DISTRIBUTION, LOCALISATION AND PATHOLOGY OF REGULATORY PEPTIDES IN MAMMALIAN GENITOURINARY TRACT, HEART, SKIN AND GALLBLADDER

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ABSTRACT

The occurrence and distribution of peptide-containing nerves and neuroendocrine cells in the urinary bladder, the male and female genital tracts, the heart, the skin and the gallbladder of several mammalian species including man was studied in both health and disease. The distribution of neuronal markers, such as neuron-specific enolase (NSE) and S-100, was also investigated for the purpose of comparison. The methods used included immunocytochemistry combined with radioimmunoassay, histology, histochemistry and electron microscopy. Seven peptides were detected in the organs, and their distribution patterns were examined, which often more indicative of their actions. Original findings made during these studies also included the discovery that the blood vessels in the penis were very densely surrounded by nerves containing the vasodilatory polypeptide (VIP). These nerves were found to be markedly reduced in quantity in penile impotence, thus suggesting VIP as the long sought neurotransmitter involved in penile erection. VIP-immunoreactive nerves were also decreased in the unstable bladder. Since VIP is known to have potent muscle relaxant properties, this finding may help to further elucidate the pathogenesis of this poorly understood disease. Schwann cell hyperplasia, demonstrated by an increase in S-100 immunoreactivity, in the dysfunctional bladder of patients with multiple sclerosis (MS) suggests that MS might involve not only the central but also

the peripheral nervous system. The heart was found to be innervated by a large number of nerves containing a newly discovered vasoconstrictor peptide, neuropeptide Y (NPY) as well as by nerves containing VIP and substance P. In addition to being present in nerves, somatostatin and bombesin immunoreactivities were demonstrated in mucosal endocrine cells in the guinea-pig biliary duct. Although no peptides have yet been identified in the neuroendocrine (Merkel) cells of the skin, these cells together with melanocytes and their derivative tumours were found to react to NSE or S-100 antibodies. Thus, immunostaining of NSE and S-100 provides a new approach to the identification of both cells and tumours which have hitherto presented formidable problems. The findings made in this study provide a new morphological basis for a better understanding of the regulatory peptides as a whole and of their significance in the investigated organs.

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ABBREVIATIONS

ACh	- acetylcholine
AChE	- acetylcholinesterase
ACTH	- adrenocorticotrophic hormone
APP	- avian pancreatic polypeptide
APUD	- amine procursor uptake and decarboxylation
ATP	- adenosine triphosphate
A-V	- atrial-ventricular
BQS	- benzoquinone solution
CCK	- cholecystokinin
ChAT	- choline-acetyl transferase
C-terminal	- carboxyl terminal
DAB	- di-aminobenzidine
DNES	- diffuse neuroendocrine system
DOPA	- dopamine
DβH	- dopamine-β-hydroxylase
FIF	- formaldehyde-induced fluorescence
FITC	- fluorescein isothiocyanate
FSH	- follicle stimulate hormone
GABA	- gamma aminobutyric acid
GIP	- gastrin inhibitory peptide
GRP	- goat anti-peroxidase
H&E	- hæmatoxylin and eosin
HPLC	- high performance liquid chromatography
5-HT	- 5-hydroxytryptamine
IF	- immunofluorescence
IR	- immunoreactive
KLH	- keyhole limpet haemocyanin
β-LPH	- β-lipotropin
MS	- multiple sclerosis
NA	- noradrenaline
NNE	- non-neuronal enolase
NPY	- neuropeptide tyrosine
NSE	- neurone-specific enolase

N-terminal - amino terminal
6-OHDA - 6 hydroxy-dopamine
PAP - peroxidase anti-peroxidase
PBS - phosphate buffered saline
PHI - peptide histidine isoleucine
PLL - poly-L-lysine
PYY - peptide YY
RIA - radioimmunoassay
RIG - rabbit immunoglobulin G
S-A - sino-atrial
SEM - standard error mean
SPG - sucrose-potassium phosphate- glyoxylic acid
TH - tyrosine hydroxylase
VIP - vasoactive intestinal polypeptide

Chapter 1: INTRODUCTION

1.1. BACKGROUND

The field of regulatory peptides is one of fascination and extraordinary complexity. After more than half a century of semi-standstill since the discovery of the first bioactive peptide, secretin (Bayliss & Starling 1902), the development of greatly improved chemical purification techniques in the sixties has led to the discovery and isolation of a series of bioactive regulatory peptides, which has in turn stimulated the relevant fields to grow at a dizzying rate.

Research has expanded the area of regulatory peptides to such an extent that it now seems that the peptides play a role of major importance in almost every organ. The peptides, some of which were originally found in the gut, are seen not only in endocrine cells of the gut but also in neurons. By 1980, when the present study was initiated, some 35 bioactive polypeptides had been shown to be present in the mammalian body and at least 22 of them were reported to occur both in endocrine cells and in nerves (Pearse 1980). Since then many more have been discovered. These peptides may act in a true endocrine manner, ie. the peptide is delivered via the blood circulation to the target tissue. They may be paracrine, in which case the peptide diffuses from the cell through intercellular spaces to its target. They may act as neurotransmitters, in which case they are released from neurons across a synaptic gap to the target, or they could have a neurocrine action, ie. the peptide is released from

neurons and reachs its target through the circulation (for review see Bloom & Polak 1981). A vast new field has been opened up for further investigation.

The improvement of another sophisticated technique, immunocytochemistry, which was initiated and subsequently modified by Coons and co-workers in the fifties (Coons & Kaplan 1950, Coons et al 1955), rapidly developed its potential when put to use in the localisation of the regulatory peptides. This technique, based on the principle of specific antibody-antigen reaction, is able to pinpoint the location of peptides not only to endocrine cells but also to neurons and their fine fibres at both light and electron microscopical levels (for reviews see Larsson 1982; Van Noorden & Polak 1983). As a result, a large amount of new information has been obtained and some 'classical' views have been challenged.

Many cells and nerves previously thought to be unrelated have now been linked together by their common feature, their regulatory peptide content. These peptides have divergent biological actions but seem to work together in a co-ordinated way (for review see Bloom & Polak 1981). Further evidence for the close inter-relationship between peptide-containing elements also comes from their ultrastructural similarities, their enzyme content and other common cytochemical features. To encompass these scattered and yet inter-related elements, the name 'diffuse neuroendocrine system (DNES)' has been commonly used to highlight the functional integrity of these nerves and cells (for review see Polak & Bloom 1979).

The root of the concept "DNES" dates back to half a century ago when Feyrter (1938) created the term "diffuse endocrine system" to define a group of scattered, poorly stained (clear) cells, which seemed to act, no matter where they were located, in a similar paracrine manner. This concept was later developed by Pearse (1966, 1969) who initiated the acronym APUD (amine precursor uptake and decarboxylation) to designate cell types, including the 'clear' cells, which are widely distributed in the body, but share the ability to synthesize biogenic amines from amine precursors. Certain common ultrastructural and cytochemical features, including the content of peptides, were assigned to these cells and it was postulated at the time that they might have a common embryonic origin (Pearse 1969). Though the hypothesis of a common origin has been subjected to criticism (for review see Le Douarin & Fontaine-Perus 1981), the internal linkage of these separate peptide-containing cells has been widely recognised. It was later found that the common features of the APUD cells are also shared by most of the neurons and vice versa. The field was therefore further widened to include the newly discovered, widely distributed and yet poorly identified nerves which also contain regulatory peptides.

1.2. PEPTIDE-CONTAINING NERVES

The first peptide to be localised in peripheral nerves was substance P (Pearse & Polak 1975a; Nilsson et al 1975; Hökfelt et al 1975a), although its discovery was made 44 years earlier (von Euler & Gaddum

1931). Almost simultaneously, somatostatin was found in the autonomic nerves (Hökfelt et al 1975b) and this was followed by vasoactive intestinal polypeptide (VIP) (Bryant et al 1976; Larsson et al 1976) and the enkephalins (Elde et al 1976). It is now common knowledge that such peptide-containing nerves exist in nearly every organ of the body, although many aspects concerning their distribution pattern, origin and relationship with adrenergic and cholinergic nerves have not been fully clarified. This is particularly true for a number of newly discovered neuropeptides.

It was long thought that the autonomic nervous system consisted, neurochemically, of cholinergic and adrenergic nerves, using acetylcholine (ACh) and catecholamines respectively as their neurotransmitters (for review see Williams & Warwick 1980a). However, evidence of the heterogeneity of this system, both functionally and morphologically, was presented more than a century ago. Heidenhain (1872) observed that the vasodilatory effect in the submandibular gland elicited by parasympathetic nerve stimulation was atropine resistant. A similar finding was made by Undritz (1929) in the nasal mucosa. Langley (1898) observed atropine resistant relaxation of the stomach after vagal nerve stimulation. A considerable amount of pharmacological and electrophysiological evidence has since been documented to show that non-adrenergic, non-cholinergic mechanisms are functioning in neurons of many peripheral organs, such as the gut (Ambache 1951; Biber 1973; Goyal & Rattan 1975; Gabella et al 1979), the respiratory tract (Coburn & Tomita 1973; Malm 1973; Coleman & Levy 1974; Änggärd 1974;

Richardson & Bouchard 1975; Richardson & Beland 1976; Chesrown et al 1980), the salivary glands (Bhoola et al 1965), the tongue (Hilton & Lewis 1958; Nagai & Pleschka 1981) and the genitourinary tract (Burnstock 1972; Lindblom et al 1979; Ottesen 1981; Ottesen & Fahrenkrug 1981; Bolten et al 1981).

The morphological divergence of the peripheral nervous system was also noticed by several observers. Nerve terminals, which are ultrastructurally distinct from the adrenergic and cholinergic types, were first described by Taxi (1965) in the amphibian gut. It was reported that there were nerves containing a high proportion of large (80-160nm in diameter) dense-cored vesicles which were quite different from the small, spherical or flattened, agranular and granular vesicles of the adrenergic and cholinergic neurons. This type of nerve was later seen in the mammalian gut and was then given the name 'p-type' (Baumgarten et al 1970), referring to the similarity between the large vesicles contained in these nerves and those present in the peptide-containing nerves in the hypothalamus (Bargmann et al 1967). Further studies on these neurons in the gut of guinea-pig (Gabella 1972, 1979; Cook & Burnstock 1976) and rabbit (Komuro et al 1982) suggested that morphologically different subpopulations of p-type nerves exist. Eventually it was confirmed, using the technique of immunocytochemistry, that these p-type nerves in both central and peripheral nervous systems contained a variety of peptides in their vesicles. In the peripheral nervous system, VIP was localised to p-type nerves in the colon (Larsson 1977a), substance P was localised in the taenia coli (Leander et al 1981)

and the ureter (Sikri et al 1981) and enkephalin was localised in the taenia coli (Leander et al 1981). More recently, using a colloidal gold-labelled antibody immunostaining technique (Varndell et al 1982), Probert et al (1981, 1983) localised substance P, VIP, somatostatin and enkephalin to different sized p-type vesicles and established that each of the peptides occurred in its own type of vesicle possessing a specific morphological profile.

The above findings, together with a considerable number of investigations on the neuronal distribution of peptide at the light microscopical level, have made regulatory peptides strong candidates for neurotransmitters or neuromodulators.

Certain criteria must be fulfilled to qualify putative candidates as neurotransmitters or modulators. A neurotransmitter is a chemical substance which is synthesised and stored in nerves and released upon stimulation to affect the activity of the effector cells through specific receptors for a limited period of time. A neuromodulator usually refers to a chemical substance which may be released from nerve terminals together with a neurotransmitter, or which may arrive from the circulation or from other local tissues to modify the release or action of a neurotransmitter (for review see Burnstock 1982). Many neuropeptides have been shown to fulfill most of the criteria (Burnstock et al 1979; Furness & Costa 1980). VIP, as an example, has a selective distribution in both the central and the peripheral nervous systems (for reviews see Said 1980; Hökfelt

et al 1982). Specific high affinity receptors for VIP have been demonstrated in certain regions which roughly marked the distribution of VIP (Robberecht et al 1978; Taylor & Pert 1979; Dupont et al 1981). On subcellular fractionation of homogenates of hypothalamus, striatum and cerebral cortex, VIP was concentrated in synaptosome-rich preparations (Giachetti et al 1977) or in synaptosomal vesicles (Emson et al 1978). VIP was released by electrical (Fahrenkrug et al 1978), chemical (Giachetti et al 1977; Bitar et al 1979) and mechanical (Chayvialle et al 1978) stimulation of nerves or, indirectly, of local tissues. A wide range of actions of VIP has been demonstrated on the central nervous system and on the periphery, including vasodilatation, smooth muscle relaxation and stimulation of endocrine and exocrine secretion (for review see Said 1981). The demonstration of VIP-containing secretory granules in the nerve terminal (Probert 1981, 1983) further supports the role of neurotransmitter for this peptide. A similar situation is also found with many other peptides, such as substance P (for review see North et al 1982)

Much evidence has been presented to show that neuropeptides may also act as neuromodulators. For example, enkephalin inhibits the release of noradrenaline (NA) and ACh from nerve terminals in vas deferens. Somatostatin has a similar inhibitory effect in guinea-pig ileum. On the other hand, the release of NA and ACh is potentiated by substance P, bombesin, cholecystokinin (CCK) and angiotensin (Regoli 1982).

The increasing number of neuropeptides discovered makes one wonder if there are any roles other than or in addition to neurotransmitter or neuromodulator which they may play. Indeed, alternative and additional roles have been suggested. One such role could be that of neurohormone released from neurons and reaching the targets via the circulation. Plasma VIP is one such example. It may be released into the circulation from non-adrenergic, non-cholinergic inhibitory nerves and be involved in, for example, the reflexes of receptive-relaxation of the stomach or the dilation of the lower oesophageal sphincter (Fahrenkrug et al 1978). Another postulated role for neuropeptides is as neuronal trophic factors, which are released from nerve terminals and have long-term trophic effects on the development or regeneration of local cells or nerves (Varon & Skaper 1981; Burnstock 1982). One such example, which is particularly worthy of mention, is the trophic effect of substance P. A number of phenomena, observed independently by different research groups, indicate that substance P may play a role in regulation of neuronal development and regeneration. The substance P level in the neurons of the gut appeared to be increased after surgery (Costa et al 1981) and during the regenerating process in culture (Jessen et al 1980). Jonsson & Hallman (1982) found that substance P counteracts neurotoxin, 6-hydroxydopamine (6-OHDA), damage on noradrenergic neurons in rat brain, probably by preventing degeneration or stimulating regrowth of these cells. Interestingly, a similar role for substance P is also indicated by one of the findings made in the present study (Section 3.1.5.).

The problem of understanding the roles of the neuropeptides is further complicated by the existence of a variety of other neurotransmitter candidates which can also meet the criteria. These include gamma aminobutyric acid (GABA), glycine, histamine, dopamine 5-hydroxytryptamine (5-HT or serotonin) and ATP (for reviews see Burnstock 1972; Krnjevic 1974; Snyder & Bennett 1976). It is not uncommon to find more than one neurotransmitter candidate co-stored within a single neuron, e.g., ACh and a peptide, a monoamine and a peptide, or two peptides (for review see Lundberg et al 1982a). The simple concept of each neuron having only one transmitter, first formulated by Dale et al (1935), has been severely questioned. Morphological and pharmacological evidence points to a multiplicity of neurotransmitters in the central and peripheral nervous systems. Why so many are needed is not yet clear.

1.3. PREVIOUS WORK ON DISTRIBUTION OF PEPTIDE-CONTAINING ELEMENTS

It is clear, however, that the first step to understand fully the physiological and pathological significance of these peptide-containing nerves and cells is a comprehensive and detailed survey of the occurrence and distribution of these elements in different organs. Extensive work has been carried out on this aspect in many regions of the body and particular attention has been paid to the gastrointestinal tract. This is due to the fact that a

great number of peptide-containing epithelial cells and nerves are present in the gut, which was also the primary site from where many peptides were extracted. It is now increasingly evident that the gastrointestinal tract is controlled by an extremely complex nervous system containing, in addition to the classical neurotransmitters, at least 8 neuropeptides (for review see Polak & Bloom 1983), let alone the other putative neurotransmitters. The peptide-containing nerves in the gut have recently been shown to be involved in pathological disorders of this organ, such as Crohn's disease (Bishop et al 1980), Hirschsprung's disease (Bishop et al 1981a), Chagas' disease (Long et al 1980) and grass sickness of the horse (Bishop et al 1982a).

In comparison with the gut, knowledge on other peripheral organs remains fragmentary. The heart was investigated mainly by Professor W.G.Forssmann's group. They reported the presence of substance P- and neurotensin-containing nerves in the heart of man (Reinecke et al 1981b; Weihe et al 1981) and VIP-, substance P- and neurotesin-containing nerves in the heart of other mammals (Reinecke et al 1980, 1981a, 1982; Weihe & Reinecke 1981; Forssmann et al 1981). The investigation of substance P immunoreactivity in the guinea-pig heart was carefully extended by Wharton and co-workers (1981a) using the methods of immunocytochemistry, histochemistry and radioimmunoassay. Recently, the occurrence of enkephalins in the guinea-pig heart was reported by Lang et al (1983).

The occurrence and distribution of peptide-containing nerves in the

genitourinary tracts were described in several articles. However, most of these were preliminary reports. VIP was found in nerves of the ureter (Wharton et al 1981), the urinary bladder (Alm et al 1977, 1980a; Hökfelt et al 1978) and the male (Alm et al 1977, 1980a; Larsson 1977; Valasti et al 1980; Willis et al 1981; Larsen et al 1981) and female (Alm et al 1977, 1980b; Larsson et al 1977; Goodnough et al 1979; Lynch et al 1980; Helm et al 1981; Ottesen et al 1981a; Ström et al 1981) genital tracts. Substance P-containing nerves were reported to be present in the ureter (Wharton et al 1981), the urinary bladder (Hökfelt et al 1978; Lundberg et al 1980) and the male and female genitalia (Lundberg et al 1980). Met- and leu-enkephalin-containing nerves were observed in the genitourinary organs (Lundberg et al 1980), particularly in the seminal vesicle and the prostate gland (Valasti et al 1980). Somatostatin-containing nerves were also reported to exist in the genitourinary tract of the cat (Hökfelt et al 1978). Most of these investigations, however, were conducted on experimental animals, apart from those made by Goodnough et al (1979), Alm et al (1980b), Valasti et al (1980), Lynch et al (1980), Helm et al (1981), and Larsen et al (1981) to find VIP and enkephalins in the human male and female genitourinary systems.

For another large and important organ, the skin, no comprehensive investigation has been carried out, except that substance P-, VIP- and neurotensin-immunoreactive (IR) nerves have been observed in the skin of some mammalian species (Hökfelt et al 1975a; Cuello et al 1978; Hartschuh et al 1983).

The involvement of these peptides in pathological changes has been tackled mainly in tumours and diseases of the gut (for review see Polak & Bloom 1982). This is not surprising since in other organs in man, even the normal distributions of these peptides have not been fully elucidated.

1.4. PURPOSES OF THE PRESENT STUDY

The present study therefore focusses attention on a number of organs which have been examined less thoroughly. These include the urinary bladder, the male and female genitalia, the heart, the skin and the gallbladder. The assumption made in this study is that if a peptide is retained in certain nerves or cells with a characteristic distribution during evolution but is not required in other tissues, then it may have a specific function in those nerves or cells. A thorough investigation of the occurrence and distribution of these peptide-containing elements in both health and disease would provide the necessary basis for further evaluation of the physiological and physiopathological significance of these peptides. A better understanding of the normal function and abnormal state of the investigated organs should thereby be achieved. The present study was therefore designed to investigate the following points:

1. The occurrence and distribution patterns of peptide containing elements in the peripheral organs of interest. Attempts were also

made to study the origin of the peptide-containing nerves in the tissues and to compare their distribution with that of the adrenergic and cholinergic nerves.

2. The possible involvement of these peptide-containing elements in pathology.

1.5. MAIN PEPTIDES AND NON-PEPTIDE SUBSTANCES TO STUDY

1.5.1. GENERAL

Certain peptides were chosen for investigation for the following reasons.

1. They were previously reported or were likely to be present in the organs of interest.

2. After general immunocytochemical screening of up to 40 peptide antigens for their possible occurrence in the investigated organs, they were either found to be present or suspected to be present.

3. They are newly discovered peptides which have not been properly investigated in the relevant organs.

4. The characterised specific antibodies against them were available.

Thus, the investigations described here were mainly concentrated on the following peptides; VIP, substance P, neuropeptide tyrosine (NPY), peptide histidine isoleucine (PHI), somatostatin$_{14}$, met-enkephalin and bombesin.

The occurrence and distribution of a few non-peptide substances were also examined for the purpose of comparison with those of peptides. These substances included neuron-specific enolase (NSE), S-100, tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DβH), noradrenaline (NA) and acetylcholinesterase (AChE). The investigations of these substances were intended to add to the findings on peptide-containing elements in different ways. The study on the distributions of NSE was done in order to gain some information on the overall distribution pattern of the entire system of neuronal and neuroendocrine elements. The immunostaining of S-100 marked out the distribution of the Schwann and satellite cells, which are often related to peptide-containing neurons. The investigations on DβH, TH, NA and AChE were intended to reveal the lay-out of nerves containing classical neurotransmitters, i.e., catecholamine and ACh, and give some clue to the relationship between peptide-containing elements and classical nerve types. Significant findings for the non-peptide substances themselves were also obtained when systematic investigations were carried out on their distribution in some previously untackled areas.

Details of these peptides and non-peptide substances are given

below.

1.5.2. VASOACTIVE INTESTINAL POLYPEPTIDE (VIP)

VIP is a 28 amino acid peptide first isolated from porcine duodenum in 1970 (Said & Mutt 1970) and its molecular structure was subsequently determined in 1974 (Diagram 1) (Mutt & Said, 1974). It has recently been shown that VIP extracted from the guinea-pig gut slightly differs in its amino acid sequence from that of porcine VIP (Furness et al 1982a). However, antisera raised against porcine VIP have been found to react with VIP of the guinea-pig as well as with VIP of other mammals including man. A precursor molecule, preprovasoactive intestinal peptide, has been discovered in pancreatic VIP-producing tumours (Bloom et al 1983) and in a neuroblastoma cell line (Itoh et al 1983).

VIP has been found to be one of the most ubiquitous neuropeptides, with an extensive distribution throughout the body (for reviews see Said 1980; Hökfelt et al 1982). In the periphery, in addition to the gastrointestinal tract where VIP-IR nerves are found in tissues from the salivary gland to the rectum (Bryant et al 1976; Larsson et al 1976; Fuxe et al 1977; Wharton et al 1979a; Jessen et al 1980; Schultzberg et al 1980), nerves containing VIP have been reported to occur in the male and female genitourinary tracts (for references see Section 1.3.), the heart (Weihe & Reineke., 1981), the respiratory tract (Uddman et al 1980; Dey & Said 1980), the eye

Diagram 1

AMINO ACID SEQUENCES OF VIP, PHI AND RELATED PEPTIDES

	N-terminal							Amino acid sequential number																			C-terminal
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
VIP	HIS	SER	ASP	ALA	VAL	PHE	THR	ASP	ASN	TYR	THR	ARG	LEU	ARG	LYS	GLN	MET	ALA	VAL	LYS	LYS	TYR	LEU	ASN	SER	ILE	LEU	ASN	
PHI	HIS	ALA	ASP	GLY	VAL	PHE	THR	SER	ASP	PHE	SER	ARG	LEU	LEU	GLY	GLN	LEU	SER	ALA	LYS	LYS	TYR	LEU	GLU	SER	LEU	ILE		
SEC	HIS	SER	ASP	GLY	THR	PHE	THR	GLU	LEU	SER	SER	ARG	LEU	ARG	ASP	SER	ALA	ARG	LEU	GLN	ARG	LEU	LEU	GLN	GLY	LEU	VAL	GLU	
GLU	HIS	SER	GLN	GLY	THR	PHE	THR	SER	ASP	THR	SER	LYS	THR	LEU	ASP	SER	ARG	ARG	ALA	GLN	ASP	PHE	VAL	GLN	TRP	LEU	MET	ASN	THR

VIP- Porcine Vasoactive Intestinal Polypeptide, 28 amino acids.

PHI- Porcine Peptide Histidine Isoleucine, 27 amino acids.

SEC- Porcine Secretin, 27 amino acids.

GLU- Porcine Glucagon, 29 amino acids.

Identical amino acid sequences are underlined.

(Terenghi et al 1982), the superior and inferior mesenteric ganglia (Hökfelt et al 1977c), the sciatic (Giachetti & Said 1979) and the vagus nerves (Said & Rosenberg 1976; Lundberg et al 1979a) and also to be associated with exocrine glands (Bryant et al 1976; Wharton et al 1979; Lundberg et al 1980a; Hökfelt et al 1982). Ganglia, both intrinsic (Schultzberg et al 1978; Jessen et al 1980; Lundberg et al 1980; Alm et al 1980a; Wharton et al 1981) and extrinsic (Hökfelt et al 1977c; Lundberg et al 1978, 1979a) to the innervated organs, have been suggested to be the origins for the VIP-containing nerve fibres in the organs. It is interesting to note that, more recently, there has been evidence to show that VIP-like immunoreactivity is also present in the primary sensory neurons of the spinal cord (Jancso et al 1981).

It has been known that a number of physiological events may be affected by the biological actions of VIP, ranging from smooth muscle contraction to body metabolism. VIP has a powerful vasodilatory effect on blood vessels, including the peripheral, the splanchnic and the coronary vessels. It can relax smooth muscle contraction, thus resulting in bronchodilation, relaxed sphincters and relaxed smooth muscles of stomach, colon, gallbladder and urinary bladder. VIP can also stimulate secretion of both endocrine and exocrine glands. Finally, VIP can influence the metabolic rate, eg., by stimulation of glycogenolysis, lipolysis and adenylate cyclase activity (for review see Said 1981).

While VIP can satisfy most of the requirements for a

neurotransmitter (see Section 1.2.), an opposing view exists. Yahasake et al (1981) reported that after desensitation of VIP receptors, the inhibitory response, thought to be exerted by VIP, still existed. Mackenzie & Burnstock (1980) observed that the antagonism of the inhibitory response by apamin did not vary the action of VIP. A specific antagonist to VIP has not yet become available to verify its role as a neurotransmitter.

VIP-IR nerves and AChE-positive nerves have often been found to be similarly or identically distributed. For example, in the lumbar 7 sympathetic ganglion of cat, about 10-15% of the ganglion cells were found to be both VIP-IR and AChE-positive (Lundberg et al 1979). In the cat submandibular ganglia, a large proportion of ganglion cells were also found to contain both neuronal substances (Lundberg et al 1980a). Therefore, it has been suggested that VIP may be co-stored with ACh. However, this co-storage, in most of the cases, is only speculative because of the unspecificity of the commonly used histochemical methods for identifying cholinergic neurons (see Section 1.5.12.). More convincing evidence for their co-storage was derived from the study on sphenopalatine ganglion, where VIP was found to be present in virtually all of the ganglion cells (Lundberg et al 1981). Resection of this ganglion led to a parallel loss of VIP and choline acetyltransferase (ChAT) in the nasal mucosa. Ultrastructurally, VIP immunoreactivity has been demonstrated in large, dense-cored vesicles (Johansson & Lundberg 1981) in what were previously assumed to be cholinergic nerve endings, which also had many small, empty vesicles known to contain ACh. In addition, both

VIP and ACh were simultaneously released upon stimulation of the same nerves and the two substances seemed to interact in evoking vasodilatation and exocrine secretion (Lundberg 1981; Lundberg et al 1981a, 1981b, 1982a).

Pathological changes of VIP-containing elements have been investigated mainly in the gastrointestinal tract. VIP release from the gut has been reported as a result of intestinal ischaemia (Modlin et al 1978) and during haemorrhagic shock (Sakio et al 1979). VIP-IR nerves have been found to be increased in the gut in Crohn's disease (Bishop et al 1980) and decreased in Hirschsprung's disease (Bishop et al 1981) and Chagas' disease (Long et al 1980). VIP-IR nerves almost vanished from the gut of horses suffering from grass sickness (Bishop et al 1982a). However, the pathological involvements of VIP-containing nerves in many other organs has not been established.

1.5.3. SUBSTANCE P

Substance P was discovered accidentally by Von Euler & Gaddum in 1931. Thirty nine years later it was isolated from bovine hypothalamus (Chang & Leeman 1970). Its molecular structure was determined as an undecapeptide (Chang & Leeman 1971) (Diagram 2). Substance P was the first brain-gut peptide discovered and, like VIP, it has an extensive distribution within the central and peripheral nervous systems (for reviews see Hökfelt et al 1976a). In the brain, high concentrations of substance P occur in the

Diagram 2

AMINO ACID SEQUENCES OF SUBSTANCE P, BOMBESIN AND GRP

	N-terminal	C-terminal
Substance P (mammalian, 11 amino acids)	ARG-PRO-LYS-PRO-GLN-GLN-PHE-PHE-GLY-LEU-MET	
Bombesin (amphibian, 14 amino acids)	PYR-GLN-ARG-LEU-GLY-ASN-GLN-TRP-ALA-VAL-GLY-HIS-LEU-MET	
GRP (mammalian)	ALA-PRO-VAL-SER-VAL-GLY-GLY-GLY-THR-VAL-LEU-ALA-LYS-MET-TYR-PRO-ARG-GLY-ASN-HIS-TRP-ALA-VAL-GLY-HIS-LEU-MET	

Identical amino acid sequences are underlined.

substantia alba, the pineal gland and the hypothalamus, and it may be co-stored in 5HT-containing neurons (Chan-Palay et al 1978; Pelletier et al 1981). In the spinal cord, it is concentrated in the dorsal horn (Lembeck 1953), notably in primary sensory neurons (Hökfelt et al 1977a). In the periphery, substance P-IR nerves were particularly dense in the myenteric and submucosal plexuses of the gut. Nerve transection and tissue culture experiments showed that these nerves were predominantly intrinsic (Pearse & Polak, 1975a; Nilsson et al 1975; Sundler et al 1977; Jessen et al 1980; Schultzberg et al 1980). Substance P-containing nerves have also been reported to be present in the heart and the peripheral vascular system (Reinecke et al 1980; Weihe & Reinecke 1981; Wharton et al 1981a; Furness et al 1982b), the urogenital tract (Alm et al 1978; Hökfelt et al 1978; Lundberg et al 1980; Wharton et al 1981; Sikri et al 1981), the respiratory tract (Wharton et al 1979; Lundberg et al 1980),the skin (Hökfelt et al 1975a), the vagus (Gamse et al 1979) and the sympathetic abdominal ganglia (Schultzberg 1980). There is evidence that certain substance P-containing nerve fibres in the periphery originate from the sensory neurons of the spinal and nodose ganglia (Gamse et al 1979; Lundberg 1979; Lundberg et al 1979a; Dalsgaard et al 1982; Matthews & Cuello 1982; Sheppard et al 1983a). Substance P was also reported to occur in endocrine cells of the gut (Pearse & Polak 1975a).

The physiological role of substance P is at present not fully understood. A diversity of actions has been reported. Substance P has been shown to cause smooth muscle contraction, vasodilation, hypotension and salivation. It can also affect pancreatic and choleretic secretion and release histamine from mast cells (for review see Powell & Skrabanek 1981). Substance P exerts effect on

gut motility. However, this may either be due to a direct action on smooth muscle cells or to involvement in cholinergic (Holzer & Lembeck 1980) or serotoninergic mechanisms (Holzer et al 1980).

Substance P was the first peptide to be proposed as a neurotransmitter (Lembeck, 1953). Otsuka and Konishi (1976) presented further evidence to suggest that substance P may be involved in sensory neurotransmission. Substance P was released when the dorsal roots were electrically stimulated, and exerted an excitatory action on spinal neurons. Ligation of the dorsal root of the cat caused an accumulation of substance P on the ganglion side and a reduction on the spinal side, suggesting that substance P is synthesized in the ganglion cells and transported to the spinal cord. Neonatal application of capsaicin, a neurotoxic agent which produces permanent loss of small diameter sensory neurons (Jancso et al 1977; Lawson & Nickels 1980), depleted certain substance P-IR nerves (Jancso et al 1967; Jessell et al 1978), further supporting a role for substance P as a sensory neurotransmitter. Electrophysiological and behavioural experiments suggested that substance P is an excitatory chemical mediator of nociception (Henry 1976, 1982).

It has recently been revealed that, in addition to the afferent transportation, the major part of the substance P synthesized in the dorsal root and nodose ganglia is transported rapidly to their peripheral branches (Keen et al 1982; Hökfelt et al 1980; Brimijoin et al 1980; Terenghi et al 1983a). Therefore, a role for substance P other than sensory neurotransmission was sought. Indeed, substance P has been found to be released from the peripheral sensory nerve

endings upon stimulation, which may possibly result in antidromic vasodilatation, plasma extravasation and, consequently, inflammation, and thereby help to overcome the peripheral damage (for review see Lembeck 1982).

Specific receptors for substance P binding have been detected in both the central nervous system and the periphery (for review see Skrabanek & Powell 1980). A number of substance P analogues have been synthesized and a few substance P antagonists have been made available (Folkers et al 1981; Rosell et al 1981). All of these antagonists are also peptides.

1.5.4. NEUROPEPTIDE TYROSINE (NPY)

NPY is a 36 amino acid peptide recently isolated from extracts of porcine brain by a novel method for detecting peptides possessing a C-terminal α-amide (Tatemoto 1982; Tatemoto et al 1982). Its molecular sequence is very similar to that of pancreatic polypeptide (PP) and peptide YY (PYY) (Tatemoto 1982), the three together forming a peptide family - the PP family (Diagram 3). PYY has been localised to endocrine cells of the gut (Lundberg et al 1982) and avian PP (APP)- and bovine PP (BPP)-like immunoreactive materials have been reported to occur in nerves in a number of organs (Loren et al 1979; Lundberg et al 1980a; Olschowka et al 1981). However, the true nature of the PP-like immunoreactivity had not been fully elucidated until the present study.

Diagram 3

AMINO ACID SEQUENCES OF PEPTIDES IN PP-FAMILY

N-terminal

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
NPY (neuropeptide Y)	TYR	PRO	SER	LYS	PRO	ASP	ASN	PRO	GLY	GLU	ASP	ALA	PRO	ALA-
PYY (peptide YY)	TYR	PRO	ALA	LYS	PRO	GLU	ALA	PRO	GLY	GLU	ASP	ALA	SER	PRO-
PPP (porcine pancreatic polypeptide)	ALA	PRO	LEU	GLU	PRO	VAL	LEU	PRO	GLY	ASP	ASP	ALA	THR	PRO-
BPP (bovine pancreatic polypeptide)	ALA	PRO	LEU	GLU	PRO	GLU	TYR	PRO	GLY	ASP	ASN	ALA	THR	PRO-
APP (avian pancreatic polypeptide)	GLY	PRO	SER	GLN	PRO	THR	TYR	PRO	GLY	ASP	ASP	ALA	PRO	VAL-

C-terminal

	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
NPY	GLU	ASP	LEU	ALA	ARG	TYR	TYR	SER	ALA	LEU	ARG	HIS	TYR	ILE	ASN	LEU	ILE	THR	ARG	GLN	ARG	TYR
PYY	GLU	GLU	LEU	SER	ARG	TYR	TYR	ALA	SER	LEU	ARG	HIS	TYR	LEU	ASN	LEU	VAL	THR	ARG	GLN	ARG	TYR
PPP	GLU	GLN	MET	ALA	GLN	TYR	ALA	ALA	GLU	LEU	ARG	ARG	TYR	ILE	ASN	MET	LEU	THR	ARG	PRO	ARG	TYR
BPP	GLU	GLN	MET	ALA	GLN	TYR	ALA	ALA	GLU	LEU	ARG	ARG	TYR	ILE	ASN	MET	LEU	THR	ARG	PRO	ARG	TYR
APP	GLU	ASP	LEU	ILE	ARG	PHE	TYR	ASP	ASN	LEU	GLN	GLN	TYR	LEU	ASN	VAL	VAL	THR	ARG	HIS	ARG	TYR

Identical amino acid sequences are underlined.

NPY has been found to be present in nerves with an extensive distribution and has turned out to be one of the most abundant neuropeptides discovered so far. Distribution studies have revealed this peptide to occur in the brain (Allen et al 1983; Adrian et al 1983), the splanchnic ganglia (Lundberg et al 1982b) and the innervation of the gut (Lundberg et al 1982a; Ferri et al 1983). Based on the results obtained from immunocytochemistry combined with 6-hydroxydopamine (6-OHDA) treatment which is known to destroy the adrenergic innervation, Lundberg et al (1982b) suggested that NPY may occur in a subpopulation of adrenergic neurons of the superior cervical, stellate and coeliac ganglia. NPY was also found to occur in the adrenal gland (Allen et al 1983a) and phaeochromocytomas and ganglioneuroblastomas (Adrian et al 1983a).

Little is known about the physiological role of this new peptide. Intravenous injection of NPY inhibited secretin-induced pancreatic exocrine secretion (Tatemoto 1982). NPY has also been found to have a dose-dependent vasoconstrictor activity in rat and mouse submandibular glands (Lundberg et al 1982b). In the isolated perfused rabbit heart, NPY caused a striking reduction in coronary flow, being more potent on a molar basis than noradrenaline (Allen et al 1983b). Stjernquist et al (1983) showed that NPY inhibited the smooth muscle contraction of rat cervix in vitro. Electrically induced, but not noradrenaline-induced, smooth muscle contraction of rodent vas deferens was inhibited by NPY in a dose-dependant manner (Allen et al 1982; Lundberg et al 1982b). It has therefore been proposed that NPY may be a neuromodulator which is slow in onset and long-lasting in action. Nevertheless, much concerning this peptide

has still to be discovered.

1.5.5. PEPTIDE HISTIDINE ISOLEUCINE (PHI)

PHI is another newly discovered peptide. It consists of 27 amino acid residues and was originally isolated from porcine intestine and brain (Mutt 1978). Its name was derived from its molecular structure, of which the N-terminal resembles histidine and the C-terminal resembles isoleucine. It has remarkable sequence homologies with VIP, secretin, glucagon and gastric inhibitory peptide (GIP) and thus is a new member of the secretin-glucagon family (Diagram 1). PHI has been detected in the gut where it was almost exclusively localised in the lamina propria, submucosa and muscle layer with very low concentrations in the mucosa (Christofides et al 1983). In porcine gut, PHI was found to be co-localised with VIP to a proportion of intrinsic ganglion cells (Bishop et al 1984).

The biological actions of PHI are very similar to those of VIP, but the former is less potent. PHI can bind competitively to VIP receptors in rat liver and intestine. It can also stimulate cyclic AMP production, induce insulin release, increase pancreatic exocrine secretion, relax gallbladder wall and inhibit gallbladder fluid absorption. PHI, however, is 1000-fold less potent than VIP as a vasodilatory substance (for review see Tatemoto 1982).

Because of the similarities between PHI and VIP in both molecular

structure and biological action as well as their co-existence in certain tumours and neurons, it has been speculated that these two peptides might be co-produced from the same precursor molecule (Christofides et al 1982). This postulate has been proven to be true by the discovery of a precursor molecule, preprovasoactive intestinal peptide, in neuroblastoma cell line (Itoh et al 1983) and in pancreatic VIPoma (Bloom et al 1983). This molecule contains the amino acid sequences of both VIP and PHM, the human counterpart of PHI. Apart from the intestine, the histological distribution of PHI-containing nerves in other peripheral organs of various mammalian species has not been investigated.

1.5.6. SOMATOSTATIN

Somatostatin was originally isolated from extracts of sheep hypothalami as a growth hormone inhibitor and its structure determined as a cyclic tetradecapeptide (Brazeau et al 1973). Seven years later, Pradayrol et al (1980) isolated an extended molecular form of the somatostatin$_{14}$ from porcine intestine and determined its amino acid sequence as somatostatin$_{28}$ (Diagram 4). Both of these molecular forms have similar effects although differences have been observed in the potency of these two peptides (Vayssel et al 1981).

Somatostatin$_{14}$ has a widespread distribution both in endocrine cells of the gut, neuroendocrine cells of the hypothalamus and nerves of both the central and the peripheral nervous systems. It has been localised to the amacrine neurons in the retina (Yamada et al 1980)

Diagram 4

AMINO ACID SEQUENCES OF SOMATOSTATIN (14) AND SOMATOSTATIN (28)

	N-terminal														C-terminal
SOM_{14}															ALA-GLY-CYS-LYS-ASN-PHE-PHE-TRP-LYS-THR-PHE-THR-SER-CYS
SOM_{28}	SER-ALA-ASN-SER-ASN-PRO-ALA-MET-ALA-PRO-ARG-GLU-ARG-LYS-														ALA-GLY-CYS-LYS-ASN-PHE-PHE-TRP-LYS-THR-PHE-THR-SER-CYS

Identical amino acid sequences are underlined.

the primary sensory neurons, the dorsal root ganglia, the sympathetic ganglia, the vagus, the sciatic nerves, the nerves in the salivary glands and the thyroid (Hökfelt et al 1975b), the parafollicular cells of the thyroid (Yamada et al 1977; Van Noorden et al 1977), and, in particular, the submucous and myenteric plexuses of the gut (for review see Patel 1979). The occurrence of somatostatin-IR nerves in the feline urogenital tract was reported by Hökfelt et al (1978). Subcellularly, somatostatin$_{14}$ was localised to synaptosomal fractions (Epelbaum et al 1977). Somatostatin$_{28}$ has not yet been found in nerves.

Somatostatin$_{14}$ was the peptide investigated in this study. It has a wide range of inhibitory biological actions. It inhibits both exocrine and endocrine secretions including that of almost all the endocrine peptide hormones. It also inhibits gastric and biliary motility and the activation of neurons (for review see Patel et al 1981). Specific receptors for this peptide have been demonstrated in the brain (Schonbrum & Tashjian 1978; Leitner et al 1979; Srikant & Patel 1980).

1.5.7. ENKEPHALIN

The enkephalins comprise a pair of pentapeptides differing only in their COOH-terminal amino acid residues. The one with a C-terminal methionine was named met-enkephalin and one with a leucine, leu-enkephalin (Diagram 5). They were originally isolated from porcine brain (Hughes et al 1975) and subsequently found in many other mammalian species (Ross et al 1977). Together with three

Diagram 5

AMINO ACID SEQUENCES OF MET- AND LEU-ENKEPHALIN

	N-terminal				C-terminal
Met-enkephalin (5 amino acids)	MET-	PHE-	GLY-	GLY-	TYR
Leu-enkephalin (5 amino acids)	LEU-	PHE-	GLY-	GLY-	TYR

Identical amino acid sequences are underlined.

endorphins, they were known as opioid-peptides because of their potent opiate receptor agonist activity. Based on their amino acid homologies, it was once thought that β-lipotropin (β-LPH), which is the precursor for endorphins, might also be the precursor for enkephalins. It is now known that enkephalins have separate precursors which contain the amino acid sequences of both met- and leu-enkephalins. The precursor molecule of the enkephalins is quite distinct from β-LPH (Clement-Jones et al 1980; Lewis et al 1980), in spite of the fact that enkephalins and endorphins are rather alike in both their molecular structures and their biological actions.

In addition to the central nervous system, enkephalins have been found to be extensively distributed in the gut including the gallbladder (Elde et al 1976; Polak et al 1977; Alumets et al 1978; Linnoila et al 1978; Schultzberg et al 1978, 1980; Jessen et al 1980). The enkephalin-containing nerve fibres in the gut may be constituted by elongations both from intrinsic neurons located within the gut wall and from extrinsic ganglia by way of vagal and pelvic nerves (Lundberg 1979). Other regions reported to contain enkephalin immunoreactivity include the adrenal medulla (Schultzberg et al 1980), sympathetic ganglia (Schultzberg et al 1980; Hervonen et al 1980), the urinary bladder (Hökfelt et al 1978) and the prostate gland (Valasti et al 1980). In ultracentrifuge studies, enkephalins were found in association mainly with the synaptosomal fraction, thus indicating a role of neurotransmitter or neuromodulator (Queen et al 1976). Electron microscopical study using a gold-labelled antibody method localised enkephalin to electron-dense granules in the p-type neurons (Probert et al 1983).

In both the central and the peripheral nervous systems, the distributions of met- and leu-enkephalins are parallel to each other or at least partially overlapping(Linnoila et al 1978; Schultzberg et al 1980; Elde et al 1976), although differences between the two may exist in certain regions (Larsson et al 1979).

Biological actions of enkephalins mimic those of morphine in many ways. In the periphery, enkephalins have been shown to increase gastric secretion, enhance intestinal muscle tone, slow intestinal transit, delay gastric emptying, inhibit pancreatic secretion and increase intestinal blood flow (for review see Konturek et al 1979; Corder & Rees 1981). There is evidence to show that the inhibitory effect of enkephalins might be achieved by suppressing the release of ACh (Paton 1957; Waterfield et al 1977) and, possibly also, the release of substance P (Gintzlor & Scalisi 1982; Bartho et al 1982), and by the activation of myenteric neurons (North & Williams 1976). Microelectro-physiological study of the myenteric ganglia of the guinea-pig gut revealed that enkephalins cause presynaptic inhibition by hyperpolarisation of the nerve cell membrane (North et al 1979). The N-terminal tyrosine of the two C-terminally diverted enkephalins has been found to be the most essential part responsible for their biological activity (Morgan et al 1976). Nevertheless, met-enkephalin was reported to be more potent than leu-enkephalin in inhibiting gut motility (North & Williams 1976; Sakai et al 1978). Specific receptors for opiate-peptides have been demonstrated (Snyder & Simantov 1977) with a distribution which largely matched that of enkephalins (Pasternak et al 1976). Met-enkephalin was the peptide investigated in this study. The antisera used against met-

enkephalin was found to cross-react with leu-enkephalin to a certain extent (Table 2d).

1.5.8. BOMBESIN

Bombesin is a 14 amino acid residue peptide first isolated from European frog skin (Anastasi et al 1971). This peptide together with a number of structurally related amphibian skin peptides and their mammalian counterparts, including substance P, form a family of bombesin-like peptides (Diagram 2) (for reviews see Erspamer 1980; McDonald 1981). The mammalian bombesin consists of 27 amino acids and was initially termed 'gastrin-releasing peptide' (GRP). The amino acid sequence of this peptide is strikingly similar to that of amphibian bombesin; nine of the ten amino acids in the C-terminals of both peptides are identical (Diagram 2) (for review see Ghatei 1982). The antisera against the C-terminals of the amphibian bombesin and the mammalian bombesin, i.e., GRP, have been found to cross-react with each other.

The amphibian and mammalian bombesins have similar and wide spectra of action on various tissues in mammals as well as in amphibians and avians (for review see McDonald 1981). They can cause smooth muscle contraction of the gut and the gallbladder, vasoconstriction, gastric acid and pancreatic secretion and release of many gut hormones (Erspamer & Meclchiorri 1973, 1975; Bloom et al 1979). In the eliciting of the gallbladder contraction and the relaxation of the choledochoduodenal junction, it remains to be

ascertained if bombesin acts directly on the effector cells or indirectly through the release of CCK (Erspamer & Melchiorri 1975).

Bombesin immunoreactivity has been localised to nerves in both the central nervous system and the periphery including the gut and the lung. In these organs, bombesin immunoreactivity was also found to occur in epithelial endocrine cells (Polak et al 1978a; Wharton et al 1978).

1.5.9. NEURON-SPECIFIC ENOLASE (NSE)

NSE is an acidic soluble protein with a molecular weight of 78,000 daltons, and was originally isolated from rat brain (Marangos et al 1975). It is identical to the previously described bovine 14-3-2 protein (Moore 1972) and rat antigen alpha (Bennett & Edelman 1968). NSE is a dimeric acidic protein composed of two identical gamma subunits, which is structurally distinct from another major brain enolase isoenzyme, non-neuronal enolase (NNE), which contains two alpha subunits, (Marangos et al 1978). Initially, NSE was exclusively localised to neurons (Pickel et al 1975) and established as a neuron-specific isozyme of the glycolytic enzyme enolase (Bock & Dissing 1975; Marangos et al 1978). The axonal transport of NSE appeared to be a relatively slow process (Marangos et al 1975a) and associated with slow component b (Brady & Lasek 1981). In contrast, NNE was localised mainly in glial elements (Schmechel et al 1978). The exact action of NSE is not yet known. It has been found that NSE levels are correlated to neuronal differentiation in

the brain and there may be a switch from NNE to NSE during neuronal maturation (Marangos et al 1981).

It was recently shown that NSE also occurs in peptide-containing endocrine cells, eg. gastrointestinal endocrine cells, Langerhans cells of pancreatic islet, thyroid parafollicular cells, adrenal medullary chromaffin cells and pituitary cells (Schmechel et al 1978a). Therefore, NSE provides a unique general marker to illustrate the overall layout of both the endocrine cells and the innervation (Bishop et al 1982; Ferri et al 1983a; Sheppard et al 1983, 1984). Furthermore, the possibility of using NSE as a probe to identify new putative neuroendocrine cells logically follows.

1.5.10. S-100 PROTEIN

S-100 is an acidic protein with a molecular weight of about 20,000-22,000 daltons (Moore 1982). It was originally extracted from bovine brain and its name derived from its solubility in 100% saturated ammonium sulphate at neutral pH (Moore 1965). It was later reported that S-100 protein was a mixture of two similar proteins, S-100a (α, β subunits) and S-100b (β, β subunits), which are composed of 93 and 91 amino acid residues respectively (Isobe et al 1978; Isobe & Okuyama 1978, 1981). S-100 has molecular structure common to the calcium binding proteins (Isobe & Okuyama 1978). However, its precise function is not known. S-100 was first thought to be present exclusively in the glial elements of the brain including astrocytes, oligodendrocytes and ependymal cells (Cicero

et al 1970; Haglid et al 1976; Ludwin et al 1976; Matus & Mughal 1975; Møller et al 1978; Cocchia 1981). Later, it was found that it also occurs in a number of peripheral cells including Schwann cells of the peripheral nerves (Cocchia & Michetti 1981; Stefansson et al 1982a), satellite cells of the sympathetic ganglia and the adrenal medulla (Cocchia & Michetti 1981), reticular cells of the lymphoid organs (Takahashi et al 1981; Nakajima et al 1982a; Cocchia et al 1983), Langerhans cells and melanocytes of the skin (Cocchia et al 1981; Nakajima et al 1982a), chondrocytes (Stefansson et al 1982b), adipocytes (Michetti et al 1983), myoepithelial cells (Nakajima et al 1982) and histiocytosis X cells (Nakajima et al 1982, 1982a). However, some controversial evidence exists. Some investigators showed that S-100 might be present in neuronal nuclei (Dravid & Burdman 1968; Miani et al 1972; Michetti et al 1974; Sviridov et al 1972; Tabuchi et al 1976), neuronal perikarya (Hyden & Lange 1970; Tabuchi et al 1976), synaptosomes (Donato & Michetti 1974; Donato et al 1975; Donato 1976, 1977), post-synaptic densities (Haglid et al 1974) and might also be transported in peripheral axons (Miani 1971; Miani et al 1972). This protein has recently been employed as a marker to map out the distribution of Schwann and glial cells in the peripheral tissues including the gut and the lung (Ferri et al 1982; Sheppard et al 1983).

Wallerian degeneration of rabbit optic nerve (Perez et al 1970) and retrograde degeneration of neurons in the dorsal thalamus (Cicero et al 1970a) are accompanied by an increase in the amount of S-100. S-100 levels are elevated in the cerebrospinal fluid and in the plasma of patients after strokes and during the course of other

neurological and non-neurological diseases including multiple sclerosis (Michetti et al 1980; Moore 1982). This may be of particular relevance to one of the findings made during the present study.

1.5.11. TYROSINE HYDROXYLASE (TH) AND DOPAMINE-β-HYDROXYLASE (DβH)

TH and DβH are two of the four enzymes acting in the sequential biosyntheses during catecholamine production. TH catalyses the conversion of tyrosine to DOPA and DβH hydroxylates dopamine to noradrenaline. Both enzymes have been purified and used to produce corresponding antibodies. Subsequently, their antibodies have been employed in immunocytochemistry for mapping the distribution of catecholamine-containing elements (for review see Gombos & Aunis 1982), as in the present study.

TH has been demonstrated in the cell bodies of dopaminergic, noradrenergic and adrenergic neurons in both the central and the peripheral nervous systems (Pickel et al 1975a; Halasz et al 1977; Hökfelt et al 1973, 1974, 1976, 1977). Immunoelectron microscopical study showed that TH is diffusely distributed in the cytoplasm and more selectively near membranes of the endoplasmic reticulum and Golgi apparatus of noradrenergic neuron perikarya (Pickel et al 1975a). On the other hand, TH in the axons and terminals of noradrenergic and adrenergic neurons appeared to be less concentrated or less accessible to the antibody than that in the dopaminergic neurons (Pickel et al 1976). However, it has been

shown that TH was immunocytochemically detectable in noradrenergic and adrenergic nerve endings in certain areas when optimal conditions were used (Hökfelt et al 1973, 1974, 1976).

DβH has been commonly used as a marker for the catecholaminergic system. In the central nervous system, good correlations between DβH levels and noradrenaline and, in some cases, dopamine and TH levels have been demonstrated (Bouclier et al 1977; Reis & Molinoff 1972; Saavedra et al 1974, 1975; Jacobowitz et al 1975; Zivin et al 1976). DβH-containing neurons have been detected by immunocytochemistry throughout the body with distributions parallel to that of noradrenaline (Hartman et al 1972; Frydman & Geffen 1973; Hartman 1973; Nagatsu 1974; Morrison et al 1978). Ultrastructural study in the locus coeruleus neurons showed that DβH was present throughout the cytoplasm and more concentrated in the vesicles, Golgi apparatus and endoplasmic reticulum (Coyle & Kuhar 1974).

1.5.12. ACETYLCHOLINESTERASE (AChE)

AChE is a degrading enzyme which hydrolyses acetylcholine (ACh). AChE appears to be present exclusively in neurons, primarily cholinergic neurons. Histochemical demonstration of AChE has been widely used to identify cholinergic neurons and to trace cholinergic pathways (for review see Silver 1974). However, low concentrations of AChE also occur in some or all adrenergic fibres (Koelle 1963; Bloom & Siggins 1975). In addition, there has been evidence that AChE may hydrolyze peptides such as substance P (Chubb et al 1981).

An ultrastructural study using a histochemical method indicated that AChE activity was mainly found on neuronal plasma membranes and endoplasmic reticulum (Butcher 1978).

In the present study, the relatively specific method modified by El-Badawi and Schenk (1967) was chosen to identify the presumed cholinergic innervation. However, it should be borne in mind that none of the histochemical procedures so far invented is satisfactorily specific for cholinergic neurons. Nevertheless, it has been shown that most, if not all, of the AChE-positive nerves demonstrated by the El-Badawi-Schenk method are cholinergic in nature, particularly when a short (1-2 hours) incubation time is used (for review see El-Badawi 1982). Although it is now possible to raise specific antibodies to purified AChE and ChAT (Choline acetyltransferase) (for review see Gombos & Aunis 1982), these methods are still at their experimental stage and they were not available at the time of the study.

Chapter 2: TECHNIQUES

2.1. GENERAL

The main technique employed in this study was immunocytochemistry, applied at the light microscopical level. This uses specific antibodies to demonstrate the presence of investigated substances in the tissues of interest. The sites of tissue antigens were visualised under the transmitted light or ultra-violet light microscope by coloured or fluorescent labelling substances on the antibodies. The basic technical principles followed in this study were based on those of the indirect immunofluorescence method of Coons et al (1955) (mainly for staining nerves) and the peroxidase anti-peroxidase (PAP) method of Sternberger (1974) (mainly for staining cells). When necessary and possible, simultaneous studies using histology, histochemistry, electron microscopy, electron-microscopical immunocytochemistry, radioimmunoassay and chromatography were also carried out in order to deepen the understanding of the immunocytochemical results at the light microscopical level. The electron-microscopical investigation was performed by Dr L.Probert and Mr F.Tapia, Histochemistry Unit, Dept. of Histopathology, Royal Postgraduate Medical School, London. The radioimmunoassay and chromatographical analyses were carried out by workers from Professor S.R.Bloom's laboratory, Hammersmith Hospital, London. To study the nature and origin of the peptide-containing nerves, surgical and pharmacological manipulations were conducted on experimental animals. These

included nerve transection and administration of neurotoxic agents, such as capsaicin and 6-hydroxydopamine (6-OHDA).

2.2. TISSUES

The organs investigated included the genitourinary tract, the heart, the skin and the gallbladder. Human tissue was the main interest of the study. Human tissues were obtained from the following sources.

Human penes, testes, epididymides, vas deferentia and attached spermatic cords were obtained at gender reassignment operations by Mr P.Philips, Charing Cross Hospital, London.

Biopsies from corpus cavernosum of penis from impotent men were obtained during insertion of semi-liquid prostheses by Mr R.Morgan and Mr J.P.Pryor, St Peter's, Whittington and Shaftesbury Hospitals, London.

Normal and diseased urinary bladders were obtained at cystectomy or per-urethral biopsy by Mr A.R.Mundy, Dept. of Surgery, Guy's Hospital, London; Mr M.Hehir and Mr A.Deane, Shaftesbury Hospital, London; and Mr R.Ewing, St Thomas Hospital, London.

Biopsies of human ureters and bladders were also provided by Mr G.Williams, Hammersmith Hospital, London.

Tissues of human cardiac biopsy were provided by Dr P.Stovin,

Papworth Hospital, Cambridge, U.K..

Human female genitalia were obtained at hysterectomy by Professor M.Elder, Hammersmith Hospital, London.

Human skin samples were provided by Dr A.Chu, Hammersmith Hospital, London.

Tissues taken from autopsies were also used. Details of surgical and post-mortem tissues are described in Appendix 1.

Tissues of several mammalian species were studied. The species examined included rat (Wistar or Sprague Dawley, body weight 100-250g), Mouse (C57-B, body weight 40-100g), guinea-pig (Dunkin/Hartley or Himalayan, body weight 200-400g), cat (body weight 2-3.5kg) and pig (adult).

2.3. BASIC TECHNIQUES FOR IMMUNOCYTOCHEMISTRY

The basic techniques in immunocytochemistry, which are applied in this laboratory (Histochemistry Unit, Dept. of Histopathology, Hammersmith Hospital, London) as well as in many others, generally include tissue preparation, fixation, cutting sections, immunostaining, result assessment and photography. These techniques have been constantly optimised and widely used in daily immunocytochemical practice. The results obtained by using these methods have been generally satisfactory. Detailed procedures of the techniques employed are presented in Appendix 2.

Before conducting the study, however, a systematic survey was carried out to verify, or modify if necessary, the existing techniques, and to develop some new methods in order to establish the most appropriate approaches to the proposed investigations. This survey included selection of suitable fixations, determination of the tissue antigen degradation rate, modification of the indirect immunofluorescence staining method, development of a light microscopical gold-labelled immunostaining technique, adaptation and modification of the layer separation technique, and setting up of a marking method to assess the immunostaining results.

2.4. TISSUE FIXATION

As most of the antigens investigated in this study were peptides and are thus water soluble, they were rendered insoluble and 'fixed' in situ while surrounding tissue structures were well preserved. Under fixation, the antigen's immunoreactivity, ie. the immunodeterminant structure of the antigen, has to be well protected, in order to react with the antibodies during immunostaining. The conventional histological fixatives, eg. formalin, were found to alter the antigenicity of some peptides and cause false negative results and were therefore not ideal for the present study. A number of cross-linking agents, such as benzoquinone (BQ), which can render peptides insoluble with very little alteration in their antigenicity, have been used in peptide immunocytochemistry (Pearse & Polak 1975; Bishop et al 1978) and these were found to suit the present study best. These fixative reagents can be used either in solution (Bishop et al 1978) (Appendix 2.1.5., 2.2.1.) or in vapour form on freeze-dried tissues (Pearse and Polak 1975) (Appendix 2.2.2.,2.2.3.). The conventional formalin fixations, however, have

the advantage of simplicity and availability, although they are not suitable for some of the peptides. Nevertheless, in certain circumstances, the formalin fixations were of vital importance, because earlier histopathological specimens, on which retrospective studies needed to be done, were mostly fixed in formalin.

An experiment was carried out to determine the most suitable and easily available fixative for individual antigens. Five different fixatives were tested for 7 relevant antigens on gut, genitourinary tract and skin of mammals including man. The results are summarised in Table 1.

In brief, for most of the examined antigens present in nerves, 0.4% benzoquinone solution (BQS) was found to be the most suitable fixative. 4% paraformaldehyde solution was nearly as good. On comparison of liquid and vapour phase fixations, it was found that the liquid fixation was preferable for nerves than the vapour fixation, particularly in combination with the immunofluorescence method on cryostat sections. Conventional formalin fixation indeed diminished the antigenicities of most of the antigens but, fortunately, it was found suitable for NSE and S-100 immunostainings, thus permitting retrospective studies using antibodies to these two proteins on conventionally fixed histopathological specimens.

2.5. ESTIMATION OF TISSUE ANTIGEN DEGRADATION

A large quantity of human tissue was used which had been taken at surgical operations, endoscopic biopsies and autopsies. It was sometimes impossible to obtain the tissues immediately after surgery or within a short time of death. It was therefore necessary to

Table 1

COMPARISON OF VARIOUS FIXATION METHODS

(for antigens in nerves)

	BQS	BQV	FV	MFF	F
VIP	excellent	good	good	excellent	NS
Substance P	excellent	good	good	good	NS
Somatostatin	poor	NS	NS	good	NS
NPY	good	acceptable	acceptable	excellent	NS
DβH	good	acceptable	acceptable	good	NS
NSE	excellent	good	good	excellent	good
S-100	excellent	good	good	good	good

BQS: Benzoquinone solution (0.4% in PBS, 50-70 mins).

BQV: Benzoquinone vapour (60°C, 3 hours).

FV: Paraformaldehyde vapour (80°C, 1 hour).

MFF: 4% methanol-free formaldehyde solution.

F: 5% phosphate buffered formalin.

NS: No staining.

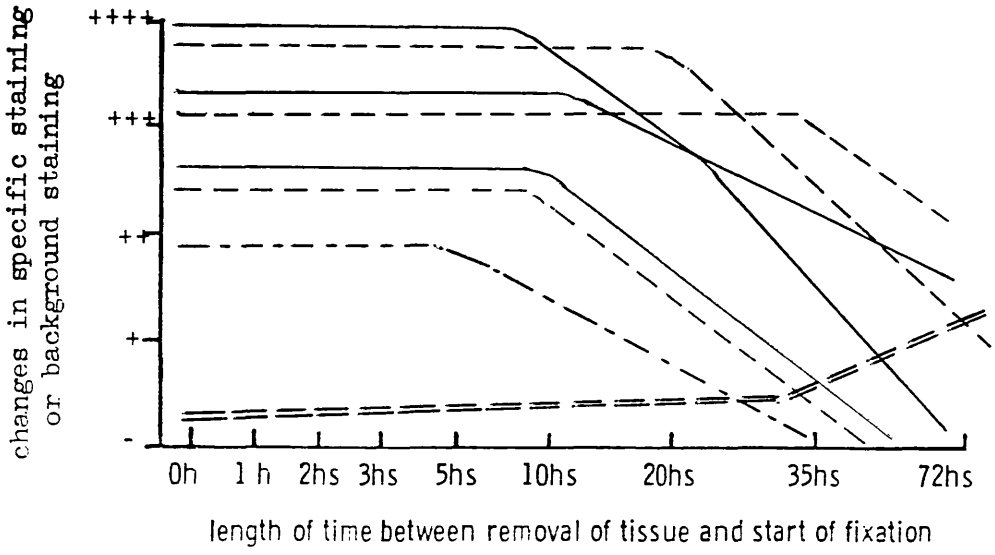
establish whether any degradation of the immunocytochemical reactivity of the antigens occurred prior to fixation. For this purpose, tissues from human and guinea-pig gut and genitourinary tract were dissected and fixations started at different periods of time, up to 72 hours after the tissues had been removed from a living body and stored at room temperature. Immunostainings with routine procedures (Appendix 2.4.2., 2.4.6.) using relevant antibodies were performed on these fixed tissues and the results obtained from tissues of different freshness were compared.

Using both human and guinea-pig tissues, it was found, for all the antigens examined, that there was little difference between freshly fixed tissues and those where fixation had been delayed for 7-10 hours after removal. After 10 hours, some antigens began to degrade and this was evidenced by deterioration in the brightness and sharpness of the specific staining. Meanwhile, the background staining began to rise slightly. The increase in the background staining, however, appeared not to be due to the diffusion of the antigenic materials from their original sites, since in tissues devoid of these antigens, such increased background staining was still shown. Among the antigens investigated, VIP and substance P in particular appeared to be best able to survive delayed fixation. For these peptides, even when the fixation was started 30 hours after removal, only a slight decrease was detected immunocytochemically. In general, the decrease in tissue antigen immunoreactivity occured earlier in the gut than in the genitalia. The different degradation curves for the immunocytochemical reactivity of the studied antigens are shown in Diagram 6.

The degradation curves for the tested antigens, as shown in the

Diagram 6

DEGRADATION OF IMMUNOCYTOCHEMICAL REACTIVITY OF RELEVANT ANTIGENS



Key :
- : No specific staining, or little background staining.
+ - +++ : Levels of specific staining, or increase of background staining.
++++ : Very abundant and bright specific staining, or very high background staining.

VIP
substance P
somatostatin
NPY
DβH
NSE
S-100
background staining

The curves represent the means of the results obtained with 6 specimens of BQS fixed colon stained by the indirect immunofluorescence method.

diagram, were found to be useful in the estimation of the staining results obtained on tissues in which the fixation started at different lengths of time after removal or death. Nevertheless, most of the tissues were fixed freshly or within 10 hours after removal or death. The significance of this survey may be beyond the scope of the present study, since there has been no information of similar type available apart from a very recent study on spinal cord in which only the degradation rate of substance P was worked out (Helme & White 1983).

2.6. LAYER SEPARATION

This technique was developed jointly with Dr W.Cai, Dept. of Anatomy, University College, London.

Nerves in the gallbladder wall are known not to be uniformly distributed in the different anatomical layers. They are mainly concentrated at the two plexuses, ie. the submucous plexus and the myenteric plexus (Onada & Miyazaki 1980). In paraffin and cryostat sections, the nerves are often too scarce to examine unless the section is cut through just the right plane. For the investigation of peptide-containing nerves in the gallbladder, a procedure of layer separation was therefore adapted from that of Costa et al (1980) and that of Ferri et al (1983b), and was subsequently modified to suit the present study.

The procedure was as follows; after removal of the gallbladder and the biliary duct from the guinea-pig, the tissues were immersed in 0.4% BQS. Using microscissors, the gallbladder was opened in the

fixative to display the lumen and then pinned onto a small cork-board which was immersed in the fixative. The different layers of the gallbladder were then peeled off gently under a dissecting microscope using a pair of fine watch-maker's forceps and great care was taken to preserve the integrity of the separated layers. After immersion in the fixative for one hour, the layers were washed overnight at 4°C in 7% sucrose in PBS. Prior to immunostaining, the preparations were dehydrated and rehydrated in xylene and graded alcohols for approximately 1 minute in each solution. After rinsing in PBS, each preparation was placed, on a poly-L-lysine (PLL)-coated glass slide, with the surface to be examined uppermost, and stretched lightly when almost dry. The immunostaining was done on the whole mount stretch preparation using an antiserum twice the concentration used for routine indirect immunofluorescence. After 30-40 hours incubation at 4°C, the free-floating preparations were rinsed in PBS, 3 times, 20 mins each. The second layer fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit antiserum was applied at double strength (1:50) and incubated for 3 hours.

This technique not only exposed the desired plane of the tissue for investigation, but also preserved the interconnections and the relationships of the different neurons inside the tissue. When examining the results under the light microscope, it was possible to trace individual nerve fibres to a certain extent by focussing up and down through the tissue. The organisation of the plexus could thus be evaluated.

Similar techniques of layer separation have been used previously in the small intestine (Costa et al 1980; Ferri et al 1983b). In this

study, the layers were separated in the fixative so that both the degradation of the antigen due to excessive handling of the tissue prior to fixation and the difficulty of separating post-fixed tissue, which was often rigid, were avoided. The combination of this layer separation technique and the modified indirect immunofluorescence staining method (see Section 2.7.) has been found to be ideal for revealing certain sparsely distributed peptides such as somatostatin.

2.7. MODIFIED IMMUNOFLUORESCENCE STAINING PROCEDURE

The indirect immunofluorescence technique has been the most frequently used method in the present study. This was because it could not only provide a good contrast between specific and background staining but it was also best suited to the liquid phase fixation and cryostat sections which are ideal for peptide preservation, as shown in Section 2.4.. For this reason, the indirect immunofluorescence method of Coons et al (1955) has been widely employed in this laboratory as well as elsewhere. The stainings have generally been found to be satisfactory. It was thought, however, that the sensitivity of the indirect immunofluorescence method was not yet optimal and many modifications were made. These included optimising the antibody concentrations, prolonging the incubation time (Brandtzaeg 1981; Scott et al 1981), raising the incubation temperature (Huang et al 1976; Portmann et al 1976; Dorsett & Joachim 1978) and treating the tissue with detergent (Hartman et al 1972; Hartman 1973). Considerable improvement was achieved by such modifications.

In an effort to optimise the immunofluorescence staining procedure during the present study, it was found that repeated application of the first layer antiserum could result in much better staining. There was sharper contrast between specific and background staining and apparently a greater abundancy of explored antigenic sites. To assess the full potential of this novel procedure, an experiment was designed and carried out to compare the proposed improvement with the routine staining procedure and with some other modifications, including prolonging the incubation time with the first layer antiserum, changing the concentration of the first layer antibody, treating the sections with a detergent, Triton X-100, and combinations of these variations.

The specimens chosen were guinea-pig ileum (n=4) and human (n=5) and feline (n=6) urinary tract including the lower portion of the ureter, and the dome and trigone of the bladder. All the tissues were either fixed in BQS or freeze-dried and fixed in benzoquinone vapour and processed according to the routine procedures described in the Appendix (2.2.). Consecutive cryostat or paraffin sections, 5 µm thick, were prepared for the experiment.

The antisera used were those against VIP, DβH, glucagon, serotonin and NSE. On serial sections from each area, the following procedures were carried out.

(1). Routine procedure, i.e., sections were incubated with the first layer antiserum for 12-16 hours at 4°C, the antiserum being

optimally diluted (that is, either higher or lower dilutions would impair the quality of the staining), generally at 1:500-4000 in PBS, pH 7.2, for antigens of peptides, amines and enzymes. The second layer, FITC-conjugated antibody, diluted 1:100 in PBS, was applied for 30-45 mins at room temperature. After each step the slide was washed in PBS three times, 5 mins each. The stained sections were mounted in PBS/glycerine (1/9) and examined immediately. (for detailed procedure see Appendix 2.4.4.).

(2). Increasing or decreasing the concentration of the first layer antiserum.

(3). Pre-treating the cryostat sections with 0.2% Triton X-100 in PBS for 30 mins or using this solution instead of PBS to dilute the antisera.

(4). Prolonging the incubation time with the first layer antiserum up to 72 hours at room temperature or 4°C.

(5). Repeating the first layer antiserum application for 2-20 hours at room temperature or repeating 2-3 times, each time for 2-6 hours.

(6). Combinations of the above procedures.

(7). The same modifications were attempted using the unlabelled antibody-enzyme, peroxidase anti-peroxidase (PAP) method (Sternberger 1974).

The controls included preabsorption of the first layer antisera with their appropriate antigens, using normal rabbit serum as the first layer or simply omitting the first layer antiserum (see Section 2.9.).

The immunostained sections were examined blindly with no indication of which procedure was used on each section. The relative abundance and brightness of the specific staining and the background staining were assessed using a marking system with the same criteria throughout (see Section 2.10.). The degree of contrast between the specific staining and the background staining was regarded as a measure of the quality of the immunostaining.

The results are shown diagramatically in Diagram 7 and are summarized below.

a). Repeating the first layer incubation, prolonging the incubation time with the first layer antiserum and treating the section with Triton X-100 all improved the quality of the staining by noticeably increasing the specific staining with or without a small increment of the background staining.

b). The improving effect was most marked when the first layer was repeated and the total time of the first layer antibody incubation was equal to that of the routine procedure (Figs. 1.2.). This improvement was still apparent when the first layer antiserum was further (double) diluted. Repeating incubation with the first layer antiserum more than once achieved little further improvement.

c). The improvement after repeating the first layer was more obvious on immunostained nerves (VIP, DβH and NSE) than on cells where the antigens are generally more abundant. The improvement was less noticeable when immunostaining by the PAP method or when using

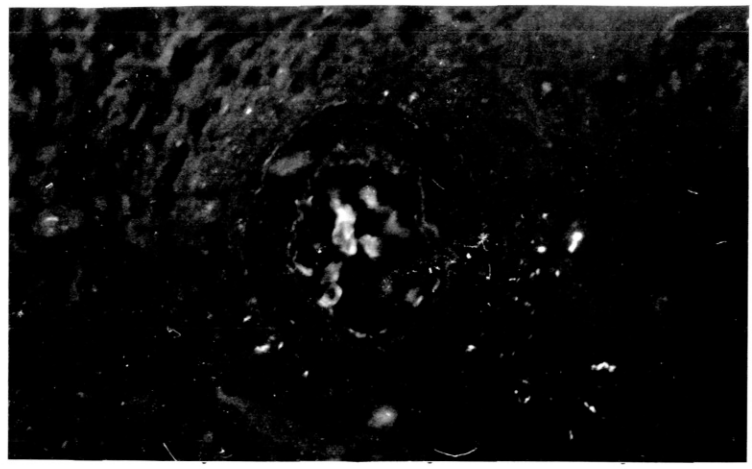


Fig. 1. A blood vessel in normal human ureter. The routine procedure for the indirect immunofluorescence method was used with antiserum to DβH. Sparse DβH immunoreactivity was identified (x250)

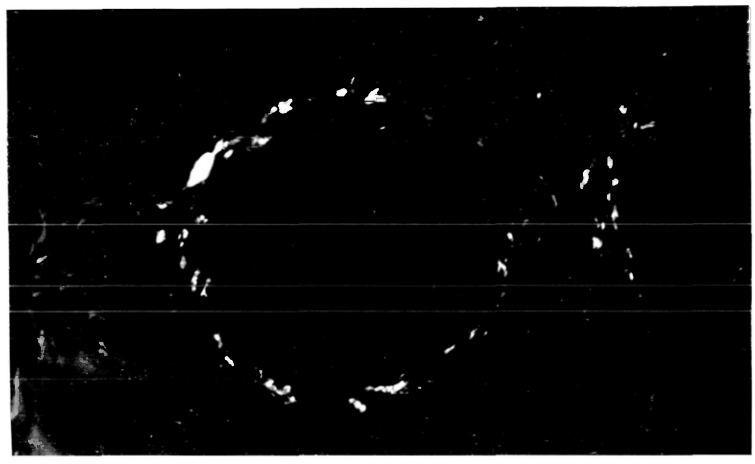


Fig. 2. An adjacent section of the same thickness (8μm) to Fig.1. When the first layer antiserum was repeated once for 4 hours, more DβH-IR nerves were immunostained (x250).



Fig. 3. An adjacent section (8μm) to Fig.2. When the combined modifications were used, many more DβH-IR nerves were identified (x250).

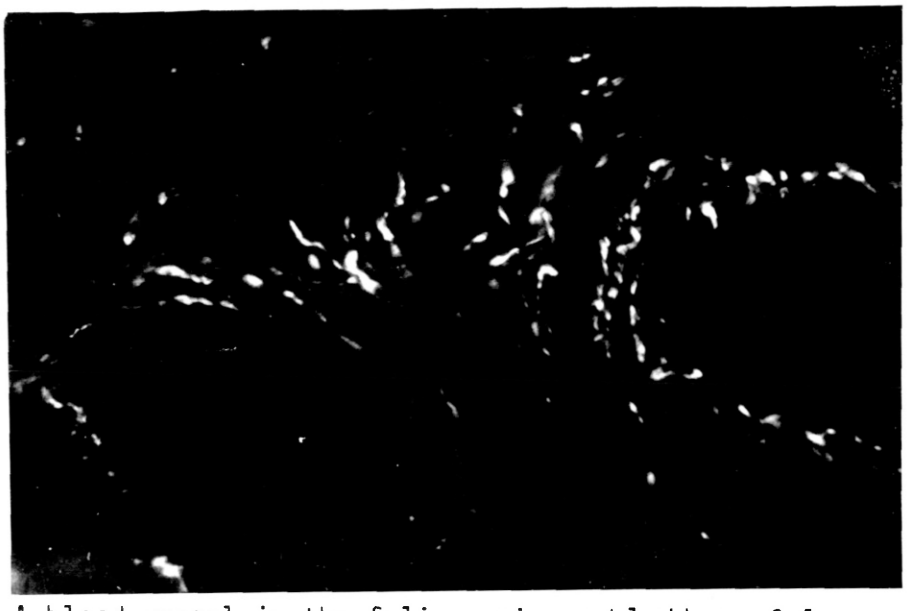


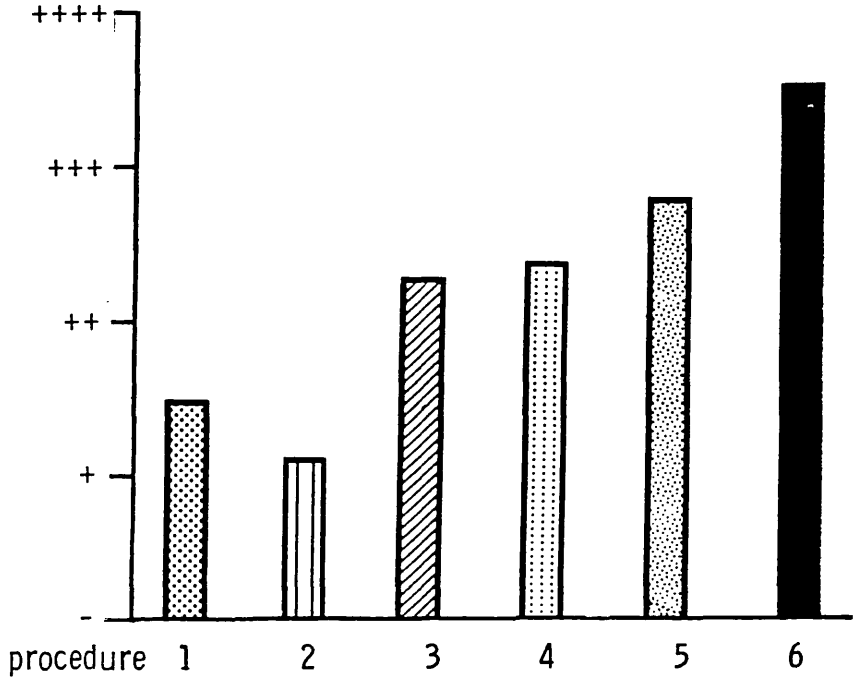
Fig. 4. A blood vessel in the feline urinary bladder. Only a small number of immunostained nerves were demonstrated when the routine procedure for the indirect immunofluorescence method was used (x420).



Fig. 5. An adjacent section (8μm) to Fig.4. When the combined modification was carried out, many more VIP-IR nerves were revealed (x420).

Diagram 7

EFFECTS OF MODIFICATIONS ON THE INDIRECT IMMUNOFLUORESCENCE PROCEDURE



The height of the bars represents the quality of the immunostaining, ie. the net results obtained by marking the abundancy and brightness of the specific staining(0-4+) after subtraction of the background staining(0-4+).

Procedure 1. routine procedure.

Procedure 2. changing the concentrations of the first layer antibodies.

Procedure 3. using Triton X-100 to pretreat the sections or to dilute the antiserum.

Procedure 4. prolonging the incubation time with the first layer antiserum.

Procedure 5. repeating the first layer antiserum application.

Procedure 6. combination of procedure 3, 4 and 5.

paraffin wax rather than cryostat sections of the same tissue. Presumably, this may be due to the higher sensitivity of the PAP technique compared with that of the indirect immunofluorescence procedure. The reason for the lack of improvement on paraffin sections is not known.

d). The best result was obtained using a procedure of combined modifications (Figs. 3,4 & 5), ie. pre-treating the sections with 0.2% Triton X100 in PBS for 30 mins, repeating the first layer incubation for 4 hours and prolonging the incubation time with the first layer antibody to a total of about 30 hours, along with increased washing time after each staining step (three times, 10 mins each). This procedure sometimes changed an apparently negative staining result into a positive one and revealed many more antigenic sites than the rountine procedure.

e). Increasing the concentration of the first layer antibody generally increased both the specific staining and the background staining, resulting in reduced contrast and a worse picture.

f). Finally and most importantly, the validity of the new procedure was tested by appropriate controls which showed that the modification did not create any non-specific positive staining but only compensated for the deficiencies of the conventional technique.

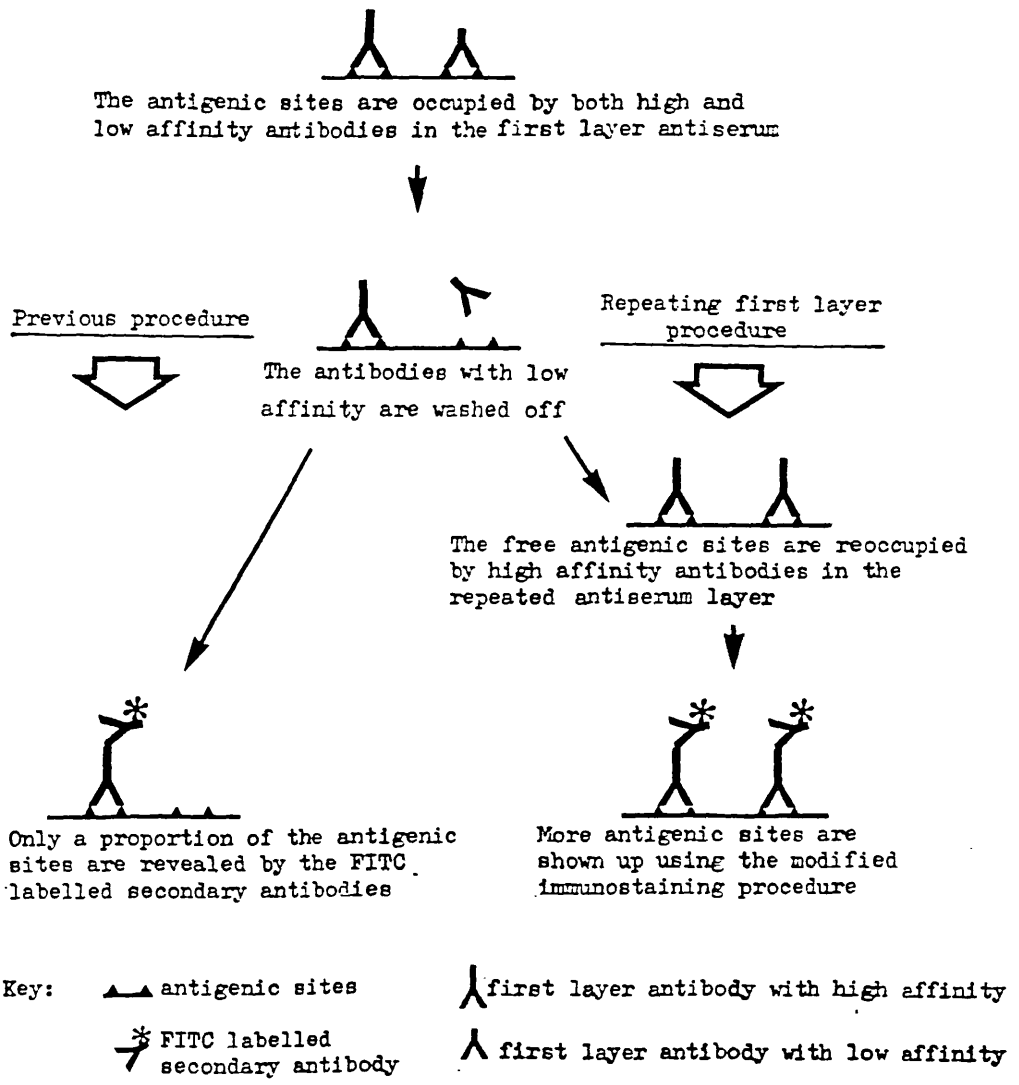
The possible mechanism of this improvement has not yet been fully clarified but can be speculated upon. It was possible that the first layer antiserum consisted of both high-affinity and

low-affinity specific antibodies. During the first incubation with the first layer antiserum, all the antigenic sites on the section were occupied by one or other of the antibody types. In the subsequent washing, the low-affinity antibodies were washed off and consequently the fluorescent label was only built up at the proportion of the antigenic sites which were initially bound by high-affinity antibodies. The vacated antigenic sites were largely re-stained by the second application of the fresh first layer antiserum, thus revealing more antigenic sites (Diagram 8).

Another speculation is that the antibodies were uniformly distributed in the antiserum and the antigenic sites were not uniformly distributed on the tissue section. The antibodies above the antigenic sites were soon used up and the slow diffusion of antibodies from neighbouring areas took time. In the routine staining procedure, incubation was stopped before the diffusion was complete. This might explain the fact that longer incubation time gave much better staining. Repeating the first layer, i.e., replacing the half-used antiserum with fresh antiserum, provided another antibody concentrate and left few free antigenic sites on the tissue, thus achieving much better staining result. The second assumption is in agreement with a recent study by McMillan (1982) who found that rocking the slides during incubation, ie. to speed up the diffus ion of the antibodies in the antiserum, could considerably improve the immunostaining results. It is of course possible that, apart from the above postulated mechanisms, some other factor or factors might also have contributed to the improvement resulting from the present modification.

Diagram 8

THE POSSIBLE MECHANISM IN THE REPEATING FIRST LAYER PROCEDURE



The combined procedure, ie. procedure No. 6 (Appendix 2.4.5.), was commonly used in this study, particularly where the antigenicity was sparse. In subsequent immunostaining, it has been further proved that this procedure is superior to the previous technique both in the sensitivity of the method in terms of the antibody concentrations used, and in the quality of the staining in terms of the contrast between the specific and background stainings achieved.

2.8. LIGHT MICROSCOPICAL GOLD-LABELLED ANTIBODY METHOD

The immunogold staining method is a novel method for light microscopical immunocytochemistry. It was developed in the present study when attempts were made to search for different methods for better immunostaining. Unfortunately, the immunogold staining method did not find direct application in mapping the distribution of peptide-containing elements in the present study. However, it remains as an alternative, useful and prospective technique in light microscopical immunocytochemistry, particularly when double and multiple immunostainings are to be attempted.

The conjugation of colloidal gold to immunoglobulins was first introduced by Faulk & Taylor (1971). It was subsequently modified and successively employed in immunoelectron microscopy (Romano et al 1974; Horisberger et al 1975; Geoghegan & Ackerman 1977). The superiority of this new approach was soon shown as it is highly precise and sensitive, so that antigenic sites can be punctately

labelled by very small (5-40nm in diameter) highly electron-dense gold particles without being shadowed by patches of immunoreaction products as in the case of PAP procedure (for review see Varndell et al 1982). However, the possibility of the application of the immunogold staining at the light microscopical level had been neglected. In the present study, the immunogold staining method was found to be suitable for light microscopical immunostaining and to possess many advantages including: 1, the immunogold staining method is simpler to apply than the PAP method since incubation with the enzyme substrate is unnecessary; 2, the final coloured product develops gradually so that the reaction can be easily controlled under the light microscope; 3, it gives a permanent, bright red colour with very low background which is highly suitable for double staining (Fig. 6a, b).

The immunogold staining method uses the colloidal gold labelled second and/or third layer antibodies instead of the FITC and peroxidase labelled ones. Under the transmitted light microscope, the investigated antigenic sites were visualized because of the accumulation of immunoglobulin-coated colloidal gold particles. It was carried out in three ways. First, a two step method was used in which the first layer antibody was raised in rabbit and the second layer was goat anti-rabbit IgG adsorbed onto gold particles (GAR/Au20). Secondly, a three step method was employed where the second layer was unlabelled goat anti-rabbit IgG and the third layer was a rabbit IgG adsorbed onto the gold particles (RIG/Au20) and acting as a gold-labelled IgG antigen. The third method combined the first two methods using GAR/Au20 as the second layer and

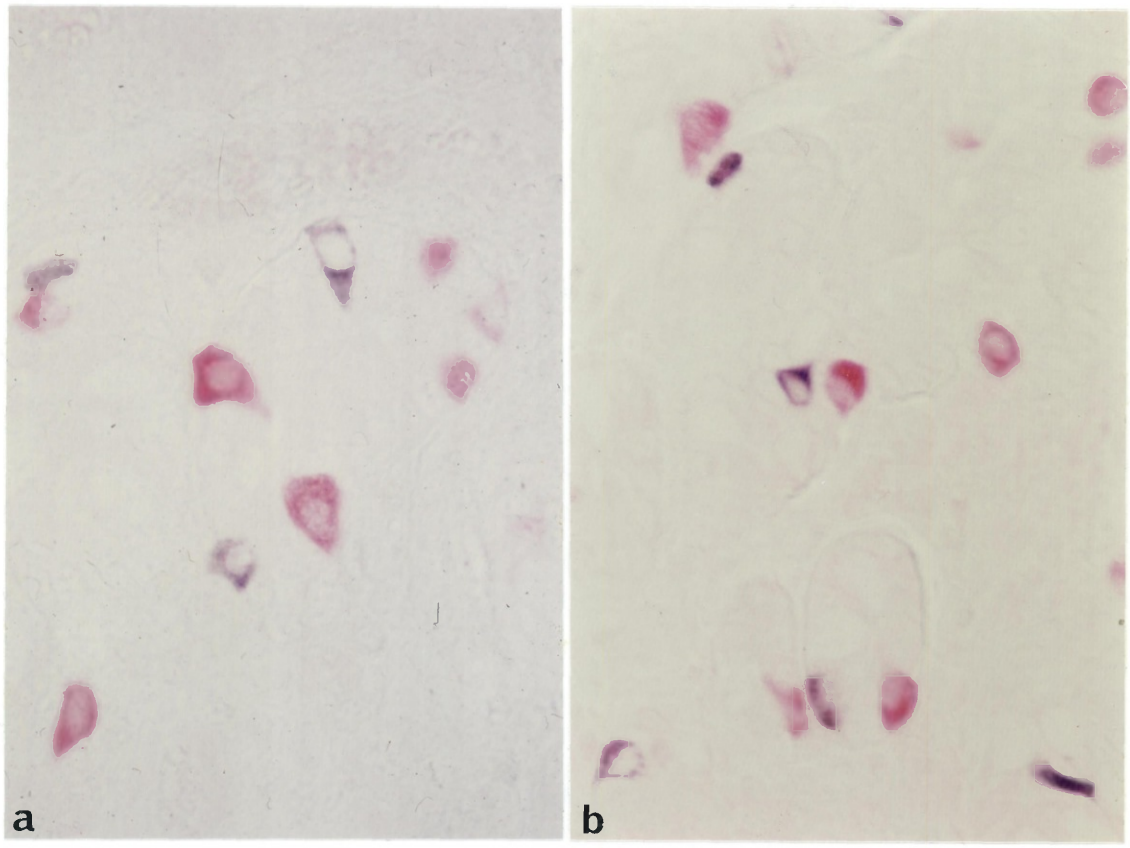
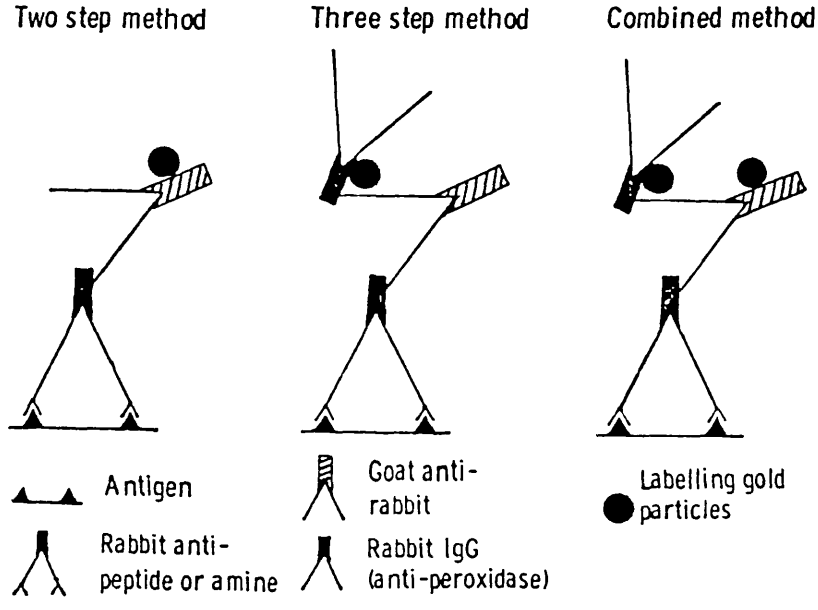


Fig. 6a, b. Double immunostaining of gastrin- and 5HT-IR cells in the porcine duodenum. The gastrin-IR cells (red) were stained with the immuno-gold method and the 5-HT-IR cells (dark-blue) were stained with PAP (4-chlorol-1-naphthol) method. (x620 -a; x500-b).

RIG/Au20 as the third layer, thus increasing the amount of bound gold and enhancing the red colour to provide a better picture. The third method has been found to be superior to the other methods, particularly when the antigen was scarce. The procedures are described in Appendix 2.4.7.. The principles of the three different ways to apply the immunogold staining method are illustrated in Diagram 9.

The gold-labelled antibodies were prepared by Dr J.De Mey and his colleagues in the Dept. of Life Science, Laboratory of Oncology, Janssen Pharmaceutica Research Laboratories, Belgium. The procedure was based on a modification of the methods of Geoghegan et al (1977) and Horisberger et al (1975). Uniformly sized colloidal gold particles were adsorbed onto the rabbit IgG by the negative charge of the gold particle formed in a high pH (pH 9.0) buffer. The immunoreactivity of labelled immunoglobulin was not impaired. Under the electron microscope, the gold particles are easily recognisable, highly electron-dense small round objects. Under the transmitted light microscope, the accumulation of the colloidal gold gives a bright red colour over the antibody-antigen reactive sites. The labelling gold particles are uniformly sized, and available in grades from 5 - 40 nm in diameter. It was found during the experiments that at the light microscopical level, the larger the size of the labelling gold particles, the greater the sensitivity of the immunogold staining method, ie. the lower the concentration required. This might be due to the fact that a small number of large sized particles would provide a total amount of gold equal to that provided by a large number of small sized ones.

Diagram 9


Two step method
Three step method
Combined method
Antigen
Rabbit anti-peptide or amine
Goat anti-rabbit
Rabbit IgG (anti-peroxidase)
Labelling gold particles

In addition, it was interesting to find that the cells and nerves on semi-thin sections stained with the immunogold staining method could also be visualised under a dark-field microscope. The gold particles bound to the tissue section reflect the light, shining like a mirror with bright golden colour even when the red stains were very weak under a transmitted light microscope. This novel dark-field approach gave the stained antigenic sites a very strong contrast and a very high resolution, thus further increasing the sensitivity of the immunogold staining method (Fig. 7). Since the staining can be carried out on very thin sections (1 μm), it may be used as a bridge method to correlate the immunocytochemical investigations at the light and electron microscopical levels.

As the final coloured products are bright red and the intensity of this colour can easily be controlled, the immunogold staining method has been found to be very suitable for light microscopical double immunostaining. A new double immunostaining procedure has thus been developed combining the PAP method and the immunogold staining method. Between the two stages of the double staining procedure for the two different antigens, the antibody complexes bound on to the first antigenic sites were eluted in glycine-hydrochloric acid solution at pH 2.2 (5ml of 0.1 NHCl, 95ml of glycine in NaCl solution (0.757% glycine in 0.5844% NaCl)) for 2 hours (Nakane 1968). This step has been found to be necessary for avoiding the cross-reaction of the antibodies to the second antigen with the antibody complex on the first antigenic site. The 4-chloro-1-naphthol method of Nakane (1968) was modified (for detail

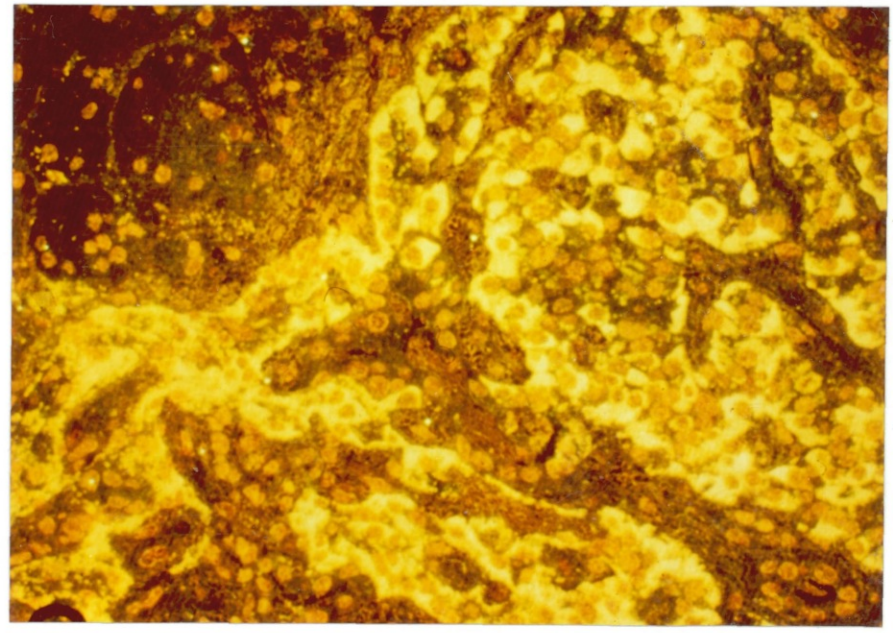


Fig. 7. Dark-field immuno-gold stained preparation.
Glucagon-containing A cells in the porcine pancreas. The tissue was embedded in araldite and the section was 1µm thick. Under transmitted light, the staining is weak. The resolution is greatly improved when viewed with the dark-field technique. (x200).

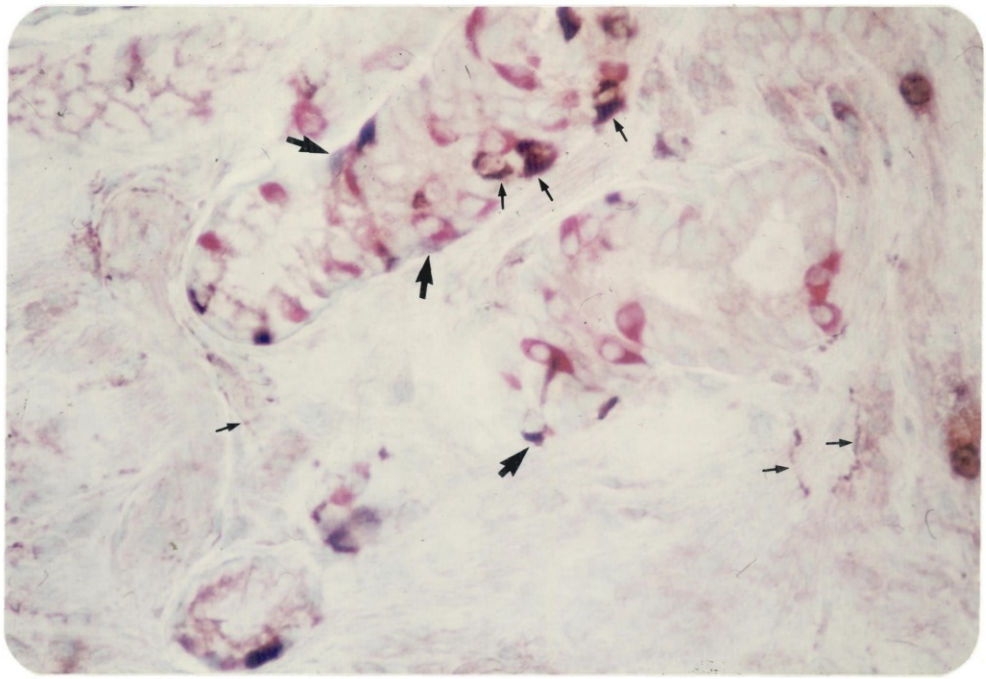


Fig. 8. Multiple immunostaining of gastrin-, somatostatin- and 5-HT-containing cells and VIP-IR nerves in the porcine duodenum. The gastrin-containing cells (red) were stained with the immuno-gold method. The somatostatin-containing cells and VIP-containing nerves (brown, small arrows) were stained with the PAP (DAB) method. The somatostatin-containing cells (dark-blue, big arrows) were stained with the PAP (4-chloro-1-naphthol) method. The relationships between different elements can be seen on a single section (x100).

see Appendix 2.4.7.4.) and used to visualise the PAP complex which gave a dark blue colour, forming an excellent contrast to the red stain of the gold (Fig. 5). The validity of the double staining method was established by appropriate controls including positive and negative controls specially designed for double immunostaining (see Section 2.9.). The immunogold staining method can also be used in multiple immunostaining to study the locations of several antigens simultaneously on the same tissue section (Fig. 8).

2.9. ANTISERA AND SPECIFICITY OF THE IMMUNOSTAINING

The antisera used in this study were raised in New Zealand white rabbits by intraperitoneal injection of peptide immunogens. The peptide immunogens normally consisted of purified or synthetic peptides which were conjugated to large carrier proteins by coupling agents to increase the immunogenicity. The immunoresponses of the rabbits were subsequently boosted with one or more subcutaneous injections of the same immunogen. The blood samples of the rabbits were harvested and the sera which contained high concentrations of specific antibodies were selected by, in the case of immunocytochemistry, immunostaining on sections with known antigens and preabsorption tests with the antigen molecule and its various fragments (see below). For simplicity, the characteristics of the antisera used are presented in tabular form (Table 2a-f).

The specificity of the immunostaining, ie. whether or not the revealed sites on the tissue section really are the residues of the

antigen of interest, is one of the most important issues in immunocytochemistry. Unspecific staining can occur for several reasons and, generally, it can be prevented. The following have been found to be key points for successful immunostaining.

a) Any serum taken from an animal immunised with a certain antigen may contain any number of different antibodies. Diluting the antiserum as much as possible whilst retaining specific immunostaining will remove many such sub-populations of immunoglobulins. Also, the antiserum may include antibodies against irrelevant antigens present in the sections to be subsequently immunostained. The Fc portion of antibodies may bind to receptors in the tissue. This non-specific attachment of immunoglobulins may be prevented by incubating the sections with normal serum (from the same species as the donor of the second antibody) prior to application of the primary antibody. Some non-specific binding may also be prevented by absorbing the antiserum with substances, e.g. liver powder, which do not contain the specific antigen. This will remove the unwanted antibodies.

b) The specific antibodies in the antiserum can be isolated from the unspecific ones by antigen-specific affinity chromatography. In this case the antiserum is incubated with specific antigens which have been coupled to a certain gel. The serum-gel mixture is poured into a column and washed with buffer at neutral pH to separate the specifically bound antibodies from the rest of the serum. These specific antibodies are subsequently removed from the antigens by eluting with acid buffer (pH 2.8). The acid eluent is then

neutralized and the specific antibodies are thereby purified (for review see De Mey 1983).

c) Full characterisation of each antiserum is essential since without this the immunocytochemical results are meaningless. Many peptides belong to "families", related by their possession of identical amino acid sequences. These common amino acid sequences may consist of a fraction of the peptide length or, conversely, two peptides may only differ by one or two amino acids. Since antibodies can be raised against specific regions of a molecule, it is important to know the structure of each antigen and the antigenic sites on each molecule which are recognised by each individual antiserum. This information can be obtained by pre-incubation of the antiserum with different peptide fragments. The fragment that abolishes the immunostaining probably has molecular sequences identical or similar to that of the tissue antigen to which the specific antibodies react.

d) The specificity of the immunostaining can only be validated by appropriate controls. These include; 1, use of non-immune serum in place of the first layer antiserum; 2, omission of the first layer antiserum; 3, absorption of the primary antisera with appropriate antigens prior to immunostaining. The specificity of the immunostaining can only be established if all the above controls give negative results. In some circumstances when two or more peptides had similar molecular structures, cross-preabsorption tests were carried out. In this case, the antiserum was incubated with undesired antigens which had amino acid sequences similar to the specific antigen, prior to immunostaining. The possibility of

antibody cross-reaction can be ruled out by unaffected positive staining obtained with the pre-absorbed antiserum.

In addition, special controls were designed to verify the specificity and sensitivity of the double immunostaining. They are described below.

In order to determine whether all the immunoreative antigens were immunostained with the double procedure, three serial sections were stained as follows. Section 1: only the first antigen was immunostained. Section 2: was processed using the double immunostaining procedure. Section 3: only the second antigen was immunostained. The three immunostained sections were compared. A sensitive double immunostaining procedure should give staining for each antigen of equal quality to those stained independently.

The efficiency of the elution of the antibody complexes from the in situ antigens, which was performed between the two stages of the staining for two different antigens, was tested in the following way: Firstly, the PAP technique was carried out on control sections, but the visualisation with the substrate was omitted. Then the antibody complexes on the tissue sections were eluted in glycine-hydrochloric acid solution for 2 hours. After rinsing in PBS, the sections were incubated again with the second and the third layer antisera, i.e. the unconjugated goat anti-rabbit antiserum followed by PAP complex, then 4-chloro-1-naphthol was applied with hydrogen peroxidase for visualisation of the complex. Efficient removal of the primary antibody complexes was demonstrated by the negative results obtained with this control.

Table 2a

DETAILS OF ANTISERA USED IN THIS STUDY FOR IMMUNOCYTOCHEMISTRY

Antisera (raised in rabbits) to	Ref.No.	Carrier/Conjugate		Specificity	Dilution		Quenching
					IF	PAP	nmol/ml
VIP (natural porcine)	652	KLH	BDB	whole	1:2,000	1:20,000	5.0
Substance P (synthetic)	910	BSA	Glut	C-term.	1:500	1:5,000	10.0
NPY (natural porcine)	820	KLH	BDB	whole	1:400	1:4,000	0.1
PHI (natural porcine)	938	BSA	Glut	C-term.	1:400	1:4,000	0.1
$Somatostatin_{14}$(synthetic)	744	BSA	CDI	whole	1:1,000	1:4,000	5.0
Met-enkephalin (synthetic)	869	BSA	Glut	C-term.	1:400	1:4,000	1.0
Bombesin (synth. amphibin)	627	BSA	Glut	whole	1:400	1:4,000	0.1
Gastrin (natural porcine)	899	KLH	BDB	C-term.	1:500	1.5,000	1.0
Serotonin (synthetic)	644	BSA	Formalin	?	1:1,000	1:10,000	1.0
Neurotensin (synth. bovine)	810	BSA	Glut	whole	1:800	1:1,600	0.1
ACTH (natural bovine)	133	BSA	CDI	whole	1:200	1:2,000	2.0
NSE (natural human)	666	-	-	?	1:200	1:2,000	1.0
NSE (natural rat)	841	-	-	?	1:200	1:2,000	1.0
S-100 (natural bovine)	704	-	-	?	1:800	1:4,000	2.0
DβH (natural bovine)	945	-	-	?	1:200	1:2,000	5.0
TH (natural rat)	871	-	-	?	1:400	1:2,000	5.0

Key - overleaf

Table 2a (continued)

Key:

BDB - Bis-diazobenzidine.

BSA - Bovine serum albumin.

CDI - Carbodiimide.

C-term.-The immunostaining can be quenched by the C-terminal portion of the antigen.

Glut - Glutaraldehyde.

IF - Indirect immunofluorescence method.

KLH - Keyhole limpet haemocyanin.

PAP - Peroxidase anti-peroxidase method.

Quenching - Quantity of antigen required in the pre-absorption test to abolish the immunostaining.

Ref.No. - Reference number of the antisera.

Specificity - Specific reacting portion of the antigen molecule to specific antibody, shown by pre-absorption test with peptide fragments or whole peptide.

Whole - The immunostaining can only be quenched by the whole molecule of the antigen.

Table 2b.

CROSS-ABSORPTION TEST OF VIP AND PHI ANTISERA

antisera to	antigen (nmol/ml)*	results**
VIP	VIP (5.0)	quenched
VIP	PHI (10.0)	not quenched
PHI	PHI (0.1)	quenched
PHI	VIP (10.0)	not quenched

* nmol antigen/ml of diluted antiserum

**Immunostaining results obtained using the antisera which have been pre-incubated with their own or related antigens

Table 2c

CROSS-ABSORPTION TEST OF ANTISERA TO SUBSTANCE P AND BOMBESIN

antisera to	antigen (nmol/ml)*	results**
substance P	substance P (10.0)	quenched
substance P	bombesin (10.0)	slightly decreased
substance P	bombesin (50.0)	same as above
bombesin	bombesin (0.1)	quenched
bombesin	substance P (10.0)	not quenched
bombesin	substance P (50.0)	not quenched

* & ** see table 2a

Table 2d

CROSS-ABSORPTION TEST OF MET- & LEU-ENKEPHALINS

antisera to	antigen (nmol/ml)*	results**
met-enk	met-enk (1.0)	quenched
met-enk	leu-enk (1.0)	not quenched
met-enk	leu-enk (10.0)	partially quenched
met-enk	leu-enk (50.0)	same as above

* & ** see table 2b

Table 2e

CROSS-ABSOPTION TEST OF NPY & APP ANTISERA

antisera to	antigen (nmol/ml)*	results**
NPY	NPY (0.1)	quenched
NPY	APP (10.0)	not quenched
APP	APP (1.0)	quenched
APP	NPY (1.0)	quenched

* & ** see table 2a

Note that the APP immunostaining was quenched by NPY antigen.

Table 2f

CROSS-ABSORPTION TEST OF NSE, NNE & S-100

antisera to	antigen (nmol/ml)*	results**
NSE	NSE (1.0)	quenched
NSE	NNE (up to 20.0)	not quenched
NSE	S-100 (up to 20.0)	not quenched
S-100	S-100 (2.0)	quenched
S-100	NSE (up to 20.0)	not quenched
S-100	NNE (up to 20.0)	not quenched

* & ** see table 2b

2.10. ASSESSMENT OF IMMUNOSTAINING AND ANALYSIS OF DATA

Assessment of the immunostaining results, particularly in a quantitative manner, could be very subjective. Certain criteria, therefore, have to be set up and used consistently throughout. In the present study, a 1-4 plus-marking system was used in which one plus, ie. +, represented a very small amount of positive staining, and four pluses, ie. ++++, represented extremely abundant staining. Intermediate grades represented increasing levels of positive staining. - meant negative and ± meant that the staining result was equivocal. When optimal immunocytochemical conditions were achieved for a given antiserum, the estimation was based mainly on the abundancy of the positively stained elements. The brightness or the intensity of the staining and background staining were also considered. These markings might not be absolutely objective. However, there has been no better method available during the study and such a plus-marking system has been commonly employed and generally accepted. Indeed, so many variable factors could be involved in the immunostaining process and might affect the outcome of the staining that even computerised image analysis, which is still in its embryological stage for quantifying immunocytochemical results, can hardly overcome these obstacles. Nevertheless, the plus-marking in this study was only used in a comparative manner, i.e. ++++ merely meant one grade more than +++, and + simply meant slightly less than ++. Great care was taken whenever these pluses were used. Comparisons of the abundancy of the same antigen in different tissues were made only when the processing conditions for the immunostainings were identical. Comparisons of the abundancies

of different antigens were made only when the optimal conditions were achieved for the immunostaining on each of them.

All the quantitative statements made in this thesis were based on consistent results obtained from tissues of at least 6 different human beings or of 6 animals in largely identical conditions. Significant individual variations are otherwise separately stated.

Dottograms are sometimes provided for clearer illustration of the results. Diagrams and histograms are often used to illustrate and summarise the significant findings. The radioimmunoassay data were statistically treated, often by paired or unpaired Student t-tests, as appropriate.

2.11. OTHER TECHNIQUES

2.11.1. 6-hydroxydopamine (6-OHDA)

To study the possible occurrence of peptide immunoreactivities in adrenergic and noradrenergic nerves, 6-OHDA was used. 6-OHDA is known to act as a false catecholamine neurotransmitter which can be taken up unspecifically by a high affinity uptake system on the presynaptic membrane of the amine-containing nerves, but, unlike physiological neurotransmitters, it can not be released again from the synapses (Thoenen & Tranzer 1971). Its accumulation produces selective degeneration of aminergic neurons. The effectiveness and selectiveness of this treatment can be checked by the depletion of

adrenergic and noradrenergic, but not cholinergic, nerves which can be demonstrated by histochemical and immunocytochemical staining methods. If any peptides were stored in the depleted nerves, they should also disappear following an effective treatment with 6-OHDA.

Adult rats of both sexes were treated with 6-OHDA (6-OHDA HCl, Sigma chemicals) by intraperitoneal injections (50 mg/kg body weight each, in saline) 8, 2 and 1 days before samples of urinary bladder and female genitalia were taken for examination. The tissue samples were then processed for immunocytochemistry and histochemistry according to currently employed procedures described in the Appendix (2.2., 2.3., 2.4.).

2.11.2. CAPSAICIN

Capsaicin is an ingredient of Hungarian red peppers and its local application can produce analgesic effects on animals (Jancso 1966) and impair neurogenic inflammation involving sensory nerves (Gamse et al 1980). It was thought to produce degeneration of unmyelinated afferents (Jancso et al 1967, 1977; Nagy et al 1980; Scadding 1980). The association of substance P and sensory neurons in the central nervous system and in the periphery was shown by the depletion of substance P-IR nerves following capsaicin treatment (Jessell et al 1978; Furness et al 1982b; Matthews & Cuello 1982; Sikri et al 1981; Papka et al 1981). It has also been shown that the disappearance of substance P immunoreactivity may not necessarily be due to the destruction of the sensory nerves but to

the liberation of substance P from primary afferent terminals followed by long term depletion of substance P from these nerves (Jessell et al 1978; Ainsworth et al 1981). Ultrastructural studies showed that the spinal dorsal root ganglion cells were intact following capsaicin treatment and the only apparent damage was in the mitochondria (Joo et al 1969). In the present study, capsaicin treatment was carried out on rats to destroy or damage their sensory nerves in the hope of depleting the peptide content in these nerves. Thereby, the sensory function of certain peptide-IR nerve types can be established.

Adult female rats (n=6) were examined. These rats were injected subcutaneously with capsaicin (50mg/kg body weight) dissolved in Tween 80 (polyoxyethylene sorbitan mono oleate, Sigma chemicals), ethyl alcohol and isotonic saline (1:1:8, vols) on day 2 after birth. The frequency and distribution of different types of peptide-IR nerves were examined and compared with those of control rats (n=6), which were injected with vehicle only.

2.11.3. SURGICAL NERVE TRANSECTION

To study the origin of peptide-IR nerves in the urinary bladder and the female genitalia, surgical sectioning of nerve supplies to these organs were performed under general anaesthesia with sodium pentobarbitone. The operations were performed by Dr J.M.B.Morrison, Dept. of Physiology, Leeds University, Leeds. The details of the operations are presented in Chapter 3 (see Section 3.2.2.).

2.11.4. ELECTRON MICROSCOPY

Conventional electron microscopy and immunocytochemistry at the electron microscopical level were performed by Dr L.Probert (human male genitalia) and Mr F.Tapia (skin), Dept. of Histochemistry, Hammersmith Hospital, London. For technical details, please refer to Probert et al 1983 and Van Noorden & Polak 1983.

2.11.5. RADIOIMMUNOASSAY AND CHROMATOGRAPHY

The concentrations and the identities of peptide immunoreactivities in the investigated tissues were examined by radioimmunoassay and chromatography respectively. These were carried out by workers from Professor S.R.Bloom's laboratory, Dept. of Medicine, Hammersmith Hospital, London. For technical details, please refer to Bloom & Long 1982.

2.11.6. HISTOCHEMICAL METHODS

To demonstrate cholinergic nerves, the relatively specific method of El-Badawi & Schenk (1967) was used. For identifying catecholamine-containing nerves, the modified glyoxylic technique, the SPG (sucrose-potassium phosphate-glyoxylic acid) method (De La Torre & Surgeon 1976), was employed. The melanocytes in the skin were demonstrated with the L-DOPA method of Becker et al (1935). The details of the staining procedures are presented in the Appendix (2.4.8., 2.4.9., 2.4.10., 2.4.11.).

2.12. SUMMARY

1. The main technique used in this study was immunocytochemistry at the light microscopical level. Results obtained from other methods, complementary to the immunocytochemical findings, are also presented. These techniques include histology, histochemistry, electron microscopy, electron-microscopical immunocytochemistry, radioimmunoassay and chromatography. To study the nature and origin of the peptide-containing nerves, surgical and pharmacological manipulations were performed on experimental animals. Prior to the study, pilot experiments were carried out to verify the reliability and sensitivity of the techniques employed.

2. Normal and abnormal human tissues were the main interest of the study. These were obtained at surgical operations, biopsies and autopsies. Tissues from animals were also investigated. The species studied included rat, mouse, cat, guinea-pig and pig. Since it was sometimes impossible to obtain the tissue immediately after surgery or within a short time of death, degradation rates of tissue antigens of relevant peptides and substances were studied. This was carried out by immunostaining different gut and genitourinary tissues on which the fixations started at different periods of time after removal from a living body. Little difference between freshly fixed tissue and those fixed 7-10 hours after removal was found, in terms of the intensity and abundancy of the immunostaining and the background staining. VIP and substance P were able to survive longer than the others. The degradation curves were produced and found useful in assessing the immunostaining results in subsequent studies.

3. As the peptides are water soluble, they were rendered insoluble by fixation while surrounding tissue structures were also well preserved. A number of fixatives were tested. It was found that a number of cross-linking agents, such as benzoquinone, which can be used in solution or in vapour form, were superior to formalin in preserving the antigenicity of the peptide antigen. The conventional formalin fixations, however, were found suitable for the immunostaining of the neuronal proteins, NSE and S-100.

4. To explore the network of nerves in the guinea-pig gallbladder, a layer separation technique was adapted from previous procedures and modified to suit the present study. The different histological layers of the gallbladder were separated in the fixative, so that the antigenicity loss due to the excessive handling of the tissue and the hardening effect of the fixation on the tissue were avoided.

5. The indirect immunofluorescence staining procedure was modified by repeated application of the first layer antiserum. It was found that this procedure increased the sensitivity of the immunostaning, resulting in a better picture. The possible mechanism of the improving effect of this modification is discussed. A novel procedure, which combined various modifications, was found to give the best results for staining nerves on cryostat sections. In this procedure, sections were pretreated with 0.2% Triton X-100, the incubation of the first layer antiserum was prolonged up to 30 hours at room temperature or 4°C, the first layer incubation was repeated for 4 hours and the washing between each step was more thorough. The

specificity of the staining obtained using the new procedures was established with appropriate controls

6. When searching for different methods to improve immunostaining, a light microscopical colloidal gold-labelled antibody method was developed. In this method, the second or third layer antibodies were labelled with small (5-40 nm in diameter), highly electron-dense gold particles by the negative charge of the gold formed in a high pH buffer. Under the light microscope, the accumulation of the gold particles gave a bright red colour which was ideal for double and multiple staining. Other advantages of this method include simplicity and reliability. On semi-thin sections (1-2μm), the immunogold staining can be visualised using dark-field microscopy, in which the gold particles reflect the light with a bright golden colour, greatly improving the resolution. Three procedures of applying the immunogold method were developed and a double immunostaining technique was introduced.

7. The specificity of the immunostaining techniques and the characteristics of the antisera were well established by various tests and appropriate controls. Problems in immunocytochemistry and their resolution are discussed.

8. A 1-4 plus-marking system was used to assess the immunostaining results.

Chapter 3: THE URINARY BLADDER

3.1. NORMAL URINARY BLADDER

3.1.1. INTRODUCTION

It has been known since the mid-sixties that the mammalian urinary bladder is innervated by both sympathetic and parasympathetic nerves, whilst part of the external sphincter of the bladder neck is innervated by somatic nerves. Both efferent and afferent fibres are contained in these nerves and they enter or leave the bladder mainly accompanying the vesical arteries at the lateral-posterior portion of either side of the organ. Nerves from each side are distributed mainly in their own half of the bladder and up to about one third of the total number of nerves spread across the midline into the opposite half. The primary sympathetic motor neurons are located at the lower thoracic and upper lumbar segments of the spinal ventral horn, which give off axons across the sympathetic chain to form bundles of nerves running down to the superior hypogastric (in man) or the inferior mesenteric (in other mammals) plexus. Two elongated plexuses are derived from the superior hypogastric plexus, i.e. the left and right hypogastric plexuses. In these plexuses, many of the fibres from primary ganglia synapse with the secondary sympathetic ganglia and their dendrites; a certain proportion of the primary sympathetic fibres go through the plexus without interruption and synapse with neurons in the pelvic plexus, vesical plexus or inside the vesical wall. In the bladder wall, the secondary sympathetic nerve fibres are distributed in all the regions but are

particularly concentrated in the base of the bladder, where they innervate the blood vessels and smooth muscle cells. Some of the nerve fibres in the submucosa are also derived from the sympathetic nerves.

The parasympathetic pathway originates from ganglia present in the ventral horn of the upper and middle sacral segments of the spinal cord. The secondary ganglia are located predominantly in the pelvic and vesical plexus and rarely inside the vesical wall. Their nerve fibres spread more or less evenly in all the regions of the bladder and are mainly related to detrusor muscle and blood vessels. In addition, the network of nerve fibres in the submucosa, known as the submucosal plexus, is in part constituted by afferent nerve terminals running in the parasympathetic pathways (for reviews see Sundin 1978; El-Badawi 1982).

According to the classical view, the sympathetic efferent nerves of the secondary ganglia are functionally and histochemically adrenergic or noradrenergic in nature, whilst all the others, including primary sympathetic efferent, parasympathetic efferent and afferent and somatic nerves, are cholinergic (for review see Williams & Warwick, 1980a).

The above view on the innervation of the urinary bladder, initially based on the human urinary bladder, has been found to be largely applicable to other mammals, although species variations exist. Many experiments have been carried out in order to help in the understanding of the vesical innervation. Some explanations have

been put forwards to illustrate the mechanisms of the neural control of the urinary bladder.

It is still puzzling, however, that the excitatory effect of exogenous ACh on the bladder is antagonized by atropine, whereas the bladder response to nerve stimulation is not, as shown by Langley & Anderson almost a century ago (1895). Further studies demonstrated that the response to electrical stimulation, at physiological frequencies, of either the pelvic or the hypogastric nerves was unaffected by adrenergic blockers and was only partially reduced (about 60%) by cholinergic blockers (Alm & Elmer 1975). In addition, the contractile responses of the rat bladder to stimulation of the pelvic nerve at low frequencies or to short transmural stimulations were completely atropine-resistant (Elmer 1975a; Carpenter 1977). It has been suggested, therefore, that these nerves may release a purine nucleotide, e.g. adenosine triphosphate (ATP), instead of ACh as a neurotransmitter (Burnstock 1971, 1972; Downie et al 1976). However, ATP is known to exist in virtually all the neurons (Nathanson 1977; Richards & Da Prada 1977; Iijima 1981) and, in vitro, no response was elicited by ATP in the rat urinary bladder (Clark & Mitchelson 1974).

The occurrence and distribution of peptide-containing nerves have been reported in the urinary bladder and been put forward to explain the non-adrenergic, non-cholinergic response (Hökfelt et al 1978; Alm et al 1980a). However, the findings were based on examination of the urinary bladders of only 3 cats and a few guinea-pigs. The description of the distribution was very preliminary. In addition,

information on the distribution of such nerves in human urinary bladder has not been previously available. In the present study, the occurrence, distribution and origin of the peptide-IR nerves and their relationships with previously recognised nerve types in the urinary bladder of five mammalian species was investigated in a systematic manner. The implications of the findings are discussed.

3.1.2. EXPERIMENTAL DESIGN

The urinary bladders of man (n=25), rat (n=36), cat (n=8), mouse (n=14) and guinea-pig (n=12) were investigated. The human urinary bladder tissues were obtained fresh at surgery for tumours, open bladder biopsy, per-urethral biopsies and from post-mortem examinations within 10 hours after death. The human bladder tissues were dissected into trigone, dome and lateral walls. The animals were killed by exposure to excess ether and the urinary bladders were dissected immediately. The bladders were divided into trigonal area and the dome. The tissues were fixed in BQS (Appendix 2.2.1.) and the modified indirect immunofluorescence method was carried out on 8 μm thick cryostat sections (Appendix 2.4.5.). The antisera employed included those against VIP, substance P, NPY, PHI, somatostatin$_{14}$, met-enkephalin, bombesin, NSE, S-100, TH and DβH. The distribution of nerves containing classical neurotransmitters was demonstrated by histochemical methods (Appendix 2.4.8., 2.4.9., 2.4.10) as well as by immunostainings of the enzyme markers.

Radioimmunoassays of VIP, substance P, NPY, PHI and

somatostatin$_{14}$ were also performed, by workers from Prof.S.R.Bloom's laboratory, on the bladders of man, cat and rat to measure the concentrations of the above peptides.

In order to establish the possible sensory nature of some peptide-immunoreactive (IR) nerves, the urinary bladders of capsaicin- and vehicle-treated rats were investigated (see Section 2.11.2.). To study the possible occurrence of peptide immunoreactivities in noradrenergic nerves, the urinary bladders of 6-OHDA-treated rats were examined (see Section 2.11.1.).

3.1.3. PEPTIDE-IMMUNOREACTIVE (IR) NERVES IN NORMAL HUMAN URINARY BLADDER

In the present study, four types of peptide immunoreactivity were demonstrated in nerves of the human urinary bladder. They were VIP, PHI, NPY and substance P. In addition, the immunoreactivity of another peptide, somatostatin, was detected by radioimmunoassay alone with a very low concentration which might be beyond the limit of immunocytochemical detection.

Among these four types of peptide-IR nerves, VIP- and NPY-IR nerves were the most abundant, whilst the PHI-IR nerves were much fewer in number and the substance P-IR nerves were encountered only occasionally.

VIP-IR nerve fibres presented a varicose appearance and, in some areas, formed networks. A considerable number of such nerves were distributed in the submucosal regions beneath the transitional

epithelium and constituted, at least in part, the submucosal plexus. No penetration of the transitional epithelium by fibres was seen (Fig. 9). These nerves were more abundant in the trigonal base and the neck of the bladder than in other regions. Two populations of VIP-IR nerves were present in the submucosa. One, the major population, ran freely in the connective tissue without apparently innervating any muscular or glandular structures, and was often seen in the close vicinity of the basement membrane of the epithelium. The other was closely associated with blood vessels, particularly the arteries and arterioles. No VIP-IR nerve bundles were present in these regions.

In the muscular coat, VIP-IR nerves were also frequently seen. They occurred both between and inside the smooth muscle blocks (Fig.10). Those running in the connective tissue between the muscle blocks were isolated fibres or small bundles with fewer varicosities (Fig. 11). Inside the muscle blocks, separate, highly varicose VIP-IR nerve fibres were seen, usually running parallel to the smooth muscle cells but with occasional bifurcate cross-fibres (Fig. 10). Single VIP-IR nerve fibres seemed to be accompanied by many smooth muscle cells. A larger number of VIP-IR nerve fibres were shown in the muscle layer when more sensitive immunostaining techniques were used (see Section 2.7.), possibly indicating a relatively low concentration of VIP immunoreactivity present in a larger number of nerve terminals. Such nerves occurred more or less uniformly in the detrusor muscle of all the regions with a slightly higher frequency towards the base and the neck of the bladder. VIP-IR nerves also surrounded most blood vessels (Fig.12).



Fig. 9. VIP-IR nerve fibres in the submucosa of the human urinary bladder. The nerves are distributed close to the transitional epithelium (x185).

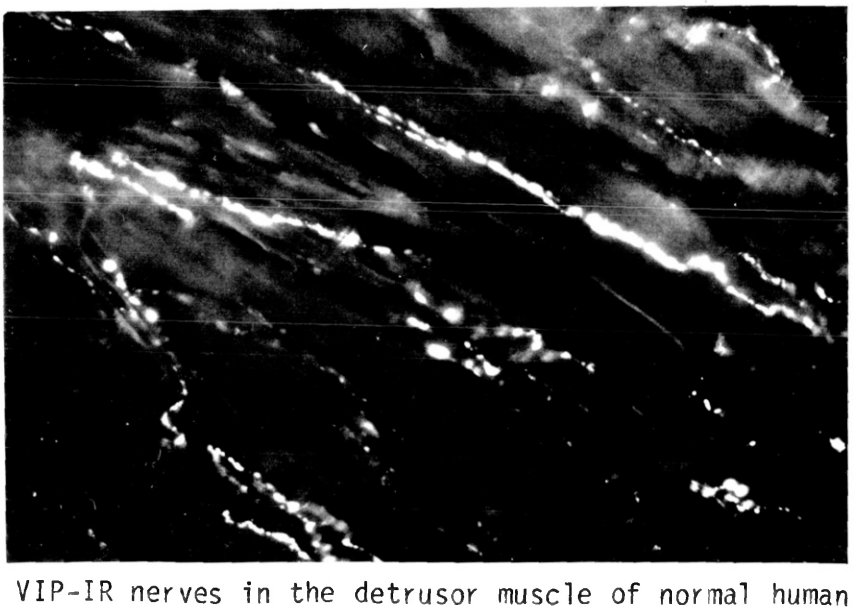


Fig. 10. VIP-IR nerves in the detrusor muscle of normal human bladder (x385).

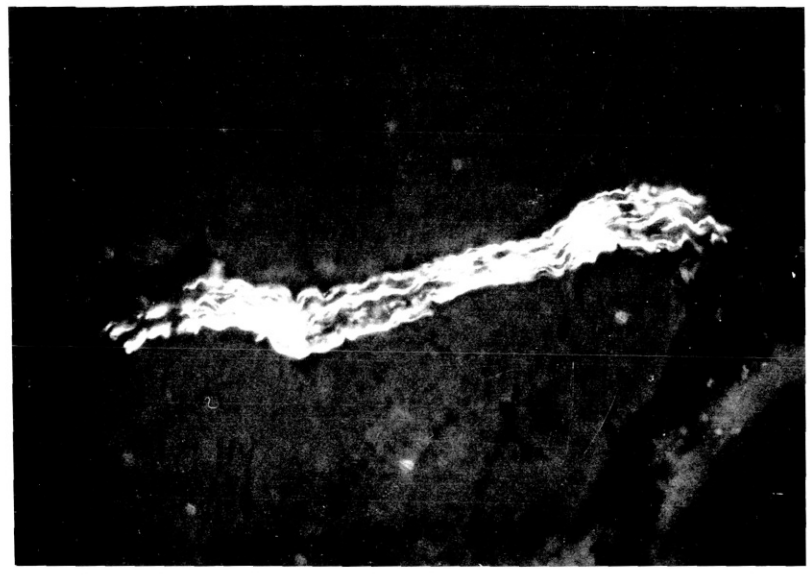


Fig. 11. A bundle of VIP-IR nerve fibres running in between smooth muscle blocks in the human bladder (x250).

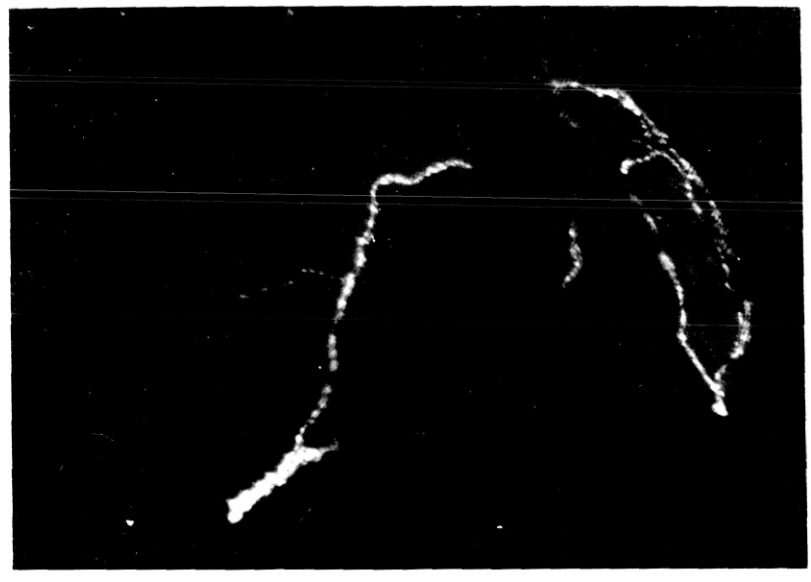


Fig. 12. VIP-IR nerves in the submucosa of the human bladder. A blood vessel is surrounded by such nerves. (x250).

The adventitia of the urinary bladder contained a fairly large number of VIP-IR nerve bundles and isolated fibres which were often vascular-related. The VIP-IR nerve bundles were more frequently seen around the lateral basal region where the vesical arteries entered the organ together with most of the nerves. VIP-IR fibres constituted the total or, sometimes, a certain proportion of fibres in each nerve bundle (Fig. 13a, b). Scattered ganglion cells were encountered, often adjacent to VIP-IR nerve bundles in the adventitia (Fig.14) and occasionally in the muscle layer. Such ganglion cells were more common at the base of the bladder than in other areas, but their overall population was very small.

The smooth muscle around the ureterovesical junction was not particularly dense in VIP-IR nerves, whereas the submucosa had a richer network of VIP-IR fibres. The internal sphincteric smooth muscle was richly innervated by VIP-IR nerves but the striated muscle of the external sphincter was virtually devoid of such nerves. The distribution pattern of VIP-IR nerves in the human urinary bladder is shown in Diagram 10.

PHI-IR nerves had a distribution pattern similar to that of the VIP-IR ones but were less numerous and only formed approximately one third of their number. No PHI-IR ganglion cell bodies were convincingly demonstrated in the bladder wall.

The distribution of varicose NPY-IR nerves was distinct from that of VIP-IR nerves mainly in that they were rarely present in the

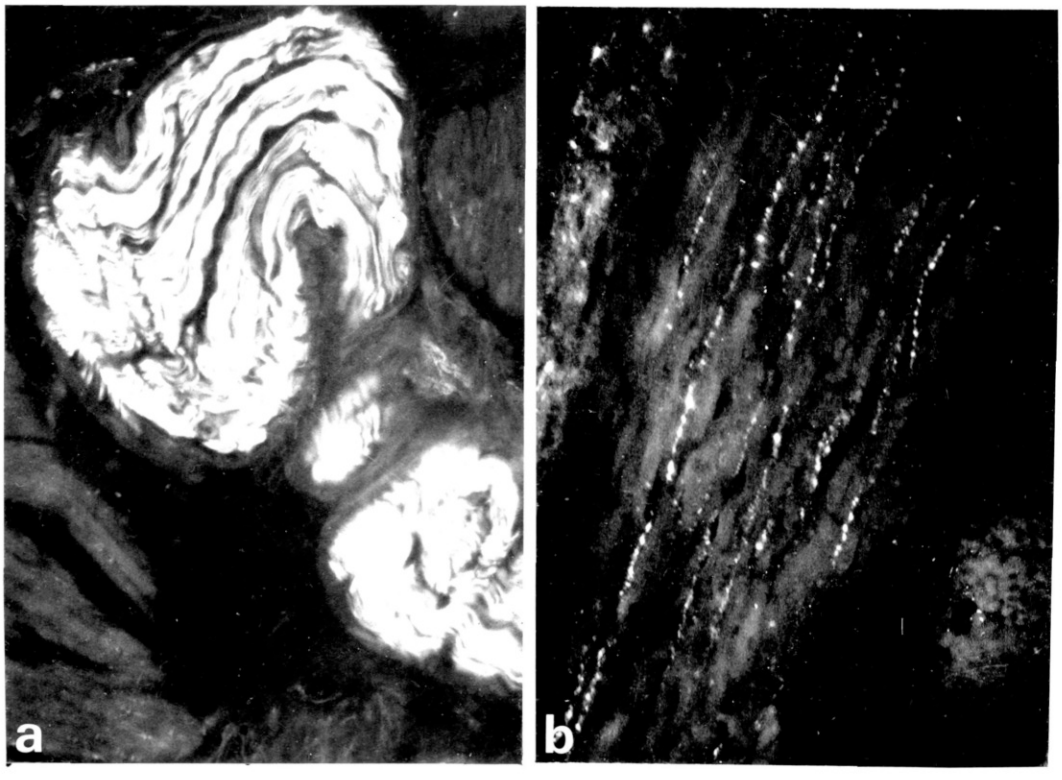


Fig. 13a. A bundle of nerves immunostained with NSE antiserum in the human bladder. Note that all the nerve fibres contain NSE immunoreactivity. (x320).

Fig. 13b. VIP-IR nerve fibres in a nerve bundle in the human bladder. Note only certain fibres contain VIP immunoreactivity (x320).



Fig. 14. A VIP-IR neuronal cell body (small arrow) adjacent to a nerve bundle. There is, possibly, a non-IR neuronal cell body (open arrow) surrounded by VIP-IR nerve fibres (x325).



Fig. 15. NPY-IR nerve fibres in the detrusor muscle of human urinary bladder (x400).

Diagram 10

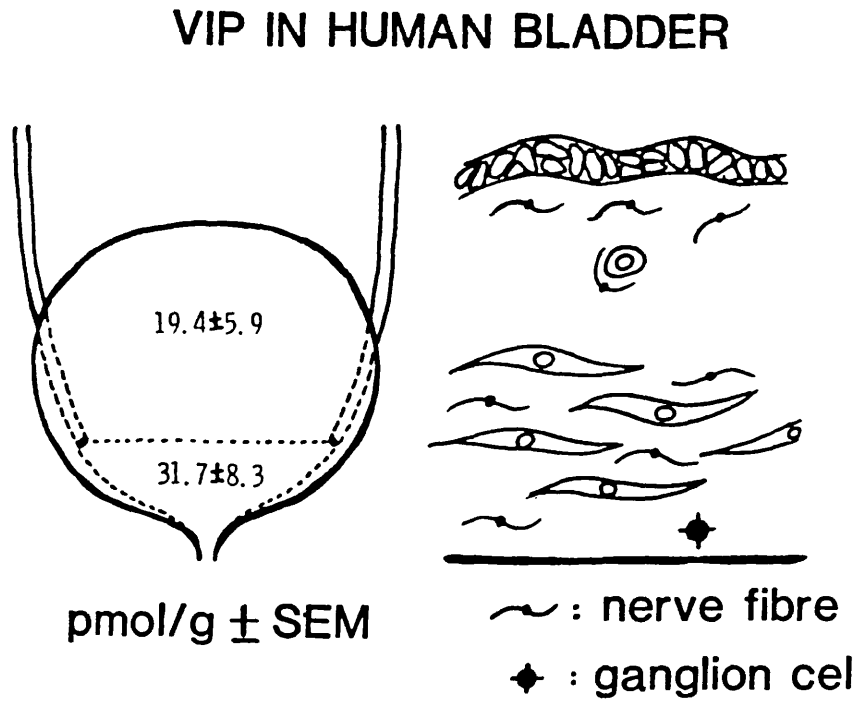
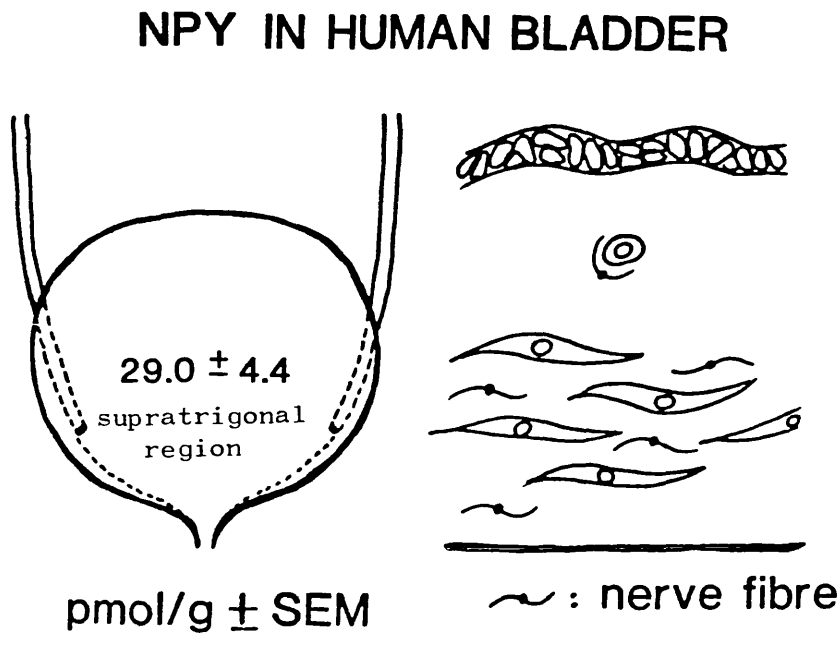

VIP IN HUMAN BLADDER
19.4±5.9
31.7±8.3
pmol/g ± SEM
: nerve fibre
: ganglion cell

Diagram 11


NPY IN HUMAN BLADDER
29.0 ± 4.4
supratrigonal
region
pmol/g ± SEM
: nerve fibre

submucosa, except when associated with blood vessels. NPY-IR nerves were mostly concentrated in the muscle layer (Fig.15) and many more occurred in the smooth muscle of the base than in rest of the bladder. At the base of the bladder, NPY-IR nerves were denser than VIP-IR ones. A moderate number of nerve bundles were found to contain NPY immunoreactivity and no NPY-IR ganglion cell bodies were encountered. Most of the blood vessels, particularly arteries and arterioles, were surrounded by NPY-IR nerve fibres (Fig.16) which sometimes had fewer varicosities than those innervating non-vascular smooth muscle. The distribution pattern of NPY-IR nerves in the human urinary bladder is shown in Diagram 11.

Substance P-IR nerves were very rare in the human urinary bladder. When encountered, they lay freely in the submucosa, between the muscle bundles and in the vicinity of blood vessels (Fig.17).

Radioimmunoassay detected five peptide-immunoreactivities, including somatostatin, in the same batches of tissue (Diagram 12). The results were generally in agreement with the immunocytochemical findings. Although the overall concentrations of somatostatin were slightly higher than those of substance P, they might be widespread within a large number of nerves, thus resulting in a much lower local density which was not detectable by immunocytochemistry. The differences in the antisera (see Section 9.1.) used in immunocytochemistry and radioimmunoassay might also be responsible for the differences in the results for somatostatin. However, this was not very likely in this study since when the same antisera were applied to the gallbladder of guinea-pig and male genital tract of man,

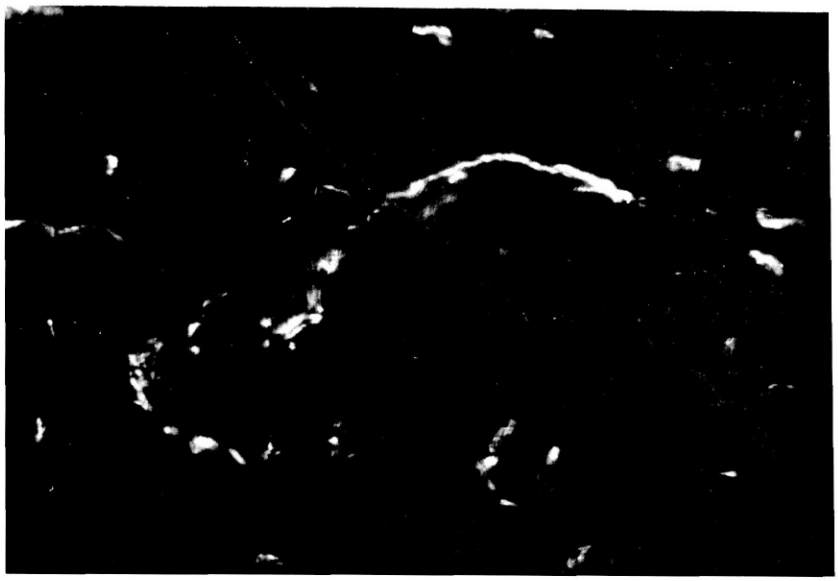
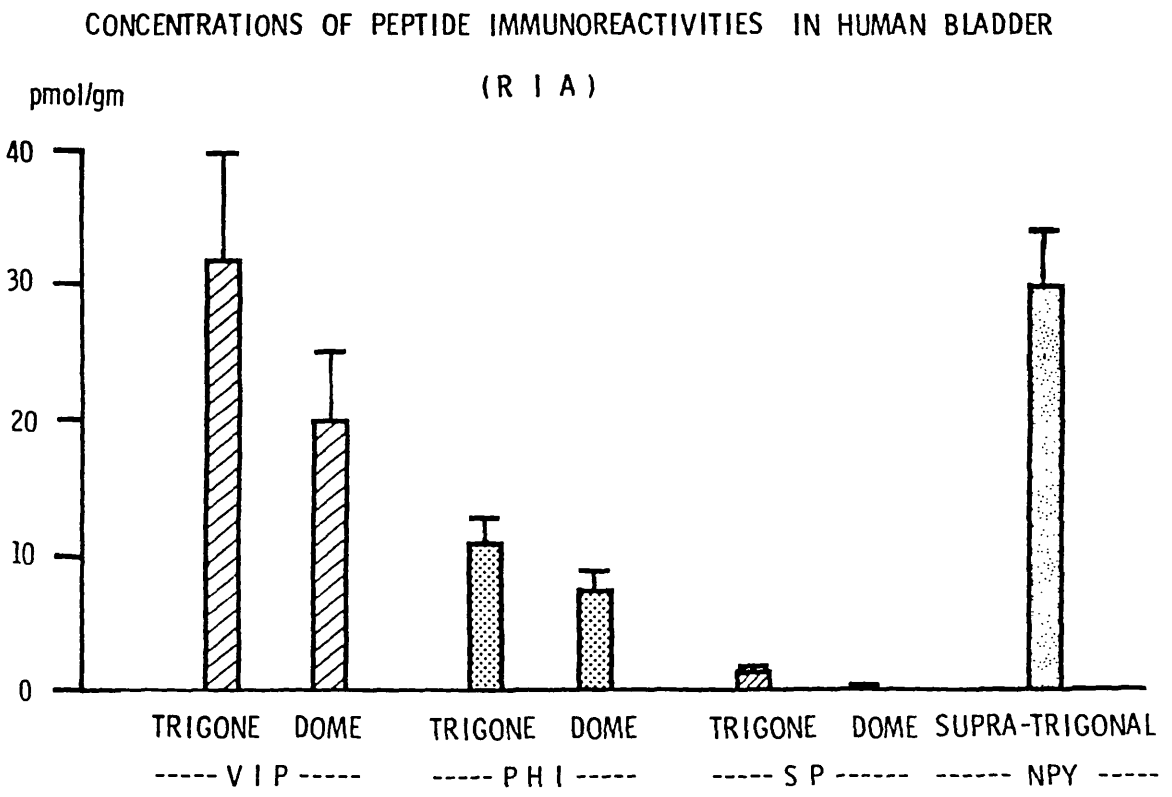


Fig. 16. Arterioles surrounded by NPY-IR nerves in human urinary bladder (x285).



Fig. 17. Substance P-IR nerve fibres running freely in the submucosa of the human urinary bladder (x325).

Diagram 12


CONCENTRATIONS OF PEPTIDE IMMUNOREACTIVITIES IN HUMAN BLADDER
(R I A)
pmol/gm
40
30
20
10
0
TRIGONE
DOME
TRIGONE
DOME
TRIGONE
DOME
SUPRA-TRIGONAL
----- V I P -----
----- P H I -----
----- S P ------
------ NPY -----

somatostatin immunoreactivities were detected by both radioimmunoassay and immunocytochemistry.

3.1.4. PEPTIDE-IR NERVES IN THE URINARY BLADDER OF OTHER MAMMALS

Peptide-IR nerves of the four types, i.e. those containing VIP, PHI, NPY and SP immunoreactivities, were also found in the urinary bladders of rat, cat, mouse and guinea-pig; the distribution patterns of each type were, in general, similar to those in the human bladder. However, certain characteristics were noted for each species.

In the urinary bladder of the cat, substance P-IR nerves were more dense than those in man, although they were still much fewer than VIP- and NPY-IR ones. In fact, all types of peptide-IR nerves detected were more numerous in feline than in human bladder, with the differences in substance P-IR nerves being the most apparent. In addition, more intrinsic VIP-IR neuronal bodies were found in the cat bladder than in the bladders of other species, including man.

The frequency and distribution of peptide-IR nerves were similar in the urinary bladder of rat and mouse and were also very similar to the findings made in man. The SP-IR nerves also appeared to be slightly more abundant in these two species than in man, whereas, the number of VIP-IR nerves was less. Nevertheless, a small number of scattered VIP-IR neuronal cell bodies were still observed in the adventitia of the rat bladder.

In the bladders of the rats which were neonatally treated with capsaicin to deplete the sensory C-fibres, the substance P-IR nerves had disappeared completely from all the regions of the bladders examined. The number of the freely distributed nerve terminals in the submucosa which were immunoreactive to the VIP antiserum also seemed to be depleted (Fig. 18a,b), whilst the VIP-IR nerves related to vascular and non-vascular smooth muscles were largely unaffected.

The most striking characteristic of peptide-IR nerves in the urinary bladder of the guinea-pig was the large number of substance P-IR nerves. The abundance of these nerves nearly matched that of VIP- and NPY-IR ones. The nerves were present in the submucosa, the muscle layer and, more conspicuously, around blood vessels. As a consequence, some blood vessels in the guinea-pig urinary bladder were associated with more than two types of peptide-IR nerves, ie., VIP-, NPY- and substance P-IR nerves, and, sometimes also, with PHI-IR nerves. No substance P-IR intramural ganglia were observed. NPY-IR nerves appeared to be less numerous in the bladder of guinea-pig than in other species.

3.1.5. NERVES CONTAINING CLASSICAL NEUROTRANSMITTERS

The noradrenergic innervation of the human urinary bladder was demonstrated by immunocytochemical and histochemical methods. Immunostaining with the antiserum against DβH identified a

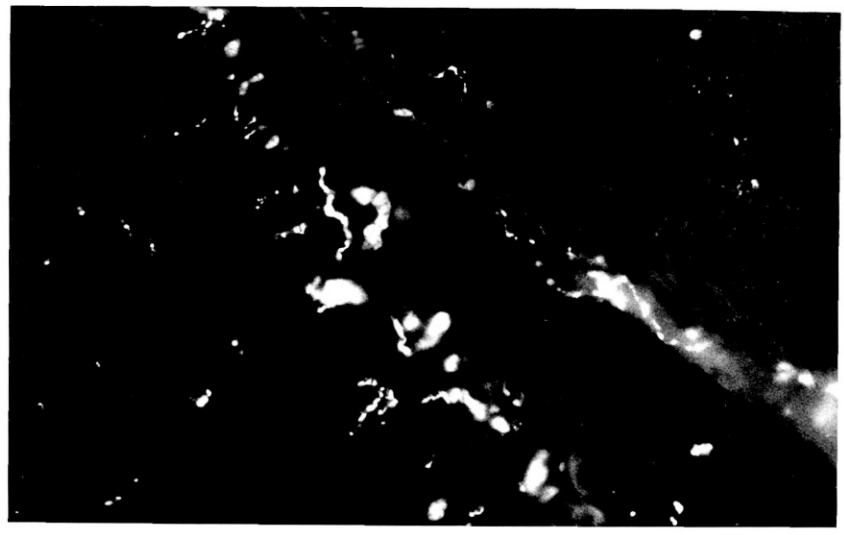


Fig. 18a. VIP-IR nerve fibres in the submucosa of the urinary bladder from an untreated rat (x185).

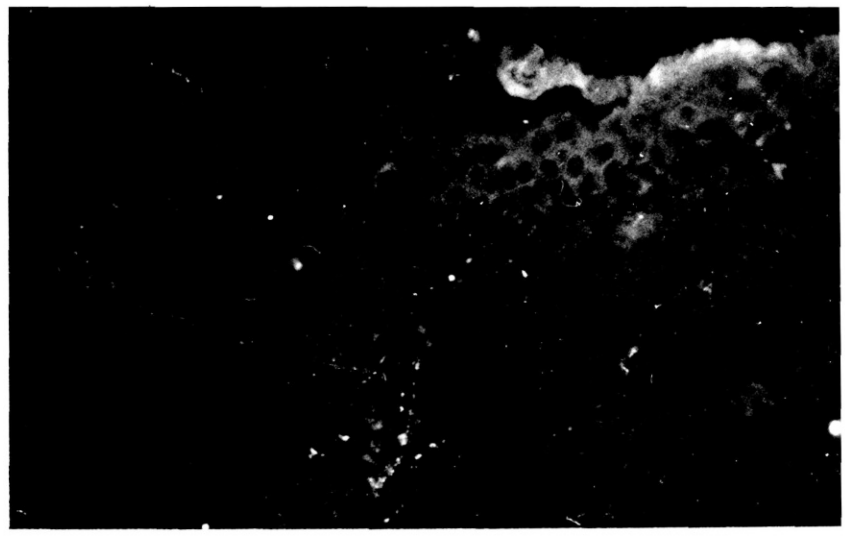


Fig. 18b. VIP-IR nerve fibres are markedly depleted in the submucosa of the bladder from a capsaicin-treated rat (x185).

population of nerves with distribution patterns almost identical to those revealed by the histochemical (SPG) method. In contrast, the specific antibody against TH often failed to immunostain, or only weakly reacted with, the neuronal structure in the bladder of all the species examined. For simplicity, the DβH/SPG-positive nerves are referred to as noradrenergic nerves in the subsequent descriptions.

The distribution patterns of noradrenergic nerves found in this study were, in general, in keeping with previous studies using histochemical methods (Falck et al 1962; Dahlström 1978) and also, interestingly, rather similar to that of NPY-IR nerves described above. The similarity between noradrenergic and NPY-IR nerves was shown by the finding that they both occur abundantly in the muscle bundles at the base of the bladder. The general lay-out of these two types of nerve in relation to blood vessels was also similar. These findings invite the postulate that NPY and catecholamine might be co-stored in the same neurons. However, it was also noticed that noradrenergic nerve fibres of the non-vascular type in the submucosa were more numerous than the NPY-IR nerves and in the detrusor muscle NPY-IR nerves were more abundant.

The possible co-storage of NPY and catecholamine was further investigated on the urinary bladder of rats which had been treated with 6-OHDA to destroy the noradrenergic nerves (see Section 2.11.1.). It was found that the number of NPY-IR nerves in the bladders treated with 6-OHDA was decreased as compared with the control level. The decrease was not overwhelming but was

significant and mainly confined to vascular-related NPY-IR nerves, whereas the majority of the NPY-IR nerves in the muscle layer seemed to be unaffected, although SPG staining and DβH immunostaining showed that the noradrenergic nerves in the bladder had been destroyed.

Another interesting phenomenon observed during the experiment of 6-OHDA treatment on rat bladder was that, accompanying the disappearance of noradrenergic nerves and the decrease of NPY-IR nerves, was an apparent increase in number of nerves immunoreactive to substance P. The increased SP-IR nerves were weak in staining but were very abundant, forming networks over the submucosa and the muscle layer of the bladder. They contrasted strikingly with the sparsity of such nerves in the control bladders. No changes were seen in the VIP- and PHI-IR nerves.

The AChE-positive nerves occurred in the submucosa and formed the submucosal plexus, which increased in density towards the base and the neck of the bladder. In addition, they were distributed in the detrusor muscle, the adventitia and around blood vessels in the same way as the VIP-IR nerves. However, the AChE-positive nerves were, in general, thicker than VIP-IR ones and had less varicosities. The population of AChE-positive nerves seemed to be marginally greater than the VIP-IR population.

3.1.6. DISCUSSION

Earlier reports on peptide-containing nerves in the urinary bladder came from two research groups. Alm and associates investigated the urinary bladder of 3 cats and described the distribution of VIP-IR nerves as being associated with smooth muscle, blood vessels, and present in the submucosa, being quite numerous towards the trigonal area (Alm et al 1980a). Hökfelt et al reported that three types of peptide-IR nerves, i.e. those containing VIP, substance P and somatostatin, were present in the urinary bladder of guinea-pig and each seemed to have its own distribution pattern. In addition, a few somatostatin-IR ganglion cell bodies were observed in the bladder wall (Hökfelt et al 1978).

In this study, five different peptides were found to be present and four of them were localised to nerves in the urinary bladder of rat, cat, mouse, guinea-pig and man by immunocytochemistry. The difference in the results, with respect to the somatostatin-IR nerves in the guinea-pig bladder, might be due to the differences between the antiserum employed in this study and that used by Hökfelt et al (1978).

VIP-IR nerve fibres in the urinary bladder appear to be comprised of 3 populations: one, which mainly innervates the detrusor and trigonal smooth muscles; another, which mainly surrounds the blood vessels; and the third, distributed beneath the transitional epithelium without innervating any apparent tissue structure.

The occurrence of varicose VIP-IR nerves in the detrusor muscle implies a muscular effect of these nerves, and one might postulate that VIP is responsible for the well-known non-cholinergic, non-adrenergic response of the urinary bladder (Alm et al 1977). Levin & Wein (1981) demonstrated that VIP can inhibit the contraction of the rabbit detrusor muscle whereas Choo & Mitchelson (1980) failed to initiate any activity with VIP in the rat bladder. Erspamer and co-workers (1981) found only a negligible effect of VIP on muscle strips from human urinary bladder. However, it is noteworthy that these experiments were performed in vitro, and that the findings may not be reproduced in an in vivo situation, with vascular and nervous connections and, especially, with intravesical tension higher than the threshold level which is necessary to facilitate the firing of the nerves innervating the bladder (de Groat et al 1982).

The distribution of VIP-IR nerves in the submucosa is quite characteristic. There are no apparent effector structures except the closely apposed transitional epithelium, the movement of which, however, is known to be passive. Therefore, a sensory function for these nerves is presumed. A number of facts support this assumption. Firstly, the freely distributed nerve endings in the submucosa, no matter which of the two classical neurotransmitters they contain, have been assumed to be sensory nerve terminals by previous observers (Gosling & Dixon 1974; El-Badawi 1982). Secondly, these nerves were affected by capsaicin treatment which

selectively destroys the C-fibres of the sensory neurons (Jancso et al 1977; Lawson & Nickels 1980). Thirdly, VIP-IR ganglia were reported to be present in the corresponding segments of the spinal dorsal root ganglion which give off branches to the urogenital organs (Jancso et al 1981). It is not certain, however, if VIP immunoreactivity is present in the previously claimed sensory cholinergic or adrenergic terminals. Nevertheless, it seems logical to assume that the freely distributed VIP-IR nerve terminals in the submucosa of the bladder subserve a function of sensory transmission.

The co-storage of VIP and ACh has been suggested in many organs (for references see Section 1.5.2.). However, the histochemical evidence has never been conclusive because of the unreliability of the various histochemical staining methods for localising ACh or AChE. In this study, a relatively specific AChE staining method was employed on human and rat bladders; it was found that the distribution of the AChE-positive nerves was largely comparable to that of VIP-IR nerves. Therefore, VIP immunoreactivity might occur in the cholinergic nerves in the urinary bladder. Nevertheless, AChE-positive and VIP-IR nerves were not entirely identical in that the former were thicker and more abundant than the latter. This might, to a certain extent, be attributed to the nature of the AChE staining method which is known to stain, in addition to cholinergic neurons, substance P-containing nerves and, sometimes, noradrenergic nerves and Schwann cells (for references see Section 1.5.12.).

PHI is a peptide possessing many similarities to VIP. A similar

distribution of PHI- and VIP-IR nerves in the urinary bladder was found in this study. It is important to note that, in spite of the close resemblance between the molecules of PHI and VIP, the immunoreactivities of these two were not the result of antiserum cross-reaction (Table 2). Judging from the proportions of VIP- and PHI-IR nerves, it seems that PHI immunoreactivity may, in fact, occur in a subpopulation of VIP-IR nerves, or the two nerve types may overlap as seen in the myenteric ganglia of the gut (Bishop et al 1984). However, more proof is needed to establish their co-storage. It has been postulated that the two peptides are co-produced (Christofides et al 1982). In human VIPomas (Bloom et al 1983) and neuroblastomas (Itoh et al 1983), it has recently been shown that the prepro-VIP molecule contains the amino acid sequence of PHI. The similar distribution patterns of VIP and PHI immunoreactivities in the mammalian urinary bladder therefore support the common precursor theory. The significance of PHI in the urinary bladder remains to be clarified.

The distribution of the newly discovered peptide, NPY, is principally muscular-oriented, i.e., mainly related to non-vascular and vascular smooth muscle. The effect of NPY on smooth muscle has been shown in the rodent vas deferens where it was found that NPY has an in vitro inhibitory effect on smooth muscle contraction (Lundberg et al 1982b; Allen et al 1982). It is noteworthy that this effect was rather slow in onset and long-lasting, and occurred only when the contraction was induced by electrical stimulation but

not by noradrenaline. This points to a role for NPY as a neuromodulator rather than a neurotransmitter. The action of NPY in the urinary bladder has not yet been evaluated.

The co-storage of NPY and catecholamine in the same neurons was previously suggested by Lundberg et al (1982a, b). They found that both NPY-immunoreactivity and catecholamine-positivity were diminished in the same splanchnic ganglia following 6-OHDA treatment. In the present study, however, the majority of the NPY-IR nerves survived while the noradrenergic nerves were destroyed by 6-OHDA. This suggests that the co-storage, if it occurs, is only partial and may be mainly confined to vascular innervation.

At this stage, however, one may argue that the disappearence of DβH immunoreactivity and SPG positivity does not necessarily mean that the whole noradrenergic neurons were destroyed, because both DβH and catecholamine have their confined ultrastructural locations which are not entirely identical with that of NPY in the neurons. If NPY is indeed stored in noradrenergic nerves, the persistence of some NPY after the depletion of DβH/SPG positivities may simply suggest that the local concentration of 6-OHDA was not high enough, or the time course was not long enough, to destroy all the microstructures of the noradrenergic innervation, although catecholamine could have been completely substituted and DβH synthesis ceased. Since 6-OHDA was transported to the organ by blood flow, a higher concentration of 6-OHDA was accumulated more easily in the nerves closest to the blood flow, i.e. the vascular nerves, than in the distant non-vascular ones. This may be one of the reasons why the

destruction was mainly confined to the vascular NPY-IR nerves. On the other hand, a reduction in NPY-IR nerves after noradrenergic denervation might not be the absolute proof of the identity of these NPY-IR and noradrenergic nerves, since noradrenergic nerves may have a trophic influence on other nerve types, and any changes in this influence might lead to changes in separate nerve types, such as those containing NPY.

Nevertheless, a similar finding has been made in the gut of rats and guinea-pigs, where 6-OHDA treatment, either intraperitoneally or subcutaneously, diminished vascular but not non-vascular NPY-IR nerves (Lundberg 1982b). It seems reasonable to postulate that, in the urinary bladder of rat, NPY and catecholamine are partially co-stored in identical neurons which mainly innervate blood vessels. There may be NPY-IR nerves which do not contain catecholamine, such as those innervating detrusor muscle, and noradrenergic nerves which do not contain NPY, such as those in the lamina propria.

Most blood vessels in the bladder of all the species studied were surrounded by both NPY- and VIP-IR nerves. This arrangment may be meaningful because of the opposing effects of NPY and VIP on vascular smooth muscle. NPY has been shown to have a dose-dependent vasoconstrictor activity in rat and mouse submandibular glands (Lundberg et al 1982b); VIP, as its name indicates, has a powerful effect of vessel dilatation. It is tempting to assume that these two peptides released from separate nerves surrounding the same

blood vessel act as agonist and antagonist in a co-ordinated manner, joining the classical neurotransmitters to regulate the local blood flow.

Substance P-IR nerves in the urinary bladder were generally very sparse and were sensory in nature, as shown by the experiment of neonatal capsaicin treatment. However, the unexpected sharp increase in substance P-IR nerves, after noradrenergic denervation with 6-OHDA, needs some rational explanation. The idea of compensatory nerve outgrowth is not satisfactory, since it can not explain why only substance P-IR nerves were increased. Presumably, the newly emerged substance P-IR nerves might be a result of outgrowth from previously existing substance P-containing nerves, of raised substance P concentration in previously immunocytochemically 'invisible' substance P-containing nerves, or of appearance of substance P immunoreactivity in previous non-substance P-containing nerves. It was noted, however, that substance P-IR nerves were not increased following any type of surgical nerve transection (see section 3.2.3.); this may indicate that the increase of substance P-IR nerves is related to 6-OHDA or to the selective destruction of noradrenergic nerves. Coincidentally, it was recently found that substance P-IR nerves were markedly increased in the eye of rabbit following selective local sympathectomy (Cole et al 1983). Jonsson & Hallman (1981) reported that substance P prevented neuronal destruction caused by 6-OHDA in the rat brain. On the other hand, Hökfelt et al (1982a) showed that an antagonist of substance P had a destructive effect on neurons. All these recent findings suggest that substance P may have a long-term trophic effect on neuronal

response to damage or on integrity of nerve connections, particularly noradrenergic ones, in addition to a role of neurotransmitter or neuromodulator.

From the findings of this study, it can be concluded that in the normal mammalian urinary bladder, five types of peptide immunoreactivity are present and at least four of them occur in nerves; each has a characteristic distribution pattern, suggesting a set of physiological actions, although much of the significance of these peptides in the urinary bladder still remains to be determined.

3.2. ORIGIN OF VIP- AND SUBSTANCE P-IR NERVES IN URINARY BLADDER

3.2.1. INTRODUCTION

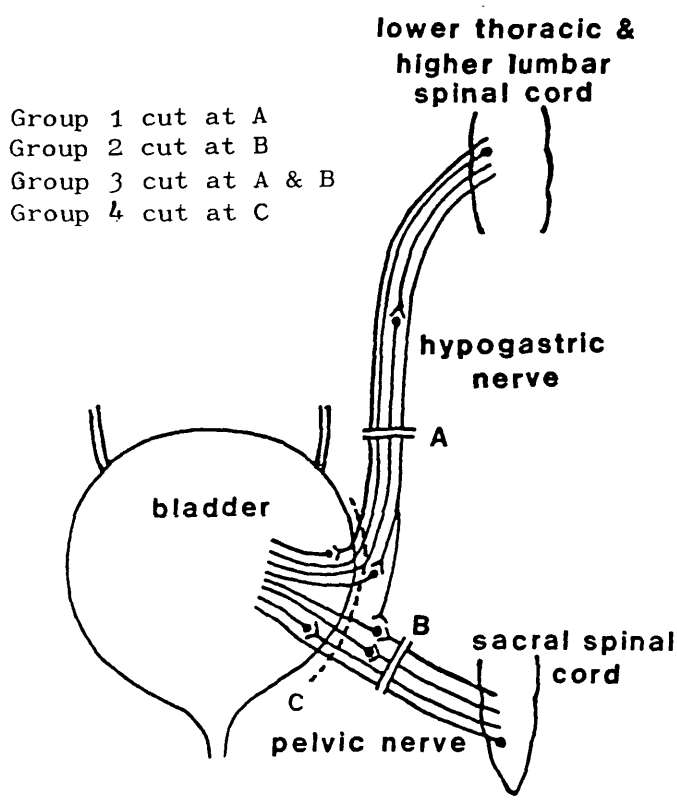
After revealing the occurrence and distribution of peptide-IR nerves in the mammalian urinary bladder, the origins of such nerves were studied. Prior to this, there had been no information on the origin of the various types of peptide-IR nerves in the urinary bladder avaliable. Undoubtedly, clarification of the organisation of these nerves would help us enormously in understanding the neural control of this organ. A number of experiments were therefore carried out to determine the origins of VIP- and substance P-IR nerves in the urinary bladder. Rats were used as models for investigation.

3.2.2. EXPERIMENTAL DESIGN

The design of the experiments was based on the current view of the urinary bladder innervation (for reviews see Williams & Warwick 1980b; El-Badawi 1982), which holds that the secondary ganglia of the sympathetic system lie mainly in the inferior mesenteric plexus, and those of the parasympathetic mainly in the pelvic plexus; in addition, the sympathetic 'short neurons' also exist in the close vicinity of the bladder. After the hypogastric and pelvic nerves meet to form the pelvic plexus, sympathetic and parasympathetic postganglionic nerves, if both exist as has been suggested, are randomly mixed. Thus, cutting within or distal to the pelvic plexus

Diagram 13

DESIGN OF SURGICAL SECTIONING
OF NERVE SUPPLY TO RAT BLADDER



would give little information as to which pathway had been interrupted. Therefore, in this study, the cutting points were mostly proximal to the pelvic plexus, which left the plexus undisturbed.

Surgical transections of the nerves to the urinary bladder of rats were carried out in four different ways and each was experimented on 6 rats. In one group of rats, the sympathetic pathways were bilaterally transected at the points just before they join the pelvic plexus. In another group, the parasympathetic pathway was cut unilaterally at a point just before the hypogastric nerve joins in. In this way, however, certain parasympathetic and sympathetic ganglia were inevitably left intact on the distal side of the sectioning points. For them, only the preganglionic fibres were discontinued; thus decentralization may be the more appropriate word to use. In a third group, both sympathetic and parasympathetic pathways were cut at the above points, as in groups one and two. The fourth group of rats were bilaterally extrinsically denervated at points very close to the bladder and, thus, distal to the pelvic and vesical plexuses. The nerve trunks entering the bladder were further crushed with forceps around both sides of the bladder base to ensure complete disconnection of all nerves entering the bladder wall. Sham-operations were carried out on the fifth group (n=6) as controls. In all the operations, damage to the blood vessels to and from the urinary bladder was carefully avoided. The design of the operations is shown in Diagram 13.

Previous experiments have shown that a decrease of AChE-positive

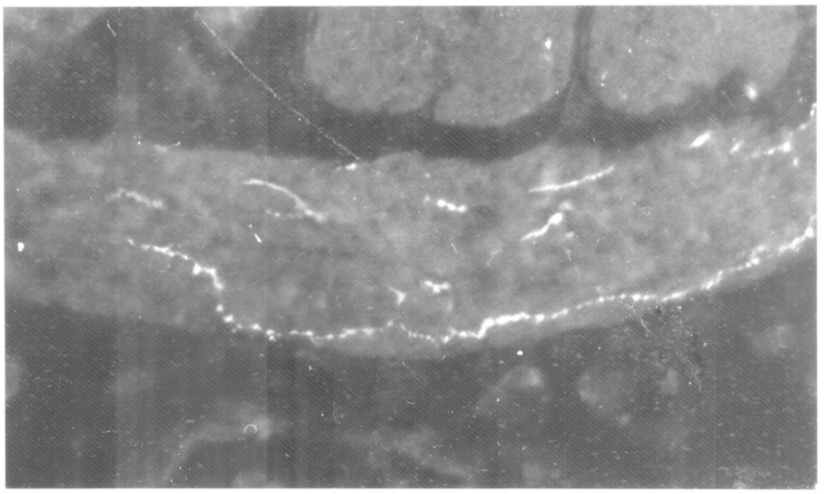


Fig. 19a. VIP-IR nerves in the detrusor muscle of sham-operated rat bladder (x200).

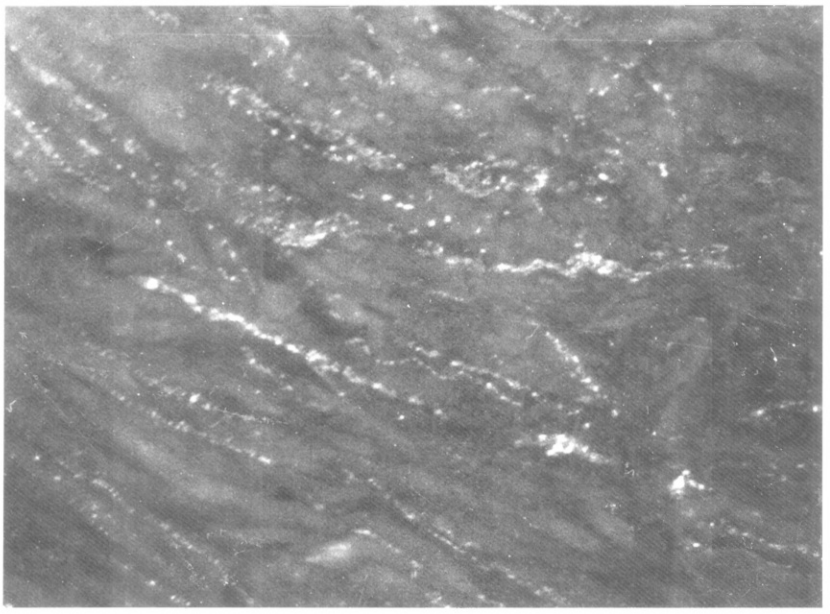


Fig. 19b. VIP-IR nerves in the detrusor muscle of rat bladder (operated side) after the pelvic nerve supply has been unilaterally sectioned. Note that the number of VIP-IR nerves is increased (200).

Diagram 14

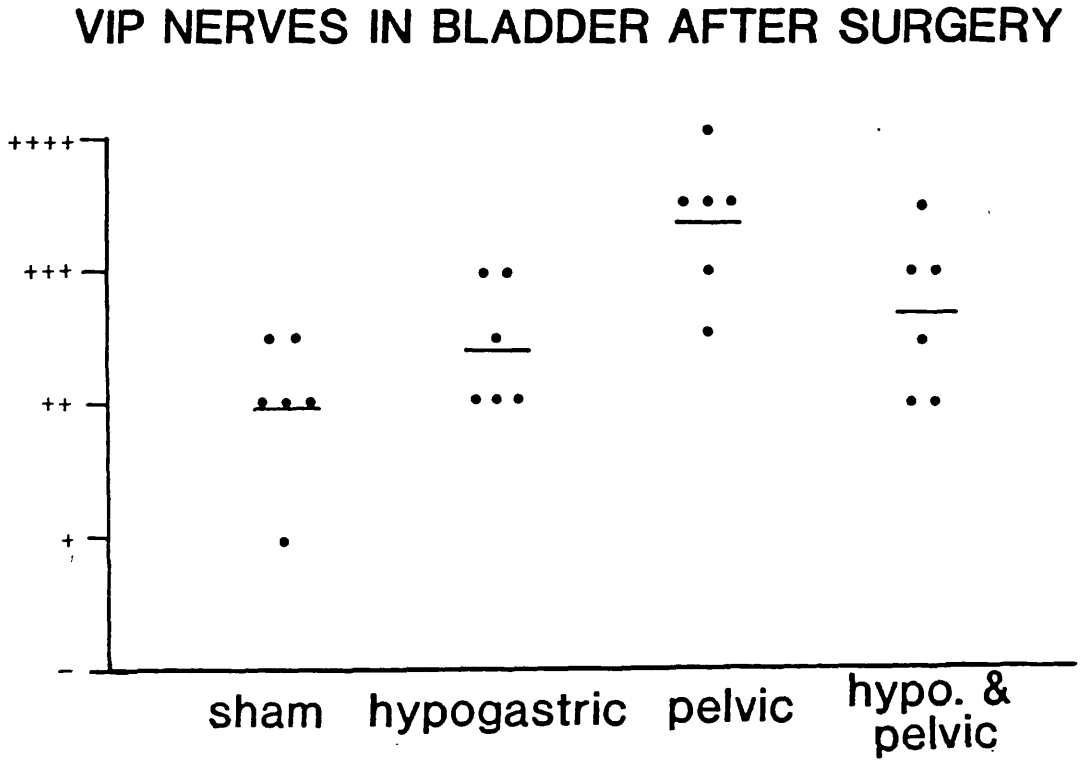


Diagram 15

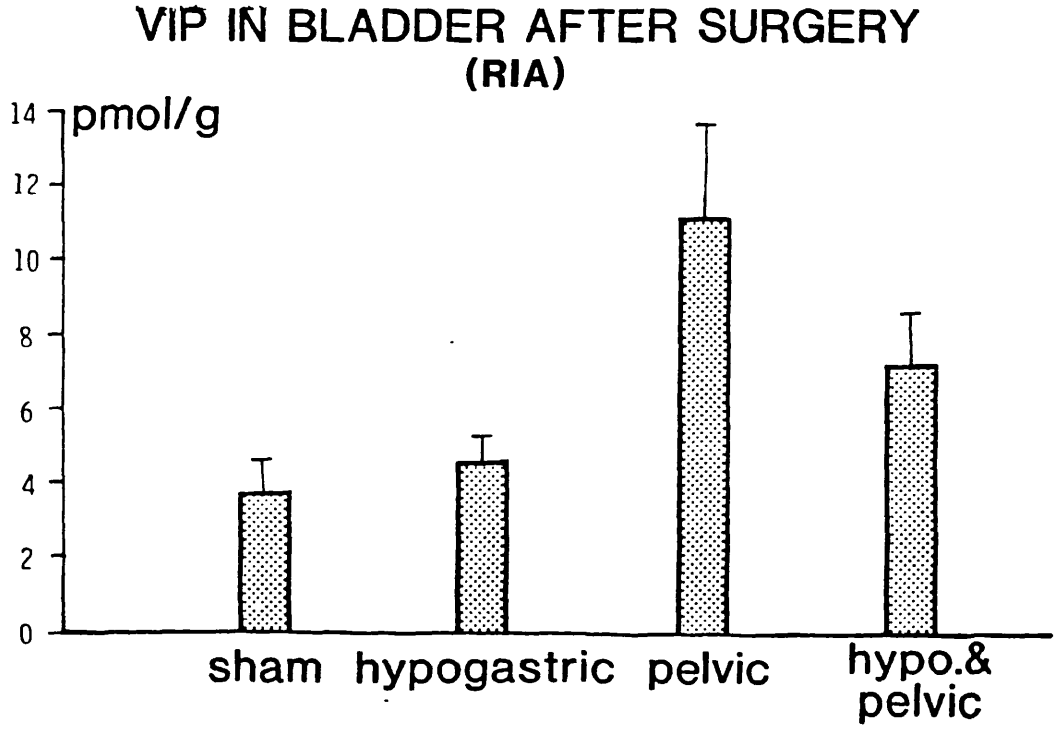
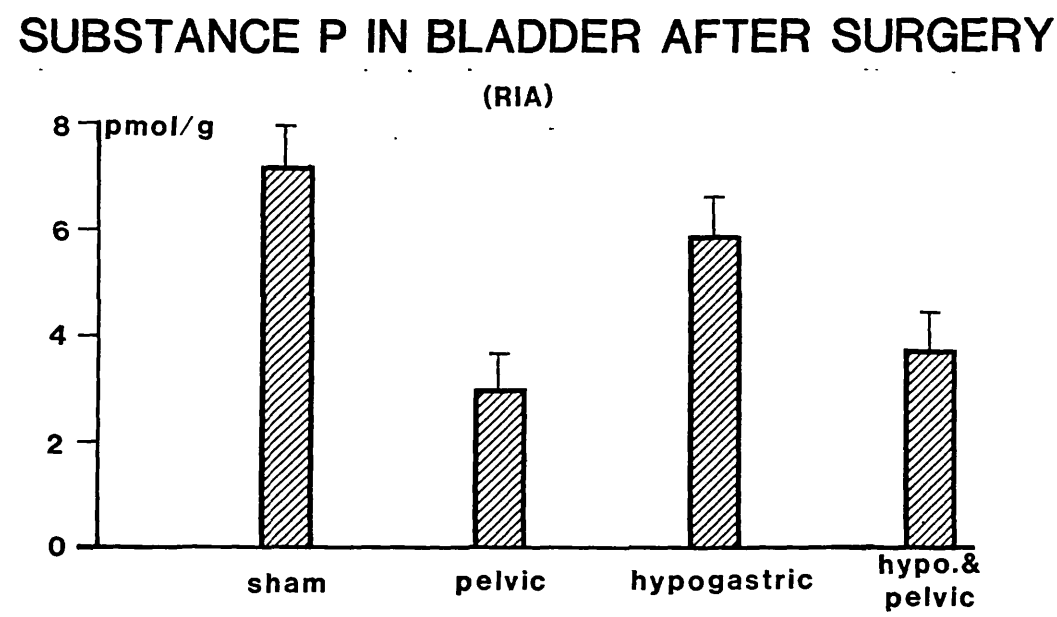


Diagram 16


SUBSTANCE P IN BLADDER AFTER SURGERY
(RIA)
8
pmol/g
6
4
2
0
sham
pelvic
hypogastric
hypo.&
pelvic

nerves occurred within one week of the pelvic denervation of the rat bladder; from two weeks onwards outgrowth of the same nerve type appeared (Alm & Jörgen 1981). Therefore in this study, the time length chosen to investigate the changes after surgery was 8 days, in the hope of finding the full morphological effects of the surgical denervation before it was obscured by regeneration from the disconnected axons. The average velocity of motor fibre regeneration has been found to be about 2 mm per day (Guth 1956); thus, eight days would be insufficent time for regeneration of transected axons in the bladder. The experiment was also based on the concept that the discontinuation of the postganglionic axons would lead to a rapid destruction of their distal terminals in the organ, whilst sectioning of the primary ganglionic, or preganglionic, axons would have little morphological effect on their postganglionic nerve terminals in the innervated organ in this short period (Waller 1850; Allt 1976).

The possible changes in NPY and PHI immunoreactivities were not examined in this study, because the experiment was performed before their antisera became available.

3.2.3. CHANGES ON VIP- AND SUBSTANCE P-IR NERVES AFTER SURGICAL SECTIONING OF NERVE PATHWAYS

It was found by immunocytochemistry that the number of VIP-IR nerves was increased after the first three types of operations, but to different degrees. After unilateral parasympathetic nerve

transection, the number of VIP-IR nerves was increased on both sides of the bladder, and particularly on the denervated side (Fig. 19a, b). The bilateral hypogastric nerve transection caused a slight increase in the number of VIP-IR nerves and this was most apparent in the trigonal region. The transection of both nerve types led to a considerable increase in VIP-IR nerves but was less marked than that caused by parasympathetic nerve transection alone. In the fourth group of rats, in which the nerves were cut closer to the bladder, VIP-IR nerves were markedly decreased following the sectioning of pelvic and hypogastric pathways. The results are shown in Diagram 14.

The substance P-IR nerves were decreased markedly after either hypogastric or pelvic nerve transection and completely disappeared when both nerves were cut.

Radioimmunoassay detected a similar increase of VIP concentration in the decentralised bladders from 3.5±0.8 pmol/g (mean±SEM) in the sham-operated group to 4.3±0.7 pmol/g, 10.8±2.6 pmol/g (operated side) and 7.1±1.5 pmol/g(operated side) after hypogastric, pelvic and combined nerve transections, respectively (Diagram 15). After any of the surgical transections the levels of substance P immunoreactivity fell by more than 50% from the control level (Diagram 16).

3.2.4. DISCUSSION

It was found in this study that VIP-IR nerves in the rat bladder were increased in number, following the interruption of hypogastric

and/or pelvic pathways proximal to the pelvic plexus. To check these results, a further group of rats and a group of cats were treated similarly, and almost identical findings were obtained Thereby, the reproducibility of the present results was fully established. The implications of this are not immediately obvious, due to lack of information on the response of peptide-IR neurons to nerve injury, and to the complexity of the organisation of the bladder nerve supply. However, it suggests that the changes in VIP-IR nerves after denervation are indicative of their origin.

That the majority of intrinsic VIP-IR nerves do not originate from ganglia located proximal to the junction of pelvic and hypogastric nerves is shown by there being no decrease in VIP immunoreactivity, following the disconnection of the nerves at these points. This indicates that some of the VIP-IR postganglionic axons, probably most of them, were not interrupted by the operations. Therefore, the majority of the VIP-IR nerves probably originate from ganglia which lie in the plexuses close to the bladder or within the bladder wall. This is in agreement with another finding of the present study that a few intrinsic ganglion cells in the bladder contained VIP immunoreactivity. This is also in keeping with recent reports by El-Badawi (1982) and El-Sadr (1982) who described the occurrence of intrinsic ganglion cells in rat bladder, although it used to be believed that no ganglion cell bodies were contained in the bladder wall of rat. However, judging from the small number of VIP-IR ganglion cells within the rat bladder wall, the main sites for the VIP-containing neuronal cell bodies seem likely to be the pelvic and vesical plexuses. This assumption was supported by the results

obtained from the fourth group of rats in which the denervations were done very close to the bladder, i.e., distal to the pelvic and vesical plexuses, and this gave a diminished number of VIP-IR nerves.

The marked increase of VIP immunoreactivity in the bladder, after pelvic decentralisation, indicates that the preganglionic parasympathetic influence may be primarily excitatory to VIP release. Therefore the loss of such influence after surgery led to an accumulation of VIP in the postganglionic endings. In contrast, the hypogastric sympathetic influence may have an inhibitory component. Thus, its removal would cause a release of VIP and result in a decrease, no change, or, when in combination with other factors, a slight increase in the VIP content in the bladder.

The increase in VIP-IR nerves after various nerve transections may also, presumably, represent a compensatory phenomenon, i.e., re-innervation or 'repairing' of the lost nerves by outgrowth from the neighbouring intact nerve fibres (for review see William & Warwick 1980). If that is the case the varying extents of increased VIP-IR nerves after different operations may simply reflect the various degrees of nerve loss. The hypogastric nerves mainly contribute to the trigonal region of the bladder, which represents a smaller proportion of the bladder innervation in comparison with the pelvic nerves. Therefore, transecting the sympathetic pathway led to a compensatory increase in VIP-IR nerves less marked than that caused by sectioning of pelvic or both pathways.

The increase of VIP-IR nerves in the bladder, following pelvic nerve sectioning alone, was less marked when the hypogastric nerves were also sectioned. This suggests that part of the former increase was dependent on the integrity of the hypogastric nerves. The VIP-containing neuronal cell bodies in the pelvic and vesical plexuses, or inside the bladder wall, may be influenced by sympathetic transmission. Cutting the hypogastric nerve, i.e., taking away the sympathetic influence, would impair the activity of outgrowth from the VIP-IR ganglia. Indeed, in the cat, a large proportion of the non-adrenergic ganglion cell bodies in the urinary bladder were found to be supplied by adrenergic nerve terminals (Hamberger & Norberg 1965). It has also been found that the sympathetic efferents facilitate or inhibit transmission through some pelvic parasympathetic ganglia located close to the bladder (de Groat & Saum 1972; De Sy et al 1974; de Groat & Theobald 1976; Craggs & Stephenson 1981). Purinton et al (1973) observed that the hypogastric nerve gave off a branch to accessory ganglia located cranioventral to the major pelvic ganglia in the rat. These findings indicate that, not only previously presumed adrenergic ganglia, but also, certain non-adrenergic ganglia are influenced by a sympathetic mechanism. The VIP-containing ganglia proximal to or within the bladder wall may be influenced by such neurons; thus the disconnection of the sympathetic pathway would partially suppress the outgrowth of VIP-containing nerve terminals. It is also possible that certain VIP-IR nerves originate from ganglia located higher in the sympathetic pathway, e.g., the hypogastric plexus or the sympathetic chain. This would be in keeping with the finding that large numbers of VIP-IR ganglia were present in the hypogastric

plexus of the guinea-pig (Hökfelt et al 1978). This could also explain the observation in this study that whenever the sympathetic pathways were severed, the increase in VIP-IR nerves was lessened, in which case the compensatory increase was reduced by the loss of certain VIP-IR nerves caused by hypogastric denervation.

Thus, the majority of the VIP-IR nerves in the urinary bladder of the rat appear to originate from ganglia located close to or within the bladder.

Substance P-IR nerves were found to be decreased, following all types of nerve sectionings. This suggests that substance P-IR ganglion cells are principally extrinsic. In view of the sensory nature of the substance P-IR nerves, it seems likely that substance P-IR nerves run in both hypogastric and pelvic pathways and probably have their origin in or close to the spinal cord. This is in keeping with the previous finding that substance P-containing ganglia occur in the spinal dorsal root (Elfvin & Dalsgaard 1977; Gamse et al 1979; Lundberg et al 1979a; Lundberg 1979; Dalsgaard et al 1982; Mattews & Cuello 1982). This assumption was recently confirmed by Sharkey et al (1983) who found, using the retrograde-labelling technique combined with immunostaining, that certain populationsof dorsal root ganglion cells, which supply the urinary bladder, contained substance P immunoreactivity.

3.3. PATHOLOGY OF THE URINARY BLADDER

3.3.1. UNSTABLE BLADDER

3.3.1.1. INTRODUCTION

The field of peptide involvement in bladder pathology is an unexplored territory. No information at all had been provided along this line until the present study, although attempts had been made to evaluate the roles of classical neurotransmitters in certain bladder disorders. There are a number of urogenital disorders of which the pathogenic mechanisms are hardly understood. Bladder instability is one of them.

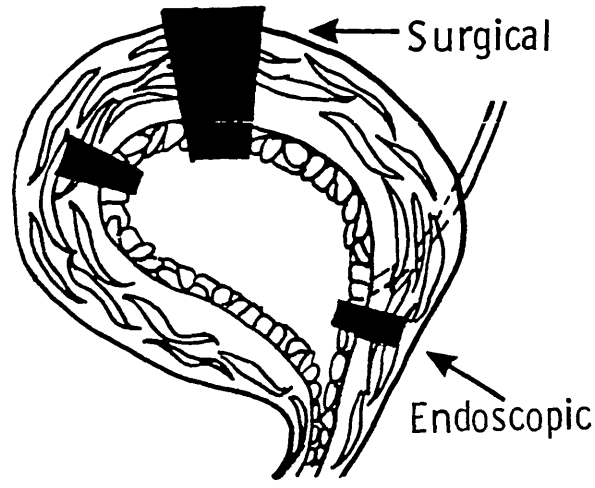
The term 'unstable bladder', or 'bladder instability', was defined by Bates (1970) as the occurrence of detrusor contractions which the patient was unable to inhibit. In such an abnormal state, the contractile activity elevates detrusor pressure to above 15 cm H_2O during the filling phase, often resulting in nocturnal enuresis, urge incontinence, or stress incontinence. It is common in association with neuropathy or outlet obstruction. This may also occur with no detectable neuropathy or other pathological changes, and is then defined as idiopathic unstable bladder. The mechanism of this disorder is unknown, and the management of the this disease has been difficult. Upon the discovery of a large number of VIP-IR nerves in the human urinary bladder and the report of inhibitory effect of VIP on detrusor muscle (Levin & Wein 1981), the possible involvement of this peptide in the pathogenesis of the unstable

bladder was therefore investigated. Bladder materials were collected from patients with detrusor instability. VIP-IR nerves were assessed both by immunocytochemistry and by radioimmunoassay. For the purpose of comparison, NSE (a marker for nerves of all types) and S-100 (a marker for Schwann cells in the bladder) immunoreactivities were also examined by immunocytochemistry. NPY and PHI immunoreactivities were not investigated because the antisera were not available at the time. Substance P-IR nerves were also assessed and no significant changes were found. This, of course, could have been due to the fact that only a few such nerves were present in the normal human bladder and any convincing decrease was difficult to establish.

3.3.1.2. EXPERIMENTAL DESIGN

Bladder tissues from 168 patients were studied. Both surgical (n=35) and endoscopic biopsy (n=133) bladder tissues were taken from patients suffering from idiopathic (n=55) and secondary (n=47) bladder instability and from control patients with no detectable urodynamic abnormality (n=66), undergoing surgery for carcinoma or other causes, or endoscopic investigation. 'Normality' in the control group was assessed by lack of symptoms, a normal voided volume chart, a normal flow rate and absence of residual urine. The clinical details of the patients are presented in Appendix 1.2.. The surgical specimens were obtained from the dome of the bladder; they contained the whole thickness of the wall across an area of about 1 cm^2. The endoscopic tissues were taken

Diagram 17



intravesically from the dome and the trigone. The samples were about 0.2 cm^3 in size and consisted of mucosa, plus about one third of the muscle layer. The regions where the tissues were taken are shown in Diagram 17. The tissues were fixed in BQS and the modified indirect immunofluorescence staining method was carried out (Appendix 2.4.5.). One section from each sample was stained with haematoxylin and eosin (H&E) to assess the histology. No significant changes were observed in the H&E preparations of the unstable bladders.

3.3.1.3. DECREASE OF VIP IMMUNOREACTIVITY IN THE UNSTABLE BLADDER

VIP, NSE and S-100 immunoreactivities were observed in all the control samples (Fig. 20a) and in most of the unstable bladders (Fig. 20b). There was a marked decrease in the abundance and the brightness of VIP-IR nerves in most of the unstable bladders, particularly the idiopathic ones, in comparison with the controls. This decrease was more marked in the muscle layer than in the lamina propria and around the blood vessels. NSE-IR nerves also showed a decrease in the unstable bladder but to a lesser extent. The decrease in NSE immunoreactivity was mainly confined to those cases where general neuronal damage was to be expected, such as spina bifida, trauma and multiple sclerosis (see Section 3.3.2.). The results are shown in Diagrams 18 & 19. The concentration of VIP in the control bladders, as detected by radioimmunoassay, was 36.52 ± 4.8 pmol/g; and in the unstable bladder, it was 7.62 ± 1.84 pmol/g (p 0.01). Radioimmunoassay detected a more severe decrease in VIP

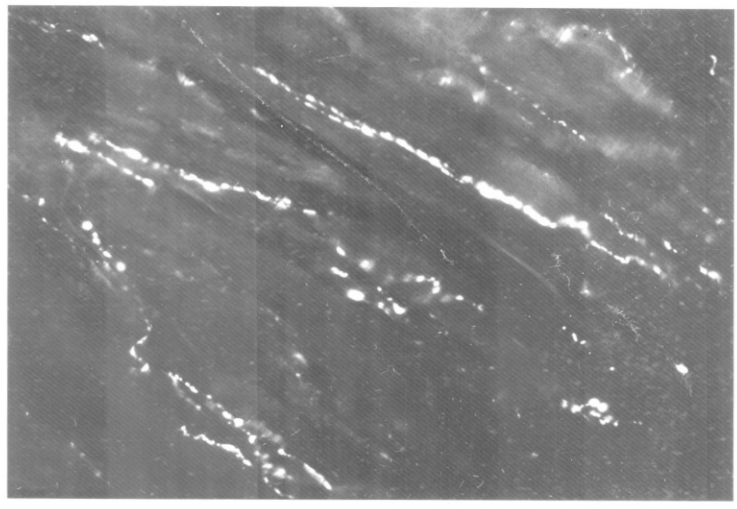


Fig. 20a. VIP-IR nerves in the detrusor muscle of normal human urinary bladder (x300).



Fig. 20b. VIP-IR nerves in the detrusor muscle of idiopathic unstable bladder. Note the number of VIP-IR nerves is decreased (x300).

Diagram 18

VIP-IR NERVES IN THE UNSTABLE AND STABLE URINARY BLADDER

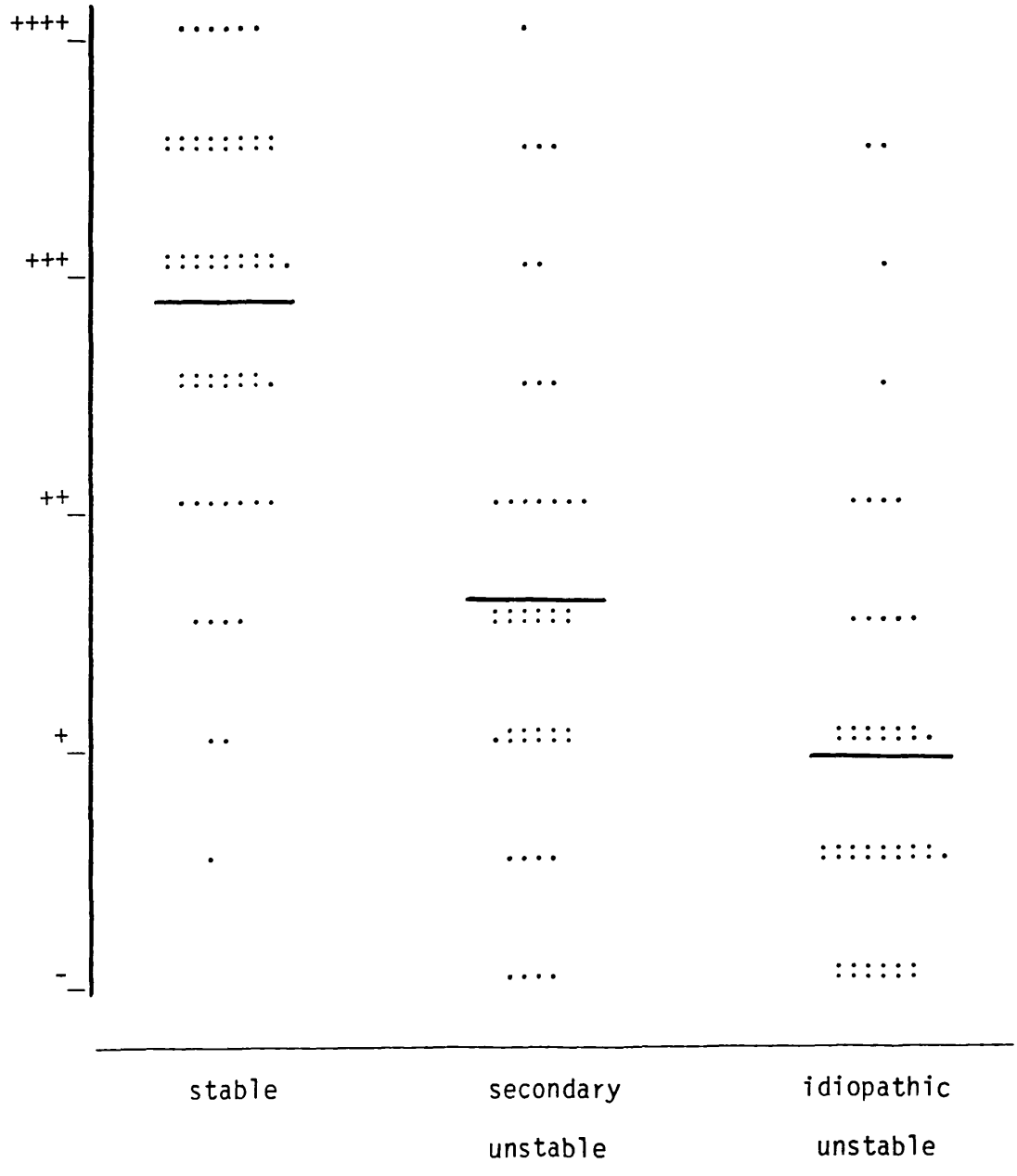


Diagram 19

NSE-IR NERVES IN THE UNSTABLE AND STABLE URINARY BLADDER

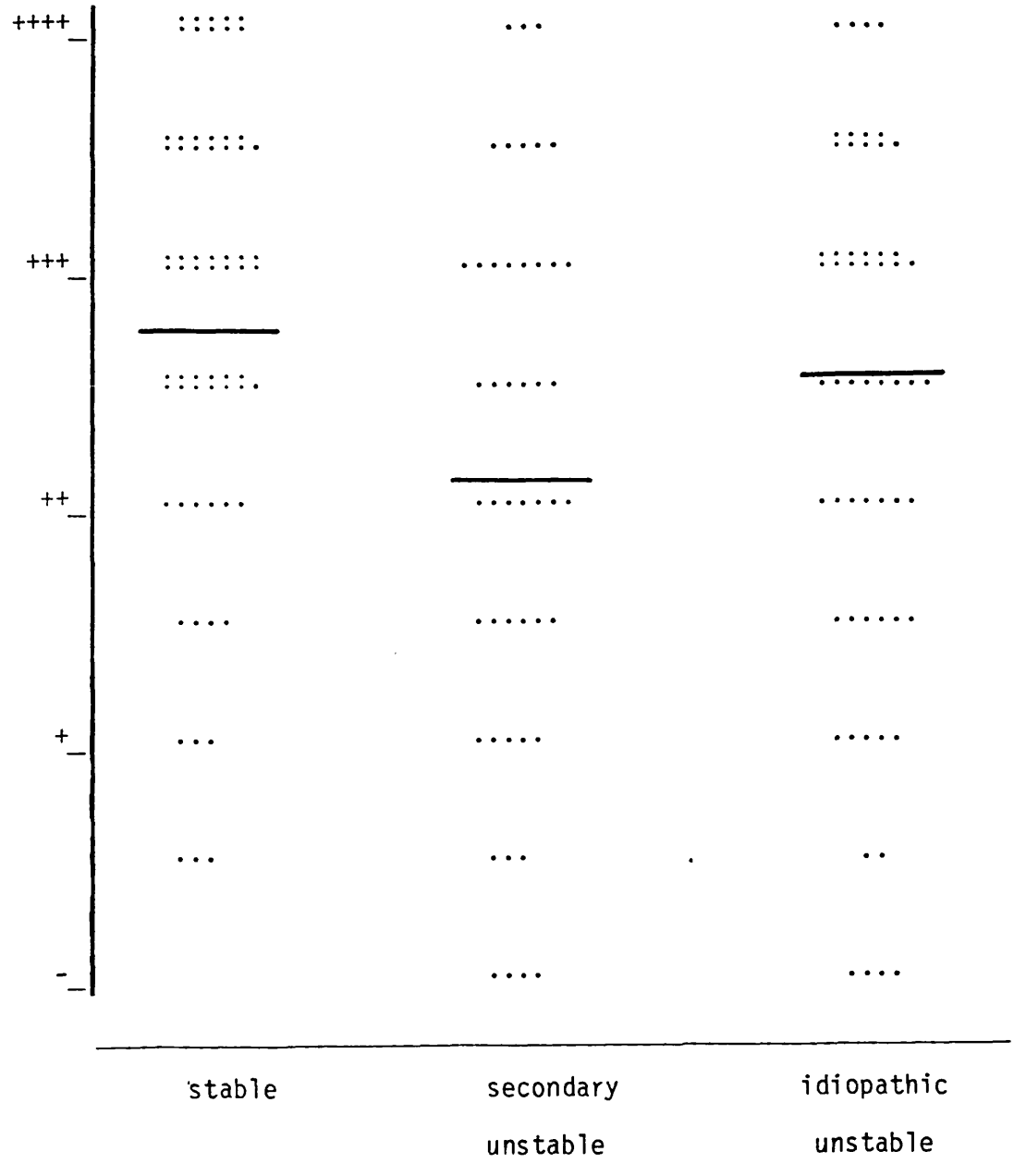


Diagram 20

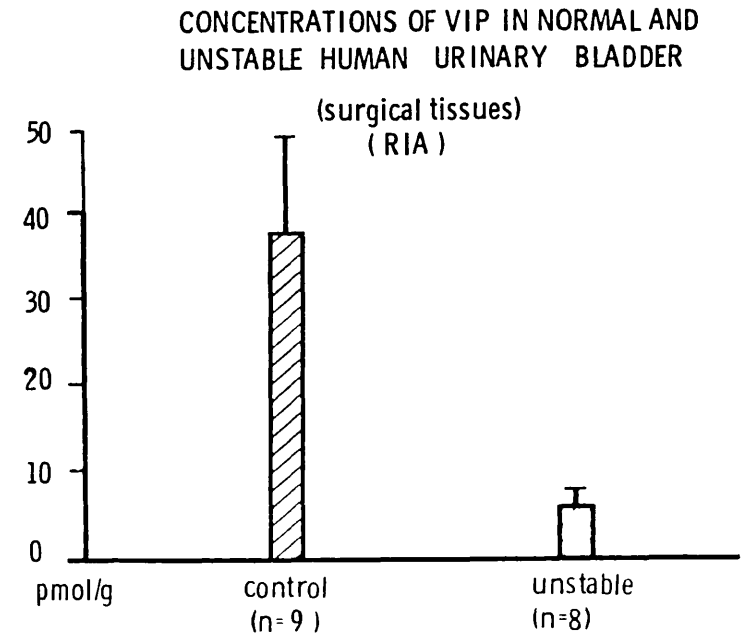
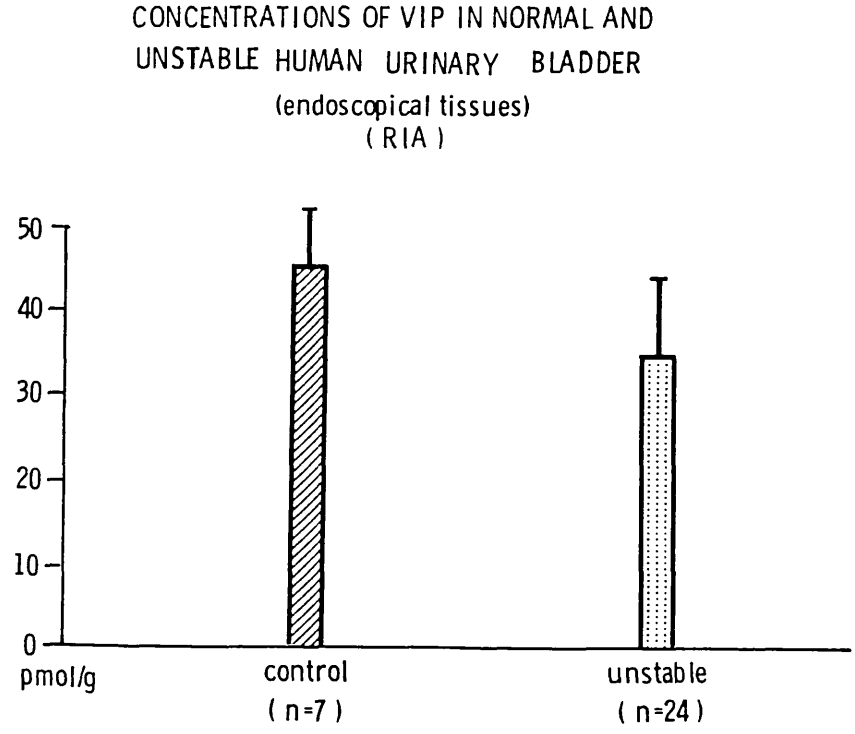


Diagram 21



immunoreactivity in the full wall thickness specimens than in the intravesical biopsies. The results of radioimmunoassay are illustrated in diagrams 20 (surgical tissues) and 21 (endoscopic tissues). The frequency and distribution of S-100 immunoreactivity was largely unchanged or slightly decreased in the unstable bladders in comparison with the controls.

3.3.1.4. DISCUSSION

The results of the present study reveal that there was a dramatic decrease in VIP immunoreactivity in the idiopathic and secondary unstable bladders in comparison with the controls. Immunocytochemistry showed that this decrease occurred mainly in the muscle layer.

If the decrease in VIP immunoreactivity reflected a nonspecific depletion of all nerves, one would, logically, expect a more severe change for the general neuronal marker, NSE. However, the decrease in the NSE-IR nerves was minimal. The largely unchanged S-100 immunoreactivity might indicate that no Schwann cell hyperplasia was taking place at the time of biopsy. This suggests that the decrease in VIP content may represent a specific pathological change in the unstable bladder, rather than a general neuronal destruction.

The aetiology of detrusor instability in patients without outflow obstruction or neuropathy is unknown. Studies of the distribution and activity of the classical neurotransmitters in specimens from

the bladders of such patients have been unrewarding (A.R.Mundy, Consultant Urologist, Guy's Hospital, London, personal communication). Clinical studies have shown that although the bladder of normal man is atropine sensitive, the mechanism that leads to involuntary detrusor contractions in patients with idiopathic detrusor instability rarely is (Brindley & Craggs 1975).

VIP produces, amongst other effects, smooth muscle relaxation. An inhibitory effect on detrusor contraction was demonstrated in rabbit bladder by Levin & Wein (1981). It may be that in the bladder it acts to modify the background activity of the detrusor smooth muscle, keeping it relaxed and maintaining normal bladder compliance until a cholinergic voiding detrusor contraction is voluntarily initiated. If this were so, a deficiency of VIP might be expected to lead to an unrelaxed and irritable detrusor. In such a state the response of the detrusor to anticholinergic medication would be limited, as the basic abnormality would be unaffected by such medication.

The findings presented here are compatible with our present understanding of idiopathic detrusor instability and with the hypothesis outlined above. It suggests that VIP is involved in the bladder pathology, and lack of VIP may play a role in the pathogenesis of bladder instability. The present findings call for further investigation on the nature and significance of the decrease in VIP-IR nerves in this hitherto poorly understood dysfunction.

3.3.2. MULTIPLE SCLEROSIS (MS) AND S-100

3.3.2.1. INTRODUCTION

Multiple sclerosis (MS) is known as a disease of the central nervous system. Its lesions are primarily found in the white matter of the brain and the spinal cord. The pathological changes are mainly demyelination and excessive formation of scars, both of which impair the normal neural conduction. The disease runs a relapsing and remitting course and is generally progressive. The aetiology of MS is uncertain, and specific therapy is not available. There is some evidence however to suggest a possible cause of virus infection combined with a dysfunctional immune system. A number of peripheral symptoms are associated with MS, such as those of the urinary bladder and the gastrointestinal tract; all of them have been attributed to the lesions in the central nervous system, presumably being caused by damage in the central neural control of the affected organ. No primary peripheral morphological changes have been reported in MS patients (for reviews see Lampert 1978).

Following an MS attack, the urinary bladder may become unstable or, alternatively, completely lose its contractility. In an effort to examine the changes in peptide, NSE and S-100 immunoreactivities in the unstable bladder, bladder tissues from MS patients were also collected and examined with H&E staining and immunocytochemistry.

3.3.2.2. EXPERIMENTAL DESIGN

Tissues from 23 dysfunctional bladders of MS patients were investigated. The samples were collected and processed in the same way as for the study of the unstable bladders. Antisera against VIP, NSE and S-100 were employed. The results were compared with those of the dysfunctional bladders from non-MS patients (n=136) as well as with those of the normal bladders (n=21). H&E staining was also performed on sections from each case to assess the morphology of the tissue. The results were examined and graded without knowing the diagnosis for each case.

3.3.2.3. INCREASE OF S-100 IMMUNOREACTIVITY IN BLADDERS OF MS PATIENTS

No apparent changes were observed on the H&E preparations (Fig. 21a), except that in some cases Schwann cell hyperplasia was suspected. An infiltration by lymphocytes, polymorphonuclear cells and macrophages was seen in cystitis and, occasionally, in other types of dysfunctional bladder, indicating acute or chronic local inflammation. In the bladders from MS patients, however, a slight or moderate cellular infiltration was evident in only 5 out of the 23 cases (Fig. 21b).

In MS bladders, VIP- and NSE-IR nerves were reduced as in other types of unstable bladder, but the decrease appeared to be more general, i.e., all nerve types were reduced. This was reflected, in

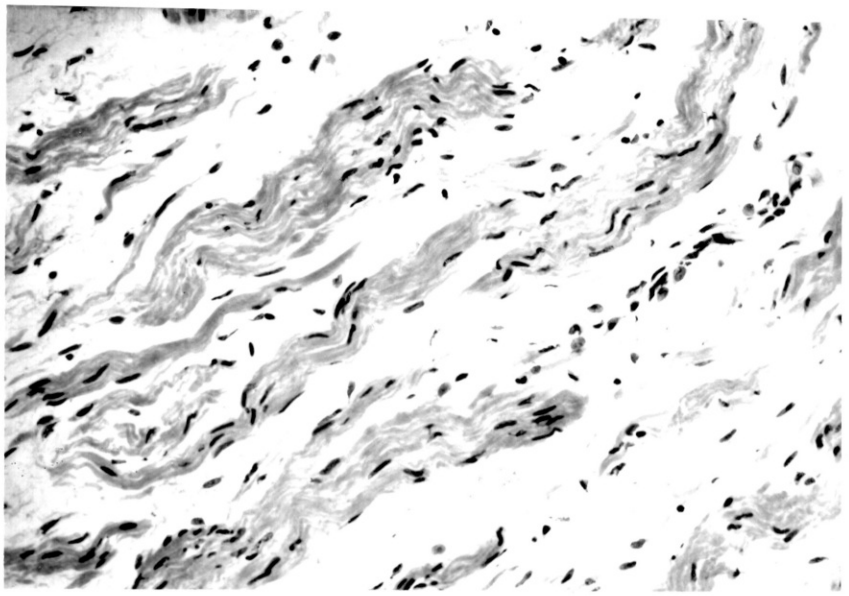


Fig. 21a. H&E preparation of the detrusor muscle from an MS patient. No abnormality can be seen. (x320).

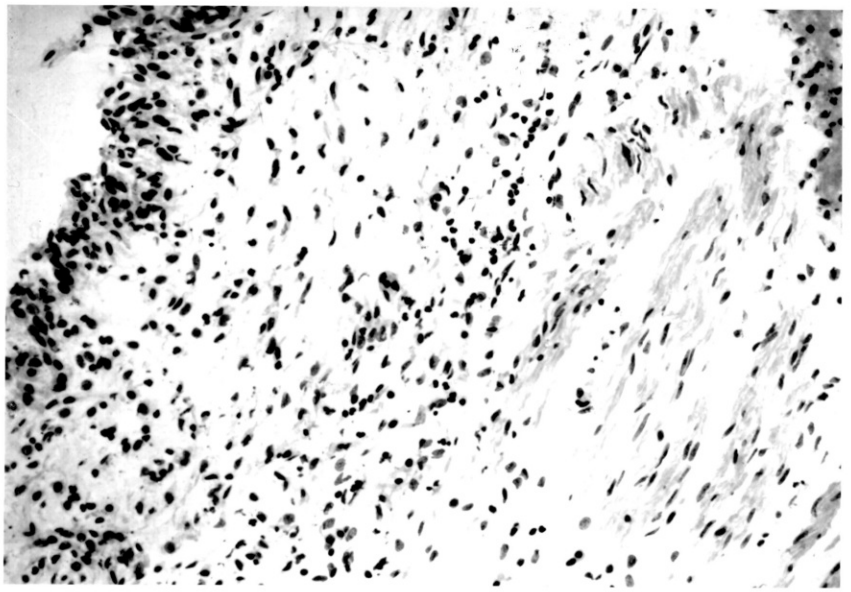


Fig. 21b. H&E preparation of the transitional epithelium, the submucosa and the muscle layer from an MS patient. Cellular infiltration is present mainly in the submucosa, but this only occurs in a small proportion of the MS patients. (x250).

most cases, by a severe decrease in number of NSE-IR nerves. In a few cases, however, the decrease in NSE-IR nerves was not significant and, thus, the variation in the results obtained from the MS group was higher than that from idiopathic and obstructive unstable bladders.

There was one change which was strikingly different from that in other unstable bladder groups. This was that S-100 immunoreactivity was significantly increased in almost all the bladders from MS patients as compared with those from other dysfunctional bladder patients and from controls (Fig. 22a, b). The extent of this increase varied in different cases. In some patients, at least 3-5 fold increase in the abundance of S-100 immunoreactivity was observed. The increased S-100 immunoreactivity in the MS bladder was particularly concentrated in the smooth muscle bundles, where they formed networks of fibres with swellings containing unstained nuclei, strongly suggestive of Schwann cell hyperplasia. The amount of S-100 immunoreactivity in the normal bladders was quite consistent, being apparently lower than that in the MS patients. The amount of S-100 immunoreactivity in the dysfunctional bladders from non-MS patients showed a larger variation, but was mostly in the normal range. In this group, the amount of S-100 immunoreactivity was apparently increased in 26 cases, particularly those of cystitis, and was absent in 6 cases, in whom the bladders were paralysed and distended. In contrast, the increase in S-100 immunoreactivity occurred in most of the bladders from MS patients, of which only 5 showed local inflammation. In one MS case, in whom the bladder was distended and nonfunctional, the S-100

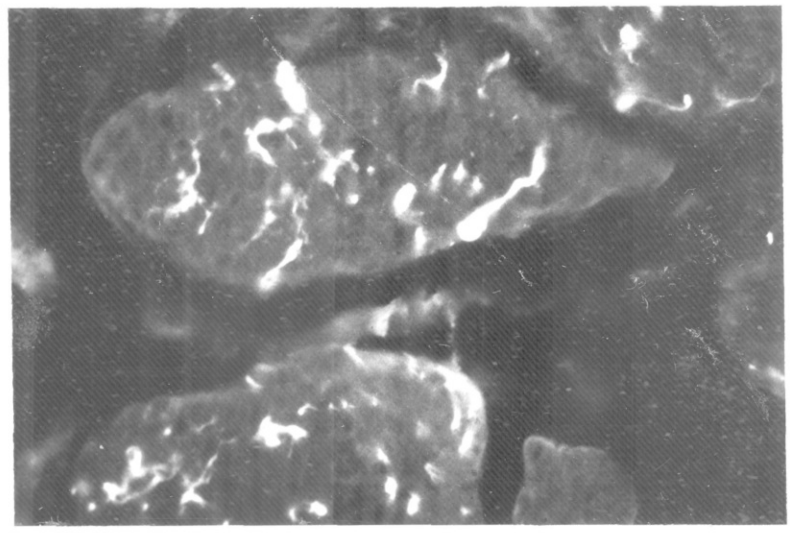


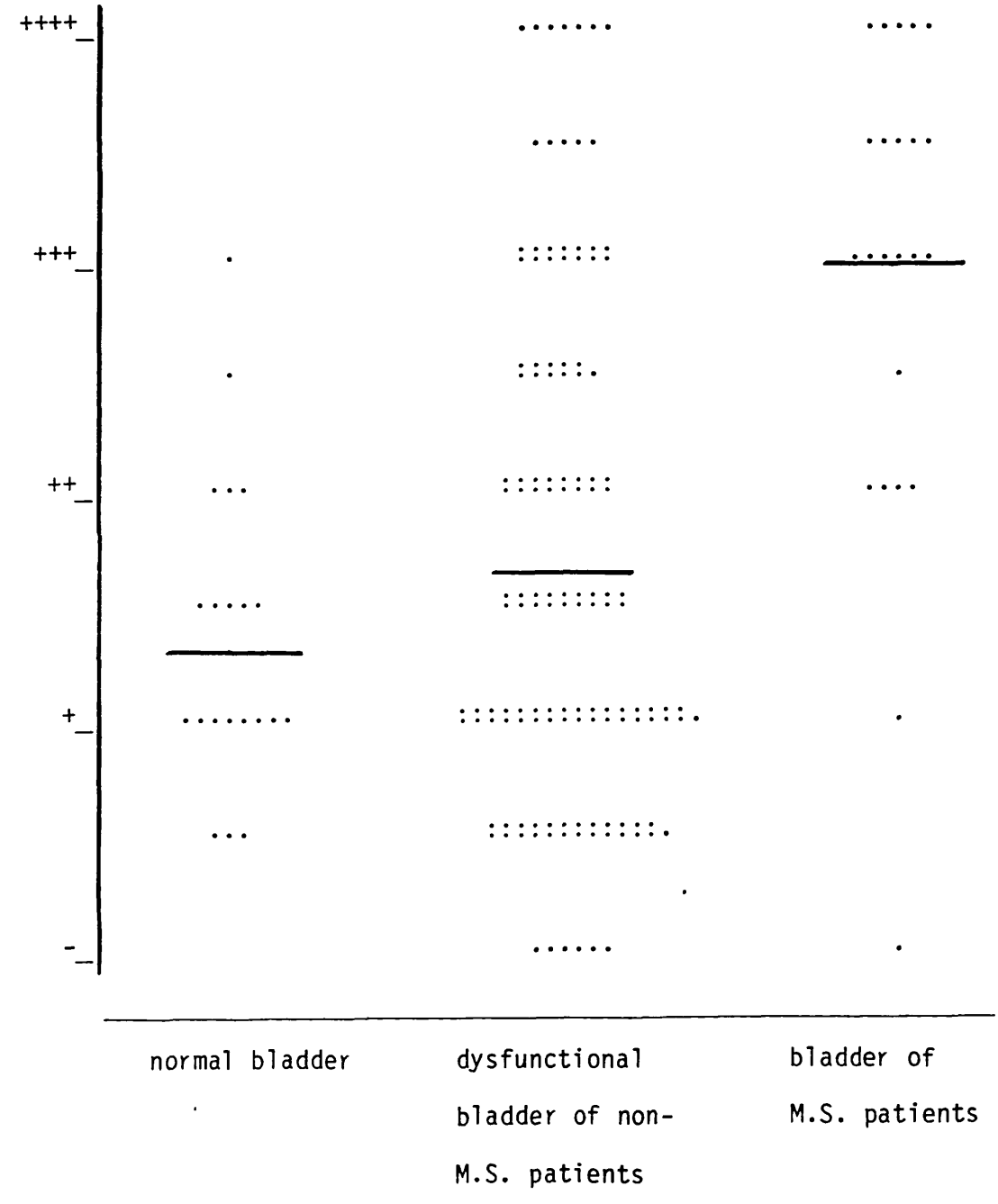
Fig. 22a. S-100 immunoreactivity in the smooth muscle of a normal human urinary bladder. (x120).



Fig. 22b. S-100 immunoreactivity in the smooth muscle of a dysfunctional urinary bladder from an MS patient. Note that the level of such immunoreactivity is markedly increased from the normal range. (x120).

Diagram 22

S-100-IR NERVES IN THE BLADDERS OF M.S. AND NON-M.S. PATIENTS



immunoreactivity was absent. The results are shown in Diagram 22.

3.3.2.4. DISCUSSION

In the present study, it has been found that the abundance of S-100 immunoreactivity was markedly increased in the urinary bladders from MS patients as compared to the control tissues. S-100 has been reported to occur in a number of cellular components (Cicero et al 1970; Ludwin et al 1976; Møller et al 1978; Cocchia 1981; Cocchia & Michetti 1981; Cocchia et al 1981; Takahashi et al 1981; Nakajima et al 1982, 1982a; Stefansson et al 1982; Michetti et al 1983).

Numerous investigations have been carried out to study the cause and the pathogenesis of MS and some have sought to elucidate why the lesions occurred exclusively in the central nervous system (for reviews see Cook & Dowling 1980; Lisak 1980). It has been postulated that certain unsuppressed immunoreactions occurred between antibody and brain tissue antigen (mainly myelin), which might be caused by a number of factors. However, none of the theories are conclusive, and there are many controversial opinions on the pathogenesis of this disease.

Nevertheless, much evidence has been presented that the lesions are likely to be caused by an autoimmune reaction (for review see Lisak 1980). If that is the case, the peripheral myelin may be as vulnerable as the central, since no immunogen specific to the central nervous system of MS patients has been convincingly identified.

It has been shown that neuronal damage such as that found in Wallerian degeneration of rabbit optic nerve (Perez et al 1970) and retrograde degeneration of neurons in the dorsal thalamus (Cicero et al 1970) is accompanied by an increase in the amount of S-100. S-100 levels are elevated both in the cerebrospinal fluid and in the plasma of patients after strokes and during the course of other neurological and non-neurological diseases including MS (Michetti 1980; Moore 1982).

In the adult urinary bladder Schwann cell hyperplasia indicated by increased S-100 immunoreactivity may be presumed to occur in two sets of circumstances, apart from Schwannoma: one is the regeneration process, following nerve fibre destruction; and the other is nerve demyelination, which is the principal change in the brain lesion of multiple sclerosis. Both these processes indicate that there are structural lesions in the neurons of the urinary bladder. In some cases, these nerve damage might be caused by local inflammation. This was supported by the finding of marked increases in S-100 immunoreactivity in the bladders with cystitis. However, in the majority of the MS bladders, no signs of inflammation were found and the Schwann cell hyperplasia was still present. Therefore, it is likely that histopathological changes in MS are not restricted to the central nervous system but also occur in the peripheral nerves. The bladder symptoms may thus be caused directly by the neuronal lesion in the bladder, instead of, or in addition to, indirectly by the lesions in the central nervous system. It is unlikely that the change in the bladder is secondary to the lesions in the central nervous system, since there have been no reports that damaged central neurons lead to a severe proliferation of Schwann cells in the controlled organ, although

transneuronal atrophic changes may, in a few instances, occur in the secondary ganglia and their axons (for review see Williams & Warwick 1980a). The possible destruction of nerve fibres in the MS bladder was also indicated by decreases in VIP- and NSE-IR nerves in most MS specimens as described in Section 3.3.1.3..

The cytochemical composition of the central and the peripheral myelins differs significantly (for review see Gregson 1976). However, one protein common to both the central and the peripheral myelins, the basic protein (Brostoff 1972; Greenfield 1973), has been found to be the antigen responsible for inducing autoimmune diseases in the brain (Laatsch et al 1962). The fact that the central and the peripheral glial elements can react with the same S-100 antibody suggests that immunogens common to both sites exist. Therefore, the destruction of myelin may occur in the peripheral nerves as well as in the central nervous system as a result of the same pathogenic causes. Pette and collaborators (Pette et al 1965; Pette 1969) observed that an autoimmune response against peripheral myelin can be provoked in experimental animals by injection of homogenized Schwann cells and Freund's adjuvant. This may give some indication to the possible immunopathogenic mechanism of the peripheral lesions in MS patients. Pette et al found also that severe demyelination occurred in the peripheral nerves of rabbits, 9 - 12 days after inoculation with cultures of Schwann cells from monkeys and rabbits. It is noteworthy that this demyelination took place in the absence of histiocytic and lymphocytic infiltration of the affected nerves. An identical lack of severe cellular infiltration in the urinary bladder of MS patients was found in this study.

One of the differences between peripheral and central myelin is that in the periphery one internodal myelin segment is formed by one Schwann cell, and its destruction is readily repaired. In contrast, in the central nervous system, a single oligodendrocyte is connected to many myelin segments by long cytoplasmic projections (Hirano & Dermbitzer 1967; Bunge 1968). The damage to one oligodendrocyte could cause the disintegration of many myelin segments and result in more widespread and lasting demyelination, which is far more difficult to repair (Lampert 1978). This might be one of the reasons why the peripheral lesions in multiple sclerosis patients are minor in comparison with the central damage and have been neglected.

Another important fact is that the urinary bladder is often one of the earliest organs in the periphery to develop symptoms in a MS patient. The bladder involvement is so common that Prineas estimated that it existed in 78% (Prineas 1970) of the MS patients. It is not suprising to find that the bladder dysfunctions are often closely correlated with the severity of MS (Miller et al 1965), since the pathogeneses in the periphery and in the central nervous system may be more or less the same. Presumably, a mild MS attack would demyelinate and damage nerves such as those containing VIP, to a certain extent, and cause bladder instability resulting in enuresis and urge incontinence. A severe attack would destroy more nerves and cause cessation of the neural response in the bladder, resulting in a distended bladder and urine retention.

The present finding provides evidence to show that primary peripheral neuronal lesions may exist in multiple sclerosis. Therefore, the concept of multiple sclerosis being a disease wholly of the central nervous system should be re-examined.

3.4. SUMMARY

1. Five types of peptide immunoreactivities, i.e., VIP, NPY, PHI, substance P and somatostatin, were found in human urinary bladder, and four of these were localised in nerves with VIP- and NPY-IR nerves being more abundant. The distribution pattern of each type of peptide-IR nerve was described. Scattered VIP-IR ganglion cell bodies were found mainly in the adventitia of the bladder.

2. The same four types of peptide-IR nerves were found in four other mammalian species, i.e. rat, cat, mouse and guinea-pig. The distribution pattern of each type was similar to that in man, but species variations existed. Many more substance P-IR nerves were found in guinea-pig bladder than in the human bladder, and also, albeit not so numerous, more in the cat and rat bladders. NPY-IR nerves, however, were less abundant in the guinea-pig bladder than in the other species. VIP-IR ganglion cell bodies were seen in the bladders of cat and rat, being more frequent in the former.

3. The distributions of VIP- and NPY-IR nerves were more or less similar to those of AChE-positive and noradrenergic nerves respectively. However, differences in abundancy between peptide-IR nerves and nerves containing classical neurotransmitters were also noted. An experiment using 6-OHDA, which selectively destroys noradrenergic nerves, suggested that NPY may be partially co-stored with noradrenaline in certain vascular nerves in the rat bladder.

4. The origins of the peptide-IR nerves in the bladder were studied

by means of surgical nerve transection combined with immunocytochemistry. The results indicate that the majority of VIP-IR nerves may originate from ganglia located very close to or inside the bladder wall. Some VIP-IR nerves may have their origin in a higher position along the sympathetic pathway or, at least, they may be influenced by preganglionic sympathetic fibres. Substance P-IR nerves may originate in spinal cord and run along the pelvic and hypogastric nerves.

5. The distribution patterns of the peptide-IR nerves are indicative of their functional significance. The VIP-IR nerves in the smooth muscle may have a relaxing effect on the bladder and those in the submucosa may subserve a sensory function. The vascular VIP- and NPY-IR nerves may be functionally co-ordinated to regulate local blood flow by their opposing vascular effects. Substance P-IR nerves were absent in capsaicin-treated rat bladder, indicating a sensory nature for these nerves. In addition, a possible trophic effect of substance P-IR nerves is also speculated on, since the number of substance P-IR nerves was apparently increased following noradrenergic denervation by 6-OHDA in the rat bladder.

6. The involvement of peptide-IR nerves in the pathology of urinary bladder was studied. It was found that VIP-IR nerves were, possibly specifically, decreased in the unstable bladders, of which the pathogenic mechanism had been completely unknown. The decrease in VIP may contribute to the development of the abnormal urodynamics and, thus, result in the various bladder symptoms in this disorder. The morphological change of peptide-containing elements in bladder

pathology is herein established.

7. S-100 immunoreactivity was found to be increased in the dysfunctional bladders of MS patients, possibly denoting Schwann cell hyperplasia. Therefore, demyelination or neuronal destruction may occur in the bladder of an MS patient. This suggests that the lesion of MS may not be restricted to the central nervous system as hitherto thought, but also occurs in the periphery, such as in the urinary bladder. The common bladder symptoms of an MS patient may, therefore, directly result from a local neuronal lesion, rather than indirectly from the damage in the central nervous system. The concept of multiple sclerosis being a disease wholly of the central nervous system should be re-examined.

Chapter 4: THE MALE GENITAL TRACT

4.1. NORMAL MALE GENITALIA

4.1.1. INTRODUCTION

The classical view of the autonomic nerve supply to the human male genitalia is similar to that of the urinary bladder in that it is also mainly innervated by nerves derived from the pelvic plexus. The meshwork of nerve fibres and ganglionic cell bodies extendsout from the plexus to form vesical and prostatic plexuses. The former gives off branches to innervate, in addition to the bladder, the seminal vesicles and the vas deferens whilst the latter innervates the prostate gland, the seminal vesicles, the ejaculatory ducts, the bulbo-urethral glands and the penis. In addition to the secondary parasympathetic neurons, the so-called 'short neurons' comprising groups of secondary sympathetic ganglia, are located close to and inside the wall of the pelvic organs (Sjöstrand 1965; Owman & Sjöstrand 1965). Somatic nerves contribute to the genital innervation via pudendal nerves to control mainly the involuntary sphincter muscle. The sensory fibres run in the hypogastric and pelvic nerves. The sensory messages from the superficial region of the external genitalia are conducted to the spinal cord via fibres in the pudendal nerves (for review see Williams & Warwick 1980c).

Both cholinergic and adrenergic nerves have been demonstrated in the male genitalia. However, reports concerning their abundancy and distribution have been conflicting. Some observers have described a

sparse adrenergic innervation in the penes of man and rabbit (Klinge & Penttilä 1969; Shirai et al 1972), whereas others reported that in the penes of man and other mammals, adrenergic nerves were numerous in the corpora cavernosa, where they were related to the cavernous spaces, and very few in the corpus spongiosum (Benson et al 1980; McConnell et al 1979; Dail & Evan 1974; Baumgarten et al 1969). The cholinergic nerves, demonstrated by AChE staining method, were said to be scant in the corpora cavernosa and sparse or even lacking in the corpus spongiosum (Baumgarten et al 1969; McConnell et al 1979; Benson et al 1980). But, in the rabbit and human penes, the cholinergic innervation was described as dense and extensive (Klinge & Penttilä 1969; Shirai et al 1972). Nevertheless, most observations agreed that both nerve types tend to relate to blood vessels and, in general, except in the rabbit and the bull (McConnell et al 1979; Klinge & Penttilä 1969), more nerves are present in the corpus cavernosum than in the corpus spongiosum.

Similarly, the prostate gland, the seminal vesicle, the testis and the epididymis are supplied by sympathetic and parasympathetic nerves consisting of adrenergic and cholinergic nerves respectively. The vas deferens, however, is innervated mainly by sympathetic nerves (for review see Williams & Warwick 1980c).

Based on the existing knowledge on the nerve supply to the male genital tract, many physiological and parmacological experiments have been conducted to study the neural control of the male sexual physiology. In spite of this, the mechanisms of a number of physiological events are still poorly undertood. One such is the

mechanism of penile erection. It has been known that electrical stimulation of the sacral parasympathetic nerves induces penile erection in dog, cat and rabbit (Eckhard 1876; Benson 1981) and atropine can not block this neural elicited erection completely (Dorr & Brody 1967; Henderson & Roepke 1933). Infusion of ACh, the classical neurotransmitter of parasympathetic nerves, does not produce erection in dog (Dorr & Brody 1967) or in cat (Domer et al. 1978). Thus it seems that ACh is not the neurotransmitter directly involved in the penile erection. On the other hand, hypogastric nerve stimulation produces penile erection in rabbit (Eckhard 1876) but not in dog (Eckhard 1876) and cat (Semans & Langworthy 1938; Root & Bard 1947; Langley & Anderson 1895). In fact, it was reported that stimulation of lumbar or abdominal sympathetic nerves in cat caused contraction of penile arteries and resulted in flaccid penis (Semans & Langworthy 1938; Langley & Anderson 1895). Noradrenaline infusion does not elicite penile erection in dog (Dorr & Brody 1967) or in cat (Domer et al 1978). In contrast, adrenaline was shown to cause penile artery contraction in dog (Elliott 1905). Therefore, adrenaline is not the principal neurotransmitter acting in the penile erection reflex either. Numerous other neurotransmitter candidates have been tested. These include substance P, histamine, bradykinin, ATP, serotonin and prostaglandins, but none of them provided the answer (Dorr & Brody 1967; Domer et al 1978; Kilinge & Sjöstrand 1974). Nevertheless, erectile response to nerve stimulation can be completely abolished by the ganglionic blocking agent, hexamethonium (Door & Brody 1967). Clearly, the principal neurotransmitter involved in one of the major male sexual responses, the penile erection, has not been discovered.

Prior to the present investigation, a few peptides have been reported to be present in the mammalian male genitalia. VIP-IR nerves were reported to occur in the vas deferens and the seminal vesicle of guinea-pig, rabbit, mouse and cat (Alm et al 1977, 1980a). Substance P-IR nerves were also found in these tissues and described as being very few (Alm et al 1978). Enkephalin-IR nerves were described in the vas deferens and the seminal vesicle of cat (Alm et al 1981). In man, only the prostate gland and the seminal vesicle were investigated and some nerves in these regions were found to contain VIP and enkephalin immunoreactivities (Valasti et al 1980).

In this study, the occurrence and distribution of a range of peptide immunoreactivities in different regions of the male genitalia of man was thoroughly investigated.

4.1.2. EXPERIMENTAL DESIGN

Human penis, vas deferens, epididymis and testis (n=33) were obtained from patients aged 21-50 years undergoing gender reassignment operation. The patients received estrogen treatment prior to surgery. Prostate glands (n=5) and seminal vesicles (n=5) were obtained at postmortem, from men aged 22-46 years, within 10 hours of death. Histological analysis of the specimens was carried out, and only apparently normal specimens were included in the study.

The occurrence and distribution of a number of peptides were studied using the method of immunocytochemistry. These were VIP, substance P, PHI, NPY, somatostatin, met-enkephalin, CCK_8, bombesin, neurotensin and APP. For comparison, the distributions of presumed cholinergic and adrenergic nerves were also examined by means of immunocytochemistry and histochemistry. NSE was used as a marker for all the neuronal elements in the tissue. The concentrations of the peptide immunoreactivities were measured by radioimmunoassay. Immunostaining of VIP was also carried out at electron microscopical level using the immuno-gold method (performed by Dr L.Probert). Gel permeation chromatography of APP was performed on the surgical samples using a 0.9x60 cm. Sephadex G-50 superfine column with 0.06 M phosphate buffer (pH7.4) as eluate to elucidate the true nature of the APP-like immunoreactivity in the male genitalia. Reverse-phase high performance liquid chromatography (HPLC) of NPY and PHI was also carried out to verify the existence of the two newly discovered peptides in the human male genitalia. Porcine gut NPY and PHI, and avian PP were used as standards. The radioimmunoassay and chromatography were performed by workers from Prof.S.R.Bloom's laboratory.

4.1.3. PEPTIDE-IR NERVES IN THE HUMAN MALE GENITALIA

VIP, PHI, substance P, somatostatin, NPY, DβH, TH and NSE immunoreactivities were demonstrated by immunocytochemical methods. AChE and SPG positivities were shown by histochemical methods. The peptides and enzymes were localized to neuronal components within

the tissues investigated. No immunoreactivity was observed with antisera to other peptides examined apart from APP which will be discussed later. All the controls for immunocytochemistry were negative. The term 'noradrenergic' was used refering to the similarly or identically distributed DβH/TH-IR and SPG-positive nerves.

VIP-IR nerves were extremely abundant, with the highest concentration in the erectile tissue toward the proximal end of the corpus cavernosum. Scattered VIP-IR neuronal cell bodies were encountered in the erectile tissue of the proximal corpus cavernosum (Fig. 23) and, more frequently, in the interstitial tissue of the prostate gland (Fig. 24). VIP-IR varicose nerves were found in association with arteries (Fig. 25) and arterioles (Fig. 26) of all sizes, particularly those in the erectile tissue of the penis. Veins and sinuses were devoid of such nerves. The non-vascular smooth muscle, particularly the circular muscle of the corpus spongiosum (Fig. 27) and the smooth muscle in the prostate gland and the seminal vesicles, was also associated with VIP-IR nerves but to a lesser extent. In the prostate gland and the seminal vesicle, VIP-IR nerve fibres were also seen in the connective tissue and followed the folds projecting into the lumen of the glands (Fig. 28). In the vas deferens and the epididymis, non-varicose VIP-IR nerve fibres ran closely beneath the basement membrane of the epithelium (Fig. 29a) and were only occasionally seen in the muscular coat. The distribution pattern of VIP-IR nerves in the human male genitalia is shown in Diagram 23a.

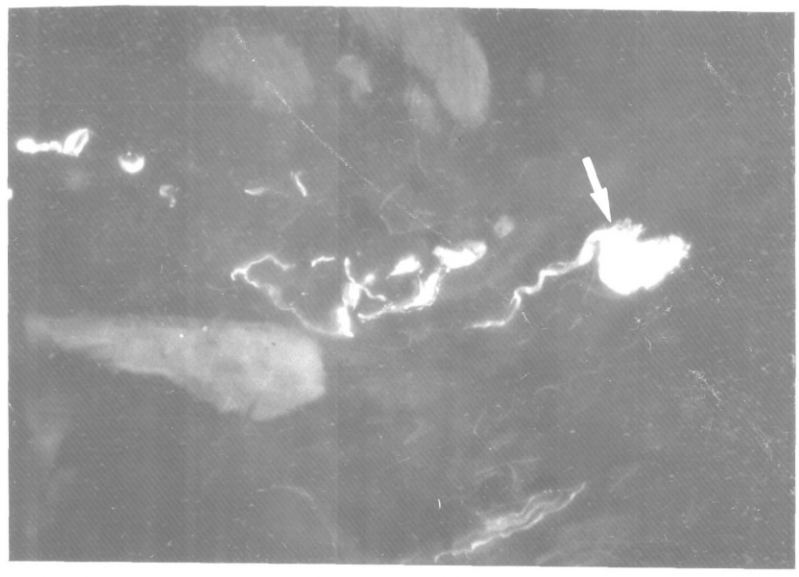


Fig. 23. A VIP-IR ganglion cell (arrow) in the erectile tissue of the corpus cavernosum. (x550).

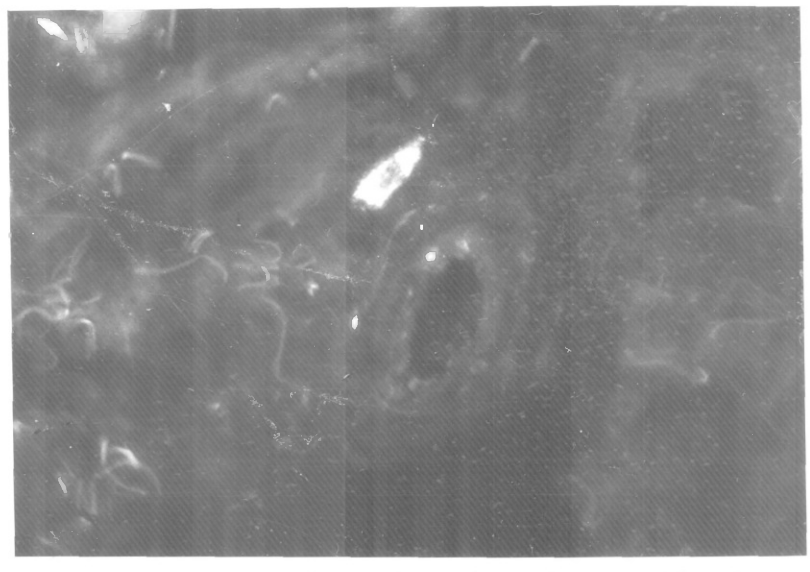


Fig. 24. A VIP-IR neuronal cell body adjacent to a blood vessel in the prostate gland. (x280). Such VIP-IR neurons were not immunostained on the control sections using VIP antiserum pre-incubated with synthetic VIP.

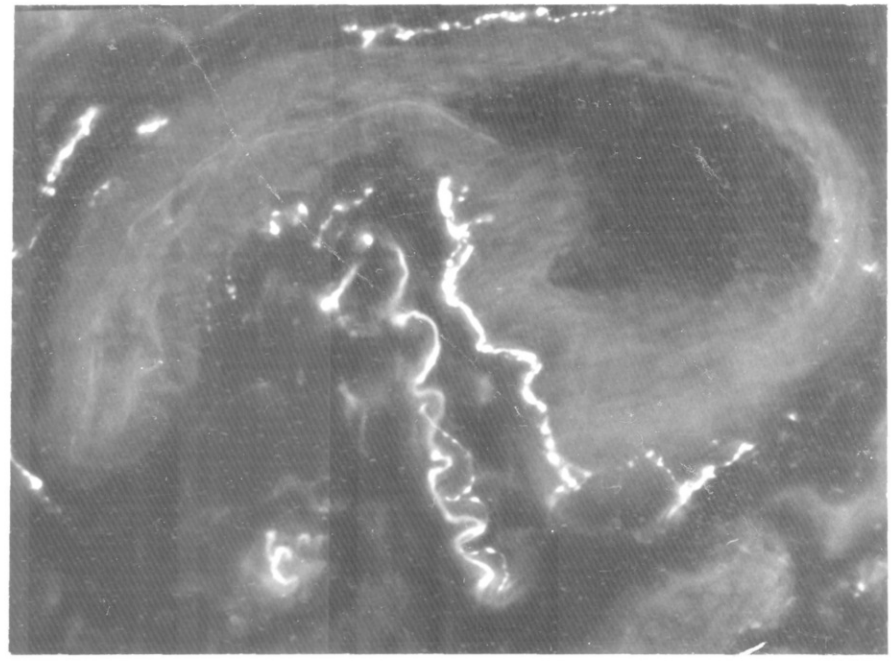


Fig. 25. VIP-IR nerve fibres surrounding an artery in the erectile tissue of the corpus cavernosum. (x415).

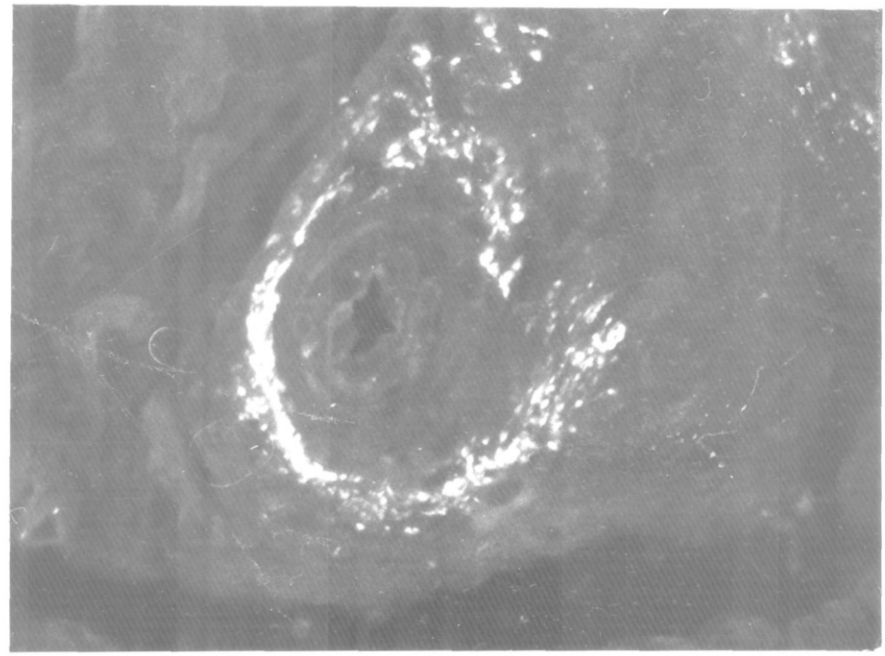


Fig. 26. VIP-IR nerves densely surrounding an arteriole in the erectile tissue of the corpus cavernosum. (x320).

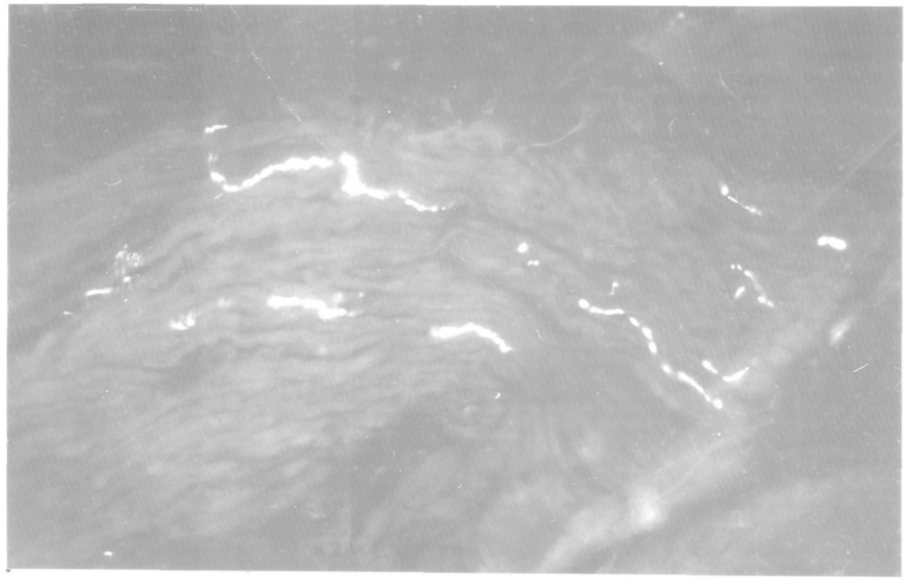


Fig. 27. VIP-IR nerve fibres in the circular muscle of the corpus cavernosum. (x320).

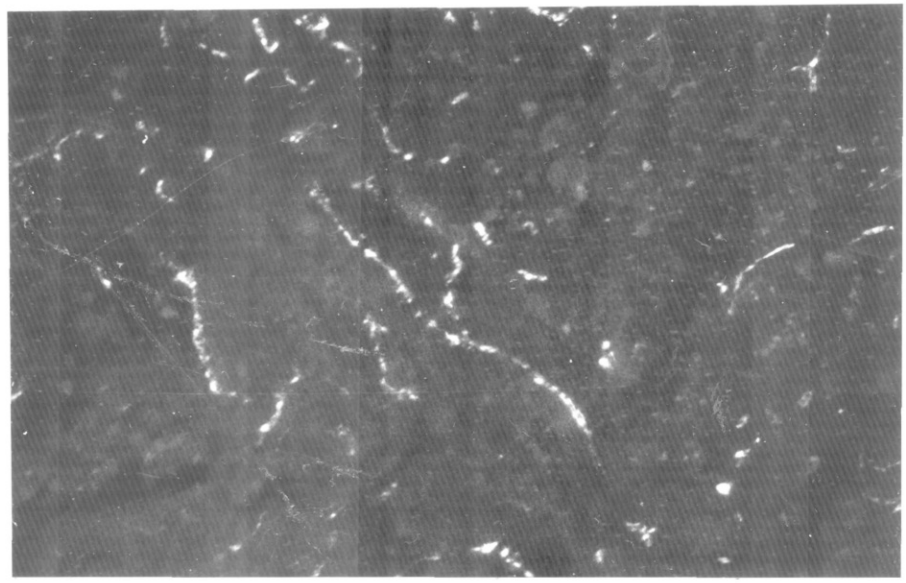


Fig. 28. VIP-IR nerve fibres in the connective tissue in the folds between the glandular structures in the seminal vesicle. (x285).

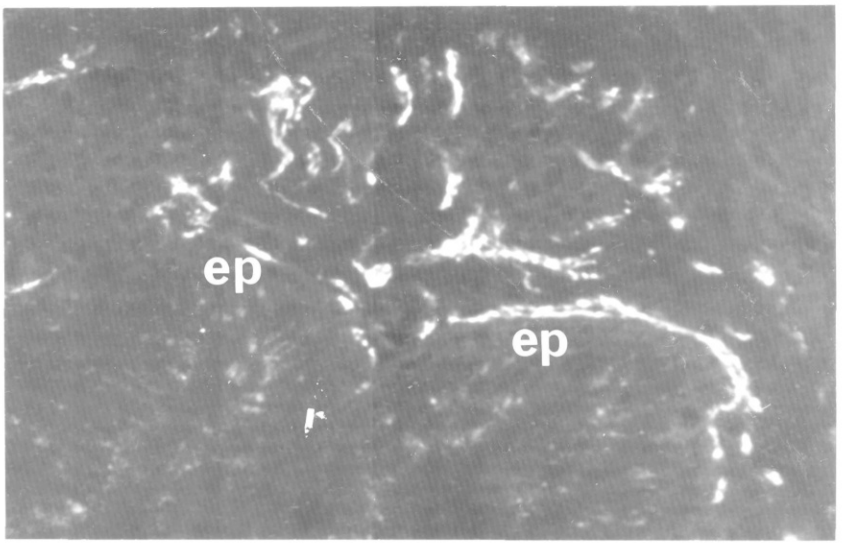


Fig. 29a. VIP-IR nerves in the human vas deferens. They are distributed in the submucosa, closely following the basement membrane of the epithelium (ep) and very few occur in the muscle layer. (x350).



Fig. 29b. AChE-positive nerves in the human vas deferens. Their distribution pattern is very similar to VIP-IR nerves but thicker. (x100).

Diagram 23a

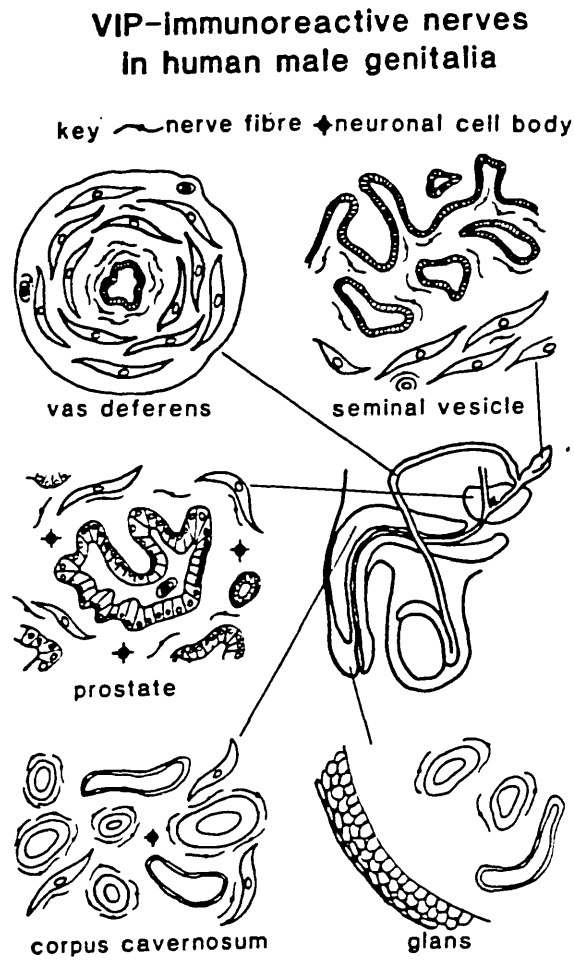
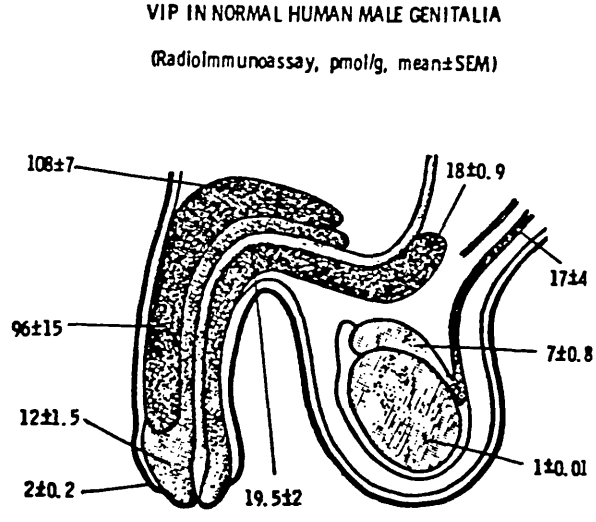


Diagram 23b



Using immunoelectron microscopy, VIP was localized to the large (diameter 97±20 nm, mean±SEM), spherical dense-cored secretory vesicles (Fig. 30), resembling those present in the VIP-IR p-type nerves of the gut (Probert et al 1981). Conventional electron microscopy revealed that in these areas, some terminals containing these large dense-cored vesicles also contained different numbers of small (diameter 40±60 nm), agranular vesicles (Fig. 31) similar to those found in classical cholinergic terminals. In the areas studied, typical cholinergic terminals could also be seen in the areas studied within the same nerve bundles as the p-type nerves.

PHI-IR nerves were demonstrated in the tissue with a distribution similar to that of VIP but less numerous. No PHI-IR ganglion cell bodies were convincingly identified. The distribution pattern of PHI-IR nerves is shown in Diagram 24.

Substance P-IR nerves were scarce but were more concentrated in groups of nerve fibres in the corpuscular receptors underneath the epithelium of the glans penis (Fig. 32). Substance P-IR nerves were also occasionally encountered in the connective tissue of the seminal vesicles. No neuronal cell bodies containing substance P immunoreactivity were found. The distribution pattern of substance P-IR nerves is illustrated in Diagram 25a.

NPY-IR nerves were detected throughout the male genitalia but were particularly dense in the muscular coat of the vas deferens (Fig. 33) and in the interstitial tissue of the prostate gland and the seminal vesicles. In the penis, NPY-IR nerves were much sparser than VIP-IR or AChE-positive nerves. They were found to be

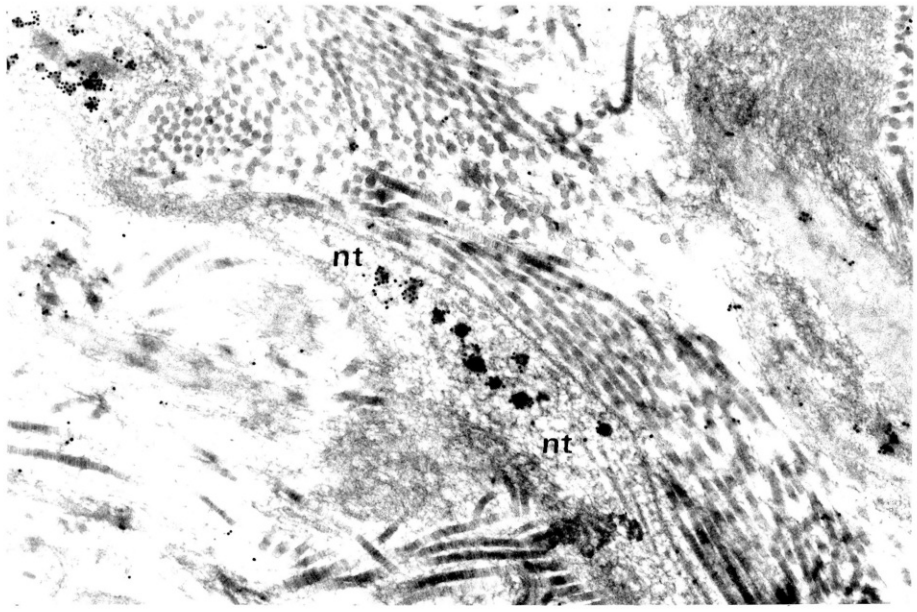


Fig. 30. With the immuno-gold method, VIP immunoreactivity is shown to be localised to electron-dense granules, at the electron-microscopical level, in nerve terminals (nt) in the corpus cavernosum. The labelling gold particles (20nm in diameter) are the very small dense spots, (x40,000).

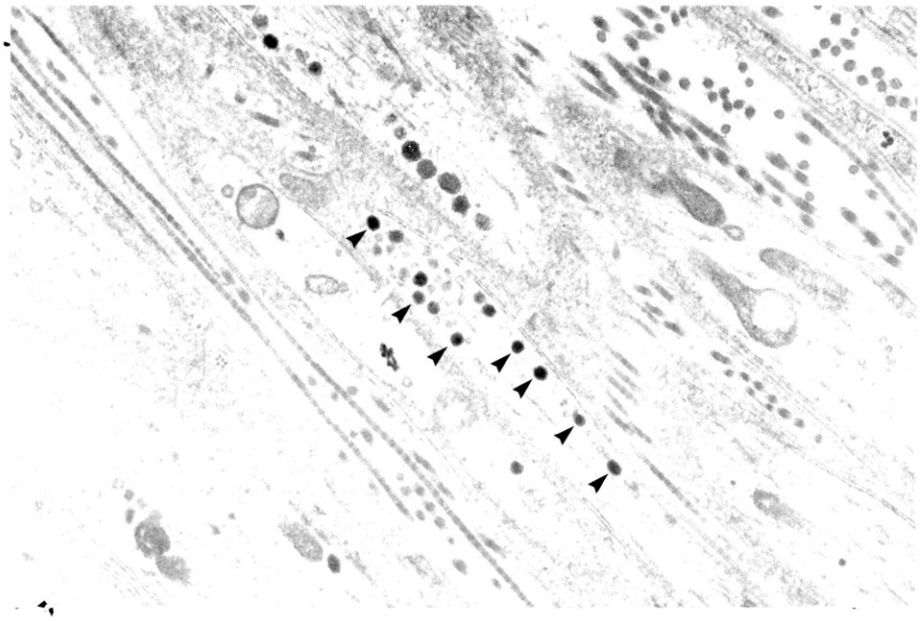


Fig. 31. Conventional electron-microscopy shows that the nerve terminals containing p-type granules (black arrows) also contain cholinergic-type granules (white arrows). (x40,000).

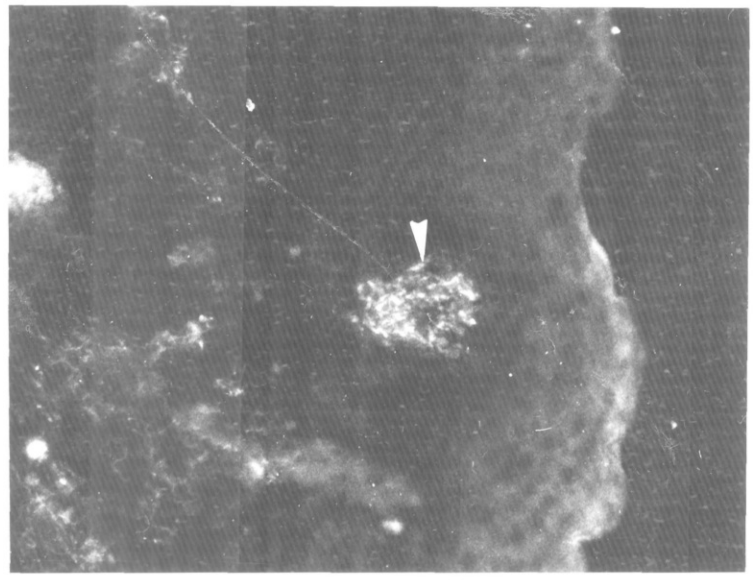


Fig. 32. Substance P-IR nerves (arrow) beneath the epithelium of the glans penile skin. (x200).

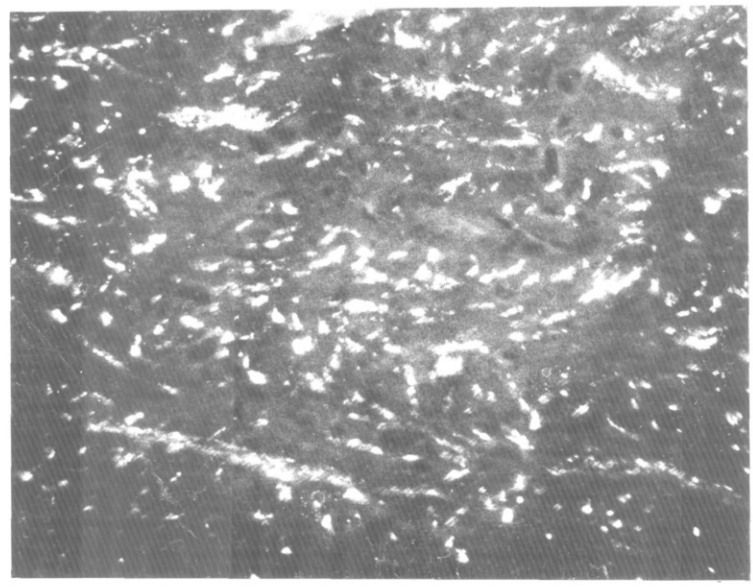


Fig. 33. Abundant NPY-IR nerve fibres in the muscular coat of the vas deferens in man. (x315).

Diagram 24

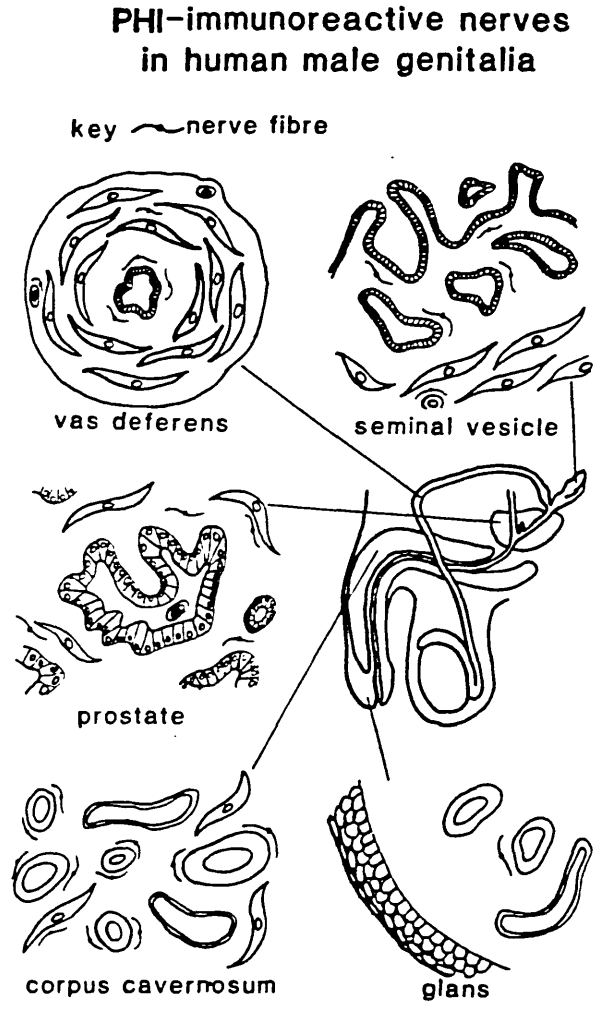

PHI-immunoreactive nerves
in human male genitalia
key nerve fibre
vas deferens
seminal vesicle
prostate
corpus cavernosum
glans

Diagram 25a

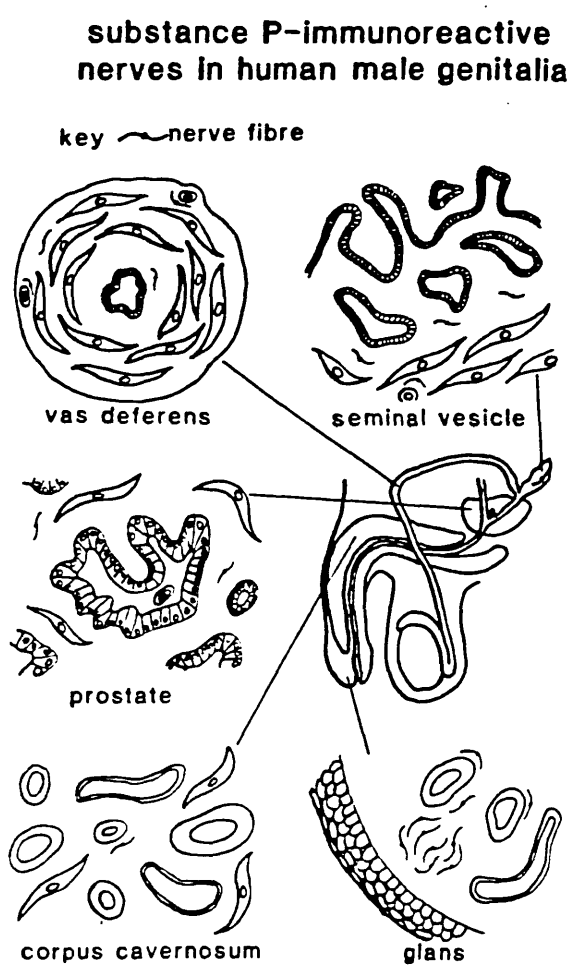
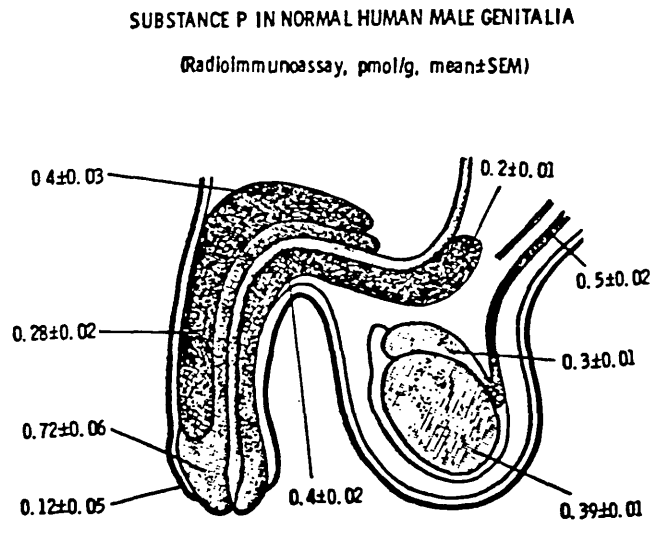


Diagram 25b



associated with both vascular and, more obviously, non-vascular smooth muscle. Scattered neuronal cell bodies in the seminal vesicles were found to react with antiserum to NPY. The distribution pattern of NPY-IR nerves is shown in Diagram 26a.

A few somatostatin-IR nerve fibres were seen in the interstitial tissue and among the smooth muscle of the prostate gland. A small number of them were found in the muscular coat of the vas deferens. The distribution pattern of these nerves is illustrated in Diagram 27a.

Certain nerves were immunoreactive when the antiserum against APP was applied. The distribution of these nerves was very similar to that of NPY. The APP-like immunoreactivity was abolished by addition of NPY antigen (10nmol/ml of diluted antiserum) in the APP antiserum prior to immunostaining.

Radioimmunoassay detected VIP, substance P, NPY, PHI and somatostatin immunoreactivities in the human male genital tissues with distribution in the various regions largely comparable to that found by immunocytochemistry. VIP had the highest concentration, being 108±7 pmol/g, mean±SEM, in the erectile tissue of the proximal corpus cavernosum. The results are shown in Diagrams 23b, 25b, 26b & 27b. No APP immunoreactivity was detected by radioimmunoassay. Chromatography confirmed the existence of NPY and PHI in the tissue. However, no APP was identified.

Diagram 26a

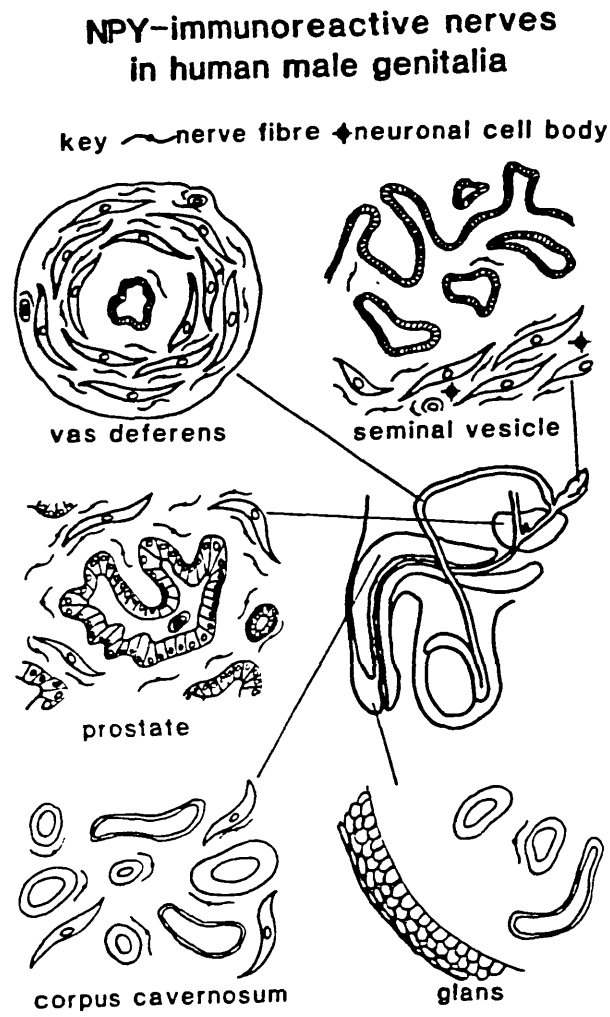


Diagram 26b

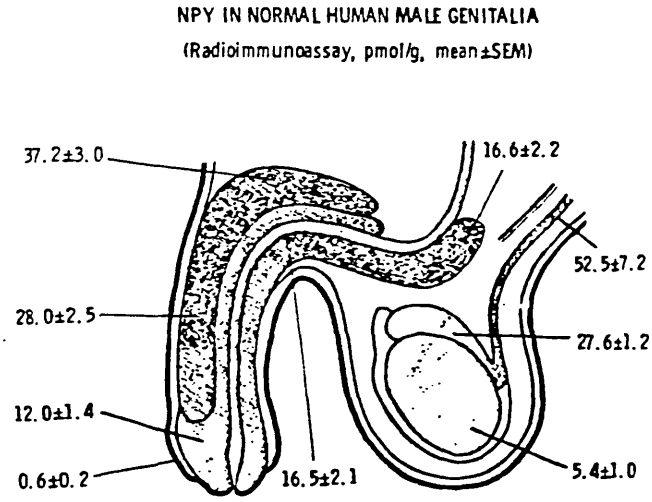


Diagram 27a

somatostatin-immunoreactive nerves in human male genitalia

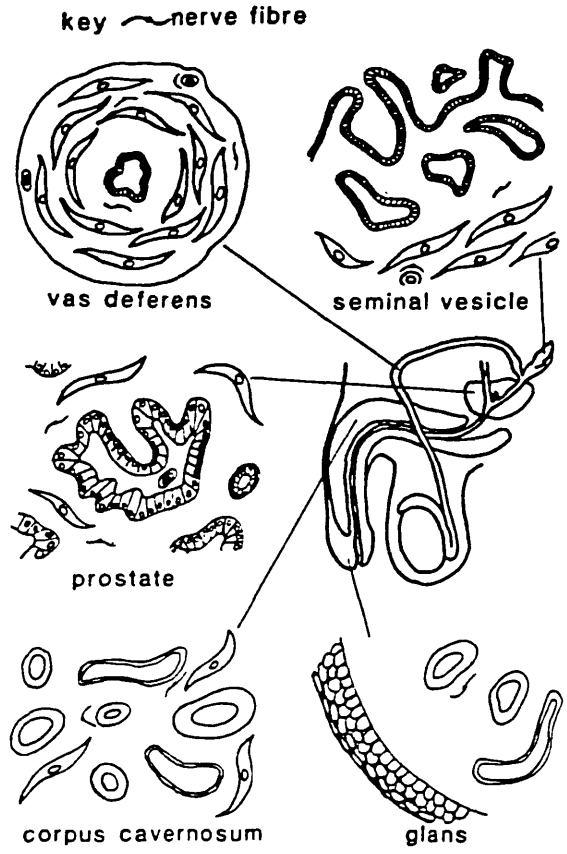
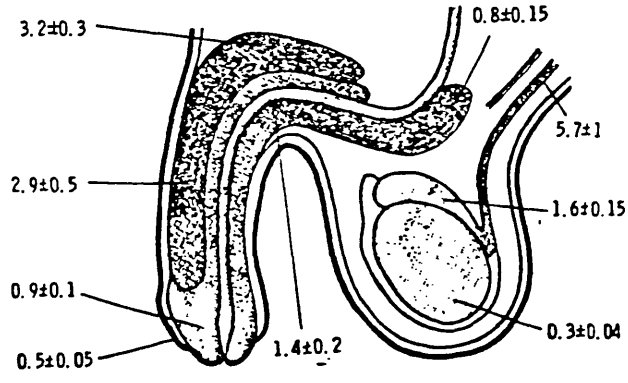


Diagram 27b

SOMATOSTATIN IN NORMAL HUMAN MALE GENITALIA

(Radioimmunoassay, pmol/g, mean±SEM)



4.1.4. NON-PEPTIDE NEURONAL SUBSTANCES IN HUMAN MALE GENITALIA

AChE-positive nerves and adrenergic nerves were distributed densely in most of the areas. They were associated with both vascular and non-vascular muscle. In general, the distribution pattern of AChE-positive nerves was similar to that of VIP, densely surrounding arteries and arterioles and running underneath the epithelium of the vas deferens (Fig. 29b) and the epididymis. However, in some neuronal elements, for example, the corpuscular receptors in the glans penis and some large nerve bundles in the trabeculae of the corpus cavernosum, only AChE-positive nerves were detected. Conversely, a small number of VIP-IR neuronal cell bodies were found in the prostate gland and none of these were demonstrated with the AChE staining method.

Adrenergic nerves were fewer than AChE-positive and VIP-IR nerves in the penis, whereas, in the vas deferens, the prostate gland and the seminal vesicles, they were more frequent. Similarity between the distributions of noradrenergic and NPY-IR nerves was observed, in that both were densely distributed in the smooth muscle of the vas deferens, the prostate gland and the seminal vesicle. However, the overall population of nerve fibres containing NPY immunoreactivity was larger than that of the adrenergic ones.

NSE immunoreactivity appeared to occur within the whole range of nerves in the various regions (Fig. 34). The relative abundance of innervation in different areas was clearly shown by NSE immunoreactivity. It was found that the outer erectile tissue of the corpus cavernosum was more densely innervated than the core and

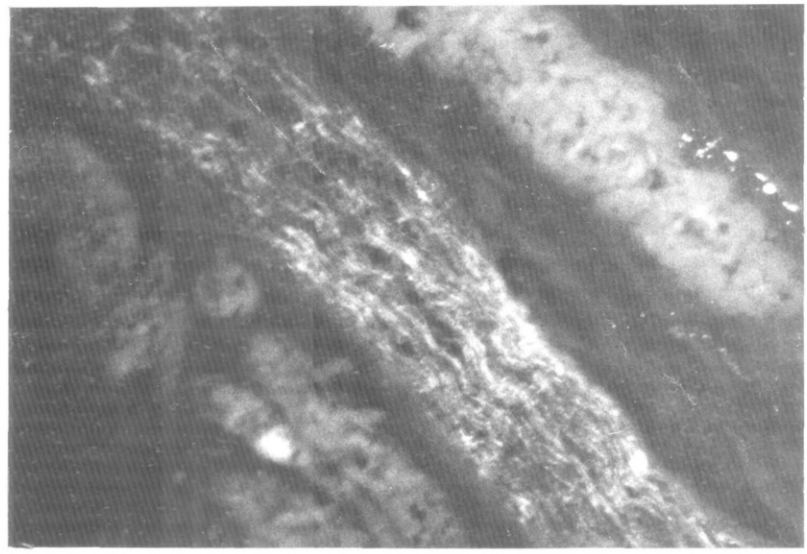


Fig. 34. A bundle of nerves containing NSE immunoreactivity in the corpus cavernosum. Note that all the nerves are immunostained by NSE antiserum. (X185).

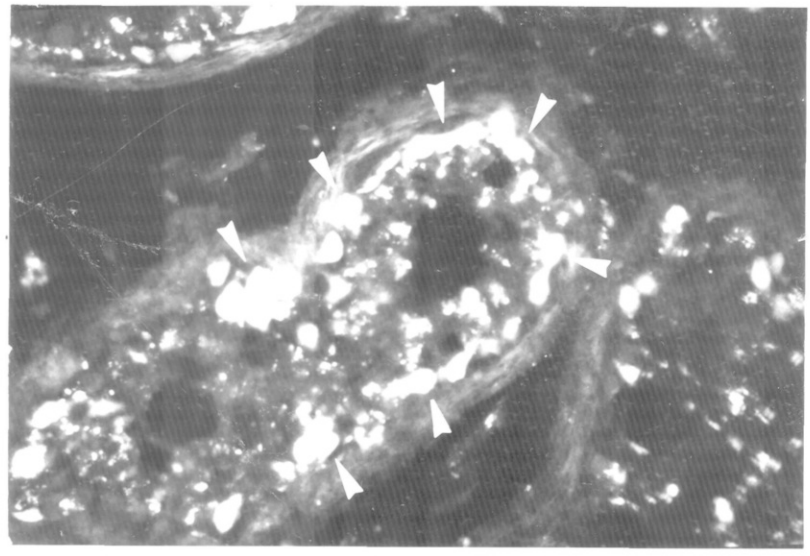


Fig. 35. NSE-IR cells (arrows) are found in the seminiferous tubules of the testis. These cells appear to be Sertoli cells. (x140).

that the proximal end of the penis was more densely innervated than the distal end. Immunostaining of NSE also showed that there were fewer nerves in the corpus spongiosum than in the corpus cavernosum. However, the variations in the intensity of the NSE immunostain were larger than those of peptides.

In the testis, some epithelial cells in the periphery of each seminiferous tubule were found to be NSE-positive, with uniformly stained cytoplasm and large, round, positively stained nuclei. These cells were round, elongated or triangular in shape, lining the inner face of the membrane of the seminiferous tubules and appeared to be Sertoli cells or the basal part of Sertoli cells (Fig. 35). Such cells were not immunostained with NSE antiserum which had been pre-absorbed with NSE antigen.

4.1.5. DISCUSSION

It has been demonstrated in the present study that the human male genitalia were supplied by 5 types of peptide-IR nerves containing VIP, PHI, substance P, NPY and somatostatin immunoreactivities respectively, and each of them possessed a characteristic distribution pattern. The specificity of the method of immunocytochemistry used in this study has been validated with appropriate controls and very high dilutions of the antisera (Table 2). Enkephalin, reported previously in the prostate and the seminal vesicle of man (Valasti et al 1980), was not detected in this study. This might be due to differences in the antisera used.

VIP-IR nerves appeared to be the major peptide-containing neuronal

component in the human male genitalia. The main characteristic of these VIP-IR nerves was that they were extremely dense around blood vessels, particularly in the erectile tissue of the proximal corpus cavernosum. Some VIP-IR ganglion cell bodies were found in the prostate and the root penis, indicating that at least some of the VIP-IR nerves had an intrinsic origin.

The significance of high concentration of VIP immunoreactivity in the penis is that this peptide may be the neurotransmitter involved in penile erection. This was implicated by its close relationship with blood vessels, particularly arteries and arterioles. One of the major actions of VIP is vasodilatation, which is the initial step in penile erection. It was reported recently that VIP was released during penile erection in man (Virag et al 1982). Manually induced penile erection elevated VIP level in the penile blood flow (Dixson et al 1984). VIP has also been shown to have a dose-related relaxing effect on penile smooth muscle in vitro (Larsson et al 1977; Willis et al 1981). It is therefore possible that when the neural excitatory impulse reaches the VIP-containing neurons during sexual arousal, VIP is released from nerve terminals which contain VIP-IR granules. This rapidly dilates the innervated arteries and arterioles to initiate and maintain an erection. In this way, VIP may play a key role in the control of this major male sexual response.

Another characteristic distribution pattern of VIP-IR nerves in the male genitalia is that they closely followed the basement membrane of the vas deferens and the epididymis. No smooth muscle was in the

vicinity of these nerves, so that they might participate either in sensory transmission, as has been proposed for the nerves in the submucosa of the urinary bladder, or in the control of columnar epithelial cell secretion, as has been found in the gastrointestinal and respiratory tracts (for review see Said 1981). The functional significance of VIP-IR nerves closely beneath the epithelium of the vas deferens and the seminal vesicles has not been elucidated so far.

The network of the presumed cholinergic nerves demonstrated in this study in the human male genitalia was in agreement with reports by some groups (Klinge & Penttilä 1969; Shirai et al 1972) and contrary to others (Baumgarten et al 1969; McConnell et al 1979; Benson et al 1980). It should be emphasised however that AChE-positive nerves cannot simply be interpreted as cholinergic nerves since the method used was not entirely specific for cholinergic innervation (see Section 1.5.12.).

The possible co-storage of VIP and ACh was implied by the similar distribution of VIP-IR and AChE-positive nerves. This was further supported by the results obtained from ultrastructural studies and immunoelectron microscopy using the immuno-gold staining method. VIP was localized to the large, round, electron-dense granules in the nerve terminals. Conventional electron microscopy revealed that some of these terminals also contained different numbers of small, agranular vesicles similar to those found in the classical cholinergic terminals. This finding indicates that VIP immunoreactivity and ACh may be co-stored, at least in part, in the

same neurons.

Judging from its distribution and density, PHI immunoreactivity in the human male genitalia may occur in a subpopulation of VIP-IR nerves. However, further evidence is needed to confirm this hypothesis.

A small number of substance P-IR nerve terminals were seen mainly in the corpuscular receptors of the glans penis, which, in the cat, have been shown to be mechanoreceptors (Krane & Siroky 1980). Thus, substance P in the penis may well be a sensory transmitter, as has been previously suggested (for references see Section 1.5.3.). These nerves may be derived from the dorsal root ganglion, possibly via the internal pudendal branches.

NPY-IR nerves are extremely dense in the muscular coat of the vas deferens and the seminal vesicle. Recently, NPY has been shown to inhibit electrically induced, but not noradrenaline induced, smooth muscle contraction of the rodent vas deferens (Allen et al 1982). This effect may be achieved through modulating the release of noradrenaline, possibly indicating a role of neuromodulator rather than neurotransmitter. The distribution and pharmacological studies suggest that NPY may be involved in the neural control of emission and ejaculation. The emission and ejaculation were thought to be mainly adrenergic events (Hucovic 1961; Swedin 1971) and the mechanisms underlying the biphasic contraction of the vas deferens and the contractile response of the seminal vesicle under transmural electrical stimulation in vitro are still poorly understood (Ambache

& Zar 1971; Fedan et al 1977). The finding of numerous NPY-IR nerves innervating the non-vascular smooth muscle, the contraction of which enhances the expelling force in emission and ejaculation, warrants further investigation on the possible role of NPY in these physiological activities.

The vasoconstrictor activity of NPY, as demonstrated in the cat submandibular gland, may also contribute to the control of penile erection, possibly antagonising the vasodilatory effect of VIP, since both NPY- and VIP-IR nerves often surround the same blood vessels, although the NPY-IR nerves were much sparser.

The possible co-storage of NPY immunoreactivity and noradrenaline could not be reliably evaluated in this study although they were similarly distributed in many regions of the organ. It appeared, however, that they were not entirely identical, since, in general, more NPY-IR than noradrenergic nerves were detected. This is different from the previous report on the splanchnic ganglia, where NPY-IR ganglia only accounted for a subpopulation of the total number of noradrenergic neurons (Lundberg et al 1982b).

NSE immunostaining has been found to be a useful method to investigate the innervation of these tissues. The variations in the intensity of the NSE immunostain might indicate that NSE content was indeed related to the physiological state of the nerves, as had been speculated by Marangos and associates (1981).

The demonstration of NSE immunoreactivity in, possibly, the Sertoli

cells is interesting. A putative peptide hormone named 'inhibin' has been extracted from the testis and had the effect of decreasing plasma follicle-stimulating hormone (FSH) (Setchell & Jacks 1974). It has been suggested that inhibin is produced by Sertoli cells (Christiansen 1975) and this was further confirmed in tissue culture study (Steinberger & Steinberger 1976). The finding of NSE immunoreactivity in Sertoli cells provides another clue to the location of this putative peptide hormone. Nevertheless, the possibility that a substance which was present in these cells and which shared a common molecular structure with NSE and reacted with NSE antiserum, can not be ruled out.

4.2. PATHOLOGY OF THE MALE GENITALIA

4.2.1. INTRODUCTION

Penile impotence is a common dysfunction in men. It may occur for a number of reasons. Apart from the vascular and psychological causes, neuronal damage is known to contribute to the development of impotence (Ellenberg 1971). However, the mechanism of this neuronal involvement is not known. This is not surprising since the normal neural control of the penile erection was only partially understood. The finding of abundant vascular-related VIP-IR nerves in the erectile tissue of the penis, described in the previous section (4.1.), suggests that VIP may be the long sought-after non-adrenergic, non-cholinergic neurotransmitter that monitors penile erection. This postulate has recently been supported by further experiments. It was found that VIP relaxed smooth muscle contraction in the corpora cavernosa of the penes of various mammals in vitro (Willis et al 1981, 1983); furthermore, that the plasma level of VIP was elevated in the local blood flow of the penis during tactile-induced erection (Dixson et al 1984).

A very recent preliminary report based on one case of human diabetic impotence described a decrease in the number of VIP-immunoreactive nerves as compared to non-diabetic impotence. No potent penes were investigated by the authors (Crow et al 1983). In the present study, the changes in peptide-immunoreactive nerves, particularly those containing VIP immunoreactivity, in the penes of men impotent from a variety of causes were studied using immunocytochemistry. The findings

were compared with those obtained by investigating potent penes. Antisera to NSE and S-100 were also employed in order to study the distribution of the entire innervation and related Schwann cells.

4.2.2. EXPERIMENTAL DESIGN

Twenty four patients with penile impotence were studied. Biopsies of the erectile tissue from the middle and proximal parts of the corpora cavernosa were obtained from impotent men at the time of insertion of semi-rigid penile prostheses. All patients had an erectile deficiency which prevented penile penetration for periods ranging from 2 to 37 years. Full investigations of these patients suggested that the impotence was due to a variety of conditions, including diabetes mellitus (n=12), atherosclerosis (n=4), trauma (n=3), spina bifida (n=1), priapism (n=1) and psychogenic which did not response to psychosexual conselling (n=2). The ages of the patients ranged from 22-54 years (average 46 years). Details of the patients are presented in Appendix 1.3.. Corpora cavernosa tissue was also obtained from the penes of potent patients with the same age range and taken at the time of gender reassignment (n=6) and at operations to correct the erectile deformity of men with Peyronie's disease (n=2).

For immunocytochemistry, the tissues were fixed in 0.4% BQS (Appendix 2.2.1.). The modified indirect immunofluorescence method (Appendix 2.4.5.) was used with antisera to VIP, NPY, PHI, substance P, somatostatin, NSE and S-100. The controls included preabsorption of the antisera with appropriate antigens, using normal rabbit serum as the first layer and omission of the first layer. H&E staining was also

performed on one section from each case.

Additional information on the concentrations of VIP in the impotent and control tissues were provided by radioimmunoassay (Prof.. S.R.Bloom's laboratory). This was carried out on the penile tissues from 12 impotent men suffering from diabetes mellitus and 8 potent men. Conventional and immunocytochemical electron microscopy was also carried out (Dr L.Probert) on the tissues taken from impotent penes of diabetic patients.

4.2.3. DECREASE OF VIP-IR NERVES IN IMPOTENT PENIS

On the haematoxylin and eosin stained sections there was no apparent abnormality in the impotent penes, except that thickened capillary walls were sometimes seen in tissues from diabetic and atherosclerotic patients and smooth muscle often showed varying atrophic changes.

In the potent penes, including those from the gender reassignment operations and the cases of Peyronie's disease, VIP-immunoreactive nerves were abundant and were distributed throughout the erectile tissue, especially around the blood vessels. The number of VIP nerves in the tissues from impotent men was markedly decreased in almost all the cases as compared to the controls (Figs. 36 &37). This decrease, however, was not confined to any particular disease. The extent of the decrease varied from case to case and was most obvious when the impotence was severe or of long duration. Many blood vessels had lost their surrounding VIP-immunoreactive nerves. In 6 out of the 24 cases, the VIP-immunoreactive nerves were almost absent. The results are shown in Diagram 28.

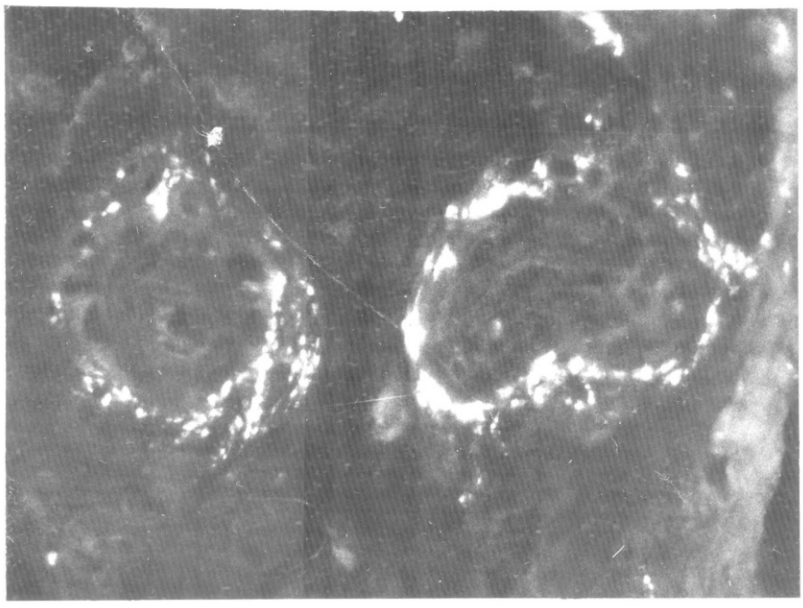


Fig. 36 . The normal amount of VIP-IR nerves surrounding blood vessels in the erectile tissue of the corpus cavernosum in man. (x300).

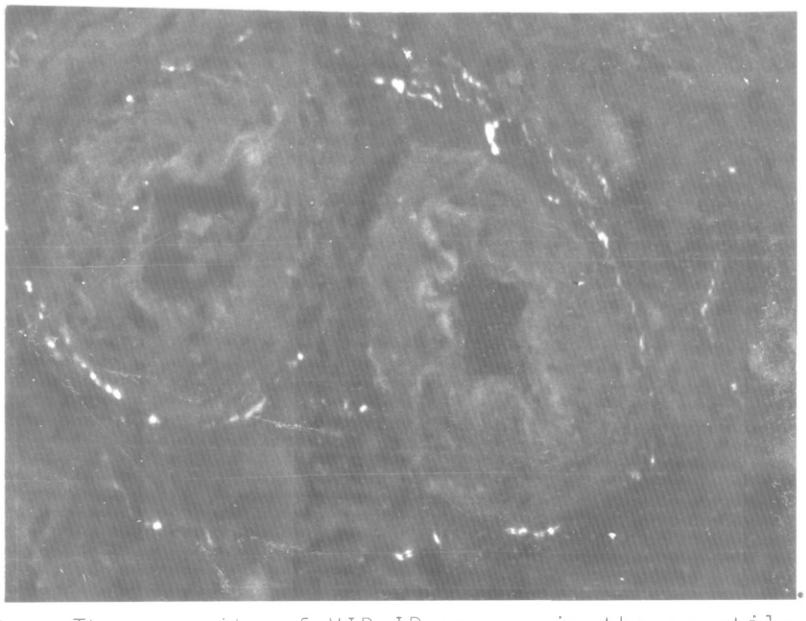
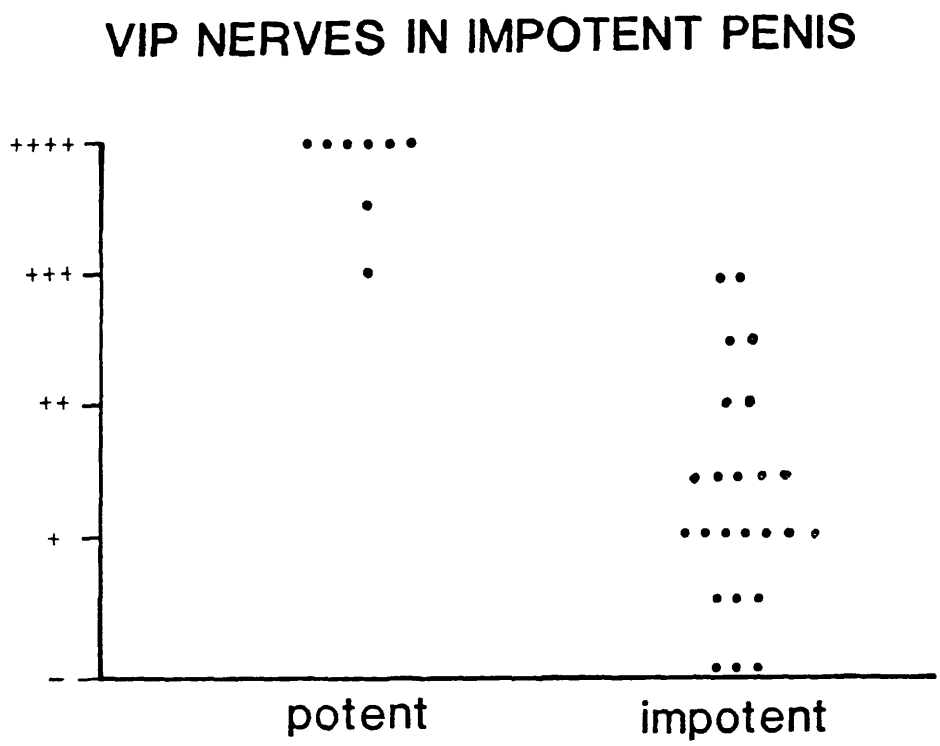


Fig. 37 . The sparsity of VIP-IR nerves in the erectile tissue of the corpus cavernosum from an impotent penis due to diabetes. Note that the number of VIP-IR nerves is markedly reduced. (x300).

Diagram 28



The other types of peptide-IR nerves were also decreased in number. However, the decreases were not as obvious as that of VIP-IR nerves. This may be attributed to the fact that even in potent penis, nerves containing other types of peptide immunoreactivity were not abundant in the erectile tissue. The densities of NSE and S-100 immunoreactivities were also reduced in impotent penes. The decrease in NSE was very apparent, and the extent was equal to or greater than that of the VIP-immunoreactivity in each case. Often there were only a few fragments left, in contrast to the rich network of fibres and bundles in the control tissues. The severity of the decrease in S-100 immunoreactivity varied from case to case and was often not as apparent as that of NSE.

Electron microscopical investigation found that nerve terminals in the penile tissues from patients with diabetic impotence were generally scanty and showed degenerative changes. Many of the nerve profiles appeared swollen (Fig. 38). A marked depletion of structural contents, including vesicles, was evident. No VIP immunoreactivity was observed in four out of the five cases of diabetics investigated and, in the fifth, the number of nerve endings containing VIP-IR vesicles was apparently reduced. The lumina of the capillaries in the diabetic erectile tissue were distorted and sometimes narrowed by markedly thickened basement membrane, measuring up to 2.5 μm in width as compared with around 0.06 μm in normal tissue. In addition, Schwann cells present in small nerve bundles in the diabetic specimens also showed lysis of the cytoplasm and loss of structural contents.

Similar decreases in VIP immunoreactivity were detected by



Fig. 38. A small nerve bundle in the erectile tissue of the penis from a patient with diabetic impotence. A nerve profile (arrow) appears to be very swollen and lacks structural contents (counterstained with uranyl acetate and lead citrate). (x40,000).

With the permission of Dr L.Probert

radioimmuoassay. The extent of the decrease was in general agreement with that found by immunocytochemistry in each case. In the control penises the level for VIP was 189.9±45.9 pmol/g (mean±SEM) (n=6), while in the diabetic impotence (n=12) the average concentration was only 33.7±6.7 pmol/g; in these cases, the decreases in VIP-immunoreactive nerves were severe. The control value of VIP immunoreactivity detected here is higher than that demonstrated in normal human penis, described in Section 4.1.3.. This might be due to the fact that the samples extracted for assay in this study were taken exclusively from the erectile tissue where the highest concentration of VIP occurs.

4.2.4. DISCUSSION

It has been found that the number of VIP-immunoreactive nerves and the concentration of VIP immunoreactivity was markedly decreased in impotent penes as compared to the normal level. The extent of the decrease was largely in parallel with the severity of the symptom in each patient.

Impotence may be caused by neural lesions. A significant number of patients, after sympathectomy or with complete lower motor neuron lesion, develop erectile insufficiency (Whitelaw & Smithwick 1951; Bors & Comarr 1960; Comarr 1970). In a previous study, Melman et al (1980) measured the content of the two classical neurotransmitters in erectile tissue of men with impotence. They found that the noradrenaline content was decreased in nearly all the impotent tissue, the most severe reduction occurring in the insulin-dependent diabetics. It was therefore assumed that dysfunction of the sympathetic

nerves may play a role in the development of impotence. It is, however, difficult to understand how lack of local noradrenaline could lead to impotence, since adrenaline has, in fact, been shown to cause penile blood vessel contraction (Elliott 1905). A logical postulate might be that the lack of noradrenaline in the impotent penis may reflect a general reduction of neural elements or neurochemical content in the tissue, and the loss of a substance not yet identified may be responsible for the development of impotence.

In a recent publication, Crow et al (1983) reported their investigation using immunocytochemistry in one case of diabetic impotence. They described a decrease of VIP-immunoreactive nerves in the penis in comparison with three cases of non-diabetic impotence. As no normal human penile tissues were examined, their findings may reflect merely the different extents of VIP decrease in impotent penes of varying aetiology and severity of symptoms.

Since VIP may be the principal neurotransmitter involved in the initiation and maintenance of penile erection, a deficiency in VIP may be, at least in part, responsible for the development of impotence. This hypothesis was supported by the present finding that the fewer the VIP-immunoreactive nerves found the more severe the impotence appeared to be. However, the decrease in VIP-immunoreactive nerves may not be a specific profile, as has been speculated in the unstable bladder (Section 3.3.1.4.), but simply part of a general degranulation or destruction of all neural elements in the penis. This was suggested by the finding that the immunoreactivity of the general neuronal marker, NSE, was also severely depleted. Electron microscopical

immunocytochemical findings supported this point and showed that general neuronal lesions existed in the impotent tissue of, at least, diabetic men. Nevertheless a large proportion of the vessel-related NSE-immunoreactive nerves seem to contain VIP immunoreactivity (see Section 4.1.3., 4.1.4.). The decrease in S-100 immunoreactivity was possibly caused by a decrease in Schwann cells, indicating that the neuronal damage was severe and little regenerative activity had taken place at the time of the biopsy.

The depletion of VIP-immunoreactive nerves in the impotent penis may be either a primary or secondary change. In the present study, it may be the result of neuropathy, ischaemia, compression or denervation from different causes. However, the decrease in VIP in the erectile tissue provides a new neurological basis for penile impotence and may play a key role in its development.

4.3. SUMMARY

1. Five types of peptide immunoreactivities were found in nerves in the human male genitalia, i.e., VIP, NPY, SP, PHI and SOM. Among these, VIP-IR nerves were the most abundant in the penis, particularly in the proximal corpus cavernosum, where a few VIP-IR ganglion cells were encountered. NPY-IR nerves were also numerous and were more concentrated in the vas deferens, the seminal vesicle and the prostate where NPY-IR ganglion cell bodies were seen.

2. The arteries and arterioles in the penis were densely surrounded by VIP-IR nerves. At electron microscopical level, VIP immunoreactivity was localised to electron-dense p-type granules in the nerve terminal by immunostaining. Considering the powerful vasodilatory action of VIP, and the physiological and pharmacological evidence, it is postulated that VIP is the principal neurotransmitter, responsible for penile erection, which had long been sought-after but never identified.

3. The non-vascular smooth muscle in the vas deferens, the seminal vesicle and the prostate gland were densely innervated by NPY-IR nerves. Together with its biological action on smooth muscle, particularly on that of the rodent vas deferens, NPY may play a role in the control of emission and ejaculation.

4. Substance P-IR nerves were sparse and were concentrated in the sensory receptor structures beneath the epithelium of the glans. This is in keeping with its postulated role of a sensory

neurotransmitter.

5. Somatostatin- and PHI-IR nerves were rare. The former were more frequent in the vas deferens and the latter had a distribution pattern similar to VIP-IR nerves, thus being seen more often in the penis. The significance of these nerves in the male genitalia remains to be elucidated.

6. VIP-IR and AChE-positive nerves had similar distributions, as did NPY-IR and noradrenergic nerves. Using electron microscopy, the large electron-dense granules immunoreactive to VIP antiserum and the small agranular vesicles of presumed cholinergic type were identified in the same nerve terminals. Therefore, VIP and ACh may be co-stored in certain nerves, as may NPY and noradrenaline. However, the co-storage, if it exists, does not seem to be ubiquitous, since each type of nerve had its own characteristic distribution pattern in addition to being similar to the other nerve type.

7. The previously reported APP immunoreactivity was also demonstrated with immunocytochemistry when antiserum against APP was applied. However, radioimmunoassay and, in particular, chromatography detected no APP in the tissue, indicating that there was no true APP in the human male genital organ. Preabsorption test by immunocytochemistry revealed that the so-called APP antiserum cross-reacted with NPY and thus the 'APP immunoreactivity' reported previously was likely to be caused by NPY.

8. Peptide-immunoreactive nerves and peptide contents in the erectile tissue of impotent patients with a variety of causes were examined. It was found that the number of VIP-IR nerves in particular was decreased to varying extents which were largely in parallel with the severity of the impotence regardless of cause. The decrease of VIP immunoreactivity might be caused by general neuronal lesions in the penis. However, since VIP may be the chief neurotransmitter to elicit and maintain penile erection, the lack of VIP may play a important role in the development of penile impotence.

Chapter 5: THE FEMALE GENITAL TRACT

5.1. INTRODUCTION

The mammalian female genital organs are well supplied by sympathetic and parasympathetic nerves. The former originate from the T_{10}-L_2 segments of the spinal cord and run in the inferior hypogastric nerves. On reaching the base of the broad ligament, the inferior hypogastric plexus gives rise to the uterovaginal plexus, from which some nerves pass down to the vaginal wall, the erectile tissue of the vestibular bulbs and the clitoris, while others pass upward to the cervix, the uterus and the Fallopian tube. Adjacent to the cervicovaginal junction , there are small ganglia, the paracervical ganglia. Some of the ganglion cells are the so-called 'short neurons' with which certain preganglionic sympathetic fibres synapse, whereas the other preganglionic sympathetic fibres may change neurons in higher sites along the sympathetic pathway, but the precise location is not clear at present. The preganglionic parasympathetic nerves arise in segments S_{2-4} of the spinal cord and relay in the paracervical ganglia. The postganglionic parasympathetic fibres are bipartite; the majority run up to innervate the cervix, the uterus and part of the Fallopian tube, while the others pass down to supply the vagina, the vestibular bulbs and the clitoris. The nerve supply to the ovary and the Fallopian tube in the female is rather like that to the testis, the epididymis and the vas deferens in the male. The ovary is supplied by nerves arising from the ovarian plexus which is composed of both

sympathetic and parasympathetic fibres and is formed by branches from the renal and aortic plexuses and the superior and inferior hypogastric plexuses. The Fallopian tube is innervated by the ovarian nerve at the ovarian half and the uterine nerve at the uterine half. The two nerves overlap in the middle portion of the tube (for review see Williams & Warwick 1980d).

Both adrenergic and cholinergic nerve fibres have been demonstrated in the female genital organ with the former being predominant (Owman et al 1974; Sjöberg 1968; Brundin 1965; Thorbert et al 1977). Ganglionic cells of both types were described in the paracervical ganglia (Owman & Sjöberg 1967; Rosengren & Sjöberg 1967; Sjöberg 1968; Bell 1974) with the cholinergic ganglia more abundant (Thorbert et al 1977).

The existence of unidentified neurotransmitters mediating non-adrenergic, non-cholinergic responses in the female genital tract, such as relaxation of oviductal smooth muscle and increase in vaginal blood flow during sexual arousal, has been known for some time (Lindblom et al 1979; Wagner & Levin 1980; Wagner & Ottesen 1980; Helm et al 1981; Ottesen et al 1981a). Nerves containing peptide immunoreactivities have been reported to occur in the female sexual organs and such nerves have been thought to be responsible for the non-adrenergic, non-cholinergic response. A considerable number of studies have been carried out on the distribution and biological actions of VIP in these regions (Larsson et al 1977; Alm et al 1977; Hökfelt et al 1978; Goodnough et al 1979; Alm et al 1980a, 1980b; Lynch et al 1980; Helm et al 1981; Ström et al 1981).

Substance P has been found in the female sex organs of guinea-pig and cat but not in rat (Alm et al 1978). Enkephalin has also been reported to be present in these organs (Alm et al 1981). Very recently NPY-IR nerves were described in the rat female genitalia (Stjernquist 1983). However, peptides other than VIP have not been thoroughly examined. Comparative numerical and anatomical distribution of peptide-containing nerves in the female genital tract of several mammalian species has not been systematically investigated. Therefore, the present study was carried out to investigate the occurrence and distribution of peptide-IR nerves in the female genitalia of several mammalian species.

5.2. EXPERIMENTAL DESIGN

The species examined included women (n=23), cats (n=8), guinea-pigs (n=6), rats (n=20) and mice (n=9).

The possible occurrence of a number of peptides was examined. These were VIP, substance P, NPY, PHI, somatostatin, met-enkephalin, CCK, bombesin and β-endorphin. Only the first four were found to be present in the investigated organs; the others were absent.

The human tissues, including the upper portion of the vagina, the whole uterus, the Fallopian tube and the ovary, were obtained from patients undergoing hysterectomy for varying reasons such as leiomyoma, ovarian endometrioma and prolapse. Only specimens with grossly and histologically normal appearance were included in the

study. Ninteen patients were pre- or peri-menopausal (average 41.9 years old). Three of them were pretreated with hormones, ie norethisterone and/or progesterone. The phases of the menstrual cycle were determined on H&E stained sections by light microscopy as well as by calculation from the first day of the last menstrual cycle. Four patients were post-menopausal (average 71.7 years old). The details of the patients are shown in Appendix 1.4..

For immunocytochemistry, the tissues were fixed in 0.4% BQS (Appendix 2.2.1.) and the modified indirect immunofluorescence method (Appendix 2.4.5.) with routine controls was carried out on cryostat sections of 8 µm thick. The morphology of the specimens was examined by routine histology and no apparent differences were observed between the tissues taken from hormone treated and untreated patients.

Radioimmunoassay of different peptides was also performed (see Section 2.11.5.) on specimens taken from the female genitalia of the various species investigated.

The same regions of the female genital tract from other mammalian species were collected and processed in a way similar to that for human tissues. In addition, genital tissues were also collected from capsaicin-treated rats (n=6) to establish the possible occurrence of peptide immunoreactivities in sensory C-fibres (see Section 2.11.2.). The results obtained from these rats were compared with those of controls (n=20).

The origin of peptide-IR nerves in the female genitalia was studied in rat. The hypogastric nerves and/or pelvic nerves were surgically sectioned as described in Chapter 3. Only operation procedures 1,2,3 & 5 were performed (see Section 3.2.2.). Therefore, the hypogastric and pelvic nerves were cut either separately or together at points proximal to the pelvic and paracervical plexuses. The uterus and the vagina from the denervated and sham-operated rats were collected 8 days after the surgery. The frequency and brightness of different types of peptide-IR nerves in the tissues from pre- and post-operative rats were examined.

5.3. PEPTIDE-IR NERVES IN HUMAN FEMALE GENITALIA

Immunoreactivities of VIP, NPY, PHI and substance P were found in nerves in different regions of the female genitalia in the various species. As in the urinary bladder and the male genitalia, VIP- and NPY-IR nerves were more numerous than the other two. Different nerve types were often distributed in the same regions, but on consecutive sections, each nerve type was distinct from the others, except that PHI-IR nerves closely mimicked VIP-IR nerves in distribution pattern, although the former were much sparser than the latter.

In human uterus, VIP-IR nerves were more concentrated in the cervix where they spread through the lamina propria and the muscle layer, with a close relationship to arteries, arterioles (Fig. 39) and smooth muscle cells. The cervical glands were often loosely

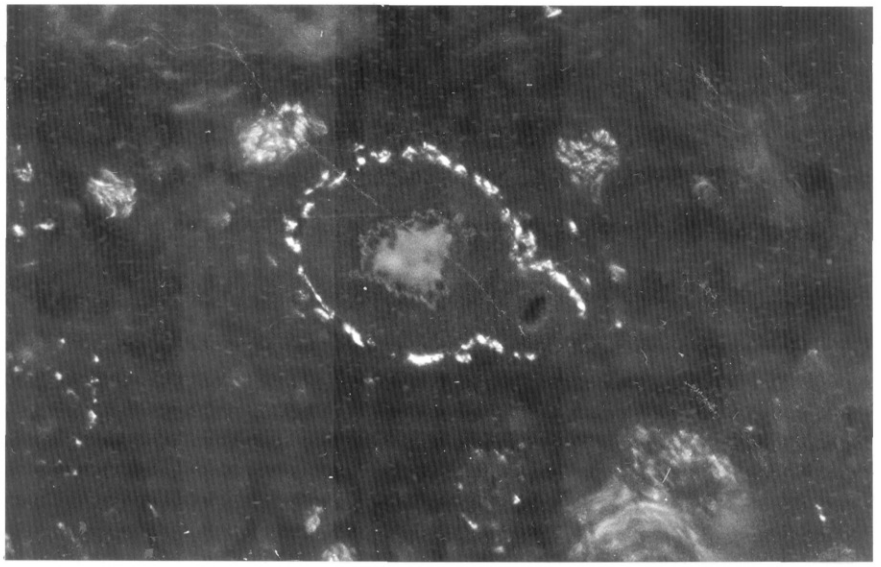


Fig. 39. VIP-IR nerves surrounding an arteriole in the submucosal region of the human cervix. (x200).

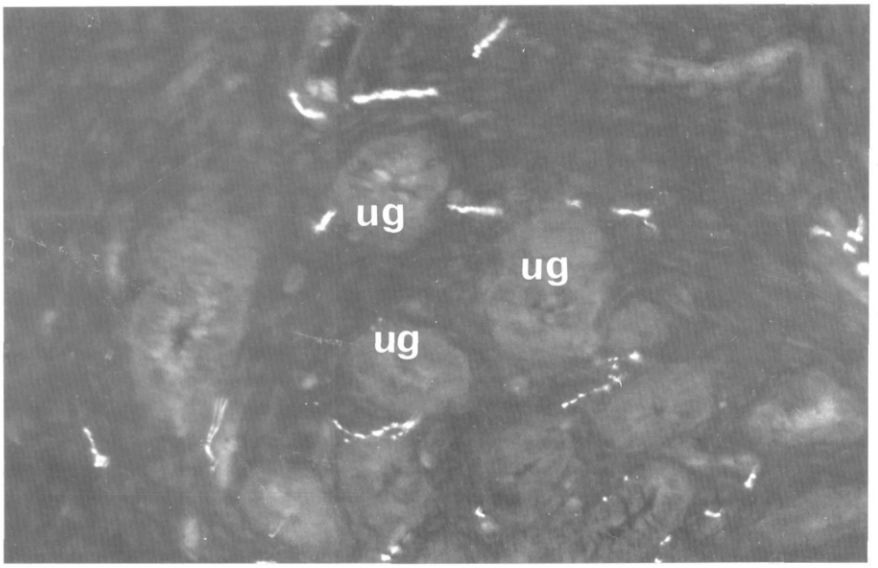


Fig. 40. VIP-IR nerve fibres adjacent to the uterine glands (ug) in the endometrium of the human uterus. (x185).

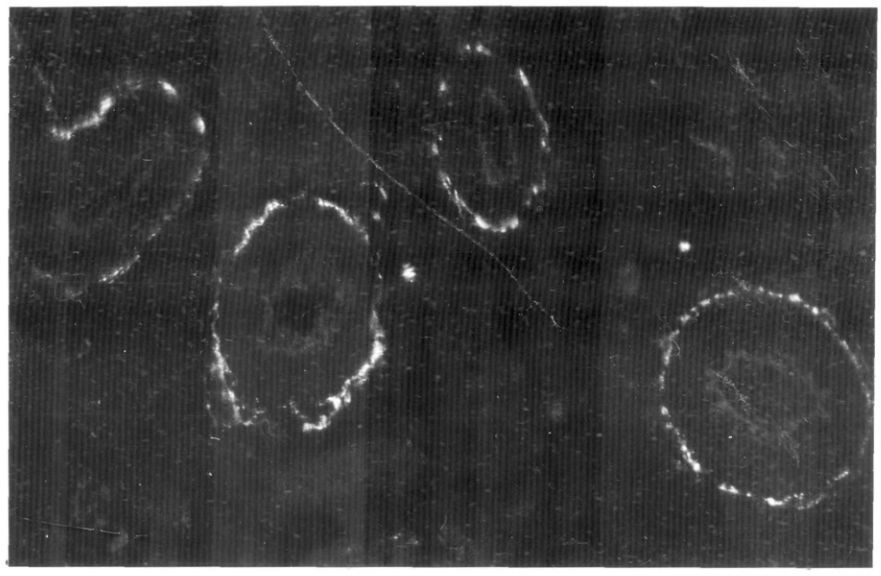


Fig. 41. VIP-IR nerves closely surrounding all the arterioles in the wall of the human vagina. (x185).

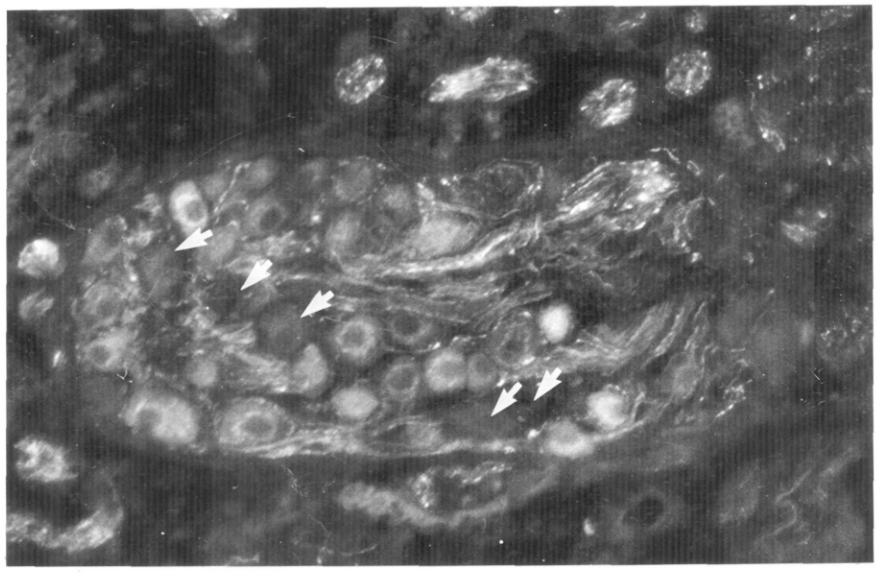


Fig. 42. A large number (about 80%) of ganglion cell bodies in the paracervical ganglion of the human female genitalia show VIP immunoreactivity of variable intensity. A few ganglion cell bodies appear to be non-immunoreactive (arrows). (x185).

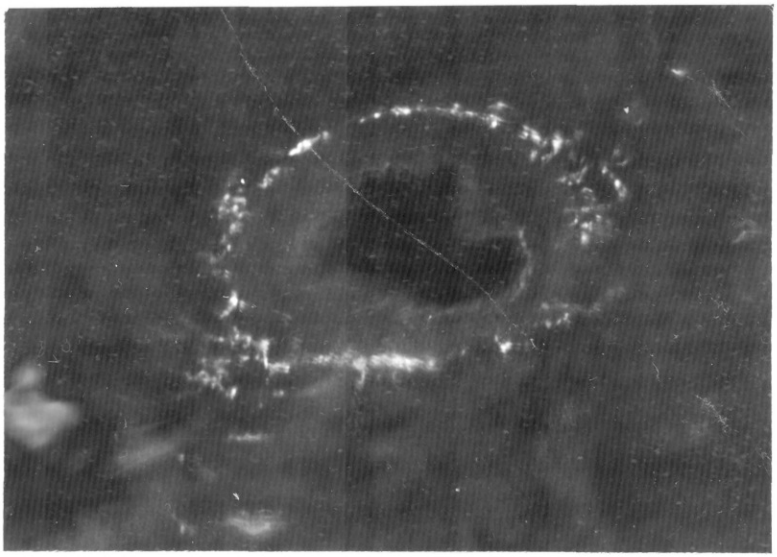


Fig. 43. NPY-IR nerves surrounding an artery in the human endometrium. (x350).

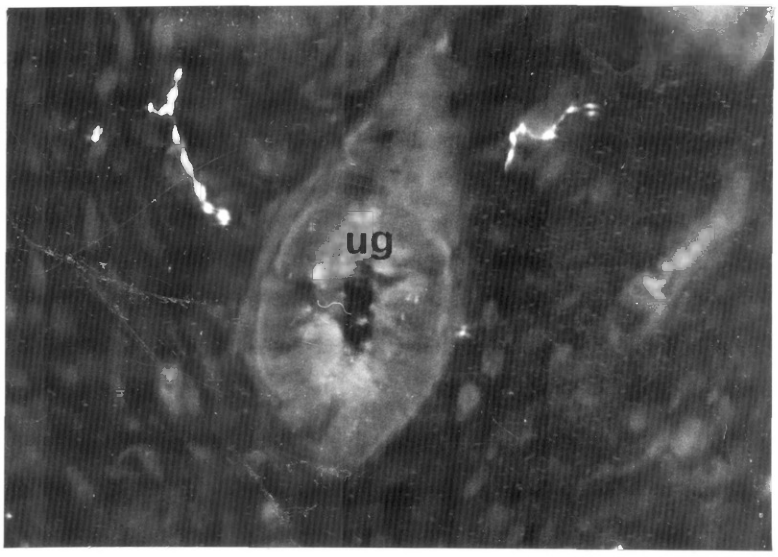
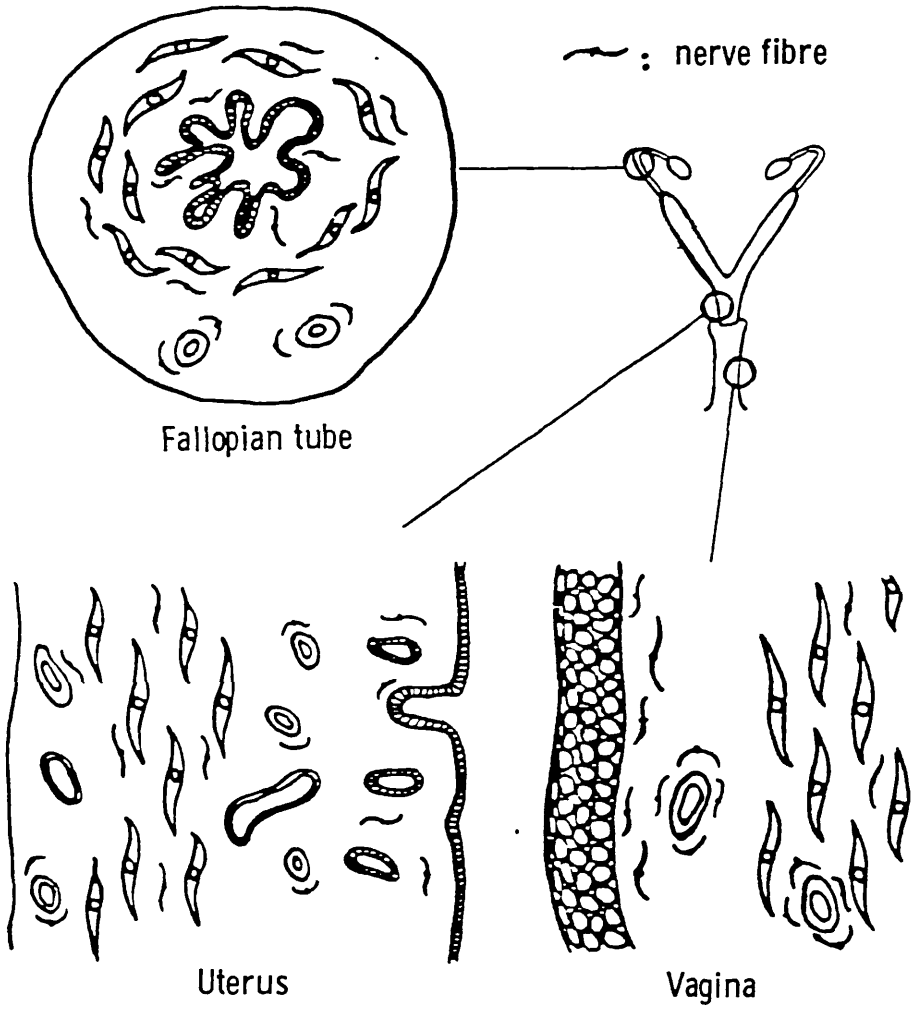


Fig. 44. NPY-IR nerve fibres in the endometrium of the human female genitalia. The nerves run close to a uterine gland (ug), but no direct contact is seen. (x400).

Diagram 29

DISTRIBUTION OF VIP-IMMUNOREACTIVE NERVES

IN MAMMALIAN FEMALE GENITALIA



associated with VIP-IR nerves. However, no direct contact between the nerves and the glandular epithelium was seen. In the rest of the uterus, VIP-IR nerves were scattered in the submucosa, the muscle layer and the adventitia. A few VIP-IR nerves were seen accompanying blood vessels in the endometrium and running close to the uterine gland (Fig. 40). However, the relationship between the nerves and the glands was not as intimate as that between the nerves and the blood vessels; the blood vessels were usually closely surrounded by VIP-IR nerves (Fig. 39). In the myometrium, most VIP-IR nerve fibres ran parallel to the muscle cells. In the circular muscle surrounding the opening of the Fallopian tube at the uterotubal junction, VIP-IR nerves were denser than in the rest of the uterine body. In the Fallopian tube, VIP-IR nerves ran mainly parallel to the circular muscle cells with a density similar to that in the myometrium. In the ovary, only a few VIP-IR nerves were seen, being mainly associated with the blood vessels and without direct contact with the follicles and the corpus luteum. The vagina was richly supplied by VIP-IR nerves, which surrounded the blood vessels running in the muscle layer and the submucosa (Fig. 41). VIP-IR nerves were also seen in the vicinity of the epithelium. At the cervicovaginal junction, clusters of 5-15 ganglion cells were found and more than half of the cells were immunoreactive to VIP antiserum with varying intensity. The VIP-IR ganglion cells are randomly mixed with the non-IR ones (Fig. 42). The distribution pattern of VIP-IR nerves in the human (as well as in other mammalian) female genitalia is shown in Diagram 29.

NPY-IR nerves were distributed in various regions with densities similar to those of VIP-IR ones, except in the cervix where NPY-IR

nerves were less dense than the VIP-IR ones. In the endometrium, more NPY-IR than VIP-IR nerves were found to surround blood vessels (Fig. 43). A few nerves which almost reached the innermost surface of the endometrium also showed NPY immunoreactivity (Fig. 44). The distribution pattern of NPY-IR nerves in the human (as well as in other mammalian) female genitalia is shown in Diagram 30. A few ganglion cells in the paracervical ganglion were found to contain NPY immunoreactivity (Fig. 45). On consecutive sections these neurons were distinct from those containing VIP immunoreactivity. The relationships between VIP-, NPY- and non-peptide-IR neurons in the paracervical ganglion are shown in Diagram 31.

PHI-IR nerves were similarly distributed to VIP-IR nerves but the former had a population of about one third of that of the later. In the paracervical ganglion, PHI immunoreactivity was very weak and could not be established with certainty. Substance P-IR nerves were not detected in the human female genitalia.

In general, the peptide-IR nerves were denser in the pre-menopausal than in the post-menopausal patients. Nevertheless, a considerable number of peptide-IR nerves still remained in the female genitalia of patients up to 83 years of age.

There were no apparent differences in the frequency and distribution patterns of the peptide-IR nerves in different phases of the menstrual cycle, nor were there any differences concerning the distribution and frequency of peptide-IR nerves between the patients pretreated and non-treated with hormones. There were no significant variations in peptide-IR nerves in tissues taken from patients with

Diagram 30

DISTRIBUTION OF NPY-IMMUNOREACTIVE NERVES
IN MAMMALIAN FEMALE GENITALIA

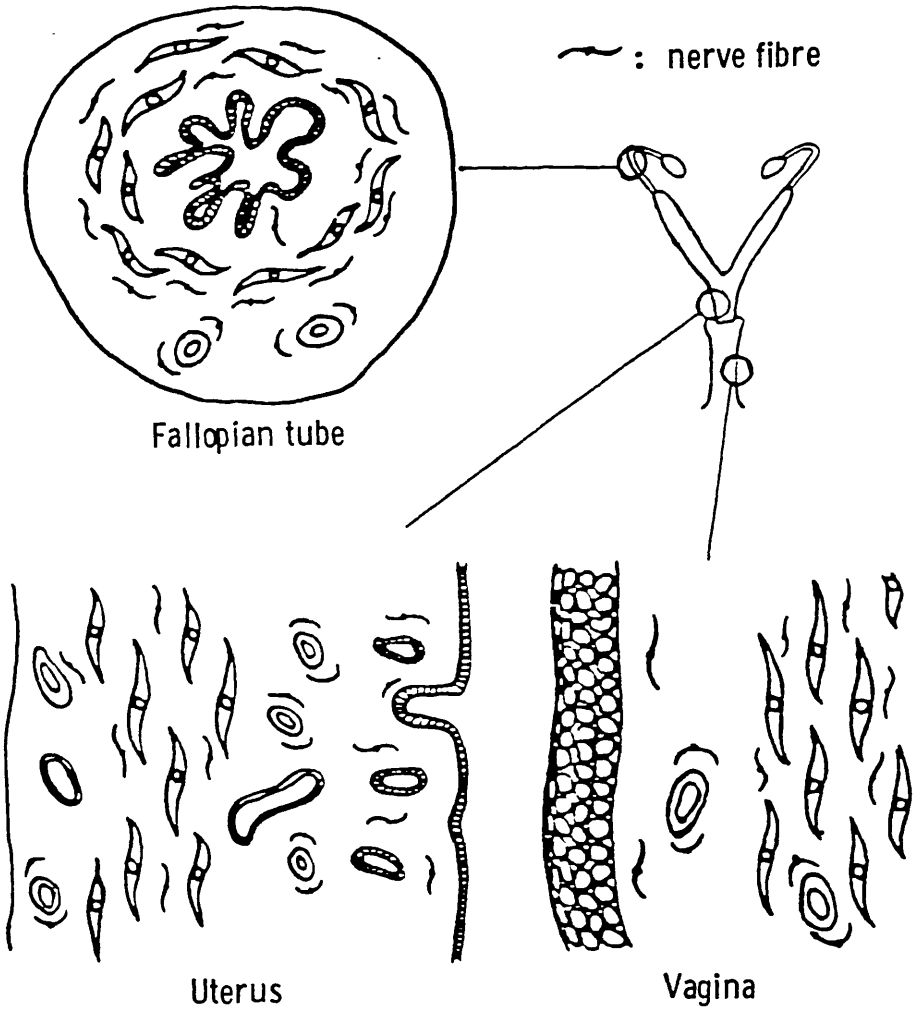
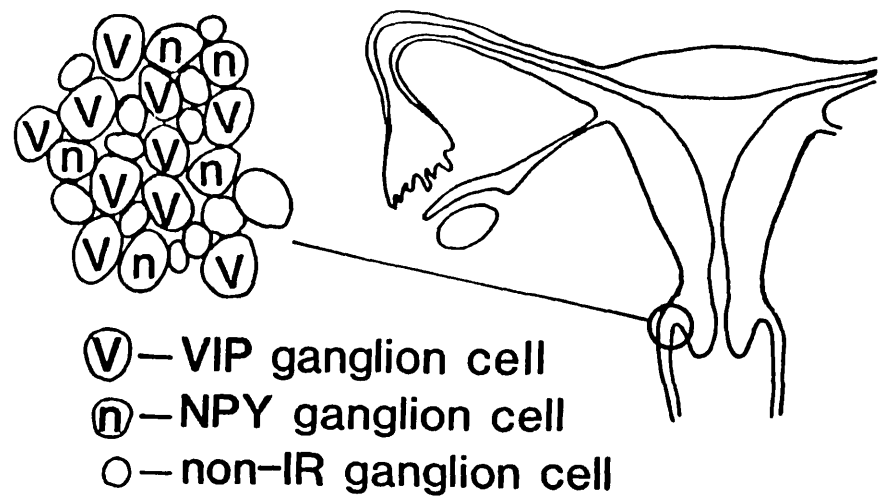


Diagram 31

PEPTIDE NEURONS IN FEMALE GENITALIA



different tumours or diseases.

Radioimmunoassay also detected the three peptide immunoreactivities with concentrations in various regions largely comparable to the findings of immunocytochemistry. The radioimmunoassay results are shown in Diagrams 32, 33 and 34.

5.4. PEPTIDE-IR NERVES IN THE FEMALE GENITALIA OF OTHER MAMMALS

Four types of peptide-IR nerves were found in the female genital organs of cat, rat, mouse and guinea-pig. These were VIP-, NPY-, PHI- and substance P-IR nerves. The distribution patterns of the former three were essentially similar to those in the human tissue, as shown in Diagrams 29 & 30, but species variations existed.

The VIP-IR nerves were most abundant in the female genital organ of cat (Fig. 46) as compared to the other species. Apart from the paracervical ganglion cells, there were scattered ganglion cell bodies in vaginal wall of the cat, but not in other species. The frequency of VIP-IR nerve fibres in the female genital organs of mouse and rat was similar to that in woman. Such nerves in the guinea-pig genital organs were fewer than in the other species, but they were still the most prodominant nerve type among the peptide-IR nerves.

The densities of NPY- and PHI-IR nerves in different species followed the same order as that for VIP-IR ones, i.e., cat > mouse > human > rat > guinea-pig.

Diagram 32

VIP in human female genitalia
pmol/g SEM

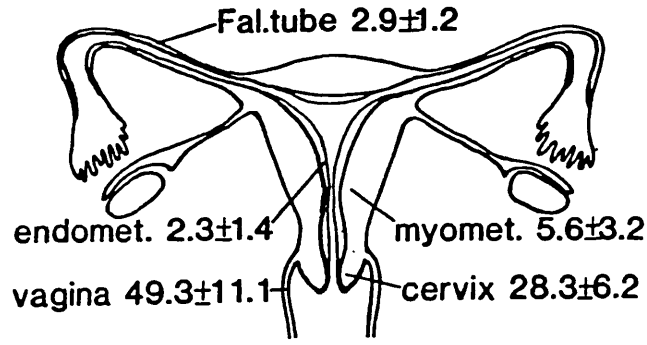


Diagram 33

PHI in human female genitalia
pmol/g±SEM

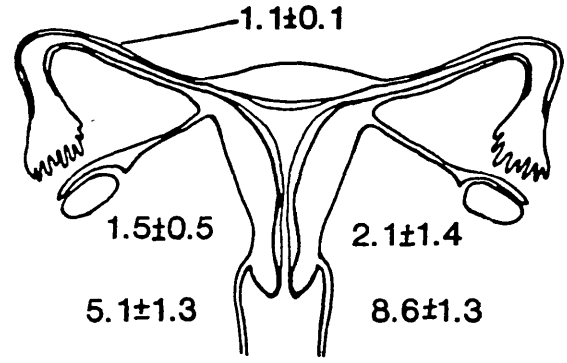
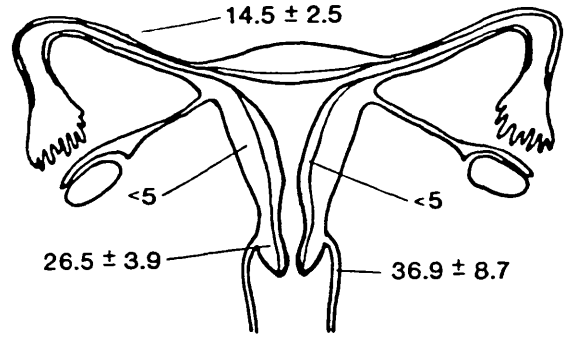


Diagram 34

NPY in human female genitalia
pmol/g±SEM



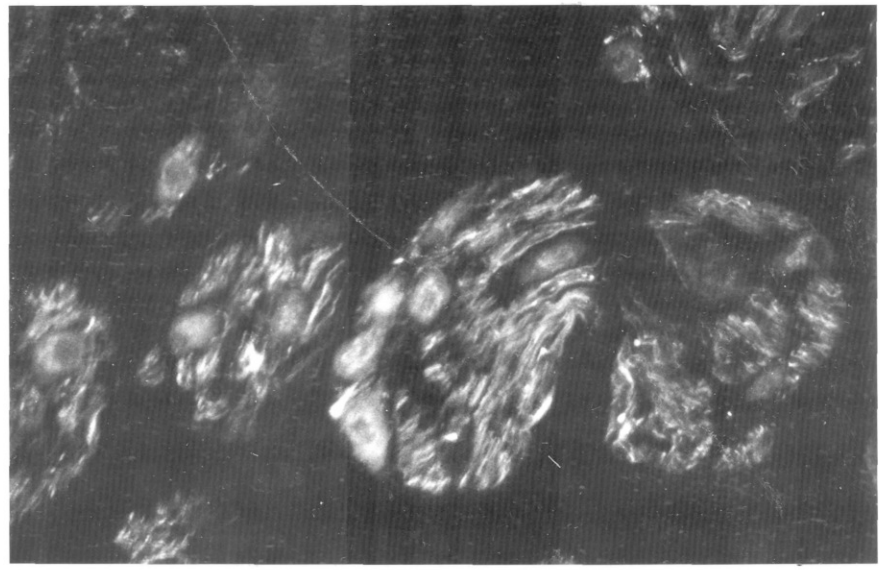


Fig. 45. A few ganglion cell bodies (about 10-15%) and associated nerve fibres contain NPY immunoreactivity in the human paracervical ganglion. (x250).

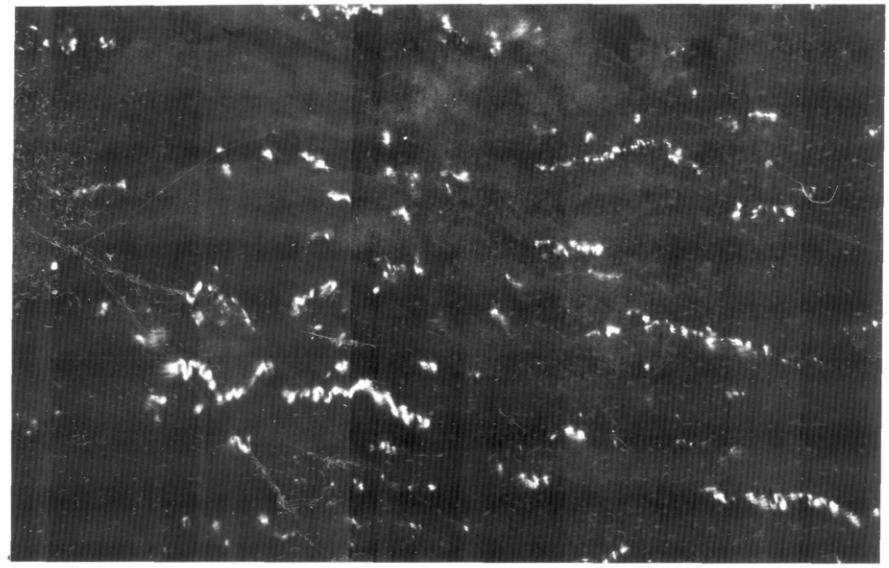


Fig. 46. Abundant VIP-IR nerve fibres running along the smooth muscle in the cervix of the feline female genitalia (x350).

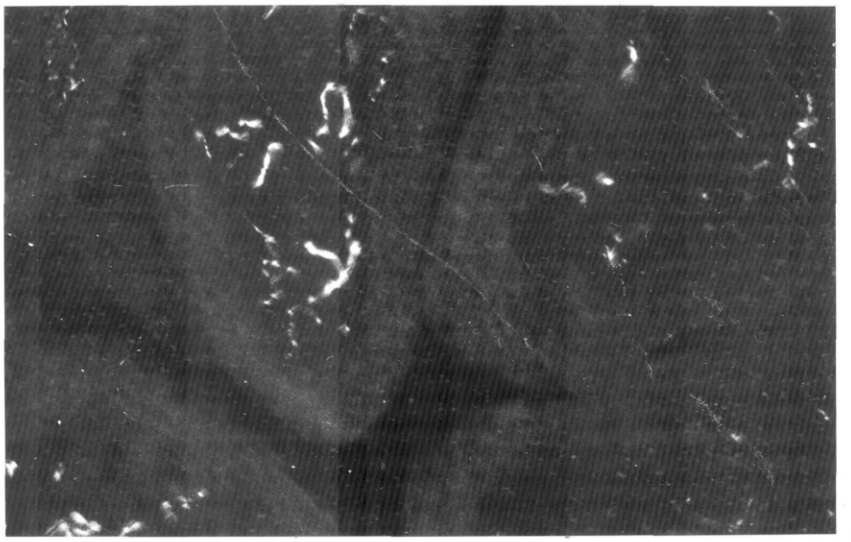


Fig. 47a. Substance P-IR nerves in the vagina of guinea-pig. Such nerves are distributed mainly beneath the squamous cell epithelium. (x125).

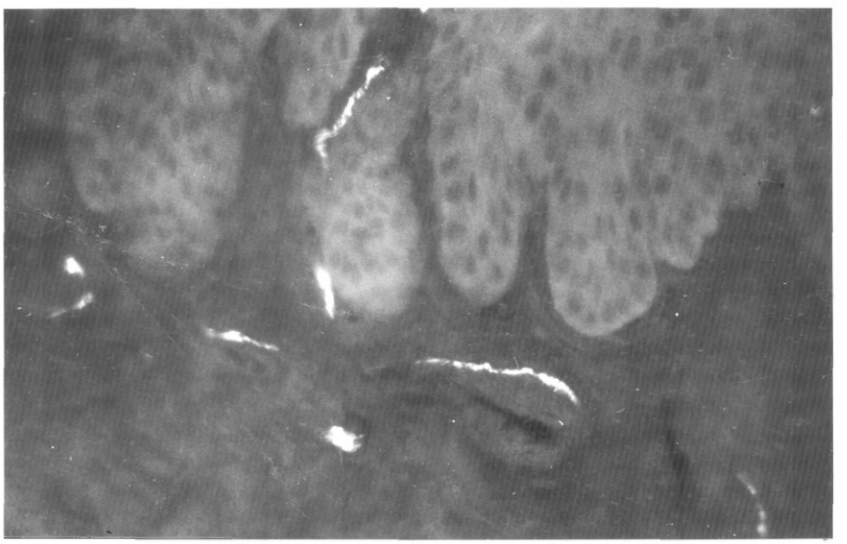


Fig. 47b. Substance P-IR nerves in the vagina of guinea-pig. Some nerve fibres appear to penetrate the basement membrane and enter the epithelium. (x350).

Diagram 35

Concentrations of peptide immunoreactivities in female genitalia of mouse

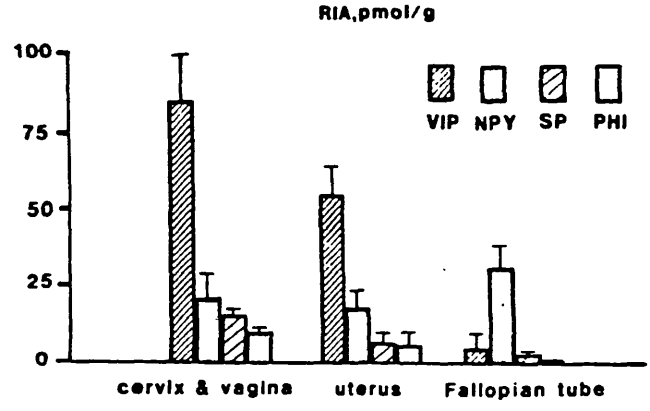
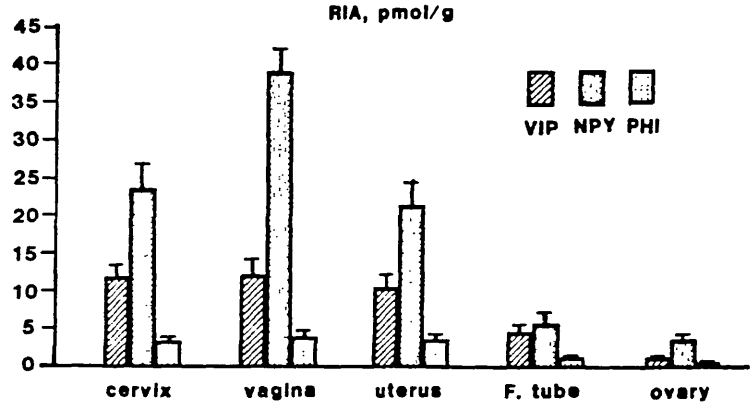


Diagram 36

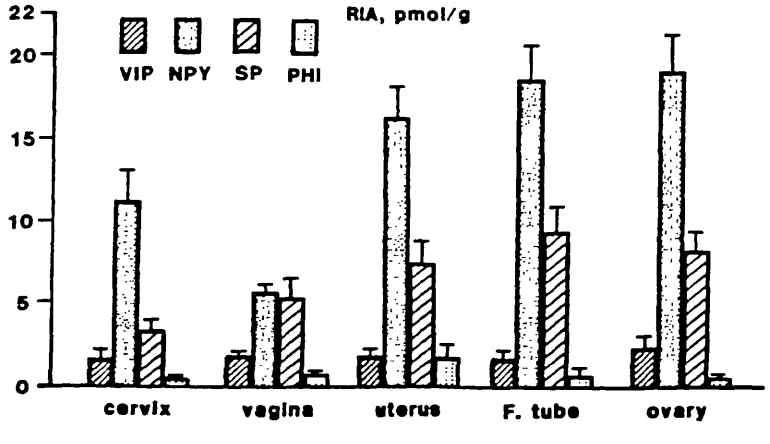
Concentrations of peptide immunoreactivities in female genitalia of rat



Substance P was not detected by RIA.

Diagram 37

Concentrations of peptide immunoreactivities in female genitalia of guinea-pig



In contrast, substance P-IR nerves possessed the highest frequency in the guinea-pig female genitalia. They were less so in cat and mouse and were sparse in rat. In addition to being associated with most blood vessels and smooth muscle, such nerves were particularly abundant in the submucosa beneath the vaginal epithelium (Fig. 47a, b). A few substance P-IR nerve fibres penetrated the basement membrane and entered the vaginal epithelium. Noticeably, such pattern was nearly absent in the cervical epithelium.

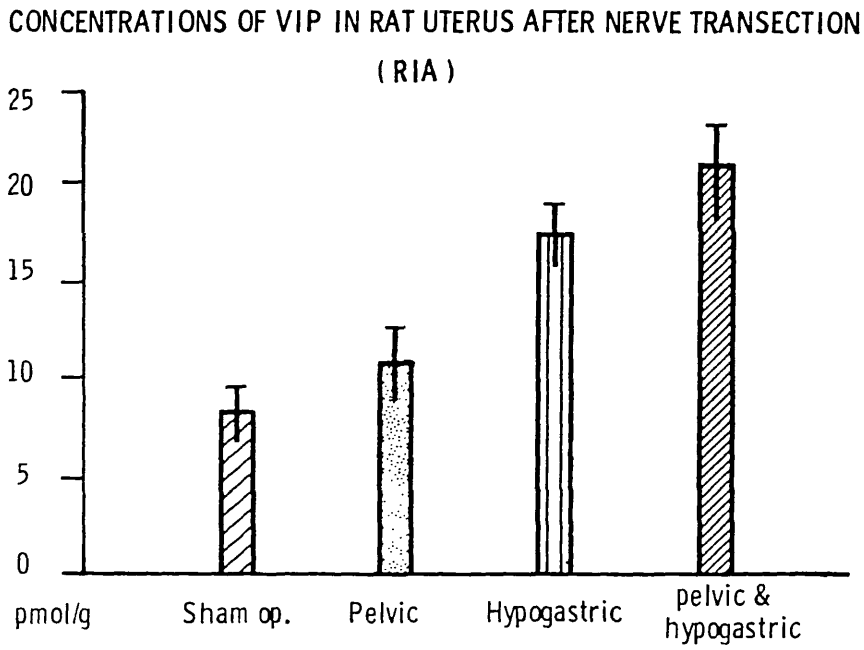
In the female genitalia of capsaicin-treated rats, substance P-IR nerves disappeared from all the regions, while the other peptide-IR nerve types were not affected morphologically.

The concentrations of the four types of peptide immunoreactivity in the female genitalia of mouse, rat and guinea-pig detected by radioimmunoassay are shown in Diagrams 35, 36 and 37.

5.5. ORIGIN OF PEPTIDE-IR NERVES IN THE FEMALE GENITALIA OF RAT

It was found that after transections of nerve supply, the number of VIP- and PHI-IR nerves in the uterus and vagina was increased, particularly when both hypogastric and pelvic nerves were cut. NPY-IR nerves were largely unchanged. Substance P-IR nerves were very scarce in the undenervated uterus and vagina, with hardly any left when either of the nerve supplies was severed and complete absence when both nerve pathways were cut. Similar results were obtained by radioimmunoassay. The changes in the concentrations of VIP immunoreactivity are shown in Diagram 38.

Diagram 38


CONCENTRATIONS OF VIP IN RAT UTERUS AFTER NERVE TRANSECTION
(RIA)
25
20
15
10
5
0
pmol/g
Sham op.
Pelvic
Hypogastric
pelvic &
hypogastric

From the results, it would appear that VIP-, PHI- and NPY-IR nerve fibres were not sectioned by the surgery and thus may originate mainly from ganglia located distal to the cutting point, i.e. close to or within the female genital organ. It is most likely that their ganglion cell bodies are located either in the pelvic or in the paracervical ganglia. The mechanisms of the post-operative changes may be essentially the same as in the post-operative changes in the bladder (see Section 3.2.4.). Therefore, the increase in VIP- and PHI-IR nerves may be a result of compensatory hyperplasia to the lost nerve fibres due to the surgery, or a result of an accummulation of VIP and PHI in the nerve terminals due to the loss of preganglionic excitatory impulse. It is possible that a similar increase in NPY-IR nerves from intact NPY-IR ganglia adjacent to the organ also occurred, but it was obscured by the loss of certain NPY-IR nerve fibres which originated from a position higher than the cutting point and were thus sectioned by the surgery. The local origin for VIP- and NPY-IR nerves was also demonstrated by the finding that a large number of VIP- and NPY-IR ganglion cells existed in the paracervical ganglion, which gave off nerve branches to supply the uterus and the vagina. Whatever the explanation for the post-operative increases might be, that the majority of the VIP-, NPY- and PHI-IR nerves had their ganglion cell bodies in the pelvic and/or the paracervical ganglia was clearly indicated by the present findings.

Because of the sparsity of substance P-IR nerves in the rat uterus (in another group of normal rats radioimmunoassay failed to detect any substance P immunoreactivity in the uterus, see Diagram 36), the

post-operative changes in substance P-IR nerves were difficult to assess. However, substance P-IR nerves appeared to be decreased when either or both pathways were cut. This may indicate that these nerves have their origin higher than the cutting points. Together with the possible sensory nature of these nerves, which was indicated by the results obtained from the capsaicin-treated rats, substance P-IR nerves may originate from the dorsal root ganglia.

5.6. DISCUSSION

In the present study, four types of peptide immunoreactivities, i.e., those of VIP, substance P, NPY and PHI, have been localised in nerves of the female genital organs of five mammalian species. Their distribution patterns were studied.

VIP-IR nerves appeared to be the most abundant nerve type of all the species studied. The distribution pattern is in general agreement with those reported previously in human being, cat and rat (Alm et al 1977, 1980a, 1980b; Goodnough et al 1979; Helm et al 1981; Hökfelt et al 1978; Larsson et al 1977; Lynch et al 1980; Ottesen et al 1981a; Ström et al 1981). The novel finding for VIP-IR nerves in this study was that five species were examined in a comparative manner so that species variations in the frequency and distribution of this peptide were better illustrated.

Although a number of investigations have been carried out, the precise role of VIP in the female genital organs is not yet known

with certainty. On the studies of actions of VIP on smooth muscle, both in vivo and in vitro, a dose-dependent inhibitory effect on the electrical and mechanical activities of smooth muscle from the uterine body, the cervix, the vagina and the Fallopian tube was demonstrated (Ottesen et al 1979, 1981a, 1981b; Ottesen 1981; Bolton et al 1981; Helm et al 1981). Bolton et al (1981) further suggested that the primary action of VIP is on the economy of the myometrial smooth muscle cell, possibly to accelerate sequestration and/or extrusion of calcium from the cell; this may be associated with inhibition of the generator potential, hyperpolarization and a slight increase in permeability of the smooth muscle membrane to potassium. In addition, the effect of local intra-arterial infusion of VIP in the myometrium of rabbit and goat was to increase local blood flow (Carter et al 1981; Ottesen & Fahrenkrug 1981). During sexual arousal VIP concentrations were found to be increased in the female genital circulation (Ottesen et al 1982b). The muscular and vascular effects of VIP on the female genital organs of rabbit and guinea-pig were not affected by various blocking agents (Ottesen & Fahrenkrug 1981; Bolton et al 1981), indicating that VIP acts directly via specific receptors. Indeed, specific, high affinity binding sites for ^{125}I-labelled VIP has recently been identified in a subcellular fraction of the myometrium containing plasma membranes of smooth muscle by Ottesen et al (1982c). Furthermore, the receptor binding of VIP and the smooth muscle relaxation response have been found to correspond closely. Fahrenkrug & Ottessen (1982) also found that electrical stimulation of the pelvic or hypogastric nerves induced an upsurge of VIP level in the local venous effluent. This was not altered by adrenergic and cholinergic

receptor antagonists, but was completely abolished by the ganglion blocking agent, hexamethonium. The above findings, together with the distribution pattern described in the present study, point to a role for VIP as a possible neurotransmitter which may be involved in the neural control of the female sex organs.

The newly discovered neuropeptide NPY was almost as abundant as VIP in all the regions of the female genital organs. It has been shown recently that NPY inhibited neurally evoked contractions of cervical smooth muscle from estrogen-treated spayed rat in a dose-dependent manner (Stjernquist et al 1983). Elsewhere, NPY has been shown to cause vasoconstriction and inhibit electrically induced smooth muscle contraction of the vas deferens (Lundberg et al 1982b; Allen et al 1982). A close relationship with blood vessels, smooth muscle and, to a lesser extent, exocrine glands may suggest an influence of NPY on these structures. For example, the relatively rich supply of NPY-IR nerves in the muscular coat of the Fallopian tube together with its smooth muscle effects suggests that NPY may play a role in the neural control of oviductal peristalsis, thereby controlling transportation of the ovum. Since NPY-IR nerves often innervated tissue structures which were also associated with VIP-IR nerves, it is possible that the local neural control is balanced by the often opposing actions of these two peptides.

The close resemblance of PHI- and VIP-IR nerves supports the concept that these two peptides are derived from the same precursor (Christofides et al 1982). In human VIPomas (Bloom et al 1983) and neuroblastomas (Itoh et al 1983) it has been shown that the

prepro-VIP molecule contains the amino acid sequence of PHI. With similar distribution patterns and biological actions to VIP, PHI may have effects on the female genitalia similar to those of VIP. However, there have also been reports that the two peptides may have separate neuronal locations and distinct local effects (Lundberg & Tatemoto 1982; Hökfelt et al 1983; Bishop et al 1984). The significance of PHI in these tissues as well as its relationship with VIP remains to be elucidated.

Substance P-IR nerves in the female genitalia, like those in the male genitalia, were in general very sparse. Those detected were mainly distributed in the sensory regions of the vagina, suggesting a sensory role for these nerves. This was confirmed by the results obtained from capsaicin-treated rats, in which all the substance P-IR nerves disappeared from the female genital tissue.

The finding of four types of peptide immunoreactivities in nerves in the female genital tracts from five mammalian species suggests that regulatory peptides may play important roles in the neural control of the female sexual organs.

5.7. SUMMARY

1. The immunoreactivities of four peptides, i.e. VIP, NPY, substance and PHI were localised to nerves in the female genitalia from five mammalian species including human. VIP- and NPY-IR nerves were much more frequent than the other two. They were particularly dense in the vagina and the cervix, less so in the Fallopian tube and sparse in the uterine body and the ovary. The peptide-IR nerves were related to both vascular and non-vascular smooth muscles, to exocrine glands and to vaginal epithelium. Species variations on the frequency of the four types of peptide-IR nerves exist.

2. The distribution pattern of VIP-IR nerves and the known actions of VIP suggest that this peptide may participate in the neural control of a number of physiological functions, such as genital smooth muscle relaxation and local blood flow regulation.

3. NPY-IR nerves had a frequency similar to, and a distribution pattern distinct from those of the VIP-IR nerves. The two nerve types often mixed in different regions of the organ. It is possible that NPY balances the neural effects of VIP with its often antagonistic action.

4. Substance P-IR nerves were more abundant in the female genitalia of guinea-pig than in other species. These nerves were likely to be sensory in nature. This was supported by the observation that substance P-IR nerves, although scarce, were mainly distributed beneath the epithelium of the vagina, and disappeared following capsaicin treatment.

5. Separate ganglion cell bodies in the paracervical ganglia at the vaginocervical junction were found to contain VIP or NPY immunoreactivity, suggesting that most of the VIP- and NPY-IR nerves in the female sex organ may originate from them. This was particularly true for VIP-IR nerves, since pelvic and/or hypogastric decentralisation produced significant increases in the number of VIP-IR nerves in the uterus and the vagina. The increase of NPY immunoreactivity was less apparent. This may indicate that a certain population of the NPY-IR nerves may have their origins closer to the spinal cord.

Chapter 6: THE HEART

6.1. INTRODUCTION

The mammalian heart is well supplied by autonomic sympathetic and parasympathetic nerves which harmonize the rate, force and output of the cardiac rhythmical activities. A large volume of literature has been devoted to the study of cardiac innervation.

The preganglionic sympathetic neuronal cell bodies are located in the intermediolateral columns of T_{1-6} spinal cord (Henry & Calaresu 1972). Their fibres ascend in the paravertebral chains and funnel through the stellate ganglia (Levy & Zieske 1966). After passing through the dorsal or ventral limbs of the ansa subclavia, they join the vagus nerves to form the cardiac plexus (Szentivanyi et al 1967; Tollack et al 1971). The synapse between the preganglionic and the postganglionic sympathetic nerves may take place in the cervical and the third and fourth thoracic sympathetic ganglia or anywhere near or within the cardiac wall (Mitchell 1953; Brown 1967; Smith 1970). The location of the secondary sympathetic ganglion cells varies from species to species and the precise site of such neurones in man is still uncertain.

The vagal preganglionic fibres proceed from their medullary nuclei in the brainstem, coursing down the neck in the carotid sheath on each side. The preganglionic axons leave the vagus as its cardiac branches and reach the cardiac plexus without interruption. The

secondary parasympathetic ganglion cells usually occur in the cardiac plexus formed by efferent vagal and sympathetic fibres in the thorax. The plexus runs to the heart along the large blood vessels.

Both adrenergic and cholinergic nerves have been demonstrated in the mammalian heart with the methods of histochemistry. The nodal and the conductive tissues in the heart have been found to be more densely innervated by both nerve types, with cholinergic nerves predominant (For review see Forssmann et al 1982). The adrenergic nerves have been found to be more concentrated in the right atrium and ventricle than in the left. The presumed cholinergic nerves have been described as being frequent in the atria and around the conductive tissues, and rather sparse in the ventricles (James & Spence 1966; Bojsen-Moller & Tranum-Jensen 1971; Kent et al 1974).

While the heterogeneity of the granules contained in nerve terminals in the heart had been noticed (Yamauchi 1973), the existence of peptides in the cardiac innervation was not known until recently. Nerves in the human heart were found to contain substance P and neurotensin immunoreactivities (Reinecke et al 1980, 1981a, 1981b, 1982; Weihe et al 1981; Forssmann et al 1981) and those in the hearts of other mammals were found, in addition, to contain VIP and enkephalin (Weihe & Reinecke 1981; Lang et al 1983). APP-like immunoreactivity demonstrated by immunocytochemistry was also reported to be present in the cardiac innervation (Lundberg et al 1980). However, the nature of APP-like immunoreactivity in the heart was never confirmed by chromatography and such

immunoreactivity in other organs, such as the male genitalia, has been shown to result from antiserum cross-reaction (see Section 3.1.3.). The most abundant peptide detected in the heart, prior to this study, was enkephalin with a concentration of around only 9 pmol/g in the guinea-pig heart (Lang et al 1983). This is much lower than the level of peptides found in many other organs, for example, the gastrointestinal tract (for review see Polak & Bloom 1983), the respiratory tract (Wharton et al 1978, 1979; Sheppard et al 1983) and the genitourinary tracts (see 3.1.3., 3.1.4., 4.1.3., 5.3. & 5.4.). Thus, one of the most dynamic organs in the body, the heart, had been known as an organ where very few peptide-containing nerves exist, until the present study.

6.2. EXPERIMENTAL DESIGN

Hearts of man, rat, mouse and guinea-pig were investigated. The human heart tissues were obtained at cardiac biopsies (n=8) and postmortem (n=6). The sino-atrial (S-A) node and atrio-ventricular (A-V) nodes, the left and right atria, the left and right ventricles, the atrial and ventricular septa, the circular coronary artery and the root of the aorta were dissected and fixed in BQS (Appendix 2.2.1.). The whole hearts of rat (n=8), mouse (n=8) and guinea-pig (n=6) were removed and fixed intact. Five µm thick cryostat sections were cut through different regions of the human heart and in a coronary plane from the base of the hearts of other mammals. A number of antisera were applied to the sections using the modified immunofluorescence method. These included the antisera

to NPY, VIP, substance P, PHI, met-enkephalin, somatostatin, neurotensin, bombesin and NSE.

To measure the concentrations of NPY, substance P, VIP and neurotensin, radioimmunoassay was carried out on the cardiac tissues of man and rat. Fractionation of NPY-like immunoreactivity from the human and mouse hearts was carried out on an HPLC reverse phase column (Waters Bondapak C-18: 3.9x300 mm), by means of a linear gradient elution system (35%-45% solvent A 0.12% trifluoracetic acid/water; solvent B 0.1% trifluoracetic acid/acetonitrile) and a flow rate of 2ml/min. One ml fractions were collected for subsequent radioimmunoassay. Recoveries were all between 88% and 94%.

6.3. NPY-IR NERVES IN HUMAN HEART

Immunoreactivities of NPY and substance P were localised in nerves of the human heart. However, in comparison with NPY-IR nerves, the latter were so sparse that they could only occasionally be found as single nerve fibres or spots, mainly in association with the blood vessels and the nodal tissues. In contrast, NPY-IR nerves with numerous varicosities were seen in abundance throughout the cardiac tissues. These nerves were more dense in the regions of the S-A and A-V nodes (Fig. 48) than in the surrounding tissues. Immunostained nerves were also found to surround the coronary blood vessels, particularly the medium to small arterial branches (Fig. 49). In the atrium, the ventricles, the papillary muscles and the

entricular and atrial septa, NPY-IR nerves were closely associated with most cardiac muscle cells (Fig.50). The myocardium was thus more densely innervated by these nerves than the endocardium and the epicardium. Such nerve fibres also occurred in the valve, diminishing in number towards the free edge of the cusps. Large nerve bundles were found to be unreactive to the NPY antiserum.

When the antiserum to NSE was applied, numerous nerve fibres were immunostained. The NSE immunoreactivity showed that the cardiac muscle in the atrium was more densely innervated than that in the ventricles (Fig. 51), and the most heavily innervated regions were those around the nodes. Most blood vessels, particularly the arteries and arterioles, were surrounded by such nerves. In general, the overall density of NSE-IR nerves was about two to three times that of NPY-IR ones. No ganglion cells immunoreactive to NSE or NPY were seen in any regions of the human heart examined.

NPY immunoreactivity in the human heart was also detected by radioimmunoassay, which showed that the tissue around the A-V node had the highest concentration of 21.8±3 pmol/g, mean±SEM, and the tissue around the circular artery 18.7±6 pmol/g. The other regions contained NPY immunoreactivity at concentration ranges of 10-15 pmol/g. The results of radioimmunoassay are shown in Diagram 39.

Chromatographic analysis using HPLC revealed a peptide in a position similar or identical to that of pure porcine NPY (Diagram 40). NPY immunoreactivity from mouse brain also eluted in an identical position. No APP was identified.

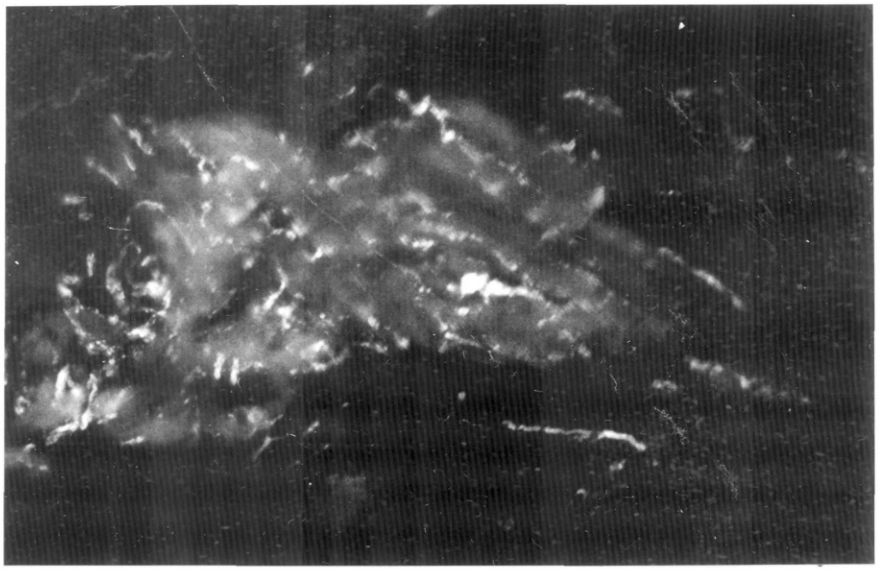


Fig. 48. The region of the S-A node densely innervated by NPY-IR nerves. The nodal tissues are taken from an autopsy of an 60 year old man 8 hours after death. (x225).

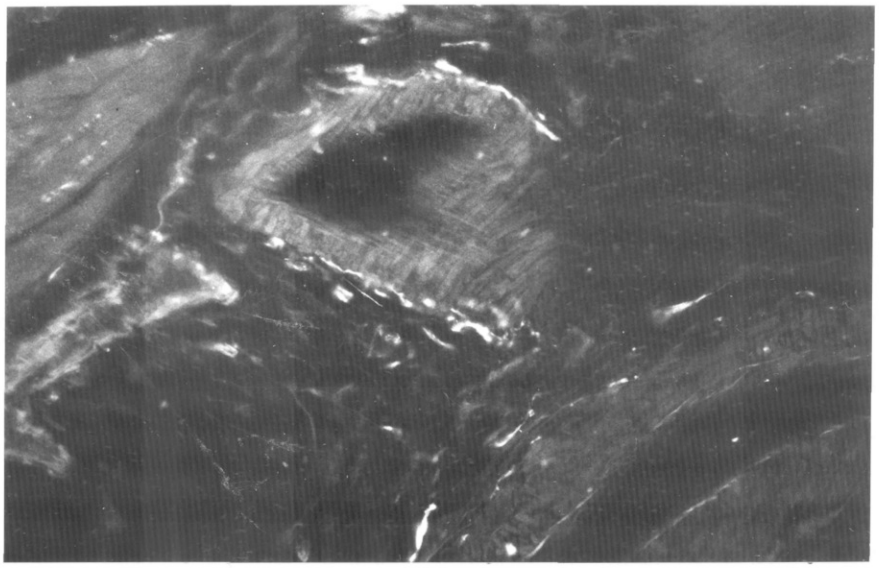


Fig. 49. A branch of the coronary artery in the human heart. Most such vessels are surrounded by NPY-IR nerves. (x300).

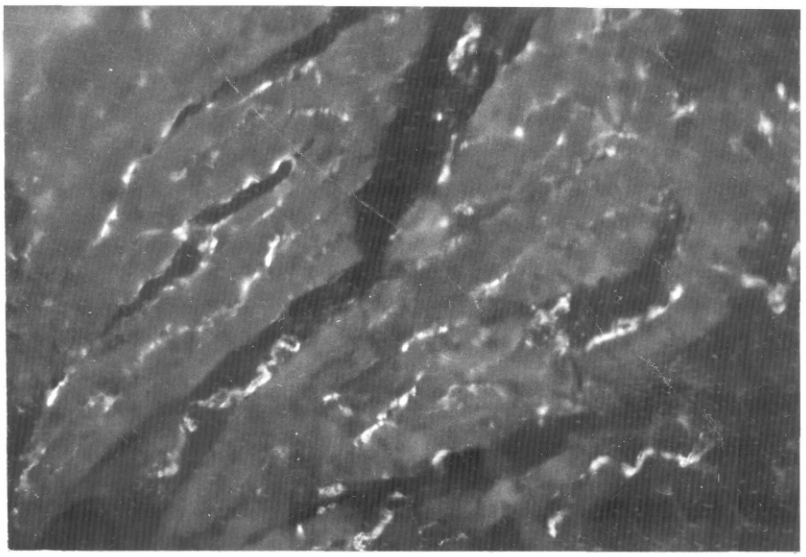


Fig. 50. NPY-IR nerve fibres running along the cardiac muscle in the left ventricle of man. (x350)



Fig. 51. The innervation of the S-A node region in man demonstrated with NSE immunostaining. (x300).

Diagram 39

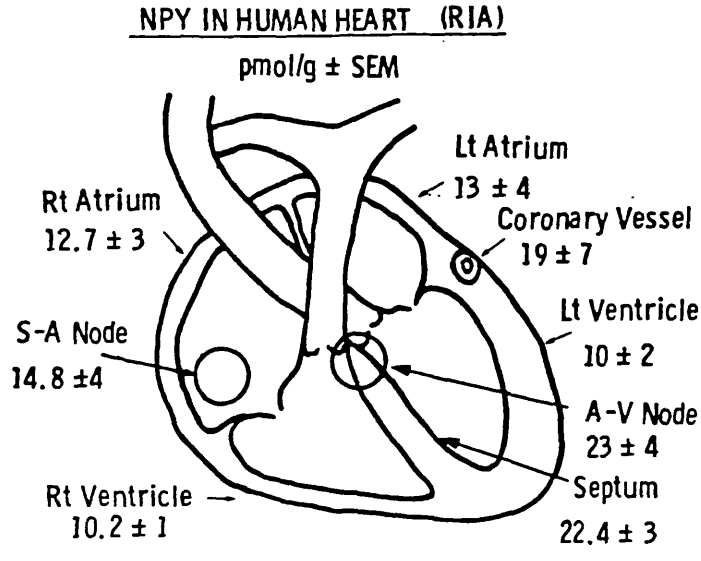
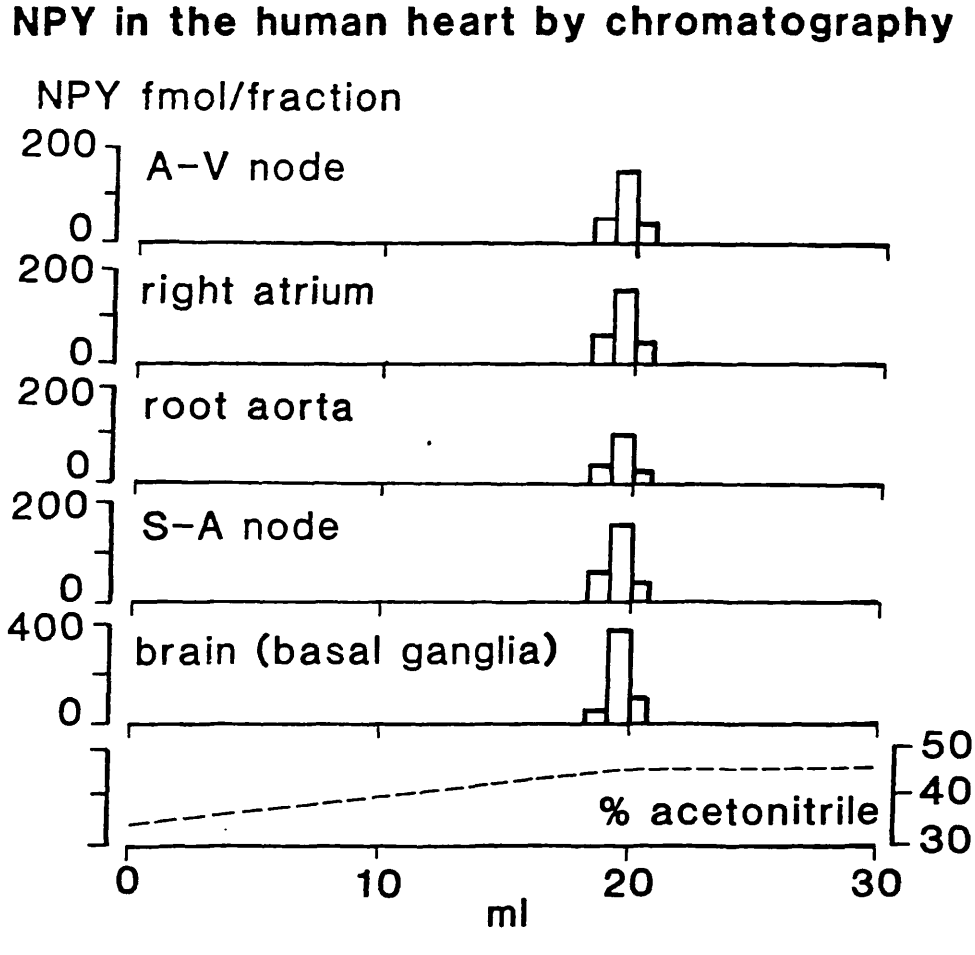


Diagram 40



VIP and substance P immunoreactivities in different cardiac regions were also detected by radioimmunoassay at very low concentrations of 0.01-0.7 pmol/g and 0.01-0.3 pmol/g respectively. Neurotensin immunoreactivity was undetectable.

6.4. NPY-IR NERVES IN THE HEARTS OF RAT, MOUSE AND GUINEA-PIG

The abundance of NPY-IR nerves, demonstrated with immunocytochemistry, in the hearts of rat and mouse was higher than that in the human heart. In comparison with NSE-IR nerves, NPY-IR nerves in the rat and mouse hearts seemed to represent more than half of the total population of cardiac nerves. Nevertheless, the distribution patterns of NPY-IR nerves in the rodent hearts (Figs. 52 & 53) were still the same as that in man. Intrinsic ganglia were immunoreactive to NSE antiserum (Fig. 54). At least two such ganglia of relatively large size, each consisting of 10-30 ganglion cells, could be seen in a heart and each ganglion was adjacent to a node. A few small ganglia and single ganglion cells were sometimes encountered mainly in the right atrium and around the atrio-ventricular junction. In general, about 50-70% of the ganglion cells and adjacent nerve fibres in each ganglion were strongly or moderately immunoreactive to NPY antiserum and the rest were very weak or negative (Figs. 55 & 56). The immunoreactivity occured in the cytoplasm and sometimes showed a granular appearance.

Radioimmunoassay detected very high concentrations of NPY

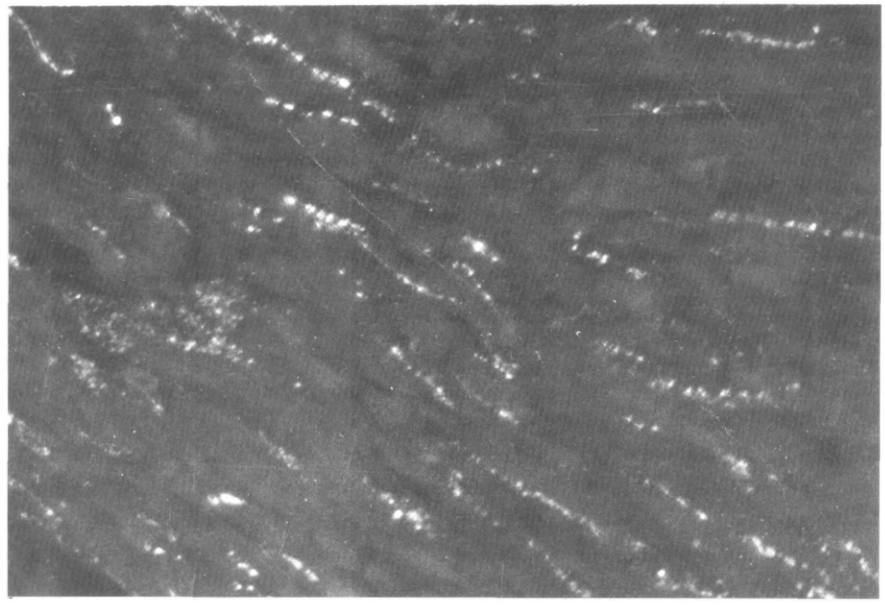


Fig. 52. NPY-IR nerve fibres running along the cardiac muscle in the ventricular septum of the mouse heart. (x350).

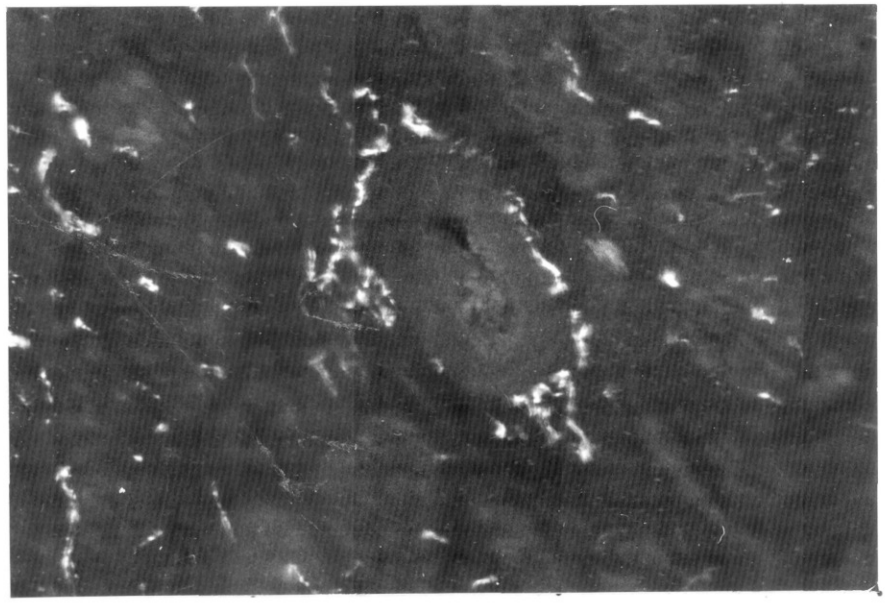


Fig. 53. NPY-IR nerves surrounding a coronary arteriole branch and distributed in the myocardium of rat. (x285).

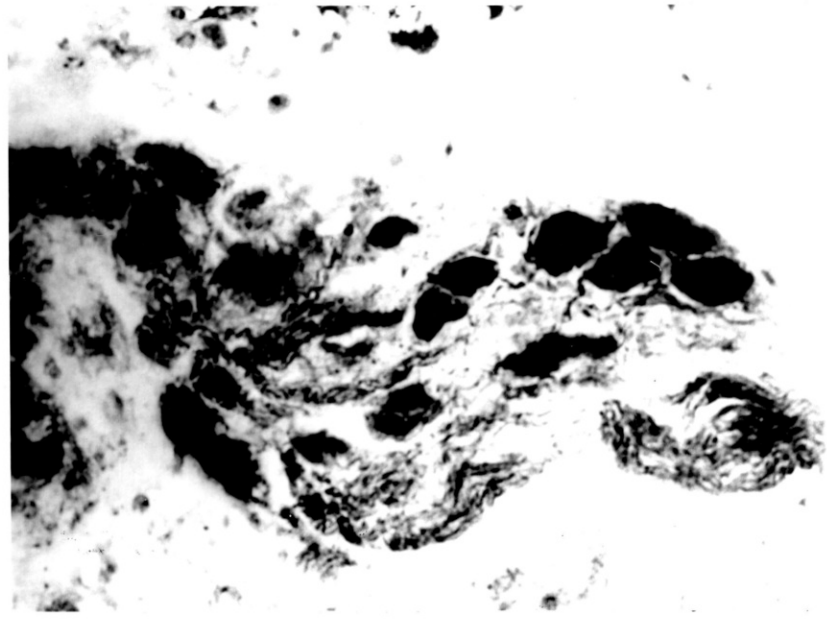


Fig. 54. The intrinsic ganglion and associated nerve bundles demonstrated by NSE immunostaining in mouse heart. (PAP method, x300).

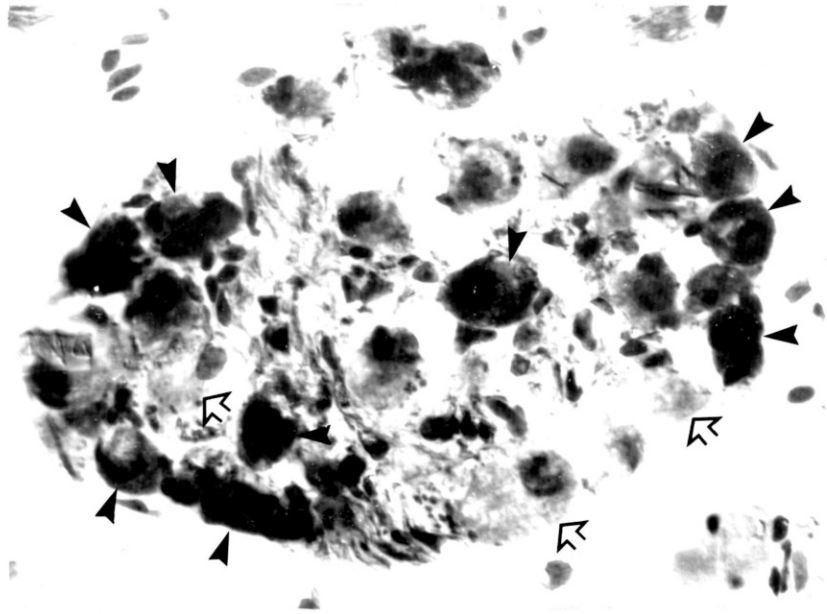


Fig. 55. Most (about 50-80%) of the intrinsic ganglion cell bodies in the mouse heart contain NPY immunoreactivity (black arrows). A few ganglion cells appear to be negative (open arrows). (PAP method, counterstained with haematoxylin, x400).

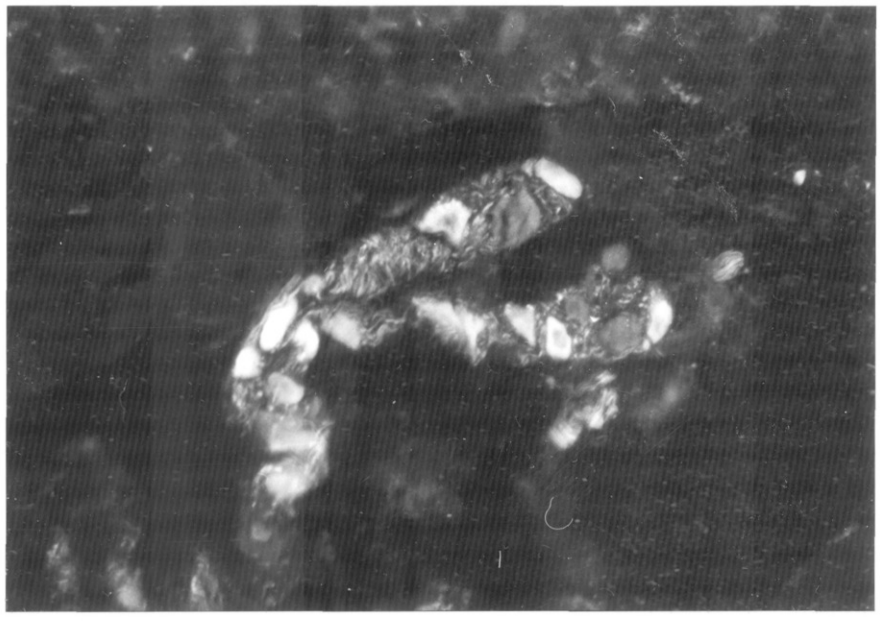


Fig. 56. NPY-IR intrinsic ganglion cell bodies and associated nerve fibres in the mouse right atrium. Note the three ganglion cells appear to be non-immunoreactive to NPY antiserum. (x185).

Diagram 41

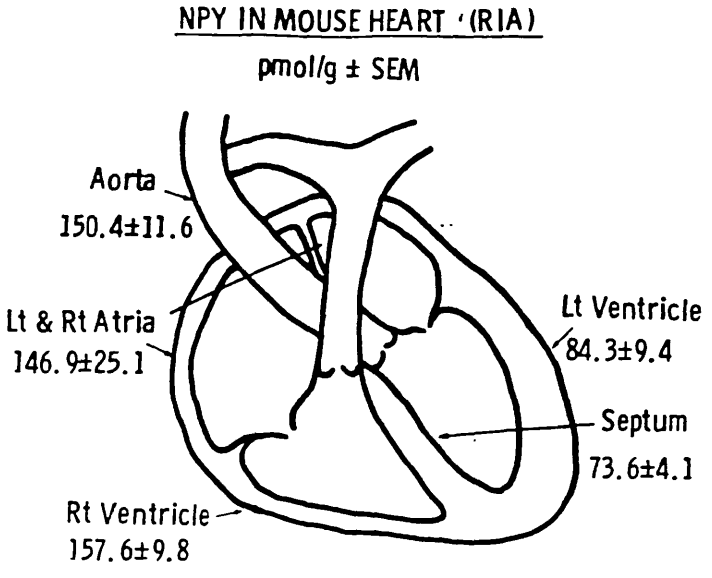
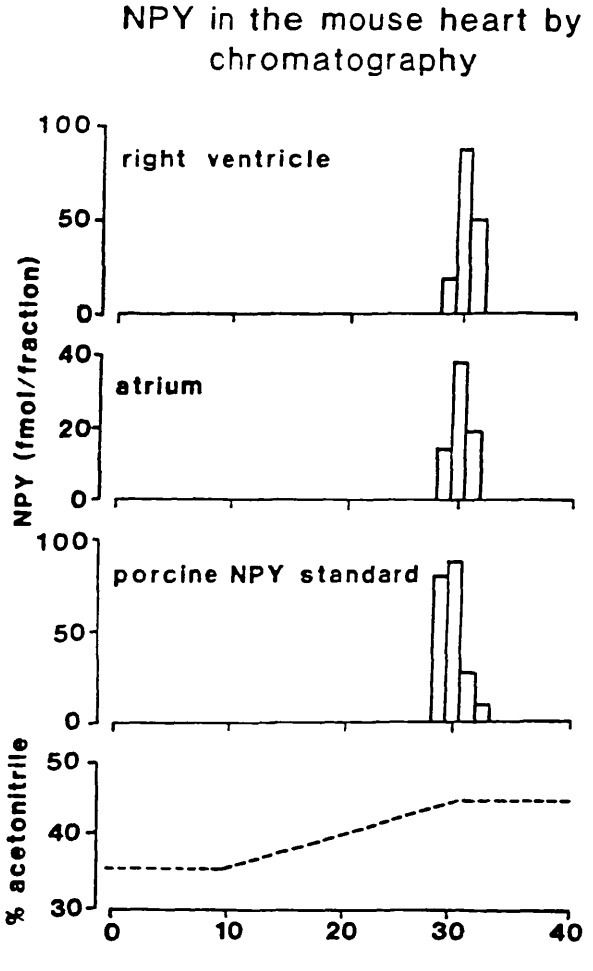


Diagram 42



immunoreactivity in the mouse heart, being 150 pmol/g in the ventricles (Diagram 41).

The true nature of the NPY immunoreactivity was confirmed by chromatography which showed that it eluted in an identical position to that of pure porcine NPY (Diagram 42). Again, no APP was detected.

The heart of guinea-pig had much fewer NPY-IR nerves than the heart of the other two species. Nevertheless, the basic distribution patterns of the few NPY-IR nerves remained the same.

The substance P-IR nerves were also demonstrated in the guinea-pig heart. These nerves were more abundant than the NPY-IR nerves in this species and the distribution pattern was in agreement with that reported by Reinecke et al (1980) and that by Wharton et al (1981).

6.5. DISCUSSION

Considerable quantities of the novel peptide, NPY, have been found in the heart of man, rat, mouse and guinea-pig. It was, as in other organs, localised exclusively in neuronal elements. The identity of this peptide was confirmed by HPLC as being true NPY. The concentrations of NPY immunoreactivity detected by radioimmunoassay in the heart by far surpassed those of other peptides. The comparison of NPY- and NSE-IR nerves indicates that the former may represent the major component in the cardiac innervation. Although

APP-like immunoreactivity has been reported to be present in the rat and cat hearts (Loren et al 1979; Lundberg et al 1980b), in this study no peptide was identified in the position of APP by HPLC. The cross-absorption test suggested that the APP-like immunoreactivity might be a result of antiserum cross-reaction.

In spite of the extensive studies devoted to the neural control of the heart, the functional mechanism of the cardiac innervation has not been fully explained. For example, stimulation of the vagus nerves after the administration of atropine evokes a cardiac acceleration (Kabat 1940) and this has been suggested as a sympathetic mechanism mediated by anomalous sympathetic fibres running within the vagus nerves (Antonaccio 1974). However, in the study of Alter et al (1973), a fast and a slow component of the response were identified; while the fast component disappeared after beta-receptor blockade with practolol, the slow component persisted. Another example is that neurally mediated coronary spasm may not always involve the adrenergic system (Maseri & Chierchia 1982). Both phenomena indicate that neural mechanisms other than cholinergic and adrenergic ones may be involved.

The finding of a massive amount of NPY-IR nerves in the heart provides a new candidate for the novel neural controlling factor. It was recently found that NPY caused a striking reduction in coronary flow in the isolated perfused rabbit heart, which was more potent on a molar basis than ergometrine or noradrenaline (Allen et al 1983b). In addition, it had a negative inotropic effect but no chronotropic influence. The vasoconstrictor activity of NPY was

also shown in the cat submandibular gland where NPY perfusion induced a dose-dependent vasoconstriction (Lundberg et al 1982a, 1982b). A role of neuromodulator for NPY is more likely since only the electrically induced, but not noradrenaline-induced, contraction of the rodent vas deferens was inhibited by this peptide (Lundberg et al 1982b; Allen et al 1982). It is known that stimulation of sympathetic or parasympathetic nerves, previously thought to be solely adrenergic and cholinergic in nature respectively, can produce effects on heart rate, atrioventricular conduction and myocardial contractility, and can also interact with each other in cardiac responses (Lee & Shideman 1959; Levy & Martin 1979). The possible involvement of NPY in these actions should now be considered.

The finding of a large number of cardiac nerves containing the newly discovered bioactive peptide, NPY, provides a new neurological basis for the understanding of cardiac physiology.

6.6. SUMMARY

1. In addition to a small number of VIP- and substance P-IR nerves, a massive number of nerves containing the newly discovered peptide, NPY, was demonstrated in the hearts of man, rat and mouse (being about 150 pmol/g in the mouse heart by radioimmunoassay). These nerves were associated with the arteries and arterioles, the cardiac muscle cells and, in particular, the nodal tissues. The majority of the intrinsic ganglion cells in the mouse and rat hearts were found to be immunoreactive to NPY antiserum. In comparison with NSE-IR nerves, it appeared that NPY-IR nerves in the mouse and rat hearts represent more than half of the total population of nerves. The identity of NPY in the human and mouse heart was confirmed by chromatography.

2. The distribution patterns of NPY in the cardiac innervation suggest that this peptide may be involved in the control of coronary blood flow, the heart beat rate and the cardiac contractility.

Chapter 7: THE SKIN

7.1. PEPTIDE-IR NERVES IN NORMAL SKIN

7.1.1. INTRODUCTION

The linkage between the skin and the regulatory peptides was first provided by Erspamer & Melchiorri (1973) who found the presence of several pharmacologically active peptides in the amphibian skin. It was subsequently found that each of these amphibian skin peptides has its counterpart in mammals, but of these only substance P has been reported to be present in the innervation of the mammalian skin (Hökfelt et al 1975a; Cuello et al 1978; Hartschuh et al 1983).

The skin is known to be richly supplied by numerous sensory nerve endings which form the encapsulated, unencapsulated and non-corpuscular cutaneous receptors conducting a variety of sensory messages to the central nervous system. Autonomic nerves, sympathetic in particular, also innervate the skin, monitoring local blood flow, exocrine secretion and arrector muscle contraction. Nerves containing peptide immunoreactivities have been reported to occur in mammalian skin. These included substance P-, VIP- and neurotensin-IR nerves (Hökfelt et al 1975a; Cuello et al 1978; Hartschuh et al 1983). However, prior to the present study, detailed information concerning the distribution pattern of peptide-IR nerves in various mammalian species had not been forthcoming. This study was therefore undertaken to examine the

occurrence and distribution of a number of peptide immunoreactivities in the skin of four mammalian species including man.

7.1.2. EXPERIMENTAL DESIGN

Skin samples of adult pig (n=4, body weight 30-35 kg), cat (n=6), rat (n=6) and man (n=8) were investigated. The pigs, cats and rats were killed by an overdose of sodium pentabarbitone and skin specimens were taken from nose, lip, front and rear foot, rump and anus. Small pieces of human skin of abdomen, back, thigh, arm and neck were obtained at various operations for tumour or other skin diseases and only grossly and histologically normal regions were included in the study. The tissues were fixed in BQS (Appendix 2.2.1.) and immunostaining with the modified indirect immunofluorescence method (Appendix 2.4.5.) was carried out on 8 µm thick cryostat sections. The antisera used included those against VIP, substance P, NPY, PHI, somatostatin and bombesin.

The concentrations of VIP, substance P, somatostatin and bombesin immunoreactivities in different regions of the pig and cat skin were also measured by radioimmunoassay. The chemical nature of VIP, substance P and somatostatin immunoreactive materials in the skin was analysed with HPLC.

7.1.3. PEPTIDE-IR NERVES IN THE SKIN

Substance P, VIP, PHI and NPY immunoreactivities were found by immunocytochemistry in nerves in the skin of all the four species studied. Various populations of peptide-IR nerves were present in all the regions examined but they were more readily detectable in the footpad and nose of the cat, and the snout and lip of the pig. The general distribution patterns of the peptide-IR nerves in the four species were similar to each other but the skin of pig and cat contained more peptide-IR nerves than the skin of rat or man. In man, the peptide-IR nerves were generally scarce. This may be due to the fact that the sensory regions of the skin, eg. the nose, the lip and the fingertip, were not available for investigation and the tissues often consisted of a thin layer of epidermis with few glands and blood vessels included.

Substance P-IR nerves were quite abundant and were mainly concentrated in the superficial layer of the skin. This was particularly apparent in the snout and the lip of the pig where they were located just beneath the epidermis, running both parallel and perpendicular to it into the folds of the dermal ridges (Fig. 57). In the skin of cat, rat and man, substance P-IR nerves were not mainly confined to the subepidermal region as seen in the pig, but also extended deeper into the dermis and surrounded the sweat glands (Fig.58) and, to a lesser extent, the blood vessels. Such a pattern was more predominant in the footpad of the cat than in other areas.

VIP-IR nerves were fairly numerous and, unlike substance P, were

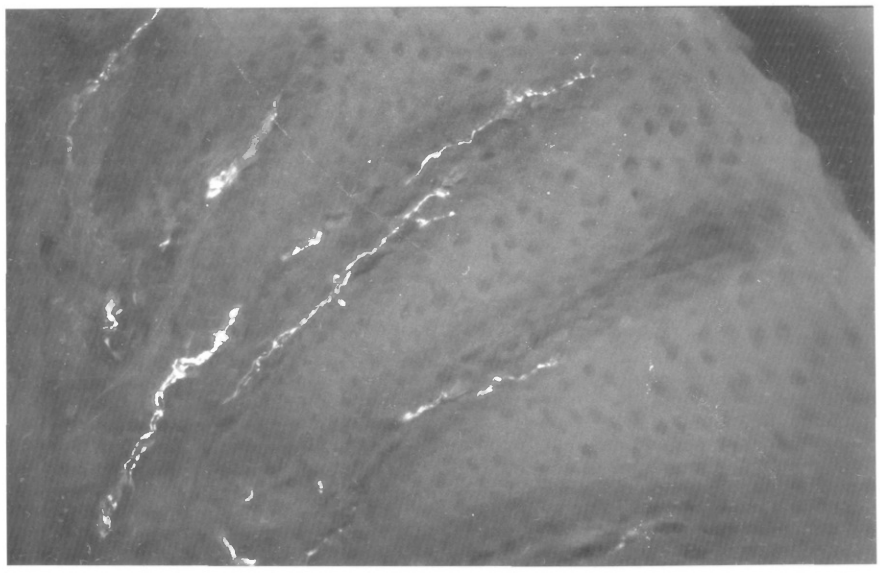


Fig. 57. Substance P-IR nerve fibres in porcine facial skin. Some nerve fibres appear to penetrate the basement membrane and enter the epithelium. (x200).

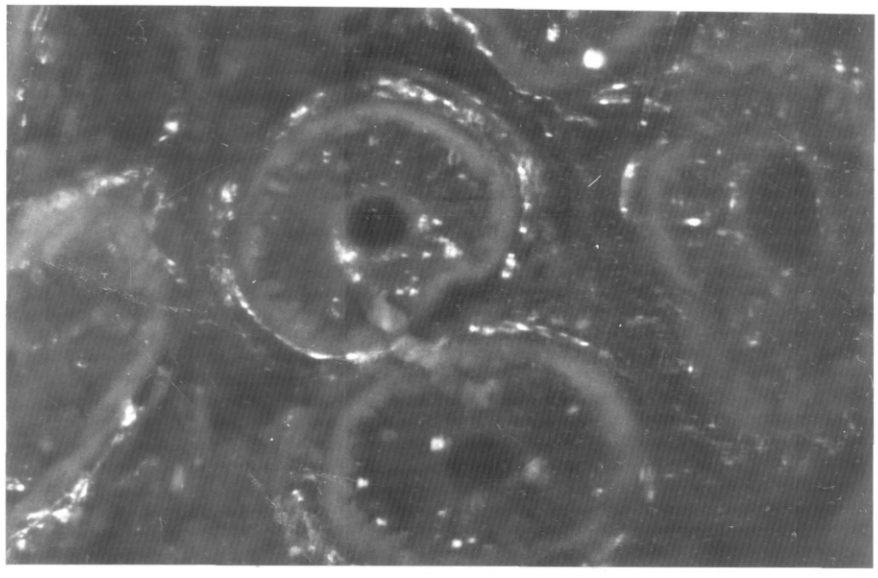


Fig. 58. In the feline skin, substance P-IR nerves are not only distributed beneath the epithelium but are also related to the sweat glands. (x420).

mainly concentrated in the deeper region of the skin. These nerves ran mostly around sweat glands (Fig. 59), blood vessels and, occasionally, sebaceous glands. The blood vessels surrounded by VIP-IR nerves were mainly arteries, arterioles and the arterial portion of the arteriovenous anastomoses. This pattern was densest in the snout and lip of pig and the nose and footpad of cat. Other areas contained roughly equal number of VIP- and substance P-IR nerves. Nerves around blood vessels always showed more immunoreactivity to VIP than to other peptides. Often, the arrector muscle was also seen to be accompanied by a few parallelly distributed VIP-IR nerve fibres. The distribution patterns of substance P- and VIP-IR nerves in the porcine and feline skin are illustrated in Diagrams 43, 44, 45 & 46.

PHI-IR nerves, as in other organs, mimicked the distribution of VIP-IR ones. It was estimated that the number of PHI-IR nerves was less than half that of the VIP-IR ones.

A small number of NPY-IR nerves were demonstrated around blood vessels mainly in the deeper region of the skin of all the species studied (Fig. 60). These nerves also occurred occasionally around the glands. In general, the population of NPY-IR nerve fibres was smaller than that containing substance P or VIP immunoreactivity.

The four peptides were also detected in the pig and cat skin by radioimmunoassay with concentrations largely comparable to the numbers of peptide-IR nerves found in each region by immunocytochemistry. In addition, radioimmunoassay detected

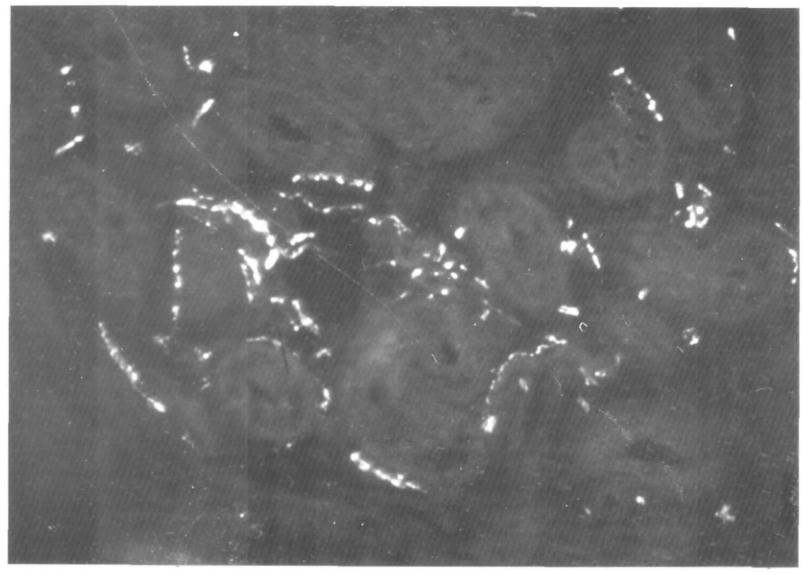


Fig. 59. VIP-IR nerves closely related to sweat glands in porcine skin. This is the main characteristic in the distribution of such nerves in the skin of pig. (x200).

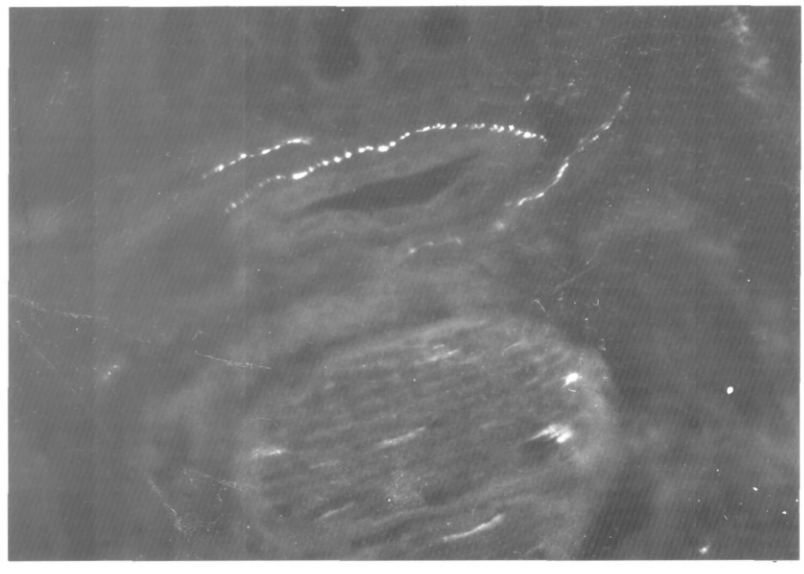


Fig. 60. NPY-IR nerves associated with a blood vessel in the skin from a human forearm. (x315).

Diagram 43

DISTRIBUTION OF SUBSTANCE P-IMMUNOREACTIVE NERVES IN PORCINE SKIN

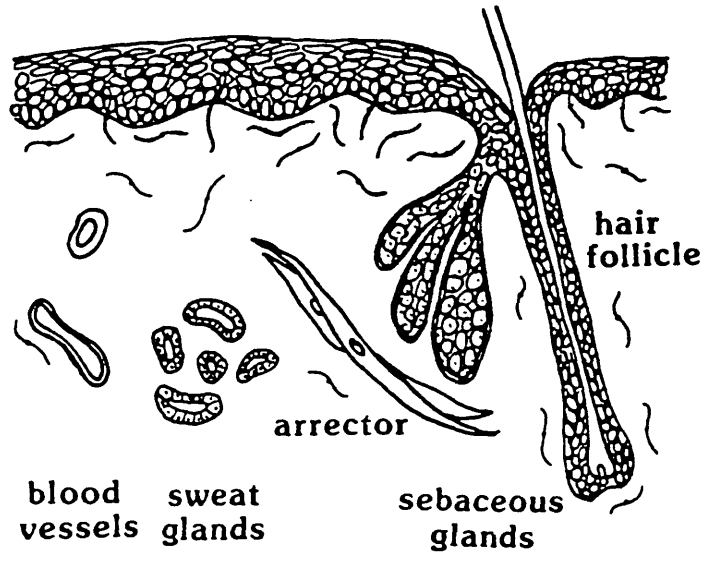


Diagram 44

DISTRIBUTION OF VIP-IMMUNOREACTIVE NERVES IN PORCINE SKIN

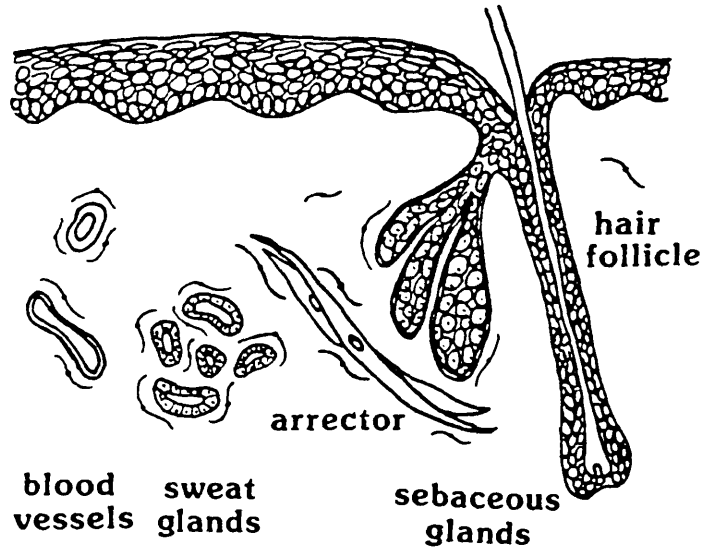


Diagram 45

DISTRIBUTION OF SUBSTANCE P-IMMUNOREACTIVE NERVES IN FELINE SKIN

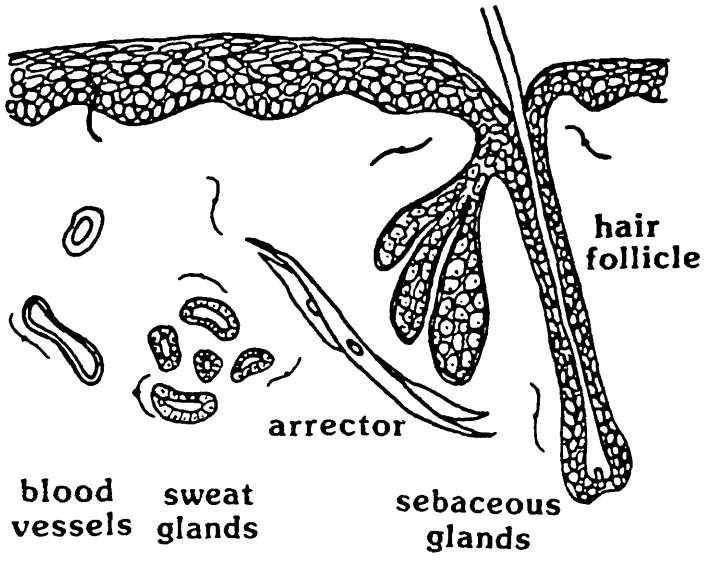


Diagram 46

DISTRIBUTION OF VIP-IMMUNOREACTIVE NERVES IN FELINE SKIN

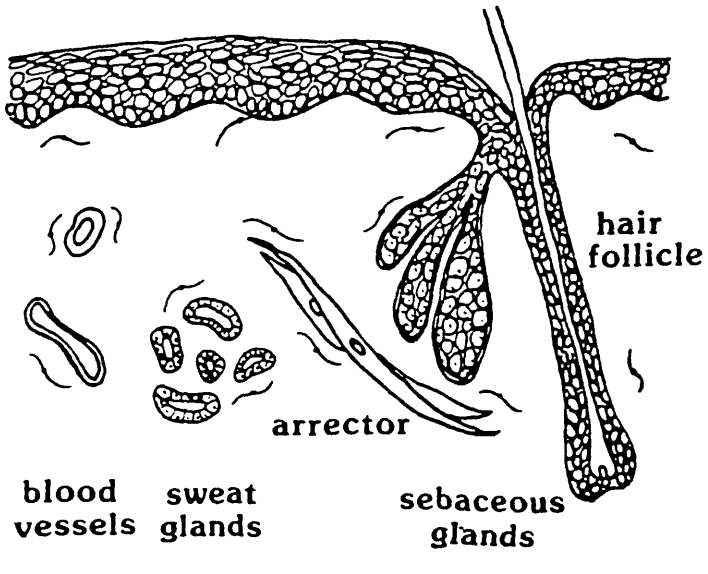


Table 3

CONCENTRATIONS OF PEPTIDES IN PORCINE SKIN

(RIA, pmol/g±SEM, n=4)

Region	bombesin	somatostatin	substance P	VIP
Upper snout	1.5±0.5	3.1±0.7	11.1±1.3	12.0±5.0
Lower snout	1.3±0.6	3.0±0.7	13.5±3.6	7.7±1.6
Cheek	0.9±0.4	0.9±0.3	2.9±0.5	1.4±0.4
Ear	1.6±0.7	1.2±0.6	3.9±1.7	0.9±0.2
Neck	0.9±0.1	1.0±0.4	3.6±0.9	0.9±0.3
Thorax	0.7±0.2	0.9±0.4	4.0±1.1	0.3±0.03
Forelimb	0.9±0.4	1.2±0.5	4.1±0.7	0.5±0.1
Abdomen	0.7±0.2	0.6±0.2	4.3±1.2	0.6±0.3
Hindlimb	0.9±0.3	0.7±0.2	5.3±1.7	0.6±0.1
Buttocks	0.7±0.2	2.0±1.2	8.1±1.6	2.1±1.1
Tail	1.0±0.3	1.9±0.7	6.5±1.7	0.1

Table 4

CONCENTRATIONS OF PEPTIDES IN FELINE SKIN

(RIA, pmol/g, n=5)

Region	bombesin	somatostatin	sutstance P	VIP
Nose	0.1	0.4±0.02	2.3±0.5	5.4±1.6
Lip	0.13±0.08	0.3±0.06	0.8±0.1	1.7±0.3
Body (rump)	0.1	0.5±0.09	0.3±0.06	0.05
Front footpad	0.17±0.02	0.3±0.05	5.0±0.9	17.0±2.0
rear footpad	0.24±0.09	0.4±0.09	3.9±1.2	29.0±6.0

somatostatin and bombesin immunoreactivities in these two species but with very low concentrations which were probably below the limit of immunocytochemical detection. The results of radioimmunoassay are shown in Tables 3 & 4.

7.1.4. DISCUSSION

In this study four peptides were localised, by immunocytochemistry, to nerves in the skin of man, pig, rat and cat whereas somatostatin and bombesin immunoreactivities were detected in the skin of pig and cat by radioimmunoassay alone.

Substance P-IR nerves were more frequent than other peptide-IR nerve types in skin, particularly in pig. Their distribution pattern, i.e., closely beneath the epithelium, supported the postulated sensory function for these nerves that had been suggested previously (for references see Section 1.5.3.). Hägermark et al (1978) found that cutaneous injection of substance P produced effects similar to the well-known physiological triple response (Rothman 1954), which is the result of the co-ordinated interaction of neural, cellular and vascular factors following stroking pressure on the skin. This is consistent with the localisation of substance P-IR nerves found in this study. It has recently been shown that local adminstration of capsaicin to the sciatic nerve of rat induced a depletion of substance P-IR nerves in the corresponding areas of the skin, suggesting that substance P immunoreactivity might occur in sensory C-fibres and be involved in nociception (Gibson et al 1982).

VIP-IR nerves were mainly associated with tissue structures where the motility is known to be under autonomic nerve control, ie. the exocrine glands, blood vessels and arrector muscle. Therefore VIP immunoreactivity may be present in the autonomic motor nerves and these nerves may exert their effects partially through the release of VIP which has been shown elsewhere to cause vasodilatation, smooth muscle relaxation and exocrine secretion (for references see Section 1.5.2.). It has been reported that VIP and substance P, both in vivo and in vitro, could cause histamine release from mast cells, and that this might be involved in the inflammatory response of the skin (Fjellner & Hägermark 1981). Williams (1982) demonstrated that VIP was a very potent vasodilator and oedema potentiator in the rabbit skin. The co-ordination of these actions of VIP is likely to be involved in the cutaneous mechanism of thermoregulation and imflammatory processes.

NPY and PHI immunoreactivities were localised to nerves with populations smaller than those immunoreactive for substance P and VIP. NPY, with its vasoconstrictor property, was mainly found around the blood vessels and thus it might participate in the regulation of local blood flow. PHI, with similar distribution and biological actions to VIP, may have effects on the skin similar to those of VIP.

Bombesin, the amphibian skin peptide, was detected only by radio-immunoassay in the skin of cat and pig. Bombesin has potent exocrine and motor stimulatory actions in the periphery and occurs in a variety of mammalian tissues (for references see Section

1.5.8.). Its physiological role and precise location in the skin, however, remain elusive at present.

Somatostatin has profound inhibitory effects on a wide range of physiological events (for references see Section 1.5.6.). However, its action on the skin has not been shown. It is interesting to note that the skin receives a prominent innervation to blood vessels and sweat glands from the sympathetic nerves and somatostatin has also been associated with such neurons (Lundberg et al 1982a). The possible occurrence of somatostatin in the sympathetic nerves in the skin remains to be determined.

The present study revealed that the skin, like many other peripheral organs, is richly supplied by nerves containing at least 4 different types of peptide immunoreactivity, and each peptide-IR nerve type has a distinct distribution pattern in each mammalian studied. The findings made in this study call for further investigations into the physiological functions of these peptides in the mammalian skin.

7.2. MERKEL CELL AND MERKEL CELL TUMOUR

7.2.1. MERKEL CELL

7.2.1.1. INTRODUCTION

The Merkel cell, first discovered in 1875 (Merkel 1875), is an uncommon cell type in the skin. The population of Merkel cells is

small. For example, in the dorsal skin of the rat, there are only about 150 Merkel cells/sq cm (English 1977). In various sensory receptive areas of the mammalian body, such as the nose and the fingertips, more such cells are found. Merkel cells are thought to be specialized sensory receptor cells (Iggo & Muir 1969) and tend to form groups which are mainly situated within the epithelial basement lamina of touch corpuscles (Haarscheiben) at the junction of the dermis and epidermis (Merkel 1875; Iggo & Muir 1969; English 1977).

Merkel cells can be distinguished, theoretically but not always practically, by their orientation in the epithelium. Their longest axis is usually parallel to the surface of the skin and the basement membrane so that the columnar epithelial cells immediately above them lie in a perpendicular plane (English 1977). In mammals, these cells are very often directly associated with tylotrich hairs (Mann & Straile 1965), which are longer than other hairs and have follicles surrounded by aggregations of fibroblasts. They are mostly innervated by long myelinated nerve fibres, forming the so-called epidermal Merkel cell neurite complexes (English 1977; Winkelmann & Breathnach 1973; Ovalle 1979).

The neuroendocrine nature of Merkel cells had been suggested (for review see Pearse 1980a) and the confirmation of this was still to be sought prior to the present study. There has been much doubt concerning their embryological origin. In addition, at the time of the investigation, there had been no specific staining method for Merkel cells, and the most reliable means of identifying them was to use the electron microscope to identify the electron-dense granules

in their cytoplasm (Iggo & Muir 1969; English 1977). The present study was therefore undertaken to investigate the possible occurrence of peptides and neuronal markers in these cells, whereby the neuroendocrine nature of these cells might be confirmed and a simple method for their identification at the light microscopical level might be found.

7.2.1.2. EXPERIMENTAL DESIGN

In this study, skin samples were taken from pig, cat, rat and man. The areas investigated were the same as those used for examining peptide-IR nerves. The tissues were fixed in BQS (Appendix 2.2.1.) or freeze-dried and fixed in benzoquinone vapour or formaldehyde vapour (Appendix 2.2.3., 2.2.4.). The modified indirect immunofluorescence method (Appendix 2.4.5.) and PAP method (Appendix 2.4.6.) were carried out. The antisera used included those against NSE, S-100, VIP, substance P, PHI, NPY, met-enkephalin, neurotensin and ACTH. To identify melanocytes, the DOPA oxidase method (Appendix 2.4.11.) was used.

7.2.1.3. MERKEL CELL AND NSE

Among the antisera against peptides, none was found to immunostain any cellular elements in the epithelium in which Merkel cells are known to be present.

When the antisera to human and rat NSE were applied, a number of cells, located just above the basement membrane, were strongly immunoreactive (Figs. 61 & 62). In the skin of cat, rat and man, the cells were found in very few sections, i.e. about 1 out of 20 or 30, and tended to form groups of 3-10 cells, separated by unstained epithelial cells (Figs. 61, 62 & 63). Such NSE-IR cells were far more abundant in the porcine snout. Most of the immunostained cells lay in a plane parallel to the basement membrane and thus at right angles to the columnar epithelial cells. Immunostained nerve fibres were clearly visible in the subcutaneous layer. They penetrated the basement membrane and were closely associated with the basal part of most of the immunostained cells (Figs. 61 & 62). At times the nerve fibres surrounded the NSE immunoreactive cells, and some projected further toward the surface of the skin (Fig. 62). The innervated cells tended to lie at the bottom of the basal lamina of touch corpuscles and were nearly always associated with tylotrich hairs (Fig. 62), particularly in the porcine snout. Noticeably, the nerve terminals around NSE-IR cells as seen on consecutive sections were unreactive to antisera against peptides including substance P.

In some cases, when the papillae of the skin were cut transversely, some immunostained cells with a distinctive shape could be seen in the epithelium, at the edge of the papilla. The periphery of these cells was immunostained more intensely than the center, giving the cells a ringlike appearance (Fig. 63). It was not possible, using sections cut in this plane, to show that the cells were intimately innervated, but very often one or two nerve fibres were found lying nearby in the subcutaneous layer.

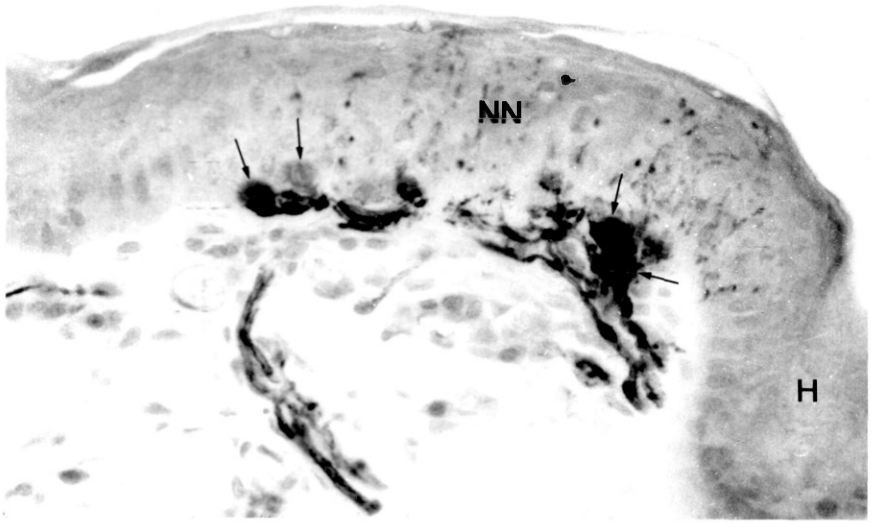


Fig. 61. NSE-IR cells in the nose skin of cat. The immunostained cells (arrows) show all the characteristics of Merkel cells which include their number, location, orientation, and association with hair follicles (H). Naked nerve endings (NN) can be seen in the epithelium. (x300).

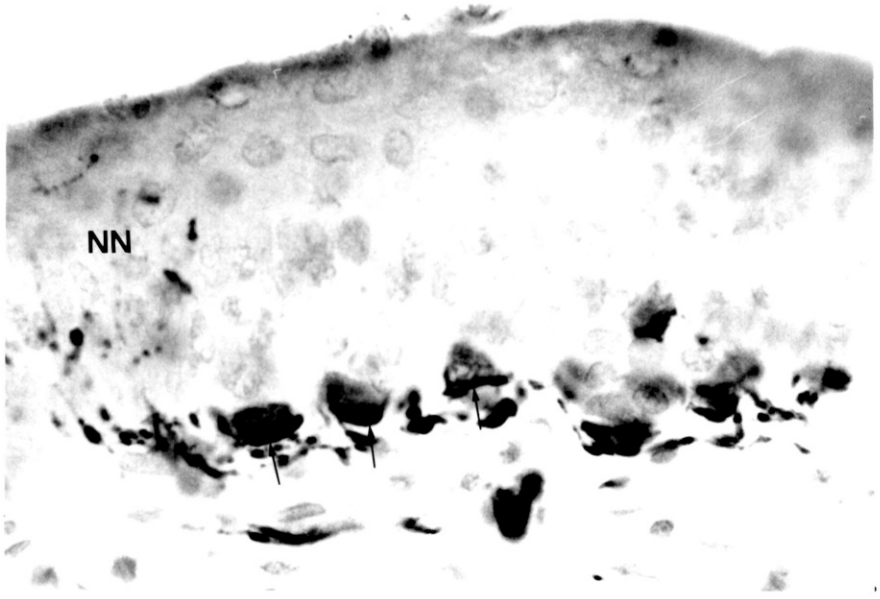


Fig. 62. A group of Merkel cells situated within the epithelial basement lamina of a touch corpuscle of feline nose skin. The cells are clearly innervated (arrows), and some nerve fibres go further toward the surface of the skin, forming naked nerve endings (NN). Both Merkel cells and the nerves are immunostained by NSE antiserum. (x400).

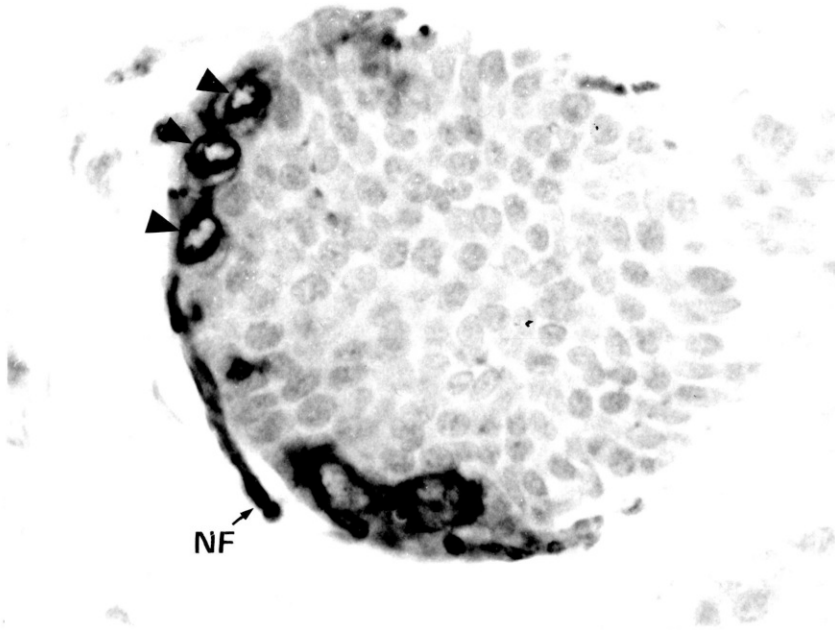


Fig. 63. A group of Merkel cells (arrow) immunostained by NSE antiserum, at the edge of a transversely cut papilla of feline nose skin. The cells have a ringlike appearance. A nerve fibre (NF) can be seen nearby in the subcutaneous layer. (x300).

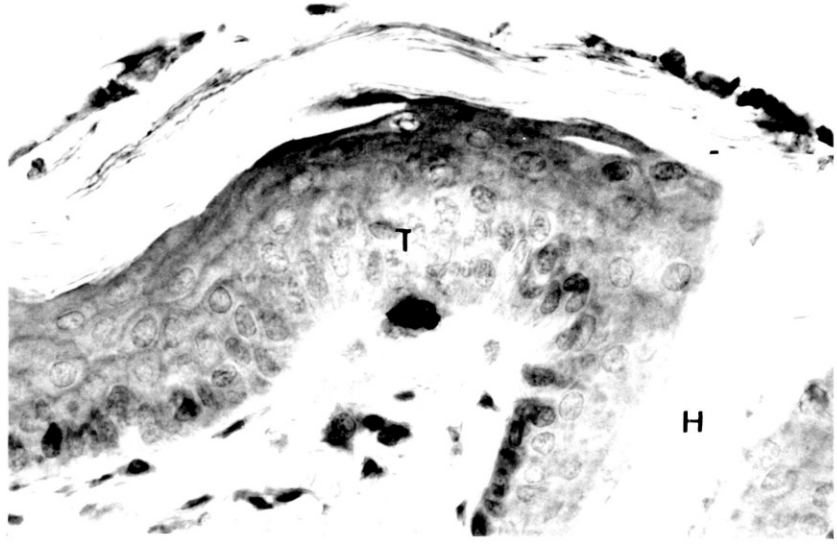


Fig. 64. Single uninnervated Merkel cell immunostained by NSE antiserum in feline nose skin. The cell is located at the junction of the dermis and epidermis, and its longest axis lies parallel to the surface of the skin. The cell is associated with a touch corpuscle (T) (Haarscheiben) and a tylotrich hair follicle (H). (x275).

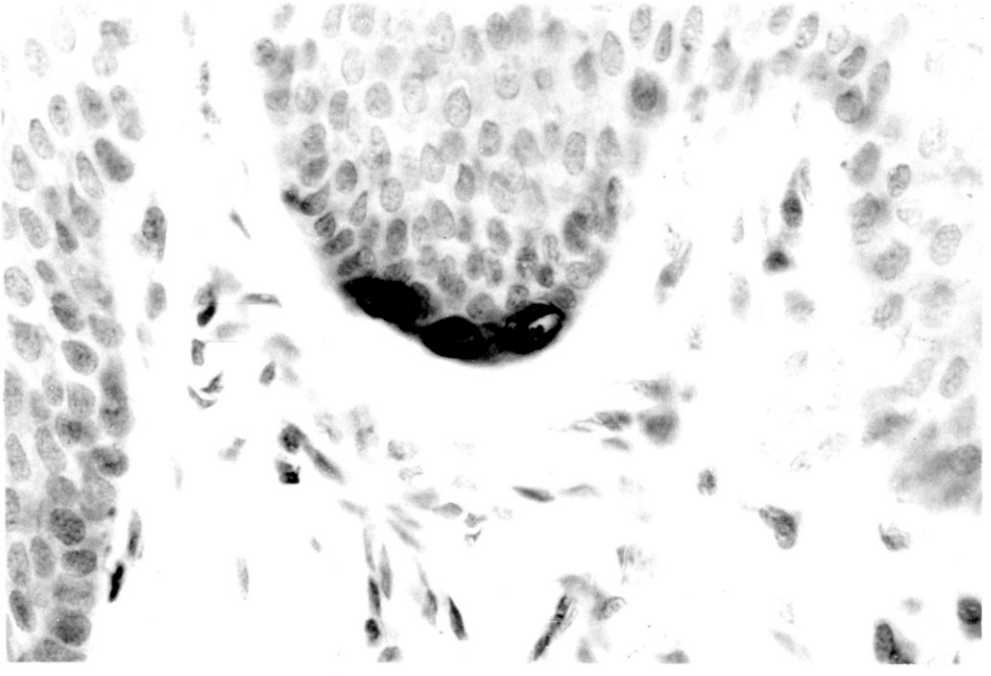


Fig. 65. Three NSE-IR cells in the layer above the basement membrane of the epithelium of feline skin. The cells show all the characteristics of Merkel cells except that they do not seem to be innervated. (x 400).

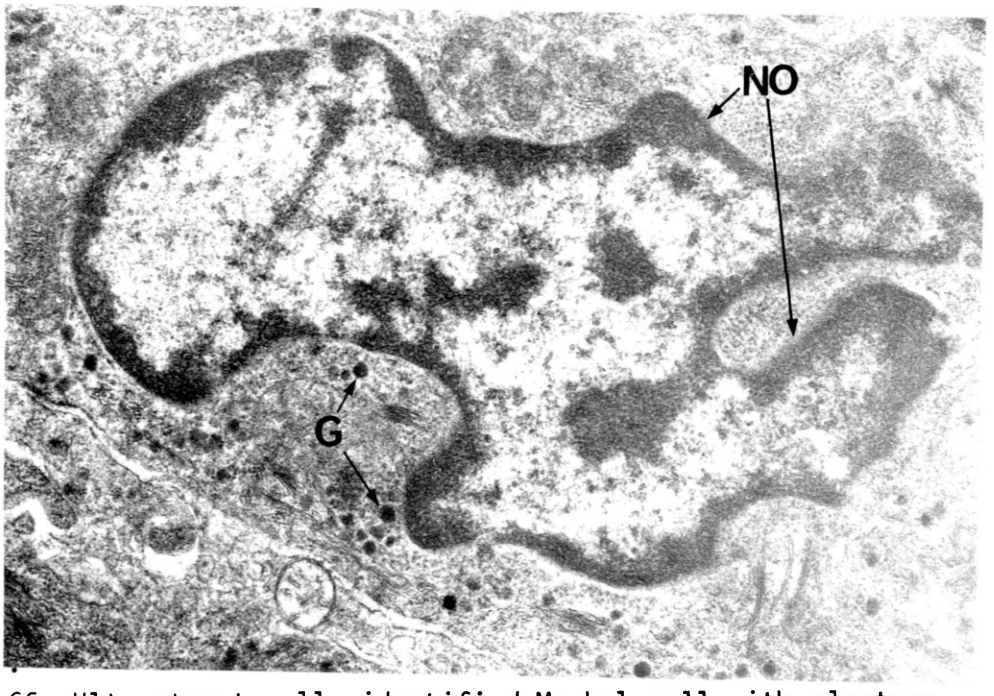


Fig. 66. Ultrastructurally identified Merkel cell with electron-dense granules (G) and irregular nuclear outline (NO) are found in the same areas. (x35000).

Occasionally, single or grouped immunostained cells (Figs. 64 & 65) were seen, lying on the basement membrane, sometimes associated with a touch corpuscle. These cells were generally not shown to be innervated. Sometimes only NSE immunostained nerve fibres were seen, penetrating the basement membrane and ending on or near the surface of the epithelium, forming naked nerve endings.

For the purpose of differentiation, melanocytes were demonstrated by the DOPA oxidase method, which stained the cells a strong brownish-black. These cells were mainly located in the basal layer of the epithelium. They had a central cell body, containing the nucleus and numerous long, thin, branching cytoplasmic processes. In addition to having a different shape, melanocytes were also far more abundant than the NSE-immunoreactive cells.

Langerhans cells were demonstrated by S-100 immunostaining. They were located superficially to the NSE-IR cells and had a few long processes extending mainly parallel to the surface of the skin; thus they were easily distinguished from Merkel cells.

Parallel ultrastructural studies on the skin of cat, rat and pig showed the presence of scattered cells with a distribution identical to that of the NSE-positive cells and having morphological features similar to those described for Merkel cells. Thus, they contained characteristic intracytoplasmic, round secretory granules, surrounded by a limiting membrane and with an average diameter of 60 nm in rat and 80-90 nm in pig. Other characteristics of Merkel

cells were also seen in these cells, including irregular nuclear outline and spindle-like cytoplasmic processes (Fig. 66).

7.2.1.4. DISCUSSION

It has been demonstrated here that NSE is present in Merkel cells. These cells were identified by the use of both light- and electron-microscopical methods.

There are three types of non-keratinocytes known in the skin, namely melanocytes, Langerhans cells and Merkel cells. The melanocytes, stained in this study by the DOPA oxidase technique and described before by other investigators (Becker et al 1935), have morphological characteristics entirely different from those of the NSE-IR cells. The Langerhans cells are located in the suprabasal layers of the epithelium, which lie in the area above the NSE-IR cells, and have a distinct star shape with long irregular processes (Langerhans 1868). The NSE-IR cells are certainly not the two cell types described above.

At light microscopical level, it would seem that since Merkel cells are the only cells in the epithelium that are characteristically innervated, the NSE-IR cells, which were intimately surrounded by nerve terminals, must be Merkel cells. In addition, these innervated NSE-positive cells display all the morphological characteristics of Merkel cells, which include association with touch corpuscles and tylotrich hairs and the tendency to form

groups. Also their location, numbers and orientation are typical of those reported for Merkel cells. Moreover, parallel electron microscopical studies showed the presence of electron-dense secretory granules, a characteristic of Merkel cells. Thus, it may be concluded that these innervated NSE-IR cells are Merkel cells.

Immunostained cells with a ringlike appearance are only seen in transversely sectioned papillae. It is likely that they are also Merkel cells, with peripherally located immunostaining giving a ringlike pattern. Associated nerve fibres could not be demonstrated in the same sections. This may be due to the plane in which the tissue was cut and may also explain the fact that there were always some nerve fibres lying nearby in the subcutaneous layer. The scattered and non-innervated NSE-IR cells are likely to be Merkel cells too since it has been shown that not all Merkel cell are innervated; neither are they all associated with touch corpuscles (Iggo & Muir 1969; English 1977; Winkelmann & Breathnach 1973; Ovalle 1979). An interesting point thus arises concerning the function of these non-innervated NSE-IR cells. The Merkel cell has been shown to be a kind of slow adapting touch sensitive cell (Iggo & Muir 1969) which needs adjacent nerve terminals to conduct the message. This action is obviously not possible for the non-innervated NSE-IR cells and thus a paracrine or endocrine function is to be sought. It is possible that the so-called 'Merkel cells' represent a group of heterogeneous neuroendocrine cells, and that skin, like gut, contains a number of neuroendocrine cells, each with its own morphological characteristic and physiological function. The absence of immunoreactivity for known peptides in

Merkel cells found in this study does not exclude the existence of peptide in this cell type. New peptides are continously being discovered and thus it is possible that in due time peptide will been found in Merkel cells.

The finding of the newly discovered biochemical marker, NSE, in Merkel cells supports the earlier suggestion that these cells are members of the diffuse neuroendocrine system (Pearse 1980a; Hartshich & Grube 1979). The presence of this neuronal antigen in these cells further supports the functional relationship between neuroendocrine cells and neurons.

The practical significance of the present finding is that NSE immunostaining introduces a simple, reliable and highly specific method for demonstrating Merkel cells at light microscopical level. Such a method was not available prior to this study.

7.2.2. MERKEL CELL TUMOUR

7.2.2.1. INTRODUCTION

Primary skin tumours thought to originate from the Merkel cell have recently been reported by a number of authors (Toker 1972; Tang & Toker 1978; Sibley et al 1980; Silva & MacKay 1981; De Wolf-Peeters et al 1980; Sidhu et al 1980). Since this tumour may histologically resemble either a malignant lymphoma or an undifferentiated carcinoma, it has frequently been misdiagnosed in the past (Tang &

Toker 1978; Sibley et al 1980; Sidhu et al 1980). Such misdiagnosis can lead to inappropriate treatment and an erroneous prognosis. This tumour type is locally aggressive, may metastasize to lymph nodes, and sometimes more distantly and may result in death. Currently, a definitive diagnosis of Merkel cell tumours can only be achieved by electron microscopy, as no stains tried have shown consistent results. A specific staining method for identifying Merkel cell tumours at light microscopical level has therefore not been available before.

Following the finding of NSE in Merkel cells, the possible presence of this isoenzyme in Merkel cell tumours was also examined in an attempt to find a simple and reliable approach at the light microscopical level for the differential diagnosis of these tumours from others that have a similar histological appearance.

7.2.2.2. EXPERIMENTAL DESIGN

Eleven Merkel cell tumours, including one local lymph node metastasis, were investigated. The tumours were found on the limbs, neck and face areas of patients older than 55 years of age. A summary of the clinical data is shown in Appendix 1.5.. All of the tumours had been previously identified as Merkel cell type by electron microscopy. Some of the cases have been previously discussed on conventional histological grounds by other investigators (Sibley et al 1980; De Wolf-Peeter et al 1980).

Sixteen non-Merkel cell skin tumours were used as controls. These included 4 cases of undifferentiated carcinoma, 4 cases of malignant lymphoma and 8 cases of basal cell carcinoma. In addition, 33 primary malignant and 25 metastatic melanomas of skin were also investigated.

Nine of the Merkel cell tumours were fixed in formol saline, one was fixed in Bouin's and another one was freeze-dried and fixed in benzoquinone vapour. The fixed samples were embedded in paraffin wax. All of the control tumours, including the melanomas, were fixed in formol saline and embedded in paraffin wax. Five µm thick paraffin sections were stained by H&E, the Grimelius technique or immunocytochemistry.

NSE immunostaining was carried out with the PAP method (Appendix 2.4.6.). To search for possible peptide content, antisera to met-enkephalin, ACTH, somatostatin, VIP, substance P, NPY, PHI, neurotensin and bombesin were also applied.

7.2.2.3. MERKEL CELL TUMOUR AND NSE

The H&E-stained preparations showed a similar appearance in all the cases. The tumours were situated in the dermis and extended into the subcutaneous or deeper tissue. They were arranged compactly and formed a diffuse infiltrate, resembling that of malignant lymphoma. They sometimes consisted of ill-defined anastomosing cords and clusters of cells. The neoplastic cells were round, uniform in size

and had very little cytoplasm. The nuclei were large, round and sharply outlined. Numerous mitoses could be seen and scattered necrotic cells were observed. Lymphocytic infiltrate was present at the periphery of some of the tumours (Fig. 67).

In each case, NSE immunoreactivity was found in all the neoplastic cells, although variations of staining intensity could be seen between different cases. Except for three cases in which the NSE immunoreactivity was rather weak, all the other Merkel cell tumours showed fairly strong NSE positivity. The NSE immunoreactivity was uniformly present in cytoplasm, which formed a rim surrounding the large, round, unstained nucleus of the cell. The positively immunostained tumour mass stood out clearly against the unstained background structures (Fig. 68). The control tumours were negative. Of the 53 primary and metastatic malignant melanomas, only six showed a variable, mostly weak and patchy, positivity with a focal distribution. This was more noticeable in the junctional zone of the tumours. The others were completely negative.

Four of the Merkel cell tumours showed staining with the Grimelius silver impregnation technique, another four reacted equivocally (borderline reaction), whereas the other three cases were completely unreactive to the Grimelius method.

Again, none of the Merkel cell tumours, or the tumours in the control group, showed peptide immunoreactivity.

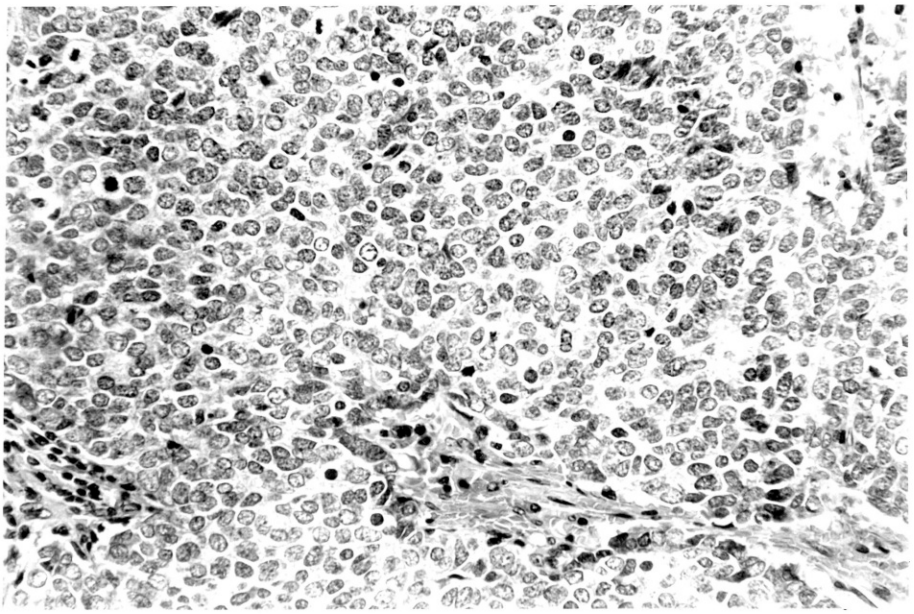


Fig. 67. H&E stained preparation of a Merkel cell tumour, showing compactly arranged, uniform neoplastic cells with large, round and sharply outlined nuclei and little cytoplasm. (x400).

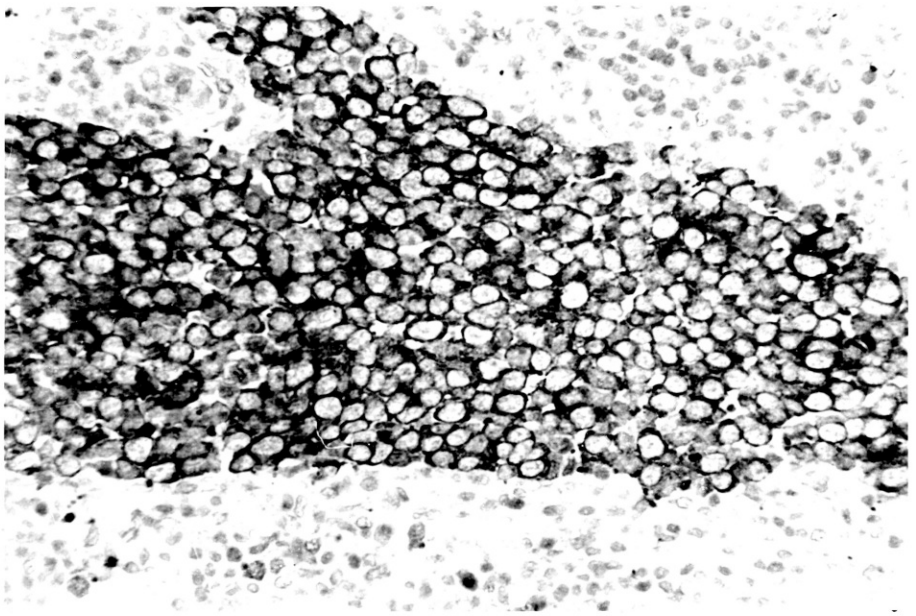


Fig. 68. NSE immunostained preparation of a Merkel cell tumour. The immunoreactivity has an even distribution in the cytoplasm of all the neoplastic cells and forms a dark rim around the large, round unstained nucleus. (uncounterstained) (x 250).

7.2.2.4. DISCUSSION

It has been demonstrated that Merkel cell tumours can be specifically identified by NSE immunostaining. The specificity of the immunostaining was validated by appropriate controls. The variations of immunostaining intensity between different tumours might reflect the variable content of NSE in each tumour, although it was also likely that they resulted from inadequate fixation and, in particular, scarcity of cytoplasm. The finding of NSE in Merkel cell tumours is in keeping with the previous finding that NSE is present in normal Merkel cells, and also with another recent report that NSE is present in most APUDomas (Tapia et al 1981).

A number of primary and metastatic tumours of the skin may morphologically resemble Merkel cell tumours. These include malignant lymphomas, certain undifferentiated carcinomas, especially cutaneous metastases of oat cell carcinoma of the lung, which can be very difficult to differentiate histologically from Merkel cell tumours. The compact arrangement of many of these tumours, with a consistently uniform cytology leads to very real diagnostic problems. To avoid such misdiagnosis, it has usually been necessary to perform electron microscopical examination to identify the characteristic electron-dense secretory granules and other ultrastructural features such as intercellular junctions. Since Toker first reported neoplasms of Merkel cell origin in 1972, under the name 'trabecular carcinoma' (Toker 1972), considerable effort has been made to distinguish Merkel cell tumours from others which are morphologically similar. A few minor differences between such

tumour types have been emphasized. It has been reported that large cell lymphomas tend to have greater irregularity of their nuclei (Sidhu et al 1980; Rappaport 1966; Lukes & Collins 1974), plasmacytomas have more prominent nucleoli (Sidhu et al 1980) and metastases of oat cell carcinomas of the lung frequently show more extensive necrosis, crush artefact and oval or spindle-shaped cells (Sidhu et al 1980). However, although such differentiating criteria can be useful in certain cases, it remains true that in practice Merkel cell tumours have often been misdiagnosed.

In the current investigation, all 11 cases of ultrastructurally identified Merkel cell tumours were found to contain NSE immunoreactivity. Together with the previous finding that NSE was not present in non-neuroendocrine tumours but was found exclusively in neuroendocrine tumours (Tapia et al 1981), it would seem that the morphologically similar tumours, such as malignant lymphomas and undifferentiated carcinomas, can be differentiated from Merkel cell tumours by NSE immunostaining.

It should, however, be emphasized that certain metastatic tumours in the skin may also show positivity for NSE immunostaining. These include metastases from islet cell carcinoma of pancreas, neuroblastomas, carcinoids and metastases from oat cell carcinomas of the lung. Granular cell tumour was also found to contain NSE immunoreactivity (Rode et al 1982). Generally, one should be able to distinguish them from Merkel cell tumours, without conducting NSE immunostaining, by taking account of both their microscopical features and clinical setting. Because of the close histological

similarity and NSE content, particular attention should be paid to the possibility that one is dealing with metastases of oat cell carcinoma. The presence of NSE in melanomas has recently been reported (Dhillon et al 1982; Dhillon & Rode 1982; Royds et al 1982). In the present study, however, NSE was found to be less frequently present in melanomas than described in these reports. Only six out of 58 tumours did show a focal, usually weak, staining (see Section 7.3.3.). Even when they lack pigment, these tumours can be distinguished by the presence of junctional activity, the different structural configurations and the distinctive cytology. In practice, therefore, the differentiation of melanoma and Merkel cell tumour is not a formidable problem.

Met-enkephalin has been reported to be present in normal Merkel cells (Hartschuh et al 1980). In this study, no peptide immunoreactivities were found in the Merkel cell tumours. Four of the tumours were argyrophilic by the silver impregnation method, three were not. The possibility, therefore, exists that the so-called Merkel cell tumours may behave like endocrine tumours of the alimentary tract, which have varying capacities for silver impregnation, different peptide contents, variously sized secretory granules (for review see Solcia et al 1981) as well as NSE immunoreactivity (Tapia et al 1981). This would be in keeping with the speculation of the heterogeneity of the Merkel cells in the normal skin (see Section 7.2.1.4.).

It can therefore be concluded that, given adequate fixation, NSE is present in most, perhaps all, Merkel cell tumours. After excluding metastatic oat cell carcinoma of the bronchus, malignant melanoma

and granular cell tumour, NSE immunostaining provides an additional, simple and reliable diagnostic tool in the recognition of Merkel cell tumours of the skin, which can otherwise pose formidable diagnostic problems.

7.3. MELANOCYTES AND MELANOMAS

(This investigation was accomplished jointly with Dr D.R.Springall, Dept. of Histopathology, Hammersmith Hospital, London)

7.3.1. INTRODUCTION

Another cell type in the skin has also been reported to contain NSE, that is the melanocyte (Dhillon et al 1982; Dhillon & Rode 1982). This, however, was denied by Royds et al (1982a, b) who found that γ-enolase (NSE) immunoreactivity was only present in intradermal naevi and in malignant intradermal melanocytes, but not in normal melanocytes. Nevertheless, both groups agreed that NSE was present in melanomas. Several other groups had reported the presence of S-100 in normal melanocytes (Cocchia et al 1981) and melanomas (Nakajima et al 1981, 1982; Gaynor et al 1980, 1981; Stefansson et al 1982c; Clark et al 1982; Cochran et al 1982). Further investigation along this line is interesting because this may not only have theoretical value but also have practical applications. Should these proteins indeed be present in melanomas, they may be useful in differentiating melanomas, amelanotic melanomas in particular, from similar tumour types. A study was therefore undertaken to look for both proteins in the same specimens of melanomas using immunocytochemistry.

7.3.2. EXPERIMENTAL DESIGN

Melanomas from 33 patients were investigated. These were 28 primary skin melanomas including 5 amelanotic, 5 skin recurrences and 25 metastases to various sites. In addition, 7 benign naevi were also examined. Some of these samples had small pieces of skin attached. A number of tumours were examined as controls. They are listed in Appendix 1.6.

All the tumours were fixed in either formol saline or Bouin's solution and embedded in wax, except for one Merkel cell tumour which was freeze-dried, fixed in benzoquinone vapour (Appendix 2.2.3.) and embedded in wax. In addition, some normal skin samples were fixed in 0.4% BQS (Appendix 2.2.1.) and cut in a cryostat. H&E staining and immunostaining using PAP or the indirect immunofluorescence method were carried out on the dewaxed paraffin or cryostat sections with routine negative controls (Appendix 2.4.3., 2.4.4. & 2.4.6.). The possible cross-reactivity of the antisera was tested by adding S-100 or NNE to anti-NSE antiserum, and adding NSE or NNE to anti-S-100 antiserum (up to 20 nmol/ml diluted antiserum) 24 hours prior to immunostaining (Table 2f).

7.3.3. S-100, NSE AND MELANOMAS

Among the 53 specimens of melanomas from the 33 patients, only 6 of

the primary melanomas showed immunoreactivity to NSE antiserum. The staining was weak and patchy (Fig. 69a). The staining was confined to the cytoplasm. On H&E stained sections, the tumours were amongst the monomorphic type of melanoma and were all melanotic. None of these NSE-IR primary melanomas had detectable metastases. No normal melanocytes, either in the normal skin samples or in those attached to the tumours, were immunoreactive to NSE antiserum.

All the melanomas, both primary and metastatic, pigmented and amelanotic, were immunoreactive for S-100 (Fig. 69b, 70a,b, 71, 72), except one which showed no reaction to either of the antisera. The immunostaining of S-100 was mostly strong, more so at the periphery of the tumour adjacent to the dermis (Fig. 70b). The immunoreactivity appeared to be present in both the cytoplasm and the nuclei. The degree of S-100 staining intensity did not correlate with the degree of pleomorphism, the extent of invasion, the size of the tumour or the amount of melanin present. However, cells which were packed with melanin did not react to S-100 antiserum. Bleaching of melanin with permanganate prior to immunostaining, which did not interfere with the immunoreaction, did not reveal any immunostaining in these heavily pigmented cells. In general, more S-100 immunoreactivity was seen in the superficial spreading melanomas than in the nodular type (Fig. 73a, b). Normal melanocytes immunoreactive to S-100 antiserum were seen only occasionally, being present in one normal skin sample and 6 others attached to melanomas.

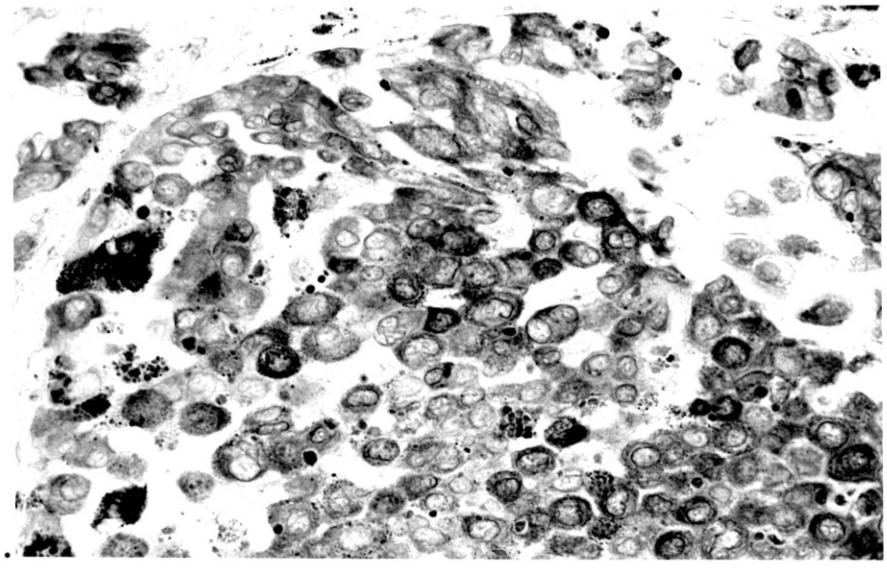


Fig. 69a. Section of a melanoma stained with antiserum to NSE (not counterstained). The staining of the tumour cells is rather weak. NSE immunoreactivity is only present in 6 out of the 53 primary and secondary melanomas from 33 patients. (x360).

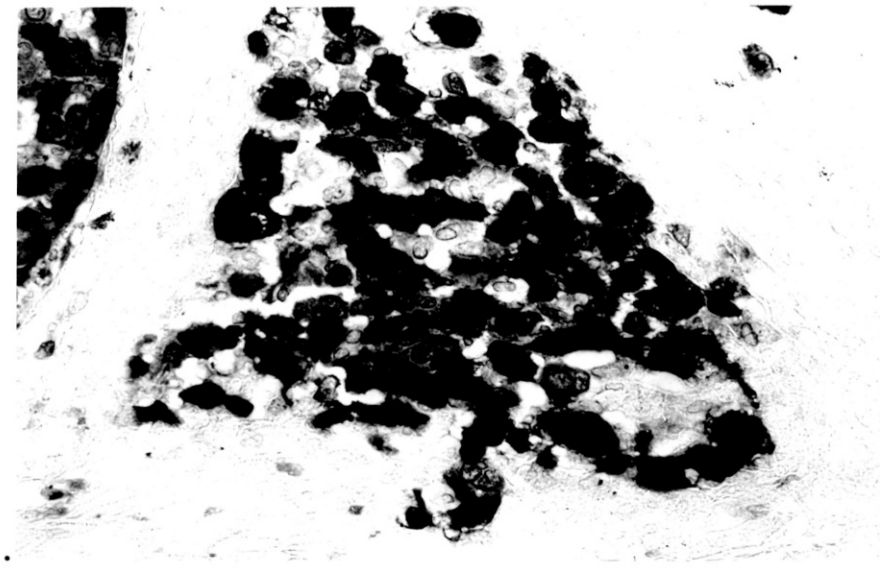


Fig. 69b. Another area from the same tumour showing strong S-100 immunoreactivity (uncounterstained) (x225).

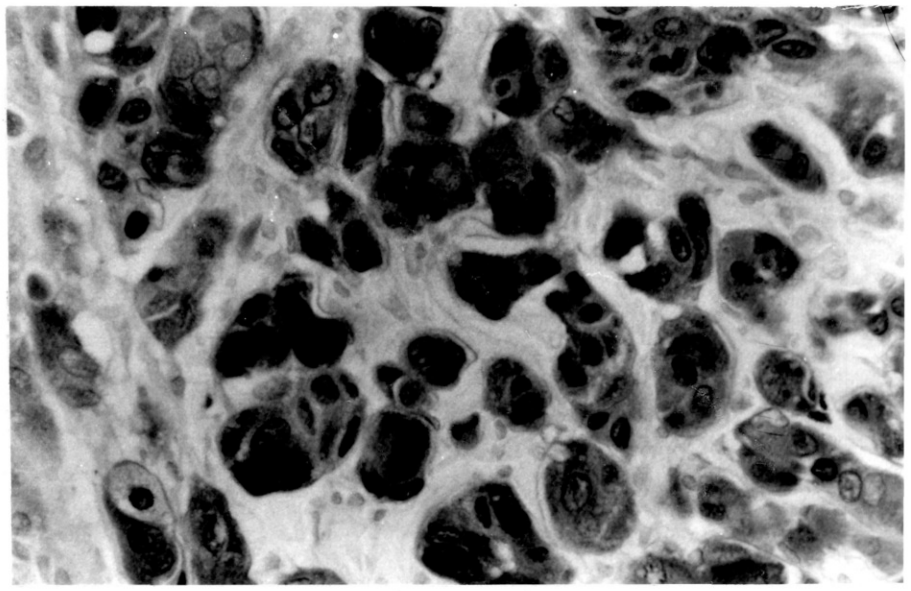


Fig. 70a. S-100 immunoreactivity in the neoplastic cells of a melanoma in the skin (uncounterstained) (x400).

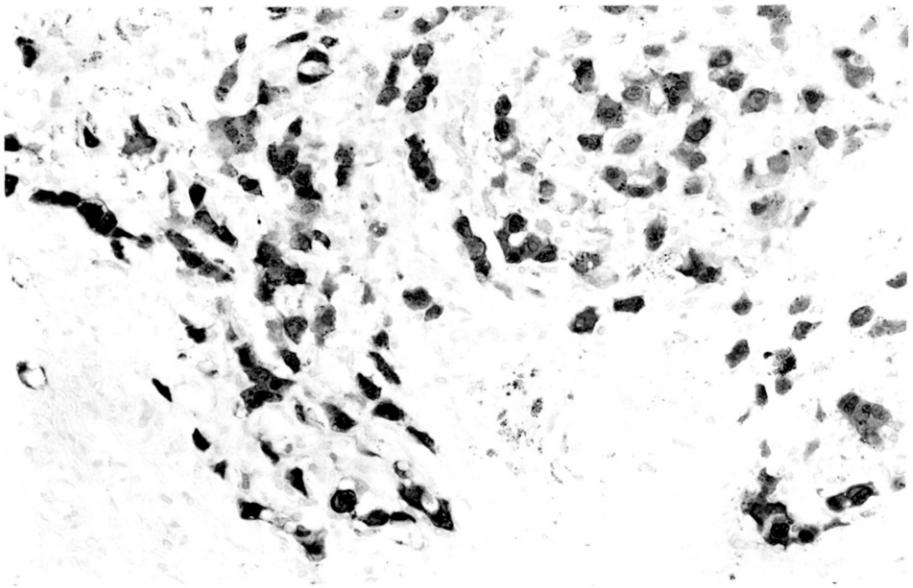


Fig. 70b. Intense immunostaining of S-100 in tumour cells at the periphery of the tumour. (x180).

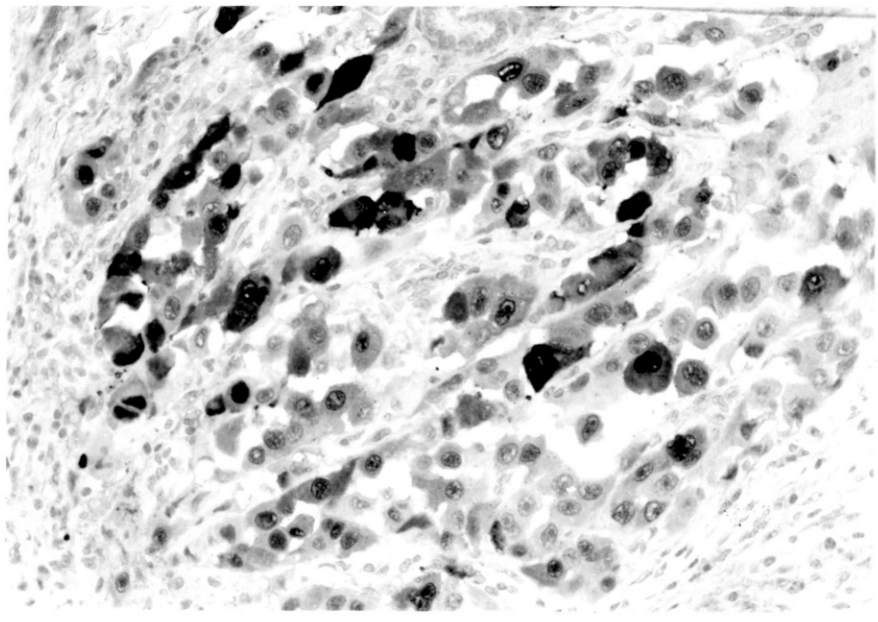


Fig. 71. Pleomorphic melanoma in skin, haematoxylin counterstained, showing immunoreactivity for S-100. (x225).

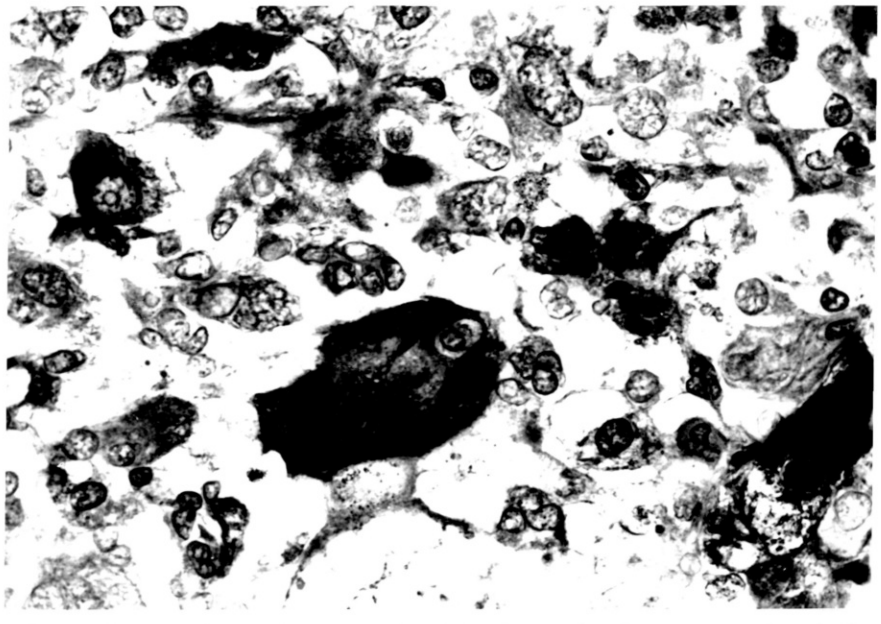


Fig. 72. Metastatic melanoma in lymph node immunostained for S-100 (counterstained with haematoxylin) (x360).

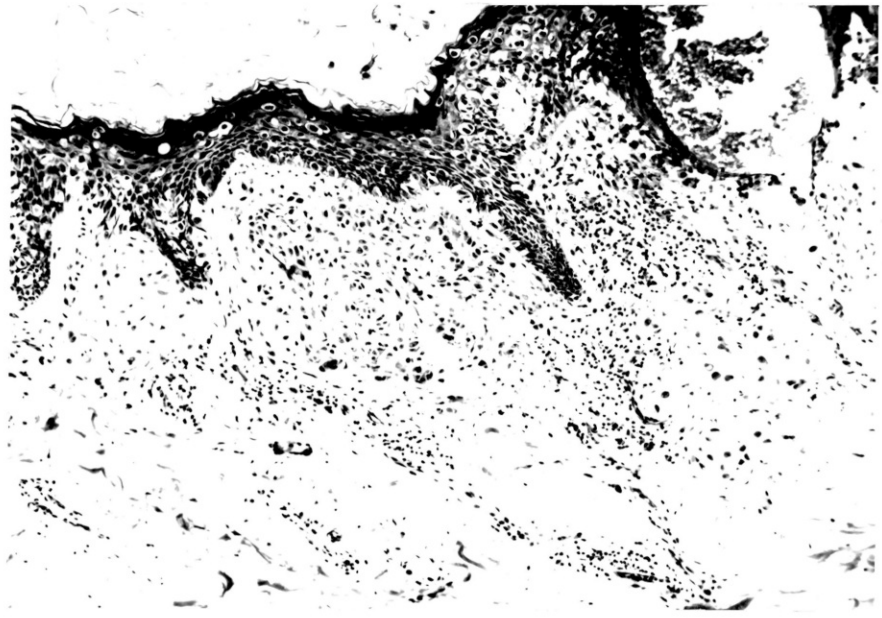


Fig. 73a. H&E stained intradermal malignant melanocytes at the lateral edge of a melanoma in skin. (x95).

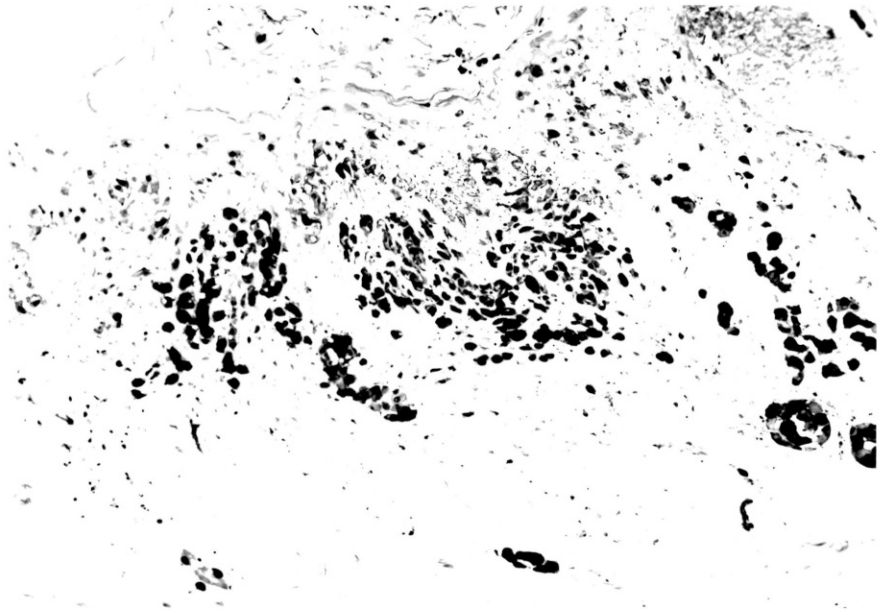


Fig. 73b. Uncounterstained section from the same tumour. The tumour cells are intensely stained for S-100. (x95).

Of the control tissues, all melanocytic naevi but one were immunoreactive for both proteins, more strongly for S-100 than for NSE. All other tumours were negative to S-100 immunostaining, whereas NSE immunoreactivity was demonstrated in small cell carcinomas (3 out of 3 case) and Merkel cell tumours (11 out of 11 cases).

The specificity of the immunostaining was validated not only by the negative results of the routine controls but also by that of the cross-preabsorption tests. Moreover, intrinsic positive controls were provided by the immunostained NSE-containing nerves and S-100-containing Schwann cells which were seen on every piece of tissue containing normal skin. This indicates that the negative results on the tumours were not caused by fixation, bleaching or the immunostaining procedure.

7.3.4. DISCUSSION

It has been found in this study that all but one of the 33 cases of melanoma contained S-100 immunoreactivity. This is in general agreement with the previous reports (Gaynor et al 1980, 1981; Clark et al 1982; Cochran et al 1982; Nakajima et al 1981, 1982; Stefansson et al 1982c). The staining of S-100 was strongest in the superficial spreading melanomas, tumour cells towards the edges of the nodular melanomas, and intradermal malignant melanocytes. The strength of the reaction may therefore reflect the availability of nutrients or the permeability of fixatives to the tumour cells,

those showing greater S-100 immunoreactivity being nearer to the nutrient supply or more easily fixed than those in the middle of the nodular tumours. Alternatively, it may indicate that the densely immunostained cells are of a more active type than those in the centre of nodular tumour. The heavily pigmented cells were found to contain less or no S-100 immunoreactivity. This is partially in keeping with the report by Nakajima et al (1982) that amelanotic melanomas contain more S-100 protein than melanotic ones. However, no definite correlation of S-100 immunoreactivity with the degree of pleomorphism of the melanomas was found.

In this study only 6 of the 33 cases examined showed NSE immunoreactivity. The discrepancy between the results of this study and those of Dhillon et al (1982) and Royds et al (1982b) could be due to differences in the antisera, fixation and immunostaining procedures used. The specificity of the immunostaining in this study has been established by both negative and positive controls. The NSE antiserum used showed no cross-reaction to NNE or S-100. In addition, the NSE antiserum was highly diluted (1:2,000) to eliminate as much as possible the unspecific antibodies from the antiserum. Nevertheless, since the NSE and the S-100 used to produce and to absorb the antisera were from natural sources, the possibility that the purity of the antigens and, consequently, the specificity of the antibodies might not have reached their perfection could not be completely ruled out.

The most obvious pointer to the diagnosis of melanoma in a skin tumour is melanin, but this does not exclude other tumours which have taken up the pigment and thus mimic a melanoma. With non-pigmented melanomas the differential diagnosis of tumours including spindle-cell sarcomas, squamous and basal cell carcinomas,

and skin metastases of undifferentiated carcinoma, becomes important, as does the site of the primary tumour in metastases. Thus a marker for distinguishing melanoma in these situations would be valuable.

Since S-100 immunoreactivity was present in most melanomas but not in the non-melanocytic (control) tumours, this protein may be a potentially useful marker for identifying melanomas, particularly amelanotic melanomas. As well as its use for differential diagnosis, it is valuable in highlighting small groups of tumour cells either spreading superficially or invading the dermis. A large number of neuronal and non-neuronal tumour types have been examined for S-100 immunoreactivity and most of them gave negative results (Nakajima 1982). A number of tumours, however, have been reported to contain S-100 immunoreactivity. These include Schwannoma, neurofibroma, amputation neuroma, granular cell tumour, ganglioneuroma, ganglioneuroblastoma, paraganglioma, chondroma, chondrosarcoma, eosinophilic granuloma, histiocytosis X, fibroadenoma (breast), duct papilloma (breast), infiltrating duct carcinoma (breast) and carcinoid tumour (Jacque et al 1979; Dhillon & Rode 1982; Nakajima et al 1982). This should be borne in mind when using S-100 as a marker to identify melanomas, although in general, most of the above S-100 positive tumour types can easily be distinguished from melanomas without conducting immunocytochemistry.

Diagnostic tests for melanoma, such as formalin-induced fluorescence and enzyme cytochemistry (Rost et al 1973), the tyrosinase method of Fitzpatrick and Clark (1964) using tissue slices, and biochemical assay of S-100, mostly require fresh or specially fixed tissue. The

immunostaining of S-100 can be performed on routinely fixed and paraffin embedded tissues as this processing does not destroy the antigenicity of S-100 in melanomas, provided that the fixation time has not been too long, say not more than two weeks. This is most important for the routine histopathology department where fresh material may be no longer available when suspicion of melanoma arises, or in retrospective studies. The antigenicities of NSE and S-100 seem to be preserved in wax blocks indefinitely.

Should S-100 prove to be released into the blood stream from the tumour, the assay of the protein in the circulation may be of value in monitoring the effectiveness of surgical ablation or other therapy, or the subsequent development of metastases, in patients with melanoma.

In conclusion, S-100 immunostaining appears to be a convenient marker for melanotic tumours, and may be used to distinguish these lesions from a variety of other tumours.

NSE immunoreactivity was not found in normal melanocytes. S-100 immunoreactivity was seen in melanocytes in only one piece of normal skin and 6 pieces of skin adjacent to melanomas. The significance of this finding is at present uncertain. To explain the absence of enolase activity in melanocytes, Royds et al (1982) speculated that it might be due to the shrinkage of the cytoplasm on fixation, which may occur in melanocytic cells of the epidermis.

7.4. SUMMARY

1. Characteristically distributed nerves immunoreactive to the antisera against substance P, VIP, NPY and PHI respectively were demonstrated in various regions of the skin in cat, rat, pig and man. Substance P-IR nerves were mainly concentrated in the sensory areas of the skin and their distribution pattern implies primarily a sensory function. VIP-, NPY- and PHI-IR nerves were mainly associated with sweat glands, blood vessels and arrector muscle. Thus they may constitute, in part, the autonomic nervous system in the skin.

2. Although no peptides have yet been demonstrated in the neuroendocrine cells of the skin - the Merkel cells, they have been found to contain the neuroendocrine marker NSE. Immunostaining of NSE can therefore be used as a simple and reliable method to identify Merkel cells at the light microscopical level. These cells could otherwise be recognised only by electron microscopy.

3. Eleven Merkel cell tumours were investigated by immunocytochemistry. Although no peptides were detected in these tumours, 8 of them were found to immunoreact strongly with NSE antiserum, while most of the control skin tumours, except, possibly, the metastases of small cell carcinomas of the lung and some melanomas, as has been found in this study and reported previously, were negative. Therefore, NSE immunostaining provides a novel, simple and reliable approach to the differential diagnosis of Merkel cell tumours from other histologically similar tumour types, which have previously presented formidable diagnostic problems.

4. Primary and metastatic melanomas from 33 patients and a number of skin tumours and normal skin samples were examined with the antisera against S-100 and NSE. It was found that all but one of the cases of melanoma showed S-100 immunoreactivity and only 6 of them reacted weakly to NSE antiserum. None of the control tumours contained S-100 immunoreactivity. Therefore, immunostaining of S-100 possesses diagnostic value in identifying melanomas, particularly the amelanotic type.

Chapter 8: THE GALLBLADDER

(This work was accomplished jointly with Dr W.Cai, Dept. of Anatomy, University College, London)

8.1. INTRODUCTION

The innervation of the gallbladder and the biliary tract has been extensively studied. It has been shown that these organs are very richly supplied by cholinergic and adrenergic nerves (Sutherland 1966; Baumgarten et al 1969; Mori et al 1971; Kyösöla & Rechardt 1973; Kyösöla 1974, 1976, 1977, 1978; Kyösöla & Penttilä 1977; Davison et al 1978; Onada & Miyazaki 1980; Cai & Gabella 1983). In a recent investigation (Cai & Gabella 1983), it has been found that three major nerve plexuses exist in the wall of the gallbladder and the biliary pathways, i.e., the mucosal plexus, the ganglionated or myenteric plexus and a plexus which lies in the adventitia of the cystic artery and its branches. Strands of nerve bundles communicate between these major plexuses.

While many peptides have been demonstrated in the nerves and endocrine cells of the gut, there have been only four peptides reported to be present in the outgrowth of the alimentary canal - the gallbladder and the biliary tracts. Nerves containing VIP have been found in the gallbladder and the sphincter of Oddi in many mammalian species (Sundler et al 1977a; Alumets et al 1979). Met-enkephalin immunoreactivity has been localised to the myenteric plexuses of the gallbladder and the cystic duct in man (Polak et al

1977). As for the mucosal endocrine cells, only those containing motilin and substance P immunoreactivities have been reported to be present in the bile duct of rabbit (Heitz et al 1977). Detailed distribution of VIP and met-enkephalin immunoreactivities in these organs, and the possible existence of nerves and cells containing other peptides, however, have not been studied. The present investigation was therefore carried out to study the occurrence and distribution of peptide-IR nerves and endocrine cells in the gallbladder and biliary pathways of the guinea-pig.

8.2. EXPERIMENTAL DESIGN

Adult (n=12, body weight 280-350g) and newborn (n=8, 1-4 days) guinea-pigs of both sexes were used. They were killed by cervical dislocation and the whole gallbladders, cystic ducts, hepatic ducts, common bile ducts and the cranial portions of the duodenum were removed immediately after death. The tissues were fixed in BQS (Appendix 2.2.1.). The gallbladders were made into whole mount stretch preparations according to the modified method described previously (see Section 2.6.). The preparations were dehydrated and rehydrated through graded alcohols and xylene and then soaked in 0.2% Triton X-100 in PBS for one hour prior to immunostaining.

Tissues of the biliary tracts and the sphincters were made into cryostat blocks and cut into 5 µm sections at -20°C. Antisera against a number of peptides, which have been identified in the gut, were used. These included those against VIP, substance P,

met-enkephalin, NPY, PHI, somatostatin, bombesin, bovine PP, motilin, neurotensin, secretin, glucagon, gastrin and gastrin inhibitory peptide (GIP). However, only the immunoreactivities of the first seven peptides were detected in the tissues.

Concentrations of the same set of peptide immunoreactivities in the same regions were measured by radioimmunoassay, except for met-enkephalin and PHI, for which radioimmunoassay was not yet available at the time of the study. The identity of the NPY immunoreactivity in the tissues was analysed with HPLC.

8.3. PEPTIDE-IR NERVES IN THE GALLBLADDER AND THE BILIARY DUCTS

Seven types of peptide immunoreactivities were localised to nerves in the specimens. They were VIP, substance P, met-enkephalin, NPY, PHI, somatostatin and bombesin. The first six were seen in most of the regions and the last one was found mainly in the sphincters. The abundance and the arrangement of the immunoreactive nerves were more clearly demonstrated in the whole mount stretch preparations than in the cut sections. Each of the seven types of peptide-IR nerves seemed to have its own characteristic distribution pattern, except that VIP- and PHI-IR nerves were similarly distributed. VIP-, substance P- and NPY-IR nerves appeared to be the most abundant in most of the regions. Somatostatin- and bombesin-IR nerves were much sparser and, sometimes, could be seen only in the whole mount stretch preparations.

VIP-, substance P- and NPY-IR nerves were predominantly localised in the ganglionated plexus and the mucosal plexus (Figs. 74 & 75) and the three nerve types were distinct from each other. A few VIP- and substance P-IR ganglion cell bodies were sometimes found in the ganglionated plexus (Fig. 76) and no NPY-IR ganglion cells were seen. The immunostained nerve fibres often formed dense mesh-like structures, surrounding non-IR ganglion cell bodies (Figs. 77 & 78). Single or bundles of VIP-, substance P- and NPY-IR nerves with few varicosities were commonly seen running along the connecting strands formed by nerve bundles linking separate groups of ganglion cell bodies. The VIP-, substance P- and NPY-IR nerves formed randomly arranged networks with numerous varicosities which were generally uniform in size with occasional larger spindle-shaped ones. Such nerves were also found to surround most of the blood vessels, particularly arteries, in all the regions.

Somatostatin-, PHI- and met-enkephalin-IR nerves were much sparser than the others described above and they were also found mainly in the plexuses (Fig. 79). In addition, somatostatin- and met-enkephalin-IR nerves were occasionally found in the lamina propria and they were not seen to be associated with blood vessels. No ganglion cell bodies were observed to contain these peptide immunoreactivities.

The different peptide-IR nerves were sparse in the muscle layer of the gallbladder, the cystic duct and the hepatic duct. The frequence of these nerves gradually increased as the muscle layer of the common bile duct reached the sphincter and became thicker

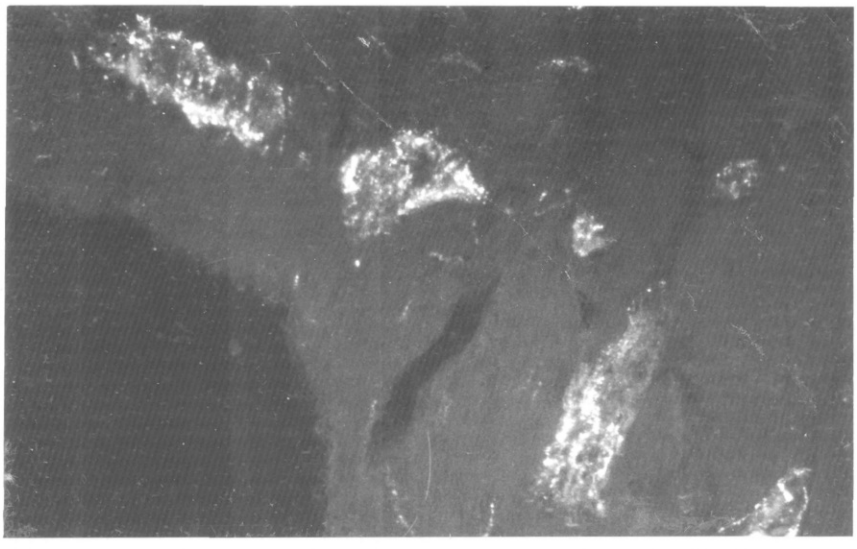


Fig. 74. Substance P-IR nerves in ganglionated plexus of gallbladder. (x150).

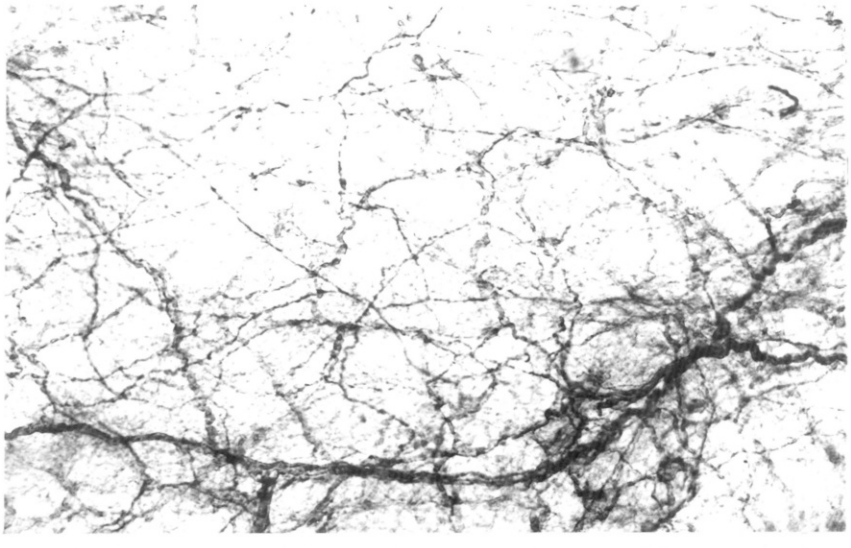


Fig. 75. Networks of VIP-IR nerves in ganglionated plexus of gallbladder (whole mount stretch preparation, PAP method). (x650).

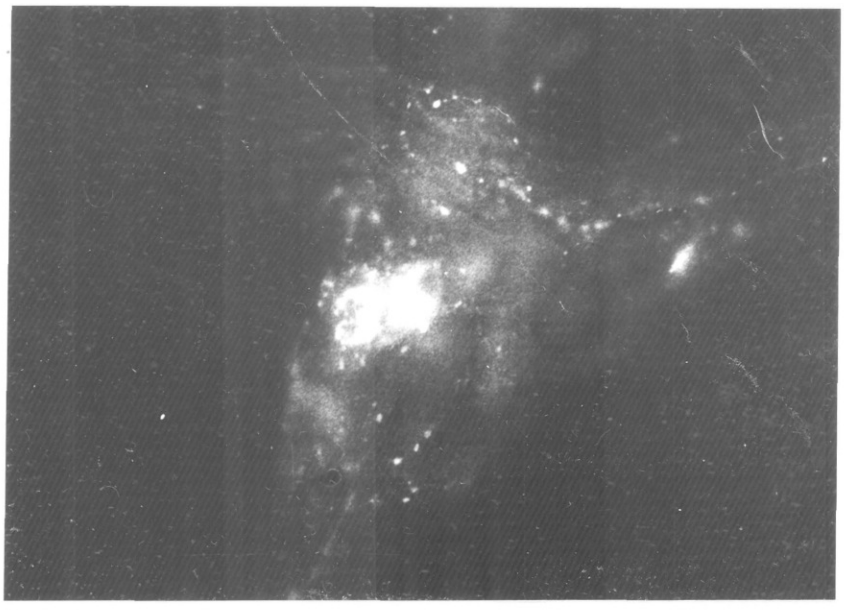


Fig. 76. Substance P-IR neuronal cell bodies in ganglionated plexus of gallbladder (whole mount stretch preparation, IF method). (x400).

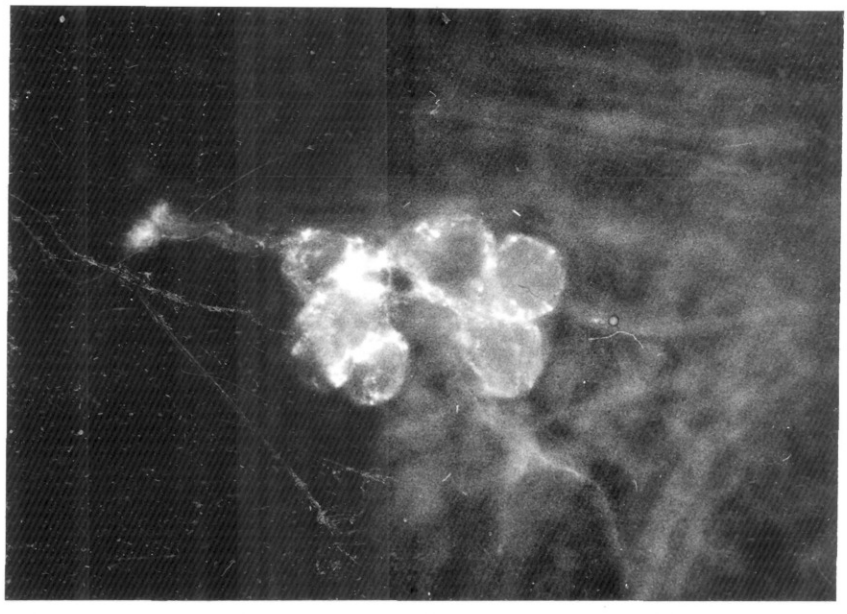


Fig. 77. VIP-IR varicose nerve fibres surrounding some neurons which do not contain the peptide (whole mount stretch preparation, IF method). (x750).



Fig. 78. Substance P-IR varicose nerve fibres surrounding some neurons which do not contain the peptide (whole mount stretch preparation, IF method). (x750).

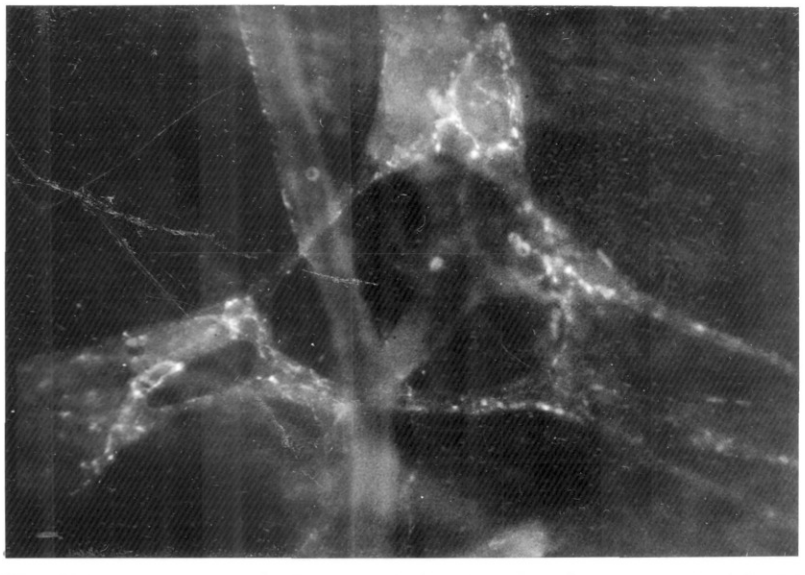


Fig. 79. Nerves containing somatostatin immunoreactivity in ganglionated plexus of gallbladder (whole mount stretch preparation, IF method). (x700).

(Fig.80).

In the sphincter of Oddi, all the six types of immunostained nerves and, in addition, bombesin-IR nerves were present, mainly in the muscle layers. The nearer to the innermost circular muscle the innervation lay, the denser it appeared to be and it was always more dense than that of the adjacent duodenal muscle. Distinct differences between the distribution patterns of the different types of peptide-IR nerves were observed in this regions. VIP-IR nerves were not only concentrated in the muscle bundles (Fig. 81) but also densely distributed in the myenteric and the submucosal plexuses. NPY-IR nerves were abundant and distributed in both the circular and longitudinal muscle (Fig. 82). Substance P-IR nerves were seen quite frequently and ran mainly along the circular muscle. PHI-IR nerves had a distribution pattern similar to VIP but were less numerous. Met-enkephalin- and bombesin-IR nerves were also associated mainly with the muscle cells (Figs. 83 & 84). Somatostatin-IR nerves were the most scarce of all and these were occasionally found in the submucous and the myenteric plexuses as well as between the muscle cells. The circular muscle of the sphincter of the duodenal papilla was found to be the most densely innervated region by, particularly, VIP-IR nerves. In this area, a few scattered VIP-IR ganglion cells were occasionally found lying outside the muscular ring of the duodenal papilla.

VIP, substance P, NPY, somatostatin and bombesin immunoreactivities were also detected by radioimmunoassay at different concentrations in various regions which were in general agreement with the findings

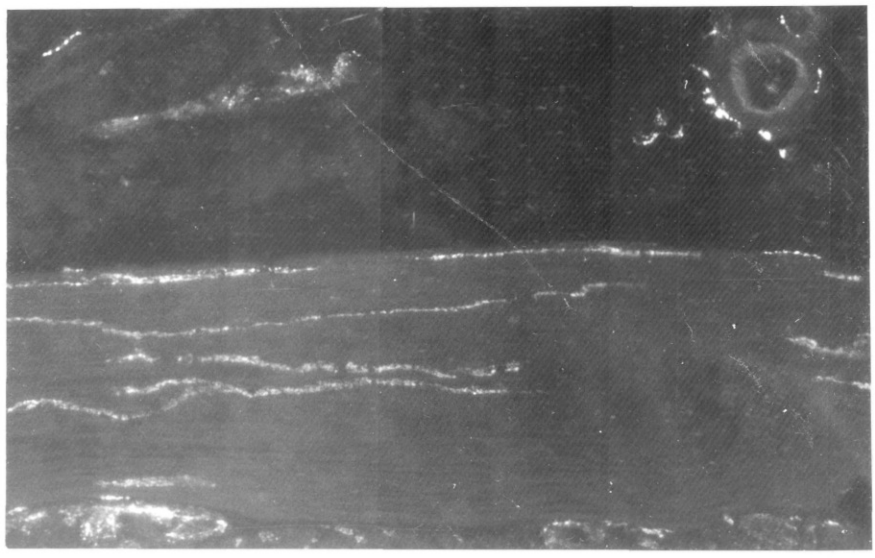


Fig. 80. NPY-IR nerves in the ganglionic plexus, the muscle layer and around a blood vessel in the common bile duct. No ganglion cell bodies were found to contain NPY immuoreactivity. (x 350).

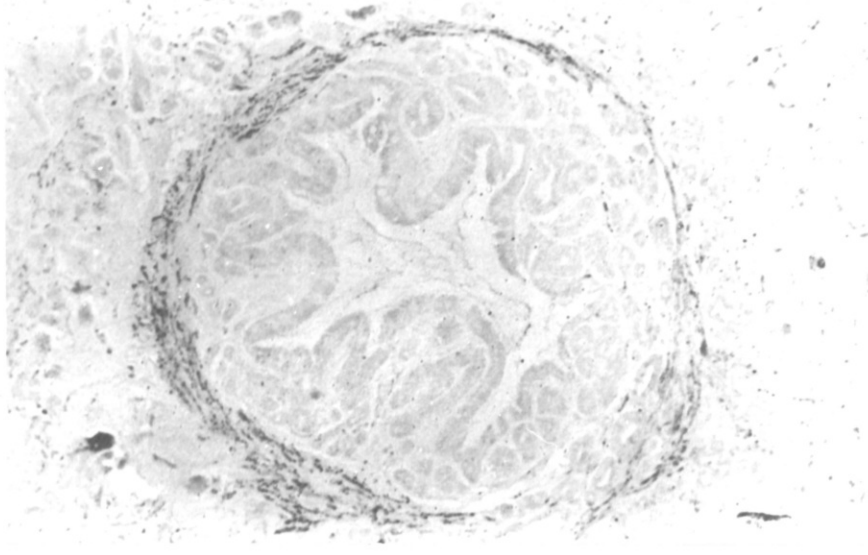


Fig. 81. Dense VIP-IR nerve fibres in smooth muscle of the sphincter of Oddi. (PAP method) (x80).



Fig. 82. NPY-IR nerves densely distributed in the circular and longitudinal muscle layers of the sphincter of Oddi (x300).

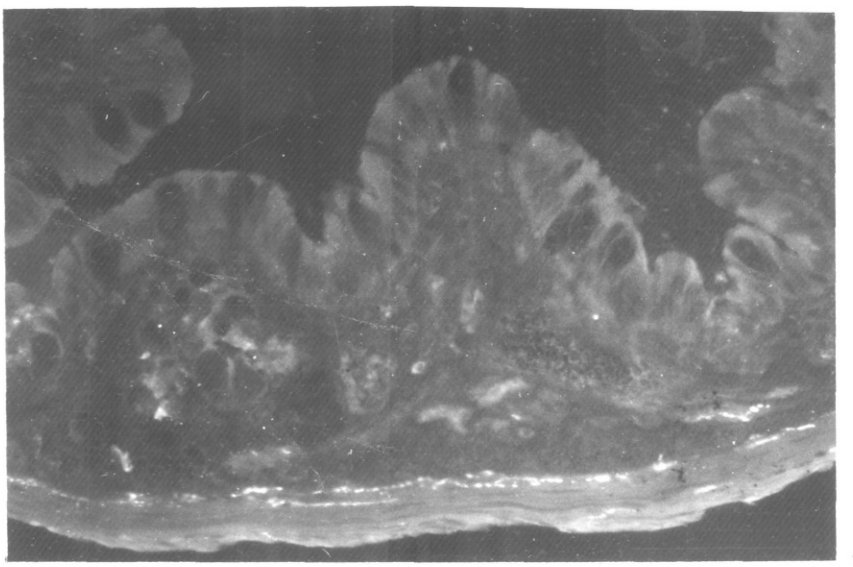


Fig. 83. Met-enkephalin-IR nerves in the muscle layer of the common bile duct adjacent to the sphincter of Oddi. (x400).

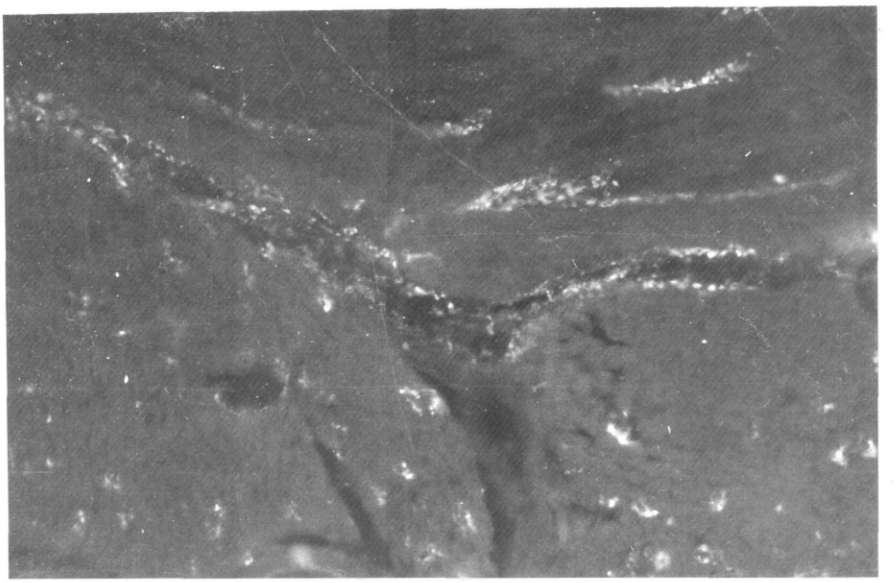


Fig. 84. Bombesin-IR nerves in muscle layer in the sphincter of Oddi. (x475).



Fig. 85. A somatostatin-IR mucosal cell in common bile duct. (x320).

of immunocytochemistry. The radioimmunoassay results are shown in Diagrams 47, 48, 49, 50 & 51.

Chromatographic analysis showed that the NPY immunoreactivity was of the same chemical nature as the original peptide extracted from the brain as it co-eluted in the same position as the porcine NPY standard. Analysis of the extract of the cystic duct revealed the majority to be coeluted at the position of the NPY standard but a small proportion eluted earlier from the column at 36% acetonitrile in the position of porcine PYY standard.

8.4. PEPTIDE-IR MUCOSAL CELLS IN THE BILIARY DUCTS

In addition to peptide-IR nerves, two populations of mucosal cells in the common bile duct, particularly in the portion close to the duodenum, were found to be immunoreactive to the antisera against somatostatin and bombesin respectively.

The somatostatin-IR cells were scattered and mostly present in the duodenal half of the common bile duct with a population of approximately 1.5 cells per 1 mm length of epithelial mucosa in 5 µm thick cryostat sections (Fig. 85). The occurrence of somatostatin-IR cells were also demonstrated by radioimmunoassay, which detected a very high concentration of somatostatin in this region, being 162±30 pmol/g, mean±SEM. The population of bombesin-IR cells was smaller, and these were mainly seen in the epithelium lining the sphincter section of the common bile duct. The

Diagram 47

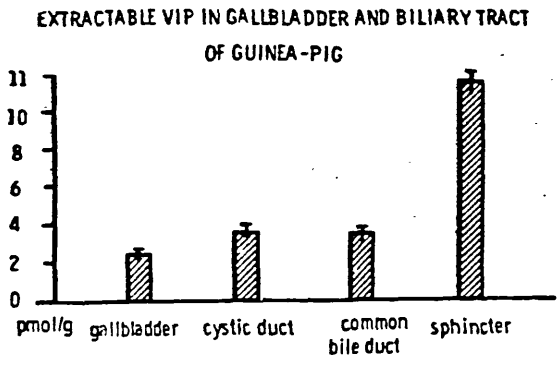


Diagram 48

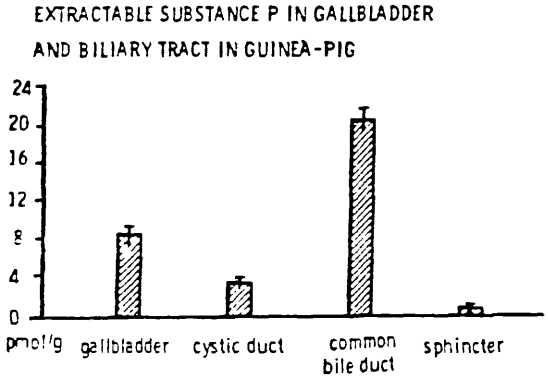
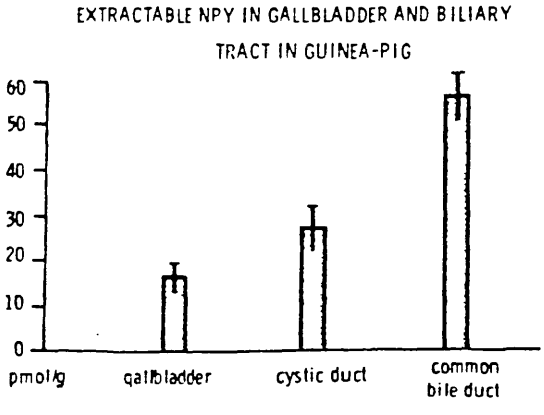


Diagram 49



The shpincter was not assayed for NPY

Diagram 50

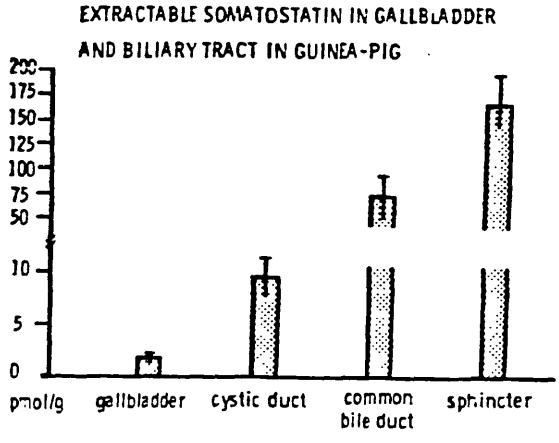
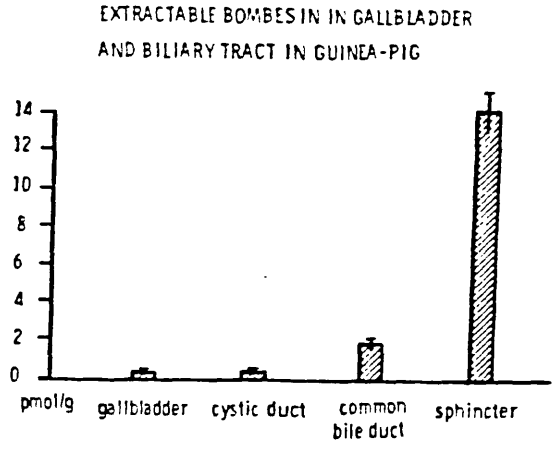


Diagram 51



concentration of extractable bombesin measured by radioimmunoassay in the region of the sphincter was 13.8±2.3 pmol/g, mean±SEM, which was considerably higher than that found in other regions.

8.5. DISCUSSION

In this study, seven peptide immunoreactivities were found in nerves in the gallbladder and biliary tract of the guinea-pig and two of them were also localised to mucosal epithelial cells. This adds new information on the innervation and neuroendocrine cells of the gallbladder and may provide a morphological basis for the previously described non-adrenergic, non-cholinergic neural effect in the gallbladder of guinea-pig (Davison et al 1978). The present findings have shown that the peptide-IR nerves in the gallbladder and the biliary pathways possess a distribution pattern similar to that in the gut (for review see Bishop et al 1982b). The finding of ganglion cell bodies reactive to VIP and substance P antisera is in agreement with the previous reports that some peptide-IR nerves in the alimentary tract have an intrinsic origin (Schultzberg et al 1980; Bishop et al 1982b; Hartman et al 1972; Polak & Bloom 1980; Costa et al 1980). As the storage of peptides in the perikarya is often poor, it is possible that the number and type of observed peptide-IR ganglion cells would be increased if axonal transport blocking agents such as colchicine or vinblastine were applied.

In this study, the peptide-IR nerves seemed to be mingled with the nerves known to contain the classical neurotransmitters described

previously (Onada & Miyazaki 1980; Cai & Gabella 1983). In general, the distribution patterns of peptide-IR nerve fibres in the gallbladder and most of the biliary tracts were similar to those found for the adrenergic innervation, whereas those in the muscle bundles of the sphincters were similar to the cholinergic innervation as regards their density and distribution pattern. The complicated pictures of different types of nerve fibres, ganglion cells and the relationships between them suggest that the gallbladder and the biliary pathways are controlled by a very sophisticated neuronal system which is more complex than had hitherto been thought.

The effects of regulatory peptides on the gallbladder and the biliary ducts have been repeatedly reported (Lonovics et al 1979; Bojornsson et al 1979; Ogoshi et al 1979; Paitras et al 1980; Nakata 1981; Johansson et al 1981; Brennan et al 1982; Jessen 1982). VIP has been shown to relax gallbladder wall and to inhibit gallbladder secretion (Takahashi et al 1982). VIP receptors have also been demonstrated in the gallbladder (Wood et al 1982). Stimulation of vagal nerves caused an increase of VIP concentration in the portal venous and peripheral arterial plasma (Schaffalitzky de Muckadell et al 1977; Dupont et al 1981). The dense network of VIP-IR nerves in various regions found in this study suggests that VIP may be one of the major neuronal components in this neuronal control system. The VIP-IR nerves seen in the lamina propria beneath the columnar epithelium of the gallbladder may be related to the inhibitory effect of VIP on gallbladder secretion (Wood et al 1982a).

The gallbladder and the bile ducts are among the organs where function can be influenced by substance P (Edwards & Bloom 1982). Substance P decreases the output of hepatic bile and amylase in a dose dependent manner. It also inhibits the choleresis induced by CCK or VIP (Powell & Skrabanek 1981).

PHI is not only similarly distributed to VIP, according to recent reports (Wood et al 1982a; Brennan et al 1982), like VIP, it can also potently inhibit gallbladder fluid absorption and relax the gallbladder wall, resulting in a fall in the intraluminal pressure. But in contrast to VIP, PHI does not cause potent vasodilatation (Lundberg & Tatemoto 1982).

As in the previously studied organs, the blood vessels in the gallbladder and the bile ducts are often surrounded by VIP-, substance P- and NPY-IR nerves. Thus the local blood flow may also be monitored by different peptidergic nerves with their often antagonist effects on blood vessel tone, in addition to adrenergic and cholinergic mechanisms.

Somatostatin immunoreactivity has now been demonstrated in both nerves and mucosal cells in the gallbladder and the biliary tract of guinea-pig. In the gut, such somatostatin-IR neuroendocrine cells have been suggested to have a paracrine function mainly because of their long processes extending from the cells and ending on other epithelial cells (Larsson 1981). Such processes however were not seen in the somatostatin-IR cells in the common bile duct in this study. Somatostatin has profound inhibitory effects on a variety of physiological functions including biliary motility and gallbladder

secretion (for review see Patel et al 1981). It has also been found to antagonise the effect of CCK on the gallbladder in man (Nakata 1981).

Little has been known about the actions of NPY, bombesin and met-enkephalin on the biliary organs. The physiological significance of the seven peptides found in nerves and mucosal cells of the gallbladder and biliary ducts awaits further investigations.

8.6. SUMMARY

1. Immunocytochemistry using specific antibodies to seven peptides, i.e. VIP, substance P, NPY, PHI, somatostatin, met-enkephalin and bombesin, localised them to nerves in the gallbladder and the biliary tracts of guinea-pig. Each of the peptides appeared to possess a characteristic distribution pattern, except that VIP- and PHI-IR nerves were similarly distributed. VIP-, substance P- and NPY-IR nerves were found to be the most frequent ones and intrinsic VIP- and substance P-IR ganglion cell bodies were found in the ganglionated plexus. The distribution patterns of peptide-IR nerves in these regions are, in general, similar to those found in the gut.

2. Two populations of mucosal cells were found to immunoreact with the antisera to somatostatin and bombesin respectively. The somatostatin-IR cells were mainly found in the epithelium of the common bile duct. Bombesin-IR cells were present exclusively in the epithelium of the common bile duct towards and within the sphincter portion.

3. Various effects of the peptides on the gallbladder and the biliary tracts have been previously reported. Together with the present findings, it appears that there is a sophisticated neuroendocrinal control system acting upon these organs. The present results provide a morphological basis for further studies in the physiological functions of these peptides in this system.

Chapter 9: GENERAL DISCUSSION

9.1. HOW RELIABLE ARE THE RESULTS ?

The present study encompassed a wide range of subjects with investigations of regulatory peptides and certain brain proteins as their internal linkage. A considerable amount of new information has been obtained. These findings are not only individually significant, but also, when seen in combination, add to our understanding of regulatory peptides and of the diffuse neuroendocrine system in general.

The present findings were dependent on the reliability of the technique of immunocytochemistry. Although the specificity and reproducibility of this technique have been fully established (see Section 2.9.), there are certain limitations that should be noted. Immunocytochemistry is based on the use of specific antibodies which react with appropriate antigens. Each antibody, however, is thought to recognise only a small fragment of the average peptide molecule, usually not more than 7-8 amino acids. The immunostained molecule may well be the peptide sought, or it is equally possible that it may be a molecule which shares common amino acid sequences and similar structural configurations with the investigated substance. This problem can be largely resolved by the technique of chromatographic analysis of the extractable material, which identifies the characteristics of the immunoreactive molecule by comparing its molecular weight or the hydrophobicity of the peptides with that of standard samples. In this study, chromatography was

only carried out on certain occasions, such as when seeking VIP in the human male genitalia or NPY in the human and rodent hearts. Therefore, the positively immunostained elements have been carefully phrased as being peptide-immunoreactive (IR), although in most of the cases it was highly likely that they were indeed the true peptides or proteins being looked for. Great care was taken when dealing with peptides belonging to the same families, i.e. sharing extensive sequence homology, such as VIP and PHI which both belong to the glucagon-secretin family (see Sections 1.5.2. & 1.5.5.). In this study, VIP- and PHI-IR nerves were always similarly distributed throughout the tissues. This suggests that the immunostains obtained with the PHI antiserum might be derived from antisera cross-reaction, ie. the antiserum raised against PHI in fact reacted with a certain part of the VIP molecule, resulting in a similarly or identically distributed but less frequent staining. Keeping this in mind, the possible cross-immunoreactivity of the two antisera was checked by adding synthetic porcine VIP to PHI antiserum and vice versa prior to immunostaining. Positive stainings in this test, comparable to those obtained using unabsorbed antisera, ruled out the possibility of antisera cross-reaction. Furthermore, chromatography detected two different identities of peptide immunoreactivities resembling standard porcine VIP and PHI respectively. Therefore, VIP and PHI immunoreactivities may either be present in two separate nerve types, which were similarly distributed, or occur in identical nerve fibres. In the latter instance, the smaller population of PHI-IR nerves may represent a subpopulation of VIP-IR nerves. Conversely, this type of antiserum absorption test revealed that the so-called APP immunoreactivity

previously reported in the male genitalia and the heart was a result of antisera cross-reaction.

The distinction between positive and negative immunostainings was made with the light microscope which has limited resolution. The non-peptide-IR nerves may contain lower amounts of peptides too small to be visualised by light microscopy. Furthermore, the results obtained from static investigations should be viewed with the functional dynamics in mind, since a given nerve fibre may be negative in one functional state, eg. after peptide release, and positive in another, eg. before peptide discharge. Two such examples might be the increase of substance P-IR nerves after 6-OHDA treatment and increase of VIP-IR nerves following nerve decentralisation in the rat urinary bladder. These increases of peptide-IR nerves might have resulted from accumulation of peptides in previously non-staining nerves which in fact contained 'invisible' amounts of peptides in their cytoplasm.

9.2. TWO APPROACHES TO ONE PURPOSE

In this study, the existence of peptide-IR nerves in a given tissue, revealed by immunocytochemistry, was mostly supported by findings obtained from radioimmunoassay of extractable tissue. The frequency of peptide-IR nerves found by immunocytochemistry and the concentrations of peptide immunoreactivities detected by radioimmunoassay were in general agreement with each other. However, this is not always the rule, since it is possible that

certain peptides may be widely distributed in a large number of nerves at very low concentrations which are immunocytochemically undetectable. One such example might be somatostatin immunoreactivity in the human urinary bladder, where the concentration of somatostatin immunoreactivity was 2.6 pmol/g in the trigone, whilst no corresponding immunoreactive nerves could be spotted. Conversely, it is equally conceivable that a small amount of peptide may be accumulated in a few nerve fibres, and although the overall tissue concentration is too low for radioimmunoassay measurement, it may be relatively easy for immunocytochemistry to detect the nerves. This was reflected by substance P immunoreactivity in the human penile glans, where the concentration of substance P detected by radioimmunoassay was only 0.72 pmol/g but a small number of substance P-IR nerve fibres were brightly immunostained. Moreover, any discrepancy between the results of immunocytochemistry and radioimmunoassay could be attributed to the different natures of these two techniques, each selecting antibodies according to its own criteria. Antibodies which are considered to be suitable for immunocytochemistry are not only highly specific but also they possess a strong binding force to the antigens so that they can survive the vigorous washing during immunostaining. Such antibodies may not be particularly desirable for the technique of radioimmunoassay, which is based on the competitive binding of labelled and unlabelled antigens (or antibodies) to specific antibodies (or antigens). Therefore, it should not be a suprise if the results obtained by immunocytochemistry and radioimmunoassay were not always closely matched. Fortunately, this problem was not

frequently encountered in the present investigation, and the compensatory and mutually supportive natures of these two techniques for studying the distribution and localisation of peptides in the investigated peripheral organs has been convincingly illustrated. Nevertheless, immunocytochemistry enjoys the advantage of visualising the precise histological locations of the tissue antigens, and played a leading role in the present study.

9.3. RELATIONSHIPS BETWEEN PEPTIDE-IR NERVES AND CLASSICAL NERVE TYPES

The peptide-containing nerves were once thought to be completely separate from those containing ACh or catecholamine and were thus regarded as the third division of the autonomic nervous system (Baumgarten et al 1970). This is no longer valid as the co-storage of peptides and classical neurotransmitters has been frequently reported (for review see Lundberg et al 1982a). The co-storage was also reflected in this study by the way the peptide-IR and the cholinergic or adrenergic nerves were often found to closely follow each other's distribution pattern. One convincing example was that the majority of ganglion cells in the paracervical ganglia of rat were VIP-IR and most of the cells in these ganglia have been known to contain ACh (Thorbert et al 1977). Therefore, in these neurons at least, certain co-storage was evident. More direct proof was provided by electron microscopical investigation of the human penis where the p-type granules and the cholinergic type granules were present in the same nerve terminals.

The possibility therefore arises that all peptides are co-stored with classical neurotransmitters and no pure peptide-containing nerves exist. The present findings, however, seem to disagree with this view. For example, in the human penis and the prostate, certain nerves and ganglion cells were immunoreactive to VIP antiserum while they showed neither AChE activity nor DβH immunoreactivity in consecutive sections. In these regions, electron microscopy demonstrated some nerve terminals packed with p-type granules alone. Thus, the co-storage appears not to be ubiquitous. Another example is the rat urinary bladder, where following the destruction of adrenergic nerves by 6-OHDA, only the vascular NPY-IR nerves were decreased but not the non-vascular ones. This suggested that the co-storage of NPY and noradrenalin was partial and held true only for the vascular noradrenergic fibres.

The present findings suggest that the picture of innervation in each peripheral organ is far more complex than that described in the textbooks a few years ago. There may be pure cholinergic, adrenergic, peptidergic or other nerve types and also nerves containing combinations of different peptides and classical neurotransmitters. Different nerve types may run in the same fasciculi nerve bundles and synapse with each other. They may originate from ganglion cells in the central nervous system or from ganglia located far from the target organs, as with substance P-IR nerves in the spinal cord. Their neuronal cell bodies may be sited very close or intrinsic to the innervated organs, such as VIP-IR

nerves in the genitourinary tract. At the light microscopical level, they may even switch from one nerve type to another, by accumulating or discharging certain chemical contents in the cytoplasm corresponding to the dynamic functional state of the organ in question. Overall, the histological design of the peripheral nervous control system appears to give no easy fit with the seemingly oversimplified concept of the bipartite autonomic nervous system.

9.4. ACTIONS OF NEUROPEPTIDES

Assessment of the actions of the neuropeptides is beyond the scope of the present study. However, the distribution patterns of these peptides are indicative of their possible roles. The present findings point to a multiple function for the peptides, that is to say each peptide may not only be different from other peptides in its actions but may also exert different effects in different locations. For example, VIP present in nerves relating to non-vascular and vascular smooth muscle in the urinary bladder may relax the smooth muscle, as has been shown by Levin & Wein (1981) on the rabbit detrusor muscle, whereas VIP occurring in the nerve fibres beneath the epithelium of the same bladder may subserve a sensory function, since, according to previous studies, there were only sensory nerves in this region (Gosling & Dixon 1974; El-Badawi 1982). The co-ordination of different peptide activities was also indicated in the finding in this study that different peptide-IR nerve types often innervate the same target tissue structure. For

example, the same blood vessels were surrounded by VIP-, NPY-, PHI- and substance P-IR nerves. The action of peptides may be additive or antagonistic to each other in regulating local blood flow.

Functions other than neurotransmitter and neuromodulator ought to be sought for the peptides, particularly for substance P. The possibly specific increase of substance P immunoreactivity following adrenergic denervation by 6-OHDA in the rat urinary bladder found in this study, as well as in the brain and the eye found by other workers independently, gave a strong hint to a possible trophic effect of this peptide on adrenergic innervation.

9.5. THE PATHOLOGICAL STUDIES

Apart from the tumours of the skin, three pathological changes were established in the present investigations, i.e. the decrease of VIP-IR nerves in the impotent penis, the decrease of VIP-IR nerves in the unstable bladder and the increase of S-100 immunoreactivity in the dysfunctional bladder from multiple sclerosis patients. These findings, although preliminary, represent the first attempt to study the pathological involvement of regulatory peptides and relevant substances in the peripheral organs investigated. The changes in peptide content might be specific for the peptide, for example, possibly, VIP in unstable bladder, or as part of a more general lesion, such as VIP in the impotent penis. Nevertheless, the causes and results of changes in the regulatory peptide levels should be taken into consideration if the pathological mechanisms

underlying these disorders are to be elucidated. In addition, information on the distribution of peptides and relevant substances in the normal state provides a comparative morphological basis for further attempts to examine abnormalities of these elements in various pathological conditions.

9.6. CONCLUDING REMARKS

A substantial number of original findings has been made during the present study. These are mainly based on the examination of the distribution of regulatory peptides and relevant neuronal proteins in a number of peripheral organs in both health and disease, of which previous knowledge had been very fragmentary. The findings of the present study add to the knowledge of the morphology of the investigated peripheral organs and of the diffuse neuroendocrine system as a whole. As a result, the gap between the knowledge of the histology and pathology of peptide-containing elements in the gut and that of other peripheral organs, such as the genitourinary tracts, the heart and the skin, has been narrowed or filled in.

It has been found that many peripheral organs are heavily innervated by nerves containing different regulatory peptides whose distribution patterns give hints to their possible functions, although many of those functions remain to be confirmed or discovered. The present study revealed the ubiquity of the regulatory peptide-containing nerves and neuroendocrine cells in the body of different mammalian species and highlighted the

significance of these peptides in neural or neuroendocrine control. The findings of this study established that morphological changes in peptide-containing elements occur in certain disorders, of which the pathogenic mechanisms had been poorly understood. This study also revealed how little was known about these control systems in comparison with their potential importance in regulating bodily functions. It appears that we are now at the stage of accumulating facts, which will contribute to a better understanding of the physiological and pathological significance of these peptides, and eventually lead to the revealing of their functional roles, which have been set up by natural selection during evolution.

Appendix 1.1.

TISSUES FROM POSTMORTEM

Ref. No.	Age	Sex	Cause of death	time length between death and dissection
H81/589	72	F	Carcinoma of colon	6 hours
H81/821	73	M	Carcinoma of pancreas	8 hours
H81/853	60	M	Perforated duodenal ulcer	6 hours
H82/39	50	F	Carcinoma of breast	10 hours
H82/489	18	M	Muscular dystrophy	6 hours
H82/491	83	F	Necrotizing enterocolitis	9 hours
H82/601	88	F	Cardiovascular accident	10 hours
H82/799	60	M	Infarcted small bowel	8 hours

Appendix 1.2.

DETAILS ON BLADDER BIOPSY TISSUES

Key: S.I.=stress incontinence. U.I.=urge incontinence. P.O.=post operative. S.W.=sphincter weakness. M.S.=multiple sclerosis. I.C.=interstitial cystitis. S.F.=sensory frequency. B.N.O.=bladder neck obstruction. C.R.=chronic retention.

Ref. No.	sex	age (years)	diagnosis	/ status	*area sampled
81/439	f	56	S.I.	/idiopathic unstable	1
81/465	f	31	normal	/stable	1
81/466	f	38	spinal compression	/acontractile	1
81/467	f	24	U.I.	/idiopathic unstable	1
81/500	f	47	M.S., U.I.	/unstable	1
81/513	f	47	S.I.	/idiopathic unstable	1
81/560	f	57	U.I.	/idiopathic unstable	1
81/561	m	59	S.W., S.I.	/stable	1
81/562	m	58	S.W., S.I.	/stable	1
81/581	m	40	M.S.	/acontractile	1
81/582	f	32	M.S., U.I.	/unstable	1
81/595	m	54	normal	/stable	1
81/596	m	75	S.W.	/stable	1
81/597	m	69	C.R., M.S.	/acontractile	1
81/598	f	50	unknown	/acontractile	1
81/675	f	31	M.S., U.I.	/unstable	1
81/676	f	36	U.I., S.I.	/idiopathic unstable	1
81/756	f	67	U.I.	/idiopathic unstable	1
81/841	m	45	U.I.	/idiopathic unstable	1
81/842	f	58	I.C., S.F.	/stable	1
82/44	f	14	spina bifida	/acontractile	1
82/45	f	33	U.I.	/idiopathic unstable	1
82/214	f	33	S.I.	/idiopathic unstable	1
82/215	m	71	normal	/stable	1
82/216	m	50	normal	/stable	1
82/217	f	40	normal	/stable	1
82/218	f	42	normal	/stable	1

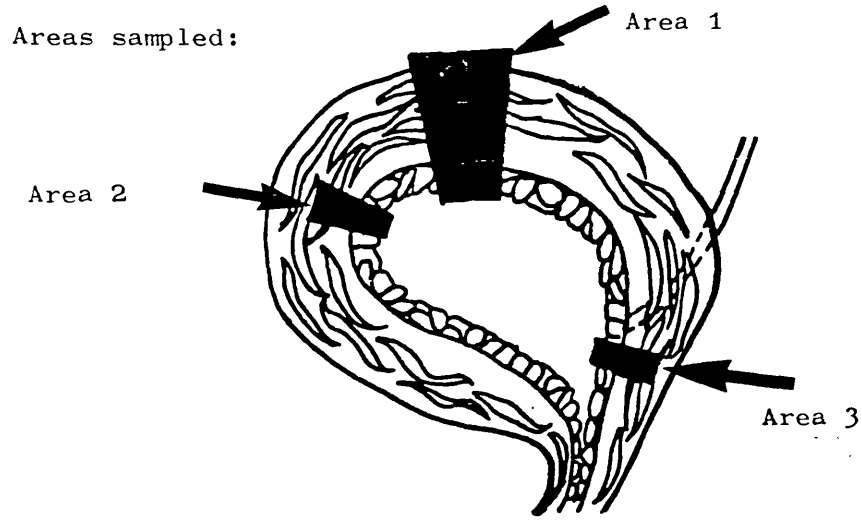
Ref. No.	sex	age (years)	diagnosis	/ status	*area sampled
82/219	m	51	brain trauma, sphincter dysergia	/unstable	1
82/220	f	37	denervated	/acontractile	1
82/221	m	64	S.W., S.I.	/stable	1
82/235	m	58	B.N.O.	/unstable	1
82/236	f	46	U.I.	/idiopathic unstable	1
82/237	f	19	spina bifida	/acontractile	1
82/319	f	54	U.I., S.I.	/idiopathic unstable	1
82/320	f	34	M.S., U.I.	/unstable	1
82/569	f	75	S.I.	/stable	2
82/570	m	40	paraparesis, urethral stricture	/unstable	2
82/571	f	65	S.I.	/stable	2
82/572	f	57	U.I.	/idiopathic unstable	2
82/573	m	61	S.W., S.I.	/stable	2
82/574	f	39	U.I.	/idiopathic unstable	2
83/586	f	5	congenital chicken box	/unstable	2
82/777	f	59	S.I.	/stable	2
82/778	f	74	normal	/stable	2
82/779	m	19	normal	/stable	2
82/780	m	78	normal	/stable	2
82/781	m	86	normal	/stable	2
82/782	m	69	normal	/stable	2
82/788	f	39	S.I.	/stable	2
82/789	m	65	normal	/stable	2
82/790	f	60	U.I.	/idiopathic unstable	2
82/13	m	61	S.W., S.I.	/stable	2
83/14	f	40	denervated	/unstable	2
83/15	f	68	I.C., S.F.	/stable	2
83/16	m	37	B.N.O.	/unstable	2
83/17	f	46	denervated, S.I.	/unstable	2

Ref. No.	sex	age (years)	diagnosis	/ status	*area sampled
83/70	f	60	B.N.O.	/unstable	2
83/71	m	56	Shy Drager Syndrome	/unstable	2
83/72	f	29	denervated	/unstable	2
83/73	f	68	I.C., S.F.	/stable	2
83/85	f	45	U.I.	/idiopathic unstable	2
83/86	m	55	B.N.O.	/unstable	2
83/87	m	56	Diabetic, B.N.O.	/unstable	2
83/112	m	40	M.S., U.I.	/unstable	2
83/113	m	38	U.I.	/idiopathic unstable	2
83/114	m	68	B.N.O.	/unstable	2
83/115	f	26	U.I.	/idiopathic unstable	2
83/116	m	33	S.F.	/stable	2
83/127	m	40	normal	/stable	2
83/128	m	50	normal	/stable	2
83/129	m	65	urethral stricture	/stable	2
83/143	f	38	normal	/stable	2
83/144	f	38	renal calculi	/stable	2
83/145	m	44	M.S., U.I.	/unstable	2
83/146	f	56	M.S., U.I.	/unstable	2
83/177	m	61	prostatic carcinoma	/stable	2
83/178	m	62	normal	/stable	2
83/179	m	69	B.N.O.	/stable	2
83/180	m	77	U.I.	/idiopathic unstable	2
83/181	m	72	B.N.O.	/unstable	2
83/231	f	55	S.I.	/stable	2
83/232	f	44	S.I.	/stable	2
83/239	f	49	S.I.	/stable	2
83/240	f	28	S.I.	/stable	2
83/241	m	41	spinal trauma, U.I.	/unstable	2
83/242	m	79	S.W., S.I.	/stable	2
83/313	m	38	U.I.	/idiopathic unstable	2
83/314	f	23	S.I.	/stable	2
83/315	f	62	paraplegic	/unstable	2
83/316	f	56	U.I.	/idiopathic unstable	2

Ref. No.	sex	age (years)	diagnosis	/ status	*area sampled
83/317	f	69	U.I.	/idiopathic unstable	2
83/461	f	69	U.I.	/idiopathic unstable	2
83/462	m	65	haemauria	/stable	2
83/463	f	46	S.I.	/stable	2
83/464	f	43	S.I., U.I.	/idiopathic unstable	2
83/466	f	47	U.I.	/idiopathic unstable	2
83/526	m	11	spina bifida	/unstable	2
83/527	m	33	urethral stricture	/stable	2
83/528	m	42	B.N.O., U.I.	/unstable	2
83/529	m	5	spina bifida	/unstable	2
83/576	m	26	U.I.	/idiopathic unstable	2
83/577	f	32	U.I.	/idiopathic unstable	2
83/587	m	28	U.I.	/idiopathic unstable	2
83/588	f	69	U.I.	/idiopathic unstable	2
83/589	f	68	S.I.	/stable	2
83/645	f	56	S.I.	/stable	2
82/32	m	26	U.I., M.S.	/unstable	2,3
82/34	m	59	U.I., M.S.	/unstable	2,3
82/35	m	48	S.F.	/stable	2,3
82/74	f	48	S.I., S.F.	/stable	2,3
82/76	f	39	S.F.	/stable	2,3
82/114	f	34	S.F.	/stable	2,3
82/163	f	24	S.F.	/stable	2,3
82/164	m	36	U.I., M.S.	/unstable	2,3
82/166	f	47	S.I.	/stable	2,3
82/167	m	68	U.I.	/idiopathic unstable	2,3
82/168	m	41	S.F.	/stable	2,3
82/169	f	54	S.F.	/stable	2,3
82/170	m	70	S.I., P.O.	/stable	2,3
82/189	f	60	U.I.	/idiopathic unstable	2,3
82/190	f	70	S.F.	/stable	2,3
82/193	m	17	U.I., M.S.	/unstable	2,3
82/200	f	32	S.F.	/stable	2,3

Ref. No.	sex	age (years)	diagnosis	/ status	*area sampled
82/201	f	64	systemic smooth muscle dysfunction	/unstable	2,3
82/202	m	37	bladder carcinoma	/stable	2,3
82/203	f	55	S.I.	/stable	2,3
82/224	m	57	U.I.	/idiopathic unstable	2,3
82/233	m	73	B.N.O., P.O.	/unstable	2,3
82/288	m	78	B.N.O.	/unstable	2,3
82/323	m	22	U.I., enuresis	/idiopathic unstable	2
82/324	m	22	U.I., P.O.	/unstable	2
82/369	m	81	U.I., P.O.	/unstable	2
82/370	m	43	U.I., M.S.	/unstable	2
82/557	f	26	C.R., M.S.	/acontractile	2
82/558	m	25	U.I., enuresis	/idiopathic unstable	2
82/559	m	34	U.I., enuresis	/idiopathic unstable	2
82/578	f	48	S.F.	/stable	2
82/579	f	40	U.I., enuresis	/idiopathic unstable	2
82/605	f	75	C.R., M.S.	/acontractile	2
82/632	f	25	S.F.	/stable	2
82/645	f	21	U.I., enuresis	/idiopathic unstable	2
82/646	f	19	U.I., enuresis	/idiopathic unstable	2
82/699	f	78	P.O.	/unstable	2
82/705	m	27	enuresis	/idiopathic unstable	2
82/706	m	25	enuresis	/idiopathic unstable	2
82/707	m	30	enuresis	/idiopathic unstable	2
82/712	m	32	U.I., enuresis	/idiopathic unstable	2
82/713	m	35	U.I., enuresis	/idiopathic unstable	2
82/714	f	44	C.R., M.S.	/acontractile	2
82/751	f	17	U.I., enuresis	/idiopathic unstable	2
82/752	f	25	U.I., enuresis	/idiopathic unstable	2
82/766	f	33	S.F.	/stable	2
82/783	m	26	U.I., enuresis	/idiopathic unstable	2
82/786	m	34	chronic cystitis	/unstable (mild)	2
83/55	m	33	U.I., brain injury	/unstable	2
83/56	m	33	enuresis	/idiopathic unstable	2

Ref. No.	sex	age (years)	diagnosis	/ status	*area sampled
83/57	m	33	enuresis	/idiopathic unstable	2
83/83	m	18	U.I., enuresis	/idiopathic unstable	2
83/84	m	17	U.I., enuresis	/idiopathic unstable	2
83/91	m	18	U.I., enuresis	/idiopathic unstable	2
83/92	m	41	normal	/stable	2
83/120	m	27	spina bifida	/unstable	2
83/158	m	34	enuresis	/idiopathic unstable	2
83/159	m	34	enuresis	/idiopathic unstable	2
83/187	m	24	U.I., enuresis	/idiopathic unstable	2
83/200	m	17	U.I., enuresis	/idiopathic unstable	2
82/581	m	74	U.I., B.N.O.	/unstable	2,3
82/611	m	74	Parkinsonism	/unstable	2,3
82/625	f	49	U.I., M.S.	/unstable	2,3
82/626	f	78	U.I.	/idiopathic unstable	2,3
82/773	f	44	normal	/stable	2,3
82/774	m	36	normal	/stable	2,3
83/96	f	24	normal	/stable	2,3
83/97	f	55	normal	/stable	2,3
83/104	f	60	U.I., M.S.	/unstable	2,3
83/105	f	53	U.I., M.S.	/unstable	2,3
83/123	f	63	U.I., M.S.	/unstable	2,3
83/124	f	45	U.I., M.S.	/unstable	2,3
83/153	f	47	U.I., M.S.	/unstable	2,3



Appendix 1.3.

DETAILS OF PATIENTS WITH PENILE IMPOTENCE

Ref No.	age (years)	total/partial impotence	duration (years)	clinic history	classification
81/775	62	total	3	non-insulin required diabetes, hyperprolactinaemia	?diabetic ?hyperprolactic
81/720	50	weak	2	after circumcision, insulin required diabetes	diabetic ?psychogenic
82/794	52	total	3	insulin required diabetes	diabetic
83/151	52	total	4	insulin required diabetes	diabetic
82/115	45	total	2	pelvic fracture, diabetes	trauma ?diabetic
82/226	62	partial	3	slow onset	idiopathic
82/297	61	total	7	hypertension	hypertensive ?psychogenic
82/382	49	total	3	priapism	priapism
82/388	54	partial	4	steroids for allergy, myopathy, hypertension	hypertensive steroids psychogenic
82/389	61	partial	2	hypertension, β-blockers	hypertensive
82/397	59	partial	3	chordee	idiopathic ?psychogenic

Ref No.	age (years)	total/partial impotence	duration (years)	clinic history	classification
83/186	52	partial	2	penile blood pressure drop	idiopathic ?vascular
83/293	57	total	37	pelvic fracture, Two neuroplasties	trauma
83/80	63	weak	6	Peyronie's disease	Peyronie's
83/381	64	weak	4	Peyronie's disease	Peyronie's
83/359	38	weak	3	Peyronie's disease	Peyronie's
83/354	60	total	5	insulin required diabetes	diabetic
83/570	23	partial	5	insulin required diabetes	diabetic
83/579	43	total	6	insulin required diabetes	diabetic
83/584	52	total	4	insulin required diabetes	diabetic

Appendix 1.4.

DETAILS OF TISSUES OF HUMAN FEMALE GENITALIA

Key: L=leiomyoma, P.E.=proliferative endometrium, A=adenomyosis,
O.E.=ovarian endometrioma, M=menorrhagia,
I.C.=irregular cycle, D=dysmenorrhoea, P=prolapse
P.M.B.=post-mentrual bleed.

Ref No.	Ages (years)	Symptoms	Length after last menstrual	Pre-treatment	Histopathology
83/101	44	M.	day 12	no	L., P.E.
83/157	44	M.	day 17	no	L., P.E.
83/210	36	M., I.C., D.	day 3	Norethisterone, Progesterone pessaries	No abnormality
83/126	40	M., I.C., D.	day 6	no	L., Secondary carcinoma from breast, O.E.
83/189	48	no	day 2	no	L., P.E.
83/350	40	P.	day 14	no	No abnormality
83/459	45	M., D.	day 21		L., A.
83/471	43	M., I.C.	day 32	no	No abnormality
83/473	42	M., D.	day 10	no	L., O.E.
83/470	42	M., D.	day 3	Norethisterone	L., O.E.
83/500	31	M., I.C., D.	day 17	no	No abnormality
83/435	39	M., D.	day 9	no	L.
83/335	36	M.	day 16	no	L.

Ref No.	Ages (years)	Symptoms	Length after last menstrual	Pre-treatment	Histopathology
83/458	43	M., I.C., D.	day 21	no	Chronic cervicitis, Normal myometrium
83/524	39	M.	day 16	no	L.
83/543	46	M., D.	day 3	no	A.
83/563	48	M.,	day 6	Primolut N	L.
83/564	41	P.	day 13	no	No abnormality
83/575	43	M.	day 11	no	Fibroids
83/325	40	M.	day 24	no	L.
83/126	40	M., I.C., D.	4 months	Medroxy progesterone acetate	Adenocarcinoma, O.E.
83/300	45	M., I.R.	4 months	no	L.
83/377	72	P.	21 years	no	Atrophic change, L.
83/99	83	P., P.M.B.	30 years	no	Atrophic change, L.
83/209	69	P.	19 years	no	No abnormality
83/139	63	P., P.M.B.	15 years	no	L.

Appendix 1.5.

DETAILS OF MERKEL CELL TUMOURS

Ref no.	age	sex	site of tumour	size of tumour at resection (diameter in cm)
	55	F	leg	2
	70	F	left leg	NA
	56	F	anterior right arm	1
	78	F	left leg	4
	74	F	neck	2
	76	F	left cheek	2-3
	77	F	anterior and interior to the left ear	2-3
	84	M	right knee	3
	63	M	left leg	1
	82	M	left cheek	massive facial involvement
	60	M	Inguinal lymph node metastasis	NA

NA: information not available.

Appendix 1.6.

MELANOMAS AND CONTROL TUMOURS STUDIED

Melanoma		number
Melanotic	primary	28
	secondary	22
Amelanotic	primary	5
	secondary	3
Naevi		
Intradermal		4
Compound		1
Blue		2

Control tumours	number
Undifferentiated Ca	2
Small cell Ca (bronchus)	1
Merkel cell tumour	11
Squamous cell Ca	3
Spindle cell sarcoma	2
Adenocarcinoma	1
Solar keratosis with CIS	1
Lymphoma	9
Basal cell papilloma	1
Dermal fibroma	1
Condyloma acuminatum	2
Basal cell carcinoma	5
Normal skin (various sites)	20

APPENDIX 2

2.1. FIXATIVES AND SOLUTIONS

2.1. 0.01M Phosphate-buffered normal saline pH 7.1 (PBS)

Used for washing sections during immunostaining and in BQS technique and buffered formalin.

NaCl 8.7gms)

KH_2PO_4 0.272gms) made up to 1 litre with distilled water.

Na_2HPo_4 1.136gms)

0.01 % bovine serum albumin (BSA) was added when used as antiserum diluent

2.1.2. PBS Sucrose

For storage of BQS (see below) fixed tissue at 4°C.

7-20% sucrose in PBS with 0.01% sodium azide.

The solution was freshly made up every week and the tissues were stored in the solution for not more than a month.

2.1.3. Formol saline (buffered) mainly for conventional histology

Commercial formalin (36%)	10ml
PBS	90ml

2.1.4. Bouin's

For conventional histology and can be used for some immunostains.

Sat. aqu. Picric acid	75ml
Formaldehyde	25ml
*Acetic acid	1ml

*Acetic acid was added just before the use of the fixative.

For immunostaining methods 5% acetic acid was used.

2.1.5. Benzoquinone solution (BQS)

For fixing tissue for immunocytochemistry

0.4% solution of purified benzoquinone (BQ) in PBS.

The solution was prepared freshly and not allowed to warm up or to be exposed to light as both procedures will oxidise the benzoquinone, rendering it unsuitable for subsequent fixation.

2.2. FIXATION PROCEDURES FOR IMMUNOCYTOCHEMISTRY

2.2.1. BQS fixation (after Bishop et al 1978)

a). 0.4% BQS was freshly prepared just before fixation allowing a tissue to solution volume ratio of approximately 1:10, using specimens no larger than $2x2x2cm^3$.

b). Tissue was fixed by immersing it in the solution for the appropriate time period, normally 0.5-2 hours, according to the size and permeability of the tissue, at 4°C in the dark.

c). Tissue was transferred to PBS sucrose and stored at 4°C for not more than a month. The fixed tissue was made into a cryostat block as soon as possible after washing overnight in PBS sucrose.

2.2.2. Tissue frozen in Arcton (or isopentane) for immunocytochemistry

a). A beaker containing Arcton was suspended briefly in liquid nitrogen to cool and allow the Arcton to freeze so that half the volume was solid and half liquid.

b). Fresh tissue was dissected into small pieces, not more than $1cm^2$

in area and 0.3 cm thick, and then placed on a nickel plated spatula, as near the tip as possible. Tissue was kept away from the Arcton and liquid nitrogen during dissection. Unfrozen tissue was handled with warm (room temperature) forceps and frozen tissue was always handled with forceps cooled in liquid nitrogen. Sudden change in tissue temperature would cause artifacts in the tissue structure.

c). To freeze the tissue, the spatula was plunged quickly into the Arton so that the specimen was completely submerged.

d). The frozen tissue was transferred to a freeze-dryer (Edwards EPD3). During storage, the tissue was not allowed to reach a temperature higher than -40°C.

2.2.3. Benzoquinone vapour (BQV) and formaldehyde vapour (FV) fixations (after Pearse & Polak 1975)

a). The frozen tissue was dried in a freeze-drier overnight at -40°C under vacuum.

b). The dried tissue was fixed in BQV (from re-crystallised BQ dissolved in toluene, 0.3-0.5 gm BQ/3ml) in a closed vessel at 60°C for three hours or in FV at 80°C for one hour.

c). Impregnation with Wax (Ralwax 1 obtained from R.A.Lamb) for 1 hour or more under vacuum at 60-65°C. Blocking out was done in the same type of wax.

2.3. PREPARATION OF SECTIONS

2.3.1. Making of cryostat blocks

a). BQS fixed tissue was blotted gently with absorbent papers before

blocking because excess water in the block causes ice crystal damage.

b). The tissue was stuck on to a cork by OCT compound, a special freezing glue. The blocks were orientated with the face to be cut uppermost.

c). Inverted blocks were dropped into precooled Arcton (isopentane) and allowed to freeze for a few minutes.

d).The cryostat blocks were stored in liquid nitrogen or -70°C deep freezer.

2.3.2. Section preparations

a). The paraffin wax blocks were cut into 5-10μm thick sections on a Reichert Microtome

b). The cryostat blocks were cut into 5-8μm thick sections on a cryostat cutting machine (SLEE fitted with Cambridge Rocking Microtome) at -20°C.

c). The sections were mounted on glass slides precoated with poly-L-lysine (PLL). The PLL used was of 36,000 dalton and at 0.01%. The PLL coating ensures section adhesion without interfering with immunostaining.

2.4. STAINING METHODS

2.4.1. Triton X-100 treatment

The tissue sections were sometimes soaked in 0.2% Triton X-100 in PBS for 30 mins to increase the permeability of the tissue to the subsequently applied antibodies.

2.4.2. Dewaxing

The paraffin sections were dewaxed in xylene and then passed through graded alcohols to water and PBS.

2.4.3. Haematoxylin & eosin (H&E) staining

a). Sections were stained in 25% haematoxylin in distilled water for 1-10 minutes.

b). Washed in tap water to remove the excess stain.

c). staining was quickly (a few seconds) differentiated in 1% hydrochloric acid in 70% alcohol for 2-3 minutes.

d). Washed in running tap water for 5-10 minutes.

e). Stained in 0.5% eosin in 70% alcohol for 2-3 minutes.

f). Washed briefly in running tap water.

g). Dehydrated through graded alcohols.

h). Cleared in xylene.

i). Mounted on glass slides with DPX and covered with coverslip.

2.4.4. Indirect immunofluorescence staining procedure (after Coons et al 1955)

a). Sections were placed on racks in covered Petri dishes containing water to create a damp chamber and avoid evaporation of the antiserum. A drop of first layer antiserum (raised in rabbits), appropriately diluted in 0.01M PBS/BSA (see 1.1.), pH 7.2, was applied to the section with a Pasteur pipette and was left overnight at 4°C or at room temperature. Only the smallest amount of antiserum was applied to the section, ie. enough to cover the section without spreading when the slide was moved.

b). The sections were rinsed in 3 changes of PBS of 5 minutes each. The slides were then wiped dry except for the area of the section and replaced in the Petri dishes.

c). A drop of second layer antiserum, ie. fluorescein-labelled goat anti-rabbit globulin, optimally diluted in PBS, was placed on the sections, which were left for 45 minutes to 1 hour at room

temperature.

d). The sections were washed as before.

The sections were mounted in buffered glycerine (glycerine:PBS = 9:1) and viewed under ultraviolet light microscope.

2.4.5. Modified indirect immunofluorescence staining procedure (Gu et al 1983)

a). As the procedure described in 4.2.a, but with incubation lasting for 30-50 hours.

b). The sections were rinsed in 3 changes of PBS, 10 minutes each, and the slides were then wiped dry except for the area of the section.

c). Application of the first layer antiserum was repeated at the same concentration and the incubated for 2-4 hours at room temperature.

d). The slides were washed in PBS three times, 10 minutes each.

e). They were then incubated with the second layer antiserum as described in 4.2.a.

f). The slides were washed in PBS as before and mounted in PBS-glycerine.

This procedure has higher sensitivity and gives better immunostainings than the routine procedure described in 4.2.

2.4.6. Peroxidase anti-peroxidase (PAP) staining method (after Sternberger 1974)

a). To block endogenous peroxidase activity in the tissue sections, slides were immersed for 30 minutes in a solution of 0.3% H_2O_2 (1 ml of 100 volume (30%) H_2O_2 per 100 ml) in PBS. For cryostat sections, gentle agitation of the solution was often necessary to remove air bubbles formed on the surface of the section.

b). PBS rinse.

c). To block possible background staining, normal goat serum, 1:30 in PBS, was applied to the section for 30 minutes. Slides were not rinsed. Serum was removed with a pipette or absorbant paper.

d). Appropriately diluted first layer antiserum (raised in rabbits) was applied to the section and incubated overnight at 4°C or at room temperature.

e). Three rinses, 5 minutes each in PBS.

f). The second layer antiserum, ie. unconjugated goat anti-rabbit globulin, normally at a dilution of 1:200 in PBS, was applied to the section and incubated for 30-60 minutes at room temperature.

g). Three rinses, 5 minutes each in PBS.

h). The third layer, ie. peroxidase anti-peroxidase (PAP) complex, at a dilution of 1:300, was applied to the section and incubated for 30-60 minutes at room temperature.

i). Three rinses, 5 minutes each in PBS.

j). The section bound peroxidase was visualised in diaminobenzidine (DAB) solution. To prepare the solution, 25 mg of DAB was dissolved in 100 ml of PBS and then 50-100 ul of 100 volume (30%) H_2O_2 added to each 100 ml of DAB solution. The slided were immersed in the solution. The development time was rather short, normally 1-5 minutes. The reaction was controlled very carefully by examining sections under the microscope until satisfactory specific staining was achieved with as little background staining as possible.

k). If the immunostaining was weak, it was possible to enhance the colour of the final reaction products by immersing the slide in a solution of OsO_4, 1% in deionised water until satisfactory staining intensity was achieved, normally in a few seconds.

l). Slides were washed well with deionised or distilled water.

m). Counterstained, if necessary, in haematoxylin.

n). Dehydrated through graded alcohols, cleared in xylene and mounted in DPX.

o). The stained sections were viewed under a transmitted light microscope. The sites of immunostaining were dark brown in colour.

2.4.7. Immuno-gold staining method at the light microscopical level (Gu et al 1981)

2.4.7.1. Two-step method

a). The de-waxed paraffin sections or cryostat sections were incubated with the first layer rabbit antiserum at a concentration similar to or slightly lower than that used in the indirect immunofluorescence method. Incubation was for 12-24 hours at 4°C or room temperature.

b). Three PBS rinses, 5 minutes each.

c). The sections were reacted with the second layer antiserum which was goat anti-rabbit conjugated with colloidal gold particles of 20nm or 40 nm diameter.. The dilution was normally 1:2 - 1:5 in PBS. The immunoreaction was checked from time to time under a transmitted light microscope. Incubation was halted when satisfactory intensity of staining was achieved, normally 30 minutes - 5 hours.

d). The slides were washed in PBS, counterstained if necessary, dehydrated (for paraffin sections) and mounted in PBS glycerine.

The end product of the immunoreaction was red in colour when viewed under a transmitted light microscope.

2.4.7.2. Three-step method

a). Same as in 4.5.1.a, but the first layer antiserum could be further diluted.

b). Three PBS rinses, 5 minutes each.

c). The sections were incubated with the second layer antiserum, unconjugated goat anti-rabbit, at a concentration of 1:200 in diluent. The incubation was for 1 hour at room temperature.

d). Rinse as before.

e). The sections were incubated with the third layer antiserum, ie., rabbit IgG conjugated to colloidal gold particles. The dilution was normally 1:2 - 1:10 in PBS. The immunoreaction was checked under a light microscope and stopped by rinsing in PBS when satisfactory staining intensity was achieved.

f). Same as in 4.5.1.d.

2.4.7.3. Combined method

a). Same as in 4.5.1.a.

b). Same as in 4.5.1.b.

c). Same as in 4.5.1.c.

d). Washed in PBS 3 times, 5 minutes each.

e). To further increase the staining intensity, the third layer antiserum, ie. the rabbit IgG conjugated to gold particles, was applied. The dilution was normally 1:2 - 1:10 in PBS and incubation was for 30 minutes - 2 hours.

f). Same as in 4.5.1.d.

This procedure gave a higher staining intensity than the previous two methods (2.4.7.1. & 2.4.7.2.).

2.4.7.4. Double immunostaining using PAP and immunogold staining method (Gu et al 1981)

a). Dewaxed paraffin sections were immunostained with PAP method to the first antigen as described in Appendix 2.4.6. The PAP complex was visualised using the 4-chloro-1-naphthol method of Nakane (1968) but modified in the following way: 4-chloro-1-naphthol was dissolved in a small amount of ethanol and further dissolved in 0.01 M PBS, pH 7.1, to a final concentration of 0.03%, the solution was heated to approximately 50°C and continuously stirred with a magnetic stirrer until completely dissolved. The solution was then filtered, H_2O_2 was added to a concentration of 0.3% and the solution was subsequently applied to the sections while still warm. The reaction was observed under the light microscope until a satisfactory level of staining was obtained (about 3-5 minutes, dark-blue in colour). For immunostaining of nerves the PAP complex was visualised using the DAB method which results in a brown end product (Appendix 2.4.6.).

b). After rinsing, the sections were placed in glycine-hydrochloric acid solution at pH 2.2 (5ml of 0.1 N HCl, 95ml of glycine in NaCl solution (0.757% glycine in 0.584% NaCl)) for 2 hours. This removes the antibody complex obtained during the first immunostaining, but leaves the blue reaction product.

c). The second antigen on the tissue section was immunostained with the immunogold method (Appendix 4.5.1-3.).

d). The stained sections were rinsed in PBS, and mounted in glycerine jelly. They could not be dehydrated because the endproduct of the 4-chloro-1-naphthol reaction is soluble in ethanol.

2.4.8. Cholinesterase staining method (after El-Badawi & Schenk 1967)

a). Air-dried cryostat sections were fixed in phosphate-buffered 10% formalin at pH 7.0 at 4°C for 10-15 minutes. Formalin or BQS prefixed cryostat sections were also used.

b). Slides were washed thoroughly in 2 changes of distilled water, 1 minute each.

c). Incubated at 37°C for 30 minutes to 2 hours in the solution described below.

Acetyl thiocholine iodide	12.5mg
0.1N (0.82%) CH_3COONa	15.8ml
0.1N (o.6%) CH_3COOH	0.5ml
0.1M (2.94%) sodium citrate	1.2ml
30mM (0.75%) $CuSO.5H_2O$	2.5ml
4mM (0.137%) iso-OMPA	0.5ml
5mM (o.165%) potassium ferricyanide	2.5ml
Distilled water	2.0ml

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

d). After staining, slides were rinsed well in distilled water.

e). Counterstained, if necessary, in haematoxylin.

f). Dehydrated, cleared and mounted as for PAP stained sections.

g). Viewed under light microscope. The sites of cholinesterase activity are dark brown.

2.4.9. Formaldehyde induced fluorescence (FIF) method (after Falk & Owman 1965)

For visualisation of catecholamines and indole amines in tissues.

a). Paraformaldehyde in a sealed container was heated in an oven at 80°C until hot, about 30 minutes.

b). Freeze-dried tissue was transferred to a pre-heated formaldehyde vapour container, sealed and heated for one hour at 80°C. As control, another piece of the tissue was placed in a jar not containing formaldehyde, but otherwise treated exactly the same.

c). Tissue was transferred to Ralwax 1 and kept under vacuum at 60°C for as short a time as possible to avoid diffusion of the fluorophore, normally 15-30 mins.

d). Tissues were embedded in wax.

e). Blocks were trimmed so that as little wax as possible surrounded the tissue. 5 µm thick sections were cut but not floated on a water bath because the fluorophore may dissolve in water. Sections were picked up directly from the knife onto a slide pre-warmed to about 60°C on a hot plate. The slide was put back on the hot plate to melt the wax and allow the sections to spread.

f). The slides were removed from the hot plate and the wax allowed to set before remounting. They were mounted in liquid paraffin and then warmed to melt the wax/paraffin mixture just before looking at it under the UV microscope. The control sections were processed identically and checked at the same time for comparison. The slides were examined as soon as possible, within 2 days at most. Noradrenaline, adrenaline and dopamine appeared green and 5-HT gave yellow fluorescence.

g). To control the specificity, catecholamine induced fluorescence

was quenched by immersing the section (after de-waxing and bringing to absolute alcohol) in 80-95% isopropyl alcohol with 0.01-0.1% sodium borohydride for 2-5 mins. Specific formaldehyde-induced fluorescence may subsequently be reproduced after exposure of the section to formaldehyde vapour (Corrode et al 1964).

2.4.10. Modified glyoxylic acid technique - The SPG method for amine containing nerves (after de la Torre & Surgeon 1976)

a). Preparation of SPG (sucrose - potassium - glyoxylic acid) solution:

1). Weighed out 10.2g sucrose (Mallincrodt) crystals, 0.200 M.

2). Weighed out 4.8g potassium phosphate monobasic (KH_2PO_4 Mallincrodt) 0.236 M.

3). Both were dissolved in 100ml distiled water.

4). 1.5g glyoxylic acid monohydride (98% free acid monohydrate, Sigma) 1%, was added.

5). The solution was titrated to pH 7.4 with 1 N NaOH (about 35 ml).

6). Distilled water was added to 150 ml.

The SPG solution was prepared freshly and used at room temperature.

b). Cryostat sections, 10µm thick, of unfixed tissue were mounted on uncoated glass slides and let to melt onto the slides for 5 minutes at room temperature.

c). The section was quickly dipped in SPG solution 3 times (1 dip/sec). Excess fluid was removed quickly from the slide with absorbaent paper.

d). The sections were then dried under cool air produced by a hairdrier.

e). The sections were then transferred and remained in an oven at

80°C for exactly 5 minutes.

f). After removing the slide from the oven, a few drops of Mineral Oil (USP, thin) were placed near the tissue and the section was coverslipped.

g). The coverslipped slides were placed for 90 sec. on a hot plate set at exactly 80°C to remove air bubbles.

h). The sections were examined immediately under an UV microscope.

2.4.11. DOPA Oxidase method (after McManus & Mowry 1960)

a). Frozen sections of fresh material or of tissue briefly fixed in formalin were placed in a solution of 0.1% Dihydroxyphenylalanine in PBS for 2-4 hours at room temperature. The incubating solution was changed every hour to prevent the deposit of the precipitate produced by spontaneous oxidation of the substrate.

b). Sections were rinsed in several changes of water and then mounted in glycerine jelly or in balsam after dehydrating by passage through alcohols and xylene.

c). Sometimes, 0.5% neutral red or very dilute eosin was used as counterstain.

d). Sites of dopa oxidase activity were brown to black under the light microscope.

e). The negative control was provided by treating boiled sections of skin as above. The negative control was colourless.

2.5. PHOTOGRAPHY

2.5.1. The UV light microscope used was Leitz Orthoplan with XBO 150 lamp.

2.5.2. The tranmitted light microscope was Reichert Polyvar fitted with Nomarski and dark-field.

2.5.3. The black and white photographs using the UV light microscope were taken on Ilford FP4 film and developed in 20% Ilford Microphen for 5½ minutes.

2.5.4. The black and white photographs using a transmitted light microscope were taken on Kodak Tech Pan 2415 film and developed in Kodak HC 110 Developer for 5 minutes.

2.5.5. The printing papers used were Kodak resin coated and were developed in Ilford contract FF developer.

APPENDIX 3

Chemicals

Fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies
(Miles Laboratories, England)

Unconjugated goat anti-rabbit antibodies
(Miles Laboratories, England)

Rabbit peroxidase-antiperoxidase complex
(UCB, Brussels, Belgium)

Recrystalised para-benzoquinone
(BDH, Chemicals LtD., Poole, Dorset, England)

3,3-diaminobenzidine tetrahydrochloride
(Aldrich Chemical, Gillingham, England)

4-Cl-1-naphthol
(Sigma, London Chemical Company Ltd., Poole, England)

Acetylthiocholine iodide
(Sigma, London Chemical Company Ltd., Poole, England)

Tetraisopropylpyrophosphoramide
(Sigma, London Chemical Company Ltd., poole, England)

Sodium pentobarbitone (Nembutal)
(May & Baker Ltd., Dagenham, England)

Gold-labelled antibodies
(Janssen Pharmaceutica, Beerse, Belgium)

Peptides and neuronal proteins

Natural porcine VIP

(A gift from Professor V.Mutt, Karolinska Institute, Stockholm, Sweden)

Antibody: made at Dept. of Histochemistry, Hammersmith Hospital, London

Synthetic substance P & neurotensin

(Beckman, Palo Alto, California, USA)

Antibody: made at Dept. of Histochemistry, Hammersmith Hospital, London

Synthetic somatostatin$_{14}$

(Bachem, Torrance, California, USA)

Antibody: made at Dept. of Histochemistry, Hammersmith Hospital, London

Synthetic sulphated CCK$_8$

(Squibb, Princeton, New York, USA)

Antibody: made at Dept. of Histochemistry, Hammersmith Hospital, London

Synthetic bombesin tetradecapeptide

(A gift from Farmatalia, Carlo Erba, SPA, Italy)

Antibody: made at Dept. of Histochemistry, Hammersmith Hospital, London

Met- & Leu-enkephalin

(Peninsula Laboratories, San Carlos, California, USA)

Antibody: made at Dept. of Histochemistry, Hammersmith Hospital, London

Natural porcine NPY and PHI

(A gift from Dr K.Tatemoto, Karolinska Institute, Stockholm, Sweden)

Antibody: made at Dept. of Histochemistry, Hammersmith Hospital, London

Natural chick PP (APP)

(A gift from Dr J.R.Kimmel, Kansas, USA)

Antibody: a gift from Dr J.R.Kimmel, Kansas, USA.

Purified rat NSE & NNE

(A gift from Dr P.J.Marangos, National Institute of Mental Health, Bethesda, Maryland, USA)

Antibody: a gift from Dr P.J.Marangos, National Institute of Mental Health, Bethesda, Maryland, USA.

Purified S-100 protein

(A gift from Dr D.Cocchia & Dr F.Michette, Istituto di Anatomia Umana Normale, Universita Cattolica del Sacro Cuore, Rome, Italy)

Antibody: a gift from Dr D.Cocchia & Dr F.Michette, Instituto di Anatomia Umana Normale, Universita Cattolica del Sacro Cuore, Rome, Italy.

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NEUROPEPTIDE TYROSINE (NPY)—A MAJOR CARDIAC NEUROPEPTIDE

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Summary A newly discovered bioactive peptide, neuropeptide tyrosine (NPY), has been found in the human cardiac nervous system. Dense concentrations of NPY-immunoreactive nerve fibres were found in association with nodal tissue (atrioventricular node 22·1±3·7 pmol/g). NPY nerve fibres were seen in close contact with cardiac muscle fibres and were also found around the coronary vessels (19·6±6·2 pmol/g). Analysis of the peptide by high-performance liquid chromatography demonstrated that it was present in a single molecular form, closely similar or identical to that of the isolated bioactive peptide. Cardiac function in man has long been known to be influenced by cholinergic and adrenergic nerves. There now appears to be a further component of the nervous system in the human heart, involving peptidergic nerves containing NPY.

Introduction

NEUROPEPTIDE tyrosine (NPY) is a 36 amino acid peptide recently isolated from porcine brain by means of a chemical assay for C-terminal amides.[1,2] Its molecular sequence bears a striking resemblance to that of the mammalian hormonal peptide secreted by the PP cells of the pancreas, pancreatic polypeptide (PP), and also to that of another active peptide localised to endocrine cells of the ileum and colon, peptide YY (PYY).[3]

The nerve supply of the human heart is known to comprise both parasympathetic fibres from branches of the vagus, and sympathetic fibres from rami of the sympathetic trunk.[4] Cholinergic and adrenergic fibres have been reported to be present in most regions of the mammalian heart.[5-8]

Neurally mediated coronary spasm, however, may not always involve the adrenergic system.[9] In many other organs such effects have been shown to be due to autonomic nerves which have regulatory peptides as their presumed neurotransmitters.[10,11] Hitherto the classic types of peptidergic nerves have not been found to any significant extent in the human heart.

We have investigated the presence of the newly isolated mammalian neuronal peptide, NPY, in the human heart.

Materials and Methods

Six hearts were obtained at necropsy, within 18 h of death. The following areas were dissected out: left and right atria, left and right ventricles, sinuatrial (S-A) and atrioventricular (A-V) nodes, atrial and ventricular septa, the tissues around coronary blood vessels, and the root of the aorta. Antisera to NPY were raised in New Zealand white rabbits against natural porcine NPY conjugated to bovine serum albumin with bis-diazobenzidine.

For immunocytochemistry the tissue was fixed in 0·4% benzoquinone solution[12] and modified methods of indirect immunofluorescence and peroxidase anti-peroxidase (PAP) staining were carried out on 5 μm thick cryostat sections, according to procedures described previously.[13,14] NPY antibodies were used at a dilution of 1:600 for immunofluorescence and 1:4000 for the PAP method. The preabsorption of the antiserum with pure or synthetic NPY, at the concentration of 1·0 nmol/ml of diluted antibody, completely abolished the staining, whereas the addition of pure or synthetic PYY, vasoactive intestinal polypeptide (VIP), neurotensin, secretin, substance P, glucagon, and glucose-dependent insulinotropic peptide (GIP) at concentrations above 30 nmol/ml of diluted antibodies had no effect on the immunostaining.

For radioimmunoassay, a representative piece from each heart region was rapidly diced and plunged into boiling acetic acid (0·5 mmol/l per g of tissue) and held at 100°C for 10 min. The solution was centrifuged and 10 μl and 1 μl volumes of the supernatant were assayed in duplicate. The assay could detect changes between adjacent assay tubes of 2 fmol with 95% confidence. No cross-reaction was seen with porcine pancreatic polypeptide (PP), PYY, or avian pancreatic polypeptide (APP) in concentrations up to 100 pmol/tube. Pure porcine NPY was used as standard. APP radioimmunoassay was performed with APP antibodies (kindly provided by Dr J. Kimmel) and I^{125}-labelled pure natural APP.

Fractionation of NPY-like immunoreactivity from the human heart was carried out on a high-performance liquid chromatography (HPLC) reverse phase column (Waters Bondapak C-18: 3·9×300 mm), by means of a linear gradient elution system (35%–45% solvent A 0·12% trifluoracetic acid/water; solvent B 0·1% trifluoracetic acid/acetonitrile) and a flow rate of 2 ml/min. 1 ml fractions were collected for subsequent radioimmunoassay. Recoveries were all between 88% and 94%.

Results

NPY-like immunoreactive nerves were seen in abundance throughout the heart, mainly running between cardiac muscle fibres (fig 1). These nerves were extremely dense around the regions of the S-A and A-V nodes (fig 2). The coronary arteries also had a particularly rich innervation (fig 3) which was most pronounced in the medium-sized arterial branches.

The highest concentration of extractable NPY detected by radioimmunoassay was 22·1±3·7 pmol/g (mean±SEM) in the region of A-V node. A similar concentration was found in the tissues around the coronary vessels. In other areas, the concentrations of NPY were in the range of 10–14 pmol/g (fig 4). APP-like immunoreactivity was undetectable (<0·5 pmol/g) in all regions.

Fractionation of heart extracts by HPLC showed more

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Fig 1—NPY-immunoreactive nerve fibres associated with cardiac muscles of left ventricle (reduced by ¼ from × 860).

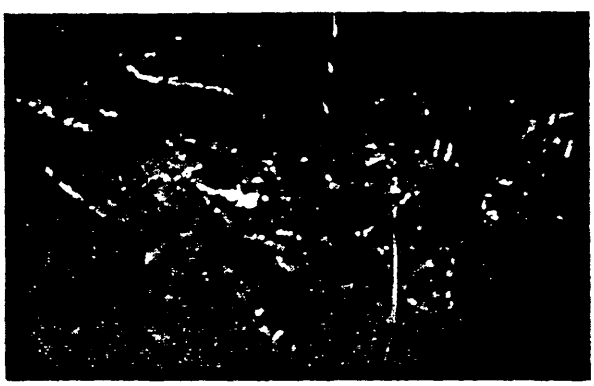


Fig 2—Numerous NPY-immunoreactive nerves in region of A-V node (reduced by ¼ from × 500).

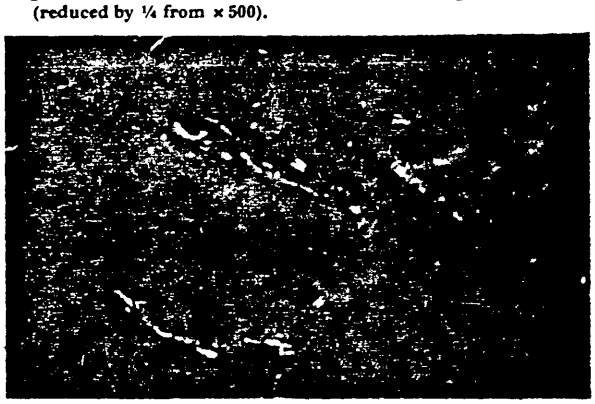


Fig 3—NPY-immunoreactive nerve fibres surrounding a coronary vessel (reduced by ¼ from × 600).

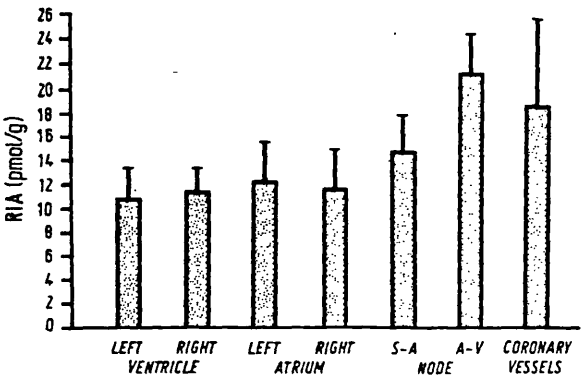


Fig 4—NPY-like immunoreactivity in extracts of various regions of 8 human hearts.

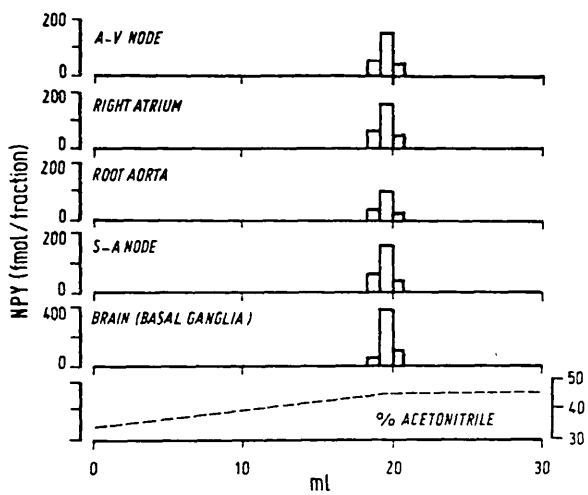


Fig 5—Histogram of NPY-like immunoreactivity of four regions of human heart, and, for comparison, human basal ganglia eluted from a Bondapak C18 column by means of a trifluoracetic acid water acetonitrile gradient.

than 90% of the immunoreactivity in a single peak in an elution position similar to that of human brain NPY (fig 5).

Discussion

Considerable quantities of a novel neuropeptide, NPY, have been found in the human heart in a dense peptidergic innervation. In other mammals VIP, substance P, and neurotensin have also been reported to be present in certain cardiac neurons, though in very small quantities.[15-17] In a similar study to that reported here, we assayed the concentrations of VIP and substance P in the human heart and found them to be very low (0·01 to 0·7 pmol/g and 0·01 to 0·3 pmol/g, respectively). Neurotensin immunoreactivity was undetectable (<0·05 pmol/g) (McGregor G. P., Gu J., Blank M. A., Polak J. M., and Bloom S. R., unpublished).

Some workers have suggested that an APP-like immunoreactivity might be present in nerves of the rat and cat hearts.[18] However, when more precise radioimmunoassay techniques were used no material with APP-like immunoreactivity was detectable. In the present study no peptide was identified by HPLC in the position of APP. It may be that such APP-like immunoreactivity, if present in the heart, is due to partial antiserum cross-reaction with the peptide NPY.

Cholinergic and adrenergic fibres originating in, or passing through, the cardiac plexus are distributed in the nodal tissue, in the atrial and ventricular myocardium, and around the coronary blood-vessels.[5-8] The functional importance of the cardiac innervation is only partly understood. NPY can induce a dose-dependent vasoconstriction in the cat submandibular gland.[19,20] This vascular response caused by combined local infusions of NPY and noradrenaline was similar to that seen upon sympathetic nerve stimulation.[20] In addition, electrically induced, but not noradrenaline-induced, contraction of the rodent vas deferens was also inhibited.[19,21] The effects were rather slow in onset and long-lasting, and thus NPY may act as a longer-term modulator of function. The presence of high concentrations of this biologically active peptide in the human heart suggests that it

may be involved in the control of myocardial contractility. Very high concentrations of NPY were found around the coronary vessels. NPY and the structurally similar peptide PYY possess visceral vasoconstrictor activity in the cat,[22] and this raises the possibility that NPY may be involved in the regulation of myocardial perfusion. Preliminary experiments in an isolated heart preparation have added support to this concept (Allen J. M., Bircham P. M. M., Edwards A. V., Tatemoto K., Bloom S. R., unpublished).

Pharmacological and physiological experiments are now required to define the full cardiac effects of this newly discovered neuropeptide.

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EFFECTS OF α-KETOISOCAPROATE AND OF LEUCINE ON NITROGEN METABOLISM IN POSTOPERATIVE PATIENTS

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Summary 21 patients undergoing major abdominal surgery were randomly assigned to one of three groups. On the day of surgery and for the succeeding 4 days each group received a daily infusion of one of the following: 10 g glucose plus 70 mmol $NaHCO_3$, 70 mmol leucine plus 70 mmol $NaHCO_3$, or 70 mmol of sodium α-ketoisocaproate (KIC). No other calories were given. Leucine infusions had no significant effect on nitrogen (N) balance, 3-methylhistidine excretion, or plasma concentrations of pre-albumin or retinol-binding protein, but they increased blood acetoacetate concentration ($p = 0 \cdot 004$). N balance was less negative ($p = 0 \cdot 002$) and 3-methylhistidine excretion lower ($p = 0 \cdot 002$) in the group receiving KIC than in those receiving glucose. Blood ketone bodies, plasma pre-albumin, and plasma retinol-binding protein concentrations at the end of the study were significantly higher in the KIC group than in the others. These N-sparing effects of KIC may be related to the heightened ketosis that followed its administration, to suppression of protein degradation, or to an effect on liver protein turnover. KIC alone in small doses diminishes N wastage in postoperative patients but under the same conditions leucine does not.

Introduction

AN infusion of branched-chain aminoacids (BCAA) is as effective as or more effective than one of a complete aminoacid mixture in maintaining nitrogen (N) balance in postoperative patients.[1–3] This finding may be related to a stimulatory effect of BCAA, particularly leucine, on muscle protein synthesis that has been observed in vitro;[4–6] but leucine supplementation in intact rats[7] or normal subjects[8,9] does not increase protein synthesis in muscle or other tissues. Another possible explanation for the effect of BCAA on N balance may be its inhibition of protein degradation in muscle or other tissues. In vitro, an unidentified metabolite of leucine (probably α-ketoisocaproate) inhibits protein degradation in muscle, but leucine itself does not when transamination to its keto-analogue, α-ketoisocaproate (KIC), is prevented.[10] BCAA inhibit whole-body protein breakdown in injured rats,[11,12] an effect which could be attributed to a metabolite of leucine.

Branched-chain ketoacid administration has apparently not been studied in injury or sepsis. However, these compounds attenuate the negative N balance that occurs with fasting;[13,14] the effect is entirely attributable to KIC.[15] This effect occurred at a very low dose (11 mmol per day) and was not seen with leucine at a dose of 33 mmol per day in the same subjects.[15] Whether this N sparing was attributable to decreased protein degradation was not determined. In patients with Duchenne muscular dystrophy a mixture of branched-chain ketoacids containing predominantly KIC, given orally for 4 days, reduces protein breakdown.[16] Leucine, given for several months, is ineffective in this

Neuron-Specific Enolase in the Merkel Cells of Mammalian Skin

The Use of Specific Antibody as a Simple and Reliable Histologic Marker

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Merkel cells are specialized skin receptor cells, characterized by their particular location in the epidermis and close association with nerve terminals. Although they can be distinguished ultrastructurally by their small, electron-dense secretory granules, there is no specific and reliable method for identifying them by light microscopy. Using antibodies to neuron-specific enolase (NSE), the authors have shown sparsely distributed groups of specifically immunostained cells and associated nerve terminals in the nose skin of cats and rats. These cells were easily distinguished from other epithelial cell types, including melanocytes and Langerhans cells and had all the morphologic features of Merkel cells and their so-called neurite complexes, including the characteristic cytoplasmic secretory granules (60 nm in diameter). NSE immunostaining is a simple and reliable method for the specific light-microscopic staining of Merkel cells and provides further evidence for NSE as a marker for the diffuse neuroendocrine system. (Am J Pathol 1981, 104: 63–68)

MERKEL CELLS, first discovered by Dr. Merkel[1] in 1875, are thought to be specialized receptor cells[2] present in the skin. The population of Merkel cells is very small. For example, in the back skin of the rat[3] there are only about 150 Merkel cells/sq cm. In various sensory receptive areas of the mammalian body, such as the nose, fingertips, etc., more Merkel cells are found, but they are still far from abundant. They tend to form groups and are mainly situated within the epithelial basement laminas of touch corpuscles (Haarscheiben) at the junction of the dermis and epidermis.[1-3]

Merkel cells can be distinguished by their orientation in the epithelium. Their longest axis is usually parallel to the surface of the skin and the basement membrane so that the columnar epithelial cells immediately above them lie in a perpendicular plane.[3] In mammals, the Merkel cells are very often directly associated with tylotrich hairs,[4] which are longer than other hairs and have follicles surrounded by aggregations of fibroblasts. They are mostly innervated by long myelinated fibers of Type 1 sensory neurons, forming the so-called epidermal Merkel cell neurite complexes.[3,5,6]

There is, at present, no specific staining method for Merkel cells, and the most reliable means of identifying them has been the electron microscope.[2,3] It has, however, been suggested that Merkel cells may belong to the diffuse neuroendocrine (APUD) system.[7,8] A newly described common characteristic for the components of this system is the presence of neuron-specific enolase (NSE).[9] NSE is an enzyme that was originally regarded as being present exclusively in neurons[10,11] but was later found in a number of APUD cells.[9]

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This study was carried out to determine whether NSE is present in Merkel cells and if so, whether immunostaining for NSE can identify these cells at the light-microscopic level.

Materials and Methods

Fresh specimens (0.2 × 0.2 sq cm) of skin were taken from the noses of cats (n = 5) and rats (n = 8) and immediately frozen in Arcton (−150 C). They were then freeze-dried overnight at −40 C, fixed in benzoquinone vapor at 60 C for 3 hours, and embedded in paraffin wax. Sections (5μ) were cut, in a plane perpendicular to the surface of the skin, and mounted on uncoated slides. In adition, some sections were cut transversely, parallel to the surface of the skin, and mounted in the same way. In this way, Merkel cells were sectioned both longitudinally and transversely.

Production of Antiserums

Neuron-specific enolase (NSE) was isolated from rat and human brain,[12,13] and antiserums to NSE were raised in New Zealand white rabbits by the intradermal injection of purified NSE emulsified in Freund's complete adjuvant.[12,13] Antiserums were also raised to rat and human nonneuronal enolase (NNE), and these antiserums showed no cross-reactivity with NSE, or vice versa.[13]

Immunocytochemistry

The unlabeled antibody enzyme (peroxidase-antiperoxidase, PAP) method[14] was performed with the use of a procedure described elsewhere.[14] Sections were incubated for 14–18 hours at 4 C in a moist atmosphere with rabbit antirat NSE antiserum, diluted 1:4000. The second layer, unconjugated goat antirabbit globulin, was used at a dilution of 1:200 for 30 minutes at room temperature. The third layer consisted of PAP complex, diluted 1:300, and incubation was again for half an hour at room temperature. The diaminobenzidine (DAB) method of Graham and Karnovsky[15] was used to visualize the final reaction product. Sections were lightly counterstained with hematoxylin.

Control sections were stained with primary antiserums preabsorbed with specific antigen. The absorbed antiserums were applied to sections serial to those that had been immunostained with antiserums to NSE. Further controls included the use of nonimmune rabbit serum as the first layer or the PAP complex alone. Sites of possible nonspecific attachment were visualized with DAB.

Enzyme Histochemistry

The modified oxidase method[16] was used to distinguish the melanocytes from the NSE-immunoreactive cells. Fresh specimens of skin (0.5 × 0.5 sq cm) were taken from the same areas and fixed in 10% formalin in phosphate-buffered saline (0.01M,pH7.1) for 2 hours at room temperature. They were then processed through graded alcohols and xylene and embedded in paraffin wax. Sections 5μ thick, perpendicular to the surface of skin, were cut and mounted on uncoated slides. The sections were then dewaxed and placed in an incubating solution, consisting of 100 mg of DL-3,4 dihydroxyphenylalanine (DL-DOPA) in 100 ml of 0.1 M phosphate buffer (pH 7.4) at 37 C for 1 hour. The sections were then changed to a fresh incubating solution possessing the same constituents for another 4 hours. After a wash in running tap water for 5 minutes, the sections were counterstained with methyl green, dehydrated, and mounted in DPX.

For electron-microscopic examination, fresh tissue was cut into 1-mm cubes and placed immediately into 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.1), then postfixed in 1% osmium tetroxide for 1 hour at 4 C. The tissue was then dehydrated through graded alcohols and embedded in Araldite plastic resin. Thin sections were stained with uranyl acetate and lead citrate, then examined on an AEI 6B model electron microscope.

Results

NSE Immunostaining

A number of cells, located just above the basement membrane, were strongly reactive with NSE antiserums (Figures 1 and 2). The cells were found in very few sections, ie, about 1 out of 20 or 30. The immunostained cells tended to form groups of 3–10, separated by unstained epithelial cells (Figure 2). Most of the immunostained cells lay in a plane parallel to the basement membrane and thus at right angles to the columnar epithelial cells. Immunostained nerve fibers were clearly visible in the subcutaneous layer. They penetrated the basement membrane and were closely associated with the basal part of each immunostained cell (Figures 1 and 2). At times the nerve fibers surrounded the NSE immunoreactive cells, and some projected further toward the surface of the skin (Figure 1). The innervated cells were situated at the bottom of the basal laminas of touch corpuscles and were nearly always associated with tylotrich hairs (Figure 1).

In some cases, when the papillae of the skin were cut transversely, some immunostained cells with a distinctive shape could be seen in the epithelium, at the edge

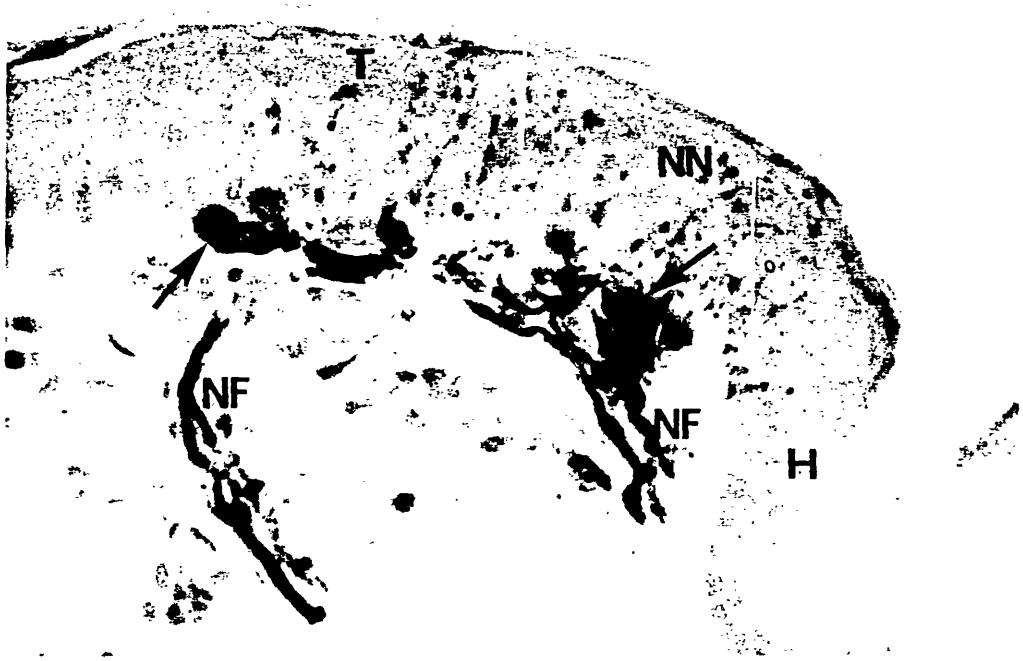


Figure 1—NSE-immunoreactive cells in the nose skin of cat. The immunostained cells (*arrow*) show all the characteristics of Merkel cells which include their number, location, orientation, and association with nerve fibers (*NF*), touch corpuscles (*T*), and a tylotrich hair follicle (*H*). Naked nerve endings (*NN*) can be seen in the epithelium. (×200)

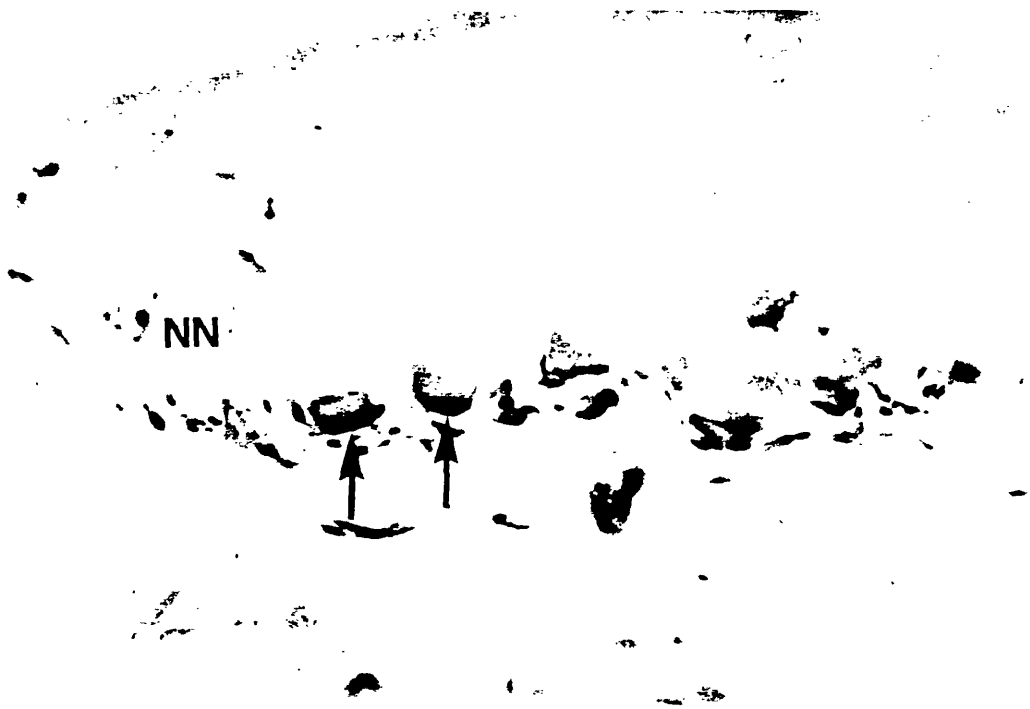


Figure 2—A group of Merkel cells situated within the epithelial basement lamina of a touch corpuscle of feline nose skin. The cells are clearly innervated (*arrow*), and some nerve fibers go further toward the surface of the skin, forming naked nerve endings (*NN*). (×400)



Figure 3—A group of Merkel cells (*arrow*) immunostained by NSE antiserum, at the edge of a transversely cut papilla of feline nose skin. The cells have a ringlike appearance. A nerve fiber (*NF*) can be seen nearby in the subcutaneous layer. (×300)



Figure 4—Single uninnervated Merkel cell immunostained by NSE antiserum in feline nose skin. The cell is located at the junction of the dermis and epidermis, and its longest axis lies parallel to the surface of the skin. The cell is associated with a touch corpuscle (*T*) (Haarscheiben) and a tylotrich hair follicle (*H*). (×300)

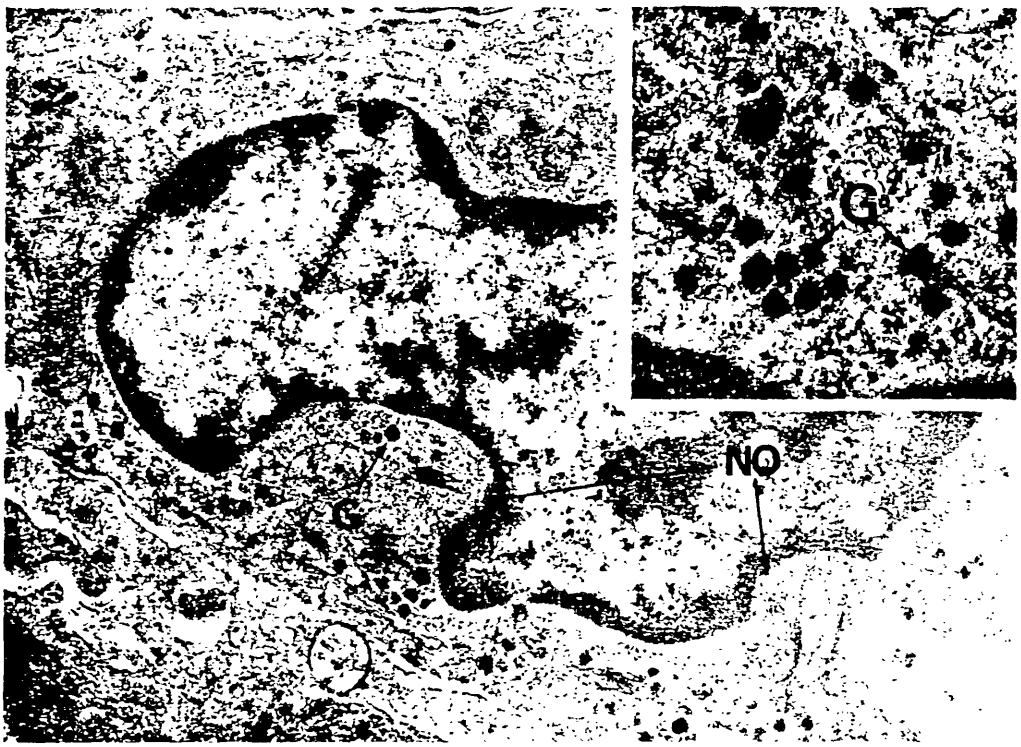


Figure 5—Ultrastructurally identified Merkel cell with electron-dense granules (*G*) and irregular nuclear outline (*NO*) were found in the identical areas. (×35,000) **Inset**—The secretory granules. (×85,000)

of the papilla. The periphery of these cells was immunostained more intensely than the center, giving the cells a ringlike appearance (Figure 3). It was not possible, using sections cut in this plane, to show that the cells were intimately innervated, but very often one or two nerve fibers were found lying nearby in the subcutaneous layer.

Occasionally, single or grouped immunostained cells (Figure 4) were seen, lying on the basement membrane, with or without association with a touch corpuscle. These were generally not shown to be innervated. Sometimes only immunostained nerve fibers were seen, penetrating the basement membrane and ending on or near the surface of the epithelium, forming naked nerve endings.

The sections incubated with preabsorbed antiserum were completely negative, as were the other control specimens.

DOPA Oxidase Method

The melanocytes were strongly stained brownish-black by the DOPA oxidase method. These cells were mainly located in the basal layer of the epithelium. They had a central cell body, containing the nucleus and numerous long, thin, branching cytoplasmic processes. In addition to having a different shape, the melanocytes were also far more abundant than the NSE-immunoreactive cells.

Electron Microscopy

Parallel ultrastructural studies showed the presence of scattered cells with a distribution identical to that of the NSE-positive cells and morphologic features similar to those described for Merkel cells. Thus, they contained characteristic intracytoplasmic, round, secretory granules, surrounded by a limiting membrane and with an average diameter of 60 nm. Other characteristics of Merkel cells were also seen in these cells, including irregular nuclear outline and spindlelike cytoplasmic processes (Figure 5).

Discussion

We have demonstrated here the presence of NSE in Merkel cells. These cells were identified by the use of both light- and electron-microscopic methods.

There are three types of nonkeratinocytes in the skin, namely melanocytes, Langerhans cells, and Merkel cells. The melanocytes, stained in this study by the DOPA oxidase technique and described before by other investigators,[16] have morphologic characteristics entirely different from those of the NSE immuno-

reactive cells. The Langerhans cells are located in the suprabasal layers of the epithelium, which lie in the area above the NSE-immunoreactive cells, and have a star shape with long, irregular processes.[17] Both of these cells can be easily distinguished from the NSE-immunoreactive cells.

At the light-microscopic level, it would seem that, since Merkel cells are the only cells in the epithelium that are characteristically innervated,[18] the NSE-immunoreactive innervated cells must be Merkel cells. In addition, these innervated NSE-positive cells display all the morphologic characteristics of Merkel cells, which include association with touch corpuscles and tylotrich hairs and the tendency to form groups, as well as the location, number, and orientation of the cells. Moreover, parallel electron-microscopic studies showed the presence of electron-dense secretory granules, a characteristic of Merkel cells. Thus, it may be concluded that these innervated NSE-immunoreactive cells are Merkel cells and not melanocytes, Langerhans cells, or any other cell type.

The immunostained cells with a ringlike appearance are only seen in transversely sectioned papillae. It is likely that they are Merkel cells, peripherally located immunostaining giving the ringlike pattern. The associated nerve fibers could not be demonstrated in the same sections. This problem may be due to the plane in which the tissue was cut and may also explain the fact that there were always some nerve fibers lying nearby in the subcutaneous layer. It has been shown that not all Merkel cells are innervated; neither are they all associated with touch corpuscles.[2,3,5,6] Thus, although the single or grouped noninnervated NSE-immunoreactive cells may represent a cell type not previously described, it seems more likely that they, too, are Merkel cells.

The finding of this newly discovered biochemical marker in Merkel cells supports the earlier suggestion that those cells are members of the diffuse neuroendocrine system.[7,8] The presence of this neuronal antigen in these cells further supports the functional relationship of neuroendocrine cells with neurons.

We therefore conclude that NSE immunostaining is a reliable, simple, and highly specific method for demonstrating not only Merkel cells but also their neurite complexes.

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Immunostaining of Neuron-Specific Enolase as a Diagnostic Tool for Merkel Cell Tumors

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Conventional histologic examination of Merkel cell tumors may result in misdiagnosis because of the close similarities these tumors bear to either malignant lymphomas or certain undifferentiated carcinomas. The authors have previously reported that neuron-specific enolase (NSE), a specific marker for neuroendocrine cells, is present in normal Merkel cells and can be used as a marker to identify this cell type. In this study, 11 Merkel cell tumors, identified employing electron microscopy, were studied using immunostaining of NSE by the peroxidase–antiperoxidase method. Varying intensities of NSE immunoreactivity were found in the cytoplasm of all the neoplastic cells in the different cases. The uniformly stained cytoplasm formed a small rim surrounding the large, unstained nucleus. Immunostaining of NSE thus provides a simple and reliable method for the differential diagnosis of Merkel cell tumors from other primary skin tumors which, with the exception of some malignant melanomas, have been shown not to contain NSE immunoreactivity.

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THE MERKEL CELL is an uncommon cell type found in the skin of many mammalian species, including humans. These cells are mainly situated in the basal layer of the epidermis, forming groups of 10 to 50 cells which are often innervated by myelinated nerve terminals. They tend to associate with hair follicles and sometimes are also found in the dermis.[1-3] The existence of typical electron-dense secretory granules in the cytoplasm of Merkel cells has characterised them as members of the APUD system.[4] However, until recently, there was no specific staining method for the demonstration of these cells at the light microscopic level, and the only reliable means for their identification has been electron microscopy. We recently reported that Merkel cells contain neuron specific enolase (NSE),[5] an isoenzyme that was first thought to be present exclusively in neurons but was later found in a number of neuroendocrine cells.[6-10] Thus, immunostaining of NSE can be used as a simple and reliable method to identify this uncommon cell type.

Primary skin tumors thought to originate from the Merkel cell have recently been reported by a number of authors.[11-16] Since this tumor may histologically resemble either a malignant lymphoma or an undifferentiated carcinoma, it has frequently been misdiagnosed in the past.[12,13,16] Such misdiagnosis can lead to inappropriate treatment and an erroneous prognosis. This tumor type is locally aggressive, may metastasize to lymph nodes, and less frequently more distantly and result in death. Currently, a definitive diagnosis of Merkel cell tumors can only be achieved by electron microscopy, as all stains tried have shown no consistent results. A specific staining method for identifying Merkel cell tumors at light microscopic level has thus not previously been available.

In this study, we investigated the possible presence of NSE in Merkel cell tumors in an attempt to find a simple and reliable approach at the light microscopic level for

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TABLE 1. Brief Clinical Patient Data

Sex/age (yrs)	Site of tumor	Size of tumor at resection (diameter in cm)
F55	Leg	2
F70	Left leg	NA
F56	Anterior right arm	1
F78	Left leg	4
F74	Neck	2
F76	Left cheek	2–3
F77	Anterior and inferior to the left ear	2–3
M84	Right knee	3
M63	Left leg	1
M82	Left cheek	massive facial involvement
M60	Inguinal lymph node metastasis	NA

NA: information not available.

the differential diagnosis of these tumors from others that have a similar histologic appearance.

Materials and Methods

Eleven Merkel cell tumors, including one local lymph node, metastasis were investigated. The tumors were found on the limbs, neck, and face areas of patients older than age 55 years. A brief summary of the clinical data is shown in Table 1. All of the tumors had been previously identified as Merkel cell type by electron microscopy. Some of the cases have been previously discussed on conventional histologic grounds.[13,15]

Sixteen non-Merkel cell skin tumors were used as controls. These included 4 cases of undifferentiated carcinoma, 4 cases of malignant lymphoma, and 8 cases of basal cell carcinoma. In addition, 10 primary malignant melanomas of skin were studied, with an average of 3 blocks per case.

Nine of the Merkel cell tumors were fixed in formol saline and one of them was fixed in Bouin's. Another one was freeze-dried and fixed in benzoquinone vapour (60°C, 3 hours) which was the method we previously used to demonstrate NSE in normal Merkel cells. The fixed samples were embedded in paraffin wax. All of the control tumors, including the melanomas, were fixed in formol saline and embedded in paraffin wax. Sections (5 μm) were cut, dewaxed in xylene and graded alcohols, and stained by hematoxylin and eosin (H & E), Grimelius technique, or immunocytochemistry.

Production of Antisera

Neuron-specific enolase (NSE) was isolated from the rat and human brain, and antisera to NSE were raised in New Zealand white rabbits by the intradermal injection of purified NSE emulsified in Freund's complete adjuvant.[9,10] Antisera were also raised to rat and human non-neuronal enolase (NNE), and these antisera showed no cross-reactivity with NSE, or *vice versa*.[10]

Immunocytochemistry

Sections fixed in formol saline were sometimes subjected to trypsinisation before immunostaining in a solution consisting of 0.1% trypsin and 0.1% calcium chloride (pH 7.8) at 37°C for 30 minutes.[17] The unlabeled antibody-enzyme (peroxidase–antiperoxidase, PAP) method was performed as previously described.[18] Sections were incubated for 14 to 18 hours at 4°C in a moist stat with rabbit antirat NSE antiserum, diluted 1:4,000. The second layer of unconjugated goat antirabbit globulin was used at a dilution of 1:200 for 30 minutes at room temperature. The third layer consisted of PAP complex, diluted 1:300, and incubation was again for 30 minutes at room temperature. The diaminobenzidine (DAB) method of Graham and Karnovsky was used to visualize the final reaction product.[19] Sections were lightly counterstained with hematoxylin. Control sections were incubated with primary antiserum preabsorbed with NSE antigen (0.5 μg in 100 μl diluted antiserum). The absorbed antiserum was applied to sections serial to those which had been immunostained with antiserum to NSE. Further controls included the use of nonimmune rabbit serum as the first layer, or the PAP complex alone. Sites of possible nonspecific attachment were visualized with DAB.

Results

The H & E-stained preparations showed a similar appearance in all the cases. The tumors were situated in the dermis and extended into the subcutaneous or deeper tissue. The tumors were arranged compactly and formed a diffuse infiltrate, resembling that of malignant lymphoma. They sometimes consisted of ill-defined anastomosing cords and clusters of cells. The neoplastic cells were round, uniform in size and had very little cytoplasm. The nuclei were large, round and sharply outlined. Numerous mitoses could be seen and scattered necrotic cells were observed. Lymphocytic infiltrate was present at the periphery of some of the tumors (Fig. 1).

In each case, NSE immunoreactivity was found in all the neoplastic cells, although variations of staining intensity could be seen between different cases. Except for three cases in which the NSE immunoreactivity was rather weak, all of the other Merkel cell tumors showed

chus and of malignant melanoma, NSE immunostaining thus provides an additional, simple and reliable diagnostic tool in the recognition of Merkel cell tumors of the skin which can pose formidable diagnostic problems.

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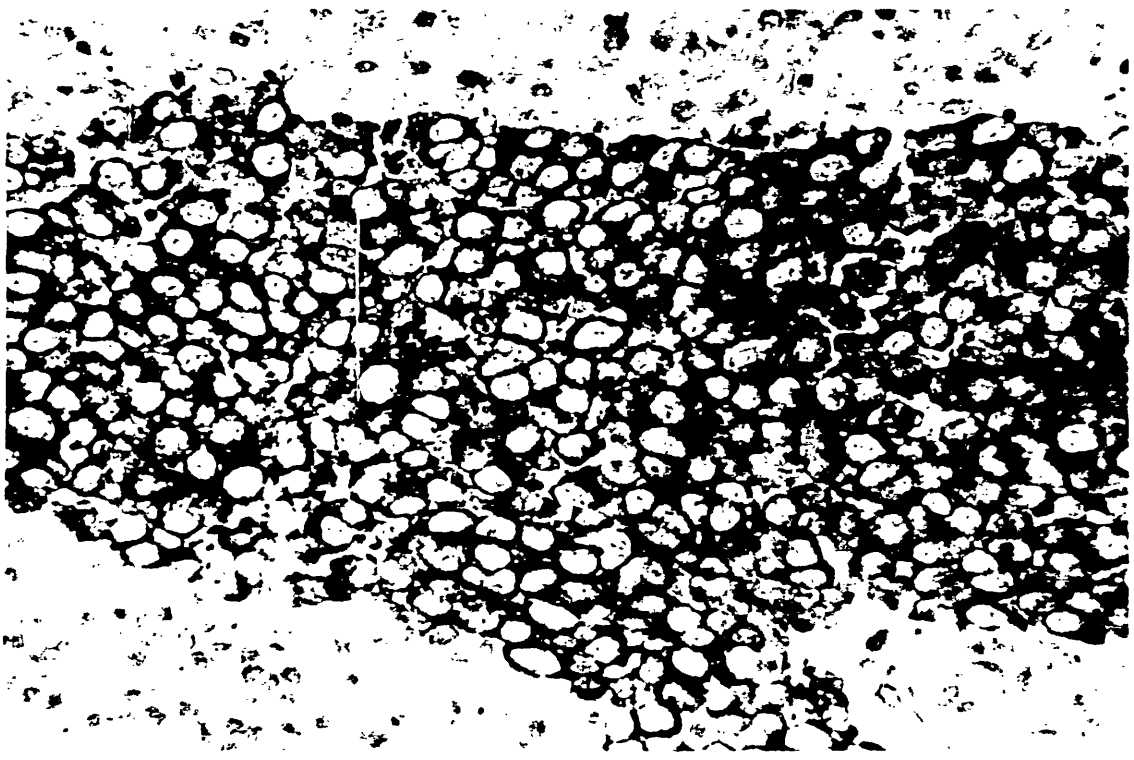


FIG. 2. NSE immunoreactivity showing an even distribution in the cytoplasm of all the neoplastic cells and forming a dark rim around the large, round unstained nucleus (×800).

tend to have greater irregularity of their nuclei,[16,21,22] plasmacytomas have more prominent nucleoli[16] and metastases of oat cell carcinomas of the lung frequently show more extensive necrosis, crush artefact and an oval or spindle-shape.[16] However, although such differentiating criteria can be useful in certain cases, it remains true that in practice Merkel cell tumors have often been misdiagnosed.

In the current study, all 11 cases of ultrastructurally identified Merkel cell tumors were found to contain NSE. Based on our previous finding that NSE was not present in non-neuroendocrine tumors but found exclusively in neuroendocrine tumors.[20] it would seem that the morphologically similar tumors, i.e., malignant lymphomas, undifferentiated carcinomas, etc., can be easily differentiated from Merkel cell tumors by NSE immunostaining.

It should however be emphasized that certain metastatic tumors in the skin may also show positivity for NSE immunostaining. These include metastases from islet cell carcinoma of pancreas, neuroblastomas, carcinoids and metastases from oat cell carcinomas of the lung. Generally, one should be able to distinguish them from Merkel cell tumors, without conducting NSE immunostaining, by taking account of both their microscopical features and the clinical setting. Because of the close histologic similarity and NSE content, particular attention should be paid to the possibility that one is dealing with metastases of oat cell carcinoma. The presence of NSE in melanomas has recently been reported.[23] We found NSE to be less frequently present in melanomas than in this report. However, five of ten tumors did show a focal, usually weak, staining. Even when they lack pigment, these tumors can be distinguished by the presence of junctional activity, the different structural configurations and the distinctive cytology. In practice, therefore, the differentiation of melanoma and Merkel cell tumor is not a formidable problem.

Met-enkephalin has been reported to be present in normal Merkel cells[24] and ACTH has been found in a Merkel cell tumor.[25] In the current study, four of the tumors were found to be argyrophilic by the silver impregnation method and three negative. The possibility, therefore, exists that the so called Merkel cell tumors may behave like the endocrine tumors of the alimentary tract which have their varying capacities for silver impregnation, different peptide contents and variously sized secretory granules[26] and, in addition, all show NSE immunoreactivity.[20]

It can be concluded that, given adequate fixation, NSE is present in most, perhaps all, Merkel cell tumors. After exclusion of metastatic oat cell carcinoma of the bron-

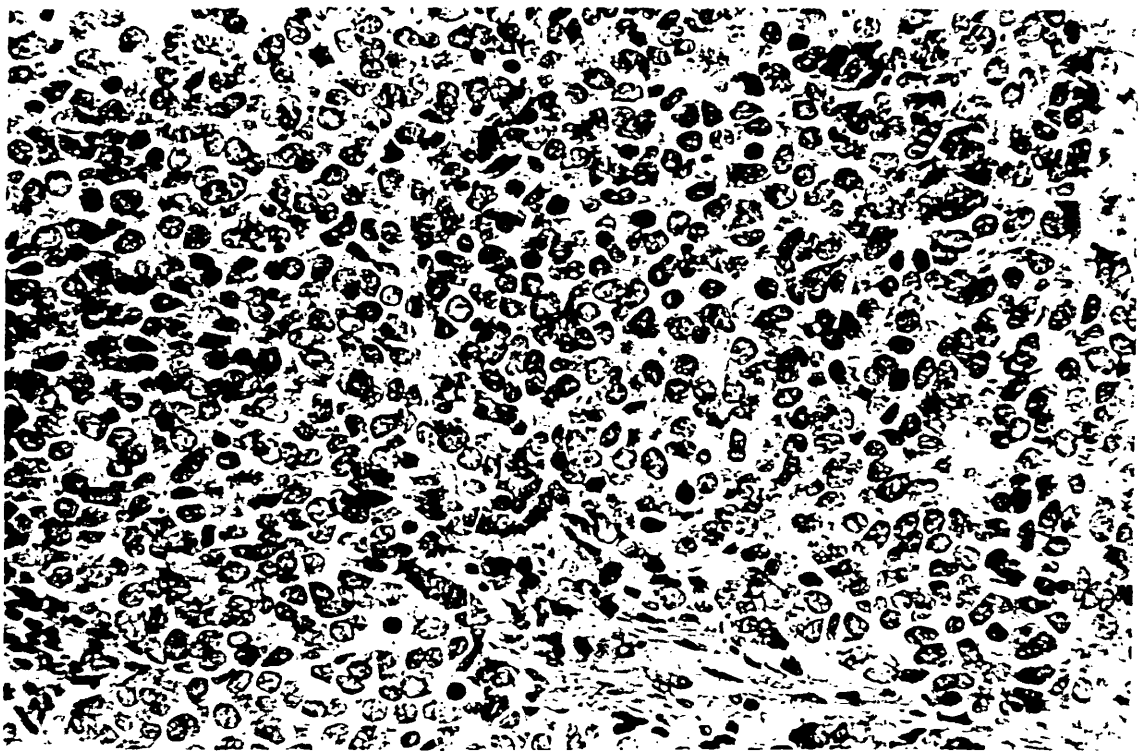


FIG. 1. Compactly arranged, uniform neoplastic cells with large, round and sharply outlined nuclei and little cytoplasm (H & E ×550).

fairly strong NSE positivity. The NSE immunoreactivity was uniformly present in the cytoplasm, which formed a rim surrounding the large, round, unstained nucleus of the cell. The positively immunostained tumor mass stood out clearly against the unstained background structures (Fig. 2). The control sections, including the 16 non-Merkel cell control tumors, were negative. Of the ten malignant melanomas, five showed a variable, mostly weak, positivity with a focal distribution. This was more noticeable in the junctional zone of the tumors. Five tumors were completely negative.

Four of the tumors showed staining with the Grimelius silver impregnation technique, another four reacted equivocally (borderline reaction), whereas the other three cases were completely unreactive to the Grimelius method.

Discussion

In this study, we have demonstrated that Merkel cell tumors can be specifically identified by NSE immunostaining. The specificity of the immunostaining was validated by appropriate controls. The variations of immunostaining intensity between different tumors might reflect the variable content of NSE in each tumor, although it seemed more likely that they resulted from inadequate fixation and scarcity of cytoplasm. The finding of NSE in Merkel cell tumors is in keeping with our previous report that NSE is present in normal Merkel cells,[5] and also with our recent finding, that NSE is present in most APUDomas.[20]

A number of primary and metastatic tumors of the skin may morphologically resemble Merkel cell tumors. These include malignant lymphomas, certain undifferentiated carcinomas, especially cutaneous metastases of oat cell carcinoma of the lung, which can be very difficult to differentiate histologically from Merkel cell tumors. The compact arrangement of many of these tumors, with a monotonously uniform cytology leads to very real diagnostic problems. To avoid such misdiagnosis, it has usually been necessary to perform electron microscopic examination to identify the characteristic electron dense secretory granules and other ultrastructural features like intercellular junctions. Since Toker first reported neoplasms of Merkel cell origin in 1972, under the name "trabecular carcinoma",[11] considerable effort has been made to distinguish Merkel cell tumors from other morphologically similar ones. A few minor differences between different tumor types have been emphasized. It has been reported that large cell lymphomas

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DISTRIBUTION OF BOMBESIN, SOMATOSTATIN, SUBSTANCE-P AND VASOACTIVE INTESTINAL POLYPEPTIDE IN FELINE AND PORCINE SKIN

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Summary

The content and distribution of several regulatory peptides in the skin of cats and pigs, freshly obtained at surgery, have been investigated. Immunoreactive bombesin was evenly distributed at low concentrations in both species, being below the detection limit in the body and nose of the cat, and showing a peak value of 1.6 ± 0.7 pmol/g in the tip of the pig's ear. Similar concentrations of somatostatin-immunoreactivity (-IR) were found but greater regional variation occurred in the pig with a low in the mid back of 0.4 ± 0.1 and the highest value in the snout of 3.1 ± 0.8. Substance-P-IR in the pig showed a marked variation in concentration, apparently parallelling skin sensitivity, with a low in the back of 0.4 ± 0.7 and higher values around the anus (8.1 ± 1.6), legs (6.8 ± 1.8) and snout (13.5 ± 3.6) whilst in the cat values ranged from 0.3 ± 0.06 in the body to 5.0 ± 0.9 in the front footpads. In contrast, vasoactive intestinal polypeptide (VIP)-IR showed greater variability in the cat, being below the assay's detection in the body and highest in the front and rear footpads (17 ± 7 and 29 ± 6 respectively), but in the pig most regions exhibited low concentrations with the exception of the snout which peaked at 12.0 ± 5.0. Immunocytochemical localisation showed the peptides to be present in nerve fibres. Substance-P-IR was particularly localised in the snout of the pig just below the epithelium while VIP-IR was more concentrated in deeper layers, often associated with sweat glands and blood vessels.

The neuropeptides bombesin, somatostatin, substance-P and VIP have been shown to be associated with the innervation of a number of peripheral tissues including the eye (1), the lung (2-4) and urogenital system (5,6). Each appears to have specific and potent biological actions and they thus represent an important part of the autonomic nervous system (7).

There has been no comprehensive investigation of the peptidergic innervation in mammalian skin. Substance-P immunoreactivity has been demonstrated in the skin of the cat paw (8). It has been located in a population of primary sensory neurones (9) which are associated with the axon reflex and it is thought to mediate, at least in part, the triple response to cutaneous injury (10). The presence of somatostatin has also been demonstrated in a distinct population of primary sensory neurones (9) as well as certain peripheral sympathetic neurones (11). However, as yet this peptide has not been localised in any peripheral sensory or sympathetic terminals. VIP has been localised in parasympathetic

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nerves of certain tissues and appears to be associated with the control of local blood flow and fluid secretions (12). Bombesin was first isolated from frog skin (13) and is one of many biologically active peptides, found in amphibian skin, which are closely homologous to certain mammalian peptides (14) including cholecystokinin (caerulein) and substance-P (physalaemin), demonstrating their ancient phylogeny.

This study has set out to investigate the relative distribution of bombesin, somatostatin, substance-P and VIP in the skin of the cat and pig. Although neither species provides a perfect model for the human skin, both possess many similarities (15) and afforded the opportunity of obtaining fresh tissue from many regions simultaneously. Specific and sensitive radioimmunoassays have been used to determine the relative levels in different regions of the skin and immunohistochemical analysis to establish the precise cellular location of these peptides.

Materials and Methods

Tissue Collection for Radioimmunoassay. Four Large White Pigs weighing between 30-35 kg and five adult cats were anaesthetised using sodium pentabarbitone. Duplicate 4mm punch biopsies were taken from seventeen regions in the pig and five regions in the cat. None of the animals were allowed to recover following this procedure. Samples were immediately weighed and extracted by boiling for ten minutes in 10 mls 0.05 M acetic acid per g tissue. Preweighed extraction tubes were used and after cooling the total contents were reweighed to establish any losses of the extraction medium during the boiling process. All extracts were stored at -20°C until the time of assay.

Radioimmunoassays. Samples were added as duplicate 50 and 10 µl aliquots to specific radioimmunoassay systems for all four peptides. Antiserum to bombesin was raised in rabbits by immunising with lysine^{3} bombesin coupled to bovine serum albumin (BSA) using glutaraldehyde as the coupling agent and the resulting C-terminally directed antibody used at a final titre of 1:640,000. Bombesin was measured by displacement of monoiodinated bombesin, prepared by chloramine-T oxidation, giving a detection limit of 0.4 fmol/tube. Somatostatin antisera were similarly raised in rabbits using the synthetic cyclic tetradecapeptide coupled to BSA by carbodiimide (CDI) condensation and an antibody selected which required the whole molecule for recognition. This was used at a final titre of 1:240,000 and the peptide measured by displacement of an $I^{125}Tyr^{11}$-somatostatin analogue (prepared by lactoperoxidase oxidation) giving a sensitivity of 0.4 fmol/tube. Antisera to substance-P were raised in rabbits immunised with synthetic peptide coupled to BSA by CDI or glutaraldehyde. This produced a C-terminally reacting antibody with a final titre of 1:8,000. The tracer for this peptide was prepared using Tyr^{8}-substance-P by chloramine-T oxidation and the assays detection limit was 0.3 fmol/tube. VIP was assayed by a modification of a previously described assay (16). None of these assays showed any significant crossreactivity with each other of any related peptide.

Chromatographic Analysis. Tissue extracts were chromatographed on Sephadex G-50 gel permeation columns and analysed by assaying the eluent for somatostatin and VIP immunoreactivity. In each run Dextran blue (void volume), cytochrome C (MW 11,600) and $Na^{125}I$ (total volume) were used as column 'markers'. Similarly extracts were chromatographed on G-25 sephadex and analysed for Sub P. Columns were eluted with 0.05 M NaPhosphate buffer, pH 7.5 containing 0.2 M sodium chloride, 1% bovine serum albumin and 0.05 M Na EDTA.

Tissue Collection and Processing for Immunocytochemistry. Five pigs and 5 adult cats were killed by overdosing with sodium pentabarbitone and skin

TABLE I

Characterisation of Antisera for Immunocytochemistry

Antigen	Carrier and coupling agent	Regional Specificity	Dilution for IF**	Quench nmol*
VIP	haemocyanin by glutaraldehyde	whole molecule	1:2000	5.0
substance P	BSA by carbodiimide	whole molecule	1:2000	10.0
somatostatin	thyroglobulin by glutaraldehyde	whole molecule	1:400	10.0
bombesin	BSA by glutaraldehyde	C-terminal	1:400	0.1

* Quantity of antigen (nmol/ml diluted antiserum) required for quenching of the relevant antiserum.
** Immunofluorescence technique.

specimens taken from nose, lip, front and rear foot, rump and anus. The samples were fixed in a 0.4% solution of benzoquinone in phosphate-buffered saline (PBS; 0.87% saline containing 0.01 M phosphate buffer pH 7.2) for 60 minutes, then rinsed and stored in PBS containing 7% sucrose and 0.01% sodium azide. The fixed tissue was frozen in melting Arcton and cryostat sections 5 to 6 um thick were cut with the block orientated so as to section at right angles to the epidermis. The sections were picked up on poly-L-lysine coated glass slides, allowed to dry for 60 minutes at room temperature, placed in PBS containg 0.2% Triton X-100 for 30 minutes and then incubated overnight at 4°C with rabbit antiserum to VIP, substance-P, bombesin and somatostatin. The dilutions used and specificities of the antibodies are shown in Table I. The slides were washed for three 5 minute changes of PBS and reincubated with the same antiserum for 2 hours at room temperature, washed as before in PBS and then incubated with FITC-conjugated goat antiserum to rabbit immunoglobulin (diluted 1:100) for 30 minutes at room temperature. After washing in PBS and mounting in PBS: glycerol (1:9), the sections were examined using a Reichert-Jung Polyvar microscope equipped with an ultra-violet source.

The controls used were preabsorption of the antibody with appropriate antigen (Table 1), the use of normal rabbit serum as first layer in place of the primary antiserum, and omission of the first layer.

Results

Radioimmunoassay. Bombesin-IR was found to be evenly distributed throughout all regions examined in the pig (Table II) although slight increases were observed in the snout and ear (1.5 ± 0.5 and 1.6 ± 0.7 pmol/g respectively) as compared, for instance, with the abdomen (0.7 ± 0.2). Low levels were similarly observed in the cat (Table III), ranging from less than the detection limit in the body and nose to 0.25 ± 0.09 in the rear footpads. In contrast VIP-IR was found to be generally low throughout the main body of the pig (less than 1.0 pmol/g in most regions) while, in the snout, levels were more than tenfold increased (12.0 ± 5.0), and in the cat varied even more from the body to the footpads (< 0.05 to 29.0 ± 6.0 respectively). Somatostatin-IR and substance-P-IR showed greater regional variability in the pig, although in general substance-P-IR levels were four to five times higher than somatostatin-IR. Concentrations for both peptides were again highest in the snout (13.5 ± 3.6 and 3.1 ± 0.7 pmol/g for substance-P-IR and somatostatin-IR

respectively) with intermediate levels in the tail and buttocks and lower levels in the main body. In the cat substance-P-IR exhibited similar variability with higher levels in the foot pads and nose as compared with the body, whereas somatostatin-IR levels were generally lower than in the pig with little variation.

TABLE II

Distribution of Neuropeptides in Pig Skin

REGION	BOMBESIN	SOMATOSTATIN	SUBSTANCE P	VIP
Upper snout	1.5 ± 0.5	3.1 ± 0.7	11.1 ± 1.3	12.0 ± 5.0
Lower snout	1.3 ± 0.6	3.0 ± 0.7	13.5 ± 3.6	7.7 ± 1.6
Cheek	0.9 ± 0.4	0.9 ± 0.3	2.9 ± 0.5	1.4 ± 0.4
Ear	1.6 ± 0.7	1.2 ± 0.6	3.9 ± 1.7	0.9 ± 0.2
Neck	0.9 ± 0.1	1.0 ± 0.4	3.6 ± 0.9	0.9 ± 0.3
Thorax	0.7 ± 0.2	0.9 ± 0.4	4.0 ± 1.1	0.3 ± 0.03
Forelimb	0.9 ± 0.4	1.2 ± 0.5	4.1 ± 0.7	0.5 ± 0.1
Abdomen	0.7 ± 0.2	0.6 ± 0.2	4.3 ± 1.2	0.6 ± 0.3
Hindlimb	0.9 ± 0.3	0.7 ± 0.2	5.3 ± 1.7	0.6 ± 0.1
Buttocks	0.7 ± 0.2	2.0 ± 1.2	8.1 ± 1.6	2.1 ± 1.1
Tail	1.0 ± 0.3	1.9 ± 0.7	6.5 ± 1.7	< 0.1

Peptide concentration (pmol/g) given as mean ± standard error of mean (SEM) (n = 4).

TABLE III

Distribution of Neuropeptides in Cat Skin

REGION	BOMBESIN	SOMATOSTATIN	SUBSTANCE P	VIP
Nose	< 0.1	0.4 ± 0.02	2.3 ± 0.5	5.4 ± 1.6
Lip	0.13 ± 0.08	0.3 ± 0.06	0.8 ± 0.1	1.7 ± 0.3
Body (rump)	< 0.1	0.5 ± 0.09	0.3 ± 0.06	< 0.05
Front footpad	0.17 ± 0.02	0.3 ± 0.05	5.0 ± 0.9	17.0 ± 2.0
Rear footpad	0.24 ± 0.09	0.4 ± 0.09	3.9 ± 1.2	29.0 ± 6.0

Peptide concentrations (pmol/g) given as mean ± SEM (n = 5).

Figure 1 illustrates representative elution profiles for somatostatin-IR, substance P-IR and VIP-IR which consistently showed co-elution of the peptide-like immunoreactivities with those of the standard peptides. Recoveries ranged from 82 to 109%. Extract concentrations of bombesin were too low to allow such chromatographic analysis.

Immunocytochemistry. Although significant concentrations of all four peptides were found in most regions examined by radioimmunoassay, only substance-P-IR and VIP-IR were present in sufficient concentration for reliable immunostaining. In both species, immunostaining was obtained with antibodies to VIP and substance P only in specialised areas, in particular the footpad and nose of the cat and the snout and lip of the pig. The abundance of nerves showing VIP- or substance P-like immunoreactivity in the different areas correlated well with the levels of these peptides found by radioimmunoassay. Furthermore, the two peptides had a distinct and different distribution of

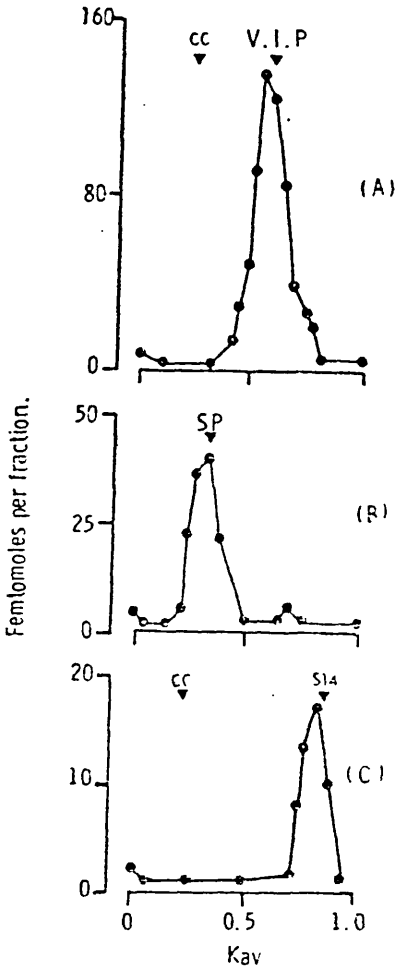


FIG. 1

Elution profiles of VIP (A) (G-50), substance P (B) (G-25) and somatostatin (C), (G-50). C.C. indicates the elution position of cytochrome C (mol. wt. 11,600). The arrow in (A) indicates the elution position of 'Standard' porcine VIP, in (B) the arrow indicates the elution position of synthetic substance P and in (C) the arrow indicates the elution position of the standard synthetic somatostatin 14 (S14).

immunostaining in each species, but this was less marked in the cat than the pig. The distribution patterns were consistent in all the animals of each species examined.

In the pig, substance P-like immunoreactivity was found in the snout, the lip and, less abundantly, in the anal skin and the foot. The immunostained nerve fibres were located beneath the epidermis, running both parallel and perpendicular to it into the folds of the dermal ridges (Fig. 2) and occasionally penetrating the basement membrane and passing into the epidermis. VIP-like immunoreactivity was found in nerve fibres that were deeper in the dermis and most abundant in the snout and lip. Here they were located principally around the sweat glands (Fig.3) and also around blood vessels. In anal and foot skin they were mainly around blood vessels.

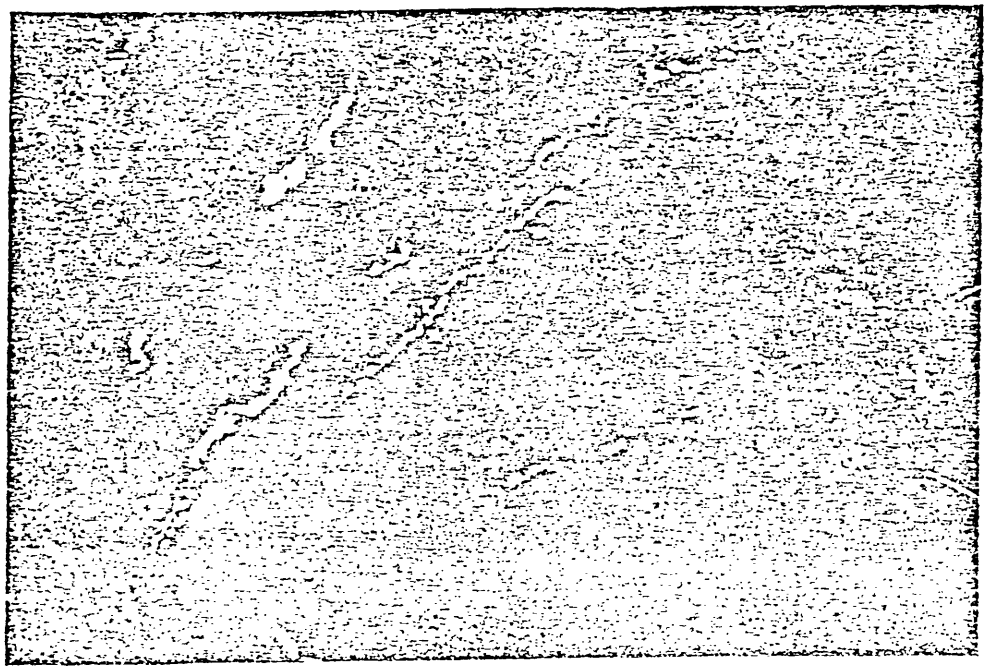


FIG. 2

Substance P immunoreactive nerves, in skin taken from the nose of a pig, shown running below the epidermis. (x 120).

In the cat, the separate intradermal distribution of the two peptides was less marked. VIP-like immunoreactivity was found in nerve fibres localised, as in the pig, around the sweat glands (Fig.4) and blood vessels with the greatest abundance in the nose and footpad. The nerves immunostained with antibodies to substance P in the cat ran perpendicular and parallel to the epidermis, as in the pig, but were fewer in number and were not confined to the subepidermal region but extended deeper into the dermis (Fig. 5). Thus, in the cat they were also found around sweat glands in equal numbers to nerves containing VIP-like immunoreactivity (Fig.6) and, to a lesser degree, around blood vessels. Again the greatest number of nerve fibres was seen in the footpad, with fewer in the nose.

In all cases, absorption of the antibody with the appropriate antigen abolished the immunoreactivity, and no immunostaining was obtained if the first layer antibody was omitted or replaced by normal rabbit serum.

Discussion

In almost all regions of feline and porcine skin significant amounts of immunoreactive bombesin, somatostatin, substance-P and VIP were found. In the pig substance-P-IR is the most abundant and present in all tissues examined. Somatostatin is present in smaller amounts but has a similar relative distribution. Both of these peptides are associated with primary sensory neurones and their relative distribution could reflect variations in cutaneous sensory structures. However the sensory modalitites which these two peptides may subserve has not been established although they appear to be associated

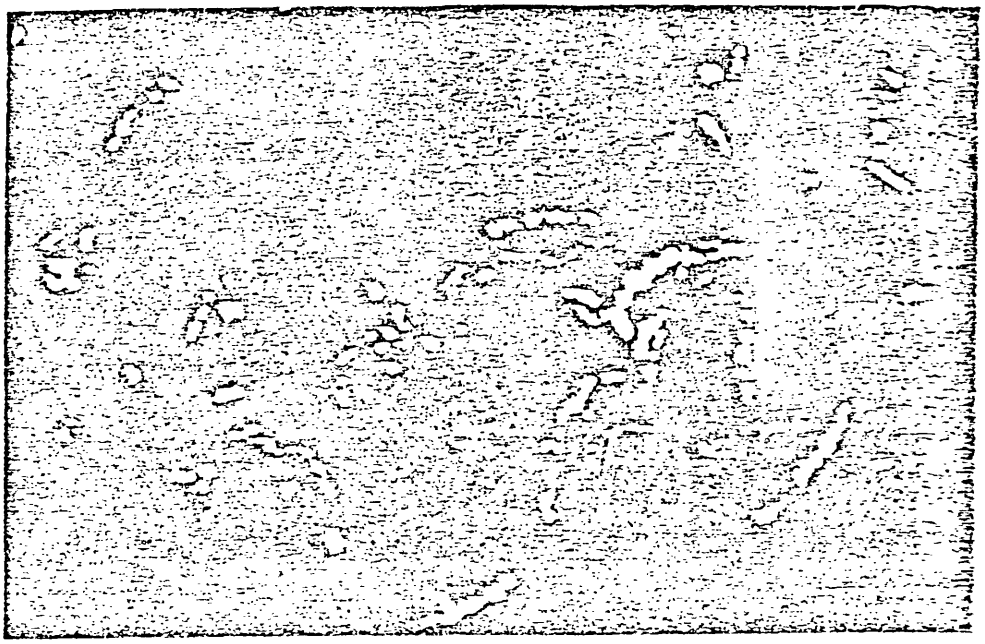


FIG. 3

Sweat glands in porcine nose skin, surrounded by immunoreactive VIP nerve fibres (x310).



FIG. 4

Skin from cat footpad containing VIP immunoreactive nerve fibres around sweat glands (x420).

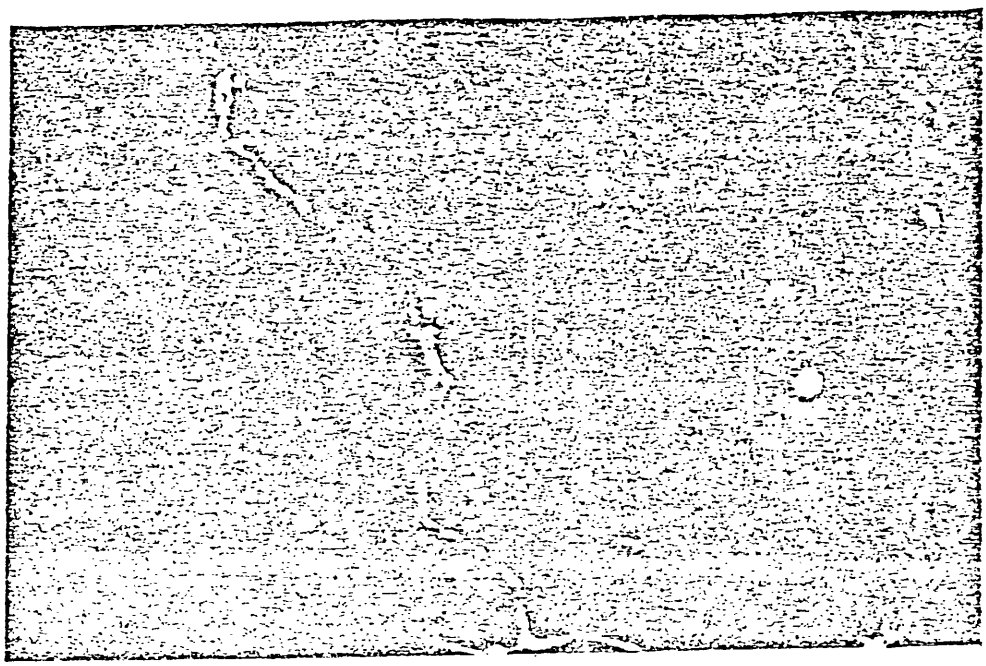


FIG. 5

Similar skin to that shown in Fig 3 but with nerve fibres immunostained for substance P running beneath the epidermis (x 230).

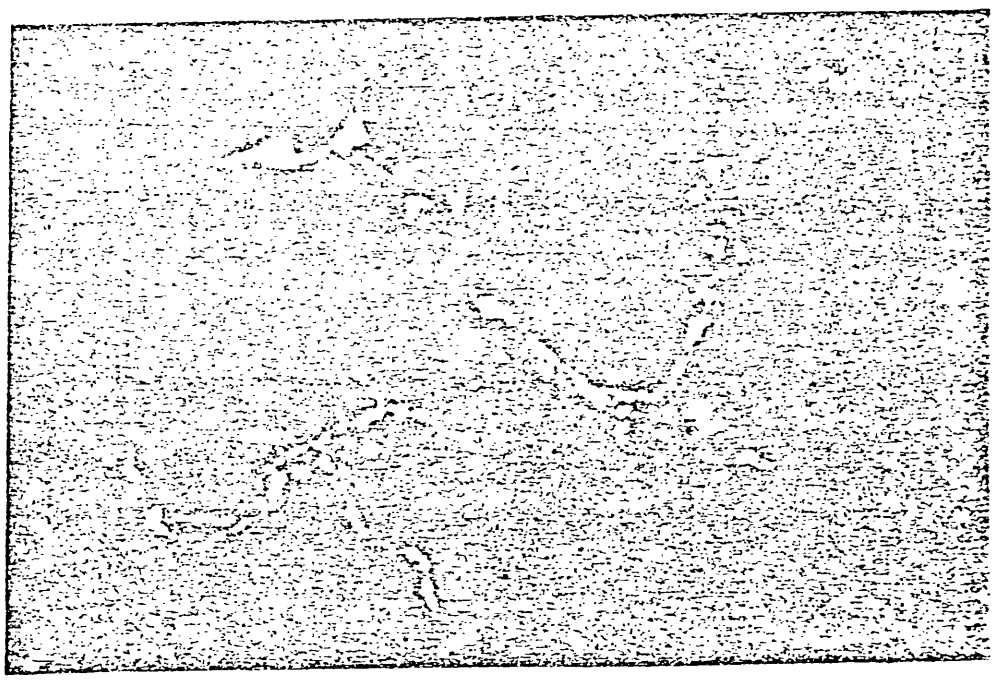


FIG. 6

Substance P immunoreactive nerves around sweat glands like these shown in Fig. 3 (x 360).

with the fine-fibre C-afferents which are thought to be nociceptive. Substance-P has been considered the central neurotransmitter of pain-sensory neurones and within the skin as the mediator of sensory axonal reflex. Cutaneous injection of this peptide produces effects similar to the triple response (17) which is consistent with our localisation of substance-P just below the epidermis. Howevever, in the cat paw we find, in agreement with Hökfelt and co-workers (6), that substance-P immunoreactivity is localised around sweat glands and blood vessels, which may indicate functional differences between species.

The skin receives a prominent innervation to blood vessels and sweat glands from the sympathetic nervous system, and although somatostatin has been associated with such neurones we have no evidence for the presence of this peptide in cutaneous sympathetic nerves. The levels of somatostatin that we find by radioimmunoassay are in fact below the detection threshold of the less sensitive immunohistochemical technique which uses a different somatostatin antibody and thus prevents determination of the location of cutaneous somatostatin.

The levels of VIP-IR in the pig snout were similar to those of substance-P but elsewhere in this species were several orders of magnitude lower. Furthermore in the cat concentrations of VIP-IR, especially in the paws, were considerably higher than those of substance-P which suggests quite separate functional roles for these two peptides. VIP has been clearly shown to influence salivation and pancreatic exocrine function (18-21). Our finding of VIP-stained nerves apparently innervating sweat glands, is therefore further evidence of its possible role in the control of exocrine fluid secretion. Exogenous administration of VIP produces a powerful vasodilatory effect (23), thus the observation that nerves staining for this peptide surround cutaneous blood vessels offers further evidence that it may control blood flow in the skin. This dual localisation of VIP-IR within the dermis suggests that it could possess an important role in the cutaneous mechanisms of thermoregulation.

Bombesin has been shown to have potent secretomotor and hormone stimulatory actions in the periphery (25) and is present in a variety of mammalian tissues, to which now can be added the skin. Such a widespread distribution probably reflects a significant physiological role, which however remains elusive at present. Porcine and feline skin, like amphibian, contains significant amounts of regulatory peptides and we have demonstrated that four of these peptides have distinct regional distributions in this tissue. We have further shown that substance-P-IR and VIP-IR are associated with different cutaneous structures. However, further functional studies are necessary to establish the physiological roles of these neuropeptides within mammalian skin.

Acknowledgements

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The Origin of VIP-Containing Nerves in the Urinary Bladder of Rat

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GU, J., J. M. POLAK, M. A. BLANK, G. TERENGHI, J. F. B. MORRISON AND S. R. BLOOM. *The origin of VIP-containing nerves in the urinary bladder of rat.* PEPTIDES 5(2) 000–000, 1984.—The innervation of the urinary bladder is known to include a considerable number of nerves containing vasoactive intestinal polypeptide (VIP). The origin of such nerves in the bladder of rat was investigated in this study using the methods of immunocytochemistry and radioimmunoassay combined with surgical sectioning of the hypogastric and/or pelvic nerves to the bladder. Eight days after pelvic nerve sectioning proximal to the main pelvic ganglion, VIP-immunoreactive nerves and VIP content were markedly increased from the level in the sham-operated rat bladder. Sectioning of hypogastric or both nerve pathways led to a less significant increase. It was therefore postulated that the majority of VIP-immunoreactive nerves originate from ganglia located either close to the bladder or within the bladder wall. It is interesting that in these experiments the VIP content of the bladder nerves is in motility that result from the nerve sections.

Denervation Ganglia Immunocytochemistry Radioimmunoassay Rat Urinary bladder
Vasoactive intestinal polypeptide

IN addition to cholinergic and noradrenergic nerves, the urinary bladder is known to be innervated by at least six nerve types containing regulatory peptides [2, 15, 17]. Of these, the nerves containing vasoactive intestinal polypeptide (VIP) are the most abundant. Although species variations in their frequency and in the populations of intramural ganglion cells exist, the distribution patterns of VIP-containing nerves are, in general, similar to each other in the bladders of man, cat, rat, mouse and guinea-pig (Unpublished observation). Anatomically, they are more concentrated in the trigone than in other regions. Histologically, they constitute the submucosal plexus beneath the transitional epithelium, innervate the detrusor muscle and surround most of the blood vessels. Scattered ganglion cell bodies immunoreactive to VIP antiserum have been seen in the bladder wall of cat [2] and man (Gu *et al.* submitted for publication). However, conclusive evidence for the origin of VIP-containing nerves in the bladder has not been forthcoming. In an attempt to gain insight into the origin of VIP-containing nerves in the bladder we carried out a variety of denervation procedures in the rat and examined the post-operative changes which may occur in the VIP innervation.

METHOD

Adult female rats (n=24) were operated under general anesthesia with pentobarbitone sodium (Sagatal). The denervation of the urinary bladder was carried out in three ways and each was performed on 6 rats. In one group, the sympathetic pathways were sectioned bilaterally at the points before they join the pelvic plexus. In the second group, the parasympathetic pathway was cut unilaterally at a point just proximal to the pelvic nerve ganglion. In this way, however, certain parasympathetic and sympathetic ganglia were inevitably left intact on the distal side of the sectioning points. For them, only the preganglionic fibres were interrupted and thus decentralization may be a more appropriate term than denervation. Thirdly, both sympathetic and parasympathetic pathways were sectioned at the above points. Sham-operations were performed on the fourth group of 6 rats as controls. The design of the operations is shown in Fig. 1.

The rats were killed with an overdose of ether 8 days after the operations and different regions of the urinary bladder were dissected. For immunocytochemistry, the tissues were fixed in 0.4% benzoquinone solution [4] for one hour. After washing overnight in 7% sucrose in phosphate-buffered saline, the tissues were made into cryostat blocks and 5 μm cryostat sections were cut. A modified indirect immunofluorescence method [12] with routine controls was performed on air-dried sections mounted on poly-L-lysine-coated slides [16]. The antiserum to VIP was used at a concentration of 1:2.000. The immunostaining was completely

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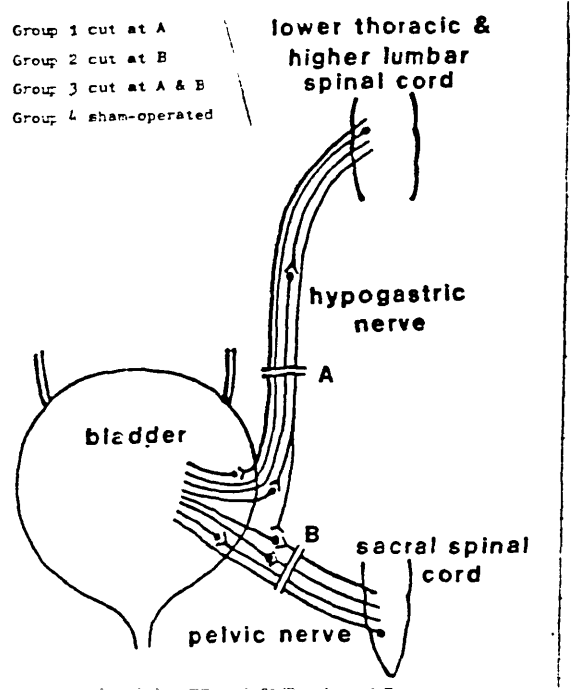


FIG. 1. Design of surgical sectioning of nerve supply to rat bladder.

abolished by adding pure natural porcine VIP to the antiserum prior to immunostaining at a concentration of 1.0 nmol/ml of diluted antiserum.

For radioimmunoassay, the tissues were extracted in boiling acetic acid (0.5 M, 10 ml per gram of tissue). Duplicate aliquots were then assayed in specific radioimmunoassays [5]. The sensitivity of the assay was 0.4 fmol/assay tube.

The antisera for immunocytochemistry and radioimmunoassay were raised in New Zealand white rabbits against natural porcine VIP conjugated to keyhole limpet haemocyanin with glutaraldehyde.

RESULTS

It was found by immunocytochemistry that the number of VIP-immunoreactive nerves was increased after all three types of denervation but to different extents. After unilateral parasympathetic denervation, the number of VIP-immunoreactive nerves was markedly increased on both sides of the bladder but particularly so on the denervated side (Figs. 2 and 3). The bilateral hypogastric denervation caused a slight increase in the number of VIP-immunoreactive nerves which was most apparent in the trigonal region (Fig. 4). Sectioning of both nerve types led to a considerable increase in VIP-immunoreactive nerves but less marked than that caused by parasympathetic denervation alone. The increased VIP-immunoreactive nerve fibres were varicose and were as brightly stained as in the control bladders. A small number of intrinsic VIP-immunoreactive ganglion cells were occasionally seen in all the different groups of rats. All the controls for immunocytochemistry were negative.

Radioimmunoassay also detected marked increases (up to 3 fold) in the VIP concentration in the denervated bladders from 3.5±0.8 pmol/g (mean±SEM) in the sham-operated group to 4.3±0.7 pmol/g, 10.8±2.6 pmol/g (operated side) and 7.1±1.5 pmol/g (operated side) in hypogastric, pelvic and both denervated rats respectively (see Fig. 5).

DISCUSSION

The experimental design was based on the current view of urinary bladder innervation [10,20] that the secondary ganglia of the sympathetic system mainly lie in the inferior mesenteric plexus and those of the parasympathetic mainly in the pelvic plexus, whereas the sympathetic 'short neurons' also exist in close vicinity of the bladder. After the hypogastric and pelvic nerves meet to form the pelvic plexus, sympathetic and parasympathetic postganglionic nerves are randomly mixed [10]. Thus cutting within or distal to the pelvic plexus would give little information on which pathway had been interrupted. Therefore, in this study, the cutting points were proximal to the pelvic plexuses and left the plexus undisturbed.

Previous experiments have shown that a decrease of acetylcholinesterase (AChE)-positive nerves in the rat bladder occurred in less than one week of the pelvic denervation, and from two weeks onwards outgrowth of the same nerve type appeared [3]. Therefore in this study, the time length chosen to investigate the changes after surgery was 8 days, in the hope of finding the full morphological effects of the surgical denervation before it was obscured by regeneration from the disconnected axons [13]. The experiment was also based on the concept that the discontinuation of the postganglionic axons would lead to a rapid destruction of their distal terminals in the organ, while sectioning of the primary ganglionic or preganglionic, axons would have little morphological effect on their postganglionic nerve terminals in the innervated organ in this short period [1,19].

It was found in this study that VIP-immunoreactive nerves in the rat bladder were increased in number following the interruption of hypogastric and/or pelvic pathways. The implications of this are not immediately obvious due to lack of information on the response of peptide-immunoreactive neurones to nerve injury and the complexity of the organisation of the bladder nerve supply. However, it suggests that the changes of VIP-immunoreactive nerves after denervation are indicative of their origin.

That the majority of intrinsic VIP-immunoreactive nerves do not originate from ganglia located proximal to the junction of pelvic and hypogastric nerves is shown by there being no decrease of VIP immunoreactivity following the disconnection of the nerves at these points. This indicates that some of the VIP-immunoreactive postganglionic axons, probably most of them, were not interrupted by the operations. Therefore, the majority of the VIP-immunoreactive nerves probably originate from ganglia which lie in the plexuses close to the bladder or within the bladder wall. The VIP-immunoreactive ganglion cells in the bladder wall may be the origin for some of the VIP-immunoreactive nerves in the bladder.

This is in keeping with recent reports by Elbadawi [10] and El-Sadr [11] who described the existence of intrinsic ganglion cells in rat bladder. We have, in addition, observed a marked VIP increase in the ipsilateral side of the urinary bladder of the cat (where cell bodies are known to occur) after unilateral pelvic decentralisation (unpublished). A contribution from extrinsic cell bodies located mainly in the pelvic and vesical plexuses cannot be ruled out since it has been noted that when denervations were done very close to the bladder wall, i.e., distal to the pelvic and vesical

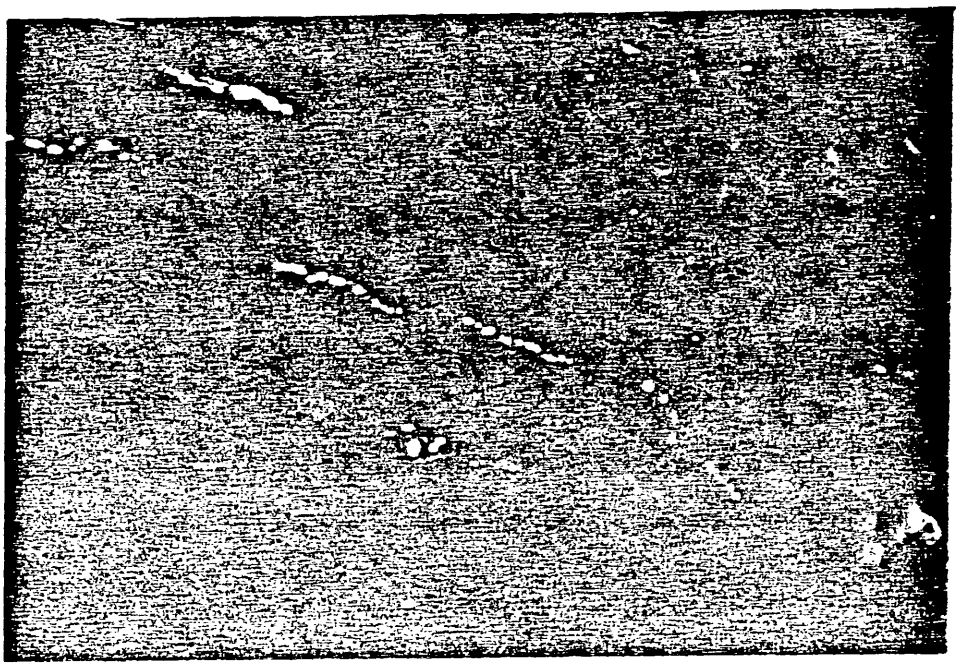


FIG. 2. VIP-immunoreactive nerves in the urinary bladder of sham-operated rat (×380).

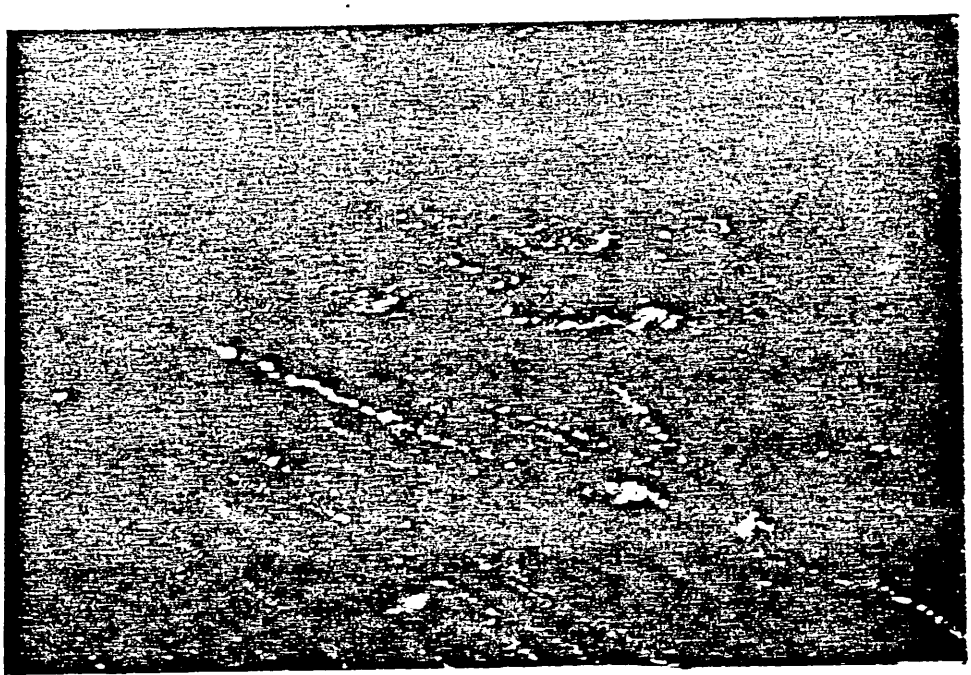


FIG. 3. VIP-immunoreactive nerves in the urinary bladder of pelvically decentralised rat (×340).

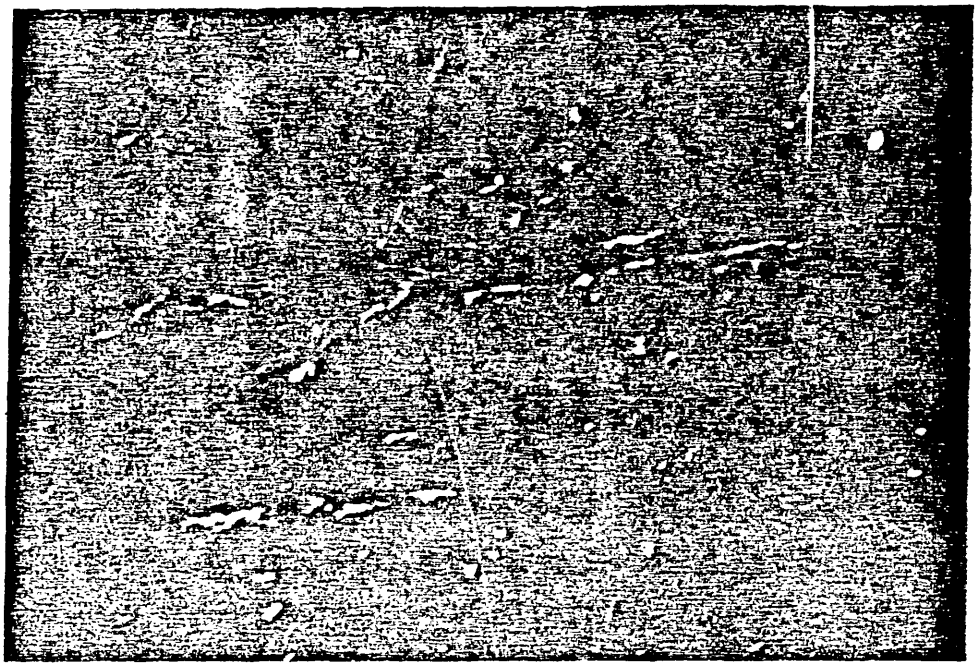


FIG. 4. VIP-immunoreactive nerves in the urinary bladder of hypogastrically denervated/decentralised rat (×400).

plexuses, the number of VIP-immunoreactive nerves was decreased.

The increase of VIP-containing nerves in the bladder following pelvic nerve section alone was less marked when the hypogastric nerves were also sectioned and suggest that part of the former increase was dependent on the integrity of the hypogastric nerves. These increases in VIP content appear to be inversely related to the changes in motility that are expected as a result of these denervations [19]. Thus the highest level of VIP occurs when the bladder has its excitatory nerve supply sectioned and has an intact inhibitory sympathetic supply; and a lower level occurs when the bladder is deprived of both extrinsic excitatory and inhibitory nerves; hypogastric nerve transection often has little effect on motility.

The increase of VIP-immunoreactive nerves after various denervations may also, presumably, represent a compensatory phenomenon, i.e., re-innervation or "repairing" of the lost nerves by outgrowth from the neighbouring intact nerve fibres [21].

Sectioning of the pelvic nerve alone resulted in an increase in VIP-immunoreactive nerves higher than that caused by sectioning both pelvic and hypogastric nerves. This may indicate that the VIP-containing neuronal cell bodies in the pelvic and vesical plexuses or inside the bladder wall are influenced by sympathetic transmission. It has been reported that the sympathetic efferents facilitate or inhibit transmission through some pelvic non-adrenergic ganglion cells located proximal to the bladders of cat and rat [6, 7, 8, 14, 18]. The disconnection of the sympathetic pathway would partially suppress the outgrowth of VIP-containing nerve terminals.

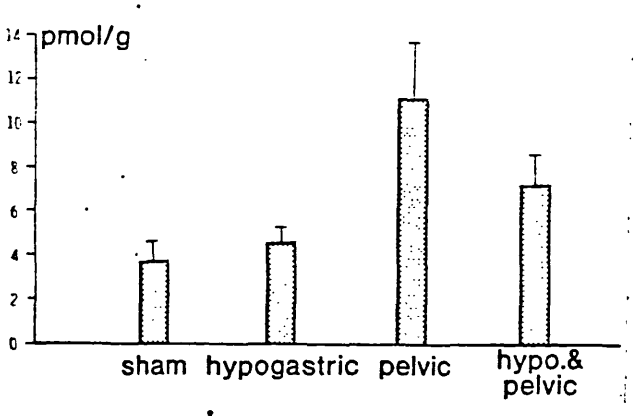


FIG. 5. VIP in bladder after surgery.

Thus, the majority of the VIP-immunoreactive nerves in the urinary bladder of the rat appears to originate from ganglia located close to or within the bladder.

ACKNOWLEDGEMENT

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Repeated application of first-layer antiserum improves immunofluorescence staining: a modification of the indirect immunofluorescence staining procedure

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Summary

Repeated application of the first-layer antiserum in the indirect immunofluorescence technique considerably improves the immunostaining. The modified method reveals more antigenic sites and increases the contrast between specific and background stainings, particularly where sparsely distributed antigenic areas are to be investigated. The effect of this novel immunostaining procedure is compared with that of the routine procedure and of other modifications. Possible mechanisms for the improving effect are discussed. A procedure of combined modifications is recommended for exploration of non-abundant antigens or for achieving a high-quality photograph.

Introduction

The indirect immunofluorescence technique of Coons (Coons *et al.*, 1955) is one of the most commonly used methods in immunocytochemistry. It consists of a first-layer antibody against the tissue antigen and a fluorescent dye-conjugated second-layer antibody against the first layer antibody, which now serves as a secondary antigen. Because one first-layer antibody molecule can react with more than one, sometimes many, second-layer antibody molecules, the indirect method is more sensitive than the direct method since the first-layer antiserum can be diluted to a greater extent. It was thought, however, that the sensitivity of the indirect immunofluorescence method had not yet reached its maximum and many modifications have been made. These include prolonging the incubation time (Brandtzaeg, 1981; Scott *et al.*, 1981), raising the incubation temperature (Huang *et al.*, 1976; Portmann *et al.*, 1976; Dorsett & Joachim, 1978), treating the tissue with detergent (Hartman *et al.*, 1972; Hartman, 1973), etc. In

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our laboratory as in many others, the indirect immunofluorescence method has been conducted using highly diluted antibodies incubated overnight at 4° C (Bishop *et al.*, 1978). The immunostaining results of this routine procedure have been generally satisfactory.

We have recently found that a second application of the first-layer antiserum for a further 2–4 h after the first incubation and washing resulted in much better staining than did the routine procedure described above. There was better contrast between specific and background stainings and apparently a greater abundancy of explored antigenic sites. In order to explore the full potential of this novel procedure, several experiments were carried out to compare the proposed improvement with our routine staining procedure and with some other modifications including prolonging the incubation time with the first-layer antiserum, changing the concentration of first-layer antibody, treating the sections with a detergent, Triton X-100 and combinations of these variations.

Materials and methods

The specimens chosen were guinea-pig ileum (n = 4) and human (n = 5) and feline (n = 6) urinary tract including the lower portion of the ureter, and the dome and trigone of the bladder. All the tissues were known to contain the antigenic sites corresponding with the antibodies applied. The tissues were fixed, within 30 min of removal, in 0.4% *p*-benzaquinone in phosphate-buffered saline (PBS) for 1 h at room temperature (Bishop *et al.*, 1978) and then washed overnight in 7% PBS–sucrose at 4° C. Tissue blocks were then frozen in Arcton and serial 5 μm cryostat sections were cut. The sections were picked up on poly-L-lysine-coated glass slides and left at room temperature for 30 min to allow them to stick firmly onto the slides. Freeze-dried, *p*-benzoquinone vapour fixed (60° C, 3 h) (Pearse & Polak, 1975), 5 μm thick paraffin sections were also prepared and treated in the same way as the cryostat sections.

First-layer antisera were raised in rabbit against vasoactive intestinal polypeptide, dopamine-β-hydroxylase, glucagon 5-hydroxytryptamine and neurone-specific enolase. The characteristics of these antisera have been described previously (Goldstein, 1972; Marangos *et al.*, 1979; Gu *et al.*, 1981; Polak *et al.*, 1981). Goat anti-rabbit IgG, conjugated with Fluorescein isothiocyanate (FITC), was used as secondary antiserum. On serial sections from each area, the following procedures were carried out.

(1) Routine procedure, that is, sections are incubated with the first-layer antiserum for 12–16 h at 4° C, the antiserum being optimally diluted (that is, either higher or lower dilutions would impair the quality of the staining), generally at 1:500–4000 in PBS, pH 7.2, for antigens of peptides, amines and enzymes. The second layer, FITC-conjugated antibody, diluted 1:100 in PBS, is applied for 30–45 min at room temperature. After each step, the slide is washed in PBS three times, 5 min each. The stained sections are mounted in PBS–glycerine (1/9) and examined immediately.
(2) Increasing or decreasing the concentration of the first-layer antiserum.
(3) Pre-treating the cryostat sections with 0.2% Triton X-100 in PBS for 30 min or using this solution instead of PBS to dilute the antisera.
(4) Prolonging the incubation time with the first-layer antiserum up to 72 h at room temperature.
(5) Repeating the first-layer antiserum application for 2–20 h at room temperature or 2–3 times, each lasting 2–6 h

(6) Combinations of the above procedures.
(7) The same modifications were attempted using the unlabelled antibody-enzyme (PAP) method (Sternberger, 1974).
The controls included preabsorbing of the first layer antisera with their appropriate antigens, using normal rabbit serum as first layer and simply omitting the first-layer antiserum incubation.

The immunostained sections were examined independently by two people who had no knowledge of the procedures used on the sections. The relative abundance and brightness of the specific staining and the background staining were separately marked from 0-8+ using the same criteria throughout. The degree of contrast between the specific staining and the background staining was regarded as a measure of the quality of the immunostaining.

Results

The results are shown diagramatically in Fig. 1 and are summarized below.

Repeating the first-layer incubation, prolonging the incubation time with the first-layer antiserum and treating the section with Triton X-100 all improved the quality of the staining by noticeably increasing the specific staining with or without a small increment of the background staining. The effect was most marked when the first layer was repeated and the total time of the first-layer antibody incubation was equal to that of the routine procedure (Figs. 2, 3, 5, 6). This effect of improvement was still apparent when

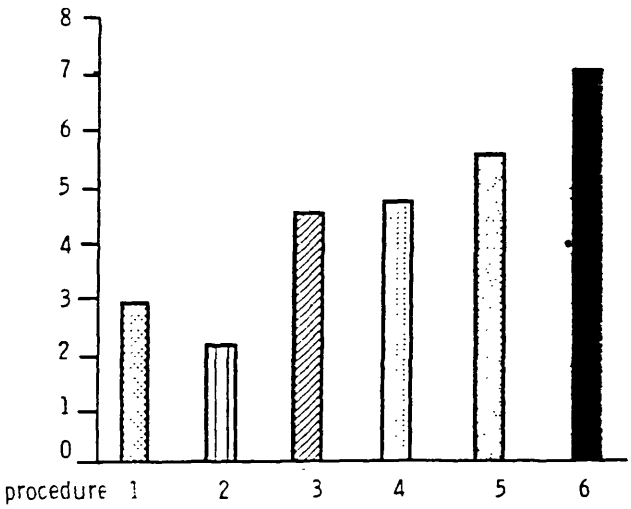


Fig. 1. Effects of modifications on the indirect immunofluorescence procedure. The height of the bars represents the quality of the immunostaining, that is, the net results obtained by marking the abundance and brightness of the specific staining (0-8+) after subtraction of the background staining (0-8+). Procedures: (1) routine procedure; (2) changing the concentrations of the first-layer antibodies; (3) using Triton X-100 to pre-treat the sections or to dilute the antiserum; (4) prolonging the incubation time with the first-layer antiserum; (5) repeating the first-layer antiserum application; and (6) a combination of procedures 3, 4 and 5.

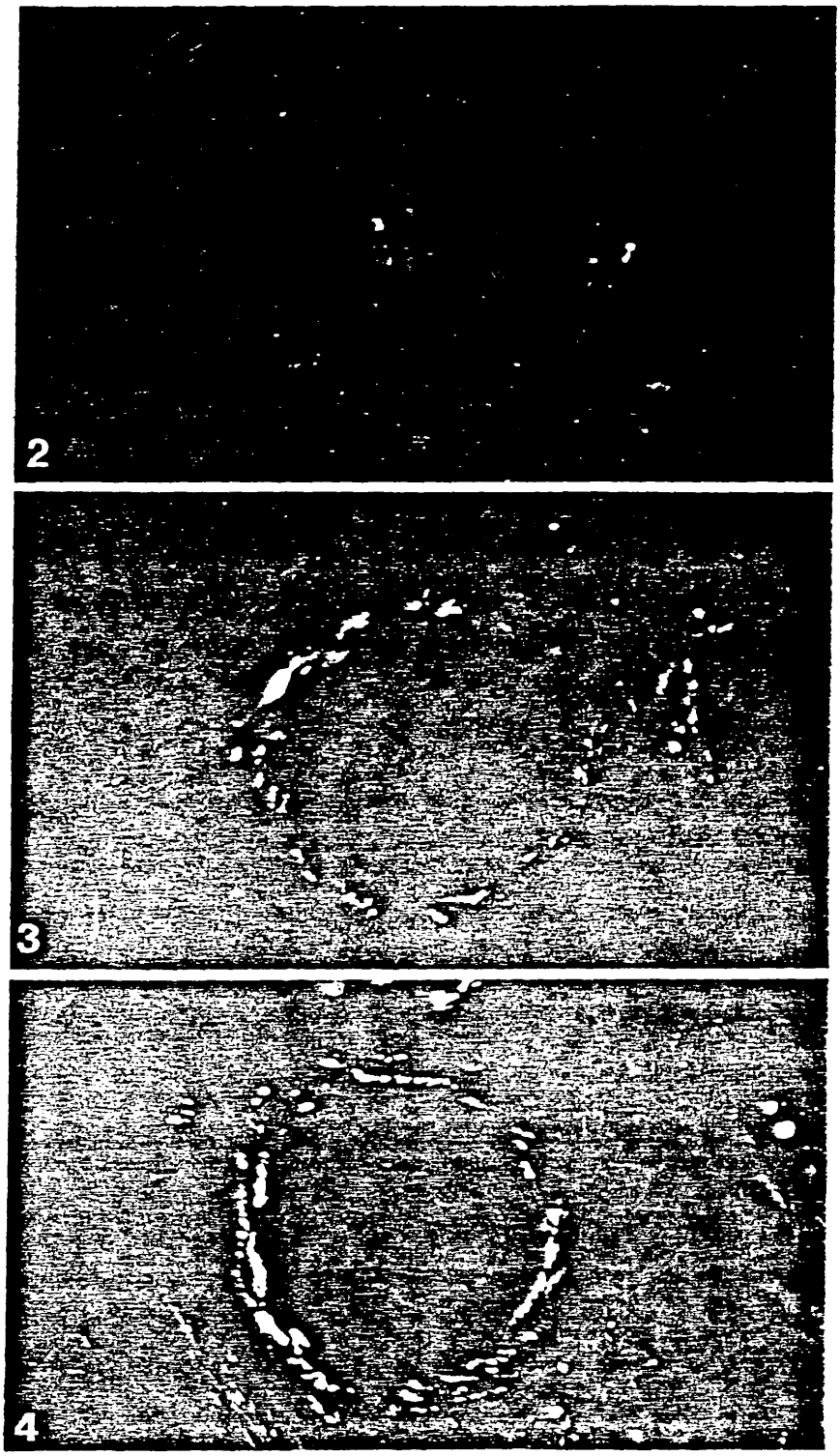


Fig. 2. Human ureter immunostained with antiserum to dopamine β-hydroxylase (DBH) using the routine procedure. Very few immunoreactive nerves were found around the blood vessel.
Fig. 3. A section serial to that in Fig. 2 immunostained with the same dopamine β-hydroxylase antiserum at the same concentration, on which the first-layer antiserum was applied twice. A considerable number of immunoreactive nerves surrounding the blood vessel were revealed.
Fig. 4. A section serial to Fig. 3 on which the combined immunostaining procedure, that is, procedure 6, was employed. Many more DBH-immunoreactive nerves were demonstrated.

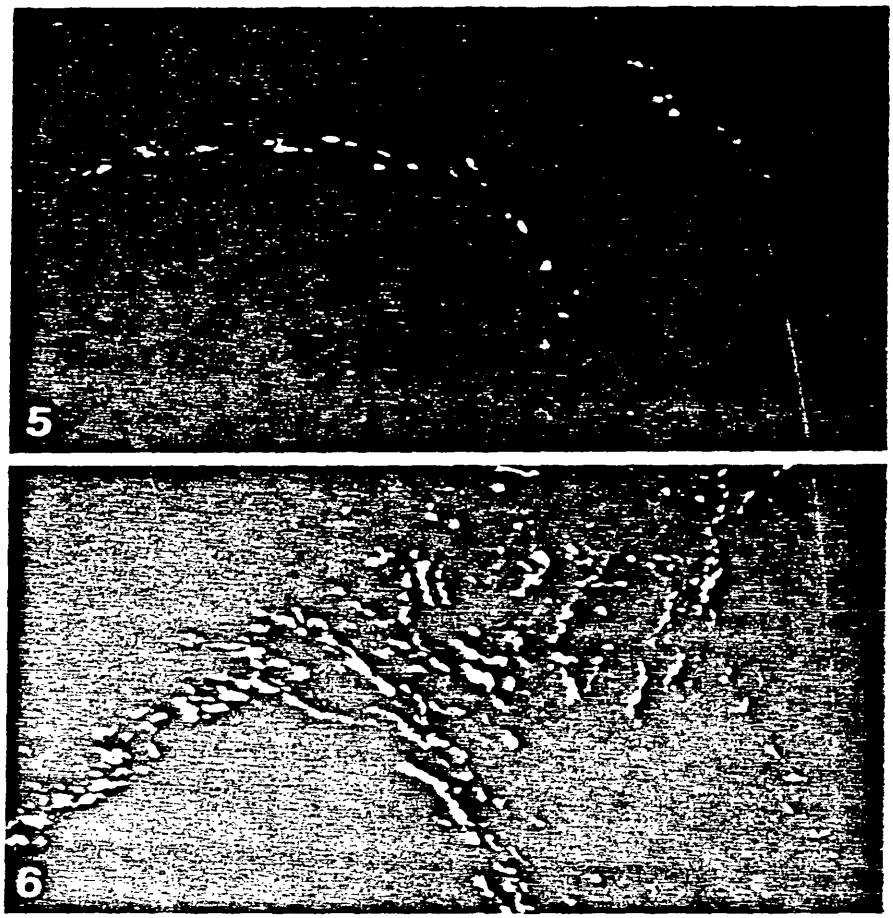


Fig. 5. A blood vessel in the trigon of feline urinary bladder. Vasoactive intestinal polypeptide (VIP)-immunoreactive nerves surrounding the vessel were demonstrated using the routine procedure.

Fig. 6. A consecutive section to that in Fig. 5 in which the incubation of the first-layer antiserum against VIP was repeated. Many more VIP-immunoreactive nerves were revealed.

the first-layer antiserum was further (triple) diluted. Repeating incubation with the first-layer antiserum more than once achieved little further improvement.

Increasing the concentration of the first-layer antibody generally increased both the specific staining and the background staining, resulting in reduced contrast and a worse picture.

The improving effect of repeating the first-layer procedure is more obvious on immunostained nerves (vasoactive intestinal polypeptide, dopamine-β-hydroxylase) than on cells where the antigens are generally more abundant. The improvement was

less noticeable when immunostaining by the PAP method or when using paraffin wax rather than cryostat sections of the same tissue.

The best result was obtained using a procedure of combined modifications (Fig. 4), that is, pre-treating the sections with 0.2% Triton X-100 in PBS for 30 min, repeating the first-layer incubation for 4 h and prolonging the incubation time with the first-layer antibody to a total of about 30 h, along with increased washing time after each staining step (three times, 10 min each). This procedure sometimes changed an apparently negative staining result into a positive one and revealed many more antigenic sites than the routine procedure (Figs. 2–4).

The controls for the various staining procedures were all negative.

Discussion

From the above results, it seems that repeated applications of the first-layer antiserum can considerably improve immunofluorescence staining on pre-fixed cryostat sections, particularly when sparsely distributed antigenic areas such as peptide-containing nerves are the targets for exploration. The improvement was still shown when the total time spent on the first layer plus the repeated layer was equal to that of the routine procedure, even when the first-layer antiserum was further diluted. The validity of the new procedure has been tested by appropriate controls which showed that the modification does not create any non-specific positive staining but only compensates for the insufficient part of the conventional technique. The improving effect of the modified procedure was less obvious by the peroxide–antiperoxidase method; this may be due to the higher sensitivity of this technique compared with that of the indirect fluorescence procedure.

The possible mechanism of this improvement has yet to be studied. It is possible that the first-layer antiserum consists of both high-affinity and low-affinity specific antibodies. During the first incubation with the first-layer antiserum, all the antigenic sites on the section are occupied by one or other of the antibody types. During the subsequent washing, the low-affinity antibodies are washed off and consequently the fluorescent label is only built up at the proportion of the antigenic sites which were initially bound by high-affinity antibodies. The vacated antigenic sites can be largely restained by the second application of the fresh first-layer antiserum, thus revealing more antigenic sites (Fig. 7).

Another speculation is that the antibodies are uniformly distributed in the antiserum and the antigenic sites are not uniformly distributed on the tissue section. The antibodies above the antigenic sites are soon used up and the slow diffusion of antibodies from neighbouring areas takes time. In the routine staining procedure, incubation is stopped before the diffusion is complete. This might explain the fact that longer incubation time gives much better staining. Repeating the first layer, that is, replacing the half-used antiserum with fresh antiserum, would provide another antibody concentrate and leave few free antigenic sites on the tissue section, thus

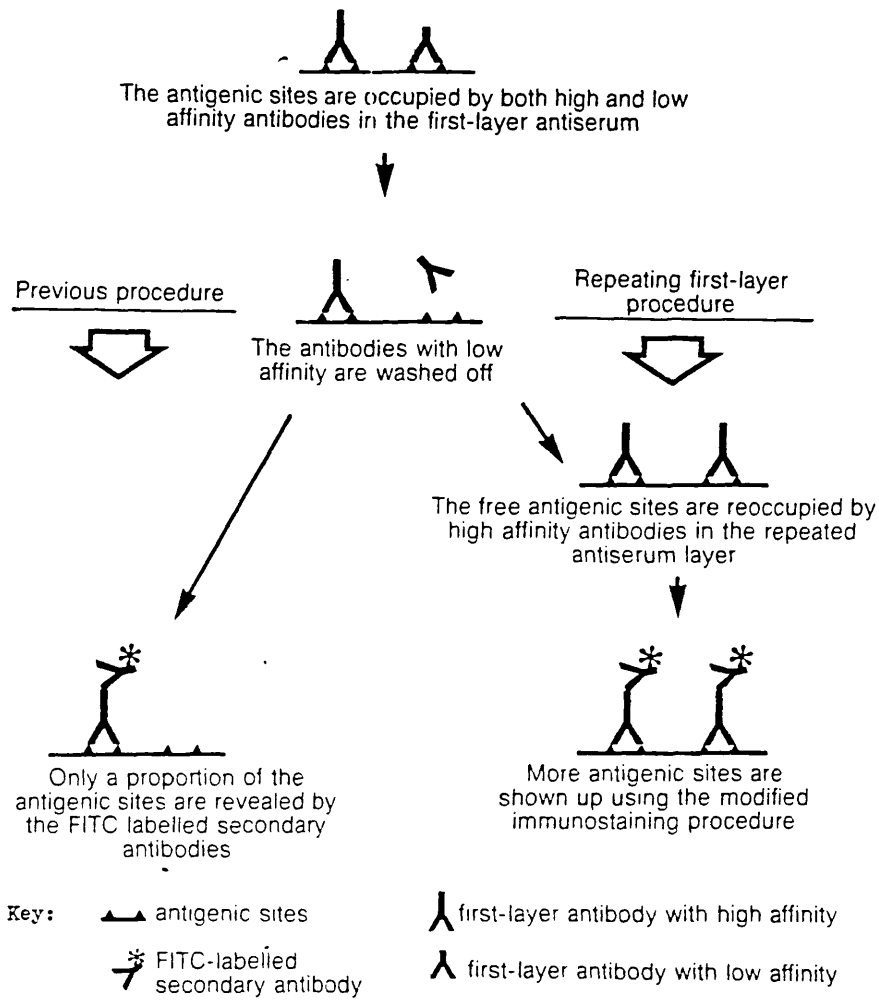


Fig. 7. The possible mechanism in the repeating first-layer procedure.

achieving a much better result. Both of the postulated mechanisms, and possibly some other factors also, may contribute to the improving effect of the modified immunostaining procedure.

Conclusion

Repeated application of the first-layer antiserum improves the indirect immunofluorescence staining for some peptides, amines and enzymes, particularly when they occur in relatively low concentrations, such as those present in nerves.

A combination of some modifications, for example, treating sections with detergent, repeating the first-layer antiserum incubation, prolonging the incubation time, etc. may be the method of choice when exploration of sparsely distributed antigenic areas and high quality photographs are the reasons for undertaking immunostaining.

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SEQUENTIAL USE OF THE PAP AND IMMUNOGOLD STAINING METHODS FOR THE LIGHT MICROSCOPICAL DOUBLE STAINING OF TISSUE ANTIGENS

Its application to the study of regulatory peptides in the gut

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SUMMARY

Double immunoperoxidase staining using different couplers can give various combinations of colours on a single tissue section to achieve a comparable picture of different antigens. However, the colour combinations achieved to date are not entirely satisfactory.

A double immunostaining procedure is introduced here, combining the peroxidase anti-peroxidase (PAP) and immunogold staining (IGS) methods. The IGS method is a new, simple, sensitive and reliable approach to immunostaining at the light microscopic level. It was carried out in three ways. Firstly, a two-step method was used in which the second layer was goat anti-rabbit IgG adsorbed onto gold particles (GAR/Au20). Secondly, a three-step method was employed where the second layer was unlabelled goat anti-rabbit IgG and the third layer was a rabbit antibody to peroxidase adsorbed onto the gold particles (RAP/Au20) and acting as a gold-labelled IgG antigen. The third method combined the first two methods using GAR/Au20 as the

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second layer and RAP/Au20 as the third layer which increased the amount of bound gold and enhanced the red colour, providing a better picture.

The use of gold-labelled antibodies in double immunostaining has great potential value for many studies including that of the diffuse neuroendocrine system of the gut.

regulatory peptides; immunocytochemistry; gastrointestinal endocrinology; endocrine cells; autonomic innervation

INTRODUCTION

Immunofluorescence and immunoperoxidase techniques have been shown to be useful tools in the study of the regulatory peptide- and/or amine-containing cells and nerves of the diffuse neuroendocrine system [10]. Most of these regulatory peptides have been localised to different populations of cells and nerves. A simple and reliable method for the double or multiple immunostaining of different peptides or amines is therefore required for the study of their interrelationships. At least 6 techniques have been introduced [12]. Immunofluorescence can be carried out using fluorescent labels of different colours, i.e. fluorescein – green, rhodamine – red. For immunoperoxidase staining, various couplers can be used to give final reaction products of different colours. One such technique [8] employs the selective elution of the antibody from the section by acid, without removing the coloured insoluble endproduct of the reaction or altering the antigenicities, and thus allows the simultaneous demonstration of multiple antigens in a single tissue section. However, for purposes of comparison, because the contrast between the colours of the different endproducts is not sharp enough, none of the various colour combinations has yet been found to be optimal.

Elsewhere, we introduce a novel light microscopic immunocytochemical method, using immunoglobulins adsorbed to colloidal gold: the immunogold staining (IGS) method (De Mey, J., Moeremans, M., Cuello, C., Milstein, C., De Brabander, M. and Polak, J.M., unpublished results). The advantages of the IGS method include its simplicity, sensitivity, and the fact that the progressive development of the red stain over the places of interest can be monitored continuously.

We wish to introduce a new double immunostaining procedure combining the unlabelled antibody enzyme (peroxidase anti-peroxidase, PAP) technique [11] and the immunogold staining (IGS) method [2] which provides an easily comparable and colourful picture.

MATERIALS AND METHODS

Specimens

Fresh specimens of fundus, antrum and colon were taken from pig, rat and man (surgical resection). A small piece of each specimen (0.5 × 0.5 cm^2) was frozen in Arcton and freeze-dried overnight at −40°C. It was then fixed in benzoquinone vapour (60°C, 3 h) or paraformaldehyde vapour (80°C, 1 h) and embedded in paraffin wax, using a previously described procedure [9]. Sections (10 μm) were cut and mounted on uncoated slides.

Preparation of gold-labelled antibodies

The procedure is based on a modification of the methods of Geoghegan et al. [4] and Horisberger et al. [7]. Purified rabbit immunoglobulin G (RIgG) was dialyzed against 2 mM borate buffer, pH 9.0. The protein solution was centrifuged at 100 000 × *g* for 1 h at 4°C to remove aggregates. The optimal amount of protein needed to stabilise the gold solution was added dropwise, but quickly, to the gold solution at room temperature [3]. After 1–2 min stirring, polyethylene glycol (PEG) stock solution (10% in water) was added to a final concentration of 0.1%. Unbound proteins were removed by 3 cycles of centrifugation (12 000 × *g*, 4°C, 1 h in 0.1% PEG/phosphate-buffered saline, pH 8.2, plus $2 \cdot 10^{-2}$ M sodium azide). The last pellet was resuspended in this PEG buffer to give a final volume 1/10 of the original volume of the gold salt. For use in immunocytochemistry dilutions of IGS reagents were made in PEG buffer. All diluted IGS reagents were centrifuged at low speed just before use in order to remove some very sticky microaggregates.

Double immunostaining

Double immunostaining was carried out by using the PAP method followed by the IGS-method.

PAP method

The PAP technique was carried out exactly as described previously [11]. Antisera against vasoactive intestinal peptide (VIP), substance P, met-enkephalin, gastrin, somatostatin, glucagon, motilin, cholecystokinin (CCK), pancreatic polypeptide (PP), secretin, gastric inhibitory polypeptide (GIP), and serotonin were used as primary antibodies at the appropriate dilutions in 0.01 M phosphate-buffered normal saline, pH 7.0 (PBS) (Table I).

Incubation lasted 14–18 h at 4°C in a moist atmosphere. After washing in PBS (three changes, 5 min each), the sections were incubated with goat anti-rabbit γ-globulin diluted 1 : 200 in PBS for 30 min. The sections were then washed as before and incubated with PAP complex diluted 1 : 300 for 30

TABLE I

Details of antisera.

Antiserum raised against	Region specificity	Dilution in IGS methods A and C	Dilution in PAP and IGS method B	Concentration of peptides used for absorption (nmol/ml diluted antiserum)
VIP	C-terminal	NU	1 : 20 000	0.1
Substance P	whole molecule	NU	1 : 16 000	2
Met-enkephalin	C-terminal	NU	1 : 2000	1
Gastrin	C-terminal	1 : 200	1 : 2000	0.1
Somatostatin	–	1 : 200	1 : 6000	1
Glucagon	N-terminal	NU	1 : 5000	2
Motilin	C-terminal	NU	1 : 600	1
CCK	9–20	NU	1 : 5000	2
PP	–	NU	1 : 12 000	1
Secretin	–	1 : 2000	1 : 6000	0.1
GIP	mid-portion	NU	1 : 12 000	1
Serotonin	amino side chain	1 : 200	1 : 2000	$6 \cdot 10^3$

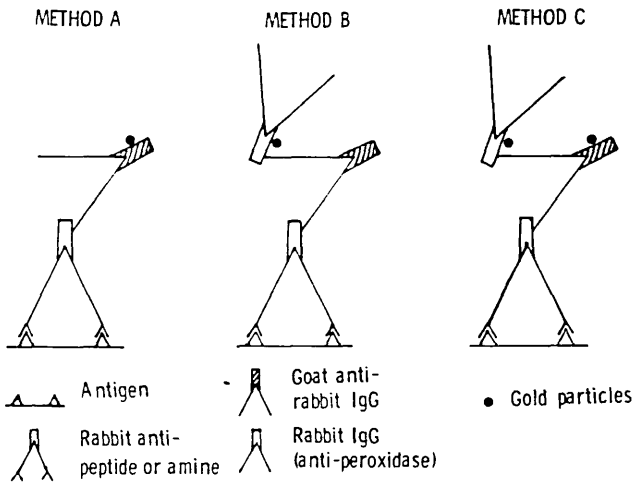
For IGS method A only antibodies to gastrin, somatostatin, secretin and serotonin were used.
NU = not used.

min. The PAP complex was visualised using the 4-chloro-1-naphthol method of Nakane [8] but modified in the following way: 4-chloro-1-naphthol was dissolved in a small amount of ethanol and further dissolved in 0.01 M phosphate buffer, pH 7.1, to a final concentration of 0.03%, the solution was heated to approx. 50°C and continuously stirred with a magnetic stirrer until completely dissolved. The solution was then filtered, H_2O_2 was added to a concentration of 0.3% and the solution was subsequently applied to the sections while still warm. The reaction was observed under the light microscope until a satisfactory level of staining was obtained (about 3–5 min). For immunostaining of nerves the PAP complex was visualised using the DAB method of Graham and Karnovsky [5] which results in a brown endproduct.

After rinsing, the sections were placed in glycine-hydrochloric acid solution at pH 2.2 (5 ml of 0.1 N HCl, 95 ml of glycine in NaCl solution (0.757% glycine in 0.5844% NaCl)) for 2 h. This removes the antibody complex obtained during the first immunostaining, but leaves the blue reaction product.

Immunogold staining (IGS) method

Serotonin, gastrin, somatostatin and secretin were selected as the second antigens to be immunostained. The colloidal gold-labelled antibodies were made by using the negative polyvalency of the colloidal gold to provide binding between the antibodies and the gold particles [4]. The IGS method was carried out in three ways. Method A used affinity-purified goat anti-rabbit/IgG adsorbed onto gold (Au) particles of 20 nm diameter (GAR/Au20). Method B was a three-step bridge method similar to the PAP method but using, instead of the PAP complex, affinity-purified rabbit anti-horseradish peroxidase adsorbed onto gold particles of some 20 nm diameter (RAP/Au20). Method C combined methods A and B using GAR/Au20 as the second layer and RAP/Au20 as the third layer in order to increase the final amount of gold bound to each antigenic site. In this case the gold-labelled third layer antibody (RAP/Au20) was an antibody to horseradish peroxidase, but any gold-labelled rabbit IgG would have done as well. The principles of these three methods are illustrated in Scheme I:



Scheme I.

For method A, PAP immunostained sections were incubated with diluted antisera to the second antigens (Table I) for 4 h at room temperature. Sections were then washed in PBS followed by polyethylene glycol (20 000 MW) buffer (0.5 mg PEG/ml of 0.05 M phosphate buffer, pH 7.1) which prevents aggregation of the colloidal gold-labelled protein [4]. The sections were then incubated for 1–2 h with GAR/Au20, diluted 1 : 3 in the PEG buffer. The resultant reddish colour of gold developed gradually and was monitored under the light microscope. When satisfactory staining was ob-

tained the preparations were rinsed in PEG buffer and PBS, and mounted in glycerine jelly. They could not be dehydrated because the endproduct of the 4-chloro-1-naphthol reaction is soluble in ethanol.

For method B, the first and second layer antisera were applied in exactly the same way as in the PAP procedure. After washing in PBS and PEG buffer, they were reacted with RAP/Au20 diluted 1 : 5 in PEG buffer for 2 h. The sections were rinsed in PEG buffer and PBS, and mounted as before.

Method C was a three-step method in which the first and second layer antisera were applied in the same way as in Method A. The antigenic sites of the second immunostain could then be seen as red under the light microscope. After washing in PEG buffer (three changes, 5 min each), the sections were reacted with RAP/Au20 diluted 1 : 5 for another hour. This enhanced the red colour and provided better contrast than Methods A and B (see Scheme I).

Controls

All the conventional controls, including preabsorption of antisera with appropriate antigens, were carried out (Table I). In addition, some special controls were needed for double immunostaining.

(1) In order to determine whether all the immunoreactive antigens were immunostained with the double procedure, three serial sections were stained in the following order. Section 1: Only the first antigen was immunostained. Section 2: Processed using the double staining procedure. Section 3: Only the second antigen was immunostained. The three immunostained sections were compared.

(2) The efficiency of the elution of the antibody complexes from the in situ antigens was tested in the following way: Firstly, the PAP technique was carried out on control sections, but the visualisation with substrate was omitted. Then the antibody complexes were eluted in glycine-hydrochloric acid solution for 2 h. After rinsing in PBS, the sections were incubated again with unconjugated goat anti-rabbit antiserum, followed by PAP complex, then 4-chloro-1-naphthol was applied with hydrogen peroxidase for visualisation of the complex. Efficient removal of the primary antibody complexes was demonstrated by the negative results obtained with this control.

RESULTS

All the antigens immunostained first were stained a dark blue colour by the chloronaphthol if they were in cells and brown by DAB if they were in



Fig. 1. Mucosa of porcine antrum double-immunostained to show the different distributions of gastrin-containing (G) cells (red) and serotonin-containing (EC) cells (dark blue). The EC cells were mainly located in the basal 1/3 of the mucosa, whereas the G cells were mainly in the middle zone. The nuclei were counterstained with methyl green (Magnification, X112).

Fig. 2. Mucosa of porcine duodenum double-immunostained for serotonin (red) and gastrin (dark blue). (Magnification, X500). The insert shows an example of close interrelationship between an EC cell (red) and a G cell (dark blue). (Magnification, X600).

nerves, whereas the antigens immunostained second with the IGS were bright red (Figs. 1 and 2). The three-step IGS method B was found to be superior to the two-step method as it gave both higher sensitivity and complete absence of background staining. Method C, i.e. the combination of the two-step and the three-step methods, gave the strongest staining because of the extra amount of gold bound to the antigen and thus provided the best contrast with other colours (Figs. 1 and 2). All the positive controls of single peptide or amine immunostaining gave results comparable to those obtained after double staining, i.e. the number and distribution of the cells and nerves was equal to that demonstrated by double immunostaining. All the other controls including the staining on the acid-washed sections were absolutely negative.

Some anatomical relationships between different peptide- or amine-containing cells and nerves were seen, such as between the cells containing serotonin and gastrin, somatostatin and serotonin and gastrin and somatostatin (Fig. 2, insert). There was also close proximity between the serotonin-containing cells and the VIP-containing nerves. Close associations were clearly demonstrated between these differently immunostained elements.

DISCUSSION

The IGS method has previously been employed at the ultrastructural level to demonstrate antigen sites [7]. The recently introduced IGS method for light microscopy [2,4] offers new possibilities for the immunostaining of antigens in histological sections. The technique has a number of advantages for example: (1) The IGS method is simpler to apply than the PAP method since incubation with the enzyme substrate is unnecessary; (2) The final coloured product develops gradually so that the reaction can be easily controlled under the light microscope; (3) It gives a permanent, bright red colour with very low background which is highly suitable for double staining. For double immunostaining, it was found more reliable to immunostain the second antigen rather than the first with gold-labelled antiserum. The thorough rinsing in glycine-hydrochloric acid solution between the two stages of immunostaining is necessary in order to avoid binding of the antibodies to the second antigen on the sites of the first antigen resulting in a confusing picture of mixed colours. After elution of the first antigen/antibody complex, mixed colours indicate the genuine presence of two antigens in the same cell. The amount of time required for rinsing varies from antiserum to antiserum and should be judged carefully since any remaining antibodies may interfere with subsequent reactions and elution with acid for an excessive time as shown by Nakane [8], and may denature the tissue antigens to be localised

in succeeding steps. Considering the gradual denaturation of tissue antigens during the long process, an optimal sequence of steps should always be followed, with either the least abundant antigen being immunostained first or the least sensitive antiserum being applied first. The nerves should, generally, be immunostained first, since the amount of peptide contained in nerves is small and it is relatively difficult to stain them a strong colour. The brown product of diaminobenzidine (DAB) [5] is not always easily distinguished from the red colour of gold obtained with the IGS so it is not an ideal coupler to use for comparison. *o*-Dianisidine dihydrochloride as shown by Calman [1] although capable of producing a contrasting green colour is complicated to develop. The Hanker–Yates mixture [6] also gives a blue-brown colour. The dark blue colour produced by 4-chloro-1-naphthol, however, provides an excellent contrast with the bright red and with the combined method a distinction can easily be made between different populations of immunostained cells and nerves. It is a logical extension to the double immunostaining method to achieve triple or multiple immunostaining in a similar manner. DAB, although not ideal, can still be used with the IGS method. We have already immunostained multiple antigens in the same section; for example, in a section of mammalian duodenum, VIP-containing nerves and somatostatin-containing cells were immunostained brown, serotonin-containing cells were immunostained blue and gastrin-containing cells were immunostained red with the IGS method. The background was also counterstained using methyl green, to give green nuclei.

The use of gold-labelled antibodies has great potential value for double immunostaining particularly in those cases concerning investigation of anatomical relationships between various tissue components.

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High Concentrations of a Novel Peptide, Neuropeptide Y, in the Innervation of Mouse and Rat Heart[1]

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A newly discovered bioactive peptide, neuropeptide Y (NPY), has been found in the innervation of the mouse and rat heart by immunocytochemistry. NPY-immunoreactive nerves were very dense around the nodal tissues. They also surrounded the coronary arteries and arterioles and were found in close association with the cardiac muscle. The distribution of NPY-containing nerves paralleled that of noradrenergic fibers, demonstrated by the use of antibodies to the catecholamine-converting enzymes tryosine hydroxylase and dopamine β-hydroxylase. Furthermore, NPY was seen to be present in a proportion of intrinsic neurons mostly found in the atria and in close proximity to the nodal tissue. The concentrations of extractable NPY-immunoreactive material (about 150 pmol/g in whole mouse heart) by far surpasses those of the other peptides so far reported in the cardiac tissue. High performance liquid chromatography demonstrated the NPY immunoreactivity to elute in a single sharp peak, in an identical position to brain NPY.

KEY WORDS. Rodent heart; Immunocytochemistry; Nerves Neuropeptide Y; Radioimmunoassay; Regulatory peptides.

Introduction

The mammalian heart is known to be innervated by postganglionic neurons from both sympathetic and parasympathetic nerves derived from the sympathetic trunk and the vagus, respectively (7,36). The classical concept, that the postganglionic fibers of the sympathetic nerves are adrenergic and those of the parasympathetic are cholinergic, has been until now the most accepted view of the cardiac innervation (4,6,7,10–12,15,22,32).

During the past decade, new types of autonomic nerves, using regulatory peptides as their presumed neurotransmitters or modulators, have been recognized in nearly every organ of the body. The classical view of a bipartite autonomic nervous sytem, i.e., adrenergic and cholinergic, has been completely revised to accommodate these bioactive peptides. However, in the heart, only a very few such nerves, containing substance P, vasoactive intestinal polypeptide (VIP), neurotensin, or enkephalin (13,23–26,33–35), have been reported, with the greatest amount of peptide in the guinea pig heart being met-enkephalin (approximately 9 pmol/g) (13).

In 1982, a 36 amino acid peptide, neuropeptide tyrosine (NPY), was isolated from extracts of porcine brain by a novel method for detecting peptides possessing a C-terminal α-miade (29–31). Its molecular sequence was very similar to that of pancreatic polypeptide (PP) and peptide YY (PYY) (30,31), the three of them forming a peptide family—the PP family. We have now been able to raise an antibody against NPY and have investigated the occurrence and concentration of this peptide in mouse and rat heart using the methods of immunocytochemistry and radioimmunoassay.

Materials and Methods

Mice (n = 10) and rats (n = 10) were killed using an overdose of ether. Their hearts were removed immediately and fixed intact in freshly prepared 0.4% benzoquinone solution for 2 hr at 20°C (5). After washing overnight, each heart was made into a cryostat block.

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Table 1. *Characterization of antisera*[a]

Antiserum to	Carrier/coupler	Dilution		Quench[b] (nmol)	Sensitivity (RIA)
		IF	PAP		
NPY	BSA/BDB	1:600	1:2000	1.0	2 fmol/tube
DBH	Unconjugated	1:400	1:4000	1.0	—[c]
TH	Unconjugated	1:400	1:2000	1.0	—[c]

[a]IF, indirect immunofluorescence staining; PAP, peroxidase–antiperoxidase method; BSA, bovine serum albumin; BDB, bis-diazobenzidine.
[b]Quantity of corresponding antigen (nmol/ml diluted antisera) required for complete abolition of specific staining.
[c]Not done.

Consecutive 3–5 μm cryostat sections were cut and separately mounted on glass slides. After soaking in 0.2% Triton X-100, made up in phosphate-buffered saline (PBS), for half an hour, serial sections were immunostained with antisera against NPY, tyrosine hydroxylase (TH), or dopamine β-hydroxylase (DβH). Both TH and DβH are known to be associated with catecholamine synthesis and are thus regarded as markers for noradrenergic neurons (21,27). Immunocytochemistry was carried out using the peroxidase–antiperoxidase (PAP) method (28) and a modified immunofluorescence procedure (8) in which the first layer incubation was repeated to achieve an enhanced brightness of the specific staining and a greater contrast. The antisera used were raised in New Zealand white rabbits. The one to NPY was rasied against natural porcine NPY conjugated to bovine serum albumin with bis-diazobenzidine. The immunostaining of NPY was completely abolished by preabsorption of the antiserum with natural or synthetic NPY at a concentration of 1.0 nmol/ml of diluted antiserum, whereas the addition of natural or synthetic PYY, vasoactive intestinal polypeptide (VIP), neurotensin, secretin, substance P, glucagon, and glucose-dependent insulinotropic peptide (GIP) at concentrations above 30 nmol/ml of diluted antisera had no effect on the immunostaining. Details of the antisera against DβH and TH as well as that against NPY are shown in Table 1. The controls included preabsorption of the first layer antisera with appropriate antigens and the use of normal rabbits' serum or PBS as the first layer. Viewing of the serial sections was carried out using a Polyvar microscope equipped with transmitted, fluorescence, and dark-field illumination and Nomarski optics. Photographs were taken using Kodak Technical Pan 2415 (transmitted light and Ilford FP4 (ultraviolet light) film.

For radioimmunoassay (RIA), the mice hearts (n = 10) were dissected and a small piece from each region was rapidly diced and plunged into boiling acetic acid (0.5 M, 5 ml per gram of tissue) and held at 100°C for 10 min. The solution was centrifuged and 10 and 1 μl aliquots of the supernatant were assayed in duplicate. The assay was shown to detect changes between adjacent assay tubes of 2 fmol with 95% confidence. No cross-reaction was seen with porcine PP, PYY, or avian PP (APP) in concentrations up to 100 pmol/tube. Pure porcine NPY was used as standard.

Fractionation of NPY-like immunoreactivity from the mouse heart was carried out on a high performance liquid chromatography (HPLC) reverse phase column (Waters Bondapak C-18: 3.9 × 300 mm) using a linear gradient elution system (35–45% solvent A—0.12% trifluoroacetic acid/water; solvent b—0.1% trifluoroacetic acid/acetonitrile) and a flow rate of 2 ml/min. Fractions of 1 ml were collected for subsequent radioimmunoassay. Recoveries were all between 88 and 94%.

Results

Very densely distributed NPY-immunoreactive nerves were seen. These nerves were more dense in the regions of the

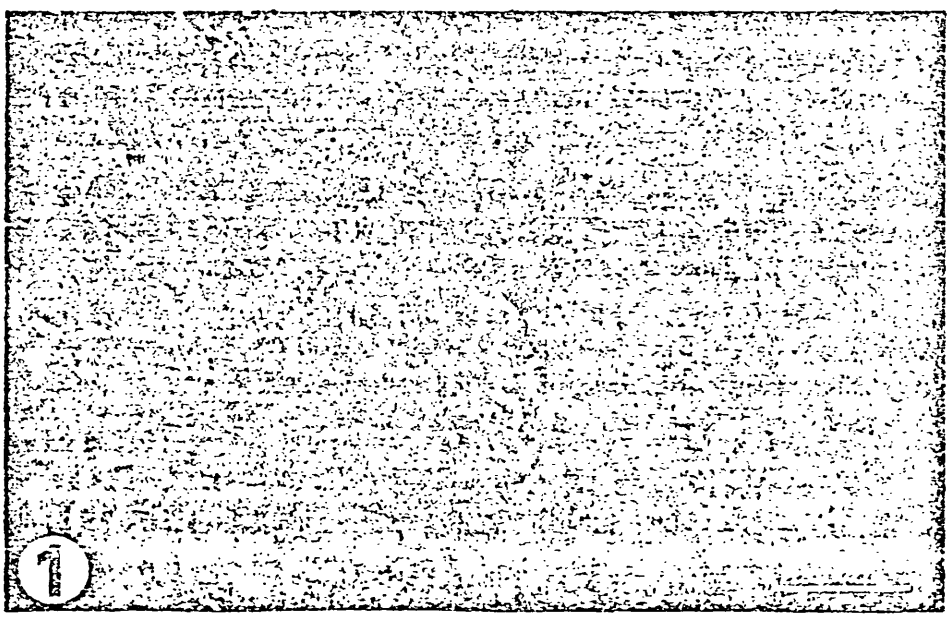


Figure 1. NPY-immunoreactive nerves surround coronary arteries in the ventricle. Original magnification ×300. Bar = 100 μm.

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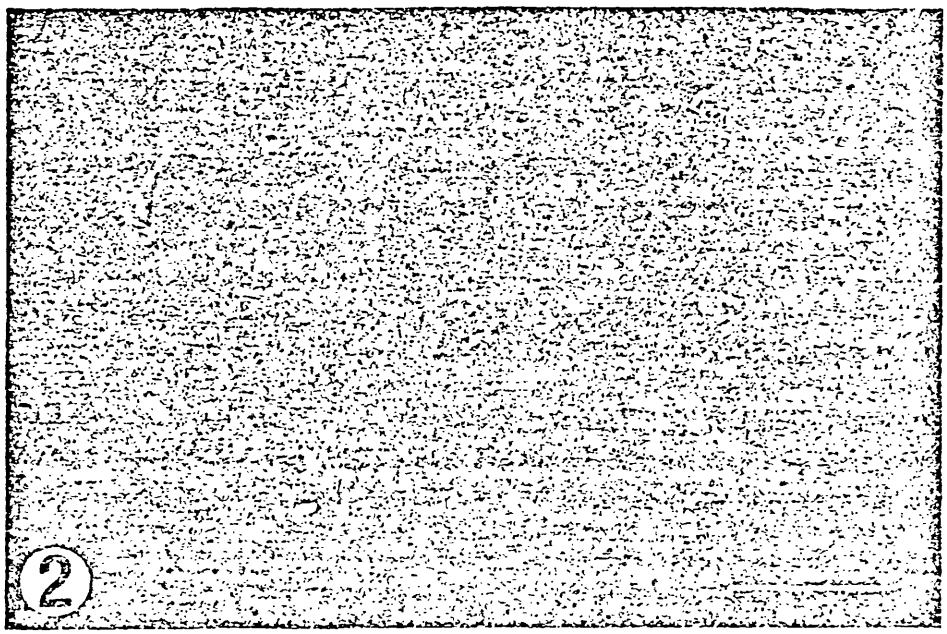


Figure 2. NPY-immunoreactive nerves associated with the cardiac muscle in the left ventricle. Original magnification ×350. Bar = 100 μm.

sino-atrial (S-A) and atrio-ventricular (A-V) nodes than in the surrounding tissues. The immunostained nerves were also found to surround the coronary blood vessels, particularly the medium to small arterial branches (Figure 1). In the atria, the ventricles, the papillary muscles, and the ventricular and atrial septa, NPY-immunoreactive nerves were closely associated with most cardiac muscle cells (Figure 2). The myocardium was thus more densely innervated than the endocardium and the epicardium. These nerve fibers also occurred in the valves, diminishing in number towards the free edge of the cusps. Large nerve bundles were found to be unreactive. The distribution of NPY-immunoreactive nerves paralleled that of noradrenergic fibers demonstrated by the use of antibodies against TH and DβH.

Neuronal cell bodies immunoreactive to NPY antibodies were found in groups of 10–20 or scattered singly from the dome of the right atrium, close to the large vessels, to the junction between the atria and the ventricles, particularly in the regions adjacent to the nodal tissues. The immunoreactive cells accounted for more than half of the total population of



Figure 3. A group of intrinsic ganglion cells in the right atrium close to the S-A node. NPY-immunoreactive ganglia (black arrows) are mixed with non-immunoreactive ones (open arrows). Original magnification ×700. Bar = 50 μm.

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intrinsic neurons (Figure 3). Using 3 μm serial sections it was found that these NPY-containing neurons were also reactive, although weakly, to antibodies to either TH or DβH, indicating their possible noradrenergic nature. The specificity of the immunostaining was shown by the negative results of the controls.

A very high concentration of NPY (up to 150 pmol/g of mouse heart tissue) was detected by radioimmunoassay (Figure 4). The distributions of NPY revealed by radioimmunoassay and immunocytochemistry were comparable.

Chromatographic investigations using HPLC revealed a peptide in a position similar or identical to that of pure porcine NPY (Figure 5). NPY-like immunoreactivity from mouse brain also eluted in an identical position.

Discussion

Several types of peptide-containing nerves have been reported to be present in the mammalian heart, including that of the rat (13,23–26,33–35). However, judging from the concentrations of the peptides and the frequency of the peptide-immunoreactive nerves so far reported in the heart, these nerves represent only a very small proportion of the cardiac innervation. The very high concentration of extractable NPY and the large number of NPY-immunoreactive nerves found in this study make this peptide one of the most conspicuous components of the rodent cardiac innervation. The specificity of the immunostaining was validated by appropriate controls. Confirmation of the nature of the NPY immunoreactivity was provided by HPLC, which showed that the peptide in the mouse heart was identical or closely similar to purified porcine NPY.

APP-like immunoreactivity has also been reported in several organs, including the heart (3,9,17,19), and it has recently been postulated that the immunoreactivity found using antisera raised to natural APP may in fact be due to the presence of NPY antibodies (3,9,19).

The coexistence of neuropeptides and classical neurotransmitters has been previously described (18). Lundberg et al. recently reported the occurrence of NPY in a subpopulation of adrenergic neurons of the superior cervical, stellate, and coeliac ganglia using immunocytochemistry combined with 6-OHDA (6-hydroxydopamine) treatment, which is known to destroy the adrenergic innervation (20). We have found that NPY occurs in gnaglia intrinsic to the heart that may be adrenergic, as demonstrated by immunocytochemistry of the enzymes TH and DβH associated with catecholamine synthesis. The extent of this costorage is now being investigated.

The precise role of NPY in the heart awaits further investigation, but in the isolated perfused rabbit heart, NPY caused a striking reduction in coronary flow, being more potent on a molar basis than ergometrine or noradrenalin (2). In addition it had an unexpected negative inotropic effect but no chronotropic influence. It has recently been shown that NPY can induce a dose-dependent vasoconstriction in the cat submandibular gland, and can also inhibit electrically induced, but not noradrenalin-induced, contraction of the rodent vas deferens

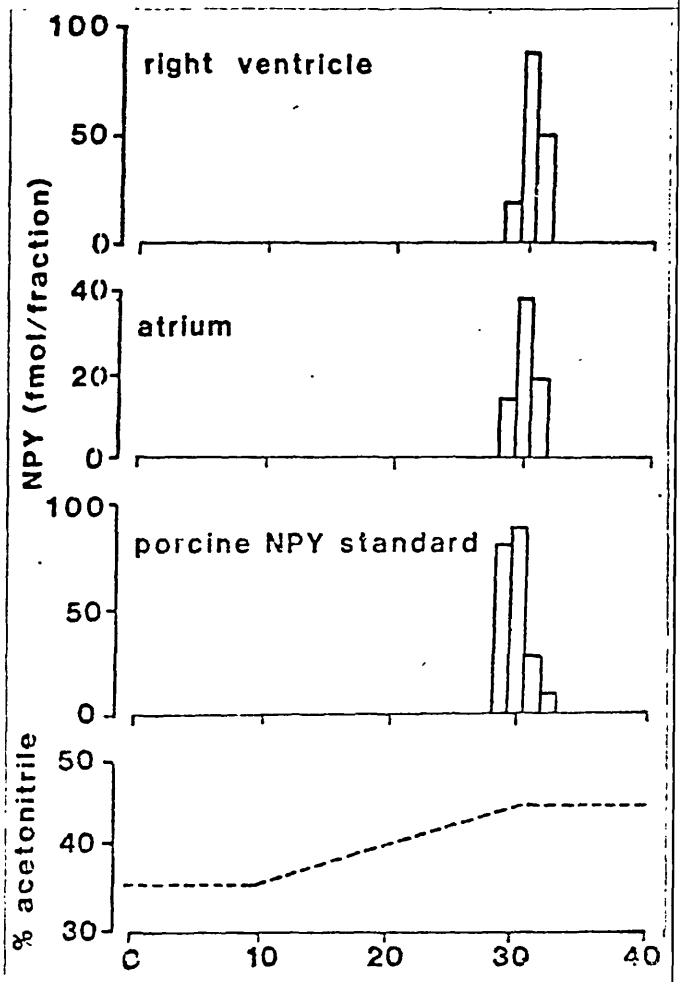
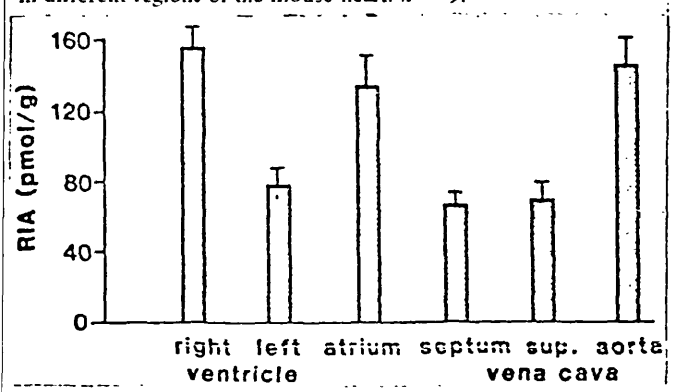


Figure 5. Chromatographic analysis of NPY-like immunoreactivity of tissue extracts from two regions of mouse heart. The position of porcine NPY standard is shown for comparison.

Figure 4. Concentrations of NPY-immunoreactivity detected by RIA in different regions of the mouse heart. $n = 9$.



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(1,20). It has been suggested that NPY might be acting as a modulator that is slow in onset and long lasting in action. It is known that stimulation of the sympathetic or parasympathetic nerves, previously thought to be solely adrenergic and cholinergic in nature, respectively, can produce effects on heart rate, atrio-ventricular conduction and myocardial contractility and can also interact with each other in cardiac responses (14,16). The action of NPY in the heart may be partially responsible for these effects.

The finding of a massive system of NPY-containing nerves in the cardiac innervation provides further information in the understanding of cardiac physiology.

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PEPTIDERGIC INNERVATION OF THE HUMAN MALE GENITAL TRACT

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ABSTRACT

Four peptides—vasoactive intestinal polypeptide, substance P, somatostatin and a peptide-like avian pancreatic polypeptide—have been found in nerves of the human male genitalia using highly sensitive and specific methods of immunocytochemistry and radioimmunoassay. Five other peptides (met-enkephalin, leu-enkephalin, neurotensin, bombesin and cholecystokinin-8) were absent. Vasoactive intestinal polypeptide was the most abundant peptide, its highest concentration being in the proximal corpus cavernosum. Immunoelectron microscopy localized this peptide to large (97 ± 20 nm), round, electron-dense granules of p-type nerve terminals. Vasoactive intestinal polypeptide-immunoreactive neuronal cell bodies were found in the prostate gland and the root of the corpus cavernosum. Substance P immunoreactive material was present in smaller concentration and was mainly localized in nerves around the corpuscular receptors of the glans penis. Somatostatin immunoreactive nerves were associated mainly with the smooth muscle of the seminal vesicle and the vas deferens. When antiserum to avian pancreatic polypeptide was applied, certain nerves were stained, particularly in the vas deferens, the prostate gland and the seminal vesicle. However, chromatography detected no pure avian pancreatic polypeptide suggesting the presence of a structurally related substance, possibly neuropeptide Y, which cross-reacts with the avian pancreatic polypeptide antiserum. Similar distributions between vasoactive intestinal polypeptide-immunoreactive and acetylcholinesterase-positive nerves and between avian pancreatic polypeptide-immunoreactive and adrenergic nerves were observed. A general neuronal marker, neuron-specific enolase, was used to investigate the general pattern of the organ's innervation. The abundance and distribution patterns of these peptide-immunoreactive nerves indicate that they may play important roles in the male sexual physiology.

A nonadrenergic, noncholinergic component of the autonomic nervous system is believed to play an essential role in the mechanism of many physiological functions, including those of the male reproductive system.[1] There is substantial evidence that this component may contain neuropeptides.[2-4] Vasoactive intestinal polypeptide (VIP) immunoreactive nerves have been described recently in the male genitalia of several mammalian species including man,[5-10] and enkephalin-containing nerves are reported to be present in the human seminal vesicle and the prostate gland.[10] Physiological and pharmacological evidence to date suggest that VIP may function as a neurotransmitter in the male genital tract.[4]

A multiple peptide-containing nerve supply has been described for peripheral organs including the gut, lung, skin and eye.[11] However, information on the possible occurrence of peptides other than VIP and enkephalin in the human male genital tract has not been previously available. We therefore investigated the possible occurrence of 9 neuropeptides in the entire human male genital tract using the methods of immunocytochemistry and radioimmunoassay and found that 4 of them were present. The density and distribution patterns of the peptide-containing nerves were compared with those of the acetylcholinesterase (AChE)-positive and adrenergic nerves. In addition, antibodies to a newly recognized neuronal marker, neuron-specific enolase (NSE),[12] were used in an attempt to reveal the entirety of the innervation in sections of the investigated tissues.

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MATERIALS AND METHODS

Human male external genitalia (no. = 33) were obtained from patients aged 21–50 years undergoing gender reassignment operation. The patients received estrogen treatment prior to surgery. Prostate glands (no. = 5) and seminal vesicles (no. = 5) were obtained at postmortem from men aged 22–46 years within 10 hours of death. Histologic analysis of the specimens was carried out, and only apparently normal specimens were included in the study.

The antisera for immunocytochemistry and radioimmunoassay were raised in New Zealand white rabbits against natural porcine VIP, synthetic substance P, natural avian pancreatic polypeptide (APP), synthetic bovine somatostatin, synthetic met-enkephalin and leu-enkephalin, synthetic cholecystokinin-8 (CCK), synthetic amphibian bombesin, synthetic bovine neurotensin, natural bovine dopamine-β-hydroxylase (DBH) and natural rat NSE. DBH is an enzyme catalyzing the conversion of dopamine to noradrenaline and was used here as a marker for adrenergic nerves. NSE is a newly characterized enolase present in central and peripheral neurons, serving here as a general neuronal marker to investigate the entirety of the neuronal elements in the male genital tissue.[12] The characteristics of the antisera are shown in table 1.

For light microscopic immunocytochemistry, a modified indirect immunofluorescence staining method was used on cryostat sections according to a procedure described previously.[13] The controls included preabsorption of the 1st layer antisera with appropriate antigens, the use of nonimmune rabbit serum as 1st layer in place of a primary antiserum and omission of the 1st layer.

TABLE 1. *Characterization of antisera for ICC and RIA*

Antisera to	Regional Specificity ICC	Regional Specificity RIA	Dilution for ICC	Quench* (nmol.)	Dilution for RIA	Sensitivity (RIA, fmol./tube)
VIP	whole molecule	middle-C terminal	1/2,000	5.0	1/400,000	0.2
substance P	C-terminal	C-terminal	1/2,000	10.0	1/5,000	0.3
somatostatin	whole molecule	whole molecule	1/400	10.0	1/240,000	0.3
APP	whole molecule	?†	1/1,000	10.0	1/160,000	0.8
met-enkephalin	C-terminal	n.d.‡	1/500	5.0	n.d	n.d
leu-enkephalin	C-terminal	n.d	1/500	5.0	n.d	n.d
CCK-8	whole molecule	C-terminal	1/200	10.0	1/160,000	0.2
bombesin	C-terminal	C-terminal	1/400	0.1	1/640,000	0.4
neurotensin	whole molecule	C-terminal	1/800	1.0	1/80,000	0.3
NSE	?	n.d	1/800	1.0	n.d	n.d
DBH	?	n.d	1/600	5.0	n.d	n.d

* Quantity of antigen (nmol./ml. diluted antiserum) required for complete abolition of specific staining.
† Not known
‡ Not done

To demonstrate possible cholinergic nerves, the cholinesterase staining method of El-Badawi and Schenk[14] was used. To identify adrenergic nerves, in addition to DBH immunostaining, the modified glyoxylic acid technique—SPG (sucrose-potassium phosphate-glyoxylic acid) method[15]—was also employed and results were compared with those obtained with DBH immunostaining. The sections were viewed using a Reichert-Jung Polyvar microscope equipped with an ultraviolet light source and bright-field Nomarski interference contrast optics.

Immunostaining of VIP was carried out at the electron microscopic level using a gold-labeled antibody method according to a previously described procedure.[16] The sections were examined with a Zeiss EM 10 CCR electron microscope.

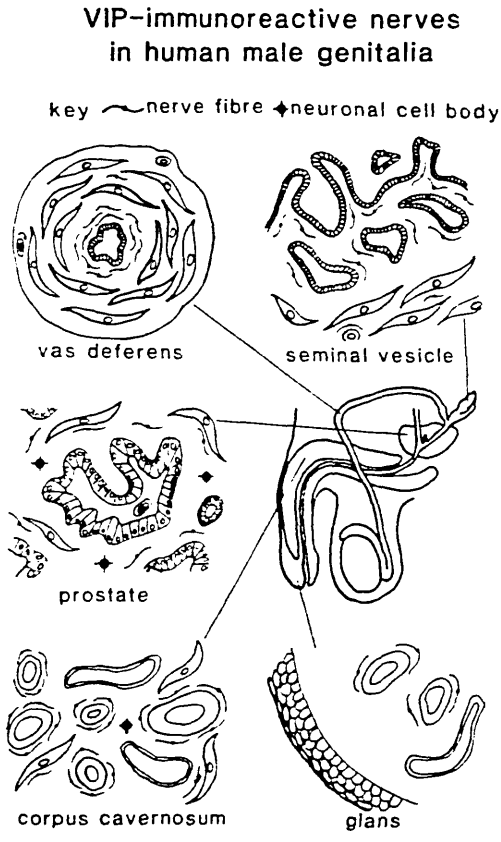
For radioimmunoassay, tissue from each region was dissected, weighed, rapidly plunged into boiling acetic acid (0.5 M) (5 ml per gram of tissue) and maintained at 100C for 10 minutes. The solution was centrifuged and 10 μl and 1 μl aliquots of the supernatant were assayed in duplicate (table 1).

Gel permeation chromatography of APP was carried out using a 0.9 × 60 cm Sephadex G-50 superfine column with 0.06 M phosphate buffer pH 7.4 as eluate. The column was eluted at 3.7 ml. per hr. and 0.7 ml. fractions were collected.

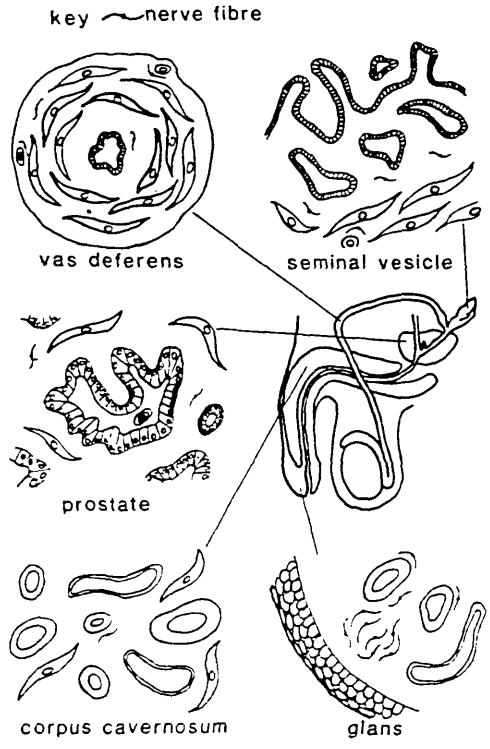
RESULTS

VIP, substance P, somatostatin, DBH, NSE and APP-like immunoreactivities were demonstrated by immunocytochemical method and AChE and SPG positivities by histochemical methods. The peptides and enzymes were localized to neuronal components within the tissue investigated. The relative frequency and the main characteristics of the distribution pattern for each type of peptide-immunoreactive nerve are shown in figures 1–4. No immunoreactivity was observed with antisera to the other peptides examined. All the controls for immunocytochemistry were negative. Since DBH immunoreactivity and SPG positivity were almost invariably found in nerves with very similar distribution patterns on adjacent sections, we here refer to the DBH/SPG-positive nerves as adrenergic.

VIP-immunoreactive nerves were extremely abundant, with the highest concentration in the erectile tissue toward the proximal end of the corpus cavernosum. Scattered VIP-immunoreactive neuronal cell bodies were encountered in the erectile tissue of the proximal corpus cavernosum and, more frequently, in the interstitial tissue of the prostate gland (fig. 5). VIP-immunoreactive varicose nerves were found in association with arteries and arterioles of all sizes, particularly those in the erectile tissue of the penis (fig. 6). Veins and sinuses were devoid of such nerves. The nonvascular smooth muscle, particularly the circular muscle of the corpus spongiosum and the smooth muscle in the prostate gland and the seminal vesicle, was also associated with VIP-immunoreactive nerves. In the prostate gland and the seminal vesicle, VIP-immunoreactive nerve fibers were also seen in the connective tissue and followed the folds projecting into the lumen of the glands (fig. 7). In the vas deferens and the epididymis, nonvaricose VIP-immunoreactive nerve fibers ran closely beneath the basement membrane of the epithelium and were only occasionally seen in the muscular coat. Immunoelectron microscopy showed that VIP was localized to the large (diameter 97 ± 20 nm, mean ± SEM), spherical, dense-cored secretory vesicles (fig. 8), resembling those present in the VIP-immunoreactive p-type nerves of the gut.[17] Conven-



substance P-immunoreactive nerves in human male genitalia



tional electron microscopy revealed that in these areas, some terminals containing these large dense-cored vesicles also contained different numbers of small (diameter 40 ± 60 nm), agranular vesicles (fig. 9) similar to those found in classic cholinergic terminals. Typical cholinergic terminals could also be seen in the areas studied running within the same nerve bundles as the p-type nerves.

Substance P immunoreactive nerves were scarce but were more concentrated in groups of nerve fibers in the corpuscular receptors underneath the epithelium of the glans penis (fig. 10). These nerves were also occasionally encountered in the connective tissue of the seminal vesicle. No neuronal cell bodies containing substance P immunoreactivity were found.

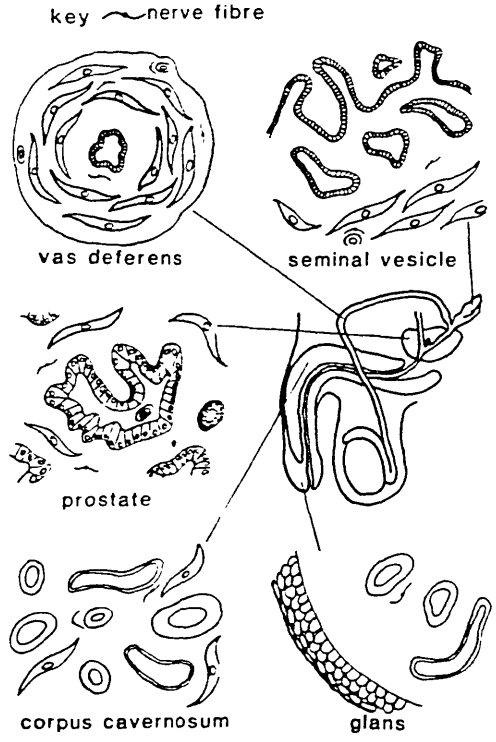
Certain characteristically distributed nerves were immunoreactive when the antiserum against APP was applied. However, in this study, the gel filtration chromatography of the tissue recorded no discrete peak in the position of pure APP. Therefore the term "APP-immunoreactive" will be used to refer to the material which reacts with antibody to APP. The APP-immunoreactive nerves were detected throughout the male genitalia but were particularly dense in the muscular coat of the vas deferens and in the interstitial tissue of the prostate gland and the seminal vesicle. They were found to associate with vascular and, more obviously, nonvascular smooth muscle. Scattered neuronal cell bodies in the seminal vesicle were found to react with antiserum to APP. A few somatostatin-immunoreactive nerve fibers were found in the interstitial tissue and among the smooth muscle of the prostate gland. A small number of them were seen in the muscular coat of the vas deferens.

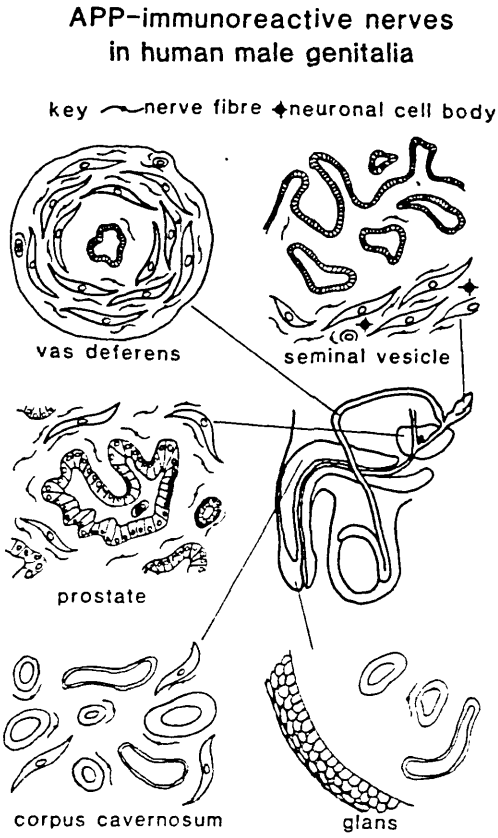
AChE-positive nerves and adrenergic nerves were distributed densely in most of the areas. They were associated with both vascular and nonvascular muscle. In general, the distribution pattern of AChE-positive nerves was similar to that of VIP, in terms of densely surrounding arteries and arterioles, underneath the epithelium of the vas deferens and the epididymis. However, in some neuronal elements, for example, the corpuscular receptors in the glans penis and some large nerve bundles in the trabeculae of the corpus cavernosum, only AChE-positive nerves were detected. Conversely, a small number of VIP-immunoreactive neuronal cell bodies were found in the prostate gland and none of these were demonstrated with the AChE staining method.

Adrenergic nerves were fewer than AChE-positive and VIP-immunoreactive nerves in the penis, whereas in the vas deferens, the prostate gland and the seminal vesicle, they were more frequent. Similarity between the distributions of adrenergic and APP-immunoreactive nerves was observed, in that both were densely distributed in the smooth muscle of the vas deferens, the prostate gland and the seminal vesicle. However, the overall population of nerve fibers containing APP-like immunoreactivity was larger than that of the adrenergic ones.

NSE-immunoreactivity appeared to occur within the whole range of nerves in the various regions (fig. 11). The relative abundance of innervation in different areas was clearly shown by NSE-immunoreactivity. It was found that the outer erectile tissue of the corpus cavernosum was more densely innervated than the core and that the proximal end of the penis was more densely innervated than the distal end. Immunostaining of NSE also showed that there were fewer nerves in the corpus spongiosum than in the corpus cavernosum. However, the variations

somatostatin-immunoreactive nerves in human male genitalia





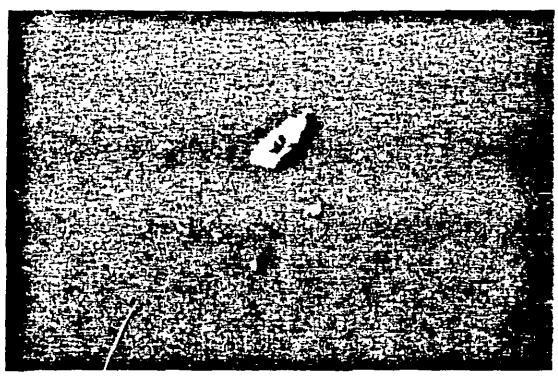


FIG. 5. VIP-immunoreactive neuronal cell body found near a blood vessel in the prostate gland. (× 1,150)

in the intensity of the NSE immunostain were larger than those of peptides.

In the testis, some epithelial cells in the periphery of each seminiferous tubule were found to be NSE-positive, with uniformly stained cytoplasm and large, round, positively stained nuclei. The cells were round, elongated or triangular in shape lining the inner face of the membrane of the seminiferous tubules and seemed to be Sertoli cells.

The distributions and relative amounts of extractable VIP, substance P and somatostatin detected in various regions by RIA are largely in agreement with the results obtained by immunocytochemistry (table 2). VIP appeared to be by far the most abundant peptide (108 ± 7 pmol./gm., mean ± SEM, in the proximal corpus cavernosum, no. = 15). Substance P was mainly concentrated in the glans (0.72 ± 0.06 pmol./gm., no. = 15), and somatostatin was mainly found in the vas deferens (5.6 ± 1 pmol./gm., no. = 15). APP was not detected by RIA, although immunocytochemistry found relatively abundant APP-immunoreactive nerves, particularly in the vas deferens, the prostate gland and the seminal vesicle.

DISCUSSION

We have demonstrated in the present study that the human male genitalia are supplied by 4 types of peptide-immunoreactive nerves containing VIP, substance P, somatostatin and APP-like immunoreactivities, and each of them possess a characteristic distribution pattern. The specificity of the methods of immunocytochemistry and radioimmunoassay used in this study has been validated with appropriate controls and very high dilution of the antisera. Enkephalin, reported previously in the prostate and the seminal vesicle,[10] was not detected in the present study. This might be due to differences in the antisera used.

VIP-immunoreactive nerves appeared to be the major peptide-containing neuronal component in the human male genitalia. The distribution of VIP-immunoreactive nerves in the

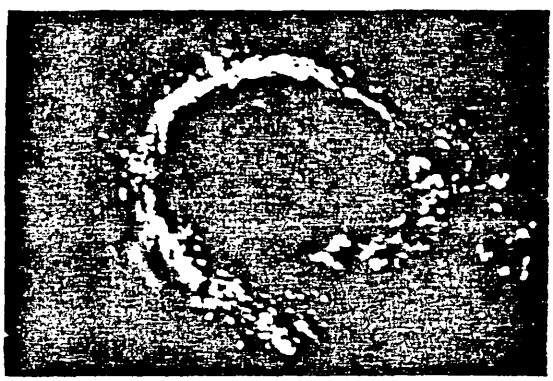


FIG. 6. Arteriole densely surrounded by varicose VIP-immunoreactive nerves in the proximal corpus cavernosum. (× 800)

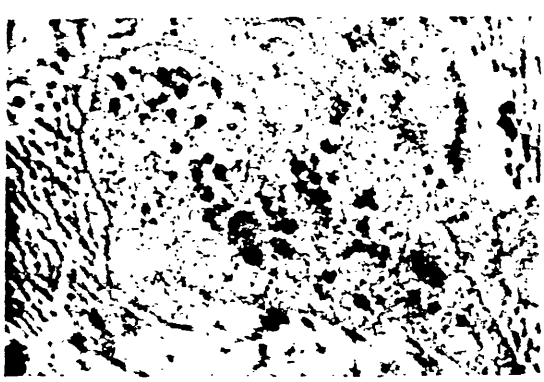


FIG. 7. Using gold-labeled antibody technique, VIP immunoreactivity was localized in the large (diameter 97 ± 20 nm, mean ± SEM), electron-dense, round vesicles of the nerve terminals in the corpus cavernosum. Highly electron-dense, small particles are the 20 nm diameter colloidal gold labeled onto the secondary antibodies (× 40,000)

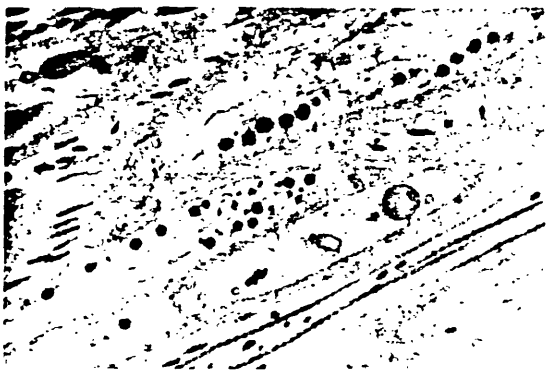


FIG. 8. Conventional electron microscopy revealed that some of the nerves containing the large, electron-dense granules also contain small, agranular vesicles similar to those found in the classic cholinergic terminals. (× 20,000)

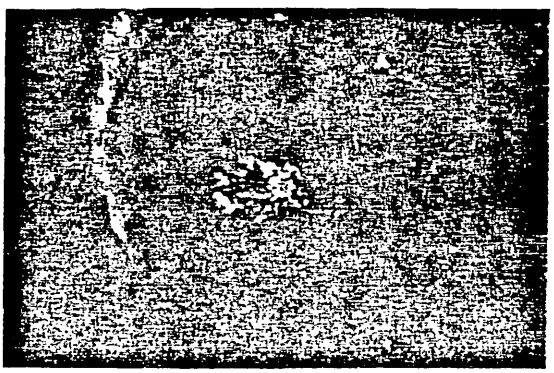


FIG. 9. Substance P immunoreactive nerve fibers found in the corpuscular receptors of the glans (× 150)

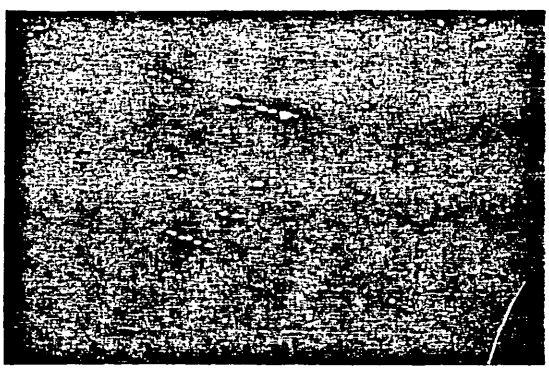


FIG. 10. APP-immunoreactive nerves running along the smooth muscle of the vas deferens. (× 600)

human male genitalia revealed in this study is in agreement with previous reports[5-10] except that in addition we have located some VIP-immunoreactive neuronal cell bodies in the prostate gland, indicating that at least some of the VIP-immunoreactive nerves had an intrinsic origin. It has been previously suggested that the significance of VIP-containing nerves in the penis is that they are involved in the control of penile erection and ejaculation. This has been further supported by the finding that VIP has an inhibitory effect on penile and vas deferential smooth muscle contraction.[4,9] The intrinsic VIP-containing neurons are likely to be controlled by the pelvic parasympathetic nerves, since pelvic nerve stimulation induces penile erection.[16]

The rich network of nerves, presumed to be cholinergic, is in agreement with recent reports by other groups.[18,19] Since the AChE staining may not be entirely specific for cholinergic neurons, the coexistence of VIP and AChE in some nerve fibers cannot be safely assumed solely from their similar distributions at the light microscopic level. AChE has been reported to occur without acetylcholine in some peripheral cell bodies and has also been found in some adrenergic ganglion cell bodies. AChE can also be demonstrated on the membrane of some Schwann cells.[20] In addition, there has been evidence that AChE may hydrolyze peptides such as substance P.[21] Thus, the AChE-positive nerves demonstrated by the histochemical method cannot be simply interpreted as cholinergic nerves. Although immunostaining of choline acetyl transferase, regarded as a superior and more specific method for the demonstration of cholinergic fibers, is nowadays possible, these antibodies were not available during the present investigation.

The possible co-storage of VIP and acetylcholine was, however, further supported by the results of ultrastructural studies and immunoelectron microscopy using the gold-labeled antibody method. VIP was localized to the large, round, electron-dense granules in the nerve terminals. Conventional electron microscopy revealed that some of these terminals also contained different numbers of small, agranular vesicles similar to those

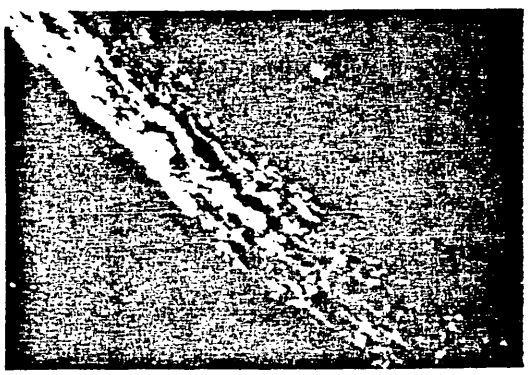


FIG. 11. A nerve bundle in the corpus cavernosum demonstrated by NSE immunostaining. All the nerve fibers in the bundle were positively immunostained with antiserum to NSE. (× 200)

TABLE 2. *Peptides detected by radioimmunoassay (N = 15)*

	Peptides*			
	VIP	Substance P	Somatostatin	APP
Glans penis	12 ± 1.5	0.72 ± 0.06	0.9 ± 0.1	—†
Foreskin	2 ± 0.2	0.12 ± 0.05	0.5 ± 0.05	—
Cavernosum body	96 ± 15	0.28 ± 0.02	2.9 ± 0.5	—
Cavernosum root	108 ± 7	0.4 ± 0.03	3.2 ± 0.3	—
Spongiosum body	19.5 ± 2	0.4 ± 0.02	1.4 ± 0.2	—
Spongiosum root	18 ± 0.9	0.2 ± 0.01	0.8 ± 0.15	—
Testis	1 ± 0.01	0.39 ± 0.01	0.3 ± 0.04	—
Epididymis	7 ± 0.08	0.3 ± 0.01	1.6 ± 0.15	—
Vas deferens	17 ± 4	0.5 ± 0.02	5.7 ± 1	—
Prostate gland‡	7.8, 10, 13, 84, 69	0.05, 0.07, 0.3, 0.07, 0.08	1.2, 1.1, 3.5, 1.4, 1.2	
Seminal vesicle‡	191, 49, 31, 34, 36	0.14, 0.04, 0.04, 0.08, 0.17	2.2, 6.2, 3.5, 1.8, 2.9	—

* Values are expressed as mean ± standard error of the mean

† Not detected

‡ These data are presented individually because of the lower number of samples available from these areas

found in the classic cholinergic terminals This finding indicates that at least some VIP-containing nerves may be identical to cholinergic ones.

A small number of substance P-immunoreactive nerve terminals were seen mainly in the corpuscular receptors of the glans penis, which, in the cat, have been shown to be mechanoreceptors.[18] Thus, substance P in the penis may well be a sensory transmitter, as has been previously suggested.[22] These nerves may be derived from the dorsal root ganglion, possibly via the internal pudendal branches.

The origin and the possible physiological implications of the few somatostatin-immunoreactive nerve fibers are uncertain. Somatostatin-containing neuronal cell bodies, as described in the bladder wall of the guinea pig,[23] were not seen in the human male genitalia. APP-immunoreactive nerves have been found throughout the human male genitalia However, chromatographic analysis of APP and the discrepancy between the APP concentrations detected by immunocytochemistry and radioimmunoassay suggest that a structurally related substance exists which cross-reacts with the APP antiserum. It is interesting to note that a 36 amino acid peptide, neuropeptide Y (NPY), has recently been isolated from the mammalian brain by means of a chemical assay for C-terminal amides, and was found to bear major sequence similarities to APP.[24] The possible identity of APP-like material with NPY in the male genitalia is now under investigation.

The distribution pattern of the adrenergic nerves revealed by both histochemical and immunocytochemical methods in this study is similar to that described previously.[16, 19] In addition, APP-immunoreactive and adrenergic nerves were found to be similarly distributed in most of the regions. However, using the present technique, we cannot evaluate the coexistence in the same neurons of the APP-like material and adrenalin or noradrenalin as has been suggested previously for some peripheral neurons of cats and rats.[17]

NSE immunostaining has been found to be a useful method to investigate the innervation of these tissues. The variations in the intensity of the NSE immunostain may indicate that NSE content is indeed related to the physiological state of the nerves, as had been speculated by Marangos and associates.[12]

The presence of NSE in Sertoli cells is interesting A putative peptide hormone named "inhibin" has been extracted from the testis and had the effect of decreasing plasma follicle-stimulating hormone (FSH).[26] It has been suggested that inhibin is produced by Sertoli cells.[27] The occurrence of NSE in Sertoli cells provides another clue to the location of this putative peptide.

The present study reveals that the neuronal (or neuroendocrine) control of the human male genitalia is more complex than had been hitherto assumed. The functional significance of these peptide-containing neurons awaits further investigation.

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We felt that it was reasonable to begin this investigation with anticonvulsant doses of phenobarbitone, since the resultant serum level should have predictable and slight side-effects and might still provide neuroprotection. The dosage schedule selected was based on the report of Lockman et al.[30] Since IVH often occurs before 24 h of age,[31] we limited the study to infants to whom we could administer phenobarbitone before they were 6 h old. We timed the dose intervals so as to achieve loading 12 h after the start of phenobarbitone treatment. This schedule should establish the desired phenobarbitone serum level before the time at which IVH is most likely to occur.[3] Eight infants (five treatment, three control) died before they were 18 h old; IVH occurred in one treated infant and in three controls. The statistical significance of the difference between the two groups is not changed if these patients are removed from the analysis. However, the relatively high incidence of early IVH indicates that dosages and dose intervals should be more closely investigated. Side-effects were not evident in any of the infants on phenobarbitone. In particular, respiratory depression and hypotension were no more common in treatment infants than in control infants (table III).

Although a brief course of phenobarbitone treatment in the neonatal period has no known long-term effects upon cognitive development, we intend to follow-up the infants in this study. We will evaluate infant development and cognitive function, and perform routine medical and neurological assessments. The ability of phenobarbitone to prevent IVH in the population studied is promising, since it suggests that the occurrence of haemorrhage can be modified by drug therapy in the first hours of life. However, before we can recommend phenobarbitone treatment of infants at risk for IVH we need to know what the long-term outcome is in those who have received phenobarbitone treatment as infants.

We thank the house officers and nurses of the Holden Intensive Care Nursery for their help; Dr M. A. Schork, for statistical analyses; and Mrs Betty Passon and Mrs Paula Donn for technical assistance. The study would not have been possible without the cooperation of the Department of Radiology, Diagnostic Ultrasound, University of Michigan Medical Center.

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VIPERGIC NERVES IN THE PENIS

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Summary High concentrations of vasoactive intestinal polypeptide (VIP) were detected by immunocytochemistry and radioimmunoassay in thirty surgical specimens of male external genitalia. VIP was found exclusively in fine autonomic nerves. VIPergic nerves were most densely concentrated in the penis around the pudendal arteries and in the erectile tissue of the corpus cavernosum. Considerable numbers of VIP nerve fibres were also seen in the vas deferens and epididymis. VIP is known to exert regulatory actions on blood-flow, secretion, and muscle tone. Its presence in considerable amounts in the male genital tract suggests that this newly discovered peptide neurotransmitter may be important in the nervous control of male external genitalia.

Introduction

VASOACTIVE intestinal polypeptide (VIP) is a newly discovered regulatory peptide composed of 28 aminoacids.[1] It relaxes smooth muscle and is a powerful vasodilator. It also stimulates secretion in the pancreas and intestine.[2] VIP has been demonstrated, by the use of specific antibodies, in autonomic nerves supplying many peripheral tissues.[3] VIPergic nerves form a major part of the newly recognised class of non-adrenergic non-cholinergic (peptidergic) nerves. Despite these discoveries, little attention has been paid to the

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possible presence of VIPergic nerves in the human male genital tract,[4,5] which is richly supplied by autonomic nerves.[6] We used immunocytochemistry and radioimmunoassay to investigate the genitalia of thirty men.

Material and Methods

Specimens

Thirty surgical specimens of male genitalia were obtained fresh at operation (gender reassignment) from patients aged 21–46 years. The specimens collected included most of the penis (average 12 cm in length—i.e., the distal 4/5 of the corpus cavernosum and the distal 2/3 of the corpus spongiosum including the urethra), the scrotum (testis and epididymis), and the attached spermatic cord (average 8 cm in length including the vas deferens and blood-vessels). The skin of the penis and scrotum was not removed.

Radioimmunoassay of Tissue Extracts

The specimens were divided into the following areas—glans, corpus cavernosum and spongiosum (body and root), urethra (distal, body, and root) testis, epididymis, and vas deferens—and extracted in 0·1 mol/l formic acid. VIP was measured by a specific radioimmunoassay by means of an antibody reacting to the whole peptide molecule, at a dilution of 1:320 000. The assay has a detection limit of 0·4 fmol/assay tube.[7]

Immunocytochemistry and Histochemistry

The whole penis was cut transversely—i.e., perpendicular to the axis of the urethra—into 5 mm thick discs, which were fixed in a solution of 0·4% benzoquinone in 0·01 mol/l phosphate-buffered saline (PBS), pH 7·0, for 1 h at room temperature.[8] After fixation, the tissue was washed overnight in buffered sucrose (7% sucrose, 0·01% sodium azide in PBS) and frozen for sectioning at 5 μm in a cryostat. The same procedure was followed for the scrotum and spermatic cord; thus consecutive sections of spermatic cord at different levels and consecutive sections across the testis and the epididymis were prepared. Immunocytochemistry was carried out by means of the indirect immunofluorescence (IF) method of Coons et al.[9] or the peroxidase antiperoxidase (PAP) method of Sternberger.[10] The accompanying table gives details of the specificity of the antibodies used in the control tests. The regional specificity of the VIP antiserum was the middle C terminal. VIP antiserum was used at a dilution of 1:2000 for IF and 1:20 000 for PAP.

Cholinesterase activity was demonstrated by the method of El-Badawi and Schenk[11] on cryostat sections from benzoquinone-fixed blocks. The sections were post-fixed in 10% formalin for 15 min. Adrenergic nerves were identified in different blocks by de la Torre and Surgeon's method of glyoxylic acid condensation on unfixed tissue.[12]

Results

VIP was found in considerable quantity in all areas of the male genital tract (fig. 1). It was localised to autonomic

CONTROL EXPERIMENTS

Controls	Concentration (nmol/ml diluted antiserum)	Results
Immunoabsorption with:		
VIP	0·1	–
Secretin	0·1	+
Glucagon	2	+
GIP	1	+
Substance P	2	+
Met-enkephalin	1	+
Somatostatin	1	+
Non-immune rabbit serum as first layer	..	–
FITC-labelled second layer alone	..	–
PAP third layer alone	..	–

FITC = fluorescein isothiocyanate.
GIP = gastric inhibitory polypeptide.

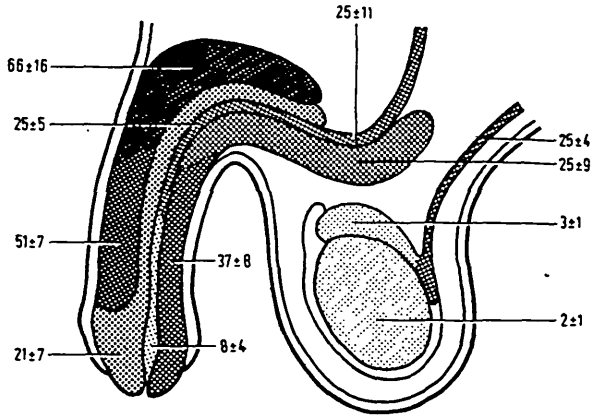


Fig. 1—Schematic representation of quantitative distribution of VIP immunoreactivity in male genital tract (pmol/g).



Fig. 2—VIP immunoreactive nerves in close association with blood-vessels in erectile tissue of corpus cavernosum.

Reduced by ½ from ×378.

nerves, particularly those in close association with blood-vessels (fig. 2). VIPergic nerves were found mainly around arterioles and smooth muscle and were numerous around the pudendal arteries (occasionally penetrating the adventitia to reach the longitudinal smooth muscle bundles of the inner layer), in the erectile tissue of the corpus cavernosum, and beneath the epithelial layer of the vas deferens. Local ganglia reacting with VIP antibodies were seen in the penis, especially near blood-vessels of the corpus cavernosum.

Glyoxylic-acid staining demonstrated the classic pattern of adrenergic nerves, with a distribution completely unrelated to that of nerves containing VIP. However, cholinesterase-containing fibres were found in some areas alongside VIP fibres—e.g., around some blood-vessels in the corpus cavernosum and spongiosum and beneath the epithelium of the vas deferens and epididymis. In other areas VIP and cholinesterase containing fibres seemed to be distributed independently.

Discussion

This study demonstrates that the male genital tract is richly innervated by autonomic nerves of a newly recognised class, and indicates that existing ideas on the innervation of this organ should be revised. The male genital tract is well innervated,[6] but to date there has been only preliminary

evidence for the presence of this new peptidergic component of the autonomic system in man.[5]

VIP was first discovered and isolated because of its very potent vasodilatory action.[13] Investigation of its effect on organ blood-flow led to the demonstration of a role of VIP as a neurotransmitter.[14,15] It has now been established that VIP is responsible for several atropine-resistant vasodilatory responses which occur after parasympathetic nerve stimulation.[15,16] These responses can also be mimicked by the local injection of very low concentrations of pure VIP.

VIP concentrations were particularly high in the pudendal arteries as well as in the erectile tissue of the corpus cavernosum. The high concentrations of VIP in the male genital tract are comparable to those found in the hypothalamus,[17] which is a crucial control centre for neuroendocrine regulation of sexual behaviour. The finding of VIP immunoreactive nerves within the muscular cushions of the pudendal arteries is relevant to the need for rapid dilation of the blood vessels at the start of erection (opening the "sluice-gates") and could also be important in the maintenance of active arterial dilation.

VIP-immunostained local ganglia were detected and were concentrated mainly in the corpus cavernosum. This accords with the general observation that peripheral VIP nerves often originate from local ganglia.[18,19] The finding of VIP immunoreactive fibres in the circular muscle lining the inner face of the albuginea of the testis suggests that VIP could also have a role in the control of testicular temperature. Changes in the scrotal surface area (cold contraction, heat relaxation) which are largely responsible for heat dispersion or conservation are under nervous control.[20]

The role of the classic adrenergic and cholinergic nerves in the control of ejaculation is well established,[20] though only ganglion-blocking agents reliably result in impotence.[21,22] The present demonstration of an extensive VIP innervation in the circular muscle of the corpus spongiosum and the epithelium of the vas deferens adds another factor to our knowledge of the nervous control of male external genitalia.

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RESPONSE TO SINGLE DOSE OF TETANUS VACCINE IN SUBJECTS WITH NATURALLY ACQUIRED TETANUS ANTITOXIN

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Summary Tests among 410 Indians not artificially immunised against tetanus showed that 80% had measurable antitoxin. Single doses (100 Lf or 250 Lf) of a potent tetanus toxoid were given to such individuals with naturally acquired antitoxin. The 100 Lf dose produced on average a ten-fold rise in antibody level, and the 250 Lf dose a twenty-fold rise. In adults who had been artificially immunised, a 5 Lf dose produced a four-fold to ten-fold rise in antibody level. In infants three doses of triple vaccine produced satisfactory antitoxin concentrations. The levels of antibody achieved after a single 250 Lf dose should protect for 5 years. Single-dose vaccination may be better than the conventional three-dose scheme for a population that is unlikely to comply with a three-dose regimen and in whom naturally acquired antitoxin is associated with partial tolerance to tetanus toxoid. Naturally acquired antitoxin in Indians is probably the result of chronic clostridial contamination of the small bowel. This contamination can induce immune tolerance in the gut and systemically and may be the reason for the poor responses to vaccination in all except infants.

Introduction

THE past decade has shown that tetanus antitoxin can be found in individuals who have neither been artificially immunised against tetanus nor had the disease. Protective levels of tetanus antitoxin (≥0·01 IU/ml) have been found in people living in rural areas and in domestic and farm animals in Brazil.[1,2] In India the antitoxin titres have been mostly well below the protective level.[3-6] Nevertheless, if naturally acquired antitoxin is widespread in the community, even at a low level, it can be of great significance in conferring

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Peptide immunoreactive nerves and cells of the guinea pig gall bladder and biliary pathways

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SUMMARY Using the methods of immunocytochemistry and radioimmunoassay, five peptides (vasoactive intestinal polypeptide (VIP), substance P, somatostatin, met-enkephalin, and bombesin) have been found in the gall bladder and the biliary tracts of guinea pig and each of them possesses a characteristic distribution pattern. Networks of nerves containing each peptide were found in the smooth muscle, around blood vessels and, occasionally, in the lamina propria. The distribution of the peptide immunoreactive nerves in the gall bladder and biliary tract is similar to those found in the gut. Vasoactive intestinal polypeptide (11±1·5 pmol/g in the sphincters, mean ± SEM) and substance P (21·5± 1·8 pmol/g in the common bile duct) were found to be the most abundant peptides and a few VIP and substance P immunoreactive neurones were localised in the ganglionated plexus. Bombesin immunoreactive nerves were mainly seen in the sphincter of Oddi, where the mean concentration of extractable bombesin was 14·6±2 pmol/g. Somatostatin immunoreactive mucosal endocrine cells were identified in the epithelium of the common bile duct and the sphincter. The extractable somatostatin in these regions were 76±19 pmol/g and 162±30 pmol/g respectively.

The gall bladder and the biliary pathways have been known for a long time to be innervated by cholinergic and adrenergic nerves.[1-13] As these organs are outgrowths of the alimentary canal and the latter is known to contain a large number of peptidergic nerves and peptide containing endocrine cells,[14-17] one might expect that peptide containing nerves and cells are also present in the gall bladder and the biliary tract. Indeed, it has been recently reported that non-adrenergic, non-cholinergic nerves are present in the gall bladder of the guinea pig[11] and nerves containing vasoactive intestinal polypeptide (VIP) have been found in both the gall bladder and the sphincter of Oddi in many mammalian species.[18 19] Nerves containing met-enkephalin-like immunoreactivity have been shown in the myenteric plexuses of the gall bladder and the cystic duct in man.[20] Mucosal endocrine cells containing motilin and substance P have been reported to be present in the bile duct of rabbit.[21]

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Information on the detailed distribution and concentration of VIP and met-enkephalin containing nerves in the gall bladder, and the occurrence of nerves and cells containing other peptides, however, has not been available. We therefore investigated the precise distribution of VIP containing nerves and the occurrence of other types of peptide containing nerves and mucosal endocrine cells in the gall bladder and biliary pathways of guinea pig, using the methods of immunocytochemistry and radioimmunoassay.

Methods

ANIMALS

Twelve adult (body weight 280–350 g) and eight newborn (one to four days) guinea pigs (Dunkin/Hartley) of both sexes were killed by cervical dislocation and the whole gall bladders, cystic ducts, hepatic ducts, common bile ducts, and the cranial portions of the duodenum were removed immediately after death. The specimens were fixed by immersion in 0·4% benzoquinone in 0·01M phosphate buffered saline (pH 7·4) (PBS) for 30

minutes at room temperature.[22] The samples were then washed in 7% sucrose in PBS at 4°C overnight. The fixed samples were made into cryostat blocks by freezing them on to cork mats and 5 μm thick sections were cut at −20°C. The sections were picked up on to poly-L-lysine coated glass slides and air dried.

Specimens from six guinea pigs were also frozen in melting Arcton (−150°C) and freeze dried overnight at −40°C. The specimens were then fixed in benzoquinone vapour at 60°C for three hours and embedded in paraffin wax. Sections 5 μm thick were cut on a Jung microtome.

In order to investigate the complete distribution pattern of the peptide containing nerves, whole mount, stretch preparations of the specimens were also made. These were prepared using benzoquinone fixed tissues. The lumen of the gall bladder or the biliary duct was opened using microscissors and the different layers were removed gently under a dissecting microscope using a pair of fine watchmaker's forceps. The preparation was then placed on a poly-L-lysine coated glass slide and stretched lightly when almost dry.

Tissues from each region were also fixed in 10% formalin and embedded in paraffin wax. Five micrometre thick sections were cut, dewaxed, and stained with haematoxylin and eosin.

The antisera for immunocytochemistry and radioimmunoassay were raised in New Zealand white rabbits against natural porcine VIP, synthetic substance P, synthetic bovine somatostatin, synthetic met-enkephalin, and synthetic amphibian bombesin respectively. The specificities of the antisera are shown in the Table.

The indirect immunofluorescence method of Coons and coworkers[23] was carried out on the cryostat sections and the peroxidase antiperoxidase (PAP) method of Sternberger[24] was performed on the dewaxed paraffin sections as previously described. The dilutions of the primary antisera are shown in the Table.

The whole mount, stretch preparations were pretreated by dehydration and rehydration through graded alcohols and xylene and by soaking in 0·2% Triton X-100 in PBS for one hour.[25] All preparations were incubated with primary antiserum for 24 hours at 4°C. They were then washed in PBS three times, 10 minutes each, and incubated with the second layer antiserum, fluorescein isothiocyanate conjugated goat anti-rabbit globulin (1:100 in PBS) for four hours at room termpature. After washing as before, the preparations were mounted in PBS/glycerine (1:9) and examined under a Reichert-Jung Polyvar widefield photomicroscope equipped with bright field, dark field, phase contrast, and ultraviolet light source.

The controls for immunostaining included preabsorption of the primary antisera with appropriate antigens (Table), the use of normal rabbit serum as first layer in place of a primary antiserum, and omission of the first layer.

For radioimmunoassay, a representative piece of tissue was removed from each region, plunged into boiling acetic acid (0·5M) (5 ml/g tissue) and maintained at 100°C for 10 minutes. Ten and 1 μl aliquots of extract solutions were assayed in duplicate for VIP, substance P, somatostatin, and bombesin. Details of the antibodies for radioimmunoassay are shown in the Table.

Results

Using the method of immunocytochemistry, networks of nerves containing VIP-, substance P-, somatostatin-, and met-enkephalin-like immunoreactive material were found in nearly all the regions of the gall bladder and the biliary pathways, in both the whole mount stretch preparations and the tissue sections. Bombesin immunoreactive nerves were

Table *Details of antisera*

Antiserum raised against	*Region specificity*	*Dilution*			*Concentration of peptides used for absorption and abolishing staining**
		IF	*PAP*	*RIA*	
VIP	Middle-C terminal	1/2000	1/20 000	1/400 000	0·1
Substance P	Whole molecule	1/1000	1/10 000	1/8000	2
Met-enkephalin	C terminal	1/500	1/5000	—	1
Somatostatin	Whole molecule	1/2000	1/10 000	1/240 000	1
Bombesin	C terminal	1/2000	1/20 000	1/640 000	1

* nmol/ml diluted antiserum.

Cross reactivity of antisera to substance P and bombesin has been tested with cross preabsorption controls – that is, incubate bombesin antigen with substance P antiserum and *vice versa* before the immunostaining. The positive immunostaining results established the non-cross reactivity of these two peptide antisera. This has been further confirmed by their distinct concentrations and distributions in the investigated tissue.

mainly seen in the sphincters. The abundance and the arrangement of the immunostained nerves were shown more clearly in the whole mount, stretch preparations than in the sections. Each of the five types of immunostained nerves seemed to have its own characteristic distribution pattern. Among them, VIP- and substance P-like immunoreactive nerves appeared to be the most abundant in most of the regions (11±1·5 pmol VIP/g wet weight of tissue, mean ± SEM, in the sphincter; 21·5±1·8 pmol substance P/g in the common bile duct). Nerves containing somatostatin and met-enkephalin-like immunoreactivities were much sparser and, sometimes, could be seen only in the whole mount stretch preparations. This was reflected by the low level of somatostatin immunoreactivity found by radioimmunoassay in the gall bladder: 1·8±0·2 pmol/g. Densely packed bombesin-like immunoreactive nerves were mainly found in the region of the sphincter, where the mean concentration of bombesin immunoreactivity was 14·6±2 pmol/g. All the controls for immunocytochemistry were negative.

Vasoactive intestinal polypeptide- and substance P-like immunoreactive nerves were predominantly localised in the ganglionated plexus and the mucosal plexus (Figs 1 and 2), and a few VIP- and substance P-like immunoreactive cell bodies were sometimes observed in the ganglionated plexus (Figs 2 and 3). The immunostained nerve fibres were often seen forming dense, mesh-like structures and surrounding ganglion cells which showed no immunoreactivity to the antisera (Figs 4 and 5). Single or bundles of VIP- and substance P-like immunoreactive nerves were also commonly found running along the connecting strands formed by the nerve bundles linking separate groups of one to four neuronal cell bodies. Vasoactive intestinal polypeptide and substance P-like immunoreactive nerves



Fig. 2 *Networks of VIP immunoreactive nerves in ganglionated plexus of gall bladder. (Whole mount stretch preparation, PAP method.) (×800, original magnification.)*

formed randomly arranged networks with numerous varicosities which were uniform in size with occasional larger spindle-shaped ones. These nerves were also frequently found surrounding blood vessels, particularly arteries, in all layers.

Somatostatin- and met-enkephalin-like immunoreactive nerves, although more sparse, had similar distribution patterns to those containing VIP and substance P – that is, they were also found mainly in the plexus (Fig. 6) and were occasionally seen in the lamina propria. They were not, however, found to be associated with blood vessels. No met-enkephalin or somatostain immunoreactive neuronal cell bodies were found.

The muscle layer of the gall bladder, cystic duct, and hepatic duct were only sparsely innervated by different peptide immunoreactive nerves. The density of these nerves gradually increased as the muscle layer of the common bile duct reached the sphincter and became thicker.

In the sphincters, which included the sphincter of the common bile duct, the ampulla, and the



Fig. 1 *Substance P immunoreactive nerves in ganglionated plexus of gall bladder. (Indirect immunofluorescence (IF) method.) (×250, original magnification.)*



Fig. 3 *Substance P immunoreactive neuronal cell bodies in ganglionated plexus of gall bldder. (Whole mount stretch preparation, IF method.) (×700, original magnification.)*



Fig. 4 *VIP immunoreactive varicose nerve fibres surrounding some neurones which do not contain the peptide. (Stretch preparation, IF method.) (×1000, original magnification.)*



Fig. 6 *Nerves containing somatostatin immunoreactivity in ganglionated plexus of gall bladder. (Stretch preparation, IF method. (×800, original magnification.)*

sphincter of papilla, the distribution patterns of the four types of immunostained nerves and, in addition, bombesin immunoreactive nerves, were essentially similar to those found in the muscle layer of the duodenum. The nearer to the innermost circular muscle the innervation lay, the denser it appeared to be and it was always more dense than that of the duodenal muscle. There were certain distinct differences between the distributions of the five types of immunoreactive nerves in the sphincters. Vasoactive intestinal polypeptide immunoreactive nerves were concentrated not only in the muscle bundles (Fig. 7) but also densely distributed in the myenteric and the submucous plexuses. Met-enkephalin and bombesin immunoreactive nerve fibres, on the other hand, were distributed mainly among the muscle cells (Figs 8 and 9). Somatostatin immunoreactive nerves were the most scarce of all and, in certain sections, none could be found. When present, somatostatin immunoreactive fibres were seen in the submucous and in the myenteric plexuses, as well as between the muscle cells.

Some substance P immunoreactive nerves were mainly related to the smooth muscle. Separated somatostatin immunoreactive cells could be observed in the mucosal epithelium of the common bile ducts (76±19 pmol/g) and the sphincters (162±30 pmol/g) (approximately 1·5 cells per 1 mm length of epithelial mucosa in 5 μ thick section) (Fig. 10) but not in the epithelium of the gall bladder. The region most denseley innervated by peptide immunoreactive nerves, particularly VIP immunorective nerves, was the circular muscle of the sphincter of the duodenal papilla. In this area, a few VIP immunoreactive neurones were occasionally seen, lying outside the muscular ring of the duodenal papilla.

The results obtained using radioimmunoassay, for the concentrations of extractable VIP, substance P,



Fig. 5 *Substance P immunoreactive varicose nerve fibres surrounding some neurones which do not contain the peptide. (Stretch preparation, IF method.) (×1000, original magnification.)*

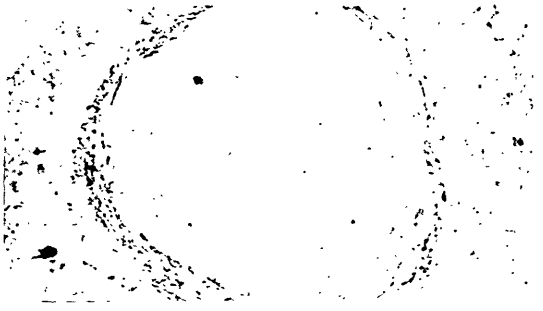


Fig. 7 *Dense VIP immunoreactive nerve fibres in smooth muscle of the sphincter of Oddi. (IF method.) (×80, original magnification.)*



Fig. 8 *Met-enkephalin immunoreactive nerves in the muscle layer of the common bile duct adjacent to the sphincter of Oddi. (IF method.) (×400, original magnification.)*

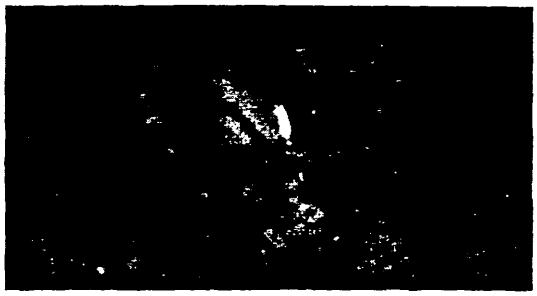


Fig. 10 *A somatostatin immunoreactive mucosal cell in common bile duct. (IF method.) (×300, original magnification.)*

somatostatin, and bombesin (Figs 11–14) were largely in agreement with the density and distribution of each type of peptide containing nerve and cells as shown by immunocytochemistry. For example, high concentrations of somatostatin were detected by radioimmunoassay in the regions of the common bile duct and the sphincters, where immunocytochemistry showed, in addition to a few somatostatin immunoreactive nerves, a number of epithelial cells containing somatostatin immunoreactivity.

Discussion

We have shown here that VIP, substance P, somatostatin, and met-enkephalin immunoreactive nerves are present in the gall bladder and the biliary pathways of the guinea pig. Bombesin immunoreactive nerves were found only in the region of the sphincters. Our results showed that the peptide immunoreactive nerves of the gall bladder and the biliary pathways possess a pattern similar to that of the gut,[16] although each type of peptide immunoreactive nerve has its own characteristic distribution. The finding of peptide immunoreactive neuronal cell bodies in the gall bladder is in agreement with previous reports that most peptide immunoreactive nerves in the alimentary tract have an intrinsic origin.[15 16 25–27] As the storage of peptides in the perikarya is often poor, it is possible that the number of observed peptide immunoreactive neurones would be increased if axonal transport blocking agents such as colchicine or vinblastine were used.

The whole mount stretch preparations used in this study were found to give superior results to those obtained using tissue sections when the organs under investigation were poorly innervated – for example, the gall bladder and the bile ducts, where the immunostained nerves were sometimes hardly



Fig. 9 *Bombesin immunoreactive nerves in muscle layer of sphincter of Oddi. (IF method.) (×600, original magnification.)*

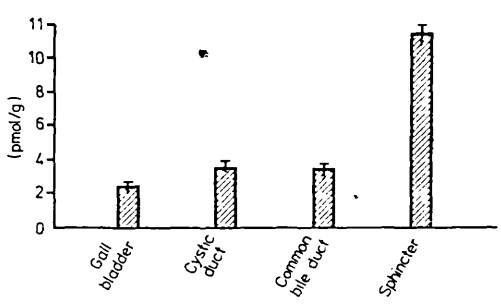


Fig. 11 *Extractable VIP in gall bladder and biliary tract of guinea pig.*

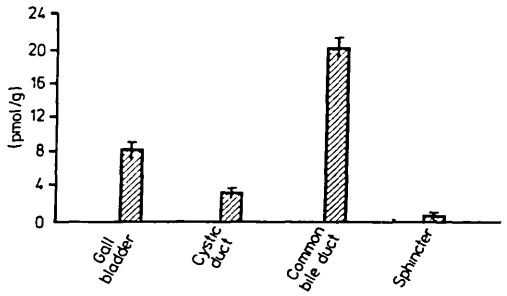


Fig. 12 *Extractable substance P in gall bladder and biliary tract of guinea pig.*

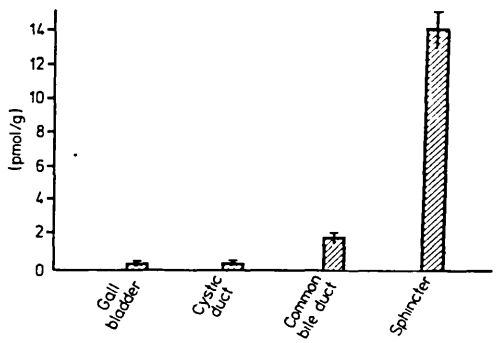


Fig. 14 *Extractable bombesin in gall bladder and biliary tract in guinea pig.*

visible on the tissue sections. In some regions – for example, the sphincter – however, this approach was not feasible.

In a previous investigation,[13] it has been found that there are three major nerve plexuses in the wall of the gall bladder and the biliary pathways – that is, the mucosal and ganglionated plexuses and a nerve plexus which lies in the adventitia of the cystic artery and its branches. Communications between these plexuses exist. Abundant adrenergic and cholinergic nerves with characteristic distributions have been shown in the gall bladder and the biliary tract.

In the present study, the nerves known to contain the classical neurotransmitters seemed to be mingled with peptide immunoreactive nerves. The distribution patterns of peptide immunoreactive nerve fibres in the gall bladder and the biliary pathways were similar to those found for the adrenergic innervation whereas the peptide immunoreactive nerves in the muscle bundles of the sphincters were similar to the cholinergic innervation as regards their density and distribution pattern.[12,13] The networks of different types of nerve and the relationships between peptide and non-peptide containing neurones indicate that there is a sophisticated neuronal control system acting upon the gall bladder and the biliary tract.

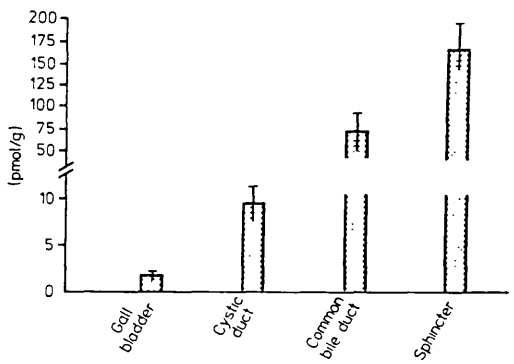


Fig. 13 *Extractable somatostatin in gall bladder and biliary tract in guinea pig.*

Many peptides have been reported to have effects on the gall bladder and the biliary tract.[28–35] Only VIP and met-enkephalin immunoreactive nerves, however, have been reported to be present in these regions.[18–20] In the gall bladder, VIP has been shown to cause relaxation of the wall and inhibition of secretion.[36] Vasoactive intestinal polypeptide receptors have also been found in the gall bladder.[37] Stimulation of vagal nerves led to an increase of VIP concentration in the portal venous and peripheral arterial plasma, indicating that VIP is likely to act as a neurotransmitter.[38,39] In this study, VIP immunoreactive nerves were found to be abundant throughout the regions examined, suggesting that VIP may be one of the major neuronal components in this control system. The occurrence of VIP immunoreactive nerves in the lamina propria, underneath the columnar epithelium of the gall bladder, may be related to the inhibitory effect of VIP on the gall bladder secretion.[36]

Subtance P has been shown to have many physiological and pharmacological effects on various organs including the gall bladder and the bile duct.[40] It decreases the output of hepatic bile and amylase in a dose dependent manner. It also inhibits the choleresis induced by CCK or VIP.[41]

As in many other organs, the blood vessels in the gall bladder and the bile ducts are often surrounded by VIP and substance P immunoreactive nerves.[16,26] Thus, the local blood flow may also be monitored

by, in addition to adrenergic and cholinergic nerves, peptidergic ones.

In the present study, somatostatin, met-enkephalin and bombesin immunoreactive nerves have also been demonstrated in the gall bladder and the biliary tract. These nerves are less abundant than those containing VIP and substance P immunoreactivities. Somatostatin has been reported to antagonise the effect of CCK on the gall bladder in man,[33] although, in guinea pig and dog, this effect has been shown to inhibit acetylcholine release from the myenteric plexus of the intestine.[42] The occurrence of met-enkephalin immunoreactive nerves indicates that such a mechanism may also exist in the gall bladder.

Somatostatin has been shown previously in neuroendocrine cells of the hypothalamus, endocrine cells of the gut and pancreas, as well as in the central and peripheral nervous system. Somatostatin plays a mainly inhibitory role in, for example, endocrine and exocrine secretion, gastric and biliary motility, splanchnic blood flow, intestinal absorption.[43] The significance of the somatostatin immunoreactive cells found in the biliary tract of the guinea pig remains to be elucidated.

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The value of S-100 immunostaining as a diagnostic tool in human malignant melanomas

A comparative study using S-100 and neuron-specific enolase antibodies

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Summary. The brain proteins S-100 and neuron-specific enolase have been reported by separate groups to be present in human malignant melanomas. There is no systematic study comparing the occurrence of these proteins in the same tumour specimens. We have examined 33 primary malignant melanomas, including 5 which were amelanotic, and 25 metastatic melanomas using immunohistochemical methods with specific, non-cross-reacting antibodies to S-100 and NSE. We found S-100 immunoreactivity to be present in all cases but one, whereas NSE immunoreaction was very weak and patchy, and present in only 6 cases.

S-100 immunoreactivity was not demonstrated in 40 control tumours, either primary or metastatic in skin, including basal- and squamous-cell carcinomas, spindle-cell sarcomas, lymphomas and Merkel cell tumours. All intradermal ($n=4$) and compound ($n=1$) naevi were positive for S-100, 2 blue naevi showing much less reaction. NSE immunoreactivity was detected in Merkel cell tumours ($n=8$), undifferentiated ($n=2$) and small cell ($n=1$) carcinomas, and all melanocytic naevi.

It is suggested therefore that antibody to S-100 is the reagent of choice for demonstration of melanocytic tumours, and may be especially valuable in the diagnosis of amelanotic melanoma or metastatic tumours of doubtful origin where melanoma is suspected.

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Key words: Brain proteins – Immunohistochemistry – Melanoma – Neuron-specific enolase – S-100 – Skin – Tumour markers

S-100 is a small, acidic, Ca^{2+}-binding protein, originally isolated from bovine brain (Moore 1965). It is found in glial elements of the brain (Perez et al. 1970), and Schwann cells and satellite cells of dorsal root and autonomic ganglia in the peripheral nervous system (Stefansson et al. 1982a). It also occurs in other tissues, including Langerhans cells and melanocytes of the skin (Cocchia et al. 1981), interdigitating reticulum cells of human lymph node (Takahashi et al. 1981) and human chondrocytes of both neural crest and mesodermal types (Stefansson et al. 1982c).

Enolase, a glycolytic enzyme, is a dimer of three possible subunits: α, β and γ. The α and β subunits, collectively termed non-neuronal enolase (NNE), are found in liver and epithelial tissues (α), and muscle including heart (β). The γ-γ form, or neuron-specific enolase (NSE), is found exclusively in neurons, peripheral and central neuroendocrine cells (Schmechel et al. 1978; Bishop et al. 1982; Gu et al. 1983) and tumours deriving from them (Tapia et al. 1981).

Complement fixation assay shows that S-100 occurs in melanoma cell cultures (Gaynor et al. 1980) and melanoma tissues (Gaynor et al. 1981), at levels up to 80-fold that found in normal human skin (Cocchia et al. 1981). Immunohistochemical studies have demonstrated S-100 in the majority (Nakajima et al. 1981, 1982; Stefansson et al. 1982b) or all (Gaynor et al. 1981; Clark et al. 1982; Cochran et al. 1982) of melanomas, and in normal melanocytes (Cocchia et al. 1981).

NSE immunoreactivity was detected immunohistochemically (Dhillon et al. 1982) in human malignant melanomas, in benign naevi, except Spitz naevi (Dhillon and Rode 1982), and in some normal melanocytes in skin. However, Royds et al. (1982a, b) found γ-enolase (NSE) immunoreactivity in intradermal naevi and in malignant intradermal melanocytes, but not in normal melanocytes.

In view of these conflicting reports of NSE and S-100 immunoreactivity in melanocytic tumours, we considered it important to look for both proteins in the same specimens using specific and non-cross-reacting antibodies. Such a study has not hitherto been made, and might establish whether an interrelationship exists between NSE and S-100 immunoreactivity in each melanoma specimen, and the relationship of both proteins to histological classification of the tumours compared with naevi and control tumours in skin.

Materials and methods

Tissues

Tissues from 33 patients with malignant melanoma, including 5 amelanotic tumours, were obtained from the files of the Departments of Histopathology at St. Helier Hospital, Carshalton, Surrey, and The Royal Marsden Hospital, Fulham Road, London. The material was all formol-saline fixed and wax-embedded. It included the skin tumour(s) in all cases, and

skin recurrences ($n=5$) and metastases to various sites ($n=25$) if they were available. Seven cases of benign naevi were obtained from the same sources.

Control tissues were normal skin from 20 patients, and 40 biopsies of various primary or metastatic tumours in skin (Table 2). The normal skin specimens were obtained fresh and processed in three ways: formol saline fixation followed by wax embedding, Bouin's fluid fixation followed by wax embedding, and 0.4% p-benzoquinone fixation (Bishop et al. 1978) followed by freezing for cryostat sectioning.

Sections (5 μm thick) were mounted on glass slides coated with poly-L-lysine (mol. wt. 350,000) to ensure good adhesion (Huang et al. 1983), and dried overnight at 37° C. One slide from each block was stained with haematoxylin and eosin to study tumour morphology and for correlation with the immunohistochemistry results.

Antibodies

Purified NSE was prepared from rat brain as previously described (Marangos et al. 1978) and antiserum was raised in New Zealand white rabbits (Marangos et al. 1975). Antiserum to S-100 extracted from ox brain was raised in rabbits and characterised according to Zuckerman et al. (1970).

Immunohistochemistry

Dried sections were dewaxed in xylene and taken to water through graded alcohols. Endogenous peroxidase was blocked using a 0.15% solution of hydrogen peroxide in PBS (phosphate-buffered saline – 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.3). The staining procedure used was the unlabelled antibody enzyme (PAP) method of Sternberger (1979). Both primary antibodies were used at a dilution of 1:4,000 in PBS containing 0.1% bovine serum albumin, and were applied for 16–18 h at 4 C.

Sections were then incubated with goat anti-rabbit IgG (Miles Laboratories) at 1:200 dilution for 30 min, followed by rabbit PAP complex (UCB Bioproducts) diluted 1:300 for 30 min. Peroxidase activity was revealed by either diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (Graham et al. 1965). The latter gave a brick-red colour which was found to contrast well with melanin, unlike the brown reaction product obtained from DAB. Sections were lightly counterstained with haematoxylin and mounted in glycerine jelly (amino ethylcarbazole) or dehydrated, cleared and mounted in Styrolite (DAB).

Initially, duplicate sections from 10 melanoma cases were immunostained, one with DAB, the other with amino ethylcarbazole as chromogen. This was done for two reasons. Firstly, to ensure that there was no loss of sensitivity when using amino ethylcarbazole rather than DAB. Secondly, since the DAB reaction product is not affected by hydrogen peroxide, the melanin in heavily pigmented tumours could be bleached in order to see whether it was masking any immunostaining. Bleaching was also performed prior to immunostaining, by placing sections in 0.25% aqueous potassium permanganate for 20 min, followed by 1 min in 1% aqueous oxalic acid.

The immunostaining controls used included the incubation of sections with non-immune rabbit serum or PBS instead of primary antibody, and antibody absorbed with homologous antigen (NSE – 2.5 nmol ml; S-100 – 5.0 nmol/ml of diluted antibody) or cross-absorbed (anti-NSE absorbed with S-100 and anti-S-100 absorbed with NSE, 5.0 and 2.5 nmol/ml respectively).

Results

Haematoxylin and eosin stained sections of melanomas were examined initially without reference to immunostaining results, and tumour grading and staging were evaluated. Results of immunostaining are given in Table 1.

Six of 33 cases of malignant melanoma showed positive immunoreactivity for NSE, and these were amongst the monomorphic tumours. The immu-

Table 1. Intensity of immunostaining for S-100 and NSE in melanomas and melanocytic naevi

Tissue		Number of cases	Immunostaining intensity[a]									
			S-100					NSE				
			0	+	2+	3+	4+	0	+	2+	3+	4+
Melanoma												
Melanotic	1°	28	1	–	4	7	16	22	3	3	–	–
	2°	22	–	1	3	10	8	22	–	–	–	–
Amelanotic	1°	5	–	–	1	2	2	5	–	–	–	–
	2°	3	–	–	1	1	1	3	–	–	–	–
Naevi												
Intradermal		4	–	–	–	2	2	–	–	3	1	–
Compound		1	–	–	–	1	–	–	1	–	–	–
Blue		2	1	–	1	–	–	–	1	1	–	–

[a] 0 signifies no immunostaining; + to 4+ represent increasing number of cells and intensity of immunostaining

nostaining in these cases was usually very patchy and weaker than that for S-100 (Figs. 1 and 2A).

Thirty-two of 33 malignant melanomas, both primary and metastatic, pigmented and amelanotic, were immunoreactive for S-100. Immunostaining of S-100 was strong in most cases (Figs. 2B and 3), more so at the periphery of the tumour adjacent to the dermis (Fig. 3B) and in intradermal tumour cells (Fig. 4). This pattern was consistent in serial sections. The degree of overall staining intensity did not correlate with degree of pleomorphism, level of invasion or absolute thickness of the tumour, or with the amount of melanin present. However, cells containing much melanin were not reactive with antibodies to S-100. Bleaching of melanin, using 10% hydrogen peroxide, after immunostaining, did not reveal further reaction product, showing that there was no masking effect of the pigment. Bleaching the sections with permanganate before immunostaining did not affect the immunoreactivity, and this method again showed S-100 immunoreactivity to be absent from heavily pigmented malignant melanocytes.

The superficially spreading melanomas immunostained for S-100 more strongly than the nodular type.

All of 25 metastatic melanomas were immunoreactive for S-100 (Fig. 5B and C). The skin metatases ($n=1$) or recurrences ($n=5$) and the metastases to lung, liver, bone, breast, peritoneum, stomach and thyroid (13 in all) and lymph node ($n=6$) tended to have the same type of staining pattern as the primary tumour. None of the primary melanomas which reacted with anti-NSE had demonstrable metastases.

Intrinsic positive controls in each section were provided by nerves or supportive cells in normal skin adjacent to the tumour. They showed reac-

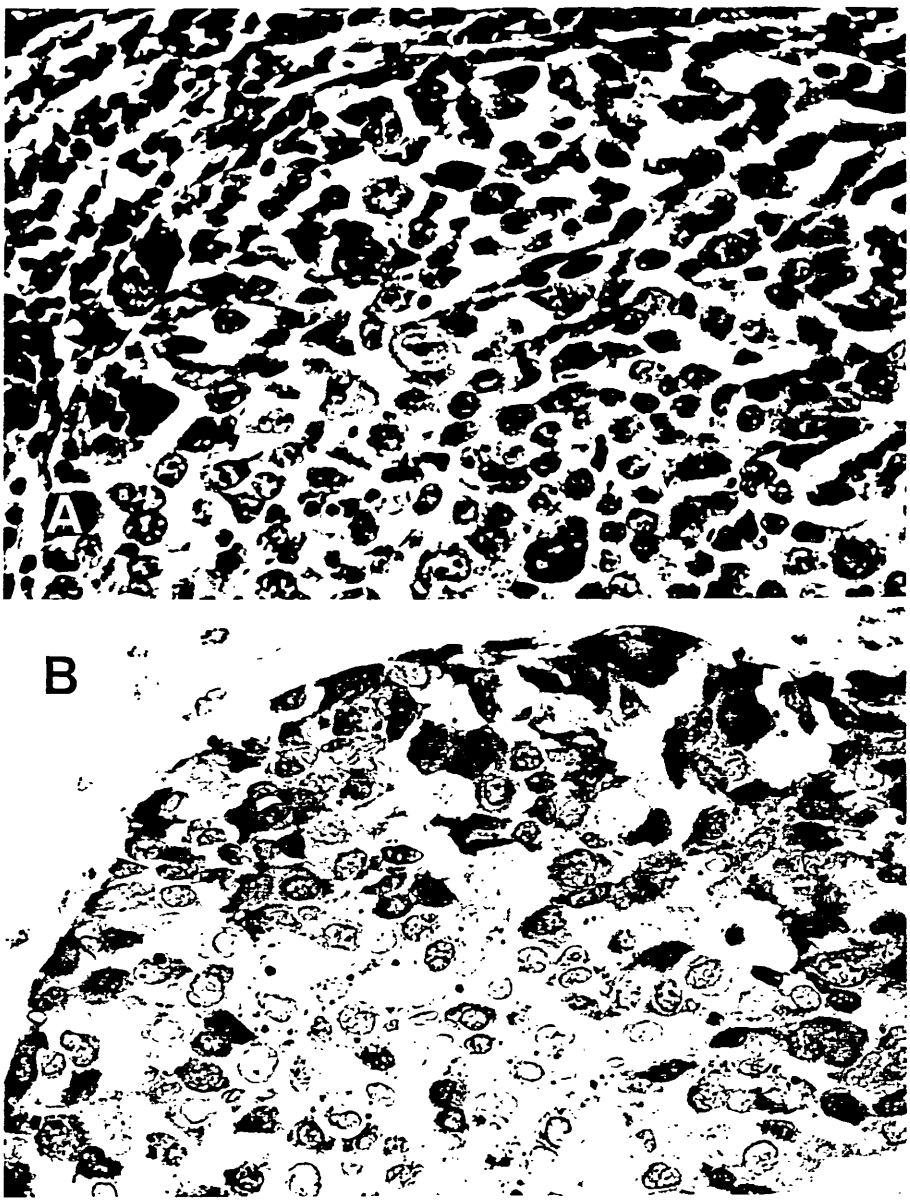


Fig. 1A, B. Melanoma in skin. Adjacent sections **A** stained by haematoxylin and eosin, **B** immunostained for S-100. The immunostained section is not counterstained and clearly reveals tumour cells in the heavy inflammatory reaction. Compare with NSE immunostaining in Fig. 2. (Both × 360)

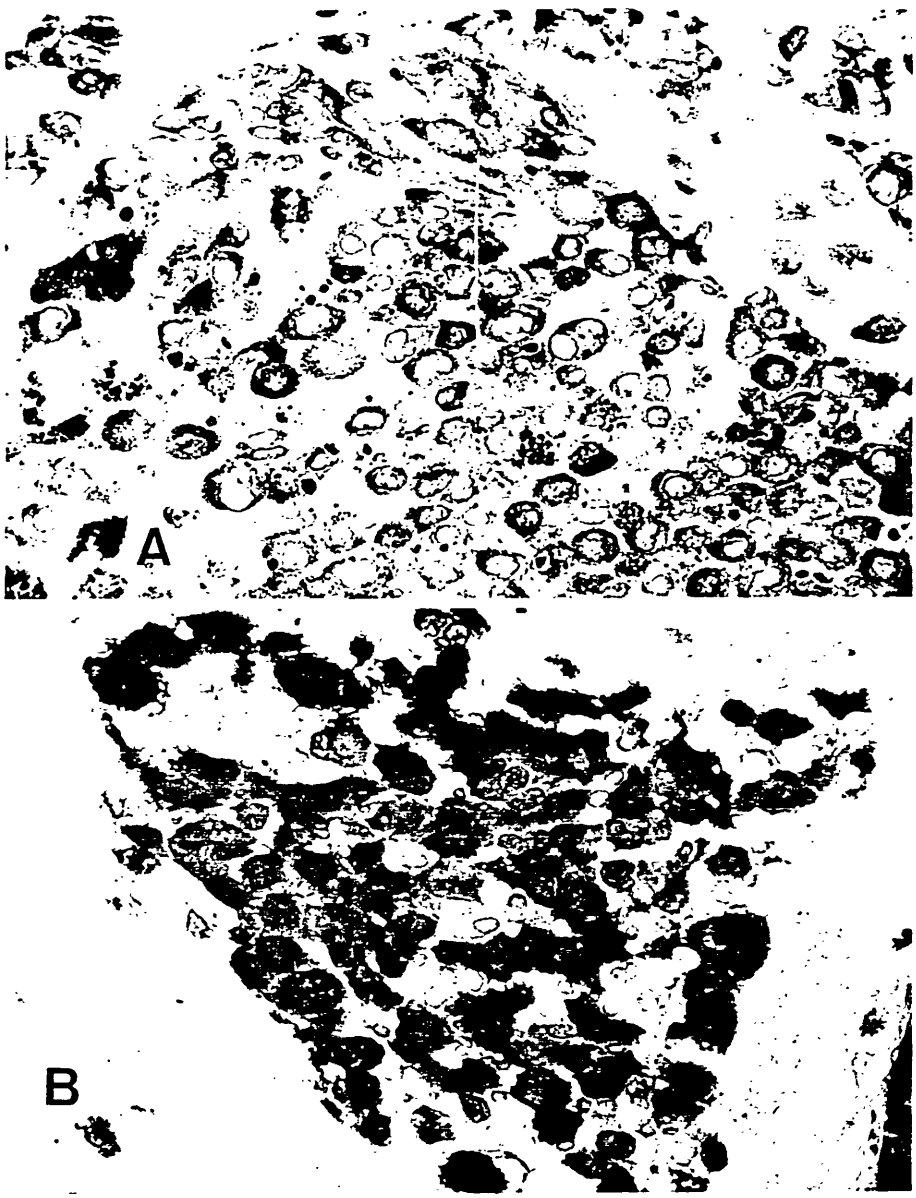


Fig. 2. **A** Adjacent section to Fig. 1, immunostained for NSE. The reaction is much weaker than that with anti-S-100 (Fig. 1). **B** Another area from the same tumour showing strong S-100 immunoreactivity. (Both uncounterstained, **A** ×360, **B** ×225)

tion with the appropriate antibody in all cases. This demonstrated that fixation had not significantly destroyed the antigenicity of the NSE and S-100. Use of non-immune rabbit serum or PBS in place of primary antiserum gave no immunostaining. Absorption controls showed no reaction in tumour or nerves. Cross-absorbed antibodies gave the same reaction as

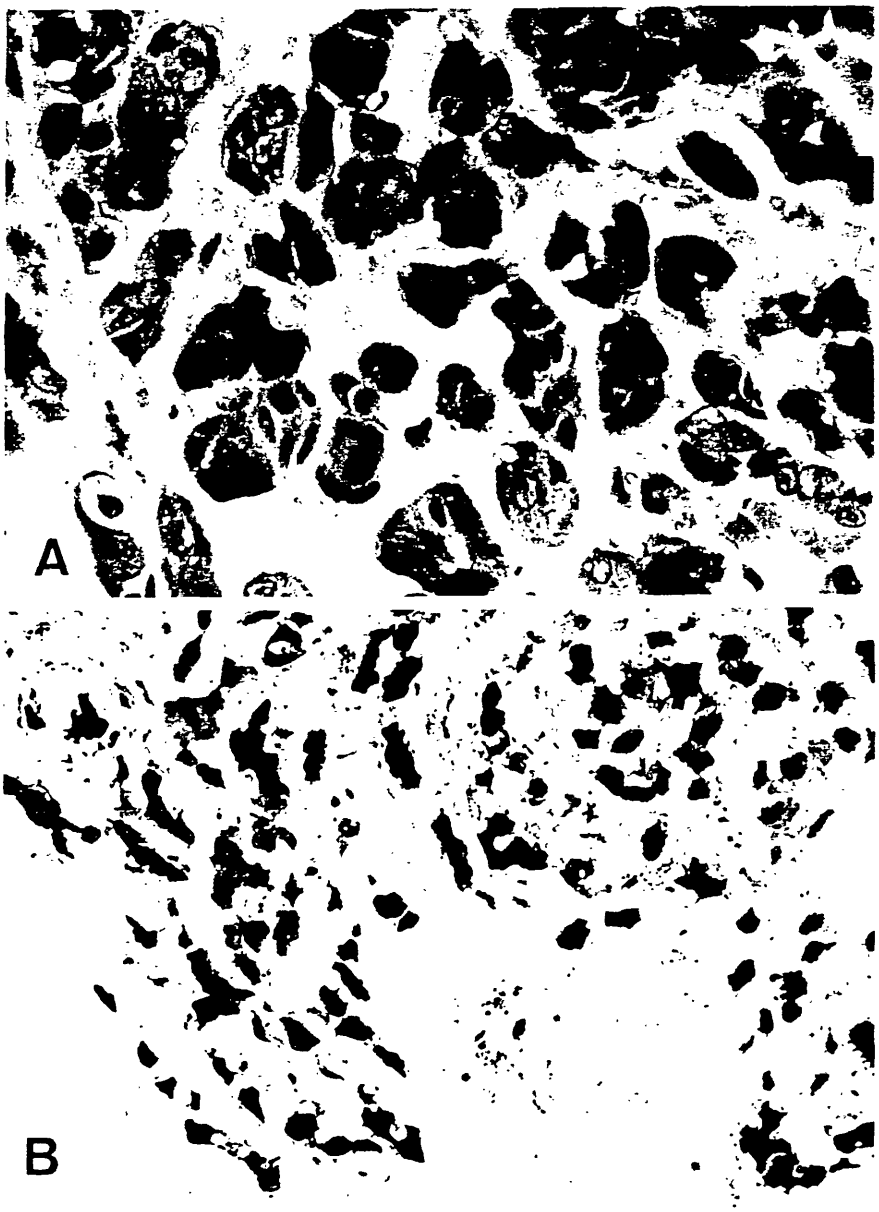


Fig. 3A, B. Melanomas in skin. A S-100 immunoreactivity in tumour cells, B intense immunostaining of S-100 in tumour cells at the periphery of the tumour. (Both uncounterstained. A ×360, B ×225)

their unabsorbed counterparts, demonstrating that the antibodies were specific and did not cross-react with inappropriate antigen.

Of the control tissues (Table 2), all melanocytic naevi but 1 were immunoreactive for both proteins; intradermal and compound naevi were more strongly reactive to S-100 antibodies than the blue naevi, and all types

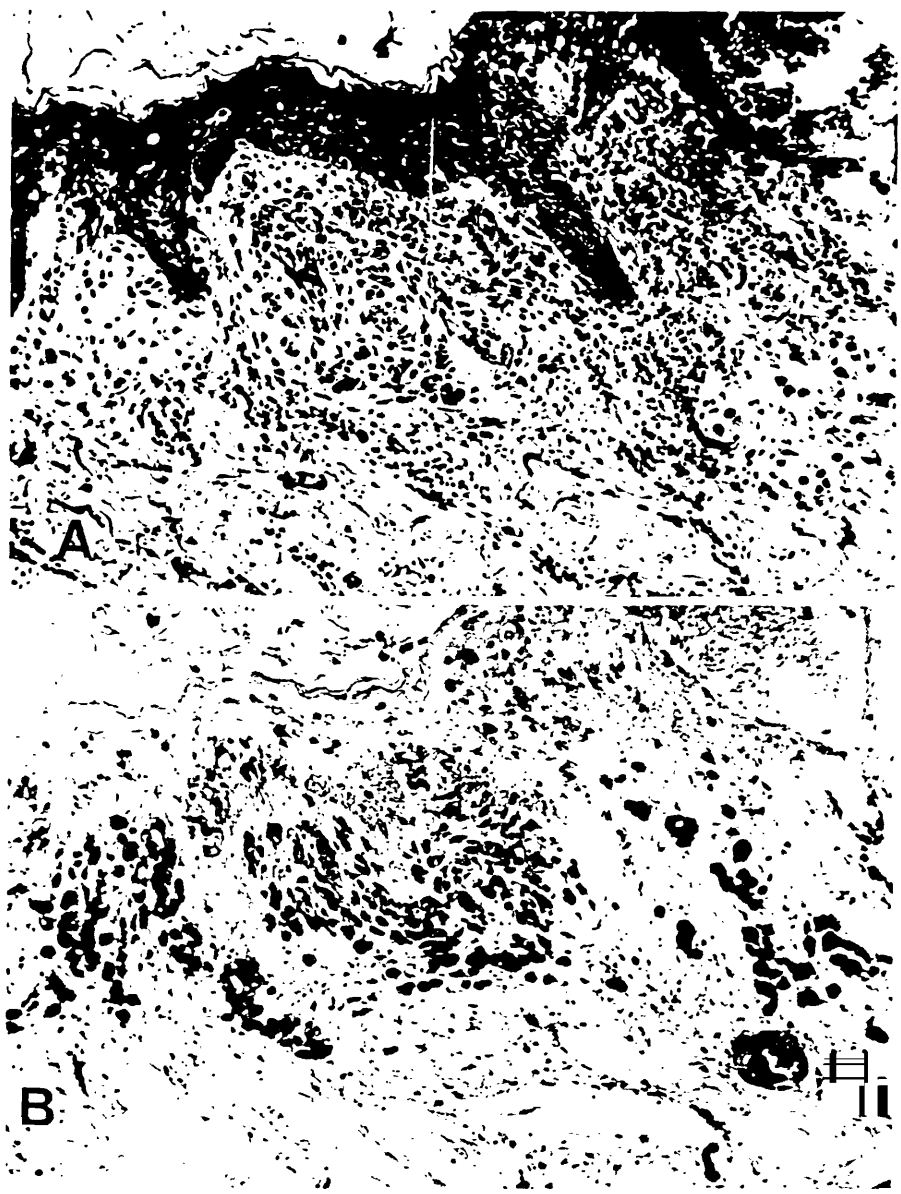


Fig. 4A, B. Intradermal malignant melanocytes at the lateral edge of a melanoma in skin. A H&E. B uncounterstained section immunostained for S-100. The intradermal tumour cells are intensely stained. (Both ×95)

were less strongly reactive to anti-NSE than to anti S-100. Eight of 11 Merkel cell tumours were immunoreactive for NSE, but all were S-100 negative. The lymphomas and normal lymph nodes were negative for both proteins (except interdigitating reticulum cells which were S-100 immunoreactive), as were squamous cell carcinomas, basal cell carcinomas and spindle cell sarcomas.

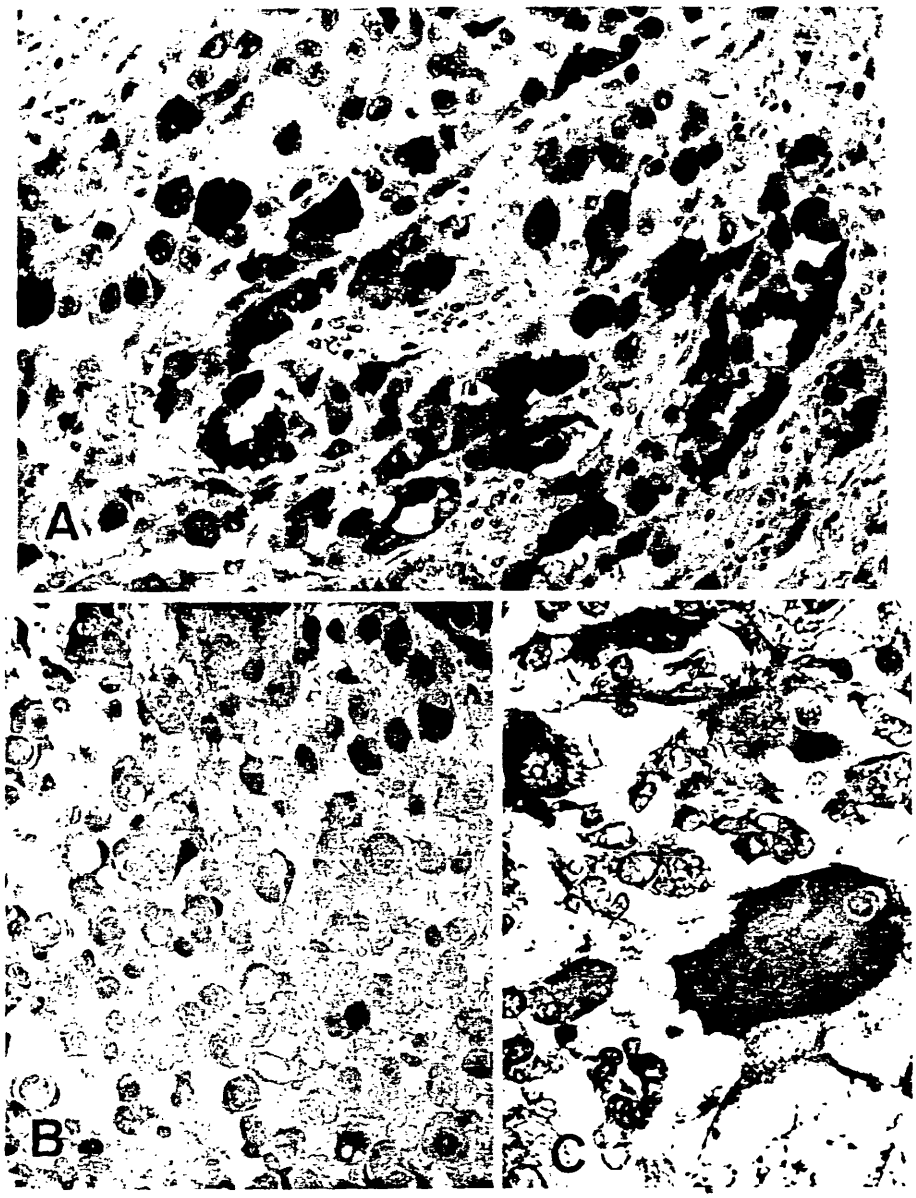


Fig. 5. A Pleomorphic melanoma in skin, haematoxylin counterstained showing strong immunoreactivity for S-100; **B** and **C** metastatic melanoma in lymph node immunostained for S-100. (**A**, **B** × 225, **C** × 360)

In 1 normal skin specimen and 6 melanomas, normal melanocytes in the epidermis or hair follicles were immunostained by S-100 antibodies, but never by anti-NSE. This was true of normal skin whether fixed in formol saline, Bouin's fluid or benzoquinone solution.

Table 2. Immunostaining of control tumours in skin for S-100 and NSE

Tissue	Number		Number with positive staining	
			S-100	NSE
Undifferentiated Ca	2		–	2 (trace)
Small cell Ca (bronchus)	1		–	1 (trace)
Merkel cell tumour	11		–	8
Squamous cell carcinoma	3		–	–
Spindle cell sarcoma	2		–	–
Breast carcinoma	1		–	–
Adenocarcinoma, site unknown	1		–	–
Solar keratosis with CIS	1		–	–
Lymphoma	9		–	–
Basal cell papilloma	1		–	–
Dermal fibroma	1		–	–
Condyloma acuminata	2		–	–
Basal cell carcinoma	5		–	–
Normal skin (various sites)	20	Melanocytes	1	–
		Langerhans cells	20	–

Discussion

Using routinely fixed histological material, we have detected S-100 immunohistochemically in all but one of 33 cases of melanoma examined, including amelanotic tumours, and in all of 5 intradermal and compound naevi. These results agree with those of other workers (Gaynor et al. 1981; Clark et al. 1982; Cochran et al. 1982; Nakajima et al. 1981, 1982; Stefansson et al. 1982b). It is also predictable since we found some normal melanocytes in normal skin and skin adjacent to melanomas to immunostain for S-100, as was also shown by Cocchia et al. (1981); tumours deriving from other S-100-containing cells also contain the protein (e.g. Schwannomas – Stefansson et al. 1982b). Why normal melanocytes are not all immunoreactive for S-100 is uncertain; possibly the protein is only present in amounts detectable by immunohistochemistry at certain stages of cell life or of activity. That melanin-containing cells in melanomas do not react with anti-S-100 supports this view, as does the strong S-100 immunoreactivity of most superficial spreading melanomas, malignant melanocytes towards the edges of nodular melanomas, and intradermal malignant melanocytes. The strength of this reaction may mean that such cells are of a more active type than those in the middle of a nodular tumour. Alternatively it may reflect the availability of nutrients to the tumour cells, those cells showing greater S-100 immunoreactivity are nearer to the nutrient supply than those in the middle of nodular tumours. However, although there is an increase in the amount of immunostaining of S-100 in the progression from normal melanocytes to naevocytes and malignant melanocytes, our results do not show that there is any correlation of S-100 immunoreactivity with the degree of pleomorphism or melanisation of the malignant melanomas.

We have found NSE immunoreactivity in only 6 of 33 malignant melanomas examined. These were amongst the more monomorphic specimens. None of the NSE-immunoreactive tumours had metastases, suggesting that they may be more benign.

Dhillon et al. (1982) found NSE to be detectable immunohistochemically in 15 out of 16 cases of melanoma, and observe that NSE immunostaining increases in strength from benign to malignant lesions (Dhillon and Rode 1982). This was also suggested by Royds et al. (1982b). However, the latter authors found NSE immunoreactivity in 18 of 20 melanomas, whereas only 6 of our 33 cases reacted with antibodies to NSE.

This variation between our findings and those of Royds and Dhillon and their co-workers could be due to differences in fixation of tissues (although we also observed NSE-immunoreactive nerves in all our sections), or a difference between antibodies or the dilutions used for immunohistochemistry. We raised the concentration of our anti-NSE serum in an attempt to increase the degree of immunostaining, but even at a dilution of 1:800 no improvement was obtained.

In common with Royds et al. (1982b), we were not able to detect NSE immunoreactivity in normal human melanocytes. NSE immunoreactivity was also absent from cat and rat melanocytes (Gu et al. 1981). Identical results were obtained with formol-saline-, Bouin-, or benzoquinone-fixed material. All naevi reacted with antibodies to NSE. Our results suggest that NSE immunoreactivity is present in transformed melanocytes and then decreases with progression to malignancy.

The most obvious pointer to the diagnosis of melanoma in a skin tumour is melanin, but this does not exclude other tumours which have taken up the pigment and thus mimic a melanoma. With non-pigmented melanomas the differential diagnosis of tumours including spindle-cell sarcomas, squamous and basal cell carcinomas, and skin metastases of undifferentiated carcinomas becomes important, as does the site of the primary tumour in metastases. Thus a marker for distinguishing melanoma in these situations would be valuable.

We feel that since we have not found S-100 immunoreactivity in non-melanocytic (control) tumours this protein is a potentially useful marker. As well as its use for differential diagnosis it is valuable in highlighting small groups of tumour cells either spreading superficially or invading the dermis.

Immunohistochemical detection of S-100 has other advantages. Diagnostic tests for melanoma such as formalin-induced fluorescence and enzyme cytochemistry (Rost et al. 1973), the tyrosinase method of Fitzpatrick and Clark (1964) using tissue slices, and biochemical assay of S-100 mostly require fresh or specially fixed tissue. This study has shown that routine fixation and embedding does not destroy the antigenicity of S-100 in melanomas. This is most important for the routine histopathology department, permitting as it does the application of the test in cases where fresh material is no longer available when suspicion of melanoma arises, or in retrospective studies. Thus the need to use non-routine techniques 'just in case' is ob-

viated. However, work in our laboratory (unpublished results) shows that the antigenicity of both NSE and S-100 is diminished if tissue is left in fixative for long periods (but up to 1 week in formol saline is satisfactory) before blocking in wax. The time of storage of wax blocks seems to be unimportant for NSE and S-100 immunoreactivity.

If S-100 should prove to be released into the blood stream from the tumour, the assay of the protein in the circulation may be of value in monitoring the effectiveness of surgical ablation or other therapy, or the subsequent growth of metastases, in patients having melanoma.

In conclusion, S-100 immunostaining appears to be a convenient and specific marker for melanocytic tumours, thus distinguishing these lesions from a variety of other tumours.

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SUBSTANCE P-CONTAINING SENSORY FIBRES IN THE RAT BLADDER ORIGINATE FROM PELVIC AND HYPOGASTRIC NERVES

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SUMMARY

The origin and nature of substance P-immunoreactive fibres in the bladder was investigated by immunocytochemistry and radioimmunoassay following denervation of the bladder and neonatal capsaicin treatment. After pelvic and hypogastic nerve resection a decrease of substance P-immunoreactivity was observed, mainly in the mucosal layer. A similar depletion of substance P-immunoreactive nerves was observed in capsaicin treated animals. These findings indicate the sensory nature of a subpopulation of substance P-immunoreactive nerves in the bladder, within and originating from the pelvic and hypogastric nerves, both of which contain a sensory component.

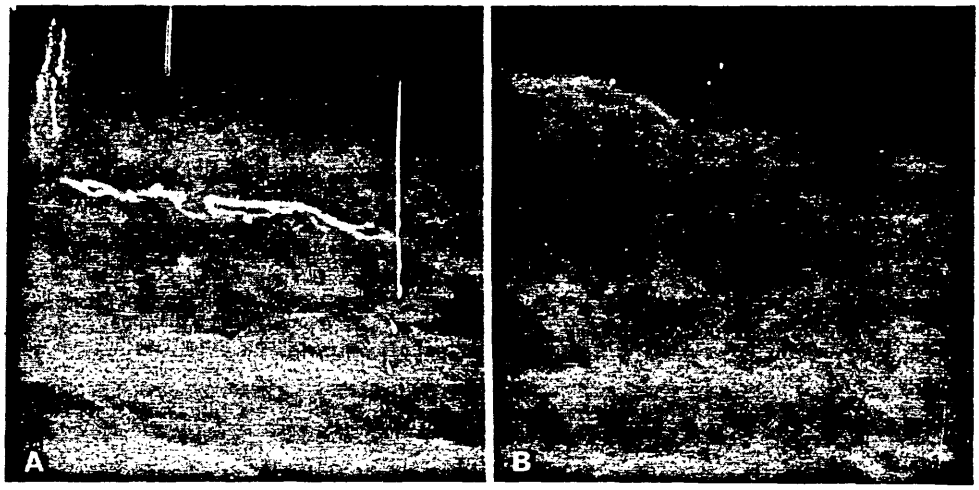
Substance P-immunoreactive nerves in many peripheral tissues are part of the sensory system, with origin from primary sensory neurons in the dorsal root or cranial ganglia (1,2). In previous studies the bladder has been found to contain numerous substance P-immunoreactive fibres (3). However, the origin and nature of these substance P-containing fibres is still a matter of speculation.

Selective surgical denervation of the bladder was carried out on adult rats as follows: unilateral parasympathetic (pelvic nerve) resection proximal to the pelvic ganglion(n=12); post-ganglionic bilateral sympathetic (hypogastric nerve) resection distal to the inferior mesenteric ganglion(n=12); a combination of the two types of denervation (n=12); normal controls consisted of unoperated rats (n=6). Samples of the right and left sides of the bladder were collected from all animals at 7 days post-operatively. Separate groups of rats of the same litter were injected at day 2 after birth with capsaicin (50 mg/kg body weight) (n=12) or vehicle solution (Tween 80, saline, absolute alcohol, 1:8:1 vols) (n=12). The animals were sacrificed at 3 months of age and samples of the bladder were collected for investigation.

For immunocytochemistry, the bladder was fixed in 0.4% benzoquinone solution (4). Cryostat sections were immunostained by the indirect immunofluorescence method using substance P antiserum (dil. 1/1000). Radioimmunoassay was carried out on the whole bladder wall as previously described (5), using a C-terminally directed substance P antiserum (dil. 1/8000).

By immunocytochemistry, numerous very fine nerve fibres were observed in the normal bladder; these fibres were mainly localised in the lamina propria, in direct contact with the transitional epithelium (Fig. A x 450), and in the muscle layer, interspersed with the smooth muscle fibres. Following pelvic denervation, a considerable decrease of substance P-immunoreactive fibres was observed in the lamina propria (Fig. B x 450), where the depletion was almost complete. In contrast, in the muscle layer no noticeable change was observed in the number of substance P-immunoreactive nerves or in the intensity of immunostaining. Very similar results were observed after resection of either the hypogastric nerves or the combination of hypogastric and pelvic nerves. However, on subjective interpretion, the decrease of mucosal substance P-immunoreactivity appeared less marked following hypogastric denervation compared to that observed after pelvic nerve section. In the bladder of capsaicin-treated animals a decrease of substance P-immunoreactive nerves was also seen, particularly evident in the lamina propria.

Radioimmunoassay results are summarised in Table 1: a significant but not complete decrease of substance P-immunoreactivity was detected after all types of denervation and capsaicin treatment, which is consistent with the partial depletion of substance P-containing fibres observed by immunocytochemistry.



These results clearly demonstrate that a subpopulation of substance P-immunoreactive fibres present in the mucosal layer of the bladder originates from pelvic and hypogastric nerves. The depletion of substance P-immunoreactivity following capsaicin administration indicates that these fibres have a sensory component (6), which is consistent with their observed localisation beneath the epithelium and their demonstrated origin from primary sensory neurons of the dorsal root ganglion (7). These findings are also in agreement with the view that both sympathetic and parasympathetic pathways to the bladder could contain sensory fibres (8).

TABLE 1: Radioimmunoassay. Substance P-immunoreactivity (pmoles/g ± SEM) in the bladder of control and experimental rats. Statistical analysis by Student's t-test.

	control	experimental	
Unilateral pelvic denervation	7.4 ± 1.1	5.3 ± 0.6	P<0.05 paired
Bilateral hypogastric denervation	6.7 ± 0.7	2.9 ± 0.4	P<0.01 unpaired
Pelvic + hypogastric denervation	6.7 ± 0.7	3.5 ± 1.0	P<0.05 unpaired
Neonatal capsaicin	6.6 ± 0.8	0.46 ± 0.09	P<0.001 unpaired

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SUBSTANCE P-CONTAINING NERVES IN THE MAMMALIAN GENITALIA

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ABSTRACT. Substance P immunoreactivity was found in nerves supplying both male and female genitalia of several mammalian species by means of immunocytochemistry and radioimmunoassay. These nerves were generally associated with blood vessels and smooth muscle and, in particular, they occured in the submucosa beneath the epithelium of the cervix, vagina and clitoris of the female and around the sensory receptors in the glans penis of the male. In rats, extrinsic section of pelvic and/or hypogastric nerves by surgery and capsaicin treatment support the contention that these nerves are sensory in nature and originate from ganglia extrinsic to the genitalia. They run in the parasympathetic (pelvic), sympathetic (hypogastric) and somatic (pudendal) pathways.

The mammalian genitalia are densely innervated by autonomic (sympathetic and parasympathetic) and somatic nerves (1). In addition to the classical neurotransmitters, several neuropeptides have been found to be present in these nerves as their presumed neurotransmitters or modulators (2,3,4,5,6). We have investigated specifically the occurrence, distribution and origin of substance P-containing (SP) nerves in the male and female genitalia of rat, guinea-pig, cat, mouse and man using the methods of immunocytochemistry and radioimmunoassay. Surgical denervations were performed on the hypogastric and/or pelvic (including pudendal) nerves to the uterus of the rat. The genital tract was taken 7 days after the operation. Capsaicin (50mg/kg body weight) or vehicle solution (Tween 80, saline, absolute alcohol, 1:8:1 vols) were applied intraperineally in separate groups of rats at day 2 after birth. The genitalia were taken at 3 months of age. Human tissues were obtained at surgery and postmorten. The details of the antiserum, and the methods used have been previously described (6).

SP nerves were found in most anatomical regions of both the male and female genital tracts of all the species investigated. In general, these nerves were sparsely associated with blood vessels and some smooth muscles. More such nerves were seen in the female than in the male except in man where SP nerves were rarely encountered in female genital organ. In the female, these nerves were particularly concentrated in the submucosa around the cervix (Fig.1) and in the region beneath the epithelium of the vagina and the clitoris. SP nerves were only occasionally seen in the uterus, the Fallopian tube and the ovary (cat only). There were more SP nerves in the female genitalia of mouse than in the other species. In the male, these nerves were more frequently seen in association to the sensory receptors of the glans penis (Fig.2) than in other regions. These structures in the cat have been known as mechanoreceptors innervated by pudendal nerves (7). A small number of SP nerves was also found in the interstitial tissue of the prostate gland. No neural cell bodies reactive to substance P antiserum were found in the genitalia of either sex.

Following bilateral hypogastric denervation in the rat, substance P immunoreactivity in the uterus was decreased significantly ($p<0.05$, unpaired T-test) from 1.6 ± 0.2pmol/g to 0.75 ± 0.1pmol/g, mean$\pm$SEM. After unilateral pelvic denervation, the immunoreactivity was reduced ($p<0.05$, paired T-test) from 1.6 ± 0.4pmol/g (normal side) to 1.0 ± 0.2pmol/g (denervated side). Denervation of

the entire extrinsic nerve supply led to a complete disappearance of SP nerves in the genitalia. The application of capsaicin also diminished the substance P immunoreactivity in the organ further supporting the postulation of substance P being a putative sensory neurotransmitter.

Our findings show that SP nerves are present in the mammalian genital organs of both sexes. These nerves originate from ganglia extrinsic to the genitalia and located higher than the sectioning points, possibly in the spinal ganglia. Most of these nerves are sensory in nature, running along the parasympathetic (pelvic), sympathetic (hypogastric) and somatic (pudendal) pathways.

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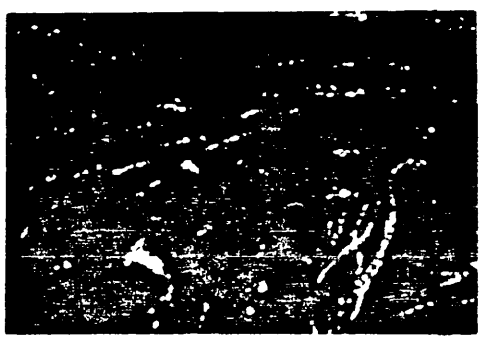


Fig. 1. Substance P-immunoreactive nerves in the cervix of mouse (x300).



Fig. 2. Substance P-immunoreactive nerves in the glans penis of man (x450).

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Immunogold Staining Procedure for the Localisation of Regulatory Peptides

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VARNDELL, I. M., F. J. TAPIA, L. PROBERT, A. M. J. BUCHAN, J. GU, J. DE MEY, S. R. BLOOM AND J. M. POLAK. *Immunogold staining procedure for the localisation of regulatory peptides.* PEPTIDES 3(3) 259–272, 1982.—The use of protein A- and IgG-conjugated colloidal gold staining methods for the immuno-localisation of peptide hormones and neurotransmitters at light- and electron microscope level are described and discussed. Bright-field and dark-ground illumination modes have been used to visualise the gold-labelled antigenic sites at the light microscope level. Immunogold staining procedures at the ultrastructural level using region-specific antisera have been adopted to localise specific molecular forms of peptides including gastrin (G17 and G34), glucagon and pro-glucagon, insulin and pro-insulin, in normal tissue and in tumours of the gastroenteropancreatic system. Similar methods have been used to demonstrate the heterogeneity of p-type nerves in the enteric nervous system. Vasoactive intestinal polypeptide (VIP) has been localised to granular sites (mean±S.D. granule diameter=98±19 nm) in nerve terminals of the enteric plexuses and in tumour cells of diarrhoeogenic VIP-producing neoplasias (mean±S.D. granule diameter=126±37 nm) using immunogold procedures applied to ultraviolet-cured ultrathin sections. Co-localisation of amines and peptides in carotid body type I cells and in chromaffin cells of normal adrenal medulla and phaeochromocytomas has also been demonstrated. Advantages of the immunogold procedures over alternative immunocytochemical techniques are discussed.

Immunogold staining methods Regulatory peptides Light microscopy Electron microscopy
Protein A-gold Molecular forms P-type heterogeneity Co-localisation

THE number of peptides isolated and characterised from animal tissues, and for which regulatory functions have been proposed, has increased dramatically in recent years. It is well recognised that most of these regulatory peptides are stored in distinct types of endocrine and neural cells which are distinguished from other cell types and from each other by the presence of electron-dense secretory granules varying in their shape, size and the form of their limiting membrane [39]. Most peptides have also been found to exist, both in blood and tissue, in multiple molecular forms, the ratios of which vary according to the dynamic state of the cell.

Evidence is accumulating to implicate abnormalities of peptide synthesis and release in disease states in man and other mammals [2]. Therefore, knowledge of the precise localisation of peptides in various tissues in health and disease is important and immunocytochemistry has played a key role in these investigations for many years.

The classical immunofluorescence [6] and peroxidase [26,40] techniques have been modified and further developed into a variety of procedures so that immunocytochemistry is now a very precise and sensitive tool [43]. One revolutionary step was the application of immunoperoxidase [26] and peroxidase-antiperoxidase [40] techniques at the electron microscope level, which revealed, conclusively, a granular localisation for regulatory peptides. Unfortunately, certain drawbacks inherent to the peroxidase techniques are now becoming apparent. Of particular concern in this respect is the homogeneous deposition of the electron-dense final reaction product over the antigenic structures. It is increasingly evident from ultrastructural studies that the enormous heterogeneity of secretory granule structure observed among neuronal and endocrine cell types may have some functional importance with respect to the peptides they contain. Therefore, it is important to be able to investigate the ultrastructure of immunoreactive organelles. The use of the semi-thin serial sectioning technique partly overcame the problem of antigenic site obfuscation, however, with increasing evidence for the co-existence of peptides and other antigens in single organelles, a more precise electronimmunocytochemical technique for the ultrastructural localisation of tissue-bound antigens was sought.

In 1971 Faulk and Taylor [11] conjugated colloidal gold to

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immunoglobulins and suggested the value of this technique in immunological procedures at the electron microscope level. Successive modifications [9, 13, 18, 34] have produced a highly efficient immunogold technique for the localisation of antigens at both light- and electron microscope level. In parallel, colloidal gold conjugated to a staphylococcal protein, protein A, which has a marked reactivity with the Fc component of IgG molecules, was developed as a highly specific immunocytochemical technique [31, 33, 35].

Using these techniques, we have been able to localise a wide variety of regulatory peptides in nerves and endocrine cells in health and disease at both light- and electron microscope levels.

METHOD

Experimental animals used in this study included cats, piglets, horses, guinea pigs and albino rats (Sprague-Dawley).

Adult cats, weighing between 1.8 and 2.0 kg, were anaesthetised by an intraperitoneal overdose of sodium pentobarbitone ("Euthatal", May and Baker Ltd., 200 mg/ml; 1 ml per kg) and were perfused, via the ascending aorta, according to the procedure detailed below. In addition, guinea pigs and rats were anaesthetised by "Penthrane" (Abbott Labs.) inhalation and perfusion-fixed as follows.

Tissue Preparation

Exsanguination was achieved with 150 ml of ice-cold 0.1 M sodium phosphate buffer, pH 7.3 (50 ml/min^{-1}) and was followed immediately by fixation with an ice-cold solution of 1% formaldehyde (prepared from its *para*-polymer) plus 2% glutaraldehyde in 0.075 M sodium phosphate buffer (pH 7.3; 720 mOsm) for 15 min (50 ml/min^{-1}).

Small blocks of tissue (carotid body, adrenal medulla, pancreas, regions of gut from stomach to colon) were excised and fixed by immersion in the latter solution for a total time of two hours. Half of the blocks were rinsed in buffer and osmicated (1% osmium tetroxide in Millonig's buffer, pH 7.2 for one hour at 4°C), whereas the other half were merely rinsed in buffer.

Some of the non-osmicated blocks were dehydrated and embedded in a hydrophilic resin (Lemix; EMscope Labs., U.K.). The remaining blocks were dehydrated in a graded series of ethanols and infiltrated with Araldite epoxy resin. Some of the Araldite blocks were cured by ultraviolet irradiation (10 days to 3 weeks; room temperature).

Silver to silver-grey sections were cut on a Reichert-Jung Ultracut and were collected on uncoated 300-mesh nickel grids. Semithin (0.5–1.0 μm) sections were also cut, mounted on clean glass slides and stained with a solution of Azure II and methylene blue in borax buffer.

A second group of twelve adult cats, eight guinea pigs, six rats, and six neonatal piglets, aged one day to three weeks, were killed by pentobarbitone overdose and dissected fresh. In addition, pieces of tissue were excised from the adrenal glands and gut of five horses (two normal, three with grass sickness). Small blocks from each organ under investigation were fixed either in the above fixative or in 3% buffered glutaraldehyde and processed for electron microscopy. Larger blocks of fresh tissue were snap-frozen in melting Arcton-12, freeze-dried, and fixed in *p*-benzoquinone vapour. The final blocks were then embedded in Ralwax, sectioned at 3–7 μm, and mounted on poly-L-lysine-coated glass slides.

Finally, normal and pathological tissue was removed from human subjects at surgery or autopsy and fixed for light- or electron microscopy according to the methods recorded above. Only human material showing good morphological preservation when studied by conventional transmission electron microscopy was investigated immunocytochemically.

Preparation of Gold Reagents

Colloidal suspensions of gold particles were prepared by reduction of tetrachloroauric acid with white phosphorus (particle diameter 5–12 nm), according to the method of Faulk and Taylor [11], with modifications [17, 34, 38], or with *tri*-sodium citrate [12,38] (particle diameter 16–20 or 30–40 nm). Goat anti-rabbit immunoglobulins were linked to colloidal gold [9], according to the principles of Geoghegan and Ackerman [13]. The protein A-gold complex was prepared as described by Roth and co-workers [35] and Slot and Geuze [38] and uniformly sized subfractions of gold reagents were prepared as described by the latter authors.

Antisera

The antisera used were raised in rabbits against synthetic peptides and, in the case of dopamine-β-hydroxylase, against purified bovine enzyme [36]. Characteristics of the antisera used are presented in Table 1. Antiserum specificity was verified by the lack of staining in adjacent sections which had been incubated in antisera preabsorbed with the specific antigen (nmol/ml of the diluted antiserum).

Although we have endeavoured to ensure the specificity of all the antisera employed, cross-reaction with a closely related, but so far unidentified antigen cannot be discounted.

Electronimmunocytochemistry

The grid-mounted sections of unosmicated tissue were dried overnight and then etched in 10% hydrogen peroxide for ten minutes. This step was later found not to be always necessary. Thorough washing of the grids in 0.05 M Tris-buffered saline (TBS), pH 7.3, was followed by incubation in normal goat serum (NGS) for 30–60 min at room temperature. Normal goat serum was not applied to grids subjected to the protein A-gold technique. The NGS was drained from the grids, each of which were then incubated in 10 μl of primary antiserum for 20–24 hr at 4°C. The antisera were diluted with TBS containing 1% bovine serum albumin (TBS/BSA) pH 7.2.

Control sections were incubated in normal (pre-immune) rabbit serum at the same dilution as the primary antiserum or in first layer antisera preabsorbed with pure antigen.

After thorough washing in TBS/BSA (pH 8.2) buffer the grids were transferred to the gold-labelled solutions for one hour at room temperature. The final dilutions of the gold-labelled solutions in comparable conditions are given in Table 2. This incubation was followed by thorough washing in large volumes of TBS/BSA buffer. Finally, the grids were rinsed in Millipore-filtered distilled water, dried, counterstained as for electron microscopy with uranyl acetate and lead citrate and viewed with a Zeiss 10CR electron microscope operating at 60 kV.

Light Microscope Immunocytochemistry

Araldite-embedded tissue: Semi-thin (0.5–2.0 μm) sections of Araldite-embedded tissue were mounted on poly-L-

TABLE 1

CHARACTERISTICS OF THE ANTISERA USED

Antiserum raised against	Region specificity	Dilution	Concentration of antigen used for absorption (nmol/ml diluted antiserum)	Ref.*
Gastrin .	C-term.	1:2000	0.1	[15]
Glucagon	N-term.	1:2000	2	
Glicentin	N-term.‡	1:1000	2	[3]
Insulin	—	1:800	2	[3]
C-peptide	—§	1:400	5	
Met-enkephalin	C-term.	1:4000	1	[43]
Substance P	Whole molecule	1:8000	3	[29]
VIP	C-term.	1:6000	5	[29]
Somatostatin	—	1:6000	1	[15]
Secretin	—	1:6000	1	[15]
Serotonin	Amino side chain	1:2000	6.10^3	[15]
Dopamine-β-hydroxylase	Bovine enzyme	1:2000	0.5†	[36,43]

*References cited in text.

†Refers to enzyme units (Sigma; Type III) required per ml of diluted antiserum.

‡From Dr. A. J. Moody, Novo Research Institute, Denmark.

§From Prof. N. Yanaihara, Shizuoka School of Pharmacy, Japan; this antiserum (R2303) is known to cross-react extensively with pro-insulin.

lysine-coated glass slides and dried overnight at room temperature. The sections were de-resinated in sodium ethoxide and washed in a large volume of PBS. Wax-embedded tissue: Sections, 3–7 μm in thickness, mounted on glass slides, were de-waxed and hydrated to PBS.

An immunogold staining technique was applied to the slide-mounted sections, based on the on-grid method described above. Gold particles (40 nm) conjugated to goat anti-rabbit sera were used for the light microscope visualisation of tissue-bound antigens. The gold-labelled antiserum was applied for one hour at room temperature and the final preparations were viewed by dark-ground (semi-thin) and bright-field (thick) microscopy with a Reichert-Jung Polyvar (British American Optical Co.).

The peroxidase-antiperoxidase (PAP) procedure (after Sternberger, [40]) was applied to sections of wax- and resin-embedded tissue [32]. The reaction product was developed either in a filtered solution of 0.05% 3,3′-diaminobenzidine (Aldrich) in phosphate-buffered saline pH 7.2, containing 0.06% hydrogen peroxide, or in a solution of 0.03% 4-chloro-1-naphthol, pH 7.1, containing 0.03% hydrogen peroxide, as previously modified by us from the method of Nakane [15,26].

RESULTS

We have used the protein A- and the immunoglobulin-conjugated colloidal gold methods for the immunolocalisation of regulatory peptides at both the light- and electron microscope level and some of our results are summarised below.

Light Microscope Level

The immunofluorescence [6] and a variety of peroxidase techniques, including the peroxidase-antiperoxidase (PAP) method [40] have been used routinely in a large number of laboratories for the immunohistochemical localisation of regulatory peptides. Recently, however, we have applied immunogold staining procedures for the immunohistochemical localisation of peptide hormones and neurotransmitters at the light microscope level [8, 9, 15] and some of our results are presented below.

TABLE 2

DILUTION OF COLLOIDAL GOLD-LABELLED SOLUTIONS CORRELATED WITH GOLD PARTICLE SIZE*

Particle size (nm)	Gold conjugated to Protein A	Gold conjugated to Immunoglobulin
5	1:50–1:80	1:20
12	1:20	1:8
20	1:8–1:20	1:4
40	—	1:1

*Dilution factors based on high granule:background ratio staining of cat pancreatic α-granules by glucagon antiserum 499 (see Table 1).

Immunoreactive sites in thick (5–7 μm) sections, from wax-embedded tissue, appear red when viewed by bright-field microscopy following immunogold staining. The sequential use of the PAP and the immunogold staining methods [15] has facilitated the development of double- and multiple staining techniques for the simultaneous localisation of tissue-bound antigens. Gold-labelled immunoreactive material in semi-thin sections, however, is not usually visible by bright-field microscopy. Observation of such preparations by dark-ground illumination reveals the gold-labelled structures. Figure 1 exhibits a semi-thin section of a cat pan-

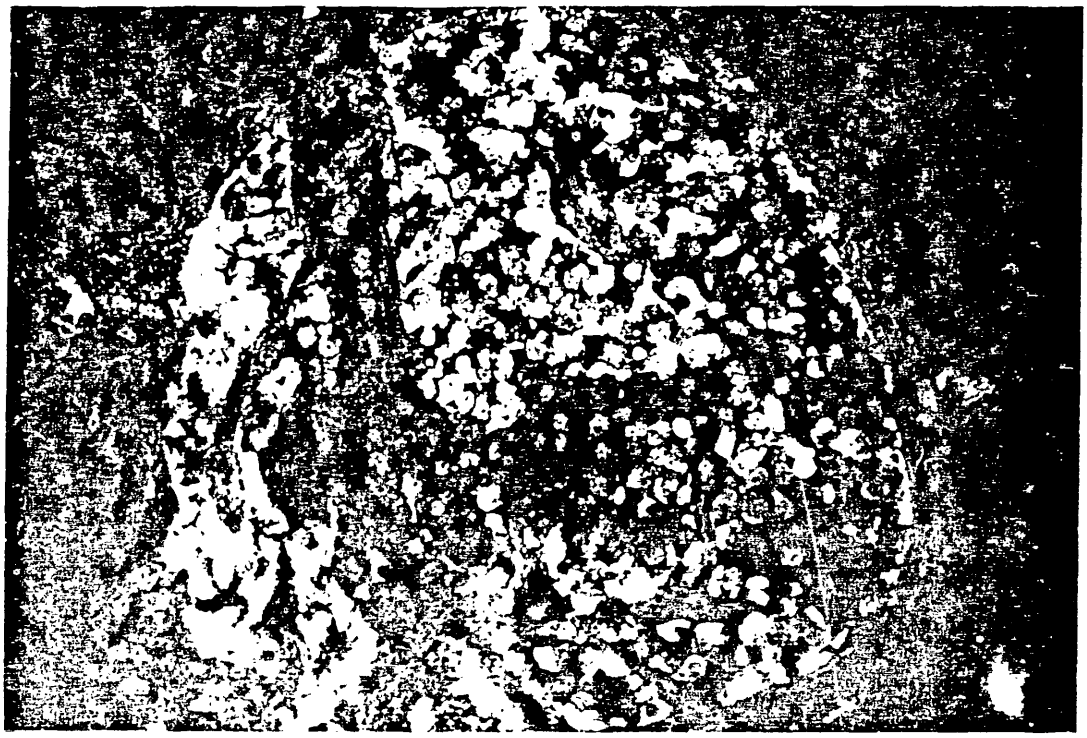


FIG. 1. Semi-thin (2 μm) section of a cat pancreatic islet immunostained for glucagon with the immunogold technique and visualised with dark-ground illumination. ×300.

creatic islet immunostained for glucagon with an immunogold staining procedure and photographed with dark-ground illumination.

Electron Microscope Level

The immunogold staining procedures have considerable advantages over alternative techniques when applied at the electron microscope level for the immunocytochemical localisation of regulatory peptides, as discussed later. In this section we describe some results of our investigations on the ultrastructural localisation of regulatory peptides in endocrine cells and nerve fibres in health and disease.

Immunocytochemical demonstration of peptide molecular forms. It is now well established that most of the regulatory peptides occur in a variety of molecular forms [10]. This suggests the existence of precursor molecules or prohormones which can be distinguished from their bioactive catabolites by the application of antibodies raised to specific regions of the peptide (for reviews see [10,22]). The use of region-specific antisera has permitted the immunocytochemical identification of the sites of production and storage of different molecular forms of a single peptide. Examples of this technology in combination with the immunogold staining procedures include:

(1) The localisation of gastrin to two types of endocrine cells containing different secretory granule populations has been achieved by the use of region-specific antisera directed to the N-terminals of gastrin 17 and gastrin 34. G cells in the antrum, which is known to store mainly the smaller molecular form of gastrin, G17, are characterised by their large (mean±SD, 340±54 nm) electron-lucent secretory granules. In contrast, G cells in the intestine, which is known, at least in man, to store predominantly the larger molecular form of gastrin, G34, are characterised by small (mean±SD, 175±21 nm) spherical, electron-dense secretory granules [3]. Thus, it would appear that the predominance of a particular molecular form determines the structure of the secretory granules [28]. This phenomenon is also evident in tumours, as cells from the rare gastrinomas known to produce predominantly gastrin 17 contain a preponderance of the antral gastrin type of secretory granules. Conversely, secretory granules in tumours producing gastrin 34 as the predominant molecular form are mostly small and electron-dense (Fig. 2a and b) resembling those of the intestinal gastrin cells.

(2) Using the protein A-immunogold staining method in combination with antibodies to glicentin (proglucagon) and glucagon, Ravazzola and Orci [30] first localised both molecular forms to different areas of the pancreatic A cell granule. We have recently provided further evidence to corroborate this finding using both the protein A- and IgG-immunogold staining methods [28]. Glicentin (proglucagon) is localised in the outer portion of the secretory granules (Fig. 3a) and glucagon in the core (Fig. 3b). The halo of the α-granules had previously been distinguishable from the inner portion by its distinct reactivity to the Grimelius silver impregnation technique [14]. This apparent topographic separation of molecular forms within one granule indicates the existence of post-translational enzymatic processes involved in the conversion of proglucagon into the smaller bioactive peptide, glucagon. However, no evidence has been obtained from immunocytochemistry to determine whether these enzymatic processes have taken place prior to granule formation in the Golgi apparatus or following release from that organelle. Molecular forms of glucagon are not only found in normal pancreatic cells but also in tumours which frequently

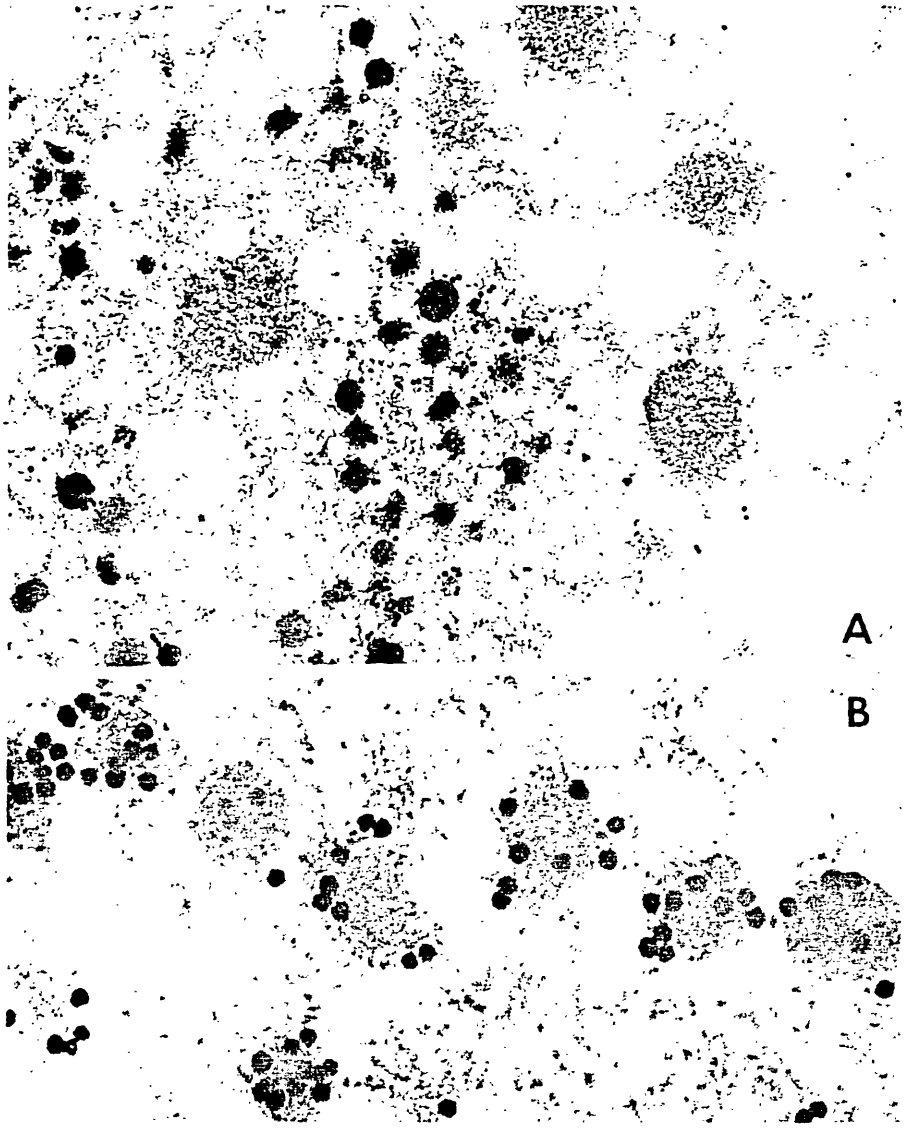


FIG. 2. Secretory granules in a tumour producing predominantly intestinal gastrin (G34). Immunostained using the immunogold technique. a, ×30,000; b, ×150,000.

show mixed populations of secretory granules. In addition to classical α granules which are immunoreactive to glucagon antisera (mean±SD, 250±25 nm) some glucagonoma cells contain granules resembling those of the intestinal L cells which contain enteroglucagon (mean±SD, 140±35 nm) (Figs. 4a and b). In these tumours approximately 60% of the cells are also immunoreactive to pancreatic polypeptide (Fig. 4c). These cells contain granules of similar morphology to those containing glicentin. Using the immunogold technique we can now distinguish between the different peptide-containing cells within these tumours.

(3) Molecular forms of insulin have also been localised to B cells of normal pancreas and also to different types of granule found within cells of insulin-producing tumours (insulinomas). The bioactive peptide, insulin, is enzymatically catabolised from its precursor molecule, proinsulin, with the liberation of a 29 amino acid molecule, C-peptide. It is now well established [21] that two distinct types of secretory granule are present in normal pancreatic B cells, one type possessing a crystalline core with a distinct limiting membrane and one type with a homogeneous core. The ratio between granule types observed within pancreatic B cell populations varies considerably between species. Both granule types are immunoreactive to insulin antisera (Fig. 5), but only the granule type with a homogeneous core is immunoreactive to pro-insulin (C-peptide) antisera (Fig. 6). It is interesting to note that only granules with a homogeneous core are observed in the Golgi apparatus, thus the immunogold staining methods have been instrumental in demonstrating a putative maturation process of granules within the cytoplasm following release from the Golgi apparatus. In our studies we have studied four insulinomas which displayed immunoreactivity to insulin, pro-insulin (C-peptide) and pancreatic polypeptide antisera. In addition to the typical β-granules (crystalline and dense, homogeneous core), some insulinomas are composed of atypical cells immunoreactive

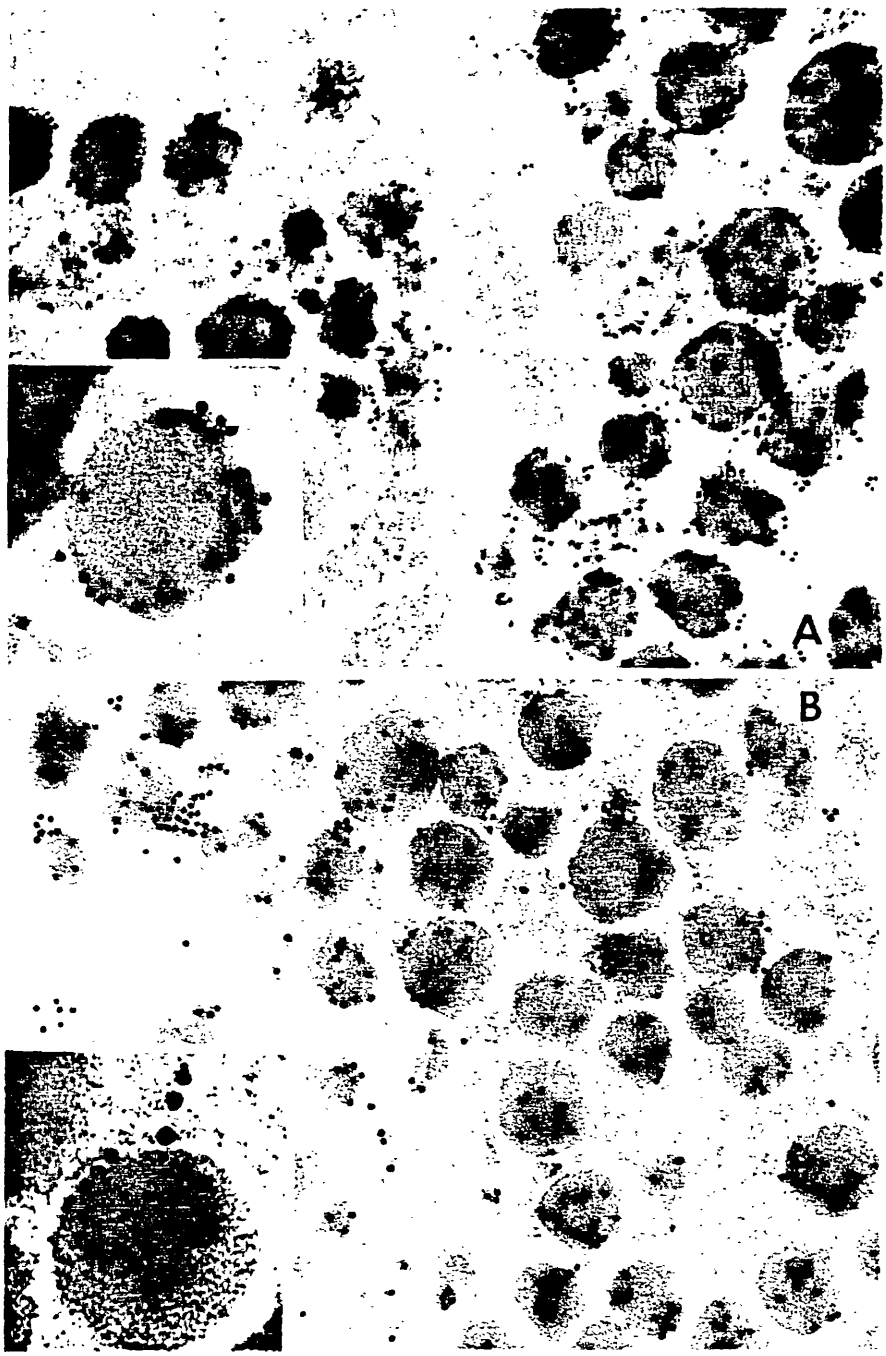


FIG. 3. A-cell granules from human pancreas immunostained by the immunogold technique for, a, glicentin (immunoreactivity is localised to the halo of the granules); b, pancreatic glucagon (immunoreactivity is localised to the core of the granules). ×60,000; inset ×136,000.

to insulin or are a combination of typical and atypical insulin cells [7]. These atypical cells have secretory granules (mean±SD, 148±33 nm) morphologically similar to those containing pancreatic polypeptide (PP) (mean±SD, 145±26 nm) which are also very common in many insulinomas. Using the immunogold techniques we can now distinguish between cells containing PP-immunoreactivity and those containing insulin-immunoreactivity.

Heterogeneity of peptidergic nerves. In 1965 Taxi [42] described enteric nerve profiles which were ultrastructurally distinct from the adrenergic and cholinergic types. The nerves were characterised by the presence of large, granular, secretory vesicles similar to those containing vasopressin and oxytocin in the posterior pituitary, which led Baumgarten and co-workers [1] to describe them as p-type (peptidergic) neurones. Subsequently, morphological heterogeneity

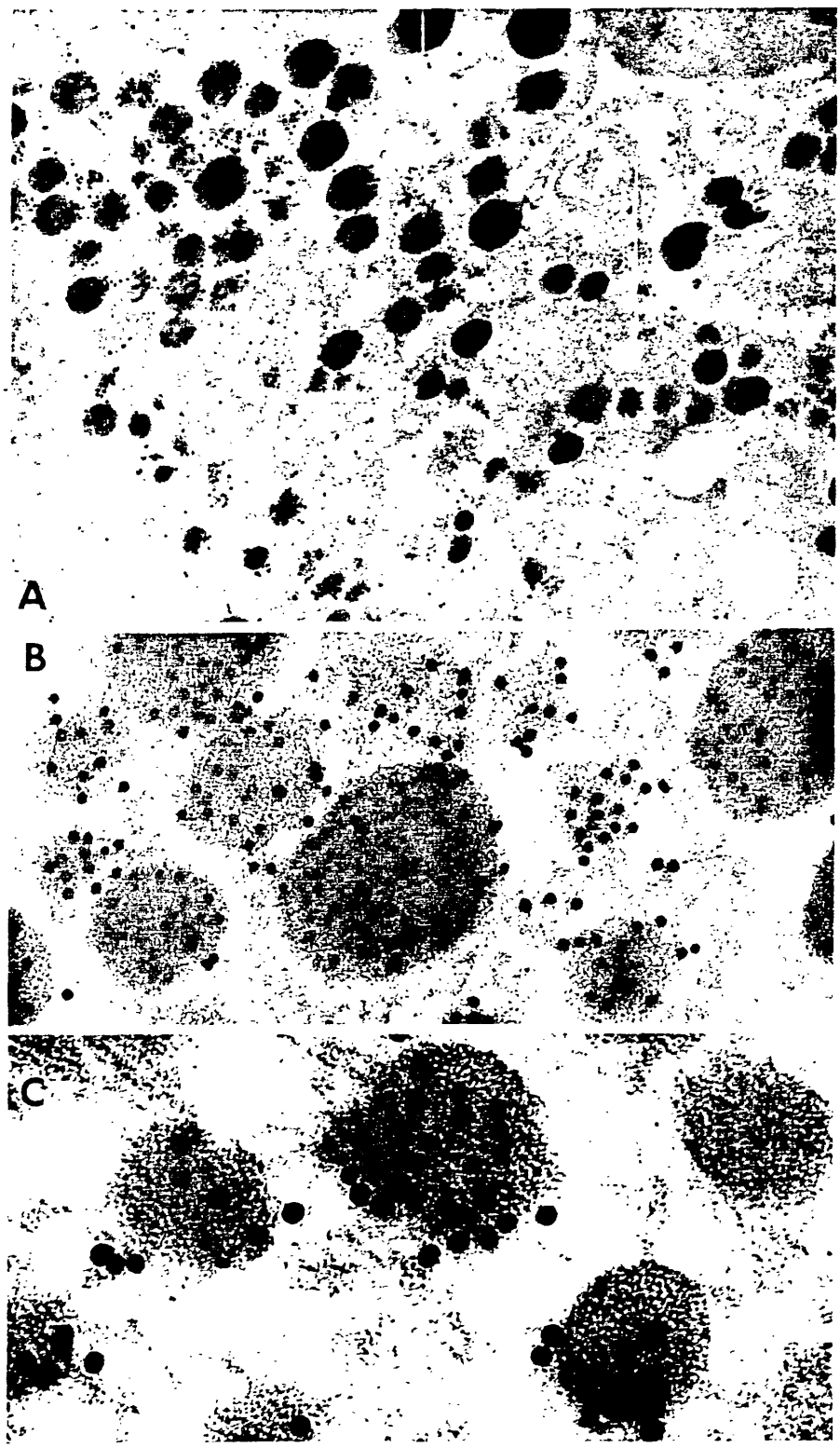


FIG. 4. A glucagonoma containing, a and b. glicentin-immunoreactive granules (a, ×34,500; b, ×138,000); c, PP-immunoreactive granules ×138,000.

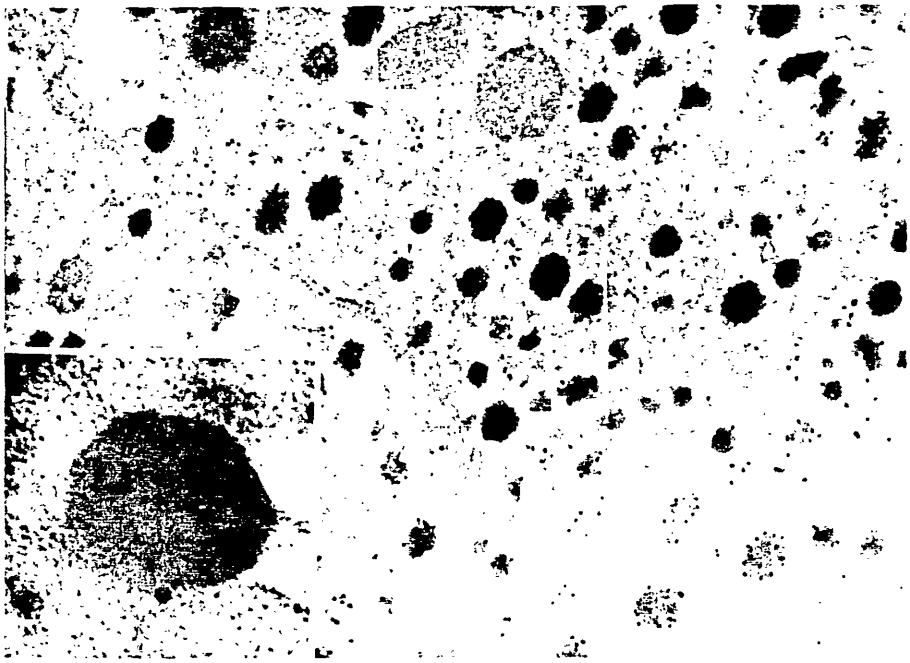


FIG. 5. An insulinoma cell containing β-granules immunoreactive for insulin. ×30,000; inset ×125,000.

within this extensive non-adrenergic, non-cholinergic group was described, based on the electron microscope appearance of these secretory vesicles [4,5]. Our observations using immunogold staining methods at the electron microscope level corroborate the claim of a marked heterogeneity among the p-type components of the autonomic nervous system. We have been able to localise substance P and vasoactive intestinal polypeptide (VIP) to distinct sub-populations of p-type neurones in the enteric nervous system [29]. Details of the electronimmunocytochemical localisation of VIP are discussed below. Morphologically, the separate neurone populations can be distinguished by the size (mean±SD, substance P: 85±15 nm, VIP: 98±19 nm) [29], and appearance of their granular secretory vesicles (Fig. 7a and b). Analysis of serial ultrathin sections immunostained for substance P and VIP reveals a significant proportion of distinct p-type nerves which remain unlabelled with the immunogold procedures. Our findings provide circumstantial evidence to corroborate immunohistochemical investigations which have shown that at least six neuropeptides, other than substance P and VIP, are found in nerve fibres in the enteric nervous system [19], including bombesin, cholecystokinin, enkephalin, neurotensin, somatostatin and thyrotropin releasing hormone (TRH).

Preservation of antigenicity by low temperature processing. By far the majority of regulatory peptides, against which antisera have been raised, can be stained with either of the immunogold procedures described here when applied to ultrathin, grid-mounted sections of aldehyde-fixed, non-osmicated tissue. However, some regulatory peptides, notably VIP, are heat-labile in the presence of resin components and consequently have proved difficult to visualise at both light- and electron microscope levels. One particular example is the localisation of VIP in VIP-producing tumours of the pancreas. It was assumed that the lack of immunocytochemical staining in such tumours was due to the dynamic state of the VIP-producing cells, releasing peptide without storage. However, considerable amounts of extractable VIP were measured by radioimmunoassay in some of these tumours. We have now been able to localise VIP to dense-cored granules (mean±SD, 126±37 nm; Tapia and co-workers, [41]) within tumour cells in VIPomas embedded in Araldite epoxy resin which was cured with ultraviolet irradiation at room temperature (Fig. 8).

Co-storage of peptides with other bioactive molecules. Several reports of the co-existence of peptides and classical neurotransmitters have been published in recent years [16, 24, 25, 37], based mainly on observations made at the light microscope level. One of the most interesting reports suggests the co-existence of VIP with acetylcholine [24]; this is corroborated by the electron microscopical identification of agranular cholinergic-type vesicles in VIP-immunoreactive nerves of the submandibular gland [20] and gut (Polak and Probert, 1981, unpublished observations). Double staining immunocytochemical techniques at the electron microscope level, necessitating the use of the pre-embedding PAP method, are required to investigate this proposed co-existence in more detail.

Recently, Pelletier, Steinbusch and Verhofstad [27] demonstrated the co-localisation of substance P and serotonin in the same secretory granules of raphe nuclei and in the dorsal horn of the spinal cord. Using the immunogold staining procedures we have obtained ultrastructural evidence to support the concept of co-localisation of peptides and amines by visualising enkephalin-like immunoreactive material in dense-cored granules of catecholamine-containing cells [44]. Dopamine-β-hydroxylase-like and methionine enkephalin-like immunoreactivities have been localised to small (mean±SD, 115±5 nm), electron-dense granules found in the type I cells of cat and piglet carotid bodies (Fig. 9) and also to the heterogeneous population of large, (mean±SD, 250±50

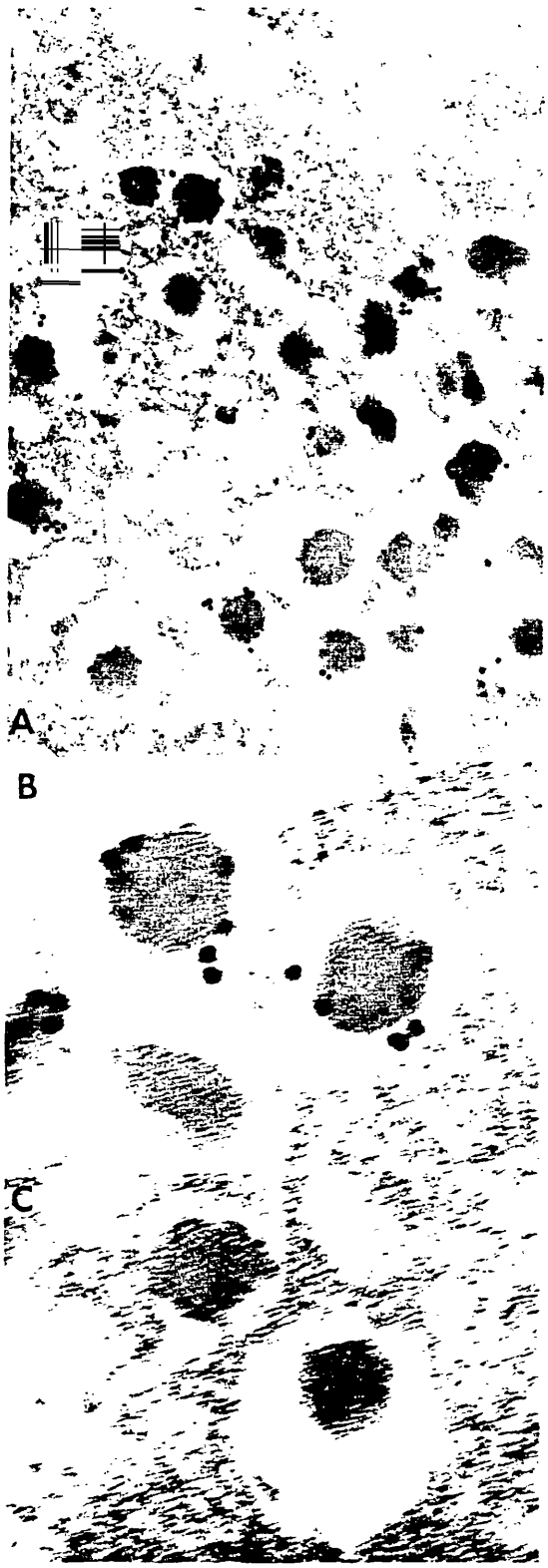


FIG. 6. Insulinoma cell showing pro-insulin C-peptide immunoreactivity within β-granules. a, ×51,000; b, immunoreactive granules with crystalline core, ×106,250, and c, non-immunoreactive homogeneous core granules, ×106,250.

nm), electron-dense granules characteristic of both chromaffin cell types of the adrenal medulla (Fig. 10) of several mammalian species, including man. Enkephalin-like immunoreactivity has also been localised to the pleiomorphic granules characteristic of phaeochromocytoma cells.

DISCUSSION

For many years the peroxidase-antiperoxidase method, modified from Sternberger [40], has been the technique of choice for the immunocytochemical localisation of tissue-bound antigens. The standard procedure involves the formation of several immunological complexes and the peroxidase-mediated deposition of an amorphous, electron-opaque oxidation product of 3,3′-diaminobenzidine (DAB), which in the presence of osmium tetroxide undergoes cyclic polymerisation to the electron-dense osmium black [23]. Apart from the toxicity of DAB, one drawback of the procedure is that the fine structure of the immunostained organelles is largely obliterated by the homogeneous deposition of final reaction product. Furthermore, conventional counterstaining with heavy metal salts cannot be performed profitably on the immunostained sections which prejudices the precise interpretation of tissue morphology.

We report here the application of two immunogold staining methods for the light and electron microscopic localisation of peptide hormones and neurotransmitters. Both the staphylococcal protein A- and the immunoglobulin-conjugated gold methods have considerable advantages over the alternative techniques in that they are composed of fewer immunological steps with less toxic reagents which are easy and cheap to prepare. Antigenic sites are not obliterated, therefore organelle ultrastructure is observable and, in addition, conventional counterstaining can be performed on colloidal gold immunostained sections. These factors have proved to be crucial when the topographic distribution of two distinct molecular forms of one peptide within a single granule, for example glucagon and glicentin (proglucagon) in pancreatic α-granules, are considered. The core of the α-granule, now shown by immunogold staining to contain glucagon-like immunoreactive material, is distinguishable by conventional uranyl acetate and lead citrate staining from the outer portion by a marginally greater affinity for these heavy metal salts. Conventional ultrastructural morphology in combination with immunocytochemical staining for specific peptide molecules has also proved instrumental in the investigation of p-type neurone heterogeneity. Co-existence of amines and peptides within single granules from catecholamine-containing cells of the carotid body and adrenal medulla has been demonstrated using the immunogold technique. In this context it is expedient to introduce one further advantage of the immunogold staining methods which is the development of double- and multiple staining methods at the electron microscope level utilising the different sizes of gold particles available. At present there are some technical problems to be overcome before two or more antigenic sites within one cell, or even one granule, can be visualised. The potential applications of double-labelling techniques are limitless.

To date, most regulatory peptides against which specific antibodies have been raised, have been visualised with the on-grid immunogold staining procedures; the localisation of heat-labile peptides, for example VIP, requiring only minor modifications to the preparative procedure (ultraviolet-cured resin, [29]).

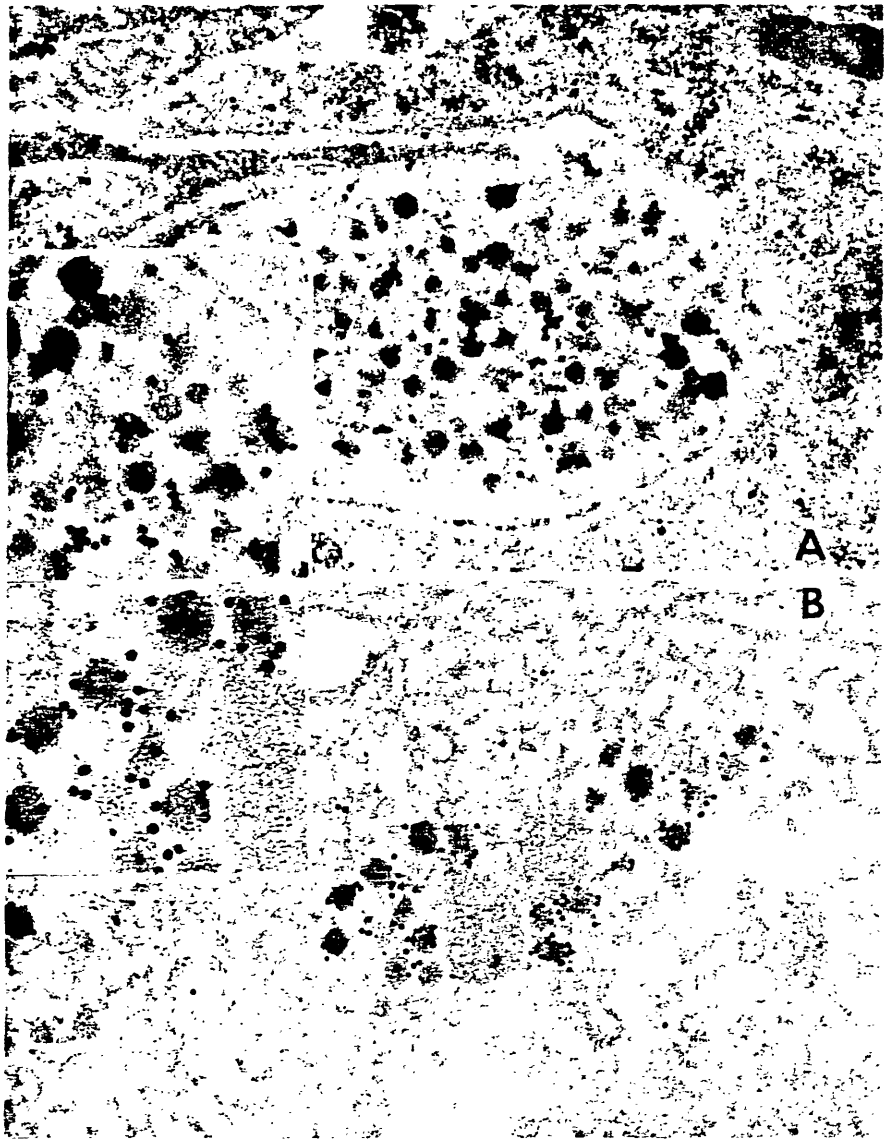


FIG. 7. Ultrathin sections of guinea-pig myenteric plexus showing nerve terminals a, immunostained with Substance P, ×50,000; inset ×75,000, b, immunostained with VIP, ×50,000; inset ×75,000. Note the specific gold labelling over the large, granular, p-type vesicles present in the nerve terminals shown.

By far the most important advantage of the immunogold staining procedures over the alternative techniques is the suitability of punctate labelling for quantification purposes. Ratios of granular immunoreactive sites to background sites can now be calculated by simple point-counting techniques and expressed in statistical terms; thus subjective criteria need not be used when describing the specificity of immunoreactions.

The application of immunogold staining methods at the light microscope level [15] have demonstrated the advantages of these techniques in double- and multiple staining procedures using bright field illumination. A new application for the colloidal gold methods, based on the observation of immunostained semi-thin sections by dark-ground illumination, has provided a very important link between light and electron microscope immunocytochemistry and further illustrates the advantages of gold-labelling techniques.

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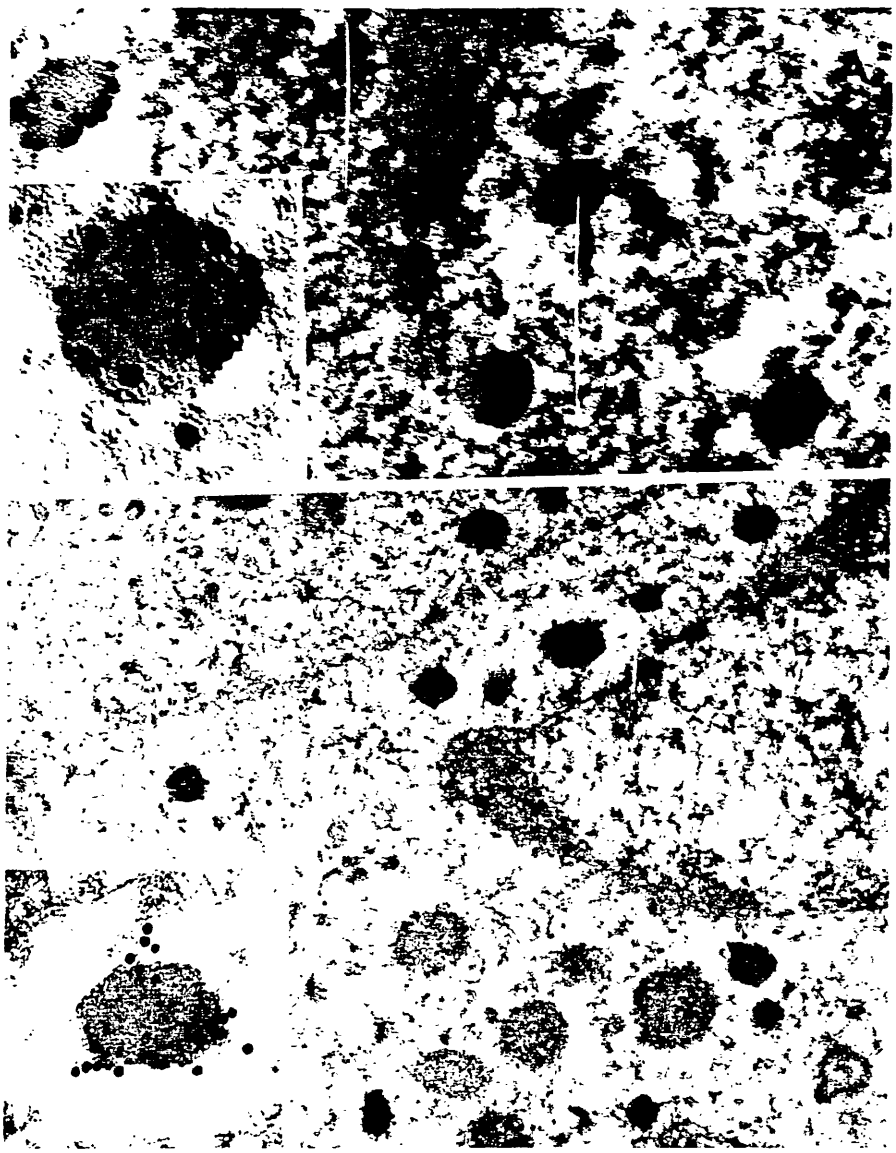


FIG. 8. a. VIP-immunoreactive granules in a pancreatic diarrhoeogenic tumour. ×56,000; inset, ×200,000. b. PP-immunoreactive granules in the same tumour. ×36,000; inset ×128,000.

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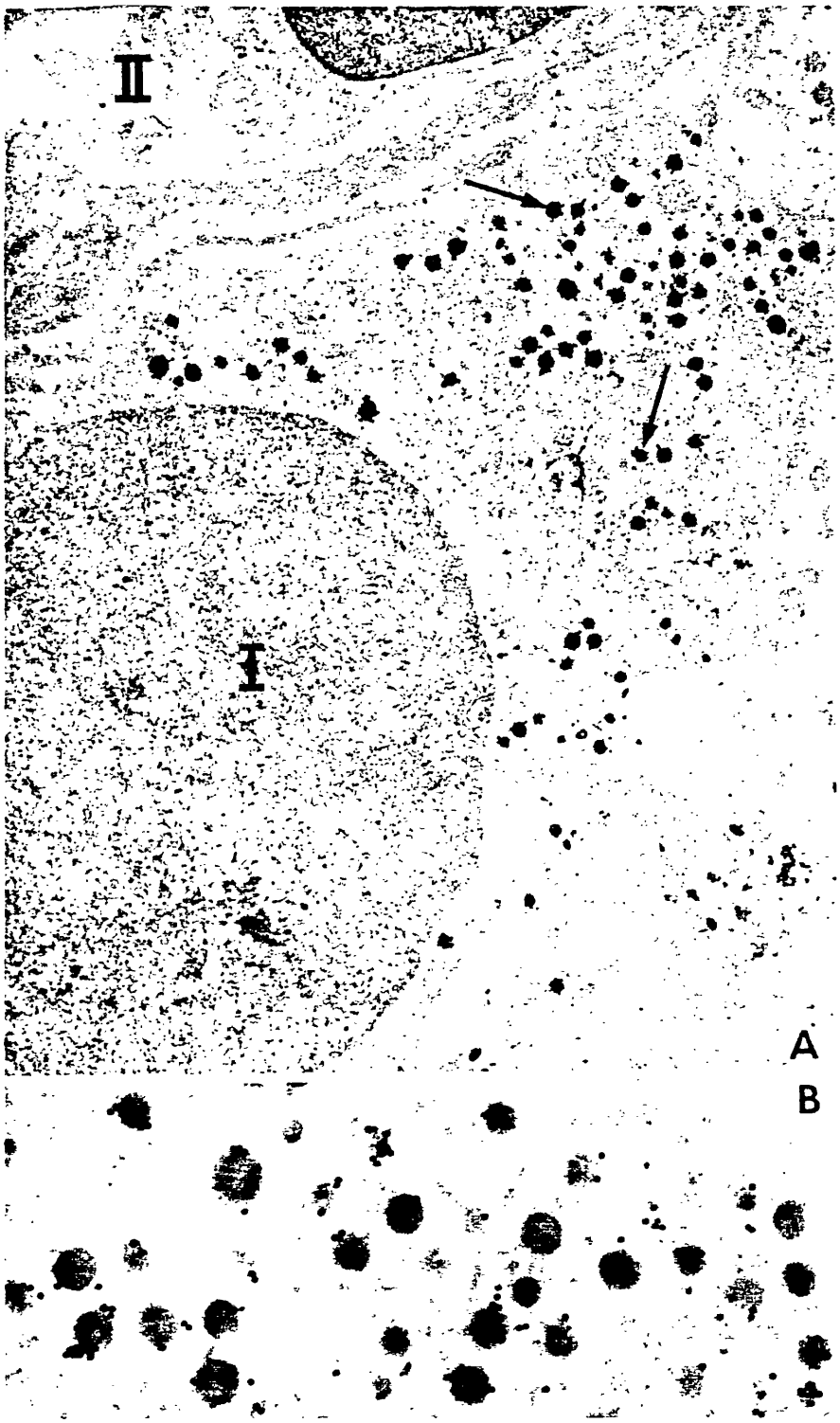


FIG. 9. a. Met-enkephalin-like immunoreactivity localised to electron-dense granules (arrows) in a type I cell (I) of the cat carotid body. Part of a type II glomus cell (II) is visible in the upper half of the micrograph. ×15,000. b. Higher magnification of the cat carotid body type I cell granules immunoreactive to met-enkephalin. ×46,000.

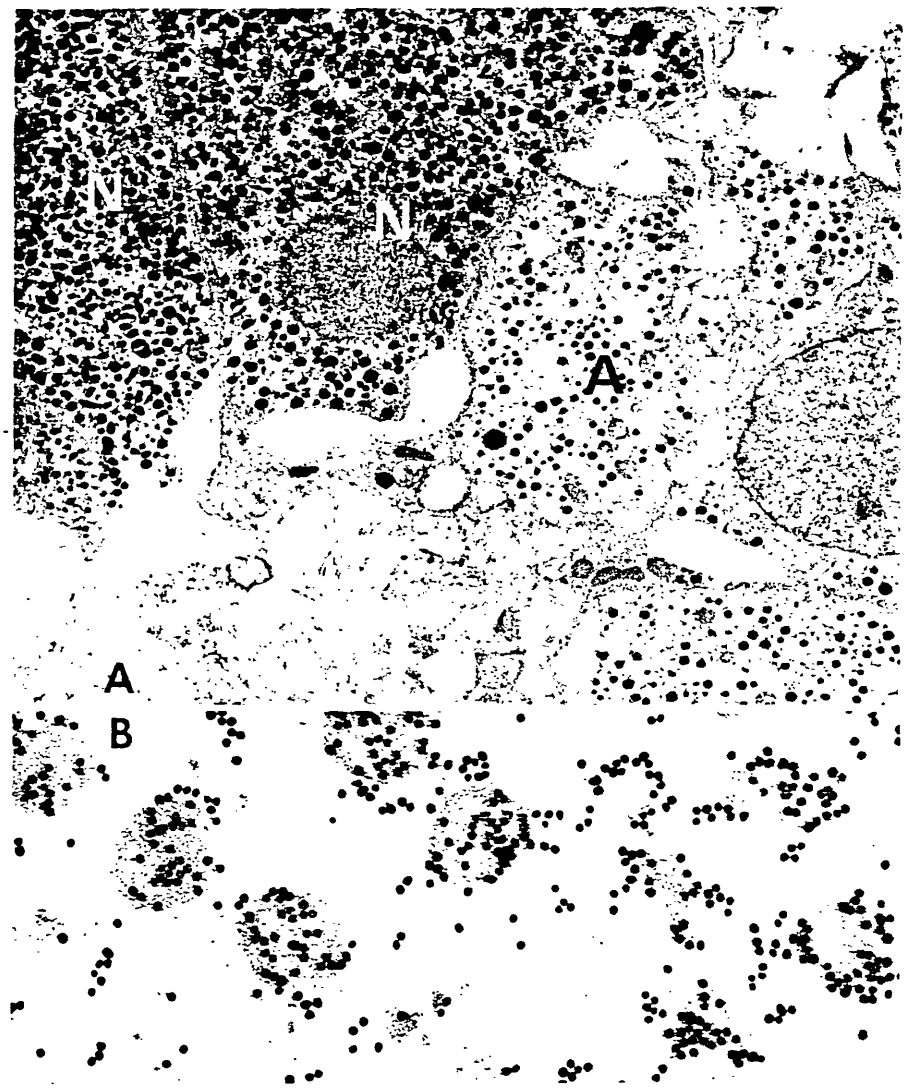


FIG. 10. a. The electron-dense granules of both (A, adrenergic; N, noradrenergic) chromaffin cell types of the horse adrenal medulla are immunostained for met-enkephalin using the immunogold technique. ×7,000. b. Higher magnification of a. ×75,000.

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