ONE

GENERAL INTRODUCTION

CHAPTER ONE

GENERAL INTRODUCTION

PREAMBLE

It has, for a long time, been recognised that the Autonomic Nervous System is the sole source of gastro-intestinal innervation. It was shown to consist of two opposing subunits acting in a see-saw manner. Further studies have shown that the autonomic control of the stomach is not quite as simple as was first conceived. Most of such studies have been on the sympathetic nervous system, but more recently, the parasympathetic innervation has generated a lot of interest among researchers mainly because of its involvement in the pathophysiology and treatment of peptic ulcers in man. The present study is therefore a follow-up of such recent studies that sought to understand the autonomic control of the stomach in the ferret.

THE FERRET AS AN EXPERIMENTAL MODEL

Most of the experiments that are designed to study human body systems cannot be carried out on human subjects. Animal models have therefore to be chosen. If the results obtained from such experiments are to be applicable to man, the system that is being studied must be comparable to that in man.

Similarly, in order that the ferret stomach may be a suitable model for the human stomach, there must be a reasonable combination of points of similarity between the two with respect to their anatomical and physiological characteristics and their innervation. Various studies of ferret gastrointestinal tract have shown a number of similarities between its stomach and that of man.

a. Anatomical Characteristics

The morphological characteristics of the stomach are very similar in man and the ferret (Stephens and Pfeiffer, 1968; Poddar and Murgatroyd, 1976). The ultrastructure of the gastric mucosal surface is also identical in the two animals (Pfeiffer, 1970). Further studies have also shown that the anatomy of ferret stomach is such that the distribution of food in it immediately after a meal is similar to that demonstrated in man, with approximately 80% of the food being accommodated in the body and fundus (Andrews, Grundy and Scratcherd, 1980).

b. Gastric Innervation

The gross vagus nerve distribution to the stomach is, in the main, similar in both man and the ferret although it is much simpler in the latter (Mackay and Andrews, 1983).

c. Physiological Characteristics

The stomachs of both man and the ferret contain cholinergic excitatory neurons and non-cholinergic non-adrenergic inhibitory neurons, both of which can be activated by unmyelinated preganglionic vagal fibres (Andrews and Scratcherd, 1980). A number of the vago-vagal reflexes that have been observed in man have also been seen in the anaesthetised ferret (Andrews, Grundy and Lawes, 1980).

As far as the secretory behaviour of the ferret stomach is concerned, this animal, like man, is a basal secretor of acid and proteolytic enzymes in the fasting state (Pfeiffer and Peters, 1969).

A combination of all of these similarities makes the ferret a more suitable animal model than other common laboratory animals which also display some, but not all, of these features. Hence the ferret was used in the present study.

PHYSICAL CHARACTERISTICS OF THE FERRET

The ferret, which descended from the wild polecat, belongs to the family *Mustilidae* of the order *Carnivora*. It was kept by man and trained to kill rats and mice and to help in catching rabbits. The mean nose to base of tail length of the ferret is about 400 mm and a tail length of about 130 mm. The adult male weight ranges

from 700 g to 2100 g. The females are generally of lower weight than the males. The species that are commonly seen are Albino, Fitch and Mixed based on the colour of their fur.

ANATOMY OF THE VAGUS NERVE AND THE STOMACH

a. CENTRAL CONNECTIONS OF THE VAGUS NERVE

The vagus nerve is the 10th cranial nerve and belongs to the parasympathetic division of the Autonomic Nervous System. The microscopic anatomy of the central connections of the vagus nerve in the ferret is yet to be documented. The connections in other animals, including man, are summarised here as a basis for future comparison with those of the ferret. The central connections of the vagus nerve is an area of on-going research, but at the moment it is generally accepted that the vagus nerve consists of five functional fibre types with associated nuclei in the brain stem. These are:-

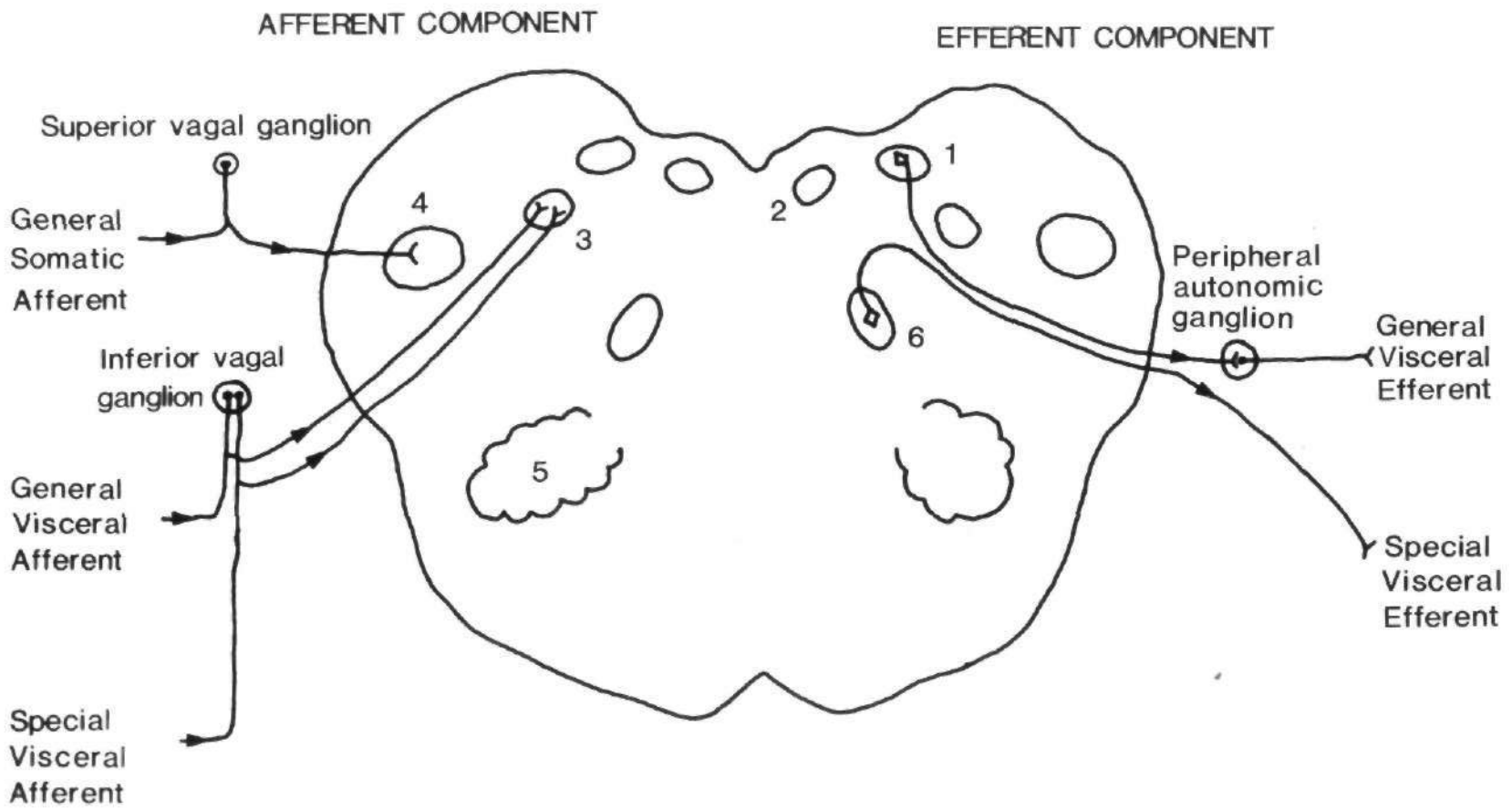
1. The Special Visceral Efferent (SVE) fibres which arise from the nucleus ambiguus (Porter, 1963), a longitudinally positioned nucleus in the medulla oblongata anterolateral to the dorsal motor nucleus of vagus nerve (Figure 1.1). The fibres from this nucleus pass backward and then bend sharply forward and laterally to reach the surface of the medulla behind the olive where they join the other fibres of the vagus in a row of

FIGURE 1.1

Diagram of the transverse section of the medulla oblongata at the level of the inferior olive. It shows the afferent and efferent components of the vagus nerve and their associated nuclei.

1. Dorsal motor nucleus of vagus
2. Hypoglossal nucleus
3. Nucleus of tractus solitarius
4. Spinal trigeminal nucleus
5. Inferior olive
6. Nucleus ambiguus

FIGURE 1.1


AFFERENT COMPONENT
EFFERENT COMPONENT
Superior vagal ganglion
General Somatic Afferent
Inferior vagal ganglion
General Visceral Afferent
Special Visceral Afferent
Peripheral autonomic ganglion
General Visceral Efferent
Special Visceral Efferent
1
2
3
4
5
6

rootlets. Along with similar fibres from the glosso-pharyngeal and cranial accessory nerves, they supply the striated muscles of the pharynx and larynx, and the upper part of the oesophagus.

2. The General Visceral Efferent (GVE) Fibres. These arise from the dorsal motor nucleus (DMN) of vagus nerve in the floor of the 4th ventricle of the medulla oblongata (Getz and Sirnes, 1949). The nucleus is longitudinally placed with about half the length rostral to and the other half caudal to the obex. The cranial half is more laterally placed than the caudal half, and at all levels, the nucleus is posterolateral to the hypoglossal nucleus.

From the DMN, the fibres travel antero-laterally, to reach the surface of the medulla. These fibres are preganglionic and supply glandular structures and smooth muscle such as the broncho-constrictor muscles of the lungs, the lower part of the oesophagus, the stomach, the pancreas, the gall bladder, the small intestine and the large intestine down to the left colic flexure.

3. The General Somatic Afferent (GSA) fibres arise from the back of the ear and the auditory tube. They travel in the spinal tract of trigeminal nerve before terminating in the spinal nucleus of the same nerve. Their cell bodies reside in the superior vagal ganglion.

4. The Special Visceral Afferent (SVA) fibres are taste fibres from the posterior third of the tongue, with their cell bodies in the inferior vagal ganglion. The afferent projections terminate in the caudal part of the nucleus of tractus solitarius. Some of the fibres pass to the spinal trigeminal nucleus and reticular formation (Culberson and Kimmel, 1972).

5. The General Visceral Afferent (GVA) Fibres. These fibres convey information about general sensation from visceral structures. Their cell bodies, like those of the special visceral afferent fibres, reside in the inferior vagal ganglion. Together with similar fibres from the glossopharyngeal nerve, they bend in a caudal direction within the medulla and form a conspicuous fibre bundle, the solitary tract, situated lateral to the DMN of the vagus and somewhat deeper. The fibres terminate in the nucleus of the tractus solitarius located lateral to the tract (Porter, 1963; Biscoe and Sampson, 1970 a and b).

b. THE PERIPHERAL COURSE OF THE VAGUS NERVE IN THE FERRET

The rootlets which are seen on the lateral surface of the medulla oblongata, posterior to the olive, unite to form a single nerve trunk on each side of the medulla. The trunk passes beneath the flocculonodular lobe of the cerebellum and leaves the skull through the jugular foramen in company of the glossopharyngeal and accessory nerves. At the level of the jugular foramen is located the superior vagal ganglion which contains the cell bodies of the GSA fibres.

Below this level, the vagus nerve has an inferior vagal (Nodose) ganglion which contains the cell bodies of other afferent fibres. The trunk of the nerve then descends in the neck in the carotid sheath, first with the internal carotid artery and then with the common carotid artery and the internal jugular vein. The two nerves enter the thorax and break up into branches which form anterior and posterior plexuses around the bifurcation of the trachea (Mackay and Andrews, 1983). Immediately below this point, the left and right vagus nerves reform from the posterior pulmonary plexus. The left vagus nerve, as it descends, passes gradually to the ventral surface of the thoracic oesophagus, lying slightly to the left of the midline. The right vagus nerve similarly passes to the dorsal surface of the oesophagus. In these positions, they descend through the oesophageal hiatus of the diaphragm into the abdomen. In the lower part of the thorax, midway between the base of the heart and the diaphragm, there is a consistent branch which passes supero-inferiorly from the left vagus nerve to join the right vagus nerve, passing on the dorsal surface of the oesophagus. This is the "crossing" branch which has no equivalent in man (Figure 1.2).

In the abdomen, the ventral trunk gives small branches to the oesophagus and divides into hepatic and gastric branches. The hepatic branch further divides

FIGURE 1.2

Diagram showing the thoracic and abdominal trunks of the vagus nerve in the ferret. The plexuses have been left out in order to enhance clarity of the diagram.

into a branch to the porta hepatis and another one (the pyloric branch) which descends in the free edge of the lesser omentum to supply the pyloric antrum, the pylorus, the first part of the duodenum and the pancreas (Mackay and Andrews, 1983). The gastric division, on the other hand, passes along the lesser curvature to supply the stomach.

The dorsal trunk which lies to the right of the midline gives numerous small branches of supply to the oesophagus and then divides into coeliac and gastric divisions. While the coeliac branch passes to the coeliac plexus and there mixes with sympathetic nerves, the gastric division passes along the dorsal surface of the lesser curvature to supply the stomach.

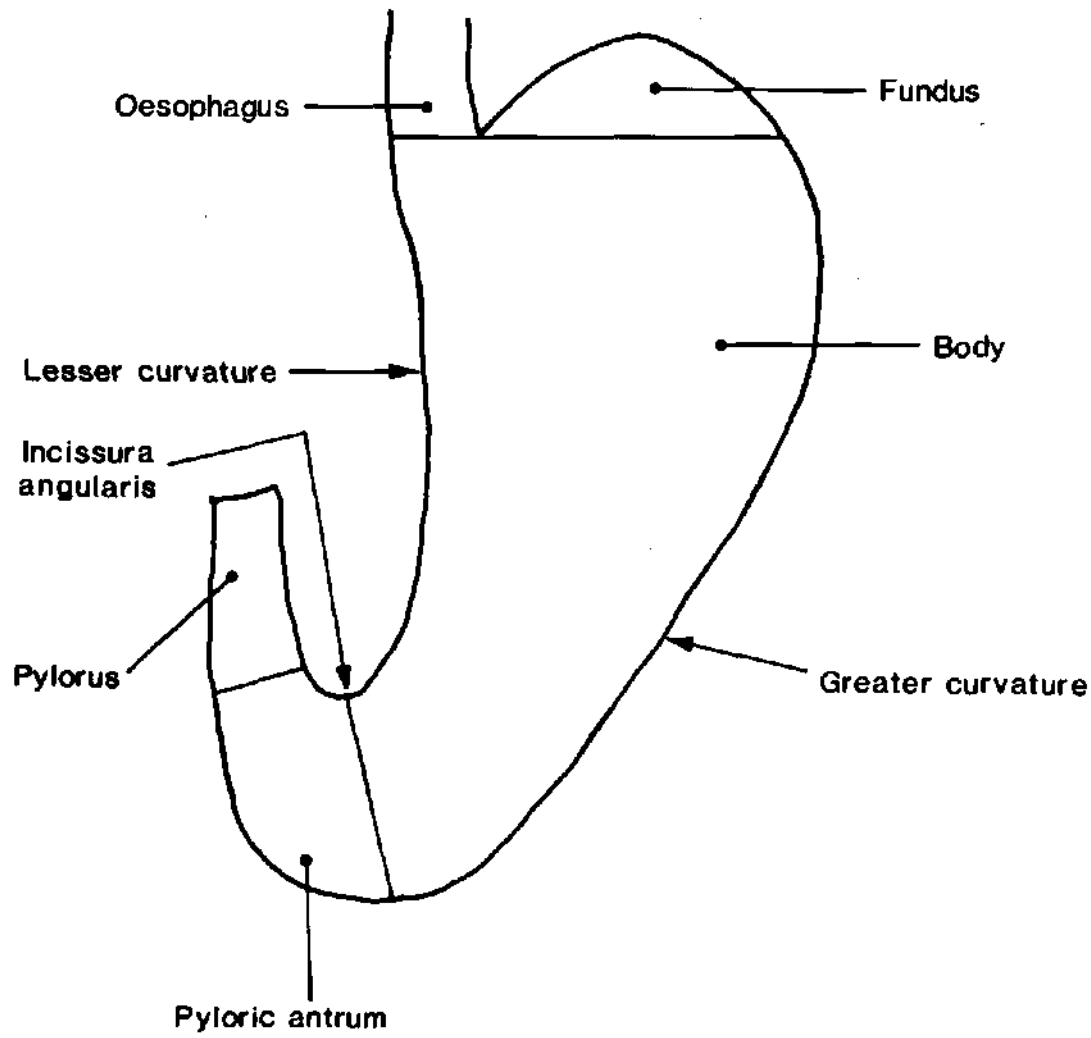
c. GROSS ANATOMY OF THE STOMACH OF THE FERRET

The stomach of the ferret, like those of other carnivores and man, is a single chamber organ. When empty, it lies nearly horizontally in the left upper quadrant of the abdomen. It is J-shaped when full. The two orifices, cardiac and pyloric, are connected by a right border or lesser curvature and a left border or greater curvature (Figure 1.3). The stomach can be divided into cardiac and pyloric parts by a line extending from a point on the lesser curvature, the incisura angularis, to the most dependent part of the greater curvature. There is a clearly identifiable

FIGURE 1.3

This diagram is a drawing from the live ferret stomach to show the gross anatomical features. It has been shown in the J-shape position which it assumes when full.

FIGURE 1.3


Oesophagus
Fundus
Body
Lesser curvature
Incissura angularis
Pylorus
Greater curvature
Pyloric antrum

fundus above the level of the cardia. The mucous membrane is thrown into folds or rugae which are predominantly longitudinal.

GASTRIC PHYSIOLOGY

The major function of the stomach is the digestion of food materials and the passage of the end-product of digestion to the small intestine. This function can be classified into

a. motor functions, and

b. secretory function.

a. Motor Functions

These functions are three-fold:-

1. The stomach acts as a RECEPTACLE and STORAGE organ for the food that reaches it from the oesophagus. It stores the food for a long enough time to allow gastric phase of digestion to be completed.

2. It MIXES the food with gastric secretions until it becomes a semifluid mixture called chyme.

3. It slowly EMPTIES the food into the small intestine at a rate suitable for proper digestion and absorption by the small intestine.

Embodied in the mechanisms of these functions is the phenomenon of gastric motility, which has to be finely controlled and regulated for a proper functioning of the stomach.

b. Secretory Function

The mucosal glands and cells of the stomach secrete a mixture of digestive enzymes and other products which make up the gastric juice.

The chief cells of fundic (gastric) glands secrete pepsinogen which is activated to digest protein. They also secrete rennin (in the calf) which curdles milk, lipase which splits fat, and gelatinase (in the pig) which splits gelatin. Other less important digestive enzymes which have been detected in gastric juice include lysozyme which splits carbohydrates, urease which hydrolyses urea, and carbonic anhydrase which is present in parietal cells (Davenport, 1939), and plays a role in the process of hydrochloric acid (HCl) formation.

The parietal cells, which are in abundance in the body of the stomach, secrete HCl, water, and intrinsic factor. The HCl, in addition to its probably insignificant role (at least in man) of hydrolysing starches and disaccharides, maintains a pH range of 2-3 which is essential for protein digestion by pepsin.

The antral mucosa contains Gastrin-secreting (G) cells which secrete the hormone gastrin which in turn plays a significant role in the control and regulation of gastrointestinal secretion and motility. The pyloric and cardiac gland cells as well as the mucous neck cells of gastric (fundic) glands and the layer of mucous cells

between the mucosal glands, secrete mucus which protects the stomach wall from digestion by the gastric enzymes.

CONTROL OF GASTRIC SECRETION

Gastric secretion occurs in various stages from the moment food is sighted to the time it reaches the small intestine. A combination of hormonal and neural factors is responsible for the secretion of gastric juice at each stage or secretory phase. The phases of gastric secretion are as follows:-

a. Cephalic Phase

This phase of gastric secretion occurs even before food enters the stomach. It results from the sight, smell, thought or taste of food. Neurogenic signals causing the cephalic phase of secretion may originate in the cerebral cortex or in the appetite centres of the amygdala or hypothalamus. They are transmitted through the DMN of the vagi to the stomach (Hightower and Janowitz, 1979). Experiments in dogs with gastric fistula have shown that the latent period of this phase of gastric secretion is less than 5 minutes and that secretion continues for 30 to 120 minutes after sham feeding.

b. Gastric Phase

Once food enters the stomach, it excites the gastrin mechanism, which in turn causes a low rate of secretion of gastric juice that continues throughout the several hours that the food remains in the stomach. This is as a result of chemical stimulation of G-cells and the most potent stimulants are peptides and aminoacids in the partially digested food.

In addition, the gastric distension produced by the presence of food in the stomach causes

a. local reflexes in the intramural plexus of the stomach, and
b. vago-vagal relfexes that pass to the brain stem and back to the stomach.

Both of these reflexes cause cholinergic stimulation of the gastric glands, and add to the secretion produced by the gastrin mechanism.

c. Intestinal Phase

This phase of gastric secretion is a result of chemical stimulation. It has long been observed in dogs that the presence of certain food substances in the small intestine stimulates gastric secretion. The following substances have been observed to excite gastric secretion when placed in the small intestine:- water, extractive substances of meat, products of protein digestion such as peptone and aminoacids, milk, alcohol, histamine, saponin,

adrenaline, 0.1N HCl, 10% glycerine solution, and magnesium sulphate. The latent period of this phase is 2-3 hours but once gastric secretion is initiated, it may last for 8-10 hours.

The exact mechanism of this chemical stimulation of gastric secretion is unknown but it is probably hormonal (Gregory and Ivy, 1941; Sircus, 1953 and 1958).

CONTROL OF GASTRIC MOTILITY

Gastric motility is controlled by, among other factors,

a. myogenic, and

b. neurogenic mechanisms.

a. Myogenic control of Gastric Motility

Gastric smooth muscle has certain intrinsic properties which enable it to undergo internal changes that react with changes in its environment to cause "spontaneous" or "myogenic" contractions (Thomas and Baldwin, 1968). For example, the circular muscle is functionally an electrical syncytium because of electrical coupling between the constituent smooth muscle fibres (Tomita, 1970; Holman and Neild, 1979). This accounts for the three-dimensional spread of excitation and associated contraction from the site of stimulation within the muscle segment after the stimulus has been removed.

There are also rhythmic myogenic pacemaker mechanisms (electrical slow waves) which initiate organised excitation of the network of electrically coupled cells (Wood, 1981). The circular muscle is highly excitable and responsive to the pacemaker so that in the absence of neural influence, each slow wave cycle triggers a synchronous circumferential contraction that propagates along the length of the isolated bowel. But in the intact bowel, the responsiveness of the circular muscle to the pacemaker, and the spread of the resulting excitation within the syncytium, are modulated by excitatory and inhibitory intrinsic neurons, but probably more so by the latter (Wood, 1981). Antral peristalsis depends mainly on the gastric slow wave (Wood, 1981).

Longitudinal muscle on the other hand, is strongly influenced by excitatory motor neurons and does not appear to receive any input from intrinsic inhibitory neurons (Wood, 1981).

b. Neurogenic Control of Gastric Motility

The neurogenic mechanism for the control of gastric motility may be divided into

(i) Intrinsic, and

(ii) Extrinsic Nervous Systems.

(i) The Intrinsic Nervous System lies between the longitudinal and circular smooth muscle layers (myenteric plexus) and in the submucosa (submucosal plexus). It

consists of groups of interconnected nerve cells which make up the intramural ganglia. Projecting in a complex manner to them by way of collaterals and to the Central Nervous System are afferent neurons whose dendritic terminals lie amongst the layers of the gastric wall (Figure 1.4). Most of these neuron cell bodies lie mainly in the inferior vagal ganglion (Ranson, 1921; Mohuiddin, 1953; Evans and Murray, 1954), and are concerned with the regulation of gastrointestinal function. They are the vagal afferent neurons. Other afferent fibres travelling with sympathetic fibres (Kuntz, 1953) have their cell bodies in spinal root ganglia (Head, 1893) and mediate pain sensation (Youmans et al., 1941). Vagal afferent fibres project mainly to the caudal part of the nucleus of tractus solitarius which, in the cat and rat, gives off axons or collaterals to the nucleus ambiguus and DMN of vagus (Morest, 1967; Cottle and Calaresu, 1975; Norgren, 1978). In this way, with the efferent vagal fibres, they form a "long reflex arc". The afferent collateral fibres, together with the intramural communicating ganglionic neurons and the postganglionic efferent neurons, constitute a "short" reflex arc. These complex reflex arcs modulate myogenic motility patterns and also respond to stimuli from the gastrointestinal lumen and from other sites. The intrinsic nervous system can function completely independently of the extrinsic nerves. Hence it has been described as a "little brain" (Wood, 1981). But like the other reflex

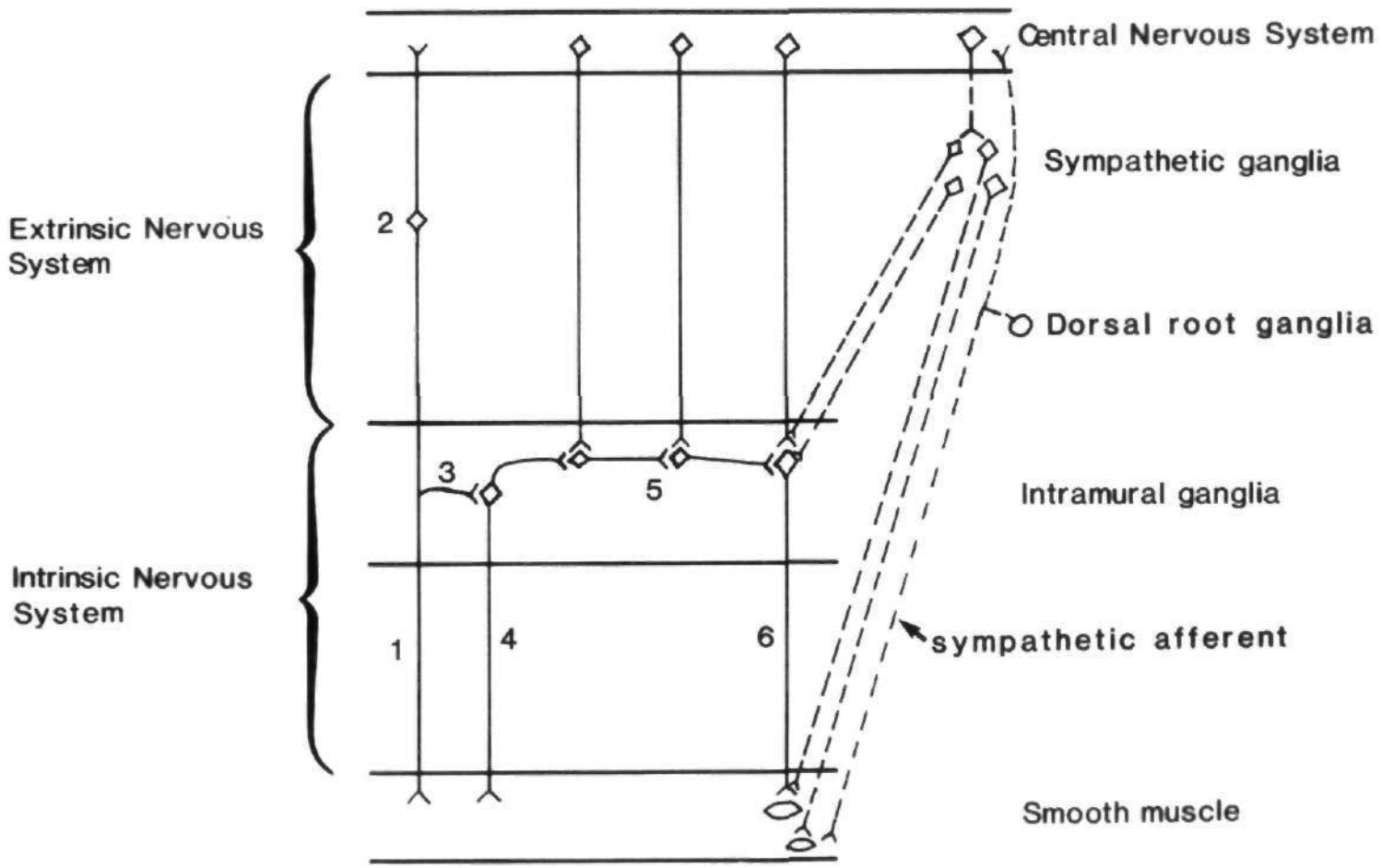
FIGURE 1.4

This is a simplified schematic diagram of the components and connections of the extrinsic and intrinsic nervous systems of the gastrointestinal tract in mammals. For the sake of clarity, the intramural ganglia have been separated from the smooth muscle. Nerve fibres shown in broken lines are sympathetic and solid line fibres are vagal parasympathetic.

1. Long vagal afferents, with their cell bodies in:-
2. Inferior vagal (nodose) ganglia and probably the dorsal root ganglia
3. Collateral branch from long afferent neuron
4. Short afferent neurons
5. Interneurons
6. Final common efferent neuron

Sympathetic afferent fibre which functions in reflex inhibition of gastric motility is shown.

FIGURE 1.4


Central Nervous System
Sympathetic ganglia
Extrinsic Nervous System
2
Dorsal root ganglia
3
5
Intramural ganglia
Intrinsic Nervous System
1
4
6
sympathetic afferent
Smooth muscle

arcs elsewhere in the body, the intrinsic nervous system is subject to facilitation and inhibition by nerve impulses which, in the case of the stomach, reach it by way of the vagus and sympathetic nerves (James, 1957).

(ii) The Extrinsic Nervous System

The extrinsic nerves reaching the stomach are post-ganglionic sympathetic and pre-ganglionic parasympathetic (vagal) fibres. Vagal fibres are now considered not to innervate gastric smooth muscle directly, but rather to synapse with efferent neurons in the intrinsic reflex arc which form the common final path for the innervation of the gastrointestinal smooth muscle (Kuntz, 1922; Bulbring et al., 1958). They may also facilitate or inhibit local reflexes in which the cells of the myenteric plexus are involved (Thomas and Baldwin, 1968). The vagus nerves may thus be regarded as connecting links between the reflex centres in the Central Nervous System responsible for regulating gastric functions and the local reflex centres in the gastric wall through which the activity of the muscle is co-ordinated and controlled.

The exact relationship of the sympathetic nerves to the intrinsic mechanism is not entirely clear. While it is possible that the postganglionic nerves terminate directly on the smooth muscle, recent findings suggest that the intramural plexuses are the primary loci for adrenergic innervation of the gut (Marks et al., 1962; Norberg, 1964; Jacobowitz, 1965; Hirst and McKirdy,

1974). Histofluorometric studies have also shown that only a sparse number of adrenergic nerves is seen in the smooth musculature of the gut as compared to the dense cholinergic innervation (Jacobowitz, 1965).

Sympathetic inhibition may therefore be at any of the following sites (see Figure 1.4):-

(i) preganglionic parasympathetic nerve terminal,
(ii) intramural ganglion communicating neurons,
(iii) common final path efferent neuron cell body,
(iv) common final path efferent nerve terminal, or
(v) directly at the neuromuscular junction.

Sympathetic afferents with their cell bodies in dorsal root ganglia are also believed to play a role in reflex inhibition of gastric motility (Kuntz, 1938; Kuntz and Saccomanno, 1944; Schapiro and Woodward, 1959).

Since the end of the last century (Langley, 1898), it has been known that stimulation of the vagus nerves may cause contraction or relaxation of the wall of the stomach. Martinson and Muren (1963) and Martinson (1964) have further shown that the vagus nerve of the cat has two groups of fibres:-

a. those that mediate contraction of the isolated stomach, having lower threshold of excitation than
b. those fibres that lead to relaxation.

The inhibitory fibres are more effective in the corpus

and fundus of the stomach whereas the excitatory ones are more effective in the pyloric portion. Neuro-effector transmission from the excitatory fibres of the vagus is cholinergic in the dog (Dale and Feldberg, 1934), cat (Harrison and McSwiney, 1936; Martinson and Muren, 1963), guinea pig (Greeff and Holtz, 1956; Paton and Vane, 1963) and in the mouse (Paton and Vane, 1963). The neurotransmitter for the inhibitory component is a subject of continuing research, but it is probably one of many peptides (Bishop et al., 1981), the most likely of which is vasoactive intestinal polypeptide (Fahrenkrüg et al., 1978; Costa et al., 1980; Edwards and Bloom, 1981).

Sympathetic efferents to gastric musculature also consist of low-threshold excitatory fibres which are cholinergic and high-threshold inhibitory fibres which are probably adrenergic. The adrenergic fibres synapse in the sympathetic ganglion, whereas the cholinergic fibres synapse at some point distal to these (Thomas and Baldwin, 1968).

VALUE OF VAGOTOMY

Vagotomy has come to be accepted as the definitive treatment for duodenal ulcer (DU). When it was first introduced, the vagus nerves were sectioned above the diaphragm before they divided into their abdominal branches (Dragstedt and Owens, 1943). This procedure was called Truncal Vagotomy. It was followed by complications, the

most debilitating of which was gastric retention. In order to overcome this, the technique was combined with gastric drainage procedures which were associated with equally debilitating side effects. The drainage procedures included various forms of gastrectomy, gastro-jejunal anastomoses and pyloroplasty. The dumping syndrome that accompanied gastrectomy and gastro-jejunal anastomoses was so troublesome that it became necessary to modify the technique of vagotomy so as to avoid the need for drainage procedures. At present, the technique used is either selective vagotomy with drainage or highly selective vagotomy. Johnson and Reynolds (1979), classified them simply as follows:-

1. Selective vagotomy (SV) in which all vagal branches to the stomach are cut leaving intact the branches to the hepatic plexus and the pyloric branches that arise from them. Additional drainage procedure is often necessary.

2. Highly Selective Vagotomy (HSV), Proximal Gastric Vagotomy (PGV), Parietal Cell Vagotomy (PCV), Selective Proximal Vagotomy (SPV), or Proximal Vagotomy (PV). In this procedure, all vagal branches to the stomach are cut except the terminal branches of the anterior and posterior nerves of Latarjet which are the motor nerve supply to the antral muscle.

The aetiology of duodenal ulceration is multifactorial but the most known of them, which is also the one most

amenable to treatment, is excessive secretion of HCl (Dragstedt, 1956; Lam and Sircus, 1975; Sircus, 1975; Wormsley, 1975). The excessive gastric acid secretion may be as a result of

a. direct, cholinergic stimulation of parietal cells by the vagus nerve, or
b. increased activity of G-cells initiated by various factors including the vagus nerve. The G-cells then excessively or incessantly secrete gastrin which in turn stimulates the parietal cells to secrete HCl (Arnold et al., 1974; Fuchs et al., 1974).

The rationale for the use of vagotomy for the treatment of DU was that it resulted in a decrease in the gastric acid hypersecretion that was characteristic of the ulcer (Dragstedt and Owens, 1943; Kennedy, 1974 and Thompson, 1974).

CONSEQUENCES OF VAGOTOMY

Most of the early experiments involving the sectioning of autonomic nerves and studying the effects on the denervated organs were done on the sympathetic nervous system especially the superior cervical ganglion, following transection of the cervical sympathetic trunk. Following such transections in the cat and rabbit (Butson, 1950), functional recovery was observed within 2 to 4 weeks as measured by the disappearance of the operatively produced miosis, ptosis, and vasodilatation of the face. Also following transection of the cervical sympathetic trunk above the superior cervical ganglion, the great majority of synaptic endings in the ganglion degenerated and finally disappeared while the post-synaptic neurons in the ganglion showed little change. Under the electron microscope, the denervated, vacated, synaptic sites were seen to persist for a long time.

However when the presynaptic (preganglionic) fibres in the trunk were allowed to regenerate, synapses began to reappear after 1 month, and after 2 months, their numbers reached normal levels (Raisman et al., 1974).

Similar experiments have been done on the vagus nerve but the results were sometimes conflicting. The commonly used techniques for assessing the immediate success of vagotomy and its long-term consequences as a treatment for DU are:-

1. Clinical assessment of symptoms and signs of duodenal ulceration.
2. Intra-operative and post-operative measurements of gastric acidity in terms of its volume or pH either as base values or following the administration of secretagogues such as insulin and pentagastrin.
3. Stimulation of vagus nerve and measuring myo-electric complexes or intragastric pressure changes immediately after surgery and at varying times after the operation.
4. Histological studies of the vagus nerve and the stomach wall for evidence of degeneration and regeneration.

Truncal vagotomy was believed to be a successful treatment for duodenal ulceration because the ulcers healed, remained healed, and did not recur in the majority of patients (Kragelund et al., 1973; Roland

et al., 1974; Moberg and Hedenstedt, 1975). The cases of recurrent ulcers were believed to be due to failure to divide all fibres in the vagal trunk. They supported Dragstedt's theory that if a relatively large branch of the vagus nerve escaped division at operation, the whole glandular apparatus of the stomach could be reactivated via the intramural plexus, with a return of high acid secretion (Dragstedt et al., 1947).

Selective vagotomy and PGV which are techniques of incomplete vagotomy have become very popular in recent years. There have been conflicting reports about the long-term success of these procedures in the treatment of DU. By stimulating the incompletely divided cervical vagus nerves in the cat and measuring intragastric pressure changes, Clark (1964) demonstrated a functional recovery of the vagus 4 to 6 weeks after nerve section. In 1970, Burge et al. reviewed the 700 patients they operated upon for DU between 1957 and 1969. Selective vagotomy and gastric drainage were the primary operations done, using Burge's intra-operative test (see Appendix 1.1) to ensure complete division of all gastric branches. Nine of these patients were found to have developed recurrent DU in spite of the completeness of the vagotomy as determined by the intra-operative test. At a second operation in each of these patients, intact vagal fibres were seen innervating the stomach. Notes recorded at the first operation indicated that there had been problems

with using Burge's test in these patients and that the authors were not satisfied with the negative result of the test at that time. In this way they showed that the intra-operative test was not infallible. They also reaffirmed their support for Dragstedt's theory although no attempt was made to explain the exact mechanism for the vagal functional recovery.

Wastell and Zervos (1976) relied mainly on clinical assessment of their patients after PGV and recorded an increasing incidence of recurrent ulceration with each year of follow-up. Their result was supported by the findings of Joffe and Bapat (1979) that following PGV, the ulcer-producing effects of secretagogues were prevented for only 5 days in the rat.

On the contrary, some workers neither found an increase in the area of stomach innervated and in the amount of acid secreted in rats 1 year after incomplete vagotomy (Jones and Griffith, 1970; Legros and Griffith, 1970) nor any sign of ulcer relapse or recurrence in their patients after administration of secretagogues (Griffith, 1980).

In all of the follow-up accounts given above, the techniques used have been limited to the assessment of clinical signs and symptoms, various types of acid test and studies of intra-gastric pressure changes in response to vagal stimulation. Because of the difficulty of carrying out acid study procedures under precisely

repeatable physiological states in different subjects and in the same subject at different times, the results are sometimes contradictory. The problem with nerve stimulation procedures is that the nerves are being stimulated in a non-physiological manner so that results from such experiments are difficult to correlate with physiological events in the subject. It would therefore be desirable if less variable indicators of vagal functional recovery were studied. It was only recently that Loup et al. (1980) used a histological technique in addition to gastric acid and motility studies to demonstrate vagal recovery after truncal vagotomy and proximal gastric vagotomy. They showed in man and the dog that whether or not vagotomy was complete, the vagus nerve proximal to the ligature was capable of sending normal axons that by-passed the vagotomy ligatures to reach the denervated stomach. Following a study of gastric secretion after vagotomy for DU in 29 patients, Butterfield et al. (1982) concluded that there is an incidence of recurrent ulcer after vagotomy even when an early insulin test has indicated a complete vagotomy. The recurrent ulcers occur only in patients whose insulin stimulated secretion rises with time.

A recent experiment in the ferret which involved the measurements of changes in intragastric pressure caused by the stimulation of the vagus nerve by discrete electric square waves via platinum electrodes,

showed that there is a significant increase in the intragastric pressure 2 weeks after unilateral abdominal trunk vagotomy (Andrews, Lawes and Bower, 1980).

There is therefore overwhelming evidence, although mainly physiological, that the vagus nerve recovers its functions after vagotomy.

AIMS OF THE RESEARCH

The tripartite aims of this research were:-

1. Using ELECTROPHYSIOLOGICAL TECHNIQUES, to determine the Time-Course of FUNCTIONAL RECOVERY of the vagus nerve in the ferret.

2. Using AUTORADIOGRAPHIC TECHNIQUES, at light microscope level, to determine the normal distribution of the vagus nerve terminals in the stomach wall and to see if there is any change in the distribution pattern after unilateral abdominal vagotomy. This may reveal evidence of any ANATOMICAL RECOVERY.

3. Using ELECTRON MICROSCOPE and STEREOLOGICAL TECHNIQUES to estimate the NORMAL FIBRE COMPOSITION of each cervical and abdominal trunk of the vagus nerve.

CHAPTER TWO

THE TIME COURSE OF RECOVERY OF INTRAGASTRIC PRESSURE RESPONSE TO VAGAL STIMULATION IN THE FERRET

CHAPTER TWO

THE TIME COURSE OF RECOVERY OF INTRAGASTRIC PRESSURE RESPONSE TO VAGAL STIMULATION IN THE FERRET

INTRODUCTION

Gastric function may be studied by means of one of three parameters, namely:-

1. acid secretion,
2. electric activity of the smooth muscle, and
3. gastric motility patterns in response to appropriate stimuli.

The function parameter that was used in this study was "gastric motility" which was in turn measured by the amount of intragastric pressure that accompanied it as a result of electrical stimulation of the vagus nerves in the neck.

Andrews, Lawes and Bower (1980) measured the intragastric pressure that was developed in response to the stimulation of cervical vagus nerves in three groups of ferrets. In the first group (the control), the abdominal vagus nerves were intact. In a second group, intragastric pressure changes were measured immediately after alternating unilateral abdominal trunk vagotomy. In the third group of animals, the pressure changes were measured two weeks after unilateral abdominal trunk vagotomy - chronic vagotomy. Their results showed that:-

a. after acute and chronic ventral abdominal trunk vagotomies, the intragastric pressures were neither significantly different from each other nor from the control value,

b. after acute dorsal abdominal trunk vagotomy (DTV), stimulation of the cervical vagus nerves produced a significant fall in the intragastric pressure,

c. following chronic DTV, there was a significant rise in the intragastric pressure towards the control value.

They have therefore shown a functional recovery of the stomach two weeks after abdominal vagotomy in the ferret.

The Aim of this Experiment

The aim of this experiment was to measure intragastric pressure responses to cervical vagus nerve stimulation in normal and post-acute vagotomy ferrets and in other groups of ferrets after unilateral chronic abdominal vagotomy of varying duration. It was hoped that it would then be possible to localise the onset of, and determine possible mechanisms for the functional recovery of the stomach.

MATERIALS AND METHODS

a. ANIMAL GROUPING

Sixty-seven ferrets were used (see Table 2.1). They were divided into two main groups. In one group, only ventral abdominal trunk vagotomy (VTV) was done while the other group had only DTV. The animals in each group were subdivided into groups of 4 to 6. The first subgroup was used for control and post acute vagotomy experiments. The remaining subgroups had chronic vagotomy with a survival period of either 3, 5, 7, 21, 28 or 84 days. The animals were adults of either sex and ranged in weight between 700 g and 1800 g. The cross section of these weights was represented in each group of animals. Any animal which became ill at any time during the period of the experiment was rejected.

b. ANAESTHESIA

Prior to doing vagotomy, the animals were anaesthetised with intraperitoneal injections of pentobarbitone sodium (Sagatal; May and Baker Ltd.) in doses of 60 mg per kg.

At the end of the survival period, the animals were given intraperitoneal doses of ethyl carbamate (Urethane; Sigma Chemicals Ltd.) 1.5 gm per kg in the form of 50% Urethane in 0.9% sodium chloride (normal saline). Supplementary doses of 25% Urethane were given intravenously if required to maintain deep anaesthesia.

TABLE 2.1

Type of Vagotomy	Control and Acute	Period of Survival after Vagotomy					
		3 days	5 days	7 days	21 days	28 days	84 days
Dorsal Abdominal Trunk	6	6	6	6	6	6	4
Ventral Abdominal Trunk	5	Nil	Nil	6	5	5	6

Preparation of Urethane Anaesthetic

See Appendix 2.1.

c. SURGERY

Two types of surgery were done, namely

(i) abdominal vagotomy, and

(ii) surgery for intragastric pressure recording.

(i) Abdominal Vagotomy

The animals were deprived of food overnight before the surgery. The abdomen was opened by a vertical midline incision. The stomach was exteriorised to improve access to the vagus nerves alongside the abdominal oesophagus. The ventral or dorsal vagus nerve trunk was identified and carefully separated from surrounding tissues as high up in the abdomen as possible. Two silk ligatures, 1 cm apart, were placed round the nerve trunk. The nerve was then cut between these ligatures and the cut ends allowed to retract from each other. The abdomen was closed in layers with catgut (size 2/0) and silk (size 2/0) sutures. The animal was allowed to recover from anaesthesia while being kept adequately warm under table lamps. The animals were restricted to a diet of water on the first post-operative day, milk on the second day, and dog meat or dead rats with fluid thereafter.

(ii) Surgery for Intragastric Pressure Recording

The animals were taken off food for 24 hours prior to the experiment. Each animal was laid on its back and an anterior midline incision made in the neck.

Tracheostomy was done and a wide-bore tube inserted and securely kept in place with a silk ligature that went round the trachea. This tube allowed for unimpeded ventilation and also permitted adequate suction of the trachea to keep it clear of mucus.

The right external jugular vein was cannulated to provide a route for subsequent doses of Urethane anaesthetic. The cervical oesophagus was dissected free from the surrounding tissues and a silk ligature loosely placed round it. The abdomen was opened by a midline incision and the distal end of the pylorus was occluded with a ligature. The stomach was intubated via the mouth with a wide-bore, open-ended plastic tube. Inside this tube was another open-ended tube through which the intragastric pressure would be transmitted (Figure 2.1). The lower end of this tube was 0.5 cm short of the end of the outer tube. This ensured that the end of the inner tube would not be in contact with and get blocked by the gastric wall. Having ensured that the tips of the tubes were lying freely in the cavity of the body of the stomach, they were kept in place with the ligature that was previously placed round the cervical oesophagus. The abdominal incision was then closed with Michel's suture clips.

Each cervical vagus nerve was exposed and carefully separated from the surrounding tissues. The connective tissue between the vagus nerve and the carotid artery

FIGURE 2.1

Schematic diagram to show the connections of the tubes and apparatus for vagal stimulation and recording of intragastric pressure changes in the ferret.

PC = Pressure calibrator

PT = Pressure transducer

PA = Pressure amplifier

D = Digitimer

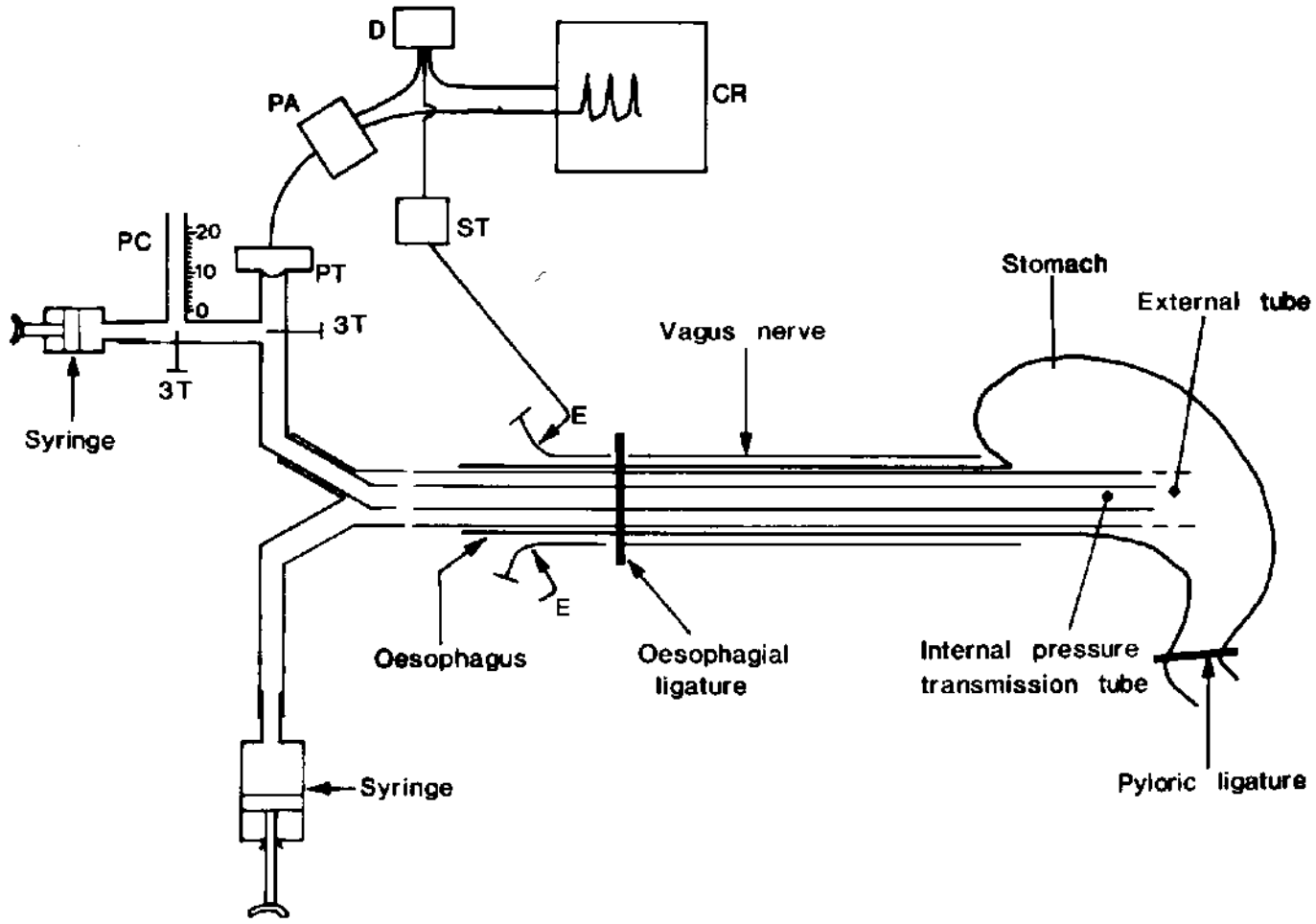
CR = Chart recorder

ST = Isolated stimulator

E = Electrode

3T = 3-way tap

FIGURE 2.1


D
PA
CR
ST
PC
20
10
0
PT
3T
3T
Syringe
E
Vagus nerve
Stomach
External tube
E
Oesophagus
Oesophagial ligature
Internal pressure transmission tube
Syringe
Pyloric ligature

was gently peeled off in order to separate the two structures for a distance of about 1-2 cm. A ligature was lightly placed round the vagus nerve as rostrally as possible. The nerve was then sectioned rostral to the ligature and kept moist with warm liquid paraffin at 37°C. The body temperature of the animal was kept at 37°C to 39°C by means of radiant heat which was achieved by a combination of hot water circulating through a hollow copper plate on which the animal was laid, and table lamps.

Each of the cervical vagus nerve trunks, 5 mm distal to the ligatures, was placed over a bipolar platinum electrode in a pool of warm liquid paraffin.

d. INTRAGASTRIC PRESSURE RECORDING

The outer of the gastric tubes was attached to a 20 ml syringe, containing 20 ml of normal saline, via one limb of a Y-junction tube. The other limb carried the inner tube and by it, the latter was connected to a Pressure Transducer (SEM 4-88, SE Labs. Ltd.) and the pressure calibration part of the apparatus by means of a three-way tap (see Figure 2.1). The Pressure Transducer was connected to the Pressure Amplifier (EMMA, SE Labs. Ltd.) which moved the recording pen on a Bryans chart recorder 28000 to display the pressure signals. Vagus nerve stimulation was achieved by means of a Devices Isolated Stimulator which was precisely timed by the Digitimer D4030 to which it was connected. The

Digitimer also timed recording chart movement.

The inner gastric tube was flushed with normal saline while keeping the outer tube open so that the whole system of tubes would be free of air bubbles. The recording chart was then calibrated as follows:-

A vertically placed calibrated glass tube was connected to the pressure transducer and a 40 ml syringe by means of a system of two 3-way taps. The syringe was filled with normal saline. An arbitrary mark was chosen on the tube and set on the same horizontal plane as the pressure transducer. This mark was called the zero mark. The glass tube was filled up to the zero mark with normal saline from the syringe. The column of saline thus produced was allowed to exert pressure on the pressure transducer. This pressure was amplified by the pressure amplifier and displayed on the chart recorder. This pressure was called 0 cm H_2O being the pressure due to 0 cm of saline in the tube. The tube was then filled to a point 20 cm above the zero mark and the pressure due to this column of saline was similarly recorded as 20 cm H_2O. This enabled the chart to be calibrated for pressure in cm H_2O.

The pressure calibrator was turned off and the cavity of the empty stomach was allowed to be in direct continuity with the pressure transducer. The pressure in the empty stomach was therefore recorded. Twenty millilitres of normal saline was placed in the stomach

by step inflation through the outer gastric tube. This gave a new level of pressure which was recorded on the chart recorder. The stomach was allowed to rest for 30 minutes before nerve stimulation was commenced. This was to allow the effects of the sudden distension of the stomach to wear off and the stomach to settle to the new pressure in it (Dr. I. N. C. Lawes, personal communication).

e. VAGUS NERVE STIMULATION

Stimuli of 20 volts and a width of 0.5 milliseconds were delivered from the stimulator at a frequency of 10 Hz. Periods of stimulation of 10 seconds were used. An interval of 3 minutes was allowed between two stimuli in order that the stomach might recover from the effects of the previous stimulation and also that the intragastric pressure might reach a stable level before a second stimulus was delivered. These parameters were found to produce a single gastric contraction of maximal amplitude (Andrews, Lawes and Bower, 1980).

The cervical vagus nerves were stimulated separately and together. The sequence of stimulation was varied in order to prevent order effect. The electrodes were moved caudally at intervals in order to prevent damage to the nerve by prolonged stimulation at one site.

Following stimulations in control animals in which the abdominal vagal trunks were intact, the animals were

immediately subjected to dorsal or ventral vagotomy and cervical nerve stimulations were repeated to give intragastric pressure changes due to acute unilateral abdominal vagotomy. At the end of the survival period, the cervical vagus nerves of animals with chronic abdominal vagotomy were also stimulated.

f. POST RECORDING CHECKS

At the end of the cervical vagus nerve stimulation in each animal, the abdominal vagus nerve trunk that was not cut at the first operation, was transected and the cervical trunks stimulated again in order to check if the unilateral chronic vagotomy was complete. If it was complete, stimulation of the cervical vagi would not give rise to increased intragastric pressure above the baseline value.

Post mortems were also done to examine the sites of the chronic vagotomies as well as the sites of the surviving nerve trunks that had just been cut for test purposes. Animals in which there was evidence of previously incomplete chronic vagotomy were rejected from the experiment.

The fluid in the stomach after the pressure recordings was drained, measured and compared with the 20 ml of normal saline introduced into the stomach prior to nerve stimulation. The stomach was removed, cut open, cleaned out and weighed.

RESULTS

Intragastric Pressure Response to Cervical Vagal Stimulation

Figures 2.4 A, B, C, D and E show typical tracings of amplitudes of intragastric pressure in the presence or absence of vagal or gastric stimulation. The empty stomach had a pressure, E, relative to atmospheric zero. When the stomach was filled with 20 ml normal saline in a single step (step inflation) the intragastric pressure increased (S) and fell to a resting level (RP) which was higher than E. Thirty minutes after step inflation, intragastric pressure reached a steady level. Stimulation of cervical vagal trunks resulted in increased intragastric pressure above resting level (L, R and B). This was followed by gastric smooth muscle relaxation (RX) as shown by a fall in intragastric pressure below resting level. The return of intragastric pressure towards resting level progressed with time but varied from one animal to the other (Figures 2.4A and 2.4D).

Evoked Intragastric Pressure Changes after Abdominal Trunk Vagotomy

Tables 2.2a, b and c show the intragastric pressures resulting from separate and combined left and right cervical vagal stimulations in control group animals and at varying periods after dorsal trunk vagotomy. The mean and standard errors of the mean (S.E.M) are shown for each group of animal. See table 2.3 and figure 2.2.

Tables 2.4a and b show the intragastric pressures due to separate and combined left and right cervical vagal stimulations in control group animals and at varying periods after ventral trunk vagotomy. See table 2.5 and figure 2.3.

FIGURE 2.4A

Shows tracings of intragastric pressure in animals with intact abdominal vagal trunks (Control).

E = unevoked pressure in the empty stomach at the beginning of the pressure recording experiment.

S = intragastric pressure due to step inflation with 20 ml normal saline.

RP = resting intragastric pressure 30 minutes after step inflation.

RX = measure of smooth muscle relaxation following cervical vagal stimulation.

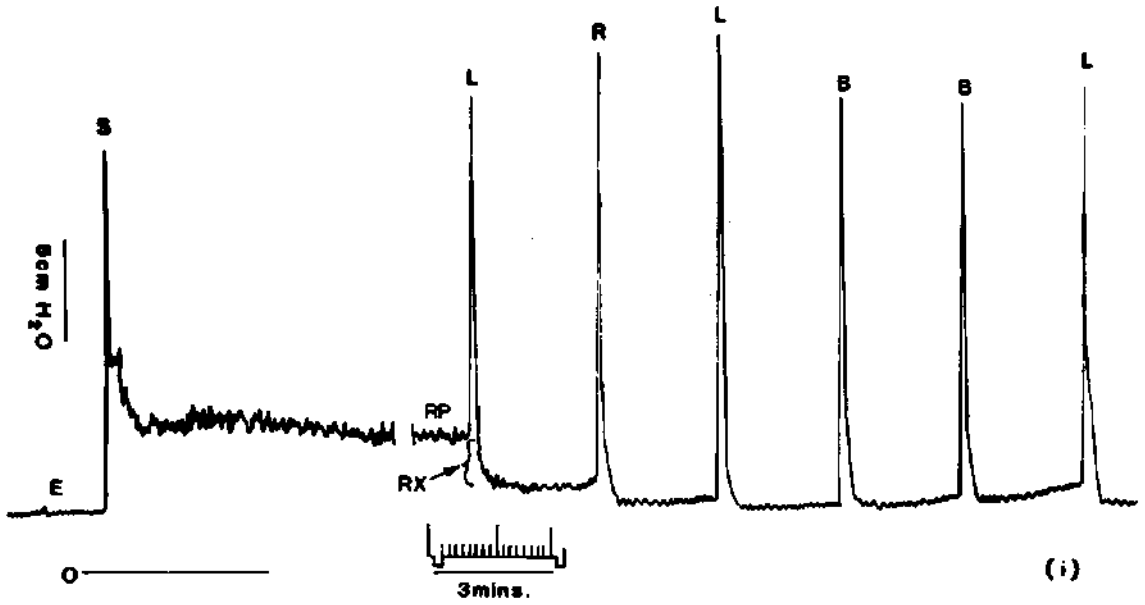
L = amplitude of intragastric pressure response to left cervical vagal stimulation.

R = amplitude of intragastric pressure response to right cervical vagal stimulation.

B = amplitude of intragastric pressure response to simultaneous left and right cervical vagal stimulation.

(i) and (ii) are from two separate control animals. The level of intragastric pressure after relaxation returned only slowly towards baseline but did not reach it before the next stimulus was delivered. (ii) shows that the longer the stomach is allowed to rest, the more the post-relaxation intragastric pressure approaches baseline resting value. Andrews and Scratcherd (1980) showed that the resting pressure is reached in 10 to 11 minutes after the initial relaxation. Six minutes gap only was allowed between two stimulations in this experiment (ii).

FIGURE 2.4A



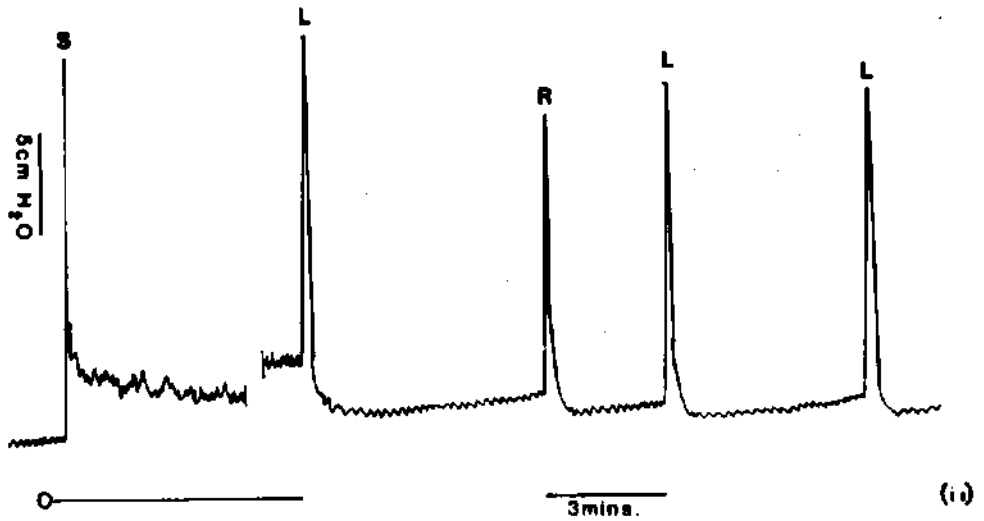
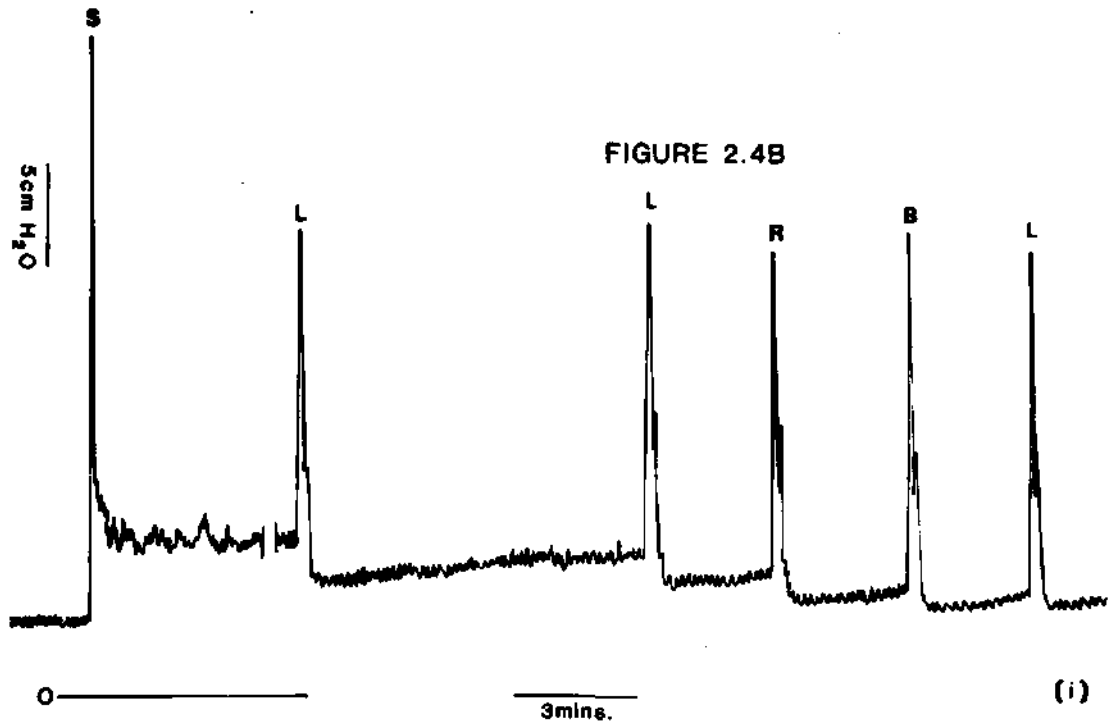


FIGURE 2.4B

These are tracings of intragastric pressures from two animals immediately after dorsal trunk vagotomy. The post-relaxation pressure is seen to approach the resting level 9 minutes after the initial relaxation (i). The amount of recovery of intragastric pressure after relaxation did not seem to affect the amplitude of subsequent evoked intragastric pressure (i).

FIGURE 2.4B



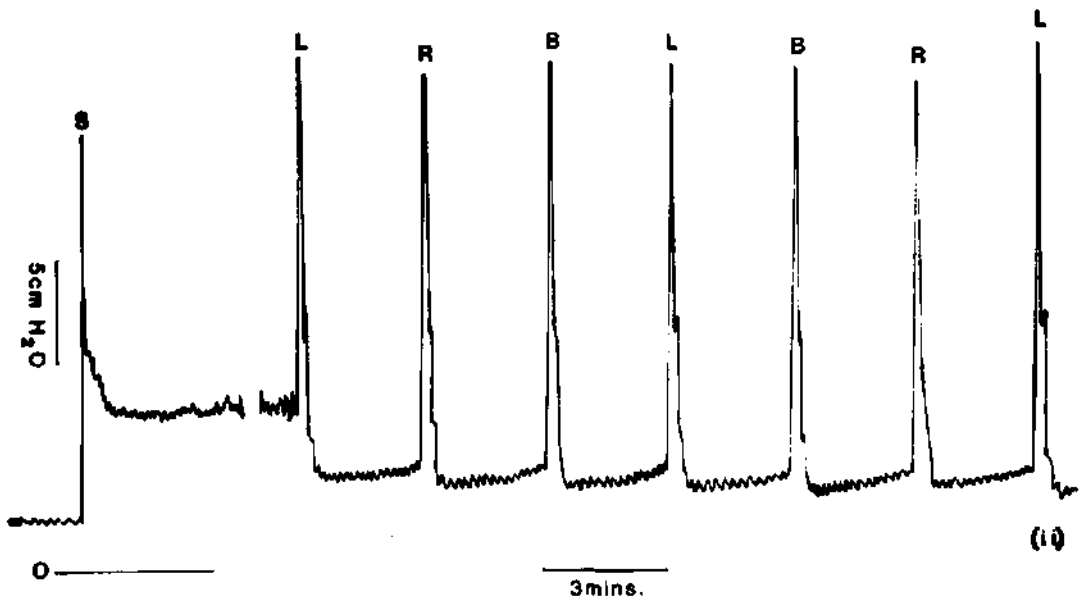


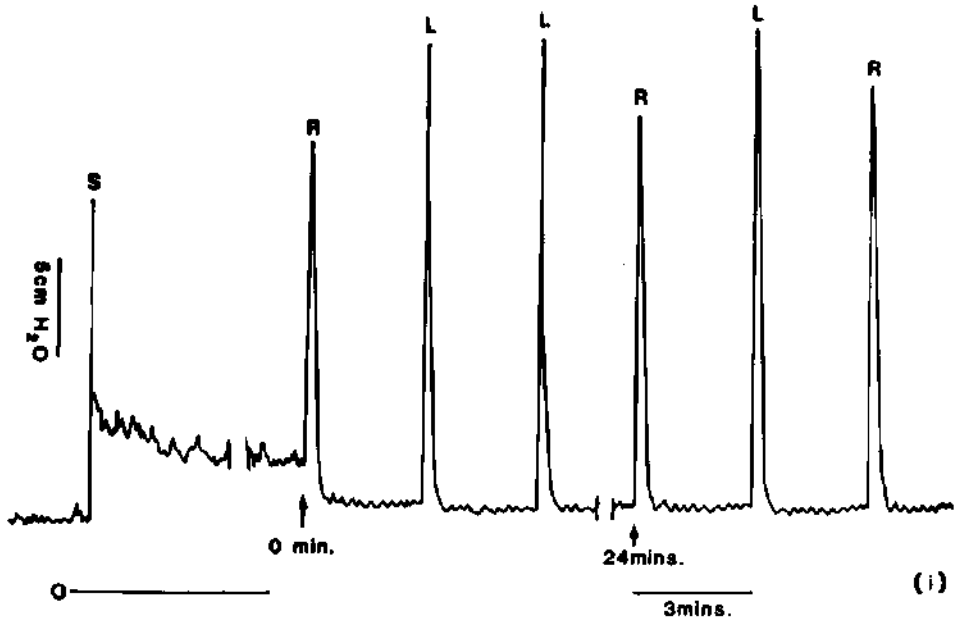
FIGURE 2.4C

These are tracings of intragastric pressure in animals 3 days after dorsal trunk vagotomy (i), and 7 days after ventral trunk vagotomy (ii). After the left cervical stimulation in (i), the stomach was still relaxing and subsequent return of intragastric pressure towards the resting value is not dramatic. The second animal (ii) again showed marked spontaneous gastric contractions.

FIGURE 2.4D

These are tracings of intragastric pressure in two animals 28 days after dorsal trunk vagotomy. They show marked inter-animal variations. Animal (ii) shows relatively higher spontaneous gastric contractions right from the beginning of the experiment and these increased as expected after step inflation. (ii) shows greater return of intragastric pressure towards resting value than (i) and has recovered sufficiently to produce a spontaneous gastric contraction within two minutes of an evoked gastric contraction (SP).

FIGURE 2.4D


S
R
L
L
R
L
R
5cm H_2O
0 min.
24mins.
3mins.
0
(i)

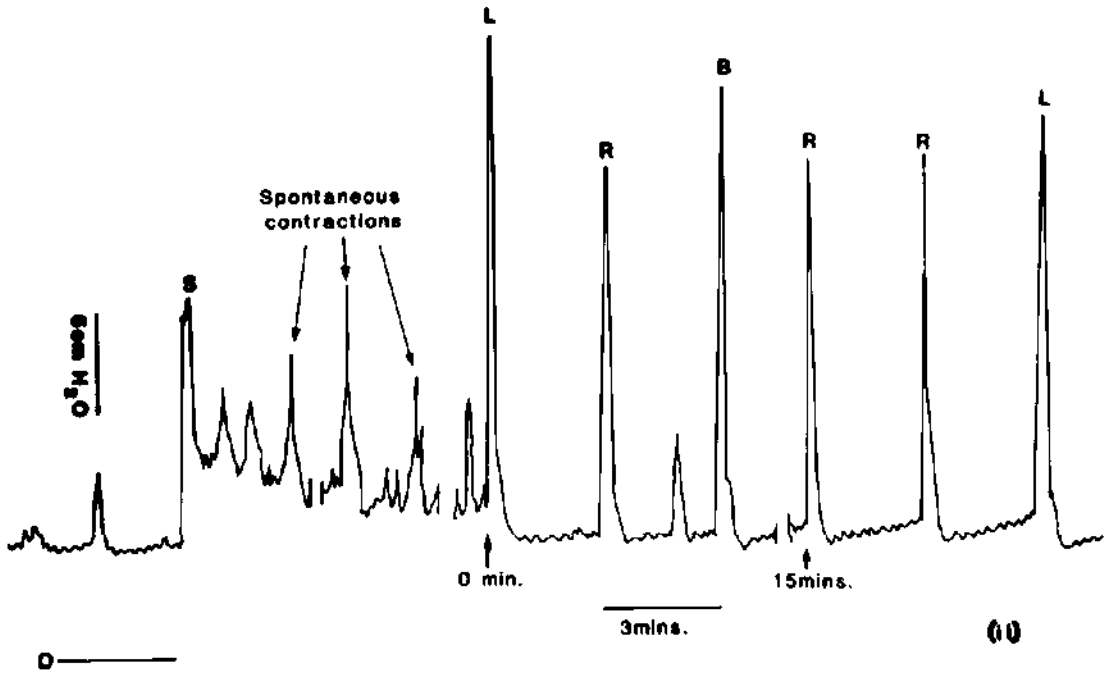
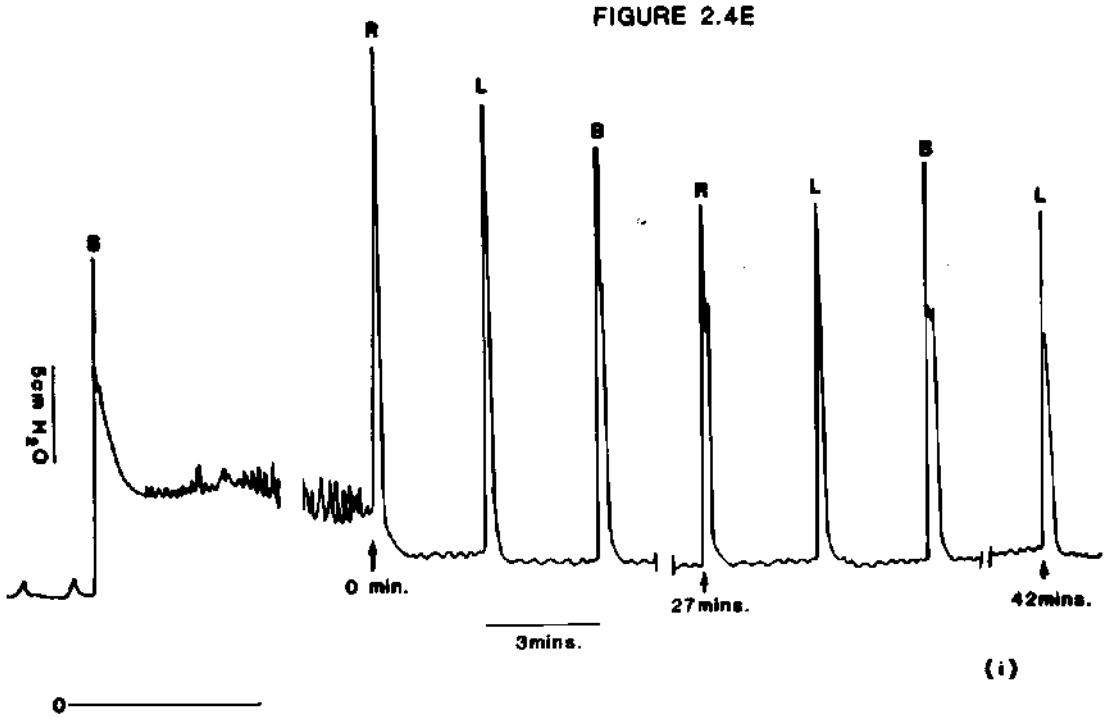
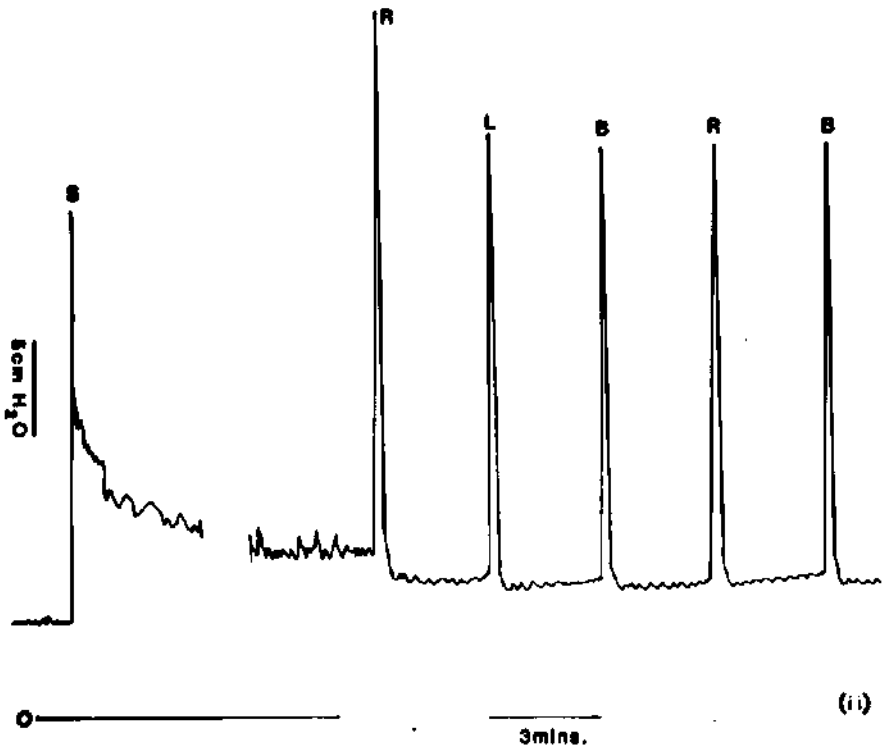

Spontaneous contractions
S
L
R
B
R
R
L
5cm H_2O
0 min.
15mins.
3mins.
0
(ii)

FIGURE 2.4E

These are tracings of intragastric pressure from two animals 3 months after dorsal (i) and ventral (ii) trunk vagotomy. The amplitude of intragastric pressure response to first cervical vagal stimulation is strikingly higher than subsequent ones. This may mean that the stomach was in an excited state and required a longer time than 30 minutes before commencing cervical vagal stimulation.

FIGURE 2.4E


R
L
B
R
L
B
L
S
5cm H_2O
0 min.
27mins.
42mins.
3mins.
0
(i)


R
L
B
R
B
S
5cm H_2O
0
3mins.
(ii)

TABLES 2.2a, b and c

Show the amplitudes of evoked intragastric pressure (in cmH_2O) in control animals and in different groups of animals after dorsal vagotomy.

Mean and standard error of the mean (S.M.M) are shown for each group.

TABLE 2.2a

Site of Stimulation	Evoked intragastric pressure (in cmH_2O) in control animals						
	1	2	3	4	5	6	Mean
Left Cervical	18.50	23.25	21.38	26.25	22.30	35.45	24.52 ±2.42
Right Cervical	18.67	25.08	21.75	24.75	26.90	25.95	23.77 ±1.23
Left and Right Cervical	18.75	22.50	19.50	26.13	26.60	28.95	23.74 ±1.69

Site of Stimulation	Evoked intragastric pressure (in cmH_2O) after dorsal trunk vagotomy													
	0 Day (Acute vagotomy)							3 Days						
	1	2	3	4	5	6	Mean	1	2	3	4	5	6	Mean
Left Cervical	18.92	11.75	13.25	7.75	13.90	9.05	12.44 ±1.62	9.60	5.50	14.60	16.37	23.60	13.05	13.77 ±2.51
Right Cervical	17.75	12.15	11.50	5.75	7.95	7.85	10.49 ±1.75	11.42	6.40	15.20	16.68	19.00	13.95	13.78 ±1.80
Left and Right Cervical	18.63	9.25	13.63	8.25	11.40	10.80	11.99 ±1.53	15.57	7.70	14.60	15.33	23.30	15.07	15.26 ±2.02

TABLE 2.2b

Site of Stimulation	Evoked intragastric pressure (in cmH_2O) after dorsal trunk vagotomy						
	5 Days						
	1	2	3	4	5	6	Mean
Left Cervical	14.18	17.00	16.20	29.31	24.00	21.98	20.45 ±2.33
Right Cervical	14.00	15.50	12.00	21.02	19.25	21.35	17.19 ±1.59
Left and Right Cervical	14.20	15.50	17.10	32.88	23.67	20.35	20.60 ±2.58

	7 Days						
	1	2	3	4	5	6	Mean
Left Cervical	20.25	16.50	17.00	20.70	26.75	18.40	19.93 ±1.53
Right Cervical	19.69	19.55	17.19	22.50	26.94	19.80	20.95 ±1.38
Left and Right Cervical	22.70	20.08	18.38	25.25	22.00	20.40	21.47 ±0.98

	21 Days						
	1	2	3	4	5	6	Mean
Left Cervical	17.80	24.75	18.75	25.44	23.17	23.50	22.24 ±1.30
Right Cervical	21.05	25.75	18.75	29.19	22.00	22.70	23.24 ±1.51
Left and Right Cervical	21.70	27.19	16.25	28.06	23.42	24.50	23.50 ±1.74

TABLE 2.2c

Site of Stimulation	Stimulated intragastric pressure (in cmH_2O) after dorsal trunk vagotomy											
	28 Days							84 Days				
	1	2	3	4	5	6	Mean	1	2	3	4	Mean
Left Cervical	20.04	21.80	19.25	12.57	20.87	23.83	19.73 ±1.57	20.93	19.90	20.80	18.77	20.10 ±0.50
Right Cervical	18.75	20.21	24.06	16.21	18.27	19.61	19.52 ±1.07	15.75	20.36	19.86	16.35	18.08 ±1.18
Left and Right Cervical	19.75	22.95	23.45	20.22	20.28	24.20	21.80 ±0.79	26.40	20.88	20.22	21.90	22.35 ±1.39

TABLES 2.4a and b

Show the amplitudes of evoked intragastric pressure (in cmH_2O) in control animals and in different groups of animals after ventral trunk vagotomy. Mean and standard error of the mean (S.E.M) are shown for each group.

TABLE 2.4a

Site of Stimulation	Evoked intragastric pressure (in cmH_2O) in control animals					
	1	2	3	4	5	Mean
Left Cervical	21.25	16.75	28.00	23.50	24.75	22.85 ±1.88
Right Cervical	18.00	14.75	26.20	22.38	24.50	21.17 ±2.10
Left and Right Cervical	17.50	15.00	26.80	23.58	23.98	21.37 ±2.20

Site of Stimulation	Evoked intragastric pressure (in cmH_2O) after ventral trunk vagotomy												
	0 Day (Acute vagotomy)						7 Days						
	1	2	3	4	5	Mean	1	2	3	4	5	6	Mean
Left Cervical	19.15	16.25	23.25	18.50	18.50	19.16 ±1.14	20.40	20.63	22.13	16.98	21.15	24.65	21.01 ±1.02
Right Cervical	18.88	14.50	21.80	17.98	19.23	18.48 ±1.78	20.15	20.50	22.69	18.75	23.73	20.85	21.03 ±1.67
Left and Right Cervical	15.42	16.63	19.10	20.77	21.98	18.78 ±1.23	18.75	18.42	23.75	19.25	24.53	26.05	21.79 ±2.37

TABLE 2.4b

Site of Stimulation	Evoked intragastric pressure (in cmH_2O) after ventral trunk vagotomy					
	21 Days					
	1	2	3	4	5	Mean
Left Cervical	21.71	22.20	23.42	23.25	28.43	21.80 ±1.82
Right Cervical	21.63	19.38	21.58	22.00	17.30	20.38 ±0.90
Left and Right Cervical	22.81	22.19	22.50	24.80	18.45	22.15 ±1.03

	28 Days					
	1	2	3	4	5	Mean
Left Cervical	17.38	17.10	21.92	23.15	20.21	20.02 ±1.25
Right Cervical	16.13	18.25	23.88	24.80	18.64	20.34 ±1.69
Left and Right Cervical	16.63	18.94	26.00	22.00	21.80	21.27 ±1.45

	84 Days						
	1	2	3	4	5	6	Mean
Left Cervical	23.28	27.85	26.07	23.83	20.38	20.00	23.57 ±1.26
Right Cervical	23.08	28.05	24.01	24.00	19.75	27.20	24.50 ±1.22
Left and Right Cervical	12.95	27.80	26.57	24.63	23.00	24.20	25.03 ±0.73

a. Evoked Intragastric Pressure Changes after Dorsal Trunk Vagotomy

These experiments showed that immediately after DTV, stimulation of the cervical vagus nerves, separately and together, gave rise to an intragastric pressure which was significantly less than the control value (Figure 2.2). Three days after DTV, the intragastric pressure had increased from 50% in acute experiments to only about 60% of the control value, an increase which did not reach a statistically significant 5 percent level. On the fifth day after DTV, the intragastric pressure had significantly risen above the acute value to 80% of the control value ($P < 0.02$; Table 2.3). This value of intragastric pressure as well as values for 7, 21, 28 and 84 days after DTV were not significantly different from one another and from the control value.

b. Evoked Intragastric Pressure Changes after Ventral Trunk Vagotomy

Intragastric pressure response to cervical vagus nerve stimulation immediately after VTV was reduced by only 16% of the control value (Figure 2.3; Table 2.5). This reduction did not reach a statistically significant level. After 7, 21 and 28 days, the intragastric pressure had still not altered significantly. Three months (84 days) after VTV, intragastric pressure increased significantly above the acute value only

FIGURE 2.2

Histogram showing intragastric pressure changes in response to cervical vagal stimulation in normal animals and after different survival times following dorsal (abdominal) trunk vagotomy.

Survival time 0 = intragastric pressure change following acute dorsal trunk vagotomy

 = Left cervical vagal stimulation

 = Right cervical vagal stimulation

 = Simultaneous left and right cervical vagal stimulation

See Table 2.3 for significant levels of intragastric pressure changes.

FIGURE 2.2

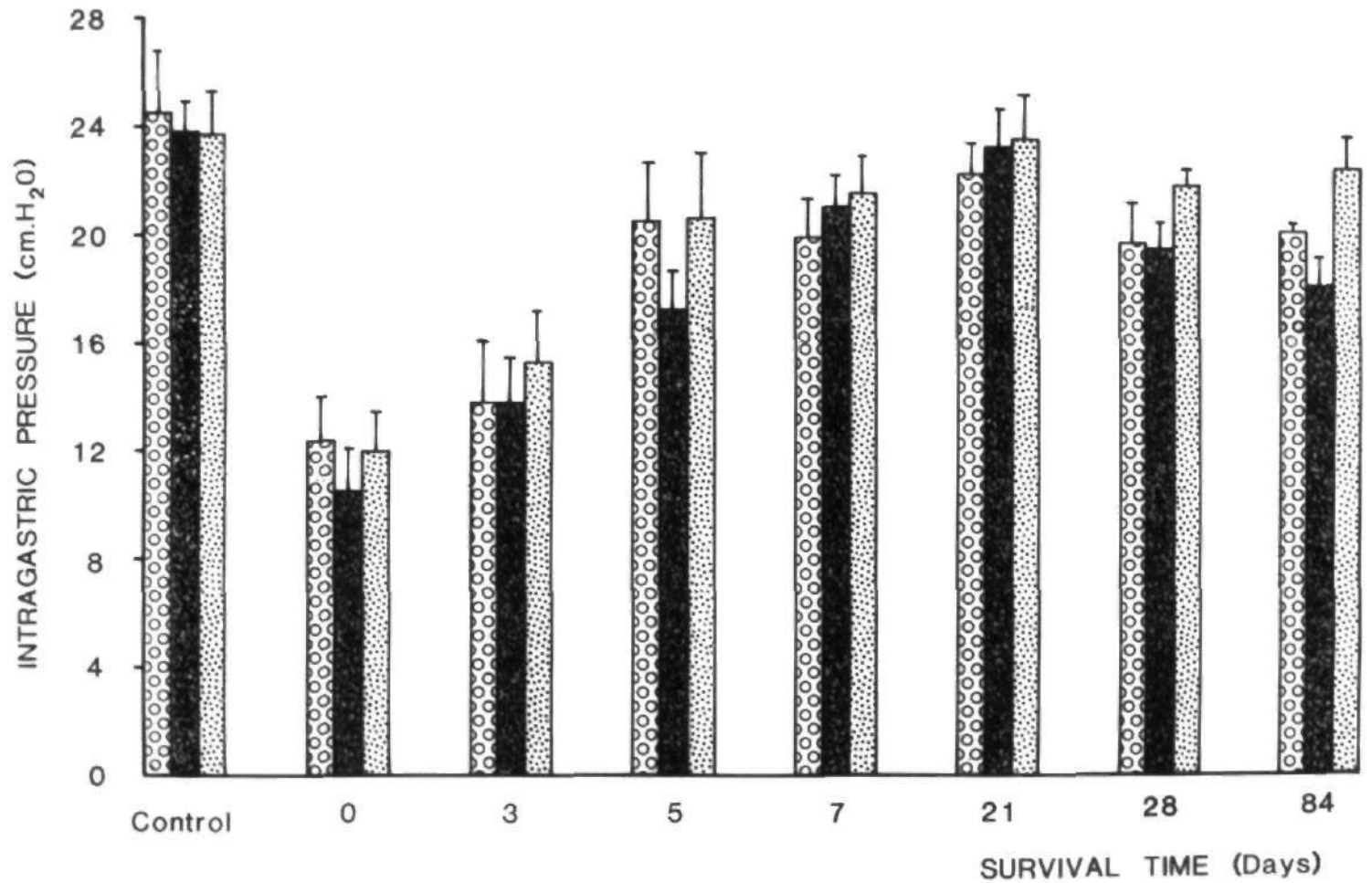

INTRAGASTRIC PRESSURE (cm.H2O)
28
24
20
16
12
8
4
0
Control
0
3
5
7
21
28
84
SURVIVAL TIME (Days)

TABLE 2.3

The effect of dorsal trunk vagotomy on intragastric pressure response to cervical vagal stimulation is represented by the mean and standard error of the difference in amplitude between acute post-vagotomy and normal, and between acute and chronic post-vagotomy intragastric pressure responses. The differences are obtained by subtracting the amplitudes after acute dorsal vagotomy from the amplitudes in normal animals and the amplitudes after acute dorsal vagotomy from the amplitudes after chronic dorsal vagotomy respectively. Significance levels from paired sample t-tests (after acute vagotomy) and unpaired sample t-tests (after chronic vagotomy) are shown by S2 = $P < 0.02$; S1 = $P < 0.01$; S0.1 = $P < 0.001$; NS = Not significant.

TABLE 2.3

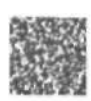
Site of Stimulation	Changes in Amplitude (in cm H_2O) brought about by Dorsal Abdominal Trunk Vagotomy after:						
	0 Day	3 Days	5 Days	7 Days	21 Days	28 Days	84 Days
Left cervical	-12.1 ± 2.0 SO.1	+1.3 ± 3.0 NS	+8.0 ± 2.8 S2	+7.5 ± 2.2 S1	+9.8 ± 2.1 SO.1	+7.3 ± 2.3 S1	+7.7 ± 1.7 S1
Right cervical	-13.3 ± 2.1 SO.1	+3.3 ± 2.5 NS	+6.7 ± 2.4 S2	+10.5 ± 2.2 SO.1	+12.8 ± 2.3 SO.1	+9.0 ± 2.1 S1	+7.6 ± 2.1 S1
Left and Right cervical	-11.8 ± 2.3 SO.1	+3.3 ± 2.5 NS	+8.6 ± 3.0 S2	+8.4 ± 1.8 SO.1	+11.5 ± 2.3 SO.1	+9.8 ± 1.7 SO.1	+10.4 ± 2. SO.1

FIGURE 2.3

Histogram showing intragastric pressure changes in response to cervical vagal stimulation in normal animals and after different survival times following ventral (abdominal) trunk vagotomy.

Survival time 0 = intragastric pressure change following acute ventral trunk vagotomy

 = Left cervical vagal stimulation

 = Right cervical vagal stimulation

 = Simultaneous left and right cervical vagal stimulation

See Table 2.5 for significant levels of intragastric pressure changes.

FIGURE 2.3

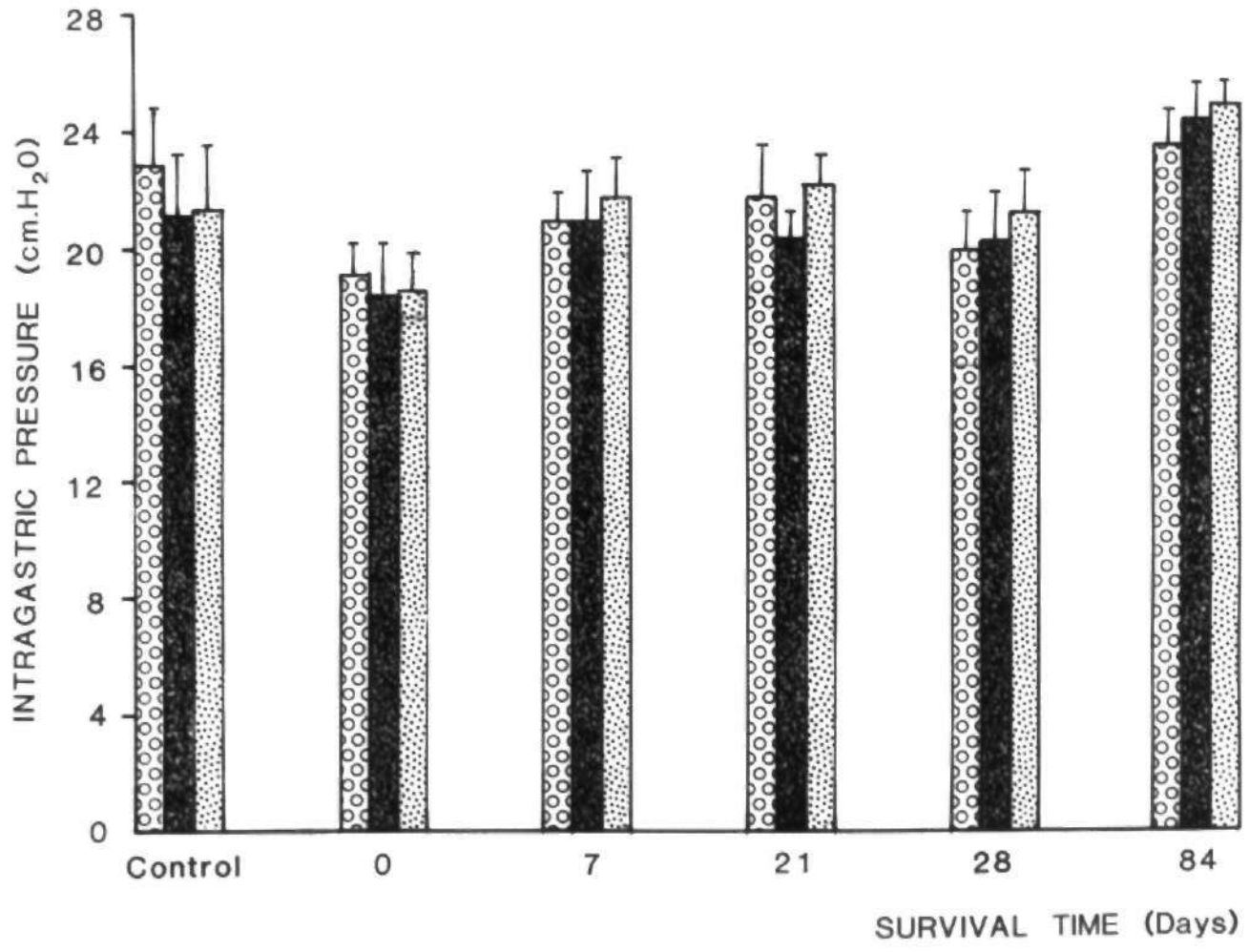

28
24
20
16
12
8
4
0
INTRAGASTRIC PRESSURE (cm.H2O)
Control
0
7
21
28
84
SURVIVAL TIME (Days)

TABLE 2.5

The effect of ventral trunk vagotomy on intragastric pressure responses to cervical vagal stimulation is represented by the mean and standard error of the difference in amplitude between acute post-vagotomy and normal, and between acute and chronic post-vagotomy intragastric pressure responses. The differences are obtained by subtracting the amplitudes after acute ventral vagotomy from the amplitudes in normal animals and the amplitudes after acute ventral vagotomy from the amplitudes after chronic ventral vagotomy respectively. Significance levels from paired sample t-tests (after acute vagotomy) and unpaired sample t-tests (after chronic vagotomy) are shown by S5 = $P < 0.05$; SO.1 = $P < 0.001$; NS = Not significant.

TABLE 2.5

Site of Stimulation	Changes in Amplitude (in cm H_2O) brought about by Ventral Abdominal Trunk Vagotomy after:				
	0 Day	7 Days	21 Days	28 Days	84 Days
Left cervical	-3.7 ± 2.2 NS	+1.9 ± 1.5 NS	+2.6 ± 2.2 NS	+0.9 ± 1.7 NS	+4.4 ± 1.7 S5
Right cervical	-2.7 ± 2.8 NS	+2.6 ± 2.4 NS	+1.9 ± 2.0 NS	+1.9 ± 2.5 NS	+6.0 ± 2.2 S5
Left and Right cervical	-2.6 ± 2.3 NS	+3.0 ± 1.8 NS	+3.4 ± 1.6 NS	+2.5 ± 1.9 NS	+7.2 ± 1.4 SO.1

($P < 0.05$). Examination of the abdominal trunks of the vagus nerves in each of the animals in that 3-month subgroup revealed a tissue bridge which by-passed the ligatures and the ends of the cut to link the proximal and distal portions of the ventral trunk. Electron microscopy of the tissue bridge revealed normal, mainly non-myelinated axons (Plate 2.1).

Because ventral trunk vagotomy did not appear to have caused any significant changes in intragastric pressure response to cervical vagus nerve stimulation, at least in the first 28 days after vagotomy, experiments were not done for 3 and 5 days survival periods. For the same reason, the following results were derived from the experiments on animals with chronic dorsal trunk vagotomy.

c. Relative Effects of Left and Right Cervical Vagus Nerve Stimulations

The intragastric pressure responses to separate stimulations of the cervical vagus nerve were not significantly different from each other. The pressure resulting from combined stimulation of left and right cervical vagus nerves was not a summated value of the separate pressure responses. In fact the intragastric pressure responses to the stimulation of the nerves separately and together were not significantly different from each other (Tables 2.3 and 2.5; Figure 2.4). Therefore, there was a functional overlap of one vagus nerve by the other.

PLATE 2.1

Electron micrograph of tissue bridge linking cut ends of the ventral vagal trunk 3 months after vagotomy. Normal nerve fibres with prominent neurotubules (Tub), neurofilaments (Fil) and mitochrondria (Mit) are present.

N-Ax = Non-myelinated axon

Col = Collagen fibres

End = Endoplasmic reticulum

Magnification = x 24000

PLATE 2.1

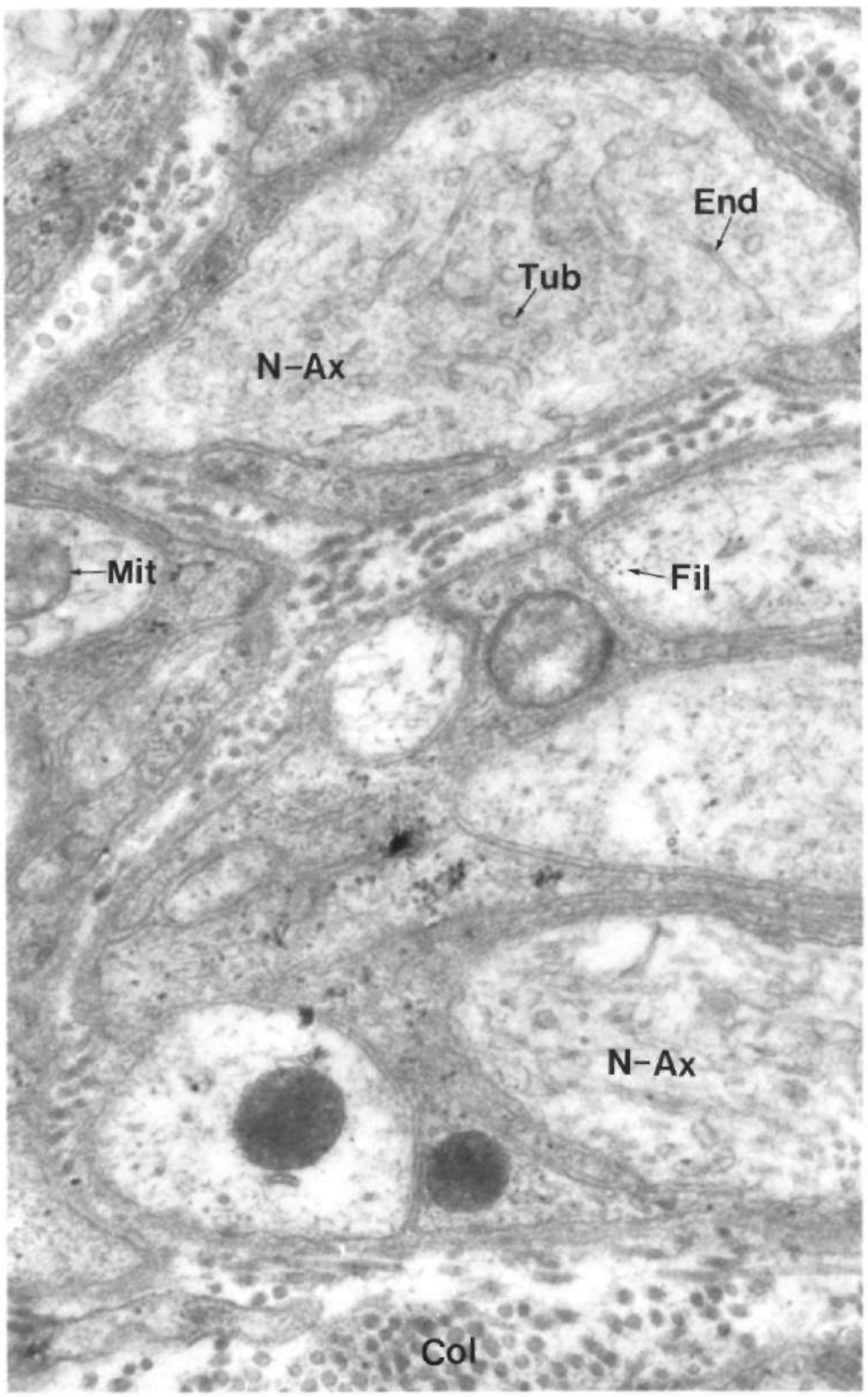


FIGURE 2.5

This shows the effect of vagotomy on intragastric pressure modalities. The survival time was plotted as logarithmic values of the days in order to give a visual impression of the rate of change of intragastric pressure responses to cervical vagal stimulation.

A = time-course of intragastric pressure changes due to step inflation

B = time-course of resting intragastric pressure changes

C = time-course of pressure in the empty stomach

D = time-course of relaxation after the first cervical vagal stimulation.

The thickness of the copper plate on which the animals were placed (2.2 cm) was subtracted from the recorded intragastric pressure values of B and C in order to obtain their true values relative to atmospheric pressure.

d. Effect of Dorsal Trunk Vagotomy on Intragastric Pressure Modalities (Figure 2.5)

(i) Intragastric Pressure due to Step Inflation

The difference between the height of intragastric pressure due to step inflation with 20 ml of normal saline and that in the empty stomach was measured and plotted against the survival time of the animals after vagotomy (Figure 2.5A). The pressure due to step inflation was about 17 cm H_2O in control animals (Table 2.6). It rose to a significantly higher level of 22 cm H_2O immediately after DTV ($P < 0.05$). It remained high up to the third day after vagotomy (24 cm H_2O). By the fifth day, the intragastric pressure had fallen to 18 cm H_2O which is not significantly different from the value in control animals. It remained more or less unchanged throughout the remaining part of the three months covered by the experiment.

(ii) Resting Pressure

This was the stable, baseline intragastric pressure reached 30 minutes after step inflation and just before cervical stimulation was begun. The resting intragastric pressure was about 6 cm H_2O in control animals (Table 2.7; Figure 2.5B). It rose through the acute phase of vagotomy to a significant level of about 8 cm H_2O on the third day after vagotomy ($P < 0.01$). It then fell to about 7 cm H_2O which is not significantly different from the control value. It remained low for the remaining part of the three months period.

FIGURE 2.5

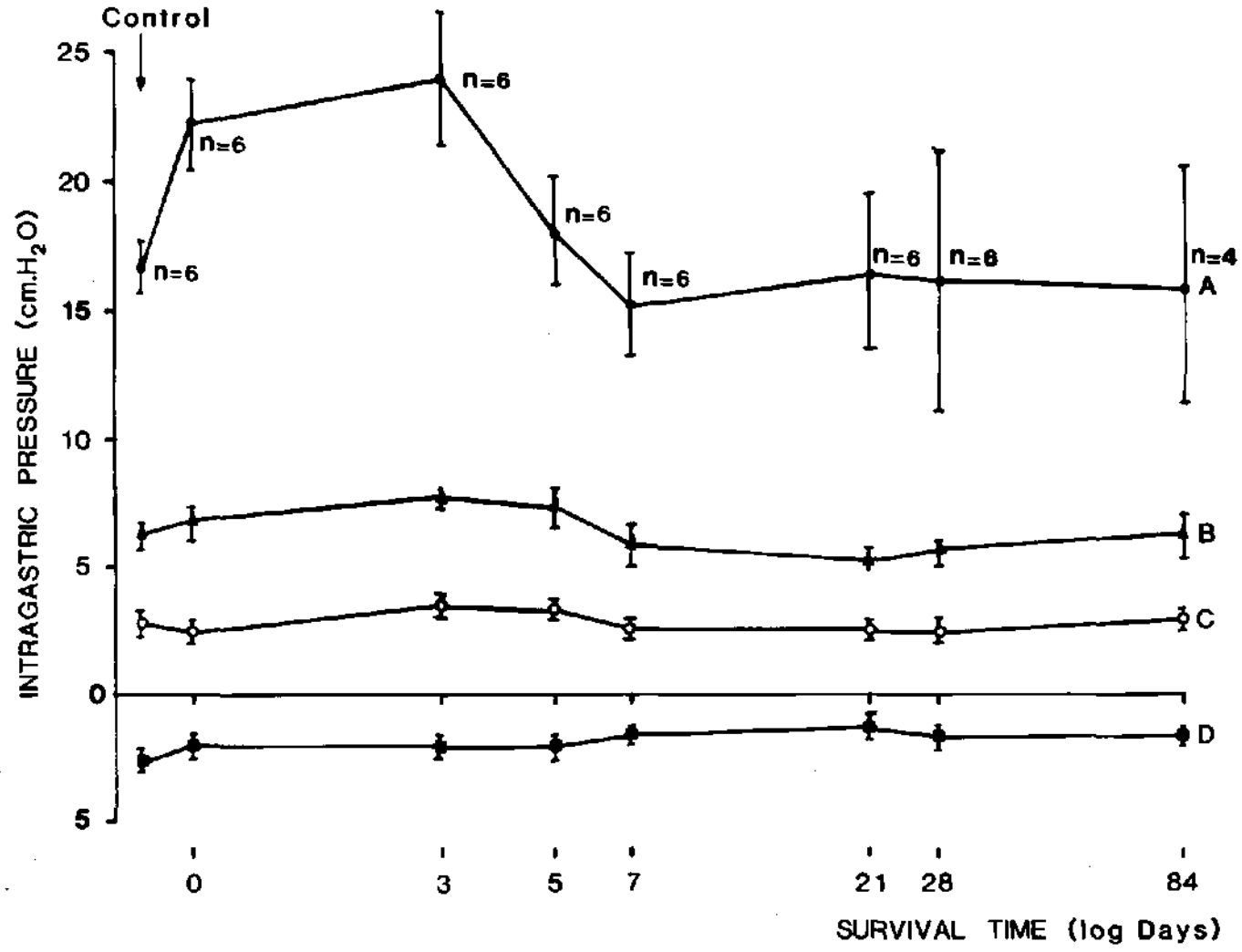

Control
25
20
15
10
5
0
5
INTRAGASTRIC PRESSURE (cm.H2O)
n=6
n=6
n=6
n=6
n=6
n=6
n=8
n=4
A
B
C
D
0
3
5
7
21
28
84
SURVIVAL TIME (log Days)

FIGURE 2.5

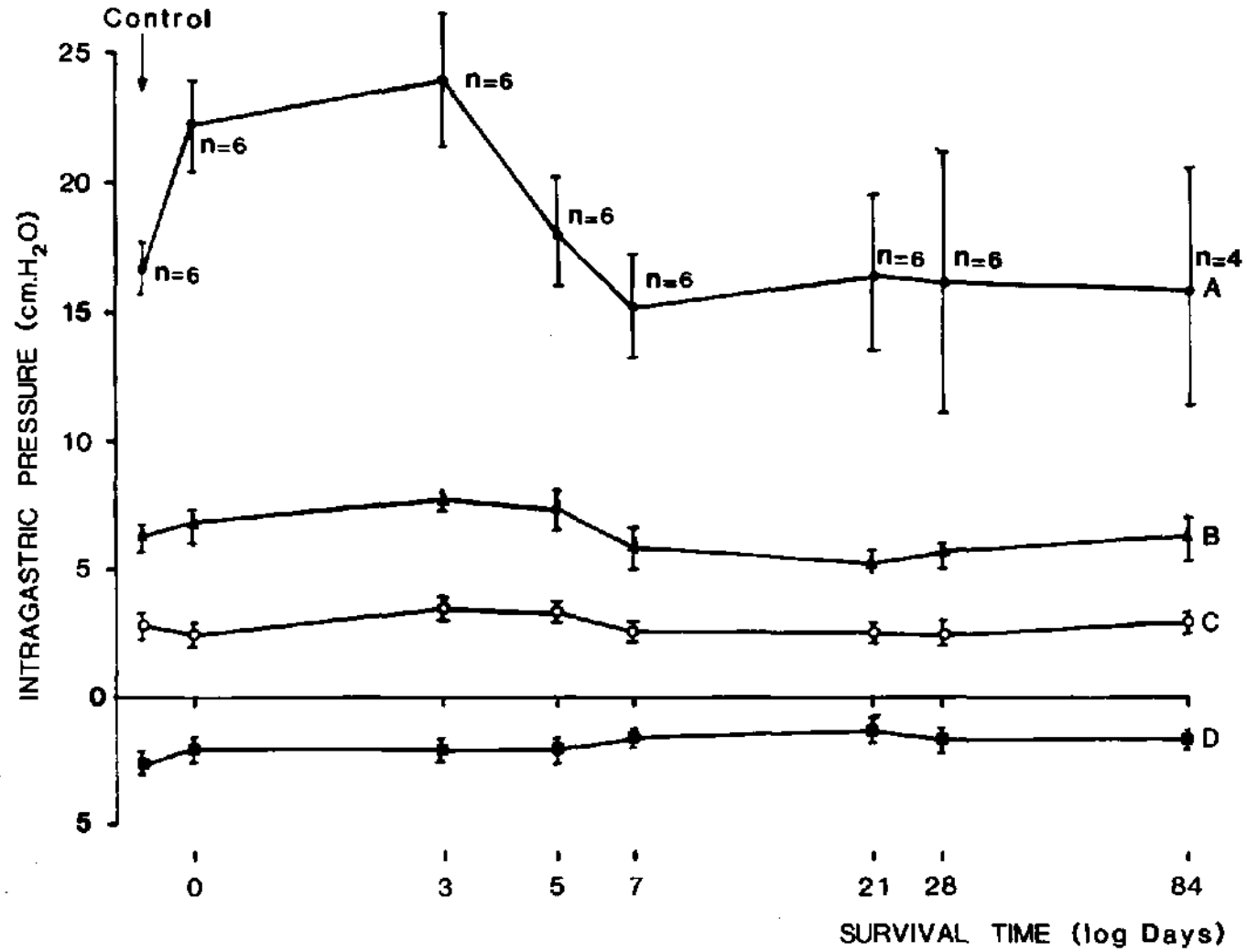

Control
25
20
15
10
5
0
5
INTRAGASTRIC PRESSURE (cm.H2O)
n=6
n=6
n=6
n=6
n=6
n=6
n=6
n=4
A
B
C
D
0
3
5
7
21
28
84
SURVIVAL TIME (log Days)

FIGURE 2.5

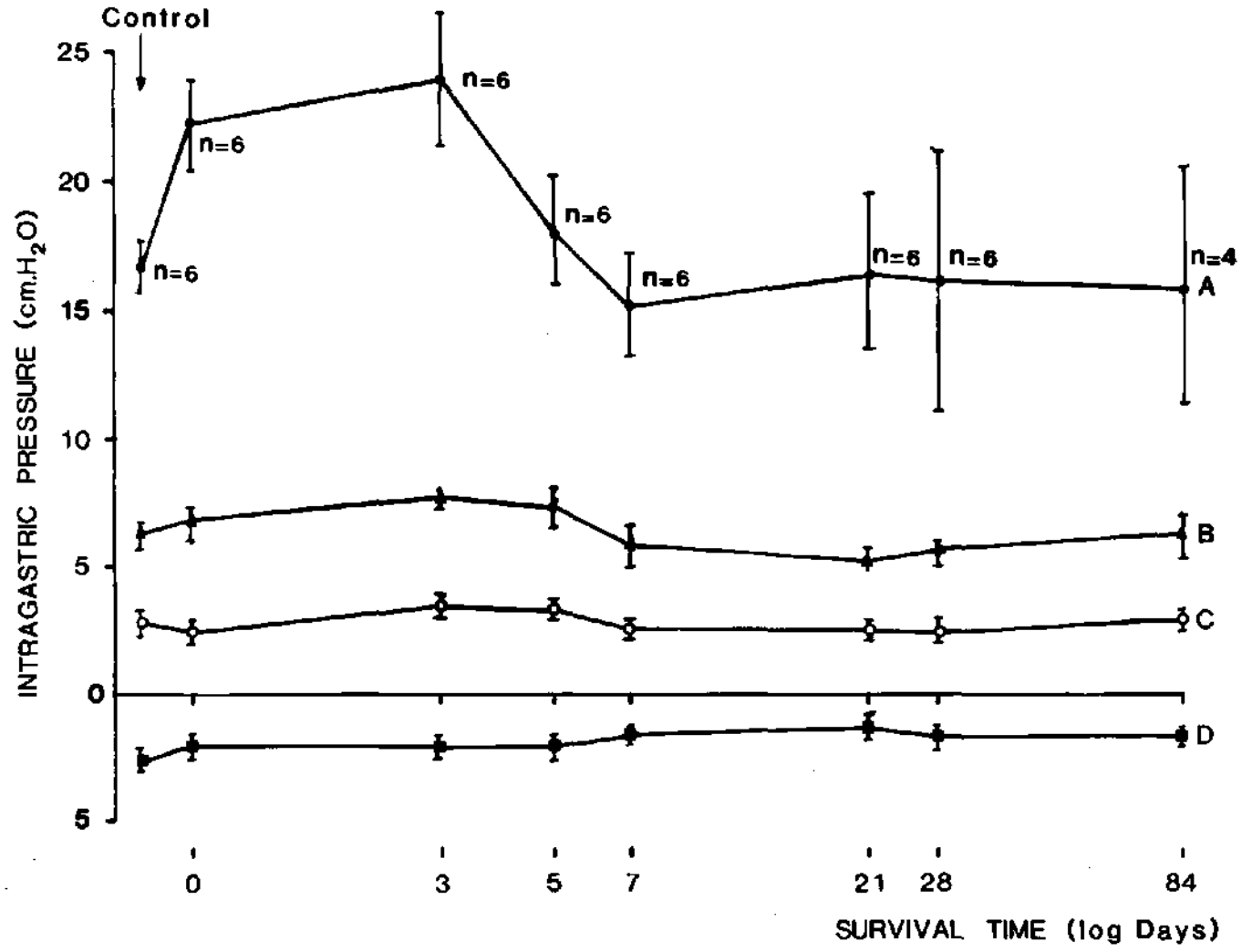

Control
25
20
15
10
5
0
5
INTRAGASTRIC PRESSURE (cm.H2O)
n=6
n=6
n=6
n=6
n=6
n=6
n=6
n=4
A
B
C
D
0
3
5
7
21
28
84
SURVIVAL TIME (log Days)

FIGURE 2.5

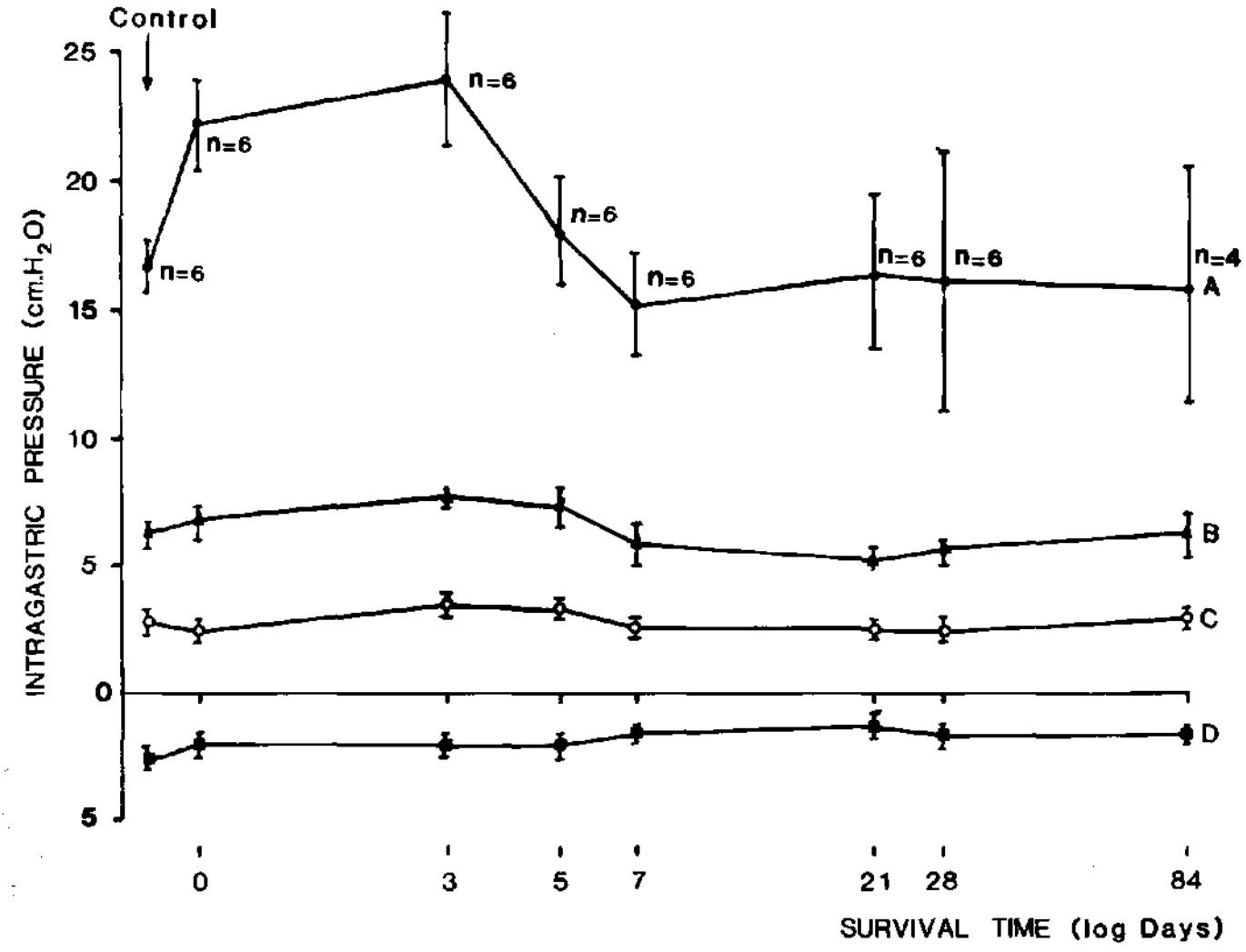

Control
25
20
15
10
5
0
5
INTRAGASTRIC PRESSURE (cm.H2O)
n=6
n=6
n=6
n=6
n=6
n=6
n=6
n=4
A
B
C
D
0
3
5
7
21
28
84
SURVIVAL TIME (log Days)

TABLE 2.6

This shows the intragastric pressure (in cm H_2O) due to step inflation of the stomach with 20 ml normal saline. See figure 2.5A.

TABLE 2.6

Animal Serial Number	Intragastric pressure (in cmH_2O) due to step inflation in different groups of animals							
	Control	Acute	3 days	5 days	7 days	21 days	28 days	84 days
1	19.20	16.60	33.00	14.25	11.50	25.00	8.90	8.80
2	19.00	27.00	21.50	10.00	16.00	12.75	12.75	8.80
3	14.00	18.75	16.25	20.00	11.75	6.40	16.50	28.00
4	13.00	21.25	30.20	22.25	22.25	25.00	9.50	17.90
5	18.00	26.75	21.40	24.00	10.00	14.25	41.00	-
6	16.25	23.00	21.60	18.00	19.40	15.50	8.60	-
Total	99.45	133.35	143.95	108.50	90.90	98.90	97.25	63.50
Mean ±S.E.M	16.58 ±1.07	22.23 ±1.72	23.99 ±2.57	18.08 ±2.13	15.15 ±2.00	16.48 ±2.98	16.21 ±5.11	15.88 ±4.58

TABLE 2.7

Shows the resting intragastric pressure (in cm H_2O) obtained 30 minutes after step inflation with 20 ml normal saline. See Figure 2.5B.

TABLE 2.7

Animal Serial Number	Resting intragastric pressure (in cmH_2O) in different groups of animals							
	Control	Acute	3 days	5 days	7 days	21 days	28 days	84 days
1	5.40	4.40	8.50	7.80	4.80	5.30	5.00	4.60
2	6.80	7.20	6.80	5.30	4.30	6.05	5.55	5.10
3	7.50	8.00	7.80	9.30	5.05	3.30	6.40	7.20
4	6.00	7.75	7.80	7.30	0.05	6.30	3.55	8.00
5	6.75	8.00	7.55	9.30	4.05	5.80	8.05	-
6	4.75	4.75	8.25	5.05	7.80	5.05	5.00	-
Total	37.20	40.10	46.70	44.05	35.05	31.80	33.55	24.90
Mean ±S.E.M	6.20 ±0.41	6.68 ±0.68	7.78 ±0.24	7.34 ±0.76	5.84 ±0.85	5.30 ±0.44	5.59 ±0.62	6.23 ±0.82

(iii) Intragastric Pressure of Empty Stomach

This was the pressure in the empty stomach relative to atmospheric pressure. It did not change either in response to acute vagotomy or with increasing survival time after vagotomy (Table 2.8; Figure 2.5C).

(iv) Relaxation of Gastric Smooth Muscle

After step inflation of the stomach with 20 ml normal saline, the intragastric pressure returned to the resting pressure. Stimulation of the cervical vagus nerve was followed by a rise in intragastric pressure and a fall below the resting level (Table 2.9; Figure 2.4). This relaxation which was plotted against survival times (Figure 2.5D), reduced from the control value (2.7 cm H_2O) to a significantly low level (1.7 cm H_2O; $P < 0.05$) on the seventh day. It remained low throughout the 3 months period of the experiment.

e. Effect of Resting Intragastric Pressure on Gastric Smooth Muscle Relaxation following Chronic Dorsal Trunk Vagotomy

The resting pressure in all 34 animals which had DTV were plotted against corresponding relaxation associated with the first cervical vagus nerve stimulation (Figure 2.6). The amount of relaxation decreased as the resting pressure decreased. For a 1 cm H_2O decrease in resting pressure, the relaxation decreased by about 0.4 cm H_2O (Regression coefficient = 0.40). The tested

TABLE 2.8

Animal Serial Number	Intragastric pressure (in cmH_2O) in empty stomach of different groups of animals							
	Control	Acute	3 days	5 days	7 days	21 days	28 days	84 days
1	2.00	1.40	2.30	3.80	2.05	2.80	3.00	2.60
2	4.00	3.60	2.80	3.30	1.30	2.80	3.30	2.20
3	2.75	2.50	4.80	3.30	2.80	2.60	3.40	3.40
4	2.50	3.00	2.80	3.55	3.55	3.30	1.05	3.80
5	3.00	2.00	4.05	4.30	2.30	2.80	1.80	-
6	1.75	2.00	4.05	2.05	3.60	2.05	2.80	-
Total	16.00	14.50	20.80	20.30	15.60	16.35	15.35	12.00
Mean ±S.E.M	2.67 ±0.33	2.42 ±0.32	3.47 ±0.40	3.38 ±0.31	2.60 ±0.37	2.73 ±0.17	2.56 ±0.43	3.00 ±0.37

TABLE 2.9

Animal Serial Number	Gastric relaxation measured as a depression in intragastric pressure (in cmH_2O) below the resting value in different groups of animals							
	Control	Acute	3 days	5 days	7 days	21 days	28 days	84 days
1	3.00	1.40	3.00	2.25	1.25	1.25	1.20	0.80
2	2.40	1.20	1.40	0.50	1.50	1.25	1.75	1.50
3	3.50	3.00	2.25	2.75	1.25	0.30	2.00	1.90
4	2.75	3.00	2.30	2.00	2.50	2.75	0.60	2.40
5	2.75	2.50	2.30	3.25	1.25	1.00	3.50	-
6	1.50	1.00	1.70	1.75	2.40	1.25	0.80	-
Total	15.90	12.10	12.95	12.50	10.15	7.80	9.85	6.60
Mean ±S.E.M	2.65 ±0.27	2.02 ±0.38	2.16 ±0.23	2.08 ±0.39	1.69 ±0.24	1.30 ±0.33	1.64 ±0.43	1.65 ±0.28

FIGURE 2.6

Scatter diagram showing the relationship between resting intragastric pressure and relaxation after chronic dorsal trunk vagotomy.
The linear regression equation is

$$y = -0.78 + 0.40x$$

Correlation coefficient = 0.84

TABLE 2.6

This shows the intragastric pressure (in cm H_2O) due to step inflation of the stomach with 20 ml normal saline. See figure 2.5A.

TABLE 2.6

Animal Serial Number	Intragastric pressure (in cmH_2O) due to step inflation in different groups of animals							
	Control	Acute	3 days	5 days	7 days	21 days	28 days	84 days
1	19.20	16.60	33.00	14.25	11.50	25.00	8.90	8.80
2	19.00	27.00	21.50	10.00	16.00	12.75	12.75	8.80
3	14.00	18.75	16.25	20.00	11.75	6.40	16.50	28.00
4	13.00	21.25	30.20	22.25	22.25	25.00	9.50	17.90
5	18.00	26.75	21.40	24.00	10.00	14.25	41.00	-
6	16.25	23.00	21.60	18.00	19.40	15.50	8.60	-
Total	99.45	133.35	143.95	108.50	90.90	98.90	97.25	63.50
Mean ±S.E.M	16.58 ±1.07	22.23 ±1.72	23.99 ±2.57	18.08 ±2.13	15.15 ±2.00	16.48 ±2.98	16.21 ±5.11	15.88 ±4.58

TABLE 2.7

Shows the resting intragastric pressure (in cm H_2O) obtained 30 minutes after step inflation with 20 ml normal saline. See Figure 2.5B.

TABLE 2.7

Animal Serial Number	Resting intragastric pressure (in cmH_2O) in different groups of animals							
	Control	Acute	3 days	5 days	7 days	21 days	28 days	84 days
1	5.40	4.40	8.50	7.80	4.80	5.30	5.00	4.60
2	6.80	7.20	6.80	5.30	4.30	6.05	5.55	5.10
3	7.50	8.00	7.80	9.30	5.05	3.30	6.40	7.20
4	6.00	7.75	7.80	7.30	0.05	6.30	3.55	8.00
5	6.75	8.00	7.55	9.30	4.05	5.80	8.05	-
6	4.75	4.75	8.25	5.05	7.80	5.05	5.00	-
Total	37.20	40.10	46.70	44.05	35.05	31.80	33.55	24.90
Mean ±S.E.M	6.20 ±0.41	6.68 ±0.68	7.78 ±0.24	7.34 ±0.76	5.84 ±0.85	5.30 ±0.44	5.59 ±0.62	6.23 ±0.82

(iii) Intragastric Pressure of Empty Stomach

This was the pressure in the empty stomach relative to atmospheric pressure. It did not change either in response to acute vagotomy or with increasing survival time after vagotomy (Table 2.8; Figure 2.5C).

(iv) Relaxation of Gastric Smooth Muscle

After step inflation of the stomach with 20 ml normal saline, the intragastric pressure returned to the resting pressure. Stimulation of the cervical vagus nerve was followed by a rise in intragastric pressure and a fall below the resting level (Table 2.9; Figure 2.4). This relaxation which was plotted against survival times (Figure 2.5D), reduced from the control value (2.7 cm H_2O) to a significantly low level (1.7 cm H_2O; $P < 0.05$) on the seventh day. It remained low throughout the 3 months period of the experiment.

e. Effect of Resting Intragastric Pressure on Gastric Smooth Muscle Relaxation following Chronic Dorsal Trunk Vagotomy

The resting pressure in all 34 animals which had DTV were plotted against corresponding relaxation associated with the first cervical vagus nerve stimulation (Figure 2.6). The amount of relaxation decreased as the resting pressure decreased. For a 1 cm H_2O decrease in resting pressure, the relaxation decreased by about 0.4 cm H_2O (Regression coefficient = 0.40). The tested

TABLE 2.8

Shows the intragastric pressure in the empty stomach of different groups of animals before step inflation with normal saline. See Figure 2.5C.

TABLE 2.8

Animal Serial Number	Intragastric pressure (in cmH_2O) in empty stomach of different groups of animals							
	Control	Acute	3 days	5 days	7 days	21 days	28 days	84 days
1	2.00	1.40	2.30	3.80	2.05	2.80	3.00	2.60
2	4.00	3.60	2.80	3.30	1.30	2.80	3.30	2.20
3	2.75	2.50	4.80	3.30	2.80	2.60	3.40	3.40
4	2.50	3.00	2.80	3.55	3.55	3.30	1.05	3.80
5	3.00	2.00	4.05	4.30	2.30	2.80	1.80	-
6	1.75	2.00	4.05	2.05	3.60	2.05	2.80	-
Total	16.00	14.50	20.80	20.30	15.60	16.35	15.35	12.00
Mean ±S.E.M	2.67 ±0.33	2.42 ±0.32	3.47 ±0.40	3.38 ±0.31	2.60 ±0.37	2.73 ±0.17	2.56 ±0.43	3.00 ±0.37

TABLE 2.9

Shows the gastric relaxation following the first cervical vagal stimulation. This was measured as the reduction in intragastric pressure below the resting value in cm H_2O. See Figure 2.5D.

TABLE 2.9

Animal Serial Number	Gastric relaxation measured as a depression in intragastric pressure (in cmH_2O) below the resting value in different groups of animals							
	Control	Acute	3 days	5 days	7 days	21 days	28 days	84 days
1	3.00	1.40	3.00	2.25	1.25	1.25	1.20	0.80
2	2.40	1.20	1.40	0.50	1.50	1.25	1.75	1.50
3	3.50	3.00	2.25	2.75	1.25	0.30	2.00	1.90
4	2.75	3.00	2.30	2.00	2.50	2.75	0.60	2.40
5	2.75	2.50	2.30	3.25	1.25	1.00	3.50	-
6	1.50	1.00	1.70	1.75	2.40	1.25	0.80	-
Total	15.90	12.10	12.95	12.50	10.15	7.80	9.85	6.60
Mean ±S.E.M	2.65 ±0.27	2.02 ±0.38	2.16 ±0.23	2.08 ±0.39	1.69 ±0.24	1.30 ±0.33	1.64 ±0.43	1.65 ±0.28

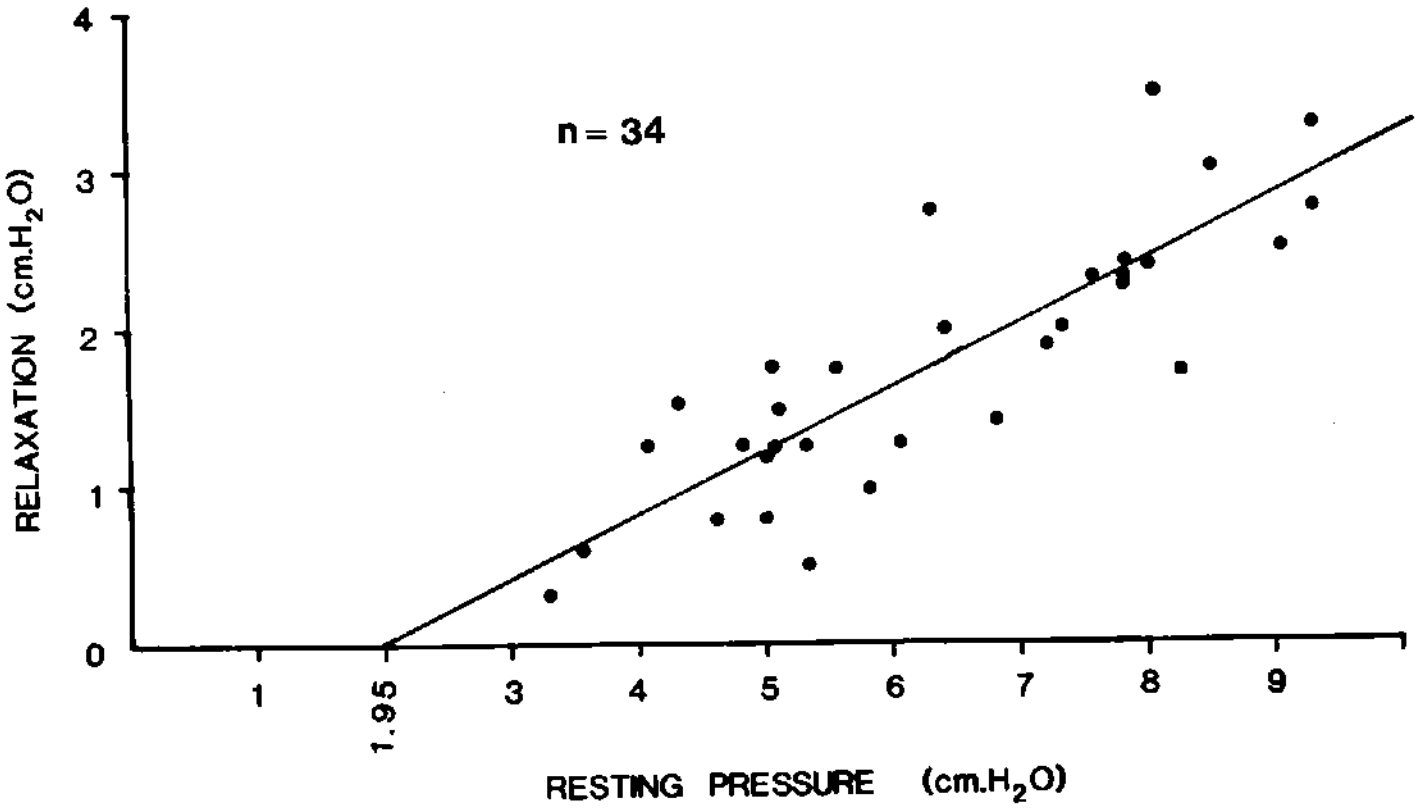
FIGURE 2.6

Scatter diagram showing the relationship between resting intragastric pressure and relaxation after chronic dorsal trunk vagotomy.
The linear regression equation is

$$y = -0.78 + 0.40x$$

Correlation coefficient = 0.84

FIGURE 2.6


n = 34
RELAXATION (cm.H2O)
0
1
2
3
4
1
1.95
3
4
5
6
7
8
9
RESTING PRESSURE (cm.H2O)

correlation between resting pressure and relaxation was highly significant ($P < 0.01$) with a correlation coefficient of 0.84.

f. Effect of Resting Intragastric Pressure on Vagally Evoked Intragastric Pressure

It was shown in section c above that intragastric pressure responses to separate left and right cervical vagus nerve stimulations were not significantly different from each other. Therefore in plotting the scatter diagram in Figure 2.7, the intragastric pressure response to left and right cervical vagus nerve stimulations were used against paired resting pressures for each group of animals so as to obtain a larger number of plotted points. The figure shows that intragastric pressure response to cervical vagus nerve stimulation increased as resting pressure decreased (Regression coefficient = -2.57). The inverse relation of the two parameters was significant at the 1 percent level (Correlation coefficient = -0.83).

g. Volume of Fluid in the Stomach before and after Nerve Stimulation

Twenty millilitres of normal saline were injected into the stomach before vagal stimulation. At the end of the experiment, the fluid volume increased by an average of 5 percent.

h. Body and Stomach Weights of Ferrets after Chronic Vagotomy

The body weights were taken just before the animals were anaesthetised for the nerve stimulation experiments. There was no relationship between the two weights.

FIGURE 2.7

Scatter diagram showing the inverse relationship between resting intragastric pressure and vagally evoked intragastric pressure after chronic dorsal trunk vagotomy. Mean group values of intragastric pressure following separate left and right cervical vagal stimulations were plotted against paired mean group resting intragastric pressures.

Regression equation is $y = 35.40 - 2.57x$

Correlation coefficient = 0.83 and is significant at the 0.1 percent level.

FIGURE 2.7

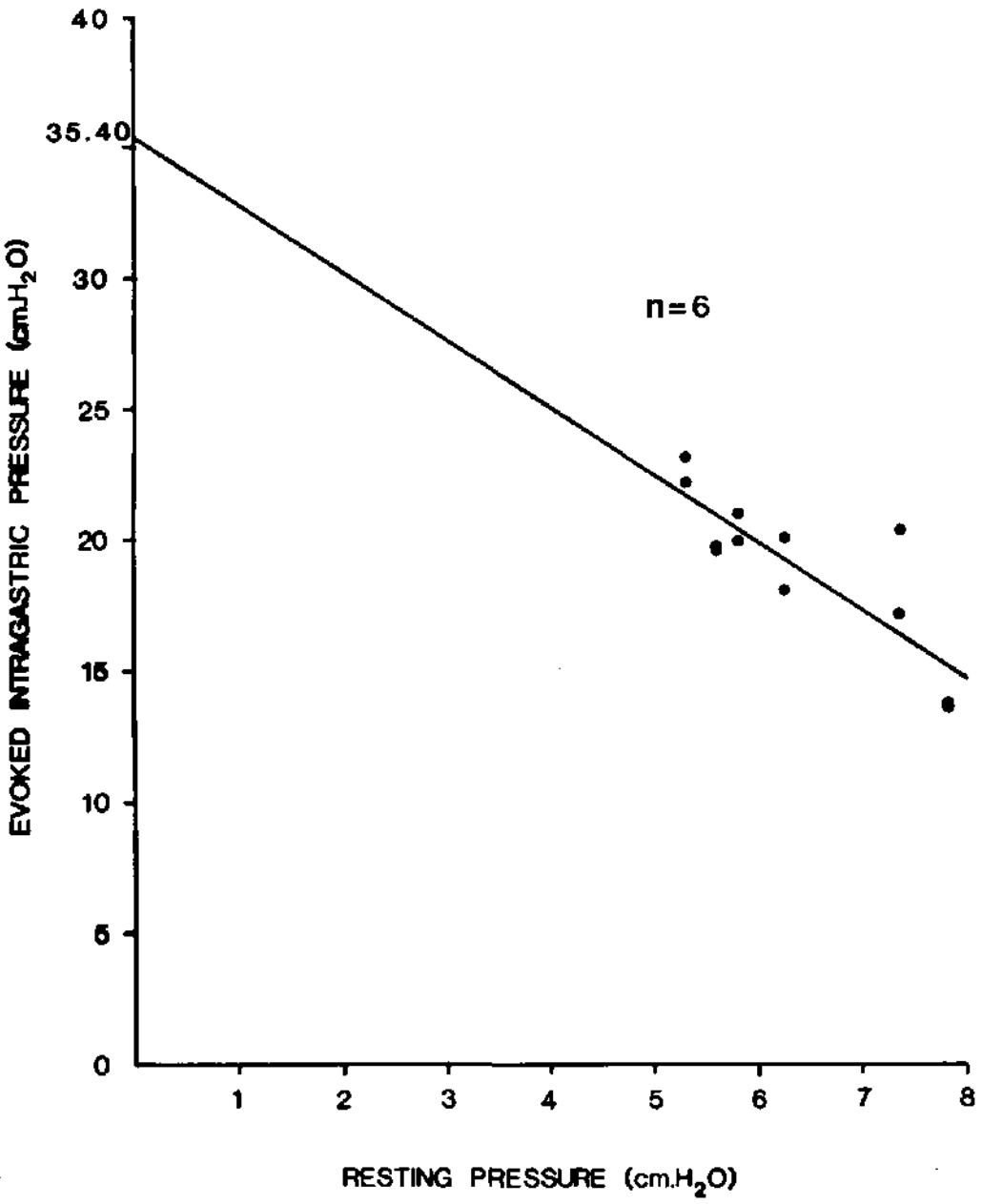
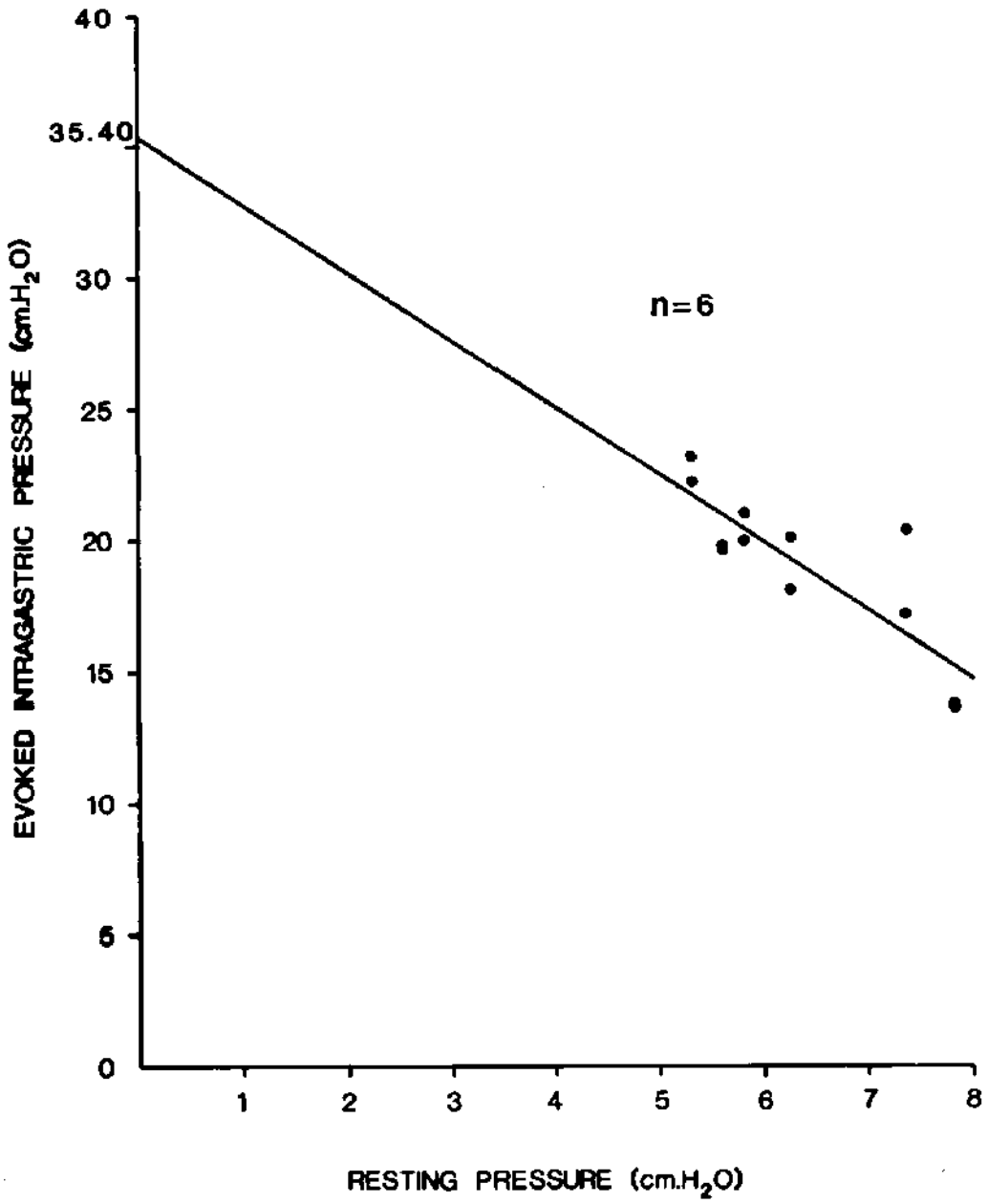

40
35.40
30
25
20
15
10
5
0
n=6
EVOKED INTRAGASTRIC PRESSURE (cm.H2O)
1
2
3
4
5
6
7
8
RESTING PRESSURE (cm.H2O)

FIGURE 2.7


40
35.40
30
25
20
15
10
5
0
n=6
1
2
3
4
5
6
7
8
EVOKED INTRAGASTRIC PRESSURE (cm.H2O)
RESTING PRESSURE (cm.H2O)

DISCUSSION

One important short-coming of nerve stimulation techniques is that the nerves are being stimulated in a completely different way from the physiological stimulation that normally takes place in the animal. For example, as outlined in the introductory chapter, the vagus nerve responds to central and peripheral (gastric) stimuli and stimulates the gastric smooth muscle to undergo organised motility patterns which in turn regulate movements that are involved in gastric digestion and emptying. This physiological stimulation is understandably different from that resulting from placing the vagus nerve trunk on an electrode and stimulating with electric waves. In spite of this difference, electric stimulation of neurons is widely used to obtain information on certain aspects of the physiological behaviour of the target organ in response to stimuli arising in the neurons that innervate it. In so far as the stimulation parameters are adjusted to produce a response that is reproduceable, and as long as pathological effects are not produced in the target organ or in the mammalian subject, the response observed in the target organ can give an insight into the normal behaviour of the animal in response to nerve stimulation within the limits of the experimental condition. In the present experiments, the parameters used produced a single gastric smooth muscle contraction of maximal amplitude. Stimulation at these

parameters did not cause pathological changes in the vagus nerve or the gastric smooth muscle as demonstrated by the fact that the intragastric pressures returned to pre-stimulation levels after only three minutes. Moreover, subsequent stimulations of the same nerve trunk with the same parameters produced intragastric pressures of similar amplitude. Stimulating at a supra-maximal voltage (20 volts in these experiments) has the advantage that all vagal fibres were being stimulated in all animals so that intragastric pressure response to these stimulations in all animals were reproduceable and comparable to one another. Reproducibility was also enhanced by the fact that the cervical vagus nerves were transected and the distal portions stimulated. In this way, central influences were eliminated.

It is expected that intragastric pressure will vary with the volume of saline injected into stomachs of varying sizes. A large stomach containing 20 ml saline will have a lower baseline intragastric pressure than a smaller stomach with an equal volume of saline in it. Unfortunately, there is no direct relationship between animal weight and stomach weight in post-vagotomy animals. Therefore fluid volume cannot be adjusted to stomach weight in order to obtain a constant baseline intragastric pressure for all animals. In order to minimise the effect of possible variations in baseline intragastric pressure on evoked intragastric pressure responses, the animals were distributed into the different groups so that

each group contained a cross-section of all weights. Moreover, 50 ml of fluid is within the physiological range of the ferret (Andrews, Grundy and Scratcherd, 1980), so that the presence of 20 ml of fluid should not cause serious changes in gastric resting tone due to muscle fibres stretching, even in small stomachs of adult ferrets.

The vagus nerve trunks were stimulated in the neck. Andrews, Lawes and Bower (1980) observed that animals with cervical vagotomy displayed ipsilateral ptosis indicating the intimate relationship between the vagus and the cervical sympathetic nerve trunks in the ferret. Therefore, apart from stimulating vagal branches to thoracic organs, the sympathetic nerves to these organs were probably being stimulated as well. This could affect the whole physiological state of the animals. During cervical vagal stimulation, the heart rate slowed but returned to normal after the stimulation. This indicated that vagal, rather than sympathetic, stimulation was predominant. Muren (1957), suspecting that gastric motor responses to vagal stimulation in the neck could be due to the pronounced fall in blood pressure, transected both vagus nerve trunks above the diaphragm. After this procedure, the fall in blood pressure was not accompanied by gastric responses to cervical vagal stimulation in the dog. It may therefore be claimed that the general effects per se, produced by stimulating vagal branches to thoracic organs, cannot be wholly responsible for the increase in intragastric pressure response to cervical vagal stimulation in the ferret.

Changes in Intragastric Pressure following Chronic Abdominal Vagotomy

One, three and four weeks after ventral trunk vagotomy, intragastric pressure response to cervical vagal stimulation was not significantly different from control or acute values. This is in agreement with Andrews, Lawes and Bower (1980) who also found that two weeks after VTV, the intragastric pressure response to cervical vagal stimulation was not different from control and acute values in the ferret. It is reasonable to ask what is the function of ventral vagal trunk and how much control does it have over gastric function. If the total number of vagal efferent fibres in the ventral trunk reaching the intramural plexus is smaller than the number of efferent fibres from the dorsal trunk, sectioning these fewer fibres in the ventral trunk will be expected to produce more limited changes in intragastric pressure response to cervical vagal stimulation immediately after vagotomy. Whatever changes take place in the vagal pathway for gastric innervation which may be responsible for the increase in intragastric pressure above acute value will be expected to be minimal after chronic VTV since the number of denervated postganglionic neurons will probably be few. Therefore, the recovery that will follow these changes may also be expected to be minimal. But because of the complexity of neural connections and neurotransmission in the intrinsic nervous system, the precise degree of the influence of the preganglionic efferent fibres of the ventral trunk in the myenteric plexus is difficult to determine.

Nevertheless, the fact that VTV had so little effect on intragastric pressure response to cervical vagal stimulation will suggest that this influence may be small.

Three months after VTV, intragastric pressure response to cervical vagal stimulation increased significantly above acute values. Two factors may be involved in this finding. Firstly, the changes which may take place soon after vagotomy, either in the nerve or in the target organ, and which are responsible for the slight increase in evoked intragastric pressure after seven days, may also be at work in the third month. Secondly, the proximal portion of the transected nerve was seen to have regenerated normal axons which by-passed the ligatures to reach the stomach three months after VTV. A proportion of these regenerated axons might have made functioning synaptic contacts with intramural neurons. These two factors acting together may be responsible for the marked increase in evoked intragastric pressure demonstrated three months after VTV.

Acute DTV resulted in a significant loss in evoked intragastric pressure response to cervical vagal stimulation. After chronic DTV, intragastric pressure response to cervical vagal stimulation increased to normal value by the fifth day in the ferret. This value was maintained throughout the three months period of the experiment, thus agreeing with the return to normal value observed after two weeks by Andrews, Lawes and Bower (1980).

A study of intragastric pressure changes in the ferret

following stimulation of the cervical vagus nerves is an indirect method of studying the motor functions of the vagus nerve in relation to the stomach. Although studies in which intragastric pressure is similarly used are scanty in the literature, the function of the vagus nerve has been studied using various other parameters. For example studies of gross electric activity of gastric smooth muscle (Kelly and Code, 1969), changes in the inter-digestive myoelectric complex (Ruckebusch and Bueno, 1977) and gastric emptying time (Wilbur and Kelly, 1973) have been used. Vagal secretory function has also been studied by measuring gastric acid output after vagal stimulation (Basso et al., 1971; Andrews and Bower, 1977; Loup et al., 1980) or following administration of secretagogues (Joffe and Bapat, 1979). But vagal function is so intimately related to gastric function that the two cannot be divorced one from the other. Therefore, for the purpose of this discussion, vagal and gastric functions are used interchangably.

Since the latter part of the last century, it has been known, at least in the peripheral nervous system, that denervated organs lose their functions only temporarily and after periods that vary from one animal to the other and from one nerve-organ system to the other within the same animal, the denervated organs begin to recover their functions. Recent experiments have shown that recovery after lesions of the central nervous system is also possible (Stein et al., 1974; Finger, 1978) and in fact does take

place. These studies in the CNS have led to the development of the theory of "Neuronal plasticity" whereby a single neuron or a group of neurons is capable of remodelling, both functionally and structurally, in response to denervation of the target organ with a return of function, or in response to certain stimuli in the environment of a developing nerve-organ system. The extent to which neuronal plasticity takes place in the parasympathetic division of the autonomic nervous system is still a subject of continuing research. Far more studies have been done on the sympathetic division where, for example, re-innervation of a previously denervated superior cervical ganglion of the cat and rabbit has been demonstrated (Butson, 1950; Raisman et al., 1974).

In the clinical realm, selective vagotomy and proximal gastric vagotomy are now being supplemented with either Burge's or Grassi's intra-operative tests in order to test the completeness of the denervation of the parietal cells (Burge and Frohn, 1969; Grassi, 1975). In some cases where such tests have confirmed the completeness of parietal cell denervation, recurrent ulcerations have been seen several months or years later (Wastell and Zervos, 1976; Storey et al., 1981). The usual explanation given in these cases is that the vagotomy had inadvertently been incomplete, but no satisfactory explanations have been given as to why an incomplete vagotomy that gave a negative response to intra-operative test gradually gave rise to recurrent ulceration.

A number of theories have now been set forth to explain the functional recovery that has been so consistently noticed after incomplete peripheral (and central) neurectomy. These include

(a) hypersensitivity.

(b) nerve sprouting and regeneration,

(c) unmasking of synapses,

(d) increased circulating neurotransmitter,

(e) target organ hypertrophy.

(a) Hypersensitivity

It has been recognised for a long time that when the nerve supply to a structure, such as a muscle, is transected, the immediate response is that of loss of activity in the muscle. But a few days later (usually within a week), the muscle becomes excessively responsive to the action of the neurotransmitter whether this be excitatory or inhibitory to the organ in question (Guth, 1956). The exact mechanism for this hypersensitivity (supersensitivity) has not been fully worked out but any of the following either singularly or collectively, could contribute to it:-

(i) increase in the number of receptor sites upon which the neurotransmitter of the transected nerve acts,

(ii) widespread distribution of the receptors over the denervated muscle, contrary to the localised distribution (at synaptic sites) that characterises the normal muscle,

(iii) because of the pathology at the endings of transected nerves, there may be a defect in transmitter re-uptake mechanism. This will lead to hypersensitivity to normal levels of neurotransmitter.

The phenomenon of hypersensitivity has been observed not only in adrenergically innervated organs but in cholinergically innervated organs as well (Hillarp, 1960). Smooth muscles are most irritable when deprived of their postganglionic innervation and somewhat less hypersensitive if deprived only of their preganglionic innervation (Noback and Demarest, 1981). In the present experiments, preganglionic vagotomy was done, thus denervating postganglionic neurons. It would therefore be expected that the hypersensitivity of gastric smooth muscle would not be as profound as it would have been if the postganglionic fibres were transected. But if hypersensitivity lasts for only about two to four weeks before it starts to decline (Murray and Thompson, 1957; Muren, 1959), then it is unknown, within the limits of this experiment, how much of it could have contributed to the functional recovery in the third month.

(b) Nerve Sprouting

There is some confusion in the literature as to what is sprouting and what is regeneration. For example, some authors have referred to the growth from the tip of the proximal segment of a transected nerve as regeneration

(Guth, 1956), while other forms of nerve growth are called sprouting. But for the purpose of this discussion, the classification by Duce and Keen (1980) as given below will be used.

Sprouting is used to cover all mechanisms by which a denervated organ is re-innervated whether or not the growing nerve fibres pass via the original neurilemmal path to reach the denervated tissue. When an organ is partially denervated, the adjacent nerves that survive project some fibres to the denervated area to compensate for the neuronal loss. This has been referred to as "reactive" sprouting (Figure 2.8). This type of sprouting may arise as a collateral branch high up along the nerve ("collateral reactive" sprouting), or it may arise from the terminal branches of the nerve ("terminal reactive" sprouting). The proximal part of a transected nerve may also react to the neuronal loss by sprouting. This is called "regenerative" sprouting. When the sprout arises high up along the nerve, it is a "regenerative collateral" sprout whereas when it arises from the region of the cut it is a "regenerative terminal" sprout.

These terminal sprouts may by-pass the ligatures, when these are present, to reach the denervated organ (Loup et al., 1980). In myelinated axons, these sprouts arise from nodes of Ranvier (Keynes et al., 1983) just proximal to the cut or ligature. In the absence of ligatures, particularly when the gap between the cut ends of the nerve is small, the regenerative sprouts may arise as growth cones or filopodia

FIGURE 2.8

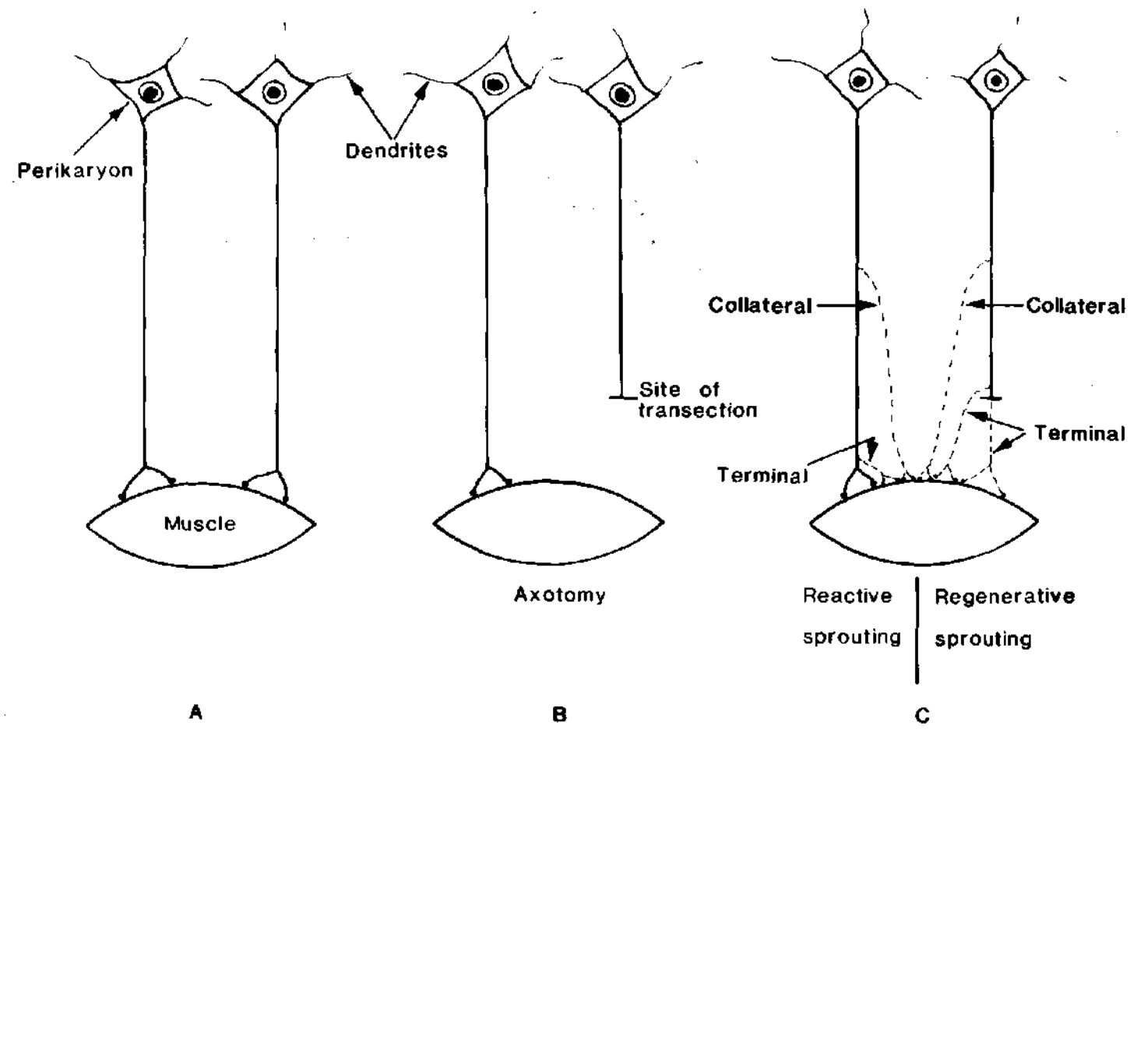
Simplified illustration of classification of nerve sprouting.

A - Normal dual (or multiple) innervation of the target organ (muscle)

B - Partial nerve transection

C - Types of sprouting that may take place in response to partial denervation

FIGURE 2.8


Perikaryon
Dendrites
Muscle
Site of transection
Axotomy
Collateral
Collateral
Terminal
Terminal
Reactive sprouting
Regenerative sprouting
A
B
C

from the tip of the proximal segment of the nerve, and grow through the original neurilemmal path to reach the denervated organ. Another variety of sprouting which has been observed in the detrusor muscle of the cat and human bladder arises from intramural ganglion cells (Dahlström, 1978). Although nerve sprouts have been shown to actually make synaptic contacts with neurons in the target organ, not much is known yet about their functional consequences. In some instances, there is evidence from electrophysiological studies that the newly formed synapses are indeed functioning (Lynch et al., 1973; Steward et al., 1974; Murakami et al., 1976). One factor that may affect the functional status of reactive sprouts is the possible acquisition of a transmitter that is different from that of the degenerated terminals. Sprouts have been found, particularly in the autonomic nervous system, which belong to fibres equipped with a different transmitter from the terminals of the degenerated fibres (Moore et al., 1971).

If indeed axonal sprouting takes place in the vagus nerve subsequent upon partial denervation of the stomach, this phenomenon might have been contributory to the increase in intragastric pressure recorded in response to cervical vagal stimulation in the ferret. How soon after partial denervation the sprouts make synaptic contact with denervated intramural neurons and become

functional, will depend on how soon degenerating vagus nerve terminals disappear. But delays of up to 20 days in the rabbit and 13 to 16 weeks in man have been demonstrated between the formation of the sprouts and the time they become functional (Duncan, 1934).

In the series of animals in which VTV was done for three months, regenerative sprouts were found by-passing the ligatures to reach the partially denervated gastric wall. In these animals, intragastric pressure response to cervical vagal stimulation was significantly greater than the acute value. The regenerative sprouts might have made functioning synaptic contacts with intrinsic neurons, thus increasing gastric response to cervical vagal stimulation. Such regenerative sprouts were not seen in the animals in which DTV was done for three months. This could be because the proximal portion of the vagus nerve retracted a long way from the stomach. The effect of this could be that because of this gap, the sprouts needed a longer time to reach the stomach. It could also be that the "sprouting factor" which is supposed to be released from denervated cells was, in this instance, not having sufficient impact on the proximal nerve segment because of the distance of the latter from denervated cells of the gastric wall.

The factors that are responsible for neuronal sprouting are still a subject of intensive research, but they may include the following.

(i) "Sprouting factor" released by degenerating target cells stimulates surviving axons to sprout (Brown et al., 1981).

(ii) Destruction, during degeneration, of normally occurring factors that suppress the tendency to sprout which normally exists in neurons (Diamond, 1979).

(iii) Nerve growth factor (NGF), a protein substance with a property of enhancing growth and differentiation in sensory and sympathetic nerve cells. It is a normal constituent of the embryonic sensory and the embryonic and mature sympathetic nerve cells, and it is also present in many organs and tissue extracts. It is found in trace amounts in the serum of all mammals including man (Cohen and Levi-Montalcini, 1956). Its action has not yet been demonstrated in parasympathetic nerve cells.

(c) Unmasking of Synapses

This theory says that there are some synapses which are normally dormant or ineffective but which may be activated to function as a result of anatomical and/or functional loss of adjacent synapses, for example following nerve section or nerve block (Wall, 1977). Unlike sprouting which presupposes morphological changes in neural connectivity and synaptic patterns and correlates these with functional changes, overt structural alterations are

not required for functional recovery to be obtained by way of unmasking of synapses. This phenomenon has been demonstrated in physiological experiments only. It is difficult to demonstrate such normally inactive fibres anatomically. It appears from present-day knowledge that whereas the main cell groups and major fibre connections in the nervous system are present at birth, the finer and more detailed patterns develop largely postnatally. It is these local neuronal circuits (Schmitt et al., 1976), which can be influenced environmentally by proper use and stimulation (partial denervation in this case). Berman and Sterling (1976) have shown an immediate unmasking of the effects of certain optic nerve terminals in the cat superior colliculus which are held inhibited by a drive from the occipital cortex. Although this phenomenon has been studied in the brain and spinal cord afferent systems, unmasking of ineffective synapses in the myenteric plexus is considered to be one of the possible explanations for the functional recovery that has been demonstrated in the vagus nerve. Whether or not this indeed takes place is an open question.

(d) Changes in the Level of Neurotransmitter

It has been postulated that in response to denervation, the surviving neurons may release more of the neurotransmitter (acetylcholine) as a compensatory mechanism (Schmid et al., 1979).

(e) Muscle hypertrophy has also been suggested as a possible mechanism for the recovery of gastric motility. Even in the absence of nerve growth, hypertrophic muscle will contract more to compensate for the effect of neuronal loss.

Relative Effects of Left and Right Cervical Vagal Stimulations

It was observed that stimulation of the left and right cervical vagus nerves separately and together gave rise to intragastric pressures that were not significantly different from one another. Andrews, Lawes and Bower (1980) thought that this meant that the two vagal trunks completely overlapped each other functionally. But recent study by I. N. C. Lawes (personal communication) revealed that if the nerves are stimulated with graded voltages beginning with low values and increasing gradually to supramaximal values, the intragastric pressure response to simultaneous left and right cervical vagal stimulation shows that the two nerves potentiate one another. Therefore the previously claimed total functional overlap was purely due to the fact that the nerves were being stimulated supramaximally. But this does not explain why the amplitude of intragastric pressure after separate left and right cervical vagal stimulations are of similar value.

Effect of Partial Vagotomy on Intragastric Pressure Modalities

The ferret stomach maintains a certain amount of pressure in its cavity relative to the atmospheric pressure.

Under Urethane aneasthesia, this pressure was a constant value, for each animal, for as long as the stomach remained empty. Even when spontaneous contractions occurred, the intragastric pressure always returned to that constant base-line value. The level of this intragastric pressure was not changed by acute partial vagotomy. Irrespective of the survival period after vagotomy, this level of intra-gastric pressure in the empty stomach remained unchanged. It would appear that the unstimulated gastric smooth muscle has a low tonus which is unaffected by partial vagotomy. But as soon as the gastric wall is stimulated either by distension or by extrinsic vagal stimulation, the character-istics of the muscle tone change and become influenced, to some extent, by events related to acute vagotomy and the duration of chronic vagotomy. This is evidenced by the time course of intragastric pressure response to step infla-tion with normal saline (Figure 2.5A). Distension of the stomach wall by injecting water into its cavity stimulates tension receptors in the corpus (Andrews and Scratcherd, 1980). This reflex is channelled via the vagal cholin-ergic excitatory, vagal non-cholinergic, non-adrenergic inhibitory fibres as well as splanchnic nerves (Andrews, Grundy and Lawes, 1980). Immediately after partial vagotomy, intragastric pressure response to step inflation begins to increase and reaches its maximal amplitude by the third day. It then decreases to the normal value by the fifth day. It will therefore appear that with

increasing time after vagotomy, either the tension receptors become less excitable (decreasing hypersensitivity), or the interplay of vagal excitatory and inhibitory as well as splanchnic pathways for the control of gastric motility results in a net decrease in response to step inflation.

The relationship between resting intragastric pressure and relaxation is represented in Figure 2.6 and shows that the two decrease or increase together. The present experiment also shows that although resting intragastric pressure temporarily increased up to 3 days after vagotomy, this pressure and smooth muscle relaxation reduced to control values 5 and 7 days respectively after vagotomy (Figure 2.5). Decreasing resting pressure facilitates excitatory response of gastric smooth muscle to vagal stimulation. This is consistent with the finding that if the tone in a smooth muscle is high, stimulating it produces relatively small contraction and vice versa. For example, if the stomach is distended and then stimulated via the vagus nerves, inhibitory relaxation seems to play a more prominent role and the amplitude of intragastric pressure is reduced (Andrews and Lawes, 1982). It has been shown in the present experiment (Figure 2.7) that intragastric pressure response to vagal stimulation increases as resting pressure decreases. Therefore one must ask if the vagal functional recovery demonstrated is completely due to diminishing resting pressure. This is unlikely to be the only causative factor because by the third day after vagotomy when

resting intragastric pressure was at its peak, evoked intragastric pressure was already on the increase although this increase had not reached statistically significant levels. It would therefore appear that other factors may be at play in addition to diminished resting intragastric pressure.

Twenty millilitres of normal saline is within the physiological limits of the ferret stomach (Andrews, Grundy and Scratcherd, 1980). The increase in intragastric fluid at the end of stimulation experiment was about 5 percent (1 ml). The total volume is still well within the physiological limits of the stomach and is unlikely to unduly influence gastric tone and intragastric pressure.

CHAPTER THREE

AUTORADIOGRAPHIC STUDY OF GASTRIC VAGAL INNERVATION

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INTRODUCTION

a. Development of Autoradiographic Technique

Although the first autoradiograph was obtained in 1867 by Niepce de St. Victor who noticed a blackening produced on emulsions of silver chloride and iodide by uranium nitrate and tartrate, and although Henri Becquerel (1896) repeated and extended Niepce's observations, a correct interpretation of the phenomenon was not forthcoming. It was not until 1898 that radioactivity was first demonstrated. Therefore autoradiography is in fact older than the knowledge of radioactivity itself, and contributed directly to its discovery. In 1924, Lacassagne and Lattès began to use this response of photographic emulsions to ionising radiations in a scientific study of the distribution of Polonium in biological specimens.

After the Second World War, nuclear emulsion - a photographic emulsion with special characteristics, which recorded the tracks of charged particles with greater precision and sensitivity - was discovered. Initially, the sectioned specimen was placed in direct contact with a lantern plate (Hamilton, Soley and Eichorn, 1940; Leblond, 1943). Later liquid emulsion was painted on

the section with paintbrush (Bélanger and Leblond, 1946), but in 1955, Joftes and Warren described dipping slides in molten nuclear emulsion, a technique which is the basis for present-day liquid emulsion methods which have been used in the experiments described here. In contrast to the early days when only a few biologically related radioactive elements were available, an increasingly wide range of compounds is now available, labelled with a radioactive isotope, thus opening up new possibilities in the study of living systems.

Three main techniques are available for the detection of radioactivity. The first is a group of electrical methods in which the ionisation caused by the passage of a particle or X-ray through the sensitive volume of a counter is recorded as an electrical pulse. The geiger tube, the ionisation chamber, and the gas-flow counter are all examples of this approach. The second group relies on the property possessed by a number of materials which enable them to absorb energy from the incident radiation and re-emit this in the form of visible light. These minute flashes of light in a scintillation counter, are converted into electrical pulses which are amplified and registered. The main disadvantage of these two techniques is that they do not discriminate between areas of the tissue which may have different concentrations of radioactivity.

Autoradiography, which is the third technique for

detecting radioactivity, differs from the pulse counting techniques in several important respects. Each crystal of silver halide in the photographic emulsion is an independent detector, isolated from the rest of the emulsion by its capsule of gelatin (Bélanger and Lebland, 1946). Each crystal can respond to the passage through it of a charged particle, with the formation of a latent image that persists throughout the counting or exposure period, and is made permanent by the process of development. The record provided by the nuclear emulsion is cumulative and spacially accurate. By responding in this strictly localised fashion to incident charged particles a nuclear emulsion is ideally suited to studies of the distribution of radioactivity within a sample, a function that the pulse counters cannot perform. But while the emulsion can and does respond in a quantitative fashion to radiation, it is often a slow and difficult process measuring the overall activity of a sample in this way, by comparison to the speed and simplicity of the pulse counters.

b. Axonal Transport

In 1850, Waller discovered that if an axon was interrupted, the portion distal to the lesion degenerated. Cajal (1928) put forward a concept that the soma of a neuron constituted its trophic centre for the maintenance of its various processes. But it was not until 1948 that the explanation for distal degeneration was convincingly given by Weiss and Hiscoe. They demonstrated

a continuous proximo-distal flow of axoplasm down the individual nerve fibres, and introduced the term "axonal flow" for this phenomenon. One of the first definitive experiments using axonal transport for tracing connections in the nervous system was carried out by injecting a small amount of tritium-labelled amino acid (|^{3}H|Leucine) into the vitreous body of a series of mice, and subsequently following the movement of the labelled protein autoradiographically (Taylor and Weiss, 1965). In this way, they clearly demonstrated the central projection of the retinal ganglion cells and showed the practicability of this approach for neuroanatomical studies. Droz and Leblond (1963) had earlier shown that when rats were given intraperitoneal injections of |^{3}H|Leucine, the label accumulated first in the region of the neuronal perikarya and then at successively later periods after the injection, the label could be traced out into the axons and dendrites. It has now been established that the label is preferentially taken up by the neuron cell body and incorporated into the protein synthesis system of the cell (Cowan and Cuénod, 1975). By way of axoplasmic flow, the label is transported to the axon and dendritic synaptic terminals (Hendrickson, 1969; Schonbach et al., 1971). The implication of this is that radioactive - labelled materials taken up by the neuron cell body can only be transported in the anterograde direction. Although some labelled amino acids may be taken up by the axon, they are not actively

transported over any substantial distance in the axon since in the absence of ribosomes, messenger ribonucleic acid (mRNA) and other essential perikaryal components, the uptake of the amino acid is not followed by protein synthesis (Heuser and Miledi, 1970).

c. Axon Transport Systems

There are two, and perhaps three, axon transport systems classified on the basis of the rate of transport of axoplasmic contents to the axon terminal (Grafstein, 1975).

(i) Slow Phase

The materials involved in this phase are transported at rates between 0.5 and 5 mm/day. The identified constituents of this slow component include microtubule protein (Grafstein, 1978), soluble enzymes (Karlsson and Sjöstrand, 1971b, Di Giamberardino et al., 1973) and mitochondria (Hendrickson, 1972; Droz et al., 1973; Di Giamberardino et al., 1973).

(ii) Fast Phase

The fast phase of axon transport proceeds at the rate of 400 mm per day in mammalian vagus nerves (Hendrickson and Cowan, 1971; Karlsson and Sjöstrand, 1971a; Grafstein, 1978). The constituents of this fast component include glycoproteins (Forman et al., 1972; Bennett et al., 1973), glycolipids (Forman et al., 1972; Rösner et al., 1973), mitochondria (Kirkpatrick

et al., 1972), amino acids (Csanyi et al., 1973), amino sugars (Forman et al., 1971), synaptic transmitter vesicles and neuro-secretory granules (Grafstein, 1978).

(iii) Intermediate Phase

It seems evident from studies done by Lasek (1970) Schonbach and Cuénod (1971) and Willard et al., (1974) that there are one or two components of axon transport that are of intermediate rates, but their characteristics have still not been clearly defined (Grafstein, 1978).

It has been established that the fast component consists largely of particulate materials, whereas a large proportion of the slow component (perhaps as much as 50%) consists of soluble protein (McEwen and Grafstein, 1968). It has also been shown that materials carried by the fast transport are predominantly (though not exclusively) destined for the nerve terminals whereas the materials carried by the slow transport tend to be deposited along the axon trunks (Schonbach et al., 1971 and 1973; Hendrickson, 1972; Droz et al., 1973). Furthermore, a large proportion of the materials transported by the fast transport system turn over very rapidly, whereas those transported at the slow rate mostly have a long life time. As a result of these differences in composition between the two components, a view has been expressed which regards axonal transport as a dual process in which the bulk of the axoplasm becomes displaced at the rate of the slow component, while particulate cell structures make their way

through the slowly moving axoplasmic mass at the much higher rate characteristic of the fast component. The exact way in which the intermediate component might fit into this picture is not yet clear.

The availability of the fast axon transport component has made it possible for the survival time between the injection of labelled material and sacrifice of the animal to be substantially reduced to a few hours rather than several days.

d. Advantages of Autoradiographic Technique for Tracing Axon Terminals

Before the autoradiographic technique for studying neuron connectivity was fully developed, the main techniques that were used in such studies included

(i) metallic impregnation method of Camillo Golgi, and

(ii) selective impregnation of degenerating fibres (Nauta and Gygax, 1951) subsequent to suitably placed lesions of nerve processes.

The advantages of the autoradiographic technique over these methods are as follows:-

1. Autoradiography uses anterograde axonal transport which is based upon an essential physiological property of nerve cells and does not involve the destruction of neural tissue or the occurrence of various pathological changes in response to such an injury.

2. There is no retrograde transport of label following the injection of radioactive-labelled amino acid into the region of termination of a neural pathway. In this way any label found in a nerve terminal must have been anterogradely transported there from specific neuron cell groups.

3. Axons passing through an area into which a labelled amino acid has been injected are unable to incorporate the label into protein. This adds to the specificity of the technique in relation to the pathway being studied.

4. A further advantage of autoradiographic method is its suitability for developmental studies. Attempts to follow the development of neural pathways in embryonic or neonatal material with reduced silver methods (either in normal preparations or following the placement of appropriate lesions) have shown that the fibres are not often consistently impregnated and traceable over appreciable distances. Autoradiographic method can be applied to the developing nervous system by labelling selected regions with an appropriate isotope and following, in embryos of successively older ages, the growth and development of the relevant pathways.

5. Autoradiographic method is, in some situations, more effective in revealing projections than the degeneration methods. For example until recently, it has

been extremely difficult to provide convincing evidence for a direct retino-hypothalamic projection with the various degeneration methods (Nauta and Haymaker, 1969). But this proved to be surprisingly easy with the autoradiographic technique (Hendrickson et al., 1972; Moore and Lenn, 1972; Meier, 1973).

e. Disadvantages of the Autoradiographic Method for Tracing Axonal Terminals

1. At light microscopic level, autoradiography does not tell one much about the actual morphology of the fibres and terminals being studied, since all that is actually observed is the distribution of silver grains in the emulsion overlying the section due to the radioactivity in the most superficial 2-3 μm of the section (Cowan and Cuénod, 1975). This limitation makes autoradiography inferior to the metallic impregnation techniques.

2. Autoradiography is not suitable for studying projections which have a broad or diffuse origin, for example the spinothalamic tract where it is impossible to label more than a small proportion of the total population of contributing neurons.

3. A third major limitation of autoradiography is that it is not well suited for studying short axonal connections. The main difficulty is the spread or diffusion of the injected isotope. This diffusional

spread commonly extends over a distance of 100 μm or more and it is impossible to distinguish this, in an autoradiograph, from the transport of the label within dendrites or a group of locally ramifying axons.

4. It is difficult to trace lightly labelled pathways over long distances. Silver grains are small (in the order of 2-3 μm) and it is necessary that the pathway is heavily labelled by injection of a large amount of radioactive material or with a long post-injection survival period which enables more slowly transported proteins to be distributed in the pathway.

5. It is still not known how heavily labelled a cell has to be before a significant amount of radio-activity can be detected in its axon and axon terminals.

In spite of these limitations, autoradiography is still the best technique for studying the distribution of the vagus nerve in the stomach wall. Firstly, the cells of origin of vagal efferent fibres that supply the stomach are fairly well localised to form the DMN of vagus and can all be labelled with radioactive-labelled amino acids. Secondly, it is difficult to selectively study vagal efferent fibre distribution to the stomach using silver impregnation or nerve degeneration techniques.

f. Autoradiographic Background

In autoradiographs, silver grains can appear in the developed emulsion which are not due to radiation from the experimental source, but to other causes. These grains make up the background. In order to differentiate genuine radioactivity from background, it is necessary to compare grains over an experimental source with those found over a source that is known to be unlabelled (control).

The common causes of background include the following:

1. Overdevelopment of Emulsion

As the strength, temperature and duration of development increase, the amount of background also increases irrespective of the degree of exposure of the emulsion (Caro and van Tubergen, 1962).

2. Exposure to Light

Exposure of the emulsion to visible light before development causes the formation of background, the level of which increases with increasing exposure to light.

3. Background due to Pressure

Pressure due to fingerprints, scratches or other insults to the emulsion will cause background grains to develop. If the emulsion is dried rapidly after coating tissue sections, the gelatin in the emulsion contracts and results in the formation of stress (pressure) artefacts.

4. Contamination of the emulsion with chemicals, either

at the time of manufacture or afterwards, and with metal ions such as copper and silver, can result in the formation of silver grains.

5. Chemography

There are many reactive groups in a biological specimen, particularly those that are reducing agents, which are capable of producing a latent image in silver halide crystals by direct, chemical action. This phenomenon is known as positive chemography. Negative chemography may also occur in which certain reactive groups in the biological tissue result in very rapid fading of the latent image in the adjacent crystals. This results in an area of emulsion that is unable to register the passage of a charged particle. Tissues that have been through the processes of fixation, dehydration, embedding in paraffin wax, sectioning and subsequent dewaxing are less likely to give rise to chemography than fresh tissues sectioned, for instance, on a cryostat. Nevertheless, the presence of this type of artefact in an autoradiograph is a possibility. Exposure at low temperatures reduces the rate at which reactive molecules diffuse from the tissue into the emulsion and the rate at which they react with the silver halide crystals (Rogers and John, 1969).

When viewed under high magnification, silver grains due to positive chemography are often large, irregular, and sometimes clumped together. Their distribution over the specimen is often thicker and more even than would be

expected from radioactivity, and are usually not arranged into definite patterns.

6. Spontaneous Background

The nuclear emulsions used in autoradiography are highly sensitised products and as a result of this, an occasional silver halide crystal will develop a latent image speck spontaneously. Ilford K5 is an example of very highly sensitised emulsions and the rate of formation of spontaneous background is high with this emulsion. High temperature and increasing age of the emulsion tend to increase the rate of formation of spontaneous background.

g. Characteristics of Tritiated Leucine

Leucine is an amino acid which is taken up by neuron perikarya and incorporated into their protein synthetic mechanism. The synthetised proteins are transported from the cell body to the axon, dendrites and their terminals either as trophic substances for the growth and maintenance of the axon, or as messengers which play a significant role in the traffic of information along the axon or to the postsynaptic cells (Weiss and Hiscoe, 1948; Cuénod et al., 1972; Jeffrey and Austin, 1973). Tritiated leucine is leucine that has been labelled with tritium which is a radioactive isotope of hydrogen. In this combination, tritium is used as a tracer. It is a low energy isotope of maximum initial energy of 18.5 keV. It has a half life

of 12.5 years. It releases β particles which have a range that seldom exceeds 2 μ in biological specimen or 1 μ in nuclear emulsion. Tritium can give autoradiographs with very high resolution which improves if the source of the radioactivity (tissue section) is thinner than 2 μ.

The Aims of the Study

The broad aim of this study was to see if there were anatomical changes in the distribution of the gastric branches of the vagus nerves resulting from chronic incomplete vagotomy, which might help to explain the observed recovery of gastric motility after chronic incomplete vagotomy in the ferret. This aim was attempted through three sub-aims which were:

1. Using normal animals, to determine, by means of silver grain distribution, the area of gastric wall innervated by each abdominal trunk of the vagus nerves,

2. in animals that had chronic unilateral abdominal trunk vagotomy, to determine the area of gastric wall innervated by the surviving vagus nerve trunk,

and

3. to compare the two areas of gastric wall innervated by each abdominal vagal trunk before and after chronic vagotomy in order to see any change that might be due to compensatory innervation by the surviving vagal trunk.

MATERIALS AND METHODS

a. Principles of the Method

If one or the other of the two abdominal trunks of the vagus nerve of the ferret is transected (vagotomy), the distal portion of the transected nerve will degenerate. If a radio isotope-labelled amino acid is injected into the cells of origin of the efferent fibre component of the vagus nerves (DMN of vagus), the label will be transported anterogradely down both trunks of the nerve. But because one trunk has been transected, the label will only reach the stomach wall via the surviving branch. Axon terminals in which radioactivity is detected in the stomach wall could therefore only have arisen from the surviving vagal trunk. In this way, the area of stomach that is innervated by the surviving vagal trunk can be determined. A repeat of this procedure after vagotomy on the opposite side will make possible the determination of the area of stomach innervated by the second vagal trunk.

If DMN is labelled immediately after unilateral vagotomy, the result obtained will be the normal distribution in the gastric wall. If labelling is then carried out several days or weeks after unilateral vagotomy, the result will show the presence or absence of changes in the area innervated by one vagal trunk consequent upon the loss, by vagotomy, of the other trunk.

b. Experimental Groups

Fourteen adult ferrets of either sex were used. These were divided into three main groups. The first group of two animals was the control group in which the brainstems and stomachs were processed for autoradiography without radioactive isotope injected into the animals.

In the second group, unilateral vagotomies were done immediately before injecting radioactive isotope into the animal. This group (acute experimental group) was subdivided into two. One subgroup with three animals had acute VTV and a second subgroup also with three animals, had acute DTV. The third group of six animals was similarly subdivided into the chronic VTV and chronic DTV subgroups each of which contained three animals (Table 3.1).

c. Anaesthesia

Prior to surgery for vagotomy and injection of radioactive siotope, the animals were anaesthetised with pentobarbitone sodium (Sagatal; May and Baker Ltd.) given intraperitoneally in a dose of 60 mg kg^{-1}. When the animals were to be sacrificed, they were anaesthetised with intraperitoneal ethyl carbamate (Urethane; Sigma Chemicals Company Ltd.) in a dose of 1.5 g kg^{-1} in the form of 50% Urethane in 0.9% saline. This produced a deeper and longer anaesthesia than Sagatal.

TABLE 3.1

Control	Acute Vagotomy		Chronic Vagotomy	
	Ventral Trunk	Dorsal Trunk	Ventral Trunk	Dorsal Trunk
2	3	3	3	3

d. Tritiated Leucine Stock

Tritiated leucine (Amersham International Ltd.) was used as the tracer material from which radioactivity was detected. It was supplied in the Laevo rotatory form (L -|4,5-^{3}H|Leucine) in 1-ml or 5-ml ampoules with a radioactive concentration of 1.0 mCi/ml (37.0 MBq/ml) and a molecular weight of 135 at this specific activity.

e. Freeze Drying and Concentration of Tritiated Leucine

One ml of tritiated leucine was carefully taken out of the ampoule with a syringe and needle. The leucine was placed in a small round-bottomed flask which was then swirled round in a jar of liquid nitrogen so that the leucine spread onto the side walls of the flask and there became frozen. The flask was then attached to the freeze-drying apparatus in a fume cupboard (Figure 3.1), care being taken to make the apparatus air-tight. The suction pump was switched on. The content of the flask was checked after 30 minutes to see if it had become fluid which would indicate air-leak into the drying system. If it remained frozen, the drying procedure was allowed to continue and usually in another one hour or so, the leucine became dry leaving only a small white powder in the flask. The suction pump was switched off and air gently and slowly let into the apparatus. A sudden surge of air into the flask would cause the fine powdery leucine to be blown out of the flask.

FIGURE 3.1

Diagram of the freeze-drying apparatus for concentrating tritiated leucine.

C = Clamp

T = Tritiated leucine in round-bottomed flask

W = Water extracted from tritiated leucine during drying and condensed in the drying flask unit

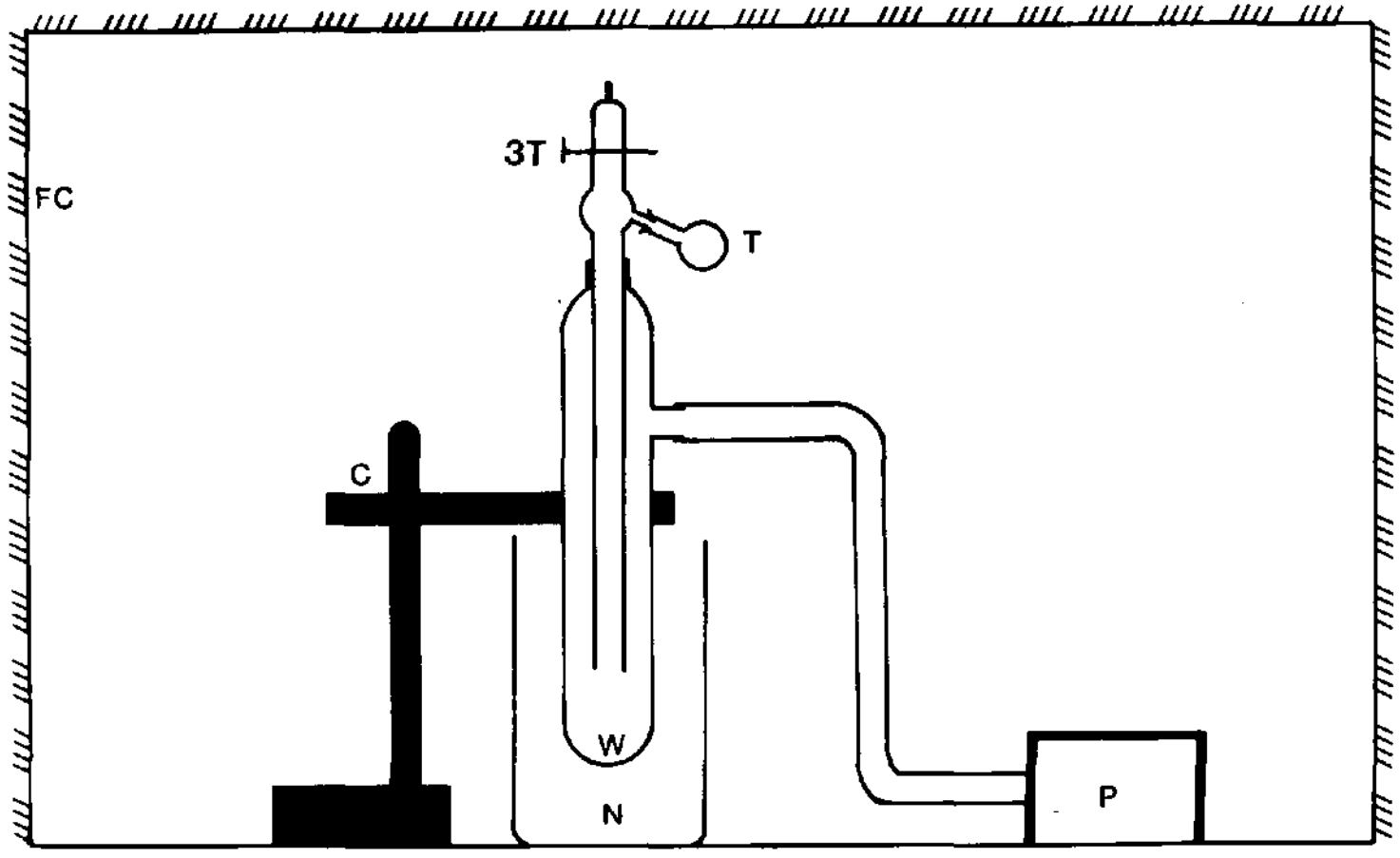
N = Liquid Nitrogen in polystyrene jar

P = Vacuum pump

FC = Fume cupboard

3T = 3-way tap

FIGURE 3.1



The flask was then removed and kept in a beaker. Normal saline (0.02 ml) was taken with a syringe and needle and introduced into the flask which was gently rotated to enable the saline to collect and dissolve the leucine powder. One millicurie (mCi) of tritiated leucine which was formerly in 1 ml of solution was thus reconstituted (concentrated) into 0.02 ml of normal saline. The flask was covered with a self-sealing, transparent film and kept in the freezer compartment of a refrigerator for a maximum of three days within which time the tritiated leucine was used. Any $|^{3}H|$leucine left was discarded at the end of this period.

f. Surgery and Injection of Tritiated Leucine

Two types of surgery were done.

(i) Surgery for Vagotomy

Each animal was deprived of food for about 18 hours before vagotomy. After the animal had been anaesthetised, the abdomen was opened by a midline incision and ventral or dorsal vagotomy was done depending on the experimental group into which the animal was to be placed. The appropriate vagus nerve trunk was isolated and two ligatures separated by a distance of 1 cm were placed around it. The nerve was sectioned between the ligatures. The cut ends were seen to retract from each other. The rostral ligature was placed as high up in the abdomen as possible so that no gastric branch would be missed by the cut. The animals that were to be

injected with tritiated leucine for the study of normal vagal distribution in the gastric wall, were immediately subjected to the next type of surgery. Those that were to be used to study the effect of chronic vagotomy were allowed to recover from anaesthesia and survive for 21 days before being subjected to surgery for the injection of leucine. A survival period of 21 days was chosen because that was, at the beginning of the experiments, the time of maximal change in the amplitude of intragastric pressure responses to cervical vagal stimulation.

(ii) Surgery for Injection of Tritiated Leucine

Each animal was again starved overnight, anaesthetised with Sagatal, and set up on a stereotaxic frame (Plate 3.1A). The animal was held face down and the head fixed above the operating table with metal bars which were passed into the external auditory meatuses. The head was then flexed with a third bar lying horizontally in front of the animal, in order that the space between the basi-occiput and the atlas might be widened. A midline incision was made on the back of the head and neck and the muscles of the neck were split down to the atlanto occipital membrane (Plate 3.1B). The muscles were retracted to expose a wide area of the membrane which was carefully nipped away to expose the 4th ventricle and the surrounding brain tissue (Plate 3.1C).

Cerebrospinal fluid was absorbed with lint. Tritiated leucine which had been allowed to melt and warm

PLATE 3.1

This shows the operative steps leading to the injection of tritiated leucine into the dorsal motor nucleus of vagus in the medulla oblongata.

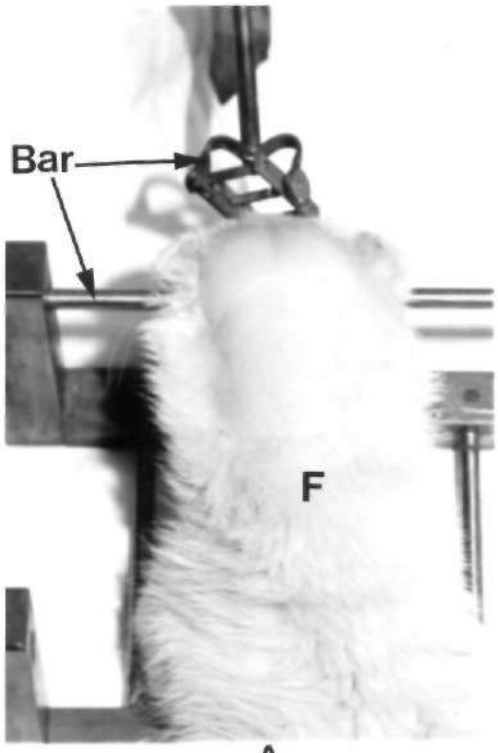
A - Animal (F) set up on stereotaxic frame. Ear and neck flexing bars are shown.

B - Atlanto-occipital membrane (M) is exposed.

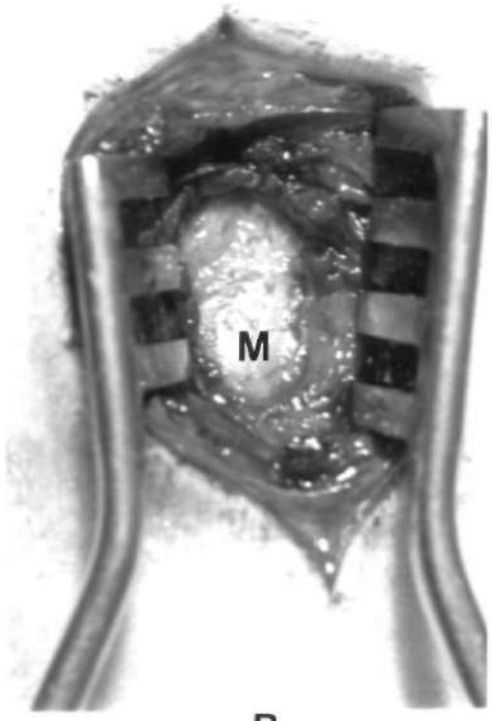
C - The medulla oblongata (Med) is exposed.
Ce = cerebellum
4th vent = 4th ventricle.

D - Injection of tritiated leucine with a glass micropipette (Pip).

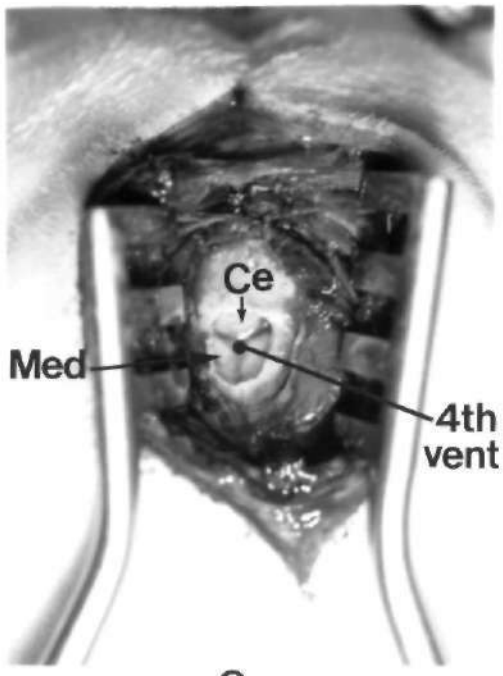
PLATE 3.1



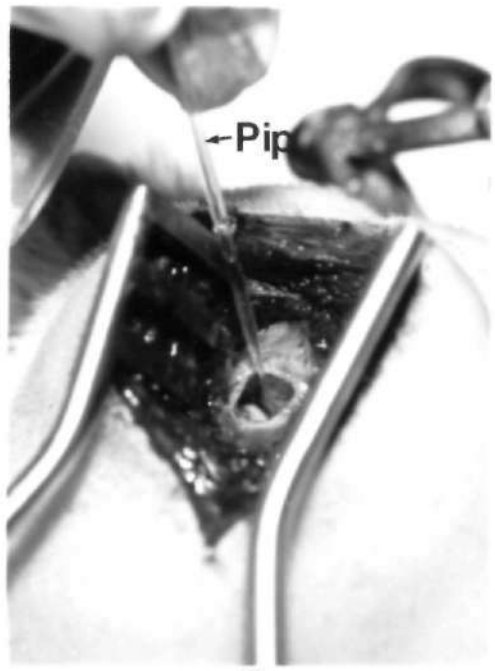
A



B



C



D

up to room temperature was taken into a glass micropipette to a height of about 1 cm. By means of a 3-way tap, a column of air was admitted into the syringe without splitting the leucine. The tip of the micropipette was inserted into the obex of the medulla oblongata on one side (Plate 3.1D), just deep enough to reach the location of the DMN of vagus nerve which had been previously determined with the aid of histological sections of the medulla oblongata (Figure 3.4). The plunger of the syringe was gently and slowly pushed in and the meniscus of the leucine watched descending as |^{3}H|leucine was injected into the medulla. Before all of the leucine had been injected, the plunger of the syringe was given a pull to prevent air from being pushed into the brain stem. A rush of air into the brain stem caused immediate death of the animal. In some cases, as a result of the injection of |^{3}H|leucine into the medulla, the animals went into respiratory arrest. Such animals were given artificial respiration and adequately warmed up with table lamps until they fully recovered. Injections were similarly made into the other side of the obex and bilaterally into the rostral and caudal portions of the DMN of vagus nerve. The muscle and skin incision was sutured in layers with the application of a mixture of neomycin sulphate BP, Polymyxin B sulphate BP and Bacitracin Zinc BP antibiotics (Polybactrin powder spray, Calmic Medical Division, The Wellcome Foundation Ltd., London) to the muscle layer and

FIGURE 3.4

Light micrograph of the medulla oblongata illustrating the position of the dorsal motor nucleus of vagus in the ferret at

A = rostral end of the medulla

B = obex of the medulla, and

C = caudal end of the medulla

DMN = Dorsal motor nucleus of vagus nerve

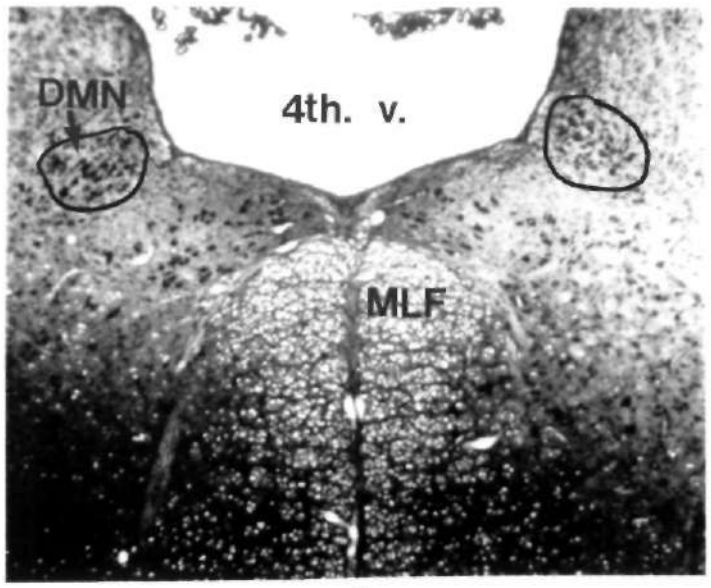
MLF = Medial longitudinal fasciculus

XIIn = Nucleus of hypoglossal nerve

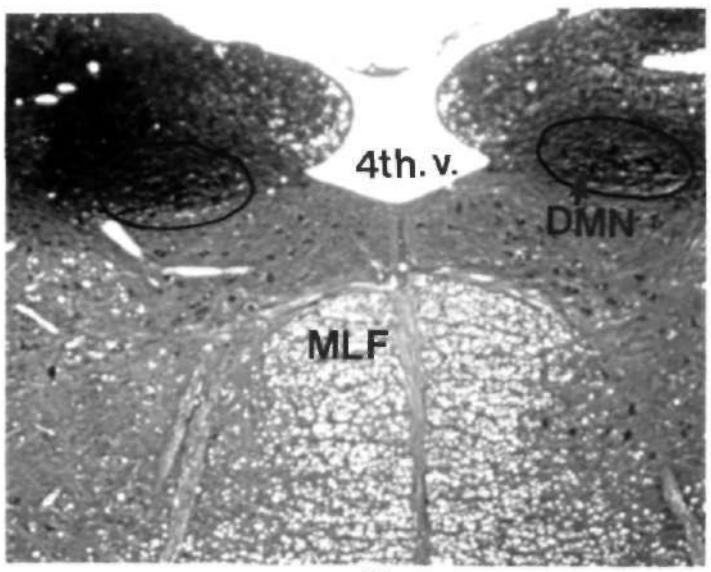
4th.v = 4th ventricle

C = central canal.

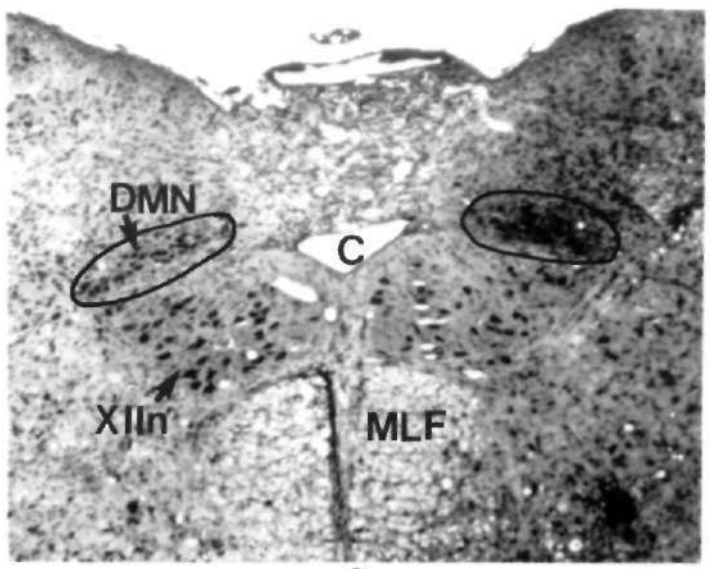
FIGURE 3.4



A



B



C

Plastron (Plastic wound) spray to the skin wound. The animals were allowed to recover from anaesthesia and survive for 26 to 30 hours. During this survival period, the animals were allowed free access to water only.

g. Perfusion Fixation

At the end of the survival period, the animals were sacrificed by perfusion with Formaldehyde fixative.

Preparation of Perfusion Solution

See Appendix 3.1.

h. Perfusion Apparatus

Two thousand millilitres of normal saline and 3000 ml of 10% formal saline were placed in separate large bottles which were connected to each other as shown in Figure 3.2 (Bower, 1981). The apparatus permitted sequential perfusion of tissues with two or more different fluids without interruption of the perfusion pressure.

i. Perfusion Technique

The animal was anaesthetised with Urethane and laid on its back on a slanting aluminium tray over a sink. With the aid of the 3-way tap and the manometer, the fixative was driven through the cannula to expel air from the system. The fixative line was shut off and the saline was allowed to flow through. The saline line was then shut off.

A thoraco-abdominal incision was made and the heart was cleared of surrounding fat. A loose ligature was

FIGURE 3.2

Diagram illustrating the perfusion apparatus.

F = Fixative fluid

NS = Normal saline

C = Cannula

M = Manometer

P = Manometer hand pump

3T = 3-way tap

placed round the heart. The apex of the heart was incised down to the cavity of the left ventricle. The cannula was passed through the apical incision via the left ventricle into the beginning of the ascending aorta. The ligature was tightened to keep the cannula in place. The superior and inferior vena cavae were transected close to the heart in order to permit a free outflow of perfusate. The saline line was opened and normal saline was driven through, keeping the driving pressure at 80 mm Hg. A litre of saline was driven through the vascular system of the animal in order to wash out all blood which might otherwise clot and prevent an adequate flow of fixative through the tissues.

The saline line was shut off and fixative driven through in the usual way. Two litres of fixative were driven through each animal. The first evidence of passage of fixative into the tissues was a twitching of the legs, tail and incised tissues. The neck and limbs later became stiff and the tissues, including the stomach, were firm to touch. When perfusion was complete, the cannula was removed. The brainstem and stomach were removed. The stomach was washed out with fixative and filled with 10% gelatin in water in order to distend it but without stretching the wall. The brainstem and stomach were then placed in fresh fixative in separate bottles. These were covered and kept in the refrigerator at a temperature of about 4^{0}C for 1 to 2 days. Post-mortem

was done to ascertain the completeness or otherwise of vagotomy that was done previously.

j. Tissue Dehydration

The brain stem was the first to be dehydrated. The midbrain, pons and spinal cord were trimmed off, and the medulla was placed in a perforated metal tray which was in turn placed in a 2L Processor (Shandon and Southern). The processor was programmed to take the tissue through graded concentrations of alcohol, chloroform and wax in 24 hours as shown below:-

Solution	Number of Hours in Solution
70% Alcohol	1
70% Alcohol	2
70% Alcohol	2
90% Alcohol	1
90% Alcohol	2
Absolute Alcohol	2
Chloroform	3
Chloroform	2
Chloroform	2
Wax	3
Wax	2

The medulla was processed for autoradiography and the sections were studied to see if the injected $|^{3}H|$leucine was in all areas of the DMN of vagus. Only if this was

so was the stomach placed in the processor for dehydration. The stomach was cut into rings which were marked in such a way that the anterior and posterior surfaces could be identified and the rings re-arranged to form the complete stomach. Having removed gelatin from the rings, they were subjected to a similar 24-hour programme in the processor.

k. Tissue Embedding

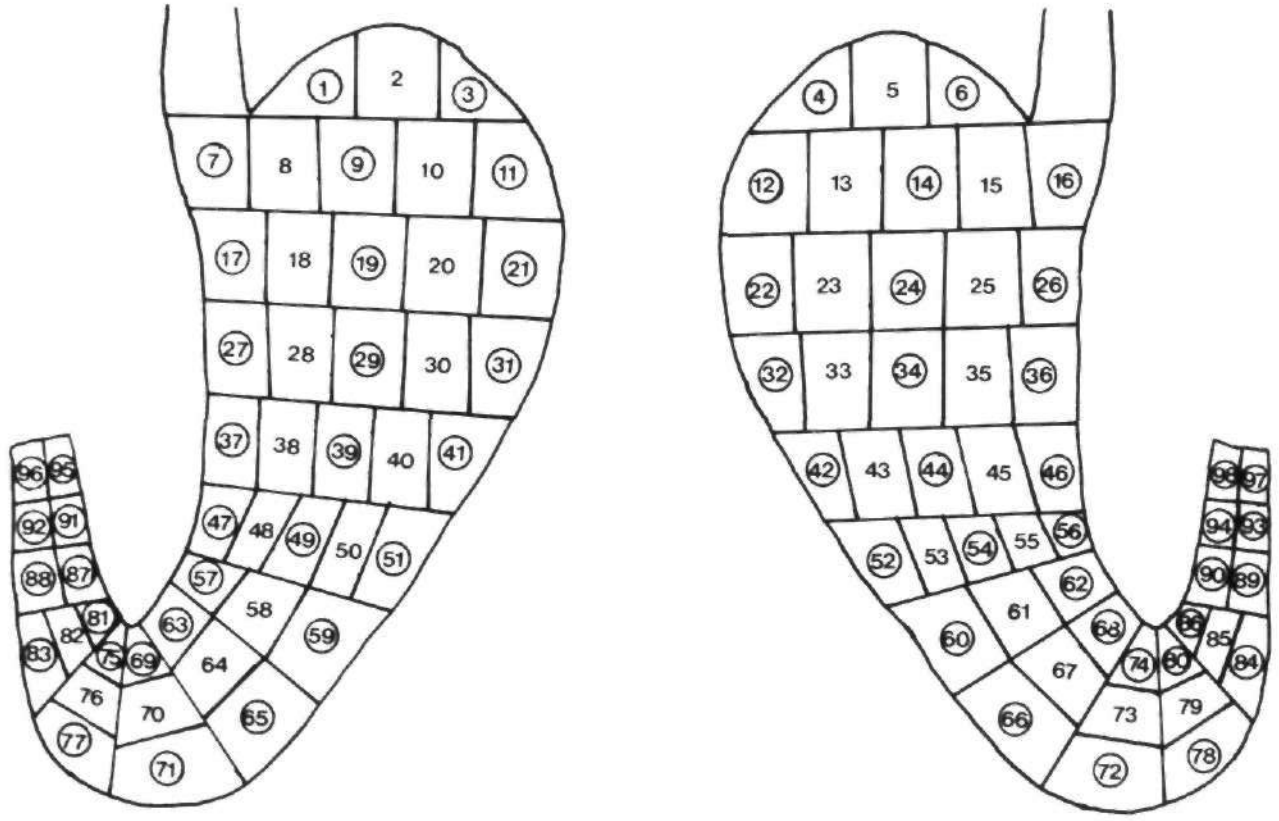
Both brain and stomach tissues were embedded in wax. Filtered molten wax at 60^{0}C was dispensed from a Wax Dispenser into plastic embedding troughs. The wax was allowed to stay for a few seconds in order that a thin film of partially hardened wax might form at the bottom of the trough. The medulla was orientated vertically in the wax with the rostral end in contact with the hardening film of wax. The trough was labelled and allowed to cool and harden under water, a procedure which is believed to promote a more uniform hardening which in turn improves block cutting.

The stomach rings were divided into anterior and posterior halves. Each half was divided into 3 or 5 blocks of about 5 mm width. Blocks 1, 3, 4 and 6 or 1, 3, 5, 6, 8 and 10 were taken and embedded in a similar way to the medulla (Figure 3.3). With this sampling method, 60 to 70% of the stomach was sampled. The orientation of the stomach blocks was such that they

FIGURE 3.3

Diagram of the ventral and dorsal surfaces of the stomach to illustrate block sampling from the stomach. The tissue blocks with ringed numbers were sampled. This method usually resulted in sampling about 65 percent of the stomach.

FIGURE 3.3



Ventral surface

Dorsal surface

could be cut progressively from the lesser curvature all the way along the anterior and then the posterior surface of the stomach and back to the lesser curvature. The blocks were labelled so that they could be reconstructed into the whole stomach.

l. Tissue Sectioning and Mounting

The tissue sections were mounted on subbed microscope glass slides; therefore the slides were subbed before cutting the tissue blocks. The gelatin in the subbing solution enhances adhesion of both sections and emulsions to the slide and also helps to reduce pressure artefacts which result from lateral displacements on drying the emulsion (Sawicki and Pawinska, 1965).

Preparation of Subbing Solution

See Appendix 3.2.

m. Procedure for Subbing Microscope Glass Slides

Eight hundred to 1000 slides were subbed at a time. They were individually hand washed into a large bowl of soap water (diluted Teepol). They were left to soak in the soap for about 8 hours and were then rinsed out 2 to 3 times. They were left under running tap water overnight and rinsed out again before subbing. A wide table was chosen for drying out the slides after subbing. Test tube racks were arranged in double or triple rows on the table which was lined with a thick layer of tissue paper. Clean glass rods were arranged horizontally

across the racks, and held down to the latter with Sellotape.

About 500 ml of subbing solution at room temperature was placed in a bowl. A few glass slides at a time were taken out of the batch, allowed to drain and placed in the subbing solution. A slide at a time was taken out of the subbing solution and supported obliquely on the glass rods so that both sides of the glass slide could remain coated with subbing solution and get dry free of dust or other contaminants. Because the subbing solution got diluted with increasing number of slides transferred into it from water, the subbing solution was replaced with a fresh one after about 3 to 4 hundred slides had been subbed. The slides were allowed to dry overnight, stored away in packets to be used as required.

n. Procedure for Sectioning and Mounting the Tissues

The tissue blocks were cut into 10 µm thick sections using a Reichert's microtome. Transverse sections of the medulla and longitudinal sections of the stomach were cut after trimming the block until a complete cross-section of the appropriate tissue was obtained. A systematic sampling technique was used to obtain sections from the tissue. Two adjacent sections were taken. The first section was mounted on glass slide A and the second on slide B. In the case of the medulla, the next five sections were rejected and the next two sections were taken and mounted as above. This procedure was repeated until the whole of

the medulla had been cut. Four to five sections were mounted on a slide. The slides were numbered serially. A and B series were taken so that the loss of one section or slide could be replaced by an adjacent one which was nearly identical.

In the case of the stomach, sections were taken and mounted as for the medulla except that a hundred sections were rejected in between samples.

The sections were expected to adhere to subbed slides, but this was not the case. Therefore, a small amount of albumen was spread over the surface on which the sections were to be mounted. This enhanced tissue adhesion to the slide. A small quantity of distilled water was placed on the slides and the sections placed in the water. The slides were then placed on a Hearson's hot plate at 50^{0}C for about a minute. The heat enabled the wax and tissue to spread and adhere to the glass. Any unyielding wrinkles were removed before the section was fully set, with the use of two metal probes. A moist tissue paper was used to remove excess water from the slide. The capillary action of the tissue paper also removed any air bubbles and particulate matter from underneath the sections. The slides were then left on the hot plate for 24 to 48 hours so that the sections could be firmly adherent to the slides. Care was taken to ensure that the bottles containing distilled water and albumen were clean and were always covered when not in use.

o. Procedure for Coating Tissue Sections with Emulsion

This can be divided into

(i) Preparatory stages;

(ii) Coating Technique.

(i) Preparatory Stages

Glassware Treatment

The glassware used had to be washed clean in order to avoid the introduction of artefacts which would interfere with the result. They were usually kept in a solution of chromic acid when not in use. They were taken out of the acid cleansing solution and rinsed in running tap water. They were then left in running tap water for 15-18 hours before allowing them to drain and dry on tissue paper.

Dewaxing and Rehydration of Sections

The slides were placed in plastic racks and soaked in xylene overnight in order to dewax the sections. They were transferred into fresh xylene for another 2 to 3 hours before rehydrating them. Rehydration was achieved by passing the slides successively through absolute alcohol, 90% alcohol and 70% alcohol for about 2 minutes in each solution. They were then washed in running tap water for 30 minutes and transferred into distilled water.

Preparation of Emulsion

This as well as coating of the sections was done in the darkroom under safelight (Kodak Safelight filter

series 2 or Ilford safelight filter 906.ISO, both of which are dark red). Three types of Ilford Nuclear Research Emulsions in gel form were used at different times during the experiment. These were K2, K5 and L4. The change from K2 to K5 was because the purity of the batches of K2 that were forthcoming could no longer be guaranteed. Emulsion L4 was preferred to K5 because of the higher level of background associated with the latter. Regardless of the type of emulsion used, the formulation of the working solution was the same. Prior to use, the emulsions were stored at 5^0C..

Glycerine was used as a plasticiser which would reduce the shrinkage of gelatin during drying. An initial 2% aqueous solution of glycerine was prepared as follows:

Distilled water	24.50 ml
Glycerine	0.50 ml

These were mixed in a 50 ml measuring cylinder and transferred into a 50 ml conical flask with flat bottom (dipping jar). The jar was placed in a thermostatically controlled water bath at 43^0C. The 50 ml and 25 ml marks were re-marked on a 100 ml measuring cylinder using a black marker which was visible under safelight. The cylinder was filled to the 50 ml mark with emulsion gel. The cylinder was placed standing in the water bath and the emulsion stirred slowly in a circular motion with a clean glass rod at a rate of one circle per second. This was

continued until the emulsion melted down to the 25 ml mark. The whole of the emulsion was poured into the 25 ml of glycerine solution in the dipping jar. These were mixed to give 50 ml of emulsion with a final glycerine concentration of 1% (Waller, 1961; Edwards and Hendrickson, 1981). The emulsion was left standing in the water bath for about 10 minutes. During this time, any bubbles in the diluted emulsion would rise and be expelled. An empty clean glass slide was dipped into the emulsion, taken out and examined for bubbles. If there were bubbles, the emulsion was left standing for a further 1 or 2 minutes and tested again with a clean glass slide. Since a bubble might settle on a part or whole of a section and prevent direct contact between the section and the emulsion, it was absolutely essential that the emulsion was free of bubbles. Only when this was so, were the experimental slides dipped in the emulsion.

(ii) Coating Technique

One slide at a time was dipped into the molten emulsion. The dipping was slow and of uniform speed. The sections were well below the surface of the emulsion. The slide was withdrawn slowly and excess emulsion was allowed to drop on tissue paper with which the emulsion on the back of the slide was wiped off. A uniform movement of the slides in and out of the emulsion was necessary to produce as uniform a coat of emulsion as possible on each section.

The slide was placed flat, with the section-carrying surface upwards, on a metal plate which was cooled with ice packs. This rapid cooling made the emulsion to gel so that initial drying of the emulsion could be gentle and so reduce stressing of halide crystals which would have increased the level of background (Sawicki and Pawinska, 1965).

After all slides had been similarly treated, they were left in cardboard trays to dry in the dark. The trays were then packed one on top of the other in black cardboard boxes. Some calcium chloride crystals were placed in an otherwise empty cardboard tray and placed on the other trays. The calcium chloride was to complete the drying of the emulsion during storage. The boxes were wrapped in foil, labelled, and kept in the refrigerator. The medulla sections were thus stored (exposed) for 3 to 4 days and the stomach sections for 4 weeks, at the end of which they were ready for development.

p. Slide Development and Fixing

Kodak D-19 Developer was used. The stock solution was prepared by dissolving the pre-packed contents of Kodak D-19 in 5000 ml (final volume) of water at 38°C. The volume of working solution prepared from the stock depended on the number of slides to be developed. Four hundred millilitres of working solution was used to develop two batches of 25 slides. The working solution

was made up of

Filtered Kodak D-19 Developer Stock	600 ml
Distilled water	600 ml

The temperature of the developer was taken and that of the distilled water was adjusted so that mixing the two would give 1200 ml of working developer at 18°C.

The fixer was 30% sodium thiosulphate which was made of

Sodium thiosulphate	240 g
Distilled water to make	800 ml

Three batches of 25 slides were fixed in each 400 ml of fixer. The slides were transferred from cardboard trays into plastic racks each of which contained 25 slides. The slides were developed, with no agitation, for 8½ minutes when Ilford K2 emulsion was used and for 5 minutes when K5 or L4 was used. Developing was arrested by transferring the slides into distilled water for about 30 seconds. Fixation was complete after 4½ minutes with the racks agitated for 10 seconds in every 30 seconds in order to completely dissolve the emulsion. The slides were rinsed in running tap water for 30 minutes to 1 hour.

q. Staining of the Sections

The sections from the medulla were stained with 1 percent cresyl fast violet while stomach sections were stained with Harris's haematoxylin.

(i) Staining of Brain Stem Sections

Stain Preparation

See Appendix 3.3.

Staining Procedure

The slides were placed in the stain in a staining dish for 15 to 30 minutes with some agitation every two minutes. The slides were then transferred into running tap water in order to rinse off excess stain. They were transferred into 95% alcohol and left standing, with occasional agitation, until the right depth of staining (light purple colour) was obtained as seen under the light microscope. In cases where differentiation with 95% alcohol did not progress, a special cresyl fast violet differentiator was used. This was made up of:

95% Alcohol	500 ml
Chloroform	10 ml
Acetic acid	0.25 ml

The sections were then transferred into two changes of absolute alcohol for about 30 seconds to 1 minute, and then into xylene. They were mounted in DPX mounting medium and covered with microscope glass cover slips.

(ii) Staining of Stomach Sections

Stain Preparation

See Appendix 3.4.

Staining Procedure

The sections were stained with haematoxylin for

5 minutes and excess stain was washed off in running tap water. They were placed in 1 percent hydrochloric acid for 10 seconds to further remove excess stain. The sections were rinsed in water and transferred to a dish containing a few drops of liquid ammonia for one minute with the dish covered. The ammonia converted the golden colour of the stained tissue to blue. The slides were washed in running tap water for about 30 minutes and left to dry in a dust-free atmosphere. The sections were mounted in DPX mounting medium and covered with microscope glass cover slips.

r. Analysis of Tissue Sections

The brain and stomach sections were examined and analysed with Vickers M17 Microscope (type E-dual illumination) and Ortholux 2 (Leitz Wetzlar Germany) as described by Bower et al. (1975). These microscopes had incident and transmitted light facilities which permitted various combinations of darkground and brightfield illumination. All brain stem sections were examined under darkground illumination in order to see if the rostrocaudal extent of the DMN of vagus contained silver grains. The presence of silver grains in the neurons of DMN at all levels was the criterion for successful injection.

The stomach sections were also examined under darkfield illumination. Since the radio isotope travelled to the preganglionic axon terminals of the vagus nerve in the

stomach wall, evidence for labelled axons was sought. There were some background grains which had to be distinguished from silver grains arising from labelled axons. Whereas the former were uniformly scattered throughout the tissue section, the latter could either form rows of silver grains with the beaded appearance or aggregates of silver grains located at intervals along the intramural plexus or a combination of both patterns. The silver grains were derived from silver halides in the nuclear emulsion responding to radiations from the radio isotope in separate loci in one or more axons.

s. Criteria for Identification of Labelled Axons

As mentioned above, two patterns of silver grains are recognised. The aggregates of silver grains are distinct from the background grains. They overlie tissue structures that are not smooth muscle fibres and which are situated in the space between the outer longitudinal and inner circular smooth muscle layers. These structures, the nature of which is not clearly seen in longitudinal tissue sections under light microscope, are presumed to be intramural ganglia. When background grains are phased out by increasing the transmitted light, the aggregates of silver grains are still clearly visible.

Rows of silver grains forming the beaded appearance have to be further differentiated from some technical artefacts which look like silver grains. The following criteria were used to identify rows of true silver gains.

1. The rows of silver grains must not be perfectly straight, a distinguishing factor from α-tracks.

2. The silver grains should be distinct and identifiable as separate grains.

3. The row of silver grains must overlie a tissue structure rather than lie in cracks in the tissue (Edwards and Hendrickson, 1981).

4. The "shine" of the silver grains should not be glittering.

5. When background grains are eliminated by slightly increasing the transmitted light, true silver grains are still visible as distinct grains, whereas contaminants or black specks are seen in the position occupied by background grains (Rogers, 1973).

The smooth muscle layers and the interspace between them, the submucosa and the mucosa of each stomach section were examined. Labelled axons seen as rows of silver grains in the outer longitudinal smooth muscle layer were recorded as belonging to the myenteric plexus. Those axons seen in the inner circular smooth muscle layer were assumed to be on their way to the submucosal plexus and so were classified with that plexus. A reconstruction map of the stomach was drawn and the result of each tissue section examined was recorded on the appropriate part of the stomach from which the section was taken. In this way, the area of stomach with labelled axons

after acute or chronic unilateral abdominal vagotomy was mapped out.

RESULTS

a. Microscopic Examination of Sections from Medulla Oblongata

Examination of the transverse sections of the medulla oblongata of the ferret under brightfield illumination revealed the DMN as a group of neurons situated postero-lateral to the hypoglossal nucleus. The two nuclei are close to one another and maintain this relationship at all levels in the medulla except that the hypoglossal nucleus extends more cranially than the DMN while the latter extends more caudally than the former. As a result of the close-ness of the two nuclei, the hypoglossal nucleus was almost always labelled in addition to the DMN.

In control animals, the cells of the DMN had clear cytoplasm surrounding the nuclei which stained purple. When examined under darkfield illumination, the cytoplasm remained clear. But in animals injected with $|^{3}H|$leucine, the cells appeared dark under brightfield illumination due to the presence of dark, grain-like materials within the cell boundary. Examination of these materials under dark-field illumination showed that they were silver grains (Plate 3.2). The concentration of silver grains over each perikaryon was much higher than over the extra-perikaryal tissue. Therefore, it was possible to identify each

PLATE 3.2

Light microscope autoradiograph of dorsal motor nucleus (DMN) of vagus nerve as seen under the dark-field illumination.

a - low power autoradiograph of the cross section of DMN of vagus (black circle) below the level of the obex.
C is the central canal.
Magnification = x 250

b - high power autoradiograph of a section of the neurons in "a" showing the silver grain-filled neuron cell bodies (N).
Magnification = x 1000

perikaryon which in turn made it possible for the boundary of the DMN to be defined. In addition to the present of silver grains over the neuron cell bodies of the DMN, silver grains were also seen in other areas of the medulla. The area of tissue outside the DMN overlaid with silver grains varied with the volume of $|^3H|$leucine injected and the position of the tip of the micropipette. The concentration of silver grains was highest at the site of injection and gradually decreased towards the periphery. A fibre tract was seen arising from each DMN and passing anterolaterally to the surface of the medulla oblongata. These were the efferent vagal fibres (Plate 3.3). Similar tracts were seen arising from the hypoglossal nuclei but these were more medially placed than the tracts from the DMN. In some sections, the neuron cell bodies of nuclei gracilis and cuneatus were labelled with silver grains. In such sections, the fibres of the medial lemnisci which arose from these nuclei, were also labelled.

b. Microscopic Examination of Sections of the Gastric Wall

Individual silver grains were not counted. The identification of silver grains either in the aggregated or beaded form in a given tissue block, was taken as an indicator of positive axon labelling in that block (Plates 3.4 and 2.7). The number of such positive indicators of labelling was entered against the corresponding tissue block (Figures 3.5, 3.6 and 3.7). The rows of silver grains varied widely in length, each labelled axon containing from

PLATE 3.3

Shows a section of the grain-filled fibre tract (T) of the vagus nerve within the medulla oblongata. This is evidence that $|^{3}H|$leucine is transported from the neuron cell bodies of the DMN along the nerve fibres.

Magnification = x 1000

PLATE 3.4

Light microscope autoradiograph of the myenteric plexus of the dorsal wall of the stomach after ventral abdominal trunk vagotomy. Two main patterns of labelled axons are shown.

a - an aggregate of a mixture of separate silver grains and the wavy, beaded appearance.
s - single silver grains
bd - beaded arrangement of silver grains

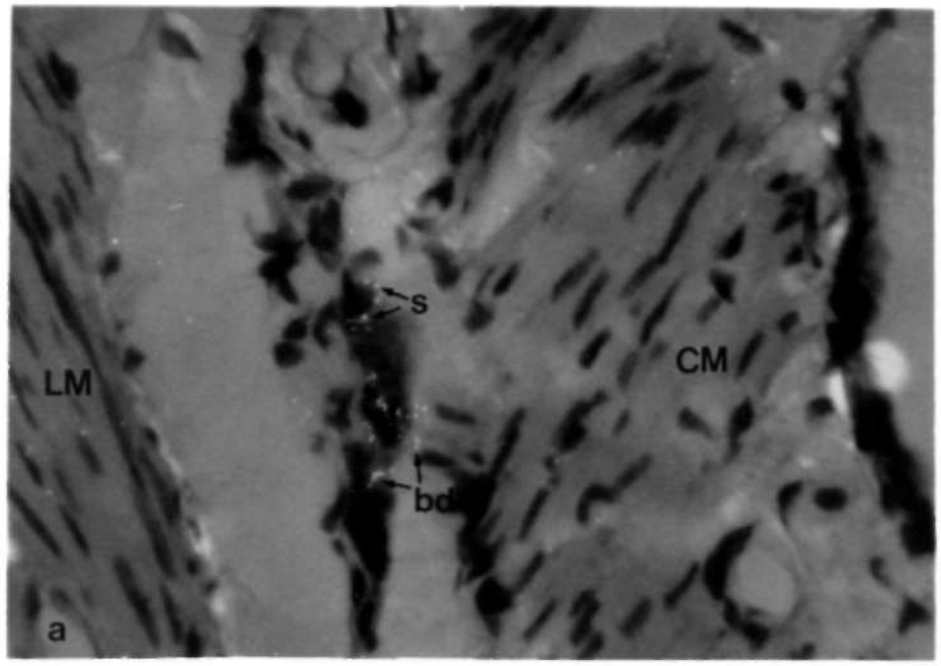
b - profile of the beaded pattern of silver grains between outer longitudinal and inner circular smooth muscle layers

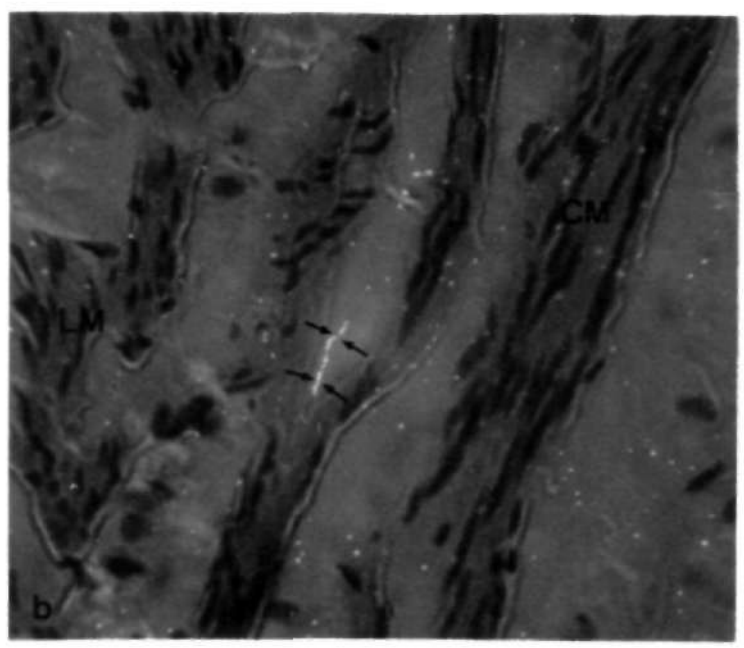
LM = Longitudinal smooth muscle layer.

CM = Circular smooth muscle layer.

Magnification = x 1000

PLATE 3.4





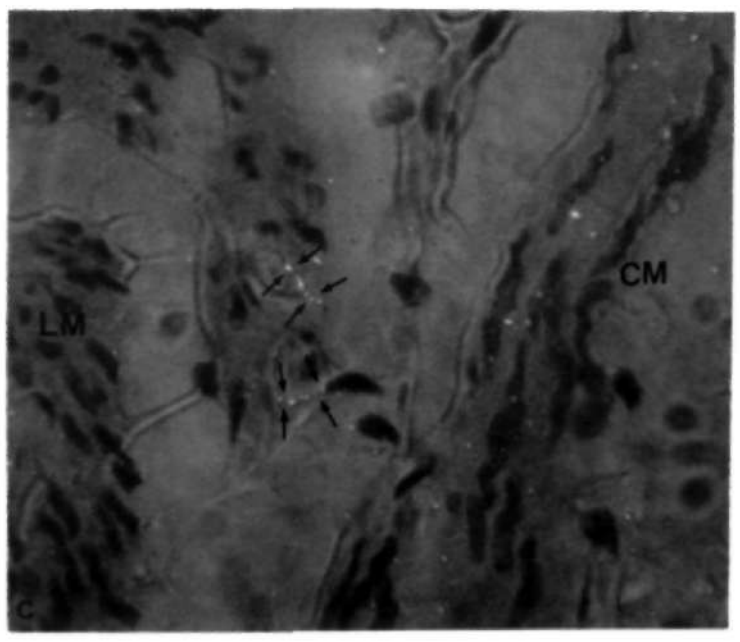


PLATE 3.7

Shows labelled axons in the submucosal plexus of the dorsal wall of the stomach after ventral abdominal trunk vagotomy (silver grains between the arrows in a and b).

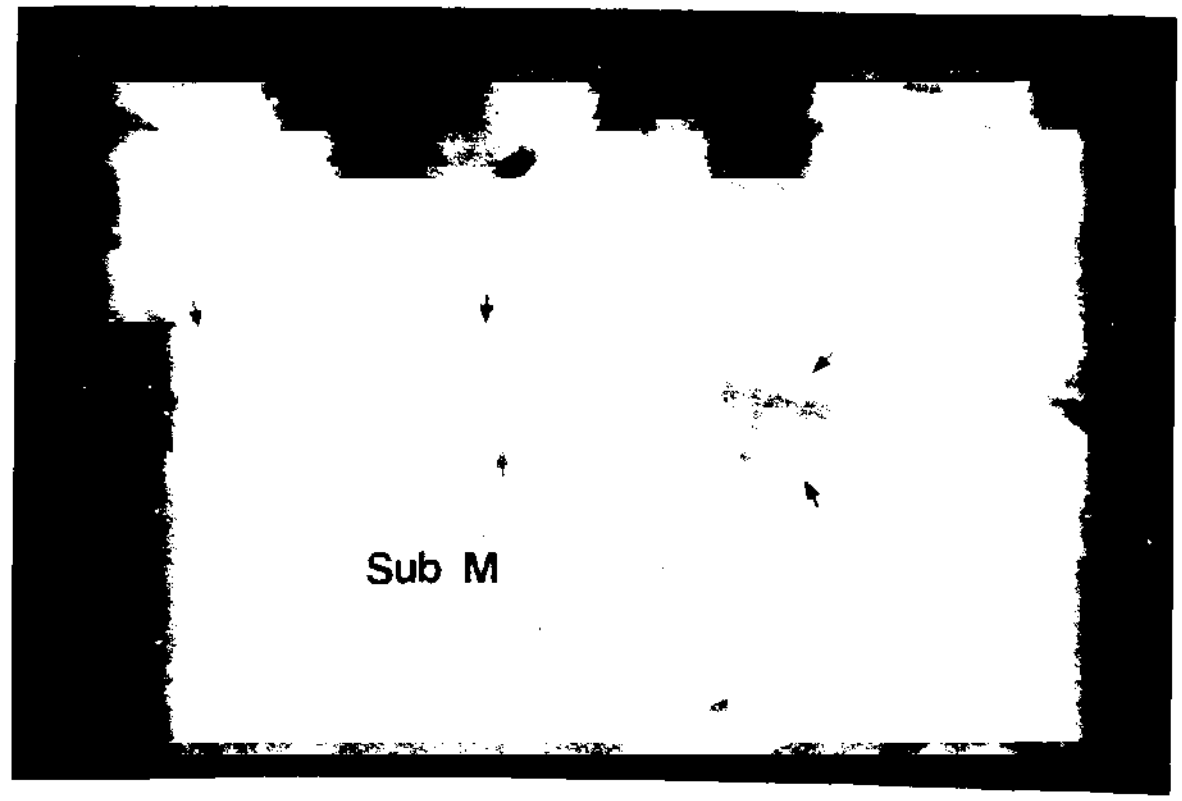
CM = Circular smooth muscle layer.

Sub M = Submucosa.

LM = Longitudinal smooth muscle layer.

Magnification = x 1000

PLATE 3.7



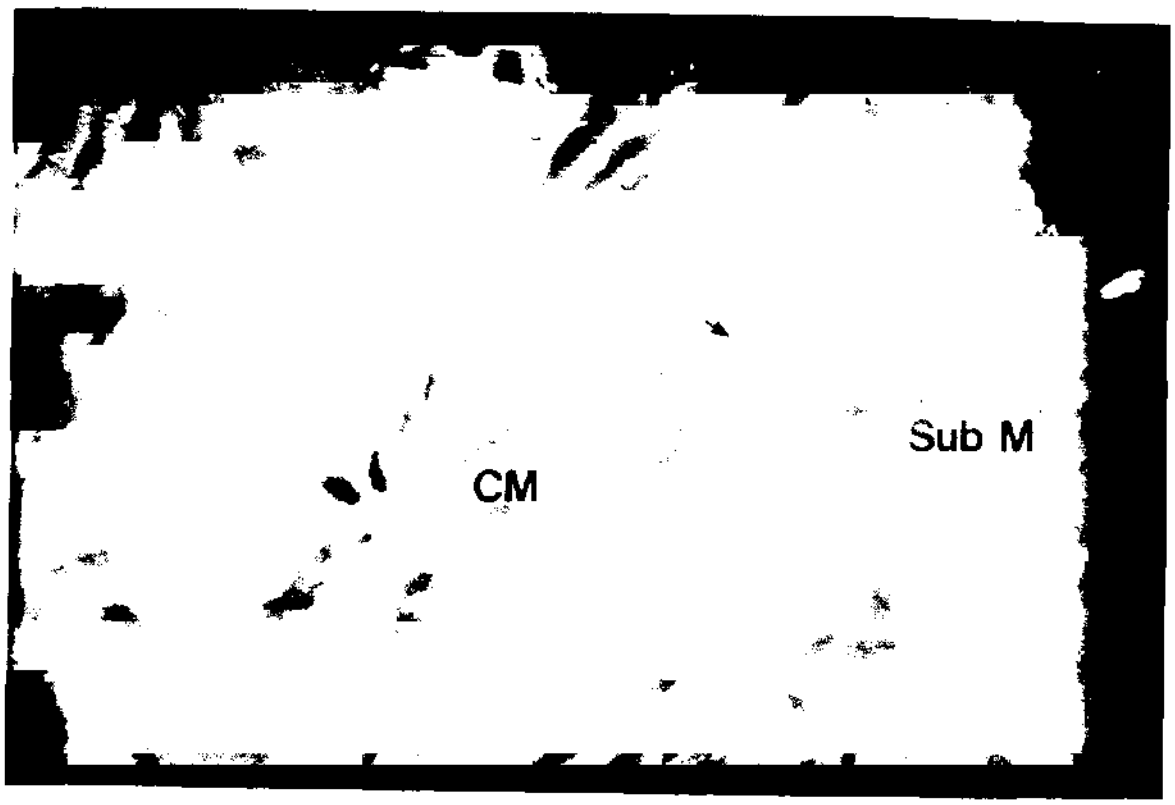


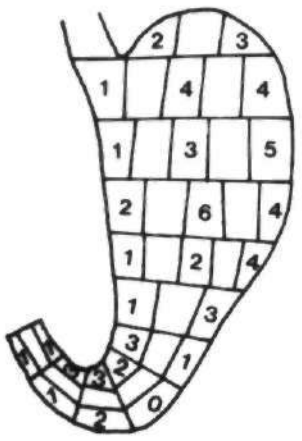
FIGURE 3.5

(i) shows the normal distribution of the ventral vagal trunk on the ventral wall of the stomach in 3 ferrets (1, 2 and 3). The numbers are those of areas of positive labelling in the sampled blocks which ranged from 3 to 5 mm square.

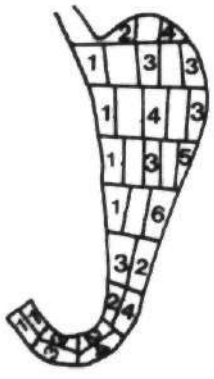
(ii) shows the distribution of the ventral vagal trunk 3 weeks after dorsal trunk vagotomy in 3 ferrets (1, 2 and 3).
The dorsal wall of the stomach is not shown because it contains no labelled axons of ventral origin.

FIGURE 3.5

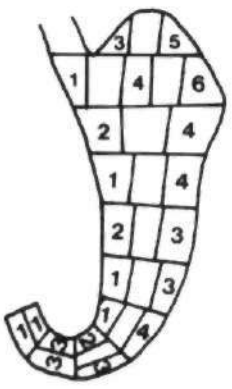
(i)



1. Ventral surface

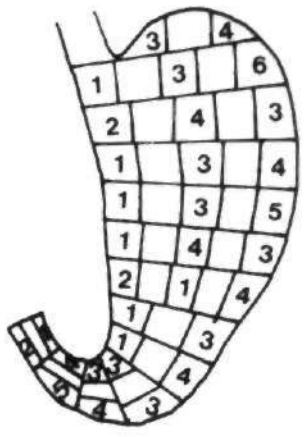


2.Ventral surface

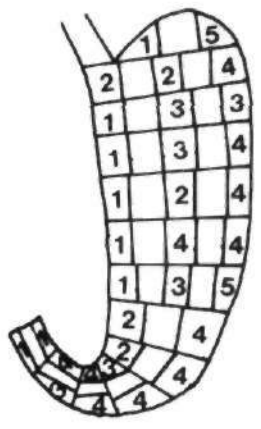


3.Ventral surface

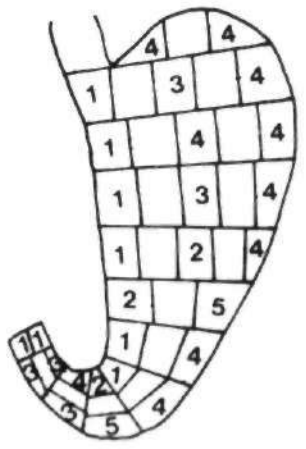
(ii)



1.Ventral surface



2.Ventral surface

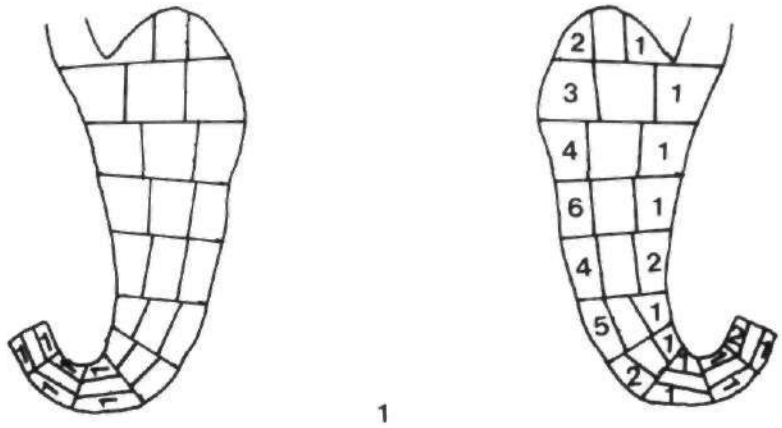


3.Ventral surface

FIGURE 3.6

Shows the normal distribution of the dorsal vagal trunk in the dorsal and ventral walls of the stomach in 3 animals. The numbers are those of areas of positive axon labelling in each 3, 4, or 5 mm square block of stomach tissue.

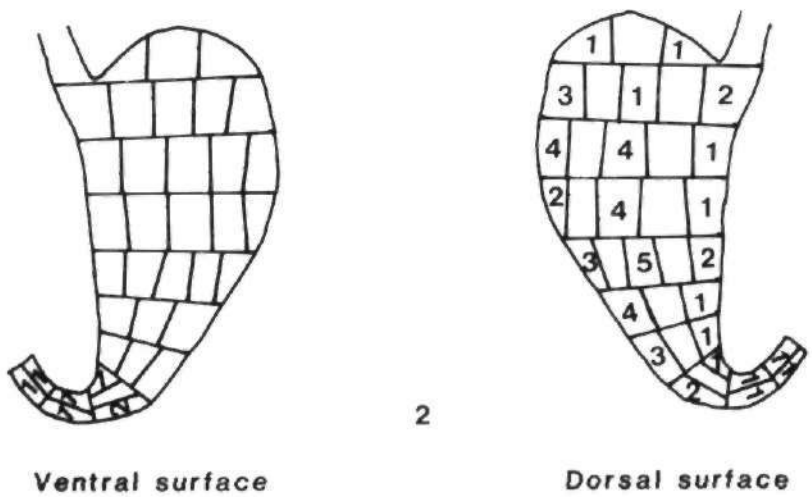
FIGURE 3.6



1

Ventral surface

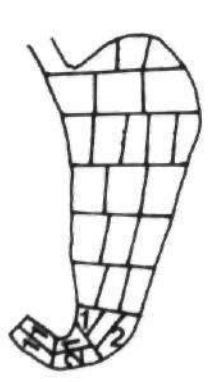
Dorsal surface

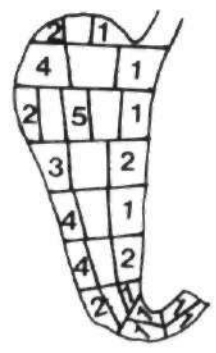


2

Ventral surface

Dorsal surface





3

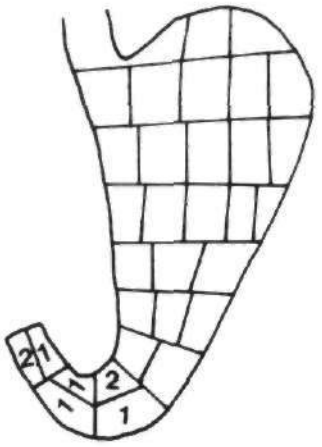
Ventral surface

Dorsal surface

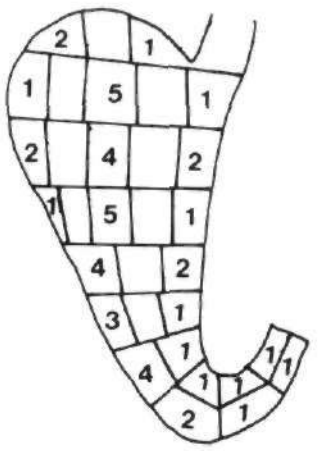
FIGURE 3.7

Shows the distribution of the dorsal vagal trunk in the dorsal and ventral walls of the stomach in 3 animals, three weeks after ventral trunk vagotomy. The numbers are those of areas of positive axon labelling in each stomach block.

FIGURE 3.7

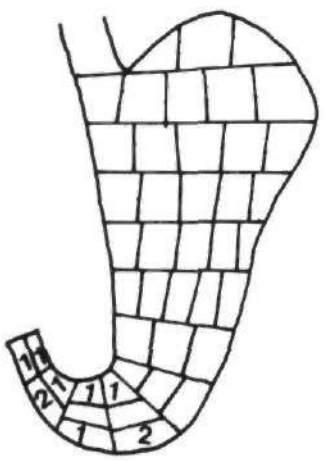


Ventral surface

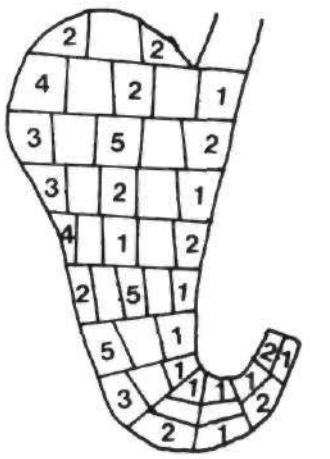


Dorsal surface

1

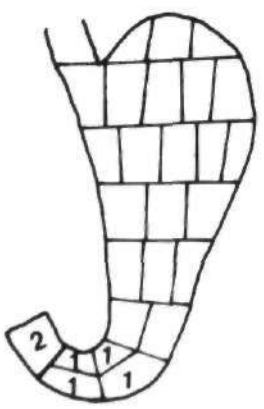


Ventral surface

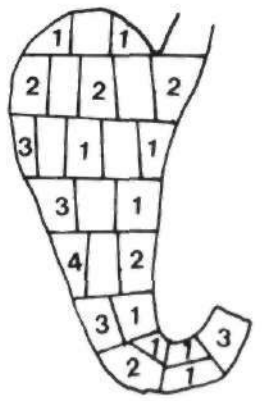


Dorsal surface

2



Ventral surface



Dorsal surface

3

about 4 to 15 silver grains. The grains were arranged in wave form and also satisfied the other relevant criteria listed above. Isolated rows of silver grains were seen at intervals along the intramural plexus but groups of two or three rows were sometimes seen (Plate 3.4 b and c). Although most of the axons labelled in this way were seen in the space between the smooth muscle layers, some were seen overlying the muscle layers themselves (Plate 3.5).

The labelled axons which were seen as aggregates of silver grains were limited to the submucosa and the space between the muscle layers and in relation to intramural ganglia as described above. The grains in this pattern were believed to arise from several axons around the myenteric ganglia, cut head-on.

The silver grains in the two patterns of label were much smaller in size when the tissue sections were coated with L4 than with K2 and K5. Patterns of silver grains similar to those described here were not seen in stomachs of control animals (Plate 3.6).

c. Normal Distribution of Abdominal Vagal Trunks in the Myenteric (Auerbach's) Plexus

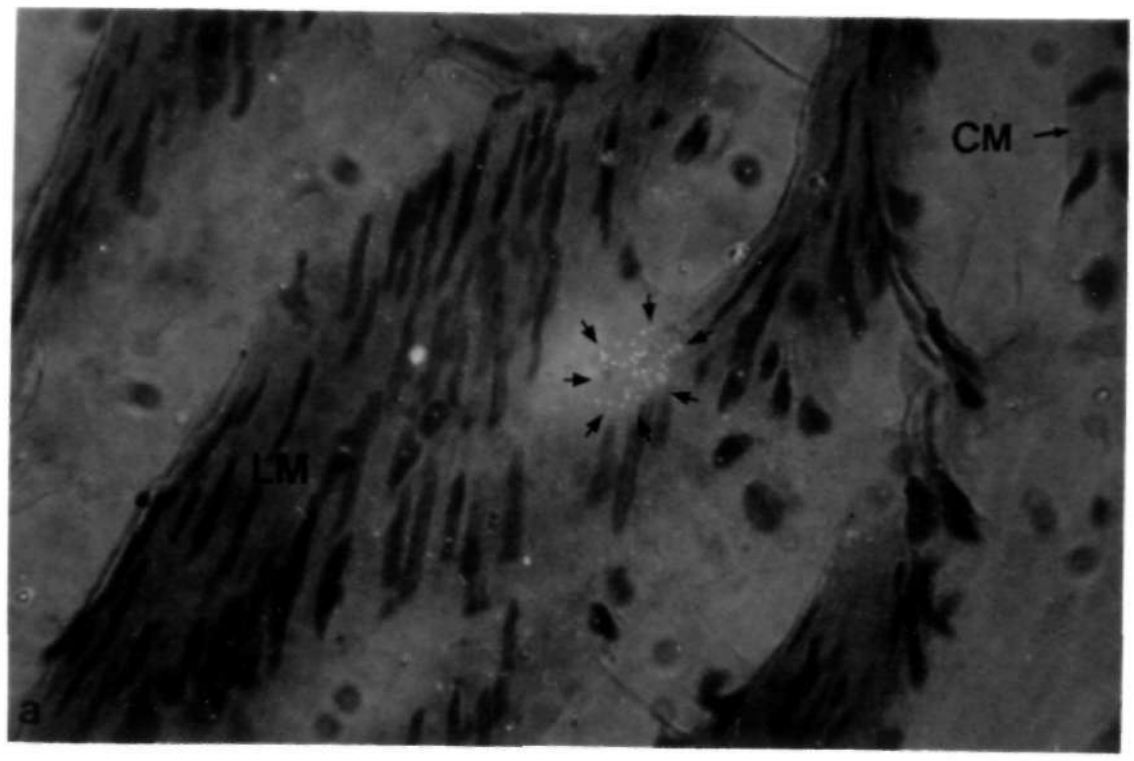
These results were obtained from animals which had |^{3}H|leucine injected into their medulla immediately after unilateral abdominal vagotomy. A table was drawn in such a way that every tissue section from every tissue block was represented by a box. The presence or absence of labelled axons in each section was entered into the appropriate box. The number of isolated or groups of beaded silver grains and of aggregates of silver grains in each section was represented

PLATE 3.5

Micrographs a and b show aggregates of labelled axons overlying the outer longitudinal smooth muscle layer (LM). These were presumed to belong to the myenteric plexus. The adjacent border of the circular smooth muscle layer is just visible at the right top corner of each micrograph.

Magnification = x 1000

PLATE 3.5



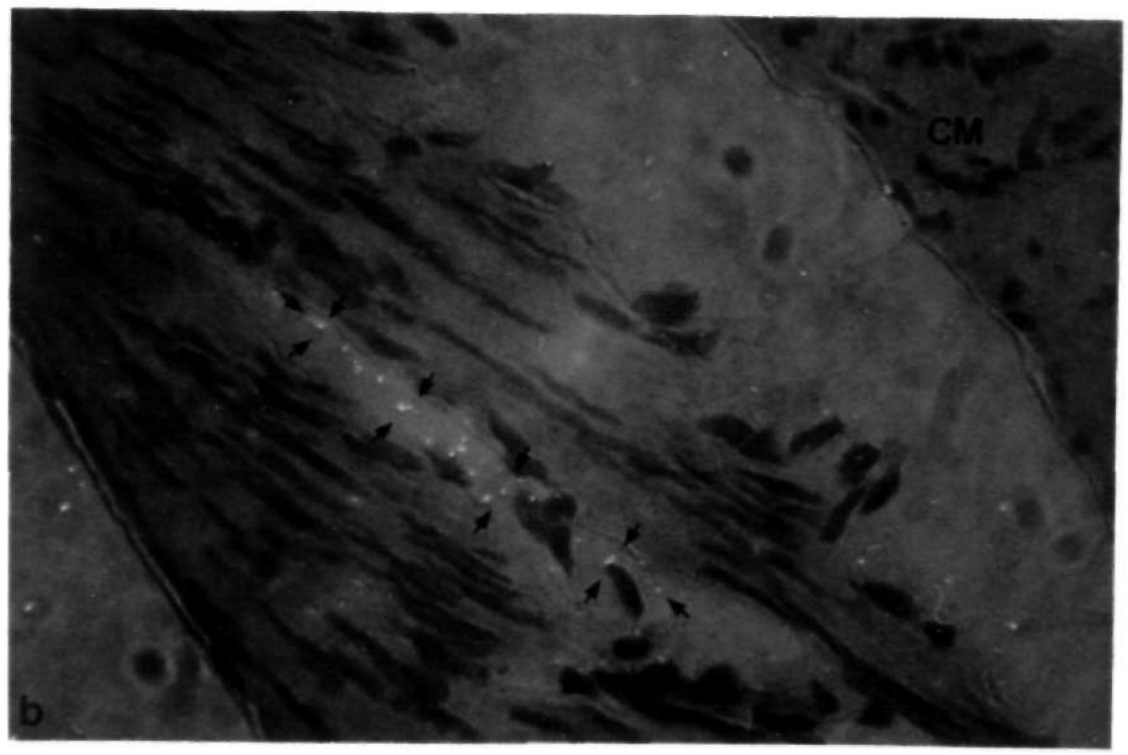


PLATE 3.6

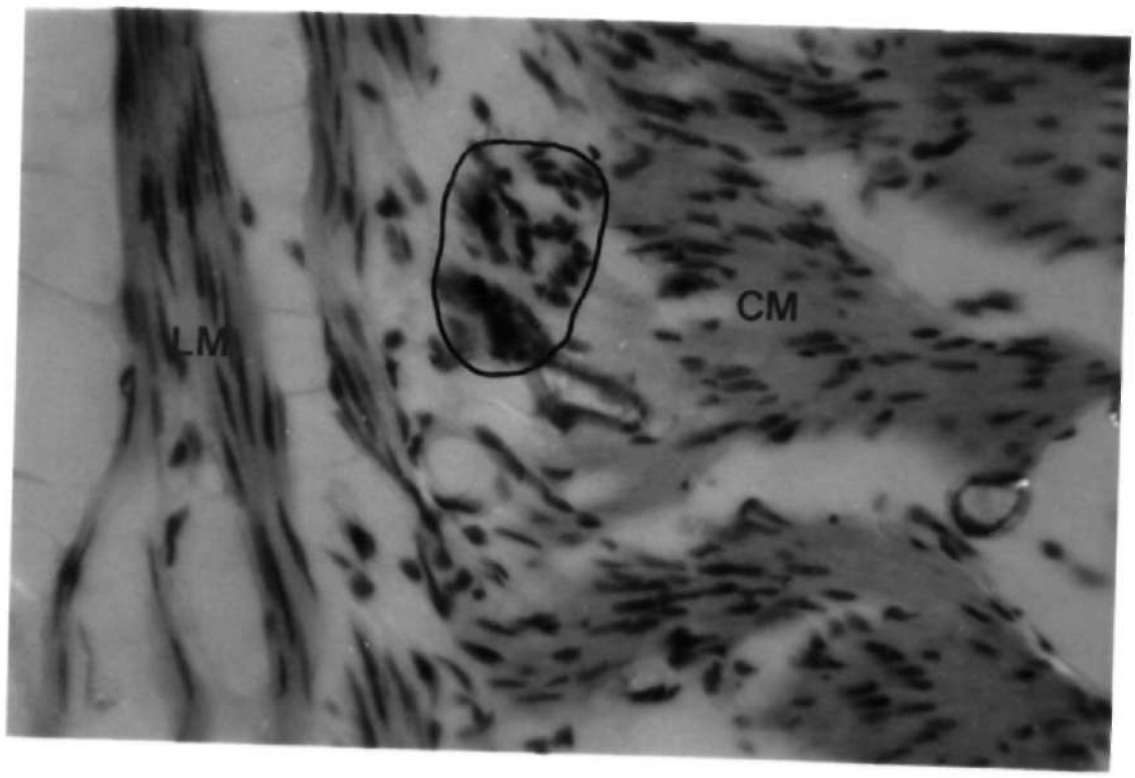
Light microscope autoradiograph of the myenteric plexus of the stomach of a control animal. The area within the circle contains no silver grains. Similar areas in experimental stomachs contain silver grains. Compare Plate 3.4a.

LM = Longitudinal smooth muscle layer.

CM = Circular smooth muscle layer.

Magnification = x 625

PLATE 3.6



by a corresponding number of positive (+) signs. Labelled axons were not seen in each and every tissue section taken from a tissue block. But the presence of axons in any of the sections was taken to mean the presence of labelled axons in the whole of the tissue block. If a sampled tissue block contained labelled axons, this positive result was extended to include the adjacent halves of the unsampled area of stomach on either side of the sample. The number of positive signs in each tissue block was used to represent the density of nerve axons in the block. Drawings of the anterior and posterior surfaces of the stomach representing the true stomach of the corresponding ferret were prepared and the results were entered onto the appropriate parts of the drawings. In this way, the stomach and the distribution of vagus nerve branches in it were reconstructed (Figures 3.5, 3.6 and 3.7).

(i) Distribution of Ventral Vagal Trunk

The three animals in this group had DTV before $[^3H]$ leucine was injected, therefore the axons that were labelled were those of ventral vagal trunk. In the first stomach examined, labelled axons were seen in all areas of the ventral surface except for a small area of about 5 mm square at the lower end of the body of the stomach adjacent to the greater curvature (Figure 3.5i). In the second and third stomachs, the whole of the ventral surface contained labelled axons. When the three stomachs were taken together, the positive labelling of all areas of the ventral

surface in the last two overlapped the area of the first stomach which contained no labelled axons (Figure 3.5 ii and iii). Therefore, it was concluded that the ventral vagal trunk was distributed throughout the fundus, body, pyloric antrum and the pylorus of the stomach in the ferret. The number of areas of positive axon labelling in each 5 mm square of stomach wall was determined (Table 3.2; Figure 3.8A). The fundus, the half of the body nearer the greater curvature and the pyloric antrum contained twice as many areas of labelled axons as the rest of the ventral surface. Labelled axons were not seen on the dorsal surface of the stomach in this group of animals.

(ii) Distribution of Dorsal Vagal Trunk

Ventral trunk vagotomy was done in the three animals in this group. Examination of the dorsal surface of each of the stomachs showed that axons were present in all areas of that surface. The number of positive areas per 5 mm square of stomach wall was similarly determined (Table 3.3; Figure 3.8C and D). The half of the dorsal surface of the body adjacent to the greater curvature has areas of positive labelling similar in number to the corresponding area on the ventral surface supplied by the ventral trunk. The fundus, the rest of the body, the pyloric antrum and the remaining part of the pylorus had lower numbers of positive areas.

FIGURE 3.8

This is a summary figure showing the mean number of areas of positive labelling per 5 mm square of tissue in different anatomical regions of the stomach.

A = normal distribution of the ventral trunk.

B = distribution of ventral trunk after chronic dorsal trunk vagotomy.

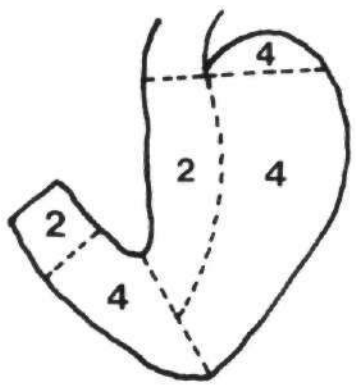
C = normal distribution of dorsal trunk on the ventral wall of the stomach.

D = normal distribution of dorsal trunk on the dorsal wall of the stomach.

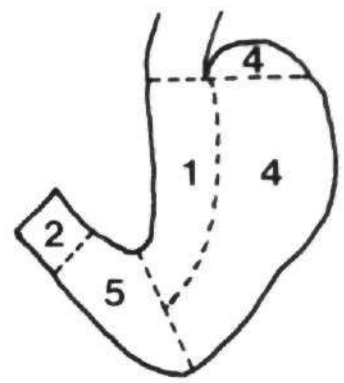
E = distribution of the dorsal trunk on the ventral wall after chronic ventral trunk vagotomy.

F = distribution of the dorsal trunk on the dorsal wall after chronic ventral trunk vagotomy.

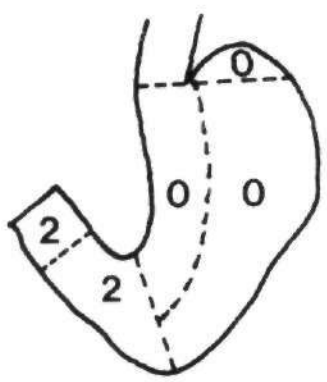
FIGURE 3.8



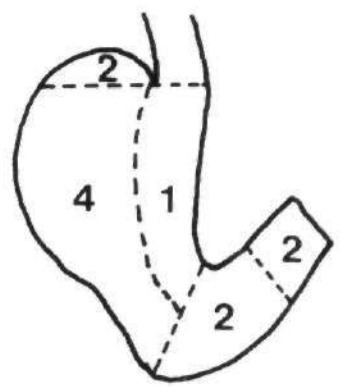
A



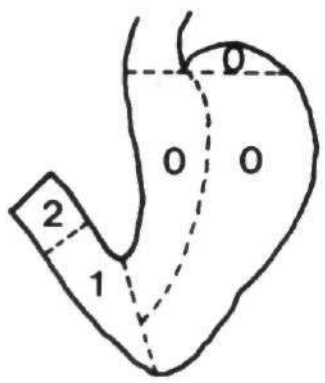
B



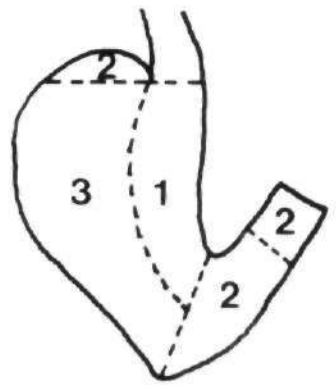
C



D



E



F

TABLE 3.2

Shows the number of areas of positive ventral trunk axon labelling per 5 mm square of different anatomical regions of the stomach wall. These numbers were derived from the raw data in Figures 3.5(i) and (ii) taking into account the number of millimetre square of each stomach block.

TABLE 3.2

Subdivision of Stomach	Normal distribution of ventral trunk				Distribution of ventral trunk after chronic dorsal vagotomy			
	Serial number of animal				Serial number of animal			
	1	2	3	Mean	1	2	3	Mean
Fundus	3	4	4	4	4	4	4	4
Body: near greater curve	4	5	4	4	4	4	4	4
Body: near lesser curve	2	2	2	2	1	1	1	1
Antrum	3	5	5	4	5	6	5	5
Pylorus	2	2	2	2	3	2	1	2

TABLE 3.3

Shows the normal distribution of the dorsal vagal trunk on the dorsal and ventral walls of the stomach. The numbers are those of areas of positive axon labelling per 5 mm square of different anatomical regions of the stomach. The numbers were derived as for Table 3.2.

TABLE 3.3

Subdivision of Stomach	Normal Distribution of Dorsal Trunk							
	Dorsal surface				Ventral surface			
	Serial number of animal				Serial number of animal			
	1	2	3	Mean	1	2	3	Mean
Fundus	2	1	2	2	0	0	0	0
Body: near greater curve	4	4	4	4	0	0	0	0
Body: near lesser curve	1	1	1	1	0	0	0	0
Antrum	2	2	2	2	2	2	3	2
Pylorus	3	2	2	2	2	2	2	2

The ventral surface of the pyloric antrum and the pylorus contained two areas of labelled axons per 5 mm square of stomach wall (Figure 3.8C). Therefore this part of the ventral surface of the stomach in the ferret is innervated by the dorsal vagal trunk as well.

d. Normal Distribution of Ventral and Dorsal Vagal Trunks in Submucosal (Meissner's Plexus)

Labelled axons were seen amongst the deep layers of the inner circular smooth muscle or immediately deep to the muscle, within the submucosa. The characteristic beaded and wave-like appearance of labelled axons as well as aggregates of silver grains in relation to submucosal ganglia were seen (Plate 3.5) but not regularly enough to permit a detailed reconstruction of the distribution of the dorsal or ventral vagal trunks in the submucosa. The number of labelled axons was very few. Labelled axons were seen only on the ventral surface of the stomach in animals with intact VT, whereas in those with intact DT, axons were seen on the dorsal surface of the stomach as well as on the ventral surface of the pylorus.

e. Innervation of the Gastric Mucosa

Examination of the gastric mucosa did not reveal any labelled pre-ganglionic efferent axons either in relation to the muscularis mucosae or the mucosal glands. Pre-ganglionic fibres did not reach the mucosa of the ferret stomach.

f. Effect of Unilateral Vagotomy on Gastric Vagal Innervation

Three weeks after dorsal trunk vagotomy, the distribution of the ventral vagal trunk was not different from normal. Labelled axons were not seen on the dorsal surface of the stomach (Table 3.2; Figure 3.8B). The number of areas of positive axon labelling is essentially the same as in the normal animal.

Three weeks after ventral trunk vagotomy, the pattern of distribution of the dorsal vagal trunk was similar to the normal pattern. Labelled axons were seen throughout the dorsal surface and on the ventral surface of the antrum and pylorus. There were no labelled axons on the ventral surface of the body and fundus of the stomach in this group of animals (Table 3.4; Figure 3.8E and F). The number of areas of positive axon labelling per 5 mm square of stomach wall is also similar to normal.

Labelled axons were scanty in the submucosal plexus and no detailed pattern of distribution could be made out for them. They were limited to the areas of the myenteric plexus in which their corresponding vagal trunks were found. Preganglionic axons were not seen in the mucosa.

TABLE 3.4

Shows the distribution of the dorsal vagal trunk three weeks after ventral trunk vagotomy. The numbers are those of areas of positive axon labelling per 5 mm square of different anatomical regions of the stomach wall.

TABLE 3.4

Subdivision of **Stomach**	Distribution of dorsal trunk after chronic ventral trunk vagotomy							
	Dorsal surface				Ventral surface			
	Serial number of animal				Serial number of animal			
	1	2	3	Mean	1	2	3	Mean
Fundus	2	2	1	2	0	0	0	0
Body: near greater curve	3	4	3	3	0	0	0	0
Body; near lesser curve	1	2	1	1	0	0	0	0
Antrum	1	2	2	2	1	2	1	1
Pylorus	1	2	3	2	2	2	2	2

DISCUSSION

The method of injecting $|^3H|$leucine into the DMN was not precise. For example, it was impossible to know precisely when the tip of the micropipette was exclusively within the DMN. Therefore leucine was almost always injected into the deeper and more superficial structures as well as into the DMN. Fortunately, as far as is known, the cell bodies of vagal efferent neurons to the stomach are exclusively located in the DMN. Therefore, labelling other pathways did not invalidate the result. Moreover, leucine is transported only in the anterograde direction. Therefore, injecting leucine into the nucleus of tractus solitarius, for example, would not have labelled GVA fibres in the vagus nerve. General visceral efferent fibres only would be labelled.

Since precise injection into DMN was not essential in order to label vagal efferent fibres to the stomach with $|^3H|$leucine, other more precise but cumbersome methods of injecting tracer materials such as microsyringe (pressure) injection and iontophoretic injection (Alheid *et al.*, 1981) were not used. As noted earlier, the animals often went into respiratory arrest. It was essential therefore that an injection method which would allow rapid withdrawal of the micropipette in order to resuscitate the animal was

used. The manual insertion of the micropipette into the brain tissue and the use of an uncomplicated source of pneumatic pressure in the form of a syringe, made this possible.

It would have been ideal to block out the whole stomach in paraffin and prepare all sections from every block for autoradiography. But even with the use of light microscope, it would be impractical and too time-consuming and might not give a better result than a carefully chosen sampling technique. Hence the stomach tissue blocks were sampled as described in Chapter Three. There are two other alternative sampling methods. The sampled blocks could be regularly alternated throughout the stomach, for example, blocks 7, 9, 11, 13, 15, 17, 19, 21 and so on. The other alternative would be to take blocks 7, 9, 11, 12, 14 and 16 from the appropriate stomach ring and then take blocks 18, 20, 23 and 25 from the next ring. The next ring to that would then be sampled as in the first ring, giving for example 27, 29, 31, 32, 34 and 36. Although the sampling technique used in this experiment may have a short-coming by leaving a whole vertical strip of tissue unsampled, it gave the greatest percentage sample. Moreover, the vagal trunks lie along the lesser curvature and from there give branches that spread towards the greater curvature. By making blocks from all of the stomach adjacent to the curvatures, it was possible to follow the spread of each trunk

either vertically along the curvatures or horizontally towards the greater curvature.

Tritiated leucine injected into the brain stem has to travel a distance of over 300 mm to reach the stomach. It is known that one of the disadvantages of autoradiographic tracing technique is that it is difficult to trace lightly labelled pathways over long distances (Künzle, 1975). But it is not known how heavily labelled the neurons of origin of vagal efferent fibres must be before the pathway, and particularly the distal parts, can be sufficiently labelled for easy identification. Therefore it was quite possible that some nerve fibres would be undetectable under the microscope if the efferent neurons were not heavily labelled.

Longitudinal sections of the stomach wall will only show the depth of the myenteric plexus which is the gap between the deep surface of the outer longitudinal smooth muscle layer and the superficial surface of the inner circular smooth muscle layer. This gap is very narrow. The myenteric ganglia are thin and spread, with their major surfaces parallel to the wall of gastro-intestinal tract. Therefore the cross-sectional area of ganglion cells seen in longitudinal sections will be less than that seen in sections taken parallel to the gastric surface. This also means that the number of vagal fibres that can be seen in longitudinal sections is limited. This may lead to both qualitative and quantitative false negative results.

In spite of this short-coming, longitudinal sections are most suited to the study of the distribution of axons in the myenteric plexus. Whereas a labelled axon lying transversely may be missed by sampled sections parallel to gastric surface, each longitudinal section cuts through the whole depth of the plexus and so is more likely to show such an axon. Sections parallel to the gastric surface on the other hand, are most suited to the study of intramural ganglia and synaptic structure. But since these are not clearly seen in light microscope autoradiography, sections parallel to gastric surface are not particularly beneficial in the present study.

It has been noted that at light microscope level, it is only radioactivity in the most superficial 2 to 3 µm of the tissue section that is recorded in the emulsion layer (Cowan and Cuénod, 1975). But 10 µm sections were used in these experiments. Therefore whatever radioactivity there was in the deeper layers of the tissue section has not been detected.

Although the myenteric plexus appears as a monolayer of ganglion cells and nerve processes, the preganglionic parasympathetic (vagal) fibres approach the ganglion cells from several directions. Therefore, when a longitudinal section is made, the vagal preganglionic nerve fibres are cut in various planes. Those that are cut at right angles to the longitudinal axes of the nerve fibres will give rise to single silver grains which will appear like dots. Since these are similar to background grains, such

positive labels may be taken for background grains and so will not be counted. This is unlikely to affect a qualitative analysis of vagal fibre distribution in the myenteric plexus. But it most certainly will affect quantitative estimations. In other words, false negative results are inevitable.

In the present study, only a rough estimate of the frequency with which axons were seen in the myenteric plexus was made. Comparisons of a quantitative nature could not be made between animals because of the difficulty of injecting equal amounts of $|^3H|$leucine into the DMN of the animals. Even if this could be done, because of the difference in length, metabolic rate and transport rate of the vagus nerves of various animals, the final concentration of $|^3H|$leucine in the myenteric plexus may vary from one animal to the other.

Distribution of Ventral Vagal Trunk Fibres in the Stomach

In this study, the ventral trunk of vagus nerve was found to innervate the whole of the ventral surface of the stomach including the pylorus. The dorsal surface of the stomach was not innervated by the ventral trunk. These findings are partly in agreement with some varieties of gastric vagal innervation in the ferret found by Mackay and Andrews (1983). Although these authors found that the ventral nerve of Latarjet, whether it arose from the ventral or dorsal vagal trunk or both, did not reach the pylorus, the pyloric branch of the hepatic division of the ventral trunk was found to consistently innervate the pylorus.

The presence of this branch could account for the identification of labelled ventral vagus nerve fibres in the pylorus. But the limit of distribution of nerve fibres as defined macroscopically is not necessarily what is seen in microscopic studies. Within the myenteric plexus, the ventral nerves of Latarjet could have descended further to reach the pylorus. An absolute proof of the extent of distribution of the ventral nerve of Latarjet can be obtained by transecting the pyloric branch of the hepatic division of vagus nerve along with DTV and studying the distribution of silver grains of ventral trunk origin in the pylorus.

Distribution of Dorsal Vagal Trunk Fibres in the Stomach

Apart from innervating the whole of the dorsal surface of the stomach (including the pylorus), the DT innervates the ventral surface of the pyloric antrum and the pylorus in the ferret. This is in contrast to the finding by Mackay and Andrews (1983) that the distal limit of the distribution of the DT is invariably the posterior aspect of the pyloric antrum, which corresponds with the distribution in man. But once again it may be argued that although gross dissection revealed such a limit, the nerve fibres, once they have entered the intramural plexus, may proceed further distally to reach the pylorus. The fact that the DT innervates the ventral surface of the antrum and pylorus in the present investigation is partly consistent with the discovery of a branch that arises from the DT and passes

onto the ventral surface of the pyloric antrum in Mackay and Andrews' (1983) series. Although they found a concurrent innervation of this surface of the pyloric antrum by branches of VT and DT in less than 10 percent of their subjects, the finding is significant and the three animals in the present study may be part of that small percentage. A large number of animals may have to be used before the variations can be seen.

The other alternative is that the VTV in the present series of experiments was done below the origin of the hepatic division. In that case, the fibres of the pyloric branch of this division were labelled and gave the wrong impression that it was the intact DT that innervated the ventral surface of the pylorus. This was unlikely because care was taken to divide the VT as near the diaphragm as possible. Moreover at the time the stomach was removed, the vagal trunks were examined to ensure that adequate vagotomy had been done.

This pattern of innervation by the ventral and dorsal trunks implies that the stomach receives a greater innervation from the DT. This is consistent with the finding that the DT of vagus below the diaphragm is significantly bigger than the VT at the same level (personal observation; Mackay and Andrews, 1983). Although a large branch of the DT goes to join the coeliac plexus, the area of stomach innervated by the DT is still wider than that innervated by the VT. The plexus formation in the thorax by the two vagal trunks allows exchange of fibres by the two trunks.

After the reformation of the vagal trunks in the lower part of the thorax, each trunk still contained fibres from the other. Moreover, the ventral trunk gave a branch (crossing branch) to the DT above the diaphragm. Some of the fibres in this branch may originally be from the LCV. It is possible that these fibres in the DT pass back to the ventral surface to innervate the pyloric antrum and the pylorus. If this assumption is accepted, it may explain why, in the ferret, stimulation of LCV and RCV separately resulted in intragastric pressure of equal amplitude in spite of the difference between the ventral and dorsal trunk size and fibre counts. In other words, the left and right cervical vagus nerves send equal numbers of fibres to the ventral and dorsal vagal trunks respectively. It may also explain why a cross over of DT branches to the ventral gastric surface has not been described in other mammalian vagus nerves with no crossing branch. In order to be able to show the origin of the DT fibres that innervate the ventral surface of the pyloric antrum and pylorus, it would be necessary to combine the injection of $|^{3}H|$leucine into the DMN with alternating unilateral acute cervical vagotomy so that the precise distribution of each cervical vagus nerve could be determined.

Effect of Chronic Vagotomy on the Distribution of Vagus Nerves

Three weeks after unilateral abdominal vagotomy, there was no compensatory spread of the surviving vagus nerve trunk. This is understandable if the increase in intragastric

pressure response to cervical vagal stimulation is entirely due to hypersensitivity and/or decreased resting tone of gastric smooth muscle. In both of these situations, there is no anatomical change in the preganglionic efferent fibres. Therefore no change in the pattern of innervation will take place after unilateral vagotomy.

Reactive sprouting from the surviving vagal trunk and regenerative sprouting from the proximal part of the transected trunk were advanced as possible mechanisms for the vagal functional recovery. If these were so, compensatory innervation of the denervated part of the stomach would be expected. Sprouting has been demonstrated in the brain of the rat (Lund and Lund, 1971; Lynch et al., 1973; Zimmer 1974), spinal cord of the cat (Liu and Chambers, 1958; Illis, 1964), and sympathetic nervous system of the cat (Murray and Thompson, 1957). It appears that all regions and fibre systems of the nervous system are not identical with regard to sprouting. For example, collateral sprouts from a particular source may invade one synaptically denervated area but not another (Murray and Golberger, 1974; Lynch et al., 1974). Therefore, the nature of whatever sprouting may take place in the vagus nerve may be quite different from what has been observed in the CNS by virtue of the distance of the stomach from the latter. Regenerative sprouting has been demonstrated in the vagus nerve of the rat, dog and man (Loup et al., 1980). Joffe et al. (1981) also found normal axons in the gastric wall of the rat seven weeks after highly selective vagotomy. Consistent with these findings is the

observation in the present series of experiments (Chapter 2) that regenerative sprouting from the ventral trunk took place three months after ventral trunk vagotomy. From the foregoing, there are four possible events taking place in the vagus nerve of the ferret after chronic vagotomy.

1. It may be that the only type of sprouting which takes place in this nerve is regenerative from the transected trunk.

2. Sprouting may require a longer time in the ferret vagus nerve than the three weeks period allowed in this experiment. It is possible that it progresses very slowly in the vagus nerve so that at the end of only three weeks, no collateral sprouts could be demonstrated.

3. It is not known if heavier labelling of the vagal pathway will be necessary to ensure adequate labelling of the axonal sprouts.

4. As mentioned above, another variety of sprouting that has been demonstrated in the detrusor muscle of the cat and human bladder arises from the intramural ganglion cells following transection of preganglionic vagal fibres. If this were to happen in the gastric intramural plexus, the present series of experiments would have been unable to detect it since the radioactive label was incapable of traversing the synaptic junction to reach postganglionic neurons within the short survival time (24-30 hrs) allowed in these experiments.

CHAPTER FOUR

ELECTRON MICROSCOPIC ANALYSIS OF VAGUS NERVE FIBRE COMPOSITION

CHAPTER FOUR

ELECTRON MICROSCOPIC ANALYSIS OF VAGUS NERVE FIBRE COMPOSITION

INTRODUCTION

The first group of experiments in which intragastric pressure responses to cervical vagal stimulations were measured after unilateral abdominal vagotomy, revealed two important findings (see Chapter 2):-

1. Acute or chronic VTV did not significantly influence intragastric pressure responses to cervical vagal stimulation.

2. There was greater gastric functional loss after acute DTV than after acute VTV as demonstrated by the significant reduction in intragastric pressure response to cervical vagal stimulation after acute DTV.

A knowledge of the fibre composition of cervical and abdominal trunks of the vagus nerve may help to explain these findings.

The composition of the vagus nerve has been determined in the rat (Gabella and Pease, 1973), cat (Foley and Dubois, 1937; Jones, 1937; Agostoni et al., 1957; Cottle and Mitchell, 1966; Mei et al., 1980), rabbit (Evans and Murray, 1954; De Neef et al., 1982), dog (Kemp, 1973), sheep and goat (Iggo, 1956), and man (Schnitzlein et al., 1958). The majority of these studies were done at light microscope level which usually resulted in underestimation

of the smallest fibres (Mei et al., 1980). In contrast, electron microscopy allows a more correct evaluation of fibre populations (Serratrice et al., 1978). Moreover, the ferret is becoming increasingly popular in physiological (Pfeiffer and Peters, 1969; Basso et al., 1971; Watson et al., 1973; Andrews and Bower, 1979; Andrews, Lawes and Bower, 1980), anatomical (Pfeiffer, 1970; Poddar and Murgatroyd, 1977; Mackay and Andrews, 1983), and pathological (Pfeiffer and Roth, 1970; Florczyk et al., 1981) studies. But it does not appear that the composition of the vagus nerve in the ferret has been determined. Therefore, an electron microscope study of the fibre composition of this nerve in the ferret would be desirable.

a. Properties of Mammalian Peripheral Nerve Fibres

The functions of nerve cells are the generation, propagation, and transmission of nerve impulses from one neuron to another or from a nerve to an effector organ. The rate at which the generated impulse is propagated or conducted along the axon of the neuron depends on

1. the size of the axon diameter,
2. the presence or absence of myelin around the axon,
3. the thickness of the myelin sheath, and
4. the internodal distance.

(i) Effect of Axonal Diameter on Impulse Conduction

As early as 1939, Hursh provided data to show that in the peripheral nervous system of the cat, axons above 4 μm diameter have conduction velocities which increase in line with the axon diameter. Myelinated axons below 4 μm in diameter are found to conduct at a rate smaller than predicted. Rushton (1951) explained that this deviation from the predicted conduction rate was due to the fact that the myelin sheath of smaller fibres is inappropriately thick. But recently, Ritchie (1982) explained that the effect of axon diameter on rate of conduction is also influenced by the intermodal length and that the critical axon diameter is 1 μm.

(ii) Effect of Myelination on Impulse Conduction

Myelination is believed to be closely related to axon diameter. For example, myelinated axons below 1 μm in diameter are rare in the peripheral nervous system (Rushton, 1951; Ritchie, 1982). But quite apart from the contribution of the axon size to the conduction velocity of myelinated axons, the mere presence of the myelin around the axon increases its capacity to conduct impulses at a faster rate than non-myelinated axons. The myelin sheath, which is a protein-lipid complex made up of many layers of unit membrane from the cell membrane of Schwann cells, envelops the whole length of the axon except at its ending and at periodic constrictions called the "nodes of Ranvier".

Myelin is a relatively effective insulator and current flow through it is negligible. The axon is presumed to be in contact with extracellular fluid only at the nodes. Hence propagated impulses jump from one node of Ranvier to the next along the axon. This type of conduction is called "saltatory conduction". The thickness of the myelin sheath and the length of the internodal distance affect the rate of conduction. Internodal distance increases with the diameter of the axon. Larger axons also tend to have thicker myelin sheaths. Therefore the thicker the myelin sheath and the longer the internodal distance, the greater the rate of conduction of impulses by myelinated axons.

b. Axon Degeneration

Most experiments on nerve fibre composition involve, at some stage, the placement of appropriate lesions and allowing a group of fibres to degenerate while the other group is analysed. Despite the extremely long axons of some neurons, it is the cell body that maintains the functional and anatomical integrity of the axon (Cajal, 1928). If the axon is transected, the part distal to the cut degenerates. This phenomenon is called anterograde or "Wallerian degeneration" (Figure 4.1). This degeneration involves a breakdown of the axon cylinder, the myelin sheath when present, and the synaptic boutons. The rate of degeneration is not the same for all fibre tracts or for fibres of different calibres (Blackstad et al., 1951;

FIGURE 4.1

Diagram to illustrate the normal features of a peripheral nerve fibre and its reaction to injury.

A - Normal fibre and synaptic junction.

B - Site of injury.

C - Features of degeneration in response to nerve transection.

Walberg, 1960). Anterograde degeneration may also proceed to involve the cell body to which the transected axon conducts impulses. This phenomenon is called anterograde transneuronal degeneration.

Primary retrograde degenerative changes also take place in the dendrites (Cerf and Chacko, 1958; Grant and Aldskogius, 1967), perikaryon and the whole length of the axon proximal to the lesion (Cowan, Adamson and Powell, 1961; Grant and Aldskogius, 1967). But the changes in the central part of the axon require a somewhat longer period of time to develop than the anterograde degeneration in the distal part (Powell and Cowan, 1964). A second order and even a third order retrograde (transneuronal) degeneration has been observed in the medial nucleus of the mammillary body and ventral trigeminal nucleus of the rabbit respectively (Bleier, 1969). When the central process of a bipolar afferent neuron is transected, the portion of the transected process on the cranial side of the cut degenerates. But the peripheral process is still attached to the neuron cell body and so it does not degenerate.

c. Aims of the Experiment

The aims of this experiment were to obtain sampled electron micrographs of the transverse sections of cervical and abdominal trunks of the vagus nerve, and using these, to determine -

(i) the total number of fibres in each trunk,

(ii) the myelinated:non-myelinated axon ratio in each trunk,

(iii) the axon diameter spectrum for myelinated axons in each trunk,

(iv) the relationship between axon diameter and myelination and between the former and myelin thickness,

(v) the afferent:efferent ratio in each trunk,

and

(vi) the effect of vagotomy rostral to the inferior vagal ganglion (supranodose vagotomy) on the characteristics of surviving fibres.

MATERIALS AND METHODS

a. Experimental Groups

Twelve adult ferrets of either sex were divided into three main groups (Table 4.1). The first group consisted of 6 animals which were used to study the composition of the complete vagal trunks. Under anaesthesia, the left cervical vagus (LCV), right cervical vagus (RCV), ventral vagal trunk (VT) and dorsal vagal trunk (DT) were taken from each of the animals for electron microscopy. The second group of 3 animals had left supranodose vagotomy (LSNV). The third group had right supranodose vagotomy (RSNV). The four vagal trunks were removed from each of these last 6 animals, at a later stage, for electron microscopy.

b. Anaesthesia

All animals were deprived of food overnight before the operations. The 6 animals in the first group were anaesthetised with Urethane in a dose of 1.5 g per kg. Prior to supranodose vagotomy (SNV), the animals were anaesthetised with Sagatal in a dose of 60 mg per kg and later with Urethane before the nerve trunks were removed for electron microscopy.

c. Supranodose Vagotomy

Supranodose vagotomy was done in order that the afferent-efferent characteristics of the vagus nerve trunks could be analysed (see "Principle for derivation

TABLE 4.1

	Normal				Supranodose Vagotomy							
					Left				Right			
Sampled Trunk	LCV	RCS	VT	DT	LCV	RCV	VT	DT	LCV	RCV	VT	DT
Number of Sampled Trunk	5	6	6	4	3	3	3	3	3	3	3	3
Number of Animals	6				3				3			

of afferent-efferent fibre counts" on page 188 for details).

The animal was laid on its back. A midline incision was made in the neck. The flexor muscles of the neck were retracted from the midline and the vagus nerve was exposed on one side of the neck. The nerve and the carotid artery were traced cranially until the latter deviated medially and disappeared from view. Rostral to this point of deviation of the carotid artery, the inferior vagal ganglion was seen with the hypoglossal and glossopharyngeal nerves passing deep to it. The dissecting microscope was sometimes used to obtain a good view of these structures. The vagus nerve rostral to the inferior vagal ganglion was carefully dissected out for a distance of about 5 mm with minimal handling of the ganglion. The available space was usually so small that two ligatures could not be placed round the nerve so as to be able to divide it between ligatures. Therefore, one ligature was placed as rostrally as possible and the nerve was transected just distal to the ligature. The incision was closed in two layers. A mixture of Neomycin sulphate BP, Polymyxin B sulphate BP and Bacitracin Zinc BP antibiotics (Polybactrin powder spray; Calmic Medical Division, The Wellcome Foundation Ltd., London) and Plastron plastic wound sprays were applied. The animals were allowed to recover from anaesthesia and survive for 21 days before the nerve trunks were removed for electron microscopy.

d. Sampling of Nerve Trunks

The same sampling procedure was used to obtain nerve specimens from all animals in the three groups. The samples were taken when the animal was alive under Urethane anaesthesia. The animal was laid on its back. A midline incision was made in the neck. A tracheostomy was done and a wide-bore plastic tube inserted and kept in place with a ligature. The LCV and RCV nerve trunks were exposed. Without further dissection, 3% glutaraldehyde fixative in 0.1M sodium phosphate buffer (see Appendix 4.1 and 4.2 for preparation of phosphate buffer and fixative) warmed to 37^{0}C, was poured onto the nerves so that both cervical trunks were soaked in the fixative (Mei *et al.*, 1980; K. Kapeller, personal communication). The nerve trunks were dissected out in the pool of fixative and left in it for 5 minutes. In this way, the nerves were partly fixed *in situ* so as to avoid their shrinkage. A small piece of each cervical vagus nerve was removed with a pair of scissors, at the level of the third tracheal ring. This was laid on a ruler and two or three 1 mm long pieces of nerve were taken from the middle portion using a sharp scalpel blade. Dyck and Karnes (1981) have found that nerve fibre calibre decreases slightly from the proximal to the distal end and that even in good health, it varies considerably within one internode. In spite of this variation, more than one sample per trunk had to be taken so that accidental losses could be replaced.

A thoraco-abdominal incision was made and the tracheostomy tube was connected to a ventilator. The ventral and dorsal vagal trunks were exposed just above the diaphragm. Fixative was poured on the nerves to partially fix them. After about 5 minutes, the nerves were dissected free of most of the surrounding tissues and 1 mm long samples taken. It was ensured that the dorsal trunk sample was taken below the point where the crossing branch (see page 9 and Figure 1.2) joined it and the ventral trunk sample taken below the origin of this branch. The animal was sacrificed and the sample sites examined to ensure that nerve tissue had been removed.

e. Primary Fixation

Four specimen vials were labelled according to the samples that were to be placed in them. They were filled with 3% glutaraldehyde at 37°C and the samples were transferred into them immediately after trimming them to size on a ruler. After one hour, the fixative in the vials was replaced with fresh fixative at room temperature. This was done because glutaraldehyde fixative is believed to deteriorate with time especially in the early part of fixation (K. Kapeller; personal communication). Fixation was allowed to continue for another 2 hours. The specimen vials were always filled to ensure a much larger volume of fixative as compared with the sample size (K. Kapeller; personal communication).

f. Washing of the Sample

At the end of the fixation period, the fixative was poured out and replaced with glucose-buffer solution (see Appendix 4.3). Two changes of glucose buffer wash were done at 30 minutes interval. This was followed by two more changes at hourly interval, giving a total of 3 hours wash in the buffer wash solution. The specimen vials were filled with fresh glucose-buffer wash solution and kept overnight at room temperature. The buffer wash solution was then replaced with a fresh one for one to two hours before secondary fixation.

g. Secondary (Post) Fixation

Secondary fixation was done with 2 percent osmium tetroxide in 0.1M Phosphate buffer (see Appendix 4.4). Having removed the glucose-buffer wash solution, and with rubber gloves worn, the specimen vial was filled with about 10 ml of osmium tetroxide fixative in a fume cupboard. These precautions were necessary because osmium tetroxide volatalises readily at room temperature and the vapour is harmful to the eyes, nose and throat. It also stains the skin for a long time. The specimens were fixed for one hour before dehydrating them. Fixation over one hour, rather than longer, was done in order to minimise the morphological effects, such as shrinkage of nerve myelin (Moretz et al., 1969) and tissue swelling caused by the cell membrane damaging effect of osmium tetroxide during fixation.

h. Dehydration of the Nerve Tissue

Osmium tetroxide was removed from the specimen vial. The tissues were washed twice with 75 percent ethanol in quick succession, by pouring the ethanol in and removing it carefully so that the tissues remained in the vial throughout the process of washing. By using the same procedure, the tissues were dehydrated for 10 minutes in each of a graded series of ethanol of increasing concentration as follows:

75 percent ethanol
95 percent ethanol
100 percent ethanol
100 percent ethanol
100 percent ethanol dried over copper sulphate.

A rapid dehydration schedule such as the one above, when carried out at room temperature, is believed to minimise the lipid extraction that takes place in ethanol (Korn and Weisman, 1966; Stein and Stein, 1971).

i. Pre-Embedding of the Specimen

The tissue was taken through a pre-embedding procedure which gradually introduced it to the embedding medium. This was necessary in order to facilitate the penetration of the tissue by the embedding medium. The pre-embedding solvent used was propylene oxide (Epoxy propane; BDH Chemicals Ltd.) which was placed over the specimen in the specimen vial for 30 minutes. The solvent was removed and

replaced with a 50:50 mixture of propylene oxide and Araldite CY212, Ciba, (see Appendix 4.5 for preparation of Araldite). One drop (about 0.02 ml) of accelerator (Dodecenyl succinic anhydride; DDSA) for each ml of Araldite was added to the mixture which was mixed thoroughly before pouring it into the specimen vial. The vial was covered and placed on a rotator overnight. The continuous rotation of the specimen vial exposed the specimen to fresh Araldite throughout the pre-embedding stage. In the morning, the pre-embedding mixture was replaced with fresh Araldite to which a drop of accelerator for each millilitre of Araldite was added. The vial was placed on the rotator for 5 to 6 hours with the cover removed so that any remaining solvent could evaporate.

j. Embedding and Polymerisation of the Tissue

The tissue was embedded in such a way that it was possible to orientate the resulting Araldite block on the microtome and obtain transverse sections of the nerve. Therefore, instead of embedding the tissue in gelatin capsule, a shallow plastic pot was used. Fresh Araldite was taken and accelerator was added in the usual proportion. These were thoroughly mixed in a graduated polyethylene beaker. A small piece of paper on which the block identification (laboratory) number was typed was placed at the bottom of the pot. The pot was filled with Araldite and the specimen was transferred from the

specimen vial into the embedding pot using orange sticks. The specimen was laid horizontally on its side in the Araldite. The pot was then placed in an oven in order to polymerise the Araldite for 48 hours. The temperature of the oven was maintained at 60°C throughout the period of polymerisation.

k. Final Tissue Orientation in Araldite Block

When polymerisation was complete, the pot was removed from the oven and allowed to cool. The Araldite cast was removed and the nerve tissue was cut out leaving a tiny rim of Araldite around it. One end of an Araldite block moulded in gelatin capsule was flattened. The block was orientated vertically with the flattened surface upwards. A 1:1 mixture of Araldite rapid (Ciba-Geigy) and hardener was placed on the flat top of the block. The specimen was held vertically in the Araldite. The Araldite block and the specimen were placed vertically in an oven at 60°C for about 30 minutes. By the end of this period, the Araldite had hardened and the block was ready for cutting on the ultramicrotome.

l. Procedure for Coating Copper Grids with Formvar

Before cutting the tissue block on the ultramicrotome, copper grids (200 mesh, unmarked centre, 3.05 mm) were coated with 1 percent or 0.5 percent formvar in chloroform. The formvar was prepared by dissolving 1 mg or 0.5 mg of the powder in 100 ml chloroform in a stoppered bottle. A

microscope glass slide was dipped briefly in the formvar and allowed to dry. The thin film of formvar was floated from the glass slide onto water in a dish. The grids were individually laid on the film with the dull side of the grids in contact with it. The film and grids were lifted out of water with a filter paper, allowed to dry and kept in a covered petri-dish.

m. Sectioning of the Tissue

A trapezium-shaped block face was made by trimming off Araldite from the sides and surface of the block until nerve tissue was reached. Tissue sectioning was done on Cambridge Huxley ultramicrotome or Reichert's ultramicrotome "OM-U3". Glass knives were made on the Knife maker type 7800 (LKB, Burma) or type 7801A. The knives were converted into water boats into which the cut sections were floated. The boats were made with pressure sensitive tape (Scotch) in such a way that the edges of the boat were completed by the knife edge. Semithin (approximately 1.0 μm) sections were taken and stained with toluidine blue and examined under the light microscope. The initial examination of a semithin section was essential in order to see if

(i) the whole cross section of the nerve had been cut,

(ii) there were holes in the section, and

(iii) the cross section obtained was perpendicular to the long axis of the nerve.

When the above criteria were satisfied, two or three

semithin sections were cut and carefully floated onto strips of glass cover slips. The sections were stained, labelled and kept away so that they could be used later to measure the total cross-sectional area of the nerve trunk. A ribbon of ultrathin sections (60-80 nm) which appeared gold or silver was cut onto the surface of the water in the boat. Depending on the size of the sections, two or three sections were floated onto the dull surface of a formvar-coated copper grid by lowering the grid into the water, under the sections, using a pair of tweezers, and gently floating the sections onto the grid. The grid was placed on a moist filter paper to dry. Two or three such grids were prepared from a tissue block from each of the four vagus nerve trunks taken from each animal.

n. Staining of the Sections

(i) Semithin Sections

The sections on the supporting glass strip were dried by holding the latter in front of a gentle stream of air from a hair dryer. The drying was completed on a warm plate. A drop of toluidine blue stain (Appendix 4.6) was placed on the sections and the glass strip was placed on the warm plate until the stain was seen to dry along the edge of the glass strip, leaving a green deposit. The elevated temperature produced an adequate intensity of stain (Ito, 1965). The glass strip was removed from the plate and excess stain washed off with distilled water. The sections were further washed in a stream of

50 percent ethanol in distilled water. They were dried on a warm plate. The glass strip was placed on a microscope glass slide, mounted in DPX mounting medium, and covered with a cover slip.

(ii) Ultrathin Sections

Frasca and Parks (1965) found that double staining with uranyl acetate and lead citrate produced more contrast than either stain alone. It has also been found that alcoholic solutions of uranyl acetate give better penetration of stain into sections than aqueous solutions. Therefore these sections were stained in uranyl acetate in 50 percent ethanol in water (Gibbons and Grimstone, 1960; Epstein and Holt, 1963) and secondarily in lead citrate.

The sections were stained in petri-dishes filled with dental wax. The surface of the wax was cleaned and separate drops of uranyl acetate solution (see Appendix 4.7) were placed on it using a Pasteur pipette. In order to avoid picking up contaminants from the surface of the stain, the bulb of the pipette was squeezed firmly before inserting it into the stain. The first two or three drops of stain were rejected before putting the drops in the staining dish. A copper grid supporting the sections, was floated onto the surface of each drop of stain with the section surface facing downwards. The petri-dish was immediately covered to protect the photolabile uranyl acetate from light. The sections were stained for 15 to 30 minutes. The grids were removed one

at a time with a pair of watchmaker's tweezers. The sections were immediately dipped several times in a clean beaker of distilled water and further washed in a stream of distilled water, from a wash bottle, for about one minute. Excess water between the prongs of the tweezers was removed with a piece of filter paper and the grid was placed on a moist filter paper to dry.

The sections were next stained in lead citrate (Appendix 4.8) using a separate wax-filled petri-dish usually set apart for this purpose. Pellets of sodium hydroxide were placed in the petri-dish in order to remove carbondioxide (CO_2) from the air in the dish. A clean Pasteur pipette was taken and the bulb squeezed at a constant pressure. Briefly lifting the stopper from the bottle containing the stain, the pipette was inserted well under the surface of the staining solution. The precautions were taken in order to avoid introducing CO_2 into the staining solution or removing contaminants from the surface of the stain. The stopper was quickly replaced and the lid of the petri-dish was lifted. Having rejected the first two or three drops of stain, the required number of stain drops was quickly squeezed onto the dental wax and the lid of the petri-dish was replaced. By using a pair of watchmaker's tweezers, the grids were floated onto the surface of the stain drops, replacing the lid of the petri-dish after each grid transfer. The sections were stained for two to three minutes and washed, initially by dipping each grid several times in a clean beaker of distilled

water, and then under the jet of a wash bottle for one minute. Excess water was removed from the space between the prongs of the tweezers with filter paper, before placing the grids on moist filter paper to dry. The grids containing sections from each vagus nerve trunk were stored together in a clean, dry and labelled petri-dish lined with dry filter paper.

The procedure has been described in more than usual detail because a preliminary application of the routine procedure to the vagus nerves of the rat and ferret gave good sections in the rat but not in the ferret. Modifications were therefore made as described above.

o. Procedure for Obtaining Electron Micrographs of Nerve Sections

(i) Electron Microscope

The specimens were viewed under Philips 200 electron microscope. The microscope had an in-built camera which permitted the recording of photographs on 35 mm black and white films. The sections from each trunk of the vagus nerve were first scanned under low power so that the best section could be selected for micrography. Sections with contaminants or holes in the formvar coating were rejected. The photographs were recorded at a "constant" magnification (position 8 on the magnification dial). But because the true magnification often fluctuated with the electron beam in the microscope, a standard copper grid which contained 2160 lines per mm was photographed at the beginning of each

roll of film and photographic session. The printed micrograph of this grid was used to calculate the true magnification of the printed micrographs of the nerve trunk section.

(ii) Sampling Technique on the Electron Microscope

A preliminary scanning of sections from cervical and abdominal vagus nerves in three normal ferrets showed that the axons were not randomly distributed in terms of their calibre. For example, small myelinated fibres tended to aggregate in one area of the section (Plate 4.1). Degenerated fibres in sections from ferrets which had supranodose vagotomy also aggregated in a limited area of the section (Plate 4.2). Therefore it was necessary to use a sampling method that would not bias any fibre group. A systematic stratified sampling technique was used (Weibel et al., 1966; Drs. M. A. Williams and G. H. Cope, personal communications). The 200 mesh grid on which the sections were supported, was placed randomly in the specimen holder of the electron microscope so that the orientation of the section was random with respect to the microscope screen. But the grids were adjusted so that the grid lines could lie vertically and horizontally on the screen and so correspond with the margins of the rectangle on the screen which represented the actual area being photographed. A ready-made stratification of the section was achieved by using the grid bars. Each stratum was a square between four grid bars (Figure

4.2). Top left and bottom right quadrants of each grid square were photographed in the case of sections prepared from the cervical vagal trunks. Despite the incomplete occupation of some quadrants by axons when the former fell over the peripheral parts of the nerve trunk, photographs were taken from them. In the case of the sections taken from abdominal vagal trunks, photographs were taken from all quadrants since abdominal vagal trunks were usually so small that taking only two sample micrographs from each grid square would result in too small a sample size.

A similar procedure was used to obtain sampled micrographs from sections of nerves prepared after supranodose vagotomy.

p. Micrograph Analysis

The true magnifications of the recorded images on the films ranged between 1100 and 1200. These were magnified by a factor of about 6.5 to obtain micrographs of final magnification between 7000 and 8000. This magnification was just high enough to make the smallest axons visible. Although a high enough magnification should be used to avoid oversampling of small fibres, too high a magnification would also result in cropping of fibres, especially large ones (Bronson et al., 1978). Data were collected from the micrographs using

1. Commodore CBM Computer Model 4032 linked to
   a. Commodore CBM 5¼ inch dual drive floppy disc, model 4040,

FIGURE 4.2

This illustrates the sampling technique which was used to obtain photographs on the electron microscope. The marked top left and bottom right quadrants were sampled from cervical vagus nerves. These quadrants as well as the unmarked top right and bottom left quadrants were sampled from ventral and dorsal trunks. Incomplete frames of nerve tissue were also included.

b. Commodore Tractor Printer 4022, and

c. Bit Pad One (resolution = 0.1 mm).

2. MOP - AM03 (Kontron Messgeraete GMBH).

(i) Data Collection with Commodore Computer

A programme was prepared and fed into the computer in order to count the non-myelinated axons and obtain two circumference measurements from each myelinated axon - an external circumference round the outer edge of the myelin and an internal circumference along the inner edge of the myelin. The mathematical part of the programme assumed that the myelinated axons were circular. The micrographs were placed on the Bitpad. A predetermined button on the cursor was pressed while tracing the inner and outer boundaries of myelin surrounding the myelinated axons. The perimeters so measured were displayed in millimetres on the computer screen. From them, myelinated axon diameters and myelin thickness were calculated and displayed in micrometres and individually stored. A cumulative count of myelinated fibres was also displayed and stored. Another button was selected and pressed in order to obtain counts of non-myelinated axons. When all micrographs in the sample had been analysed, the individually stored data and total non-myelinated axon counts were printed out. By selecting and operating appropriate parts of the programme, the computer calculated and printed out histogram figures for the size-frequency distribution

of myelinated axons with respect to axon diameters and myelin thickness.

(ii) Data collection with the MOP

The MOP was used to measure the total area of the sampled nerve tissue (Total Sample Area), and the cross-sectional area of the corresponding vagus nerve trunk. The area of nerve tissue in each micrograph in the sample was measured by tracing the boundary of the tissue with a stylus. All such areas were added together to give the total sample area from the nerve trunk. The exact final magnification of each micrograph was used in measuring these areas.

A light microscope with a drawing side tube was used in conjunction with the MOP to measure the cross-sectional area of each nerve trunk. A graticule was inserted into the microscope. The lines of the graticule were relayed through the side tube of the microscope and superimposed on a graph paper placed on the Bitpad under the side tube. With the eyepiece of the microscope fixed, an objective which had been found to allow the microscope field to contain the whole nerve cross section was chosen and kept constant for all measurements. The magnification of the field was calculated from the superimposed graticule and graph paper lines. This magnification was fed into the MOP as a factor for measuring the true cross-sectional areas of the nerve trunks.

The image of a semithin section of whole nerve trunk was projected through the side tube of the microscope onto the graph paper. The outline of the inner edge of the epineurium of the nerve trunk seen on the graph paper was traced with a cursor. The area of each nerve trunk was printed out in square micrometres.

Micrographs and semithin sections of vagal nerve trunks from ferrets in which supranodose vagotomy was done were treated in exactly the same way as those from normal ferrets.

q. Data Analysis

The axon profiles in the contralateral cervical vagus nerve of animals which had unilateral supranodose vagotomy, were normal. Axon counts and measurements from such normal trunks were pooled with those from animals in the normal group.

(i) Total Fibre Count

The total number of axons in each nerve trunk was obtained by adding the number of myelinated and non-myelinated axons together.

(ii) Proportions of Myelinated and Non-myelinated Axons

The ratio of myelinated to non-myelinated axons was obtained by dividing the number of myelinated axons in each nerve trunk by the number of non-myelinated axons in the same trunk. The percentages of myelinated and non-myelinated axons were found.

(iii) Measurement of Myelinated Axon Diameter

Some axons were partly cut off by the edge of the micrograph. All non-myelinated axons which had their centres within the micrograph were counted. Myelinated axons which had their complete internal circumference within the micrograph were measured. The missing parts of the myelin in such axons were added manually with a pencil before carrying out the measurements.

The myelinated axons were assumed to be circular for the purpose of these measurements. Therefore, using the equation for the circumference of a circle,

$$2\pi r = p$$

where p = circumference measurement

r = radius

and π = a constant (3.142).

Dividing both sides of the equation by π gives

$$2r = \frac{p}{\pi}$$

But 2r is the diameter, d, of the circle (axon in this case).

Therefore axon diameter $= \frac{p}{\pi}$ mm

(measurements were primarily in millimetres).

The axon diameters were then converted to μm.

(iv) Measurement of Myelin Thickness

Two circumference measurements were obtained from each myelinated axon. The external circumference was around the outer edge of the myelin whereas the internal circumference was around the inner edge of the myelin (Figure 4.3). From the external and internal circumference measurements, corresponding diameters b and a were obtained.

Subtracing a from b,

$$b - a = 2t$$

where t = myelin thickness

Dividing this by 2 gave the myelin thickness.

(v) Afferent-Efferent Counts and Ratio

(I) Principle for Derivation of Afferent-Efferent Counts

The vagus nerve is a mixed nerve containing afferent and efferent axons. These axons cannot be differentiated from one another under the electron microscope without differential labelling. One way of counting the two types of fibres separately is to destroy one and count the other and vice versa. The abdominal afferent neurons within the vagus nerve have their cell bodies in the inferior vagal (nodose) ganglion. If the vagus nerve is transected rostral to the ganglion, the efferent fibres, with their cell bodies in the DMN, will degenerate distal to the cut. But the afferent fibres distal to the cut will remain intact because they still maintain contact with their mother cells. If sufficient time is given for

FIGURE 4.3

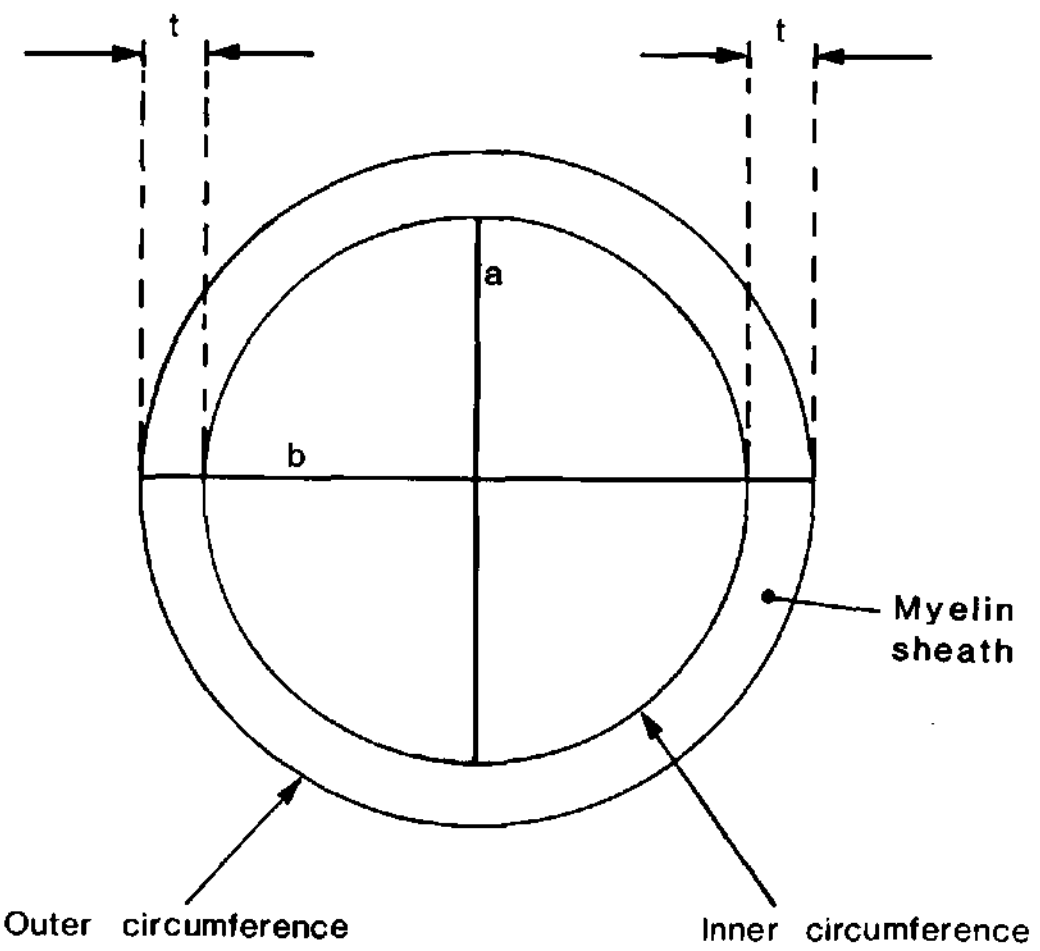
Schematic diagram of a myelinated axon assumed to be circular for mathematical purposes.

a = Internal axon diameter

b = External axon diameter

t = Myelin thickness.

FIGURE 4.3


t
t
a
b
Myelin sheath
Outer circumference
Inner circumference

the efferent fibres to degenerate, examination of the nerve trunk below the cut will show degenerated and intact axons. Transecting left and right vagus nerves simultaneously above the inferior vagal ganglion will lead to vagal efferent denervation of vital thoracic and abdominal organs such as the heart and lungs. The animal may not survive for long enough to allow all efferent fibres to degenerate. Therefore alternating unilateral supranodose vagotomy is done in different groups of animals. If a left supranodose vagotomy is done, for example, efferent fibres in the LCV will degenerate leaving afferent fibres only. But because of the mixing of fibres from LCV and RCV in each of the abdominal trunks, the ventral abdominal trunk, although it appears to derive more from the LCV (personal observation), will contain normal efferent fibres from the RCV as well.

(II) Mathematical Derivation of Afferent-Efferent Counts

a. Afferent-Efferent Fibre Counts in Cervical Vagus Nerve (Figure 4.4)

Suppose

Total number of axons in LCV = A

Number of afferent axons in LCV after LSNV = B

Then

Number of efferent axons in LCV = A - B

The same approach applies for fibre counts in RCV.

b. Afferent-Efferent Fibre Counts in Abdominal Vagal Trunks (Figure 4.4)

Suppose

Total number of axons in ventral trunk = A_V

Total number of axons in dorsal trunk = A_D

After LSNV:

Total number of axons in ventral trunk = L_V

Total number of axons in dorsal trunk = L_D

$A_V - L_V$ = Number of efferent axons in ventral trunk arising from LCV

$A_D - L_D$ = Number of efferent axons in dorsal trunk arising from LCV

After RSNV:

Total number of axons in ventral trunk = R_V

Total number of axons in dorsal trunk = R_D

$A_V - R_V$ = Number of efferent axons in ventral trunk arising from RCV

$A_D - R_D$ = Number of efferent axons in dorsal trunk arising from RCV

Therefore

Total number of efferent axons in ventral trunk = $(A_V - L_V) + (A_V - R_V)$

and

Total number of efferent axons in dorsal trunk = $(A_D - L_D) + (A_D - R_D)$

Subtracting each of these efferent axon counts from the total fibre count in the corresponding abdominal vagal trunk gives the number of afferents in each trunk. The different axon counts were expressed as a percentage of the total number of axon counts in the corresponding vagal trunk.

FIGURE 4.4

Schematic diagram to illustrate afferent and efferent fibre components of the vagus nerve in the ferret. Diagram I shows the normal cervical and abdominal vagal trunks.

Af = Afferent fibres

Ef = Efferent fibres

NG = Inferior vagal (Nodose) ganglion

RCV = Right cervical vagus

LCV = Left cervical vagus

DT = Dorsal vagal trunk

VT = Ventral vagal trunk

A_D = Total number of fibres in DT

A_V = Total number of fibres in VT

Diagram II shows the four vagal trunks with a left supranodose vagotomy (LSNV) and degenerated distal efferent fibres in LCV, DT and VT.

L_D = Number of fibres left in DT after LSNV

L_V = Number of fibres left in VT after LSNV

FIGURE 4.4

Schematic diagram to illustrate afferent and efferent fibre components of the vagus nerve in the ferret. Diagram I shows the normal cervical and abdominal vagal trunks.

Af = Afferent fibres

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RCV = Right cervical vagus

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DT = Dorsal vagal trunk

VT = Ventral vagal trunk

A_D = Total number of fibres in DT

A_V = Total number of fibres in VT

Diagram II shows the four vagal trunks with a left supranodose vagotomy (LSNV) and degenerated distal efferent fibres in LCV, DT and VT.

L_D = Number of fibres left in DT after LSNV

L_V = Number of fibres left in VT after LSNV

RESULTS

a. Qualitative Description of Microscopic Appearance of Vagus Nerve Fibres

(i) Normal Cervical Vagal Trunks

The axons seen in most microscope fields varied a great deal from one another, in their calibre, although in certain parts of the cross section, small myelinated axons outnumbered large ones. The myelinated axons were of varying shapes. Some were oval whereas others had boomerang or crenated shape (see Plates 4.3 and 4.4). The profile of a single axon is known to depend on the level along the axon from which the section is taken (Dyck and Karnes, 1981). The oval-shaped axons are from the middle portion of the internode. The crenated shape is obtained near the node of Ranvier whereas the boomerang shape is from an intermediate position between the two. In most of the irregularly shaped myelinated axons, the areas of indentation were occupied by Schwann cell cytoplasm or nucleus. One Schwann cell was not seen to surround more than one myelinated axon. On the contrary, three or even five non-myelinated axons could be surrounded by one Schwann cell (Plate 4.3). Many non-myelinated axons were seen which were as large as, or even larger than, some small myelinated axons and yet the former are not myelinated. The axons contained large number of neurotubules and neurofilaments, and were surrounded by bundles of collagen connective tissue fibre. Some blood vessels

were seen in the perineurium and epineurium of the nerve trunks (Plates 4.4 and 4.5).

(ii) Normal Abdominal Vagus Trunks

These trunks were much smaller than the cervical vagal trunks. Their axons were almost entirely non-myelinated (Plate 4.6). The non-myelinated axons were mainly in groups of two to five surrounded by a Schwann cell, but some single axon profiles were seen surrounded by separate Schwann cells. Inside the axons were neurotubules and neurofilaments. Some large non-myelinated axons were present. Blood vessels were also present in the abdominal vagal trunks. A few myelinated axons were present but they appeared mainly small.

(iii) Cervical Vagus Nerve Trunks after Unilateral Supranodose Vagotomy

After a unilateral supranodose vagotomy, the contra-lateral cervical vagus nerve was normal. The axons were similar to those of normal cervical nerves described above. The ipsi-lateral vagus nerve trunk below the vagotomy site showed areas of degenerated myelinated and non-myelinated axons, although the latter were by far the majority. Plate 4.2 shows the tendency for degenerated axons to aggregate in a part of the cross section of the nerve. These axons had lost their outline and intra-axonal structures could not be identified. Plate 4.7 shows the broken down myelin sheath of a degenerated myelinated axon. Schwann

PLATE 4.1

Light micrograph of a cervical vagal trunk showing the non-uniform distribution of myelinated (My-Ax) and non-myelinated (N-Ax) axons. Non-myelinated axons are seen aggregated along the epineurium (Ep) in the top right section of the nerve trunk.

Ves = Blood vessel

Magnification = x 150

PLATE 4.2

Electron micrograph of left cervical vagal trunk after left supranodose vagotomy showing non-uniform distribution of afferent and efferent fibres. Afferent fibres remained normal after vagotomy and are seen collected at the top right corner of the micrograph (normal side of the line). On the "degenerated" side of the line, degenerated fibres are predominant.

deg = degenerated axon

Per = perineurium

Nu = Schwann cell nucleus

Magnification = x 10100

PLATE 4.3

Electron micrograph of a cervical vagal trunk showing a variety of shapes of myelinated axons (My-Ax).

Ov = oval-shaped myelinated axon

Bo = boomerang-shaped myelinated axon

A Schwann cell (Sch5) is seen surrounding up to five non-myelinated axons (N-Ax) and another one surrounding only one non-myelinated axon (Sch1).

Magnification = x 10700

PLATE 4.3

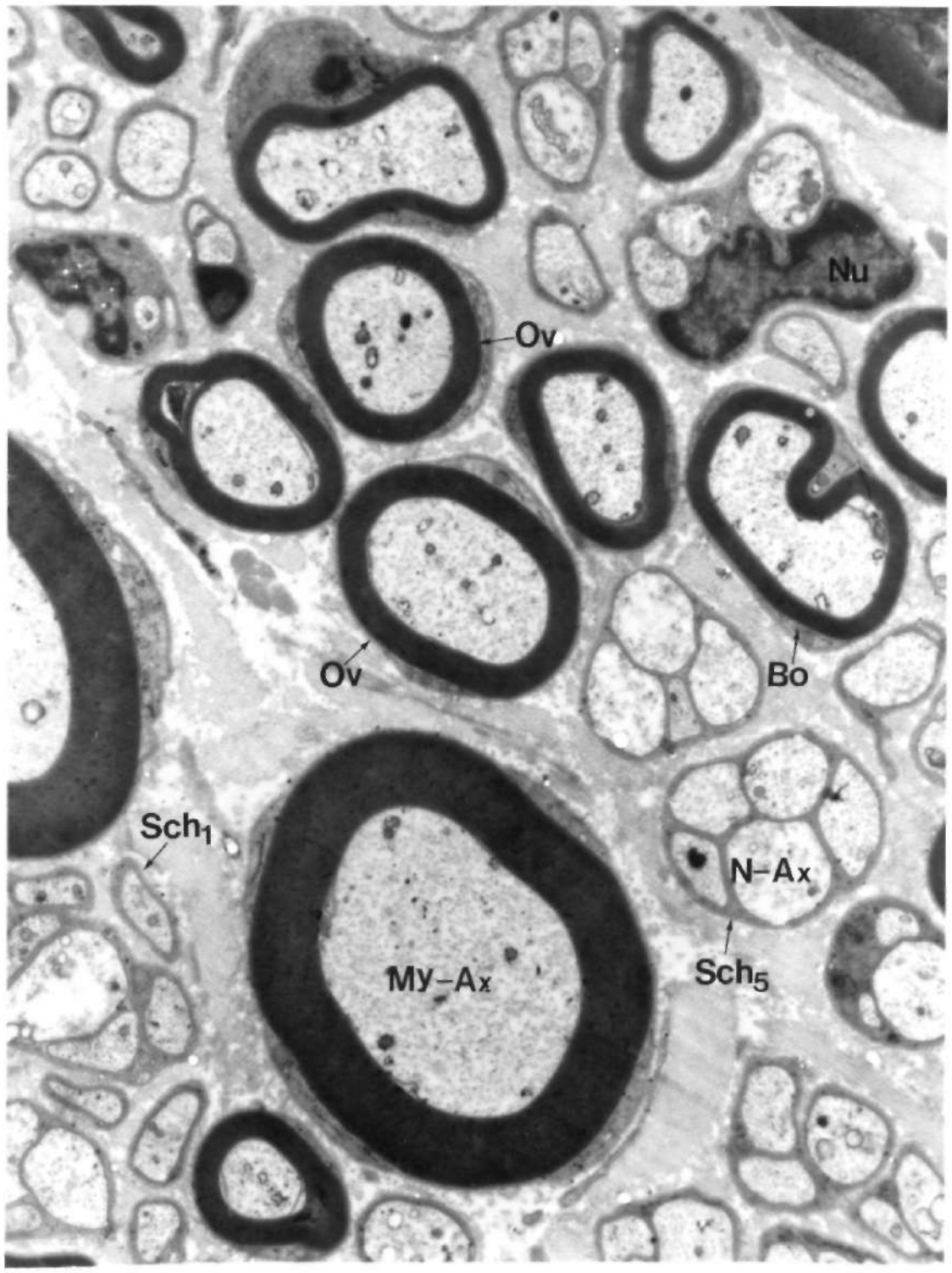


PLATE 4.4

Electron micrograph of cervical vagal trunk showing a crenated-shaped (Cr) myelinated axon and a blood vessel (Ves) in the perineurium.

Col = collagen fibres cut transversely and obliquely.

Magnification = x 9700

PLATE 4.4



PLATE 4.5

Electron micrograph of cervical vagal trunk showing a blood vessel (Ves) containing a phagocytosed nucleus of a Schwann cell (Nu).

Magnification = x 12500

PLATE 4.5



PLATE 4.6

Electron micrograph of abdominal vagal trunk showing predominantly non-myelinated fibres (N-Ax). One myelinated axon (My-Ax) only is seen in this field.

Magnification = x 11400

PLATE 4.6

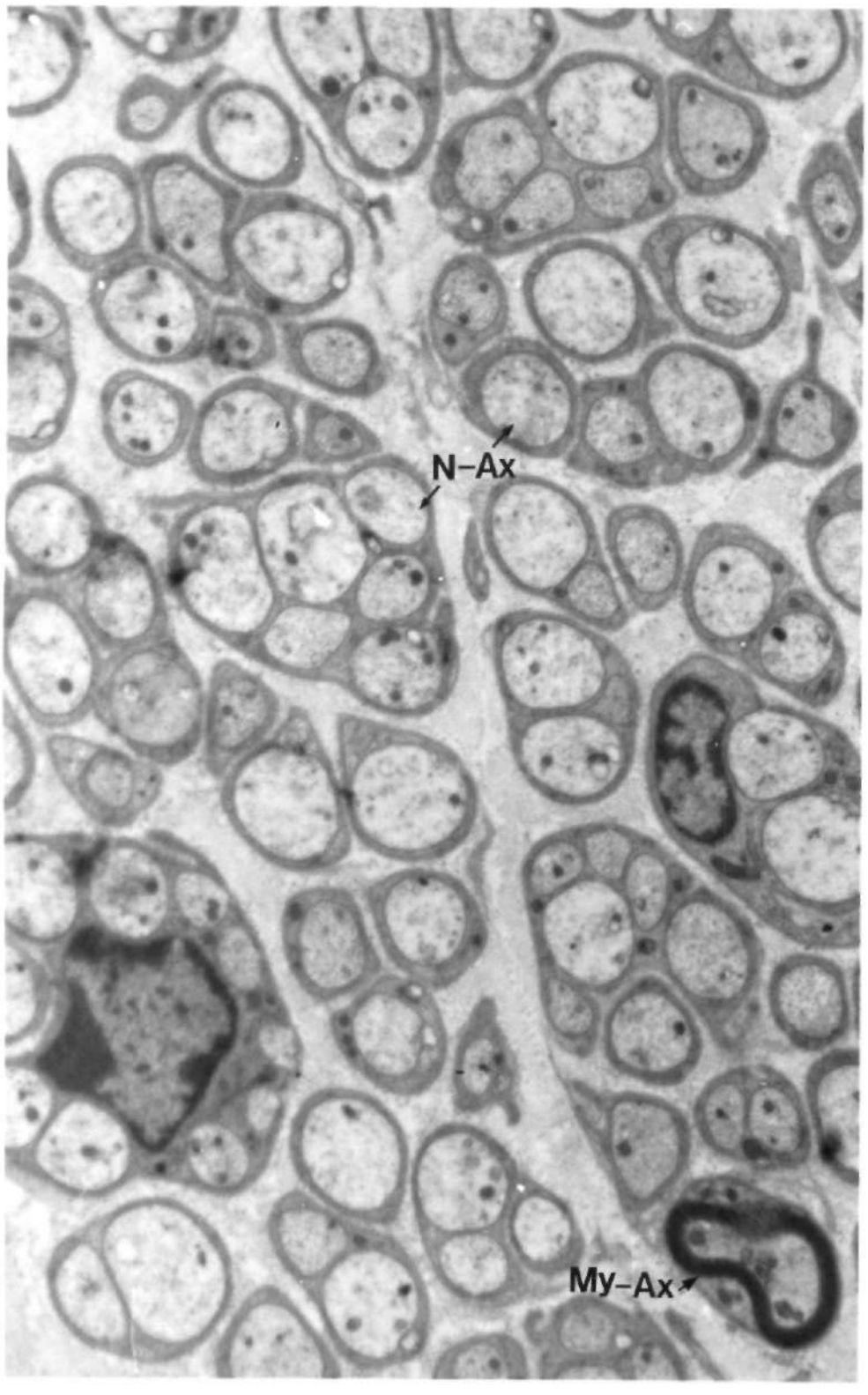


PLATE 4.7

Electron micrograph of cervical vagal trunk after supranodose vagotomy showing a degenerated myelinated fibre (Deg.My).

Magnification = x 16000

PLATE 4.7

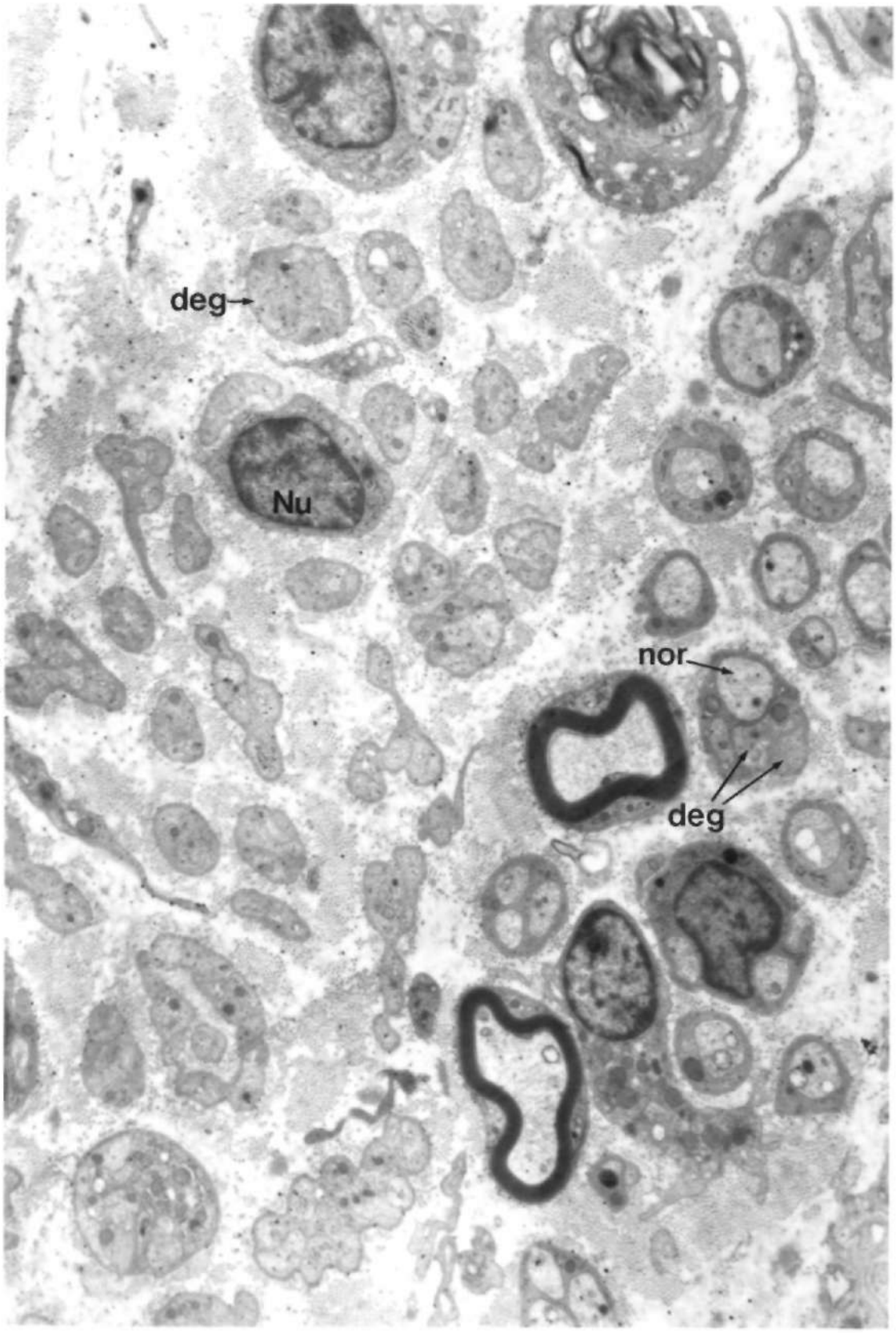


PLATE 4.8

Electron micrograph of cervical vagal trunk after supranodose vagotomy showing a group of non-myelinated fibres, some of which are degenerating (deg) while one in the group remains normal (nor). An intact nucleus of Schwann cell (Nu) is seen when the non-myelinated fibres surrounded by its cytoplasm have degenerated. Degenerated axons (deg) are again seen preferentially located together on the left side of the micrograph.

Magnification = x 9100

PLATE 4.8



cell nuclei were intact even when the axons which were surrounded by their cytoplasm had degenerated (Plate 4.8). But Plate 4.5 shows the nucleus of a degenerated Schwann cell which was phagocytosed by blood macrophages.

(iv) Abdominal Vagus Nerves after Unilateral Supranodose Vagotomy

They contained normal as well as degenerated axons. The degenerated axons were scanty and not uniformly distributed throughout the section. In a group of non-myelinated axons surrounded by one Schwann cell, one or more axons might remain intact when others had degenerated (Plate 4.8).

b. Total Fibre Composition of the Vagus Nerve

(i) Cervical Vagal Trunks

Tables 4.2 and 4.3 show that the total number of axons in the lower part of the LCV is 27,273, and 28,356 at the same level in the RCV. Although the RCV shows a slight increase in the number of fibres above the LCV, this difference does not reach statistically significant 5 percent level.

(ii) Abdominal Vagal Trunks

The total number of axons in the ventral trunk is 9,457, and 17,657 in the dorsal trunk. The total number of axons in the two abdominal trunks is 27,114 which is about 50 percent of the total number of fibres in the two cervical vagus nerves. The ventral trunk contains 35 percent of all axons in the abdominal trunks (Tables 4.2 and 4.3).

TABLE 4.2

Shows the normal myelinated and non-myelinated fibre counts in 9 animals. Fibre counts with an asterisk are pooled results from six animals with unilateral supranodose vagotomy - the left cervical counts from 3 right supranodose vagotomy animals and right cervical counts from 3 left supranodose vagotomy animals. Fibres in the ventral and dorsal trunks of these post-vagotomy animals could not be pooled with normal fibre counts because they contained degenerated as well as normal fibres.

TABLE 4.2

Animal Number	Left Cervical Trunk			Right Cervical Trunk			Ventral Trunk			Dorsal Trunk		
	Myelinated	Non-myelinated	Total	Myelinated	Non-myelinated	Total	Myelinated	Non-myelinated	Total	Myelinated	Non-myelinated	Total
1	3401	27256	30657	2880	18319	21199	72	6435	6507	25	23607	23632
2	3242	28261	31503	3453	32084	35537	87	14696	14783	141	20112	20253
3	3289	22926	26215	3203	27518	30721	38	7660	7698	180	12207	12387
4	-	-	-	3645	24592	28237	47	8805	8852	90	14265	14355
5	4691	24260	28951	3138	21311	24449	27	8993	9020	-	-	-
6	4672	19868	24540	4248	25373	29621	81	9798	9879	-	-	-
7*	4284	15958	20242	3211	23689	26900	-	-	-	-	-	-
8*	4033	19004	23037	2936	29419	32355	-	-	-	-	-	-
9*	4131	28903	33034	4831	21352	26183	-	-	-	-	-	-
Total	31743	186436	218179	31545	223657	255202	352	56387	56739	436	70191	70627
Mean ±S.E.M	3968 ±210	23305 ±1674	27273 ±1589	3505 ±216	24851 ±1842	28356 ±1434	59 ±5	9398 ±520	9457 ±522	109 ±19	17548 ±1515	17657 ±1501

TABLE 4.3

The percentage distribution of myelinated and non-myelinated fibres in the vagus nerve trunks of the ferret. The numbers shown are mean and standard error of the mean.

TABLE 4.3

Vagus Nerve Trunk	Myelinated Fibres		Non-myelinated Fibres		Total
	Number	Percentage	Number	Percentage	
Left Cervical	3968 ±210	14.5	23305 ±1674	85.5	27273 ±1589
Right Cervical	3505 ±216	12.4	24851 ±1842	87.6	28356 ±1434
Ventral	59 ±5	0.6	9398 ±520	99.4	9457 ±522
Dorsal	109 ±19	0.6	17548 ±1515	99.4	17657 ±1501

c. Proportion of Myelinated and Non-myelinated Axons

(i) Cervical Vagal Trunks

The ratio of myelinated to non-myelinated axons in the LCV is 1:6 (Table 4.3). The myelinated axons are about 15 percent of all axons in that trunk. The ratio of myelinated to non-myelinated axons in the RCV is 1:7 and the myelinated axons form only about 12 percent of all axons in the trunk. The difference seen between the proportions of myelinated and non-myelinated axons in the two trunks does not reach statistically significant levels. The myelinated axons in the left and right cervical vagal trunks form only about 13 percent of all axons in those trunks.

(ii) Abdominal Vagal Trunks

The ratio of myelinated to non-myelinated axons in the ventral trunk is 1:160 (Table 4.3). Myelinated axons in this trunk are less than 1 percent of its total number of axons. For every myelinated axon in the dorsal trunk, there are also 160 non-myelinated axons. Although the number of myelinated axons in DT is higher than that in the ventral trunk, the number of non-myelinated axons in the DT is proportionately higher than the corresponding number in the VT. Therefore myelinated axons in the dorsal trunk are also less than 1 percent of the total number of axons in that trunk. When the two abdominal trunks are taken together, myelinated axons form only 0.6 percent of all axons in the two trunks.

d. Myelinated Axon Diameter Spectrum

(i) Cervical Vagal Trunks

Figures 4.5 and 4.6 are histograms showing respectively, the size-frequency distribution of myelinated axons in the LCV and RCV and in both cervical trunks together. They all show a unimodal distribution with a distinct peak at 2 to 3 μm diameter. The distribution of the myelinated axons on either side of the peak is similar in the left and right cervical vagal trunks separately, and together. Figure 4.6 shows that about 90 percent of all myelinated axons in the cervical vagal trunks have diameters ranging from 1 μm to 5 μm. The largest axon diameter seen was 13 μm but those greater than 8 μm were only about 0.2 percent of all myelinated axons. A few myelinated axons with diameters less than 1 μm were seen but they were less than 1 percent of all myelinated axons in the cervical vagal trunks.

(ii) Abdominal Vagal Trunks

The size-frequency distribution of myelinated axons in the VT and DT is similar to that seen in the cervical vagal trunks with a unimodal distribution and a peak at 2 to 3 μm (Figure 4.7). Ninety-three percent of all myelinated axons in the ventral trunk have diameters between 1 and 5 μm whereas 86 percent of myelinated axons in the DT have diameters between 1 and 5 μm. When both vagal trunks are taken together, 90 percent of all

myelinated axons have diameters between 1 and 5 µm (compare 90 percent in the cervical trunks). In the VT, the largest axon seen had a diameter of 7 µm and those with diameters below 1 µm form 1.7 percent of all myelinated axons in that trunk. There is an increase in the number of myelinated axons with diameters below 1 µm to 8.2 percent of all fibres in the DT, and the largest axon seen in this trunk had a diameter of about 10 µm.

e. Relationship between Axonal Diameter and Myelin Thickness

Myelinated axons with diameters below 1 µm were seen. The thickness of myelin sheath increased as axon diameter increased from 1 µm (Figure 4.8). This direct linear relationship has a correlation coefficient of 0.79. It appears that an S-shaped regression curve will fit the plotted points better so that the direct relationship between axon diameter and myelin thickness becomes more emphasised when the axon diameter is above 3 µm. Whereas the latter is true, the regression equation for such a curve does not give a statistically improved correlation coefficient. In absolute terms, myelin thickness in larger diameter axons is greater than in small myelinated axons. But in relative terms, the ratio of myelin thickness to axon diameter is higher in small than in large myelinated axons.

FIGURE 4.5

Size frequency distribution of myelinated fibres in left and right cervical vagus nerves in normal animals.

A = Left cervical vagus nerve

B = Right cervical vagus nerve

FIGURE 4.6

Size frequency distribution of myelinated axons in left and right cervical vagus nerves combined. No previous vagotomy was done in this group of animals. Therefore the myelinated axons were a mixture of afferents and efferents.

FIGURE 4.6

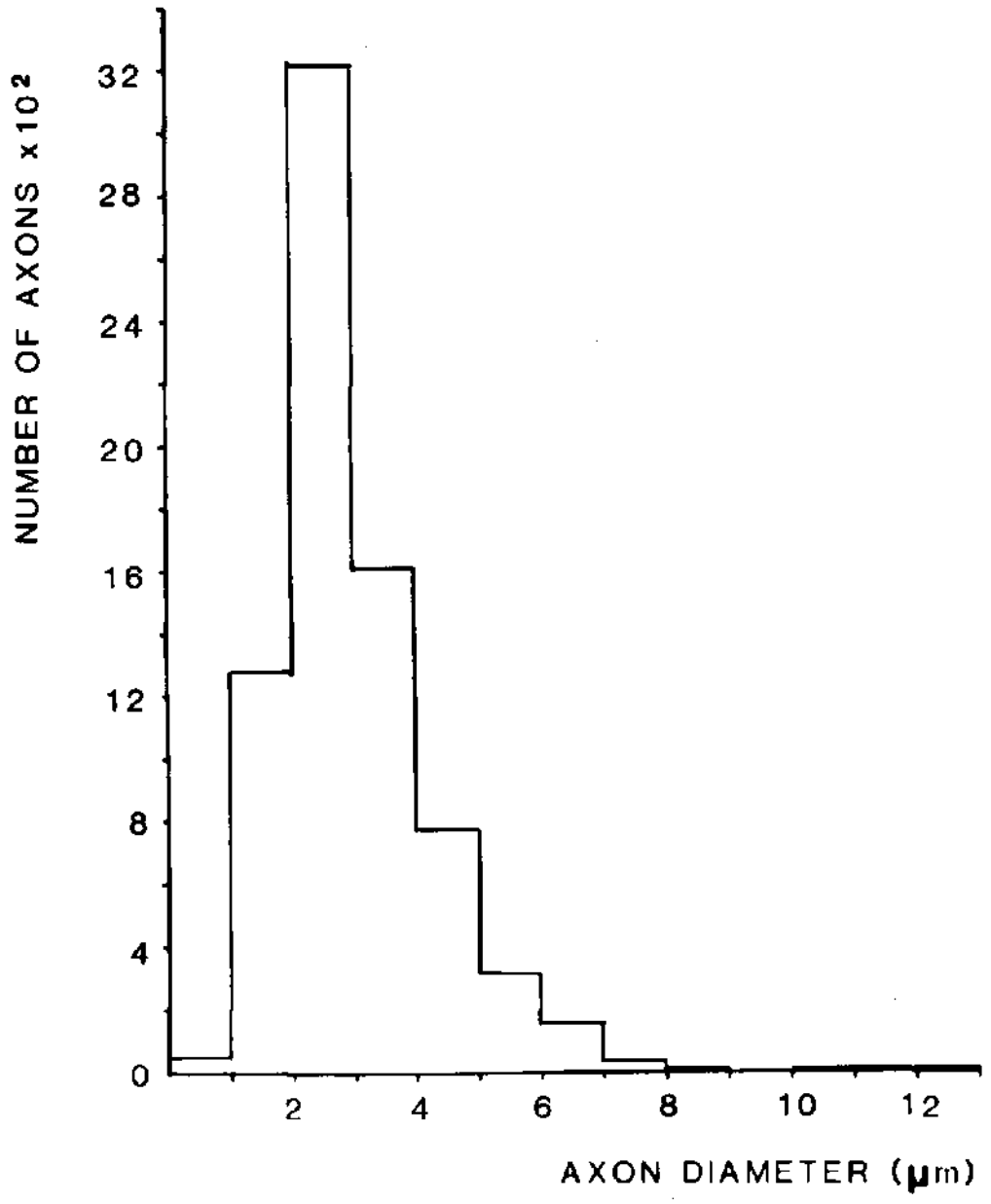

NUMBER OF AXONS x10²
32
28
24
20
16
12
8
4
0
2
4
6
8
10
12
AXON DIAMETER (μm)

FIGURE 4.7

Size frequency distribution of myelinated axons in ventral (A) and dorsal (B) trunks of vagus nerve. These data are for afferent and efferent fibres in normal nerve trunks.

FIGURE 4.7

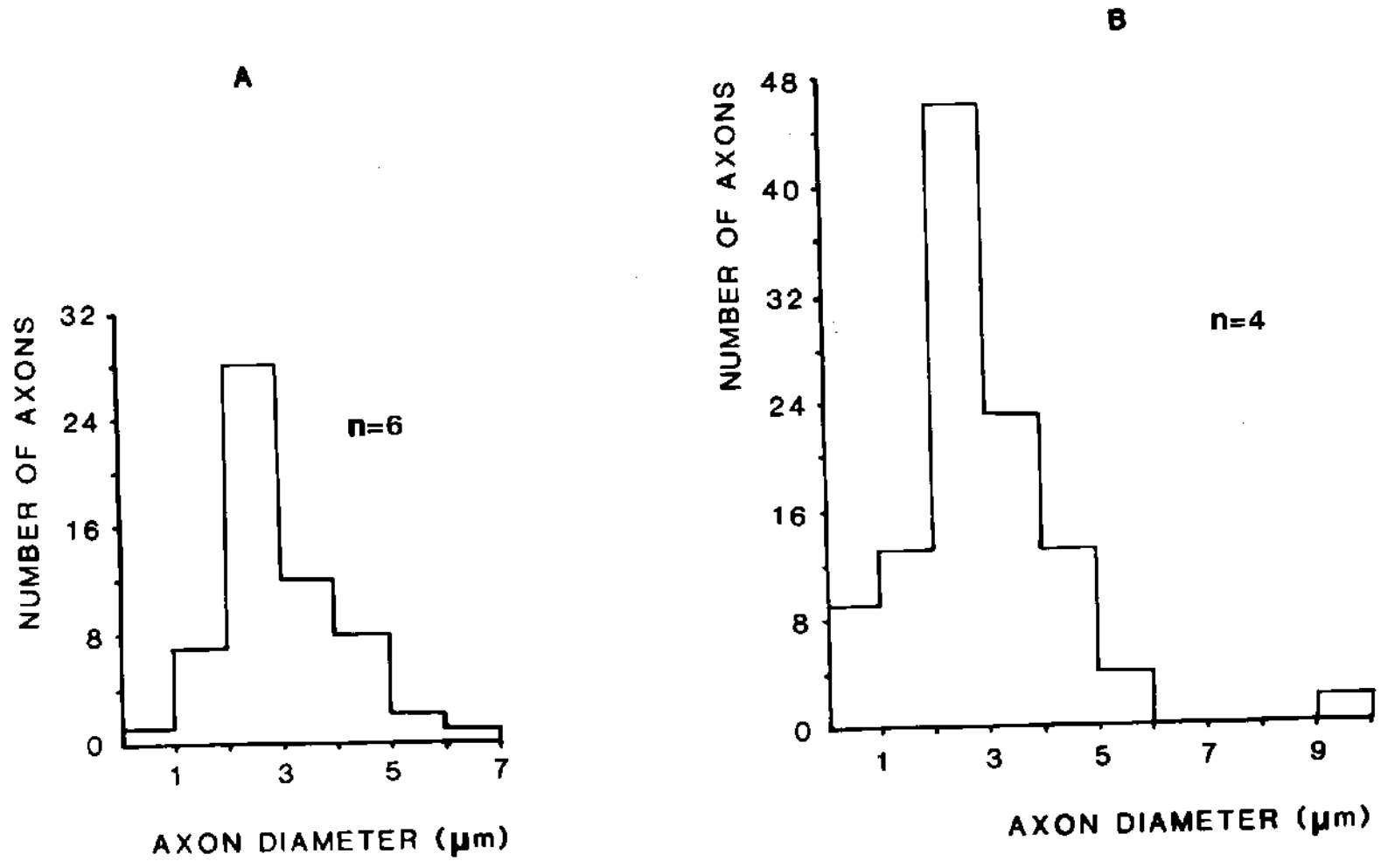
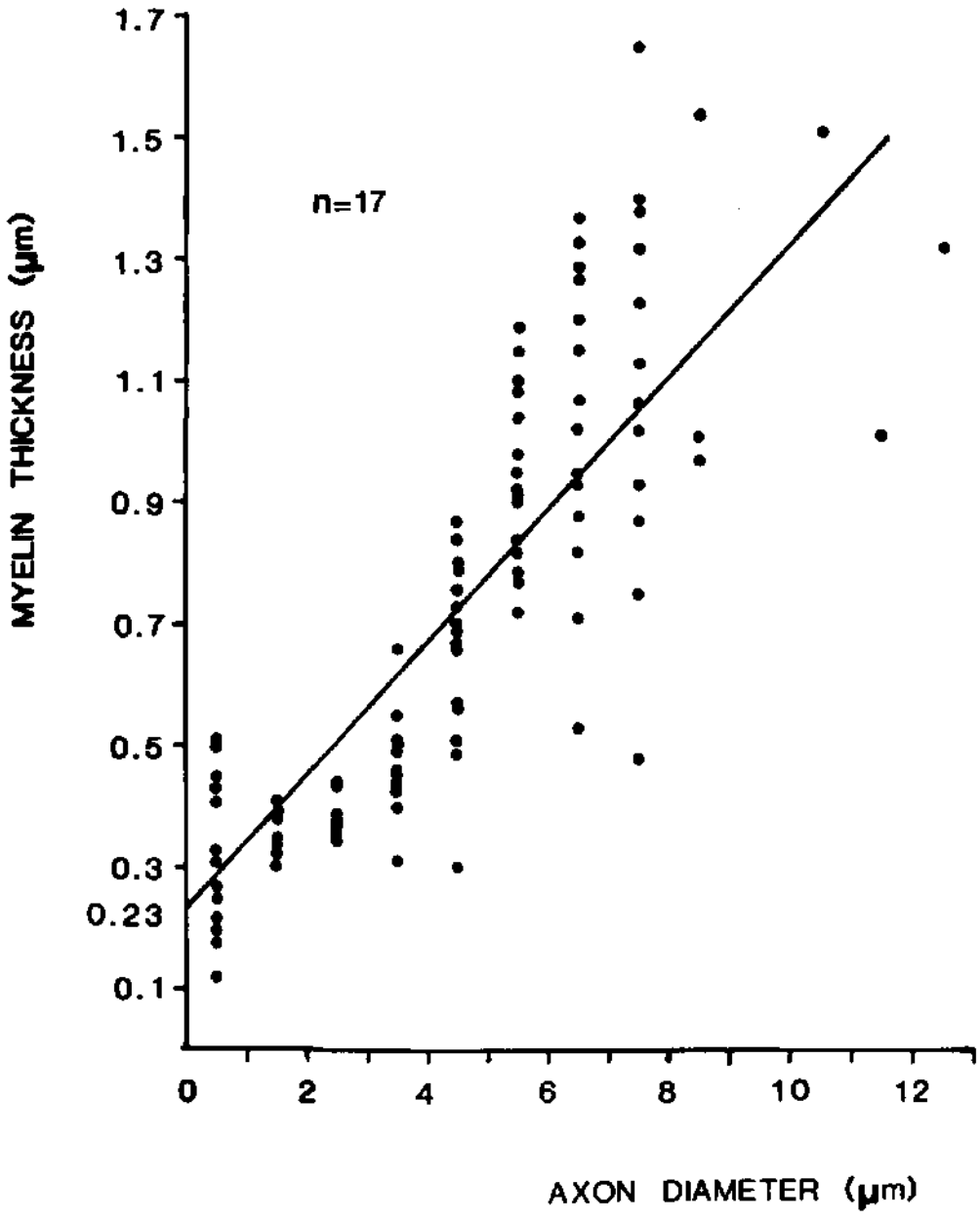

A
NUMBER OF AXONS
32
24
16
8
0
n=6
1
3
5
7
AXON DIAMETER (µm)
B
NUMBER OF AXONS
48
40
32
24
16
8
0
n=4
1
3
5
7
9
AXON DIAMETER (µm)

FIGURE 4.8

Scatter diagram showing the relationship between axon diameter and myelin thickness in cervical vagal trunks. The linear relationship with a regression equation of $y = 0.23 + 0.11x$ has a correlation coefficient of 0.79.

FIGURE 4.8


MYELIN THICKNESS (µm)
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.23
0.1
n=17
0
2
4
6
8
10
12
AXON DIAMETER (µm)

diameter. Supranodose vagotomy did not alter the relationship between axon diameter and myelin thickness.

(ii) Abdominal Vagal Trunks (Figure 4.10)

In the abdominal trunks, the size-frequency distribution of surviving myelinated fibres in the dorsal trunks after either left or right supranodose vagotomy remained similar to that seen in normal nerves. The peak of these unimodal distributions remained at 2 to 3 μm diameter. The distribution of surviving fibres in the ventral trunks is different from the normal pattern. But these fibres are very few and this fact may be responsible for the different pattern seen. In spite of the mixing of fibres from RCV and LCV in the thoracic plexus, each cervical vagus nerve seems to have contributed fibres of similar range of calibre to both abdominal vagal trunks.

TABLE 4.4

Shows the myelinated and non-myelinated fibre counts in 3 animals with left supranodose vagotomy. The fibres counted in the left cervical trunk are all afferent whereas the right cervical and abdominal trunks contain both afferent and efferent fibres.

TABLE 4.4

Animal Number	Left Cervical Trunk			Right Cervical Trunk			Ventral Trunk			Dorsal Trunk		
	Myelinated	Non-myelinated	Total	Myelinated	Non-myelinated	Total	Myelinated	Non-myelinated	Total	Myelinated	Non-myelinated	Total
1	2617	25692	28309	3211	23689	26900	58	9611	9669	-	-	-
2	2406	15960	18366	2936	29419	32355	23	7055	7078	112	18697	18809
3	2739	22987	25726	4831	21352	26183	92	11087	11179	104	15137	15241
Total	7762	64639	72401	10978	74460	85438	173	27753	27926	216	33834	34050
Mean ±S.E.M	2587 ±97	21546 ±2900	24133 ±2797	3659 ±591	24820 ±2396	28479 ±1949	58 ±20	9251 ±1178	9309 ±1197	108 ±4	16917 ±1780	17025 ±1784

TABLE 4.5

Shows the fibre counts in 3 animals with right supranodose vagotomy. Whereas the fibres in the right cervical trunk are afferents, the remaining three vagal trunks contained afferent and efferent fibres.

TABLE 4.5

Animal Number	Left Cervical Trunk			Right Cervical Trunk			Ventral Trunk			Dorsal Trunk		
	Myelinated	Non-myelinated	Total	Myelinated	Non-myelinated	Total	Myelinated	Non-myelinated	Total	Myelinated	Non-myelinated	Total
1	4284	15958	20242	2679	25739	23418	63	5359	5422	88	19283	19371
2	4033	19004	23037	2507	20848	23355	43	10989	11032	80	16848	16928
3	4131	28903	33034	2631	20111	22742	63	10071	10134	151	15995	16146
Total	12448	63865	76313	7817	66698	74515	169	26419	26588	319	52126	52445
Mean ±S.E.M	4149 ±73	21288 ±3908	25437 ±3883	2606 ±51	22233 ±1766	24839 ±1799	56 ±7	8806 ±1844	8862 ±1740	106 ±22	17375 ±985	17481 ±971

TABLE 4.6

Percentage distribution of afferent and efferent fibres in vagus nerve trunks. These results were obtained by deducting the mean fibre counts in animals with supranodose vagotomy from the mean fibre counts in normal animals. The number of efferent fibres in each trunk could not be derived from a single animal since unilateral supranodose vagotomy only was done. Therefore Standard errors of the mean could not be calculated.

TABLE 4.6

Vagus Nerve Trunk	Afferent Fibres		Efferent Fibres		Total
	Number	Percentage	Number	Percentage	
Left Cervical	24133	88.5	3140	11.5	27273
Right Cervical	24839	87.6	3517	12.4	28356
Ventral	8714	92.1	743	7.9	9457
Dorsal	16849	95.4	808	4.6	17657

TABLE 4.7

The percentage distribution of afferent and efferent fibres within the myelinated and non-myelinated fibre groups of the vagus nerve.

TABLE 4.7

Vagus Nerve Trunk	Myelinated Fibres					Non-myelinated Fibres				
	Afferent		Efferent		Total	Afferent		Efferent		Total
	Number	Percent-age	Number	Percent-age	Number	Number	Percent-age	Number	Percent-age	Number
Left Cervical	2587	65.2	1381	34.8	3968	21546	92.5	1759	7.5	23305
Right Cervical	2606	74.4	899	25.6	3505	22233	89.5	2618	10.5	24851
Ventral	55	93.2	4	6.8	59	8659	92.1	739	7.9	9398
Dorsal	105	96.3	4	3.7	109	16744	95.4	804	4.6	17548

FIGURE 4.9

Size frequency distribution of myelinated fibres in left and right cervical vagal trunks after supranodose vagotomy. Afferent fibres only are present.

A = Left cervical vagus

B = Right cervical vagus

FIGURE 4.9

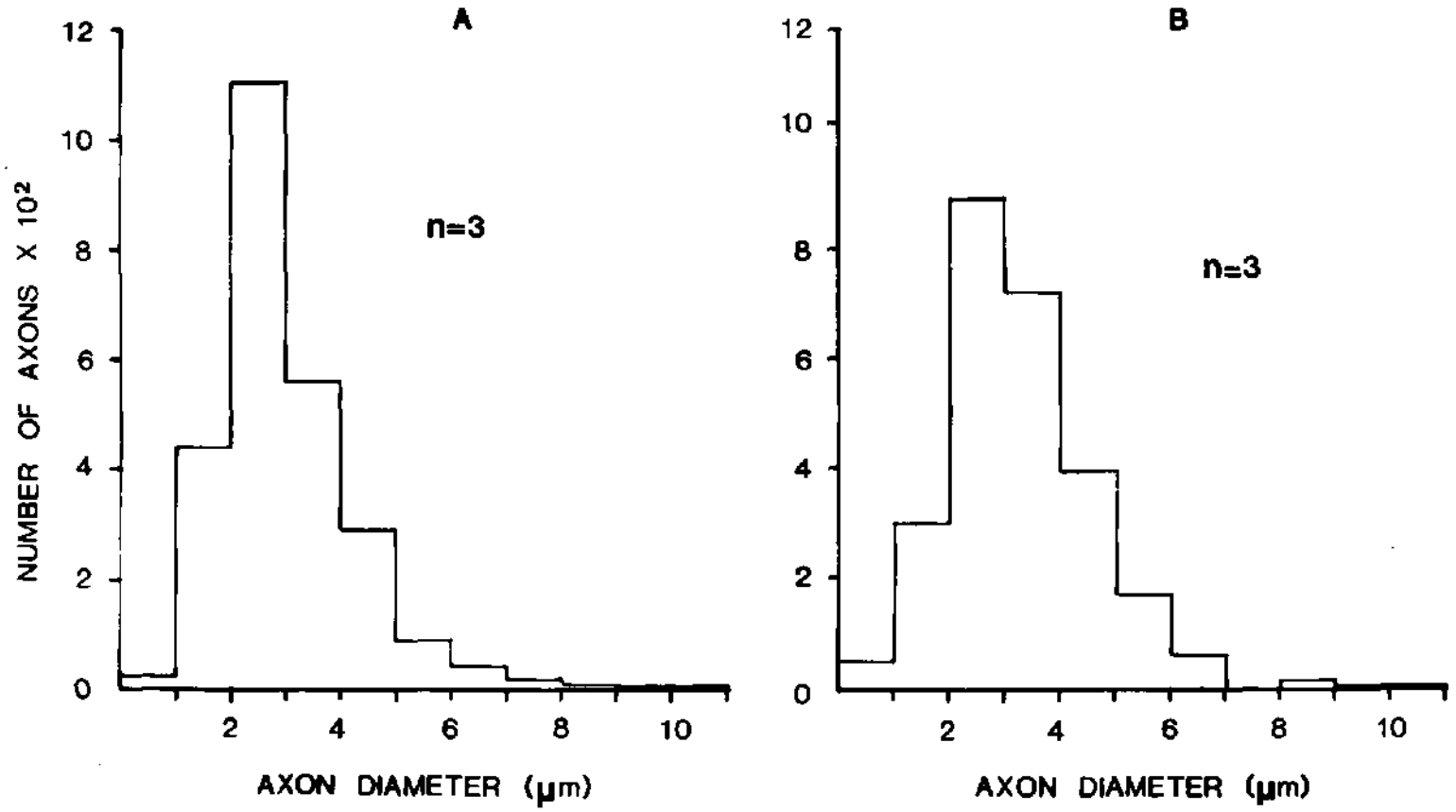

A
n=3
NUMBER OF AXONS X 10²
AXON DIAMETER (µm)
B
n=3
AXON DIAMETER (µm)

FIGURE 4.10

Size frequency distribution of myelinated fibres in ventral and dorsal vagal trunks after left supranodose vagotomy and after right supranodose vagotomy.

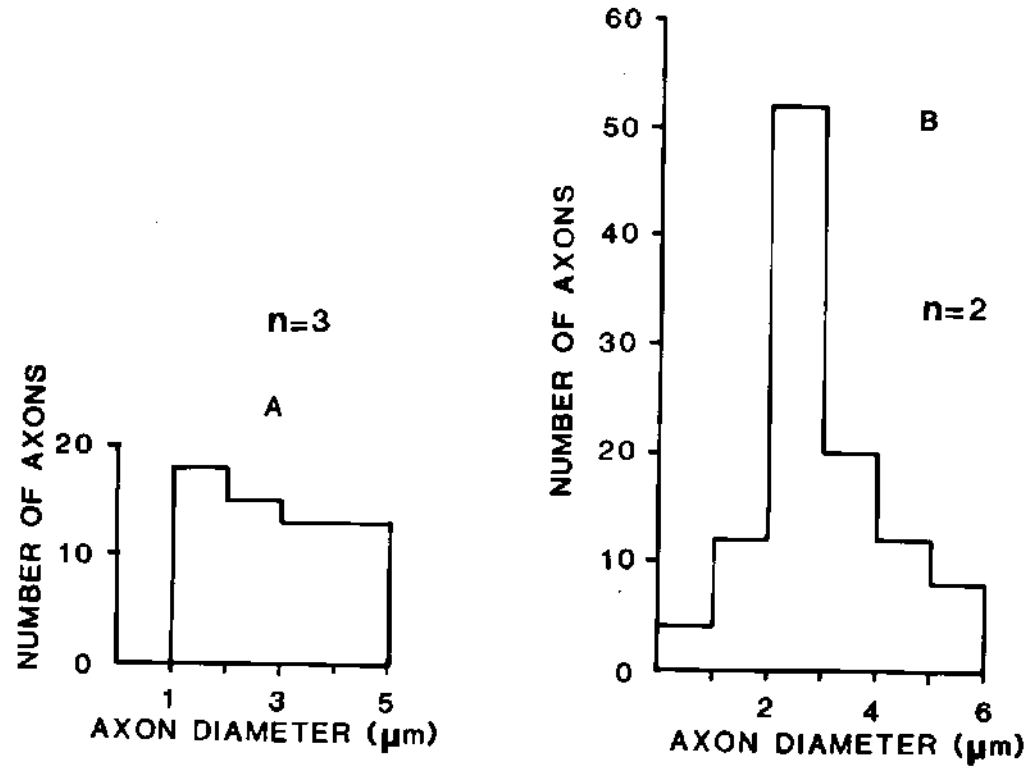
A - Ventral trunk after left supranodose vagotomy

B - Dorsal trunk after left sypranodose vagotomy

C - Ventral trunk after right supranodose vagotomy

D - Dorsal trunk after right supranodose vagotomy

FIGURE 4.10


n=3
A
NUMBER OF AXONS
0
10
20
1
3
5
AXON DIAMETER (µm)
B
n=2
NUMBER OF AXONS
0
10
20
30
40
50
60
2
4
6
AXON DIAMETER (µm)
C
n=3
NUMBER OF AXONS
0
10
20
30
1
3
5
7
AXON DIAMETER (µm)
D
n=3
NUMBER OF AXONS
0
10
20
30
40
50
2
4
6
AXON DIAMETER (µm)

DISCUSSION

ELECTRON MICROSCOPIC ANALYSIS OF VAGUS NERVE FIBRE COMPOSITION

Until recently, the evaluation of fibre populations of nerves was performed by light microscopy with osmium- or silver-impregnated preparations. This technique may be suitable for the examination of myelinated fibres, but it is not adequate for the evaluation of the non-myelinated fibres which are usually underestimated because of their generally small size (Bronson et al., 1978; Mei et al., 1980). The use of electron microscope has its own problem especially when fibre analysis is to be carried out on micrographs. It is usually impossible to photograph the whole of the tissue section. Therefore a reliable sampling technique which will eliminate bias has to be used to obtain representative micrographs. This is even more important if the fibres are not distributed uniformly throughout the section, since an inappropriate sampling technique can lead to oversampling of certain groups of fibres and under-sampling of others. When this happens, the result obtained can have no biological significance.

Nervous tissue, unlike most other tissues in the body, is highly sensitive to hypoxia. These other tissues may maintain their normal cytoarchitecture when samples are taken from them for fixation minutes after the animal has died. But the nervous tissue has to be fixed as soon as possible after death. Better results are obtained if fixation is partly done before the death of the animal. Two alternative methods are available for partly fixing

peripheral nerve tissue before the death of the animal. The animal may be perfused first with the diluted form of the appropriate fixative, and then with the normal osmolar concentration of the same fixative, or the latter may be laid on the nerve for a few minutes with the animal under anaesthesia (Mei et al., 1980). One possible problem with perfusion fixation is that if blood is not washed out of the vascular system before it clots or if the entry of fixative into the blood vessels causes vasospasm, the ultrastructure of the tissue may not be well preserved even in the presence of gross evidence of fixation such as muscle rigidity and joint stiffness. In order to prevent clot formation and vasospasm, the vascular system is first washed out with normal saline. When fixatives are laid on a nerve trunk for a few minutes, the nerve is only partly fixed. If it is not handled with care or not completely submerged under fixative for the rest of the fixation period, the tissue may not be satisfactorily fixed.

Nerve tissues are also very sensitive to changes in the osmolarity of the fixative and especially the buffer solution (Arborgh et al., 1976). Holland's (1982) study of myelinated axons in the rat showed that nerve profiles increasingly deviate from a true circle with increasing concentration of the buffer solution. He was, of course, not suggesting that choosing the right osmolarity for the animal would give axon profiles that would be true circles. Myelinated axon profiles are only rarely true circles (Duncan, 1934; Gutmann

and Sanders, 1943; Karnes et al., 1977; Fraher, 1980). Dyck and Karnes (1981) further showed that the shape of axon profiles depends on the level along the axon from which the section is taken. The nearer the section is to the node of Ranvier, the more the axon profile deviates from a circle.

Other factors which may affect the final morphometric result are the temperature and pH of the fixative solution (Dyck and Karnes, 1981). At high temperatures (within the physiological limits of the animal), tissues are more rapidly and effectively fixed (Carson et al., 1972). The final pH of the fixative solution should also be within the physiological range of the animal. Another factor that affects the morphometric result from myelinated fibres is whether or not the tissue section is truly transverse. If it is not, measured axon diameters and myelin thickness will be greater than their true values. The adverse effect of measuring oblique axon profiles is of course minimised if the section is very thin (Dyck and Karnes, 1981). For this reason, ultrathin sections 60-80 nm thick were used in these experiments.

In summary, the morphometric result obtained from myelinated axons will depend on the osmolarity, temperature and pH of the fixative solution as well as on the method of application of the fixative to the nerve segment, that is, by immersion of the tissue in fixative in a specimen bottle, laying of the fixative on the nerve in situ or perfusion fixation. If the final effect of the above is to cause a

shrinkage or swelling of the axons, the axon diameters and myelin thickness will be under- or over-estimated respectively. How far the measured axon calibres deviate from their true values will depend on what calibre estimate is used. One of several methods may be used.

1. Axon circumference;
2. Axon cross-sectional area;
3. Hypothetical axon diameters derived from either axon area or circumference; and
4. Comparison of the smallest diameter of the axons.

Circumference values remain relatively unaffected by alterations in buffer molarity, the major effect of increasing or decreasing buffer concentration being a change in shape (Holland, 1982). Bronson et al. (1978) have also argued that since the circumference of a tube is unchanged when the area is reduced by compression, circumference is a better measure of axon size. Since most authors have used axon diameters as a measure of axon size, it would be profitable, for the sake of being able to make useful comparisons, to derive axon diameters from axon circumference.

When computers and other electronic equipment are used to measure axon diamters and myelin thickness, care must be taken to ensure that the machine actually measures what it is designed to measure and that axons are not counted or measured twice. Whatever method is used to include or exclude incomplete axon profiles at the edge of the micrograph should be strictly adhered to throughout the measurements. Inconsistencies can readily invalidate the result.

The animals in which supranodose vagotomy was done were allowed to survive for three weeks before being sacrificed. This survival period was found to be long enough to permit the efferent fibres to degenerate sufficiently for them to be readily recognised. A sufficiently long survival time is essential because any confusion as to what is degenerated axon and what is not, will result in inaccurate estimations of afferent and efferent counts.

The fibre spectrum of the vagus nerve in the ferret does not appear to have been determined. Comparison can therefore only be made with results from other animals. The total vagal fibre count in the ferret varies from one animal to the other. This is consistent with the observations in the rat and cat (Mei et al., 1980). For example, the left cervical vagal fibre counts in eight ferrets ranged from about 20,000 to 33,000 (Table 4.2). The cervical vagus nerve in the ferret is unifasciculus, therefore all fibres can very easily be included in the section. This means that the variation between fibre counts in different animals is unlikely to be due to the exemption of some fibres from one cervical trunk and not from the other. It may be that total abdominal vagal fibre counts depend on stomach size such that animals with low fibre counts have small stomachs with the result that the proportion of nerve to stomach remains the same. Stomach weights were not recorded in this group of animals but this study shows that there is no

correlation between animal weight and total abdominal vagal fibre counts in the ferret.

The total fibre counts in the LCV, RCV and both trunks combined are much higher than the corresponding values obtained by light microscopy for the cat (Foley and Dubois, 1973; Jones, 1973; Agostoni et al., 1957), rabbit (Evans and Murray, 1954) and man (Schnitzlein et al., 1958). This supports the claim that underestimation of small fibres is the rule when light microscope is used. For example by using light microscope, 34,300 fibres were counted in both cervical vagus nerves of the cat compared with 55,600 fibres in the corresponding vagus nerve trunks of the ferret using electron microscope. The total fibre count in the LCV is not significantly different from the count in the RCV in the ferret. This is consistent with the result of stimulation of right and left cervical vagus nerves which produced intragastric pressures of equal amplitude.

In the ferret, the total number of fibres in both abdominal vagus nerves is about 27,000. This is similar to the corresponding fibre count in the rabbit (Evans and Murray, 1954) and the cat (Agostoni et al., 1957). Only half of the fibres in the cervical vagus nerve reach the abdomen. The remaining half take part in the autonomic innervation of such structures as the heart, lungs, larynx and pharynx. Unlike in the dog where Kemp (1973) using electron microscope techniques found identical numbers of fibres in ventral and dorsal vagal trunks, the ventral trunk in the ferret contains only 35 percent of all fibres in both abdominal trunks.

This is due to the fact that the VT which derives partly from the LCV gives the crossing branch to the DT. But this finding in the ferret is compatible with the observation that the DT innervates a greater area of the stomach than the VT. It is recognised that not all fibres counted in the abdominal trunks at the level of the diaphragm go to innervate the stomach. Some branches leave the VT to innervate the liver and its derivatives while the DT sends the largest of its branches to the coeliac plexus (Mackay and Andrews, 1983). In spite of this, it is possible that the number of DT fibres that finally reach the stomach is higher than the number of fibres from the VT. If this is true, it could explain the greater responsiveness of the DT to cervical vagal stimulation as well as the larger area of stomach innervated by the preganglionic efferent fibres of the dorsal trunk. The truth will only be known if the branches of the vagus nerve that finally reach the stomach can be selectively studied.

The cervical vagus nerve is composed mainly of non-myelinated fibres. By virtue of the fact that these fibres are devoid of myelin, their conduction velocity is slower than it would have been if they were myelinated. This velocity would of course be influenced by the diameters of the axons which, although not quantified in these experiments, were observed to be small. The abdominal vagal trunks contain an even high percentage of non-myelinated fibres than the cervical vagal trunks. Whereas 87 percent

of all fibres in the cervical vagal trunks are non-myelinated, 99 percent of all fibres in the abdominal trunks are non-myelinated. This implies that the vagal trunks give relatively more myelinated fibres to cervical and thoracic structures than non-myelinated fibres. For example only 2 percent of myelinated fibres in the cervical vagus nerve reach the abdomen. This is less than the 6 percent found by Schnitzlein et al. (1958) in the vagus nerves of man. This large number of small non-myelinated fibres in the cervical and abdominal vagal trunks explains why in vivo recording of the compound action potential from the vagus nerves of anaesthetised ferrets revealed the presence of a large component with a latency appropriate to conduction in C-fibres (Mackay and Andrews, 1983). Similar results have been obtained in the dog (Kemp, 1973). It also explains previous demonstrations that the abdominal vagus nerve in the ferret contained efferent fibres with a conduction velocity of 1.20 ± 0.29 m/sec (Andrews, Fussey and Scratcherd, 1980), and afferent fibres, originating from the stomach, with a conduction velocity of 0.91 ± 0.21 m/sec (Andrews, Grundy and Scratcherd, 1980). These figures fall within the range of conduction velocities (0.15 m/sec to 3.9 m/sec) of the majority of fibres in the abdominal vagus nerves of man (Andrews and Taylor, 1982). This is an added factor in favour of the suitability of the ferret as a model for studying the structure and function of the vagus nerve in man.

The vagus nerve of the ferret, both at cervical and abdominal levels, consists mainly of afferent fibres. In the cervical vagal trunks, only about 12 percent of all fibres are efferent. This is much less than the corresponding figure of 20 to 35 percent obtained in the cat by Foley and Dubois (1937). But these authors took their samples from the upper cervical level and therefore probably included fibres that were destined for the largely myelinated recurrent laryngeal nerves (Dubois and Foley, 1935; Evans and Murray, 1954; Murray, 1957). Efferent fibres in both abdominal trunks form 6 percent of all fibres in those trunks. This confirms Kemp's (1973) finding in the dog that the contribution of efferent fibres from the medulla to abdominal vagal trunks is small.

Although 5 and 8 percent of dorsal and ventral trunk fibres respectively are efferent, in absolute figures, both trunks contain similar numbers of efferent fibres whereas the DT contains nearly twice the number of afferent fibres in the VT. But DTV resulted in a greater loss of function as shown by the intragastric pressure response to cervical vagal stimulation. The same trunk was found to innervate a greater proportion of the stomach wall. If majority of these afferent fibres reach the stomach, then their role in the control of gastric motility needs to be explained. There are two possible explanations for these contrasting findings. Firstly, the efferent fibres in the DT may branch more profusely as they approach their target cells in the myenteric plexus and so make synaptic contacts with more

intramural ganglion cells than the VT. But there is no reason why efferent fibres in the DT alone should do this. Secondly, the effect of afferent, rather than efferent, denervation may play a greater role in the gastric functional loss that was recorded. This will imply that in the intact vagus nerve, the afferent fibres from the stomach play a more important role in the regulation of gastric motility than efferent fibres. This seems to be a reasonable proposition because as was explained in the introductory chapter, the intrinsic nervous system is the more important nervous component for the control of gastric motility, with the extrinsic parasympathetic (vagal) and sympathetic nerves serving as mediators of central influence. Much of the normal stimuli for gastric muscle and glandular activity arise from within the gastro-intestinal lumen. The afferent fibres are therefore actively involved in mediating the resulting impulses to the rest of the intrinsic nervous system and the regulatory centres in the CNS.

The unimodal distribution of myelinated axon diameters of the vagus nerve of the ferret is consistent with a similar pattern of distribution seen in the vagus nerve of the cat (Burgh, Daly and Evans, 1953). In animals, such as the rat, with bimodal distribution of vagus nerve axon diameters (Bronson et al., 1978), the second peak is usually in the large diameter range (larger than 10 μm). But less than 1 percent of the myelinated axons in the vagus nerve of the ferret have diameters above 8 μm. It could therefore

be that the second peak is lost as a result of large myelinated axons forming vagal branches high up in the neck or such large diameter axons simply do not exist in the vagus nerve of the ferret and some other mammals. The latter suggestion is further strengthened by the demonstration of a unimodal distribution of axon diameters in the vagus nerve of the cat at supranodose and infranodose levels (Mei et al., 1980).

The majority of the myelinated fibres in the vagus nerve of the ferret, by virtue of their calibre, fall within the range of A_δ fibres (see Table 4.1). Nevertheless, it is conceivable that compound action potentials with latency consistent with conduction in C-fibres were obtained from the majority of vagus nerve fibres in the ferret (Mackay and Andrews, 1983). This is because these large, A_δ, myelinated fibres are very few compared with the generally smaller, non-myelinated C fibres (Mei et al., 1980).

Myelinated fibres less than 1 μm in diameter are said to be rare in the peripheral nervous system (Rushton, 1951) whereas myelinated fibres as small as 0.2 μm exist in the CNS (Waxman and Bennett, 1972). But in the vagus nerve of the ferret, myelinated fibres less than 1 μm diameter were seen, but they form a very small percentage of all myelinated fibres. Although non-myelinated fibres were not measured in this experiment, some of these fibres were observed to be larger than some small myelinated fibres, hence there is an overlap in the spectrum of the non-myelinated and myelinated fibres (Mei et al., 1980).

Therefore the size of a fibre is not the only determinant of whether or not it is myelinated (De Neef et al., 1982).

The relationship between axon diameter and myelin thickness shows that myelin increases only slowly in thickness with increasing axon calibre up to 2 to 3 µm. After this level, myelin thickness increases more rapidly with increasing axon diameter. This is in support of Rushton's (1951) conclusion from Hursh's (1939) data on cat peripheral nerve fibres that there is a critical level of axon diameter (1 µm) when myelination begins to be of advantage in terms of nerve conduction. In other words, in the lower part of the myelinated axon spectrum, the calibre of the axon plays greater role in conduction than the myelin thickness. In fact in myelinated fibres less than 1 µm diameter, the presence of myelin becomes a disadvantage and will conduct less rapidly than a non-myelinated fibre of the same calibre (Waxman, 1983). The explanation for this comes from the studies by Brill et al. (1977) and Ritchie and Stagg (1982) in which they found that there is an optimal ratio between internodal distance and axon diameter in terms of maximizing conduction velocity at any given diameter. Ratios higher than the optimum value result in decreased conduction velocity. Ritchie (1982) noted that there is a minimal internodal length of no less than 200 µm in the peripheral nervous system (compared with 10 µm in the CNS). This means that internodal length is not entirely related to axon diameter in a linear fashion. Thus at the minimal internodal length,

axon diameters less than 1 μm will give a higher internodal length/axon diameter ratio which will result in reduced conduction velocity.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION

Intragastric pressure changes in response to cervical vagal stimulation were used as an indirect method of studying gastric motility changes in response to acute and chronic abdominal trunk vagotomy. Acute and chronic ventral trunk vagotomy did not have significant influence on gastric motility whereas chronic dorsal trunk vagotomy resulted in a significant recovery of acutely diminished gastric motility. This may be due to the fact that the ventral trunk efferent fibres innervate a smaller area of the stomach than the dorsal trunk, so that the functional loss following VTV is minimal. If this is true, then gastric motility does not have much recovery to undergo. The finding of equal numbers of efferent fibres in both ventral and dorsal trunks does not support the differential distribution of these fibres in the gastric wall. But this may be because the number of efferent fibres is so small and so varied from one animal to the other that a large number of animals will be needed in order to obtain more reliable figures. It is also true that the fibres counted at the level of the diaphragm do not all reach the stomach. In which case, if the fibres that reach the stomach are selectively counted, the differential distribution of the two abdominal trunks may be supported by the number of fibres they contain. On the other hand, the afferent fibres may be playing a more important role than has hitherto been realised. It has always been assumed that when the distal end of an acutely transected cervical vagus nerve is stimulated, the efferent

fibres only are contributing to the gastric response. But it may in fact be that there is antidromal stimulation of the afferent fibres as well.

It appears that an initially marked vagal functional loss is essential if a good level of recovery after chronic vagotomy is to take place since it was only after chronic DTV that there was a significant degree of recovery. The stimuli which initiate functional recovery must be closely related to the end-products of vagotomy or gastric denervation. Following vagotomy, the distal portion of the nerve degenerates and the appropriate portion of the stomach loses the efferent bombardment which it normally receives from the vagus nerve. The stomach then undergoes appropriate changes which will enable it to recover the lost function. The greater the loss, the greater the changes the stomach undergoes. It becomes hypersensitive to normal levels of circulating neurotransmitter and may release sprouting factor which will stimulate the proximal part of the transected nerve and other surviving, adjacent nerves to sprout. The transected nerve, on the other hand, loses its power to suppress the normal tendency to sprout that resides in the adjacent intact nerves. A combination of these factors among others probably leads to recovery of gastric motility.

One of the main aims of this study was to relate the possible factors which are contributory to the recovery of gastric motility to different periods in the time course of this recovery. Although no definite cause-effect has been

proved, the following relationships are proposed (see Figure 5.1).

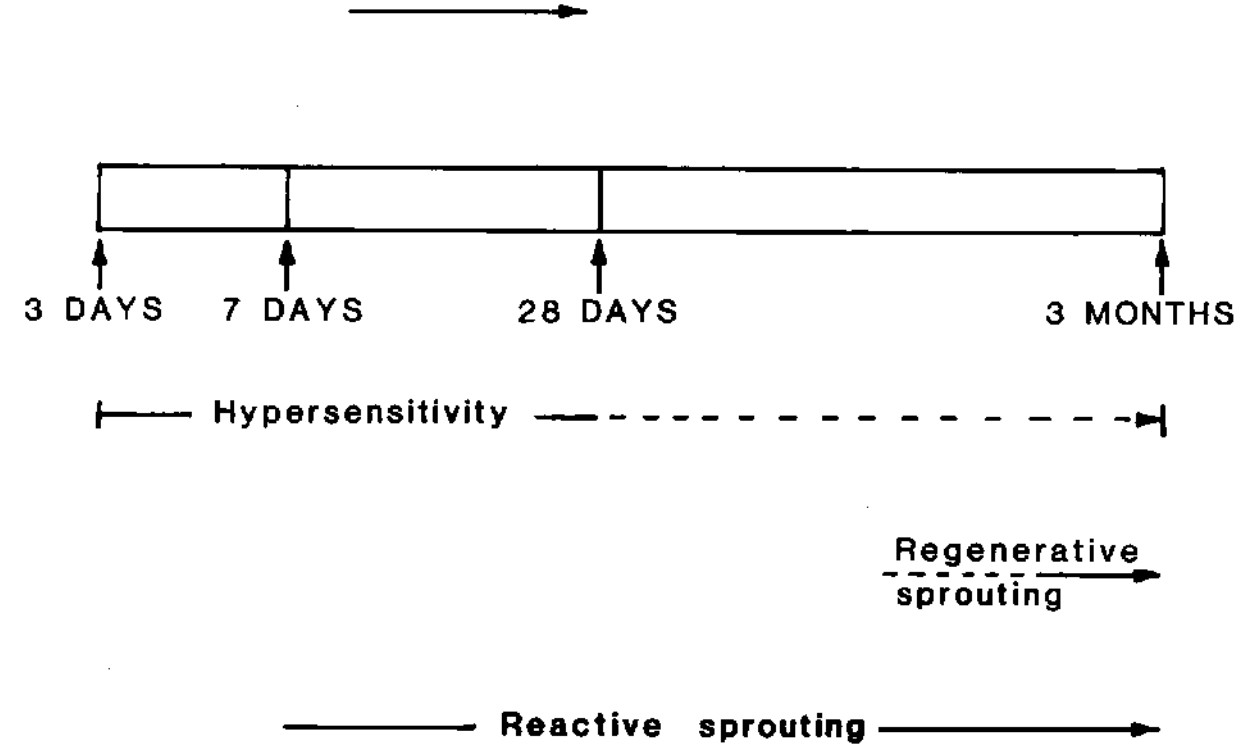
The recovery that was noted from the third day to the fourth week might be partly due to hypersensitivity of gastric muscle. But it is not known precisely when it stops being effective in the ferret. It might have contributed, to a limited extent, to the recovery in the third month. Regenerative sprouting contributed to the recovery of gastric motility three months after VTV. If the cut ends of the DT were approximated, this form of sprouting might have been seen after DTV. Reactive sprouting was not demonstrated in these experiments. It may be that the experimental technique failed to detect it, or it may require a longer time than three weeks for it to be complete. But if reactive sprouting does take place and as quickly after vagotomy as it does in some areas of the CNS, then it could have contributed to the full recovery noticed from the seventh day on. Also contributing to the recovery from the fifth day on is the decreased smooth muscle resting tone which has been shown to have an inverse relationship with vagally evoked gastric smooth muscle contraction. The muscle fibres themselves may be hypertrophic, a mechanism which can make the stomach contract more than it would have done in response to the surviving vagal fibres.

Estimates of fibre counts are at best an approximation to the true values in so far as only sampled portions of the nerve trunks are counted. If computer-based image

FIGURE 5.1

Schematic illustration of possible mechanisms which may be contributory to the recovery of gastric motility at various times after partial vagotomy in the ferret. The broken lines indicate periods when it is most uncertain how much the corresponding mechanism contributes to the recovery. Three months equal 84 days.

FIGURE 5.1


Decreasing resting pressure
3 DAYS
7 DAYS
28 DAYS
3 MONTHS
Hypersensitivity
Regenerative sprouting
Reactive sprouting

analysis methods (Dyck and Karnes, 1981) are used in place of expensive and time-consuming micrograph analysis, it may be possible to count all fibres. The vagal efferents are very few and slowly conducting. This is probably because gastric motor control does not depend exclusively on the extrinsic efferent nerves. The intrinsic nervous system appears to be the main control centre and structural changes, such as reactive sprouting, which may be contributory to the functional recovery may be at the level of the intrinsic nervous system.

As pointed out in the introductory chapter, the vagus nerve is very important to man because of its involvement in the pathophysiology of duodenal ulcer. It is cut frequently in duodenal ulcer patients. But in spite of adequate vagotomy at the time of operation, duodenal ulcer recurrences are seen. If the transected vagus nerve of man recovers structurally and re-innervates the parietal cells, then the long term value of selective vagotomy in the treatment of duodenal ulcer is put in doubt. But since ulcer recurrence does not take place in all patients, it may be that the rest that vagotomy gives to the gastric mucosa enables the majority of ulcers to heal and become less susceptible to the effects of subsequent increases in gastric acid and of other factors contributory to the causation of duodenal ulcers. In the absence of better, more permanent and acceptable forms of treatment, selective vagotomy remains the definitive treatment, especially of ulcers that do not respond to drug treatment.

But conscious efforts must at least be made when vagotomy is done, to discourage regenerative sprouting which has been shown to take place in the ferret. This may be done by removing long segments of gastric branches of the vagus nerves.

A consistent finding in the distribution of the vagal branches in the wall of the stomach is that the innervation of the ventral and dorsal walls nearer the lesser curvature is less than the greater curvature. If this pattern is confirmed by a more detailed quantitative study of vagal gastric innervation, it could mean that some of the fibres seen to go to the lesser curve pass through to distal structures without entering the myenteric plexus. Such fibres of passage have been described by Odekunle (personal communications).

CONCLUSIONS

The main aims of this work were as follows:-

1. to see if there is vagal functional recovery following chronic unilateral abdominal vagotomy in the ferret;
2. to determine the time course of such a recovery;
3. to see if there are any changes in the distribution of the surviving vagal trunk in the stomach wall following unilateral abdominal vagotomy, and
4. to determine the afferent-efferent and myelinated-non-myelinated axon ratios in cervical and abdominal trunks of the vagus nerve and correlate these with relevant findings in the above objectives.

The first two aims were attempted using physiological techniques which measured intragastric pressure responses to cervical vagal stimulation. The ventral vagal trunk was found not to significantly influence evoked intragastric pressure. But acute dorsal trunk vagotomy produced a marked loss of evoked intragastric pressure. Five days after dorsal trunk vagotomy, the intragastric pressure returned to normal. Vagal functional recovery was therefore shown to occur five days after dorsal trunk vagotomy in the ferret.

Using autoradiographic techniques at light microscope level, the ventral vagal trunk was found to innervate the whole of the ventral wall of the stomach whereas the dorsal

vagal trunk innervated all of the dorsal wall as well as the ventral wall of the antrum and pylorus. This pattern of distribution suggests that the widespread distribution of the dorsal trunk may be responsible for the greater loss of vagal function after acute dorsal trunk vagotomy. But a detailed enough quantitative study of tritium labelled axons was not done to permit any conclusive statement on the density of ventral and dorsal vagal preganglionic fibres in the myenteric plexus. Three weeks after unilateral abdominal vagotomy, there was no change in the pattern of distribution of the vagal trunks. Regenerative sprouting was demonstrated following chronic ventral trunk vagotomy but not after chronic dorsal trunk vagotomy. Therefore no explanation could be given for the vagal functional recovery that has been demonstrated. Reactive sprouting was not demonstrated in vagal preganglionic fibres.

The electron microscope study of fibre ratios in the vagus nerve of the ferret showed that the numbers of afferent and efferent fibres in the cervical trunks are similar. This supports the fact that intragastric pressures of equal amplitude were obtained in response to left and right cervical vagal stimulations. The total number of fibres in the dorsal trunk was twice those in the ventral trunk. The number of efferent fibres in the two trunks are equal. Therefore the dorsal trunk is overwhelmingly afferent. Although the fibre counts were

done at the level of the trunk rather than within the stomach wall, it is most probable that these ratios reflect the position in the stomach wall. If the vagus nerve is so predominantly afferent, the only explanation that can be given for the relative ineffectiveness of the ventral trunk as compared to the dorsal trunk is that probably the afferent fibres play a greater functional role in gastric innervation than has hitherto been recognised.

From the above account, it is clear that certain questions remain unanswered:-

1. Why is the ventral vagal trunk relatively ineffective?
2. In the absence of regenerative sprouting, what is responsible for vagal functional recovery after chronic dorsal trunk vagotomy?
3. Why is it that the dorsal trunk innervates the ventral wall of the antrum and pylorus?

and

4. Why is it that only the antrum and pylorus have double innervation?

Recommendations

In pursuance of attempts to answer the above questions, the following experiments are recommended:-

1. Quantitative study of vagal innervation of the stomach by autoradiographic techniques at light and electron microscope levels.

2. Autoradiographic study, at electron microscope level, of synaptic morphometric changes in response to chronic abdominal trunk vagotomy.

3. Combined autoradiography and unilateral cervical vagotomy to determine the vagal distribution of each cervical vagal trunk. This can be extended to determine the origin of the fibres that innervate the ventral wall of the antrum and pylorus.

APPENDIX 1.1

Burge's Intra-operative Electrical Stimulation Test

Burge and Vane (1958) developed, first in cats and later in man, a technique of electrical stimulation of the vagal nerves around the oesophagus. They measured the rise in pressure inside the stomach which had been filled with water and isolated by a pyloric clamp and an oesophageal balloon. After the surgeon had cut all the vagal nerves he could find, the electrodes were stimulated supramaximally by 40-50 volts at 10 Hz for 1-2 minutes. If there was a rise in intra-gastric pressure, the surgeon searched for persisting vagal fibres. These were then cut and the electrical tests repeated until a negative result was obtained. A ring electrode allowed each of four quadrants to be stimulated separately. The water in the stomach was later replaced by air and the kymograph by water manometer (Burge and Clark, 1958). The test was further modified by the use of a transistorised stimulator (Burge, 1960). The Mark 2 model used air pressure gauge (Burge, 1964) and the Mark 3 instrument was built into a standard sphygnomanometer box (Burge et al., 1969). A written record could be obtained with a transducer and pen recorder. The disadvantages of this test included prolongation of the operation by 15 to 20 minutes, and the need to omit atropine or hyoscine from the premedication. Occasionally, it is difficult or impossible to pass the tube, and there has been at least one death from perforation of the oesophagus (Coupland and Cumberland, 1972). Persisting fibres may be missed if they are within the ring electrode but not stimulated or outside the electrode some distance from the oesophagus (Baron, 1978).

APPENDIX 1.2

The Grassi Test

This is based on measuring acid secretion to an intravenous infusion of pentagastrin since it is known that denervated parietal cells become unresponsive to a standard concentration of circulating gastrin. The stomach is opened through a small gastrotomy and carefully washed out to remove acid and musus. By means of a pH probe, the pH of the mucosa is mapped out. A denervated area has a pH greater than 5.5. (See Grassi, 1975.)

APPENDIX 2.1

Preparation of Urethane Anaesthetic

1. Normal Saline

100 ml of normal saline was prepared as follows:

Sodium chloride (NaCl)	0.9 g
Distilled water to make	100 ml

The mixture was hand-shaken or stirred on a magnetic stirrer until the sodium chloride was completely dissolved.

2. 50% Urethane

100 ml of 50% Urethane was prepared as follows:

Urethane powder	50 g
Normal saline to make	100 ml

3. 25% Urethane

100 ml of 25% Urethane was prepared as follows:

Urethane powder	25 g
Normal saline to make	100 ml

The mixtures were warmed and stirred on a magnetic stirrer until the urethane was completely dissolved. The solutions were stored in tightly stoppered bottles.

Precaution

Urethane is believed to be carcinogenic. Therefore gloves were worn and great care was taken in its handling to avoid contact with the skin, oro-nasal mucosa and the eyes.

APPENDIX 3.1

Formulation and Preparation of Perfusion Solutions

1. Normal Saline

Sodium chloride	45 g
Distilled water to make	5000 ml

The sodium chloride was stirred into the distilled water to give 5 litres of normal saline.

2. 10% Formal-Saline Fixative

Normal saline	2700 ml
Formaldehyde	300 ml

These were mixed to give 3 litres of 10% formal-saline fixative.

APPENDIX 3.2

Preparation of Subbing Solution

1. Recipe

Gelatin	5.0 g
Chromic Potassium Sulphate (Chrom alum)	0.5 g
Distilled water to make	1000 ml

2. Procedure

Seven hundred and fifty millilitres of distilled water was placed in a 2-litre conical flask and warmed on a hot plate/magnetic stirrer unit. Gelatin was stirred into the water. When this had fully dissolved, Chrom alum was added. The mixture was stirred until a uniform colour was obtained. More distilled water was added to make 1000 ml. The solution was allowed to cool to room temperature and filtered. The solution was usually prepared just prior to use.

APPENDIX 3.3

Preparation of One percent Cresyl Fast Violet Stain

Recipe

Cresyl Fast Violet powder	5 g
Distilled water	500 ml

Procedure

The distilled water was placed in a 1-litre conical flask and warmed on a hot plate. The cresyl fast violet powder was stirred into the water. The solution was allowed to cool. It was filtered and kept in tightly stoppered bottle.

APPENDIX 3.4

Preparation of Harris's Haematoxylin Stain

1. Recipe

Haematoxylin	5.0 g
Absolute Alcohol	50.0 g
Aluminium Ammonium Sulphate (Ammonium Alum) OR Aluminium Potassium Sulphate (Potash Alum)	100.0 g
Distilled Water	950.0 ml
Mercuric Oxide	2.5 g
Glacial Acetic Acid	40.0 ml

2. Procedure

The distilled water was warmed up and the alum added and stirred until all dissolved. The haematoxylin was dissolved in absolute alcohol. The two solutions were mixed together in a 5-litre flask. It was necessary to use a flask of much larger volume than the contents on account of the frothing that took place on adding mercuric oxide. The neck of the flask was blocked with cotton wool and the mixture was allowed to boil for 1 to 2 minutes. Mercuric oxide was added and the flask was cooled rapidly in a bowl of cold water under running tap water. The stain was covered and left standing for several hours before it was filtered. Four millilitres of glacial acetic acid per 100 ml of stain (40 ml) was added before use. The acid enabled the stain to keep for some time - usually a maximum of 4 days in these experiments.

One percent hydrochloric acid in water was prepared from a 2N stock solution by adding 55 ml of the stock solution to 345 ml of distilled water.

APPENDIX 4.1

Preparation of 0.1M Phosphate Buffer

This was prepared in three stages as follows:

1. 0.2M solution of monobasic sodium phosphate

   (SOLUTION A) was prepared with

$NaH_2PO_4.2H_2O$ (sodium dihydrogen orthophosphate)	3.12	g
Distilled water to make	100	ml

2. 0.2M solution of dibasic sodium phosphate

   (SOLUTION B) was prepared with

$Na_2HPO_4.12H_2O$ (disodium hydrogen orthophosphate)	6.16	g
Distilled water to make	100	ml

3. 0.1M phosphate buffer was prepared with

Solution A	23	ml
Solution B	77	ml
Distilled water	100	ml
	200	ml

The pH of this buffer was usually about 7.2 and the molarity was about 226 mosmols.

APPENDIX 4.2

Preparation of 3 percent Glutaraldehyde Fixative

Recipe		
	Glutaraldehyde stock (25 percent in water)	12 ml
	0.1M Phosphate buffer solution	88 ml
		100 ml

Method

Eighty-eight millilitres of 0.1M Phosphate buffer solution were placed in a measuring cylinder. Twelve millilitres of stock glutaraldehyde solution were measured in a fume cupboard and added to the buffer solution. The two were mixed and stored in a clean, glass-stoppered bottle. The osmolarity of this solution was usually about 400 mosmols.

It is generally accepted that the osmolarity of the vehicle (phosphate buffer in this case) determines the effective osmolarity of the fixative. Nevertheless there are differences of opinion as to whether or not glutaraldehyde contributes to the effective osmolarity at all, and if it does, how much. Whereas Bone and Denton (1971) and Bone and Ryan (1972) believed that glutaraldehyde contributes little to the osmotic effect of the fixative, Arborgh et al. (1976) said it does not. Barnard (1976) on the other hand, suggested that the osmotic effect due to glutaraldehyde is only a half of the value of osmolarity obtained using osmometers. Therefore the osmolarity contributed by glutaraldehyde to the fixative solution

in the present experiment was calculated by subtracting the osmolarity of the buffer solution from that of the fixative solution. This value was divided by two to obtain the effective osmolarity of glutaraldehyde alone. This was added to the osmolarity of the buffer solution to give the effective osmolarity of the fixative solution. The value was adjusted to between 350 and 400 mosmols by adding sucrose (usually 0.1M sucrose).

The fixative solution was prepared fresh for use within 24 hours but if it needed to be kept for a longer period, it was stored in a refrigerator at 4^{0}C.

APPENDIX 4.3

Preparation of Phosphate Buffer Wash

Recipe

Sucrose	6.84 g
0.1M Phosphate buffer to make	100 ml

Method

The sucrose was measured out into a conical flask. About 80 ml of the buffer was added. The mixture was hand shaken until the sucrose dissolved. Sufficient buffer was added to bring the final volume to 100 ml. The osmolarity of this solution was usually in the region of 400 mosmols.

APPENDIX 4.4

Preparation of 2 percent Osmium Tetroxide

Recipe

Osmium tetroxide crystals (1 g per ampoule)	2 g
0.1M phosphate buffer	100 ml

Method

One hundred millilitres of 0.1M phosphate buffer was placed in a clean coloured bottle. Two ampoules of osmium tetroxide with ready-scored neck were broken and dropped into the bottle which was firmly stoppered. The bottle was shaken vigorously and intermittently over several hours until the crystals dissolved. The fixative was usually prepared the day before it was to be used because osmium tetroxide takes a long time to dissolve. The preparation was carried out in the fume cupboard and the fixative stored in a refrigerator at $4^{0}C$.

APPENDIX 4.5

Preparation of Araldite Embedding Medium

Recipe

Araldite Resin (CY212)	400 ml
Hardener (DDSA)	400 ml
Di-butyl phthalate (plasticisor)	40 ml
	840 ml

Method

The three components were measured into a bottle of ample size. This bottle was placed on a magnetic stirrer and the contents thoroughly mixed until there was uniformity in their colour and texture. This araldite required the addition of one drop of accelerator per ml of araldite before use.

APPENDIX 4.6

Preparation of 1% Toluidine Blue in 1% Borax

One percent borax was first prepared from

Sodium Tetraborate (Borax)	1 g
Distilled water to make	100 ml

After the borax had been completely dissolved, it was used in the preparation of the toluidine blue as follows:

Toluidine blue	1 g
1 percent borax to make	100 ml

The solution was filtered and stored in a stoppered bottle.

APPENDIX 4.7

Preparation of 3 percent Uranyl Acetate Stain

The solvent used was 50 percent ethanol rather than water.

Recipe

Uranyl acetate powder	0.3 g
50 percent ethanol in distilled water	11.0 ml

Method

The uranyl acetate was placed in a narrow specimen bottle and ethanol added. The bottle was shaken until the uranyl acetate dissolved. The resulting solution was centrifuged. Care was taken not to shake the bottle after centrifugation so as not to mix the precipitate with the clear supernatant. The bottle was wrapped in foil and either covered on the bench or stored in a refrigerator.

APPENDIX 4.8

Preparation of Reynold's Lead Citrate Stain

Analar materials were used to ensure their purity.

Recipe

Lead nitrate	1.33 g
Tri-sodium citrate	1.76 g
CO_2-free distilled water	30.00 ml
One Normal Sodium hydroxide (N.NaOH)	8.00 ml
Additional CO_2-free distilled water to make	50.00 ml

Method

The glass-distilled, CO_2-free water was placed in a 50 ml flask. The lead nitrate and tri-sodium citrate were added and the contents shaken vigorously for about one minute. The solution was allowed to stand for 30 minutes, shaking intermittently. Eight millilitres of N.NaOH was added to clear the solution of its turbidity. Sufficient distilled water was added to make a final volume of 50 ml. The solution was then mixed by inversion. Any remaining turbidity was removed by centrifugation. The stain was stored in a tightly stoppered bottle to prevent CO_2 from dissolving in it.

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