THE PEPTIDE-CONTAINING NERVES OF THE MAMMALIAN GUT AND PANCREAS IN HEALTH AND DISEASE

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ABSTRACT

Since the 1960s the field of gut endocrinology has undergone a period of rapid growth. An ever increasing number of active peptides are being discovered in tissue structures ranging from classical endocrine cells to autonomic nerve fibres. The peptides found in nerve fibres in the gastroenteropancreatic tract have been called the gut neuropeptides; these include VIP, substance P and enkephalins. It was the localisation of these substances which formed the basis of this study.

The purpose of this work was to apply and, where necessary, adapt immunocytochemical techniques in order to examine the distribution of the peptides in the nerves of the mammalian gut and pancreas, in both normal and disease states and to test the hypothesis that the peptide containing nerves undergo identifiable changes in some diseases.

An extended series of experiments established a new method for preparing tissue for immunocytochemical investigation. The distribution of the peptide containing nerves was studied in a wide range of mammalian species, particularly man and the various different types were found in each species, at all levels of the gut and in each layer of the wall. In the pancreas, peptide containing nerves were found in association with both the endocrine and the exocrine tissue.

Following the localisation of peptides to nerves in normal tissues, pathological specimens were studied from a number of different gut diseases. Distinct abnormalities of the nerves were detected. For example, changes were found in both congenital and acquired megacolon where peptide containing nerves were reduced in number. In contrast, in Crohn's disease one particular group of nerves, those containing VIP, was found to be hyperplastic.

In addition to examining the distribution of the peptides, the occurrence of two so-called 'brain specific' proteins was investigated in the gut and pancreas. Immunostaining of one of the proteins, an enzyme originally but erroneously called neuron specific enolase (NSE), was shown to be a specific means for the demonstration of all known endocrine cells and nerves of the gut and pancreas. The other protein, S-100, has no known function but has a widespread distribution in glial and Schwann cells. It was therefore used as a marker for these elements in normal and diseased tissues.

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CHAPTER 1 INTRODUCTION

BACKGROUND

There exists in the body of every animal so far examined an intricate and delicately balanced system of biologically active peptides, the anatomical locations of which exhibit a diversity (they are found in virtually all areas of the body) matched only by that of their actions. The particularly exciting aspect of the research into these substances is that, despite the 80 year period since their first description (Bayliss and Starling, 1902), it has only been comparatively recently that their singular importance has been recognised and a new era has opened up in a series of related fields, including endocrinology, physiology and pathology.

Gut endocrinology

The first substantial evidence that small peptides can play a role in the control of bodily functions emerged in 1902 (Bayliss and Starling, 1902) with the discovery of secretin. During the previous century the theories of "nervism", or the neural control of functions, predominated. The first recorded research into what is now known as the autonomic nervous system was made by Galen around AD 200 and by the nineteenth century the work of Remark, Bichat, Pavlov and other notables had established the "vegetative" nervous system as a major influence in physiological control. However, at the turn of the century, opinion was beginning to be swayed. New theories were put forward and the possibility was discussed of organs being controlled by substances produced

by distant tissue. Brown Sequard provided the first support for such a concept when he showed that a deteriorating organ could be made to function again on administration of extracts of a normal organ, a process called organotherapy. Further evidence was supplied by Bayliss and Starling who demonstrated that duodenal extracts cause a flow of alkaline juice in dogs. Their isolation of a hormone (a word derived from the Greek for "I arouse to activity"), secretin, initiated the field of endocrinology.

For a number of years after 1902, the contribution made to physiological control by the autonomic nervsous system was largely overshadowed by the great interest in endocrinology. However, as far as gut endocrinology was concerned, following the discovery of gastrin in 1905 (Edkins, 1905), there was a lull of more than 50 years during which little progress was made in determining the nature of the gut endocrine system.

Enteric nervous system

The importance and virtual independence of the enteric nervous system has long been recognised (Bayliss and Starling, 1899). In his delineation of the components of the autonomic nervous system, Langley classified the enteric nervous system as a third division, as he believed that the enteric ganglion cells received little or no input from the central nervous system (Langley, 1921). This fact, so prophetically hypothesised by Bayliss and Starling in their treatise on the movements and innervation of the small intestine, was neatly illustrated in 1917 by Trendelburg (Trendelburg, 1917). He revealed that the gut itself must contain all the necessary neural apparatus for reflex activity simply

by eliciting peristaltic contractions in vitro. The independence of the enteric nervous system has been made widely apparent by the successful application of current surgical procedures, such as vagotomy and sympathectomy, which do not affect gut motility.

Although the autonomic nervous system was classically considered to be bipartite, the first suggestion of the existence of non-adrenergic, non-cholinergic autonomic nerves came as long ago as Langley's era, in 1898, when he found that stimulation of the vagus nerve causes gastric relaxation (Langley, 1898). At the time it was thought by some workers that there may be sympathetic fibres in the vagus. However, it was soon suspected that this is not the case when the pattern of response was found to differ from that obtained after adrenergic nerve stimulation. Later, when blockers of adrenergic transmission became available, it was possible to show that the effect is not due to adrenergic nerves (Greeff et al, 1962).

The finding, in 1963, of inhibitory junction potentials in the musculature of the guinea pig ileum led Burnstock to postulate the existence of non-adrenergic inhibitory fibres (Burnstock et al, 1963). He later put forward his theory that purine nucleotides act as neurotransmitters in this type of nerve (Burnstock et al, 1970). The first suggestion, based on morphological evidence, of the existence of additional types of autonomic nerves was made one year after Langley's physiological experiments on the vagus, in 1899. Dogiel investigated the neuronal cell bodies in the autonomic ganglia of the gastrointestinal tract, using the methylene blue technique, and described three completely different types of cell which he called Types I, II and III (Dogiel,

1899). More recently, certain autonomic fibres were found, using the electron microscope, to have an ultrastructural profile which did not appear to correspond to that of either cholinergic or adrenergic nerves (Baumgarten et al, 1970). These fibres contained large, electron-dense secretory granules, which differed from the agranular vesicles found in cholinergic nerves and the small, electron-dense granules in adrenergic nerves. Baumgarten called these nerves P (peptidergic)-type because of the similarity they bore to the neurophysin and vasopressin ccntaining fibres of the hypothalamus. The term 'peptidergic' had been coined earlier by Bargmann, who used it to describe these hypothalamic neurons (Bargmann et al, 1967). Baumgarten's classification of the apparently non-cholinergic, non-adrenergic fibres as peptidergic now seems quite prophetic in the light of the subsequent localisation of peptides to fibres of this type.

Peptide containing nerves

By 1970 the list of known gut "hormones" or peptides had grown considerably. Major technical advances, such as the development of the biochemical techniques of gel chromatography and amino acid sequencing, had largely overcome the problems inherent to the study of gut endocrinology. These problems mainly ccncerned the very nature of the gut endocrine system. Instead of being localised to a single, easily removable endocrine organ, the gut peptides are found in diffuse cells, strung out along the length of the gut. Thus, it was only when the isolation, purification and synthesis of these peptides became possible that investigative methods, such as immunocytochemistry and radioimmunoassay, were able to be applied. The application of these methods yielded

a wealth of information which led to the rapid, exponential growth of the field of gut endocrinology. Peptides were localised to characteristic cell types and a seemingly endless stream of "new" peptides was discovered. However, indications of the actual complexity of the peptide containing system of the gut, and indeed the whole body, already existed. In 1931, Von Euler and Gaddum described substance P which they found in both the brain and gut of the horse (Von Euler and Gaddum, 1931). By 1975 it was evident that, in most mammals, substance P occurs in the gut, and many other tissues, in nerve fibres (Nilsson et al, 1975, Pearse and Polak, 1975). This finding was of major importance. It provided an ideal reconciliation between the theories of "nervism" and endocrinology by showing that there is an area of overlap between the nervous and endocrine systems. The two systems are related or, as has recently been suggested, they form the ends of a spectrum embodied in a single neuro-endocrine system.

Peptide containing nerves can be found in almost all areas of the body but the gut and pancreas form a major depository for this type of innervation (Bishop and Polak, 1978b, Bishop et al, 1982a). It was in the gut, where Baumgarten first found P-type fibres, that it was recently determined, again using electron microscopy, that certain non-cholinergic, non-adrenergic fibres actually contain peptides. Six years after Baumgarten's report, in 1976, Cook and Burnstock observed that the autonomic nerves of the gut represent a heterogeneous population and they described at least 8 separate ultrastructural profiles (Cook and Burnstock, 1976). Subsequent electron microscopical studies have reported the occurrence of P-type fibres in a number of systems but it was not until the advent of immunostaining at the electron micro-

scopical level that it became possible to show that peptides are present in P-type granules. Using a colloidal gold labelling method, the two most abundant peptides in mammalian enteric nerves, vasoactive intestinal polypeptide (VIP) and substance P, have been immunostained in P-type nerve fibres of the gut (Probert et al, 1981) (Fig 1b). Analysis of the size of the granules containing the two peptides shows that they, in fact, form a heterogeneous group, with each type of granule possible containing a different peptide.

Thus, peptides have been shown to be present in a particular group of enteric nerves and it is the localisation and examination of these P-type or peptide containing nerves, in health and disease, which forms the basis for the work described in this thesis.

GASTROENTEROPANCREATIC NEUROPEPTIDES

The following is an account of the main chracteristics and actions of the neuropeptides currently thought to exist in the innervation of the mammalian gut and pancreas. This list of peptides is constantly being added to, both as more are found in the gut and pancreas and as entirely new peptides are discovered.

This section is divided into two main parts. The first part contains details of those peptides which are generally considered to be present in gastroenteropancreatic nerves and are frequently found at these sites. The second part lists neuropeptides for which less or very recent evidence exists for their occurrence

in peripheral nerves.

Each account includes an outline of the relevant chemical characteristics and actions of the neuropeptides.

Neuropeptides found in gut and pancreatic nerves

Vasoactive intestinal polypeptide (VIP)

Bayliss and Starling, whilst carrying out the investigations which led to their discovery of secretin, reported the presence of a vasodepressor substance in the intestinal extracts (Bayliss and Starling, 1902). In 1969, Said and Mutt found that extracts of lung are vasoactive (Said and Mutt, 1969) and, by 1972, the peptide responsible for these actions had been isolated and termed vasoactive intestinal polypeptide or VIP by them (Said and Mutt, 1972).

VIP is made up of 28 amino acids and, because of similarities in their amino acid sequencies, has been grouped with glucagon, secretin and GIP into what is known as the secretin-glucagon family of peptides. Little is known about the biosynthesis of VIP and its precursors (see section entitled Neurotransmitter Role).

Following its discovery, there was some controversy as to whether VIP is produced by endocrine cells or nerve fibres. The rapid removal or inactivation of VIP in the circulation

(in man the half-life of VIP is about 12 minutes) (Modlin et al, 1978) suggested that it does not act as a circulating hormone. Radioimmunoassay revealed high concentrations of VIP in nervous tissues and cultured nerve cells (Said and Rosenburg, 1976) and subsequent immunocytochemistry has shown that VIP is mainly localised to nerve fibres (Bryant et al, 1976). VIP, in fact, has a widespread distribution in the autonomic nervous system and VIP containing nerves form a major component of the peptide containing nervous system.

Vascular effects

Like all regulatory peptides, VIP shows potent biological activity. As was originally established, VIP is highly vaso-active, an effect which has been demonstrated in most systems of blood vessels, including peripheral (Said, 1980), coronary, splanchnic (Said and Mutt, 1970), pulmonary (Mojarad et al, 1980) and cerebral vessels (Edvinsson, 1981). The effect VIP has on the smooth muscle of the vessel wall does not appear to involve receptors for any other known neurotransmitter ie the receptors are specific for VIP (Said, 1982). The frequent finding of VIP immunoreactive nerves around vessels in the organs where its pronounced vasodilatory actions have been demonstrated strongly suggests that VIP plays a prominent role in the control of local blood flow.

Motor effects

VIP has been shown to relax the circular smooth muscle

of all areas of the gut of various mammals (Rattan et al, 1977, Morgan et al, 1978, Anuras and Cooke, 1978). VIP containing nerves have been suggested to be the enteric inhibitory nerves found in all mammals which produce the descending inhibitory component of the peristaltic reflex (Furness and Costa, 1982). In general, the location and actions of VIP support this proposal. Stimulation of the vagal pathways which evoke the activity of enteric inhibitory neurons causes a rise in VIP concentrations in plasma or venous effluent from the relaxing region of gut (Fahrenkrug et al, 1978). Furthermore, when the oesophagus is distended so as to induce an inhibitory reflex in the stomach, VIP can be found in the gastric effluent and antiserum to VIP blocks the activation of the enteric inhibitory nerves (Goyal et al, 1980).

However, there is also some evidence against VIP being the transmitter substance in the enteric inhibitory nerves. Firstly, administration of the drug chymotrypsin causes a significant reduction in the effect of VIP whilst not causing any change in the response of the enteric inhibitory nerves to stimulation. Conversely, apamin is an antagonist of transmission in the enteric inhibitory nerves but does not affect the action of VIP (Mackenzie and Burnstock, 1980). Secondly, the VIP receptors are desensitised when repeatedly exposed to VIP but transmission in the nerves is not affected (Yahasaki et al, 1981). It is possible that VIP does play some part as a neurotransmitter in enteric inhibitory nerves but may not be the sole component. Its exact role has yet to be established.

Receptors for VIP are present on smooth muscle cells isolated from both human (Bitar et al, 1980a) and guinea pig stomach (Bitar and Makhlouf, 1980). Therefore, the effect VIP has on muscle appears to be a direct one. Intracellular c-AMP rises during VIP-induced relaxation of smooth muscle cells (Makhlouf, 1982), suggesting that this is the mechanism whereby the inhibition is induced.

A contractile effect of VIP on muscle has also been reported but only at very high concentrations of peptide. This effect, on guinea pig small intestine, was blocked by both tetrodotoxin and atropine and so appears to involve cholinergic transmission (Jaffer et al, 1974). Although the role of VIP in this contraction is yet to be determined, further evidence for the existence of the effect has been provided by experiments showing that VIP increases the firing rate of approximately 50% of the neurons of the myenteric plexus of guinea pig small intestine (Williams and North, 1979).

Secretory effects

Exocrine

The fact that VIP is a powerful stimulant of exocrine secretion has been demonstrated by perfusion studies in a range of mammals including man. High levels of circulating VIP reduce or totally abolish water and electrolyte absorption in the jejunum, ileum and colon of humans and induce secretion (Krejs, 1981). VIP is thought to mediate this effect by stimulation of adenylate cyclase activity, thus causing

a rise in c-AMP concentration (Schwartz et al, 1974). It has been shown to do this in isolated mucosal cells from rats (Amiranoff et al, 1978), guinea pigs (Binder et al, 1980), rabbits and humans (Schwartz et al, 1974). Using radioactively labelled peptide, VIP receptors have been demonstrated on mucosal cells from rat gut and similar binding affinities were present in each of the different regions examined (Laburthe et al, 1979).

In the dog, VIP inhibits food (Villar et al, 1975) and histamine (Konturek et al, 1976) stimulated acid and pepsin secretion. VIP is suggested to play a role in the regulation of gastric secretion as a reduction in gastric secretion can be seen where high levels of circulating VIP exist, but this may be secondary to other factors such as dehydration etc (Makhlouf, 1982) . VIP inhibits acid secretion in vivo but does not affect histamine induced c-AMP production in isolated fundic glands, possibly because the in vivo inhibition involves release of somatostatin (Makhlouf, 1982).

In the pancreas, vagal stimulation of water and bicarbonate secretion is accompanied by a rise of VIP in the venous effluent and is abolished by hexamethonium but not atropine (Hickson, 1970, Holst et al, 1979). Exogenous application of VIP to the isolated pancreas has the same effect (Lindkaer Jensen et al, 1978). In vivo experiments show that VIP has only a minimal effect on enzyme secretion, although it has a more potent effect on isolated acinar cells (Robberecht et al, 1976).

As in the gut, the secretory effects of VIP on the exocrine

pancreas are thought to be due to stimulation of adenylate cyclase and the binding of VIP to specific receptors on acinar cells, which correlates with its ability to stimulate increases in c-AMP concentrations (Gardner and Jackson, 1977).

Endocrine

Little is known about the effects of VIP on endocrine secretion from the gut, although it appears to inhibit gastrin secretion both in vivo and in vitro (Chiba et al, 1980[a]). In the pancreas, VIP causes release of all four pancreatic hormones, insulin, glucagon, pancreatic polypeptide (Adrian et al, 1978) and somatostatin (Ipp et al, 1978). In order to cause the release of these peptides, high concentrations of VIP must be attained. Such levels are above those found physiologically in the plasma but may result from the local release of VIP from nerve terminals.

RELEASE OF VIP

The release of VIP from the gut has been demonstrated under a number of conditions. Neural release of VIP into local venous effluent is induced by stimulation of vagal and pelvic nerves (Fahrenkrug et al, 1978[a]). It can also be induced by injection of acetylcholine (Fahrenkrug et al, 1978[b]) or neostigme (Bitar et al, 1980[b]), an inhibitor of cholinesterase. The release of VIP produced by administration of these agents is blocked by atropine thereby indicating that at least a proportion of the VIP containing neuronal

cell bodies in the gut wall are innervated by cholinergic neurons (Makhlouf, 1982) . Other factors which can induce VIP release include intraduodenal infusion of hydrochloric acid, fat or ethanol, distension of the oesophagus and mechanical stimulation of the mucosal epithelium (Makhlouf, 1982).

Substance P

Substance P was the first "brain-gut" peptide to be described (Von Euler and Gaddum, 1931). It was isolated from extracts of equine brain and intestine in 1931 but has only recently been purified, sequenced and shown to consist of 11 amino acids. The dipeptide amide at the carboxyl terminal of the substance P molecule is the same as that in bombesin.

Like VIP, substance P is thought to be present mainly in nerve fibres, although it has been reported to occur in enterochromaffin cells in certain mammals (Nilsson et al, 1975). The peptide is found in the majority of tissues in the body and has a diverse range of actions.

Sensory neurotransmission

Very little is known about the sensory innervation of the gut. However, additional information has become available with the finding that substance P may be a sensory neurotransmitter (Otsuka and Konishi, 1977). The discovery of substance P in neurons of spinal ganglia (Hokfelt et al, 1975) gave an early indication that the peptide may have such an function. Substance P is now

known to be present in primary sensory efferents and evidence is accumulating from electrophysiological experiments to show that it is involved in peripheral nociception (Polak and Bloom, 1981). Although substance P, in general, has an excitatory effect on nociceptive neurons it can also, in low doses, have an analgesic effect. As these opposite effects appear to be dose related, it is thought that the analgesia obtained with low doses of substance P is mediated by the release of endogenous opiates. Higher doses of substance P elicit a more powerful nociceptive response which outweighs the effect of the opiates (Frederickson et al, 1978).

Capsaicin, the active ingredient of red pepper, has proved to be extremely useful in the investigation of the role of substance P as a sensory neurotransmitter. This chemical releases the peptide from sensory terminals and, so, depletion of substance P after capsaicin application provides a simple marker for substance P containing sensory fibres (Skrabanek and Powell, 1980).

In the gut the distribution of sensory fibres has not been fully established but it has been suggested that those involved in the peristaltic reflex are present mainly in the myenteric plexus, where a large number of substance P containing fibres occur (Gabella, 1974) It may seem, in view of previous concepts of sensory neurotransmission, that, if substance P is a sensory neurotransmitter, its presence in nerve endings is unexpected. However, it is known that, as well as being transported to the central nervous system, neurotransmitters can have a local effect at the peripheral ends of sensory fibres. For example, sensory fibres have been shown to cause vasodilatation (Hinsey and Gasser, 1930). Substance P has vasodilatory effects and may act on local

tissues as well as in sensation. It has been reported that capsaicin does not alter the substance P content of the guinea pig gut (Holzer et al, 1980), which suggests that there are no substance P containing sensory fibres at this site. However, these findings remain unconfirmed. It may be that sensory fibres containing the peptide form only a small part of the total population of substance P nerves in the gut. If this is so then it may not be easy to detect a relatively small depletion of peptide content.

Vascular effects

Substance P is one of the most potent vasodilators known (Eklund et al, 1977). It appears to affect the precapillary resistance of all vascular systems so far studied. However, the circulation in the intestine seems to be far less sensitive to the peptide than that of other systems, such as the skin, despite there being, generally, a higher content of substance P in the gut of mammals than in the skin. This apparent discrepancy may be explained by the autoregulation which is a prominent feature of intestinal circulation. Infusion of substance P may not affect total blood flow in the gut because of local compensatory alterations (Burcher et al, 1977).

Motor effects

Substance P has been shown to stimulate peristalsis in the guinea pig gut (Holzer and Lembeck, 1978). It causes contraction of smooth muscle at very low doses and appears to be more potent than acetylcholine, histamine or serotonin (Rosell et al, 1977).

The octapeptide at the carboxyl terminal is the most active portion in this respect. The stimulatory effect of substance P on peristalsis has been demonstrated in vivo and in vitro (Skrabanek and Powell, 1980). The longitudinal muscle seems more sensitive than the circular coat and substance P affects it mainly in a direct manner as the effect is little altered by atropine, serotonin agonists or tetrodotoxin. The effect of substance P on the circular muscle however is partially antagonised by tetrodotoxin and has been suggested to involve a serotoninergic pathway ((Skrabanek and Powell, 1980).

Substance P also causes, in a dose dependent manner, depolarisation of most myenteric neurons. This has led to the suggestion that it functions as a neurotransmitter of excitatory neurons in the myenteric plexus (Katayama and North, 1978). As was mentioned previously, large numbers of substance P containing fibres are found in the myenteric plexus and so its distribution agrees with this suggestion. Non-cholinergic contractions of the gut can be observed after high frequency stimulation of the enteric nerves. It was thought that this effect may be due to substance P as it has a direct effect on muscle (Franco et al, 1979). However, it was thought that substance P was not involved in the normal neural excitation of the gut musculature but it "overflowed" from the fibres in the myenteric plexus during the high frequency stimulation (Franco et al, 1979). The presence of substance P containing nerves in the two muscle coats argues against this and the recent electron microscopical finding of substance P in nerves terminating on gut muscle fibres (L. Probert, personal communication) indicates that these nerves directly innervate

the musculature and that substance P has a primary role in controlling gut motility.

Secretory effects

Exocrine

Although substance P was first described in 1931, its identity was not made clear until 40 years later when, whilst attempting to purify corticotrophin releasing factor from bovine hypothalamus, workers isolated a peptide with powerful sialogogic actions, which turned out to be substance P (Leeman and Hammerschlag, 1967, Chang and Leeman, 1970). The sialogogic effect of substance P is somewhat paradoxical in that, although it has been found to be atropine resistant, atropine causes a slight increase in the latency of substance P induced activity of rat salivary acinar cells. Receptors for substance P have been found on acinar cells. In the guinea pig, substance P causes calcium efflux and accumulation of c-AMP with release of amylase (Powell and Skrabanek, 1981).

Endocrine

Very few effects of substance P have been demonstrated on the endocrine system. It inhibits the release of somatostatin from the isolated rat stomach (Chiba et al, 1980[a]) and has been reported to cause the in vivo release of insulin, glucagon and somatostatin from the dog pancreas (Hermansen et al, 1980). Apart from these effects, substance P may have some function as a hypothalamic releasing or inhibiting factor (Kerdelhue et al, 1978).

Release of substance P

The release of substance P has been evoked from synaptosomal fractions and brain slices using depolarising concentrations of potassium but not, as yet, from nerves supplying the gut (Nicoll et al, 1980). Opiate analgesics and endorphins prevent the release of substance P from sensory afferents (Frederickson et al, 1978).

Enkephalins

The discovery of the enkephalins was unusual in that it was preceded by that of their stereospecific binding sites. Whilst studying the actions of opium alkaloids in the brain, Pert and Snyder, in 1973, used radioactively labelled substances which revealed the existence of these sites (Pert and Snyder, 1973). If there were binding sites then it followed that there must be some previously unrecognised endogenous opiate. In fact, a whole range of endogenous opiate-like peptides was discovered (Hughes et al, 1975, Ling et al, 1976). These have subsequently been divided into two main groups, the endorphins and enkephalins.

The enkephalins were isolated from porcine brain, sequenced and shown to be two pentapeptides differing only by the presence of methionine or leucine at the carboxyl terminal (Hughes et al, 1975). They were thus called met- and leu-enkephalin. Variants of these two molecules have been found in extracts of brain and adrenal gland. These include met^5arg^6-enkephalin and met^5-arg^6phe^7-enkephalin (Stern et al, 1980). These variants have not been found in the gut and their significance has yet to be determined.

Met-enkephalin was recognised to correspond to the 61-65 portion of B-lipotrophin (B-LPH) (Hughes etal, 1975), a 91 amino acid peptide originally found in the pituitary (Li et al, 1965). Active portions of the B-LPH molecule had been sequenced. These were derived from the C terminal and called endorphins (Ling et al, 1976). The most potent of these substances was found to be B-endorphin. Although B-endorphin contains the entire sequence of met-enkephalin there is little evidence to suggest that it, or any of the other endorphins, is a precursor of met-enkephalin. In fact, a common precursor for both enkephalins has been identified in extracts of adrenal medulla (Lewis et al, 1980). This protein contains seven units of met-enkephalin and one of leu-enkephalin. Enzymatic degradation of the protein thus gives rise to a ratio of met- to leu-enkephalin of almost seven to one. There is, generally, more met-enkephalin in tissues than leu-enkephalin eg in porcine brain the ration between the two is four to one. Intermediate molecules in the probable biosynthesis of the enkephalins have also been isolated. If there is separate biosynthesis of enkephalins and endorphins it would explain some of the differences in their anatomical distributions.

In the gut, met-and leu-enkephalin have been reported to be present in separate nerve types (Larsson et al, 1979). Some reports exist of enkephalin-like material in endocrine cells of the mucosa, particularly in gastrin containing cells of the antrum and duodenum (Polak et al, 1977[b]). Enkephalin-like material has been found by radioimmunoassay in all areas of the gut and is present in the highest concentrations, in man, in the antrum and duodenum (Linnoila et al, 1978), a location which appears to agree with the postulated

existence of enkephalin in gastrin cells.

Motor effects

Enkephalins, and other opiates, have the main action in the gut of decreasing motility (Uddman et al, 1980). This effect is achieved by inhibition of acetylcholine release (Waterfield et al, 1977) and of firing of neurons in the myenteric plexus (North and Williams, 1976). It has been suggested that enkephalin acts on the nerve processes which release acetylcholine, rather than on the cell body (North et al, 1979) and opiate receptors have been found on cholinergic terminals in guinea pig gut (Bornstein and Fields, 1979). Acetylcholine release is inhibited by activation of these receptors. Naloxone is an antagonist of enkephalin receptors and can affect gut motility, providing further evidence for a role for enkephalins in the control of gut motility (Kromer and Pretzlaff, 1979).

Secretory effects

Endocrine

Low doses of both met- and leu-enkephalin stimulate the release of insulin and glucagon from isolated islets, while high doses inhibit secretion (Gleen et al, 1980, Kanter et al, 1980). Findings made in patients with non-insulin dependent diabetes have given an indication that enkephalins or other opiates may be involved in glucose homeostasis, as the patients show attenuated sensitivity to the opiates (Corder and Rees, 1981).

Exocrine

In the past, there have been conflicting reports as to the effects of opiate peptides on gut secretion. Morphine and other opiates have long been considered to inhibit gastric secretion but recent results suggest that this is not the case. Both morphine and met-enkephalin induce a dose dependent increase in basal acid secretion from the canine stomach, with no concomittant change in gastrin levels (Konturek et al, 1980). In addition, the opiate peptides increase both acid secretion and mucosal blood flow when administered during histamine or pentagastrin stimulated gastric secretion. Opiate peptides release histamine and so, as their stimulation of gastric secretion does not involve gastrin, a rise in histamine in the mucosa may provide the stimulus for the increased blood flow and secretion.

Opiates are known to inhibit pancreatic secretion but this effect may be due to contraction of the pancreatic duct rather than a direct action on the secretory cells Konturek et al 1981). Enkephalin inhibits CCK induced pancreatic enzyme secretion (Konturek et al, 1981). It is also possible that these effects may be due to the inhibitory influence on cholinergic mechanisms.

Vascular effects

Met-enkephalin appears to increase intestinal circulation but does not affect heart rate or systemic blood pressure (Konturek et al, 1978, Walis et al, 1980). The vasodilatory effect seems to be specific for the gut.

Bombesin

Comparatively little notice was taken, in 1971, of the isolation of a fourteen amino acid peptide from the skin of a European frog (Bombina bombina) (Anastasi et al, 1971). However, two years later great interest was shown in this peptide when it was found to be a potent stimulator of gastric acid release (Bertaccini et al, 1973). This stimulation of gastric acid is accompanied by a pH independent rise in the circulating levels of gastrin. The peptide, called bombesin, is now considered to be the most potent releaser of gastrin in man.

Bombesin is one of a number of peptides, including alytesin, ranatensin and litorin, which have been isolated from amphibian skin. All these peptides are structurally related but only bombesin has so far been found in mammals (Erspamer, 1980). Bombesin is also structurally related to substance P as they have the same two amino acids at the carboxyl terminal.

Bombesin-like peptides have been extracted from both porcine (McDonald et al, 1979) and chicken (Erspamer et al, 1979) intestine. Both peptides have very similar effects to bombesin and nine of the ten amino acids at the C terminal are the same as in bombesin. These two peptides have been called porcine- and chicken- GRP (gastrin releasing peptide) and may form part of an entire family of bombesin-like peptide. Bombesin is mainly found in endocrine cells in the gut of birds (Timson et al, 1979) and in autonomic nerves in mammalian gut (Polak et al, 1978). This dual localisation

suggests that the family of peptides may have a range of different modes of action. A physiological role for these peptides has been suggested as the bioactivity of each bombesin-like peptide resides in the common C terminal end of the molecule (Erspamer, 1980).

The actions of bombesin are mainly stimulatory, in contrast with those of somatostatin which are, generally, inhibitory. As well as stimulating the release of gastrin, bombesin also releases motilin, neurotensin and enteroglucagon from the gut and glucagon and pancreatic polypeptide from the pancreas (Bloom et al, 1979).

Just as it indirectly causes secretion of gastric acid, bombesin also causes enzyme rich pancreatic juice flow by releasing CCK from the duodenum (Erspamer et al, 1974). Bombesin may also have a direct effect on the pancreas as it has been shown to stimulate amylase secretion from rat pancreas in vitro and specific membrane receptors for the peptide have been demonstrated on pancreatic acinar cells (Robberrecht et al, 1975, Iwatsuki and Petersen, 1978, Jensen et al, 1978). A role has also been postulated for bombesin in the control of pancreatic growth and/or ontogenic development. Young rats injected with bombesin over a 14 day period had a significant increase in pancreatic weight. This hyperplasia was of both the endocrine and exocrine pancreas and was accompanied by an increase in pancreatic DNA and enzyme content (Lezoche et al, 1981).

Infusion of bombesin into man caused a brief increase followed

by complete inhibition of mechanical activity in the duodenum and total inhibition of mechanical activity in the jejunum throughout the infusion period (Bertaccini et al, 1974, Corazziani et al, 1974). However, this effect may be due to one of the peptides released by bombesin, for example, cholecystokinin.

Somatostatin

Somatostatin was first isolated from the hypothalamus of the sheep and characterised as a peptide having the action described by its alternative name: growth hormone release inhibiting factor (Brazeau et al, 1973). This tetradecapeptide was also found in large concentrations in the pancreas and gut, where it was shown, in 1975, to be present in what are now known to be D cells (Polak et al, 1975).

However, radioimmunoassay of somatostatin in different tissues detected a peptide larger than the tetradecapeptide first isolated, but with somatostatin-like immunoreactivity, indicating that is shared a similar molecular structure (Praydayrol et al, 1978). Subsequently, the peptide now known as somatostatin 28 (amino acids) was isolated and characterised (Praydayrol et al, 1978). Somatostatin 28 contains the entire amino acid sequence of somatostatin 14 at its C-terminal. It also appears to have similar actions to somatostatin 14 and, in certain cases, has been shown to be more potent (Vaysse et al, 1981). This indicates that somatostatin 28 is not just a pro-hormone. Its physiological role has yet to be established. It is not yet known whether there is a differential distribution of the two molecules in nerve fibres and endocrine cells.

Gut

The actions of somatostatin are mainly inhibitory. Infusion of somatostatin into mammals, including man, decreases both basal levels of gastrin and levels stimulated by a meal (Bloom et al, 1974, Phillip et al, 1977). Somatostatin also inhibits the release of a number of other gastrointestinal hormones, including secretin (Boden et al, 1975), CCK (Schlegel et al, 1977), GIP (Pedersen et al, 1975), VIP (Besser, 1976), motilin (Bloom et al, 1975) and enteroglucagon (Sakurai et al, 1975). Its effects are thus in direct contrast with those of bombesin, which are stimulatory.

Somatostatin can alter gut motility, probably in inhibition of electrically stimulated acetylcholine release from the myenteric plexus. It reduces absorption from the gut and decreased gastric acid and pepsin secretion.

Pancreas

As in the gut, somatostatin affects the release of peptides from the pancreas. It has been shown to inhibit the basal and stimulated release of insulin (Alberti et al, 1973), glucagon (Mortimer et al, 1974) and pancreatic polypeptide (Gerich et al, 1976) by direct action on B, A and PP cells. It also inhibits pancreatic bicarbonate and enzyme secretion (Boden et al, 1975).

CCK-8 (cholecystokinin octapeptide)

When first isolated, CCK (or pancreozymin) was characterised as a 33 amino acid peptide (Jorpes and Mutt, 1973), but it has subsequently been shown to exist, in blood and tissues, in several molecular forms (Dockray, 1981). The CCK-33 molecule is found in mucosal endocrine cells in the upper small intestine (Buffa et al, 1976, Buchan et al, 1978). A larger molecule, CCK-39 (Mutt, 1976), with the 33 amino acids of the slightly smaller molecule and an additional 6 at the N-terminal, has been isolated and is thought to be a precursor of CCK-33. There may be other, larger forms of CCK which have still to be described.

A small form of CCK, consisting of the 8 amino acids at the C-terminal of CCK-33 and CCK-39 (CCK-8), was originally isolated from brain (Vanderhaegen et al, 1975, Dockray et al, 1976). This molecule exhibits greater biological activity than CCK-33 and is found in both endocrine cells and nerve fibres in the gut (Dockray et al, 1981). Gut nerves containing CCK-8 have, so far, only been described in rodents (Schultzberg et al, 1980). They are more frequent in the rat than in the guinea pig, but are present only in very small numbers in this species, where they are found predominantly in the circular muscle coat.

An even smaller portion of the CCK33 molecule has been isolated (Larsson and Rehfeld 1979a, b). This is the so-called tetrin molecule which consists of the 4 amino acids at the C-terminal of all forms of CCK (these 4 amino acids also occur at the C-terminal of gastrin). Tetrin has been found in most tissues which produce

gastrin and/or CCK (Rehfeld, 1978, Rehfeld and Larsson, 1979, Larsson and Rehfeld, 1979a) and one report exists of its occurrence in colonic innervation (Larsson and Rehfeld, 1979b). However, there is a certain amount of controversy surrounding tetrin as not all researchers agree that it exists (Rehfeld, 1981). It has been suggested that this disagreement may be due to inappropriate antisera. This question is unresolved.

The main actions of CCK include stimulation of enzyme secretion from the pancreas (Jorpes and Mutt, 1966) and contraction of the gall bladder (Ivy and Oldberg, 1928). In addition, CCK is known to cause contraction of the longitudinal muscle of the guinea pig ileum. This is thought to be an indirect effect involving the myenteric plexus and the release of acetylcholine (Vizi et al, 1973, Vizi et al, 1974). It is thus possible that the CCK-8 containing nerves found in the rodent gut may act as excitatory interneurons.

Infrequently localised and "new" neuropeptides in gut and pancreatic nerves

Angiotensin

In 1971, a brain renin-angiotensin system was demonstrated, which was thought to play a part in the control of drinking and blood pressure (Ganten et al, 1971). Angiotensin II immunoreactive nerve cells have been found in the hypothalamus, amygdala and substantia gelatinosa of the spinal cord and are postulated to

control neuronal functions by acting as neuromodulators at synaptic junctions (Fuxe et al, 1976). There is some evidence that these angiotensin II containing neurons may interact with catecholamine pathways in the central nervous system (Goldstein and Brody, 1976).

However, only very few reports exist of the occurrence of antiotensin II immunoreactivity in the gut (Fuxe et al, 1977, Ganten et al, 1978, Furness et al, 1982). It was first found in the rat, mainly in the neuronal cell bodies of the myenteric plexus and around arteries. This latter location has been put forward as evidence for a direct participation of angiotensin II in the control of blood flow in the gut.

Angiotensin II has also been found in neurons of the guinea pig myenteric plexus, which have been observed to have processes extending in only an oral direction (Furness et al, 1982). This antiotensin II-like material could not however, be detected by radioimmunoassay. As yet, angiotensin II has not been found in the gut of other mammals.

Neurotensin

Neurotensin is another peptide, like somatostatin, which was first extracted from the brain (hypothalamus) (Carraway and Leeman, 1973) and later shown to be present in the gut (Kitabagi et al, 1976, Polak et al, 1977[a]). Radioimmunoassay of the neurotensin content of the body showed that only 10% is in the brain and around 80% can be found in the ileum. In this latter location,

neurotensin has been localised to characteristic cells called N cells (Polak et al, 1977a).

When isolated originally, neurotensin was shown to consist of 13 amino acids (Carraway and Leeman, 1973). It is now thought that, like CCK and somatostatin, there may be a group of neurotensin-like peptides, each containing the C-terminal region which contains the molecules' biological activity (Carraway, 1981). Variants of neurotensin have been shown to exist in the mammalian, including human, gut and brain, although they have yet be fully characterised (Carraway, 1981). The fact that they exist has been shown using region specific antisera. Antisera to the N-terminal of neurotensin measured similar concentrations of neurotensin in radioimmunoassay to those detected by antisera to the 8 residues at the carboxyl terminal. However, an antiserum to just the 4 amino acids at the C-terminal end measured much higher concentrations, indicating that there are other neurotensin-like peptides with the identical 4 amino acids at the biologically active C-terminal, but differing in structure elsewhere. No portions of the neurotensin molecule have been extracted from tissues (Carraway, 1981).

So far, neurotensin has been found, outside the central nervous system, in very few nerves of the rat gut (single report) (Schultzberg et al, 1980). This substance appears to be the tridecapeptide.

There is some dispute concerning the actions of neurotensin. In original experiments which used large doses of neurotensin, the effects obtained included hypertension with cutaneous vasodilation, cyanosis and hyperglycaemia. It is difficult to determine

whether the peptide actually has these actions because of the size of the doses used. Intravenous infusion of neurotensin at lower doses has, more recently, been shown to decrease the rate of gastric emptying (Blackburn et al, 1980) and inhibit gastric acid secretion (Andersson et al, 1976). The levels of neurotensin induced in the plasma in both of these studies were of the same magnitude as those seen after ingestion of a high fat meal and, thus, may occur physiologically.

In the pancreas, neurotensin has been found, also in low doses, to stimulate the release of pancreatic polypeptide. This effect appears to be the most sensitive of the actions so far attributed to neurotensin and its discovery has led to the peptide being cited as a component of the humoral control of pancreatic polypeptide release.

In vitro experiments on the effect of neurotensin on gut motility indicate that its actions are species specific (Kitabi and Freychet, 1978). Neurotensin relaxed the rat ileum and contracted the guinea pig ileum and taenia. Tetrodotoxin did not affect the responses to neurotensin in the rat ileum or the guinea pig taenia. However, the contraction of the guinea pig ileum was abolished by tetrodotoxin, suggesting that a neural mechanism is involved in this action. Neurotensin containing nerves have not been found in any part of the guinea pig gut and so it may be that neurotensin is present in nerves in some species in a yet to be discovered molecular form. This would certainly explain why there is only one report of its neuronal localisation in the gut.

Motilin

Although motilin was originally extracted and isolated in 1971 (Brown et al, 1971) in a single molecular form as a peptide of 22 amino acids, it is now known to exist in two forms (Christofides et al, 1980). These two forms are both present in gut and plasma. One form has been found, on gel chromatography, to co-elute with pure, natural porcine motilin and the other appears to be of a larger size and may be a precursor molecule.

As its name suggests, motilin is a potent stimulator of motility. Although its actual physiological role is not yet clear, it appears to enhance gastric emptying (Christofides et al, 1979), stimulate human colonic activity (Rennie et al, 1980) and increase the frequency of interdigestive myenteric motor complexes (Itoh et al, 1976).

Most reports of motilin being found in nervous tissue have been con fined to the brain (Yanaihara et al, 1978), with only isolated accounts of its presence in gut innervation (Nagai et al, 1980). The vast majority, if not all, motilin in the gut appears to be produced by mucosal endocrine cells (Christofides et al, 1980).

TRH (Thyrotrophin Releasing Hormone)

There have been several reports of the effects of TRH on the gut (Smith et al, 1977, Dolva et al, 1979, Bruce et al, 1979, Dolva et al, 1980), but few describing its neuronal localisation

at this site (Schultzberg et al, 1980). This small (3 amino acids) peptide was first described as an hypothalamic hormone which can stimulate the synthesis and release of pituitary thyrotrophin (Reichlin et al, 1978).

In man, TRH inhibits gastric motility induced by either distension (Dolva et al, 1980) or hypoglycaemia (Dolva et al, 1979). The mainly inhibitory effects of TRH in humans are not seen in rat (Bruce et al, 1979) or dog (Morley et al, 1979) where the peptide causes muscle contraction.

Although TRH has been extracted from the gut and pancreas of rats (Morley et al, 1977, Leppaluto et al, 1978) as yet there is no conclusive evidence of its presence in peripheral nerve fibres. It has recently been reported to be found in large amounts in pancreatic islets, in the same areas as glucagon (Koivusalo et al, 1981).

PHI

The list of gut peptides continues to grow. During the course of this study the pioneer biochemists in the field of gut endocrinology working in Sweden, discovered "new" peptides including one which appears to be biologically active and may be present in nerves in the gut.

PHI is a 27 amino acid peptide first isolated from intestinal extracts (Tatemoto and Mutt, 1980 & 1981). It was isolated using a method whereby fragments of peptides, in this case the carboxyl terminal α-amide which occurs mainly in biologically active peptides, are split enzymatically and isolated from tissue extracts.

Purification and sequencing of peptides obtained in this way from extracts of porcine small intestine revealed the existence of 2 previously unrecognised peptides, one of which was PHI. The name PHI is derived from Peptide with Histidine at the N-terminal end and Isoleucine at the C-terminal end. The structure of PHI is very similar to that of peptides of the secretin-glucagon family, which includes VIP. PHI, as well as being structurally related to VIP, also has some similar actions. Little is known about the biological activity of PHI. It binds to VIP receptors so efficiently as to inhibit the binding of VIP (Bataille et al, 1980). PHI also stimulates C-AMP activity in rat intestinal and liver membranes (Bataille et al, 1980) and acinar cells of the guinea pig pancreas (Jensen et al, 1981). It potently stimulates pancreatic secretion in birds (Dimaline and Dockray, 1980) and, to a lesser extent, cats and has some effects on endocrine cells as it releases insulin and glucagon from the isolated rat pancreas (Szecowka et al, 1980).

Dynorphin

Dynorphin is a tridecapeptide, originally isolated from the porcine pituitary, which appears to have very potent opiate effects (Goldstein et al, 1979). It exerts an opioid-like inhibition

of electrically induced contractions of isolated strips of longitudinal muscle from guinea pig gut. The finding that cross-tolerance to dynorphin does not occur in the muscle strips that have been made tolerant to morphine, although other opiates show typical cross-tolerance, suggests that there is a specific opioid receptor for dynorphin.

Recently, a heptadecapeptide was isolated from the porcine duodenum and its structure determined (Tachibara et al, 1982). The peptide has the biological characteristics of dynorphin and shows immunoreactivity with antiserum to dynorphin. The larger molecule consists of dynorphin with a tetrapeptide extension at the carboxyl terminus. Comparison of the potencies of the pituitary tridecapeptide and the heptadecapeptide, showed that the tetrapeptide has a negligible effect. This heptadecapeptide which has been called duodenal dynorphin, may be an active opiate in the gastrointestinal tract but has yet to be localised by morphological techniques.

"Brain" Specific Proteins

Neuron specific enolase (NSE)

Until recently it was virtually impossible, using available techniques, to demonstrate all neurons and endocrine structures in a single tissue section. Immunocytochemical staining of peptidergic nerves could be carried out and, by mixing of primary antibodies, it is possible to immunostain all known types in one section. However, mixing of antisera is a totally unsatisfactory method

as artefactual results can be obtained e.g. by cross-reaction of antisera or high levels of background staining. Similarly, simultaneous immunostaining of peptidergic nerves and endocrine cells cannot be achieved by this method. Further problems are encountered when demonstrating cholinesterase containing or aminergic neurons using histochemistry and immunostaining peptides in the same sections, as different fixation methods are required. Some silver stains have been used to demonstrate endocrine cells or nerve fibres, but none of them have been found to demonstrate all such structures e.g. some endocrine cells are not argyrophilic (Grimelius and Wilander, 1980) .

In order to study all components of the nervous and endocrine system at one time a universal marker was required and when an enzyme called neuron specific enolase (NSE) was discovered it seemed possible that it is such a marker.

The development, during the past several years, of advanced biochemical techniques e.g. polyacrylamide gel electrophoresis, isoelectric focussing etc. has enabled the isolation and characterisation of a number of proteins. Amongst these was a group of highly acidic soluble proteins which were isolated, in the mid 1960's, from brain extracts (Moore, 1972). These proteins were thought to occur solely in the brain and were called brain specific. Two of the proteins were purified and called S-100 (see later) and 14-3-2 protein. 14-3-2 protein was later shown to be an isoenzyme of enolase (2-phospho- D-glycerate hydrolase, E.C. 4.2.1.11) and was called neuron specific enolase (Marangos et al, 1975). The enzyme has since been shown to be a dimeric protein consisting

of two identical sub-units ($\gamma\gamma$) and have a molecule weight of 78,000 daltons (Marangos et al, 1978). Previous immunocytochemical studies localised NSE not only to neurons but also to a number of APUD (Amine Precursor Uptake and Decarboxylation) cells including those of the thyroid, pituitary, adrenal medulla, pancreatic islets (Schmechel et al, 1978), lung (Wharton et al, 1981) and skin (Gu et al, 1981). Very little information existed on the localisation of NSE in the gastrointestinal tract. Although NSE has been found in neurons and in APUD cells no characterisation of the neurons or cells had been carried out to test if NSE is really a useful marker for all such structures. From what is known about its function it seems feasible that NSE is a universal marker. The enzyme converts 2-D-glycerophosphate to phosphoenol-pyruvate and is common to virtually all living cells. In addition, the fact that it is a very stable molecule is also in favour of it being an appropriate marker substance.

S-100

The S-100 protein is so-called because of its solubility in 100% ammonium sulphate at neutral pH (Moore, 1972). The function of the protein is still to be elucidated. Like NSE, it was originally thought to be present only in the brain (Matus and Mughal, 1975). Recent information indicates that S-100 may correspond to multiple forms. The protein is present primarily in glial cytoplasm but it appears that a very small proprtion may also occur on the membranes of glia and, in addition, neurons (Tabuchi et al, 1976). Different forms of the S-100 protein may be present in glial cytoplasm than in neuronal and/or glial membranes.

The wide occurrence of S-100 in glial cells has suggested that it may provide the means for demonstration of the glial network.

Thus, it may be that NSE and S-100 together provide the opportunity for simultaneous demonstration of all endocrine cells and neurons, with their associated glial structures.

Origin of Peptide Containing Nerves in the Gut

Although peptide containing fibres have been found entering the gut from external sources (Hokfelt et al, 1977a & b, Uvnas-Wallensten, 1981), experimentation in certain mammals has shown that the vast majority of the peptide containing nerves in the gut have an intrinsic origin (Schultzberg et al, 1978, Jessen et al, 1980, Malmfors et al, 1981). Evidence for an intrinsic origin in the human gut has been obtained from pathological conditions (see chapters on pathology).

Extrinsic origin

The extrinsic autonomic nervous supply to the gut arises from the vagus nerve and sacral spinal outflow and from prevertebral sympathetic ganglia. Several peptides have been demonstrated in fibres in these inputs. Substance P, CCK, enkephalin, VIP and somatostatin have all been found in the vagus (Gamse et al, 1979, Lundberg et al, 1979, Lundberg et al, 1980). Ligation studies

carried out by one group indicate that these peptides are transported towards the periphery (Lundberg et al, 1979, Lundberg et al, 1980). However, there is some disagreement as to whether the peptides are only transported to the periphery. This is particularly because, as the vagus contains both motor and sensory fibres, if some of the peptides act as sensory neurotransmitters, as has been suggested for substance P, then they would not be expected to be transported solely in this outward direction. The same group which reported the unidirectional transport of peptides to the periphery also reported the presence of CCK, substance P, VIP and somatostatin in cell bodies of the nodose ganglion, which gives rise to the sensory vagal fibres (Lundberg et al, 1979, Lundberg et al, 1980). This finding provides weight to the argument against the transport of the peptides being solely in an outward direction.

A large proportion of the principal ganglion cells of the inferior and superior mesenteric ganglia and the coeliac ganglion contain somatostatin (Hokfelt et al, 1977a). As the cells are adrenergic this finding has been put forward as evidence for the co-existence of neurotransmitters (see section on co-existence). Also sensory fibres containing substance P may project to the gut via sympathetic ganglia as immunoreactive fibres have been found surrounding non-immunoreactive ganglion cells (Hokfelt et al, 1977b). VIP and enkephalin containing fibres have also been described in some sympathetic ganglia, surrounding non-immunoreactive ganglion cells in most ganglia (Hokfelt et al, 1977c). They may represent relays from the gut. Some principal ganglion cells in the rat superior cervical ganglion were found

to contain enkephalin.

Intrinsic origin

It has been established by experimentation in the pig (Malmfors et al, 1981), guinea pig (Jessen et al, 1980) and mouse (Schultzberg et al, 1978) gut, at least, that the vast majority of the peptide containing nerves have an intrinsic origin from the two main ganglionated plexuses. The intrinsic origin has been demonstrated using denervation and tissue culture methods. The experiments made on the guinea pig gut (work carried out by colleagues in the Department of Histochemistry) involved removing the extrinsic nervous supply, by crushing or cutting nerves, and maintaining the gut in situ. Following sacrifice of the animals, immunocytochemical and radioimmunoassay measurement of peptide content (VIP, substance P and enkephalin containing nerves have been examined) did not detect any significant decrease in peptide content, indicating a predominantly intrinsic origin of the fibres. In addition plexuses were removed separately and cultured. These studies show, that, in the guinea pig at least, VIP fibres mostly originate from the sub-mucous plexus and substance P and met-enkephalin from the myenteric plexus. It has been suggested by other workers that these results may be invalid on the basis that, in a culture system, if one type of cell predominates it might grow and swamp a minor population. This does not appear to be the case however, as immunocytochemical examination of pieces of gut removed from normal animals shows that the distribution of peptide containing ganglion cells is in agreement with these findings.

The origin of peptide containing nerves in the human gastro-intestinal tract can only be postulated from results obtained using pathological specimens. In Hirschsprung's disease (congenital aganglionosis) there is a significant decrease in peptide containing nerves in the aganglionic region (Bishop et al, 1981) (see section on pathology). This suggests that, in humans too, the majority of the peptide containing nerves have an intrinsic origin.

Neurotransmitter Role

The extensive distribution of peptides in nerve fibres leads to the obvious question as to whether they in fact act as neuro-transmitters. To begin to answer this, the established criteria for neurotransmitters must be examined and these are as follows:-

a) Presence of substance in nerve fibres and storage in sufficient quantities in presynaptic terminals.
b) Release of substance from terminal axon when nerve is activitated.
c) Exogenous application of substance to effector must mimic its effect when released by stimulation of nerve.
d) Mechanism for inactivation of substance following its release at or adjacent to the site of its action.
e) Drugs which reduce or potentiate nerve mediated responses must similarly affect responses to the exogenous substance.

Some workers have also added to this list:

f) Specific receptors for substance on post-synaptic membranes.
g) Mechanisms for synthesis of substance and existence of precursor molecules.

The main point concerning whether or not peptides in gut nerves act as neurotransmitters may not be whether they fulfill all these criteria but whether these criteria are actually appropriate. More recent consideration has led to this list of criteria being considerably shortened. This is mainly because the criteria were drawn up on the basis of what was known about neurotransmitters several years ago. They may all be characteristics of certain neurotransmitters but it has been argued that they are not necessarily essential features of all neurotransmitters. In fact, it has been proposed that the only two requirements for any substance to meet before it can be called a neurotransmitter is that it is released by a nerve and that it has the appropriate effect. This is based on the reasoning that a neurotransmitter can simply be defined as something which is released by stimulation of a nerve and has an effect on an adjacent cell (Furness et al, 1982).

Close examination of the established criteria provides good evidence for the need to prune the list. For example, the fourth criterion in the list, i.e. the presence of a mechanism for inactivation of the substance following its release, is one which receives a good deal of support from neuropharmacologists. It is based on work on neurotransmitters in the periphery where, e.g. cholinesterase can be found at the muscle endplate. Mostly, the search for an inactivating mechanism has centred on looking for a degrading enzyme, which is not invariably essential as inactivation can take place by simple extension of the neurotransmitter molecule or possibly the neurotransmitter may cease to act when post-synaptic receptors alter and no longer accept

the neurotransmitter molecule. What is also important is that there is no reason why the neurotransmitter should be inactivated anywhere near where it has its action. It is essential that the neurotransmitter is in some way inactivated but this may occur in the blood stream or in another organ after the neurotransmitter has diffused away from its site of action. Even the apparently basic requirement that the neurotransmitter should occur in nerve fibres may be irrelevant. A certain neurotransmitter may be present in nerves in an unrecognisable precursor form, only assuming its active structure at the terminal when the nerve is stimulated. Clearly then, the requirement is that the substance must be potentially available from the nerve. Alternatively, a transmitter may be present in all nerve fibres throughout the body. Those terminals which are capable of releasing the transmitter may possess specific release mechanisms not present in the other nerves. This is interesting in view of the possible co-existence of "neurotransmitters" (see section on co-existence).

Having reviewed the definition of a neurotransmitter the available information on peptides must be examined to see how confidently a neurotransmitter role can be assigned to the neuropeptides in the gut.

Vasoactive intestinal polypeptide (VIP)

A disproportionately large number of investigations have been carried out on the pharmacological and physiological roles of VIP and it is for this peptide that most evidence for a neurotransmitter role exists. Therefore, this peptide will be discussed in some detail.

Like all the other gut neuropeptides, the widespread distribution of VIP in the innervation of the gut and many other organs suggests that it has a neurotransmitter role. In addition, its specific location in fibres associated with blood vessels, muscle and secretory cells suggests that it may act as a mediator of the neuronal control of blood flow, motility and secretion (Bishop et al, 1982).

VIP can be said to be a neurotransmitter if the only requirements it must meet are that it should be released from a nerve and should have the appropriate effect. It has not yet been shown to possess all the 5 (or 7) characteristics in the classical list of criteria for neurotransmitters.

a) Presence in nerve fibres and storage in sufficient quantities in presynaptic terminals.

That VIP is present in nerve fibres is now a well accepted fact. Large, dense core secretory granules in nerve terminals have been shown to contain VIP, using immunocytochemistry at the ultrastructural level, and these granules appear to be present in significant numbers (Probert et al, 1981). VIP is probably only stored at the terminals and is supplied to the nerve endings via axonal transport in a particulate form at a rate of several mm/hour.

b) Release from terminal when nerve is activated.

VIP is known to have several potent effects in the gut and other organs (Said, 1981). Many of these actions were previously

shown to be non-cholinergic, non-adrenergic neural effects, due to their not being altered by muscarinic or adrenergic blockade, including gastric relaxation (Eklund et al, 1979) and salivary gland blood flow (Bloom and Edwards, 1980[a]).

Relaxation of the stomach follows swallowing and this has been known for some time to be a vago-vagal reflex with the vagal fibres ending on non-cholinergic intramural neurons. Electrical stimulation of the vagus or reflex activation causes a rise of VIP in the venous outflow from the stomach, with no change occurring in the arterial concentrations of the peptide (Eklund et al, 1979). Similarly, stimulation of the feline chorda tympani causes atropine resistant vasodilatation in the sub-maxillary gland which is accompanied by release of VIP. Furthermore, infusion of acetylcholine into the gland causes vasodilatation but not release of VIP, demonstrating that there is no muscarinic control of VIP release (Bloom and Edwards, 1980).

In the gut the number and variety of nerves found in any region preclude selective stimulation of a specific nerve type, a real pre- requisite for demonstration of the release of a substance from a nerve. However, it would, thus, appear from the available information that VIP is released from nerve fibres.

c) Exogenous application to effector must mimic its effect when released by stimulation of nerve.

This is virtually impossible to demonstrate in the gut innervation as, in order to mimic the effect of nerve stimulation, the peptide

would need to be applied to specific action sites in physiological amounts. As mentioned above, the complexity of the gut innervation precludes such a demonstration. However, a general effect of the proposed neurotransmitter may be observed after exogenous application. Thus, intra-arterial infusion of VIP into the stomach (Eklund et al, 1979) or sub-maxillary gland (Bloom and Edwards, 1980[a]) gives rise to gastric relaxation and vasodilatation, mimicking that seen after stimulation of the vagus and chorda tympani respectively. The finding that the VIP concentration in the venous effluent plasma attained during the infusions is similar to that seen during neural stimulation may or may not be of significance. The concentrations measured in plasma may be artefactual, mainly because VIP is very rapidly inactivated in blood (Modlin et al, 1978). In addition, the concentration of VIP in the venous outflow at any given time may simply relate more to the rate at which it is being washed out of the tissue rather than to its rate of release.

As far as can be concluded at present VIP does meet this criterion.

d) Mechanism for inactivation following release at or adjacent to the site of its action.

As was discussed earlier, there is no reason for stipulating that there should be a method of inactivation anywhere near the site of action. If a neurotransmitter passes across the synapse onto post-synaptic receptors it may have its effect and then be released, by simple diffusion perhaps, into the bloodstream where it may then be inactivated or transported to another organ for inactivation. VIP is rapidly inactivated in blood (1-2 minutes

in man and pig) (Modlin et al, 1978) and widespread sites for its destruction have been demonstrated. It seems feasible that it should be removed from action in this way rather than be broken down by some post-synaptic mechanism.

e) Drugs which reduce or potentiate nerve mediated responses must similarly affect responses to exogenous application.

At present no drugs are known to selectively affect the responses of a tissue to exogenous application of VIP.

f) Specific receptors must exist on post-synaptic membranes.

Specific VIP receptors have been demonstrated on several tissues including gut epithelial (Laburthe et al, 1979) and smooth muscle cells (Bitar et al, 1980[a]).

g) Mechanisms for synthesis and existence of precursor molecules.

As yet, little is known about precursors of VIP. Early immunocytochemical studies localised VIP to endocrine cells and/or nerve fibres in the gut. Subsequent biochemical studies have shown that, in fact, the authentic 28 amino acid VIP is only present in neurons. It has been suggested that the immunostaining of endocrine cells was due to cross-reactivity of the antisera employed with peptides belonging to the glucagon-secretin family or, possibly, with other unknown VIP-like peptides. These peptides may be precursors of true VIP. The VIP-like peptides have no vasoactive properties

and do not bind to epithelial cells. Some evidence for the existence of VIP precursors is provided by the presence of a possible tryptic cleavage point on the VIP molecule. Thus, VIP or a precursor molecule may be broken into fragments which can be detected by antibodies to certain portions of the VIP molecule.

Therefore, there is sufficient evidence to show that VIP is present in enteric nerves, is released (albeit under artificial conditions) from them and has neural effects (i.e. abolished by blockers of nerve conduction) in the gut. The peptide is, generally, considered to be a neurotransmitter but it remains to be seen whether the other criteria are met by VIP or whether reassessment of the list will lead to a new appraisal of how exactly a neurotransmitter can be defined. Or perhaps, instead of trying to see if VIP and the other peptides fit into the established category of neurotransmitter, study of their mode of actions may give rise to new functional classifications. VIP has been reported as having neuroparacrine effects. This meant to imply that it is not exactly a neurotransmitter but is released from nerve terminals and diffuses onto adjacent cells. It is apparent that, until new methods are developed, the study of the physiology of VIP will make only slow progress. An obvious beneficial development would be the production of specific VIP antagonists.

Substance P

It has long been known that substance P facilitates peristalsis both *in vivo* and *in vitro* (Skrabanek and Powell, 1980). The effects of substance P on the circular and longitudinal muscle coat appear

to be mediated quite separately. The effect of substance P on the circular muscle, with an intact myenteric plexus, is blocked by atropine, suggesting that it involves activation of excitatory cholinergic neurons. The slow excitatory postsynaptic potentials which can be recorded at neuro-neuronal junctions are similar to those elicited by substance P and both are reduced by chymotrypsin, which denatures the peptide. Substance P is present in nerve fibres in the myenteric plexus and has been shown to be released from them (Katayama and North, 1978). It thus seems possible that substance P has a role as a neurotransmitter in the stimulation of cholinergic neurons in the myenteric plexus. In addition, the peptide has a direct stimulatory effect on the gut musculature (Bury and Mashford, 1977, Yau, 1978). This effect appears to be direct as it is not prevented by atropine, tetrodotoxin or serotonin antagonists. Substance P fibres may terminate directly on muscle cells or it could be that the peptide has a paracrine action, being released in large amounts during intense stimulation, from terminals in the myenteric plexus and, "overspilling" onto the muscle. Substance P may therefore have a dual neurotransmitter and paracrine effect.

Enkephalins

Certain other gut neuropeptides have been described as neuromodulators but none more so than enkephalin. Opiates are known to reduce gut activity, causing constipation (Jaffe and Martin, 1975). There is no direct effect of enkephalin on the smooth muscle of the gut (Cook and Burnstock, 1979) and so the peptide appears to mediate its effects by neuromodulation as it inhibits

to be mediated quite separately. The effect of substance P on the circular muscle, with an intact myenteric plexus, is blocked by atropine, suggesting that it involves activation of excitatory cholinergic neurons. The slow excitatory postsynaptic potentials which can be recorded at neuro-neuronal junctions are similar to those elicited by substance P and both are reduced by chymotrypsin, which denatures the peptide. Substance P is present in nerve fibres in the myenteric plexus and has been shown to be released from them (Katayama and North, 1978). It thus seems possible that substance P has a role as a neurotransmitter in the stimulation of cholinergic neurons in the myenteric plexus. In addition, the peptide has a direct stimulatory effect on the gut musculature (Bury and Mashford, 1977, Yau, 1978). This effect appears to be direct as it is not prevented by atropine, tetrodotoxin or serotonin antagonists. Substance P fibres may terminate directly on muscle cells or it could be that the peptide has a paracrine action, being released in large amounts during intense stimulation, from terminals in the myenteric plexus and, "overspilling" onto the muscle. Substance P may therefore have a dual neurotransmitter and paracrine effect.

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both acetylcholine release (Waterfield et al, 1977) and firing of neurons in the myenteric plexus (North and Williams, 1976). The finding of opiate receptors on cholinergic nerve terminals (Bornstein and Fields, 1979) suggests that enkephalin inhibits acetylcholine release by acting at the terminal rather than the cell body. Enkephalin thus may act as a neurotransmitter but carry out its actions by neuromodulation rather than directly.

Neuromodulation

The term neuromodulator, like neuroparacrine substance, implies that the peptide in question is not a neurotransmitter in the classical sense but, in some way, modifies the actions of real neurotransmitters. However, neurotransmitter and neuromodulator are not mutually exclusive terms and should not be treated as such. Although the physiology of the gut neuropeptides has yet to be fully elucidated, there is no reason to imagine that they will not all be found to be neurotransmitters, in addition to whatever other roles they may play.

Co-existence of Peptides in Nerves with other Neurotransmitters

Several problems are encountered during the evaluation of possible co- existence of transmitters in nerve fibres and not the least of these is the definition of a neurotransmitter. As was outlined above, gut neuropeptides may act as neurotransmitters in the classical sense or may have previously unrecognised "neurotransmitter-like" modes of action. Even if there is a generally accepted definition of a neurotransmitter, such as a substance

which is present in nerves, released by neural stimulation and has postsynaptic actions on specific receptors, it is still very difficult to prove conclusively that any substance fulfills the criteria and that two such substances co-exist in the same nerve fibre.

Precursors

There are several reasons for this. Firstly, there is the question of precursor molecules. Some types of nerve may contain substances with a putative or proven neurotransmitter role which are actually the precursor molecules of the "true" neurotransmitter for that nerve. For example, an adrenaline producing nerve will have adrenaline as its neurotransmitter but may also contain nor-adrenaline and dopamine as molecules produced during adrenaline synthesis. It has been suggested that as these amines are precursor molecules and are present in adrenaline nerves in only small amounts, although they are considered neurotransmitters elsewhere, this cannot be taken as co-existence of transmitters. However, no evidence has yet been given to show that the precursor amines are not released from adrenaline nerves and do not have postsynaptic effects and so this may still prove to be a long known but unrecognised example of transmitter co-existence.

The question of precursors is, of course, irrelevant to the study of co-existence of peptides with classical neurotransmitters as they have distinctly different precursors but will be of vital importance when considering co-existence of peptide transmitters. Most of the gut peptides are thought to be produced primarily

as larger molecules which are then split by enzymes into the active peptides. It is conceivable that a peptide containing nerve may, like the adrenaline nerve, contain small quantities of precursor molecules. Little is known about this aspect. It is conceivable that, for example, nerves reported to contain tetrin may also produce small quantities of CCK-8 and they may both act as neurotransmitters. Similarly, met- and leu-enkephalin are now thought to have a common precursor molecule and it is not known whether the two are present in the same nerve fibres. There are precedents for this in the central nervous system where, in the hypothalamus, B-endorphin and ACTH are found in the same neurons (Bloch et al, 1978), although it is not known whether they are both released.

"Silent" and "False" Transmitters

The co-existence of transmitters has been postulated where one of the substances, although it fulfills a role as a neurotransmitter in other systems, can be found to occur in certain nerve fibres but have another effect (Burnstock et al, 1976). For example, dopamine-β-hydroxylase is an enzyme in the synthetic pathway of noradrenaline and adrenaline. Tests have shown that a number of substances including serotonin, are effective inhibitors of the activity of this enzyme. Thus, serotonin may occur in adrenaline or nor-adrenaline nerves only as an internal modulator of transmitter synthesis. These would not qualify as examples of co-existence as only adrenaline or noradrenaline is released.

Some nerves are known to take up transmitters. A situation

may arise whereby a nerve, although it is capable of producing only one transmitter, may actually release more than one because it has this property of taking up "false" transmitters (Thoenen and Tranzer, 1971). Such nerves can also be ignored when searching for co-existing transmitters.

Classical Transmitters and Peptides

Peptides in sympathetic neurons

Biochemical assessment of possible co-existence of transmitters could only be achieved under the ideal conditions of a single nerve cell being available with no attached tissue. This is possible when considering invertebrates with giant nerves but is not possible in mammalian tissue. Physiological evidence again depends on having an indisputably "pure" nerve preparation and so it is mainly morphological investigation which has, so far, yielded most information on the possible co-existence of transmitters.

These morphological studies are beset with problems. To begin with, staining of a single neuron for two (or more) substances involves either double staining of the neuron which is mostly unsatisfactory or cutting two sections through the same neuron. At the light microscopical level this, of course, means through the neuronal cell body. Even this may not provide a suitable means for staining two different substances as the histochemical demonstration of each may require different tissue fixation methods. So, of choice, the same techniques should be used to stain the two substances or ultrastructural studies should be carried out.

In the gut, serial sections cannot be used to see if peptides are present in adrenergic nerves as these do not have intrinsic cell bodies. The occurrence of peptides in adrenergic nerves has therefore been studied in the sympathetic ganglia supplying the gut. Here, a somatostatin-like peptide has been found in the majority of the nor-adrenaline ganglion cells (Hokfelt et al, 1977). Furthermore, ligation of the mesenteric nerves causes accumulation of this somatostatin-like peptide on the ganglion side, indicating transport of the peptide towards the gut. An enkephalin-like peptide has been found in a small number of principal cells in certain sympathetic ganglia (Schultzberg et al, 1979). This morphological evidence for co-existence was obtained by immunostaining peptides and enzymes of catecholamine biosynthesis (tyrosine- and dopamine-β-hydroxylase) in serial sections. Whether or not the peptides are functioning as neurotransmitters in the sympathetic neurons remains to be seen.

Peptides in cholinergic neurons

A similar experiment could theoretically be carried out in the gut by immunostaining serial sections for peptides and enzymes of acetylcholine biosynthesis. However, good antibodies to such enzymes have not been forthcoming and evidence for the co-existence of peptides and acetylcholine has been gathered using other methods.

It has been reported that VIP occurs in cholinergic fibres on the basis of a number of observations. These observations

include that of very similar distributions of VIP containing nerves and acetylcholinesterase rich fibres in various tissues. There is controversy regarding the specificity of acetylcholinesterase staining as a marker of cholinergic neurons (Pepler and Pearse, 1957, Silver, 1974, Lehmann and Fibiger, 1980). This enzyme is not only present in non-cholinergic neurons but has recently been shown to break down substance P (Chubb et al, 1980). Furthermore, acetylcholinesterase breaks down acetylcholine post-synaptically and so would be expected to occur on cell bodies, at least, with which cholinergic nerves synapse. Although there is doubt about the classification of acetylcholinesterase containing neurons as cholinergic, VIP has been found in acetylcholinesterase rich ganglion cells in what may be the best defined cholinergic neurons i.e. those from the stellate and L7 and S1 sympathetic ganglia (Lundberg et al, 1979). These cells are known to control sweat secretion and ganglionectomy resulted in a loss of both acetyl-cholinesterase positive and VIP immunoreactive nerves around sweat glands.

Ultrastructural observations have also been used to suggest the concept of the co-existence of VIP and acetylcholine (Johansson and Lundberg, 1981). VIP was immunostained in large dense core vesicles in nerves of exocrine glands which contained many small agranular vesicles, typical of those found in cholinergic neurons. This could be artefactual as the small agranular vesicles found in adrenergic nerves and reported to contain acetylcholine have been shown, in experiments where adrenergic nerves are loaded with noradrenaline or serotonin, to be empty granular vesicles.

Co-existence of VIP and acetylcholine has been put forward

to explain certain physiological anomalies. Stimulation of parasympathetic supply to the submandibular salivary gland causes secretion, vasodilatation and release of both acetylcholine and VIP. The salivation is entirely atropine sensitive but there appears to be two components of the vasodilatory response, one atropine positive and therefore cholinergic and the other atropine resistant. There is good evidence for VIP being the mediator of this atropine- resistant response. VIP is present in nerves around salivary blood vessels, it is released when atropine-resistant vasodilation occurs, exogenous VIP causes vasodilation and the neurally stimulated vasodilatation is blocked by VIP antisera (Lundberg et al, 1982).

The atropine sensitive component occurs at low frequency stimulation and, at moderate frequencies,vasodilatation is reduced by atropine. It is at maximal frequencies that the response is atropine resistant and therefore probably VIP induced. VIP release increases with stimulation frequency and, thus, it has been suggested that there is a proportional release of acetylcholine and VIP according to the level of stimulation. These studies suggest close interaction in the control of acetylcholine and VIP release but do not necessarily mean that the two are functioning in the same nerve fibres.

There is therefore no conclusive proof that peptides exist as transmitters with the classical neurotransmitters in nerves of the gut.

Peptides and peptides

As yet only one unsupported report has emerged of the co-existence of peptides in nerves of the gut. This was of somatostatin and gastrin/CCK-like immunoreactivity in ganglion cells of the sub-mucous plexus (Schultzberg et al, 1980). Co-existence of these peptides has not been reported in any other tissue and no common precursor of the two substances has been described.

AIMS OF STUDY

The gastrointestinal tract and pancreas have long been known to contain large quantities of bioactive peptides which were originally thought to be present exclusively in endocrine cells. Although the first 'brain-gut' peptide was described in 1931, it has only been comparatively recently that the concept has been generally accepted of these peptides also being present in a widely distributed system of peripheral nerves. This study used the technique of immunocytochemistry at the light microscopical level to investigate various aspects of this neuro-endocrine system. The aims of the study were as follows;

a) To examine and compare existing methods of tissue fixation and, if necessary, to develop a new technique in order to obtain a method which would allow optimal immunostaining of peptides in nerve fibres and endocrine cells in various different tissues.

b) Using a wide range of antisera to different peptides, to determine which of the peptides exist in the innervation of the gastrointestinal tract and pancreas.

c) The mapping of the various types of peptide containing nerves in the gut and pancreas of a number of mammalian species in order to determine their normal distribution patterns.

d) Comparison of these normal patterns of distribution with those seen in specific gastrointestinal diseases to see if any identifiable, consistent and, therefore, characteristic, changes occur.

e) Localisation of two brain specific proteins, neuron specific enolase (NSE) and S100, and characterisation of the tissue components which contain NSE.

CHAPTER 2 METHODS

At the start, the intention of this study was to examine the entire gut (or regulatory) peptide-containing central and peripheral nervous systems. The enormity of this task was not fully comprehended at the time because very little was known about the extent of the innervation containing these, mostly, newly-discovered peptides. When the work began, it soon became clear that an investigation of all peptides in the entire nervous system of the body was well beyond the scope of a single worker. Different aspects of the work were therefore handed over to other PhD students in the Department of Histochemistry while one area of particular interest was concentrated on, the gastroenteropancreatic tract and its pathology.

The investigative methods used in this study were histology, histochemistry and immunocytochemistry. Where relevant, information on the ultrastructure of the peptide containing nerves has been included. This was kindly provided by my colleague in the Department of Histochemistry, Dr Lesley Probert. Data on the radioimmunoassay measurement of peptide content were supplied by colleagues in Professor S R Bloom's laboratory, Department of Medicine, Hammersmith Hospital. I carried out all statistical analysis of the data, using Student's unpaired t-test or analysis of variance, where appropriate. Immunocytochemistry, which forms the basis of this study, is one of the most useful analytical methods currently available in the field of morphological research. As cells and nerves containing the various peptides under investigation cannot be distinguished from one another by conventional

histology or histochemistry, immunocytochemistry provides the sole means for localisation of these structures. Parallel histology and histochemistry were used to establish the overall condition of the tissues, using conventional histological standards and to visualise components of the autonomic nervous system which, because of the inavailability of appropriate antisera, cannot yet be confidently demonstrated using immunocytochemistry.

The purpose of this chapter is to describe in general the main methods used in this study and, where necessary, to show how they were developed. Further, more specific details, in particular sampling methods, are described separately in the chapters dealing with each aspect of the work.

DISSECTION AND HANDLING OF TISSUE

All dissection and fixation of tissue was carried out rapidly, as soon as possible after death or removal at surgery. Animals were sacrificed by decapitation or overdose of anaesthetic, where appropriate, and immediately dissected and the tissues fixed.

Whenever possible, human specimens were collected in person from the operating theatre or post-morten room and transported to the nearest available location for dissection and fixation. Specimens sent from other centres on a routine basis were immediately placed within a container of ice and despatched. To evaluate any possible degradation of tissues in transit from these centres, normal control tissues were despatched in exactly the same

way as the diseased specimens, so that the results could be compared.

Examination of tissues fixed at different lengths of time after post-mortem or surgery showed that obvious degenerative changes occurred, in both the morphology of tissue and the immunostaining of peptides, between 10-12 hours of death or surgical removal. Thus, a time limit of 8 hours between death or removal and fixation of tissue was established (see Fixation). As, in Britain, post-mortem examinations are generally carried out more than 8 hours after death, material taken after death was only used infrequently.

The methods of dissection and sampling of tissue were varied with the nature of each specimen and are given in detail in each section of this study. In general, whole widths of gut wall were taken and sectioned transversely, as in routine histopathological practice. This was done in order to enable comparison with routine histological findings made in the diseases studied and with a view to the possible applications of immunocytochemistry in diagnostic histopathology. One of the main onuses of this study has been the modification of what was previous considered to be a research tool into a technique of possible usefulness in routine laboratories.

CONVENTIONAL HISTOLOGY

Sections from all tissues examined were stained with haemotoxylin and eosin. As I had no previous experience of histo-

pathology, I am very grateful to Drs S Hamilton, M N Sheppard and S S Kurian and my supervisor, Dr Julia Polak, for examining the H&E preparations with me and for their patient teaching.

IMMUNOCYTOCHEMISTRY

In simple terms, immunocytochemistry can be defined as the localisation of a substance in situ by the use of labelled antibody. Many modifications have been made of the original immunocytochemistry methodology described by Coons et al in 1961 (Coons et al, 1961, Facer et al, 1980). For the localisation of substances in tissue sections, the initial immunocytochemical technique consisted of the application of a labelled antiserum to the section and the subsequent examination of the section to observe the distribution of the label and, hence, the substance. This direct method has several disadvantages, not least of these being the necessity to label all the various antisera to different substances. An improvement of this technique was the use of a second layer antiserum, raised against the immunoglobulin of the donor animal of the first antiserum. This meant that only the second layer antiserum need be labelled. This indirect method is widely used at the present time. The second layer antiserum is usually labelled with fluorescein, for the indirect immunofluorescence technique, or with an enzyme, peroxidase (Avrameas & Ternynck, 1971). In order to see the distribution of the substance under study in the indirect immunofluorescence technique, the sections have to be examined using an ultra-violet microscope. For the immunoperoxidase method, the enzyme is reacted with its substate, hydrogen peroxide, in the presence of a light invisible coupler. Various couplers can be used,

giving a range of colours in the final reaction. Diaminobenzidine is probably the most popular coupler. A refinement of the immuno-peroxidase technique is the unlabelled antibody enzyme or peroxidase anti-peroxidase (PAP) technique (Sternberger, 1974). This method employs three layers of antibodies. The first layer is the same as in the other techniques. The second layer consists of an unlabelled antibody against the immunoglobulin of the donor animal of the first layer antibody. This antibody forms a bridge between the first layer antibody and the third layer of the PAP complex (3 peroxidase molecules and 2 antibodies against peroxidase raised in the same species as that which produced the first layer antibody).
The indirect immunofluorescence and the PAP methods were both used in this study.

Antisera

For the results of immunocytochemistry to have any significance at all, only fully characterised antisera can be used. There are a number of possible sources of non-specific immunostaining, i.e. an antibody-antigen reaction other than that intended. Before attempting to raise an antiserum by immunising an animal, as most peptides are too small to produce a suitable immunoreaction, they must be coupled to a larger carrier molecule eg bovine serum albumin. Straight away, unwanted antibodies can be produced against this carrier protein. However, this can be overcome by absorbing the resultant antisera with a small amount of the carrier protein to order to precipitate the unwanted antibodies. Similarly, any serum subsequently removed from the donor animals

may contain a large population of mixed antibodies to various substances present in a fixed tissue section. Again, these can be removed firstly by diluting the antiserum as far as is compatible with strong specific immunostaining and by absorbing the antibodies with tissue components from the species being immunised which do not contain the substances being localised. This sort of non-specific immunostaining can also be remedied by applying normal, non-immune serum from the donor animal to tisseu sections. All the non-specific binding sites present in the section will thus be blocked.

Newer methods for obtaining "pure" preparations of antibodies include affinity chromatography (Dockray,1979) and production of monoclonal antibodies (Kohler & Milstein, 1975). Affinity chromatography consists of the coupling of the relevant antigen to an inert support medium. As a mixed population of antibodies passes over the medium only the required antibodies will bind to the antigen. The antibodies can subsequently be removed by elution with buffers of differing pHs. Monospecific antibodies can also be prepared by cloning of hybridoma cells, giving the added advantage of a potentially inexhaustable supply of antibodies.

However, although it is possible now to produce preparations of antibodies with a very high level of purity, the basic problem still exists of establishing exactly what that pure antibody recognises. Studies of the size of antigen binding sites on antibody molecules have shown that most antibodies will recognise between 4-8 amino acids on a peptide molecule. This means that,

whatever portion the antibody recognises, it must be found only in that one peptide. This becomes increasingly difficult to ensure as the existence of "families" of structurally related peptides and pro- and even pre- proforms of peptides becomes more evident.

It is therefore imperative to use only those antibodies for which the region specificity has been fully determined. The antibodies used in this study were all fully characterised prior to their use. This characterisation included preincubation of each antiserum with its corresponding whole antigen, with fragments of the antigen where available, and with structurally related substances. Details of each antiserum are displayed in Table **1.**

Controls

Controls were routinely applied when each new tissue was immunostained. These consisted of:-

1) The use of non-immune rabbit serum as first layer.

2) The use of either the fluorescein-conjugated globulin or the peroxidase anti-peroxidase complex alone, as appropriate.

3) Pre-absorption of each antiserum with its corresponding antigen, at a range of concentrations.

Table 1 DETAILS OF ANTISERA

Antiserum to:	HH Ref.	Dilutions IF	Dilutions PAP	Region Specificity	Results of aborption tests on antibodies to peptides in nerves: Bomb 1-14	Leu Enk	Met Enk	PHI 1-27	PHI 1-15	PHI 14-27	Som 1-14	Som 1-28	Sub P 1-11	Sub P 7-11	VIP 1-28	VIP 1-22	VIP 7-28
Bomb	362	1:400	1:1000	C-term	0.1								10	10			
	377	1:200	1:800	N-term	0.1								+	+			
	627	1:400	1:4000	C-term	0.1								10	10			
Enk-Met	497	1:200	1:800	Whole		50	0.05										
	869	1:400	1:4000	Whole		50	0.05										
PHI	938	1:400	1:4000	C-term				0.01	+	0.01					+	+	+
Som 14	744	1:2000	1:10000	Whole							0.1	0.1					
28	1082	1:800	1:2000	N-term							+	0.1					
Sub P	359	1:400	1:2000	C-term	+								0.1	1.0			
	910	1:500	1:5000	C-term	+								0.1	1.0			
VIP	324	1:800	1:2000	Whole				+	+	+					0.01	+	+
	652	1:2000	1:20000	Whole				+	+	+					0.01	$\pm$	$\pm$
Angio	470	1:100-1000		Whole													
CCK 39	899	1:200	1:5000	C-term													
Dyn	734	1:100-1:1000		Whole													
Enk-Leu	492	1:100-1:1000		Whole													
	774	1:100-1:1000		Whole													
Gas*	899	1:200	1:5000	C-term													
GIP	378	1:600	1:12000	Mid													
Gluc	499	1:1000	1:5000	N-term													
Ins**	413	1:800	-	Not known													
Mot	123	1:300	1:1500	N-term													
	407	1:400	1:1000	C-term													
NT	810	1:400	1:4000	Whole													
PP	317	1:3000	1:12000	Not known													
Sec	53	1:2000	1:6000	Not known													
Serot	644	1:1000	1:4000	Not known													
TRH	493	1:100-1000		Not known													
NSE	839	1:200	1:2000	Not known													

Figures = amount of peptide or fragment needed to completely quench immunostaining (nmol/ml diluted antiserum)

+ = No quenching of immunostaining after addition of up to 20nmol peptide or fragment/ml diluted antiserum

$\pm$ = Partial quenching of immunostaining after addition of 10nmol peptide or fragment/ml diluted antiserum

*This antibody fully cross-reacts with gastrin and CCK-39

**All antibodies were raised in rabbits except for anti-insulin which was a guinea pig antiserum

MODIFICATIONS OF BASIC IMMUNOCYTOCHEMICAL METHODS

There have been various papers describing ways of modifying and, thus, improving the two main immunocytochemical methods, immunofluorescence and immunoperoxidase. The more appropriate of these modifications were tested. Test immunostains consisted in each case of 10μm cryostat section of BQS-fixed brain, gut (antrum and colon) and pancreas immunostained with three of the antisera most frequently used in these studies i.e. anti-VIP, anti-substance P and anti-glucagon (except in the brain). The modifications described below were made to the standard techniques of indirect immunofluorescence (Coons et al, 1955) and peroxidase anti-peroxidase (Sternberger, 1974).

1) Temperature during immunostaining

All immunostains were carried out on tissue sections mounted on glass slides. During incubation of the sections with antibodies the slides were placed on racks in plastic Petrie dishes. The interior of each dish was kept moist by dampened filter paper laying across the bottom of the dish.

The antibody solutions were used at their highest possible dilutions (for reasons outlined earlier) and so sections were incubated with primary antisera for a minimum period of 12 hours, to allow maximum formation of antibody-antigen binding.

In order to see if this binding could be improved by altering the temperature at which the sections were incubated, test immunostains were set up and incubated for 16 hours at $4^{o}C$, room temper-

ature (average 22°C) and 37°C.

At 37°C, even in a well moistened incubation dish, evaporation of the antisera occurred. No variation in the quality of the immunostain could be found in the sections incubated at the 3 different temperatures. For convenience, room temperature was selected for all subsequent incubations.

2) Use of surfactant in washing buffers and/or antibody diluent

The theory behind the use of a surfactant in immunostaining, either in washing buffers or antibody diluent, is that it aids penetration of the antibody into the tissue and reduces non-specific, low affinity protein binding. Triton X100 has been found to be a useful agent for this purpose (Hartman et al, 1972). However, it has to be used at low concentrations (0.3% in Hartman et al) so as not to interfere with antigen-antibody binding.

Solutions of 0.1%, 0.2% and 0.3% Triton X100 in phosphate buffered saline were prepared. These were used to dilute antibodies and/or wash sections between antiserum incubations. Using a series of test immunostains the only significant improvement was seen in sections of brain where background staining was lower. This effect may be related to the high lipid content of the nervous tissue. Immunostaining of sections of gut and pancreas was not greatly improved. In view of the inconveniences of using a surfactant in immunostaining (mainly as loss of surface tension results in antibody solutions having to be used in larger quantities

than usual to cover whole glass slides) and the lack of significant improvement in immunostaining of gut and pancreas, Triton X100 was not used in this study.

3) Effect on immunostaining of altering salt content and pH of buffer

A possible source of unwanted immunostaining can be the binding of immunoglobulins to secretory granules by non-specific ionic interactions. It has been reported that this effect is enhanced by lowering the sodium chloride content and/or lowering the pH of the phosphate buffered saline used for diluting antibodies and/or washing the tissue sections during immunostaining (Grube, 1980).

Test immunostains were set up, as before, using the following variations in phosphate buffered saline as antibody diluent and washing buffer.

1) 0.01M phosphate buffered 0.15M saline, pH 7.1 - 7.4
2) 0.01M phosphate buffered 0.5M saline, pH 7.1 - 7.4
3) 0.01M phosphate buffered 0.15M saline, pH 8.0
4) 0.01M phosphate buffered 0.5M saline, pH 8.0

Subjectively, no variations in the quality of the immunostains could be detected. The level of background staining remained uniformly low. No additional possibly non-specifically immunostained cells or nerves could be seen in sections where phosphate buffered

saline solutions of high salt concentration or pH were used. 0.01M phosphate buffered 0.15M saline at pH 7.1 - 7.4 was used throughout these studies.

SENSITIVITY OF IMMUNOSTAINING

The sensitivity of an immunostain or its limits of dectection is impossible to assess in tissue sections. A certain number of immunostained structures cannot be correlated with the known peptide content of a tissue as many structures with little peptide will not immunostain. A cell or nerve may produce a certain peptide but, at any given time, there may be too little stored peptide to allow immunostaining of the structure. This was taken into account when evaluating the results obtained in these studies. Furthermore, the apparent intensity of an immunostain has not been shown, in any tissue, to bear a direct relationship to the peptide content of the immunostained cell or nerve. For this reason, any variation in intensity was noted only if marked and consistent in a number of specimens. It was therefore used as a relative indicator in tissues which had been immunostained in exactly the same way and not in order to provide definitive information.

FIXATION

The problems encountered at the beginning of this study mainly concerned fixation of tissue: of the many conventional fixatives available (such as formaldehyde, Bouin's solution etc) it has long been known that none gives completely satisfactory preservation of antigens for immunocytochemistry. The exact reason for this is unknown, but it is possible that the reactions of the fixatives with the proteins in the tissues may be too harsh, causing denature of the antigens to be immunostained. As immunocytochemistry is carried out using aqueous media, the substances of interest must be rendered insoluble without any loss of their antigenicity. This, of course, must be achieved without altering the morphological details of the tissue.

Bifunctional reagents

During the 1970s, bifunctional reagents, such as p-benzoquinone and diethylpyrocarbonate (DEPC), were introduced to the field of immunocytochemistry as tissue fixatives (Pearse & Polak, 1975). These chemicals form cross-links either within or between tissue proteins, as do the conventional aldehyde fixatives, but they are rather more weakly reactive. This comparative "weak" reactivity means that p-benzoquinone and DEPC do not provide good morphological preservation for electron microscopy.

Although many such chemicals have been found to be very efficient in rendering peptides insoluble, their effects can interfere with subsequent immunoreactions. As well as altering the antigenic site on the peptide molecule, thereby rendering it unrecognisable to the corresponding antibody, the chemical may fix other larger proteins

present in the cell, causing "masking" of the peptide being immunostained. In view of these varied features, it is not really possible to use a single fixation procedure for all immunocytochemical methods. However, extensive experimentation has established certain methods as being suitable for different aspects of immunocytochemistry.

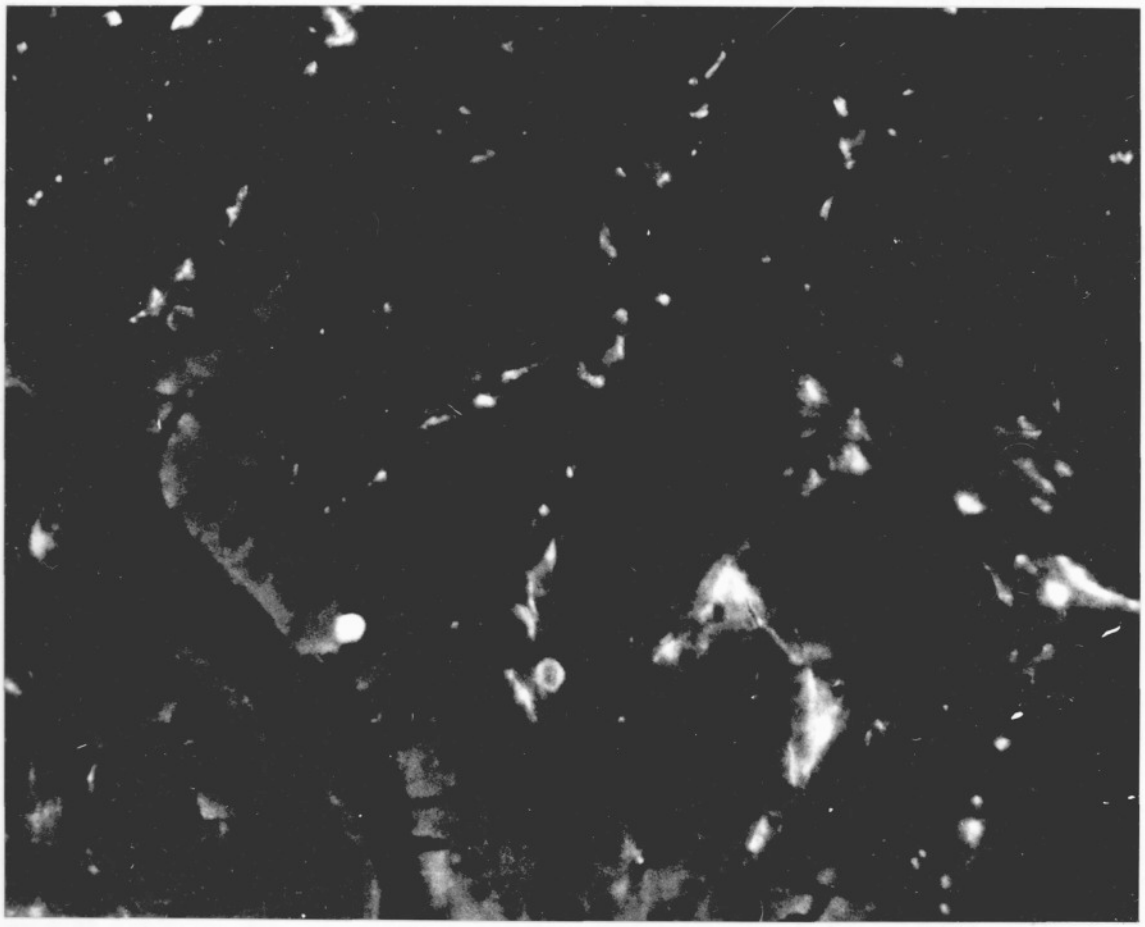
Both benzoquinone and DEPC have been found to be particularly effective when used as vapour fixatives of freeze-dried tissue to be immunostained for peptides. The technique of freeze-drying and vapour fixation, with either of the two fixatives, was the major tissue fixation method used in the Department of Histochemistry at the beginning of the study. This method appears to give optimal preservation of peptide immunoreactivity in endocrine cells in any tissue. However, neither benzoquinone nor DEPC as a vapour fixative gave optimal preservation of peptide immunoreactivity in nerve fibres. Immunostained peptide containing nerves could be seen in vapour fixed tissue, but the morphological detail was frequently obscured (Fig 1a). Individual fibres were difficult to discern and it was not possible to see the characteristic varicosities of the nerves. It is not known exactly why it should be that freeze-drying and vapour fixation give entirely satisfactory immunostaining of peptides in cells but not nerves. It may be that the storage form of the peptides in nerves is more labile than in cells. Electron microscopy shows no morphological evidence for this as peptides are stored in similar secretory granules in either location. However, if the peptide immunoreactivity in nerves is, in some way, particularly labile then it seemed possible that a less harsh process of tissue fixation was needed to obtain satisfactory immunostaining of peptide containing nerve fibres. Freeze-drying involves taking the tissue down to temperatures of -40°C and below,

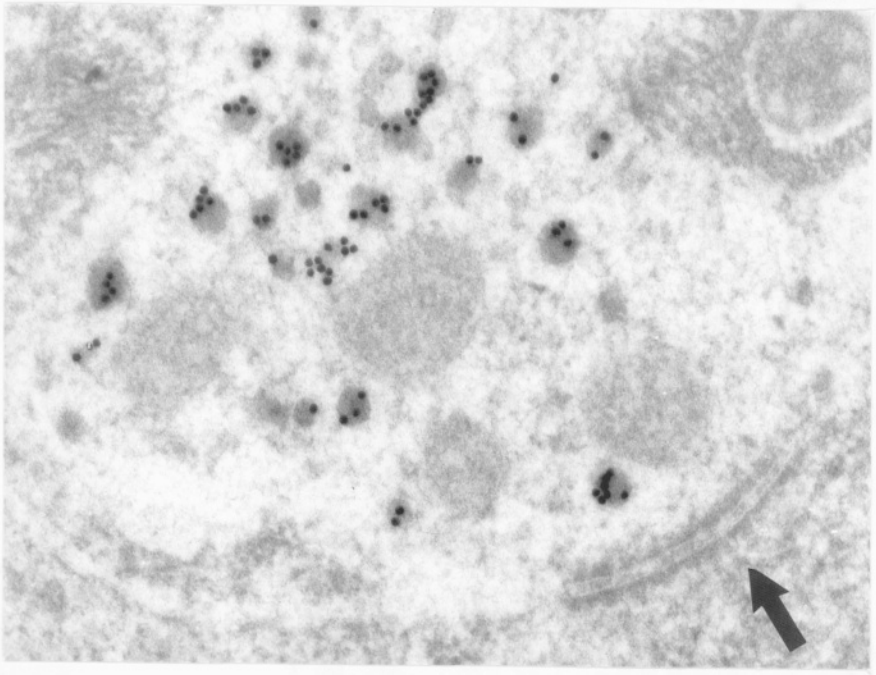
FIG 1

Colonic mucosa from a mouse immunostained for VIP with the technique of indirect immunofluorescence. Areas of immunoreactivity are seen as diffuse patches of fluorescence. Tissue was freeze-dried, fixed in benzoquinone vapour and embedded in paraffin wax (x 150).

Fig 1b

A nerve containing substance P-immunoreactive granules immunostained using the immuno-gold method. A tight junction can be seen (arrow) (x 60,000).





whilst the vapour fixation and wax embedding is carried out at 60°C and above.

Thus, as no available tissue fixation technique was found to allow immunostaining of peptides in nerve fibres to a satisfactory degree, the initial part of this study was devoted to developing an appropriate standard fixation technique.

Tissues under investigation

As was mentioned above, at the beginning of the study it was intended to examine the entire system of peptide containing nerves in the mammalian body. Thus, experiments on fixation were carried out on a range of different tissues, not only the gut and pancreas. The initial tests of fixation procedures were made on the brain, spinal cord and gut, with other tissues being examined during later experiments.

Unfixed tissues

To avoid "harsh" treatment of tissues as much as possible, unfixed specimens were examined. The necessity of fixing tissue was recognised but, in view of what seems to be a particularly labile nature of peptides in nerves, a few unfixed tissues were also examined, if only to show that fixation is required. Only rat tissues were used. Brain, spinal cord, stomach and colon (n=2) were either freeze-dried and wax embedded or frozen and cut in a cryostat. All sections were 10μm thick.

Immunostaining: Both the indirect immunofluorescence and the unlabelled antibody enzyme (PAP) (see appendix) techniques were used to immunostain the following peptides in tissues from rats;

Brain	Somatostatin, LHRH
Spinal cord	Substance P
Antrum	Gastrin, somatostatin, VIP
Colon	Enteroglucagon, VIP, substance P
Pancreas	Somatostatin, glucagon

These peptides were chosen to be immunostained in each area as there had been previous reports of their localisation (by immunocytochemistry or radioimmunoassay) to these sites.

Not only was the morphology of the tissue poorly preserved in all tissues, whether they were freeze-dried or cut in a cryostat, but the background staining was so high as to obscure whatever immunostaining had taken place.

Conventional histological fixatives

Immersion

Four conventional fixatives were used first; 10% buffered formalin, Bouin's solution, 3% glutaraldehyde, and methyl free formaldehyde (MFF) (see appendix). Brain ($1cm^3$), spinal cord (1cm), antrum and colon (both 2 x 2cm) (n=3 for each) from rats were immersed in each of the fixatives for 30, 60, 90 and 120 minutes.

Embedding

After fixation, the material was either dehydrated, embedded in wax and sectioned on a microtome or frozen and sectioned in a cryostat (appendix). The two different methods were used to see if the prolonged tissue dehydration and heat involved in wax-embedding had any adverse effect on peptide storage when compared with rapid freezing of tissue.

Immunostaining: No entirely satisfactory immunostaining was obtained with any of the antisera in any area. Generally, the background staining was very high and often obscured what little immunostaining there may have been in the sections. The best results for peptide containing nerves were obtained in tissue fixed in 10% buffered formalin and sectioned in a cryostat. In the antrum and colon, occasional cells were found to be immuno reactive for gastrin, somatostatin and enteroglucagon. It was difficult to discern any immunoreactive nerves in the tissues fixed by the other solutions. There was no distinct difference between the results obtained for the wax-embedded and frozen tissue, except for peptide containing nerves in buffered formalin-fixed specimens cut in a cryostat.

Perfusion

Some workers in the field reported the use of methyl-free formaldehyde (MFF) as a perfusion fixative for immunocytochemical staining (Elde et al, 1976 after Pease, 1962). Following their methods (appendix), 4% MFF was perfused into rats (n=2) and guinea pigs (n=2). In addition, animals were also perfused with 10% buffered formalin, Bouin's solution or 3% glutaraldehyde. Two methods of perfusion were

applied, one via the heart (left ventricle) and the other via the carotid artery (particularly for fixation of the brain) (appendix). Both methods involved "washing out" of blood vessels with isotonic buffer prior to perfusion with fixative. Tissues were either dehydrated and wax-embedded or cut in a cryostat, as for tissues fixed by immersion.

Immunostaining: This was carried out as for the immersion fixed tissues. The immunostaining obtained was notable in that it varied tremendously. Inconsistent staining was obtained for the same peptide in a single section. This variation may be a feature of perfusion but it was not mentioned by the author of the MFF perfusion technique and, as this method was reproduced as exactly as possible, it seems unlikely that the perfusion method, in all cases, was inadequate. In general some of the staining, especially in the tissue frozen and cut in a cryostat, was superior to that observed in tissues fixed by immersion. MFF and buffered formalin gave the best results. However, one of the main objects of the study as a whole was to examine human pathological specimens and, although this technique was promising as far as fixation of animal tissue was concerned, perfusion with fixative has little practical application in human pathology.

Post-fixation

A standard method of fixation for some histochemical procedures (eg staining of acetylcholinesterases) is cryostat sectioning of unfixed, frozen material followed by fixation of sections on glass slides by immersion in a fixative such as buffered formalin. In view of the

established success of this technique and the results obtained with cryostat sections of tissues fixed in conventional fixatives, post-fixed cryostat sections were immunostained. Ten μm sections of unfixed rat brain, spinal cord, stomach and colon were cut in a cryostat, picked up on uncoated glass slides, allowed to dry for 10 minutes and then fixed by immersion in MFF, Bouin's solution, 3% glutaraldehyde or 10% buffered formalin for 5, 10 or 20 minutes.

Immunostaining: This was carried out as before. Most of the sections were found to be negative and only poor immunostaining was obtained in the others. The background staining was very high but the preservation of tissue morphology was, generally, quite good.

Conclusion: It was concluded from the testing of conventional histological fixatives that perfusion and cryostat sectioning of frozen, fixed tissues allows the best immunostaining of peptides.

Vapour fixation of freeze-dried tissue with bifunctional reagents

As was mentioned above, freeze-drying and vapour fixation in para-benzoquinone was used routinely in the Department of histochemistry for fixation of tissue to be immunostained in endocrine cells. This method only gives limited immunostaining of peptides in nerves.

Slight variations in the technique (eg reduction or prolongation of fixation time, lowering of the temperature during fixation etc) gaven improvement of the immunostain. This routine method, however, gave consistent, if not entirely satisfactory, immunostaining of peptidesi nerves. The results obtained with this method were mostly superior to those obtained with the conventional histological fixatives tried previously.

In view of this, it was decided to examine the bifunctional reagents, particularly para-benzoquinone (originally purchased in an impure state and re-crystallised in the laboratory before, due to its increasing use, becoming available commercially in a readily usable, pure form) which can be purchased fairly easily (from BDH Chemicals).

p-benzoquinone as a liquid phase fixative

As cryostat sections of frozen material had provided the best results for the conventional fixatives, it was decided to try to use para-benzoquinone as a fixative in solution. This had been tried during the original experiments on the use of p-benzoquinone as a tissue fixative for immunocytochemistry (Pearse & Polak, 1975). However,the potential value of this method was obscured by two main factors. Firstly, at that time it was not widely established that peptides occur in the innervation of the gut and so only peptides in endocrine cells were immunostained during the study. Secondly, the manner in which the p-benzoquinone solution was prepared did not give the best results.

Benzoquinone (1, 4-benzoquinone) reacts with proteins to give inter-and intramolecular cross linkages (Fig **2**). It can only be used efficiently when it is in a fully oxidised form. The method described during the early work on fixatives for immunocytochemistry involved heating the benzoquinone to get it into solution. This method was tried but found to give poor results. When benzoquinone is heated, however gently, it became darker in colour, presumably due to the formation of mixed hydroquinones. In order to try to minimise reduction, nitrogen was bubbled through the solution as it was warmed. This gave no appreciable improvement.

It was noted that, when in a fully oxidised form, benzoquinone in solution has a clear, bright yellow colour. When reduced, it changes to an opaque, dark brown. This fact was found to be of great use

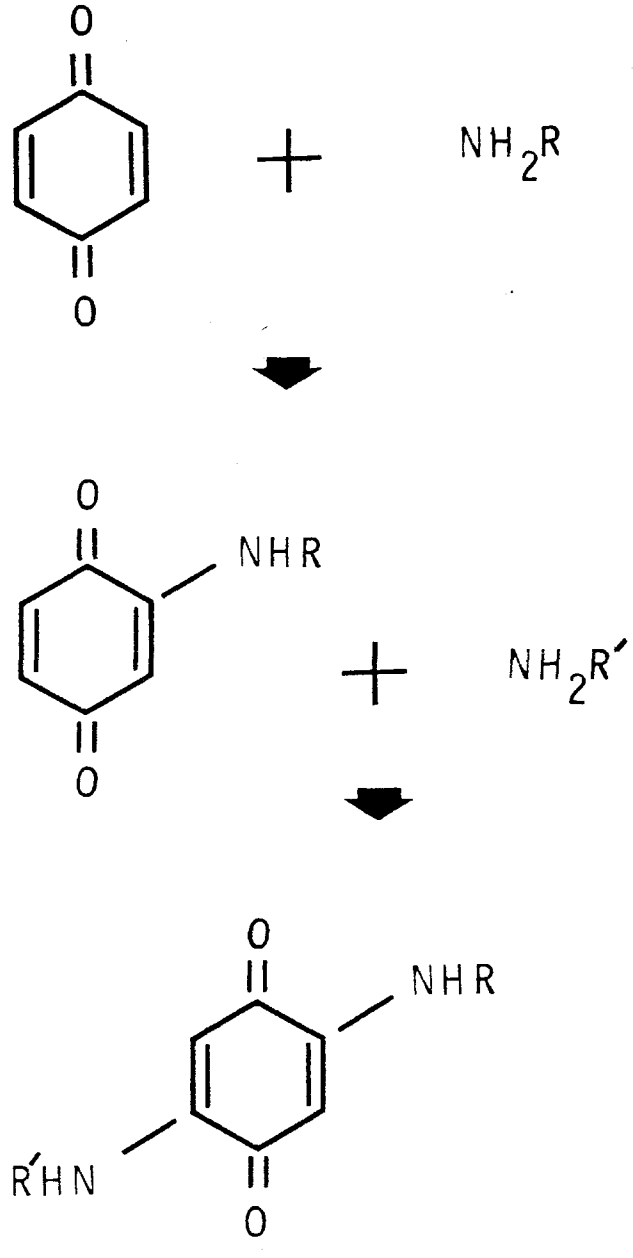


Fig 2. Para-benzoquinone substitution reaction

as it means that the benzoquinone solution has its own inbuilt indicator of its state.

Solutions of benzoquinone were prepared without heat. This proved to be difficult at first as the benzoquinone was not easy to solubilise. However, when freshly re-crystallised, benzoquinone was used, solubilisation became much easier.

Solvents

A range of different solvents (including (0.01M phosphate buffered saline, 0.1M phosphate buffer and 0.2M Sodium cacodylate buffer) were tried to see which gave the most effective fixative when mixed with benzoquinone. The fixatives were prepared by adding parabenzoquinone to the solvent and mixing for one or two minutes until a solution was obtained. The concentration of 3% was that quoted by Pearse and Polak in 1975. The solutions were used immediately and fixation was for 1, 2 and 3 hours.

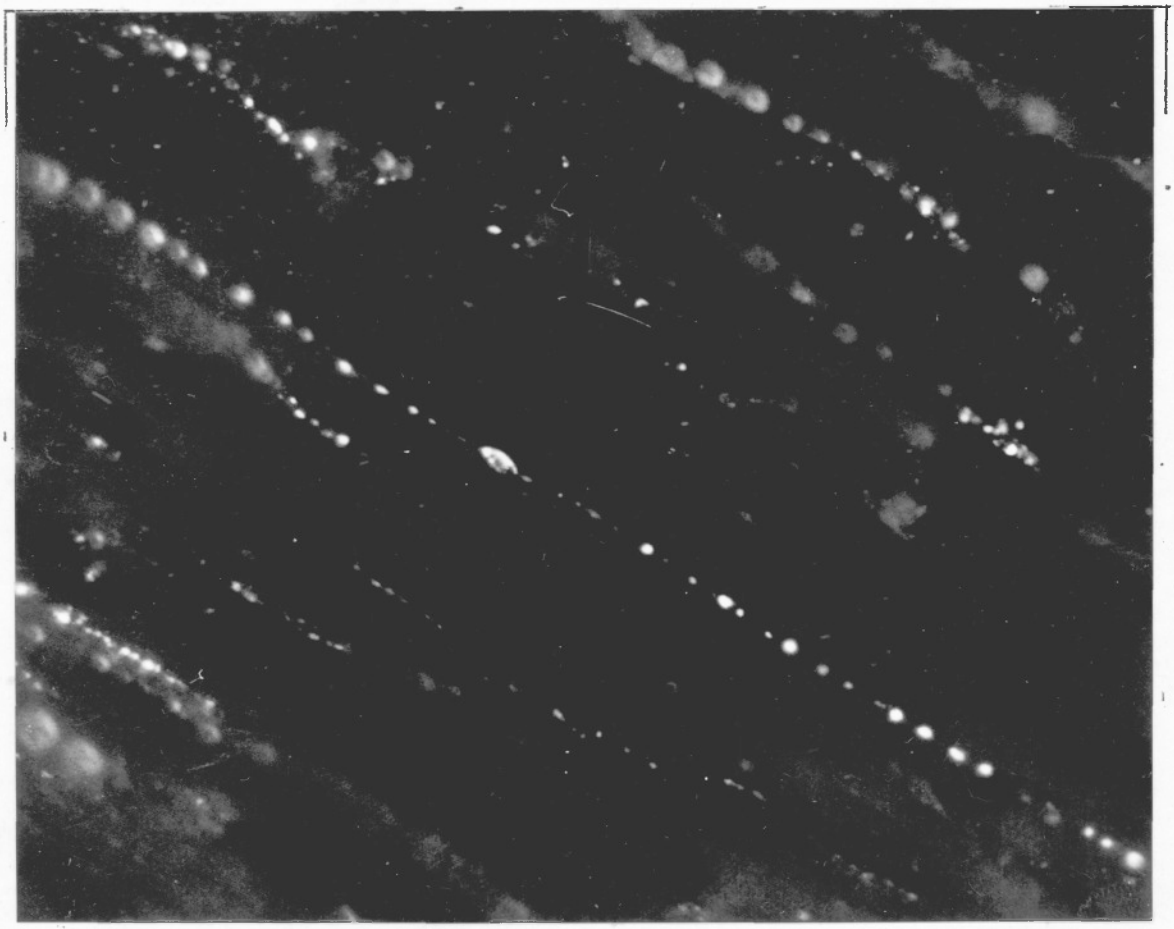
Immunostaining: It was immediately apparent from immunocytochemical examination of fixed tissues (specimens, antisera and methods were as for the previous tests using immersion in conventional fixatives) that cryostat sectioning was preferable to wax embedding and that the immunostaining of peptides in nerves was vastly superior to that seen in tissues fixed by the other methods employed in this study. Nerves could be seen in surprisingly fine detail (Fig **3**). Multiple varicosities were obvious and single fibres could be discerned within bundles. Immunoreactive

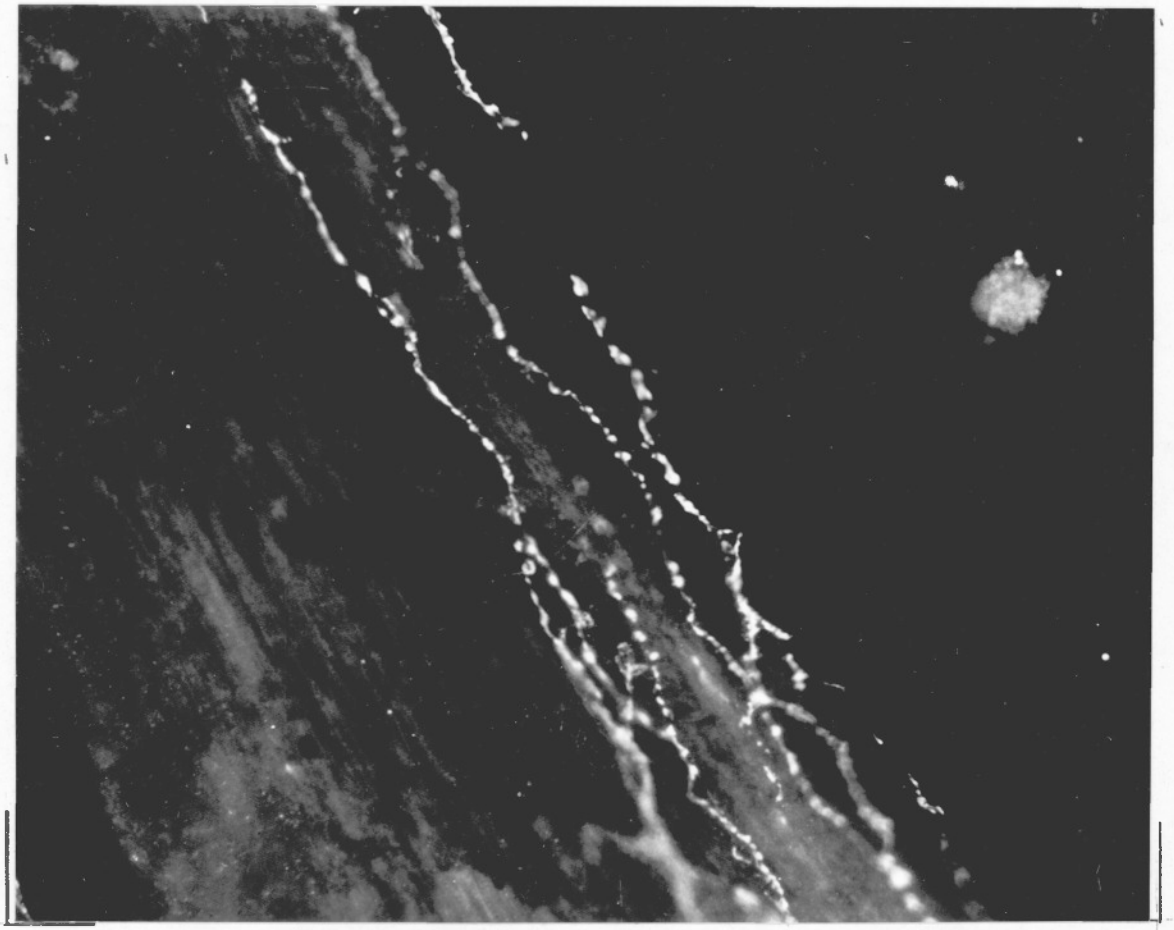
FIG 3

a) Fine, varicose VIP-immunoreactive fibres in the longitudinal muscle of the feline colon (x 600).

b) Intermeshed VIP-immunoreactive fibres at the edge of the circular muscle of the rat ileum (x 375).

Tissue was fixed by immersion in BQS and cryostat sections were immunostained using the technique of indirect immunofluorescence.





ganglion cells, where they occurred, were clearly distinguishable, as were immunoreactive fibres around non-immunoreactive ganglion cells. There appeared to be no "diffusing out" of immunoreactivity, as seen in the vapour fixed tissues. The immunostaining of peptides in endocrine cells was entirely comparable to that obtained using benzoquinone as a vapour fixative (Fig **4a**).

The nature of the diluent was shown to be irrelevant, similar results being obtained with each one. However, phosphate buffered saline was chosen as a standard diluent because of its lack of toxicity, ease of preparation and its wide usage in many laboratories (Bishop et al, 1978c Bishop & Polak, 1978a).

Concentration of parabenzoquinone and length of fixation

The very promising results obtained by the use of benzoquinone in solution as a fixative combined with cryostat sectioning of tissue prompted a further series of experiments to establish the optimal concentration of benzoquinone in phosphate buffered saline (PBS) and the optimal length of fixation time.

Benzoquinone was prepared as a solution in PBS in concentrations of 0.1%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% and 20% and the tissues (rat brain, spinal cord and human and rat antrum/stomach and colon) were fixed for 30 minutes, 1 hour, 90 minutes, 2 hours, 150 minutes and 3 hours.

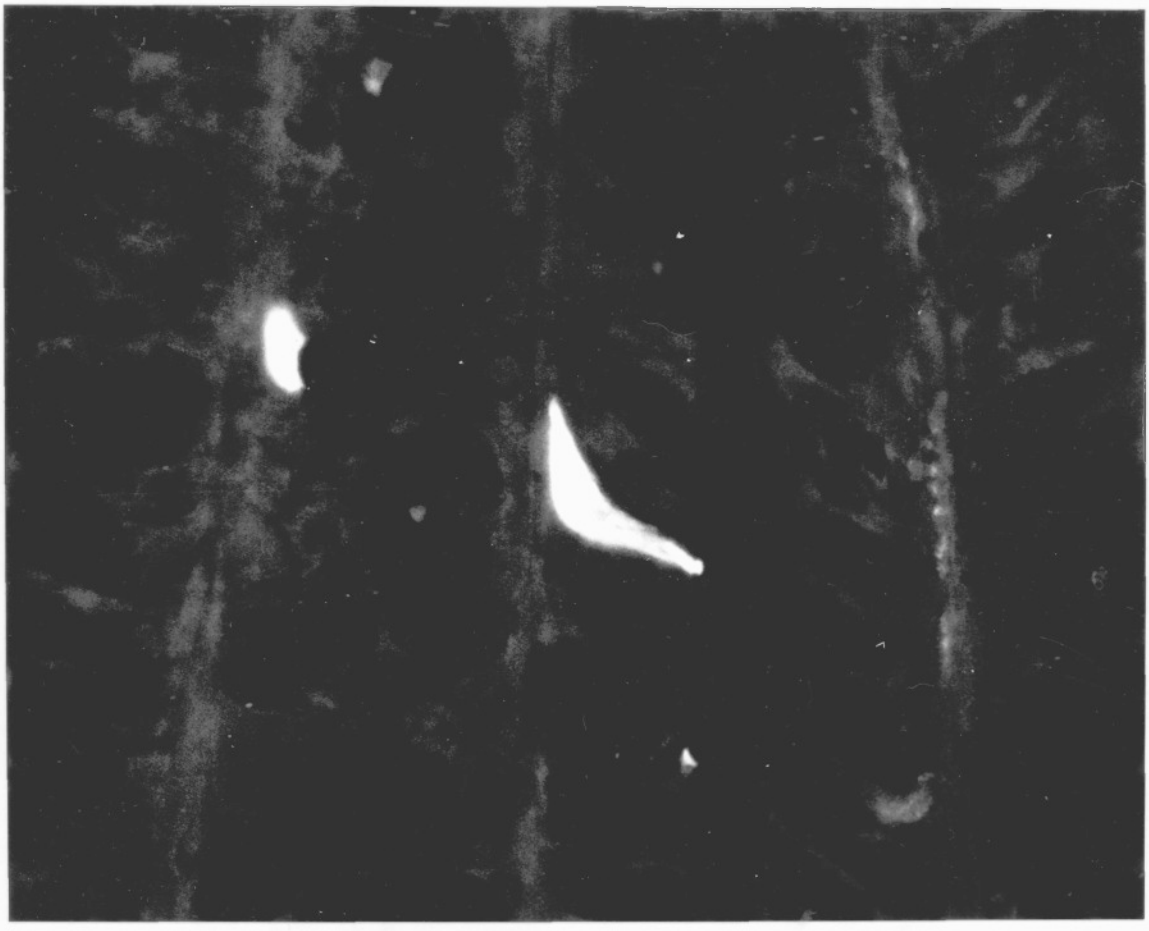
Immunostaining: This was carried out as previous described. As regards the length of time for fixation, this varied with

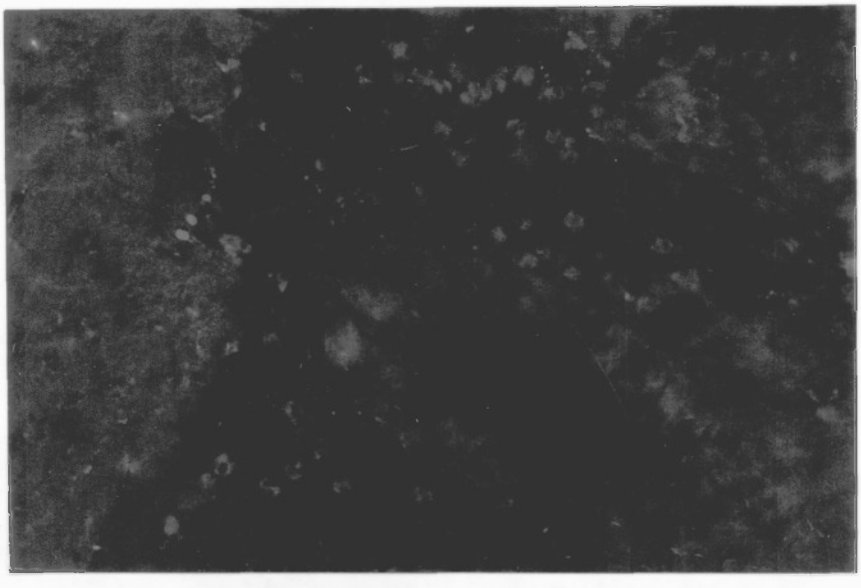
FIG 4

Enteroglucagon-immunoreactive cell in human colonic mucosa. Tissue was fixed by immersion in BQS, cryostat section, indirect immunofluorescence (x 500).

Fig 4b

A section of human colon fixed in benzoquinone solution and incubated with antiserum to VIP pre-absorbed with 0.01nmol synthetic porcine VIP per ml diluted antiserum. The immunostain was quenched. (x 250).





the nature of the tissue. The thin rat gut was fixed after 1 hour whilst human gut needed 2 hours for optimal fixation. The central nervous system tissue, on the other hand, needed extended fixation. The time needed depends, presumably, on how easily the tissue can be penetrated by the fixative. It was thus obvious that a range of fixation times was necessary.

The concentration of benzoquinone which gave the best results was 0·3-0·4% with 0·4% enabling slightly better immunostaining in certain cases. It was decided that a 0·4% solution of benzoquinone and phosphate buffered saline was the best method of fixation but more tests were needed on a wide range of tissues to establish appropriate fixation times. The results of these tests are displayed in Table **2**.

Immersion vs Perfusion

A comparison was made of the efficiency of immersion fixation with benzoquinone solution compared with perfusion. Three rats were perfused with benzoquinone in solution via the heart or carotid artery (as previously described - see Appendix) and fresh tissue (brain, spinal cord, stomach and colon) was taken from 3 sacrificed unperfused rats and immersion fixed. The perfused tissues were also post-fixed by immersion in benzoquinone solution for one hour. Immunostaining, carried out as before, revealed that the only detectable difference in the degree of immunostaining was in the brain, where perfusion gave particularly good results, probably by improving the rate of penetration. Other than this, immersion fixation was totally satisfactory.

Table 2

OPTIMAL FIXATION TIMES IN BENZOQUINONE SOLUTION (BQS)

TISSUE	MAXIMUM SIZE (cm)	TIME (h)
Endoscopic biopsies of gut	0.5 x 0.5	½
Whole gut wall -		
Thin (eg child/small animal)	2 x 2	1
Medium (eg adult human)	2 x 2	2
Thick (eg horse)	2 x 2	4
Pancreas	1^3	2
Tumour	1^3	2
Heart	2 x 2	2
Female genital tract -		
Uterus	2 x 2	2
Cervix	2 x 2	2
Vagina	2 x 2	2
Fallopian tube	1	2
Bladder	2 x 2	2
Skin	2 x 2	4
Breast	1^3	4
Brain	Whole	½ - perfusion
		4 - immersion
Spinal cord	Whole	½ - perfusion
		4 - immersion

Storage of Tissue

As there is clearly a finite length of time for fixation in benzoquinone solution, some solution was needed for washing and preservation of the tissue during storage. In routine histological methods of tissue fixation, neutral buffered solutions are used for this purpose. A solution of 7% sucrose in PBS was routinely used for storage of formalin fixed tissue in the Department of Histochemistry and this was chosen for use on benzoquinone solution fixed tissue. 0.01% sodium azide was added as an antimicrobial agent. Tissues can be safely stored in this solution for a number of years (maximum time period tested is 4 years at the time of writing) as long as they are checked for microbial growth. The sucrose in the solution has the additional advantageous effect of acting as a cryoprotectant and thereby reducing the formation of ice crystal artefacts during freezing of tissue in the preparation of cryostat blocks.

Interval between preparation of fixative and fixation of tissue

As previously noted, benzoquinone solution becomes less effective as a fixative when it is heated or just left for a period of time at room temperature, particularly in the light. An experiment was carried out to see how long the fixative could be kept as a solution.

A 4% solution of benzoquinone was made up at room temperature by mixing benzoquinone crystals in PBS. Specimens of human gut (colon measuring 2 x 2cm) were fixed in the solution immediately

after its preparation and at 30 minute intervals, up to 6 hours. Between use, the solution was stored at 4°C in the dark, with the container wrapped in aluminium foil to reduce the amount of light reaching it as much as possible. The solution was noticeably browner after one hour and became progressively darker with time.

Immunostaining: This was carried out using the methods previously described. The best immunostaining was obtained with tissue fixed immediately after the benzoquinone was prepared or 30 minutes after it was made. After this time, an obvious increase in background staining could be observed, particularly around the edge of the mucosa and the muscle membranes. At 2 hours onwards, immunostaining was significantly reduced, being totally obscurred in tissue kept in a solution which was stored for 4 hours. It was thus concluded that benzoquinone solution should be used as soon as possible after its preparation and certainly before the solution becomes brown.

Time between death/surgical removal and fixation of tissue

A limit was established for the time between death of the individual or surgical removal of tissue and its fixation by examination of various specimens of gut and pancreas. The surgical samples were fixed a minimum of 30 minutes after removal and the oldest specimen received had been removed some 18 hours before. The freshest post-mortem tissue was obtained 6 hours after death and the oldest just over 48 hours. It was felt that, by examining numerous different specimens in this way, the conditions encountered in routine pathology were reproduced rather than in an artificial situation whereby a specimen of tissue is left on the bench and sampled at intervals. Such a laboratory experiment does take into account the undoubted individual variations in

the possible retention of peptide immunoreactivity in nerves and cells nor the, frequently adverse, handling of specimens.

Immunostaining: (as before) showed an obvious reduction of peptide immunoreactivity in nerves and cells in tissue fixed between 10-12 hours after death or surgical removal. There was also marked degeneration of tissue morphology after this time. A time limit of 8 hours was set for the period between death/removal of tissue and fixation. However, great care was taken, at each stage of the study, to ensure that when pathological specimens were examined, the average time interval between removal and fixation was matched by that of the control tissue.

Tissue sampling

In the pathological specimens examined in this study, sampling would have, ideally, consisted of the removal of multiple pieces of tissue from all areas of each specimen. However, the research work described here could obviously not interfere with routine investigations. Therefore, sampling was restricted and had to be limited to those areas of each specimen which were likely to yield the most information. The sampling methods used are described in each section.

Cryostat sections

Once it had been established that fixation in benzoquinone solution followed by cryostat sectioning was the best method available for processing tissue for immunocytochemistry of

of gut neuropeptides, the method of preparing blocks and sectioning was examined.

i) Preparation of cryostat block

All gut specimens were sectioned transversely so as to obtain a complete cross-section of the gut wall. For man and the larger mammals the gut was thick enough to be cut into blocks which could stand upright. These were attached to cork mats using O.C.T. compound (R.A. Lamb) and frozen by rapidly inverting the mat and dropping it, tissue downwards, into liquid Arcton suspended in liquid nitrogen to prevent evapo ration. For thinner pieces of gut, eg rat or mouse, and endoscopic biopsies some supporting medium had to be used. The best method was found to be inserting the tissue, in a perpendicular plane, into a slit in a cube of unfixed liver tissue. This was then frozen, as for the larger pieces. As liver is easily sectioned it presented an ideal mounting medium.

ii) Thickness of sections

From the beginning of the study, tissue sections of 10μm were used. Different thicknesses (5, 8, 12, 15 and 20μm) were tried but at 10μm sufficient lengths of nerve fibres could be seen in each section and in thicker sections background staining began to obscure the immunostain. Sectionsof 10um were also stained with haematoxylin and eosin for histological examination. Allowance was made for the thickness of the sections when they were read.

iii) Section mounting and drying

Sections werepicked up on glass slides coated with either formol gelatin (1:1 v/v, 2% formalin:1% gelatin) or, later in the study, 1% poly-L-lysine. Slides were coated and stored, up to 4 weeks, prior to use. Once sections were mounted on slides they were allowed to dry for between 30 min - 1 hour. This was done at room temperature. Thirty minutes ensured that the sections were thoroughly dry. After more than 1 hour, although peptide immunoreactivity did not decrease for some considerable time, the morphology of the tissue often suffered from excess drying.

Number of specimens

In the normal and, most particularly, in diseased tissues examined in this study, it was important to examine a sufficient number of specimens in each case. The question therefore arose as to what constitutes a sufficient number. Clearly, individual variations had to be taken into account, so it was not enough to single out just a few examples. However, it was decided that a minimum number of 6 specimens should be examined in any one group of similar normal or diseased tissues. Although the morphological studies were not quantitative, parallel data were obtained for each specimen by measurement of peptide content by radicimmunoassay of tissue extracts. To allow statistical analysis of these data, a minimum of 6 specimens was needed.

General methods

Tissues examined in this study were therefore processed in the following standard way:-

1) Maximum time between death/surgery and fixation = 8 hours.
2) Sampling of multiple areas (as necessary).
3) Fixation in BQS (appendix)
4) Sectioning in cryostat (appendix)
5) Haematoxylin and eosin stain and histological examination
6) Immunostaining for regulatory peptides, enzymes etc (appendix)

Suitability of benzoquinone as a fixative for conventional histology and histochemistry

As part of this study it was intended to examine peptide containing nerves not only in isolation but to compare their distribution with that of cholinergic nerves, which are also intrinsic to the gut.

In the absence of a more specific technique, the method for the demonstration of acetylcholinesterase activity of El Badawi & Schenk (El Badawi & Schen k, 1967) was used (appendix). The acetylcholinesterase staining technique is thought not to be specific for cholinergic nerves; all cholinergic fibres have acetylcholinesterase but not all acetylcholinesterase-possessing fibres are necessarily cholinergic. It was intended

to apply this technique in order to see if peptide-containing nerves have acetylcholinesterase activity and could, thus, be cholinergic. The standard fixation method used for this technique is post-fixation of unfixed cryostat sections in 10% formal saline for 10 minutes. This method was found to allow some immunostaining of peptides in nerves, but their morphology was not satisfactory.

Tissues were fixed in 10% formal saline and then sectioned in a cryostat and immunostained for peptides or stained for acetylcholinesterase activity. The acetylcholinesterase activity was not affected but the immunostain was no better than that seen in the post-fixed cryostat sections. Cryostat sections of tissue fixed in benzoquinone solution were stained for acetylcholinesterase activity. The results obtained were entirely comparable to those obtained with the post-fixed sections, prepared as for the standard technique. Thus, it was possible to stain acetylcholesterase and immunostain peptides in serial or even the same sections. No revision of the acetylcholinesterase staining method was required.

CHAPTER 3 THE DISTRIBUTION OF PEPTIDE CONTAINING NERVES IN THE NORMAL MAMMALIAN GASTROINTESTINAL TRACT AND PANCREAS

The distribution of peptide containing nerves was studied in a variety of adult mammals; man, horse, pig, cat, dog, guinea pig, rat and mouse.

The peptides investigated fell into three main groups. Vasoactive intestinal polypeptide (VIP), substance P, met-enkephalin, bombesin and somatostatin were all generally agreed to be present in peripheral autonomic nerves. There was some controversy as to whether certain other peptides, including motilin, neurotensin and CCK-8, also existed in these nerves. In addition, a third group of peptides was made up of those, like peptide histidine isoleucine (PHI), which were discovered and characterised comparatively recently. As many peptides as possible were studied, depending on the availability of specific, reliable antisera.

In the following accounts, a description is given of the general pattern of distribution of each type of peptide containing nerve. Human tissue received the most detailed attention, mainly because of the subsequent pathological investigations. The differences in this pattern which occur in the various areas of gut and in other mammals follow the general description.

MATERIALS

For human tissues, the different areas of gut and pancreas examined were obtained at surgery, mostly for resection of carcinoma. Jejunum and ileum were also obtained at by-pass operations for obesity. Samples were removed as far from the tumour as possible and only macroscopically and histologically normal tissues were included in the studies.

Animal tissue was taken immediately after sacrifice by overdose of anaesthetic or, for the smaller animals, decapitation.

All samples were immediately fixed in benzoquinone solution, sectioned and stained with haematoxylin and eosin, for histological examination to ensure that there was no tissue degradation, and immunostained with antisera to a range of peptides (appendix). The total numbers of specimens examined are given in each section.

MAN

VASOACTIVE INTESTINAL POLYPEPITDE-IMMUNOREACTIVE NERVES

Gastrointestinal tract

Vasoactive intestinal polypeptide (VIP) containing nerves, as with all other types of peptide containing nerves, were found along the entire length of the gastrointestinal tract. They were also present in each layer of the gut wall.

Generally, the fibres were slender with multiple varicosities which could be seen clearly in those stained with either the indirect immunofluorescence or the PAP method. Sometimes, fibres with a ribbon-like appearance could be seen emerging from immunoreactive ganglion cells. In this study, only two peptides were consistently immunostained in ganglion cells of either plexus , VIP and PHI.

VIP-immunoreactive fibres were rarely found in the serosa and, when present, were associated with blood vessels. In the muscle layers the nerves usually ran in a similar plane to that of the muscle cells. Only in the circular muscle coat could VIP-immunoreactive fibres be seen to cross the plane of the muscle directly at right angles, and then only in connective tissue between the blocks of muscle. If fibres traversed the longitudinal muscle or muscularis mucosae they seemed to do so in a gradual, diagonal direction. In the longitudinal muscle immunoreactive fibres were mostly single (Fig **5**) but in the circular muscle they occurred more frequently as closely intertwined bundles of three or four (Fig **6**).

A dense network of VIP-containing fibres was present in both of the main ganglionated plexuses. In the myenteric plexus, these surrounded non-immunoreactive ganglion cells. Immunoreactive cells were found only twice in this plexus during the entire course of the studies. In the sub-mucous plexus, however, VIP-immunoreactive ganglion cells were a frequent finding in all regions of the gut (Fig **7**). These cells usually formed between 40-80% of the total number of cells in any one group. They were mostly round or ovoid, multipolar and of a similar size to the non-immunoreactive cells around them.

FIG 5

Single VIP-immunoreactive fibres in the longitudinal muscle of rat duodenum. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 300).

FIG 6

Meshes of VIP-immunoreactive fibres in the circular muscle of feline colon. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 375).

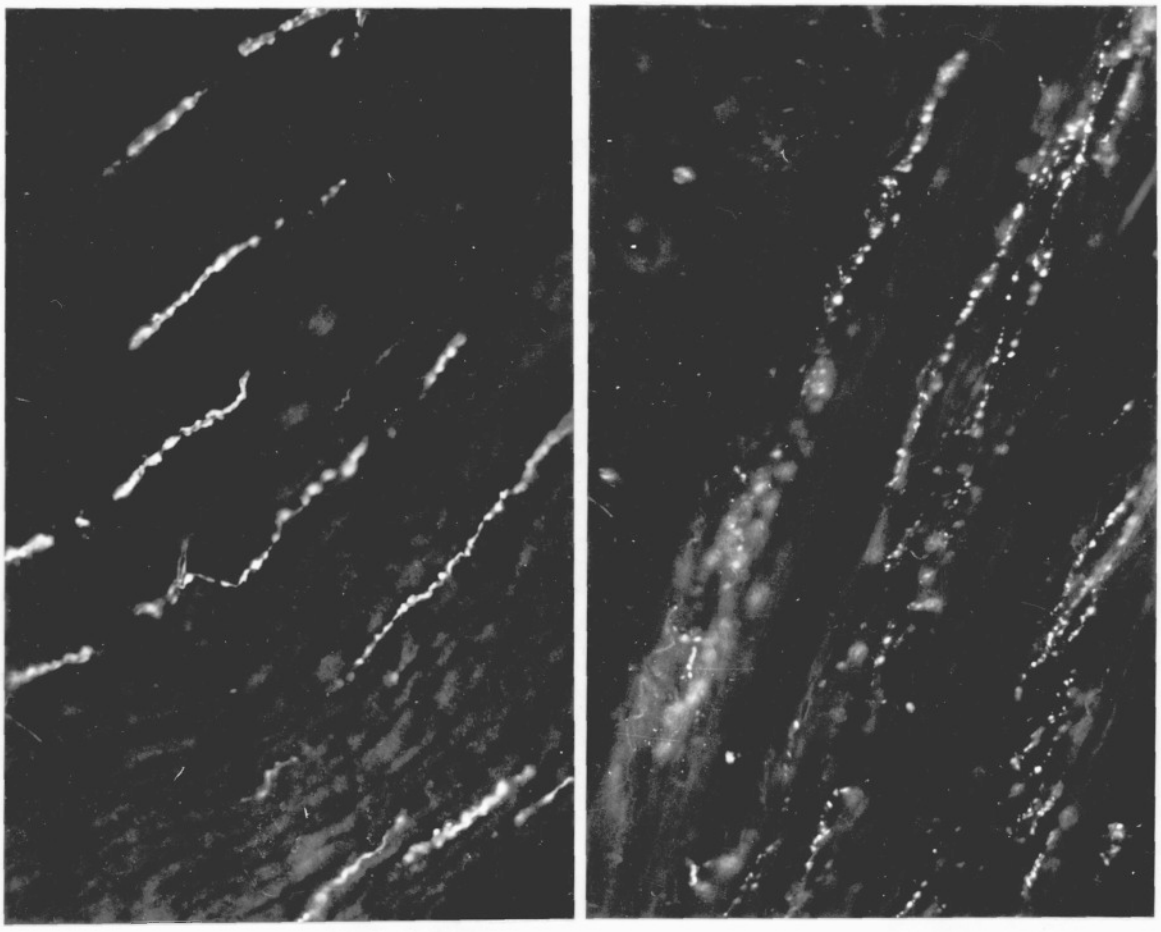
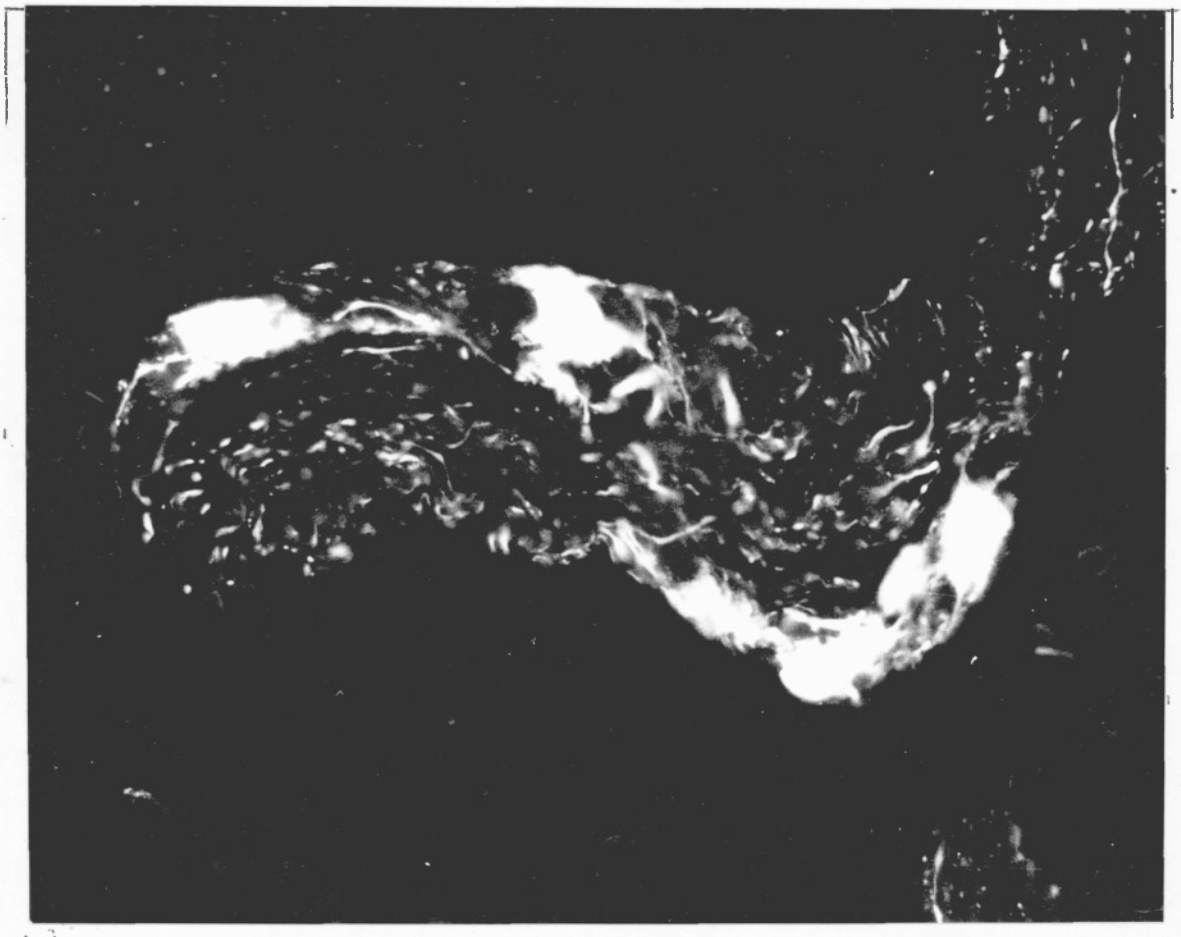


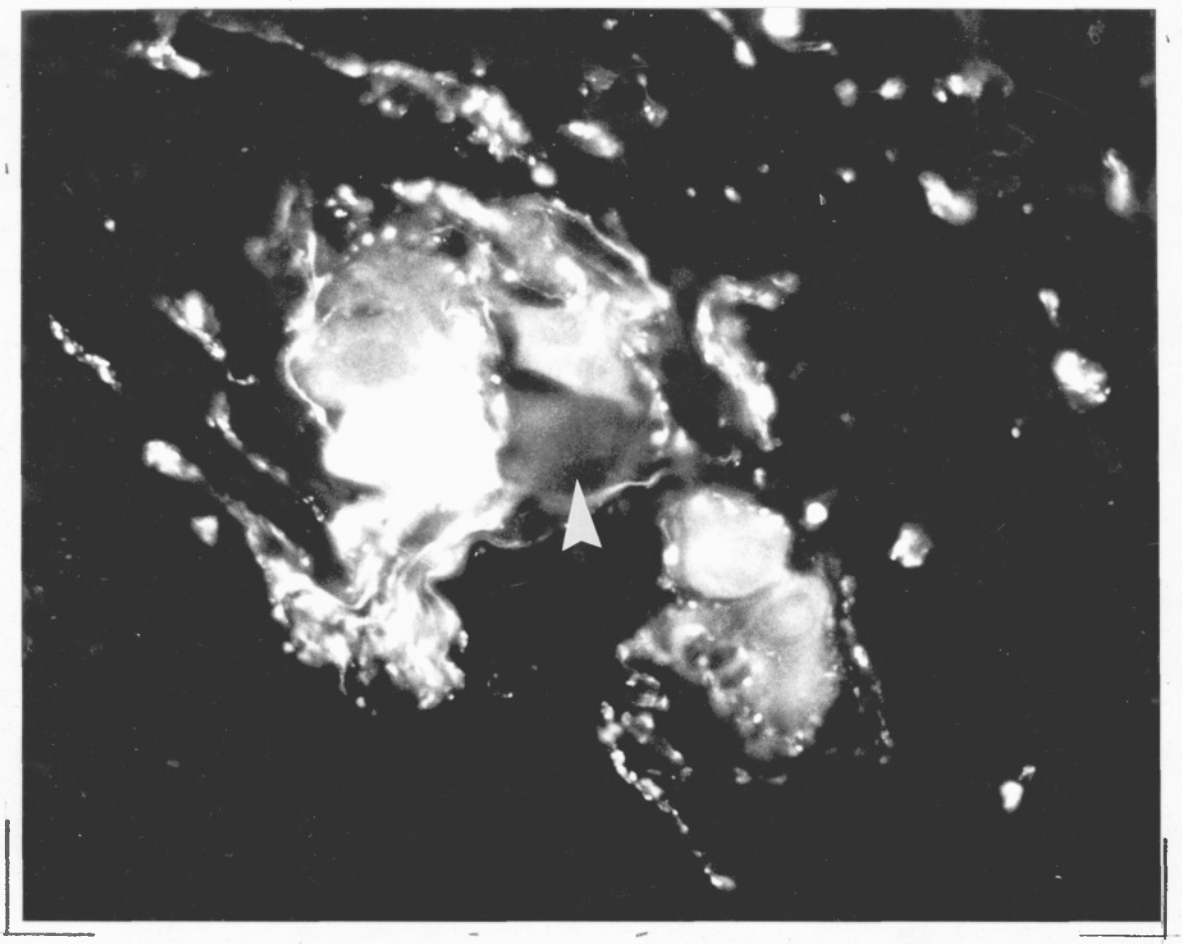
FIG 7

a) VIP-immunoreactive cell bodies and fibres in the sub-mucous plexus of human ileum (x 300).

b) VIP-immunoreactive cell bodies surrounding one non-immunoreactive cell (arrow) in the sub-mucous plexus of cat colon (x 375).

Tissue was fixed by immersion in BQS, cryostat section, indirect immunofluorescence.





In the sub-mucosa, apart from being in the plexus, VIP-immunoreactive nerves were mainly associated with blood vessels (Fig **8**). Occasional fibres were also found in scattered muscle fibres. In some specimens, it was possible to find large bundles of immunoreactive and non-immunoreactive nerves, showing no particular relationship with any other structure in the sub-mucosa. The mucosal layer was densely populated with VIP-immunoreactive fibres, which extended throughout the layer. The nerves appeared to have a noticeably close relationship with mucosal but not squamous epithelium. Immunoreactive fibres ran just under the mucosal epithelium, often appearing to touch it, perhaps terminating on it (Fig **9**). In the lamina propria, the fibres intermeshed around central blood and lymphatic vessels, projecting sometimes along the length of villi (Fig **10**). As well as projecting upwards in this way in the mucosa, some lengths of fibres could be traced along the base, just above the muscularis mucosae.

VIP-immunoreactive nerves were the most abundant type of peptide containing nerve found in man.

Oesophagus (No. of specimens examined, n=11)

VIP-immunoreactive nerves were particularly numerous in the muscle layers of the oesophagus. In the submucosal layer, they were associated with blood vessels. There did not appear to be any relationship between the fibres and the squamous epithelium.

FIG 8

Sub-mucosa of human colon with VIP-immunoreactive fibres lying around blood vesssels. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 150).

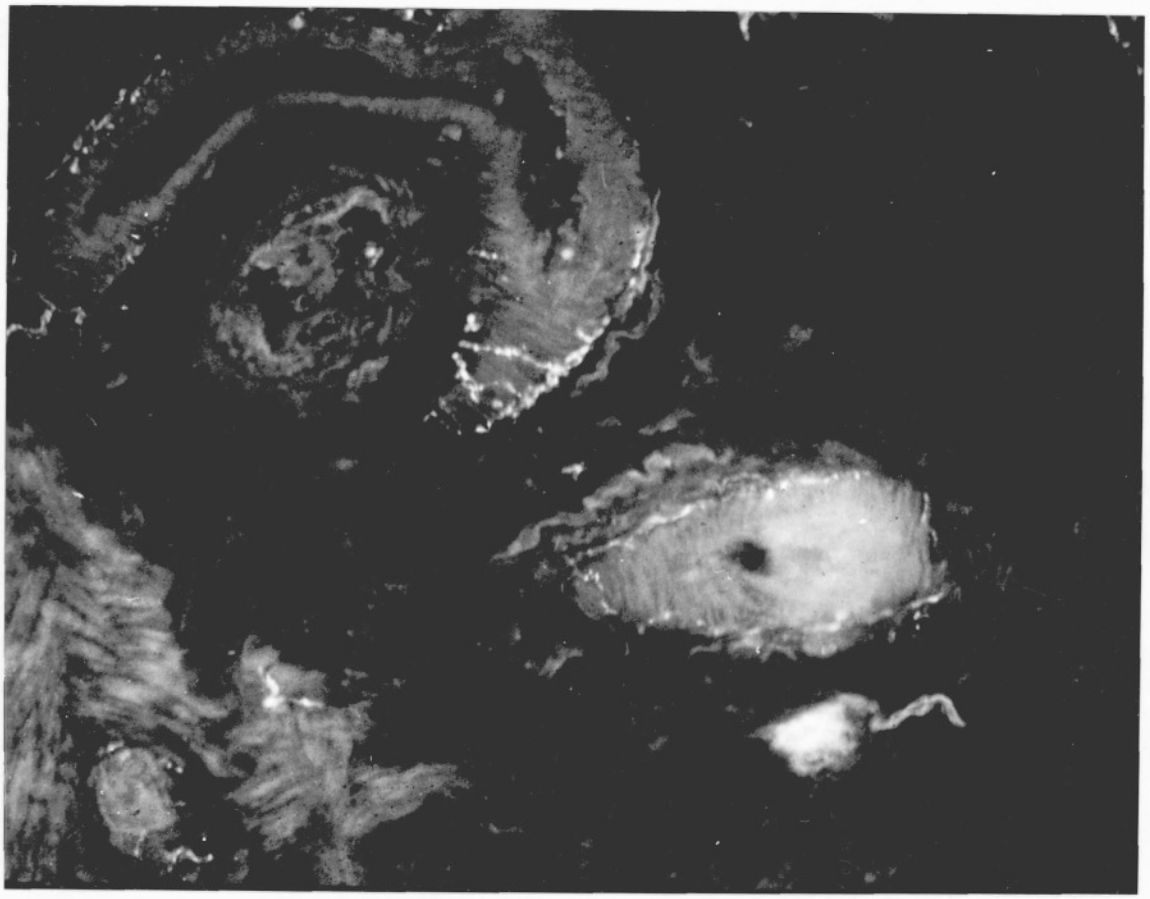
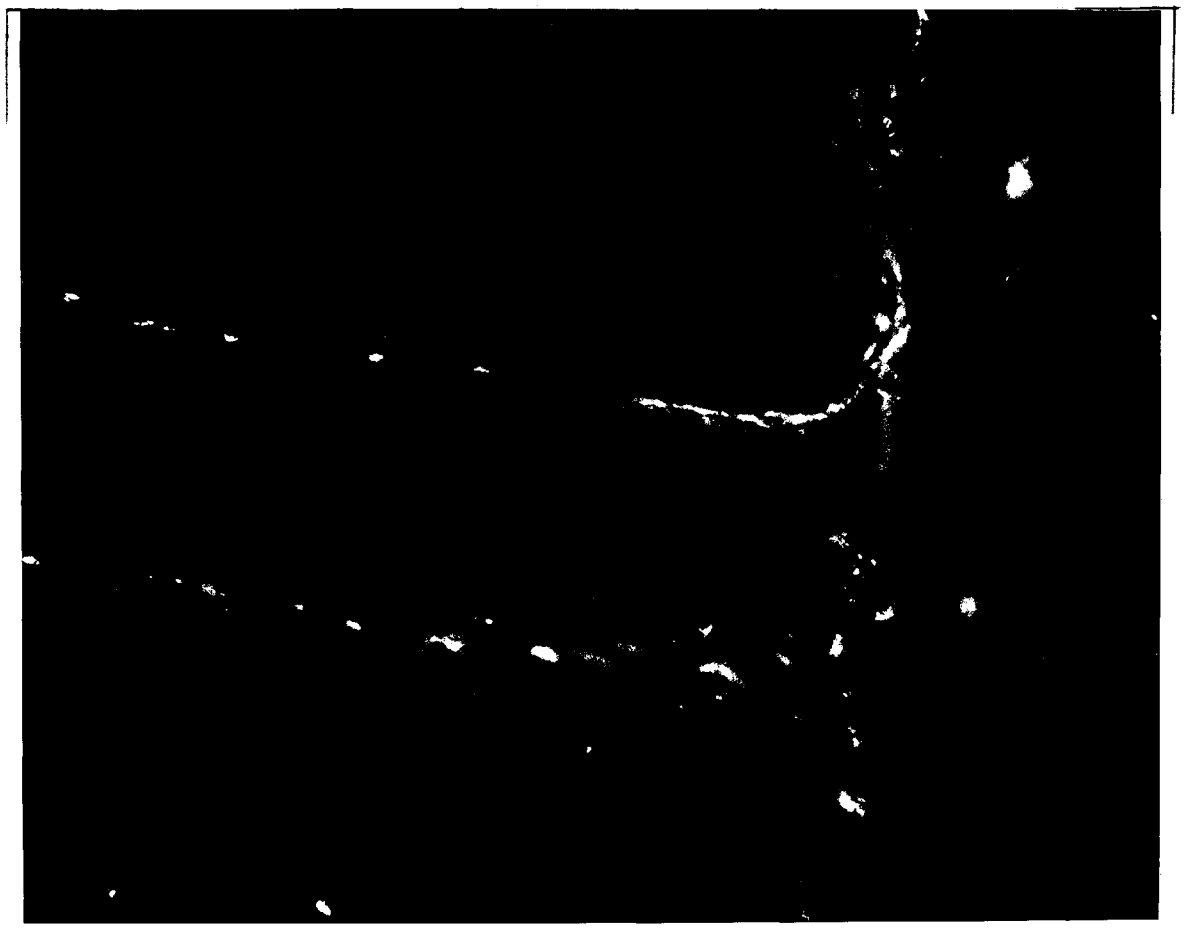


FIG 9

a) Feline colonic mucosa with VIP-immunoreactive fibres lying just beneath the epithelium (x 250).

b) Human colonic mucosa immunostained as for a) (x 350).

Tissue fixed in BQS, cryostat section, indirect immunofluorescence.



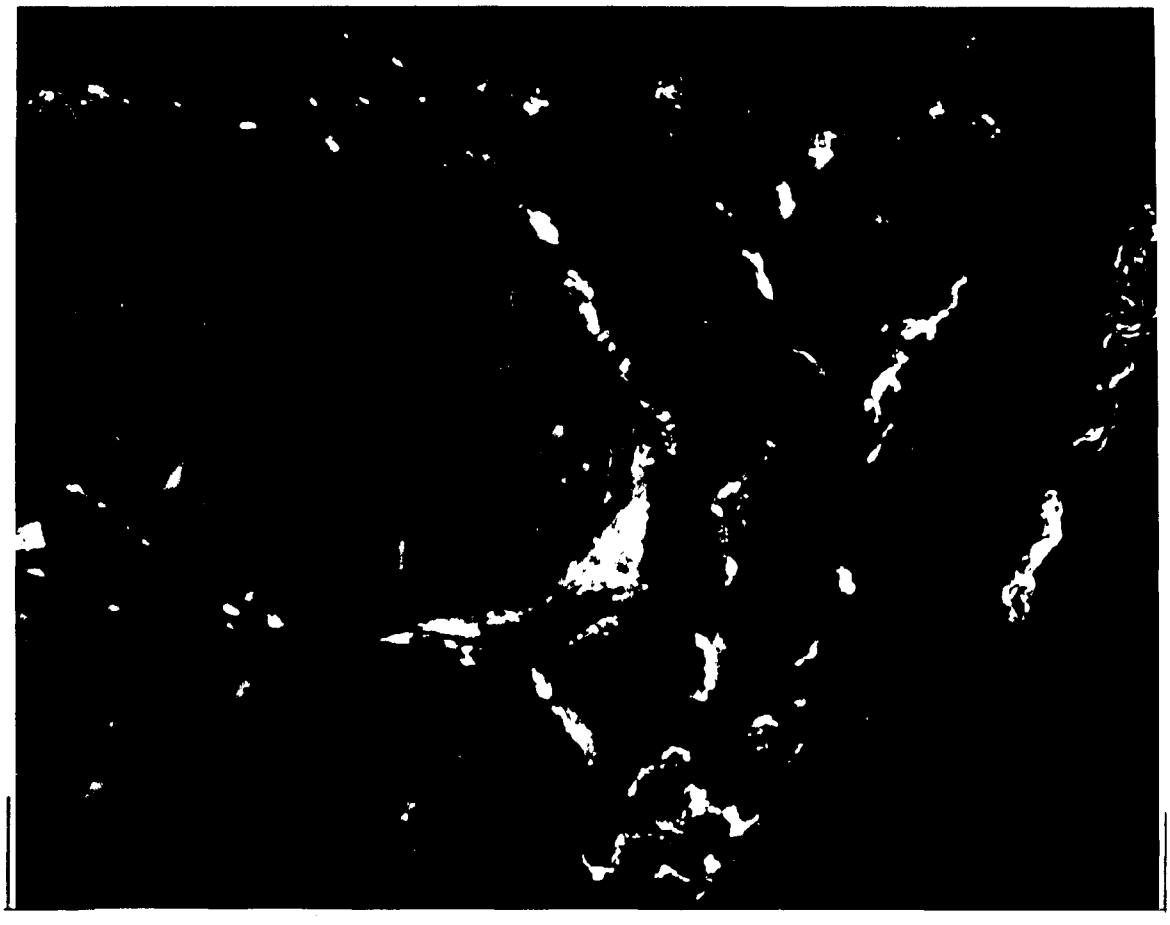
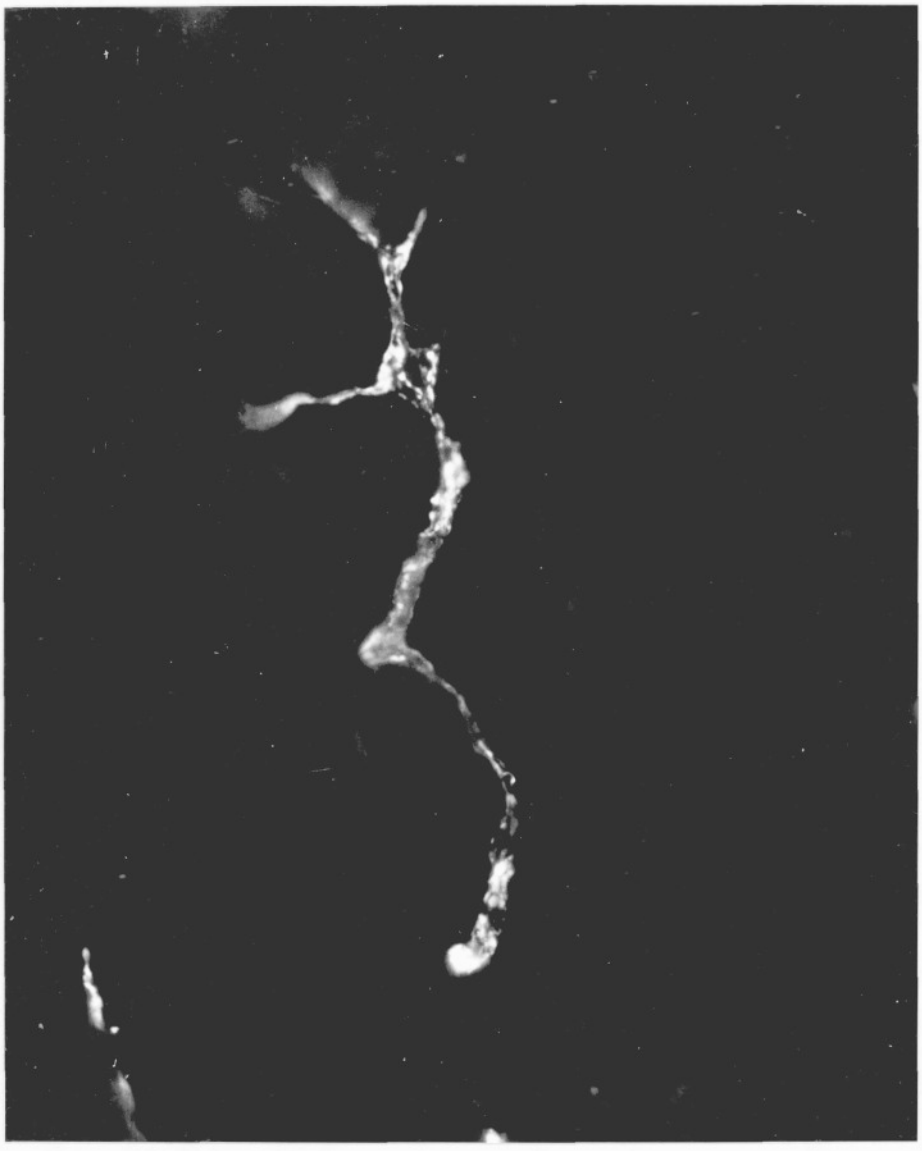


FIG 10

VIP-immunoreactive fibres around a vessel in the lamina propria of rat colonic mucosal epithelium. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 450).



Cardiac sphincter (n=3)

Stomach (n=22)

Pyloric sphincter (n=17)

Duodenum (n=12)

Jejunum (n=9)

Ileum (n=15)

Ileo-caecal valve (n=6)

Caecum (n=12)

Appendix (n=4)

Colon (n=42)

Rectum (n=26)

VIP-immunoreactive nerves had a similar distribution in each of these areas of gut. However, there appeared to be more fibres in the antrum, colon and rectum, particularly in the circular muscle and mucosa. The jejunum seemed to receive the least VIPergic innervation.

In the fundus, subjectively, there were fewer fibres in the circular muscle, compared with the distal end of the oesophagus. The antral mucosa had a particularly rich supply of VIP nerves but the sub-mucosa contained less fibres than either the fundus or duodenum. This may reflect the slightly greater width of the sub-mucosa in the antrum compared with the other two regions. In duodenum, the general pattern of VIP fibres was observed, but surprisingly few fibres were found in association with the Brünner's glands.

There was a marked difference between the levels of VIP innervation of the jejunum and ileum. The ileum had more fibres in the mucosa and circular muscle, a similar density to that seen in the colon . The

caecum had slightly less fibres than the ileum. In the appendix, there were more VIP-immunoreactive ganglion cells in the sub-mucous plexus than in any other region of gut. In addition, in two specimens, occasional VIP-immunoreactive cells could be seen in the myenteric plexus. Colon and rectum received a high level of VIP innervation. Unfortunately, it proved to be too difficult to collect fresh specimens of human anus and anal sphincters.

Pancreas (n=13 whole but some specimens were collected as head, body or tail separately)

A widespread distribution of VIP-immunoreactive nerves was seen throughout the pancreas. They were present in the exocrine tissues, between acini (Fig **11**) and around ducts and blood vessels. The endocrine cells were found to have a particularly rich supply of VIP containing nerves which enmeshed the islets forming peri-insular networks (Fig **12**). This neuro-endocrine complex was found in all specimens. Further investigation of the neuro-insular network of VIP fibres was made in the rat (see later). As in the mucosal epithelium, VIP fibres ran very close to the islets, often appearing to terminate on them. In the connective tissue, VIP immunoreactive ganglion cells seemed to be more numerous in the pancreatic ganglia than in the gut, forming at least 50% of the total number of cells. Occasional large nerves contained parallel aligned VIP fibres and were usually seen near to the ganglion cells in the connective tissue. There was no appreciable difference in the numbers or distribution of VIP nerves in the three main areas, head, body and tail of pancreas. Only one other peptide, PHI, was found in the innervation of the pancreas.

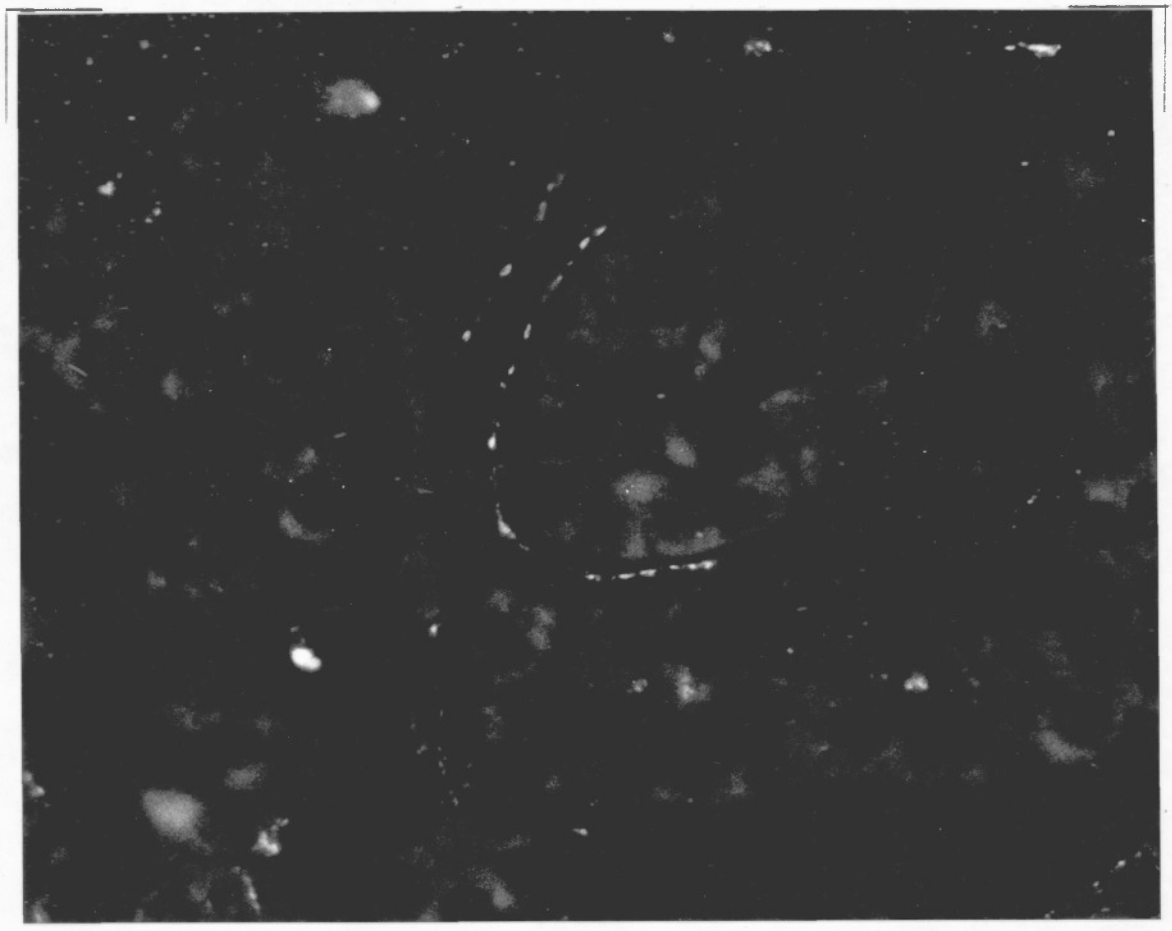
FIG 11

VIP-immunoreactive fibres in the pancreatic acinar tissue of a:

a) human

b) cat (x 400)

Tissue fixed in BQS, cryostat section, indirect immunofluorescence.



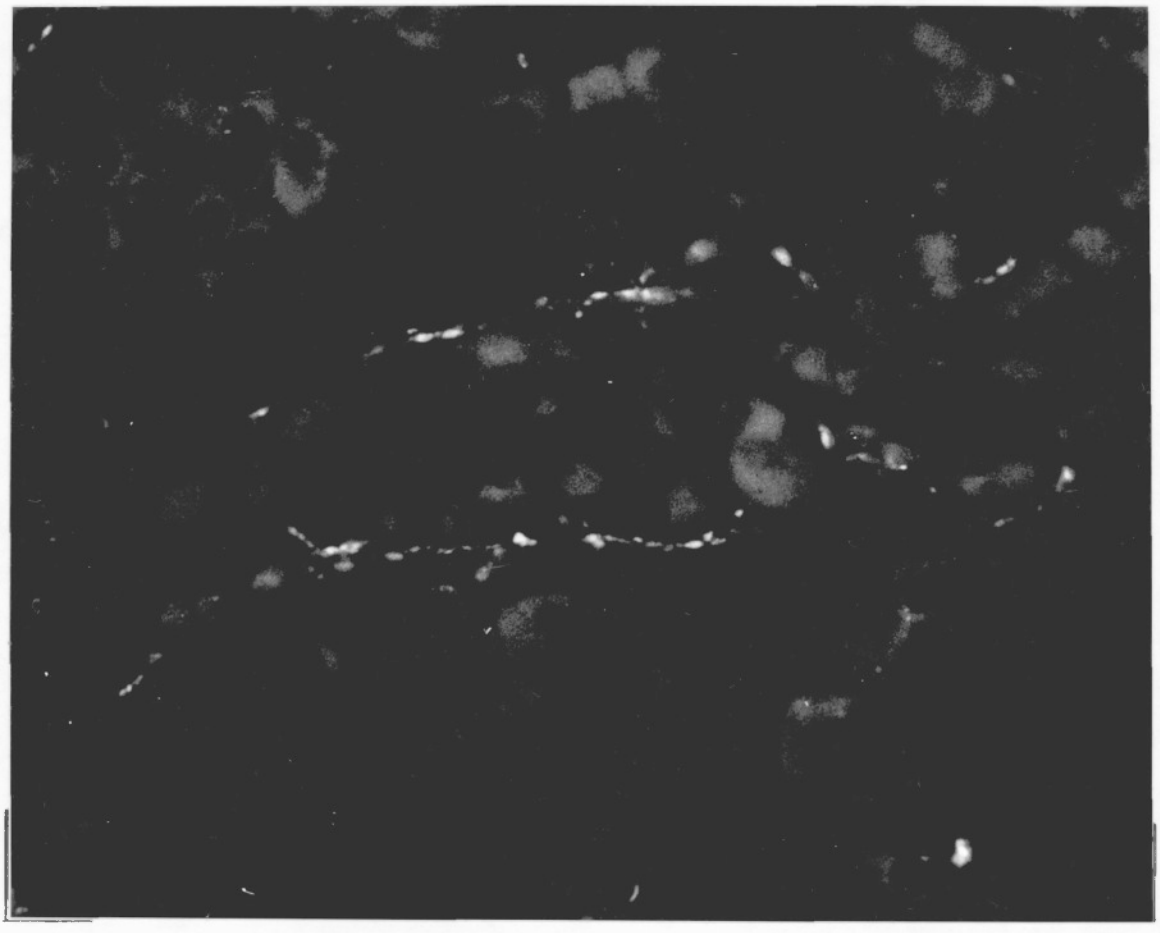
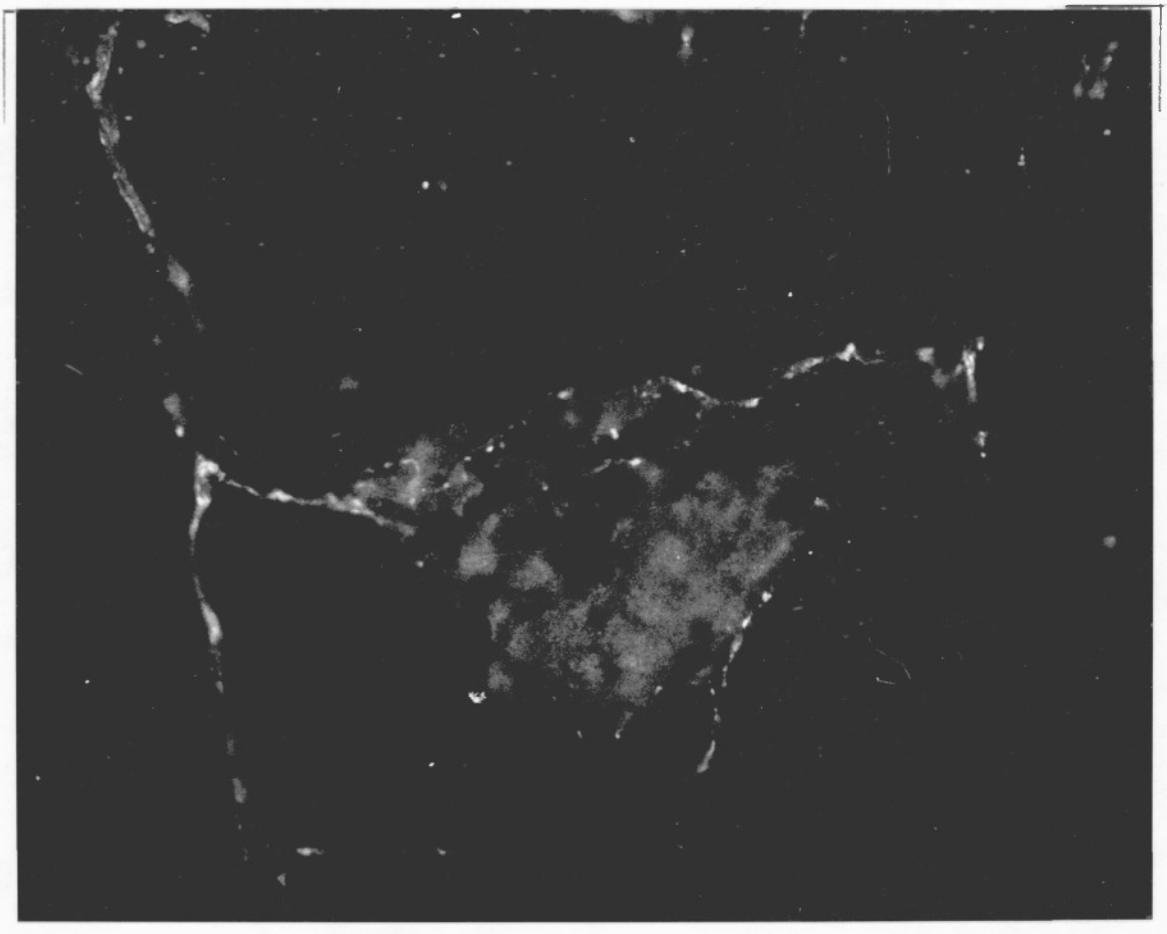


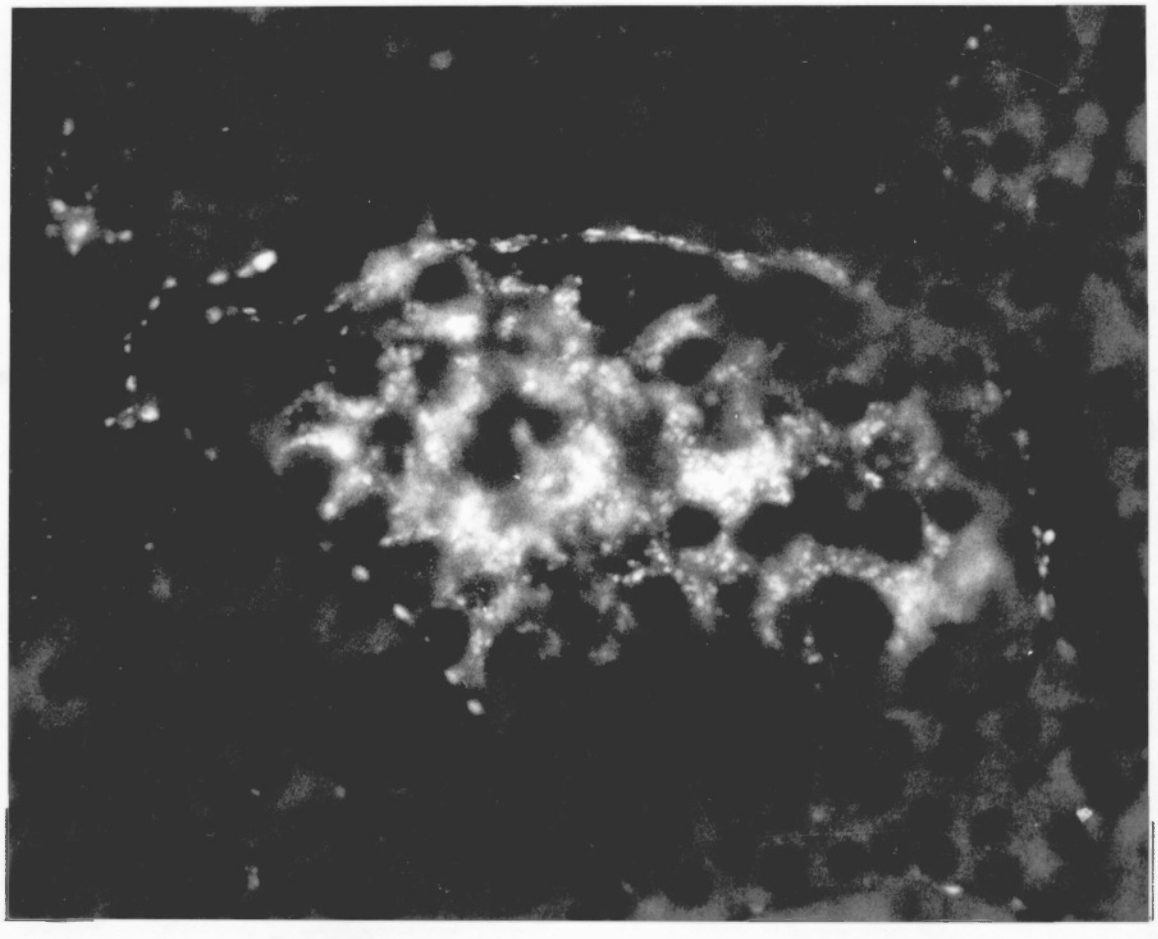
FIG 12

a) An islet in rat pancreas, showing slight autofluorescence, surrounded by VIP-immunoreactive fibres (x 250).

b) Human pancreatic islet with closely associated VIP-immunoreactive fibres (x 400).

Tissue fixed in BQS, cryostat section, indirect immunofluorescence





SUBSTANCE P

Gastrointestinal Tract

In man, substance P nerves were less abundant than those containing VIP. They did, however, have a similar appearance, being convoluted, slender with multiple varicosities. Like VIP-immunoreactive nerves, substance P nerves were present along the entire length of the gastrointestinal tract and in each layer of the wall.

Despite their similar overall distributions, there are some areas of the gut wall where there are quite distinct differences between the two populations of nerves. These are as follows;

a) In the oesophagus, substance P fibres were frequently found lying just beneath the squamous epithelium. This is in contrast to VIP fibres which rarely projected to the epithelium in this portion of the gut. The substance P-immunoreactive fibres often lay at right angles to the epithelium, projecting upwards as far as the epidermis.

b) Very occasional substance P-immunoreactive ganglion cells were found in the myenteric plexus (Fig 13). No substance P-immunoreactive cells were seen in the sub-mucous plexus.

c) Whereas VIP fibres appeared to be equally numerous in both of the main plexuses, substance P nerves were more dense in the myenteric plexus. A similar density of these fibres was seen in this plexus whether or not immunoreactive ganglion cells were present.

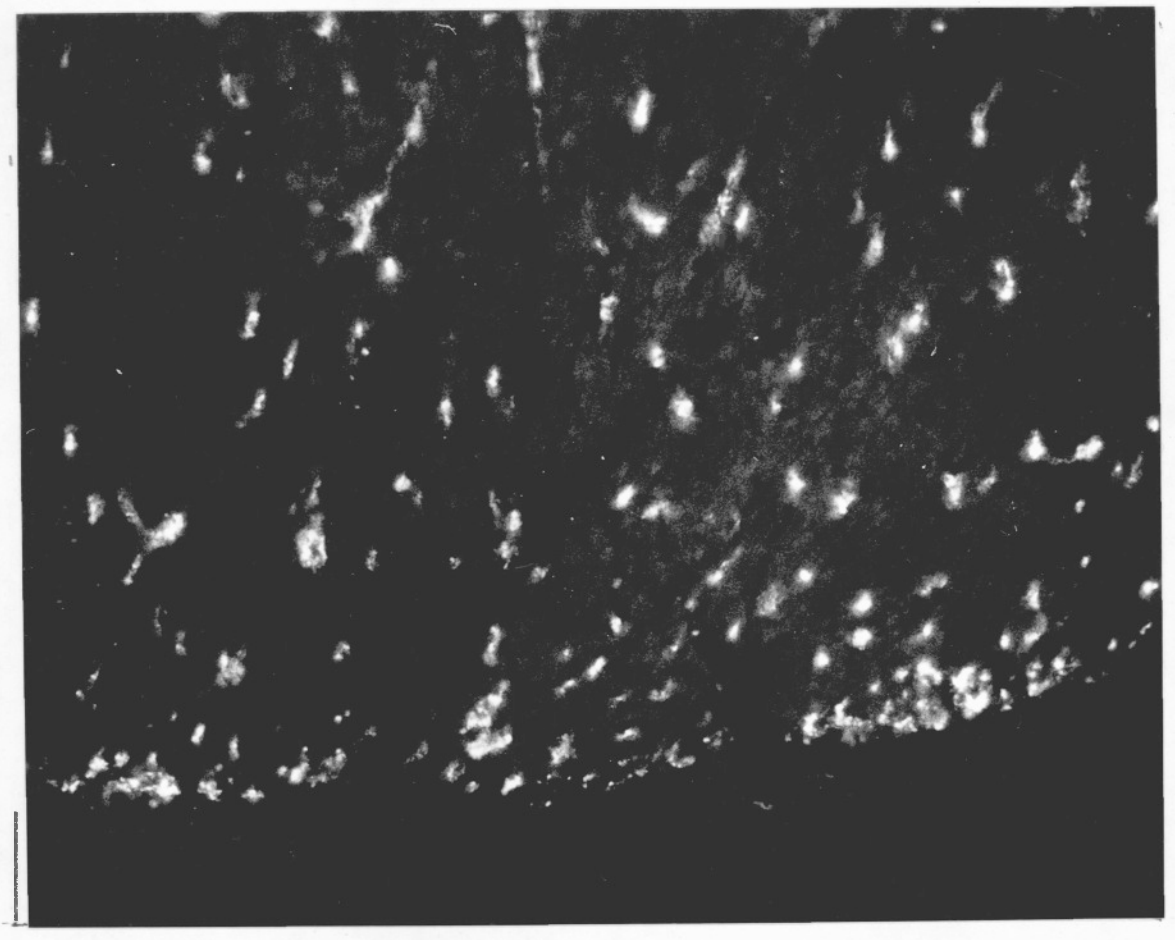
FIG 13

A single substance P-immunoreactive ganglion cell (arrow) surrounded by immunoreactive fibres and non-immunoreactive cells in the myenteric plexus of human ileum. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 450).

FIG 14

Circular muscle of feline pyloric sphincter immunostained for VIP. Immunoreactive nerves are more dense towards the inner edge of the muscle, at the base of the figure. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 300).





d) In the circular muscle, substance P immunoreactive fibres were seen mainly as single elements, whereas VIP nerves formed loose, interwoven strands of a few fibres. Both types of peptidergic nerve ran in a similar direction, roughly in the same plane as the muscle fibres.

e) Substance P nerves did not show such an obvious close relationship with the mucosal epithelium as VIP nerves. In the sub-mucosa, the substance P nerves were most numerous around the base, with fewer fibres than those containing VIP projecting towards the top of the mucosa.

PEPTIDE HISTIDINE ISOLEUCINE (PHI)

PHI nerves were found to have a very similar distribution to those containing VIP, but were present in lesser numbers in each area of bowel and pancreas examined. PHI-immunoreactivity was detected in a proportion of sub-mucosal ganglion cells but, again, these were fewer than those immunoreactive for VIP. The differential distribution of the two types of peptide containing nerves was most noticeable in the upper regions of the gut, particularly in the stomach, where fewer PHI fibres could be seen. In the fundus, hardly any PHI nerves could be found. Like VIP nerves, PHI-immunoreactive fibres were found in muscle, around blood vessels and in the mucosal layers of the gut, often in close proximity to the mucosal epithelium but they were less frequently associated with the islets.

MET-ENKEPHALIN

Fibres containing met-enkephalin-immunoreactivity were comparatively infrequent in the human bowel and were not found at all in the pancreas. Very scattered fibres were seen at each level of the bowel, although not in every specimen and they seemed to be most numerous in the appendix. Immunoreactive nerves, if found at all, were present in the muscle layers, particularly the muscularis mucosae and the myenteric plexus. No met-enkephalin immunoreactive ganglion cells were found.

BOMBESIN & SOMATOSTATIN

These two peptides were rarely found in nerves of the human gut. A few bombesin-immunoreactive nerves were seen in two specimens of antrum, in the mucosa. A few somatostatin-immunoreactive fibres were found, only once, in the muscularis mucosae of one specimen of colon. As they were so rare, it was impossible to test the immunostains by absorption of the antisera with the corresponding peptides. These results thus remain equivocal.

OTHER MAMMALS

In the main, the distribution patterns of peptide containing fibres in the other mammalian species studied, in particular pig, were similar to those seen in the human gut and pancreas. Mouse tissue, also was very like that of the rat. There were, however, some obvious differences, as follows;

GUINEA PIG (n=14)

The guinea pig appeared to have a greater supply of substance P containing nerves in its gut than any other animal studied, except the horse. This meant that, although VIP nerves were still the most numerous, the difference between the proportions of the two separate populations was less marked. There was no associated increase in the number of substance P-immunoreactive ganglion cells, compared to the human gut. Although CCK-, neurotensin- and somatostatin-immunoreactive nerves have been reported in the enteric innervation of guinea pigs (Schultzberg et al, 1980), they were not found under the conditions of the present study. Only VIP- and PHI-immunoreactive nerves were found in the guinea pig pancreas.

RAT (n=29)

In the human gut, the level of VIPergic innervation was not found to increase in the region of the enteric sphincters. It has been reported (Alumets et al, 1979),however, that all the sphincters in the feline gut do have an unusually rich supply of VIP nerves. In the rat, two sphincters did have more VIP nerves than adjacent areas of bowel, although the number of immunoreactive ganglion cells did not appear to alter. A relative increase in VIP containing nerves was observed in the pyloric sphincter and ileo-caecal valve. This increase was particularly pronounced in the deep muscular plexus, which lies along the inner edge of the circular muscle'(Bishop et al, 1980a).

Although the overall distribution of VIP fibres was similar, the rat

pancreas contained approximately 50% more nerves than the human. Evidence for the preferential innervation of islets by VIP nerves was obtained from preparations of isolated islets (Bishop et al, 1980**c**). Radioimmunoassay of the VIP content of whole rat pancreas revealed 28±7 (mean ± SEM) pmol VIP/g wet weight of tissue. In preparations of isolated islets, however, much higher concentrations of 374±30 pmol VIP/g were found.

CAT (n=12)

A relative abundance of VIP nerves was found only in the pyloric sphincter of the cat. As in the rat, the larger numbers of VIP nerves were found mainly in the deep muscular plexus (Fig **14**). Specimens of anal sphincter were obtained from the cat, enabling inspection of the peptide-immunoreactive nerves. The external anal sphincter contained only very few peptidergic fibres, mainly VIPergic, which were usually seen in association with blood vessels. Also, the peptidergic innervation of the internal anal sphincter was less than that of the proximnal portion of bowel. Fewer VIP-immunoreactive ganglion cells were seen in the sub-mucous plexus.

The cat was the only species with peptidergic nerves in the pancreas other than those containing VIP or PHI. Substance P nerves were present in very small numbers, mainly in the ganglia and connective tissue. Occasional fibres also lay close to blood vessels. Few were associated with either the exocrine or endocrine pancreas.

HORSE (n=6)

Like the guinea pig, the horse had more substance P immunoreactive fibres in its gut than man. The general distribution of these fibres was similar to that in man but the horse appeared to have a more dense network of immunoreactive fibres in the mucosa. This larger number of substance P fibres was not accompanied by an increase in the number of immunoreactive ganglion cells.

OTHER NEUROPEPTIDES

Although a whole range of antibodies was used to peptides reported or suggested to be in peripheral nerves (see Table 1), only those peptides mentioned above were found to have this neuronal localisation.

THE INTRINSIC ORIGIN OF THE VIP CONTAINING NERVES OF THE CANINE PANCREAS

VIP nerves in the canine pancreas were of a similar number and distribution to those of the human pancreas, except that the innervation of the islets was even more pronounced. The intrinsic origin of the VIP nerves was investigated in a series of experiments involving the surgical denervation of the pancreas. These experiments were carried out in collaboration with Professor RB Welbourn and Mr RA Prinz, Department of Surgery, RPMS (Prinz et al, 1983).

Materials and methods

Pancreatic denervation was carried out on 5 dogs under general anaesthesia. The animals were allowed to recover and were sacrificed 2 weeks after normal food consumption had been resumed.

Pieces of head, body and tail of pancreas were obtained from;

A) Dogs undergoing surgery for pancreatic denervation, before the procedure had began.

B) Dogs with fully denervated pancreas

C) Normal dogs sacrificed for other reasons.

Each sample was processed in either of two ways;

a) Freeze-dried and fixed in formaldehyde vapour for the formaldehyde induced fluorescence (FIF) test (Falck & Owman, 1965)(appendix).

b) Fixed in benzoquinone solution and stained with H & E or immunostained for VIP (appendix).

Results

The FIF test reveals the presence of nerves (and cells) containing amines. Thus, the aminergic (extrinsic) innervation of the pancreas could be seen in the normally innervated tissues taken from the dogs in groups A nad C. However, in the surgically denervated pancreata of group B, no FIF positive nerves were found, indicating successful denervation.

In groups A and C, large numbers of VIP-immunoreactive fibres were found throughout. VIP-immunoreactive ganglion cells were present in the

connective tissue. The pattern of VIP innervation in the denervated specimens of group B was entirely comparable to that of the normal controls. It was, therefore, concluded that the majority if not all of the VIP nerves of the canine pancreas have an intrinsic origin.

THE POSSIBLE CO-EXISTENCE OF PEPTIDES IN NERVES WITH OTHER NEUROTRANSMITTERS

PEPTIDES IN CHOLINERGIC NERVES

As was mentioned previously (Chapter 1), VIP has been reported to be present in cholinergic fibres (Lundberg et al, 1982) but research into the co-existence of these two substances has taken place mainly in exocrine glands, not the gut. There has been controversy as to whether or not the technique of acetylcholinesterase staining actually demonstrates cholinergic nerves (Pepler & Pearse, 1957, Silver, 1974). Not only does the enzyme breakdown substance P but it is also found in non-cholonergic nerves. In addition, as acetylcholinesterase causes post-synaptic degradation of acetylcholine it would be expected to occur in cell bodies with which cholinergic nerves synapse. However, in the absence of antibodies to acetylcholine or to enzymes specifically involved in its synthesis, the occurrence of VIP in nerves with acetylcholinesterase activity was investigated.

Materials and methods

As nerves are found in the gut and pancreas mainly in bundles consisting of a number of fibres, confident identification of a single fibre is impossible at the light microscopical level. For this reason,

serial sections were used to see if VIP and acetylcholinesterase exist in the same nerves. Specimens of human (n=4) and rat (n=6) antrum, colon and pancreas were fixed in benzoquinone solution and processed (appendix). Serial (4-6um) cryostat sections were cut and one of each pair stained for acetylcholinesterase activity. In order to obtain suitable serial sections through cells, the first section of each pair was picked up directly off the microtome knife. The second section was gently brushed from the knife and overturned onto a chilled surface and then picked up onto a glass slide. In this way, the same cut surface was uppermost in each section. The other section was immunostained for VIP.

Results

The distributions of ganglion cells containing acetylcholinesterase activity and/or VIP-immunoreactivity were compared in the serial sections. The distributions of the two types of fibres were similar in all tissues, except that the enzyme containing nerves were more numerous. Also they had a much thicker, coarser appearance, probably reflecting the nature of the histochemical method used. The acetylcholinesterase method seemed to stain most, if not all, of the ganglion cells in both plexuses. VIP-immunoreactive cells were restricted to the sub-mucous plexus.

Comparison of the sections showed that all of the VIP-immunoreactive cells were also positively stained for acetylcholinesterase.

CO-EXISTENCE OF PEPTIDES IN NEURONS

The co-existence of peptides in neurons had only been reported once (Schultzberg et al, 1980). This report was of somatostatin and CCK in ganglion cells of the guinea pig colon. In the present study, only VIP and PHI were consistently found in ganglion cells. Accordingly, the possible co-existence of these two peptides was investigated. Their co-existence was of interest, in addition, as the two peptides share sequence homology and certain actions (see Chapter 1)

Materials and methods

Fresh specimens of human large bowel (n=4) were obtained at surgery for removal of carcinoma. Macroscopically and histologically normal samples were removed, fixed in benzoquinone and processed (appendix). Pairs of 4-6um thick serial cryostat sections were cut as for the study of VIP and acetylcholinesterase co-localisation. One of each pair was immunostained for VIP and the other for PHI.

In view of the structural similarities between the two peptides, stringent controls for the immunostains were carried out. The immunostaining for PHI was not affected by addition of natural or synthetic VIP in concentrations of up to 20nmol/ml of diluted antiserum. It was, however, removed by addition of as little as 0.1nmol/ml of natural or synthetic PHI. Conversely, immunostaining with VIP antibodies was quenched by prior absorption with natural or synthetic VIP (0.1nmol/ml diluted antiserum) but not natural or synthetic PHI (20nmol/ml diluted antiserum) (Table 3).

Table 3

PHI & VIP ANTISERA ABSORPTION TEST

Antiserum to:	Conc. of synthetic or natural VIP added (nmol/ml diluted antiserum)	Result	Conc. of synthetic or natural PHI added (nmol/ml diluted antiserum)	Result
Natural porcine PHI	20	+++	0.1	-
" " "			0.01	±
" " "			0.001	++
Natural porcine VIP	0.1	-	20	+++
" " "	0.01	+		
" " "	0.001	++		

Results

The reversed sections allowed easy identification of serially sectioned ganglion cells. PHI and VIP were both immunostained in a proportion of the cells (Fig **15**). Some cells were, in addition, immunostained for VIP but not PHI, or showed very weak, equivocal staining for PHI. Ganglion cells with no apparent immunoreactivity formed a minor population whilst only very few contained PHI- but not VIP-immunoreactivity.

DISCUSSION

The results of these studies show that there is a high degree of correlation between the distribution of the peptide containing nerves and what is known about the actions of the peptides. This is illustrated well by the observed distribution of VIP nerves. In accordance with its three main actions in the gut ie vasodilation, muscle relaxation and stimulation of water and electrolyte secretion (Polak & Bloom, 1978), VIP-immunoreactive fibres were found around blood vessels, in muscle layers and in the mucosa. VIP nerves were found in association with the cardiovascular system throughout the body and its vasodilator action was the first property established for the peptide (Said & Mutt, 1972). Most of the VIP innervation of the muscle coat was found in the circular muscle. This is in agreement with the finding that, although VIP causes muscle relaxation in the gut (Eklund et al, 1979), it does not appear to have a direct effect on the longitudinal muscle (Williams & North, 1979). The stimulation of gut secretion by VIP may be mediated by direct release from nerve fibres onto cells in the mucosal epithelium. There has been a preliminary report of a direct contact between VIP nerves and epithelial cells

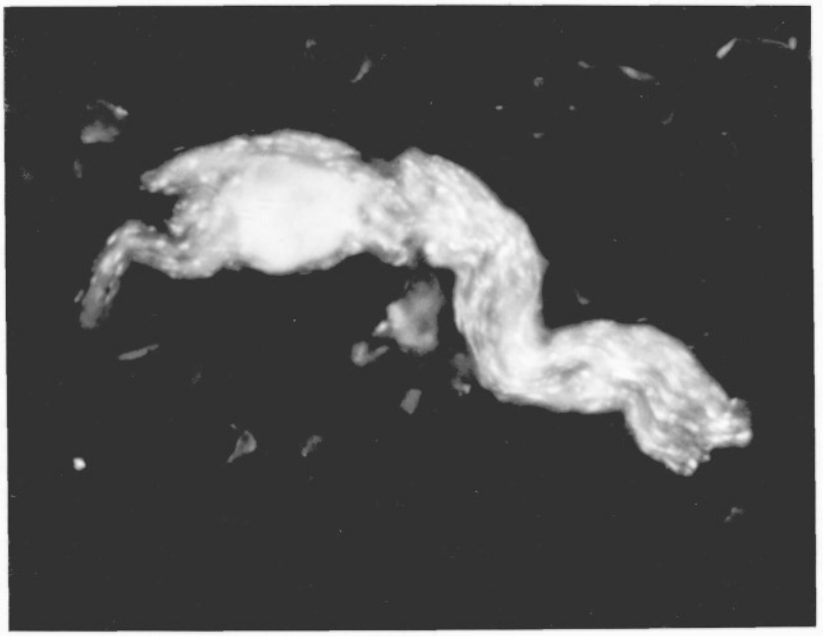
FIG 15

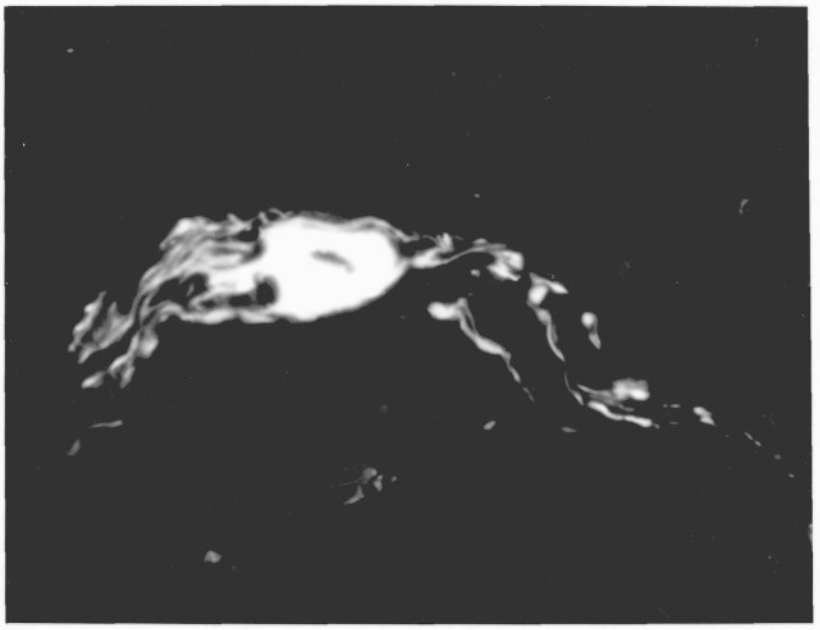
Serial cryostat sections through a ganglion cell body and its associated fibres in the sub-mucous plexus of pig colon immunostained for:

a) VIP

b) PHI (x 375)

Tissue fixed in BQS, indirect immunofluorescence.





(Rosselin, 1981). In addition, epithelial cells are known to have VIP receptors, which are coupled with adenylate cyclase stimulation (Laburthe et al, 1979).

In the exocrine pancreas, the VIP-immunoreactive nerve fibres described in this study may directly cause release of bicarbonate and pancreatic alkaline juice flow. VIP stimulates exocrine secretion from the pancreas (Lindkaer Jensen et al, 1978, Holst et al, 1979) and there are specific receptors for the peptide on the membranes of acinar cells (Gardiner et al, 1977).

A dense VIP containing innervation of the pancreatic islets was also demonstrated in this study. VIP may well act as an agent in the control of the endocrine, as well as the exocrine, pancreas. Infusion of VIP into the isolated dog pancreas releases somatostatin (Ipp et al, 1978), insulin, glucagon and pancreatic polypeptide (Adrian et al, 1978). Similarly, perfusion of the isolated pig and cat pancreas has been shown to release both insulin and glucagon (Schebalin et al, 1977, Lindkaer Jensen et al, 1978). It is interesting to note that the amounts of VIP required to release these hormones (between 1-50nmol/L) are never attained in the circulation under normal physiological conditions. However, it is possible that such concentrations could be reached during release of VIP directly onto islets from peri-insular fibres. These results, together with the finding of a predominantly intrinsic origin of the VIP innervation of the canine pancreas, indicate that VIP nerves are closely involved in the local control of both the exocrine and endocrine pancreas.

Findings made for the other peptides also illustrated this parallelism between actions and distribution. For example, substance P-immunoreactive ganglion cells, when found at all, were present in the myenteric plexus. This plexus also contains a denser network of substance P fibres than the sub-mucous plexus. The high ratio of substance P nerves in the myenteric plexus is in agreement with its postulated role as a transmitter of excitatory interneurons within the myenteric but not sub-mucous plexus (Katayama & North, 1978). No effects of substance P on the neurons of the sub-mucous plexus have been described. The observed preferential distribution of substance P nerves in the myenteric plexus may also relate to the function of the peptide as a sensory neurotransmitter. Although not much is known about the distribution of sensory fibres in the gut, those involved in the peristaltic reflex are thought to be present mainly in the myenteric plexus (Gabella, 1974). Enkephalin containing fibres, in keeping with the peptide's role as an inhibitor of neuronal activity (North & Williams, 1976) are found mainly in the myenteric plexus.

The finding of only comparatively rare ganglion cell bodies with substance P-immunoreactivity is somewhat anomalous in view of previous reports of substance P nerves being intrinsic to the bowel of mice (Schultzberg et al, 1978), guinea pigs (Jessen et al, 1980) and pig (Malmfors et al, 1981). In addition, the predominantly intrinsic nature of substance P nerves in the human bowel has been suggested by pathological conditions (see Chapter 5) where loss of intrinsic neurons results in loss of substance P-immunoreactive nerves. Possible explanations for the lack of immunoreactivity in ganglion cell bodies may be firstly, that the peptide, unlike VIP, is not stored in the cell body or is present in too low concentrations to be detected by

immunocytochemistry. Secondly, in the ganglion cell body substance P may occur in a slightly different, perhaps precursor, form which is not recognised by the antiserum used in this study.

The distribution of peptide containing nerves in man has never been examined before in any detail. Although most of the results obtained in the other mammals have been documented previously, there are some anomalies. For example, no somatostain, CCK or neurotensin containing fibres were found in the gut of guinea pigs or rats as reported by Schultzberg et al, 1980. Many of these may be explained by the use of different antisera or even, perhaps, different strains of the same species of animal. In the case of the gut sphincters, VIP nerves have been reported to be dense in these regions of the cat (Alumets et al, 1980). However, in the present studies such a relative increase in VIP innervation was only found in the pyloric sphincter in the cat (and in the pyloric and ileo-caecal sphincters of the rat). In the previous report (Alumets et al, 1980), the VIP nerves were more numerous throughout the muscle layers but in this study the increase was most noticeable along the inner edge of the circular muscle, in a region known as the deep muscular plexus. This plexus has been cited as the main location of neuro-muscular transmission.

Co-existence of peptides with other transmitters has been described previously (Lundberg et al, 1982). In this study, it was only possible to show that VIP is found in some of the ganglion cells of the gut which contain acetylcholinesterase activity. As acetylcholinesterase is no longer considered to be specific for cholinergic fibres (Pepler & Pearse, 1957, Silver, 1974), it is not possible to conclude anything about co-existence of transmitters from the results. Only one report

exists of co-localisation of two peptides. In this study a further example was demonstrated (Bishop et al, 1983). The general distribution of PHI in the bowel was found to be very similar to that of VIP. Both are present in sub-mucosal ganglion cells, thereby providng an easy means for investigation of their co-localisation to nerves. The two peptide immunoreactivities were demonstrated to be co-localised to a significant proportion of these neuronal cell bodies. Diligent testing of the antisera used showed that they were specific for their intended peptide. At the time of the study, these results were taken to suggest either co-existence of separate peptides or a common origin for VIP and PHI from a single precursor molecule. The existence of such a molecule was postulated (Christofides et al, 1982a) and subsequently characterised. Analysis of both human VIP-producing tumours (Bloom et al, 1983) and human neuroblastoma cells (Itoh et al, 1983) revealed a pre-pro-vasoactive intestinal polypeptide containing the amino acid sequences of both VIP and PHI. The usual site of cleavage of hormonal peptides from their precursors is at pairs of basic amino acids. Within the pre-pro-VIP molecule there are pairs of the basic amino acids lysine and arginine at either end of VIP but only at the carboxyl end of the PHI sequence. Theoretically, this provides the opportunity for differential expression of the two peptides. The morphological findings described here, of plenty of VIP but little PHI in the fundic region, supports the concept that there are fundamental differences in the post-translational processing of VIP and PHI.

CHAPTER 4 - DISTRIBUTION OF NSE AND S-100 IN MAMMALIAN GASTROINTESTINAL TRACT AND PANCREAS

Available methods for the demonstration of nerves can be limited and often involve specialised procedures and tissue fixation. When antibodies to the proteins NSE and S-100 became available their known presence in neural and glial structures, respectively (see Chapter 1), suggested the possible usefulness of the antibodies in delineating the nervous system of the gastroenteropancreatic tract.

NEURON SPECIFIC ENOLASE (NSE)

In this study, antibodies to neuron specific enolase (NSE) were used to establish whether the enzyme is a marker for the enteric and pancreatic innervation. In addition, as it had previously been found in endocrine cells (Schmechel et al, 1978) in other tissues, they were used to see if NSE is a common marker for nerves and endocrine cells. To do this it was necessary first to determine a suitable tissue fixation procedure which allowed not only immunostaining of the enzyme but also demonstration of the various nerves and endocrine cells.

An additional aim of this study was to test whether NSE immunostaining helped to reveal morphological integration between nervous and endocrine elements. There is known to be a high degree of functional integration between the diffuse endocrine and enteric and pancreatic nervous systems, revealed by instances of interactions (Walsh, 1981, Eklund et al, 1980, Bloom & Polak, 1980, Bloom & Edwards, 1980, Adrian et al, 1978) which support earlier anatomical descriptions of neuroendocrine complexes (Van Campenhout, 1927).

Materials

Specimens were obtained from both humans and rats. Fresh tissues from the following areas were obtained from rats (n=6), shortly after they were sacrificed by decapitation and from humans (n=6 for each area), at surgery: fundus, antrum, duodenum,jejunum, ileum, colon, rectum and pancreas (head ,body and tail separately). The human specimens were obtained at resection for carcinoma.

All tissues were fixed immediately using various methods (Table 4 & appendix) and processed for conventional histology (haematoxylin and eosin), histochemistry and immunocytochemistry for NSE, peptides and an amine (serotonin) (appendix).

Comparative Studies

To compare the distributions of NSE and peptides, antisera to a range of peptides were used as previously described (Chapter 2 & appendix). Antibodies to serotonin were also used to reveal enterochromaffin cells.

As discussed in Chapter 2, certain fixation methods are more suitable than others for the preservation of antigens in tissue. As the enzyme NSE presented a new antigen to be immunostained, further investigation of fixation techniques had to be undertaken, in addition to that previously described in Chapter 2. The range of fixation methods, including conventional histological techniques, used in this study in order to establish the best method for the demonstration of NSE and for the comparison of the distributions of NSE, peptides and

amine are shown in Table 4 . Those methods found to give optimal results are described below.

Endocrine cells

Investigations were carried out using the three most widely applied techniques for comparing the distributions of two, or more, different antigens in the same tissue component. These methods were (a) a modified version of the double-immunostaining technique of Nakane (Nakane, 1968), (b) Tramu's method of antibody elution (Tramu et al, 1978) and (c) the use of serial thin (0.5 - 2um) sections (Grube, 1980).

Nakane's technique

Sections of formaldehyde vapour fixed tissues were double immunostained following the procedure described by Nakane, but using the PAP method for added sensitivity (appendix). 4-chloro-1-napthol was used as coupler for the first immunostain and diaminobenzidine for the second.

Tramu's method of antibody elution

As with the previous method, this technique involved the double immunostaining of a single section. The PAP technique was carried out, on tissue fixed as for Nakane's technique, in order to immunostain NSE and 4-chloro-1-napthol was used as coupler (appendix).

Table 4 Preparation of tissue

Tissue	Fixative	Solvent	Concentration	Time (h)	Temperature ('C)	Embedding medium		
						Cryostat	Paraffin wax	Resin
Fresh	Unfixed	-	-	-	-	+	+	+
	Liquid phase							
	p-BQ	PBS	0.4% wt/vol	1/2-1	4	+	+	
	Bouin's	aq		1-2	4	+	+	
	Formalin	PBS	10% wt/vol	1/2-1	4	+	+	
	Glutaraldehyde	PBS	1-4% wt/vol	1/2-1	4	+		+
Freeze-dried	Unfixed	-	-	-	-	+	+	
	Vapour-fixed							
	p-BQ	Toluene	Saturated	3	60		+	+
	DEPC	-	Saturated	3	60		+	+
	Formaldehyde	-	Saturated	3	60		+	+
	Formaldehyde	-	Saturated	1	80		+	+

p-BQ = p-benzoquinone, DEPC = diethylpyrocarbonate, PBS = phosphate buffered saline, aq = water

Serial thin sections

Lastly, serial thin (0.5 - 2μm), resin-embedded sections (appendix), passing through the same endocrine cell, were immunostained, one for NSE and the other for a peptide or amine.

Demonstration of enterochromaffin-like cells: Sevier and Munger's silver impregnation method

Enterochromaffin-like cells were demonstrated in formaldehyde vapour fixed, resin embedded tissue (see above) using the silver impregnation meethod of Sevier and Munger (Sevier & Munger, 1965) and their distribution was compared with that of NSE-containing cells, immunostained in serial thin (0.5 - 2μm) sections (appendix).

Innervation

Three methods were used to provide some characterisation of neurons containing NSE. These were (a) acetylcholinesterase staining (El Badawi & Schenk, 1967) (b) immunocytochemistry for peptides and (c) the formaldhyde induced fluorescence technique (Falck & Owman, 1965), the latter in order to localise nerve fibres containing amines.

As nerves are found in the gut and pancreas mainly in bundles consisting of a number of fibres, confident identification of a single fibre is not possible using current techniques at the light microscopical level. For this reason, serial sections were used to provide some characterisation of neuronal cell bodies immunoreactive for NSE. In view of the predominantly extrinsic origin of aminergic

fibres, it was not possible, for the reasons outlined above, to obtain a definitive comparison of NSE and amine distribution in fibres. Instead, only a partial comparison was possible and the distribution of amine was compared with that of NSE-immunoreactive fibres in serial sections.

Cholinesterase staining

Pieces of whole thickness of gut (no larger than 2x2cm) and pancreas (no larger than 1cm3) were fixed in benzoquinone solution (appendix). After fixation, cryostat blocks were prepared and serial 3-5um thick sections were cut and air-dried.

Serial sections were stained for the presence of acetylcholinesterase activity (El Badawi & Schenk, 1967) (appendix) and immunostained for NSE. Photographs were taken for comparison.

Immunostaining of peptides

Again, consecutive 3-5um cryostat sections were cut from tissues fixed in benzoquinone solution and immunostained for NSE and for vasoactive intestinal polypeptide (VIP) or substance P, the most abundant of the peptides present in the gut innervation. VIP was particularly important as it is present in a large proportion of the enteric ganglion cells of the sub-mucous plexus. The same ganglion cell was examined in the serial sections and photographs were taken for comparison.

Formaldehyde induced fluorescence

For this method the tissue was freeze-dried, fixed in formaldehyde vapour and embedded in paraffin wax. Serial sections (3μm) were cut and processed for the formaldehyde induced fluorescence technique (appendix) or immunostained for NSE.

Results

Immunostaining with antibodies to NSE allowed the simultaneous demonstration of large numbers of endocrine cells and a massive network of nerves in the gut and pancreas of both humans and rats.

NSE-immunoreactivity was preserved in tissue fixed by each of the methods used, to varying degrees. Vapour fixation in formaldehyde or benzoquinone, followed by wax or resin embedding, gave the best results for endocrine cells. The use of serial thin sections successfully demonstrated the presence of NSE in all 15 currently identifiable endocrine cells types in the gut and pancreas. Fixation in benzoquinoine solution was the most suitable method for the histochemical (acetylcholinesterase) and immunocytochemical staining of nerves. Peptide immunoreactivity or acetylcholinesterase activity, or both, could be demonstrated in the majority of NSE-immunoreactive ganglion cells in the two main gut plexuses and in the pancreas.

The density of NSE and peptide or amine immunostaining was found sometimes to vary, even in cells in close proximity, or of the same type, or both. In addition, there was no obvious relationship between the density of the two immunostains.

GASTROINTESTINAL TRACT

In the fundus, NSE was demonstrated in cells, the majority of which could be identified in serial sections as enterochromaffin-like or somatostatin-containing.

NSE-immunoreactive cells were particularly numerous in the antral region of the stomach. A band of NSE-immunoreactive cells was seen lying across the base and middle parts of the pyloric glands (Fig **16**). Comparative immunocytochemistry revealed that the majority of these cells corresponded to gastrin, somatostain or enterochromaffin cells. All the immunoreactive cells were flask-shaped and many had elongations reaching the lumen.

In the duodenum, smaller numbers of NSE immunoreactive cells were observed. These were approximately equally distributed between Brünner's glands and the villus-crypt region. In the duodenum and jejunum, the immunoreactive cells showed denser staining for NSE than in the lower bowel, where fewer cells could be found. The scattered cells in the lower bowel were also mainly flask-shaped and some, particularly those shown by comparative immunocytochemistry to contain enteroglucagon or somatostatin, had basal elongations and slender processes reaching the lumen (Fig **17**). Unlike peptide immunoreactivity, which shows a polar distribution in cells, as the storage granules tend to lie near to the basement membrane, immunostained NSE was spread diffusely throughout each cell, as would be expected for a soluble cytoplasmic enzyme. A few additional NSE-immunoreactive cells were seen which could not be identified as containing either a peptide or an amine.

FIG 16

A band of NSE-immunoreactive mucosal endocrine/paracrine cells in human antrum. Freeze-dried tissue, fixed in benzoquinone vapour and embedded in paraffin wax (x 225).

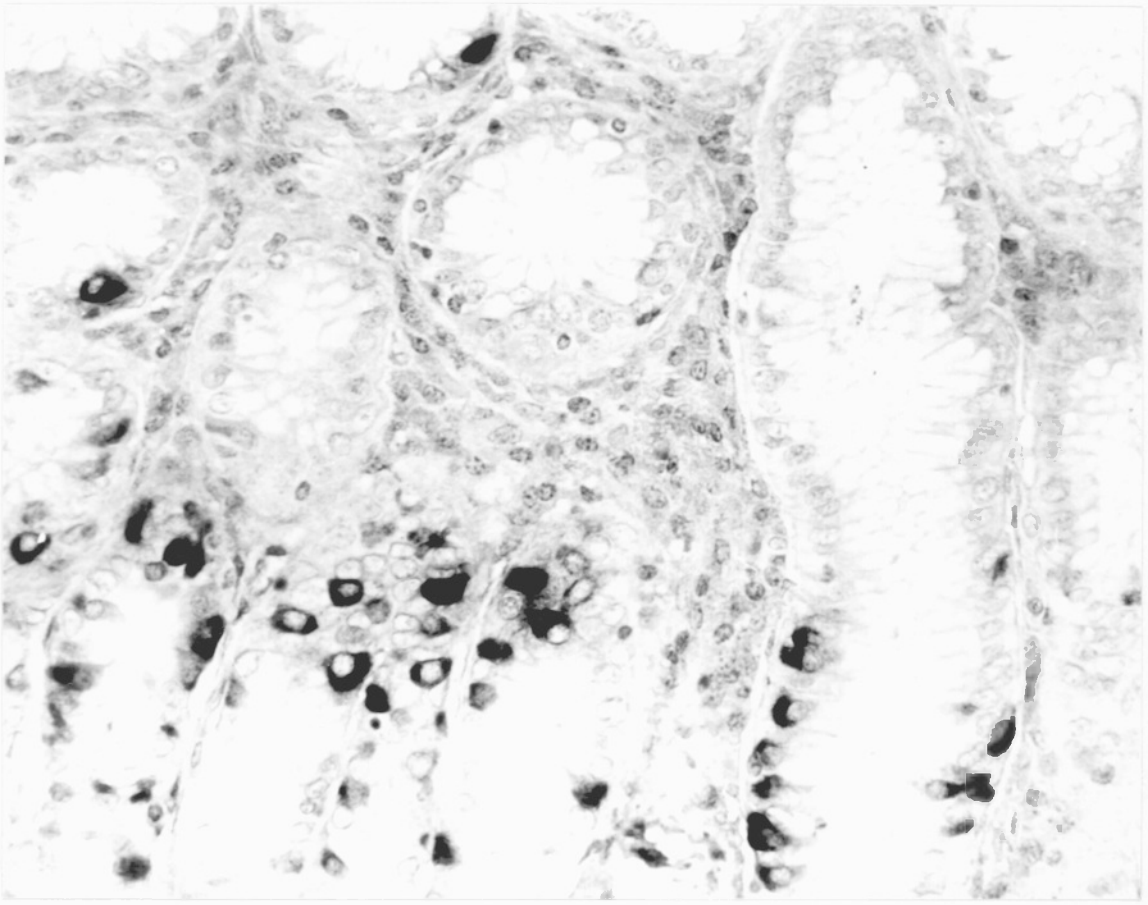
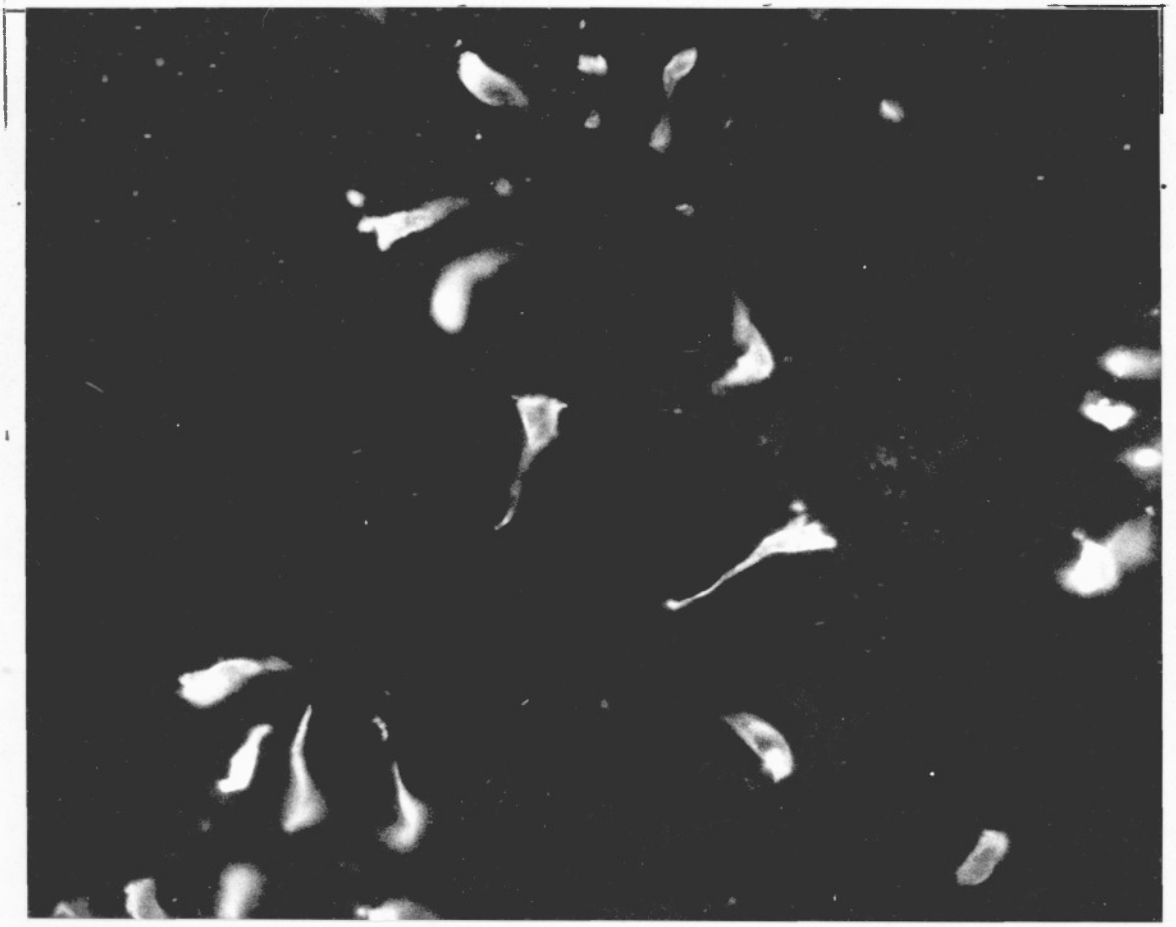


FIG 17

a) & b) Enteroglucagon-immunoreactive cells, showing basal and/or luminal elongations, in the mucosal epithelium of human rectum. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 350).





The general distribution of NSE-immunoreactive neurons was similar in all areas of the gut. Immunoreactive fibres were evenly spread across the entire width of the gut wall. They were slender in appearance but did not consistently show varicosities. All ganglion cells of the two main plexuses were immunostained to some degreee. NSE-immunoreactive fibres were clearly present in larger numbers than those that could be demonstrated by either peptide immunostaining or the histochemical methods used. Individual fibres did not appear quite as slender or as varicose as the immunostained peptide containing ones. This may be related to the diffuse localisation of NSE in cell cytoplasm rather than in discrete granules.

Using serial sections, NSE was demonstrated in all ganglion cells containing VIP (Fig 18), possibly the most abundant of all the gut neuropeptides. In addition, although acetylcholinesterase staining is not a specific marker for cholinergic nerves, it can be used to demonstrate a large proportion of the enteric nervous system. NSE immunoreactivity could be found in all regions in fibres and ganglion cells clearly outnumbering those revealed by the cholinesterase stain. Furthermore, using serial sections, co-existence of the two enzymes in neuronal cell bodies could be shown.

As fibre-to-fibre comparison of NSE and amine-containing nerves was not possible, the distributions of the two substances were compared in serial sections. NSE-immunoreactive fibres were present in all areas innervated by aminergic nerves. Aminergic nerves were found particularly in the myenteric plexus and around blood vessels.

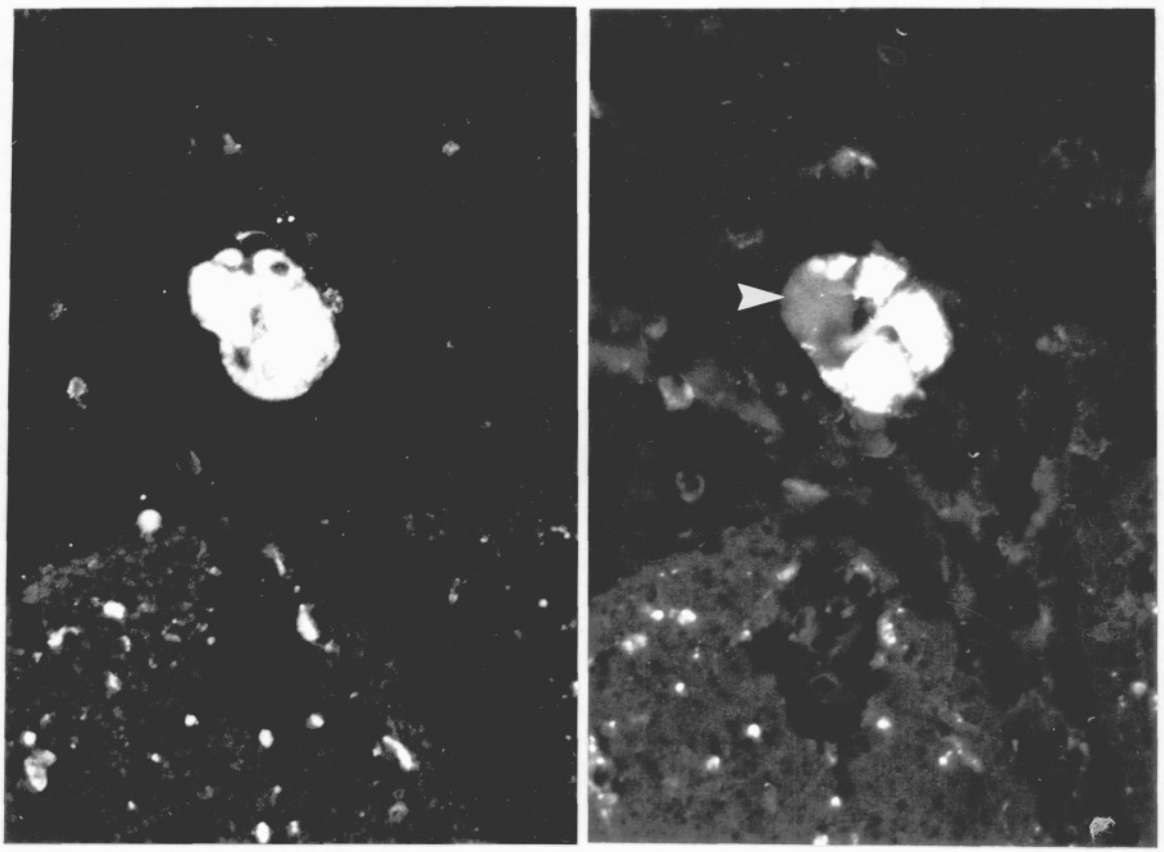
FIG 18

Serial sections of ganglion cells of the sub-mucous plexus of human colon immunostained for:

a) NSE - all ganglion cells are immunoreactive

b) VIP - all of the ganglion cells except one (arrow) are immunoreactive (x 200).

Tissue fixed in BQS, cryostat section, indirect immunofluorescence.



In the gut, it was possible to see NSE-immunoreactive endocrine cells and nerve fibres in close contact. In all regions of the gut, NSE-positive nerve fibres ran through the lamina propria up to the level of the epithelium, where they often ran very close to, and, perhaps even terminated on immunoreactive and non-immunoreactive cells.

Occasional NSE-immunoreactive cells in the lamina propria of the gut mucosa were also observed (Fig **19**). These cells were not demonstrated by peptide or amine immunostaining nor by acetylcholinesterase staining. The cells occurred singly or in small groups. Rarely, immunoreactive fibres could be seen emerging from the cells and extending towards the base of the lamina propria, away from the lumen. The full identity of these cells, as neuronal or endocrine, could not be established here.

PANCREAS

In vapour-fixed pancreatic tissue, the islet cells were densely immunostained and stood out clearly against the non-immunostained exocrine tissue. Examination of serial sections by comparative immunocytochemistry revealed that NSE is present in all four peptide-containing cell types (insulin, somatostatin, glucagon (Fig**20**), pancreatic polypeptide). In the ductal epithelium, NSE-immunoreactive cells could be seen in larger numbers than the total of all the peptide-containing cells identifiable in the same area.

Pancreatic neuroendocrine complexes could easily be distinguished in the tissue fixed in benzoquinone solution. NSE-immunoreactive fibres formed closely packed networks around the islets, which they appeared

FIG 19

NSE-immunoreactive cells in the lamina propria of human duodenum. Freeze-dried tissue, fixed in benzoquinone vapour and embedded in paraffin wax (x 200).

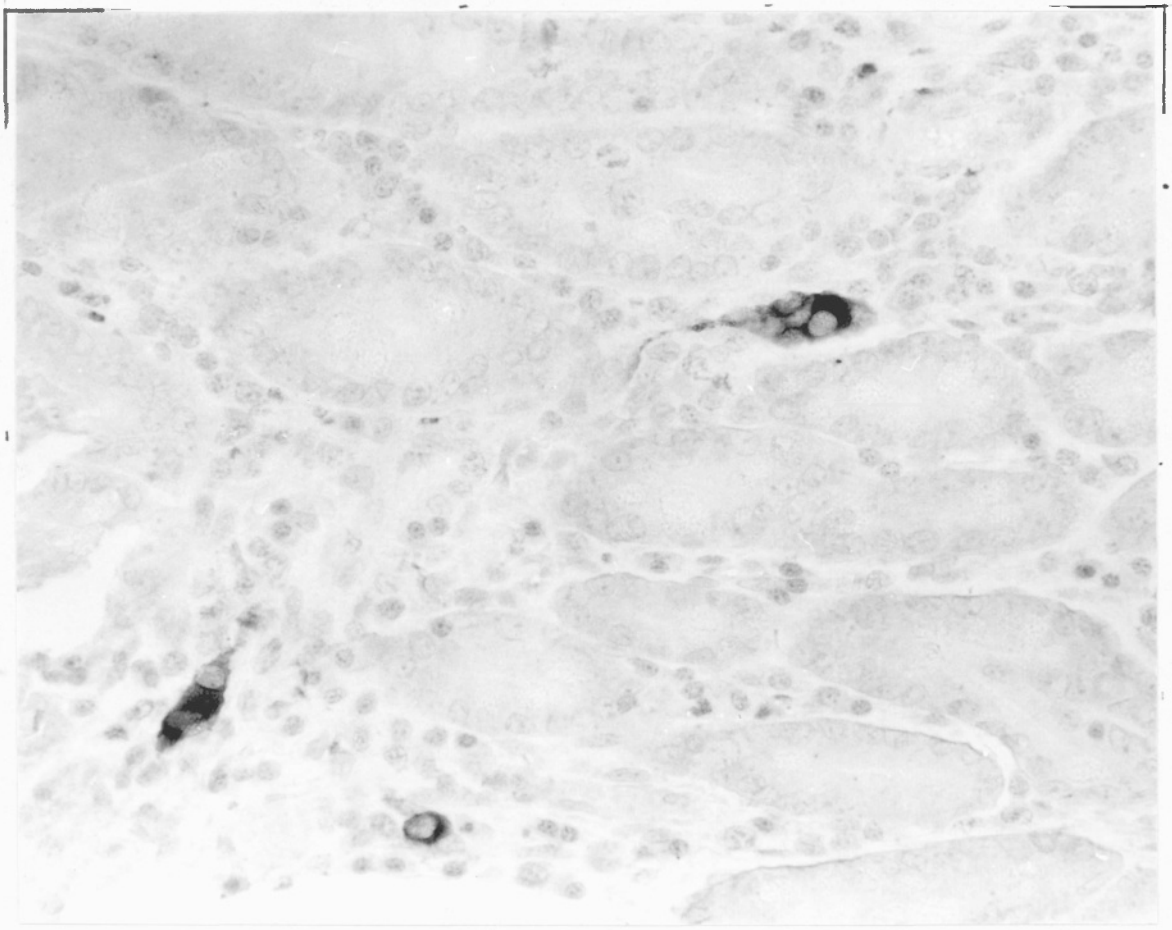
FIG 20

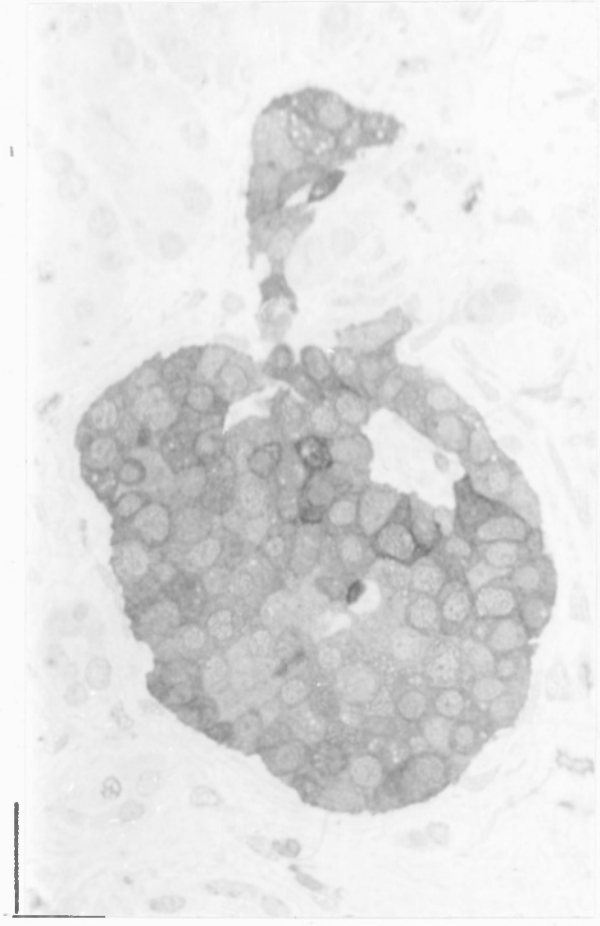
Serial sections through an islet of human pancreas immunostained for:

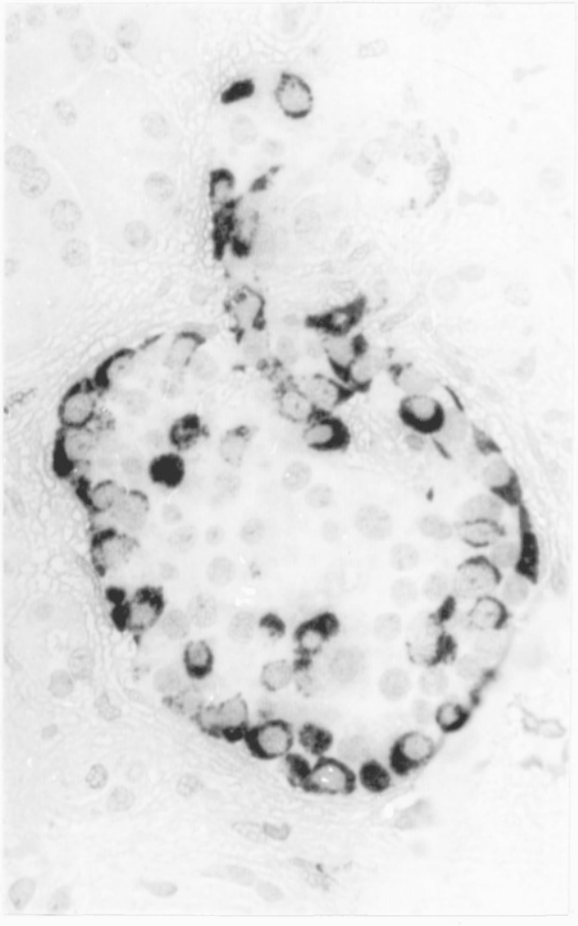
a) NSE - all cells are immunoreactive

b) PP (x 300)

Tissue freeze-dried, fixed in benzoquinone vapour and embedded in resin.







innervate preferentially. Scattered fibres lay in the exocrine pancreas and around blood vessels and ducts. In the connective tissue, groups of immunoreactive ganglion cells could be found, the majority of which showed peptide immunoreactivity or acetylcholinesterase activity. Aminergic fibres occurred mainly around ganglia and in trunks in connective tissue.

Discussion

The presence was demonstrated of a specific form of the enzyme enolase, NSE, in all 15 currently identifiable cells of the diffuse endocrine system of the gut and pancreas in humans and rats. In addition, NSE was immunostained in an extensive network of enteric and pancreatic nerves, some of the somata of which were identified as containing VIP and/or acetylcholinesterase activity. Thus, since no peptide-, amine- or acetylcholinesterase-containing cells or nerves were found that were not immunoreactive to NSE antibodies, it would appear that NSE can be regared as a marker for all differentiated endocrine cells and nerves of the gut and pancreas identified by these means.

The small number of NSE-immunoreactive cells that contained no identifiable peptide or amine product may be highly active secretors with a low content of stored peptide or they may be precursor or stem cells (Creutzfeldt, 1974). Equally the cells may contain substances which have yet to be discovered. This suggestion is supported by ultrastructural studies of endocrine cells where distinct cell types have been observed (eg the EC-like cells or the D1 cells of the gut) but for which no product has been described (Solcia et al, 1981).

Characterisation of nerve fibres in the gut and pancreas is difficult mainly because they occur predominantly in bundles consisting of a number of fibres. Thus, comparison of individual fibres by, for example, double immunostaining of a single section, is not possible. In view of this, a characterisation of neuronal cell bodies was carried out as serial sections can easily be cut through one cell. Acetylcholinesterase has not been considered to be a completely specific marker for cholinergic fibres for some time (Pepler & Pearse, 1957). More recently, the enzyme has been shown to break down substance P (Chubb et al, 1980). Immunostaining of VIP gave a more specific means of identification. As the aminergic nervous supply is mainly extrinsic, a comparison of ganglion cells could not be made within the gut wall and pancreatic parenchyma. Therefore, the formaldehyde induced-fluorescence technique was used to visualise the aminergic nerves and a partial characterisation was obtained by comparing the general distribution of these with that of NSE-containing fibres in serial sections. This method showed that a fraction of NSE immunoreactive fibres have a very similar distribution to those containing amines.

It is interesting to note that the NSE-immunoreactivity could be detected in cells in the lamina propria that did not contain peptide or amine immunoreactivity nor acetylcholinesterase activity. The occurrence of "neuroendocrine" cells in the lamina propria, characterised ultrastructurally by their content of secretory granules, has previously been described (Matusuo et al, 1976, Kataoka, 1974). The functional significance of these cells has yet to be ascertained. However, it has been suggested, on the basis of their morphological appearance, that they are related to Fujita's paraneuron or Pearse's APUD system.

The capacity of NSE to reveal entire neuroendocrine complexes may become increasingly valuable as the importance of these integrated control systems becomes more apparent. The existence of close relationships between endocrine cells and nerves in the gut and pancreas has been suggested by functional studies. For example, the influence of vagal stimulation on the release of gastrin from the stomach (Walsh, 1981) and pancreatic polypeptide from the pancreas (Adrian et al, 1978) have been known for some time. However, synaptic contacts between nerves and cells have not been observed frequently using the electron microscope . It has been reported by one group of workers (Newson et al, 1979) that membrane-to-membrane contacts between axons and cells occur in the mucosal epithelium. There has also been a preliminary report of a direct contact between VIP nerves and epithelial cells in the gut (Rosselin, 1981), which are known to have VIP receptors (Laburthe et al, 1979). NSE immunostaining may help in obtaining more information on the frequency and distribution of neuroendocrine complexes in health and disease. Some potentially important applications are highlighted by these results. Immunostaining of NSE provides a simple histological means for the rapid assessment of the overall state of the endocrine and nervous systems in a tissue section. Separate components of the systems can then be examined one by one, using specific methods such as peptide immunocytochemistry or enzyme histochemistry.

S-100

Immunostaining of S-100 was carried out in the gut and pancreas of man and rat to test the usefulness of this method in the demonstration of glial elements.

Materials

Fresh specimens of antrum, colon and pancreas (head) were obtained at surgery from humans (n=3 for each) and immediately following sacrifice from rats (n=3 for each).

Methods

Each sample, with the pieces of gut consisting of whole thickness of the wall, was fixed by immersion in benzoquinone solution (appendix). Serial cryostat sections of 6um were immunostained for S-100 and, to reveal neural structures, neuron specific enolase (NSE) (appendix).

Results

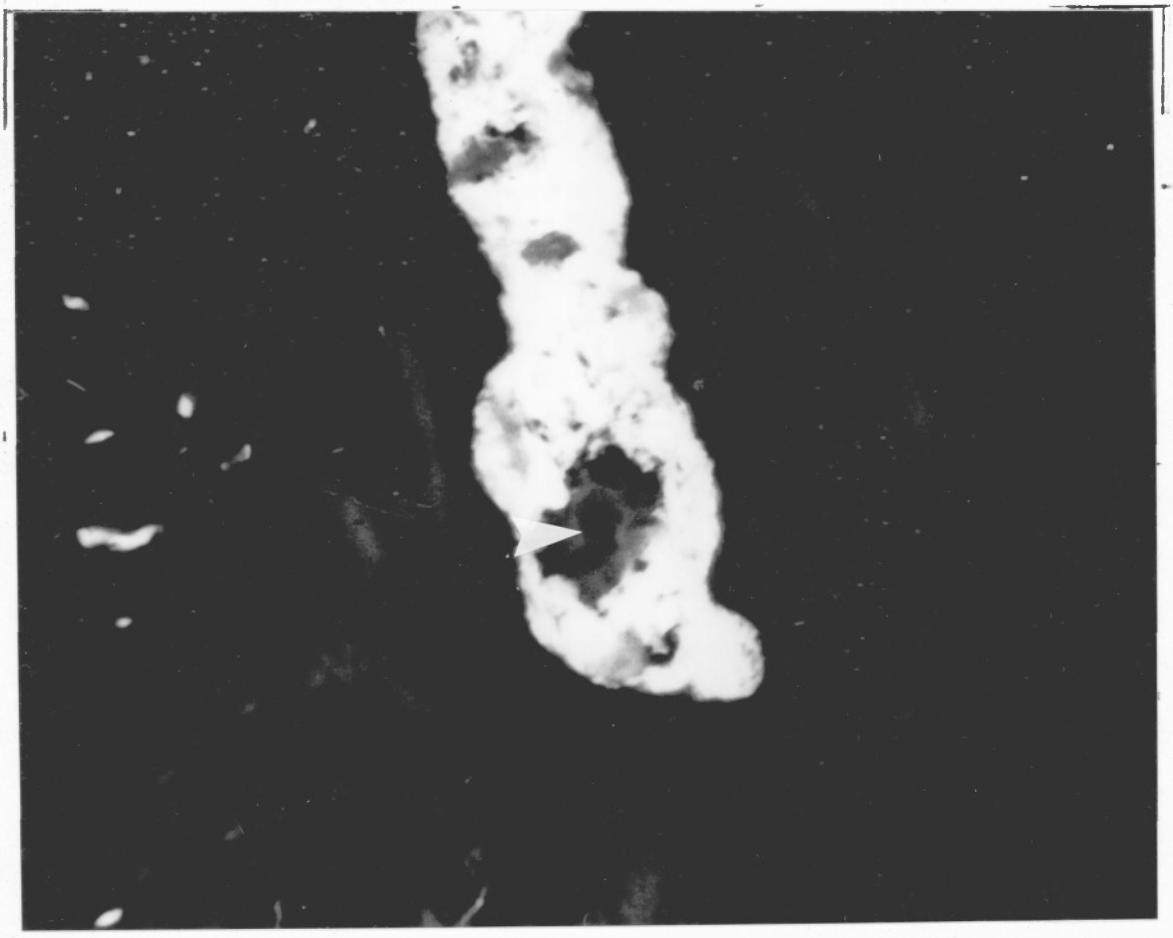
In the gut, antrum and colon, structures immunostained for S-100 were visible mostly in the two main ganglionated plexuses but also scattered in the other layers. In the plexuses, the cells were seen surrounding non-immunoreactive ganglion cells (Fig 21). The shapes of the individual immunoreactive cells were difficult to discern as the cells formed intermingled bundles around the negative nerve cells. Where they could be seen clearly, these glial cells had non-immunoreactive nuclei and almost ribbon-like projections. None of the cells showed varicosities or differing degrees of immunoreactivity. All were immunostained uniformly. The results obtained for S-100 immunostaining were in direct contrast to those obtained using NSE antibodies on serial sections. NSE occurred in the ganglion cells and nerve fibres. Thus, in the serial sections immunostained for the two proteins it was possible to distinguish

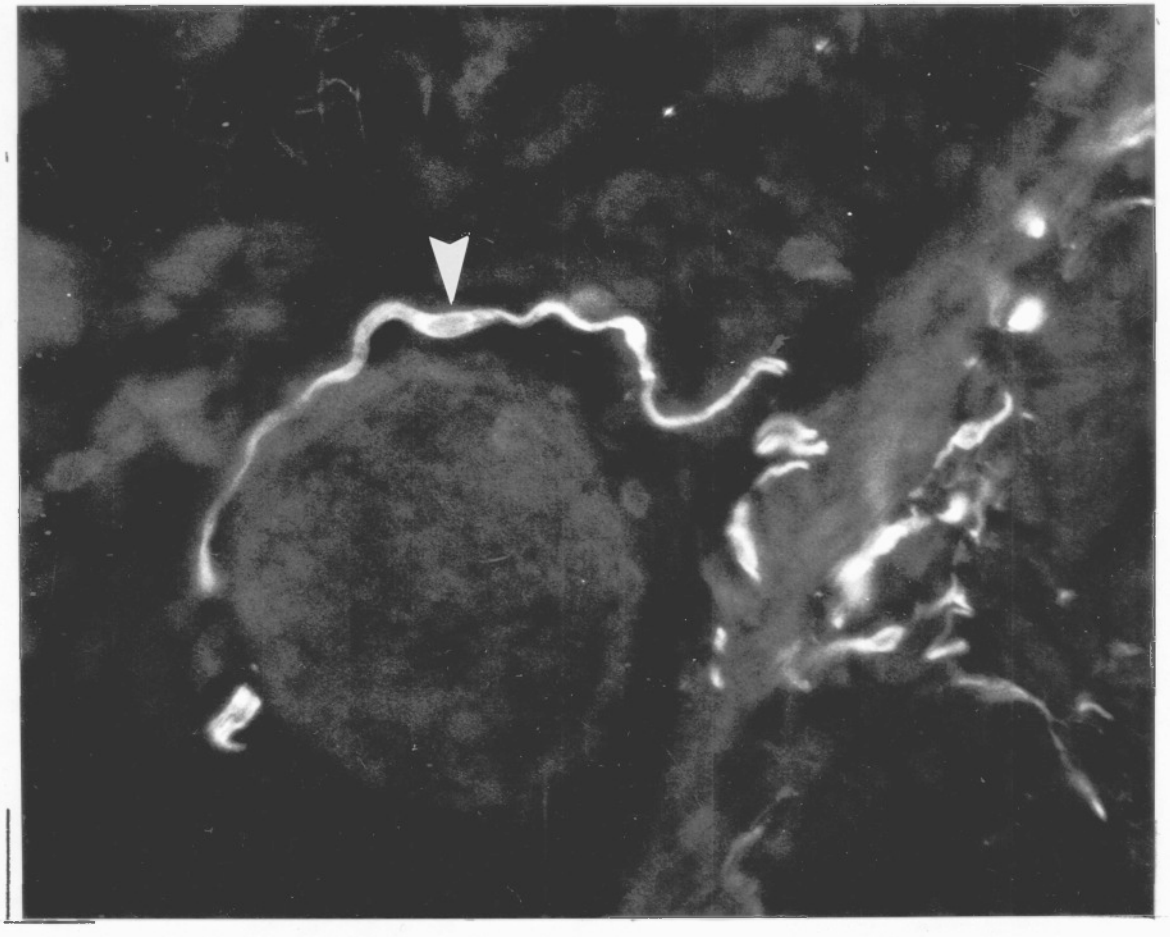
FIG 21

S100-immunoreactive cells around a non-immunoreactive ganglion cell body in the myenteric plexus of human colon. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 300).

FIG 22

S100-immunoreactive cells in the muscularis mucosae of human colon and a single cell in the sub-mucosa with bipolar projections on either side of the nucleus (arrow). Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 400).





nerves and their corresponding supportive glial cells which enveloped them. NSE-immunoreactivity, in addition, did not give the consistent level of cellular immunostaining seen with the S-100 antibodies.

Outside of the plexuses, S-100 immunoreactive elements were seen as long, slender ribbons , mainly single with overlapping ends forming ramifying lines of cells (Fig **22**). Each cell had a non-immunoreactive nucleus, frequently at the mid-length point. Narrow bundles occurred between muscle blocks but, elsewhere in the muscle layers, the cells were scattered. In the mucosa they were most numerous just beneath the muscularis mucosae (Fig **23**) but a smaller proportion projected up into the lamina propria.

In the pancreas, the S-100 immunoreactive cells were seen mostly in connective tissue surrounding what could be seen in serial sections to be NSE-immunoreactive ganglion cells. They were also scattered in exocrine tissue and showed no particular association with the islets.

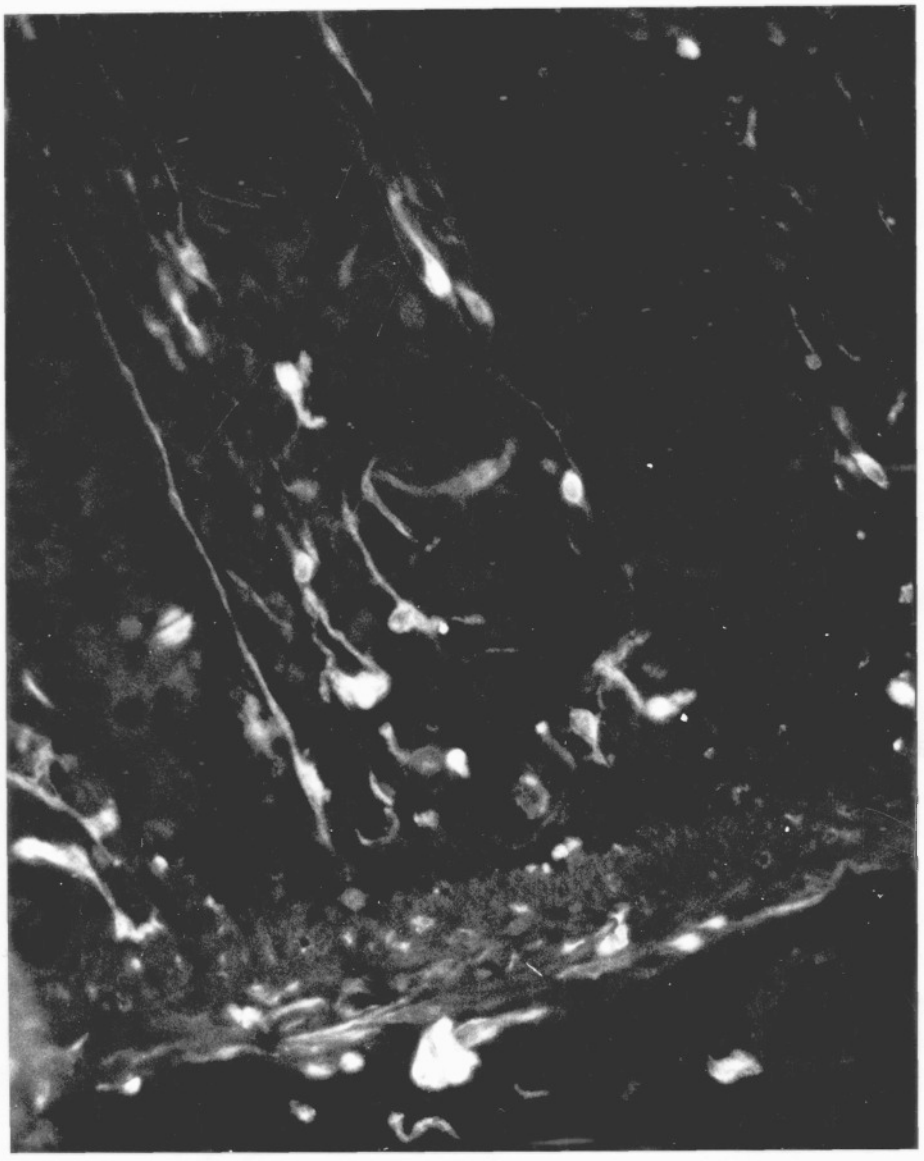
Discussion

S-100 immunoreactivity was shown to be present throughout the gastrointestinal and pancreatic specimens examined, in cells which were closely related to the nervous system and appeared to be glial cells.

Previous methods for the demonstration of glial cells in the gut have been limited to electron microscopy (Gabella, 1981). S-100 is known to be a reliable marker for glial cells in the central nervous system (Bock, 1978) and it seems that, in the gut and pancreas also, S-100 can be immunostained in cells supporting nerves.

FIG 23

Human colonic mucosa with numerous S100-immunoreactive cells in the lamina propria, muscularis mucosae and sub-mucosa. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 200).



The function of S-100 is not known. NSE, which is involved in the energy production of cells shows variable levels of immunoreactivity in cells of the same tissue section. S-100, however, was uniformly immunostained in all cells.

The results presented here provide evidence that antibodies to S-100 and NSE can provide a simple, easily reproducible means for the demonstration of the nervous and glial cells in tissues.

CHAPTER 5 MEGACOLON

CONGENITAL MEGACOLON (HIRSCHSPRUNG'S DISEASE)

INTRODUCTION

Although the pathophysiology of Hirschsprung's disease has yet to be fully defined, abnormalities of the autonomic nerve supply to the gut are considered to be the main cause (Whitehouse and Kernohan, 1948, Bodian et al, 1949). However, the neuropathology of Hirschsprung's disease is rather confused. An absence of ganglion cells from both the myenteric and submucous plexuses, in the narrowed segment and, to varying extents, in the portions of bowel immediately proximal, is generally accepted to occur in the disease (Whitehouse and Kernohan, 1948, Bodian et al, 1949). This aganglionosis apparently develops *in utero* by interruption, of unknown nature, of the cephalo-caudal migration of ganglion cells along the gut. The total absence of skip lesions in Hirschsprung's disease supports this theory.

Ehrenpreis et al (1968) studied the catecholamine nerves supplying the gut and, whilst there were normal networks of fibres around ganglion cells in the dilated bowel, in the constricted segment no such synapses were found. In the dilated and constricted segments reduced numbers of catecholaminergic fibres were also found in the submucosa and muscle layers. It was concluded that a catecholaminergic denervation occurs, as well as aganglionosis, in Hirschsprung's disease. This prompted speculation that unopposed muscle activity was responsible for the contraction of the bowel. In

direct contrast, it was also reported that the numbers of catecholaminergic fibres in the muscle layers are actually increased (Bennett et al, 1968).

Using the cholinesterase stain, Meier-Ruge (1968) found a distinct increase in positive fibres. He proposed that the constriction of the bowel in Hirschsprung's disease is due to an increased excitatory, cholinergic innervation. Cholinesterase positive fibres were also found to be increased in the mucosa and lamina propria in suction rectal biopsies from children with Hirschsprung's disease (Lake et al, 1978). This finding has been cited as a means for diagnosis of the disease.

Another study, this time investigating both cholinesterase activity and catecholamine fluorescence, revealed a rather more variable neuropathological picture (Garrett et al, 1969). The density of cholinesterase fibres in the circular muscle of the aganglionic segment varied and there appeared to be a relationship between the density of the fibres and the severity of the symptoms. The number of catecholamine fluorescent fibres also varied, being reduced in the muscle of the region between the ganglionic and aganglionic segments. It was concluded that the spasm is due to unopposed cholinergic activity, the lack of co-ordinated contraction and relaxation is due to the aganglionosis and the altered muscular innervation in the junctional region results in defective propulsion. Electron microscopy was used to provide support for these conclusions (Howard and Garrett, 1970a & b). The muscle of the aganglionic region was found to be richly supplied with nerves and that of the junctional region deficient.

Previous assessment of the neuropathology of Hirschsprung's disease has largely been in terms of excitatory cholinergic and inhibitory adrenergic influences. The findings of increased cholinesterase positive nerves and decreased adrenergic supply certainly provides an attractive explanation for constriction of the aganglionic segment. However, the simple theory of reduced inhibitory and unopposed proliferation of excitatory innervation is challenged by a number of observations. For example, animal experiments have shown that destruction of the adrenergic innervation of the colon may contribute to the constriction observed but would not affect the propulsive mechanisms (Crema et al, 1970). In order to provide an explanation for the possible interactions between excitatory and inhibitory neural influences on gut function, the existence of non-adrenergic inhibitory neurons was postulated and demonstrated by physiological experiments (Crema et al, 1968). It is known that in Hirschsprung's disease the internal anal sphincter fails to relax. Relaxation of this sphincter in response to rectal distension is under non-adrenergic control. This suggests that there is a loss of non-adrenergic inhibitory ganglion cells in this disease. Further evidence for the involvement of these nerves in Hirschsprung's disease was provided by the failure to elicit nerve mediated, non-adrenergic relaxation of the aganglionic segment (Frigo et al, 1973). A proportion, if not all, of these non-adrenergic inhibitory nerves contains peptides (Furness et al, 1980). The majority of the gut neuropeptides influence motility and VIP, the most abundant of them all, inhibits smooth muscle activity. It was therefore possible to postulate that VIP containing nerves may show a reduction in Hirschsprung's disease. Furthermore, the predominantly intrinsic nature of peptide

containing nerves had been demonstrated in other mammalian species (see Chapter 1) and therefore it seemed likely that, if these nerves are also intrinsic to the human gut, the aganglionosis in Hirschsprung's disease would cause an observable reduction in peptidergic innervation. Some preliminary evidence was available at the beginning of this study to suggest that this is, in fact, the case. In 1953, Ehrenpreis and Pernow demonstrated, using bioassay, that there is a decrease of substance P activity in the aganglionic segment of bowel taken from children with Hirschsprung's disease. More recently, a similar decrease of VIP content, as assessed by radioimmunoassay, has been reported (Freund et al, 1980).

The initial part of the study of regulatory peptides in Hirschsprung's disease was thus devoted to neonatal disease but sometimes the disease is not diagnosed until well beyond the neonatal stage. Hirschsprung's disease usually becomes apparent shortly after birth when little or no meconium is passed and discernible abdominal distension occurs. In the adult, Hirschsprung's disease is considered to be rare and relatively few cases have been published in the literature. However, it is possible that the condition occurs more frequently than is thought. Apart from the more isolated, extreme cases where the subjects with long segment involvement have been kept alive by administration of enemas, most instances of "late-onset" Hirschsprung's disease are associated with a relative mildness of symptoms. It seems rather anomalous that this disease, which can cause such severe symptoms in affected children, may sometimes be so mild as to escape detection until later in life. That the cases of adult Hirschsprung's disease

are mild forms of the disease and not just instances where full-blown neonatal disease has escaped diagnosis is evident from the fact that neonatal presentation of Hirschsprung's disease is often fatal if left untreated. An obvious possible explanation for the marked variation in disease severity would be the different lengths of the aganglionic segement. However, this is not the case. Although Garrett et al (1969) found a subjective relationship between the density of cholinesterase containing nerves in the circular muscle and the severity of the symptoms, no relationship between the disease severity and extent of aganglionosis has been found (Fairgrieve, 1963, Duhamel, 1969). A further question about the nature of "adult" Hirschsprung's disease is whether it is truely congenital agangliosis. One of the criteria of diagnosis of the "adult" disease is long-standing history of constipation, since birth or infancy, which argues against a progressive degenerative change in intrinsic bowel innervation. In addition, histology of ganglion cells in "adult" Hirschprung's disease does not reveal any evidence for neuronal degeneration. Thus, "adult" Hirschsprung's disease may have altered peptide containing nerves in the same way as the childhood disease. The major aims of these investigations were therefore:-

1) To establish whether any alteration of peptide containing nerves (and/or cells) occurs in Hirschsprung's disease.

2) If changes could be detected, to compare the nerves (and cells) in childhood Hirschsprung's disease with those in long-standing cases (defined as Hirschsprung's disease with symptoms traceable back to birth but not severe enough to warrant treatment until beyond the neonatal period).

A) Childhood Hirschsprung's disease

Preliminary Work

Before embarking on an investigation of childhood Hirschsrpung's disease, two main practical problems had to be overcome. During these studies, all previous examination of peptide containing nerves and cells of the human gut had used tissue from adults. The work carried out on gut from different mammals had shown that the time for fixation in benzoquinone solution (BQS) varied, mainly with the thickness of the gut wall. For this reason some preliminary work had to be done to establish the optimal time period for fixation of gut from neonates. At this point the second problem could be foreseen. The main source of histologically normal control bowel for these studies had been surgical resections for carcinoma. Such operations are so rare in children that another source of suitable tissue had to be found. Even the more frequent gut operations, such as those for intestinal atresia or imperforate anus, could only yield a very small number of specimens. Thus, post-mortem tissue was taken, within as short as possible time after death, and the results compared with those obtained using adult normal control bowel.

Materials

Four specimens of colon and rectum were removed from children (ages 2 - 30 months) within 8 hours of death. None of the children had any history of gastrointestinal disease. Each piece of bowel was cut into samples no more than 2 x 2cm in area and consisting of the whole width of the gut wall. Pieces from each specimen

were fixed by immersion in 0.4% benzoquinone solution (appendix) for 0.5, 1, 1.5, 2, 2.5 and 3 hours. Normal control adult bowel was routinely fixed (appendix) for comparison. All tissues were immunostained for VIP, substance P, NSE and enteroglucagon.

Results

Histology: The tissues were all found to be free from degenerative changes. The mucosa was intact and there were no signs of autolysis. No pathological features of any kind were detected.

Immunocytochemistry: Positive immunostaining was found for each of the peptides and NSE in tissue fixed for between 0.5 - 3 hours. The best results were seen after 1 hour of fixation. VIP, substance P and NSE were present in densely immunostained nerve fibres in all areas of each specimen and VIP and NSE immunoreactive ganglion cells were seen. Enteroglucagon containing cells were widely distributed in the mucosal epithelium. The results obtained in the post-mortem specimens fixed for 1 hour were indistinguishable from those in the adult specimens examined here and also those described previously (see Chapter 3).

Conclusions

From this preliminary investigation of tissues taken at post-mortem examination it was evident that the optimal fixation time in BQS for gut from children is 1 hour and that post-mortem specimens (taken within 8 hours of death) were suitable for use as normal controls. In fact, the results obtained from children after death

were far better than those from adults. One of the main reasons for this may be that the small body size of the children allows more rapid cooling, thereby slowing the processes of tissue degeneration.

MAIN STUDIES

Materials

Surgical specimens of large bowel were obtained from 8 children (ages 6 months - 2 years) undergoing resection for Hirschsprung's disease. Diagnosis of the disease was based on clinical data and results of histological and histochemical (Lake et al, 1978) examination of rectal biospies. For comparison, similar areas of bowel were obtained at necropsy from 6 children (ages 2 months - 3 years) who died as a result of non-gastrointestinal disease. All tissue was removed within 8 hours of death. All specimens were kindly provided by Dr BD Lake, The Hospital for Sick Children, London.

Samples were removed from proximal (dilated) and distal (constricted) areas of each specimen and from mid-regions. Samples were fixed and processed for conventional histology, immunocytochemistry (appendix) and radioimmunoassay. For immunocytochemistry, sections were stained with antisera to a range of peptides, NSE and S-100 (appendix).

Results

Histology

Controls: No histological abnormalities could be detected in any of the control specimens, which contained ganglion cells and nerve fibres with normal distribution and appearance. No degenerative changes could be found.

Hirschsprung's: Aganglionic segments were easily discernible in the diseased specimens and varied greatly in length (mean; 10.3cm: range; 1.5-28cms). A fairly consistent pattern of innervation could be seen. Proximal to each aganglionic segment, a region of normal or transitional innervation could be identified and the length of this region also varied. Ganglion cells, when present in the diseased specimens, were normal in appearance. In many specimens, large bundles of nerves were found in the aganglionic portions, most often in the submucosa and the interface between the two muscle layers. Some specimens showed thickening of the muscle, particularly in the proximal parts. No other distinctive histological changes were observed.

Only those specimens which showed no signs of autolysis were included in the study.

Immunocytochemistry

Controls: NSE and S-100 immunostaining revealed the total extent of nerve and glial elements across the width of each specimen.

Ganglion cells were seen in both plexuses. VIP and substance P immunoreactive fibres were seen in all specimens. VIP nerves were more abundant than those containing substance P, but their overall distributions were similar (see Chapter 3). VIP and substance P nerves were present in all areas of the gut, from the mucosa to the serosa. A small proportion of met-enkephalin and bombesin immunoreactive fibres were also found, mainly in the muscle layers. The fibres were mostly slender and varicose. In the mucosa, they ran in the lamina propria, either singly or in very fine networks, and sometimes appeared to terminate on the mucosal epithelium. Both substance P and VIP fibres could be seen in association with blood vessels in the mucosa, as in other layers.

All types of peptide containing nerves could be distinguished in the muscularis mucosae, running in the direction of the muscle fibres and interweaving with them. In the submucosa, VIP and substance P nerves were occasionally found in networks, but mostly they were present as single elements or strands. The submucous plexus contained both VIP and substance P nerves and, additionally, occasional VIP immunoreactive ganglion cells. In the circular muscle coat, both types of nerves showed a similar distribution. The fibres were present mostly as intermeshed strands lying between the blocks of muscle fibres.

VIP (Fig **24**a) and substance P nerves were abundant in the myenteric plexus, and a few substance P immunoreactive ganglion cells were found in this region. Only very few Met-enkephalin and bombesin containing fibres could be found. Bombesin nerves were scattered across the gut wall, but the met-enkephalin fibres

were only in the muscle layers. In general, the distribution of peptide containing nerves in these neonates was similar to that seen in adults.

Mucosal endocrine cells were only weakly immunostained by NSE antibodies. Enteroglucagon and somatostatin immunoreactive cells were also present in each of the specimens. Both types of cell were found distributed across the mucosal epithelium, but larger numbers were present towards the base of the mucosa. The cells had a similar morphology, both showing occasional luminal or basal elongations.

Hirschsprung's: Each specimen contained a different pattern of peptide-containing nerves. The level of peptidergic innervation was generally reduced but in the most proximal and distal areas the majority of the specimens had a level of innervation which approached that seen in the control tissues. In the aganglionic segments a consistent decrease in peptide-containing nerves was observed. This decrease varied greatly. A subjective loss of VIP and substance P nerves was apparent in all specimens, but in those with the most extensive aganglionosis, only one or two fibres could be found. In the areas with an almost total loss of peptide-containing nerves the fibres were usually present in or just beneath the muscularis mucosae or in the area which normally contains Auerbach's plexus (Fig 24b), i.e. in regions where the ganglion cells are normally found. Some VIP and substance P fibres could be found in the characteristic large nerve bundles between the two muscle layers.

NSE immunostaining demonstrated clearly the ganglionic (Fig

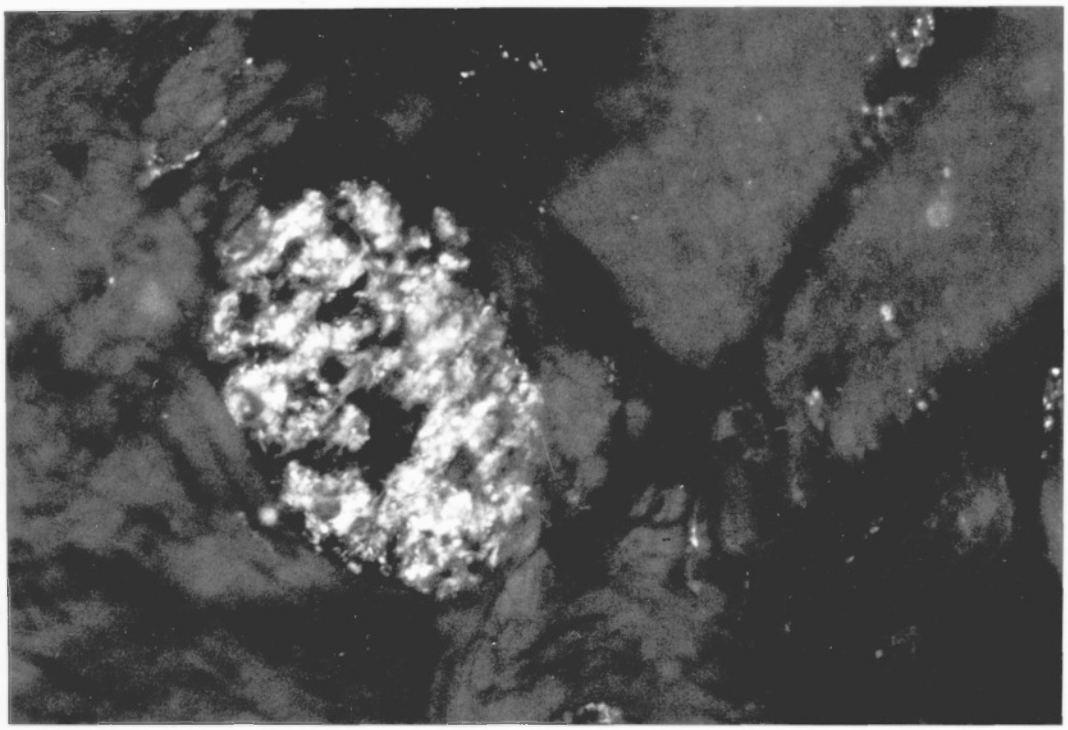
FIG 24

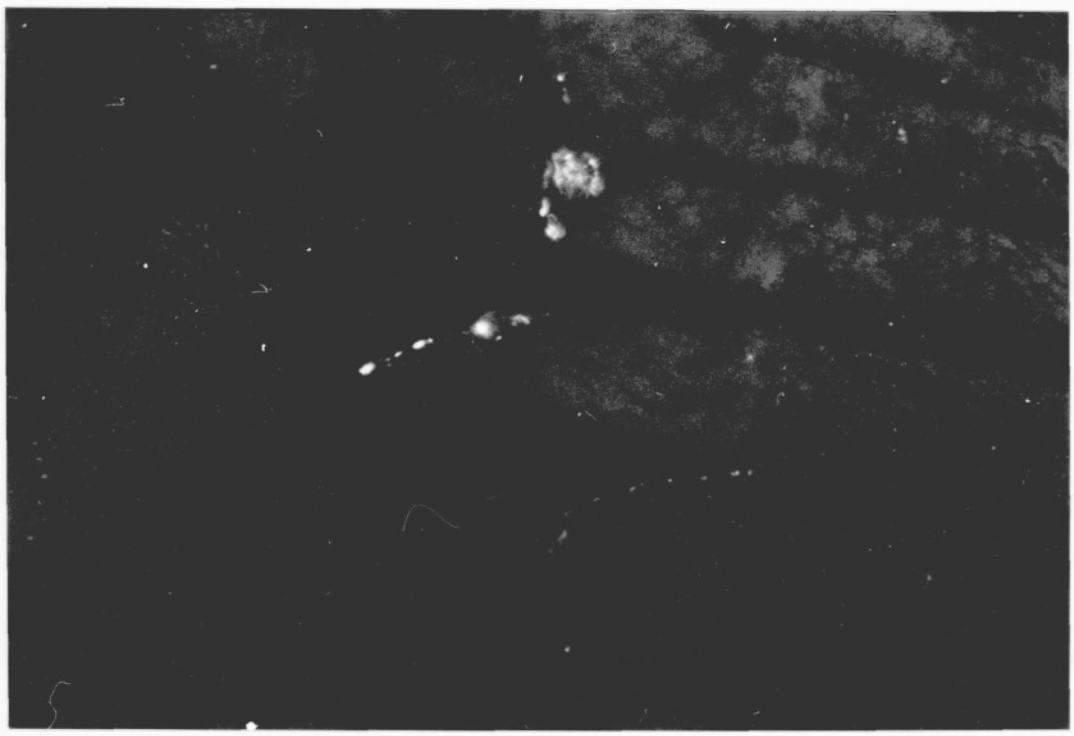
VIP-immunoreactive fibres at the interface of the circular and longitudinal muscle layers in colon taken:

a) at post-mortem examination from a child who died of non-gastrointestinal disease.

b) at surgery from a child with Hirschsprung's disease (x 400).

Tissue fixed in BQS, cryostat section, indirect immunofluorescence.





25), hypoganglionic (Fig 26) and aganglionic (Fig 27) portions of each specimen. Although absence of ganglion cells was obvious in some areas, there was no great loss of nerve fibres. No hyperplasia was revealed by the NSE antibodies. Similarly, S-100 immunostaining did not demonstrate any great change in glial or Schwann cells.

Few or no met-enkephalin fibres were found in the aganglionic segments. However, the very low numbers of fibres present in the control tissue make it impossible to tell whether or not there was a consistent decrease in these types of peptide containing nerves.

Additionally, both enteroglucagon and somatostatin immunoreactive cells were decreased in the mucosal epithelium of not only the aganglionic portion but also, most particularly, the proximal segments of diseased bowel.

Radioimmunoassay

A significant ($P<0.01$) decrease in VIP content was found by radioimmunoassay of tissue extracts of specimens from children with Hirschsprung's disease as compared with normal controls (Table 5). Not only was there an overall reduction in VIP content but there was also a significant decrease in middle, aganglionic portions of bowel as compared with the proximal and distal areas ($P<0.05$). VIP content fell by almost 50% in the proximal and

FIG 25

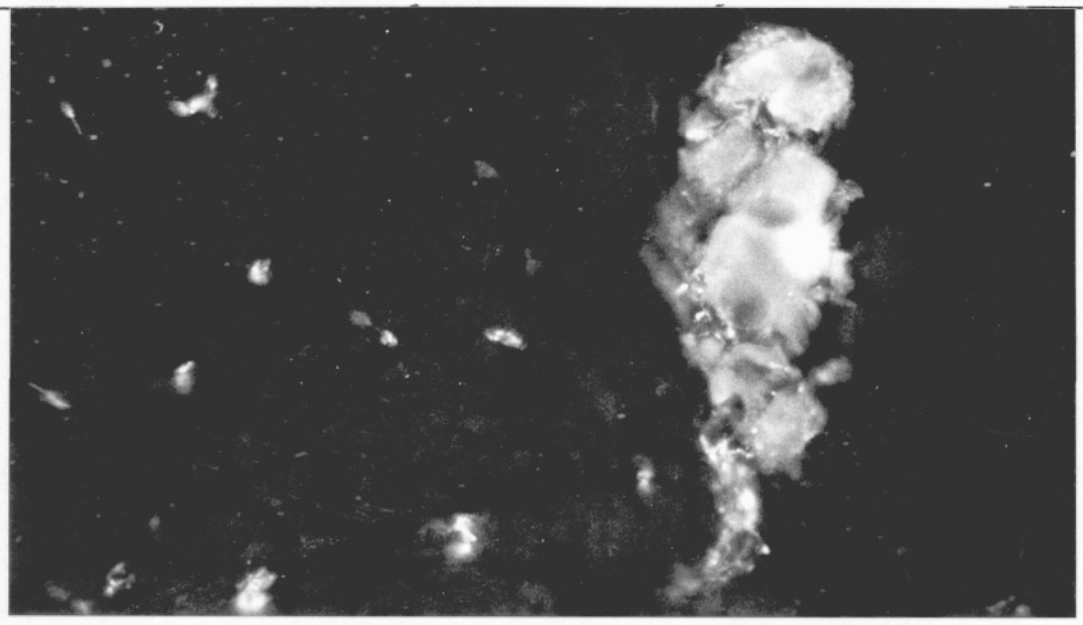
NSE-immunoreactive ganglion cells in the myenteric plexus of normal, ganglionic bowel. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 275).

FIG 26

Hypoganglionic large bowel with a single NSE-immunoreactive ganglion cell body (arrow) and numerous immunoreactive fibres in the myenteric plexus. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 225).

FIG 27

NSE-immunoreactive fibres but no ganglion cells in aganglionic bowel. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 200).



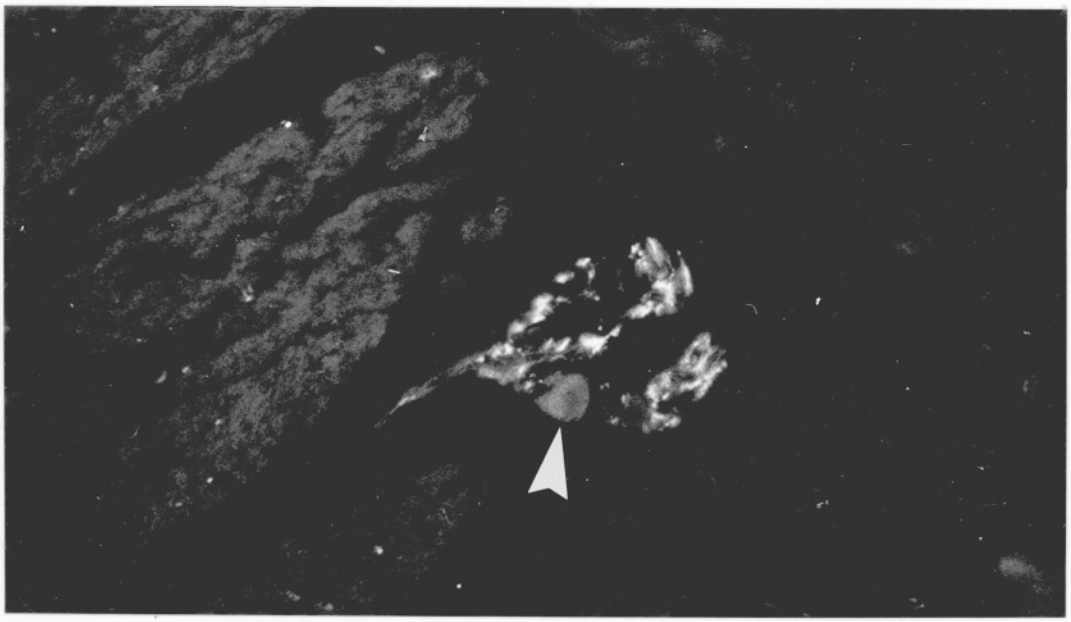
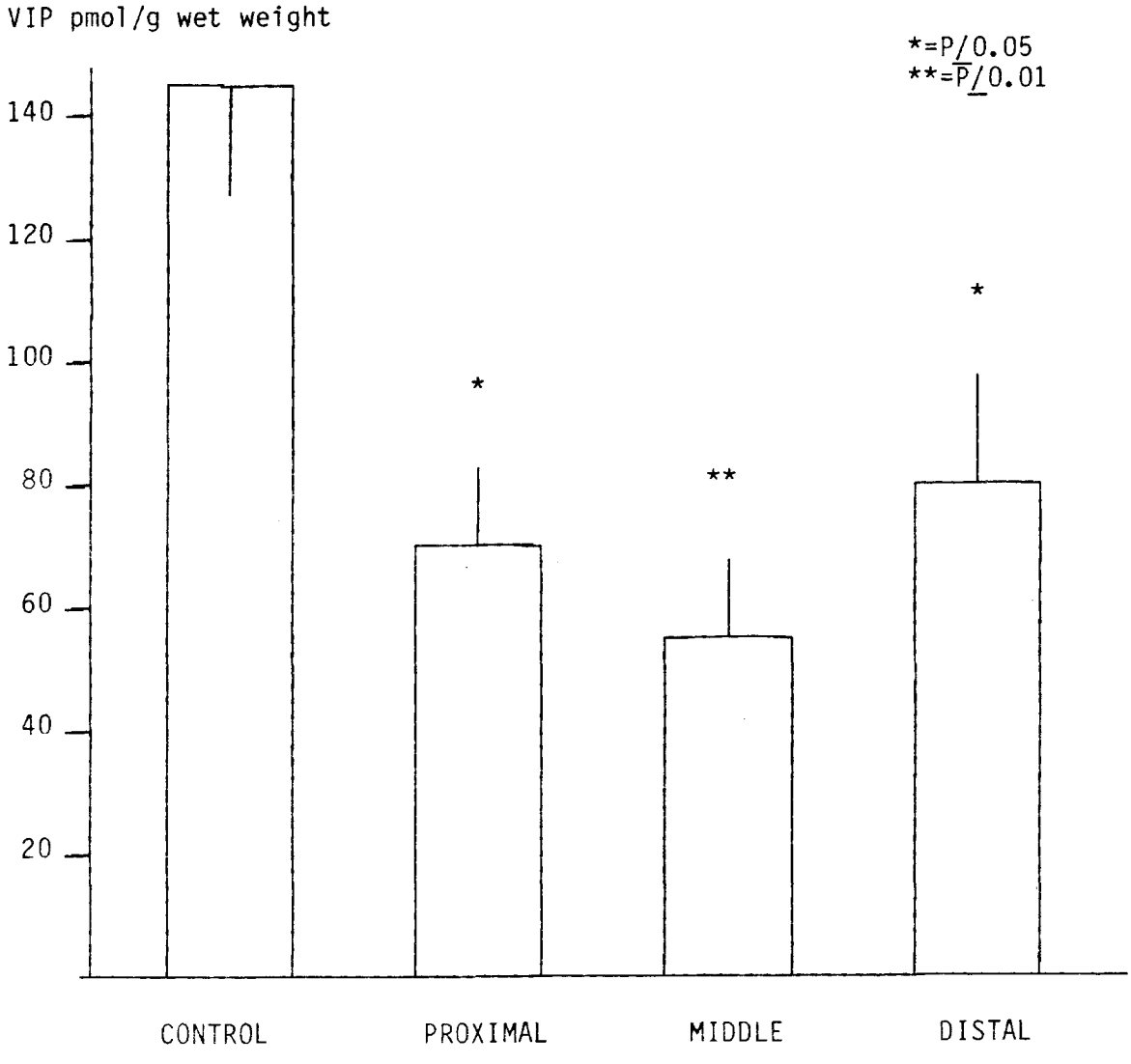




Table 5

VIP CONTENT IN HIRSCHSPRUNG'S DISEASE IN CHILDREN



distal regions, compared with the controls and the content of the middle portions was again decreased by a further 50%. Therefore, the aganglionic region contained, on average, one quarter of the VIP content of the controls.

B) "Adult" Hirschsprung's Disease

Materials

A total of 6 surgical specimens of colon/rectum were investigated from patients (ages 15 - 56 years) diagnosed, like the children, as having Hirschsprung's disease.

Eight control specimens were taken from macroscopically normal bowel during surgical resection for carcinoma (ages 23 - 75). These specimens were provided by Dr DM Preston, St Mark's Hospital, London. Both the diseased and normal bowels were processed in the same way and so the time between surgery and tissue fixation was more or less constant.

Samples were removed from proximal, middle and distal regions of each specimen. These were processed, immunostained and assayed in the same way as the specimens from children with two exceptions. The bowel was fixed for 2 and not 1 hour and radioimmunoassay was used to measure not only VIP but also substance P and enteroglucagon.

Results

Histology

Controls: The histology of all the control specimens was entirely normal. No signs of autolysis or any form of degradation could be detected.

Hirschsprung's: In the diseased bowel, a great variation in histological features could be seen. Two of the specimens had no completely aganglionic region. At the distal resection edge, very sparse ganglion cells could be identified. This may mean that either the patients had hypoganglionosis or that the small aganglionic segment may have been left in situ after surgery. The aganglionic segments in the other specimens varied in length (mean; 8.8cm: range; 1 - 16cm) and were thus slightly shorter than the average lengths of those of the children. Large nerve trunks were found in all specimens, in the submucosa and in the area between the two muscle coats, where the myenteric plexus would normally lie. Muscle thickening was also a feature of all the specimens, either in the proximal portion or throughout. Some fibrosis or slight inflammatory infiltration, reaching only as far as the submucosa, was occasionally seen but never to any significant extent.

Immunocytochemistry

Controls: A normal profile of peptide containing nerves and mucosal cells was seen in all specimens (see Chapter 3). NSE and S-100 immunostaining revealed the components of the nervous system in normal numbers and distribution. In addition, weakly immunostained NSE immunoreactive cells were found in the mucosa.

Hirschsprung's: The variability in the histological features of the diseased specimens was equalled by that of the immunocytochemical results. Unlike the specimens from children with Hirschsprung's disease, there was no consistent pattern of decreased peptide containing nerves or endocrine cells. The great variation in results is well illustrated by the two samples with the greatest aganglionic segments, 14cm and 16cm. In the specimen with a 14cm long aganglionic segment distinctly reduced VIP and substance P nerves were seen along the proximal and middle portions, in all layers of the wall, but a greater than normal density of brightly immunostained fibres was found in the distal few centimetres, particularly in the muscle. NSE immunoreactive nerves showed parallel changes in frequency, although S-100 immunostained structures appeared to be normal in number and distribution. In the other specimen, normal levels of VIP, substance P and NSE immunoreactive innervation were seen proximally, with a clear reduction over the last 5cm of the specimen. There was no apparent relationship between the level of peptidergic innervation and the numbers of endocrine cells in the mucosa.

The two cases with only hypoganglionosis and no complete aganglionosis hade normal peptidergic innervation, although NSE immunostaining demonstrated the lack of ganglion cells. One feature which was noted in most of the specimens was the presence, particularly in the circular muscle, of VIP fibres with an unusual appearance in areas where the muscle layer was thickened. Individually, these fibres were not abnormal, as in for example Crohn's disease. However, they formed wider bundles which were more numerous than usual (Fig **28**). This distinct pattern of innervation was also revealed by the immunostaining for NSE. NSE immunoreactive fibres formed similar bundles with a broad, open network. The glial structures were more frequent in the muscle but showed no distinctive pattern.

Two cases also showed rather odd results. In one, very dense S-100 immunostaining was seen in packed glial cells in the mucosa. These cells had a normal bipolar appearance with prominent nuclei and were closely intermingled. In the other specimen, a very small number of NSE immunoreactive neuronal cell bodies were found in the circular muscle of the ganglionic, proximal segment of bowel. These cells were large with a rounded or almost triangular shape. They all seemed to be bipolar, projecting along the muscle (Fig **29**).

Radioimmunoassay

The results of radioimmunoassay of VIP, substance P and entroglucagon in tissue extracts are presented in Table **6**.

FIG 28

VIP-immunoreactive fibres with normal individual appearance but in more densely packed bundles than normal in the circular muscle of colon from an adult with Hirschsprung's disease. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 300).

FIG 29

NSE-immunoreactive ganglion cell (arrow) and fibres in the colonic circular muscle of an adult with Hirschsprung's disease. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 375).



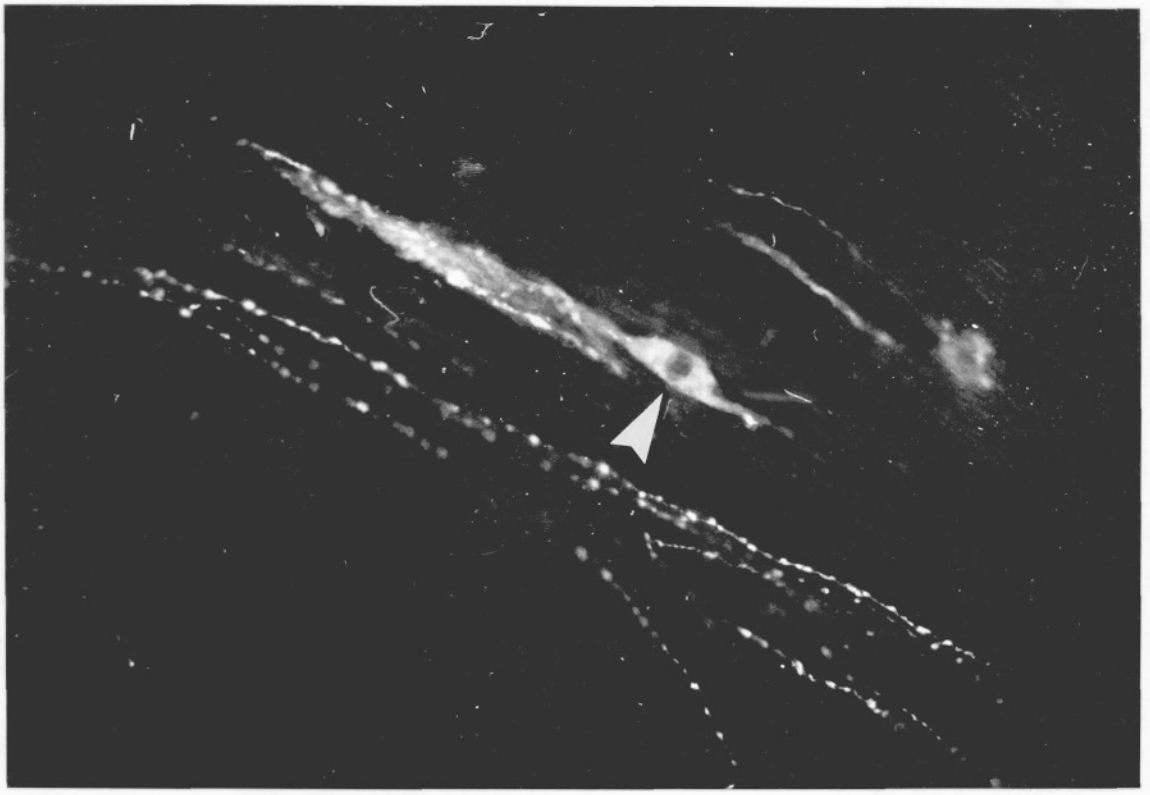
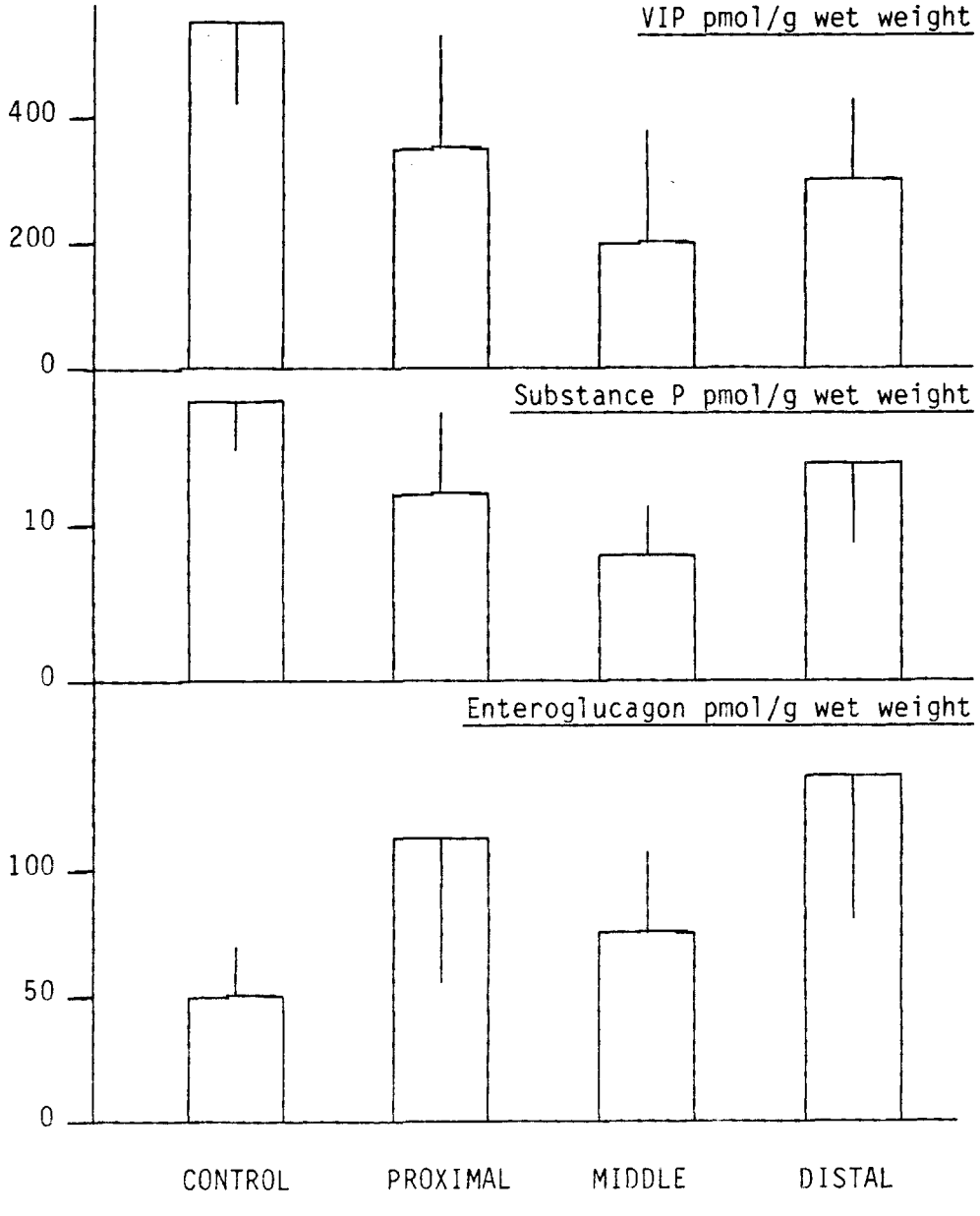


Table 6

CONTENT OF VIP, SUBSTANCE P & ENTEROGLUCAGON
IN HIRSCHSPRUNG'S DISEASE IN ADULTS



There was an enormous variation in the results for peptide content of the diseased tissues. The peptides each showed different patterns in the various areas of the specimens. The mean VIP content was lower (by 30-60%) in the Hirschsprung's disease samples than in the controls. The levels of VIP fell in the middle and distal portions compared with the proximal part. Substance P and enteroglucagon, on the other hand, showed the highest levels in the distal part and lowest in the middle. Substance P concentrations were, like those of VIP, lower (by 40-60%) in the diseased tissues than in the controls. Enteroglucagon was more concentrated in the proximal and distal parts (50 and 90% respectively) and reduced in the middle (by 30%).

Despite these apparently large changes in the tissue content of these peptides, none were found to be statistically significant. This was entirely due to the great deal of variation in the results, which was reflected in the values for standard errors of the mean (SEM) (Table **6**). This large variation is in agreement with the observed morphological changes which were similarly highly variable.

Discussion

The results of this study demonstrate clearly that there are gross abnormalities of the peptide containing enteric nerves in Hirschsprung's disease.

A consistent loss of peptide containing nerves was found in bowel from children with the disease, to a degree which appeared

to bear relation to the extent of the aganglionosis present. In individuals beyond the neonatal stage, a more confused picture of the peptidergic innervation was seen, with no obvious relationship with aganglionosis. Similarly, in children, where portions of bowel contained fewer peptidergic nerves, a reduction in the numbers of peptide immunoreactive mucosal cells was seen. This was not the case in the adults.

The lack of peptide containing nerves in Hirschsprung's disease is not surprising. Peptide immunoreactive ganglion cells are demonstrable in the human gut and the intrinsic origin of peptidergic nerves has been established in a number of mammals (Schultzberg et al, 1978, Jessen et al, 1980, Malmfors et al, 1981). It appears, therefore, entirely possible that these nerves have a predominately intrinsic origin in the human gut. Aganglionosis would mean a corresponding loss of peptidergic nerves and, consequently, peptide content of the tissue.

NSE and S-100 immunostained nerves and glial structures were reduced in those areas where peptide containing nerves were lacking, but not to the same extent. The finding that NSE immunoreactive nerves are not increased is not in agreement with the previous report of hyperplastic nerves with acetylcholinesterase activity in rectal biopsies from children with the disease (Lake et al, 1978). As has been shown (Chapter 4 & Bishop et al, 1982), all nerves demonstrated to have acetylcholinesterase activity also contain NSE immunoreactivity. This disagreement may be explained by the fact that the methods used to demonstrate acetylcholinesterase activity were not the same. However, the question still remeains

as to what structures are shown to be hyperplastic in the previous study. It is possible that they may be glial cells which have taken up cholinesterase but the results of S-100 immunostaining do not support this contention.

The development of megacolon in Hirschsprung's disease is not only due to the constriction of the aganglionic portion. This constriction only rarely causes total obstruction of the lumen. Partial obstruction may, theoretically, be overcome by the peristaltic activity of normal bowel, but this does not happen. There is a lack of propulsive activity in the proximal segment (Smith, 1972) and there is, in addition, a disturbance of the normal defaecatory reflex (Kuntz, 1945). Slow delivery of faeces into the lumen does not adequately cause contractions for evacuation, as the reflex is only initiated by rapid distension of the rectal musculature. Neurological abnormalities, taking the form of smaller and fewer than normal ganglia and reduced innervation of the circular muscle, have previously been noted (Garrett and Howard, 1981). Our findings reveal that these neurological disturbances are more extensive than previously thought as there is a reduction in the peptidergic nerve supply in the portion proximal to the constriction. This reduction, of both ganglion cells and fibres, was only partial. Abnormal innervation of the proximal portion of bowel could also bee seen at the ultrastructural level (L Probert, personal communication). In four patients, damage to nerve fibres, varying from axonal swelling to complete degeneration, could be seen in the segment of bowel immediately proximal to

the constriction. Some of these fibres were shown, by the immuno-gold staining method, to contain substance P or VIP. Although electron immunocytochemical studies have not yet been made on bowel from children with Hirschsprung's disease, conventional electron microscopy does not show any morphological abnormality of axons in any area (Garrett et al, 1970). This suggests that the neuronal damage may be due to some secondary effects of the disease, such as the chronic constipation or, perhaps, the long-term use of laxatives. If this disruption of neurotransmission is a secondary effect, unlike the aganglionosis, it may develop progressively and be one of the major causes precipitating eventual surgery in "adult" cases of the disease. The observed ultrastructural changes would obviously lead to disrupted intestinal motility.

Another ultrastructural abnormality observed was that of pleomorphic VIP immunoreactive vesicles in the narrowed segment of bowel (L Probert, personal communication). Substance P granules were normal in appearance. The significance of the altered morphology of the granules has yet to be established but the changes may explain the odd-looking VIP nerves seen in this region of bowel at the light microscopical level.

The finding of abnormal innervation in proximal, non-constricted bowel may also be important from the point of view of surgical treatment. Many cases of Hirschsprung's disease show constipation and even re-formation of megacolon after resection of the constricted segment. This may be because of the disordered peptidergic innervation in the proximal, normal looking bowel. The present findings argue for some re-consideration of existing resection procedures.

Further understanding of the pathogenesis of the bowel constriction in Hirschsprung's disease may come from the study of the internal anal sphincter. Aganglionosis in the terminal bowel is normal as it enables the automatic continence of the bowel contents. The internal anal sphincter has few or no ganglion cells and therefore is not actively involved in defaecation. Its permanent contractile state is overcome by the voluntary action of the striated muscle of the external anal sphincter, in combination with abdominal pressure. In Hirschsprung's disease, this aganglionic ring is extended and lies proximal to the region where the external sphnicter and its local reflexes can exert their effects. The contractile state cannot therefore be overcome. It was not possible, during this study, to obtain enough of the very rare human surgical specimens of anal sphincters to map the distribution of the peptidergic nerves. However, in the cat (see Chapter 3), only very few peptide immunoreactive ganglion cells could be found in the internal anal sphincter. The external sphincter, as would be expected for striated muscle, contained little peptidergic innervation, with a few nerves being found in association with blood vessels. In Hirschsprung's disease, it seems that this lower than normal peptidergic innervation of the sphincter extends proximally with the aganglionosis. The present findings emphasise the possible importance of peptide containing enteric nerves not only in Hirschsprung's disease but also in normal defaecatory processes.

As with all the work included in this study on constipation, the possible effects of laxatives on the populations of peptide

containing nerves cannot be ignored. Electron microscopical alterations in nerves have been shown in laxative abusers (Riemann et al, 1982). The use of laxatives, however, can be difficult to assess as patients are often reluctant to admit to laxative abuse. Amongst the children investigated in the present study, not all had received laxatives but they all showed consistent, similar changes in peptidergic innervation.

Comparison between the results obtained in the specimens from children and those from adults shows some interesting differences. In the children, the loss of peptidergic nerves, roughly in proportion to the extent of the aganglionosis, was consistent in all specimens. This was in spite of the fact that some of them had colonic wash-outs and/or laxatives and others had not. In the adults, there was no such relationship and, in some cases, no obvious change in peptide containing nerves at all. None of the adult cases had a history of severe symptoms before they were diagnosed as having Hirchsprung's disease. They had all "escaped" diagnosis because of the relative mildness of their condition, in spite of the fact that all but two of them had distinct aganglionic segments, just like the children. From these morphological studies it is not possible to tell whether the less extensive changes in peptidergic nerves seen in the adults provide an explanation for the mildness of their symptoms. They may have had less disordered gut motility than the childern studied becaused their peptidergic innervation was more intact. However, it has been suggested that autonomic nerves can regenerate and the peptide containing nerves may have recolonised the bowel

of the adults. The finding of apparently increased numbers of nerves in some cases may also be a feature of re-growth of nerves, coupled with the effects of the long-standing muscle distension in an effort to overcome the constriction. Muscle hypertrophy appears to result from this tension and was observed in all the cases. This change in muscle mass may explain some of the discrepancies between the results of immunostaining and peptide content. An increase in tissue weight could dilute to total content of peptides.

It is not entirely surprising that the APUD (enteroglucagon and somatostatin) cells in the mucosal epithelium should also be decreased in number in the aganglionic segments of the children. There have been several reports of the trophic effects of autonomic innervation on the maintenance of mucosal cells (Tutton, 1974, Lachat and Goncalves, 1978). A similar loss of endocrine/paracrine cells, subsequent to or concommittant with, loss of peptide containing nerves was observed in another example of megacolon, Chagas' disease (see this chapter) (Long et al, 1980).

Until more information on the functions of the enteric peptidergic nerves becomes available, the significance of these results cannot be fully evaluated. As VIP and substance P containing nerves are the most abundant of all peptidergic nerves it is logical that their absence in Hirschsprung's disease should have the greatest effect. As far as gut motility is concerned, it appears, at the moment, that the two peptides have opposite effects. VIP causes inhibiton of muscle activity and substance P stimulates it (see Chapter 1). VIP nerves have been suggested to be the non-adrenergic inhibitory nerves which have been described in

the bowel on the basis of physiological experiments (Furness et al, 1980). The inhibitory component of the peristaltic reflex is not adrenergic as sympathetic denervation and pre-treatment with reserpine do not affect propulsive activity (Crema et al, 1970). Impairment of the non-adrenergic inhibitory pathways blocks peristaltic activity (Crema et al, 1970). It, therefore, seems likely that, if VIP nerves are indeed the non-adrenergic inhibitory nerves, their absence in Hirschsprung's disease leads to the non-propulsive state of the gut. In fact, it has been shown that there is a failure to elicit nerve mediated, non-adrenergic relaxation of the aganglionic segment in the disease (Frigo et al, 1973). These various findings and the results of the present study taken together give a neat concept of the genesis of the neuromuscular disorder in Hirschsprung's disease. However, this is a rather simplified view. Firstly, there is some evidence against VIP nerves being the enteric inhibitory nerves (see Chapter 1). The most important point to be taken into account is, however, that the entire intrinsic nervous supply is missing in the aganglionic segment and so the resulting disorder can only be viewed as dysfunction of a variety of different mechanisms. Further physiological tests, on isolated bowel from patients with Hirschsprung's disease, are needed before any more conclusions can be drawn about the significance of the neural abnormalities described here. There are many factors which govern the size and extent of megacolon formation in this disease but inadequate peptidergic nervous supply is likely to contribute.

ACQUIRED MEGACOLON - CHAGAS' DISEASE

INTRODUCTION

Chagas' disease, or South American trypanosomiasis, occurs mainly in South and Central America and, to a lesser extent, in some of the southern United States. The trypanosome, T. cruzi, causes the disease by an unknown mechanism, possibly by release of a neurotoxin or by stimulating some kind of destructive autoimmune reaction in the victim. Chagas' disease is transmitted by contamination of the bite wound of the so-called assassin reduviid bug with the infected faeces of the insect.

Acute Chagas' disease occurs mainly in young children and is characterised by fever and lymphoadenopathy. Death at this stage can be caused by meningoencephalitis or acute myocarditis. In the adult, chronic Chagas' disease can be almost or completely asymptomatic. The main cause of death in chronic Chagas' disease is myocardiopathy but the other frequent manifestation of the disease is enteromegaly. This usually takes the form of megaoesophagus and/or megacolon. Histological examination of the diseased bowel reveals periganglionitis and myositis. During the course of infestation with T. cruzi the gut muscle is invaded and pseudocysts of the parasite in the leishmania phase form. These rupture, causing an inflammatory reaction. Degeneration of the intrinsic ganglion cells occurs but there is rarely a total loss of cells. According to Koberle, who made extensive studies of the histopathology of this disease, the number of ganglion cells needs to be reduced by at least 50% to create bowel dysfunction (Koberle, 1963). The denervation of the diseased gut segments leads to formation of the megabowel.

At the moment, there is no known cure for Chagas' disease. The gut lesions are usually treated by surgical resection. It is mainly poor people who contract the disease, largely because the reduviid bugs show a preference for living in a particular type of wood, from which the poor construct their slum houses. Spraying homes with a chemical (5% benzene hexachloride) is effective in killing the bugs but no programme has been launched for widespread eradication because of lack of finances.

As in Hirschsprung's disease, the abnormality of the intrinsic enteric nerves obviously suggested a possible change in the peptide containing nerves. Also, Chagas' disease was particulalry suitable for inclusion in the study of the involvement of peptide containing nerves in gastrointestinal pathology as previous studies had shown a reduction of substance P in man which correlated with the disturbances in oesophageal and intestinal motility in the disease (Hial et al, 1973). Furthermore, changes in substance P levels, as measured by bioassay, had been demonstrated in animals with the disease (Almeida et al, 1977).

Although Chagas' disease presented an ideal example of acquired megacolon for study of the peptide containing enteric nerves, the major problem was obviously the collection of specimens. Surgical resection for Chagas' disease is not a frequent occurrence in any one city in South America, as was soon discovered when enquiries were made about possible specimen collection. The study was divided into two parts. Firstly, endoscopic rectal biopsies were collected for investigation and then, once it was found that there is a major lesion of peptide containing nerves in Chagas' disease, samples of bowel were taken at

surgery and examined. This study was carried out over a period of 4 years and involved 4 separate journeys to Brasil; two were made by a colleague in Professor Bloom's department, Dr RG Long, and two by the author.

ENDOSCOPIC RECTAL BIOPSIES

The purpose of examining rectal biopsies was two-fold. Firstly, the development of megabowel in Chagas' disease is not associated with involvement of the rectum (Martin-Campus & Tafuri, 1973). If any alteration in enteric peptide containing nerves could be found then it would aid diagnosis. Secondly, not only were biopsies from Chagasics with megabowel examined but also from patients with chronic autonomic failure. This condition is characterised by widespread sympathetic and parasympathetic dysfunction, but there are rarely any bowel changes. The main lesions in chronic autonoimic failure vary but usually involve brain stem nuclei and intermediolateral-column cells (Bannister & Oppenheimer, 1972). Thus, enteric peptide containing nerves were studied in Chagas' disease, where there is a perihperal intrinsic neuronal lesion, and compared with those in the pre-ganglionic extrinsic nerve abnormality seen in chronic autonomic failure. These were, of course, all compared with normal controls. The Chagasics were all Brasilian and the patients with chronic autonomic failure British, so control tissues were taken from patients from both countries.

Materials

Endoscopic rectal biopsies were obtained from 10 patients with Chagas' disease. These patients were chosen to give a mixture of megaoesophagus and megacolon. Thus, clinical and radiological

examination showed that 4 patients had megaoesophagus, 3 had megacolon and 4 had both types of enteromegaly. The patients were all selected from endemic areas and all had positive serological tests for T. cruzi. Similar specimens were obtained from 10 patients with chronic autonomic failure, 8 with multiple system atrophy and 2 with pure autonomic failure. A total of 13 normal controls were taken from Brasilian and British subjects of similar ages to the diseased patients.

Biopsies were processed for histology and immunocytochemistry by fixation in benzoquinone solution (appendix) for 30 minutes and cryostat sectioning. Specimens from the same area of rectum were also sent for radioimmunoassay of total peptide content. Immunostaining of a range of rgulatory peptides was carried out as previously described (appendix).

Results

Histology

Controls: Haematoxylin and eosin staining showed that the biopsies all consisted of mucosa, submucosa and muscularis mucosae. The specimens were too superficial for ganglion cells to be seen. The tissues from healthy controls and the patients with chronic autonomic failure were normal.

Chagasic: Some sections from Chagasics, with or without megacolon, showed mild, chronic inflammatory infiltrate but no other notable changes.

Immunocytochemistry

Controls: Biopsies from healthy controls and patients with chronic autonomic failure showed a normal pattern of VIP, substance P and met-enkephalin containing nerve fibres, as described earlier (Chapter 3). VIP nerves were the most abundant but both VIP and substance P nerves could be found in the mucosa, around the base of the crypts and projecting upwards through the lamina propria, and in the muscularis mucosae, intertwined with muscle fibres (Fig 30a). Only very occasional met-enkephalin immunoreactive nerves could be seen, in the muscularis mucosae.

Chagasics: In the Chagasics, VIP- and substance P-immunoreactive nerves were reduced in number in all regions of the biopsies (Fig 30b). In those fibres which were immunoreactive the immunostain was weak. In some sections no immunoreeactive fibres could be found. No met-enkephalin immunoreactive fibres were seen. Enteroglucagon- and somatostatin-immunoreactive cells were the only peptide containing cells detected in the mucosal epithelium, where they were mainly distributed around the base of the crypts. The cell morphology was similar in each of the 3 groups of patients, with both types of cell having an apical shape with occasional basal and/or luminal elongations. The numbers of enteroglucagon- and somatostatin-immunoreactive cells in the Chagasics were noticeably smaller in comparison with the controls and patients with autonomic failure. There appeared to be no change in the area of the mucosa in the Chagasic specimens.

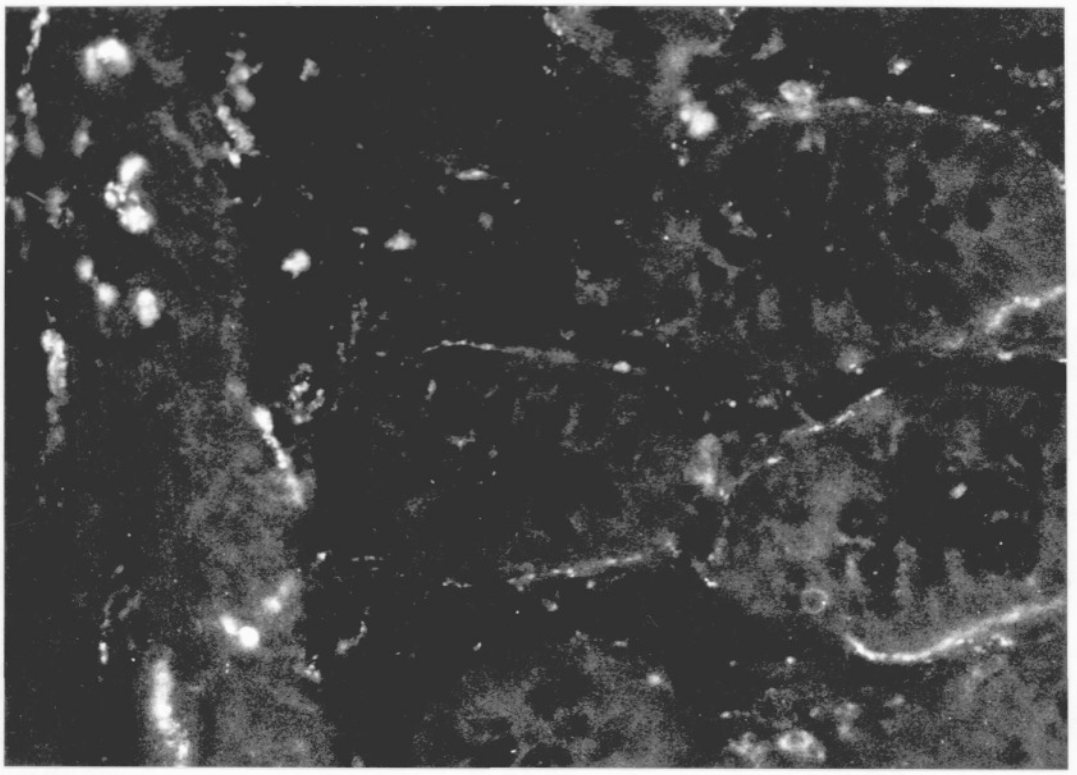
FIG 30

Substance P-immunoreactive fibres in the muscularis mucosae and at the base of the mucosa of an endoscopic rectal biopsy from:

a) a normal control

b) a patient with Chagasic megacolon. Very few immunoreactive fibres can be seen (arrow) (x 250).

Tissue fixed in BQS, cryostat section, indirect immunofluorescence.





Radioimmunoassay

Results of radioimmunoassay of peptide content agreed with the immunocytochemical findings and demonstrated a significant loss of peptides in Chagasics (Table 7).

SURGICAL SPECIMENS OF MEGACOLON

In order to examine more closely the lesion of the peptide containing nerves in Chagasic megacolon, surgical specimens were collected and immunostained.

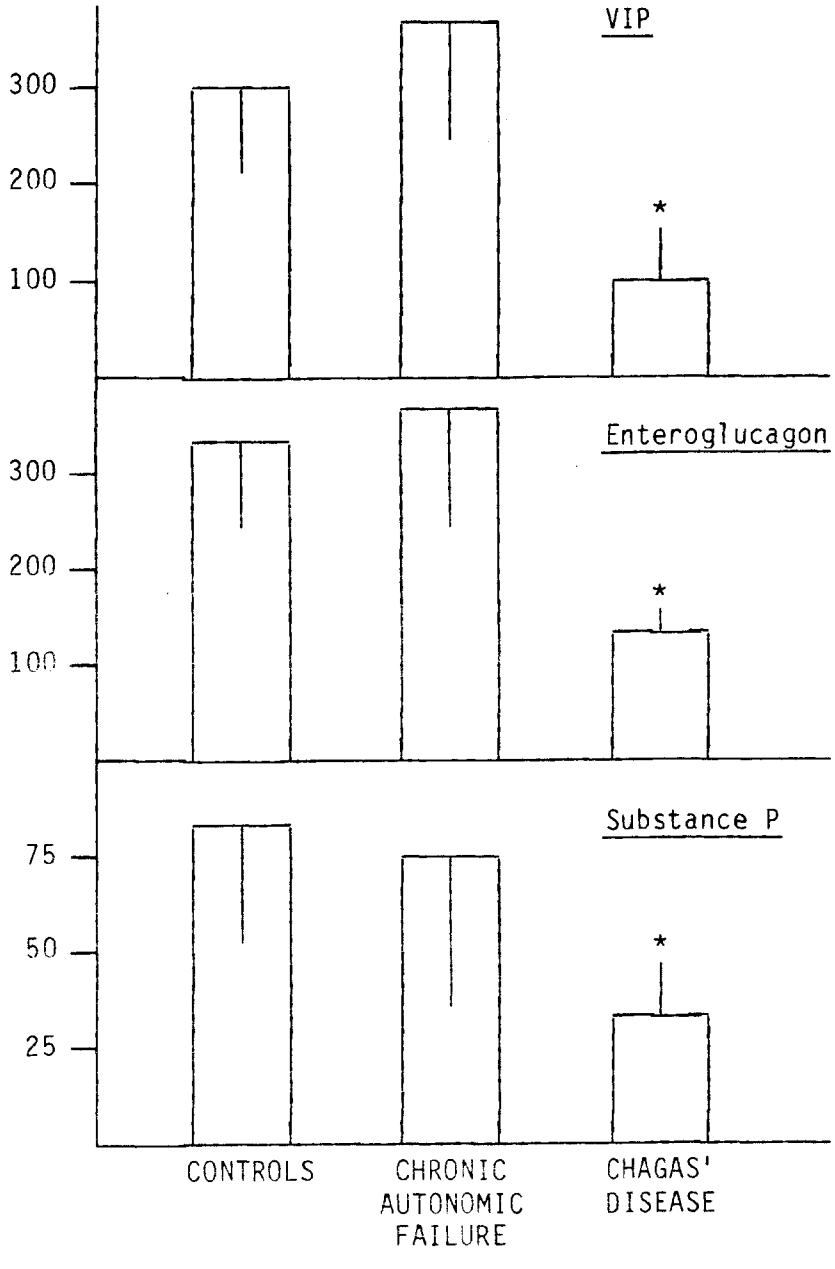
Materials

Twelve megacolons were obtained fresh at surgery. These all consisted of sigmoid colon and proximal rectum with the larger specimens containing some descending and transverse regions. Although, by travelling to different centres, it was fairly easy to obtain a sufficient number of megacolons, collection of normal surgical specimens of colon (from carcinoma resections etc) proved to be difficult in Brasil. For this reason, post-mortem colonic samples were taken from patients dying of non-gastrointestinal disease. In Brasil, as in many other countries with a hot climate, post-mortem examinations are frequently carried out shortly after death, much sooner than in Britain. It was therefore possible to obtain pieces of colon within 6 hours of death, well within the time limit of 8 hours previously established. A total of eight samples of normal colon were thus taken at surgery for carcinoma (n=4) and at post-mortem examination (n=4).

Table 7

CONTENT OF PEPTIDES IN RECTAL BIOPSIES FROM CONTROLS, CHAGASICS AND PATIENTS WITH CHRONIC AUTONOMIC FAILURE

peptide pmol/g wet weight



Samples were taken from proximal, middle and distal portions of each specimen. All tissues were bisected and half fixed in benzoquinone solution (appendix) for two hours, processed and immunostained for regulatory peptides as described (appendix). By the time this part of the investigation had begun, antibodies to neuron specific enolase (NSE) and S-100 had become available. These two markers for nerves and glial cells, respectively, were also immunostained in the tissue sections. The other half of each sample was extracted and assayed for peptide content.

Results

Histology

Controls: The normal control specimens showed no histological abnormalities. The tissues taken at post-mortem examination were indistinguishable from the surgical specimens.

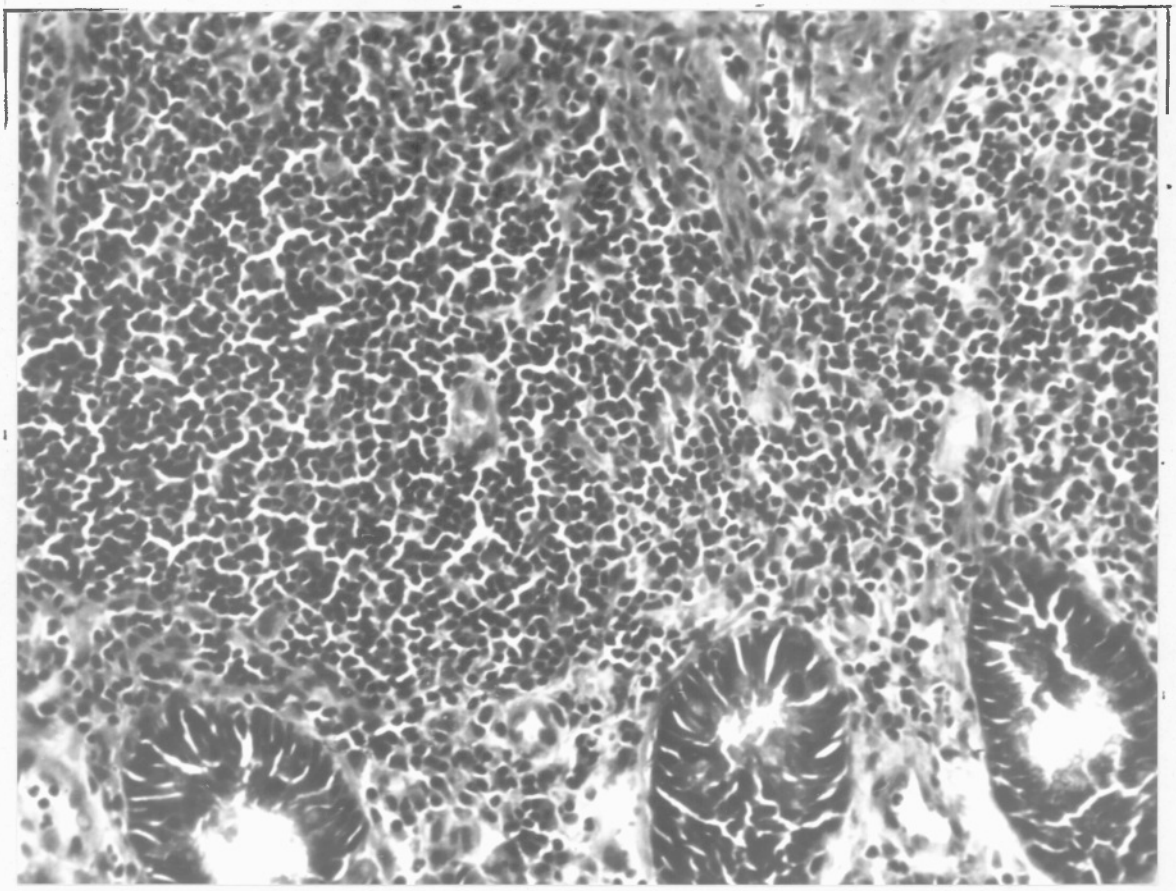
Chagasics: The histological features of the megacolons were not striking. In dilated portions the muscle wall was occasionally somewhat thinner than normal and oedema, particularly in the submucosa, was sometimes observed. Chronic inflammatory infiltration was a frequent finding, but no granulomas were seen (Fig **31**). Myositis, especially of the circular muscle layer, was often present but not to any great degree, and ganglionitis (Fig **32**) could be seen, more frequently in the myenteric rather than the submucous plexus. Although loss of ganglion cells was obvious, no specimen was found to have a reduction as great as that reported from quantitative studies (Koberle, 1956, reported a loss of up to 96% of the normal quota of ganglion cells).

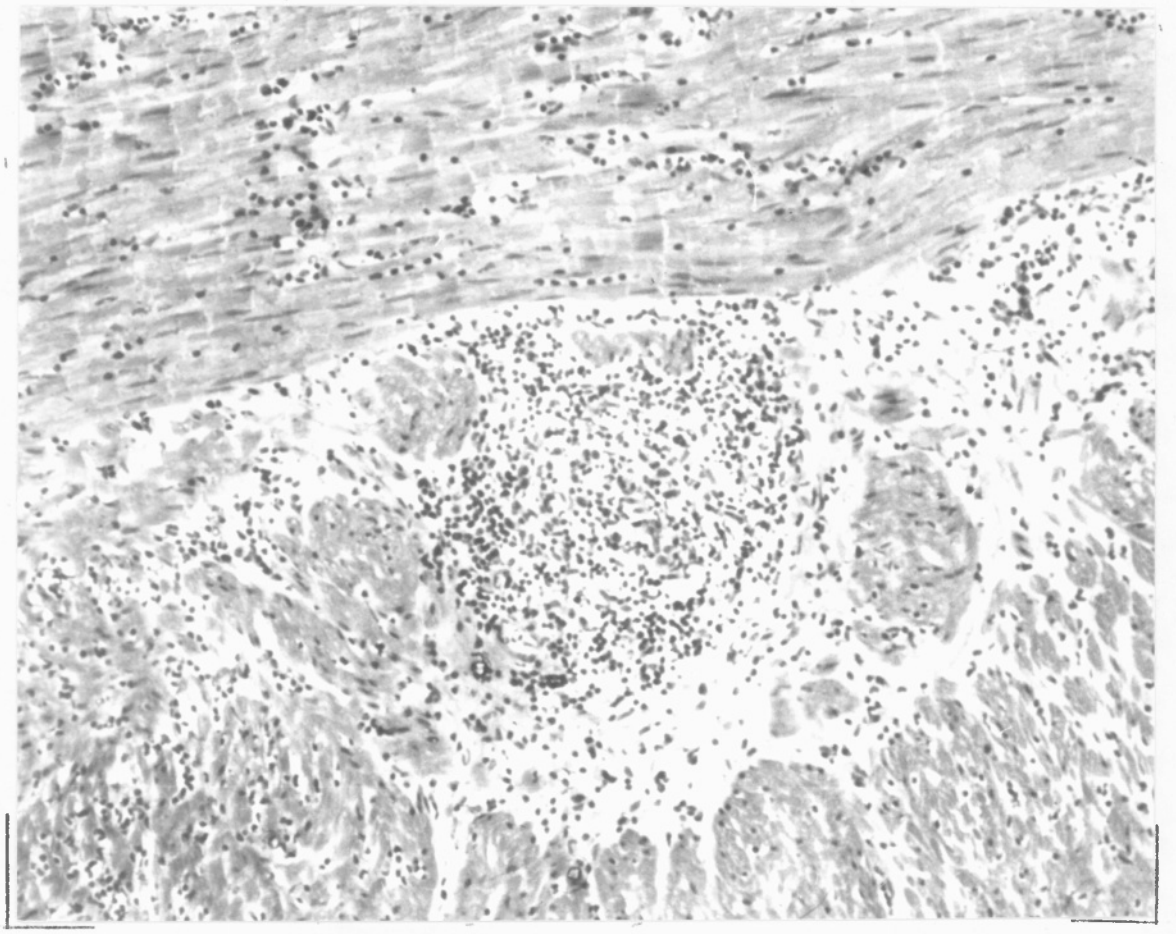
FIG 31

Haematoxylin and eosin stain of the base of the rectal mucosa, showing chronic inflammatory infiltration, from a patient with Chagasic megacolon. Tissue fixed in BQS, cryostat section (x 275).

FIG 32

Haematoxylin and eosin stain showing ganglionitis in the myenteric plexus of colon from a patient with Chagasic megacolon. Tissue fixed in BQS, cryostat section (x 200).





Immunocytochemistry

Controls: All of the control specimens showed a normal pattern of peptide containing nerves and cells, as previously described (Chapter 3). NSE- and S-100-immunoreactivities were demonstrated in large numbers of nerves and glial cells, all of which looked entirely normal (Chapter 4). The immunostaining of nerves and cells in the post-mortem specimens was comparable to that in specimens taken at surgery.

Chagasics: Both NSE- and S-100-immunoreactivities were reduced in the megacolons. A diffuse, somewhat patchy loss of nerves was revealed by the antibodies to NSE. Degeneration of ganglion cell bodies apparently involved the myenteric plexus more than the submucous plexus as NSE-immunoreactive cells were fewer at this site (Fig 33). The reduction in fibres, however, was fairly uniform across the wall. Although glial cells were also generally reduced in number, there was no change in their distribution or structure. The overall appearance of the enteric innervation, as shown by the NSE antibodies, was generally in agreement with what has previously been described in Chagas' disease.

Both VIP and substance P immunoreactive fibres could be found in all specimens examined, but in lesser numbers than those in the controls. No met-enkephalin containing fibres were found in any of the sections. The reduction in peptide containing fibres was both obvious and consistent throughout the group of specimens. Also, within each specimen, the samples taken from different areas along the length showed remarkably similar changes. There was no patchy loss of fibres, as seen with the antibodies to NSE. This is in direct contrast with

FIG 33

Ganglion cells and fibres in the sub-mucous plexus of human Chagasic megacolon showing low immunoreactivity for NSE. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 450).

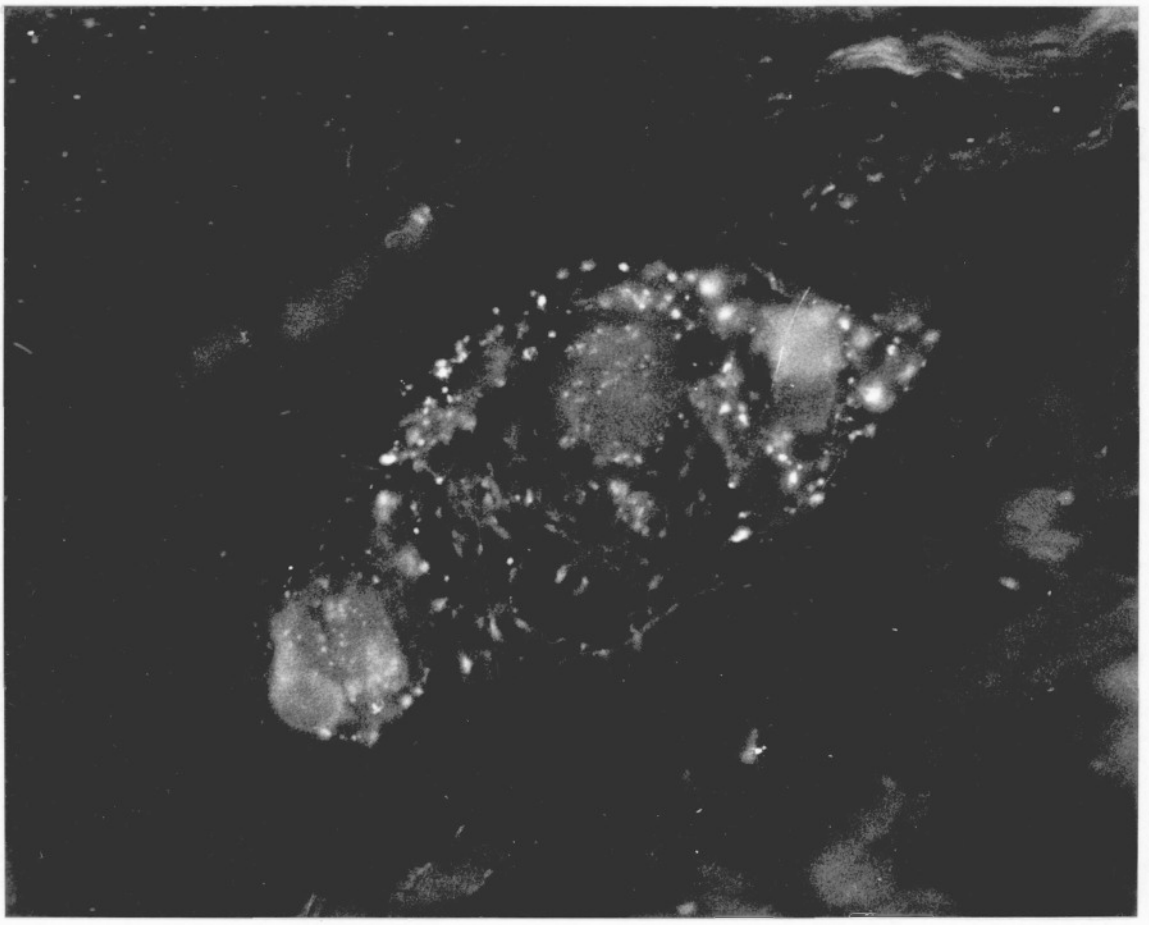
FIG 34

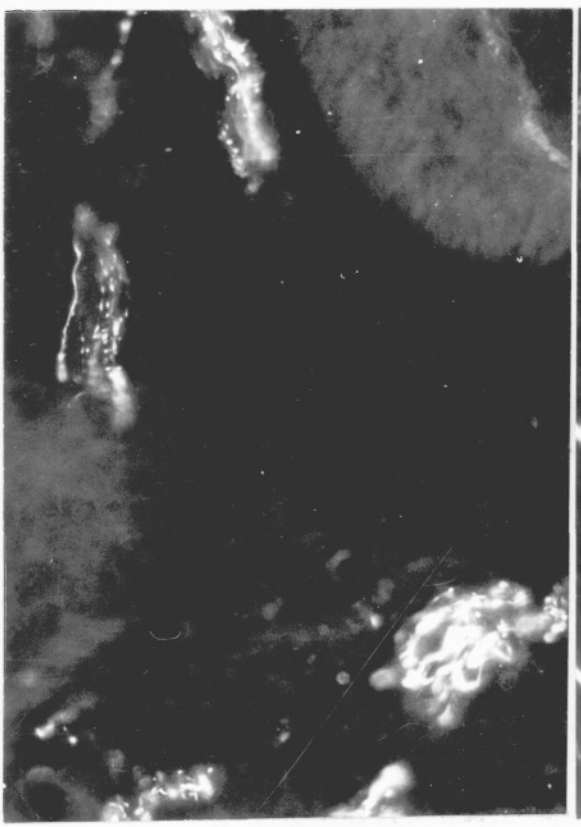
VIP-immunoreactive fibres in the sub-mucosa of colon taken from:

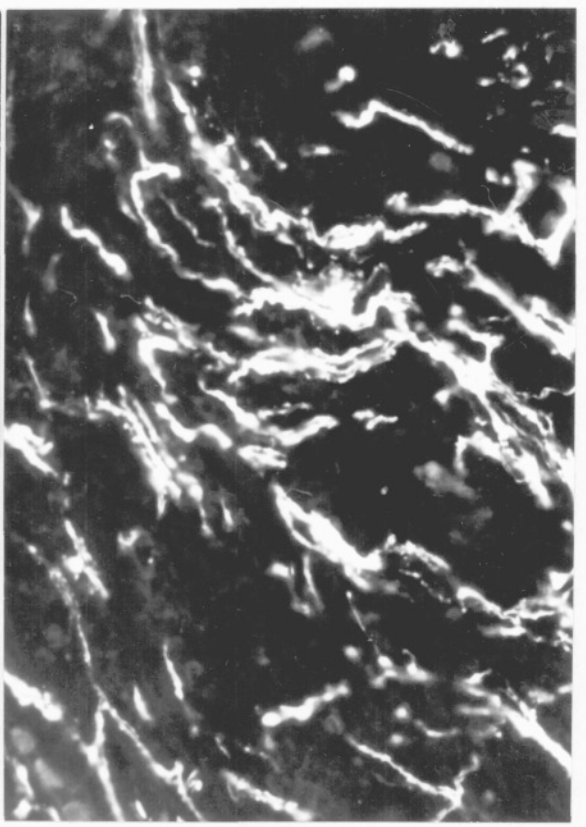
a) normal bowel removed at resection for carcinoma

b) cobblestoned bowel removed at resection for Crohn's disease (x 350)

Tissue fixed in BQS, cryostat section, indirect immunofluorescence.







Hirschsprung's disease where peptide containing fibres were gradually reduced towards the aganglionic segment.

The VIP and substance P nerves all had a typically beaded appearance, except for some VIP immunoreactive fibres at the point where they emanated from immunoreactive ganglion cells. At this site, they were more ribbon-like and non-varicose. Also, there appeared to be no change in the degree of immunostaining of individual fibres. Again, unlike Hirschsprung's disease, where remaining peptide-immunoreactive fibres occurred in areas which would normally contain ganglion cells, there seemed to be no preferential location of the VIP and substance P containing fibres in the Chagasic megacolons. The fibres were present throughout the bowel wall and the reduction in number was of a similar degree in each layer. Some VIP-immunoreactive ganglion cells could be found in the submucous plexus, but only about one third of the usual number seen in this area.

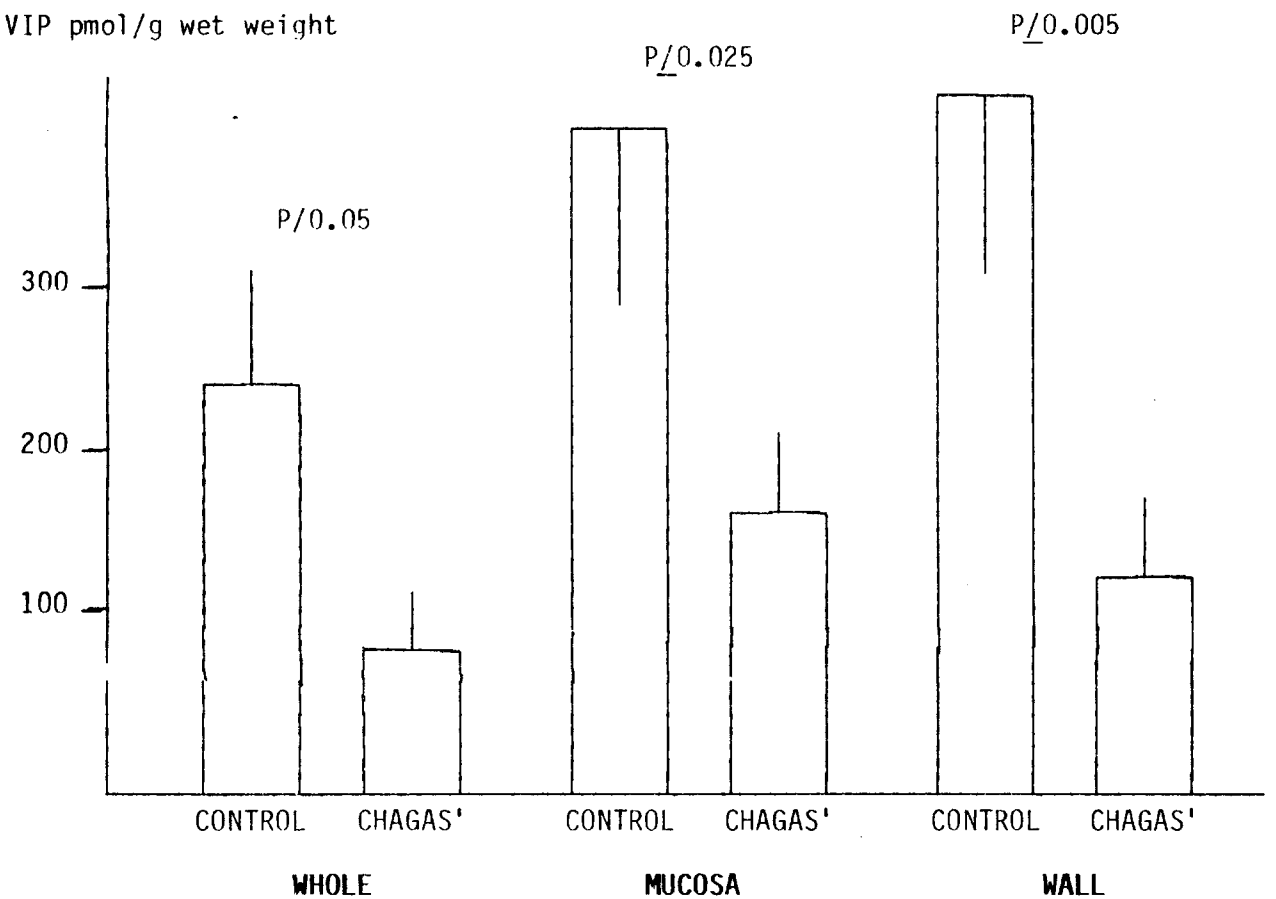
There was some loss of peptide-immunoreactive mucosal cells but this was in no way as consistent as the loss of peptide-immunoreactive nerves. Variations in the density of the endocrine/paracrine cells occurred along the length of some specimens, but there was no change in the morphology or degree of immunostaining of individual cells.

Radioimmunoassay

Measurement of VIP content of not only whole bowel wall but also the muscle coat and submucosa/mucosa separately showed a statistically significant loss of peptide in the Chagasic megacolons (Table **8**).

Table 8

VIP CONTENT OF SURGICAL SPECIMENS FROM PATIENTS WITH CHAGAS' DISEASE



DISCUSSION

The results obtained from the endoscopic rectal biopsies show that even though the clinically apparent gut abnormalities are usually localised, there is a generalised lesion of peptide containing nerves, and cells, in Chagas' disease. This can be seen from the fact that there was a decrease in nerves in the recta of patients even if they only had megaoesophagus with no clinically or histopathologically demonstrable involvement of the large bowel. A loss of intrinsic ganglion cells along the entire length of the gastrointestinal tract of Chagasics has previously been reported (Ferreira-Santos, 1961). In Chagas' disease, where the intrinsic neurons are thus affected, it followed, as in Hirschsprung's disease, that some change in the level of peptide containing nerves may occur. In chronic autonomic failure, however, the preganglionic autonomic degeneration does not appear to affect the peptide containing nerves of the bowel.

In the surgial specimens, a consistent reduction in VIP and substance P containing nerve fibres was found along the length of each megacolon. There was a loss of both NSE- and VIP-immunoreactive ganglion cells, reflecting the general degeneration of intrinsic neurons. The consistency of the changes in peptide containing innervation was somewhat surprising in view of the variable appearances of the megacolon and the patchy loss of nerves shown by NSE immunostaining. However, this consistency is probably a reflection of the very generalised nature of the lesion of peptidergic innervation, as was shown in the rectal biopsies.

The contribution of the lack of peptidergic nerves to the development of megabowel may be similar to that postulated for Hirschsprung's disease (see Discussion of Hirschsprung's disease this chapter). However, the megacolon seen in Chagas' disease is not the same as that in Hirschsprung's disease. Generally, in Hirschsprung's disease there is a dilated segment of bowel followed by a funnel-shaped transitional zone and then the constricted, aganglionic segment. In Chagas' disease, as mentioned in Materials, a megacolon may be hugely dilated but only extend a short distance or the whole colon may be elongated and dilated, but to a comparatively lesser degree. There is, therefore, no single, typical pattern. This variation does not appear to be a result of duration of constipation. It may be related to the degree of neuronal degeneration. The genesis of megacolon in Chagas' disease is also entirely different from that in Hirschsprung's disease. In the latter case, neuronal cells have never colonised the areas of affected bowel. In Chagas' disease, the enteric nervous system was originally complete and, presumably, functioned normally, before the onset of the disease. Perhaps, the mode of develpment of the megacolon governs its final form. As the intrinsic neurons degenerate the nature of the bowel dysfunction may vary with the type of neuron affected first, if this is not uniform.

One of the many unexplained features of Chagas' disease is the high incidence of enteromegaly in the colon and oesophagus, compared with the rest of the gut. This is in spite of the fact that all neurons, including peptidergic ones as shown here, are diffusely reduced in number along the gut. One explanation may be that these two regions are exposed to more mechanical stress than the rest of the gut as they both receive and contain either faeces or undigested food. In comparison,

other areas of gut deal with fluid.

As in Hirschsprung's disease, there appeared to be an associated loss of peptide (enteroglucagon and somatostatin) containing mucosal cells. It is not possible to determine, from these morphological investigations, whether the change in cell frequency is related to the loss of peptidergic, or any other types, of nerves. However, radioimmunoassay of peptides in mice inoculated with T. cruzi shows that VIP and substance P levels in the large bowel fall in the acute disease, whereas enteroglucagon and somatostatin concentrations either do not change or rise (RH Albuquerque et al, unpublished observations). This suggests that the loss of peptide containing nerves is primary to the reduction in mucosal endocrine cells.

IDIOPATHIC MEGACOLON

Introduction

Idiopathic megacolon is a less well defined disease than either Hirschsprung's or Chagas' disease and its pathophysiology is ill-understood. Unlike the other two main forms of megacolon, no neural abnormalities have been described in this disease. It, thus, seemed possible that the development of megacolon may stem from a lesion of the peptidergic innervation, which had hitherto not been examined.

Idiopathic megacolon occurs more frequently than Hirschsprung's disease (Ravitch, 1958) and is difficult to treat. Except for constipation, there are surprisingly few symptoms associated with this disease. A person with idiopathic megacolon may not pass faeces for

weeks without great discomfort or general debility, as seen in congenital megacolon. The constipation can often be traced back to childhood and faulty bowel habits, as a result of mismanagement of toilet training, has frequently been cited as the underlying aetiology of the disease (Bodian, 1952, Ganad & Richmond, 1952, Wilson, 1954). However, there is some evidence for a disordered bowel mechanism rather than a purely psychogenic cause for the disease. It may be that some individuals are born with a very large rectum. Thus, a large quantity of faeces has to accumulate before the defaecation reflex is stimulated by bowel distension, leading to the development of propulsive and sensory defects. The lack of sensation in the rectum has been demonstrated by experiments using a lax balloon. The volume of the balloon is increased until sensation is felt. In diseased patients, much greater volumes are needed to produce senstation (Porter, 1961) and this sensation may actually be abdominal rather than rectal (Goligher, 1961). The pain and difficulty experienced by a child during the passage of large stools may then lead to repression of the defaecatory urge, thereby further complicating the situation. It may, however, be argued that the lack of sensation in megacolon is purely secondary to the bowel dilation.

Whatever the pathogenesis of the disease, treatment is usually medical with surgery being used if this is not successful. The main aim is to empty the bowel and keep it empty by administration of laxatives and re-education. Rectal sensation has been reported to return after treatment (Nixon, 1961), allowing cessation of therapy.

No histological abnormality, other than muscle hypertrophy, has been associated with idiopathic megacolon (Morson & Dawson, 1972). In this

study, specimens of colon were sampled from adults undergoing surgery for idiopathic megacolon. Thus, the cases were all severe and the bowel possibly affected by prolonged laxative ingestion.

Materials

Surgical samples of megacolon were obtained from 8 patients (ages 17-47 years). All specimens were kindly provided by Dr DM Preston, St Mark's Hospital, London. Samples were removed from proximal, middle and distal portions . The pieces of resected bowel were dilated to variable degrees, with some areas of muscle thickening.

For comparison, normal colon was obtained from 10 patients (ages 23-73 years) undergoing surgery for carcinoma of the large bowel. All samples were bisected, half for histology and immunocytochemistry and half for radioimmunoassay of peptide content.

For morphological studies, tissues were fixed in benzoquinone solution for 2 hours and stained with haematoxylin and eosin or immunostained for a variety of regulatory peptides and the proteins NSE and S-100 (appendix).

Results

Histology

Controls: None of the controls contained obvious histological abnormalities.

Megacolons: The megacolons also had an essentially normal appearance. The only noticeable changes were some muscle thickening, which was niether extensive nor very great, and slight oedema of submucosa in a few sections. Ganglion cells appeared to be present in normal numbers in both plexuses.

Immunocytochemistry

Controls: The controls all had normal numbers and distributions of peptide immunoreactive nerve fibres and cells. In addition, NSE and S-100 immunostaining revealed no changes in the total populations of nerves and glial cells.

Megacolons: In general, the appearance of the peptide-immunoreactive nerves and cells was indistinguishable from that of the controls. VIP, substance P and met-enkephalin fibres could all be seen in each layer of the wall in normal numbers. Individual fibres had typical morphology. VIP-immunoreactive ganglion cells could be seen in the submucous plexus and enteroglucagon and somatostatin containing cells were frequent in the mucosal epithelium. NSE and S-100 immunostaining revealed no gross alterations in innervation.

In 2 specimens, however, slight changes in peptide-immunoreactive nerves were detected, although the total population of nerves, as demonstrated by NSE antibodies, seemed unaltered. Both specimens had particularly marked muscle hypertrophy in the proximal portion. In these regions, substance P and, especially, VIP-containing fibres in the circular muscle had an unusual pattern. VIP-immunoreative nerves were not only greater in number but formed wider bundles than normal.

The individual fibres appeared to be normal but, within the circular muscle layer, instead of the usual thin bundles of closely intertwined immunoreactive fibres there were more loosely arranged meshes. These abnormal meshes also appeared to follow a straighter course than the bundles of VIP-immunoreactive fibres usually found in this location. The substance P-immunoreactive fibres were just more numerous than in the same area of controls. The numbers of VIP-immunoreactive ganglion cells did not change in these regions of muscle hypertrophy and the fibres in the other layers of bowel were not altered. Peptide containing cells were equally numerous and brightly immunostained in both control and diseased groups.

Radioimmunoassay

Radioimmunoassay of peptide content revealed no significant differences between the VIP, substance P, enteroglucagon and somatstatin contents of the idiopathic megacolons and the controls.

DISCUSSION

The mostly normal pattern of peptide containing innervation in idiopathic megacolon provides no further explanation for the disturbed bowel function. It appears that all the intrinsic innervation in this condition is normal and there are no morphologically identifiable abnormalities. These results provide no evidence against the theory that the megacolon may be due to insensitivity to distension or may have a purely psychogenic origin. It is also possible that the condition may arise from some abnormality of neuromusclar transmission. Ultrastructural studies may yield further information on changes in sensory nerves or neuromuscular connections.

The finding of abnormal bundles of VIP-immunoreactive nerves in regions of muscle hypertrophy of two specimens may simply be secondary to the mechanical changes. The innervation in other layers of bowel was not affected. However, similar bundles were not found in any other type of megacolon examined. It is possible that, although some cases of Hirschsprung's and Chagas' disease showed muscle thickening, the overall level of peptidergic innervation was reduced, thereby precluding formation of these meshes of fibres.

The use of laxatives did not appear to affect the peptide-immunoreactive nerves. It is possible , however, that any damage to the innervation by laxatives may only be detectable using electron microscopy. Ultrastructural changes have previously been investigated in this way (Riemann & Schmidt, 1982).

CHAPTER 6 INFLAMMATORY BOWEL DISEASE

INTRODUCTION

In the search for gut diseases with known neural abnormalities in which to examine peptidergic nerves, inflammatory bowel disease may not be an obvious choice. However, neuromatous hyperplasia has been described in Crohn's disease (Morson & Dawson, 1972, Whitehead, 1974) and examination of literature on the disease revealed a report, made more than twenty years ago, of the finding of increased numbers of ganglion cells in this disease (Davis et al, 1955). In this study, a three-fold average increase was found in the number of ganglion cells in the myenteric plexus. This increase was not confined to grossly affected bowel but could also be seen in surrounding tissues which had a normal appearance. A possible explanation for the abnormal number of ganglion cells in Crohn's disease may be hypertrophy of small, underdeveloped cells in the ganglia, not easily detectable using routine light microscopy. The finding that the numbers of ganglion cells increase with the duration of the disease (Davis et al, 1955) makes this explanation more likely than that of victims having an abnormally high number of normal sized cells even before disease onset. In view of these reported abnormalities of the intrinsic gut innervation in Crohn's disease, the possible involvement of the intrinsic peptide containing nerves was postulated.

Thus, surgical specimens were obtained from patients with Crohn's disease in order to examine the peptide containing nerves. These specimens were compared not only with normal controls but also with tissues from patients with ulcerative colitis, the other major

inflammatory bowel disease. The two diseases were studied in parallel to see whether, if there were alterations of the peptide containing nerves, such alterations are a feature of inflammatory bowel diseases in general or one of the diseases in particluar. Only clear cut cases of Crohn's disease and ulcerative colitis were included in this study, which was divided into two parts; the first was the investigation of surgical specimens of bowel and the second of endoscopic rectal biopsies.

SURGICAL SPECIMENS

MATERIALS

Fresh, surgical specimens of small and large bowel were obtained from 19 patients with suspected Crohn's disease. For comparison, similar areas of bowel were taken from 6 patients with ulcerative colitis and normal tissues from 8 patients undergoing jejuno-ileal by-pass for obesity and 17 patients undergoing resection for carcinoma. All diagnoses were made on the basis of clinical, radiological and pathological findings. Each specimen was processed within one hour of removal from the patient. A macroscopic analysis was made and all relevant features, such as muscle hypertrophy, oedema, haemorrhage, ulceration and fissuring, were noted.

The Crohn's disease and ulcerative colitis specimens were sampled from the areas of maximal pathology and, where possible, from normal looking areas 1cm proximal and distal. Samples from larger specimens were taken in addition from more distant areas above and below the lesion.

Macroscopically normal samples were taken at resection for carcinoma (as far from the tumour as possible) and at jejuno-ileal by-pass for obesity. The dimensions of specimens and the distances between samples and lesion areas were also noted. All samples measured at least 2 x 2cm and consisted of mucosa with muscle wall. Each sample was bisected longitudinally. One half was processed for histology and immunocytochemistry and the other for radioimmunoasssay of peptide content. The samples were bisected so that pieces taken for each technique were from the same level of bowel. Regulatory peptides were immunostained in all samples as described (appendix). Antibodies to NSE, S-100 and PHI were not available at the time of this study.

RESULTS

Histology

Controls: Analysis of the histological features of all sections was made independently by the author and Dr S Hamilton (at St Mark's Hospital, London). Macroscopically and histologically normal pieces of bowel (n=25) were selected from the pieces of control tissue.

Diseased: Only clear cut cases of Crohn's disease (n=17) and ulcerative colitis (n=6) were chosen for further investigations. Histological assessment of the sections was made using the criteria outlined by Morson and Dawson (Morson & Dawson, 1972). In accordance with this selection procedure, typical features of each disease were seen in the abnormal specimens from patients with Crohn's disease and ulcerative colitis. Where granulomas were not found in the Crohn's specimens, other characteristics, such as transmural inflammation and normal crypt architecture around fissures, indicated

Crohn's disease. In each case, the histological observations were found not to be at variance with the clinical findings.

Immunocytochemistry

Controls: All 25 control specimens showed the characteristic distribution pattern of peptide (VIP, substance P, met-enkephalin) containing nerves (Chapter 3) except for some slight increases in the number of VIP-immunoreactive fibres in the area of the submucosa and mucosa of a few specimens (Table 9). A semi-quantitative assessment of these results was made by two independent observers (Dr S Hamilton and the author) (Table 9). Mucosal peptide containing endocrine/paracrine cells were normal in number and distribution.

Ulcerative colitis: Four of the six ulcerative colitis specimens showed a pattern of VIP fibres identical to that observed in the control specimens, with no alteration in the intensity of the immunostain nor in the number of immunoreactive ganglion cells in the submucous plexus. The remaining two cases showed, however, a moderate increase in VIP-immunostained nerve fibres, particularly in areas of gross inflammation. All other types of peptide containing nerves were normal, as were peptide containing cells of the mucosal epithelium.

Crohn's disease: In contrast with the controls and most of the ulcerative colitis specimens, all the Crohn's disease tissues exhibited transmural changes in VIP innervation. The mucosa and submucosa of each sample showed these changes, but 13 of the 17 cases studied had a total transmural increase of VIP-immunostained nerves in all areas, whether affected or macroscopically normal. The changes took the form

Table 9

Crohn's disease	Ulcerative colitis	Carcinoma resection	By-pass
AA	AA	CC	All EE
A	ACAC	CC	
A	CC	CE	
AA	C	DC	
A	CCCE	DD	
A	CDE	DD	
AABA		DD	
AAB		D	
BB		D	
B		D	
B		D	
B		DE	
B		DE	
BBBCB		DE	
BBBC		EE	
BCB		E	
CCBB		E	

Key; A=total transmural increase in number of VIP nerves, cells & intensity of immunostain; B=total transmural increase in VIP nerves, only few nerves thickened & distended; C=VIP nerves increased but only in mucosa & submucosa; D=slight increase in VIP nerves in mucosa & submucosa; E=no change, normal.

Each line in table refers to grading of single or multiple samples from each patient.

of thicker and more brightly immunostained fibres in each layer of the gut wall, with a greater population of fibres in the muscle and more densely packed and disorganised meshes in the submucosa and mucosa (Fig 34). In some specimens, immunoreactive fibres could be found in the densely packed nerve bundles observed in the granulomatous areas after conventional histological staining, an observation not made in either the control or the ulcerative colitis specimens (Fig 35).

Intensely immunostained fibres filled both the myenteric (Fig 36) and submucous plexuses, which were often enlarged and infiltrated the muscle layers. A clear increase was seen in the number of immunostained ganglion cells in the submucous plexus (Fig 37). Semi-quantitative assessment showed a range of 6-8 immunoreactive cell bodies in ganglia in the Crohn's disease specimens (0-4 in controls). These features were detected not only in the areas of maximal pathology but also in adjacent, apparently normal bowel where histological staining only revealed slight neural changes. Similar changes in VIP-immunostained nerves were observed in specimens from the ileum, colon and rectum. A semi-quantitative analysis was made of these results (Table 9).

No other type of peptide containing nerve showed any form of abnormality in these tissues and all mucosal endocrine/paracrine cells were similar to those seen in controls.

Radioimmunoassay

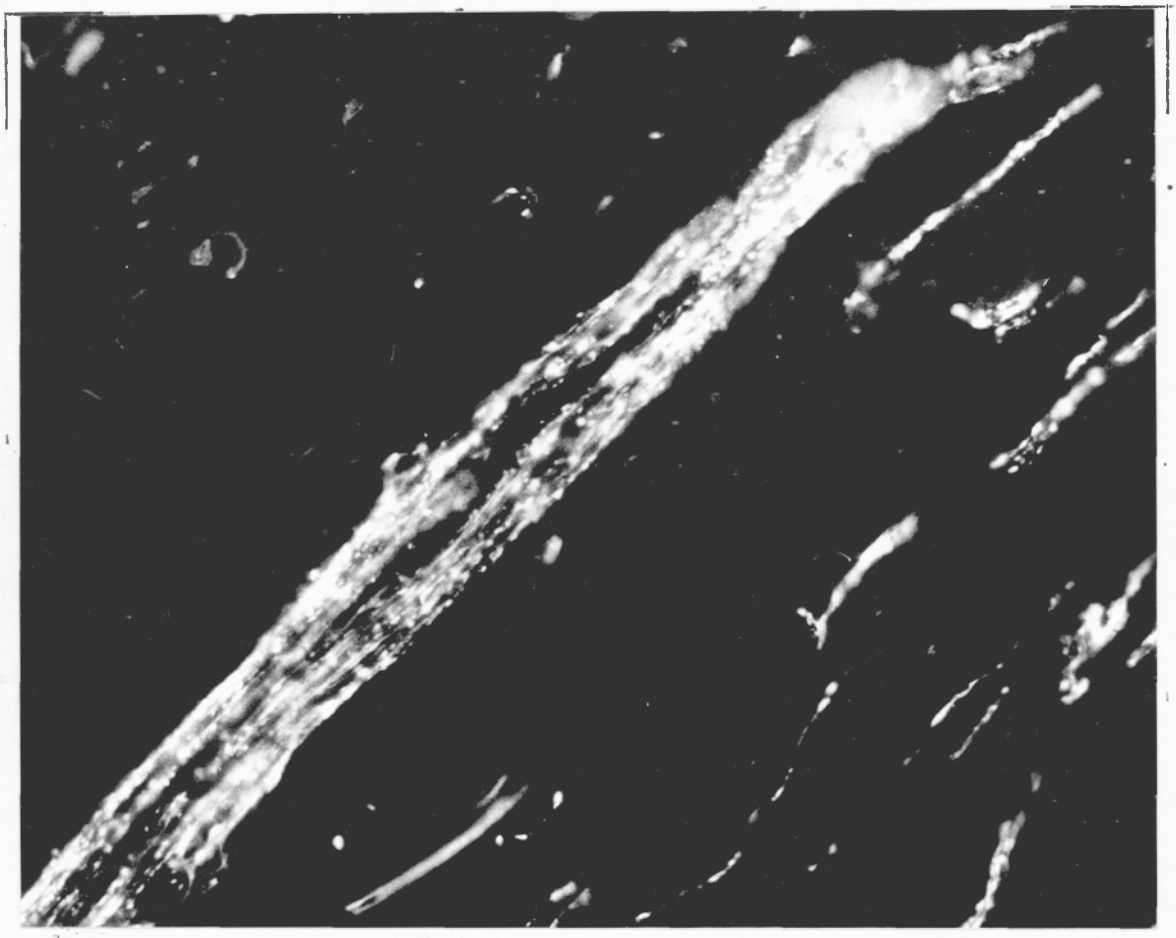
Radioimmunoassay of tissue extracts showed a large, approximately 200%, increase in VIP content in the Crohn's disease specimens in comparison with all other types of tissue investigated. Statistical

FIG 35

Thickened, very straight VIP-immunoreactive fibres forming a large, densely packed bundle in the sub-mucosa of ileum taken at surgery for Crohn's disease. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 300).

FIG 36

Disarranged, thickened VIP-immunoreactive fibres in the myenteric plexus of ileum from a patient with Crohn's disease. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 400).



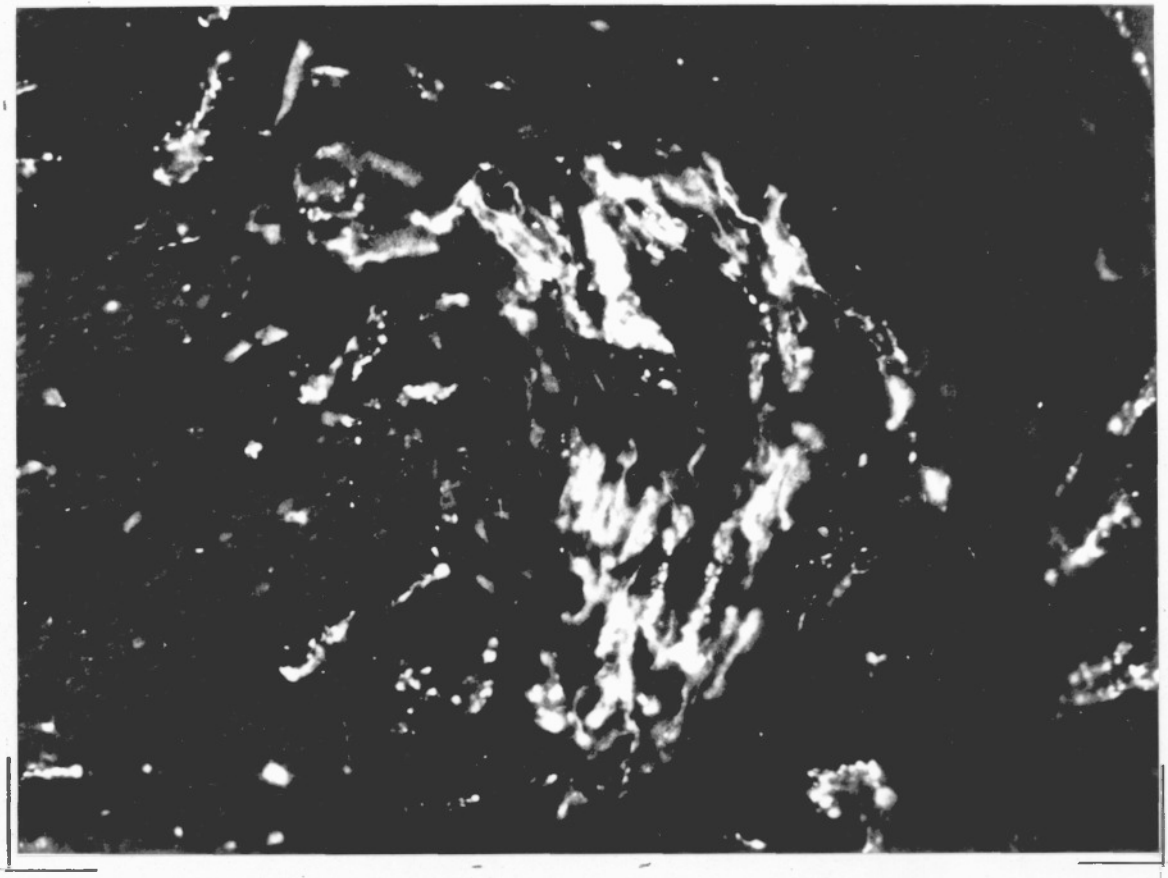
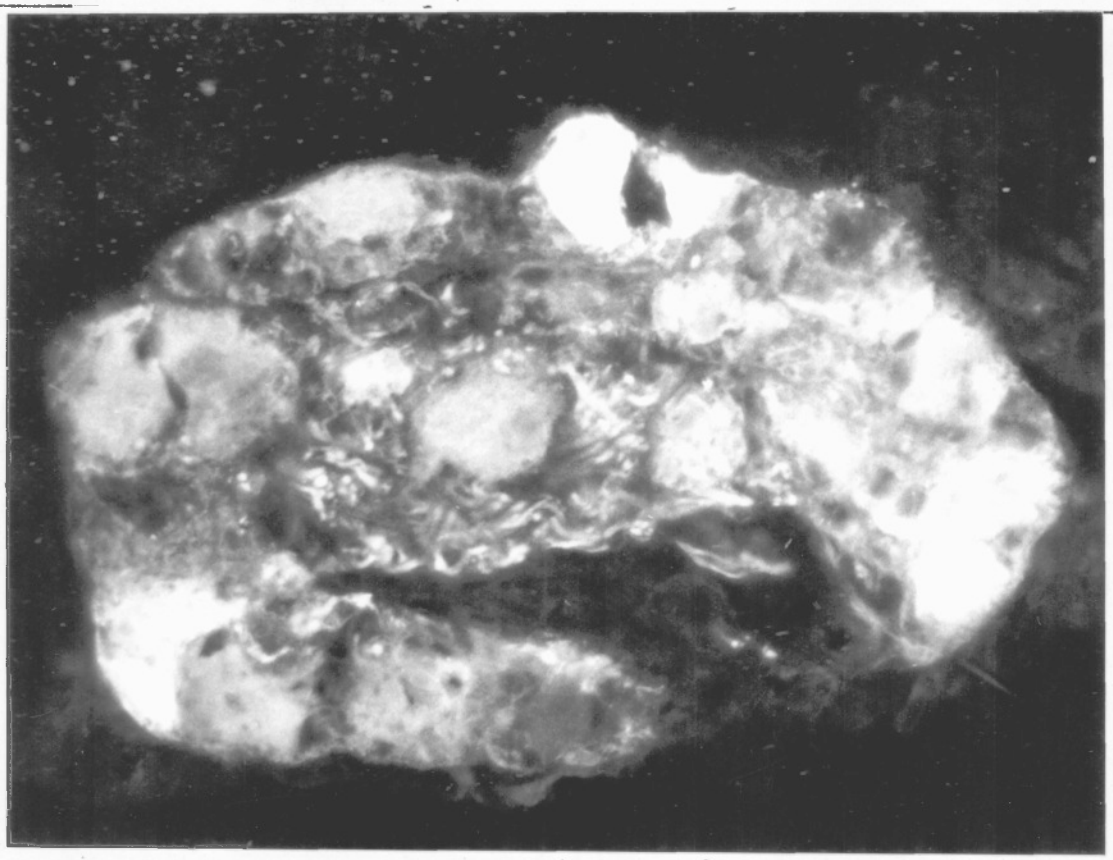


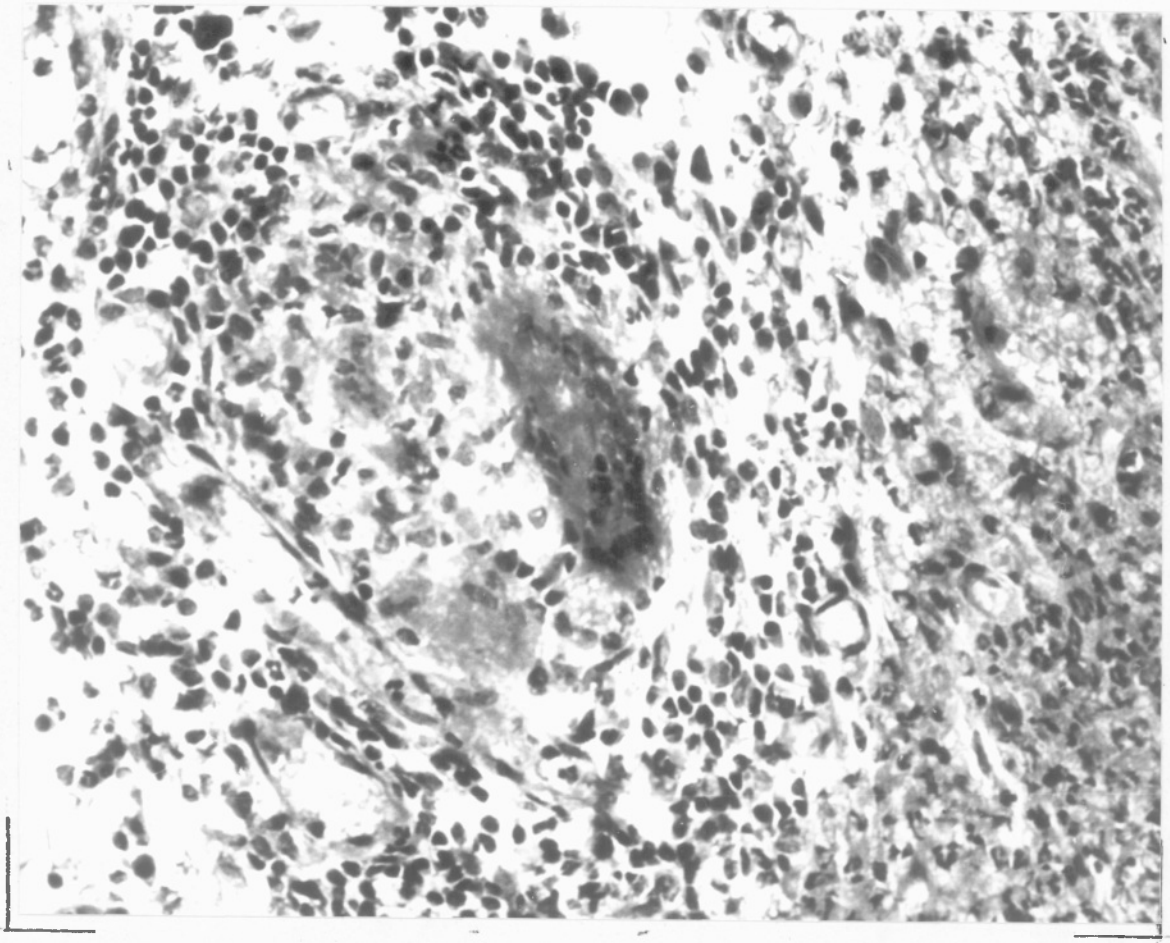
FIG 37

An increased population of VIP-immunoreactive ganglion cells in the sub-mucous plexus of ileum from a patient with Crohn's disease. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 450).

FIG 38

Haematoxylin and eosin stain showing a granuloma in the sub-mucosa of an endoscopic rectal biospy from a patient with Crohn's disease. Tissue fixed in BQS, cryostat section, (x 350).





analysis of these results showed that the VIP content of the Crohn's disease tissues was significanlty higher than that of any of the controls ($P < 0.001$). No significant difference was found between the VIP levels in the ulcerative colitis and control specimens (Table **10**).

DISCUSSION

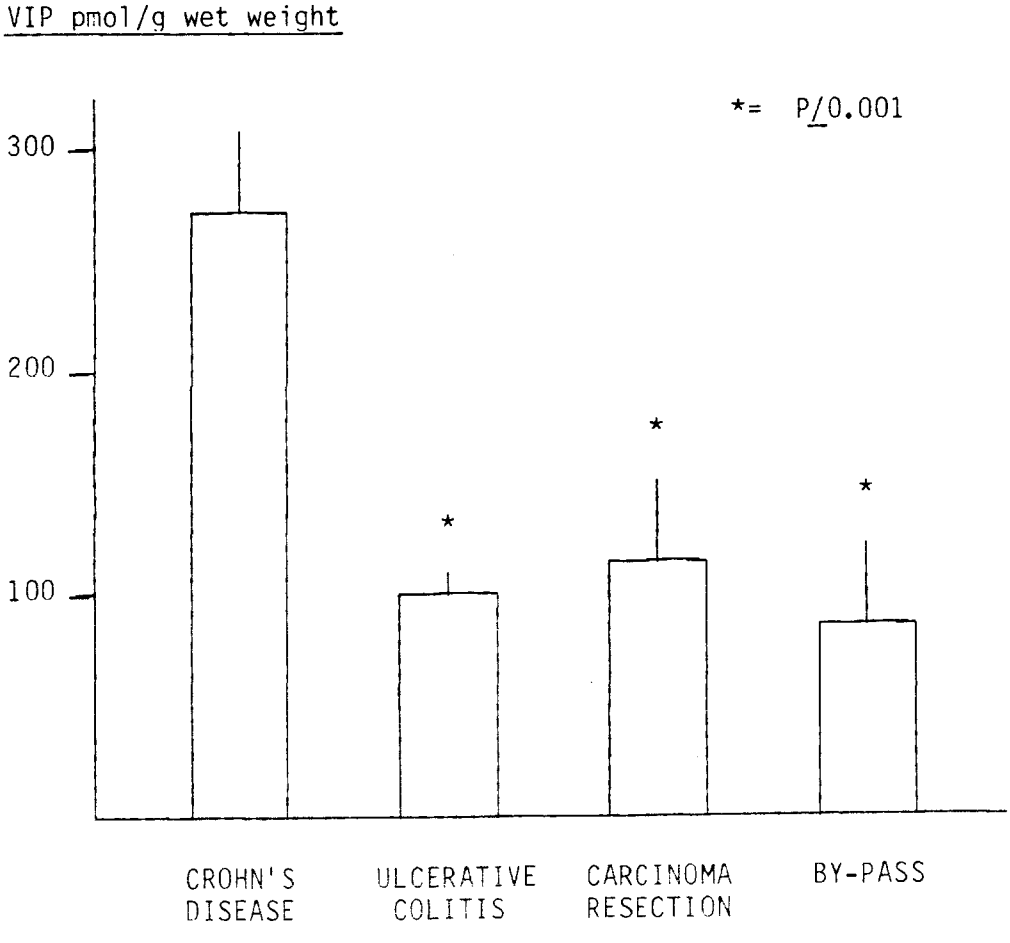
There is, therefore, a substantial increase in the population of VIP-immunostained nerves and in the content of VIP in the gut wall of patients with one type of inflammatory bowel disease, Crohn's disease, though not in the other main type, ulcerative colitis, as compared with normal controls (carcinoma resection and by-passed gut from obese patients).

The VIP nerves not only appeared to be present in greater numbers in Crohn's disease but they also had abnormal morphology. This took the form of very bright immunostaining and thickening and distortion of individual fibres. There was also an associated increase in the numbers of immunostained ganglion cells. Transmural increases in the number and size of VIP nerves was seen in areas from all specimens, with particularly consistent changes in the mucosa and submucosa. These observations represent, on average, a greater than 200% increase in VIP content, as measured by radioimmunoassay of tissue extracts.

Abnormalities of autonomic nerves in Crohn's disease, including a rise in the number of ganglion cells and a general nerve proliferation, were first noted more than 20 years ago (Davis et al, 1955). However, their significance has remained unknown. Specific VIP immunostaining indicates that these autonomic nerve changes are greater than those

Table 10

VIP CONTENT OF SURGICAL SPECIMENS



revealed by conventional histological staining and can be accounted for, at least in part, by the significantly increased content of VIP-immunoreactive nerves found in this study.

Although some increases in VIP nerves were found in two of the six specimens from patients with ulcerative colitis, radioimmunoassay did not detect any rise in VIP content. Thus, there seems to be a clear distinction between VIP nerves in Crohn's disease and ulcerative colitis. A possible explanation for this difference may reside in the severe transmural inflammatory process seen in Crohn's disease. It may be that this transmural inflammation acts as an irritant stimulus for the proliferation of intrinsic VIP ganglion cells, a stimulus possibly absent in all but the most severe cases of ulcerative colitis. However, it is interesting to note that the changes in VIP innervation in Crohn's disease can be detected in histologically normal bowel adjacent to the areas of maximal pathology. This finding argues against the changes being a mere secondary response to the irritant stimulus of the inflammatory process. Although the possibility of previous inflammation, subsequently healed, cannot be ruled out, the fact that the neural hyperplasia is confined to VIP immunostained nerves would suggest that a specific mechanism caused the changes, rather than generalised inflammation. Furthermore, the findings made here in Crohn's disease can be compared with those made in Chagas' disease, where there is also chronic, widespread inflammation. In Chagas' disease, those fibres which have not been destroyed are normal in appearance (Chapter 5 and Long et al, 1980). In Crohn's disease, the VIP nerves specifically are distorted and enlarged. This again suggests that the changes observed in Crohn's disease are not simply the result of long-standing inflammation.

Subsequent ultrastructural studies have fully corroborated these light microscopical observations (L Probert, personal communication). Electron immunocytochemistry revealed enlargement of VIP nerves and increased numbers of VIP-immunoreactive secretory granules. Substance P-immunoreactive fibres showed no changes. As with immunocytochemistry at the light microscopical level, electron immunocytochemistry also showed that these abnormal VIP nerves are present in histologically normal adjacent bowel. No changes of this kind could be found in ulcerative colitis. In the original report of larger numbers of ganglion cells occurring in Crohn's disease (Davis et al, 1955), it was also noted that the changes are present in histologically normal bowel.

Comparison between the VIP-immunostained nerve changes and the histological and clinical data revealed no real correlation. In view of the powerful effects which VIP is known to have on the gut, it seems likely that the increase in VIP innervation may have a significant effect on gut function in Crohn's disease, causing diarrhoea for example. However, the morphological studies employed here cannot show whether or not the nerves which are increased in Crohn's disease are actually functioning. The observed distortion and increased brightness of immunostaining may be results of increased storage of VIP within the nerve, which may be inactive and not secreting the peptide. The answer as to whether or not the VIP nerve hyperplasia in Crohn's disease is functional cannot really be obtained using just these morphological studies.

ENDOSCOPIC RECTAL BIOPSIES

In both of the major inflammatory bowel diseases, Crohn's disease and ulcerative colitis, abdominal colic and diarrhoea, with passage of blood, are common symptoms but the clinical presentation of both diseases varies greatly. Crohn's disease (or regional ileitis) was originally thought only to occur in the ileum, but the colon and rectum can, in fact, be affected with or without ileal disease. In their typical forms, it is possible to distinguish between the two diseases on the basis of clinical, radiological and histological data. However, because of the frequent overlap of features, it can be difficult to differentiate between colonic Crohn's disease and ulcerative colitis. In fact, there has, in the past, been considerable confusion over the relationship between the two diseases. This has led to reports of ileal Crohn's disease and ulcerative colitis in the same patient (termed combined ileo-colitis)(Yarnis, 1958, Daffner & Brown, 1958). The essentially separate natures of the two diseases have now been established but problems of differential diagnosis remain. This is especially so in recent years since effective medical treatment for both diseases has become available and surgery can be avoided. It is important to obtain a differential diagnosis from the point of view of both treatment and prognosis.

In most cases where the clinical picture is confused, radiological evidence for skip lesions will provide a diagnosis, or the appearance of a rectal biopsy. The most reliable diagnostic finding in Crohn's disease is the presence of sarcoid-like reactions. These take the form of focal aggregates of epithelioid cells with giant cells and a poorly demarcated outer zone of lymphocytes. However, such granulomas are

found in only about 60% of specimens, even if the biopsy is successfully removed from an affected area. In general, diagnosis of Crohn's disease depends on observation of a combination of histological features, including transmural inflammation, focal aggregates of lymphocytes and fissuring ulceration, all of which can, unfortunately, be found in very acute ulcerative colitis (and also, perhaps, in malignant lymphoma of the gut).

So there remains a small proportion of cases where a differential diagnosis cannot be made using existing means. In view of this, the immunocytochemical findings made in surgical specimens of inflammatpry bowel led to further investigations being undertaken.

Two particularly notable features of the VIP nerve hypertrophy in Crohn's disease were firstly its presence in histologically normal bowel and secondly its consistent appearance in the mucosa and submucosa of diseased specimens. These findings suggested that examination of VIP nerves in endoscopic rectal biopsies may help in finding a differential diagnosis for the two main inflammatory bowel diseases. A double blind study was, therefore, set up and immunocytochemistry (and radioimmunoassay) used to investigate VIP nerves and VIP content in rectal biopsies from patients with Crohn's disease, both with and without rectal involvement, in comparison with normal controls and ulcerative colitics, with or without proctitis.

Materials

A group of 56 patients, in whom rectal mucosal histology was required for diagnostic or follow-up purposes, were studied. Endoscopic

biopsies were taken from the rectum at a level below the peritoneal reflection. The group was made up of 17 patients attending the gastroenterology clinic for investigation of non-specific symptoms, in whom no rectal pathology was subsequently found, and 39 patients with some form of inflammatory bowel disease. As with the surgical specimens, only patients with clear cut disease were chosen for the study. The diseased biopsies were classified, according to the available clincial and histological data, into three separate groups: 22 were from patients with Crohn's disease, 11 patients with disease involving the rectum and 11 with intestinal Crohn's disease diagnosed on clincial and radiological grounds but with no histological changes in the rectum, and 17 patients with ulcerative colitis.

Each biopsy was bisected; half was processed for histology and immunocytochemistry of VIP (appendix) and half sent for radioimmunoassay of VIP content.

Results

Histology

Controls: Each biopsy was found to consist of mucosa with muscularis mucosae. All of the biopsies from normal controls had normal histology.

Diseased: Biopsies from patients with non-rectal Crohn's disease and most of the ulcerative colitics had normal histology. Some of the biopsies from ulcerative colitics (7 out of 17) had evidence of proctitis. Patients with rectal Crohn's disease had varying degrees of disease and granulomas (Fig **38**) were found in only 4 cases.

Immunocytochemistry

Controls: A semi-quantitative method was used to assess the immunocytochemical results. This had to be different from that used for the surgical study because of the superficial nature of the biospy specimens. Grades ranging from 1+ to 4+ were given to each biopsy as follows: 1+, normal number and distribution of VIP nerves; 2+, increase in the number of VIP nerves; 3+, increase in the number of VIP nerves and either a concomittant increase in the number of VIP-immunoreactive ganglion cells or distortion of individual fibres, taking the form of thickening with a greater density of immunostain; 4+, increase in the number of VIP nerves and ganglion cells with distortion of the fibres and greater degree of immunostaining.

The characteristic pattern of VIP nerves was seen in all the control biopsies (Chapter 3). Fibres were present in the mucosa, muscularis mucosae and submucosa and could be seen singly or in small bundles (Fig **39**). Each fibre was typically thin with multiple varicosities and followed a convoluted path through the tissue. Some ganglion cells in the submucous plexus contained VIP-immunoreactivity. All the biopsies were graded as 1+ or, occasionally, 2+.

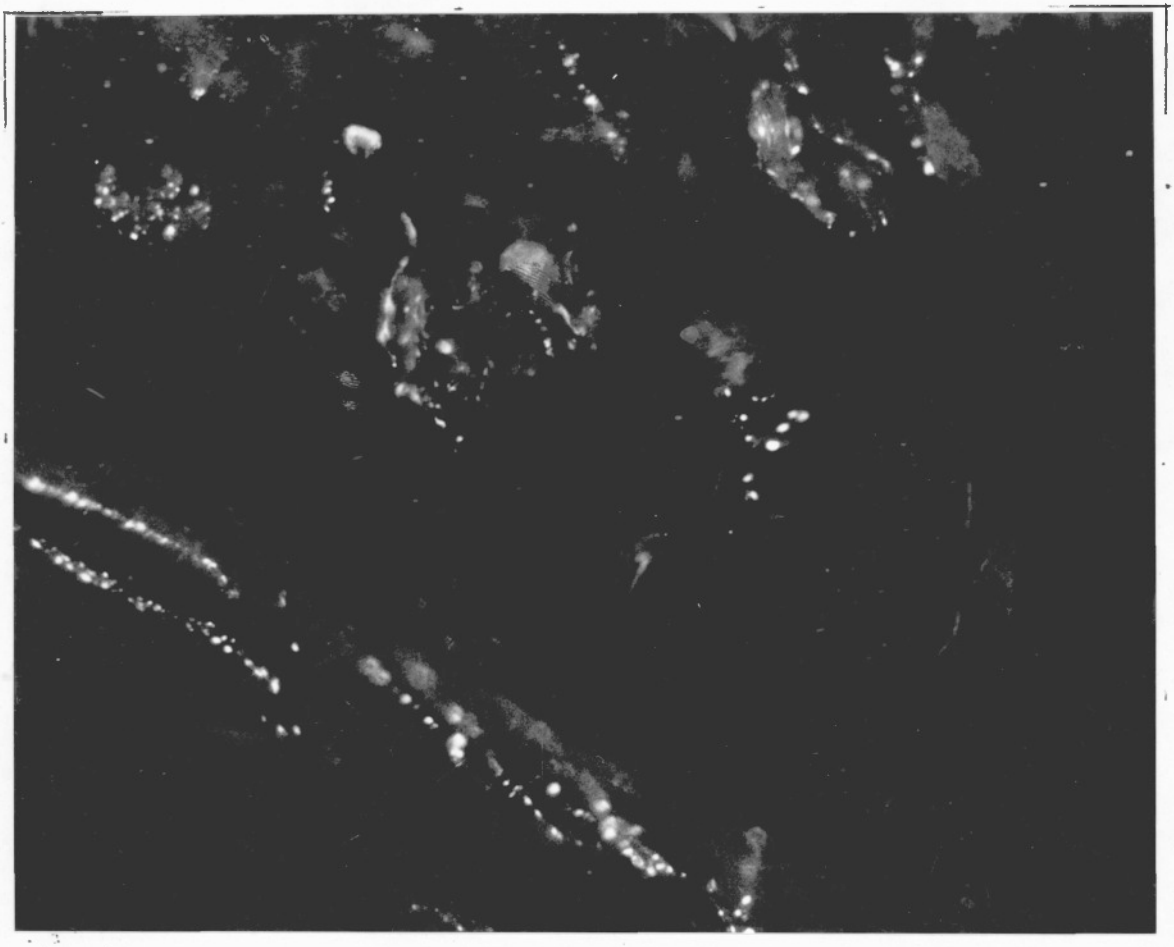
Ulcerative colitis: The majority (10 out of 17) of the biopsies from the ulcerative colitics showed a similar pattern of VIP innervation to that seen in the controls and, thus, received grades of 1+ and 2+. Five biopsies had increased numbers of VIP nerves with some distortion and were graded as 3+ and 2 biopsies had gross changes and were therefore graded as 4+. Examination of the haematoxylin and eosin stain for these biopsies showed that those with markedly increased VIP innervation had obvious active disease (Fig **40**).

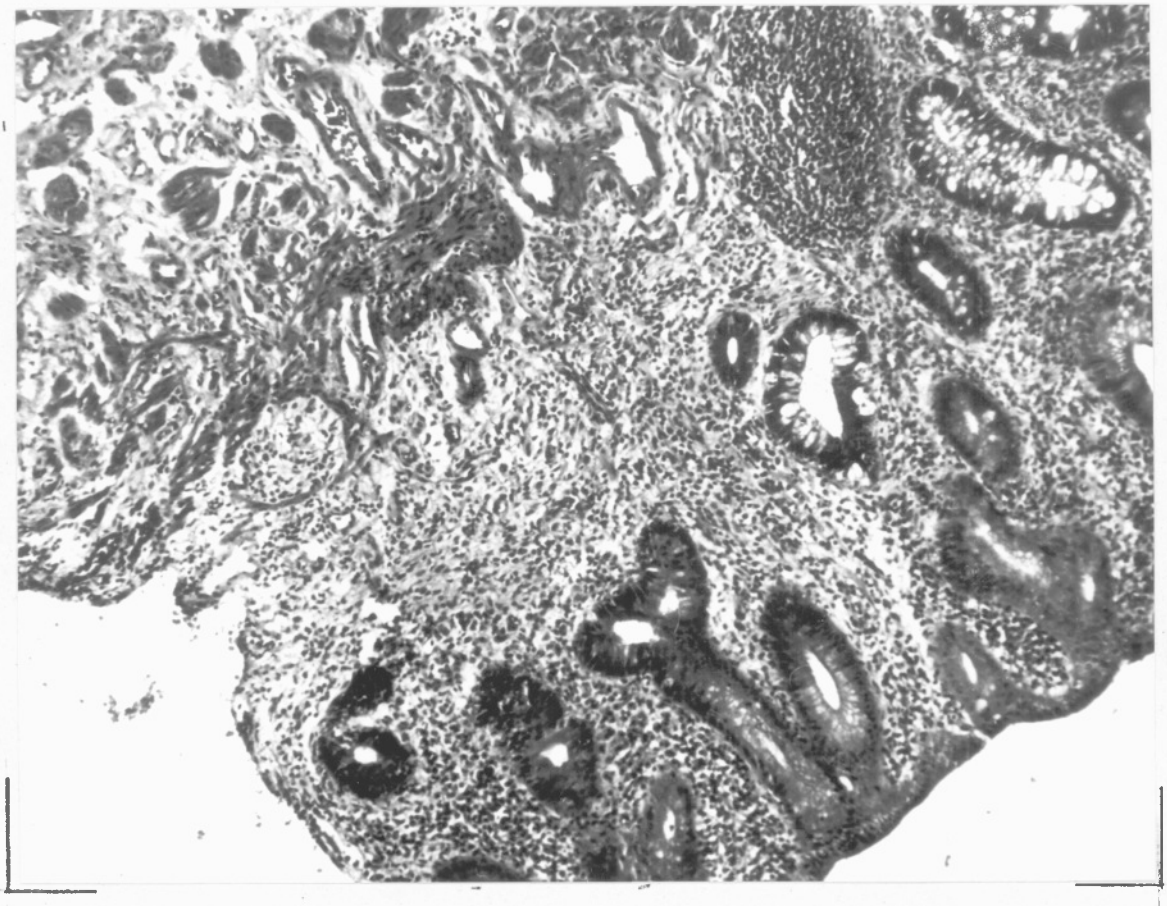
FIG 39

VIP-immunoreactive nerves in the muscularis mucosae and sub-mucosa of a normal endoscopic rectal biospy. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 450).

FIG 40

Haematoxylin and eosin stain of colonic mucosa from a patient with active ulcerative colitis. Tissue fixed in BQS, cryostat section (x 150).





Crohn's: All of the biopsies from patients with Crohn's disease had increased VIP innervation. Tissues from patients with normal histology were graded as 2+ and above, whilst all those with rectal involvement showed highly consistent, gross changes in VIP innervation and received grades of 3+ and 4+ (7 out of 11) (Fig **41**). In the biopsies with the most abnormal VIP innervation, the fibres had essentially the same distribution as those in the normal controls but both fibres and ganglion cells were markedly increased in number and degree of immunostaining. In addition, the fibres frequently occurred in a straightened, thickened form, without their characteristic varicosities, as in the surgical specimens.

Radioimmunoassay

VIP content was found in the tissues from patients with rectal Crohn's disease to be more than double that in the normal controls (Table **11**). A larger, statistically significant difference in VIP content was found between biopsies from patients with non-rectal Crohn's disease and the normal controls ($P < 0.02$). Biopsies from ulcerative colitics contained less VIP than the normal controls, but the difference between the two groups was not statistically different. There was, however, a highly significant difference between VIP levels in the colitics and the non-rectal Crohn's tissues ($P < 0.01$).

DISCUSSION

The results of this blind study show that there is a detecable increase in VIP nerves in endoscopic rectal biopsies from patients with Crohn's disease, irrespective of whether or not there is rectal

FIG 41

Increased numbers of VIP-immunoreactive fibres at the base of the mucosa in a rectal biospy from a patient with non-rectal Crohn's disease. Tissue fixed in BQS, cryostat section, indirect immunofluorescence (x 400).

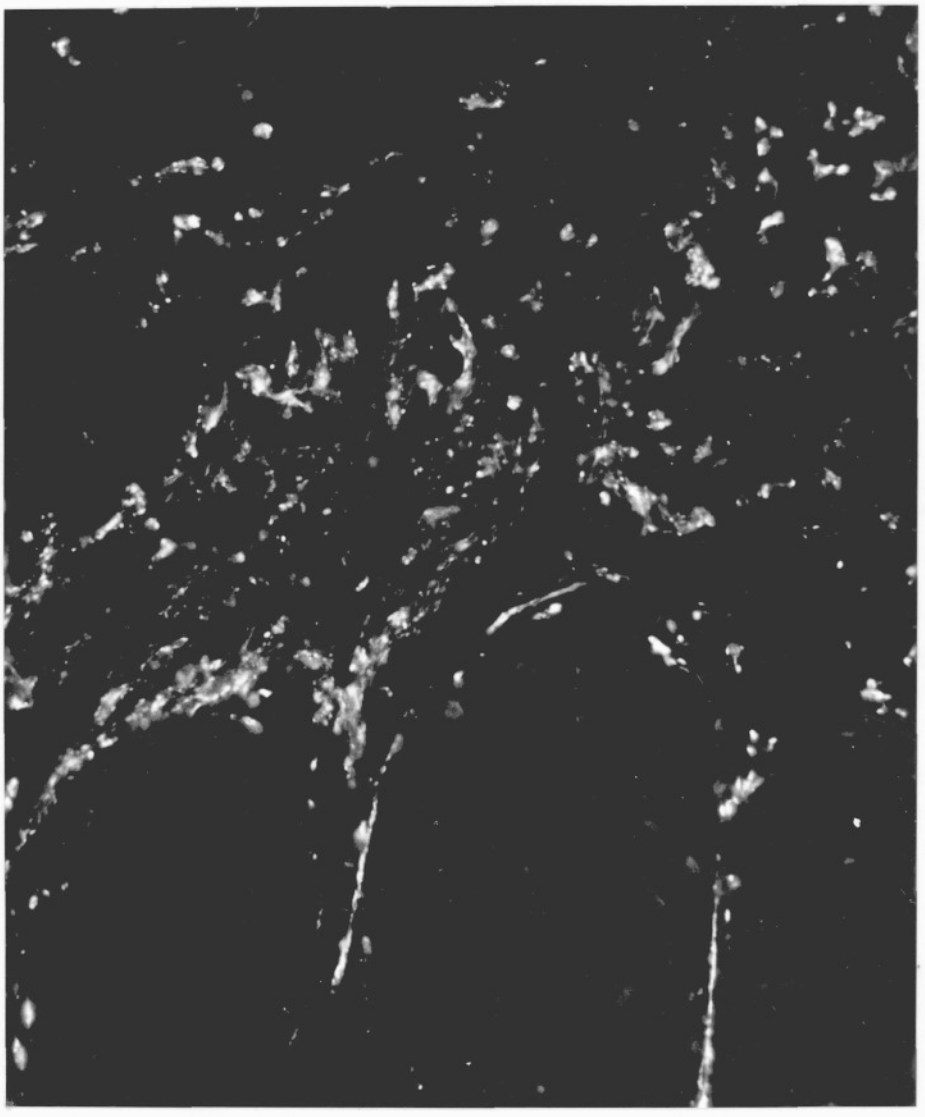
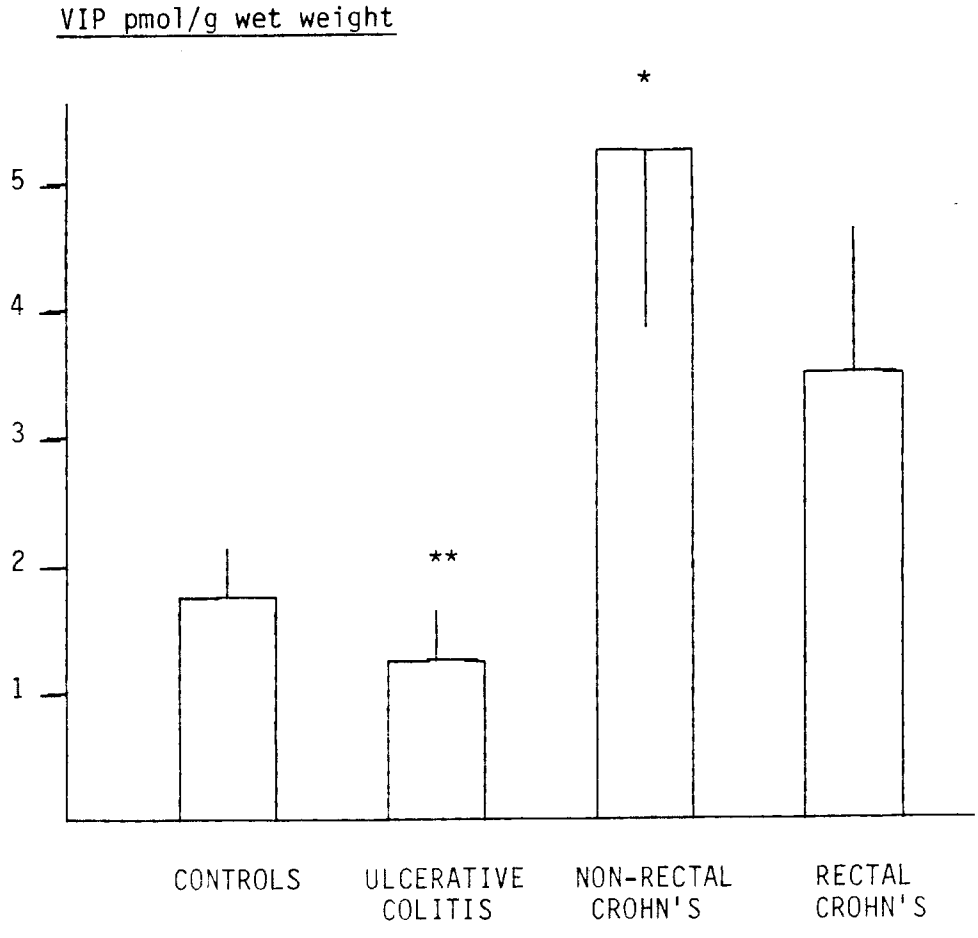


Table 11

VIP CONTENT OF RECTAL BIOPSIES



*= P/0.02 compared with controls
**= P/0.01 compared with non-rectal Crohn's

involvement. This means that evidence for the presence of Crohn's disease can be obtained from biospies which display no pathological changes on conventional histology.

All of the biopsies from patients with ulcerative colitis had levels of VIP within the normal range. On immunocytochemistry, six cases showed an increase in VIP innervation. However, examination of the histology of these biopsies revealed that they were clearly from patients with ulcerative colitis and not Crohn's disease.

Comparison of the VIP content of the biopsies showed that there was a statistically significant ($P < 0.02$) increase in tissue from patients with non-rectal Crohn's disease but not in those from patients with rectal involvement, although immunocytochemistry demonstrated marked VIP nerve hyperplasia in these specimens. This apparent disparity may be explained by the presence of areas of inflammatory infiltrate and oedema in the diseased recta, which may have diluted the total content of VIP in the tissue.

Radioimmunoassay showed no significant difference between VIP levels in the ulcerative colitis tissues and the normal controls. There was, however, such a difference between VIP in ulcerative colitics and biopsies from patients with non-rectal Crohn's disease, a finding which emphasises the obvious disimilarity between the two groups.

The significance of the VIP nerve hypertrophy in Crohn's disease remains speculative. As for the surgical specimens (Bishop et al, 1980[b]), comparison of the changes in VIP innervation with histologial and clinical data revealed no absolute correlation. The findings do not

entirely disprove the previous suggestion that the transmural inflammation characteristic of Crohn's disease may act as an irritant stimulus for the development of the apparent VIP nerve hypertrophy. However, an interesting feature of this hypertrophy, observed during the studies of surgical specimens, is that it is present in histologically normal bowel adjacent to the areas of maximal pathology. The results of the rectal biopsies support this previous finding by showing consistent increases in VIP nerves in normal recta from patients with Crohn's disease. This finding suggests that some stimulus exists other than transmural inflammation. Indeed, the results reveal that the involvement of the gastrointestinal tract in Crohn's disease is more extensive than has previously been shown using conventional histological methods. This may account for the high rate of recurrence of the disease following surgical excision, even though the ends of the resected bowel may appear normal.

Rectal biopsy remains a safe and convenient source of tissue and the use of a single immunostain, as described here, may provide a useful, cheap aid to routine diagnostic procedures in inflammatory bowel disease.

CHAPTER 7 EQUINE GRASS SICKNESS

INTRODUCTION

Grass sickness is a fatal disease of horses, the aetiology and pathogenesis of which have remained largely unknown. It is thought, however, from experimental evidence, that the characteristic gut paralysis is secondary to degeneration of extrinsic autonomic ganglia (Gilmour, 1973) and that a blood-borne neurotoxin is involved (Gilmour & Mould, 1977).

Clinically acute, sub-acute and chronic forms of the disease have been described largely as different expressions of "sympathicotonia" (Grieg, 1928). There is no known method of prophylaxis or treatment and, at the moment, the prognosis is hopeless in the acute cases and only a very small number of sub-acute and chronic cases recover. The acute cases die within 48 hours and sub-acute and chronic cases may survive for three weeks or longer, when they die from exhaustion (Keith, 1928).

The predominant gastrointestinal features of the disease are bowel stasis and massive inpouring of fluid into the stomach and small intestine (normal mean fluid volumes of 9 and 7 litres in stomach and small intestine respectively are increased to 22 and 30 litres). The colon becomes greatly dilated and compacted with dry faeces, covered in a characteristic layer of black mucus.

It seemed highly likely, in the light of the findings made in human disease, that in this gross disruption of normal bowel function there may be some peptidergic nerve abnormality. Although previous studies

(Gilmour, 1973) suggested that the major cause of the bowel disease was pathological changes in the extrinsic ganglia supplying the gut, there has been at least one description of intrinsic neuronal damage (Obel, 1955). Grass sickness in horses therefore presented an ideal example of an animal disease where lesions of enteric peptide containing nerves may be anticipated. It was chosen for study as purely an animal disease and not as a model for human disease.

As well as examining peptide containing nerves and cells in horses with the three different forms of grass sickness, compared with normal controls, tissue was also taken from horses with colic. In each case, the colic was due to small intestinal obstruction. It was important to examine tissue from these animals, to see if any detectable damage to peptide containing nerves in grass sickness may be just a secondary effect of obstruction, possibly related to faecal impaction.

This study was carried out in collaboration with the Royal Veterinary College, Camden Town and Potters Bar. Collection and examination of material took place over a period of two years.

MATERIALS

Five groups of horses were studied (Table 12). Specimens of each region of distal gut (proximal and distal ileum, right ventral and right dorsal colon, and rectum) were removed at post-mortem examination, within two hours of death, from the horses with grass sickness and normal controls. These areas were chosen for study because they show the most marked functional changes in grass sickness. Fresh surgical specimens of ileum were obtained, from pre- and post-obstruction regions, from horses with colic.

Table 12

Horses studied	No.
Acute (hours to 2 days) grass sickness	3
Sub-acute (2 to 14 days) grass sickness	9
Chronic (14 days or more) grass sickness	3
Colic (small intestinal obstruction)	3
Normal controls (age and breed matched)	9

For conventional histology and immunocytochemistry tissue samples measuring no more than 2 x 2cm^2 were fixed in benzoquinone solution (appendix). Similar areas were also analysed by radioimmunoassay of peptide content. Cryostat sections of fixed tissue were immunostained for regulatory peptides, NSE and S-100 (appendix).

Results

Histology

Controls: The normal control tissues were found to have no histological signs of gastrointestinal disease nor of degenerative changes due to autolysis or fixation. Large numbers of plasma cells and eosinophils, a feature of horses, were found in all specimens, whether normal or diseased.

Colics: None of the samples from sick animals showed gross abnormalities. As in the controls, no degenerative changes were observed which could be attributed to sampling or fixation methods. Some hypertrophy of the longitudinal and circular muscle layers could be seen in the ileum of the colics. Specimens from colics and grass sickness horses were found to have certain changes in common. These included oedema, either confined to the mucosa and submucosa or transmural, inflammatory infiltration, infrequently permeating through to the serosa, fibrosis, mainly of the submucosa, and congestion.

Grass sickness: As well as the features mentioned above, the grass sickness specimens also showed slight variations in the prominence of ganglion cells. There was no apparent relationship between inflammatory changes and ganglion cells.

Immunocytochemistry

Controls: Neuron specific enolase (NSE) and S-100 immunostaining revealed large numbers of nerves and supporting cells distributed across the whole thickness of each different area of gut. In the two main ganglionated plexuses, large, rounded ganglion cells were shown by NSE antibodies in groups of six or more in the myenteric plexus and in smaller numbers in the submucous plexus. Glial and Schwann cells showed normal morphology and distribution. Weakly NSE immunostained mucosal cells could be discerned in the ileum.

A pattern of regulatory peptide distribution was seen in the normal horse gut which was similar to that observed in other mammalian species (Chapter 3). A network of peptide containing nerves infiltrated each layer of the gut wall. The most abundant peptides found in these nerves were substance P and VIP. Unlike most other mammals, including man, where VIP nerves are most frequent, in equine ileum, colon and rectum, substance P and VIP nerves were equally numerous. A very minor population of met-enkephalin containing fibres was also found in each region of gut, mainly in the myenteric plexus. VIP nerves were present mostly in the circular muscle, myenteric and submucous plexuses and in the mucosa. They were also found, typically, around blood vessels. VIP-immunoreactive ganglion cells occurred only in the submucous plexus. Immunoreactive fibres were all slender and varicose, except where they emitted from ganglion cells. At this site a ribbon-like structure was most typical. The immunoreactive ganglion cells were, generally, round or ovoid with a large nucleus. Two or three immunoreactive ganglion cells were seen in each group of cells.

Substance P-immunoreactive fibres were particularly prominent in the myenteric plexus and circular muscle. No immunoreactive ganglion cells were found and substance P nerves were less numerous in the submucosa and mucosa than those containing VIP. No other fibres containing peptides were detected. This pattern of distribution of peptide containing nerves was consistent throughout the ileum, colon and rectum.

Peptide containing mucosal cells, on the other hand, showed a variable distribution. In ileum, cells containing neurotensin, somatostatin or enteroglucagon were present in approximately equal numbers. These all had an apical shape with the somatostatin and enteroglucagon cells sometimes showing luminal and/or basal elongations. In the colon and rectum, enteroglucagon cells were by far the most numerous cell type. Somatostatin cells were also present but no neurotensin cells were found.

Colics: The regulatory peptide containing nerves and cells in the ileum of horses with colic were indistinguishable from those of the normal controls. They all had a similar distribution and were brightly immunostained. NSE and S-100 immunostaining revealed no change in the overall level of innervation, nor in the glial and Schwann cell populations.

Grass sickness: The tissues from horses with grass sickness, whether in an acute, subacute or chronic form, had undergone a striking change in the level of innervation. NSE-immunoreactive nerves were slightly reduced in number but not in the degree of immunostaining. Ganglion cells could still be seen in both main plexuses, but in smaller numbers. There was no marked loss of glial or Schwann cells, as

demonstrated by S-100 immunostaining. The changes in peptidergic nerves were more drastic. In the ileum, VIP and substance P nerves were virtually absent. A few fibres could be found in the myenteric plexus, circular muscle and mucosa (Fig42). In addition, some VIP nerves were seen around non-immunoreactive ganglion cells in the submucous plexus.

Met-enkephalin immunoreactive fibres were only rarely seen. The ileum showed the most marked loss of peptidergic innervation. Although a loss of nerves was also seen in the colon and rectum, it was not as great. In the large bowel, as in the ileum, the remaining peptidergic nerves were mainly in the plexuses, circular muscle and mucosa, but no VIP-immunoreactive ganglion cells were found. Although peptide containing nerves were reduced in number throughout each diseased specimen, the ones which remained were brightly immunostained.

In the mucosal epithelium, there appeared to be some reduction in the number of peptide containing cells. However, this was neither as striking nor as consistent as the alteration in peptidergic nerves. The cells were never completely absent and their distribution and relative proportions did not alter.

Radioimmunoassay

Radioimmunoassay was carried out on samples from normal controls and grass sickness horses only, as too few colics were available for full biochemical investigation. Significant loss of VIP ($P/0.001$) and substance P ($P/0.02$) was found in the samples of ileum from sick horses, compared with the controls. VIP was also significantly ($P/0.01$) lower in the colon. Rectal content of neuropeptides in the diseased horses was lower than in controls but did not reach statistical

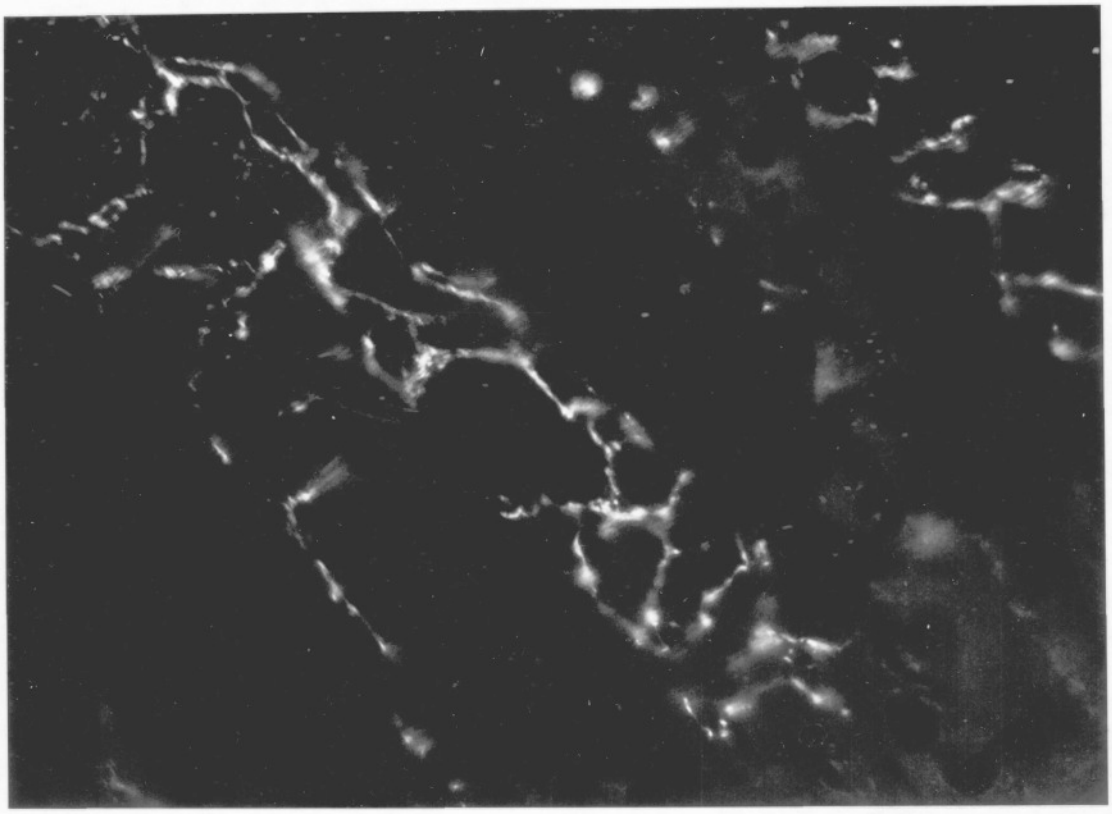
FIG 42

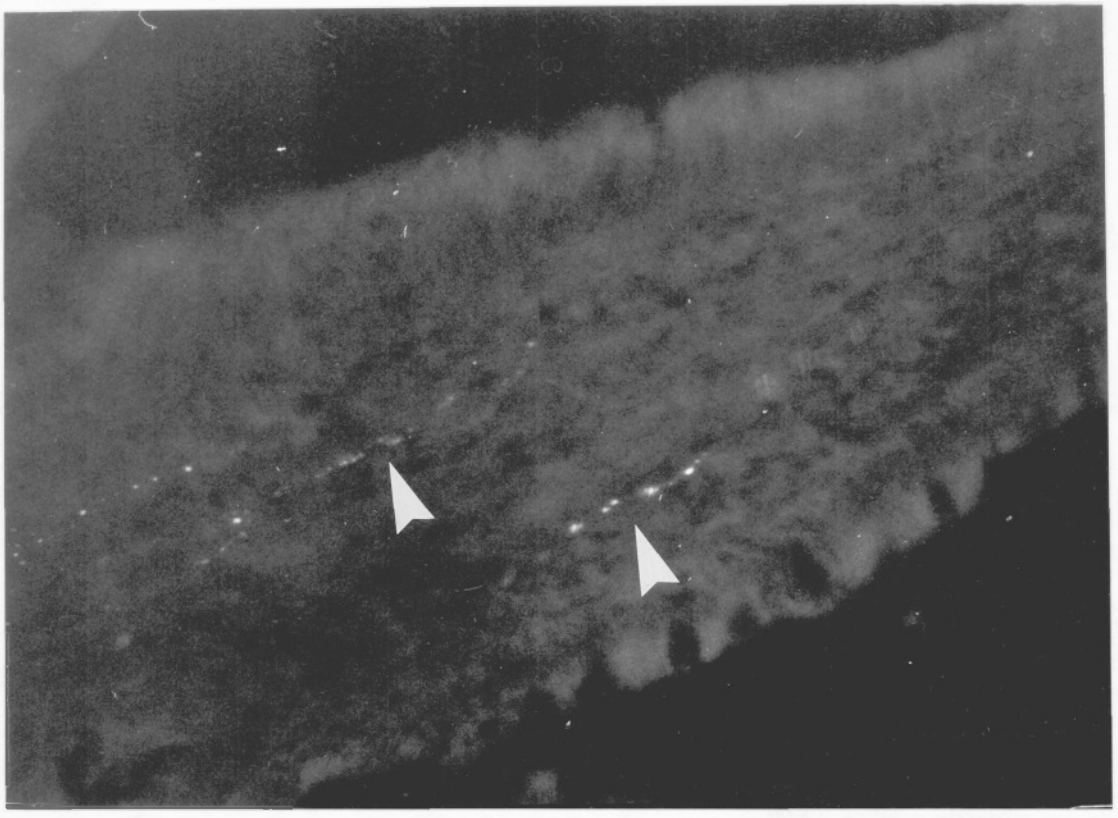
Ileal mucosa immunostained for substance P taken from:

a) a normal horse

b) a horse with grass sickness. Very few immunoreactive fibres can be seen (arrows) (x 350).

Tissue fixed in BQS, cryostat section, indirect immunofluorescence.





significance. No significant changes were found in peptide (enteroglucagon and somatostatin) content of mucosal cells in any region (Table 13).

DISCUSSION

In horses with grass sickness, whether an acute, sub-acute or chronic form, there was a consistent loss of regulatory peptide containing autonomic nerves in the distal bowel, particularly in the ileum. The general level of enteric innervation, as shown by immunostaining of NSE, was slightly lower than normal in grass sickness. However, ganglion cells could still be seen in the two main plexuses. This indicates that the cells were not all destroyed by the disease process. This finding and that of the peptide containing nerves being almost totally absent from the diseased bowel suggests a specific lesion of peptidergic nerves. Radioimmunoassay provided support for the morphological results by measuring significant loss of peptides from the ileum. In addition to the nerve abnormalities, there was also some reduction in mucosal endocrine/paracrine cells, although this was too small to be detected by radioimmunoassay of peptide content.

A similar alteration of the enteric regulatory peptide containing system was found in human diseases. In both congenital and acquired megacolon, Hirschsprung's (Bishop et al, 1981) and Chagas' (Long et al, 1980)(Chapter 5) disease, respectively, both peptide containing nerves and cells are reduced. It was concluded that, as both diseases involve absence or degeneration of intramural ganglion cells, the lack of peptide containing nerves is evidence for their predominantly intrinsic origin in the human gut. This intrinsic origin is also shown by the

TABLE 13

RESULTS OF RADIOIMMUNOASSAY MEASUREMENT OF PEPTIDE CONTENT

Peptide	Ileum		Colon		Rectum	
	Control	Grass Sickness	Control	Grass Sickness	Control	Grass Sickness
VIP pmol/g mean ± SEM:	109.2±19.8***	6.8± 1.4	85.5±16.5**	31.1± 4.9	121.0±34.9	45.6±11.8
Substance P pmol/g mean ± SEM:	65.9± 8.1*	31.3± 9.5	41.7± 7.1	27.8± 4.4	13.7± 3.9	13.6± 4.0
Bombesin pmol/g mean ± SEM:	5.4± 1.2**	0.6± 0.08	13.2± 2.0***	2.6± 0.7	5.0± 1.4	4.1± 1.4
Enteroglucagon pmol/g mean ± SEM:	78.6±12.6	85.1±16.9	22.2± 6.6	29.5± 4.5	108.3±27.5	53.6±18.9
Somatostatin pmol/g mean ± SEM:	39.6± 8.5	22.8± 2.2	17.9± 2.2	22.8± 5.4	105.5±35.2	29.7±10.5

P 0.001 ***
P 0.01 **
P 0.02 *

presence of large numbers of peptide immunoreactive ganglion cells in the two main intramural plexuses. It had previously been thought that the neuronal damage in grass sickness primarily concerned the extrinsic supply to the gut (Gilmour, 1973). However, the findings made in the present study, of peptide immunoreactive ganglion cells, suggest that in horses, as in humans and other mammals, a large proportion of the peptide containing nerves arise from intrinsic ganglion cells. Thus, the loss of these nerves in grass sickness indicates a lesion of the intrinsic nerve supply. In humans, in an example of pre-ganglionic autonomic degeneration the peptide containing innervation of the gut was unaltered (Long et al, 1980) (Chapter 5). The fact that there is a definite lesion of the enteric peptide containing nerves in grass sickness does not rule out the possibility of further peptide containing nerves arising from extrinsic sources. Immunocytochemical and radioimmunological investigations of relevant ganglia are therefore warranted.

Damage to ganglion cell bodies of the myenteric, and to a lesser extent the submucous, plexus has previously been observed in grass sickness (Obel, 1955). However, such findings have not been made consistently and, as is shown in the present study, it is not easy to detect these neural lesions with conventional histology at the light microscopical level. Staightforward immunostaining, as shown here, reveals clear abnormalities in the enteric nervous system.

The nature, but not the cause, of this damage has been further revealed by ultrastructural examination of the nervous supply (L Probert, personal communication). Ileal tissue from horses with sub-acute grass sickness was compared with similar tissue from

controls and horses with colic, using both conventional and immuno-electron microscopy. The most striking feature of the ultrastructural profiles of the enteric nerves in affected ileum was the almost complete lack of neuronal vesicles, particularly those shown by immunostaining to contain substance P and VIP. The ultrastructure of the ileum from horses with colic was normal. The absence of secretory vesicles from enteric nerves of horses with grass sickness explains the lack of immunostaining at the light miscoscopical level. As was mentioned above, NSE immunostaining demonstrated no great loss of innervation, although the peptide immunoreactive nerves were almost completely absent. Thus, the nerves were still present in the diseased bowel but their content of peptide had been lost. The most likely reason for the depletion of secretory vesicles is the release of peptides from the nerve endings and the almost total absence of vesicles suggests both a massive loss of peptides coupled with cessation of peptide production within the nerve. Electron microscopy showed that peptide containing vesicles were lost from the ileum, without any other degenerative changes, indicating that release of peptides from nerves may form one of the earliest morphologically identifiable events in grass sickness.

A massive release of peptides over a short period of time would certainly contribute to a large extent to the development of many of the gastrointestinal features of grass sickness. The large quantities of fluid found in the stomach and small intestine may be secreted as a direct result of VIP release. Copious water secretion is a characteristic feature of over-release of VIP, as is illustrated by the VIPoma syndrome (Bloom et al, 1973)(Chapter 1). As VIP and substance P both cause vasodilation, they may be responsible for the congestion

which is a frequent post-mortem finding in the gut of horses with grass sickness. In addition, as VIP and substance P containing nerves appear to form an integral part of the peristaltic pathway, their hyperactivity, with subsequent inaction, would severely disrupt bowel motility. One of the previously unexplained features of grass sickness has been the lack of pain in sub-acute and chronic cases, despite the huge distension of the gut. In colic, for example, the horses experience severe pain. As substance P is thought to be a sensory neurotransmitter, the lack of substance P nerves in grass sickness may explain the absence of pain.

The patchy reduction in the mucosal endocrine/paracrine cells may be related to the general loss of bowel activity. It may also be a consequence of the neural degeneration specifically. As previously discussed (Chapter 5), reduction of mucosal cell turnover following autonomic denervation has previously been reported (Lachat & Goncalves, 1978) and parallel loss of peptide containing nerves and cells seems to be a phenomenon of certain human gastrointestinal diseases (Bishop et al, 1981). Whatever the cause, lack of peptides produced by cells may further contribute to the bowel dysfunction, as each peptide is a potent modulator of gut activity.

Although the exact aetiology remains obscure, these findings fit well with previous concepts of grass sickness being caused by absorption, in some way, of a neurotoxin (Gilmour, 1973). If this neurotoxin exists and is ingested by the horse, it may mean that the enteric nerves are first affected, leading to development of the gastrointestinal symptoms, with the generalised neural damage occurring after full absorption of the toxin into the blood. No effective

treatment has been developed for this disease. If the loss of regulatory peptides from the gut, whether primary or secondary to the disease, can be compensated for by exogenous administration of peptides, then it may be possible to alleviate the debilitating gastrointestinal symptoms. This may not lead to a full cure, but could feasibly prolong the life of the animal. Early diagnosis of the disease, differentiating it from, in particular, colic, may be made possible by immunocytochemical examination of endoscopic rectal biopsies. Consistent loss of peptides, albeit less marked than in the ileum, could be detected in the rectal mucosa, thus providing the basis for a simple diagnostic test.

CHAPTER 8 GENERAL DISCUSSION

When substance P was discovered in 1931 and found to be present in both the brain and gut there was little idea of the existence of a whole range of different types of peptide containing nerves, forming what is possibly the largest component of the autonomic nervous system. Thus, this system can no longer be considered as bipartite in nature. It is now established that there are further divisions and a wealth of evidence indicates that peptides are neurotransmitters (or have a related function) in the majority of the non-cholinergic, non-adrenergic nerves. However, the autonomic nervous system is not simply tripartite, it is now thought to be far more complex than was previously believed. Many other substances, including ATP, dopamine and serotonin, have been suggested to be autonomic neurotransmitters and it seems that even the previous concepts of there being separate compartments (parasympathetic and sympathetic) in the system must be revised in view of the co-localisation and co-function of neurotransmitters.

The present study has examined the peptide containing nerves of the gastrointestinal tract and pancreas of man, in particular, and other mammals. The results reveal a highly integrated network of nerves, forming major components of the enteric and pancreatic nervous systems. As no specific means of demonstrating cholinergic and noradrenergic nerves was available during the time of this study, it was not possible to assess exactly what proportion of the total autonomic innervation contained peptides. The peptide containing portion of the autonomic nervous system seems to arise mainly from cell bodies intrinsic to the gut and pancreas, and thus appears, on

morphological evidence, to form largely autonomous units in these organs. This autonomy of the enteric nervous system is well established and was first demonstrated at the end of the nineteenth century by Bayliss and Starling (Bayliss & Starling, 1899).

All of the nervous and endocrine structures investigated in this work were demonstrated using immunocytochemistry. One of the main problems which exist when applying immunocytochemical techniques is tissue fixation. Many of the conventional fixatives, eg formaldehyde and glutaraldehyde, do not allow full preservation of peptide antigenicity. Although benzoquinone was established as a good vapour fixative of freeze-dried tissue (Pearse & Polak, 1975), its particular suitability as a fixative for immunostaining of peptide containing nerves has not been revealed previously. However, although benzoquinone solution gives good results in comparison with other presently available fixatives, the possibility of some loss of peptide immunoreactivity as a result of fixation damage can never be ruled out.

A further problem which is encountered when evaluating immunostaining is how the stain varies with the activity of nerves or cells and this is one of the main limitations of immunocytochemistry. Obviously it is not possible to identify a nerve or cell which is capable of producing a certain substance if sufficient amounts of the substance are not present at the time of the immunostain. Assuming that tissue fixation was suitable several things must be taken into account. Firstly, the sensitivity of the antisera must be considered. A particularly good, highly sensitive antiserum may detect low levels of a peptide in a nerve which may not be revealed by a less sensitive antibody. Another factor which may limit immunocytochemical detection of a peptide may be the synthesis of the bioactive molecule.

A nerve may produce the substance to be immunostained but if it is storing the peptide in a precursor form, the antigenic part of the molecule may be somehow masked from the antibodies by the tertiary structure. This point of molecular heterogeneity may explain why some of the results presented here are not in full agreement with those of other workers, eg no nerves were found to contain neurotensin or motilin. It is likely that the two sets of antibodies used did not recognise exactly the same molecule. The range of antibodies used in this study was characterised as far as possible, depending on the availability of peptides and their fragments, in order to demonstrate what molecule each antibody recognised.

Cellular activity will also affect the level of peptide immunoreactivity. Demonstration of the cell bodies of these nerves is often difficult as the peptides produced in the soma are rapidly transported to the nerve fibres and terminals. Several questions are thus raised. For example, does an apparently high concentration of a peptide in a nerve mean that it is not active and is therefore storing the peptide or that it is being produced in large amounts as it is rapidly released? Conversely, does a lack of peptide immunoreactivity mean that the nerve is dormant or that the peptide is being released so rapidly that it cannot build-up? Clearly, light microscopical studies are too limited to answer these points and ultrastructural investigations may clarify the situation by showing a relationship between nerve/cell activity and levels of peptide immunoreactivity. Meanwhile, it is still difficult to say confidently whether a lack of immunostaining of a certain peptide containing nerve means that the nerve is not there or that it contains too little peptide to be immunostained. In the present work, it has been possible to conclude

that lack of immunostaining of peptide containing nerves probably means a loss of the nerves in, for example, Hirschsprung's and Chagas' disease as other evidence shows that there is nerve loss. Furthermore, in this thesis, the results of radioimmunoassay measurement of peptide content of tissues was found to correlate well with the degree of immunostaining. These data provide support for the immunocytochemical findings, thereby allowing more convincing speculation to be made on the possible effects of the observed abnormalities. Electron microscopical observations have also been included in order to corroborate the light microscopical results and these provide further insight into the nature of the pathological changes.

When evaluating the significance of any immunostain, the specificities of the antisera must be clearly defined. In this way, the routine methods of omitting the primary antiserum, applying the non-immune serum instead of the primary antiserum etc were used. Most importance is attached to the successful quenching of the immunostain under test by the addition to the antiserum of its corresponding antigen and this was done for all the peptide antisera used in this study. In view of the possibility of non-specific saturation of the antibodies by excess antigen, in each case the antibodies were tested by addition of a range of concentrations of antigen, from 20nmol/ml diluted antiserum to 0.001nmol/ml. The successful quenching of the immunostains was underlined by the fact that the immunostaining returned, not always to the same degree obtained with unabsorbed antisera, when only very low concentrations of antigen were added. Where possible, further characterisation of the antisera was carried out by testing them against fragments of peptide molecules. It was particularly important to do this in cases where there was sequence

homology of the peptides. For example, subsance P and bombesin have a common C terminus. Thus, an antibody shown to recognise only the N terminus of the bombesin molecule will not cross-react with substance P.

In themselves, the morphological studies performed in this thesis cannot prove or disprove the possible neurotransmitter nature of the peptides. They, of course, show the peptides to be present in nerves, thereby satisfying one of the major criteria for neurotransmitter identification. In addition, the frequent finding of particular peptide containing nerve types in association with tissues which are known to be affected by the peptides strengthens the argument for them having an active physiological role. However, as was pointed out in Chapter 1, the previously narrow concepts of what substances in nerves can do are being broadened. The peptides present in nerves may be neuromodulators; they may even act in a neurocrine manner, releasing peptide into the bloodstream to act in a classical endocrine way. These new ideas on nervous physiology reflect the recognition, during the past few years, that the nervous and endocrine systems are not entirely separate entities and that they show considerable overlap and may even form a single unified system, the diffuse neuroendocrine system. Again, the results presented in this thesis provide some substantiation for this concept by showing peptides in nerves and in endocrine (or paracrine) cells, eg somatostatin, and also by demonstrating the same isomer of the glycolytic enzyme enolase, neuron specific enolase, in all types of nerves and endocrine cells of the gut and pancreas.

The possible co-existence of neurotransmitters (or at least substances present in nerves) is a highly controversial matter, mainly because of the present inadequacy of methods for the demonstration of cholinergic and adrenergic nerves. In the present work, because of the lack of appropriate methods, it was not possible to establish the co-existence of peptides with classical neurotransmitters, although VIP could be demonstated in acetylcholinesterase containing neuronal cell bodies, a proportion of which were probably cholinergic. It was, however, shown that the two peptides, VIP and PHI, co-exist in submucosal ganglion cells. By revealing their differential distribution in the fundic region, it was also possible to show that the synthetic pathways of VIP and PHI must be separate.

At the moment, the significance of the observed alterations in peptide containing innervation in gastrointestinal diseases is difficult to ascertain. Taking into account the previously specified limitations of immunocytochemistry as a method for demonstrating these nerves, it is only really possible to state that morphologically identifiable,and possibly characteristic, abnormalities of these nerves occur in certain diseases. The fact that, in most of the diseases, very similar changes occur in different individuals with the same condition, gives considerable strength to this statement. The main objective of the work presented here has been to provide a basis for the use of immunocytochemistry of peptide containing nerves in the diagnosis and study of gastrointestinal diseases. Although it has been possible to speculate on the contributions of the various pathological changes in these nerves to the development of each disease, it is the morphological changes which are, at this point, the most important aspect. The peptide containing nerves form a large component of the

enteric nervous system and each peptide is known to have potent actions. Therefore, the gross pathological changes described in this innervation would be expected to contribute substantially to the manifestation of each condition. However, when speculating on the extent of this contribution it must be remembered that existing knowledge of the mode and nature of action of the peptides is based largely on the results of pharmacological experiments on isolated preparations of normal animal tissues. It is therefore difficult to extrapolate with any real certainty to human pathological conditions. In view of these morphological findings, further light may be thrown on the contributions of neuropeptides to gut disease if physiological examination is made of affected tissues. Eventually, it may be possible to use peptides, or their antagonists, where necessary, in disease therapy. This, of course, must follow extensive trials, a somewhat daunting prospect in view of the potent and not fully understood effects of many peptides. However, application of peptides to horses is now being considered as a means for treating grass sickness, in order to alleviate the gastrointestinal symptoms. A further problem in giving neuropeptides as therapy will be the fact that they (most probably) act as neurotransmitters or neuromodulators. Therefore, their action is local, not via the circulation. It may be difficult to attain sufficient concentrations of the peptides at the sites of action, particularly as some of them appear to be rapidly degraded in the circulation.

These results may be helpful in the routine diagnosis of gastrointestinal disease. Antisera are becoming increasingly available commercially and so immunocytochemistry is no longer purely a research tool. Examination of surgical specimens and rectal biopsies revealed

distinct changes in peptide containing nerves in cases where conventional histology did not always provide conclusive information. Thus, routine immunostaining of surgical specimens will help to confirm or disprove diagnoses, thereby aiding determination of the prognosis and subsequent treatment. The additional value of immunostaining is in the examination of endoscopic rectal biopsies, a safe and convenient source of tissue. It is also a cheap diagnostic method and rarely causes distress to the patient. In the case of Chagas' and Crohn's disease, immunostaining of peptides in rectal biopsies provides invaluable information. However, this whole area of research is still in its infancy and it may be some time before the full value of these findings is known. Hopefully, correlative physiological and pharmacological studies carried out by other workers will soon advance the field to a stage where clinical applications can be made.

CONCLUSIONS

The purpose of this study was to examine, using immunocytochemistry, the peptide containing nerves of the gastroenteropancreatic system of man and other mammals in normal conditions and to see if changes occur in disease states. In addition, the localisation of two proteins, neuron specific enolase and S100, to neural and endocrine components of this system was also carried out. The following conclusions can be drawn from the results obtained:-

a) Tissue fixation in benzoquinone solution is a straightforward technique which appears to give suitable tissue and antigen preservation for peptide, amine and enzyme immunocytochemistry and for other histological and histochemical stains.

b) Peptide containing nerves form a large part of the enteric and pancreatic innervation and appear to arise mainly from intrinsic neuronal cells bodies within these tissues.

c) The distributions of the different types of peptide containing nerves within the gut and pancreas is in general agreement with what is known about the actions of each peptide. This provides morphological evidence supporting their postulated physiological roles.

d) Distinct, possibly characteristic, abnormalities of these nerves occur in certain diseases. The easily applied techniques of immunocytochemistry allow identification of these changes and thus may be useful in diagnostic histopathology, augmenting the information obtained from routine histological examination of specimens. The finding of clear-cut changes in peptide containing innervation in rectal biopsies in at least two of the conditions studied may further aid diagnosis of gut diseases as these specimens are an easily accessible source of material.

e) Neuron specific enolase (NSE) is present in identifiable nerves and endocrine cells of the gut and pancreas and may, therefore, be a useful marker for these structures. S100 appears to be in Schwann and glial cells in the gut and pancreas and may be a marker for these structures.

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APPENDIX 1

INDIRECT IMMUNOFLUORESCENCE TECHNIQUE

After Coons et al, 1955

1) Sections were mounted on glass slides and allowed to dry (overnight for paraffin wax embedded tissue or 1 hour for cryostat sections).

2) If wax-embedded, sections were dewaxed by immersion in xylene followed by washing in alcohol. Antisera were applied directly to cryostat sections. The glass slides were placed on racks in Petri dishes containing a small amount of water to provide a moist atmosphere. Appropriately diluted antisera (various sources, see Chapter 2) were applied to the sections in sufficient quantities to cover the sections without spreading over the surface of the slide. In this study all the antibodies used were raised in rabbits.

3) The first layer antiserum was used as dilute as possible and the sections are left overnight at 4'C to incubate. After incubation the sections were washed in 3 changes (5 minutes each) of phosphate buffered saline (PBS)(0.01M, pH 7.1-7.4). All antibody dilutions were also made in PBS.

4) After washing, fluorescein-labelled anti-rabbit globulin (Miles Labs), appropriately diluted in PBS, was applied. The sections were left to incubate at room temperature for 1 hour and then washed in PBS as before.

5) The sections were then mounted in PBS:glycerine (1:9 parts) and examined under a UV microscope.

APPENDIX 2

PEROXIDASE ANTI-PEROXIDASE TECHNIQUE

After Sternberger, 1974

1) Section preparation was the same as for the indirect immunofluorescence technique. When ready, sections were immersed in a solution of 0.3% H_2O_2 (1ml of 100 volume (30%) H_2O_2 in PBS) for 30 minutes to block endogenous peroxidase.

2) After rinsing in 3 changes of PBS (5 minutes each), possible background staining was blocked by application of normal goat serum (1:30) for 10 minutes. After this the sections were not rinsed but the goat serum was taken off with a pipette.

3) The highly diluted primary antiserum was applied and left overnight at 4'C to incubate. The sections were then washed in 3 changes of PBS as before.

4) A second layer consisting of unconjugated goat anti-rabbit globulin (Miles Labs) was placed on the sections at a dilution of 1:200 for 30 minutes at room temperature. The sections were then washed as before.

5) The final layer was the PAP complex (UCB Bioproducts) which was applied at 1:300 for 30 minutes. After washing as before the sections were developed by immersion in diaminobenzidine (DAB) solution (25mg of DAB in 100ml of PBS plus 100ul of 100 volume (30%) H_2O_2). The appearance of the brown reaction product occurred usually within a few minutes and so this stage was carefully monitored.

APPENDIX 3

FIXATION IN BUFFERED FORMALIN

1) A solution of 10% buffered formalin (or formol saline) was prepared using 1 part of commercial formalin (36%) to 9 parts of 0.01M phosphate buffered saline (PBS)(pH 7.1-7.4).

2) This solution could be prepared in bulk and stored prior to use.

3) Allowing a tissue to solution volume ratio of at least 1 to 10, tissue was fixed by immersion for the chosen time (see Chapter 2) or used as a perfusion fixative. If the tissues were perfused, they were also immersed in fixative afterwards.

4) Following fixation, tissues were rinsed, at least overnight, in a solution of 7% sucrose and 0.01% sodium azide in PBS. The tissues were then embedded in wax or made into cryostat blocks.

APPENDIX 4

FIXATION IN BOUIN'S SOLUTION

1) A stock solution was prepared from the following:

Saturated aqueous picric acid	75ml
Commercial formalin (36%)	25ml
*Glacial acetic acid	1ml

*The acetic acid was added just prior to use of the fixative.

2) After immersing the tissues in the fixative for the prescribed time (see Chapter 2) or perfusing the whole animal and then immersing tissues, they were allowed to rinse, at least overnight, in 30% 66 O.P. alcohol in deionised water, before being embedded in wax or made in cryostat blocks.

APPENDIX 5

FIXATION IN 3% GLUTARALDEHYDE

1) A stock solution of 3% glutaraldehyde was prepared from 25% Ultra-Pure glutaraldehyde in 0.1M phosphate buffer (ph 7.2-7.4).

2) After immersion, or perfusion and immersion, fixation in this solution for the prescribed time (see Chapter 2), the tissues were rinsed in washing buffer. This buffer solution consisted of 0.1M phosphate buffer containing 3.4% sucrose and 0.01% sodium azide. This method was a standard technique for fixation of tissue for electron microscopy in the Department of Histochemistry.

3) After rinsing, at least overnight, the tissues were wax embedded or made into cryostat blocks.

APPENDIX 6

FIXATION IN METHANOL-FREE FORMALDEHYDE

1) A stock solution was prepared in the following way:

   a 4gm of paraformaldehyde was dissolved in 25ml of distilled water, pre-heated to 69'C.

   b 4 or more drops of 1N NaOH were added until the precipiate in the solution cleared.

   c The pH was adjusted to 7.4 with NaOH.

   d An equal volume of 0.1M phosphate buffer (pH 7.4) was added.

2) Tisses were fixed by immersion in or perfusion with the solution, followed by immersion for the prescribed time (see Chapter 2). After fixation, they were washed, at least overnight, in a solution of phosphate buffered saline (PBS)(pH 7.1-7.4) containing 7% sucrose and 0.01% sodium azide. The tissues were embedded in wax or made into cryostat blocks.

APPENDIX 7

WAX-EMBEDDING OF LIQUID-FIXED TISSUE

1) After thorough rinsing overnight in 30% 66 O.P. alcohol in de-ionised water, the tissues fixed in Bouin's solution were transferred into fresh 30% alcohol and left on a roll mixer for 30 minutes.

2) They were then placed in 50% 66 O.P. alcohol in de-ionised water for a further 30 minutes.

3) After this Bouin's fixed tissues were placed in two changes of 70% alcohol, 45 minutes each change. All the tissues which were rinsed in buffer solution entered the dehydration process at this point.

4) The two changes of 75% alcohol were followed by two of 90% alcohol, each for 60 minutes.

5) The 90% alcohol was replaced with three changes of absolute alcohol, 2 hours each change.

6) Tissues were then transferred into xylene for two changes of 90 minutes.

7) Finally, the tissues were embedded, under vacuum in Fibrowax in a 60'C oven.

APPENDIX 8

CRYOSTAT BLOCK PREPARATION

1) Fixed tissue was blotted dry on postlip fibre-free paper before being frozen in case any excess water caused ice crystal damage. Pieces of tissue were trimmed so as to be no wider than the cork mat (standard size of 2.2cm) and no taller than the distance between the end of the cryostat microtome arm and the knife (about 1cm).

2) Tissue was orientated so that transverse sections of the gut wall could be obtained. If the gut was thin (eg gut from a small mammal) or if endoscopic rectal biopsies were to be sectioned, liver was used as a support medium. Whole pieces of bowel or small pieces in a block of liver were stuck to the cryostat corks using OCT compound (Raymond Lamb).

3) The blocks were frozen by inverting them and dropping them into a beaker of Arcton, suspended in liquid nitrogen. The block was agitated slightly in the Arcton so as to disperse any warm gaseous nitrogen which might have formed around the block.

4) Before being sectioned, the blocks were placed in a cryostat at -20'C to allow the tissue temperature to rise.

APPENDIX 9

PERFUSION METHODS

1) Tubing and cannula were attached to the rotating head of a perfusion pump (Watson Marlow Ltd) and the free end of the tubing placed in phosphate buffered saline (PBS)(ph 7.1-7.4).

2) The animal was killed by exposure to ether fumes in a sealed container and immediately laid supine on a cork dissection board where it was spread with pins through its paws. For perfusion via the heart, the abdominal wall was opened and the diaphragm cut open to expose the heart. The perfusion pump was set at a constant slow speed (setting 10-20) and the cannula was inserted into the left ventricle and manoeuvred into the ascending aorta. The right atrium was cut.

3) For perfusion via the carotid artery (to perfuse the brain), the cannula was placed in the carotid artery, pointing caudally. The artery was ligated below the cannula and ligated around the cannula to hold it in place.

4) After washing through with buffer, in order to clear vessels of blood cells as much as possible (until the liver became pale), fixative was pumped through the animal. This was continued until fixative appeared in the effluent from the right ventricle and the animal stiffened visibly (at least 30 minutes).

5) The required tissues were dissected out of the animal and placed in fixative for the appropriate time (minus the time period during which fixative was perfused into the animal).

APPENDIX 10

BENZOQUINONE SOLUTION (BQS) FIXATION

Bishop et al, 1978

1) A fresh solution of purified benzoquinone was prepared in 0.01M phosphate buffered saline (PBS)(pH 7.1-7.4). A tissue to solution ratio of at least 1 to 10 was allowed, using specimens no larger than 2x2cm.

2) The PBS was added to the benzoquinone crystals and agitated until all the crystals dissolved. The BQS was prepared a maximum of 15 minutes before use in view of its rapid degradation.

3) Tissue was fixed by immersion for the appropriate time period (see Table 2) at 4'C away from the light (ie in a refrigerator).

4) After fixation, the tissue was transferred to PBS containing 7% sucrose and 0.01% sodium azide. This solution was prepared in bulk and stored at 4'C. If the washing buffer became discoloured, it was changed. The tissues were washed at least overnight. If they were not needed straightaway, the tissues were stored at 4'C for an indefinite period, with occasional checks for possible microbial contamination.

APPENDIX 11

CHOLINESTERASE STAIN

El Badawi & Schenk, 1967

1) Cryostat sections of tissue prefixed in 0.4% benzoquinone solution were allowed to air-dry.

2) They were then incubated at 37'C for 2 hours in the following:-

Acetyl thiocholine iodide	12.5mg
0.1N CH_3COONa	15.8ml
0.1N CH_3COOH	0.5ml
0.1M sodium citrate	1.2ml
30mM $CuSO.5H_2O$	2.5ml
4mM iso-OMPA	0.5ml
5mM potassium ferricyanide	2.5ml
Distilled water	2.0ml

The constituents were added in the order listed, not more than 30 minutes before use. The pH of the final solution was 5.5-5.6.

3) Following incubation, the sections were washed in distilled water, dehydrated, cleared and mounted in DPX.

APPENDIX 12

FORMALDEHYDE INDUCED FLUORESCENCE TECHNIQUE

Falck & Owman, 1965

1) Small pieces of tissue were frozen in Arcton, suspended in liquid nitrogen, and then freeze-dried at -40'C under vacuum overnight in an Edwards-Pearse freeze-drier.

2) Freeze-dried tissue was fixed in hot (80'C) formaldehyde vapour in a sealed jar placed in an oven. The fixed tissue was transferred to Ralwax under vacuum at 60'C and embedded.

3) Wax blocks were cut as soon as possible after embedding, to ensure minimal loss of fluorophore. Microtome sections of 5um were cut in a ribbon and picked up on warm glass slides. The slides were placed on a hot plate until the wax melted and then mounted in liquid paraffin. At no time were the sections allowed to come into contact with water, thereby losing fluorphore activity.

4) The sections were immediately examined under a UV microscope.

APPENDIX 13

DOUBLE IMMUNOPEROXIDASE STAINING USING ACID ELUTION

After Nakane, 1968

1) The first antigen was immunostained using the PAP method with the reactions sites revealed with DAB.

2) The first antigen/antibody complex was eluted by immersing the sections in 0.1M glycine/HCl buffer at pH 2.2 for one hour.

3) The sections were washed thoroughly in PBS and the second antigen/antibody reaction was carried out. This time, the peroxidase was developed with 4-chloro-1-napthol, which gives a blue precipitate easily distinguishable from the brown precipitate obtained with DAB.

4) The sections were mounted in PBS:glycerine (1:9) and not dehydrated as 4-chloro-1-napthol is soluble in alcohol.

APPENDIX 14

DOUBLE IMMUNOPEROXIDASE STAINING USING ELUTION BY OXIDATION

After Tramu et al, 1978

1) The PAP technique was carried out on fixed sections and the peroxidase was developed with 4-chloro-1-naphthol, which was used instead of DAB as it can be removed more easily. Photographs were taken of the immunostained cells and the reaction product was removed by dehydration and clearing of the sections through graded alcohols to xylene.

2) Following rehydration, the antibody complex was removed by immersion of the sections in a solution of potassium permanganate ($KMnO_4$) in sulphuric acid (H_2SO_4)(1 volume 2.5% $KMn=_4$ + 1 volume H_2SO_4 + 140 volume water, pH 1.8) for 1 minute with constant agitation.

3) The PAP technique was repeated and DAB was used as a coupler.

4) Photographs of the positive cells were taken and the distributions of the two immunostained substances were compared.

APPENDIX 15

SERIAL THIN SECTIONS

After Grube, 1980

1) Small pieces of tissue were frozen in Arcton and freeze-dried overnight at -40'C in an Edwards-Pearse freeze-drier.

2) Fixation consisted of exposure to benzoquinone or formaldehyde vapour at 60'C for 3 hours and the tissue was embedded by impregnation under vacuum in araldite resin including plasticiser (Araldite 1). This was followed by the same solution with hardener added (Araldite 11).

3) The tissue block was polymerised at 60'C and serial thin (0.5-2um) sections were cut on an ultramicrotome. Resin was removed from the sections by immersion in a saturated solution of sodium hydroxide in absolute alcohol, prior to immunostaining.

APPENDIX 16

PREPARATION OF BUFFER SOLUTIONS

0.01M Phosphate Buffered Saline (PBS)

A stock solution of buffer was prepared by making the following up to 1 litre with distilled water:

NaCl	8.7gm
KH_2PO_4	0.272gm
Na_2HPO_4	1.136gm

This 10x concentrate was diluted 1 in 10 in distilled water before use. The pH of the final buffer was 7.1-7.1.

0.1M Phosphate Buffer

A stock solution of buffer was prepared as follows:

Solution A - 0.2M NaH_2PO_4 was made up in distilled water (119.98g in 1 litre)

Solution B - 0.2M Na_2HPO_4 was made up in distilled water (151.99g in 1 litre)

57.7ml of solution A was mixed with 192.5ml of solution B to give 250ml of 0.2M phosphate buffer. This was then made into 0.1M by addition of 250ml of distilled water. The final pH of the buffer was 7.1-7.4.

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