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Occurrence, distribution and origin of peptide-containing nerves of guinea-pig and rat male genitalia and the effects of denervation on sperm characteristics*

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INTRODUCTION

The pelvic organs receive nerve fibres from the sympathetic and parasympathetic pathways. Sensory nerves accompany both types of motor fibre. Since the work of Langley & Anderson (1896), it has been accepted that the parasympathetic innervation is derived from the visceral sacral outflow, via the pelvic nerves, and that the sympathetic fibres reach the pelvic organs by way of the hypogastric nerves, which arise from the inferior mesenteric ganglion. Unlike the classical concept of sympathetic innervation, the sympathetic supply to the ductus (vas) deferens and the accessory genital glands was shown to be innervated chiefly by 'short' adrenergic neurons, located close to the target organs. Thus, the hypogastric nerve contains, in addition to the sensory fibres, mainly presynaptic motor fibres, ending in nerve cells in the vicinity of these organs (Sjöstrand, 1965). The motor nerve supply to the testis is derived mainly from long postganglionic fibres from the superior spermatic nerves or spermatic plexus, which arise from the superior mesenteric ganglion and accompany the testicular artery (Hodson, 1970).

More recently, autonomic nerves, using regulatory peptides as their presumed neurotransmitters or modulators, have been found in several organs. A number of them have also been demonstrated in the mammalian male genitalia, by means of both immunohistochemistry and radio-immunoassay. Vasoactive intestinal polypeptide (VIP) was observed in the reproductive system of several species, including man (Alm, Alumets, Hakanson & Sundler, 1977; Alm *et al.* 1978; Larsson, Fahrenkrug & Schaffalitzky de Muckadell, 1977; Vaalasti, Linnoila & Hervonen, 1980; Polak, Gu, Mina & Bloom, 1981; Gu *et al.* 1983*b*).Enkephalin-immunoreactive nerve fibres (Vaalasti *et al.* 1980), substance P (Alm *et al.* 1978; Gu *et al.* 1983*a*), avian pancreatic polypeptide (Gu *et al.* 1983*b*), neuropeptide Y (NPY) (Adrian *et al.* 1984) and somatostatin (Gu *et al.* 1983*b*) have also been reported in the male genitalia of man and other mammals. Although several neuropeptides have been demonstrated in the male genitalia, a systematic survey of the nature of the peptidergic innervation has not been presented hitherto.

In the present work, the occurrence, distribution and origin of five peptides (VIP,

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peptide histidine isoleucine (PHI), neuropeptide tyrosine (NPY), calcitonin gene-related peptide (CGRP) and substance P) were investigated in guinea-pig and rat male genitalia, by means of indirect immunofluorescence. In addition, radioimmunoassay was used to measure the concentrations of VIP, substance P and CGRP. The densities and distribution patterns of the peptidergic nerves were compared with those of the noradrenergic nerves, the latter being revealed by means of immunostaining dopamine-β-hydroxylase (DβH) and tyrosine hydroxylase (TH), two enzymes associated with catecholamine synthesis. Antibodies to a newly recognised neuronal marker, neurofilament protein (NF) (Bishop *et al.* 1985), were also used in an attempt to reveal all the nerves, including the sensory and cholinergic components, supplying the male genitalia.

In order to investigate the origin of peptidergic nerves in the genital organs, a variety of denervation procedures was carried out, and the postoperative changes which occurred in the peptidergic, as well as in the noradrenergic innervation, were examined. The experimental design was based on the current view of pelvic organ innervation presented above and included decentralisation by section of pelvic or hypogastric nerves, as well as chemical sympathectomy with guanethidine (Burnstock *et al.* 1971). As decentralisation/denervation interferes with the nerve-mediated contractions of the male genital ducts and glands, impairing seminal emission (Hodson 1964, 1965), possible changes in the spermatozoal motility and morphology were also investigated in guinea-pigs and rats treated in these ways.

MATERIALS AND METHODS

Surgical decentralisation

Cutting of the hypogastric or pelvic nerves causes destruction of the peripheral end of the sensory and preganglionic autonomic fibres (decentralisation); the postganglionic fibres would be morphologically unaffected. Selective surgical decentralisation of the genital system was carried out on sexually mature male guinea-pigs, of about 700 g body weight. The animals were deprived of food, but not water, 6 hours prior to operation, and were anaesthetised by Fentanyl fluorisone (Hypnorm, JANSSEN), 1 ml/kg body weight, intramuscularly, and Diazepam (Weddel Pharmaceuticals Ltd), 2·5 mg/kg body weight, intraperitoneally. Full surgical aseptic procedures were followed.

At operation, the hypogastric nerves and the inferior mesenteric ganglion were located as described by Cross & Glover (1958). The pelvic nerve was located on the lateral surface of the rectum and observed to join the pelvic plexus. Stimulation of this nerve always caused contraction of the bladder.

In 5 animals, 1 cm of both hypogastric nerves was removed by lens scissors, and, in another 5 animals, 1 cm of one pelvic nerve was removed. Sham operations were performed on 3 animals. Normal controls consisted of 10 unoperated guinea-pigs. The incisions were sutured and 5 mg of Terramycin was injected intramuscularly into all animals. Diprenorphine (Revivon, C-VET Ltd), 0·5 mg, was then given intraperitoneally and the animals kept warm until fully recovered. The animals were killed one week postoperatively with an overdose of sodium pentobarbitone (Nembutal, Ceva Ltd).

Chemical sympathectomy

Five adult male rats were injected with guanethidine (Ismelin, CIBA), 10 mg/kg body weight/day, intraperitoneally, for four weeks and then killed by an overdose

Table 1. *Characterisation of antisera for immunohistochemistry and radio-immunoassay*

Antisera to	Antigen molecule		Dilution for		Sensitivity (95% confidence limits) (RIA) (fmol/tube)
	IC	RIA	IC	RIA (final	
Natural porcine NPY	C-Terminal		1/400	—	—
Natural porcine VIP	Whole molecule		1/2000	1/480000	0·3
Synthetic porcine PHI	C-Terminal		1/400	—	—
Synthetic substance P	C-Terminal		1/500	1/8000	0·3
Synthetic rat CGRP	Whole molecule		1/200	1/400000	2·0
Bovine DβH	Whole molecule	—	1/200	—	—
Rat TH	Whole molecule	—	1/400	—	—
Chicken NF protein	150 and 200 KD subunits	—	1/400	—	—

IC, Immunocytochemistry; RIA, Radio-immunoassay.

of sodium pentobarbitone. Controls consisted of five animals injected with physiological saline.

Immunohistochemistry

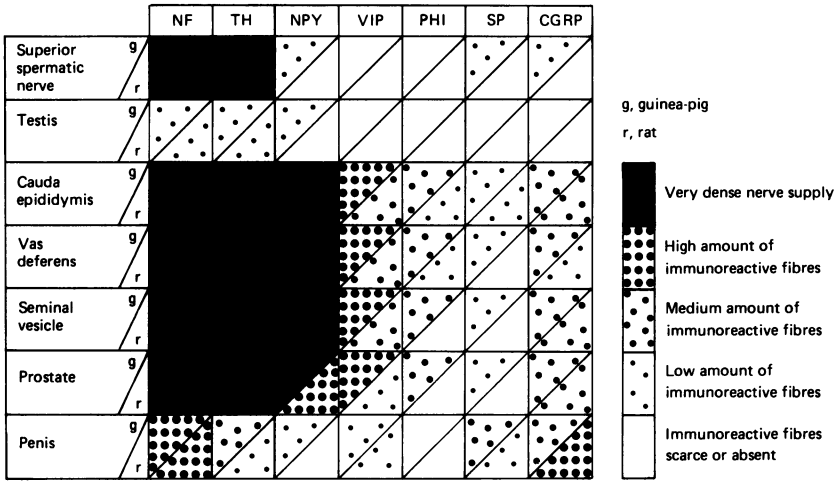
For immunohistochemistry, specimens from various parts of the genital system (testis, cauda epididymidis, vas deferens, prostate, seminal vesicle, testicular artery and vein, body of penis and glans) were dissected out and immediately fixed by immersion in 0·4% solution of benzoquinone in phosphate-buffered saline (PBS), pH 7·0, for 1·5 hours at room temperature, and then washed overnight in PBS containing 15% sucrose (w/v), at 4 °C. Tissue blocks were frozen in melting Arcton (ICI Ltd) and 10 μm thick cryostat sections cut. For light microscopic immunohistochemistry, a modified indirect immunofluorescence staining method was used (Gu, Islam & Polak, 1983).

The antisera for immunohistochemistry and radio-immunoassay were raised in New Zealand White rabbits against NPY, VIP, PHI, CGRP and substance P. Antisera to DβH, TH and NF proteins were used for immunohistochemistry and were kindly donated by Dr R. Rush (Flinders Medical Centre, Australia), Dr J. Thibault (Biochimie Cellulaire, Collège de France, Paris) and Dr D. Dahl (Spinal Cord Injury Research Unit, Massachusetts, USA), respectively. The characteristics of the antisera are shown in Table 1. For immunohistochemistry, the negative controls included the use of non-immune rabbit serum as first layer, omission of the first layer, and pre-absorption of the first layer antiserum with appropriate antigens (10 nmol/ml diluted antiserum).

Radio-immunoassay

For radio-immunoassay, representative pieces of the same animals that had been used for immunocytochemistry were taken from different regions of the genital system (testis, penis, epididymis, prostate, vas deferens and seminal vesicle). The tissues were extracted in boiling 0·5 M acetic acid (10 ml/g of tissue) and duplicate aliquots were then assayed in specific and sensitive radio-immunoassays for VIP, substance P and CGRP. The assays for VIP and substance P have been described previously (Bloom & Long, 1982; McGregor & Bloom, 1983) but brief details of the

Table 2. *Diagrammatic representation of the results of semi-quantitative assessment of the distribution and density of peptide-containing, noradrenergic (TH-immunoreactive) and NF-immunoreactive nerves in the normal guinea-pig and rat male genitalia*



antisera used are presented in Table 1. The cross-reactivity of the CGRP antiserum with related peptides such as substance P, VIP, gastrin-releasing peptide, somatostatin-28 and calcitonin was less than 0·02 %. Radio-iodinated rat CGRP was prepared by oxidative iodination (chloramine T) of the histidine residue followed by purification of HPLC. Assays were set up in sodium phosphate (0·05 M) buffer containing 3 % bovine serum albumin. Following 5–7 days incubation, the antibody-bound and free CGRP were separated by the addition to each assay tube of 8 mg activated charcoal. Differences between control and test groups were determined using an unpaired Student's *t*-test.

Sperm evaluation

Guinea-pig and rat spermatozoa were collected directly from the vas deferens and adjacent cauda epididymidis immediately after death. The ducts were compressed with forceps and the contents collected into approximately 1 ml of pre-warmed PBS, pH 7·0. To evaluate sperm motility, a drop of the fluid was placed on a warm microscope slide and immediately examined with a low power microscope objective. A subjective assessment of the proportion of motile spermatozoa and the quality of motility (0: immobile – 4: excellent progressive motility) was recorded (Emmens, 1947). In order to investigate sperm morphology, a drop of diluted semen was smeared on a pre-warmed slide, dried and fixed by immersion in buffered formol saline and stained in buffered Giemsa solution (Watson, 1975). The percentage of normal acrosomes was counted in 100 spermatozoa selected at random on each slide. A spermatozoon was considered normal if the acrosome was present, evenly stained, and had a regular smooth anterior margin. The degree of maturity of rat spermatozoa was evaluated from the percentage of spermatozoa retaining the cytoplasmic droplet (Dott & Dingle, 1968; Mann, 1975), also calculated for 100 sperm

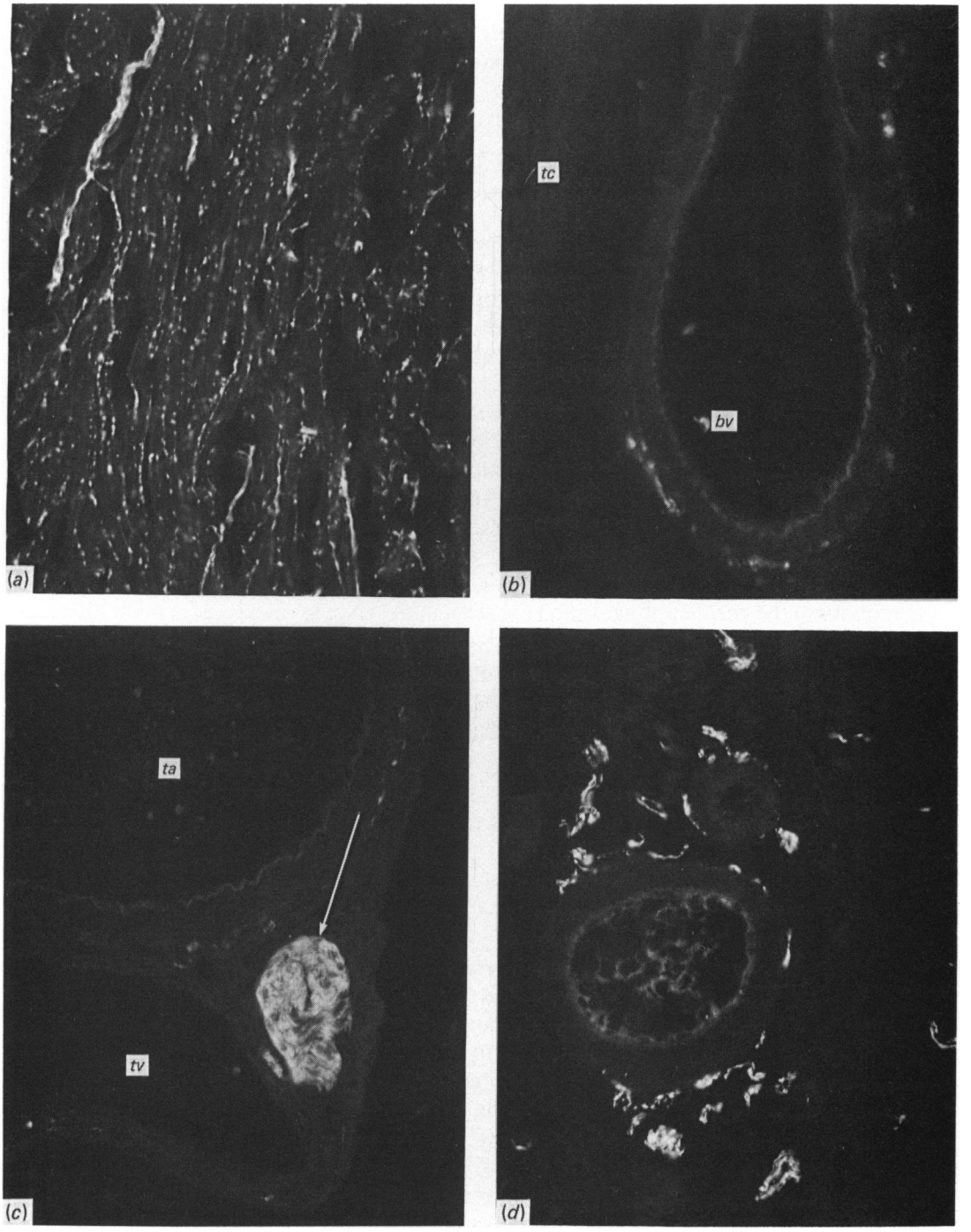


Fig. 1 (*a–d*). (*a*) Smooth layer of rat vas deferens, densely innervated by noradrenergic nerves (TH-immunoreactive) × 220. (*b*) Noradrenergic nerves (DβH-immunoreactive) surrounding a subcapsular blood vessel (*bv*) in the guinea-pig testis. *tc*, testicular capsule. × 450. (*c*) A cross section through guinea-pig superior spermatic nerve (arrow), showing also the testicular artery (*ta*) and vein (*tv*). The great majority of the fibres in the superior spermatic nerve appear to be immunoreactive to TH antiserum. × 220. (*d*) TH-containing nerves in the capsule of the corpus spongiosum, in the guinea-pig penis. × 270.

cells selected at random on each slide. Both guinea-pig and rat slides were randomised before evaluation, to eliminate observer bias. Differences between control and treated groups were examined using an unpaired Student's *t*-test.

RESULTS

Normal tissues

The results of the study of normal tissues are summarised in Table 2.

Noradrenergic nerves

Noradrenergic nerves, revealed by antibodies raised against DβH or TH, were the most abundant nerve type in the guinea-pig and rat male genitalia and showed a distribution pattern similar to that described previously (Hodson 1965, 1970). They were mainly distributed in the smooth muscle and submucosa of the vas deferens (Fig. 1*a*), cauda epididymidis, seminal vesicle and prostate. In the serous coat, as well as in the interstitial tissue of the epididymis and prostate, thick immunoreactive bundles of nerves were seen and some fibres also occurred around blood vessels. Noradrenergic nerves were found occasionally within the testicular capsule and surrounding capsular blood vessels (Fig. 1*b*). All the fibres in the superior spermatic nerves appeared to be positive for TH (Fig. 1*c*). Some noradrenergic nerves were also seen surrounding the testicular artery and vein, as well as small blood vessels in the spermatic cord. In the guinea-pig penis, they were mainly observed in the capsule surrounding the corpus spongiosum (Fig. 1*d*), in the erectile tissue and around blood vessels.

Peptidergic nerves

Immunohistochemistry

Van deferens, cauda epididymidis, seminal vesicle, prostrate

NPY-, VIP-, PHI-, CGRP- and substance P-immunoreactive nerves occurred, in different amounts, in the smooth muscle and submucosa of the vas deferens, cauda epididymidis (Fig. 2*a*), seminal vesicle (Fig. 2*b*) and prostate. Thin bundles of immunoreactive nerves were also observed in the interstitial tissue and serous coat (Fig. 2*c*, *d*). NPY- and VIP-containing fibres were found surrounding blood vessels in these organs. The NPY nerve supply was the major peptide-containing neuronal component in guinea-pig and rat vas deferens and accessory sexual glands, showing a similar distribution to noradrenergic nerves in most of the male genital tissues. A characteristic distribution pattern of PHI and VIP nerves was that they were observed mainly within the inner circular muscle layer, in the vas deferens and seminal vesicle. The former, however, were fewer in number and exhibited generally a weaker fluorescence. Except for NPY, the peptidergic nerves were revealed to be less numerous in rat than in guinea-pig internal genital ducts and glands.

Testis

Peptidergic nerves were found to be few in the testis and testicular blood vessels. NPY-immunoreactive fibres were found occasionally in guinea-pig and rat testicular capsule, surrounding subcapsular blood vessels (Fig. 3*a*) and also around the testicular artery, in the spermatic cord (Fig. 3*b*). VIP-, CGRP- and substance P-containing nerves were seen rarely within guinea-pig testicular capsule. In addition, some CGRP- and substance P-containing fibres were seen in the superior spermatic nerve and in association with the testicular artery (Fig. 3*c*).

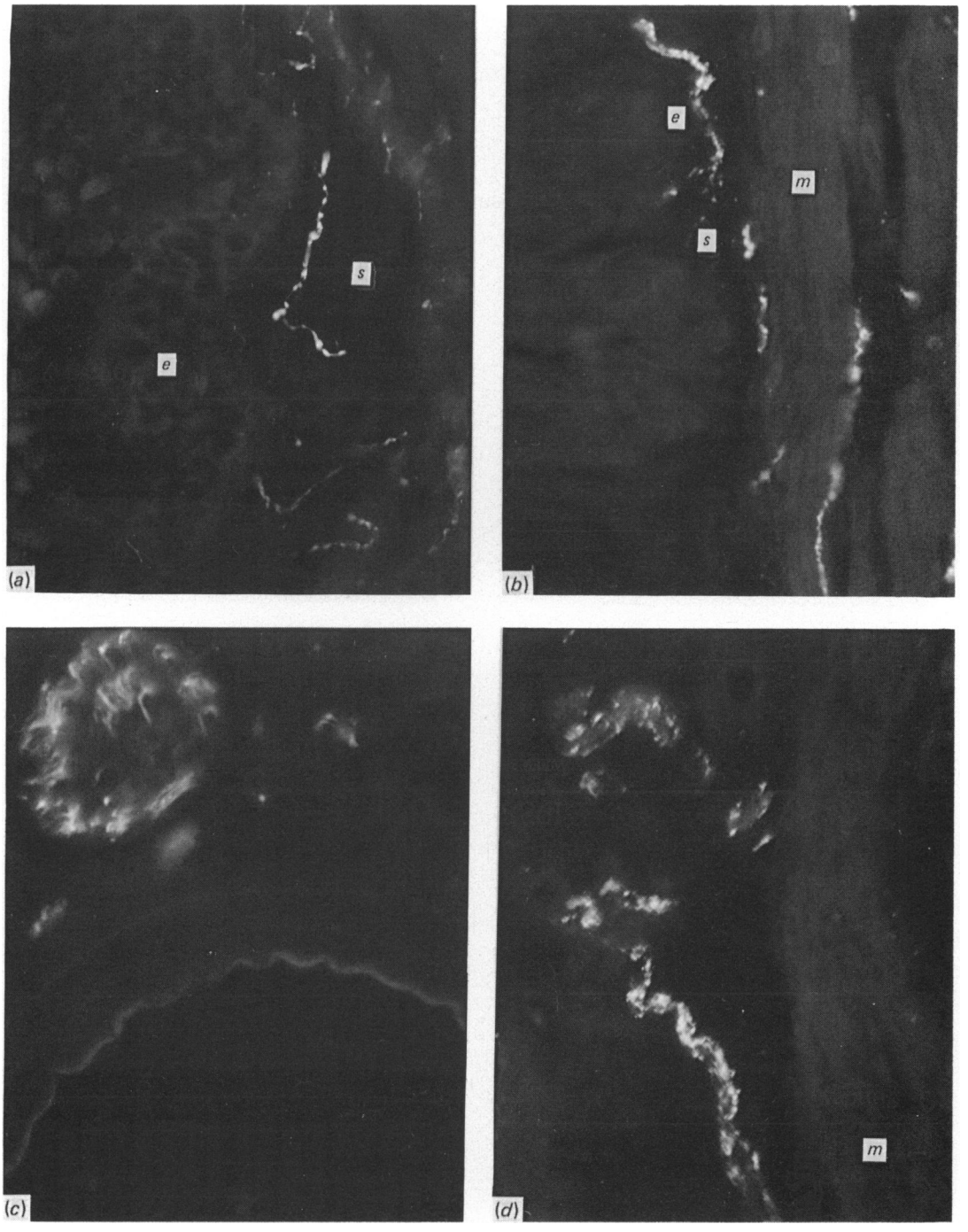


Fig. 2(*a–d*). (*a*) VIP-immunoreactive nerves in the submucosa (*s*) of guinea-pig epididymis. *e*, ductal epithelium. × 380. (*b*) PHI-containing nerves in the muscle layer (*m*) and submucosa (*s*) of guinea-pig seminal vesicle, some in the vicinity of the glandular epithelium (*e*). × 470. (*c*) A large bundle of nerves running along a blood vessel, in the serous coat of guinea-pig vas deferens. Many fibres are immunoreactive to CGRP antiserum. × 600. (*d*) CGRP-containing small bundles of nerves in the interstitial tissue of guinea-pig epididymis. *m*, ductal muscle layer. × 440.

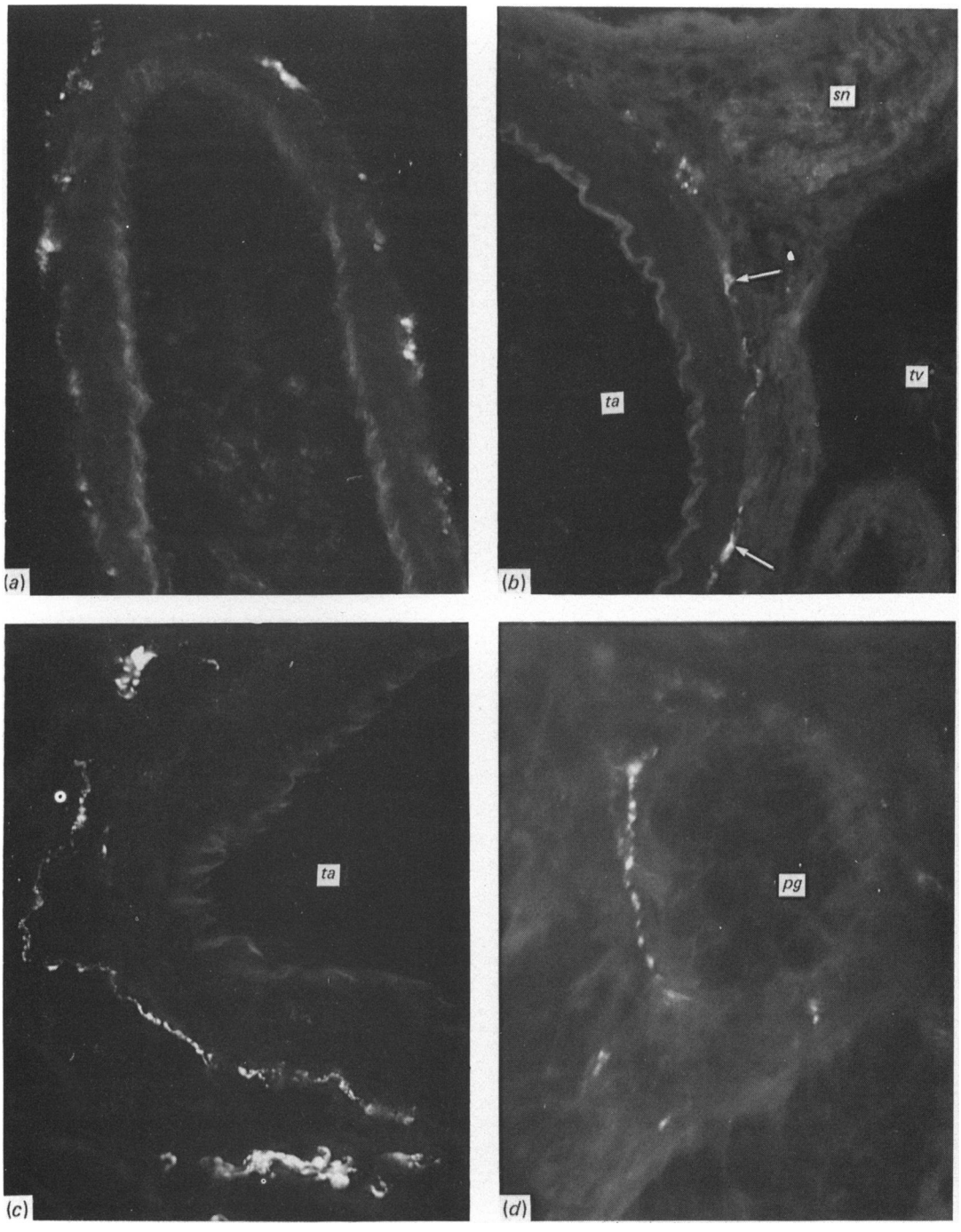


Fig. 3(*a–d*). NPY-positive nerve fibres: (*a*) Surrounding a subcapsular blood vessel in the rat testis. × 430. (*b*) Surrounding the guinea-pig testicular artery (*ta*) (arrows). The testicular vein (*tv*) and superior spermatic nerve (*sn*) show no immunoreactivity to NPY antiserum. × 360. (*c*) Substance P-containing nerves in close association with the guinea-pig testicular artery (*ta*). × 350. (*d*) CGRP-containing nerve fibres surrounding the penile para-urethral gland (*pg*) in the guinea-pig. × 520.

Table 3. *Peptide content (pmol/g, mean ± SEM) of genitalia of normal and operated male guinea-pigs*

Peptide	Area	Normal controls ($n = 10$)	Decentralisation	
			Hypogastric ($n = 5$)	Pelvic ($n = 5$)
CGRP	Testis	0·1	0·2	0·1
	Penis	11·2 ± 1·1	10·9 ± 1·1	9·5 ± 1·2
	Epididymis	15·9 ± 2·0	4·6 ± 0·6°°	3·0 ± 1·1†
	Prostate	21·4 ± 1·7	5·3 ± 1·4†	10·0 ± 3·1*
	Vas deferens	54·8 ± 5·2	23·9 ± 2·6°°	4·0 ± 2·6††
	Seminal vesicle	14·4 ± 2·7	0·26 ± 0·05††	5·4 ± 2·0
Substance P	Testis	1·5 ± 0·3	1·8 ± 0·3	1·4 ± 0·3
	Penis	1·3 ± 0·3	1·5 ± 0·1	1·6 ± 0·2
	Epididymis	4·3 ± 0·4	2·2 ± 0·4°	2·5 ± 0·5°
	Prostate	2·1 ± 0·2	1·2 ± 0·3	1·7 ± 1
	Vas deferens	6·4 ± 1·2	3·1 ± 0·3*	3·1 ± 0·4*
	Seminal vesicle	1·5 ± 0·2	1·2 ± 0·3	0·8 ± 0·5
VIP	Testis	1·8 ± 0·2	0·28 ± 0·02†	0·26 ± 0·03†
	Penis	2·7 ± 0·1	1·2 ± 0·2	1·0 ± 0·1
	Epididymis	3·9 ± 0·6	2·1 ± 0·1	2·7 ± 0·8
	Prostate	5·3 ± 0·5	3·4 ± 0·9	3·8 ± 0·2**
	Vas deferens	6·3 ± 0·6	6·7 ± 0·6	6·8 ± 0·8
	Seminal vesicle	4·7 ± 0·5	3·8 ± 0·5	3·6 ± 1·5

Statistically significant difference from controls: $P < 0·05$*; $P < 0·025$**; $P < 0·02$°; $P < 0·01$°°; $P < 0·005$†; $P < 0·001$††.

Table 4. *Peptide content (pmol/g, mean ± SEM) of genitalia of normal and chemically sympathectomised male rats*

Peptide	Area	Normal ($n = 5$)	Sympathectomised ($n = 5$)
CGRP	Testis	0·1	0·1
	Penis	2·7 ± 0·6	2·9 ± 0·7
	Epididymis	3·4 ± 0·9	3·0 ± 0·9
	Prostate	0·2 ± 0·1	0·8 ± 0·3
	Vas deferens	0·7 ± 0·2	6·3 ± 0·8**
	Seminal vesicle	0·3 ± 0·1	0·4 ± 0·1
VIP	Testis	0·3	0·3
	Penis	4·6 ± 0·2	4·5 ± 0·7
	Epididymis	4·1 ± 0·5	6·1 ± 0·4*
	Prostate	2·0 ± 0·2	1·8 ± 0·2
	Vas deferens	16·4 ± 2·4	22·3 ± 6·7
	Seminal vesicle	4·8 ± 0·4	7·0 ± 1·4

Statistically significant difference from control: $P < 0·05$*; $P < 0·005$**.

Penis

CGRP-immunoreactive nerves appeared to be the major peptide-containing neuronal component in the rat penis. They occurred in the erectile tissue and just beneath the urethral epithelium, but were particularly numerous in the dermis of the glans penis, some of them in close vicinity to the epidermal cells. Nerves containing CGRP displayed a similar distribution in guinea-pig penis, most of them being found around para-urethral glands (Fig. 3*d*) and glans dermis. Substance P nerves

also showed a similar distribution in the penis of both species, but were much less numerous. In the guinea-pig penis, some NPY-positive fibres were found in the erectile tissue, surrounding arteries and arterioles and also around para-urethral glands. They were detected only rarely in the rat penis. VIP-immunoreactive nerves were observed in the erectile tissue, beneath the urethral epithelium and para-urethral glands, as well as surrounding arteries and arterioles. PHI-containing nerve fibres were observed only rarely in the penis and, when found, were distributed in the corpus cavernosum erectile tissue and also surrounding blood vessels.

Radio-immunoassay

Radio-immunoassay of VIP, substance P and CGRP immunoreactivities in the male genitalia of guinea-pigs corroborated the morphological findings by detecting significant quantities of the peptides in most areas (Table 3). The lowest concentrations of peptides were found in the testis, where immunocytochemistry localised only very few peptidergic nerves, whereas the largest amounts were measured in the vas deferens.

In the rat, the levels of substance P were too low to detect but VIP and CGRP were found consistently in all areas except the testis, as in the guinea-pig (Table 4). Like the guinea-pig, the rat vas deferens contained the highest concentration of VIP but the epididymis and penis were the areas in the rat which contained the most CGRP.

Neurofilament protein (NF)

These results are summarised in Table 2. Neurofilament protein-immunoreactivity appeared to occur in the whole range of nerves in all the regions of guinea-pig and rat male genitalia, except, perhaps, in finest unmyelinated fibres. Neurofilament protein antiserum revealed dense innervation of the muscle layer and submucosa of vas deferens and sexual accessory glands. Thick bundles of nerves were also seen in the serous coat and interstitial tissue, in these organs. Some nerve fibres in the testicular capsule, and also surrounding subcapsular blood vessels, were immunoreactive to the NF antiserum. All the fibres in the superior spermatic nerves appeared to be NF-immunoreactive. In the penis, beside the nerves immunoreactive to DβH, TH and peptide antisera, the NF-immunostaining revealed also the presence of large bundles of nerves in the dermis and in the capsule around corpus cavernosum and corpus spongiosum. Although only a few fibres in these bundles of nerves showed immunoreactivity to TH and CGRP antisera, all of them appeared to be immunoreactive to NF.

No neuronal cell bodies in the genital system of either species were found to be reactive to any of the antisera employed. All the controls for immunohistochemistry were negatiive.

Hypogastric and pelvic decentralisation

Immunohistochemistry

Following hypogastric decentralisation in the guinea-pig, a considerable decrease of substance P-containing nerves was revealed in the vas deferens smooth muscle and submucosa (Fig. 4*a*, *b*). Hypogastric decentralisation resulted also in a marked decrease of VIP, NPY and PHI nerve supply in the seminal vesicle (Fig. 5*a–d*).

Unilateral transection of the pelvic pathway, in the guinea-pig, resulted in a subjectively assessed decrease of VIP, PHI, substance P (Fig. 4*c*, *d*) and CGRP nerve supply in the ipsilateral vas deferens and cauda epididymidis.

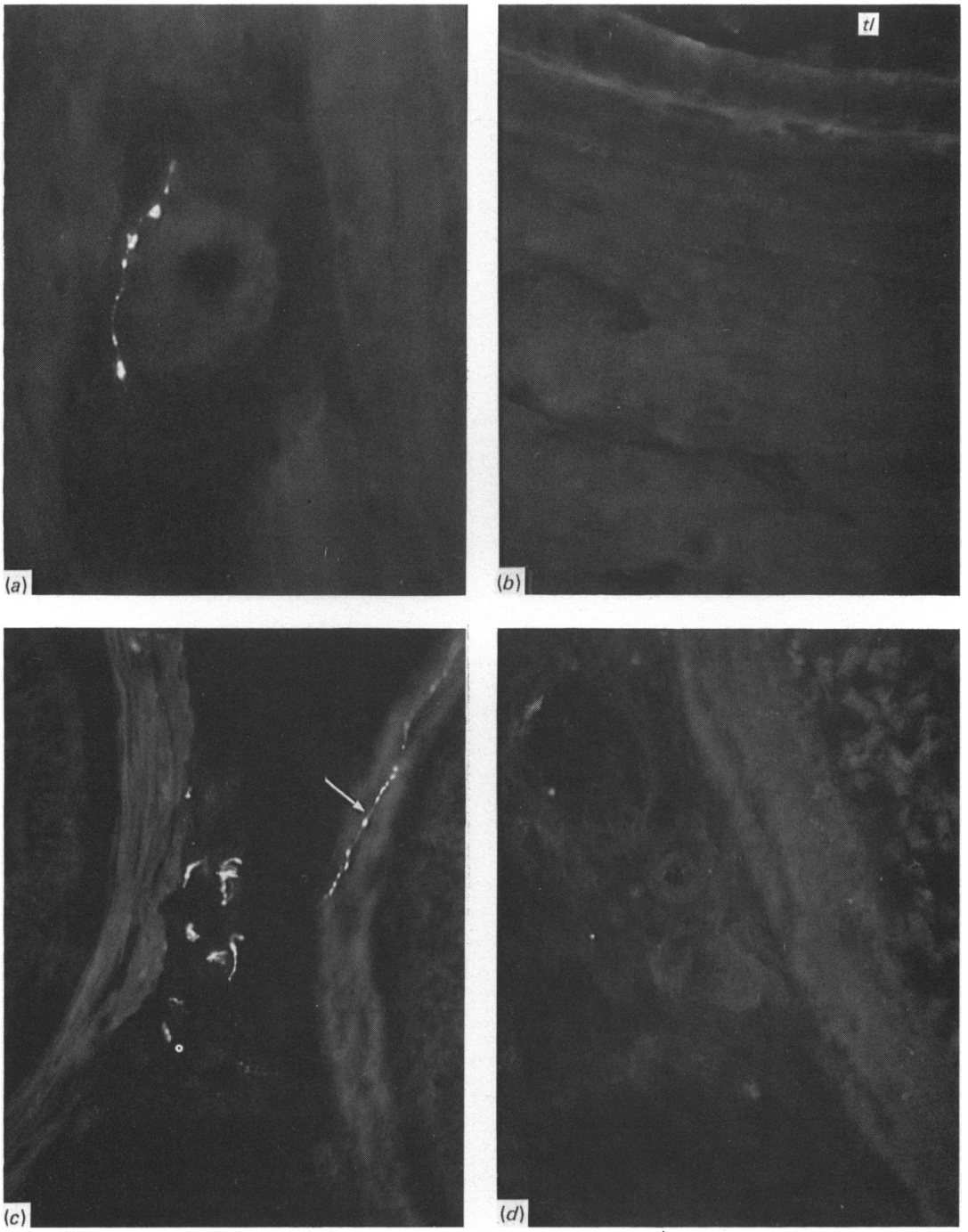


Fig. 4(*a–d*). (*a*) A varicose, substance P-containing, nerve related to a small blood vessel, in the vas deferens of a control guinea-pig. ×570. (*b*) Substance P-immunoreactive nerves are abolished in the tissue of the vas deferens from a hypogastric decentralised guinea-pig. *tl*, tubular lumen. ×570. (*c*) A small number of fine substance P-positive nerve fibres in the interstitial tissue and muscle layer (arrow) of epididymis from a control guinea-pig. ×240. (*d*) Negative immunoreaction to substance P antiserum in the interstitial tissue and muscle layer of epididymis from a pelvic decentralised guinea-pig. ×240.

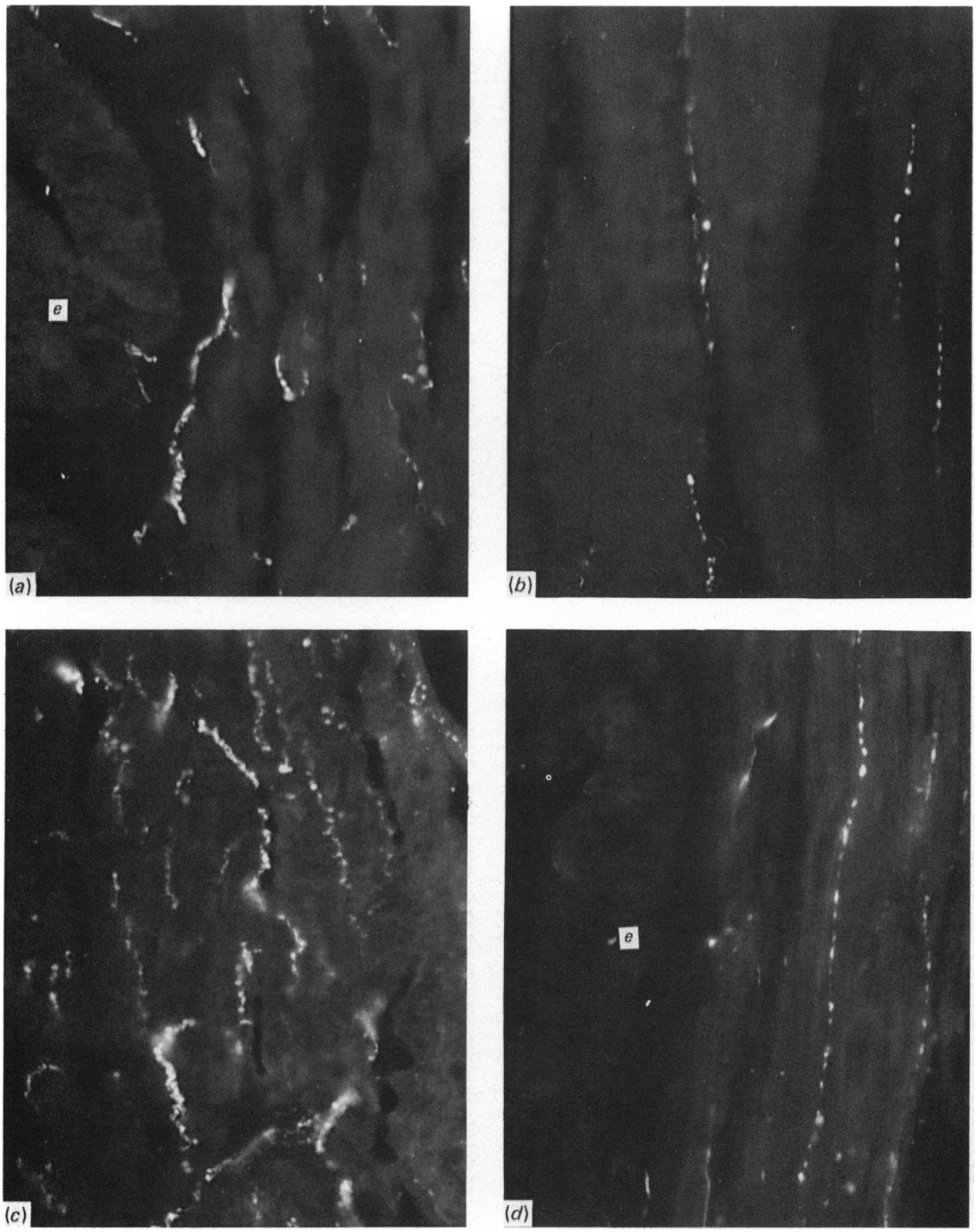


Fig. 5(*a–d*). (*a*) VIP-containing nerves in the smooth muscle and submucosa of the seminal vesicle from a control guinea-pig. *e*, glandular epithelium. × 420. (*b*) VIP-immunoreactive nerves in the seminal vesicle smooth muscle from a hypogastric decentralised guinea-pig. × 420. (*c*) Smooth muscle of the seminal vesicle from a control guinea-pig, densely innervated by NPY-containing nerves. × 420. (*d*) Only a small number of fine nerve fibres in the seminal vesicle smooth muscle from a hypogastric decentralised guinea-pig are immunoreactive to NPY antiserum. *e*, glandular epithelium. × 420.

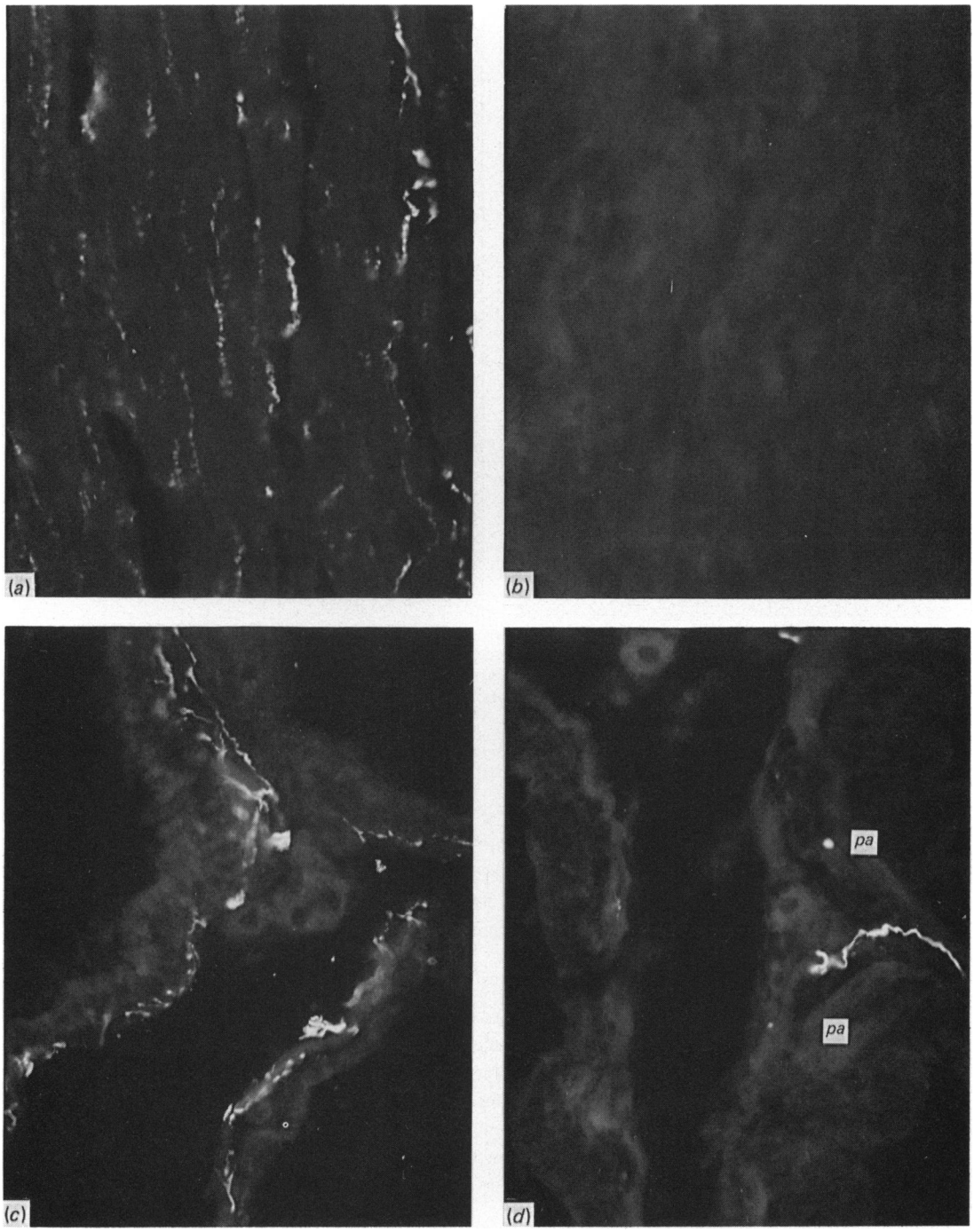


Fig. 6(*a–d*). (*a*) NPY nerve supply in the muscle layer of the seminal vesicle from a control rat. × 390. (*b*) Absence of NPY-immunoreactive fibres in the seminal vesicle smooth muscle from a guanethidine injected rat. × 390. (*c*) Noradrenergic nerves, immunoreactive to TH antiserum, in the prostate from a control rat. × 250. (*d*) Only a small number of noradrenergic nerves remain in the epididymis of the guanethidine-injected rats. *pa*, prostatic acini. × 250.

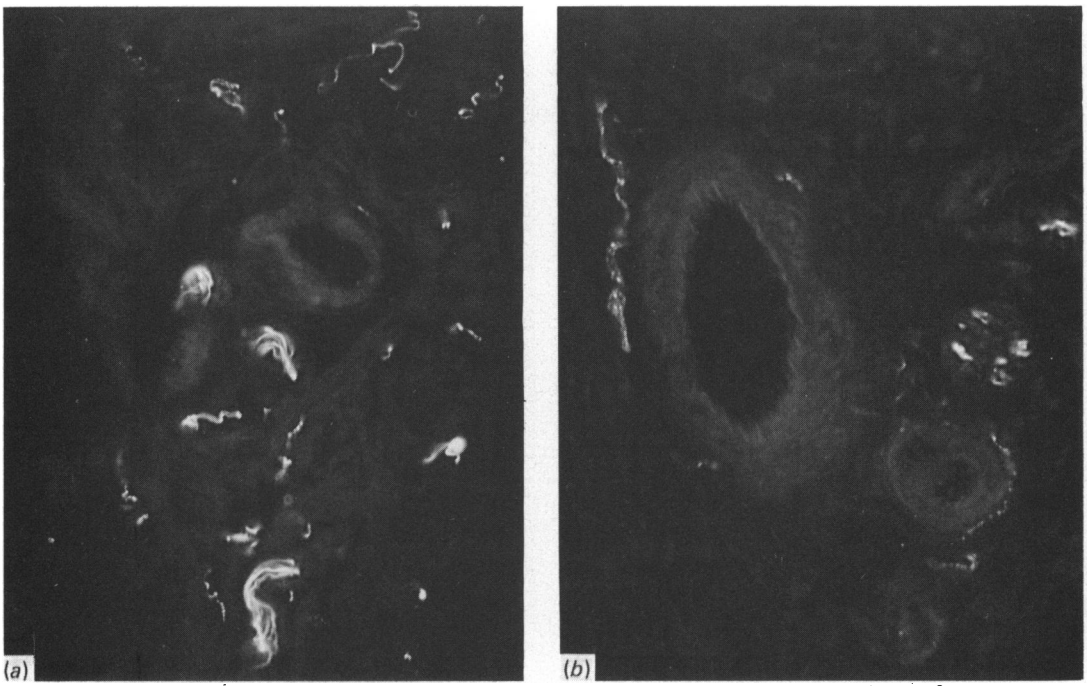


Fig. 7(*a–b*). (*a*) CGRP-containing nerves in the erectile tissue of corpus spongiosum from a control rat penis. ×240. (*b*) CGRP-immunoreactive nerve fibres in association with blood vessels in the erectile tissue of corpus spongiosum from a guanethidine-injected rat. ×240. Note a decrease in the number of CGRP-containing nerves when compared with the normal level.

Radio-immunoassay

The loss of substance P-immunoreactive nerves from the vas deferens, visualised by immunocytochemistry after hypogastric decentralisation, was reflected by a decrease in substance P content (Table 3). However, radio-immunoassay also revealed a loss of CGRP from the epididymis, prostate and vas deferens (Table 3). These changes in peptide content, which were all statistically significant, were not apparent by immunohistochemistry.

Pelvic transection also affected the concentrations of peptides in certain areas. There was again a significant reduction in the quantities of CGRP in the cauda epididymidis and vas deferens (Table 3) as was shown by immunohistochemistry. The loss of immunostained substance P nerves from the vas deferens and cauda epididymidis was corroborated by a fall in their peptide content (Table 3). On the other hand, the reduction in VIP innervation in vas deferens and epididymis shown by the morphological study did not result in a significant difference in VIP content (Table 3).

Chemical sympathectomy with guanethidine

Immunohistochemistry

Chronic treatment with guanethidine markedly reduced, or even completely abolished, the noradrenergic and NPY-containing nerve supply in the rat vas deferens, cauda epididymidis, seminal vesicle and prostate (Fig. 6*a–d*). A marked increase in the density of substance P and CGRP nerves was observed subjectively,

Table 5. *Guinea-pig sperm characteristics*

Sperm characteristics	Control ($n = 5$)	Hypogastric decentralised ($n = 5$)	Pelvic decentralised	
			Contralateral ($n = 5$)	Ipsilateral ($n = 5$)
% of motile spermatozoa	53·3±5·8	50·0±10·9	64·0±13·4	50·0±7·1
Degree of motility	1·8±0·6	1·6±1·1	2·3±0·7	1·8±0·3
% of normal acrosomes	41·0±28·0	38·4±20·9	39·7±19·2	41·8±13·4

Results are expressed as mean ±S.E.M.

Table 6. *Rat sperm characteristics*

Sperm characteristics	Control ($n = 5$)	Guanethidine-injected ($n = 5$)
% of motile spermatozoa	19·0±6·6	26·0±12·0
Degree of sperm motility	1·8±0·2	2·2±0·4
Percentage of cytoplasmic droplets	39·4±8·7	25·2±5·2*

* Significantly different from the control group ($P < 0·05$).
Results are expressed as mean ±SEM.

particularly in the vas deferens. However, the penis of the sympathectomised rats exhibited a decrease of substance P- and CGRP-containing nerves (Fig. 7*a*, *b*).

Radio-immunoassay

The substance P content of the genitalia of rats with chemical sympathectomy was undetectable, as in the normal control animals. A significant rise of CGRP content was found by radio-immunoassay in the vas deferens of sympathectomised rats (Table 4). The increase of CGRP in treated animals corresponded to the marked proliferation of CGRP nerves shown by immunohistochemistry. However, no loss of CGRP was obvious in the penis, although, morphologically, there appeared to be fewer nerves. A small but statistically significant increase in VIP content was shown in the cauda epididymidis alone. This change was not obvious by subjective assessment of the immunohistochemical results.

Sperm evaluation

Sperm evaluation carried out on guinea-pigs revealed wide 'between animal' variations in sperm characteristics, but no significant difference between control and operated groups (Table 5).

Chemical sympathectomy in the rat had no significant effect on the percentage of motile spermatozoa or the degree of sperm motility. However, a decrease in the percentage of spermatozoa exhibiting cytoplasmic droplets was evident in these animals ($P < 0·05$) (Table 6).

DISCUSSION

Our results demonstrate that nerves containing VIP, NPY, CGRP, PHI and substance P are present in the guinea-pig and rat male genital system, and each of

them possesses a characteristic density and distribution pattern. Moreover, NPY-immunoreactive fibres appeared to be the major peptide-containing neuronal component in the male genital organs. Both our evidence and that of others (Alm *et al.* 1977, 1978; Larsson *et al.* 1977; Vaalasti *et al.* 1980; Polak *et al.* 1981; Gu *et al.* 1983*b*) suggest that VIP may participate in regulating smooth muscle activity, local blood flow and epithelial function in the male reproductive organs. Nerves containing PHI were found to be present with a distribution similar to that of the VIP-containing nerves but were less numerous and exhibited a weaker fluorescence. This finding is not surprising as both peptides may be co-produced from the same precursor molecule (Bloom *et al.* 1983; Itoh, Obata, Yanaihara & Okamoto, 1983), and have similar biological actions (Anagnostides *et al.* 1982; Brennan *et al.* 1982; Ghiglione *et al.* 1982). In addition, they have been shown to co-exist in intrinsic ganglion cells of porcine gut (Bishop *et al.* 1984).

NPY immunoreactivity has been described in both mouse (Allen *et al.* 1982) and human (Adrian *et al.* 1984) male genital system. In the present investigation, it was detected throughout the guinea-pig and rat male genitalia, and NPY-containing nerves were particularly abundant in the muscle layers of ducts and glands. NPY has been shown to inhibit contractions of vas deferens muscle, possibly acting as a neuromodulator of noradrenaline release at a presynaptic level (Allen *et al.* 1982). Moreover, the occurrence of NPY in a sub-population of adrenergic neurons of the superior cervical, stellate and coeliac ganglia has been reported (Lundberg *et al.* 1982). Our results revealed a marked decrease, not only in the noradrenergic but also in the NPY-containing nerves, in the vas deferens and sexual accessory glands of chemically sympathectomised rats, thus indicating that NPY most likely co-exists with the classical sympathetic neurotransmitter, noradrenaline, in the sympathetic nerve supply of the male genital system. The selectivity of guanethidine in damaging sympathetic neurons specifically was revealed by Heath & Burnstock (1977). Low doses of guanethidine, such as we have used in the present work, have been found to damage 'short' rather than 'long' noradrenergic neurons (Evans *et al.* 1972). Thus, it seems reasonable to assume, from our results, that not only the noradrenergic nerves, but also the NPY-containing nerves originate from 'short' sympathetic neurons supplying the internal male reproductive organs. It is interesting that the noradrenergic nerves of the rat penis remained unchanged, indicating that they are probably 'long' postganglionic fibres possibly carried in the pudendal nerve or accompanying blood vessels. In the penis, some NPY-immunoreactive nerves were found to be associated with both vascular and non-vascular smooth muscle. The vasoconstrictor activity of NPY (Lundberg *et al.* 1982) has been suggested as a contributor to the control of penile erection (Adrian *et al.* 1984), possibly antagonising the vasodilatory effect of VIP.

A new neuropeptide, CGRP, was identified by Rosenfeld *et al.* (1983) and observed in sensory and motor components of the central and peripheral nervous system, but its presence in the male genital system has not been hitherto presented. We have detected CGRP-containing nerves throughout guinea-pig and rat male genitalia and they are likely to be the most abundant peptidergic nerve in the rat penis. Although the function of these nerves in the male genitalia is not yet known, the presence of CGRP in sensory neurons of trigeminal and dorsal root ganglia (Rosenfeld *et al.* 1983; Gibson *et al.* 1984; Terenghi *et al.* 1985) indicates that the peptide may play a sensory neurotransmitter role.

Penile erection is brought about by the inhibition of the vasoconstrictor nor-

adrenergic fibres in the erectile tissue and/or inhibition of the noradrenergic fibres of the retractor penis muscle (Klinge & Sjöstrand, 1977*a*, *b*). These authors, by pharmacological methods, decided that this inhibition was caused by fibres which were not cholinergic. Physiological and morphological evidence has shown recently that VIP is involved in the nervous control of penile erection in primates and other large mammals (Polak *et al.* 1981; Virag *et al.* 1982; Gu *et al.* 1983*b*; Dixson, Kendrick, Blank & Bloom, 1984). However, the present study shows that VIP is found in relatively small amounts in the guinea-pig and rat penis, and gives the first demonstration of the occurrence of CGRP-containing nerves in the erectile tissue of any species. This may be morphological evidence for CGRP being involved in the non-adrenergic, non-cholinergic mechanisms of penile erection in the rat and guinea-pig, and possibly other species.

Nerves displaying substance P immunoreactivity were detected throughout guinea-pig and rat male genitalia. It has been suggested that substance P is a sensory neurostransmitter in the urogenital tract (Gu *et al.* 1983*a*, *b*) and in the dorsal horn of the spinal cord (Hökfelt, Kellerth, Nilsson & Pernow, 1975). It thus seems possible that, as for CGRP, some, if not all, of the substance P-containing nerves may subserve a sensory function.

The presence of neuropeptide-containing neuronal cell bodies in the male genital system has been reported rarely and has been restricted to human tissues (Polak *et al.* 1981; Gu *et al.* 1983*a*, *b*; Adrian *et al.* 1984). In the present work, no neuronal cell bodies were found, in guinea-pig and rat male genitalia, to be reactive to the antisera used. In the guinea-pig, at least, the autonomic nerves supplying the male genital organs have been shown to originate from the prostate-deferential plexus, lying close to the vas deferens/seminal vesicle/prostate junction (Coujard, 1954).

The testicular autonomic innervation of most mammals has been reported to be restricted to the testicular capsule and most superficial blood vessels, and, in addition to their sensory components, they are assumed to play a role in the control of testicular blood flow and temperature (for review see Hodson, 1970; Bell, 1972). We have observed that the adrenergic and peptidergic nerve supply to the rat and guinea-pig testis is very sparse and confined to the capsule and capsular blood vessels. Close similarity between peptidergic nerves innervating the testis and those present in the superior spermatic nerves and related to the spermatic artery was also observed, thus adding further evidence to the classical concepts regarding the testicular innervation (Hodson, 1970).

By sectioning the hypogastric or pelvic pathways it was thought that only the preganglionic sympathetic and parasympathetic axons and sensory nerves to the internal genitalia would be interrupted (Sjöstrand, 1965). It was found in this study that hypogastric decentralisation in the guinea-pig resulted in a marked decrease of substance P-immunoreactive nerves in the vas deferens and of NPY-, PHI- and VIP-containing nerves in the seminal vesicle. These morphological observations were supported by biochemical evidence for a reduction in substance P and VIP content. The findings indicate that, in addition to the sensory axons, at least some of the substance P, VIP, PHI and NPY postganglionic axons were interrupted by the operation. Disconnection of the pelvic pathway, on the other hand, promoted a decrease of VIP, PHI, substance P and CGRP nerves in the vas deferens and cauda epididymidis, indicating that the cell bodies for these fibres lie proximal to the lesion. Hypogastric and pelvic decentralisation has no other noticeable effect on the amount of noradrenergic and peptidergic nerves in the guinea-pig male internal genitalia, thus

indicating that most of the postganglionic fibres were not interrupted by the operations, and suggesting that they probably originate from local ganglia, the prostate-deferential ganglia (Coujard, 1954), or even from another source.

The innervation of the penis was unaffected by both operations so it would seem that the hypogastric nerve may be relatively unimportant in the innervation of the guinea-pig penis and that, as in other mammals, large numbers of sensory and sympathetic nerves reach the penis via the pudendal nerve (Langley & Anderson, 1896). After unilateral pelvic nerve excision one pelvic nerve remained intact, so that it is possible that fibres from this innervated both sides of the penis.

In the vas deferens of guanethidine-injected rats, substance P- and CGRP-immunoreactive nerves were increased. In contrast, the penis showed a decrease in such nerves. An increase in substance P immunoreactivity was also reported in the iris of rat and guinea-pig, following both surgical and chemical (6-hydroxydopamine) sympathectomy (Kessler, Bell & Black, 1983*a*, *b*; Zhang *et al.* 1984). The putative role of substance P in regulating regeneration and development of noradrenergic neurons has been suggested (Linder & Gross, 1981; Cole *et al.* 1983) and our results support this suggestion. The increase of CGRP immunoreactivity after chemical sympathectomy may also indicate a trophic role in the neuronal response to damage, particularly of noradrenergic nerves. The decrease in substance P- and CGRP-immunoreactivity in the penis is difficult to interpret. Perhaps these fibres originate from 'short' sympathetic neurons and are damaged by guanethidine, but if so, they must differ from those in the vas deferens, which increased in response to the treatment. The suggestion of coexistence of substance P (Kessler, Adler, Bohn & Black, 1981) or CGRP with noradrenaline raises the possibility of such coexistence in the rat penis. However, this arrangement seems unlikely in view of our findings. These points deserve further investigation.

Sperm accumulation and associated enlargement of the seminal vesicle, vas deferens and cauda epididymidis were observed in the guanethidine-injected rats. Chronic treatment with guanethidine has been reported to promote damage to the adrenergic innervation of rat male genital ducts, resulting in an accumulated mass of spermatozoa due to prevention of normal nerve-mediated emission (Evans *et al.* 1972). In the present work it was observed that spermatozoa, retained in the cauda epididymidis and vas deferens of guanethidine-injected rats, showed no change in motility, but exhibited fewer attached cytoplasmic droplets. Normally the cytoplasmic droplet migrates from the neck of the spermatozoon to the distal end of the middle piece during the passage of the cell down the epididymal duct, and is usually lost by the time of ejaculation (Dott & Dingle, 1968). Our findings are consistent with the hypothesis of sperm retention in the cauda epididymidis and vas deferens of guanethidine-treated rats, allowing the ageing process to take place. It seems that the epididymal environment is nevertheless able to maintain the motility capability of ageing spermatozoa. Accumulation of spermatozoa in the vas deferens and cauda epididymidis was also observed in guinea-pigs, one week after hypogastric or pelvic nerve transection. However, no changes in sperm motility or morphology were observed. It seems reasonable to assume that the one week period elapsed between the decentralisation procedures and sperm collection was not sufficient time to allow observable changes in the retained spermatozoa.

In conclusion, our findings add further evidence of the complexity of organisation of the nerve supply to the male genitalia, and show that, although some motor peptidergic postganglionic axons seem to run along the parasympathetic (pelvic)

and sympathetic (hypogastric) pathways, most of them are likely to originate from local ganglia or even from another source. They also support the contention that at least some of the substance P- and CGRP-immunoreactive cells present in the male genitalia might have a trophic role in regulating noradrenergic neuron regeneration.

SUMMARY

A systematic immunohistochemical and radio-immunological survey of the occurrence, distribution and origin of the peptidergic nerve supply in guinea-pig and rat male genitalia is presented. Neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI), substance P and CGRP were detected in the genital organs of both species. The densities and distribution patterns of the peptidergic nerves were compared with those of the adrenergic nerves, as revealed by antibodies raised against dopamine-β-hydroxylase (DβH) and tyrosine hydroxylase (TH), and the general neuronal component, as revealed by antibodies raised against neurofilament proteins (NF). Bilateral transection of the hypogastric nerves, in the guinea-pig, resulted in a decrease of substance P-containing nerves in the vas deferens and of NPY-, PHI- and VIP-containing nerves in the seminal vesicle. Unilateral disconnection of the pelvic nerves caused a decrease of VIP, PHI, substance P and CGRP nerve supply in the ipsilateral vas deferens and cauda epididymidis in the guinea-pig. A marked reduction of noradrenergic and NPY-containing nerves was observed in the vas deferens and sexual accessory glands of rats, chemically sympathectomised by chronic injection of low doses of guanethidine. Conversely, increases of substance P and CGRP immunoreactivities were observed, particularly in the vas deferens.

After guanethidine, the cauda epididymidis and vas deferens were distended with spermatozoa, suggesting paralysis of the ducts. Spermatozoa had a decreased percentage of attached cytoplasmic droplets, indicating prolonged retention in the ducts.

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