DOCTOR OF PHILOSOPHY

Studies on the nervous system and arterial gland of the slug, Deroceras Reticulatum (Pulmonata, Limacoidea)

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STUDIES ON THE NERVOUS SYSTEM AND ARTERIAL GLAND OF THE SLUG *DEROCERAS RETICULATUM* (PULMONATA, LIMACOIDEA)

A thesis submitted for the degree of
Doctor of Philosophy at Bangor University

by

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Summary

Significant developments in gastropod endocrinology since 1969 are reviewed.

The anatomy of the CNS, distribution of nerves and the general plan of the vascular system in *Deroceras reticulatum* is described.

The central ganglia are encased in a thick perineural sheath, a matrix of collagen in which muscle fibres, blood vessels, granular cells, pigment cells and vesicular cells are embedded. The fine structure of the blood vessels supplying the CNS suggests that metabolic requirements reach the neurones by diffusion from these vessels.

Dorsal Body tissue (DBT) occurs on the cerebral ganglia and intercerebral commissure as well as other parts of the CNS. Synaps-like contact between neurosecretory axons and DBT was not found, but the close relationship with the blood system, suggests secretory material may be liberated directly into the blood.

Distinct groups of possible neurosecretory neurons were identified in the cerebral, parietal and visceral ganglia, but specialised neurohaemal areas were not found. Axons containing large numbers of elementary granules were observed in the perinueral sheath, but axons with swollen ends or evidence of exocytosis was not found. The presence of possible neurosecretory axons close to capillaries and blood spaces suggests release of neurosecretory material into the blood can take place easily.

During investigation of the CNS a tissue was discovered attached to arteries arising from the cephalic arborescence. It appeared glandular, lacked ducts and was provisionally named the arterial gland.

Electron probe microanalysis revealed an accumulation of copper within the arterial gland, but immunoelectrophoresis performed using rabbit antiserum to *H. aspersa* haemocyanin. and homogenised arterial glands gave negative results.

The arterial gland in *D. reticulatum* contains secretion throughout reproductive development. Its size is extremely variable between individuals, but neither size nor histology could be related to reproductive development.

Tissue similar to the arterial gland of A.reticulatus was found in four other gastropod species.

STUDIES ON THE NERVOUS SYSTEM AND ARTERIAL GLAND OF THE SLUG *DEROCERAS RETICULATUM* (PULMONATA, LIMACOIDEA)

PUBLISHED WORK

Significant developments in Gastropod endocrinology from 1970 – 2018.

Significant advances in our understanding of gastropod endocrinology have occurred since the work presented in this thesis was completed in 1969. The late Professor Lever and the late Professor Joosse's group at the Free University of Amsterdam carried out an enormous body of work on neuroanatomy and the neuroendocrine system of the pulmonate snail *Lymnaea stagnalis*. In addition, many of the neuropeptides produced by the *Lymnaea* CNS have been identified and their gene expression and precursors determined.

In addition peptidergic systems controlling specific pieces of behaviour and physiological processes have been unravelled, the genes encoding these peptides identified, hormones and peptides sequenced. The classic techniques of histology, extirpation and implantation have been built on and given way to molecular biology and neurophysiology.

Our understanding of the nature of the neurosecretory neuron completely altered once it become clear that peptides in neurons could function as hormones and/or neutotransmitters (Hokfelt,1980). The terms neurosecretion and neurotransmitter were replaced by, neuropeptide and the cells producing them known as peptidergic.

In *L. stagnalis for example*, the caudodorsal cell hormone (CDCH) acts as a hormone that stimulates ovulation, (Geraerts and Bohlken 1976; Geraerts et al. 1983) but also acts as a neurotransmitter to organise the behavioural sequence that underlie egg laying behaviour (ter Maat *et al*., 1989). We know a single neuron can produce many different transmitters - the paired B2 oesophageal motoneurons that modulate gut contractility in *L. stagnalis* produce a total of nine transmitters: acetylcholine, nitric oxide and seven neuropeptide co-transmitters (Park, *et al*., 1988; Perry, *at al*., 1998, 1999).

It also became apparent that many more peptidergic neurons existed in the molluscan CNS than had been mapped as neurosecret**o**ry. Benjamin (2008) estimated that at least 2,500 of the estimated 20,000 neurons, in the *L stagnalis* CNS, some 12.5%, are peptidergic. This figure is likely to be an underestimate as many of the neurons in the CNS have not been analyzed.

Most of the advances in our understanding of gastropod endocrinology derive from comprehensive studies on the freshwater gastropod *L. stagnalis*, and the marine gastropod,

Aplysia californica. Terrestrial gastropods with tough, often pigmented connective tissue surrounding the CNS, proved more difficult subjects and have been less extensively studied.

For a comprehensive review of the endocrine physiology of terrestrial pulmonates see Flari and Edwards (2003), for an extensive review of the neuroendocrine control of reproduction in hermaphrodite freshwater snails see Koene (2010) and for a detailed account of peptidergic systems in *L. stagnalis* see Benjamin and Kemenes (2013, 2018).

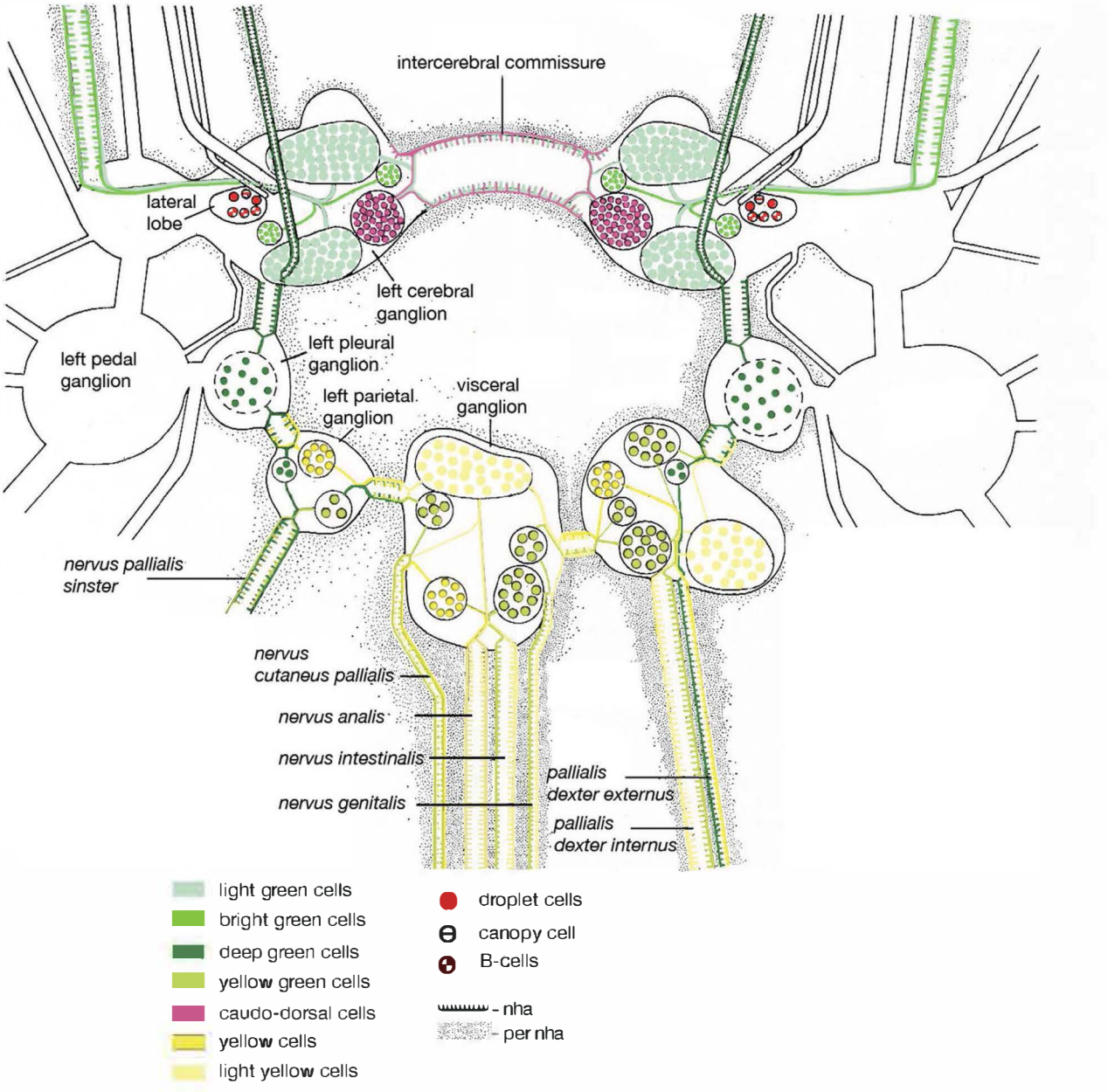
Neurosecretory Neurons

The occurrence of neurosecretory cells was first demonstrated by B. Scharrer (1935) in opisthobranch snails. Since then light microscopical studies have been carried out on numerous species of mollusc (see Gabe (1966), Simpson (1966a) and Durchon (1967)) using the classic neurosecretory stains – chrome alum haematoxylin and paraldehyde fuscin - and neurosecretory cells identified. Lever (1957) was the first to establish neurosecretion in pulmonates in *Ferissa shimekii*.

Cells that stain with the classic neurosecretory stains are known as Gomori positive, however, lipofuscin pigments and food reserves in molluscan neurons also react positively to the classic neurosecretory stains. Bern (1966) suggested that such stainability should not be the sole criterion for neurosecretory function. Boer (1965) demonstrated the value of histochemical analysis in mitigating the unreliability of the classic neurosecretory stains and suggested that evidence of possible neurosecretion should be substantiated by ultrastructural confirmation of elementary secretory granules in stained cells ((Nolte, 1965; Simpson *et al*, 1966b; Boer *et al.* 1968a). In 1964 Joosse identified possible neurosecretory cells, the caudo-dorsal cells, in the cerebral ganglia of the pond snail *L. stagnalis* that did not stain with the classic neurosecretory stains, as Gomori negative.

Building on the work of Lever, Joosse and Boer at the Free University of Amsterdam, Wendelaar Bonga (1970) carried out a meticulous study of neurosecretory neurons *in L. stagnalis*. Using the alcian blue/ alcian yellow staining technique. The neurosecretory neurons stain different shades of green and yellow, the colour differences reflecting different ratios of strong and weak acid groups in the secretory materials (Peute and van de Kamer, 1967).

Wendelaar Bonga identified 7 different neurosecretory cell groups, within what had hitherto been simply Gomori positive cells and identified 4 Gomori negative neurosecretory cell



The mottled areas (per. nha) represent parts of the perineurium and of the connective tissue which are traversed by small nerves containing neurosecretory axons. The pedal ganglia and the ventral parts of the cerebral ganglia are turned to the lateral sides. The follicle gland and the media- and latero-dorsal bodies are not indicated.

Fig. 1. The location of the neurosecretory cell groups and their neurohaemal areas in the central nervous system of *Lymnaea stagnalis* (dorsal view).
From Wndelaar Bonga (1970)

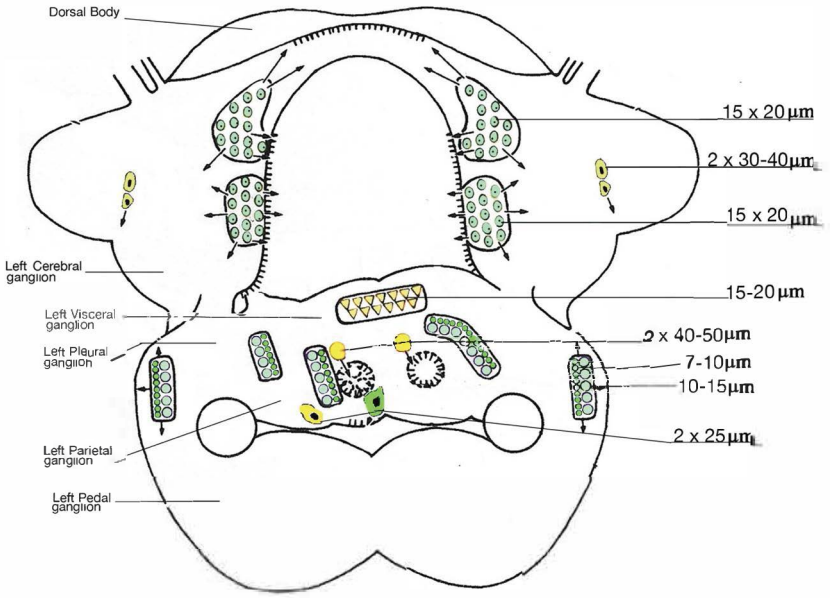
groups, not identified previously. He also showed that the histochemically differentiated secretory material of 4 different types of neurosecretory cell in the visceral ring, was reflected in differences in the size and appearance of elementary granules.

Wendelaar Bonga traced the neurohaemal areas of these neurosecretory cells and identified the network of tiny nerves supplying extensive neurohaemal areas in the perineurium and connective tissue surrounding the central ganglia. He also presented convincing evidence for exocytosis being the mechanism by which the contents of neurosecretory granules are released.

In *L. stagnalis* the appearance of clear vesicles coincides with the occurrence of omega profiles exocitosis. Wendelaar Bonga suggests the clear vesicles are formed from the remnants of the membranes of the elementary granules, which in turn fuse to form vesicular and tubular structures as part of the membrane conservation process.
The detailed map of neurosecretory neurons in the basommatophoran (now Hygrophilan) *L. stagnalis* (Fig. 1) provided a useful reference for comparison with various studies of neurosecretory neuron groupings in Stylommatophora, because, although neurosecretion had been studied histologically in a number of pulmonates, no detailed maps of their neurosecretory systems existed. A study by Duce (1976) established 10 different types of neurosecretory cells in the CNS of *D. reticulatum* using AB/AY, resorcin fuchsin (RF), CH, and PAF stains and in 1980 Wijdenes *et al*. also mapped neurosecretory neurons in the central ganglia of *D. reticulatum* and two other terrestrial pulmonates: *Arion hotensis* and *Helix aspersa* using the AB/AY technique (Figs. 2, 3).

Wijdenes *et al*. identified nine groups of neurosecretory cells in *D. reticulatu*m, eleven groups in *A. hortensis* and thirteen groups in *H. aspersa*. The neurosecretory systems of the three Stylommatophora investigated showed some differences, but overall, great similarity. On the basis of staining properties, size and location, Wijdenes *et al*. considered the neurosecretory cells in the metacerebrum of all three species (Ce-BrGC); (Ce-GC); (Ce-SGC); (Ce-BlC) to be homologous and the distinct groups of neurosecretory cells in the ganglia of the visceral ring of *D. reticulatum* and *A. hortensis* (Pl-LGC; (Vi-YGC); (Vi-GC) to also be homologous. The blue droplet cells (BIDC) scattered throughout the visceral complex of *A. hortensis* and *H. aspersa* were also thought to probably be homologous.

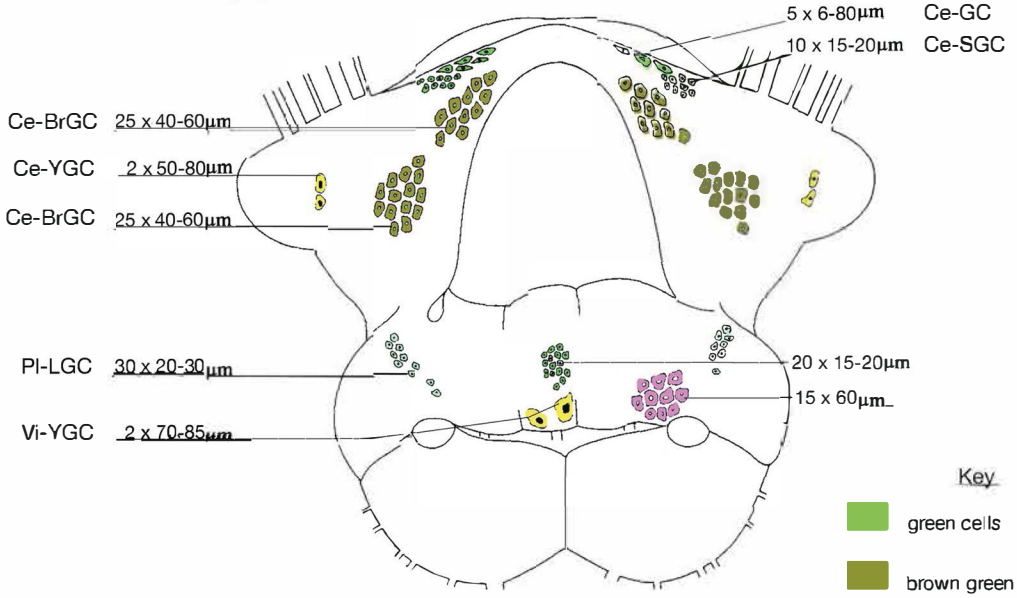
The main similarity between the three Stylommatophora investigated and *L. stagnalis* was the



Key

A + B		mid green
C		light yellow green
D +E		yellowish green
K		yellowish
F -J		bright light green
M		yellow
L		bright green

Deroceras reticulatum
After Duce (1976)

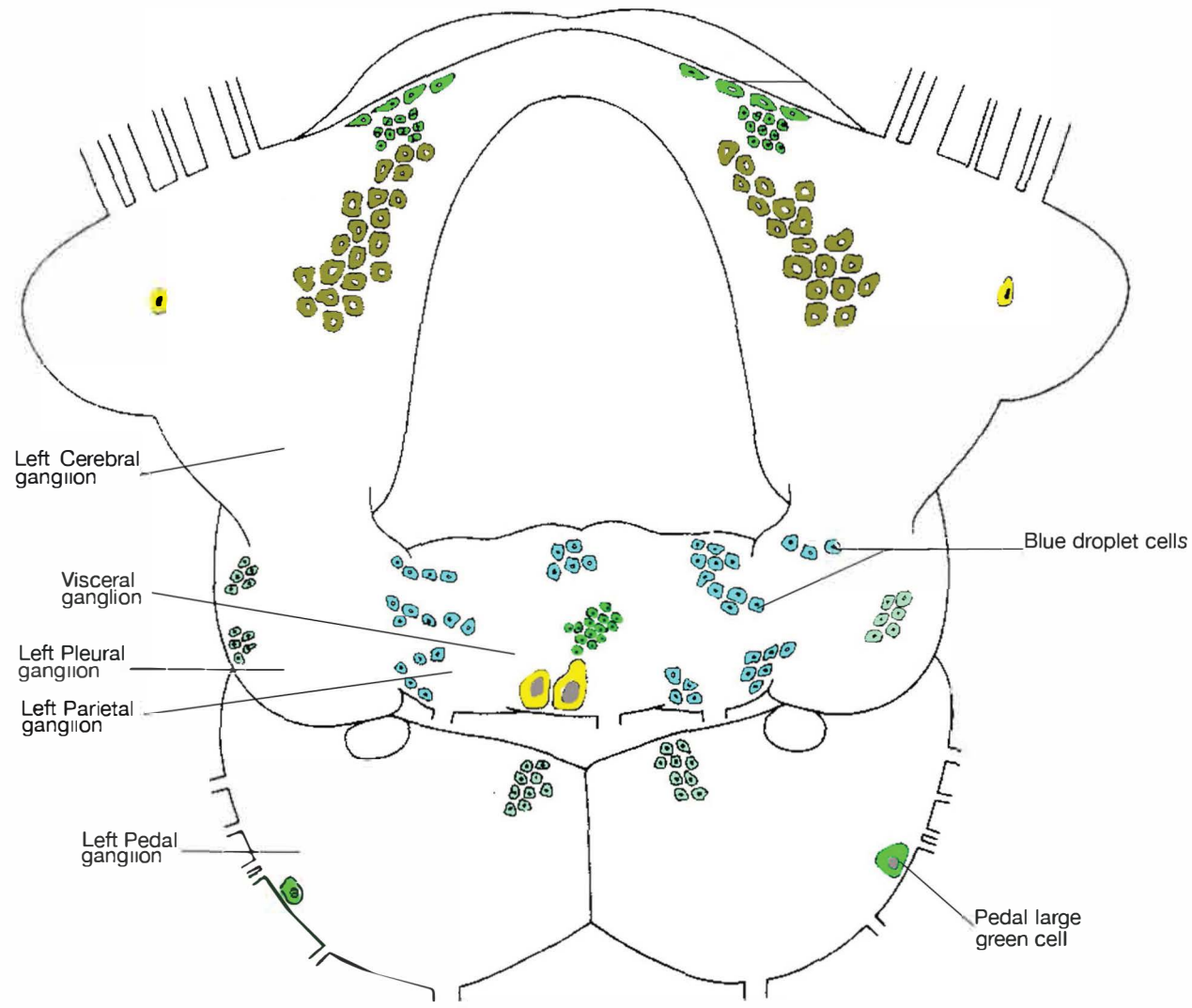


Key

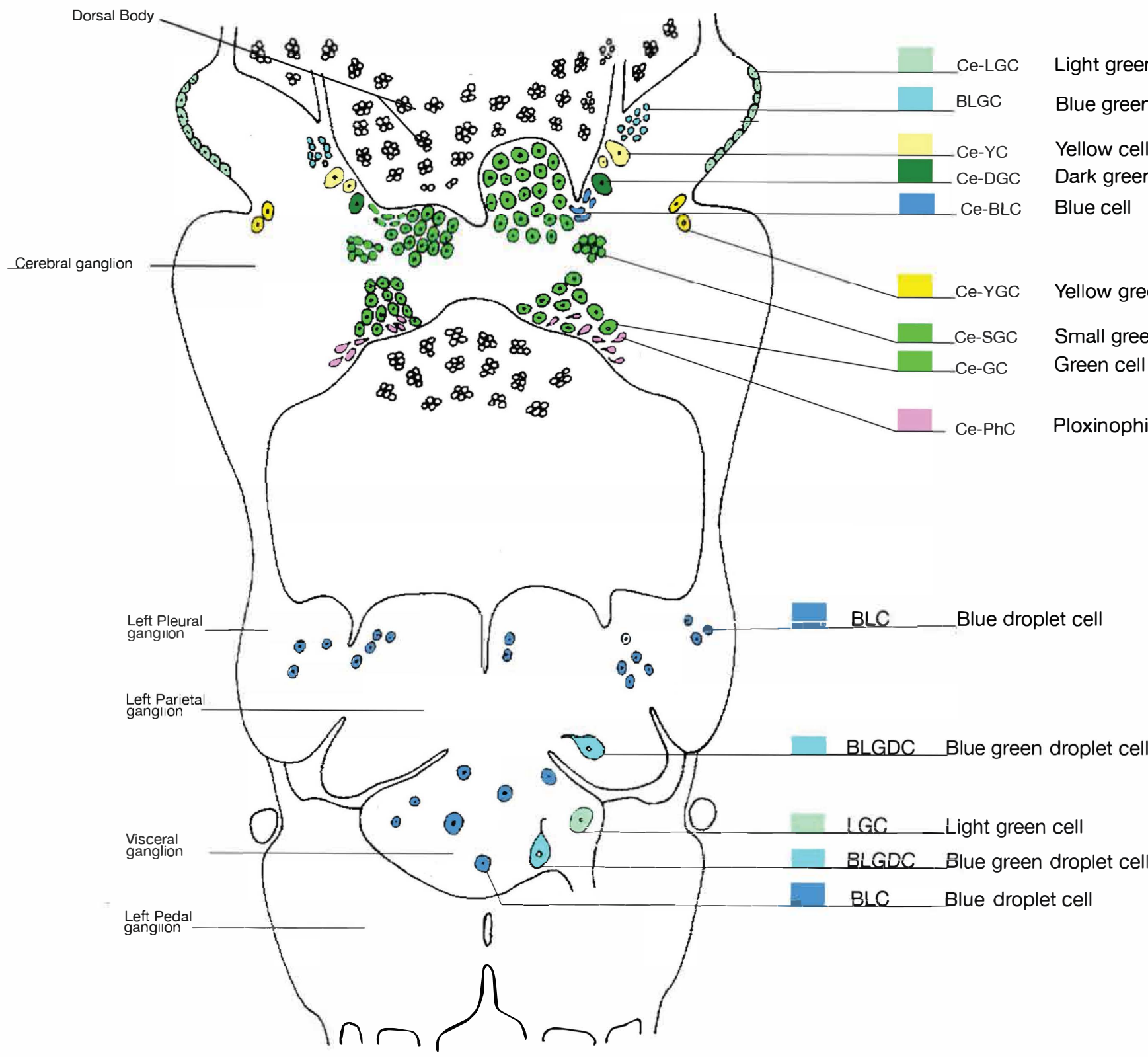
green cells	GC
brown green cells	BrGC
light green cells	LGC
yellow green cells	YGC
phloxinophilic cells	PhC

After Wijdenes *et al.*, (1980)

Location of neurosecretory cells in the CNS of *Deroceras reticulatum* after Duce, (1976) stain: AB/AY and Wijdenes *et al.*, (1980) stain: AB/AY/phloxin



Arion hortensis



Helix aspera

Fig. 3. Location of neurosecretory cells in *A. hortensis* and *H. aspersa* identified with AB/AY and phloxin. From Wijdenes *et al*., 1980).

occurrence of green staining cells in the dorsal and latero-dorsal areas of the cerebral ganglia. According to Wijdenes *et al*. these Ce-GCs in *A*. *hortensis*, and *H*. *aspersa* are morphologically and histochemically homologous to the Ce-BrGC in *D*. *reticulatum*. These cells were known to be involved in the control of growth in *D*. *reticulatum* (medial cells, Wijdenes and Runham 1977) and considered homologous with the cerebral light green cells (LGC) of *L*. *stagnalis* (Geraerts 1976) which have similar staining properties, are located in homologous areas, and produce a growth hormone.

Although Duce and Wijdenes *et al*. identified common neurosecretory cell groups, some groups identified by Duce were not found by Wijdenes *et al*. and some groups identified by Wijdenes *et al*., were not found by Duce. There were also differences in the size, number and the colours produced by staining with AB/AY (see Fig. 2). However, Duce provided evidence that confirmed the histochemical differences between cell groups identified in his light microscope investigation, reflected ultrastructural differences in their neurosecretory granules. He also identified possible neurohaemal areas, collections of swollen axon endings packed with elementary granules, throughout the perineurial sheath, particularly on the posterior surfaces of the parietal and visceral ganglia.

Neurohaemal areas have also been described in other stylommatophoran species including Arionidae (Herlant-Meewis and Van Mol 1959, Van Mol l960a, Smith 1967), Helicidae (Kuhlmann 1963), *Succinea putris* (Cook 1966) and *Helix pomatia* (Sakharov and Salanki 1971). In *H*. *aspersa*, axon tracts from the metacerebral cells have been followed into the *arteria cerebralis* and into the intercerebral nerves which terminate in the connective tissue dorsal to the cerebral ganglia (Kuhlmann 1963; Nolte 1965, 1978).

Classic extirpation and reimplantation experiments revealed the function of many neurosecretory cell groupings in *L stagnalis*. For example, in 1975 Geraerts and Bohlken showed the Caudal Dorsal Cells (CDCs) produced a hormone that controlled ovulation and in 1976 the Light Green cells (LGCs) together with the lateral lobes, were shown to be involved in the control of growth (Geraerts, 1976). Removal of the medial cells in the cerebral ganglia of *D*. *reticulatum* (Wijdenes and Runham 1977) demonstrated their involvement in the control of growth.

Neuropeptides

Investigating the mechanisms underlying the function of peptidergic cells required a multidisciplinary approach and a variety of techniques including electrophysiology, gene technology and cytochemistry, were used to explore the neural circuits involved in the integration of complex processes such as physiology and behaviour in *L stagnalis*.

L. stagnalis was a brilliant choice of experimental animal because its CNS consists of relatively few neurons - about 20,000 - arranged in prominent central ganglia and many of these neurons are of a giant size and can be easily identified. Also, many of the peptidergic sytems controlling specific behaviours and physiological processes had already been studied extensively.

Neuropeptides form the largest group of direct signaling molecules in nervous systems and control the major physiological processes such as the cardiovascular system, digestion, reproduction, growth and metabolism and ion and water regulation. They are derived from large precursor molecules which are cleaved into several smaller peptides, some acting as neurohormones, exerting effects on distant targets, such as the gonad and accessory sex organs, while others act as neurotransmitters, acting upon neurons located in the CNS (Boer and van Minnen, 1987).

The diversity of neuropeptide form and function is enormous and makes possible a high degree of complexity and finesse in neuronal communication. There are multiple gene families encoding neuropeptides. Structurally related peptides can be encoded on the same gene as can structurally diverse peptides. Alternative mRNA splicing also leads to diverse peptide expression and diversity in neuropeptide receptors allows for differential regulation. Peptides can act at the synapse, diffuse locally to mediate volume transmission (Agati et al., 2010) or be released into the blood stream at neurohemal sites and act at a distance. A single neuropeptide, or peptides from the same precursor, can act on multiple distinct receptors. Thus, the response to the neuropeptide will be dependent on the receptor with which it interacts, which in turn can be regulated by differential receptor expression (Jékely *et al*., 2018).

See Table 1 for examples of neuropeptide hormones and transmitters and Fig. 4 for a map of peptidergic neurons in the CNS of *L. stagnalis* (both after Benjamin and Kemenes, 2018).

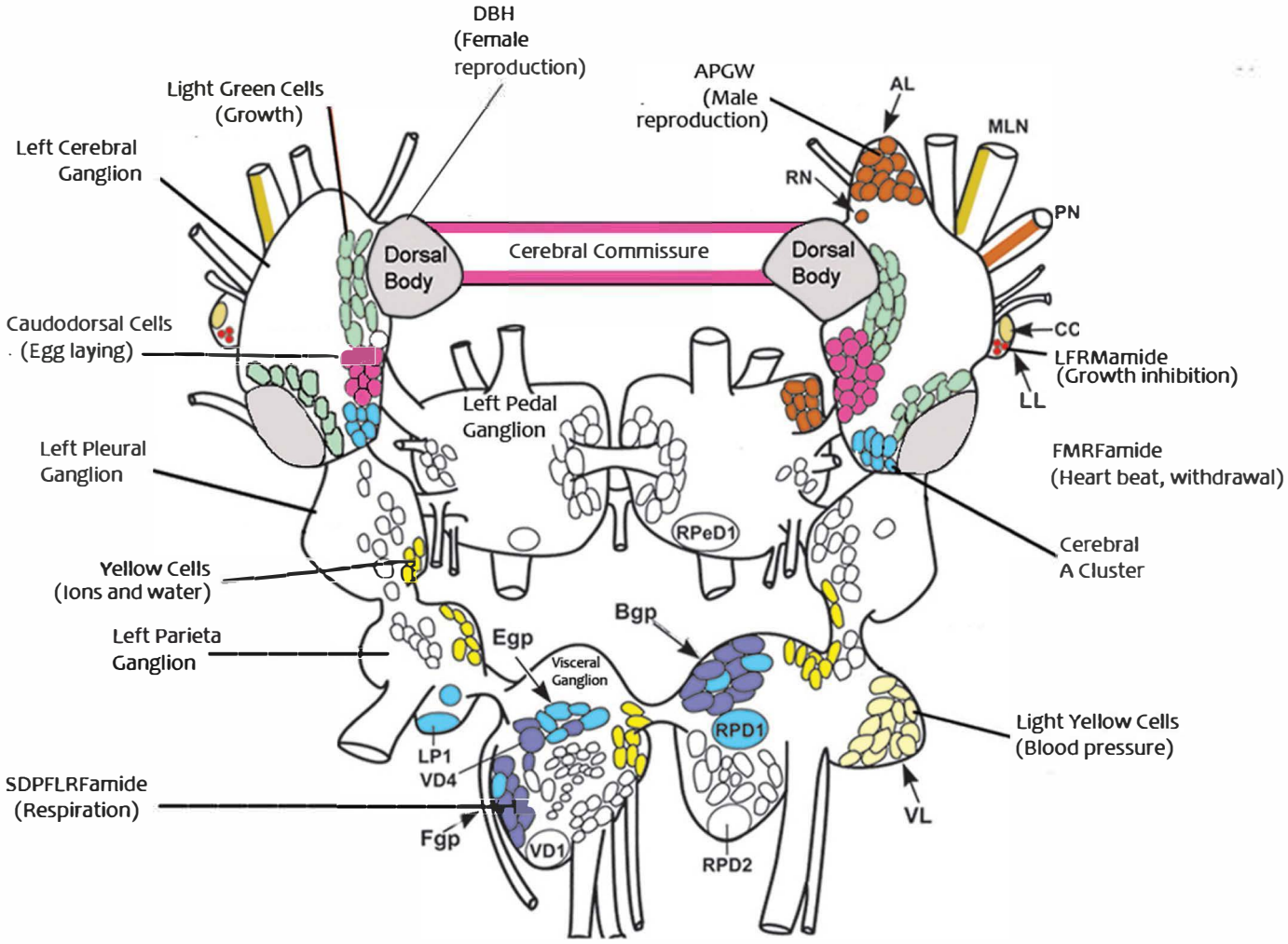


Fig.4. Map of peptidergic neurons in the CNS of Lymnaea stagnalis
From Benjamin and Kemenes (2018)

Table 1 Examples of neuropeptide hormones and transmitters

Neuropeptides	Amino acid sequence
α-peptides	DMYEGLAGRCQHHPRNCPGFN (α1) , DMVTTTRIGTGGLAGRCQHHPRNCPGFN (α2)
APGW	APGWamide
Caudodorsal cell hormone (CDCH)	LSITNDLRAIADSYLYDQHKLRERQEENLRRRFLELamide
Calfluxin (CaFl)	RVDSADESNDDGFD
FMRFamide-related peptides (FARPs)	FMRFamide, FLRFamide, SDPFLRFamide, GDPFLRFamide
F(X)RIamides	ASSFVRIamide, SPSSFVRIamide, PNSFLRIamide, YPMNRFIRIamide
Granularin	EPCEHNGVTYNPGDAYHKDQCTTCYCGEDSEAFCIPLQCDW PQCEDGASPVYLEDSCCPGCP
LFRFamides	NTLFRFamide, GTLLRFamide, GGSLFRFamide, TLFRFamide
Light Yellow Cell (LYC I-III) peptides	AFIVEEDDLTGYPTTIDAAMTTIRP (LYC I′), TPDKSILLNRL (LYC II′)
Lys-conopressin	CFIRNCPKGamide
Lymnaea cardioactive peptide (LyCEP)	TPHWRPQGRFamide
Lymnaea inhibitory peptides (LIPs)	GAPRFVamide (LIP A), SAPRFVamide (LIP B), ARPSKFVamide (LIP C)
Lymnaea leucokinin-like peptide	PSFHSWSamide
Lymnaea neuropeptide Y (LNPY)	TEAMLTPPERPEEFKNPNELRKYLKALNEYYAIVGRPRFamide
Lymnaea tetradecapeptide	GFRANSASRVAHGYamide
Molluscan insulin- related peptide (MIP)	QGTTNIVCECCMKPCTLSELRQYCP-- (A Chain) QFSACNINDRPHRRGVCGSALADLVDFACSSSNQPAMV-- (B chain) (MIP1)
Myomodulins	PMSMLRLamide, GLQMLRLamide, SMSMLRLamide,
Ovipostatin	EKDQTPSCSPDTFEANLYCTDGSVCGKYAVDWTQNVSVVQL KSFRLVFYINQIKGFRISNEEECNPVDGRSNQIPIRCIPPNAVIR LKGNAFGADFFSFDVVSPSPTVTWYPEKEHVPKILKIFIDGGR LFPGDFVFFPDLLKTDDLSLSLFDFPVTCPYFEGYDPE
Schistosomin	DNYWCPQSGEAFECFESDPNAKFCLNSGKTSVVICSKCRKK YEFCRNGLKVSKRPDYDCGAGWESTPCTGDNSAVPAVF
Small cardioactive peptides	SGYLAFPRMamide (SCP_A), $_P$QNYLAFPRMamide (SCP_B)
Sodium influx-stimulating peptide (SIS)	SRTQSRFASYELMGTEGTECVTTKTISQICYQCATRHEDSFV QVYQ-ECCKKEMGLREYCEEIYTELPIRSGLWQPN

From Benjamin and Kemenes (2018)

Neuroendocrinology of male and female reproductive behaviour

The reproduction of *L. stagnalis* has been studied extensively and is perhaps the best understood of its biological processes. The animal is a simultaneous hermaphrodite, but during mating behaviour one individual acts as the male and the other the female.
Male courtship, copulatory behaviour and egg-laying in *L. stagnalis* are examples of stereotyped behaviour consisting of a limited number of behavioural acts that are controlled by specific sets of neuropeptides.

The role of neuropeptides in male courtship and copulatory behaviour.

Most neural and hormonal information concerns the motor control of the preputium, the muscular structure that surrounds the penis. Five groups of neurons project along the penis nerve that exits from the right cerebral ganglion. The neurons of one of these groups located in the ventral part of the right anterior lobe (rAL) are normally silent but increase their spiking during preputium eversion and throughout intromission (de Boer *et al*. 1997). Artificial electrical stimulation of the rAL neurons causes eversion of the preputium in all the animals tested (de Boer et al, 1997).

Eversion of the preputium involves relaxation of preputial retraction muscle bands and the circular muscles surrounding the male gonophore. These muscles are innervated by nerve fibres in the pedal nerve from rAL neurons that contain the peptide APGWamide. Injection of the APGW peptide into intact snails causes eversion of the preputium, so one of the roles of the APGW peptide is to relax the preputial muscle bands to cause preputial eversion (de Boer et al, 1997).

The rAL neurons that contain APGW also co-express other peptides. Lys-conopressin is often co-localised with APGW. In other rAL neurons, APGW is co-expressed with LyNPY (de Lange et al, 1997) and in this type of AL lobe neuron both peptides act together as synergistic co-transmitters to relax retractor muscles. There are a number of other peptides present in the penial complex eg myomodulins (van Golen et al, 1996) and LIPs (Smit et al, 2003) that have the ability to relax the preputial retractor muscles and these, like LyNPY, may also be involved in preputial eversion, although the details of this are unknown (Benjamin and Kemenes, 2018).

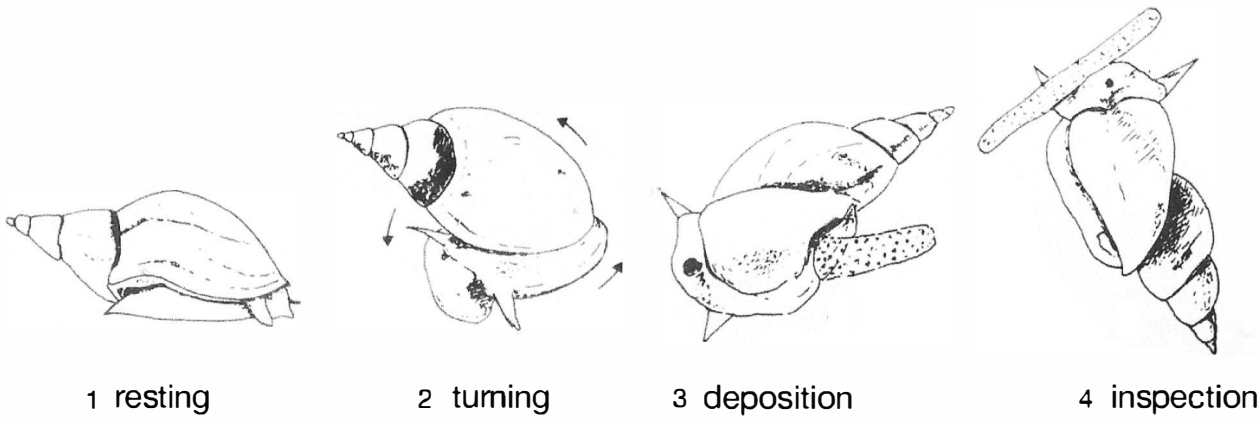


Fig. 5. Postures during the four phases of egg-laying behaviour From Geraerts et al., (1991)

The phase of mating behaviour after eversion is probing, when the fully everted preputium makes movements under the lips of the recipient shell in search of the female gonopore. The sensory mechanism needed to achieve accurate positioning may be provided by neurons that lay on the distal tip of the preputium. Several of these neurons are Lys-conopressin-containing sensory neurons with dendrites that extend through the preputial epithelium (Zijlstra, 1972). They send their axons into the penis nerve (de Lange et al, 1998a).

At the end of these courtship behaviours the penis is inserted into the female gonopore. Eversion of the penis resulting from alternate contractions of longitudinal and circular muscle layers in the penis. After the transfer of sperm and seminal fluid into the vaginal duct of the recipient the penis and preputium are retracted completing the mating sequence (Koene, 2010). Another peptide Lys-conopressin, increases peristaltic movements in the vas deferens, which transports semen to the penis, while APGW acts antagonistically to modulate peristalsis (van Golden *et al*, 1995)

Egg-laying behaviour and its hormonal control

Egg-laying behaviour in *L. stagnalis* is triggered by exposure to a clean water stimulus and consists of a sequence of behavioural events involving ovulation, packaging of fertilized oocytes, oviposition and a change in locomotion and feeding movements.
The overt stereotyped behaviour begins with a rest period when the animal stops moving around, then a turning phase characterized by counter clockwise shell movements and high frequency rasping to clean the substrate, followed by oviposition. The final phase is inspection, when the snail moves along the length of the egg-mass, brushing it with lips and tentacles (ter Maat et al., 1987) (Fig. 5).

This complex behaviour is programmed by the central release of multiple types of peptides encoded by a small family of genes. The CDCs express three different CDCH (caudodorsal cell hormone) genes, CDCH I-III, that encode related but diverse peptide hormones (Li *et al*., 1992). CDCH-1 and CDCH-2 both encode eleven different peptides. So far the structure of 9 of the CDCH-1 peptides have been confirmed: CDCH peptide, the ε peptide, the δ peptide, α, β1and β3 CDC peptides, the CTP (C-terminal peptide), the αCDCP peptide and calfluxin (Li et al., 1994; Jemenez *et al*, 2004)). Calfluin stimulates the albumen gland to produce

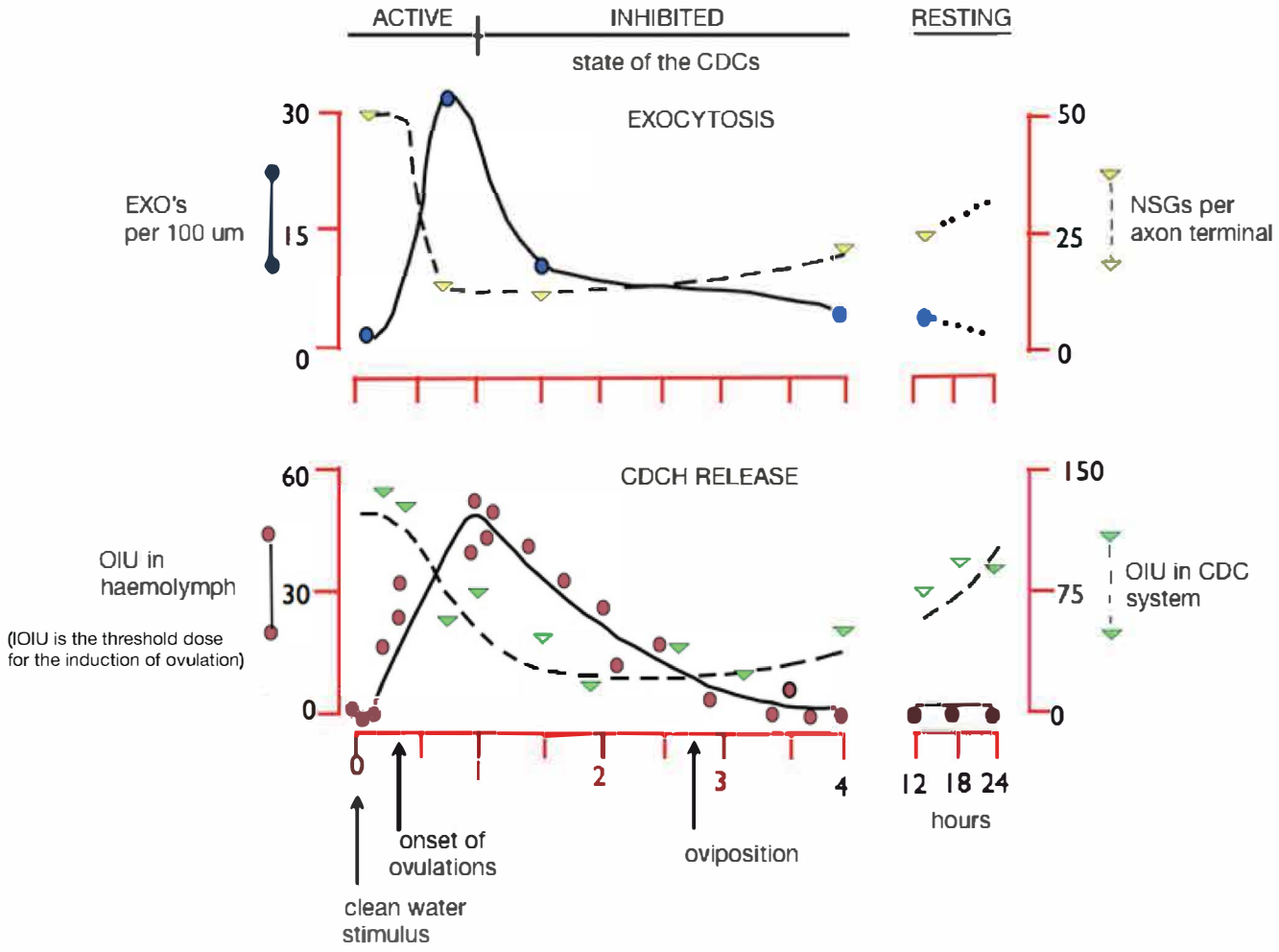


Fig. 6. Discharge, exocytosis, CDCH release and egg-laying in *L, stagnalis*
From Geraerts et al., (1991)

periviteline fluid that is deposited around freshly ovulated eggs during the egg-laying process.

The caudodorsal cells (CDCs) that control egg-laying, are located in the caudodorsal part of each cerebral ganglion (Joosse, 1964; Wendelaar Bonga, l970). Each cluster contains about 50 cells, the axons of which run to a neurohaemal area in the periphery of the intercerebral commissure. Axon branches of ventral CDCs project into the interior of the intercerebral commissure and form an extensive diffuse network, the collateral system, throughout its inner compartment and make electrotonic contact with the contalateral CDC group (Schmidt and Roubos, 1987) (Fig. 6). Ventral CDCs receive the sensory egg-laying inducing stimuli and relay them to the other CDCs via the collateral system, creating synchronous sustained electrical firing in the CDCs. (Schmidt and Roubos, 1987). This triggers the release of ovulation hormone, CDCH into the blood.

CDCH and the αCDC peptide together act as auto-transmitters and provide a self-sustaining mechanism for maintaining firing of the CDC neurons (Brussard et al, 1990), which ensures the maximum output of the system. In addition, CDCH causes ovulation and packaging of ripe eggs in the female tract. When CDCH is injected into animals it evokes ovulation, egg mass formation and oviposition (Ter Maat et al, 1989).

Release of CDCH takes place almost exclusively during the CDC discharge and is accompanied by a decrease in the CDCH contents of the CDC system. These phenomena are closely paralleled by an enormous increase of exocytosis profiles in CDC terminals of the neurohaemal area in the COM during the CDC discharge. Ovulations do not begin until the hormone has appeared in the blood (Fig. 6).

CDCH and other peptides encoded on the CDCH gene have a variety of roles in controlling the neural circuitry associated with the different phases of egg-laying behaviour (Hermann *et al*., 1997). For example, injection of β3 and α CDC peptides into intact snails increase the rate of rasping movements of the radula that occur during the turning phase of the natural behaviour. The rasping movements clean the substrate to allow the subsequent deposition of the egg mass. This is a distinct behaviour from the normal role of rasping in food ingestion and is accompanied by changes in the firing pattern of feeding motoneurons and the modulatory CGCs that are ‘re-programmed’ for their role in egg laying (Jansen et al, 1997, 1999) in Benjamin and Kemenes, 2013. Also, pedal ganglion motoneurons involved in turning behaviour are excited by the β3 CDC peptide but are inhibited by the ovulation

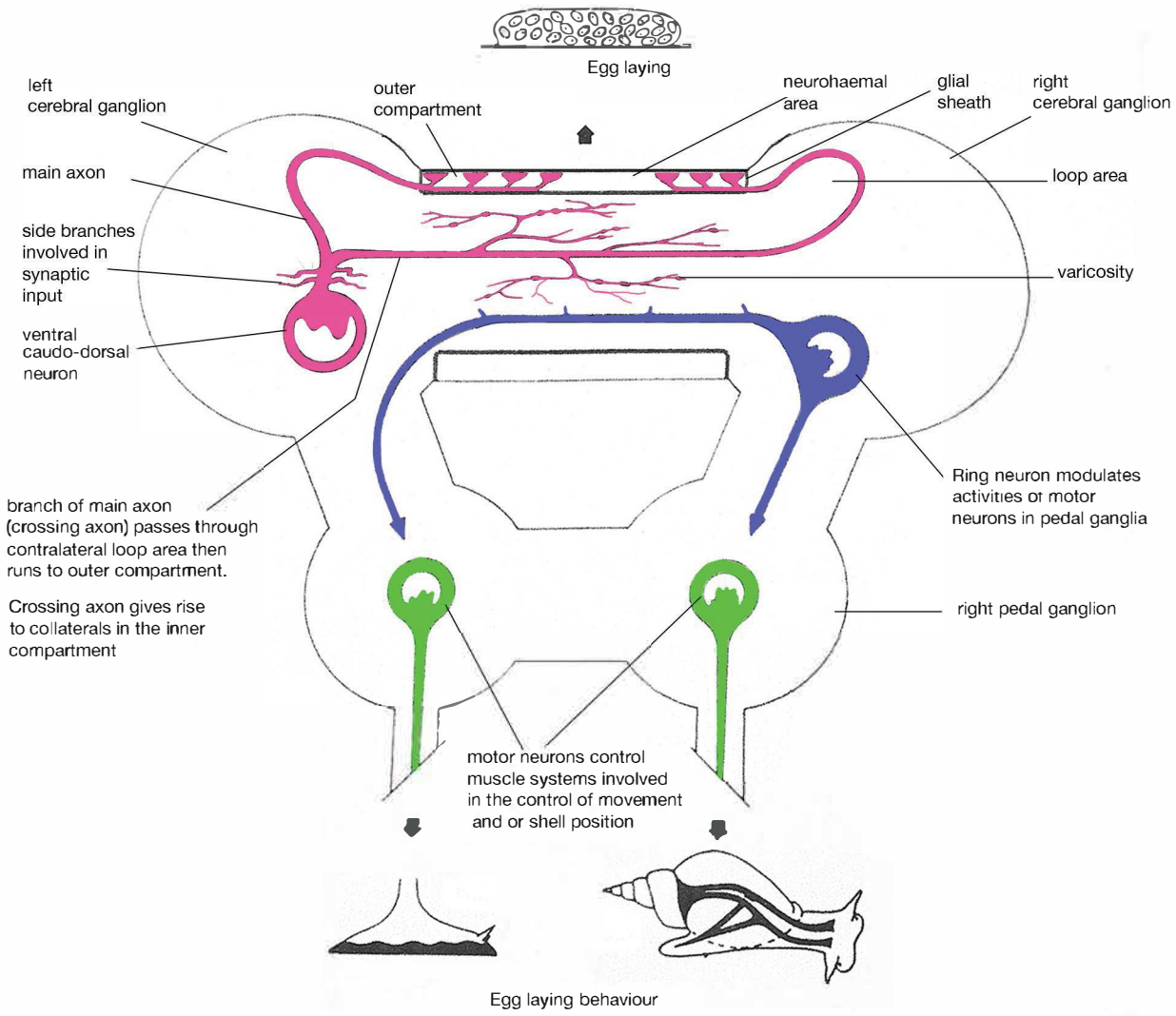


Fig. 7. Tentative scheme for the release of neurohaemal and nonsynaptic release of peptides from the CDC neurohaemal and collateral systems in the control of egg-laying behaviour in *L. stagnalis* From Geraerts *et al.*, (1991)

Diagram of the outer and inner compartments of the intercerebral commissure of *L. stagnalis* showing a single ventral caudo-dorsal neuron. From Schmidt and Roubos, (1987)

hormone to suppress turning during oviposition.

The unpaired Ring neuron in the right cerebral ganglion (projects into the pedal ganglia, traverses the pedal commissure, completing a ring (Jansen and Bos, 1984) has a modulatory role during the early phase of egg-laying. CDC discharge increase the firing rate of the Ring Neuron, which in turn inhibits the firing of the pedal motor neurons and may thus be responsible for the resting phase (Jansen and ter Matt, 1985). When enough CDCH has been released the Ring neurone is inhibited, which coincides with the transition from resting to turning phase (ter Matt., 1987). The Ring neuron also modulates the columellar muscle which control shell turning during egg-laying (Fig. 7).

Simultaneous hermaphrodite species need to regulate separate male and female function and in *L. stagnalis* this is thought to be a competitive inhibitory process. The Ring neron contains APGW (Croll and van Minnen, 1992) and there is evidence that APGW plays a key role in female suppression. Application of APGW hyperpolarizes the CDCs (Croll *et al*., 1991) and prevents their after-discharge, which is necessary for the release of CDCH and the initiation of egg-laying. Other peptides such as lys-conopressin and FMRFamide also inhibit CDC discharges and could also be involved in female suppression (Brussaard *et al*, 1991; van Kesteren, 1995a).

Although hormones are known to be involved in stylommatohoran reproductive development, to date, no chemical factor for ovulation or oviposition has been identified. However, using antibodies raised against alpha-CDCP, one of the neuropeptides encoded on the egg-laying hormone gene of *L. stagnalis,* van Minnen *et al*., (1992) identified immunoreactive neurons in two species of Stylommatophora - immunoreactive neurons in the cerebral ganglia of *Limax maximus* and immunoreactive neurons and/or fibres in the female part of the reproductive tract of *Limax maximus, Biomphalaria glabrata and Aplysia calificornica*

About 800 immunoreactive neurons were identified in the parietal ganglia and 60 cells in the cerebral ganglia of *Helix aspersa*. As the genes of Egg Laying Hormone (ELH) are well conserved among the gastropod species, this data suggests the parietal ganglia as a putative source for the egg-laying hormone in *H. aspersa*. Ovulation and ejaculation in *H. aspersa* appears to be under neural control (via fine branches of the intestinal nerve) with ACTH and seratonin acting as excitatory transmitters and FMRFamide acting as a relaxant (Geoffroy *et al*., 2005).

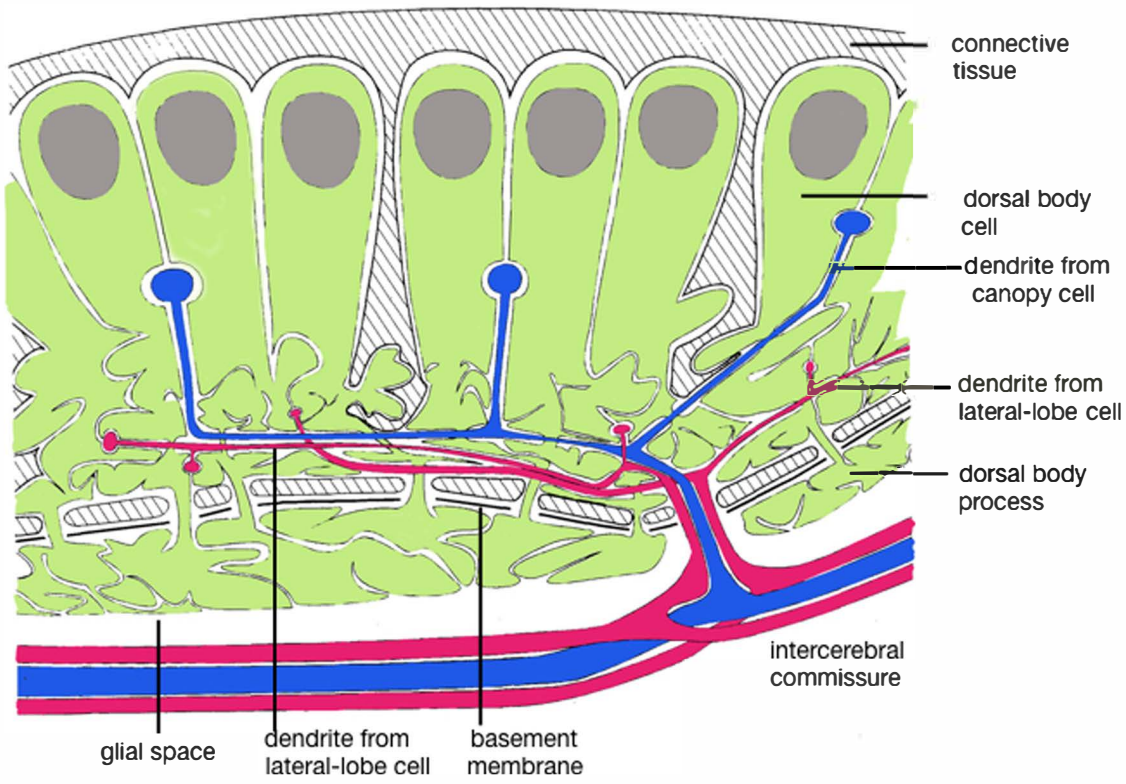


Fig. 8. Diagramatic representation of neurites from the CC and the LLC innervating the DB-cell bodies and the DB processes.

Note the DBPs pass through the connective tissue and the basement membrane into the intercerebral commissure. From Saleuddin, et al., (1996)

Dorsal Body Cells

Using classical extirpation and implantation techniques on *Lymnae stagnalis* Geraerts and Joosse (1975) demonstrated the dorsal body cells DBCs produce an endocrine factor, the dorsal body hormone (DBH) that stimulates vitellogenesis and growth of the female accessory sex organs. Exterpation of the DBCs resulted in no egg production in juveniles and low egg production in adults. Vitellogenesis was blocked, but spermatogenesis continued normally. Reimplantation of DBCs restored female function. That growth of the female accessory sex organs is entirely dependent on the presence of the DB, was clearly demonstrated by extirpation and implantation of DB in juvenile snails. Experiments by Dogterom *et al*.,1993, showed that DBH stimulates the follicle cells in the ovitestis.

Wijdenes and Runham (1976) found DBCs of the slug *D. reticulatum* were involved in oogenesis and growth and synthetic activity of the female accessory sex organs.

The precise nature of DBH is still unknown. Experimental results suggest either two hormones are produced by the DBCs - a protein and a steroid hormone or DBH is a steroid hormone together with its binding protein. It is suggested that the steroid may be ecdysone

Although neuronal axons were identified on the surface of DBCs (Nolte, 1966; Kuhlman; 1966 van Mol, 1967) synaptic junctions were not identified.

However, in 1987 Wijdenes *et al*. confirmed and described the nature of the nervous innervation in *H. aspersa*. Axons innervating the DBCs where shown to originate from the peptidergic cerebral green cells (Ce-GC). Single axons or small nerves from these cells establish synapse-like structures (SLS) with the DB cells. There is no basal lamina at these contacts, the membranes of axons and cells being only separated by a cleft. The innervation of DBCs has been described in detail in several stylommatophora, (see Flari and Edwards), but significantly, also in the basommatophoran *Helisoma dury*i. Saleuddin, *et al*. (1996) found the DBCs were innervated from three large neurosecretory cells, (one canopy cell and two lateral-lobe cells) from the right lateral lobe of the cerebral ganglia. Neurites from the canopy cell innervate the cell-bodies whereas those from lateral-lobe cells innervate the cell-processes of the DBCs (Fig. 8). Two or more types of axon innervate the DBCs tempting the suggestion of stimulatory and inhibitory control. There is certainly evidence for inhibitory control - in tissue culture experiments, denervated DB of *H. aspersa* become hyperactive and release their secretory product (Wijdenes *et al*.1983). Also, DBCs in vivo are inhibited by the peptide FMRFamide. FMRFamide has been found in some Ce-GCs, but the stimulatory factor is

unknown.

Gonads

There is no evidence that gonadal hormones play a role in reproductive development in the Hygrophila; a fundamental physiological difference to the situation in the Stylommatiophora.

Castration in limacid and arionid slugs causes the reproductive tract to remain undeveloped (Abeloos, 1943; Laviolette, 1954a; Runham *et al*.1973). In vitro tissue culture experiments by Baily (1973) suggested the presence of cerebral gonadotrophins in the CNS of *D. reticulatum*. Gonads and reproductive tracts were cultured together in various combinations: the brain-tentacle complex produced a hormonal factor necessary for successful male gametogenesis, but all three organs needed to be cultured together for successful differentiation of the prostate gland.

Sokolove, *et al*. (1984a) placed castrated *L. maximus* in long day conditions (conditions that normally trigger reproductive maturation) and found their reproductive organs didn't grow or develop, indicating the necessity of a functional gonad. Homogenates of cerebral ganglia from photoperiodically enhanced slugs didn't stimulate 3H-thymadine incorporation into the accessory sex organs of castrates (an increase in 3H-thymadine incorporation is an indicator of increased cell division). Further experiments by Sokolove *et al*. (1987) with *L. maximus,* confirmed the action of cerebral gonadorophin, but as yet, no gonadal hormone has been isolated, identified or characterised in any terrestrial mollusc.

Optic tentacles

There is evidence from tissue culture experiments that the optic tentacles of *Arion subfucas* produce an androgenic hormone, during the male phase of sexual development. It stimulates sperm production, and inhibits the differentiation of the eggs and female accessory sex organs and egg-laying, Wattez, (1978).

Experiments by Takeda *et al*., suggested the collar cells as the source of this hormone, but an ultrastructural and immunocytological study by Magdelaine *et al*., (1990), indicate this is very unlikely. The collar cells are highly glandular, producing a secretion that that passes via small ducts to the chemosensory epithelium that covers the tip of the tentacles in Stylommatophora.

They do not contain neurosecretory granules.

Runham and Edser (1983), suggest the secretion is likely to be involved in smell detection and make the point that if the collar cells produce a hormone it would require reabsorption through the sensory epithelium, back into the circulation -a highly unusual route for a hormone.

In the slug *Limax*, Sokolove *et al*., (1984) showed that long day photoperiods promote the maturation of both male and female organs. This occurred in long days even if the tentacles (and eyes) were removed, suggesting the optic tentacles are not required for maturation and that day length is perceived by an extra-ocular pathway.

The control of body growth

The light green cells are giant neurons (90μm) located in the cerebral ganglia in two paired groups of about 100 cells in each ganglion, which release their peptides into the blood via the paired median lip nerves (Joose, 1964; Wendelaar Bonga, 1970). Using classic endocrinological extirpation and implantation experiments, Geraerts, (1976a) was able to prove that the LGCs regulate the growth of the soft body parts and the shell.

These medial cells were known to be involved in the control of growth in *D. reticulatum* (Wijdenes and Runham 1977). Body growth stops when these cells are destroyed and the differentiation the reproductive organs is delayed. These authors suggest the neurosecretory medial cells may produce a growth hormone with a specific somatotrophic effect. These cells are considered homologous with the cerebral light green cells (LGC) of *L. stagnalis* (Geraerts 1976) which have similar staining properties, are located in homologous areas, and produce a growth hormone.

The LGCs produce molluscan insulin-related peptides (MIPs) that are encoded by a family of 5 related genes (MIP genes) (Smit *et al*., 1998). cDNA cloning and peptide characterization demonstrate that the LGCs express five structurally diverse peptides, MIP I-II, V-VII (amino acid sequence of MIP1 shown in Table 1). Although the population of LGCs express all five of the functional MIP genes (MIP IV and VI are pseudogenes) individual LGCs appear to contain only subsets of the gene products, suggesting that there may be functional

differentiation of the MIP peptides. (Geraerts et al. 1991).

A second endocrine centre involved in growth regulation is located in the lateral lobes (LL) of the cerebral ganglia. Cauterization of the LLs results in giant growth, whereas re-implantation of the cerebral ganglia with the lateral lobes restores normal growth showing that the LGCs are under inhibitory control by the LL (Geraerts *et al*, 1976 b). The single large neuron, the Canopy Cell (CC) present in each of the lateral lobes appears to be an ectopic LGC, expressing the MIP peptides, rather than being responsible for having inhibitory effects of the LL (Benjamin *et al*., 1976).

There is evidence that another type of peptide-expressing neuron performs this LL inhibitory function. The LFRFamide precursor mRNA (Hoek at al, 1997) is expressed in three neurons in the LL and all five of the LFRFamide peptides have been extracted from the LL and identified by mass spectrometry (Hoek et al, 2005). When any one the LFRF peptides (Table 1) e.g. GGSLFRFamide is applied to the LGCs *in vitro*, firing of the LGCs is inhibited. A rapid hyperpolarizing response is recorded on the LGCs due to the opening of K^+ channels (Benjamin & Kemenes, 2018).

It is not known for certain what physiological factors stimulate the release of MIP peptides to cause growth but *in vitro* electrophysiological experiments show that the LGCs respond to a variety of chemical messengers. These messengers include glucose (present in the blood), monoamines such as dopamine and several different types of peptides.

There is evidence suggesting that the peptide lys-conopressin could be part of the mechanism for stimulating LGC MIP release. The receptor for lys-conopressin is expressed in the LGCs and application of this peptide drives spiking activity in isolated LGCs (van Kesteren et al, 1995) by increasing their excitability.

Another peptide, schistosomin, is present in the LGCs (immunocytochemistry, Hordijk, et al, 1991) and application of the peptide *in vitro* caused an increase in LGC excitability and the induction of firing by slow depolarization (Hordijk et al, 1992). It is presumed to play a role in controlling growth in non-parasitized animals, but the details are unknown (Benjamin and Kemenes, 2019)

Ion and water regulation

The pond snail *L. stagnalis* needs to maintain its body fluid at a higher osmolarity than the surrounding environment. It achieves this by the uptake of ions and the excretion of dilute urine from the kidney. De Witt & van der Schors, (1986) injected extracts of SIS-containing neurons into the haemolymph of intact snails and demonstrated that a sodium influx-stimulating peptide (SIS) promotes Na+ ion uptake across the integument, from the ambient medium.

Immunocytochemistry and in situ hybridization showed the SIS peptide is expressed in the yellow cells (YC) in the parietal and visceral ganglia (Boer et al., 1992). There are about 25 YC cell bodies scattered in the visceral and parietal ganglia with a few others located in the proximal visceral nerves (Swindale & Benjamin, 1976)

Most YCs release SIS hormonally into the blood from fine neuritic branches that penetrate the vascular connective tissue surrounding the central ganglia (Wendelaar Bonga, 1970). The majority of the SIS peptide is released from these neurohaemal areas into the head sinus where it acts upon the sodium pumps in the epidermal cells of the head (Benjamin& Ildiko 2018). However, there is a special identified group of YCs in the visceral ganglion (Swindale & Benjamin, 1976) whose axons project along the distal processes of the intestinal nerve to innervate a number of peripheral organs including the pericardium, pericardial canal and ureter (Boer et al., 1992).

Wendelaar Bonga, used quantitative analysis of YCs at the ultrastructural level to study changes in the release activity (1972) and showed peripheral release of peptides from YC terminals in these organs were increased when snails were subjected to a hypo-osmotic environment suggesting the SIS peptide also maintains blood Na+ concentration by reducing the loss of Na+ ions in the urine by peripheral reabsorption of Na+ ions in the cardiac-renal system. DGCs also showed release of peptides when snails were subjected to a hypo-osmotic environment suggesting the DGCs contain a diuretic factor. Earlier experiments by Hekstra and Lever (1960) and of Lever et al. (1961) supported the hypothesis that the DGC stimulate diuresis.

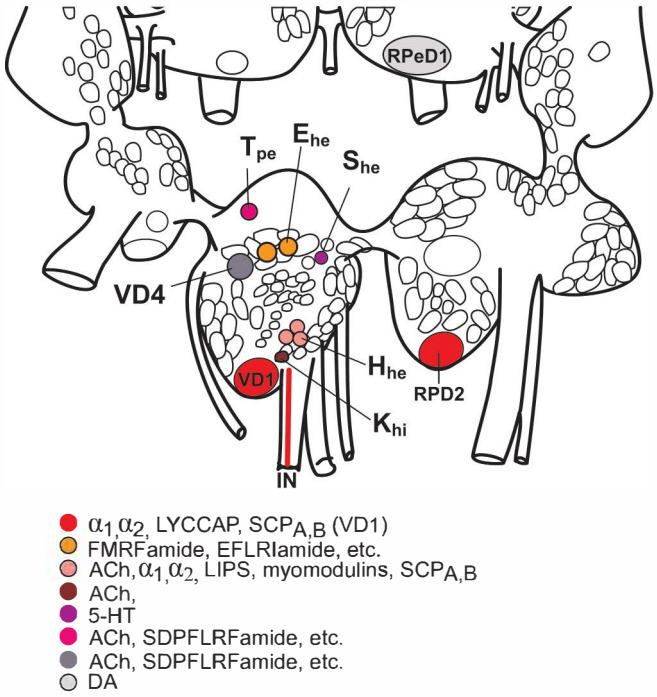


Fig. 9. Heart motoneurons.
From Benjamin and Kemenes, (2018)

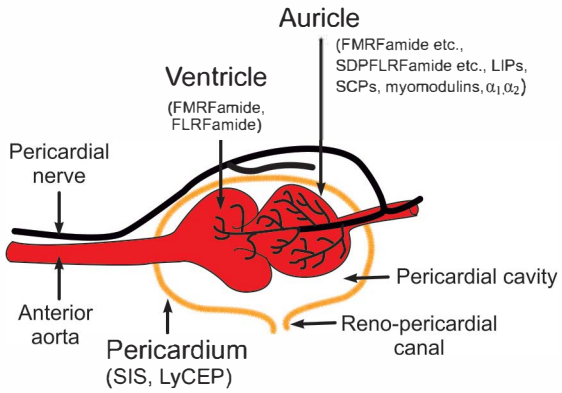


Fig. 10. Lymnaea heart.
From Benjamin and Hemenes, (2018)

Light yellow cells

Light yellow cells (LYCs) of *L. stagnalis* express a neuropeptide gene encoding three different peptides. A large cluster of LYCs (c40) is situated in the ventral lobe of the right parietal ganglion, with smaller clusters in the posterior dorsal part of this ganglion and in the visceral ganglion (c20). The cells have an extended central neurohaemal area and axons project into all nerves of the ganglia of the visceral complex, into the superior cervical and the nuchal nerves and into the connective tissue surrounding the central nervous system. LYC axon tracts also ramified between the muscle cells of the walls of the anterior aorta and of smaller blood vessels, with peripheral innervation in the muscular tissue of the ureter papilla (Boer *et al*.,1994).

These authors hypothesise that light yellow cells are involved in the control of body shape and blood pressure, regulation that is important in behaviours that involve movements of large volumes of fluid within the animal such as eating, copulation and egg laying, but the detailed mechanisms are not understood.

The Heart

The heart is myogenic, but the heartbeat is controlled by a network of 5 different types of centrally located motoneurons with diverse excitatory and inhibitory effects. These neurons are all located in the visceral and right parietal ganglia with axonal projections to the heart along the pericardial branch of the intestinal nerve (Fig. 9).

28 peptides were identified in heart and pericardial tissue arising from 8 different genes. The auricle is much more heavily modulated by peptides than the ventricle and only 1of the 8 peptide families, the Farp family (FMRFamide, FLRFamide), found in the heart have been found in the ventricle (Fig. 10).

A lot is known about the peptide effects on heartbeat but little is understood about known about they regulate cardiac function in the intact animal (Benjamin and Kemenes 2018).

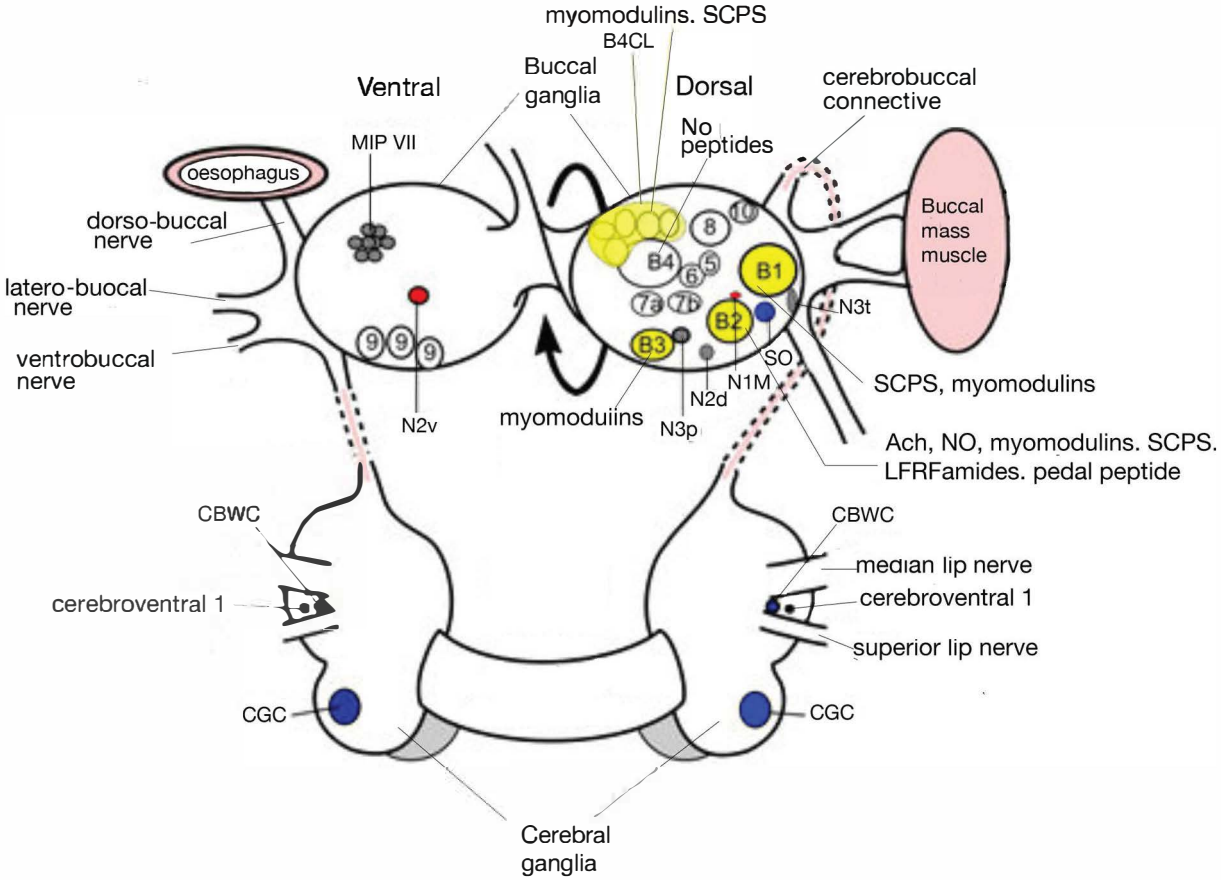


Fig. 11. Neuropeptide expression in neurons of the feeding system.

Peptide-containing neurons are colour-coded according to function.
Motoneurons - yellow, CPG interneurons - red, Modulatory interneurons - dark blue.

From Benjamin and Kemenes, 2018

Feeding

The feeding circuitry in *Lymnaea* is an example of a central pattern generator (CPG) interneuronal network that generates rhythmic feeding movements (Benjamin, 2008). About 100 neurons are known to be involved in generating feeding movements and they function as motoneurons, CPG interneurons and modulatory interneurons (Benjamin, 2012). These neurons are mainly located in the buccal ganglia but a number of interneurons in the cerebral ganglia are functionally linked to the buccal feeding network. All three of these neuron types contain neuropeptides as do their target organs the oesophagus and the buccal mass (Fig. 11). A total of 47 different peptides have been identified in the buccal ganglia arising from 12 different genes. The gaseous transmitter nitric oxide is produced by the B2 neuron (Park *et al*., 1988), but its role is unknown.

Large buccal motoneurons, the paired B4/B8s and the B4CL cells (up to six on each side) innervate buccal mass muscles that are directly responsible for the swallow and rasp phases, respectively, of the feeding ingestive cycle. The rasp phase of the feeding cycles appears to be modulated more than the swallow phase because the B4CL neurons contain myomodulin and SCP peptides but no peptides have so far been found in the B4s. The rasp phase of feeding is under more dynamic modulatory control because the strength of the bite varies according to the 'hardness' of the food substrate (Benjamin and Kemenes, 2012).

The N1, N2 and N3 CPG interneurons fire in sequence to generate the protraction, rasp and swallow phases of the rhythmic feeding cycle, respectively. The N2v has been shown to express the myomodulin and SCP peptides. None of the N3 cells have been shown to contain neuropeptides (Santama etal., 1994). Classical transmitters mediate the main synaptic effects of CPG interneurons, ACh in the N1Ms and glutamate in the N2s and so the peptides act as co-transmitters (Benjamin and Kemenes, 2012).

Peristaltic contractions of the gut are controlled by peptidergic motoneurons in the buccal ganglia. Extensive axonal projections from the paired B2 oesophageal motoneurons in the buccal ganglia run to all regions of the pro-oesophagus.

Compared to our knowledge of the neuroendocrine system and neuropeptides produced by the CNS of L. stagnalis, our knowledge in the Stylommatophora is limited. Not only are the neurons easily identified in *L stagnalis* and can be seen through the neural sheath, the cells identified with neurosecretory stains exist as distinct groups in well localized areas. In terrestrial gastropods a tough, often pigmented connective tissue surrounds the CNS and the neurosecretory cells in the pleural, parietal and visceral ganglia have a more scattered distribution than found in *L. stagnalis*. (compare Fig. 1 with Figs. 2 and 3). Also the endocrine dorsal bodies are distinct structures on the CNC of *L. stagnalis* , whereas in Stylommatophora, the form and distribution of the DBs and varies considerably and DB tissue may extend over the cerebro-pleural connectives on to the pleural ganglia as *D. reticulatum*. They are therefore more difficult experimental subjects and have been less extensively studied. The main reason, however, is the vast number of research scientists who worked on this one snail in the same laboratory over many years. Knowledge, experience and techniques could be shared as a complete picture of the biology of the animal was worked towards. This vast research resource was available to other scientists in other labs, making *L. stagnalis* an even more attractive research animal.

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General Introduction

This general introduction is to provide a context for the work presented here as papers and which was carried out between 1964 -1969.

At the time of this investigation slugs were a serious agricultural and horticultural pest, but little was known about their physiology, behaviour or ecology. It was thought a better understanding of their biology might facilitate better methods of control. This limited knowledge of slug biology made them both intriguing and potentially rewarding research subjects.

The commonest of all species of slug is probably the grey field slug *Deroceras reticulatum,* previously *Agriolimax reticulatus* (Muller), which is available in large numbers at most times of the year. It is small in size 3 to 4 cm when extended and breeds throughout the year producing about 300 eggs (Runham and Hunter 1970). It is also easily maintained in laboratory cultures. *D. reticulatum* was therefore chosen by Dr N.W. Runham as a research animal for a number of his PhD students, the idea being over time, to build-up a complete picture of the animal's biology.

My topic was the possible role of the central nervous system in the control of the animal's reproduction. As a pre-requisite a detailed study of the reproductive system, the central nervous system and the vascular system was undertaken. During this investigation a structure was discovered on the posterior pedal artery that had all the characteristics of an endocrine gland so this structure, provisionally named the arterial gland, was also investigated.

It has been stated that all endocrine secretions in molluscs, with the exception of cephalopods, are produced by neurosecretion (Higham and Hill, 1969). However in gastropods there is experimental evidence that the gonads, the dorsal bodies and the optic tentacles may be involved in the synthesis and secretion of hormones.

The Gonad

Laviolette (l954) studied the relationship between the gonad and the reproductive tract in terrestrial pulmonates. Following removal of the gonad in several species of arionoid and limacid slugs he found both the common reproductive duct and the male and/or female accessory 'sex organs underwent degenerative changes. When Laviolette transplanted gonads from mature slugs into castrated immature slugs, it resulted in maturation of the host reproductive tract, suggesting the involvement of a bloodborne gonadal hormone/s.

Dorsal Bodies

Lever (1958) was the first to suggest that Dorsal bodies have an endocrine function. They exist in all Hygrophila studied so far as discrete organs surmounting the cerebral ganglia. In the majority of Stylommatophora studied, they are not discrete organs, but diffuse groups of cells, intimately associated with the connective tissue of the brain, extending over the cerebral ganglia and down on to the pleural ganglia.
In Helix the dorsal bodies increase in size after injections of calcium or magnesium chloride (Nolte and Machemer-Rohnisch 1966) suggesting they are concerned with osmotic control (Kuhlmann 1966). Extirpation and implantation of dorsal bodies in *Lymnaea stagnalis* suggest that they produce a special factor which is necessary for vitellogenesis and for the growth and function of the accessory sex glands (Joosse and Geraerts, 1969; see also Joosse, 1964).

Optic tentacles

Pelluet and Lane (1961) were the first to suggest an endocrine function for the optic tentacles of terrestrial pulmonates, having experimentally demonstrated a clear feminisation of the gonad after removal of the tentacles in arionid slugs. They also proposed a dual hormonal control of gametogenesis, involving an optic tentacle hormone that promoted the production of male cells, and a brain hormone (later defined as the dorsal body hormone) that stimulated the female line of gametogenesis. Since then, several studies investigating a possible endocrine role for the optic tentacles have produced contradictory results.
Sanchez and Sablier found extirpation of optic tentacles in Helix aspersa had no effect on gametogenesis, as did Kuhlmann and Nolte (1967) using Helix pomatia. In contrast Meenaskshi and Scheer (1969) working with Agriolimax californicus found optic tentacle extirpation resulted in an increase in the weight of the albumen gland

and its galactogen concentration the effects of which were reversed following *in vivo* - injection of tentacular homogenates.
The majority of the evidence, however, indicates that the optic tentacles produce a factor that stimulates spermatogenesis and, simultaneously, inhibits the auto differentiation of female cells and oogenesis. It is also thought to regulate the growth and synthetic activity of female accessory sex organs (FASO) such as the albumen gland, and to be involved in the inhibition of egg laying.

Neurosecretory cells

Using the classic neurosecretory stains chrome-haematoxylin (CH) and paraldehyde-fuchsin (PF) several groups of Gomori-positive neurosecretory cells have been identified in most gastropods investigated while Gomori negative cells have only been established in a few species. For a review of the literature see Gabe, 1966; Simpson, l966a.
In the cerebral ganglia of pulmonates, groups of Gomori-positive neurosecretory cells occur at specific locations. Their secretory products are transported via axonal tracts to neurohaemal areas, where these neurohormones are released (Joosse, 1964; Rohnisch, 1964; Nolte, 1965)
The most detailed studies of neurosecretory phenomena have been on the cerebral ganglia of Lymnaea stagnalis, at the Zoology department of the Free University of Amsterdam. Gomori-positive and Gomori negative neurosecretory cells have been identified in the cerebral ganglia and their neurohaemal release sites located in the peripheries of the median lip nerve and of the intercerebral commissure, respectively (Joosse, 1964; Boer et al., 1968a).

Neurosecretory stains are useful for locating possible neurosecretory cells, but ultrastructural investigation of these cells is essential given the lack of specificity of the classic neurosecretory stains (see Bern and Knowles, 1966). So far only a limited number of ultrastructural investigations have been carried out on pulmonates (Nolte, 1965; Boer et al., 1968a; Simpson et al., 1966b) as well as the opisthobranch *Aplysia californica* (Rosenbluth, 1963a; Coggeshall, 1967).

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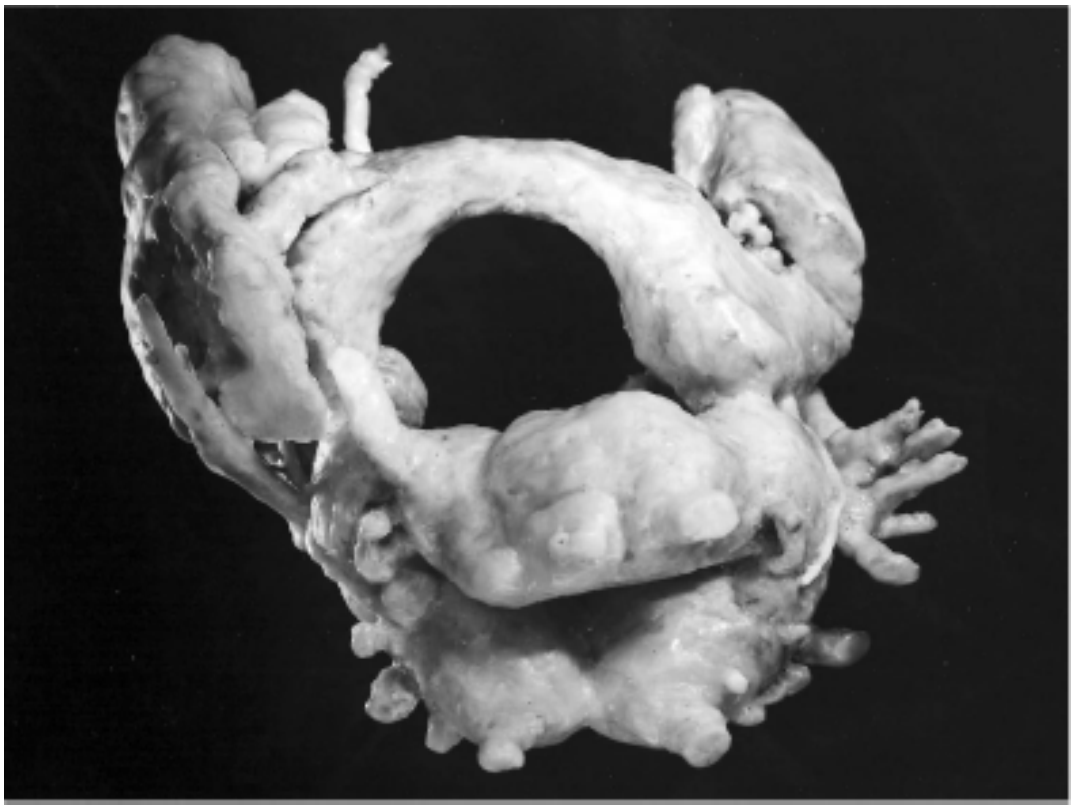
THE NERVOUS SYSTEM OF THE SLUG *DEROCERAS RETICULATUM* (PULMONATA, LIMACOIDEA)

I. The Anatomy of the Central Nervous and Vascular Systems.

SUMMARY

The Anatomy of the CNS, distribution of nerves and the general plan of the vascular system in *Deroceras reticulatum* is described. The CNS of consists of paired cerebral, pleural and parietal ganglia and a single visceral ganglion, as a double nerve ring around the oesophagus, with paired buccal ganglia situated between the oesophagus and the buccal mass. The cerebral ganglia are connected by the intercerebral-commissure, and to the pedal and pleural ganglia by ventral connectives. A sub-cerebral commissure, connects metacerebra, passing beneath the ventral surface of the cephalic artery. A certain amount of fusion has occurred between the pleural, parietal and visceral ganglia, and connectives as such do not exist between them.
The CNS is supplied with blood by interconnected vessels, allowing blood to reach a particular ganglion from different sources. It is suggested that a shunt system could route blood to where and when it was most needed. A capillary system within the central ganglia was not found. Commissural nerves ramify in the connective tissue above the cerebral-commissure in an area rich in capillaries and blood spaces. Muscle fibres in the walls of arteries and arterioles may facilitate control of blood flow. There is considerable innervation of the aortic stem, anterior and posterior aortae and cephalic artery. These nerves terminate at neuro-muscular junctions and probably play a role in the regulation of blood flow.



Model of the nervous system of *Deroceras reticulatum*

INTRODUCTION

Despite much renewed interest, the role of hormones in gastropod physiology, particularly their role in the control of reproduction, remains little understood. In 1964 it was decided to investigate this process in the grey field slug, *Deroceras reticulatum*.

Such behavioural activities as copulation and egg laying take place rapidly when compared with the maturation of the reproductive tract, which in *D. reticulatum* takes several months (Runham and Laryea, 1968). By analogy with other animals it is likely that 'fast' processes such as the former are mainly under direct nervous control, while 'slow' processes such as maturation are mainly controlled by an endocrine /neuroendocrine system. Experimental evidence certainly exists suggesting that both types of control play a role in gastropod reproduction (Laryea, unpublished). Characteristically the invertebrate nervous system plays a controlling role in endocrine regulation - itself producing hormones. Classical endocrine glands are, by comparison with the vertebrates, rare.

A detailed study of the nervous anatomy of *D. reticulatum* was a prerequisite to any experimental work. The general circulation was also studied because a close topographical relationship exists between much of the nervous tissue and the blood vascular system. The arterial supply to the cerebral ganglia was investigated in detail.

The optic tentacles and the mouth lobe glands, structures suspected of hormonal activity that have a close relationship with the nervous system, were also studied. Cook (1966) has compared the morphology of the central nervous system of *Succinea putris* with that of *Helix pomatia*, while a recent publication by van Mol (1967) deals with the physiology of the pulmonate cerebral ganglion. Much of the literature relevant to the stylommalophoran central nervous system is discussed in these works and is not therefore discussed to any great extent in this paper. The nerves of *Helix pomatia* (Schmalz, 1914; Bang, 1917; Kunz, 1917 a) and the arteries of *Helix pomatia* (Schmidt, 1916) and *Lymnaea stagnalis* (Carriker, 1946) have been described in detail and the terminology used in this paper has been adapted from these descriptions.

The histology of the central nervous system is reported in subsequent papers.

MATERIALS AND METHODS.

Slugs were killed by injection with fixative (Susa, Carnoy or Bouins) and then either the whole animal or the entire head region was transferred to fresh fixitive — Susa, 6 - I2 hours, Carnoy 18 hours, Bouins 24 hours. After two changes of Cellusolve 12 - 24 hours each, (Bouins fixed material was first washed in alcohol), the material was embedded in ester wax (Steedman 1947) and serially sectioned at 5µm or 10µm. In addition optic tentacles were serially sectioned longitudinally and mouth lobes and associated glands horizontally. Sections were stained in: Azan, Periodic acid - Schiff /celestine blue /iron alum (Casselman 1962), Bromophenol blue (Mazia *et al*., 1953), Heidenhain's haematoxylin, silver nitrate (Rowell 1963) and Alcian Blue (Zugibe *et al*., 1959).
Fresh frozen sections were stained with Sudan black B (Casselman, 1962).

For examination of the nervous system *in situ*, animals were killed either by immersion in propylene phenoxetol for 3 - 5 minutes, or by immersion in liquid nitrogen for 5 - 8 seconds. Addition of 70% alcohol, methylene blue and water in various combinations aided the dissection. The innervation of organs found on dissection was checked in serial sections.

A large reconstruction of the central nervous system was made as follows. Complete series of brain sections at 5 µm were photographed, and the negatives enlarged to give prints 160 x the original section size. The photographs were then stuck on cardboard to give a total thickness per photograph of 0. 8 mm (160 x 5 µm).
Outlines of the nervous elements in the photographs were then traced on to transparent plastic sheets. The pieces of the nervous system were then cut out of the photograph, and using the tracings to orient them, were stuck together to make a solid model of the central nervous system. The model was used as an aid to investigate the anatomy of the central nervous system.

For examination of the arterial system both anaesthetised and unanaesthetised slugs were used. Anaesthesia was produced either by immersion in a 10% solution of urethane in 0.6% saline (Chetail, 1963) for five to ten minutes, or by being placed in a closed pot on a tray above a small lump of solid carbon dioxide for five to ten minutes (Bailey, 1969). The heart and aorta were then exposed under saline and the needle of a syringe containing

diluted carbochrome ink (Gurr) pushed through the mantle into the heart and down the aorta. The ink was injected into the system by gentle pressure on the syringe, back flow being prevented by clamping the mantle tissue around the needle with forceps, or in some cases by a ligature around the aorta. The injections were discontinued either when the ink began to flow into the sinus system, or when the finer arteries began to burst. In addition, diluted carbochrome ink was injected into the haemocoel of anaesthetised slugs. After recovery slugs were killed at different time periods and were examined. Brains were removed from suitably injected animals, fixed in Susa, embedded and serially sectioned as above. The venous system was investigated by prolonged injection of the arterial system, by injection through the foot into the haemocoel and by back injection through the aorta into the heart.

OBSERVATIONS

The Anatomy of the Central Nervous System

The central nervous system of *D. reticulatum* consists of paired buccal, cerebral, pleural and parietal ganglia and a single visceral ganglion. The buccal ganglia (Fig. 1) lie between the oesophagus and the buccal mass; the other (central) ganglia occurring posteriorly as a double nerve ring around the oesophagus (Fig. 2). In life the ganglia are usually a pale cream colour although they sometimes appear translucent. The ganglia are sheathed in connective tissue which contains varying amounts of pigment granules. A connective tissue membrane dorsally pigmented, surrounds the buccal mass and extends between some of the nerves. It is probably homologous to the *membrana capito - cerebralis* described by Kisker (1924).

The buccal ganglia (Fig. 1) are slightly flattened ovoid bodies of approximately equal size and are interconnected by the buccal commissure. Cerebro - buccal connectives arise laterally from these ganglia and join them to the cerebral ganglia. All the nerves arising from the buccal ganglia are paired and innervate the buccal mass, the salivary glands and a major portion of the digestive tract (Table 1).

The cerebral ganglia (Fig. 2) are lobed symmetrical bodies and lie on, and slightly to one side of the oesophagus. They are connected to each other by the thick intercerebral - commissure, and to the pedal and pleural ganglia by the ventral connectives. Each cerebral ganglion consists of a large antero - lateral procerebrum and a much larger metacerebrum,

a morphologically distinct mesocerebral lobe being absent. A very thin commissure, the sub - cerebral commissure, connects metacerebra, passing beneath the ventral surface of the cephalic artery. No nerves arise from this commissure. There is a small conical anterior projection of the procerebrum and this probably represents the remnants of the cerebral tube (see Cook, 1966 and van Mo1, 1967 for a complete list of references).

A so - called medio - dorsal body occurs on the medio - dorsal surfaces of the cerebral ganglia and the dorsal surface of the intercerebral - commissure. It is usually separated from the neuronal and glial elements by varying amounts of connective tissue. The widespread occurrence of dorsal bodies within the Stylommatophora is now recognized (Nolte, 1965; Cook, 1966; Kuhlmann, 1966, and van Mol, 1967), the early literature was mainly concerned with its occurrence in the Basommatophora (Lever, 1958b and Joose, 1964). The dorsal body sometimes appears a dirty white colour but due to pigment contained in the underlying connective tissue often appears light to dark brown. The size and structure of the dorsal body shows considerable individual variation. In of *D. reticulatum* identical tissue to that composing the dorsal body occurs, not only on the cerebro - pleural connectives of some specimens but also on the pleural ganglia.

The cerebral nerves are with one exception paired, the major nerves innervate sense organs, the base of the reproductive tract, the rostral artery and various muscles (Table II).
A certain amount of fusion has occurred between the pleural, parietal and visceral ganglia, and although the ganglia themselves are anatomically distinct, connectives as such do not exist between them (Fig. 7). This arrangement of the visceral complex of ganglia is termed zonitoid (Bergman, 1930). The pleural ganglia are attached to the anterodorsal surface of the pedal ganglia and are linked to the cerebral ganglia by the cerebro - pleural connectives.
The nerves of the visceral complex of ganglia innervate many different tissues and are described separately under the respective ganglion (Table III).

The two large disc shaped pedal ganglia which lie ventral to the visceral complex of ganglia are connected to the cerebral ganglia by the long thin cerebro - pedal connectives. The pedal ganglia which are of equal size are interconnected by anterior and posterior commissures and each carries a single statocyst on its dorsal surface. The number of nerves arising separately from the pedal ganglia varies. The most consistent arrangement is shown in Table IV and Figs. 4 and 5. The pedal nerves innervate the skin, body and foot musculature and the base of the reproductive tract.

Variation in the origin of nerves is common to all the ganglia but variation in area of innervation and number of nerves is most pronounced amongst the nerves of the pedal ganglion. The occurrence of neuronal somata along the course of the nerves was most pronounced in the nerves of the buccal and pedal ganglia. But for minor differences such as the non - fusion of optic and tentacular nerves, the arrangement of the cerebral nerves in *D. reticulatum* is almost identical to the generalised scheme described by van Mol (1967).

The remainder of the central nervous system is very similar to that of *Helix pomatia*. There are minor differences in the arrangement of the buccal nerves (see Schmalz, 1914; Bang, 1917; Kunze, 1917 a; Cook, 1966).

The pallial nerves are also similar in the two animals. Of the two aortic nerves present in *D. reticulatum* the one originating from the right parietal ganglion is homologous to the single aortae found in *Helix pomatia*. (Kunze, 1917 a).

While no attempt was made to homoligise the nerves of the pedal ganglia of *D. reticulatum* with those of *Helix pomatia*, they are not dissimilar, although there are four cutaneus pedal nerves in *D. reticulatum* and not three. The anal and intestinal nerves from the visceral ganglion are easily homoligised with the nerve analis and the nerve intestinalis respectively of *Helix pomatia*, while the cephalic retractor muscle nerve together with the second tentacle retractor muscle nerve represents the nerve *musculi retractoris pharyngealis* of *Helix pomatia*. In *D. reticulatum* the single muscle formed by the fusion of tentacular and pharyngeal retractor muscles is here termed the cephalic retractor muscle.

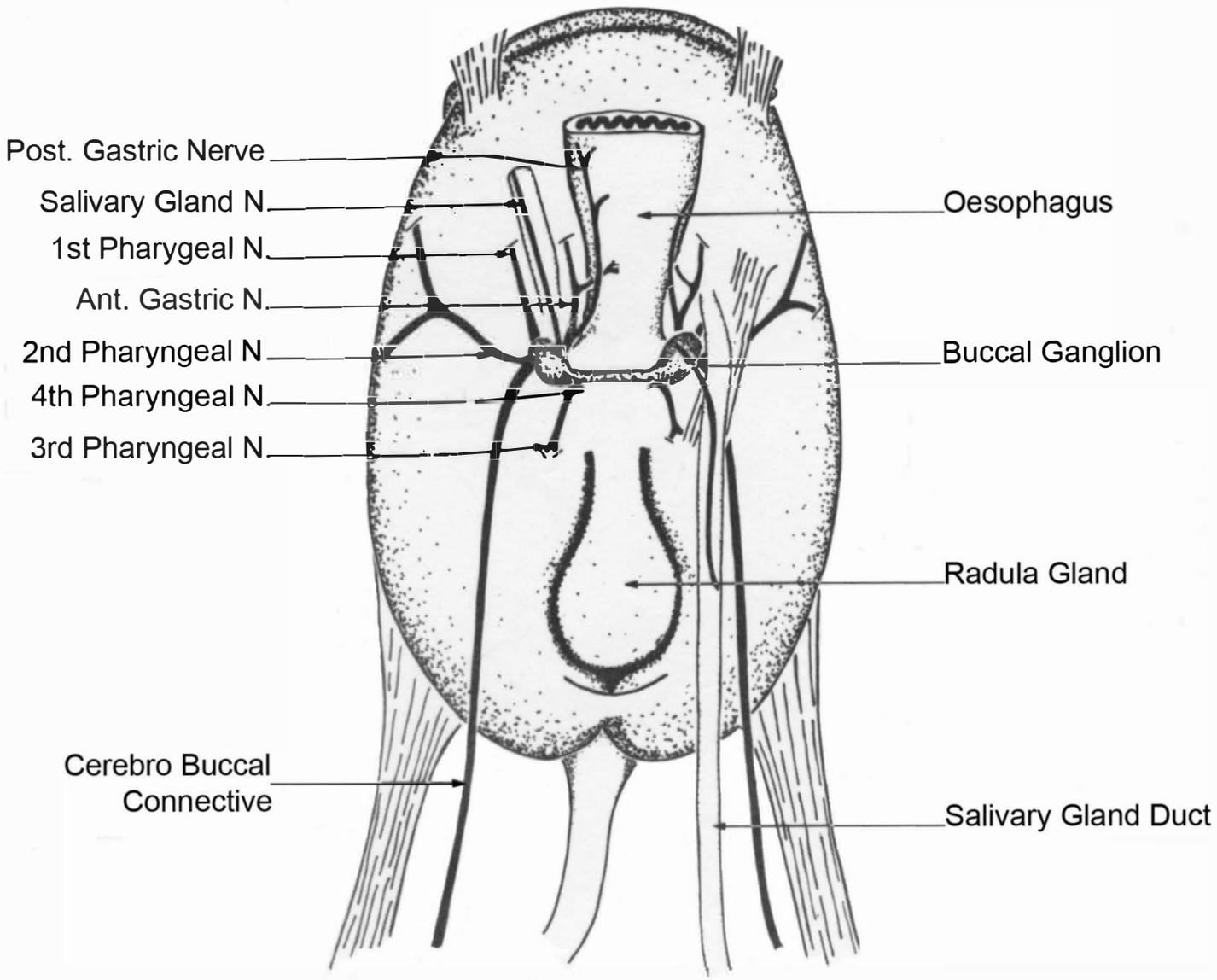


Fig. 1 The origin and distribution of the nerves of the buccal ganglion

Nerve	Origin from Buccal Ganglion	Area innervated	Notes
Anterior Gastric	antero-medial	Anterior region of oesophagus and small branches to the oesophagus adjacent to buccal gonglia	In some cases both anterior gastric nerves do not divide from the common root until some short distance from the ganglion.
Posterior Gastric	antero-medial	Posterior region of oesophogus and the crop. Major branches form a gastric plexus. Small nerves run from plexus to digestive glands and anterior part of stomach.	Runs posteriorly along lateral borders of oesophagus, left nerve giving off a ventral component; the lateral nerves divide into two on the anterior crop. Many anostamoses occur on crop but major nerves continue to stomach. Considerable gongliontion.
Salivary Gland	postero-dorsal	Salivary gland	Becomes bound to the salivary gland duct by connective tissue; following its course to the salivary gland.
Ist Pharyngea	antero-lateral	Antero-dorsal musculature of pharynx and buccal mass	In some specimens branches innervate anterior regions of the oesophogus, then the area innervated by the anterior gastric n. does not extend as for anteriorly as usual.
2rid Pharyrgeal	lateral, together with cerebro-buccal connective	Lateral and ventral musculature	Superficial; dividing into anterior and posterior of the buccal massbranches on lateral margin of buccal mass and then passes into deep musculature.
3rd Pharyngeal	poster~ventral near to origin of buccal commissure	Dorsal musculature of buccal mass in region of buccal ganglia. Small branch ramifies in musculature lateral to radula.	Runs posteriorly through surface musculature before branching, main branch then run anteriorly. Nerve cell bodies along course of this nerve are a constant and striking feature.
4th Pharyngeal	lateral and slightyl anterior to origin of 3rd Pharyngeal nerve	Musculature between oesopho-gus and anterior part of buccal mass	Thinnest buccal nerve. Passes into superficial musculature almost immediately it arises. On occasion originates from commissure.

Table I Origin and distribution of the nerves of the buccal ganglion

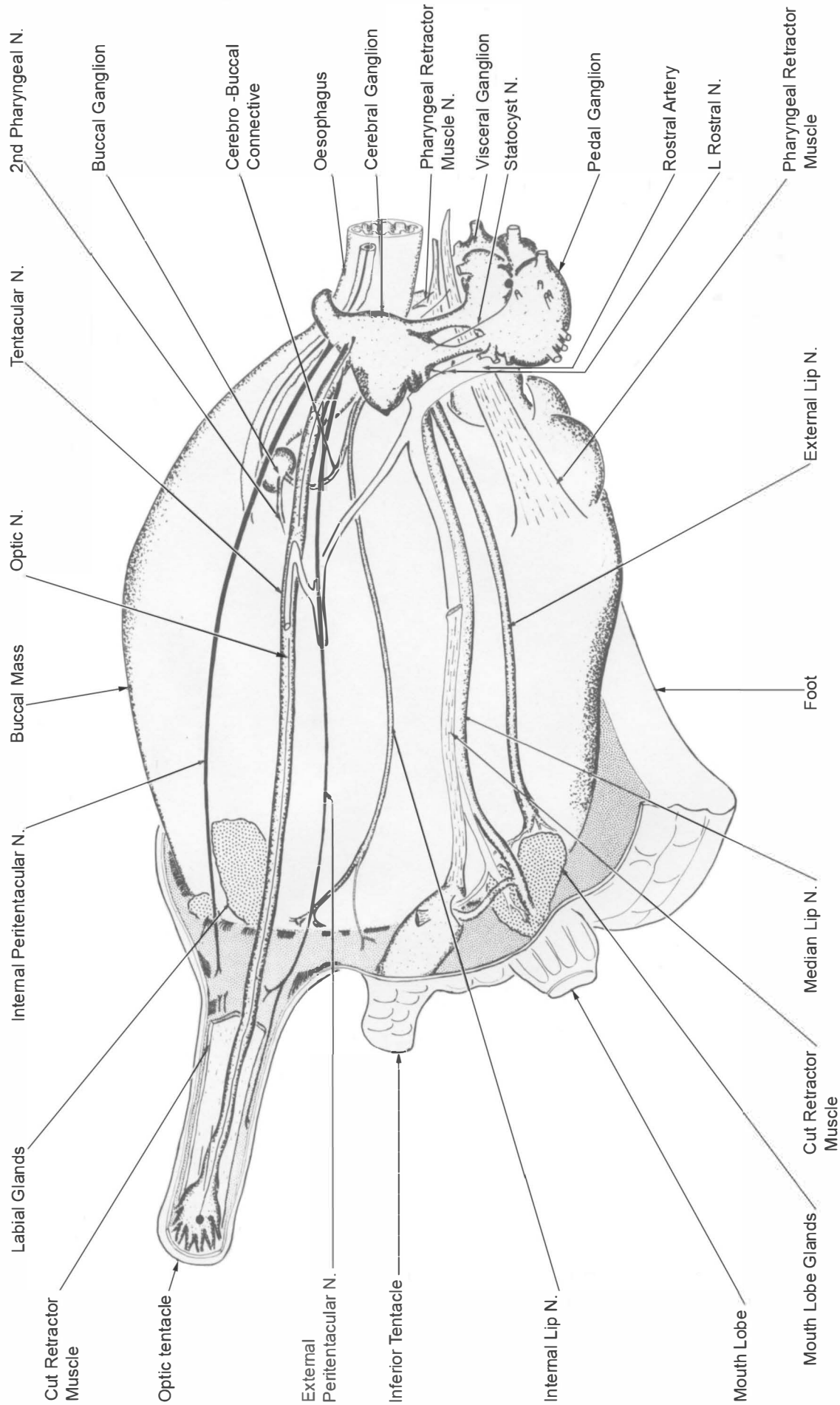


Fig. 2 Origin and distribution of the nerves of the cerebral ganglion

Nerve	Origin from cerebral ganglion	Area innervated	Notes
Internal peritentacular nerve	anterodorsal	Skin at the base of, and medial to the optic tentacle	Most medial cerebral nerve, running anteriorly in the *membrana capito-cerebralis* for most of its length.
Tentacular nerve	dorsoanterior	Forms digitate ganglion within optic tentacle, processes supply the tentacular epithelium and underlying musculature	Very thick, runs for major part of its length freely within optic tentacle. Two, sometimes three, very fine nerves originate from the tentacular ganglion to innervate the skin at tentacle base. These nerves are sheathed in a single layer of muscle fibres.
Optic nerve	slightly posterior to tentacular nerve	Retina	A very slender nerve bound to some of the fine muscle fibres attaching the optic tentacle retractor muscle to the cerebral ganglion. Infrequently it passes directly into the tentacle, but more commonly anastomoses for a short distance with the Ist tentacle retractor muscle n.
1st tentacle retractor muscle nerve	posterior to optic nerve near juncture of pro-cerebrum and meta-cerebrum	Adjacent optic tentacle retractor muscle	Runs in association with fine muscle fibres.
External peritentacular nerve	at base of, but sometimes as a branch of tentacular nerve	Medial and lateral regions of tentacle base, skin medial to optic tentacle	Attached to the outer labial artery for part of its length.
Commissural nerve	anterodorsal surface of cerebral commissure	No precise area of innervation determined but has close association with dorsal body cells and blood vessels above the commisure.	Quite a short nerve which divides into two or more branches.
Median lip nerve	anterolateral directly below pro-cerebrum	I.Forms digitate ganglion within inferior tentacle II.Columnar epithelium of mouth lobes via many small branches	Attached to inner labial artery and inferior tentacle retractor muscle, divides into two adjacent to inferior tentacle and mouth lobe glands. Branch II passes between mouth lobe glands. Aggregations of nerve cell bodies occur without significant enlargement of the nerve and continue along the branches which innervate the mouth lobe.
External lip nerve	together with but ventral to the median lip nerve	Ventolateral portions of lips and buccal musculature in this region	Forms small ganglion. There are accumulations of nerve cell bodies along the many branches into which this nerve divides.
Internal lip nerve	medial, anterior to and lateral to cerebro-pleural connective	Skin and musculature between optic and inferior tentacles and the mediodorsal parts of the lips	Thinner than the other two lip nerves. A small ganglion is present.
Penial nerve	either a branch of the median lip nerve directly posterior to its origin	base of the reproductive tract	Present on right side only. Anastamosis with 3rd cutaneous pedal nerve. Secondarily a cerebral nerve, its fibres pass through the cerebro-pleural connective and have their origin in the peal ganglion.
Statocyst nerve	Close to and lateral to the cerebro-pleural connective	Statocyst	Runs down between cerebropleural and cerebro-pedal connectives to the pleural ganglion following the contour of the ganglion to the statocyst.
Rostral Artery nerve	between the sub-cerebral commissure and the external lip nerve	Rostral artery	Very short, disappearing almost immediately it reaches the rostral artery.
Pharyngeal Retractor Muscle nerve	posterior, near tothe origin of the cerebro-pleural connective	Pharyngeal retractor muscle	Very thin, sheathed in muscle fibres. A branch from this nerve joins the base of the cerebro-buccal connective.

Nerve	Origin	Area innervated	Notes
2nd Tentacle Retractor Muscle	Posterior surface of pleural ganglion	Tentacular retractor muscle	
Left Pallial	Posterior surface of left parietal ganglion	I. Roof and left side of anterior free edge of mantle II. Anterior roof of pallial cavity	Penetrates body musculature anterior to pallial cavity
Right Pallial	Posterior surface of right parietal ganglion	I. Right side of mantle II. Lateral musculature of body wall along right margin of pallial region and posterior region of right side of anterior free mantle edge	
Cephalic Retractor Muscle	Usually a common origin from the posterior surface of the visceral ganglion continuing together in a connective tissue sheath for a short distance before separating	I. Cephalic retractor muscle II. Skin and muscle at its base	In some specimens branch II runs within the muscle to its base
Anal		I. Forms plexus in region of anus and pneumostome II. Rectum and rectal ganglia	Branches from rectal ganglia pass to rectal caecum and posterior intestine
Intestinal		I. Right salivary gland II. Aortic stem, anterior and posterior aortae, cephalic artery, mantle floor and intestine. III. Hermphrodite gland in some specimens	Anastamoses of fibres supply the intestine with components of the posterior gastric n. Runs along anterior and part of posterior aorta before supplying the hermaphrodite duct. Innervation of hermaphrodite gland could not always be established
Left Aortic	Posterior surface of visceral ganglion	Anterior portion of cephalic artery	Sometimes two nerves occur
Right Aortic	Posterior surface of right parietal ganglion		

Table III origin and distribution of the nerves of the pleural, parietal and visceral ganglia

Nerve	Origin from Pedal Ganglion	Area innervated	Notes
Ist Cutaneus Pedal	posterodorsal	Posterodorsal body musculature	
2nd Cutoneus Pedal	dorsal, directly posterior to statocyst	Body musculature below mantle	
3rd Cutoneus Pedal	lateral, posterior to cerebro-pedal connective	Left N - dorsal and lateral body musculature anterior to heart Right N - in addition to above, penis sac, genital atrium, free oviduct, base of spermatheca and possibly vas deferens	Nerve cell bodies within base of nerves on both sides
4th Cutaneus Pedal	anterodorsal	Lateral skin and musculature of head	
Ist Pedal	medial to 1st cutaneous pedal nerve	Posterior region of foot although small branches innervate more anterior regions	Often has small branch at its origin
2d Pedal	posteriorly and not infrequently below Ist pedal nerve	Middle region of foot	
3rd Pedal	lateral to 2nd pedal nerve	Foot in region of posterior pedal gland.	
4th Pedal	anterior to 3rd pedal nerve	Anterior to area innervated by 3rd pedal gland	Usually anastamoses with the 5th pedal n.
5th pedal	anterior to 4th pedal nerve	Foot in region of brain	short nerve
6th pedal	most anterior	Anterior to foot	

Table IV Origin and distribution of the nerves of the pedal ganglion

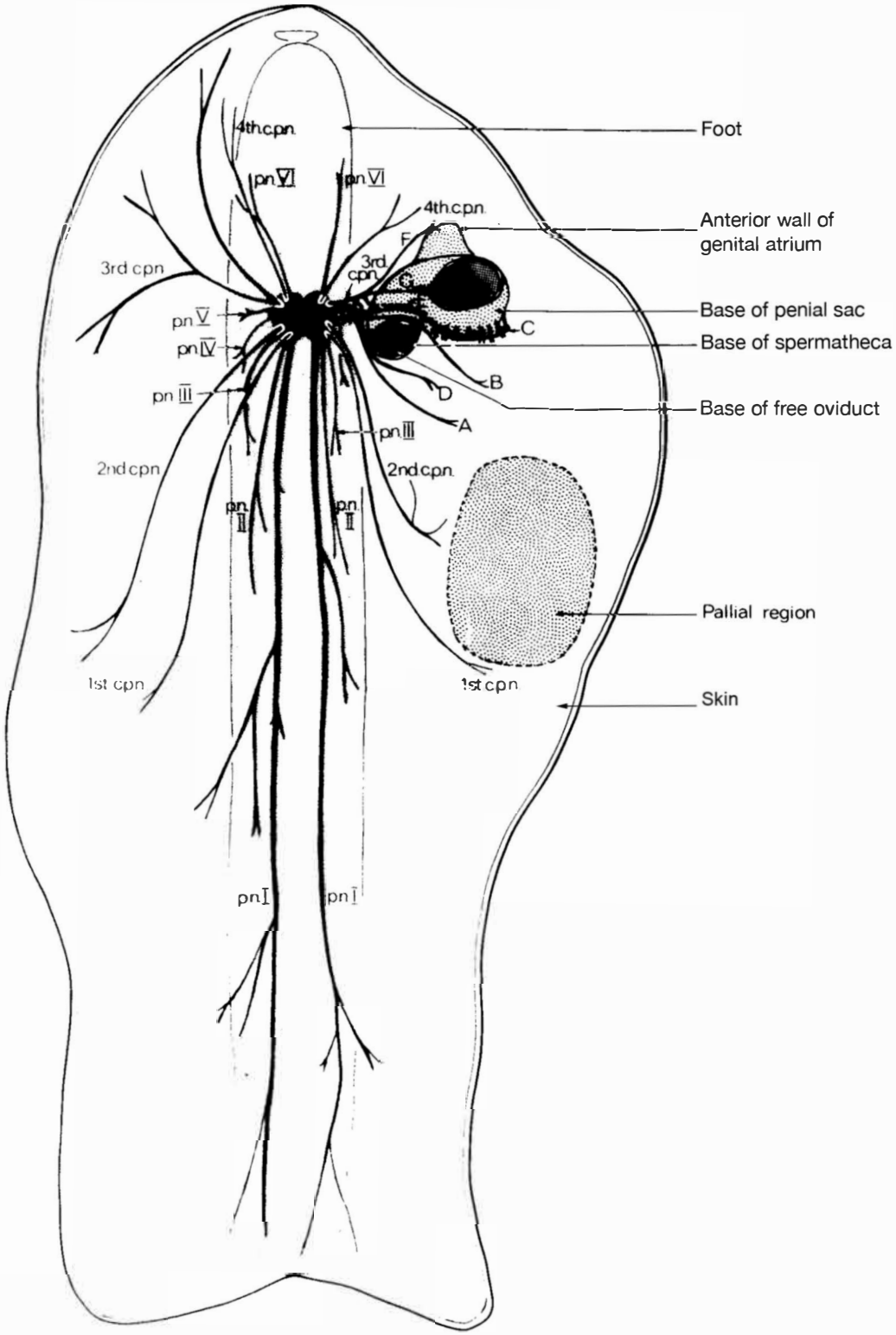


Fig. 4 Distribution of pedal nerves

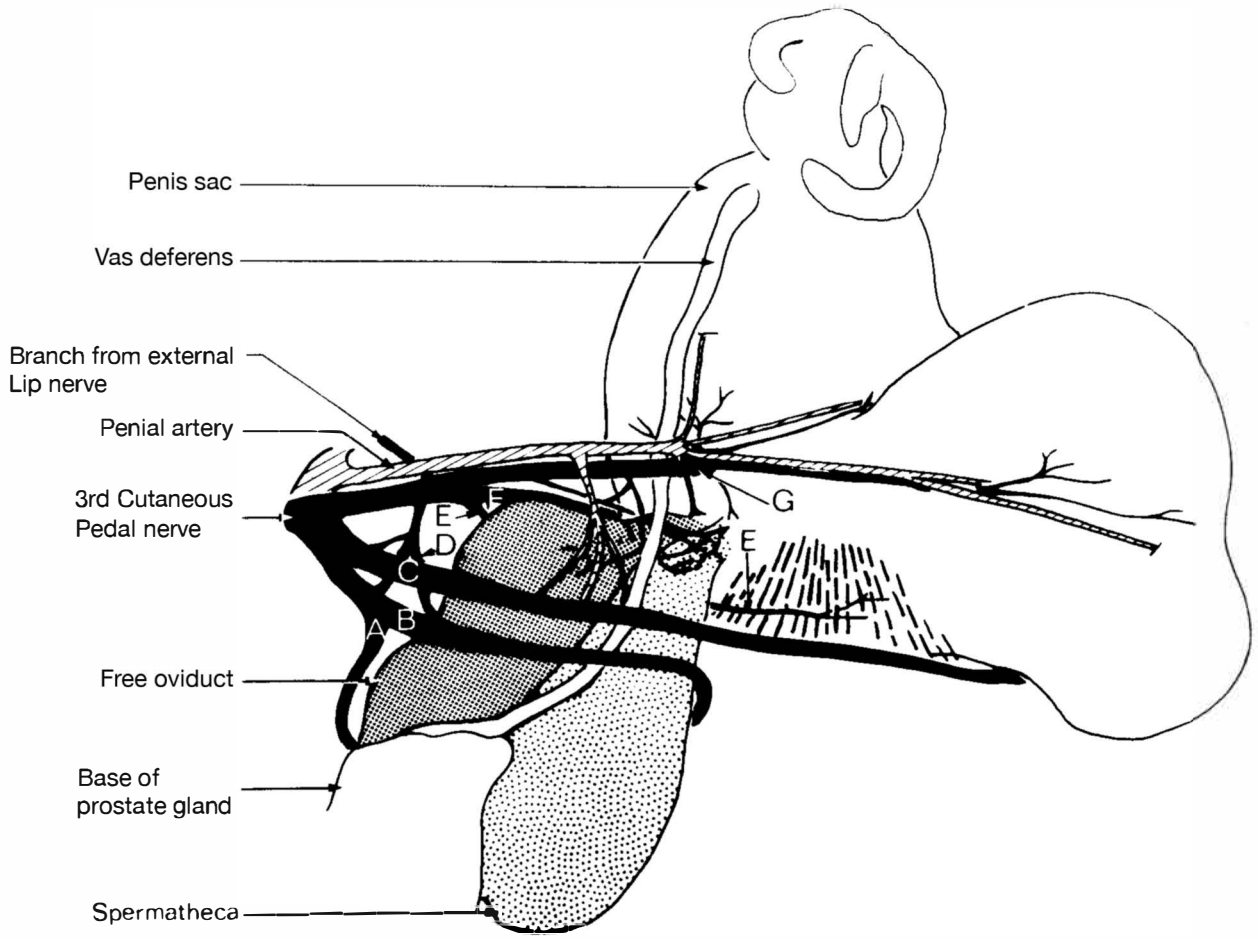


Fig. 5 Distribution of branches of the 3rd cutaneous pedal nerve to the reproductive system

Artery	Origin	Distribution	Notes
Ventral Pedal	From rostral a. near cephalic arborescence	Pedal ganglion - ventral surface	Origin of right vessel not as near cephalic arborescence as left.
Left Inner Dorsal Pedal		Left pedal ganglion medial portion of dorsal surface.	Right vessel not usually present.
Outer Dorsal Pedal		Pedal ganglion Left artery ateral portion of dorsal surface	
		Right artery - whole of dorsal surface. Fine branches supply posterior surface of right pleural ganglion.	Origin of right vessel not as near cephalic arborescence as left.
Left Pleural	From left outter dorsal pedal artery near base of cerebro-pedal connective	Left pleural ganglion - posteriolateral surface	Loops around anterior surface.
Right Pleural	From a branch of right posterior cerebral artery	Right pleural ganglion) postero- Right parietal ganglion) dorsal surface.	Vessels to right pallial nerve usually from this artery.
Left Parietal	Posterior continuation of left outer dorsal pedal artery	Left parietal ganglion - dorsal and ventral surface Left pleural ganglion mediodorsal surface Visceral ganglion - dorsal surface visceral nerve	Grooves between ganglion of visceral complex well supplied with arteries. Anstamosis with branch of right dorsal parietal artery
Right Dorsal Parietal	From right outer dorsal pedal artery	Right parietal ganglion - dorsal and posterior surfaces. Right pleural ganglion mediodorsal surface	Anastamosis with branch of right pleural artery
Right Ventral Parietal	posterior to right dorsal parietal artery	Right parietal ganglion -ventral surface	
Posterior Cerebral	From rostral artery near origin of median and external lip nerves from cerebral ganglion.	Cerebral ganglion - whole of posterior and posterolateral surface Cerebral commissure -posterior and dorsal surface	Large branch posses between cerebro-pleural and cerebra- pedal connectivesto anastamose with branch of anterior cerebral a. on anterio-medial surface of cerebral ganglia. Fine vessels run from dorsal surface of cerebral commissure into connective tissue above Passes under procerebrum
Anterior Cerebral	Dorsal to posterior cerebral artery	Similar to that of posteriocerebral artery but more extensive on cerebral commissure	
Medial Procerebral	From posterior cerebral artery near to its origin from the rostral artery	Cerebral ganglion - part of anterior surface of metacerebrum and medial surface of procerebum	

Table V Distribution of arterial system

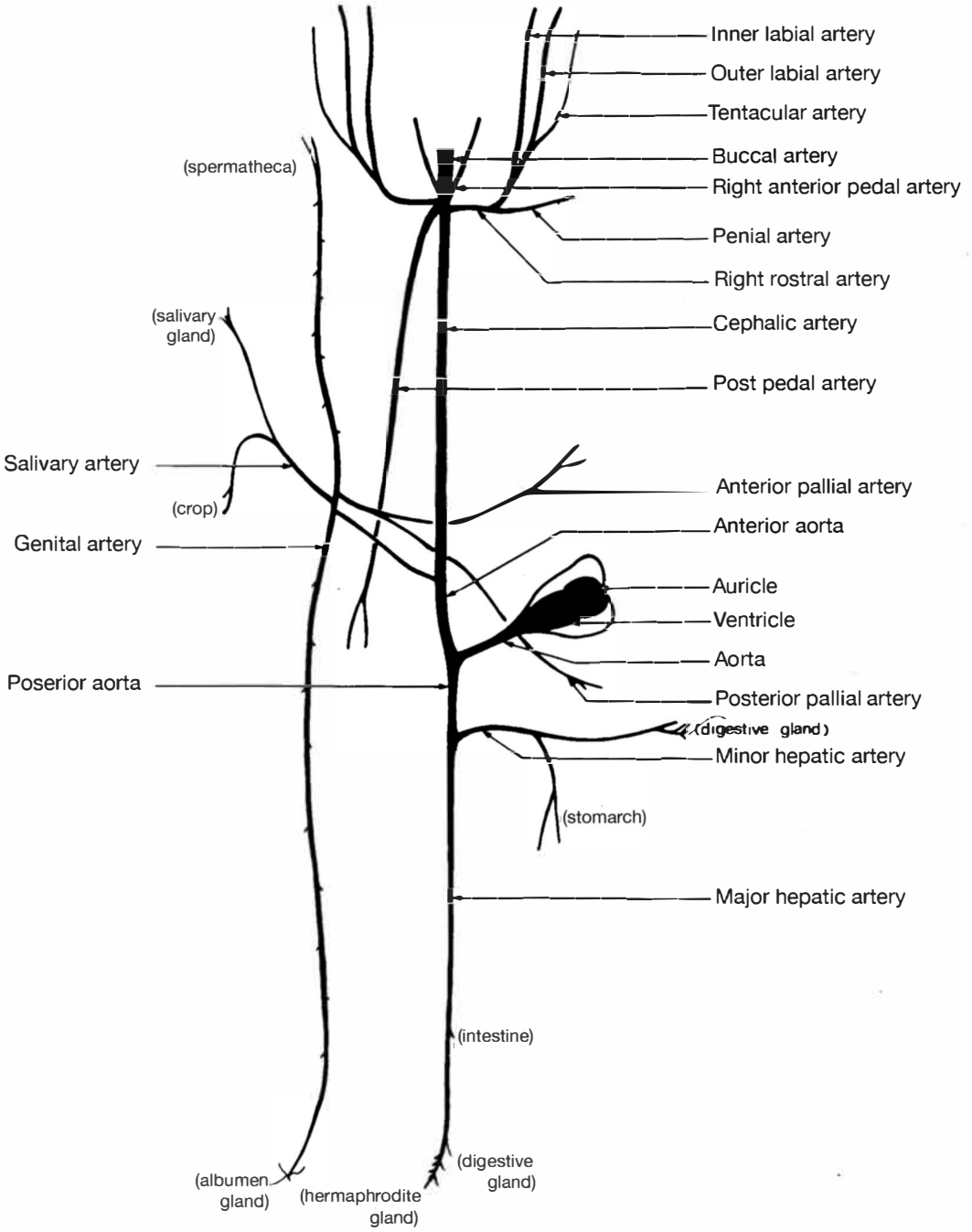


Fig. 6 Arterial System

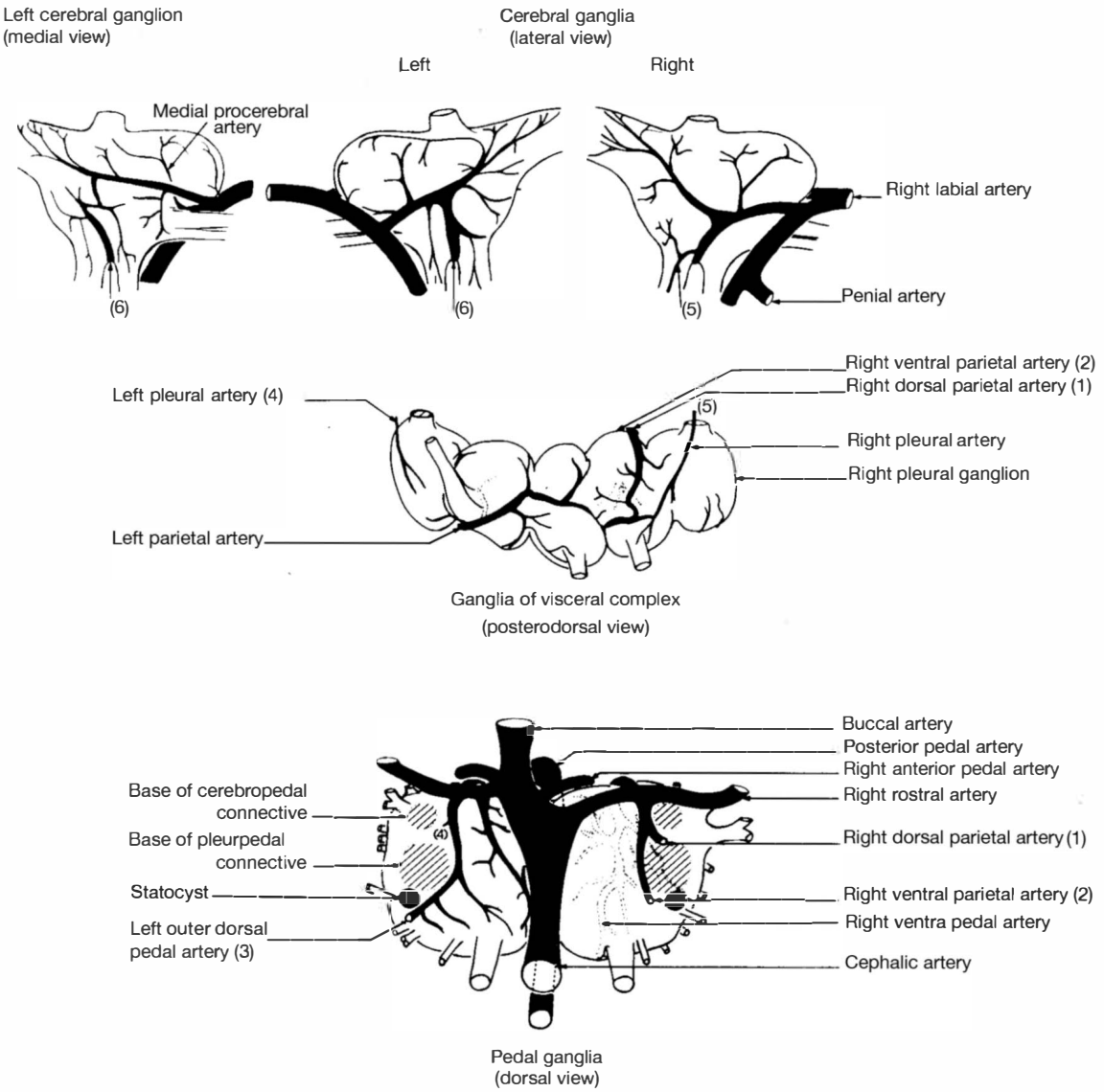


Fig.7 Arterial supply to the brain

Anatomy of the Circulatory System.

Comprehensive investigations of the pulmonate circulatory system have been carried out by a number of workers, (see Boer and Lever, 1959), although detailed information on the blood supply to the central ganglia is limited to *Ferrissia shimekii* (Boer and Lever, 1959) *Lymnaea stagnalis* (Joosse, 1964) and *Helisoma tenue* (Simpson, Bern and Nishioka, 1966b).

In this study the blood vessels have been arbitrarily designated artery, arteriole and capillary depending upon their size. The two-chambered heart is situated in the pericardial cavity which lies between two anterior lobes of the kidney. The short aortic stem arising from the ventricle curves around the prointestinal loop of the gut to divide into anterior and posterior aortae (Fig. 5).

The posterior aorta runs to the region of the stomach where it divides into the major and minor hepatic arteries; vessels from both supply the intestine. In addition the major hepatic arteries supply the posterior lobe of the digestive gland and the hermaphrodite gland while the minor hepatic artery supplies the anterior digestive gland and the stomach. No separate posterior genital artery as such exists.

The anterior regions of the body are supplied by branches of the anterior aorta. A short distance from its origin, this vessel gives rise to the much larger cephalic artery, and then continues to the surface of the common duct, where it gives rise to the genital, salivary and pallial arteries. Distally the cephalic artery passes between the pedal ganglia and the visceral complex of ganglia widening into the cephalic arborescence on the dorso - anterior surface of the pedal ganglia. Six major arteries arise from this arborescence and their origins are asymmetric (Fig. 6). A highly glandular tissue extends from the cephalic arborescence for varying distances along these arteries and is attached to their walls. This previously undescribed tissue, termed the arterial gland is described in a subsequent paper (Laryea, 1969 d).

The right and left rostral arteries arise laterally and from them arise the arteries which supply central ganglia (cerebral, pedal, visceral complex of ganglia - Table IV). These rostral arteries initially follow the curvature of the anterior surfaces of the pedal ganglia before passing antero-dorsally to give rise to inner labial, outer labial and tentacular arteries. The right rostral artery in addition gives rise to the right penial artery which supplies the penial sac and the base of the reproductive tract. In addition to the main vessels

supplying the central ganglia additional small arteries are present in some specimens (Fig. 6). Notable features of the arterial supply to these ganglia are: the asymmetry, the anastamosis not only of capillaries (Nalepa, 1883) but of arterioles, and the variability in both origin and distribution of these vessels, found in some specimens. Fig. 6 shows the commonest arrangement of the main arteries and a typical distribution of some of the arterioles. It is worth noting that fine vessels could be traced within the perineurial sheath of some of the nerves.

The paired anterior pedal arteries arise medial to the rostral arteries and run antero - laterally, branching to supply the anterior part of the pedal gland and give rise to the extensive vascularisation of the anterior foot. The posterior pedal artery arises medially, immediately loops under the pedal ganglia and passes posteriorly. It branches to supply the pedal gland and the foot posteriorly.

The single buccal artery also arises medially, dorsal to the posterior pedal artery and divides into two a short distance from its origin. A short wide vessel passes dorsally to form paired sinuses between the odontophore cartilage and the buccal musculature dorsal to it, while longer thinner vessel runs forward as the ventro - buccal artery, beneath the buccal mass and divides into two identical branches. These supply the lips and musculature of the buccal mass, as well as the buccal ganglia and part of the oesophagus. The blood supply to the buccal ganglia is symmetrical and although vessels run in or on the ganglionic sheath they do not pass into the ganglia. This is also true of the vessels supplying the central ganglia.

The arteries grade via arterioles into capillaries and these form a network in or over most of the internal organs. The pedal arteries, via capillaries, empty into ill defined pedal sinuses. The capillaries arising from the supply to the organs empty directly into a sinus system either within the tissues or surrounding them. In some preparations certain of these capillaries appeared to end blindly. These sinus systems are in communication with the general body cavity. The pedal sinus system opens directly into the lung network. Gamer (unpublished results) has found two large sinuses in the dorsal body wall tissue which return blood to the kidney and the lung. Blood flows into the auricle via a renal sinus from the kidney and via venae - cavae from the lung network.

The Optic Tentacle.

Many workers have studied the stylommatophoran optic tentacle. Chetail (1963) reviews the major work before 1959, while a more recent paper by Rohlich and Bierbauer (1966) brings the structural knowledge up to date and reviews some of the recent literature (see also Bullock, 1965). Both experimental and histological work has suggested a possible endocrine /neuroendocrine involvement of the optic tentacles in gastropods (see Simpson *et al*, 1966 a) and so it was thought pertinent to include this structure in the present study. The optic tentacle of *D. reticulatum* is very similar to the generalized stylommatophoran form described by Lane (1962), but of the so - called special cells only two of the three types, collar cells and lateral processed cells are identifiable with the light microscope.

The collar cells are large and encircle the distal portion of the tentacular nerve, below and around its enlargement to form the digitate ganglion. The cells are usually pyriform in shape with a granular nucleus. Characteristically the cytoplasm contains spheroidal granules, which sometimes extend into the elongate process of the cell. These processes run to the distal tentacular epithelium, where many can be found discharging their secretion. Basally the cells are attached either to the dermo - muscular sheath and/or to each other.

The lateral processed cells are distinguishable on the inner surface of the dermo - muscular sheath and processes from these cells also penetrate the tentacular epithelium. Lateral oval cells have not been identified in *A. reticulatus* but their presence may be revealed by subsequent work with the electron microscope.

The nature of the special cells has been the subject of much controversy, and they have been considered to be gland cells, neurones, and a special type of neurosecretory cell. This is discussed in detail by Röhlich and Bierbauer (1966) in a paper in which they present the ultrastructural evidence for the glandular nature of the collar and the lateral processed cells of *Helicella obvia*.

In some preparations of the optic tentacle of *D. reticulatum*, it was impossible to trace processes from the collar cells to the exterior and, moreover, to be certain of their relationship to nervous tissue, while in others, individual cell processes could be followed and the site of secretory discharge determined with ease. The difficulty of distinguishing the various elements of the optic tentacle with general stains, together with the poor results often obtained using more specific methods, is in large part responsible for much of the confusion surrounding the special cells.

Stainability with the neurosecretory stains does not by itself, constitute evidence for the neuronal nature of these elements, also elementary neurosecretory vesicles never occur in the collar cells and their secretory vacuoles are generally much larger and have a less dense content (Röhlich and Bierbauer, (1966). Thus, even if the collar cells are regarded as transformed neurons, they cannot be considered as neurosecretory cells in the classical sense. It is perhaps interesting that the collar cells of *D. reticulatum* take up very little, if any, of either chrome alum haematoxylin or paraldehyde fuchsin (see Laryea, 1969 c). The relationship of both types of gland cell to the nervous system in *D. reticulatum* is obscure. Nerve fibres can be found in structural association with the gland cells, but what this represents anatomically, or physiologically, is not known. Röhlich and Bierbauer feel the large number of, mainly dense-core, vesicles is suggestive of direct innervation of the gland cells, but have not found any evidence to support this suggestion.

A similarity exists between the collar cells of the tentacles and the mouth lobe glands in so far as they both surround an accessory ganglion and pour secretion on to a highly sensory epithelium. These secretions may function as lubricants and in the case of the collar cells this could certainly facilitate inversion and eversion of the tentacles. They may even be necessary for the proper functioning of the sensory elements.

The mouth lobe glands.

The mouth lobe glands are unicellular glands surrounding the distal portion of the median lip nerve, and open on to the surface of the skin at the base of the mouth lobes. The association of these glands with the mouth lobe ganglion, together wit the similar labial glands and the lip ganglia is known as Semper's organ (Echardt, 1914; Lane,1964).

In *D. reticulatum* the unicellular mouth lobe glands are aggregated into a leaf-like structure which surrounds the median lip nerve and attaches anteriorily to the skin between the buccal mass and the inferior tentacle. Similar unicellular glands the labial glands also occur aggregated into packets of variable size and number on the antero-dorsal surface of the buccal mass. No evidence for nervous innervation of any of these glandular elements was found.

Histologically the gland cells have a well-defined nucleus, and are usually drawn out into a long duct which may reach 120µm in length. These ducts penetrate the skin either at the

base of the mouth lobes in the case of mouth lobe glands or dorsal to the lips in the case of labial glands. With Azan trichrome staining, the secretory product is stained a light blue; some of the cells, however, stain a much deeper blue than others.

Lane (1964) suggested a possible endocrine function for the secretory cells of Semper's organ of *H. aspersa* for which she was unable to demonstrate an external duct: In some preparations of *D. reticulatum* it was only possible to demonstrate a few of the glands opening to the exterior, but in others sections could be found showing almost all the glands discharging secretion. In *Vaginulus langsdorfi,* van Mol (1967) has found that the glands discharge into a common duct, which then opens on to the surface. In the absence of any experimental evidence, there seems to be no reason for suspecting that any of the glandular elements of Semper's organ have an endocrine function.

A similarity between certain of the unicellular glands and cells in the pedal gland has been noted (Echardt, 1964; Lane, 1964). In *D. reticulatum* this similarity is well marked and secretions from both contain acid mucopolysaccharide components. Rotarides (1929) suggested that the glandular portion of Semper's organ is a slime gland, which pours secretion on to the surface of the head, while Hanitsch (1888) suggests their function is to keep the lips moist. Presumably voluminous secretion is required on and around the mouth lobes to keep the surface moist, and possibly also to act as a lubricant and/or protectant (Van Mol, 1967). The innervation of the mouth lobe suggests that it has primarily a sensory function with which the secretion seems likely to be connected. Whether this function is gustatory, (Garnault, 1889) or tactile (Van Mol, 1967) is not known. The mouth lobes are certainly used a great deal to probe the surface of the substrate when the animal is active. The constant association of the glands with the median lip nerve may then simply be a question of design, particularly as no innervation of these glands by this nerve was found. This would also explain the inconsistent association of the labial glands with the internal and external lip nerves. The value of the term Semper's organ for this association of ganglionic and glandular elements is therefore questionable as has also been suggested by Echardt (1914).

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DISCUSSION

Apart for minor differences such as the non - fusion of optic and tentacular nerves, the arrangement of the cerebral nerves in *D. reticulatum* is almost identical to the generalised stylommatophoran scheme described by Van Mol (1967).
The remainder of the central nervous system is very similar to that of *Helix pomatia*. Apart from minor differences the arrangement of the buccal nerves is almost identical to that pertaining in *Helix pomatia*. (see Schmalz, 1914; Bang, 1917; Kunze, 1917 a; Cook, 1966). The pallial nerves are also similar in the two animals. Of the two aortic nerves present in *D. reticulatum* the one originating from the right parietal ganglion is homologous to the single aortae found in *Helix pomatia*. (Kunze, 1917 a).

While no attempt was made to homologise the nerves of the pedal ganglia of *D. reticulatum* with those of *Helix pomatia* they are not dissimilar, although there are four cutaneous pedal nerves in *D. reticulatum* and not three. The anal and intestinal nerves from the visceral ganglion are easily homologised with the *nervus analis* and the *nervus intestinalis* of *Helix pomatia*, while the cephalic retractor muscle nerve together with the second tentacle retractor muscle nerve represents the nerve *musculi retractoris pharyngealis* of Helix pomatia. In *D. reticulatum* the single muscle formed by the fusion of tentacular and pharyngeal retractor muscles is here termed the cephalic retractor muscle.

The general plan of the vascular system in *D. reticulatum* is similar to that described by Schmidt (1916) for *Helix pomatia*, although part of the venous system appears better defined. In this paper the term cerebral artery has been restricted to the vessels that initially supply the cerebral ganglia and the term rostral artery adopted for the distributing vessels from which they arise.

In *D. reticulatum* the very fine arterial terminations were never found to have the precisely arranged muscles found around the capillary osteoles of *L. stagnalis* by Carriker (1946). Arteries opening directly into the body cavity through funnel shaped orifices, as described for *Arion rufus* by Jourdain (1879) were not observed either. As arteries were, however, found in association with muscle fibres and found to run between muscles it remains a possibility that muscles do not control the openings. The arteries and arterioles have circularly and longitudinally arranged muscle fibres in their walls and it is possible that their contractions could control blood flow.

The fact that the central ganglia are supplied with blood by several different vessels and that these are interconnected means that blood can reach a particular ganglion from one or more of several different sources. Given that muscular constriction could limit blood flow, the anastamoses of capillaries and particularly of arteries suggests that a shunt system could route blood to where it was most needed at any particular time. Evidence for muscular constriction of blood vessels is suggested by the fact that it is often not possible to completely inject the arterial system of a particular organ.

The very rich arterial supply to organs and tissues means that the maximum distance over which diffusion of oxygen, ions and nutrients etc. has to take place is very short. All this notwithstanding that fact that the organs lie bathed in venous blood. In contrast and perhaps surprisingly, considering the great demands for nutrition made by the nervous system, a capillary system within the central ganglia was not found. Diffusion across the ganglionic sheath would, however, seem to be effective. While the peripherally situated nerve cell bodies are no great distance from the arteries in the ganglionic sheath, the centre of the neuropile is a considerable distance away. It may be that glial cells assist transport in some way.

The nervous system makes intimate contact with the vascular system at a number of sites, via 'innervating' nerves. The rostral artery (*n. arteriae/cerebralis*) and the commissural nerves are already established as means by which 'neurosecretory' material is transported from the CNS of Stylommatophora and their terminations have been regarded as neurohaemal areas (Van Mol, 1960a, 1967; Kuhlmann, 1963; Nolte, 1965). The commissural nerves ramify in the connective tissue above the cerebral commissure in an area rich in capillaries and blood spaces, but whether these nerves terminate in dorsal body tissue, on capillaries, or at blood spaces in *D. reticulatum* has yet to be resolved. Accumulation of 'neurosecretory' material has been found within the sub-cerebral commissure of *Achatinella fulgens* (van Mol, 1967), and even though the sub-cerebral commissure in *D. reticulatum* is in this study considered a true commissure this does not preclude its participation in the release of secretory material.

The innervation of the aortic stem, the anterior and posterior aortae and the cephalic artery are quite considerable in *D. reticulatum* in common with the situation in *Helix* pomatia, but no information is as yet available on the physiology of pulmonate arteries. In comparison

considerable information exists on the innervation of the pulmonate heart and both inhibitory and excitatory fibres have been demonstrated (Hill and Welsh, 1966). There is evidence for cardio-regulation by neurotransmitter substances such as acetylcholine, 5-hydroxytryptamine (5-HT) and catecholamines. 5-HT is released into the heart following stimulation of the intestinal (extra cardial) nerve of *H. pomatia* (S-Rozsa and Pereny, 1966) and these substances are suspected of being the natural cardio-regulators. Some at least of the nerves innervating arteries and aortae *in D. reticulatum* certainly terminate at neuro-muscular junctions and these probably play a role in regulation of blood flow. All such nerves are, however, worth investigating as potential release sites for neurosecretory material, particularly in view of the suspected neuroendocrine regulation of cardiac activity in some snails (Jaeger, 1966; S-Rozsa and ZS-Nagy, 1966) and the terminating neuro-secretory system recently described in the heart of *Helix* (Gottrel and Osborne, 1969).

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THE NERVOUS SYSTEM OF THE SLUG
DEOSERAS RETICULUM (PULMONATA, LIMACOIDEA)

II. The Histology of the Medio - Dorsal Body, Ganglionic Sheath and Associated Blood Vessels.

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SUMMARY

The perineural sheath of *Deroceras reticulatum* consists of a matrix of dense collagen like fibres, in which are embedded muscle fibres, blood vessels, granular cells, and pigment cells. The vesicular cells which usually occur towards the outside of the sheath often form a distinct outer region several cells thick. Ultrastructurally, the vesicular cells were found to possess the same specializations of the plasma/membrane as pore cells.

The fine structure of the blood vessels supplying the central nervous system is similar to those found in some other invertebrates but they lack a distinct basement membrane and apparently endothelial cells. The structure of the blood vessels and their relationship with the elements of the sheath suggest that metabolic requirements reach the neurones by diffusion from these vessels.

The Dorsal Body occurs as a more or less compact lobe on the medio-dorsal surfaces of the cerebral ganglia and the dorsal surface of the inter-cerebral commissure, but dorsal body tissue also occurs on other parts of the central nervous system. Synapse-like contact between neurosecretory axons and dorsal body tissue cells was not found, but a close relationship does exist between the dorsal body tissue cells and the blood system. It is suggested that secretory material (vesicle laden profiles) may be liberated directly into the blood.

INTRODUCTION

The perineural sheath of *D. reticulatum* is important not only because it probably acts as a selective barrier to material diffusing from the blood, and hence determines the immediate environment of the nervous and glial tissues, but also because of possibility that some of its cellular elements may themselves liberate secretory material into the blood system (Fernandez, 1966)

Much histological and some experimental evidence exists for an endocrine role of Dorsal Body cells in some pulmonates (see text). Equivalent cells occur attached to the ganglionic sheath in *Deroceras reticulatum*, and previous study has established a close relationship between these cells and the blood supply to the central nervous system (CNS) (Laryea 1969 a).

A study of the interrelationships of the ganglionic sheath, dorsal body cells and associated blood vessels was undertaken as a prerequisite to investigation of the possible neuroendocrine role of the CNS in *Deroceras reticulatum*.

OBSERVATIONS

Histology of the ganglionic sheath

The neuronal and glial elements of the nervous system (NS) of *D. reticulatum* are encased by a connective tissue sheath, the epineurium, the cellular composition of which shows considerable variation, between individual animals and between different areas of the same NS. In the peripheral region of the NS the epineurium consists of a matrix of dense collagen fibres in which are embedded muscle fibres and a variety of connective tissue elements; pigment, granular, vesicular and fibroblast-like cells.

Large vesicular cells occur on the outside of the epineurium on the CNS and around the origins of the nerves; sometimes in vast quantities. In these areas they thus produce what appears to be a distinct outer region to the sheath (Fig. 2). Blood vessels occur in all regions of the sheath.

The innermost region of the perineurium consists of an amorphous basal lamina which is slightly more electron dense than the ground substance of the surrounding collagenous matrix (Figs 5,6). Cellular connective tissue elements are absent from the matrix for some distance from this lamina, but they occur in increasing numbers towards the outside.

Muscle fibres run in all directions within the sheath (Figs 5, 6) and are similar in structure to those found in the walls of the large arteries (Laryea, l969 a).

Pigment cells occur throughout the sheath, but are most abundant in the median area of the inter-cerebral commissure. These cells are irregularly shaped and have long processes which ramify through the collagenous matrix. They contain melanin-like pigment granules in a variety of shapes and sizes, a number of which have membranes around them (Fig 6, 7). The cell nucleus is elongate and often irregular. Vesicular cisternae of rough endoplasmic reticulum, free ribosomes, polysomes and glycogen-like material occur within the cell. Very occasionally, pigment granules have been observed within muscles and other unidentified cells.

Processes of granular cells (Fig 4) ramify between the cellular elements of the sheath particularly along the edges of blood sinuses. The cytoplasm of these cells contain numerous small vesicles in addition to large granules. Two types of granule of similar size occur; one being much more electron-dense than the other. There are slight variations in electron density between the granules of an individual cell Usually only one type of granule occurs in a cell, but occasionally the two types are found together.

Fibroblast-like cells are often spindle shaped, but frequently highly branched, with long, thin, irregular processes which sometimes interdigitate with those of similar cells. They have irregular nuclei and dense cytoplasm. The usual cell organelles occur within the cytoplasm but vesicular rough endoplasmic reticulum, numerous mitochondria and prominent Goligi bodies are the most conspicuous (Fig 3). These cells are frequently found around the walls of capillaries.

The vesicular cells (Fig 2) are large (e.g. 44 x 22 μm) and their nuclei are usually eccentric in position. Their cytoplasm is usually only faintly stained after Azan trichrome staining and so when large numbers of such cells occur they have the appearance of adipose tissue. Their cytoplasm is PAS positive (diastase sensitive) but only lightly stained by Sudan Black B or Bromophenol Blue. Ultrastracturally (Fig 5) they have numerous interconnected extracytoplasmic vesicles and peripheral pores and appear identical to the Pore cells described by Plummer (1966), and the Blasenzellen more recently described by

Wandrak (1968). The invaginations of the vesicular cells are lined by fine granular material, which is usually continuous with similar material around the outside of the cell. Vast quantities of flocculent, glycogen-like material usually occur within the cell. The endoplasmic reticulum is usually rough and occurs in large membrane bound whorls, electron dense, liposome - like structures occur within the cytoplasm.

The relative abundance of vesicular cells varies greatly between individuals. In areas of the CNS of some animals they may be completely absent (e.g. dorsal portion of the cerebral commissure in Fig.1) while in other areas they may be four or five cells deep (Fig 2). While vesicular cells usually occur towards the outside of the sheath they sometimes occur very near to the basal lamina among other connective tissue cells. Thus, the demarcation of the sheath into two regions is not always a sharp one, Similarly the outer region of the sheath is not composed exclusively of vesicular cells; limited numbers of the other connective tissue elements are also present.

Fine Structure of blood vessels supplying the CNS

The structure of the large arteries in *D. reticulatum* appears to be the same as that previous described for the posterior pedal artery (Laryea, 1969 a), and consists of an inner circularly oriented and outer longitudinally oriented layers of muscle fibres embedded in a matrix of collagen fibres. Sparsely distributed cells interpreted as fixed amoebocytes border part of the lumen. Axons and connective tissue elements occur embedded in the matrix.

The cerebral artery and other small arteries supplying the CNS are characterized by the possession of a complete layer of pericytes (Barker and Graziadei, 1965). The pericytes (Fig 8) form a single inner layer of cells which are embedded in the collagen matrix and do not actually border the lumen. The lateral surface of the pericytes interdigitate with one another and specialized contact zones, pericyte plaques occur (Gray, 1969). The pericyte plaques are regions where plasma membranes are separated by a dense extracellular matrix. The pericytes are not innervated. Mitochondria, vesicles of varying size, rough

surfaced endoplasmic reticulum and glycogen-like material occur in the pericyte cytoplasm.

In the small arteries the number of muscle fibres decreases, their arrangement becomes irregular and the amount of collagen between them increases. The initial branches into which these small arteries (arterioles) divide have an identical structure but a smaller diameter. In the finer branches of the small arteries (capillaries) the pericyte layer becomes incomplete and much of the vessel wall consists only of a thin layer of collagen fibres in ground substances ((Fig7). Where the pericyte layer is absent the lumen is usually lined by a large number of cell processes. A trabeculate cell is frequently found in such areas; the trabeculae spanning part of the lumen (Fig 6).

The Dorsal Body

Light microscopy

In *D. reticulatum* a more or less compact medio-dorsal body (MDB) surrounded by a connective tissue, occurs attached to the dorsal surface of the inte-rcerebral commissure and the medio-dorsal part of the cerebral ganglia (Fig1). Tissue identical to that of the MDB and continuous with it presents over parts of the postero-lateral surfaces of the cerebral ganglia, the cerebro-pleural connectives (Fig 2) and on to the dorsal surfaces of the pleural ganglia. The term dorsal body tissue (DBT) has been adopted here for all the tissue having a similar appearance to that of the MDB.

In adult specimens, the DBT cells are pyriform in shape (4-10µm diameter) and have one or more processes. The cell nucleus is round or ovoid. The cells are arranged in groups separated by small amounts of muscle and connective tissue elements, blood sinuses and capillaries; axons ramify between the cells. Neither the cells nor their processes are oriented in any particular direction (Fig 2). The majority of the DBT cells, particularly those of the MDB are separated from the ganglionic sheath by one or more layers of

vesicular cells (Fig 5). When vesicular cells are sparsely distributed large numbers of DTC cells attach directly to the inner region of the ganglionic sheath (Fig1). In many other Stylommatophora an extremely thick epineurium encapsulates the DBT, but in *D. reticulatum* most of the DBT lies above this and is only separate from the haemocoel by a very thin layer of connective tissue.

When stained with Azan, the nuclei of the DBT cells stain bright red and the cytoplasm pale pink with usually slightly darker staining granules. The cells stain weakly with the so-called neurosecretory stains, although sometimes, positively staining granules occur (Nolte,1965; Kuhlmann, 1966).

Ultrastructure

The most characteristic feature of the DBT cells is their numerous large mitochondria, with prominent cristae, usually arranged in whorls (Fig 9). Some of the mitochondria contain electron-dense flocculant material which is probably lipid (Fig10), while not infrequently large, clear areas occur within the mitochondria when their cristae are not so regularly arranged (Fig11). This latter condition is found in damaged cells, but when it occurs in cells that otherwise appear well fixed, it is thought that it represents a normal appearance. Whether the clear areas represent material accumulating within the mitochondria subsequently extracted during fixation or a degeneration of the organelle is not known. Small moderately electron-dense granules (Figs 7, 9) may occur within the cell body and they appear to be produced by the small Golgi body (Boer, *et al*., 1968), but large numbers of these granules occur in the cell processes. The endoplasmic reticulum usually occurs as small, elongate vesicular profiles of rough surfaced reticulum; numerous free ribosomes, polyribosomes and glycogen-like material are also present. Microtubules, filaments and lysosomes occasionally occur (Fig 9).

The surfaces of the DBT cells are irregular and interdigitate, but are not in direct contact (Fig 9). Desmosomes were not found. Because of the irregularity of the cells, there appears in some sections to be more of the main cell processes than actually exist.

Most of the cytoplasmic organelles have been found in the cell processes, but the moderately electron-dense granules and the mitochondria are the most consistent feature.

The cell processes pass between the DBT cells and they do not usually penetrate the basal lamina. Some cell processes occur in direct apposition to the capillaries, but are separated by a very thin extracellular zone containing collagen fibres (Fig 7). Interspersed between the groups of DBT cells are smooth muscle fibres, granulated cells, pigment cells, fibroblasts, nerve axons and near the inner region of the sheath vesicular cells. Axons also pass between the individual DBT cells (Fig 12) and along their surfaces and processes, but while this close anatomical relationship exists between axons and the DBT cells, innervation was not observed. The axons contain elementary granules of varying size and electron density. Some of the axons contain electron-dense elementary granules, modal diameter 1200 - 1300Å (Fig12) characteristic of neurosecretory cells identified in the cerebral and right parietal ganglia, (Laryea, 1969 c)

Because of the irregularity of the cell groups, many DBT cells border the numerous blood sinuses that permeate the DBT (Fig 1, 2), but they are always separated from the sinus by a thin layer of connective tissue, usually a membrane of collagen fibres in ground substance (Fig 4), but not infrequently, cellular connective tissue elements are present. The connective tissue sheath separating the DBT cells from the haemocoel has a similar structure.

In one specimen, the cell bodies DBT were found within the cerebral ganglion (Fig13). These DBT cells were surrounded by glial cell processes, axons and nerve cell bodies, but neither synapses nor specialized areas of contact were observed. Processes from some of these cells penetrate the cerebral ganglionic sheath (Fig14).

Discussion

The structure and composition of the perineurium is very similar to the sheath of *Helix pomatia* (Baeker, 1932) and *Helix aspera* (Fernandez 1966; Newman, *et al*.1968), and contrasts sharply with the relatively thin sheath of some Hygrophila (e.g. *Lymnaea stagnalis* and *Planorbarius corneus*; Boer, *et al*. 1968)

Some features of the fibroblasts *D. reticulatum*, such as their irregular outline, vesicular rough-surfaced endoplasmic reticulum and prominent Golgi bodies, are similar to those characterizing vertebrate fibroblasts (Porter, 1966). However, when compared with the connective tissue fibroblasts of *Arion rufus* (Wondrak, 1968), the golgi zone is not as prominent, nor the plasmalemma as invaginated. Experimental work is needed to determine if these cells are homologous with vertebrate fibroblasts; autoradiography at the electron microscope level, or tissue culture studies, since vertebrate fibroblasts play a role in collagen formation.

It has been suggested that the vesicular cells (pore cells) play a role in collagen formation (Plummer, 1966; Newman, *et al*., 1968). Wondrak (1968) states that no conclusive proof exists for the role of pore cells in collagen synthesis, hence the designation of this type of cell as a fibroblast is not justified. Obviously experimental studies similar to those suggested above are also needed on these cells.

Vesicular connective tissue cells have been described by a number of authors under a number of different names and suggested functions include: a role in glycogen and /or calcium metabolism, excretion (via accumulation) and collagen formation (see Kisker, 1923 Filhol, 1938; Carriker, 1946; Fernandez, 1966; Plummer, 1966 Wondrak, 1968). Fernandez found that the glycogen and 'lipofuscin' pigments of the vesicular cells appeared to undergo seasonal changes, but does not mention variation between specimens in the relative number of vesicular cells per sheath as occurs in *D. reticulatum*. Calcium-containing vesicular cells were not found in the ganglionic sheath of *D. reticulatum*, but do occur attached to the walls of some of the blood vessels; particularly the major and minor hepatic arteries. It will be interesting to see if these cells also possess

specialisations of the plasmalemma since the terms ‘vesicular cell’ or ‘Leydig cell’ are often used as all-embracing terms, and it may be that they have been used for cells of completely different lineage and function. Obviously structural comparisons are not enough; we need to know something of their embryology and physiology.

The blood vessels of *D. reticulatum* have been arbitrarily designated arteries, arterioles and capillaries depending on their size (Laryea, 1969 a). In this study, the differences between the large arteries, the small arteries and arterioles and the capillaries are seen to have a structural basis and these terms have been retained although since the vessels lack a true endothelium and the capillaries do not open into the veins, capillaries in *D. reticulatum* cannot be considered homologous to vertebrate capillaries. Some structural features of the blood vessels in *D. reticulatum* are similar to those previously described for other invertebrates (Goggeshall, 1965,1967; Hama, 1960; Barker & Graziadei, 1965, 1966, 1967 a, b; Stephens & Young, 1969; Gray 1969) but they lack a basement membrane and endothelial cells do not apparently exist. Gray (1969) has suggested the incomplete layer of endothelial cells found in cephalopods may be fixed amoebocytes and this view is held here. In *D. reticulatum* these cells lack the morphology of the amoebocytes seen in the blood, and are frequently attached to the luminal wall of the blood vessel by only a small portion of their cell surface. Identical cells occur scattered in the connective tissue.

The pericytes in *D. reticulatum* appear very similar to those described in the blood vessels of cephalopods (Barber & Graziadei, 1965.1966, 1967 a,b; Gray, 1969) and since the surface of these cells does not actually form the lumen of the blood vessel the name was retained. A plexus of fibres which looked like elastic fibres has been found in the arteries of various pulmonates including *Agriolimax agrestis* (Jullien, et al.1958; Fernandez, 1966). After permanganate oxidization of sections followed by paraldehyde fuchsin staining, similar fibres were found in the arteries of *D. reticulatum,* but the equivalent fibres were not seen with the electron microscope .

The extremely thin capillary walls presumably present no great barrier to the fusion of nutrients and oxygen from the blood and in view of the rich arterial supply, it is likely that

diffusion alone accounts for the provision of such materials to the ganglia. Glial cells probably play a role in the transport of material within the neuropile (see Laryea, 1969 c) and of course the nerve cell bodies are peripherally located, It is unlikely that haemocyanin passes through the wall of the capillaries owing to its large molecular size, (van Bruggen, *et al*1966) and the large number of collagen fibres and associated ground substance. There is also the considerable thickness of the acellular part of the ganglionic sheath between the cells of the ganglia and the nearest capillaries. No system of extracellular spaces comparable to those found in cephalopods Stephens and Young 1969 ; Gray 1969), which possibly facilitate transport of haemocyanin, occur in *D. reticulatum*. In cephalopods, of course, capillaries actually run within the lobes of the brain. Many of the capillaries that occur within the ganglionic sheath of the cerebral commissure of *D. reticulatum* were found to continue dorsally between the various cellular elements occurring above the commissure (Laryea, 1969 a) and presumably open into the sinus surrounding the CNS. As yet such openings have not been identified with the electron microscope.

There is considerable variation in the number, form and distribution of the MDBs and their relationship to the connective tissue surrounding the CNS within the Stylommatophora (Nolte, 1965; Kuhlman, 1966' Cook, 1966; van Mol, 1967).
The variation in the form of the MDB in *D. reticulatum* appears to reflect the unpaired condition found in most Stylommatophora (Nolte, 1965 ; Khlman, 1966; van Mol, 1967

Completely separate MDBs and lateral dorsal bodies (LDM) occur in *Succinea putris* (van Mol, 1967) but not in the majority of Stylommatophora. Similar tissue to that of the MDBs (here called DBT) does occur in other areas of the CNS. The most extensive distribution of this tissue is found in *Strophocheilus oblongus* (Kuhlman, 1966), where in addition to the presence of a very large mass on the cerebral ganglia, it also extends down the cerebro-pleural connectives to the pleural ganglia. The distribution of DBT in *D. reticulatum* is therefore very similar to that found in *Strophocheilus oblongus.* Cook

(1966) also found DBT on the exterior of the vesceral ganglionic complex in *Succinea putris,* although in this case it is isolated from the MDBs and the LDBs.

The MDB of *D. reticulatum* is similar to the dorsal bodies of Succinidae (van Mol, 1967) and *Strophocheilus oblongus* (Nolte, 1965) only being separated from the haemocoel by a thin connective tissue sheath. This is in contrast to the condition in the majority of Stylommatophora, where the ganglionic connective tissue completely encapsulates the MDB.

In some Stylommatophora, the MDBs lie directly against a thin connective tissue sheath, while in others it is separated from the inner region of the ganglionic sheath by larger amounts of connective tissue. The presence of commissural nerves, which ramify between the MDB cells, is always associated with this latter condition. It has been suggested that a functional relationship exists between the MDB and the cerebral neurosecretory cells, since in those species with commissural nerves some of the neurosecretory material leaves the cerebral ganglion via these nerves (Nolte, 1965; Kuhlmann ,1966 ;van Mol, 1967). In *D. reticulatum*, while the axons from the median cerebral neurosecretory cells ramify between MDB cells, synapses were not found and so a secreto-motor role for these axons is unlikely. Liberation of neurosecretory material into capillaries or sinuses could of course, affect the MDB cells. This is also true of the neurosecretory axons which ramify between the DBT cells on the pleural ganglia.

In some Hygrophila, MDB cell processes penetrate the basement membrane of the ganglionic sheath of the cerebral ganglia. Even in these species, where a really close morphological relationship exists between the MDBs and cerebral ganglia, only a few cells come into direct contact (although apparently not synaptic) with nerve-cell processes; the vast majority did not (Boer, *et al*, 1968). In this connection it is interesting that when MDB cells occurred beneath the perineural sheath in *D. reticulatum*, processes were found passing from some of these cells, back through the sheath.

It will be interesting to see what relationship is revealed by electron microscopy between the DBT on the pleural ganglia and cerebro-pleural connectives, and the nerve tissue in these regions.

The accumulation of secretory granules, the absence of ducts and the close relationship between DBT and the blood system described here, would seem to support the proposed endocrine nature of the DBT (see Boer, *et al.*1968). Exocytosis was not observed, but the secretory product of the DBT is presumably able to diffuse into the blood via the capillaries and/or the sinuses. This is, of course, speculation and must await experimental verification. Extirpation and implantation of DBs in *Lymnaea. stagnalis* suggest that the DBs produce a special factor which is necessary for vitellogenesis and for the growth and function of the accessory sex glands (Joosse & Geraerts, 1969; see also Joosse, 1964). In *D. reticulatum* part at least of the maturation of the accessory sex glands is known to be under the control of a blood-born factor (unpublished results). It will be possible to investigate the possible role of DBT in reproductive maturation by injection of DBT extracts, but with the distribution of DBT on the CNS widespread, and its often indistinct nature, extirpation experiments will be challenging.

Plate 1

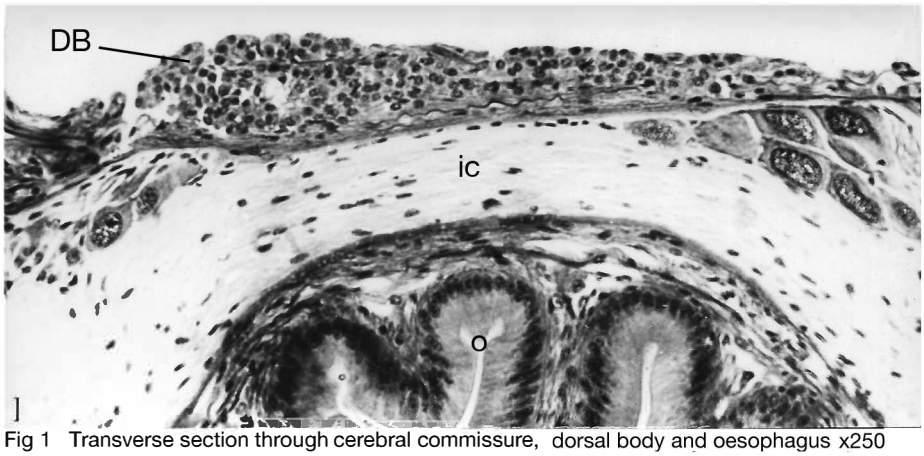

Fig 1 Transverse section through cerebral commissure, dorsal body and oesophagus x250
DB, dorsal body; ic, intercerebral commissure; o, oesophagus

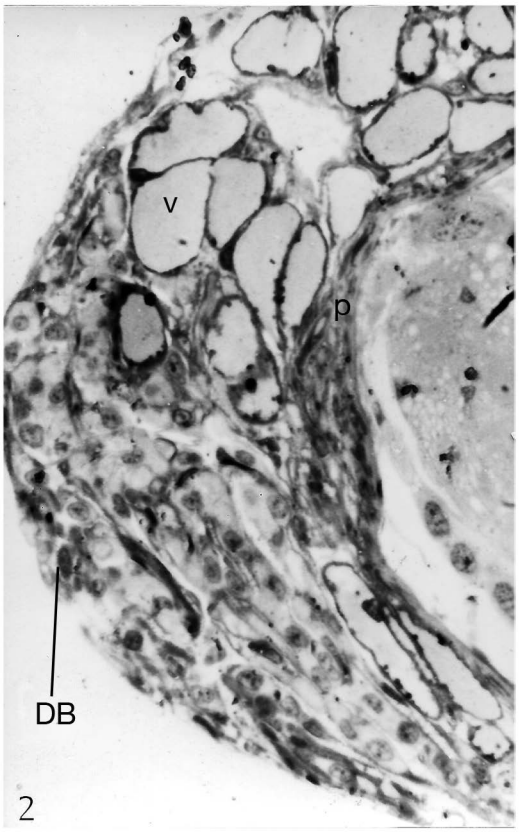

Fig 2 Vesicular cells (pore cells) and dorsal body cells. x500
DB, dorsal body cells; v;vesicular cells; p, perineurium.

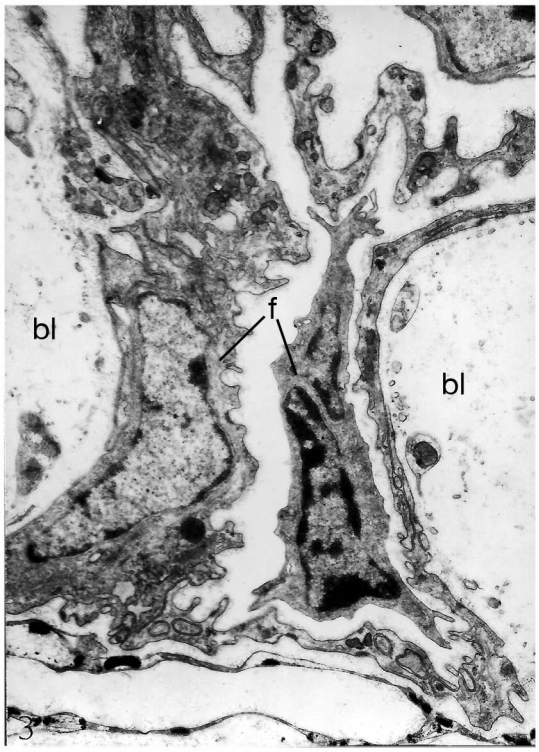

Fig 3 Fibroblasts adjacent to capillary walls. x3,300
f, fibroblast, bl, blood capillary.

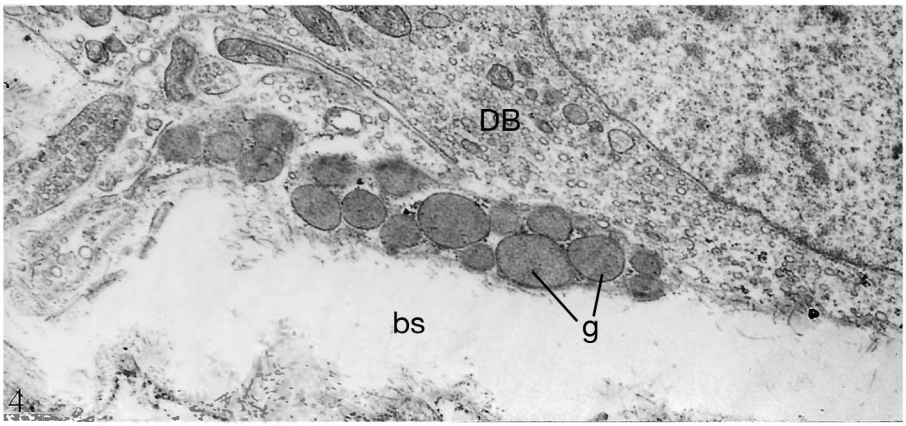

Fig 4 Granular cell processes alongside blood sinus. x16,360
bs, blood sinus; DB, dorsal body cell, g, granular cells.

Plate 2

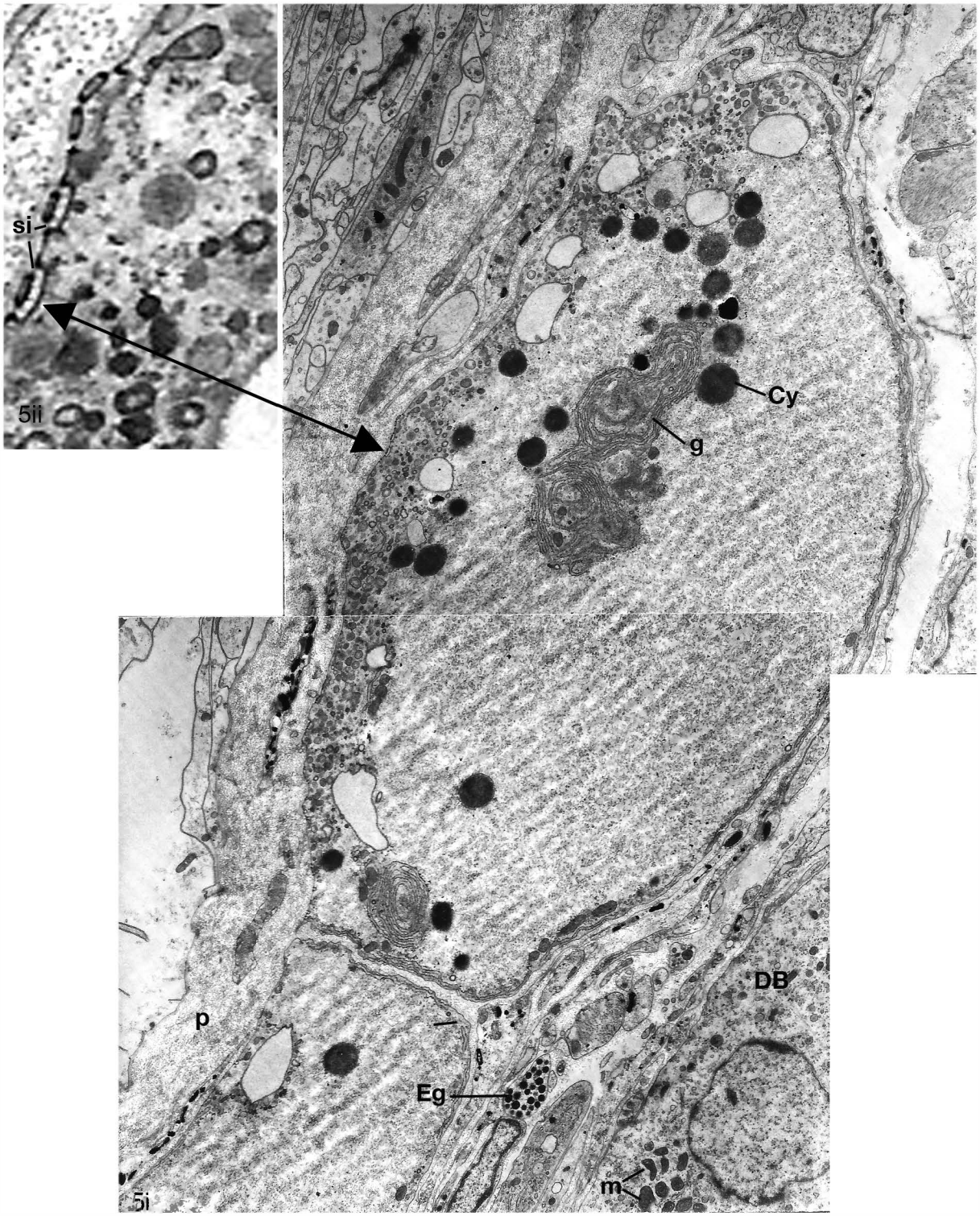

Fig. 5i Vesicular cell (pore cell) attached to the epineurium with characteristic surface invaginations, flocculent material dominating the cytoplasm and highly developed Golgi apparatus arranged in whorls.

Cy, cytosome; DB, dorsal body cell; g, Golgi body; si, surface invaginations; m, mitochondria p, perineurium; Eg, elementary granules.

Fig. 5ii Portion of fig 5i at a higher magnification showing surface invaginations.

si, surface invaginations.

Plate 3

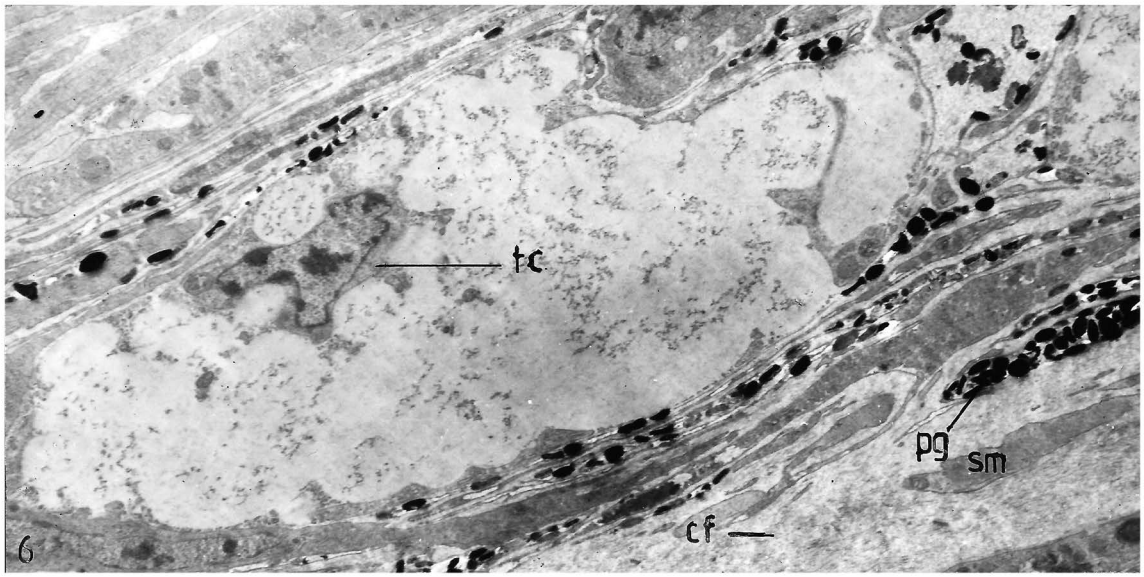

Fig 6 Part of a large artery with prominent trabeculate cell. Inner circular and outer longitudinally oriented layers of muscle fibres are embedded in a matrix of collagen.

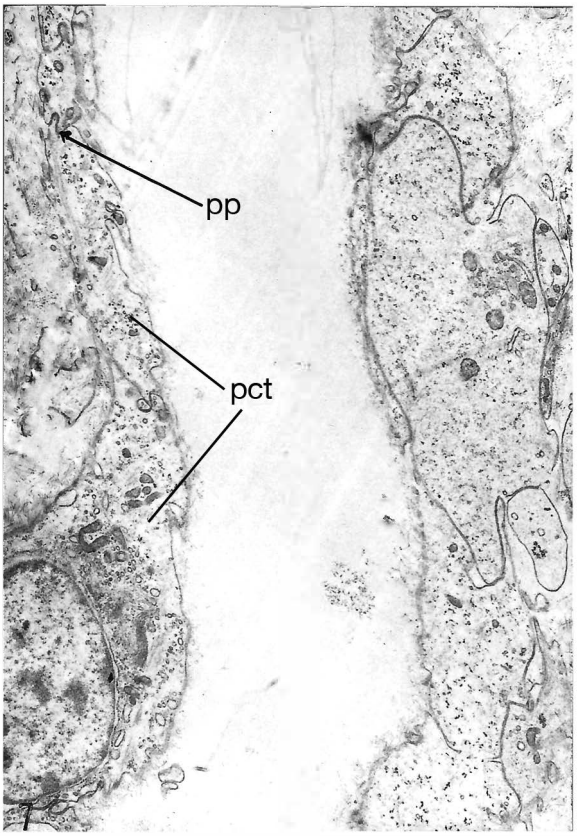

Fig 7 Pericytes forming a single inner layer of arterial wall cells.
pct, pericytes; pp, pericyte plaque.

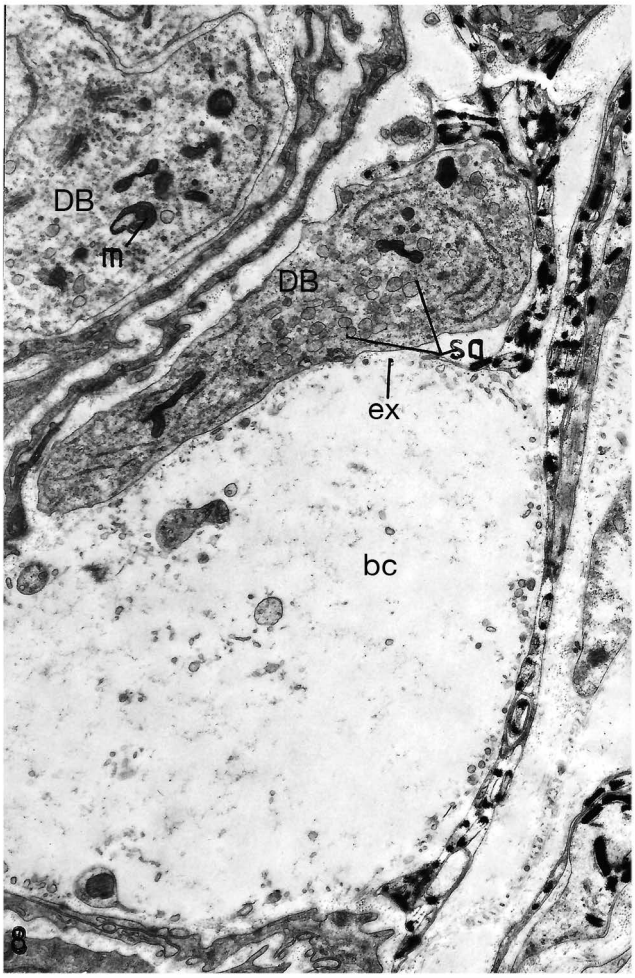

Fig. 8 Dorsal body cell process in direct apposition to a blood capillary.

DB, dorsal body cell; DBp, Dorsal body cell process;
bc blood capillary; ex, extracellular zone;
m, mitochondrion; sg, secretory granules;

Plate 4

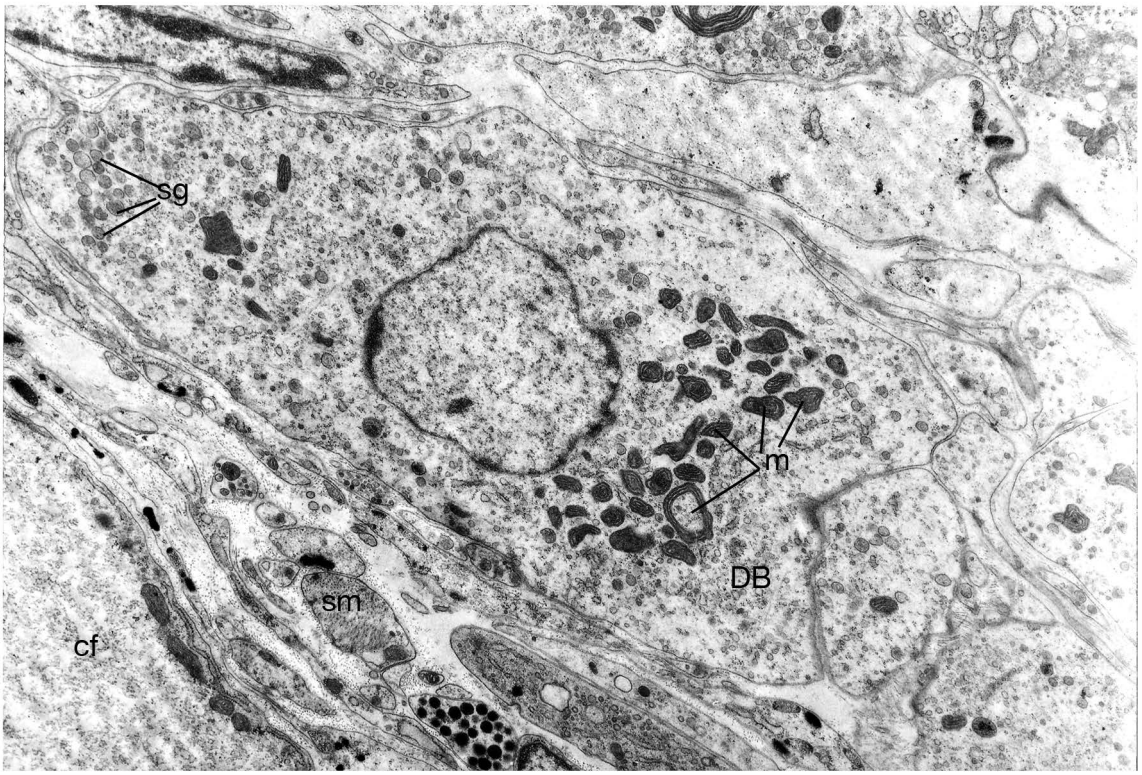

Fig. 9 Dorsal body cell with large mitochondria arranged in whorls. x 6,600
DB, dorsal body cell; cf, collagen matrix;
m, mitochondria; sg, secretory granules;
sm, smooth muscle.

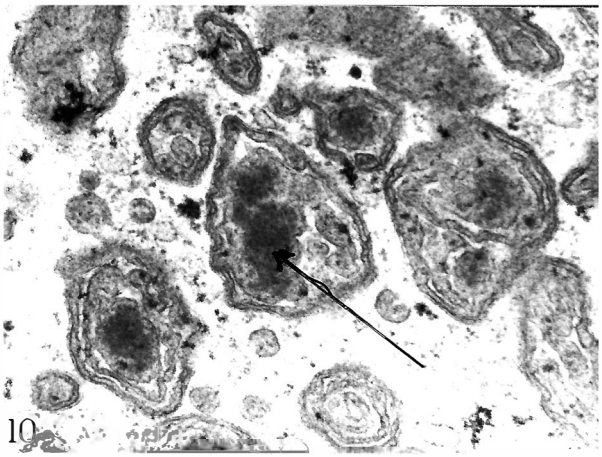

Fig. 10 Dorsal bodv cell mitochondria containing electron dense flocculent material -arrow x 19,800

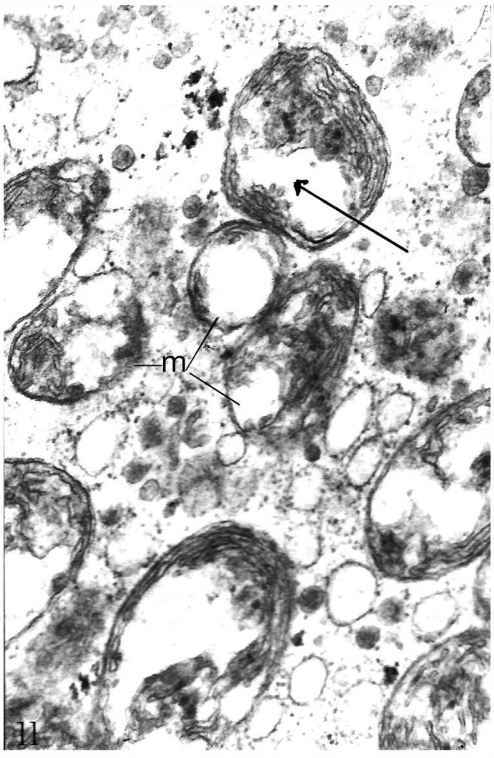

Fig. 11 Dorsal body cell mitochondria with clear areas-arrow. X 19,800
m, mitochondria.

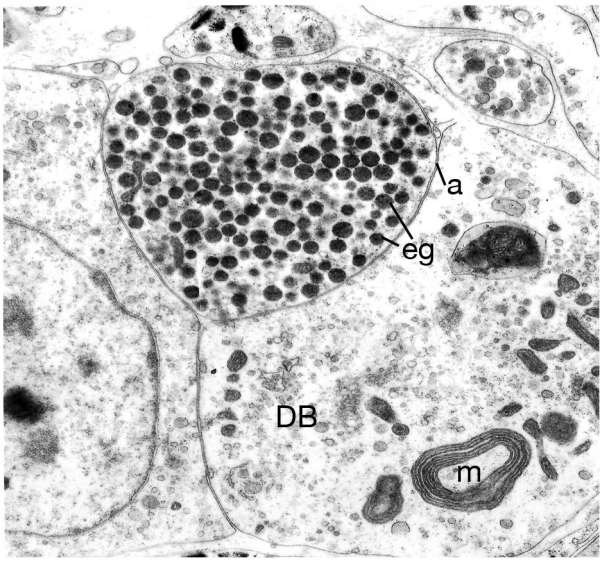

Fig. 12 Axon containing electron dense granules passing between two dorsal body cells. x 10,480
a, axon; DB, dorsal body cells; eg, elementary granules.

Plate 5

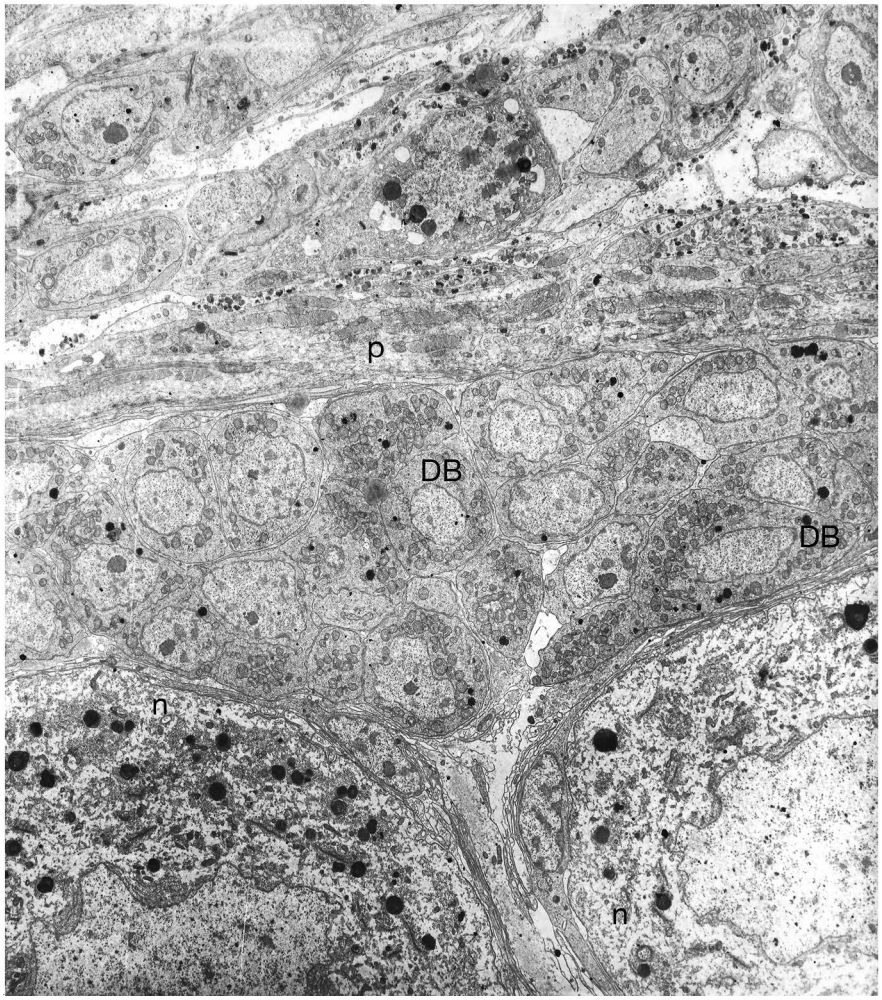

Fig. 13 Dorsal body cells within the ganglionic sheath, adjacent to neurones of the cerebral ganglia. X 2,770

DB, dorsal body cells; p, perineurium; n, neurones.

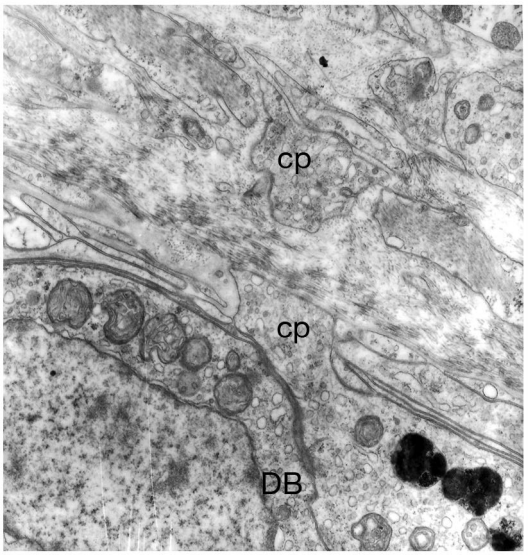

Fig 14 Dorsal body cells ventral to the ganglionic sheath, with cell processes passing back through the sheath. X10,000

Cp, cell process; p, perineurium.

Acknowledgments

It is with much pleasure that I acknowledge the help and encouragement of Dr. N. W. Runham, who suggested the topic and criticised the manuscript.

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POSSIBLE NEUROSECRETORY NEURONS IN THE SLUG *DEROCERAS RETICULATUM* (PULMONATA, LIMACOIDEA)

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SUMMARY

Distinct groups of possible neurosecretory neurons were located in the cerebral, parietal and visceral ganglia and two distinct populations of elementary granules were identified; granules with a modal diameter of 120nm in the cerebral and parietal ganglia and a modal diameter of 200nm in the visceral ganglia. This size difference may indicate a difference in the chemical nature of the two populations of granule.

Groups of axons containing large numbers of elementary granules were observed in the perineural sheath surrounding the cerebral ganglia, but specialised neurohaemal areas were not found. The presence of possible neurosecretory nerves close to capillaries and blood spaces suggests release of neurosecretory material into the blood can take place easily.

The enormously swollen cisternae of rough endoplasmic reticulum (ER) found in some neurons may represent an abnormal condition, where the production of the ER has gone into overdrive and/or the production of elementary granules has broken down. It was not possible to correlate this swollen type of ER with any physiological condition in *Deroceras reticulatum.*

INTRODUCTION

In most species of gastropods investigated, several groups of Gomori-positive possible neurosecretory cells occur. For reviews of the literature on neurosecretory phenomena in gastropods see Gabe, 1966; Simpson et al, 1966a, b; and Durchon, 1967. Gomori-negitive neurosecretory cells have also been found in *Lymnea stagnalis,* (Joose, 1964; Boer, 1965*); Milax gagates* (Quattrini, 1962) and *Succinea putris* (Cook, 1966).

These investigations using the classic neurosecretory stains chromehaematoxylin (CH) and paraldehyde-fuchsin (PF), have been criticised for their lack of specificity (Bern 1962; Boer, 1965; Gabe1966; Simpson *et al*. 1966a,b) and they are known to also

stain lipofuscin pigments and food reserves. Bern (1966) suggested that such stainability should not be the sole criterion for neurosecretory function. Boer (1965) demonstrated the value of histochemical analysis in mitigating the unreliability of the classic neurosecretory stains and suggested that evidence of possible neurosecretion should be substantiated by ultrastructural confirmation of elementary secretory granules in stained cells ((Nolte, 1965; Simpson *et al*, 1966b; Boer *et al*. 1968a).

This study was undertaken as a prelude to investigating the possible role of neurosecretory neurons in the control of reproduction in the slug *Deroceras reticulatum*. The anatomy and histology of the CNS, arterial system, perineurium and dorsal bodies of *D. reticulatum* has been described in earlier papers, (Laryea, 1969a, b).

MATERIALS AND METHODS

D. reticulatum were collected locally after night-fall, kept overnight in a closed container together with damp moss or grass and used the next day. Some animals were used from laboratory culture. For light microscopical observations animals were killed by injection of Susa's. The whole animal or the entire head region was then transferred to fresh Susa for 12-24 hours. When appropriate the hermaphrodite gland was also removed and fixed. After two changes of Cellosolve, 12-24 hours each, the material was embedded in ester wax and serial sectioned at 5-10µmm. Sections were routinely stained in Azan triple stain and paraldehyde fuchsin.

For electron microscopy slugs were killed by decapitation or injection with 1%OsO_4 and the buccal mass and nerves dissected out, placed in 1% OsO_4 and upgraded to 75% ethanol. The CNS was dissected free and the area for study removed and embedded in araldite. Sections were cut on a L.K.B. ultramicrotome.1µm sections were stained with toluidine blue and examined by light microscopy prior to sectioning at 700Å and examination with an A.E.I. EM6B. Possible neurosecretory neurons were located by the dense staining of their contents and their characteristic position. Eight specimens with white and/or translucent nerve cell inclusions so prominent that individual neurons could be easily seen in the visceral complex, were examined under the dissecting microscope. Consistent groupings of cells containing such inclusions were identified and the ultrastructure of these neurons examined.

Only the cerebral, parietal and visceral ganglia of the CNS were examined in this study. The diameters of elementary granules within possible neurosecretory neurons were measured (400 per cell type) and modal diameters determined.

Observations

The CNS of *D. reticulatum* consists of paired buccal, cerebral, pleural, parietal and pedal ganglia and a single visceral ganglion; the pleural, parietal and visceral ganglia forming a fused visceral ring. The ganglia of the CNS are ensheathed in a perineural sheath of connective tissue containing smooth muscle fibres and pigment, granular, vesicular and fibroblast-like cells. Small arteries and capillaries supply the perineural sheath which contains extracellular spaces and small nerves from the cerebral commissure. Within the ganglia the neurons lie in several layers peripheral to the central neuropile and are separated from the basal lamina of the perineurium by a layer of glial cells. The processes of these cells ensheath most of the neurons with the cytoplasm of the deeper lying glial cells reduced to a thin layer around the nucleus and numerous long processes, which penetrate deeply into the perikarya of the larger neurons (fig 4). Many of these glial cells contain thin filaments. Membrane bound dense cores granules of varying density, mean diameter 80 – 130nm occur in most neurons and in some, small clear vesicles, 35 – 50nm also occur. Both axo-axonic and axo-somatic synapses occur in the cortex and neuropile of the CNS ganglia. Two closely apposed neuronal membranes with no membrane thickening and clusters of small clear vesicles apposed to one membrane, are probably axo-axonic synapses (see Frazier *et al*, 1967) while axo-somatic synapses consist of neuronal branches with their axolemma apposed to the somatic plasma membrane (fig 1) Dorsal body tissue, often hidden by the connective tissue of the perineurium, is present on the cerebral ganglia and intercerebral commissure as well as on the cerebro-pleural connectives and the pleural ganglia.

Neurosecretory Neurons

Cerebral Ganglia

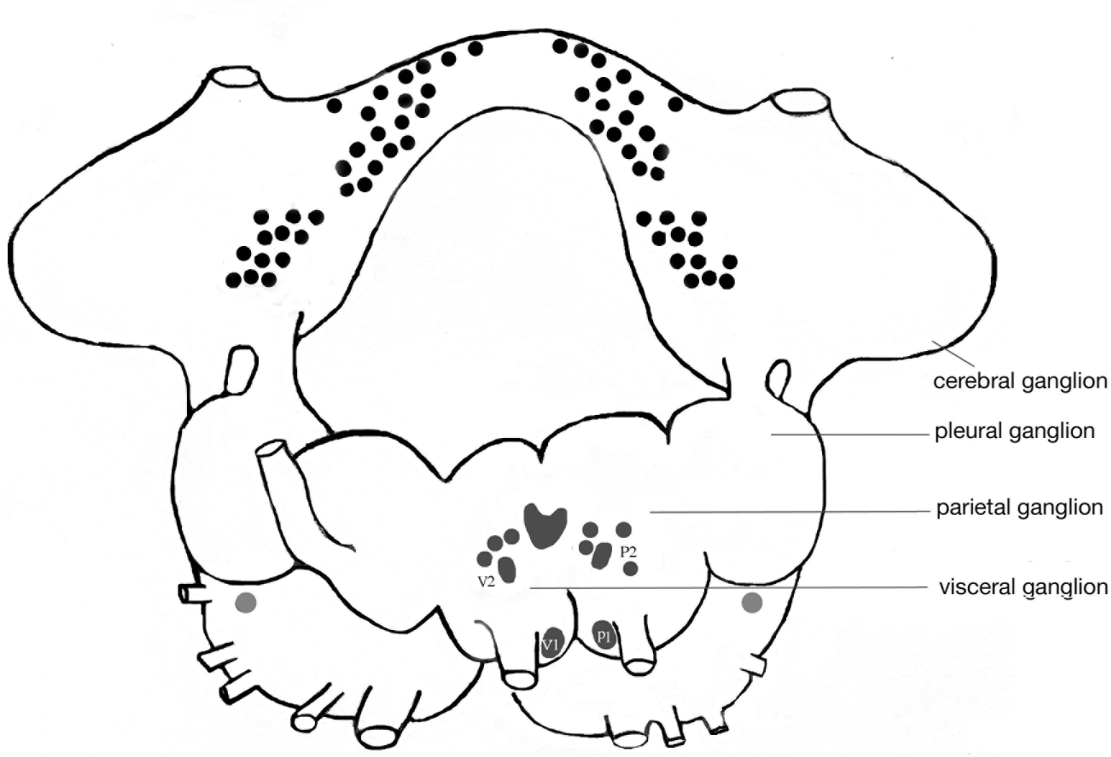
The neurosecretory neurons of the cerebral ganglia are a group of 50 – 60 cells in the

dorsal region of the intercerebral commissure, extending laterally and posteriorly into the medial and posterior regions of both cerebral ganglia. They vary in size from 20-80µm. The largest and smallest neurons tend to be the most dorsal; most have their dendrites directed towards the perineurium (fig1). The posterior neurons can form a continuous grouping of cells with the medial group, but usually occur as a separate group. The cell bodies of the neurons are irregular in outline due to penetration of glial cell processes. The nucleus is usually spherical or oval, often showing considerable indentation with a well - developed nucleolus, both large, scattered clumps and finely dispersed chromatin occur within the nuclear envelope. The cytoplasm contains clumps of membrane bound elementary granules of moderate electron density with a modal diameter of 120nm.

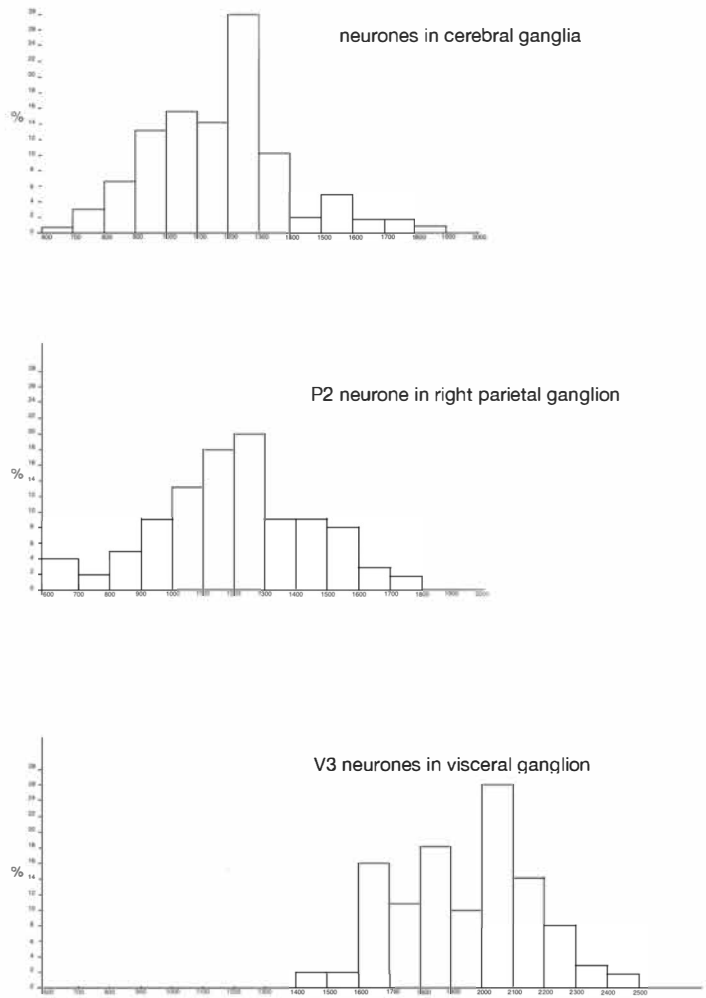
The cytoplasm frequently shows clumping of its components, resulting in scattered, irregular areas of low-density, containing filaments. The endoplasmic reticulum (ER) is predominantly of the rough variety and exists as parallel strands studded with ribosomes. Many free ribosomes exist, mainly as polysomes. Golgi bodies may be abundant, with many forming electron dense granules by budding. They can occur distributed throughout the cytoplasm, but are sometimes restricted to an area around the nucleus. The ovoid mitochondria, vary in size and number and have transverse cristae.

Lysosomes vary in number and are present in a variety of forms; numerous multi-vesicular bodies may occur as well as very large dense cytosomes, the content of which varies in appearance; (membrane - like structures and possible lipofuscin).

In several specimens neurons in the dorsal region of the intercerebral commissure, and the posterior – medial region of the cerebral ganglia were found to contain rough endoplasmic reticulum with enormously, swollen cisternae (EER) that formed large globules occupying much of the cell cytoplasm of perykaria in which they occurred (figs 2, 3). These cisternae contain a fine, homogenous granular material of low to



Diag,1 Location of neurosecretory neurones in the cerebral, right parietal and visceral ganglia of *D. reticulatum*



Diag. 2 Size distribution of the diameters of neurosecretory elementary granules in neurone cell bodies in the cerebral, right parietal and visceral ganglia of *D. reticulatum*.

moderate electron density. In outline, the giant cisternae are irregular and in places the reticulum is drawn out into smaller profiles, interspersed with the cytoplasmic organelles and filling much of the remaining space. The swelling appears to be a general feature of the whole ER (figs 3, 4) and continuous strands of swollen ER may extend a considerable distance down the axon in these neurons (fig 5).

In these specimens elementary granules are few in number and usually restricted to the vicinity of the Golgi apparatus. Many of the Golgi bodies contain material comparable in electron density to the core of these dense granules (trans surface) while the cis surface of some also contains granular material similar to that contained in the swollen ER (fig 6).

In 3% of specimens examined Orange G positive material was found within the cerebral 'neurosecretary' neurons, both as fine granular material and large crystaline like structures and this has been found in the small commissural nerves that pass into the perineurium. It is possible to identify similar inclusions in 1µm sections of material prepared for electron microscopy and stained with Toluidine Blue (see materials and methods). Examination of corresponding sections with the electron microscope revealed these inclusions to be enormously swollen components of the rough endoplasmic reticulum. It was not possible to correlate this swollen condition of the RER with a specific biological function or periodicity.

Right parietal ganglion

A single very large neurosecretory neuron P1, 60 - 80µm occurs medial and/or dorsal to the origin of the pallial nerve, near to the junction of the parietal and visceral ganglia. Electron dense granules of modal diameter 120nm occur in dense packets throughout the cytoplasm and appear to correspond to the fine granular/ flocculent Azan positive staining material and the white inclusions observed with light microscopy.

A group of 7 - 6 medium size neurosecretory neurons P2, occur on the medial dorsal surface anterior to the origin of the right pallial nerve. Sometimes this group contains 1 or 2 much larger cells.

In the anterior region of the ganglion large neurons, 35 -55µm with white inclusions have been seen under the dissecting microscope (Azan positive). From electron microscopy these inclusions appear to be large irregular shaped masses of electron dense lipid like material. Parallel lamellae of GER occur throughout the cytoplasm often arranged in layers around this lipid like material, elementary granules are also present (figs 7, 8, 9).

Visceral ganglion

A very large neurosecretory neuron 65 - 85µm V1, occurs to the right of the visceral nerve at the junction of the visceral and parietal ganglia. Electron dense granules of modal diameter 200nm occur in dense packets throughout the cytoplasm of these cells and probably correspond to the white inclusions sometimes observed with light microscopy, when the perineurium is unusually thin and few pigment granules are present.

On the medial dorsal surface, close to the juncture with the right parietal ganglion and anterior to the origin of the visceral nerve a group of 7 - 8 medium size neurons V2, occur. Sometimes these cells straddle the juncture between the visceral ganglion and right parietal ganglion and contain 2 or 3 larger cells (fig 10) The cytoplasm of these cells contain packets of electron dense granules of modal diameter 200nm.

Small commissural nerves pass from the dorsal neurosecretory cells in the intercerebral commissure through the perineurium and ramify in the surrounding connective tissue, but no evidence of specialised neurohaemal areas were found.

Axons packed with membrane bound granules in the range100 -120nm and of similar electron density to those of the dorsal cerebral ganglia neurosecretory neurons are found in close contact with dorsal body tissue and small blood vessels and blood spaces in the periganglionic sheath, but they do not appear to innervate the dorsal body cells and no specialized axonal contacts were found. Axons containing elementary granules modal diameter 120 -130nm ramify between the DBT cells on the cerebro-pleural connectives and pleural ganglia, but similarly no innervation of dorsal body cells or specialised neurohaemal areas were found.

Small aortic nerves originating in the visceral and right parietal ganglia run to the connective tissue surrounding the cephalic aorta. These axons were found to contain electron dense granules of modal diameter 190 -200nm, characteristic of the neurosecretory cells identified in the visceral and right parietal ganglia (Fig 9), but it was not possible to trace the terminations of the aortic nerves.

Discussion

In *D. reticulatum* the possible neurosecretory neurons of the cerebral ganglia always occur in the same location – the mediodorsal region of the cerebral ganglia - which is consistent with the arrangement in other gastropods that have been studied (see van Mol, 1967; Simpson *et al*., 1966a, b). In the parietal and visceral ganglia of the CNS, however, the location and arrangement of possible neurosecretory neurons in *D. reticulatum* is less consistent and differs from the arrangements reported in other pulmonates.

As well as occurring in the cerebral ganglia, Gomori-positive neurons have also been

reported in the pleural, parietal and visceral ganglia of *Lymnaea stagnalis,* (Lever *et al*., 1961); *Succinea putris*, (Cook, 1966); the pleural and visceral ganglia of Vaginula sp, (Nagabushanam and Swaranamayye, 1963); the parietal and visceral ganglia of *Ferrissia shimekii* (Lever, 1957); *Planorbarius corneus* (Rohnisch, 1964); *Australorbis glabratus* (Lever *et al*., 1965); *Helisoma tenue* (Simpson *et al*., 1966b) and the visceral ganglia of Helicidae sp., Kuhlmann, 1963). Gomori-negative cells have been identified in the cerebral ganglia of *Lymnaea stagnalis* (Joose, 1964; Boer, 1965); and the parietal ganglia of *Milax gagates* (Quattrini, 1964) and *Succinea putris* (Cook, 1966).

There appears to be no prior record of such enormously swollen ER within neurons and it is significant that this phenomenon was only observed in cells in the the medio-dorsal region of the cerebral ganglia, where the possible neurosecretory neurons are located. If this phenomenon is part of a normal production cycle, the relative lack of elementary granules and Golgi bodies, would suggest secretory material is being stored within the cisternae prior to granule production or that it has only just begun. Transport of secretory material from the ER to the Golgi apparatus has, however, been shown to be rapid, Stein and Stein (1967) found labelled protein within the Golgi elements 10 minutes after initial injection, while Jameson and Palade (1967) found labelled protein reached the Golgi apparatus within 7 minutes in *in vitro* guinea pig pancreatic slices.

It is therefore more likely that the enormously swollen ER and numerous Golgi bodies may represent an abnormal condition, one where the production of the ER has gone into overdrive or the production of elementary granules has broken down, or both. It was not possible to correlate this swollen type of ER with any physiological condition in *D. reticulatum*.

However, striking similarity exists between the enormously swollen ER in the cerebral ganglia neurosecretory cells of *D. reticulatum* and the secretory cisternae described by Sterba (1967) in the ependymal cells of the lamprey brain. Here the phenomenon appears to be part of a normal secretory cycle.

In vertebrates and invertebrates the presence in neurons of electron-dense elementary granules within the range 100 - 300nm is usually considered to indicate they are neurosecretory cells, although this criterion should not by itself be considered absolute proof (see B. Scharrer, 1967).

Boer *et al* (1968a), have suggested that in *Lymnaea stagnalis* neurons crowded with elementary granules represent an 'inactive' state (storage of granules), compared to 'active' neurons which were relatively empty of elementary granules. In this study the number of elementary granules within cells varied considerably and elementary granules, with cores of varying density, were frequently observed originating by budding from Golgi lamellae.

Two distinct populations of elementary granules were identified. 120nm in diameter in the cerebral ganglia and 200nm in the parietal and visceral ganglia. This size difference may indicate a difference in the chemical nature of the two populations of elementary granule. However, elementary granules 200nm in diameter are produced by neurons in both the parietal and visceral ganglia and it is likely these similarly sized granules differ chemically from each other. Histochemical analysis of the secretory products found in the different possible neurosecretory cell groups is needed to resolve this issue. It has been suggested that two biochemically distinct groups of neurosecretory product, peptide and non-peptide, may occur in neurosecretory neurons (Bern, 1966), but classic neurosecretory products are polypeptides bound by a carrier protein, neurophsin, and thus differ chemically from known neurotransmitters (Scharrer B., 1967)

Electron-dense elementary granules within the range 100 - 300nm have been found in some pulmonates, in locations that suggest that they contain neurotransmitters viz; at synaptic junctions in *Vaginula solea*, *Helix pomatia* and *Cryptomphallus aspersa* (see Gerschenfeld, 1963) and in fibres innervating effector organs in *Archachatina*, (Baxter and Nisbet, 1963; Amoroso *et al*., 1964) and *Lymnaea stagnalis* (Wedelaar Bonga, 1969). However, the elementary granules found in the CNS of *D. reticulatum* are more likely to be neurosecretory, than contain neurotransmitters, since the axons bundles (fig 11, 12) contain granules of the same size and appearance as those occurring in perikaryia and axonal membrane thickenings, indicative of synapses were not observed.

In Basommatophora the neurohaemal areas for the Gomori-positive cells in the cerebral ganglia are located in the lip nerve and the intercerebral commissure, Joosse (1964); Röhnisch, 1964; Nolte, 1965; Boer *et al*., 1968a*)*, but Wendelaar Bonga has also found a network of nerves containing neurosecretory material near blood spaces and capillaries forming extensive neurohaemal zones in the connective tissue surrounding the CNS of *Lymnaea stagnalis* (personal communication 1969).

In the Stylommatophora, the axonal tracts of the large neurosecretory cell groups of the cerebral ganglia were found to run to the *nervus arteriae cerebralis* (van Mol, 1960; Kuhlmann, 1963) the small nerves arising from the intercerebral commissure (Kuhlmann, 1963); the median and internal lip nerves (Kuhlmann, 1963); Cook, (1966). Neurosecretory fibres have been observed in the perineurium (Lemche, 1955; Kuhlmann, 1963; Röhnisch, 1965; Goggeshall, 1967; Sanchiz and Zambrano, 1968), but not release phenomena.

Although possible neurosecretory neurons were identified in the cerebral, parietal and visceral ganglia of *D. reticulatum*, convincing evidence for specialised neurohaemal areas was not found by staining methods or electron microscopic investigation. Groups of axons containing large numbers of elementary granules were observed in

the perineural sheath of the cerebral ganglia, but grouping of axons with swollen axonal ends or evidence of exocytosis was not found.

The presence of possible neurosecretory nerves in the perineurium of *D. reticulatum*, close to capillaries and blood spaces suggests that release of neurosecretory material into the blood can take place easily. Although neurohaemal areas were not identified in this study, it is likely that they exist in *D. reticulatum*, given their existence in other pulmonate species. Large numbers of elementary granules within distended axon endings have been found in the periphery of the intercerebral commissure of *Planorbarius corneus* Nolte, (1965) and *Lymnaea stagnalis*, Boer *et al*., (l968a).

In vertebrates and invertebrates, with few exceptions, neurosecretory substances are released from the axon, usually from bulbous endings or pre-terminal swellings (Scharrer, B. 1968), but the actual mechanism of release is uncertain. Scharrer found release sites in blattarian insects, characterized by small electron lucent vesicles clustered near the internal surface of the plasma membrane and accumulations of electron dense material on either side of this membrane; ultrastructurally similar to conventional pre-synaptic neuronal junctions. These vesicles may be formed by budding from elementary granules as suggested by Scharrer or more likely represent the remnants of membranes of elementary granules after their content has been extruded into the extracellular space by exocytosis (e.g. Weitzmann, 1969; Norman, 1969). It has been possible to correlate experimentally induced release of neurohormones with the occurrence of these vesicles in *Periplaneta americana* (Scharrer and Kater, 1969) and in *Calliphora erythrocephala* (Normann and Duve, 1969).

Despite the lack of evidence for secretory release the presence of numerous small clear vesicles found in axons with membranes apposed to blood capillaries (fig 1), may represent membrane fragmentation after the release of neurosecretory material into the blood via the sinuses and capillaries as has been suggested by Bern, (1963).

In *Lymnaea stagnalis*, Nolte found clear vesicles and membrane fragments in the lip nerves (1967) and Boer *at al*. (l968a) noted the occurrence of clear vesicles in the intercerebral commissure.

The optic glands of cephalopods (Durchon and Richard, 1967; Richard, 1969) and the dorsal bodies of the gastropods (Joosse, 1964; Joosse and Geraerts, 1969) are known to be endocrine glands involved in the regulation of the reproductive process. There is also experimental evidence for a neurosecretory role in the control of osmoregulation in *Lymnaea stagnalis* (lever *et al*, 1961; Wendelaar Bonga, 1969); *Aplysia rosea* (Vicente, 1963) and *Crassostrea virginica* (Nagabhushanam, 1964).

Blood - borne hormones are also known to be involved in the maturation of the reproductive tract in *D. reticulatum,* (Runham *et al*., 1973), but whether endocrine or neurosecretory in origin, is yet to be determined. A definite hormonal function is implicit in the concept of neurosecretion (Bern, 1966), but precisely how the different groups of neurosecretory neurons in the CNS of *D. reticulatum,* control various physiological functions is yet to be determined.

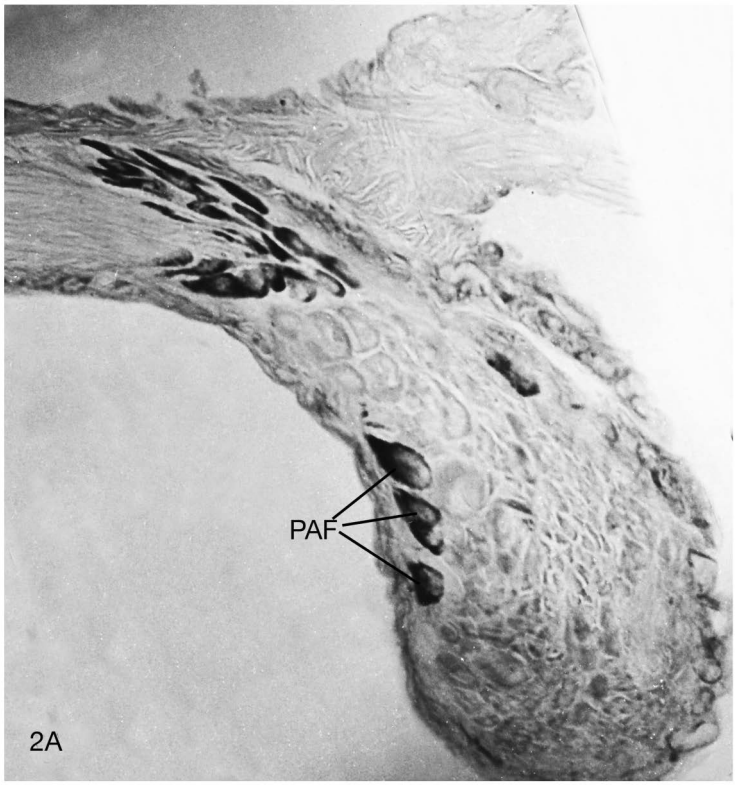


Fig. 2A P.A.F – positive cells in an oblique section through the intercerebral commissure and right cerebral ganglion.

PAF, P.A.F – positive cells

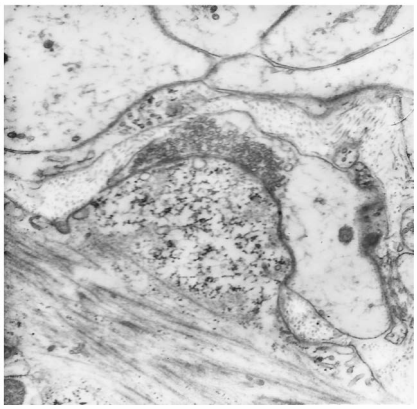


Fig. 1 Clear vesicles at synapse

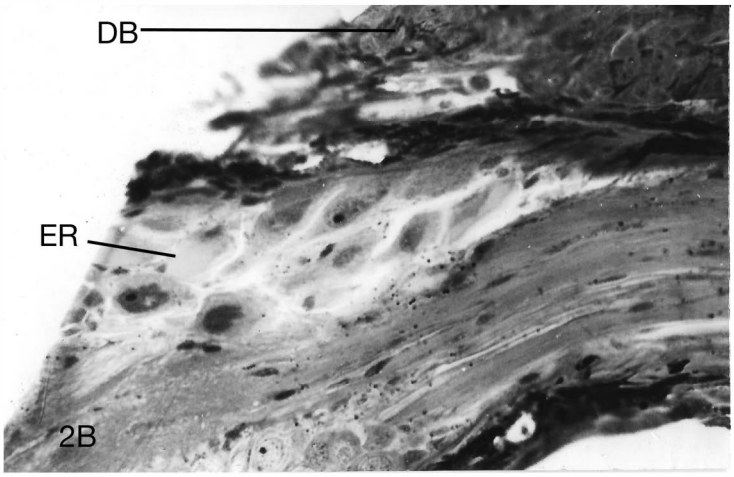


Fig. 2B Swollen cisternae of rough endoplasmic reticulum, stained with Orange G (Azan stain), in neurones in the left cerebral ganglion and intercerebral commissure.

EER, swollen cisternae; DB, dorsal body tissue.

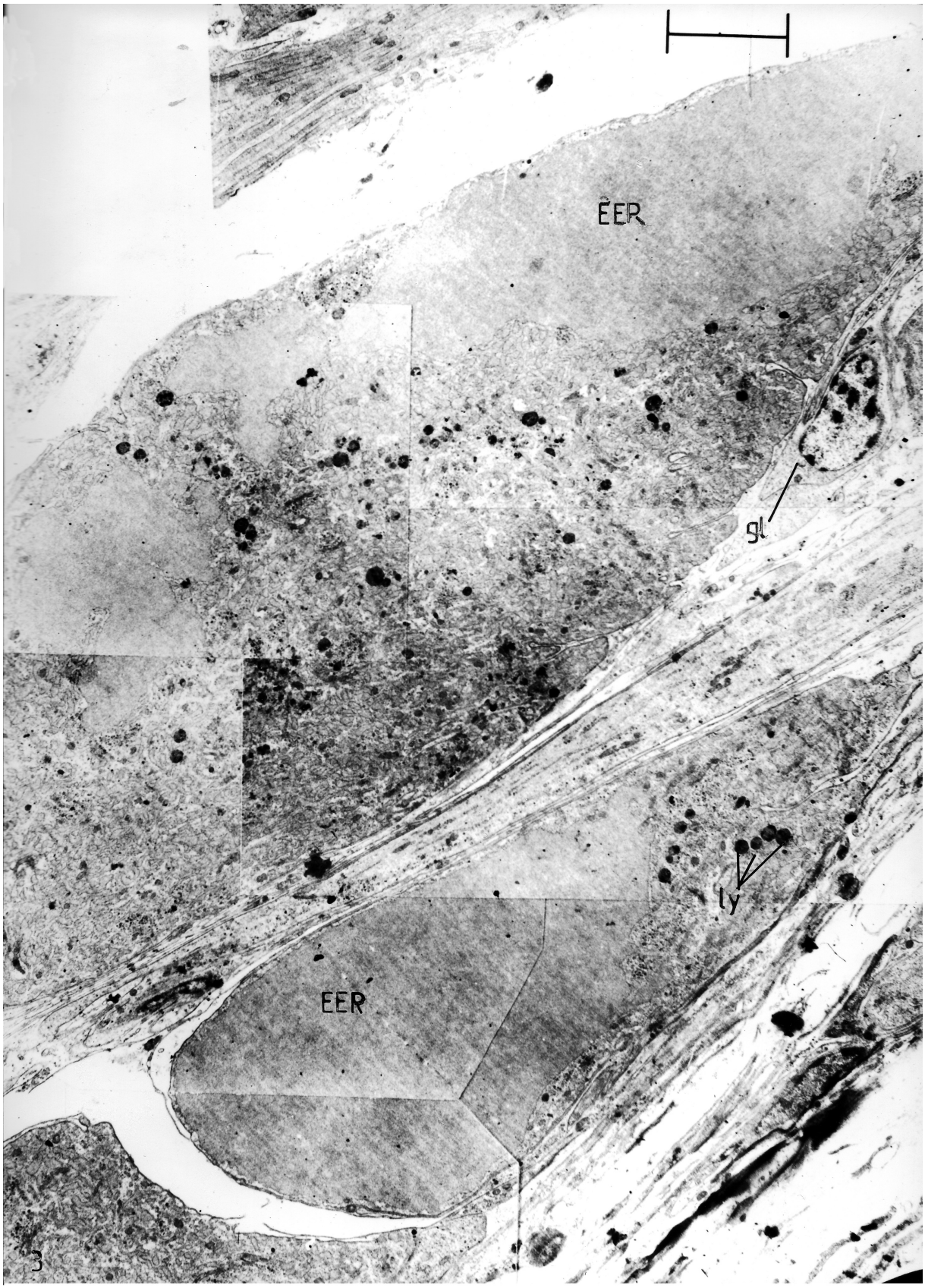
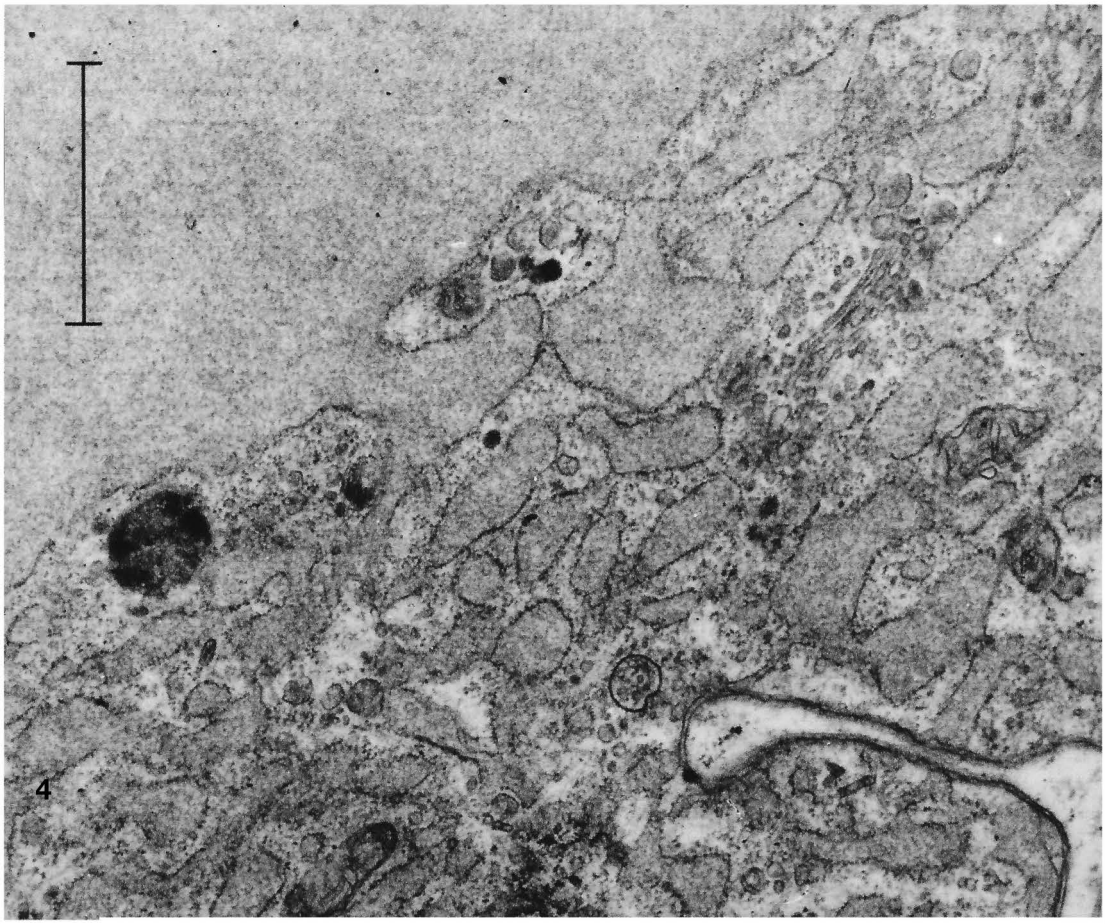


Fig. 3 Composite electron micrograph showing swollen cisternae in two neurones in the medio - dorsal region of the right cerebral ganglion. x 5,025.

EER, swollen cisternae; gl, glial cell; ly, lysosome.



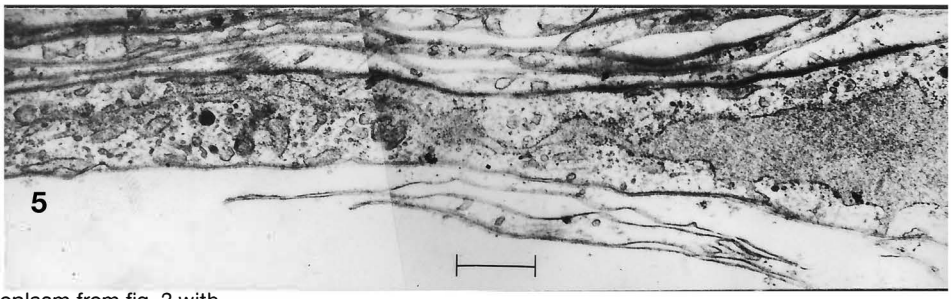


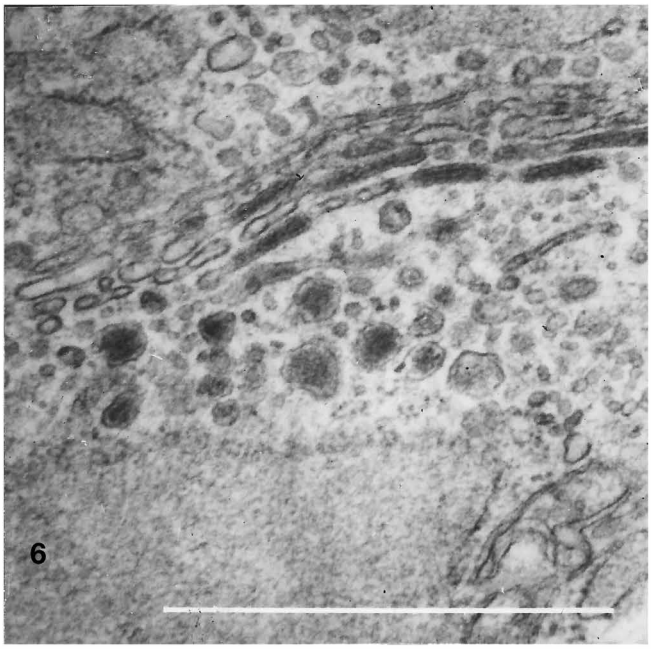
Fig. 4 Enlarged area of cytoplasm from fig. 3 with swollen cisternae. x 32,000

ly, lysosome; EER, swollen cisternae;
tr, trophospongium.

Fig. 5 A continuous strand of swollen cisternae extending into the axon of a neurone in the cerebral ganglion.

ax, axon; EER, swollen cisternae.

Fig. 6 Enlarged area of cytoplasm from fig. 3 with electron dense granules budding from a Golgi body. x 57,000
eg, elementary granules; EER, swollen cisternae;
gb, Golgi body.



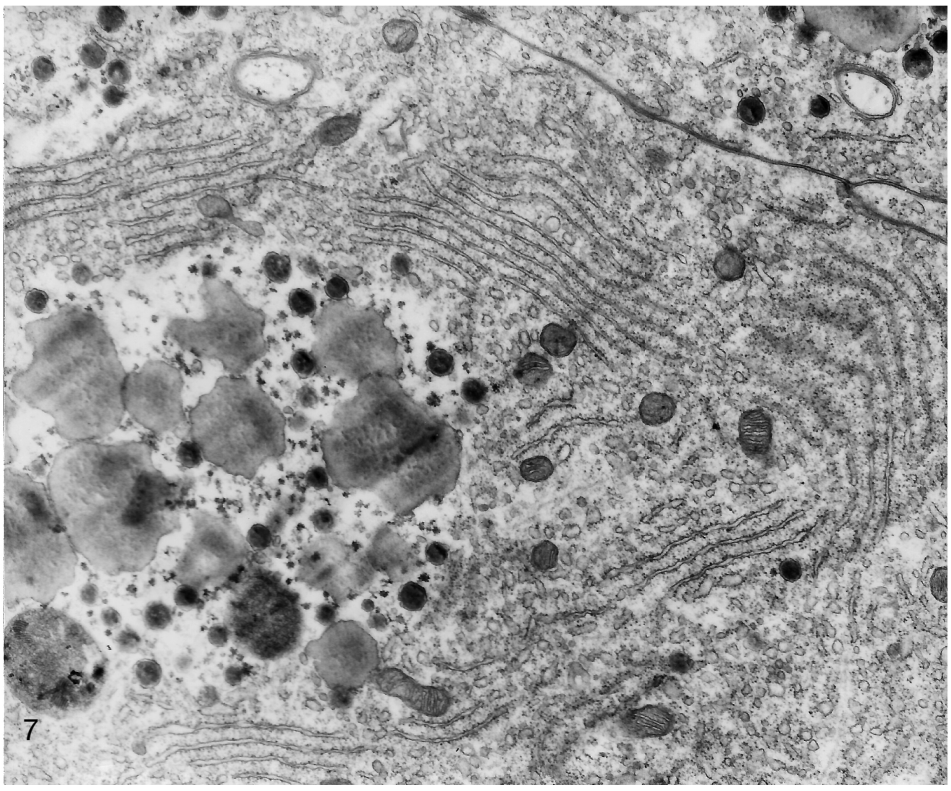
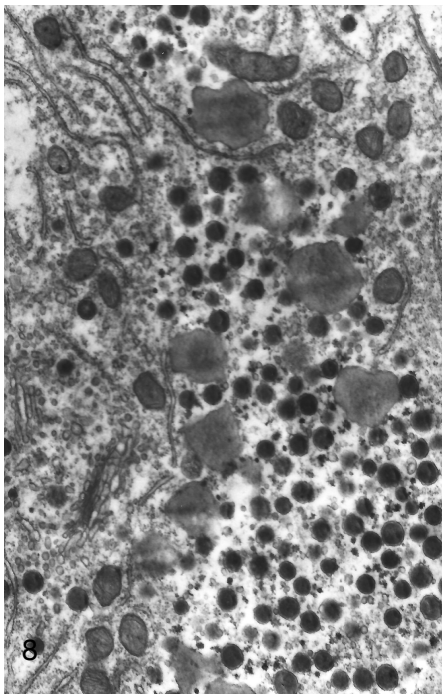


Fig. 7 Cytoplasm of P2 neuone in right parietal ganglion: lipid-like globules encompassed by parallel strands of rough endoplasmic reticulum.
eg, elementary granules; li, lipid-like globules, ly, lysosome; m. mitochondrion.



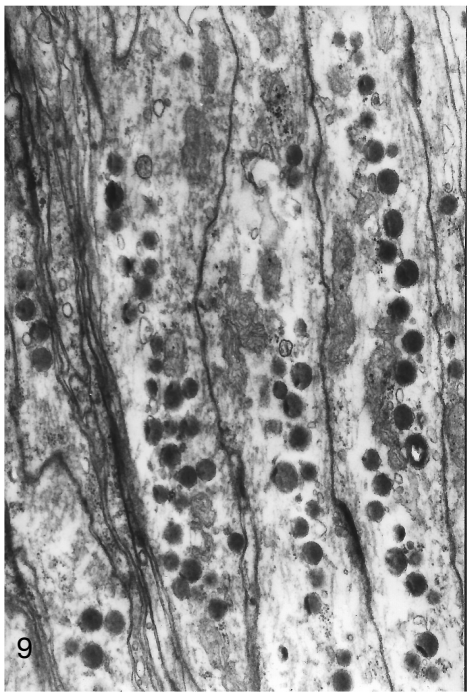


Fig. 8 Cytoplasm of a different P2 neuone with electron dense elementary granules. 100nm - 125nm dia. x 34,000

Fig. 9 Axons of P2 neuones containing electron dense granules 100nm - 125nm dia. x 34,000

eg, elementary granules; li, lipid-like globules.

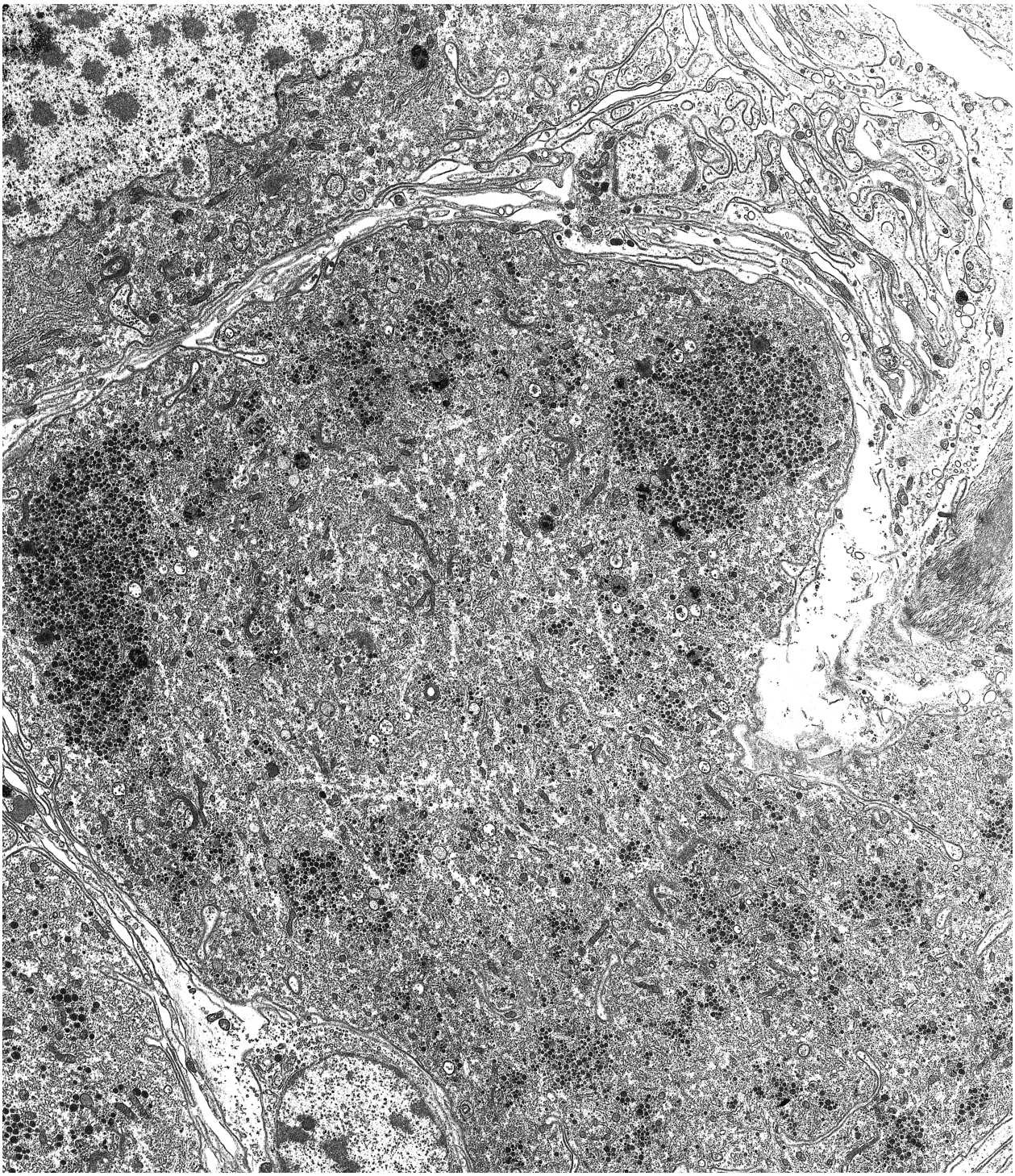


Fig. 10 Large neurone V3 straddling the juncture between visceral and right parietal ganglion containing packets of electron dense granules 180nm – 200nm dia. x 35,700

eg, elementary granules; gl, glial cell.

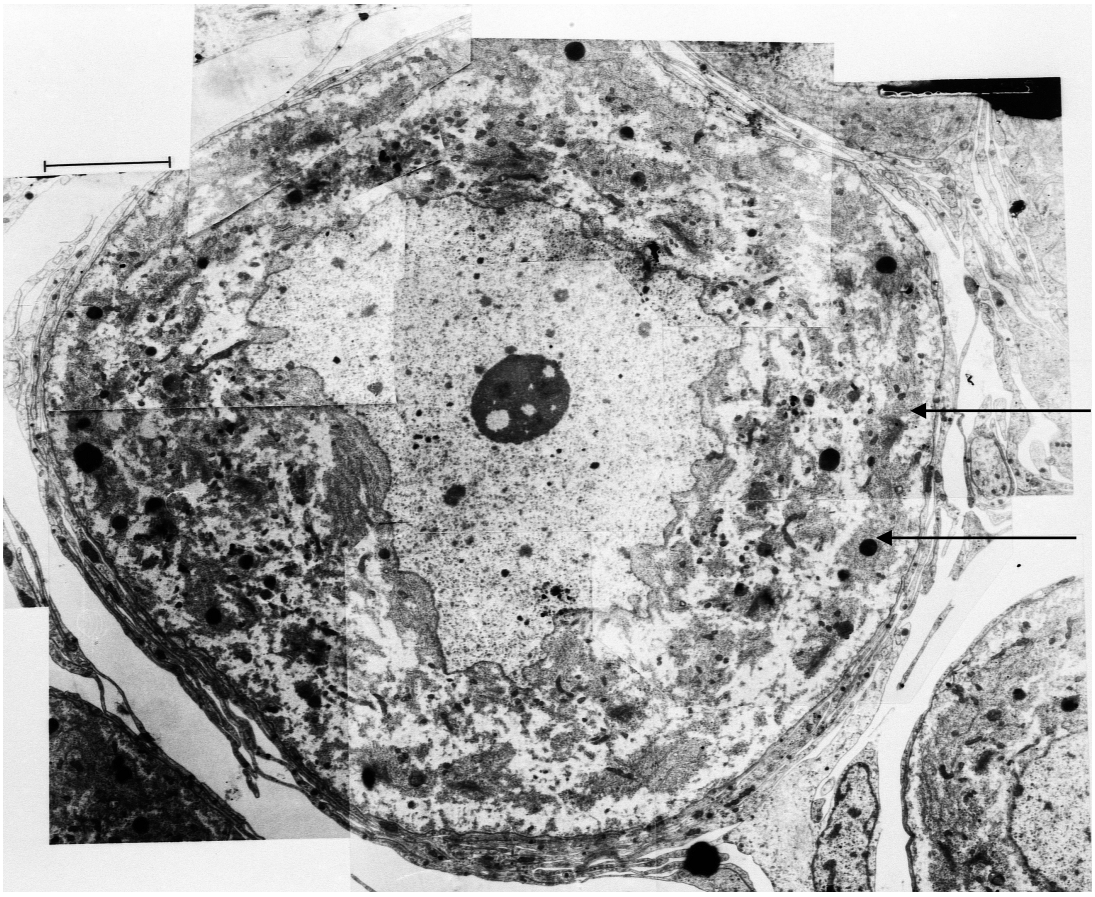


Fig. 11 Giant neurone V1 in visceral ganglion.
Arrows - neurosecretory granulesin clumps x 2,132

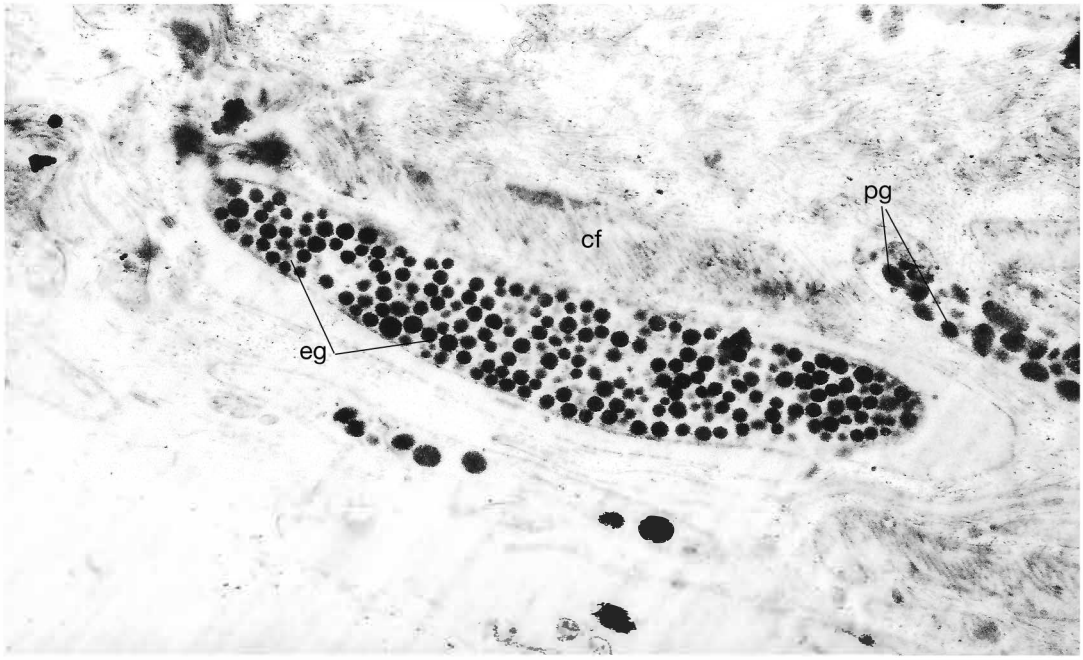


Fig. 12 Section of axon containing electron dense granules 190nm – 200nm dia. in the epineurium of the cerebral ganglion. X 11,000

cf. collagen fibres; pi, pigment granules; eg, elementary granules.

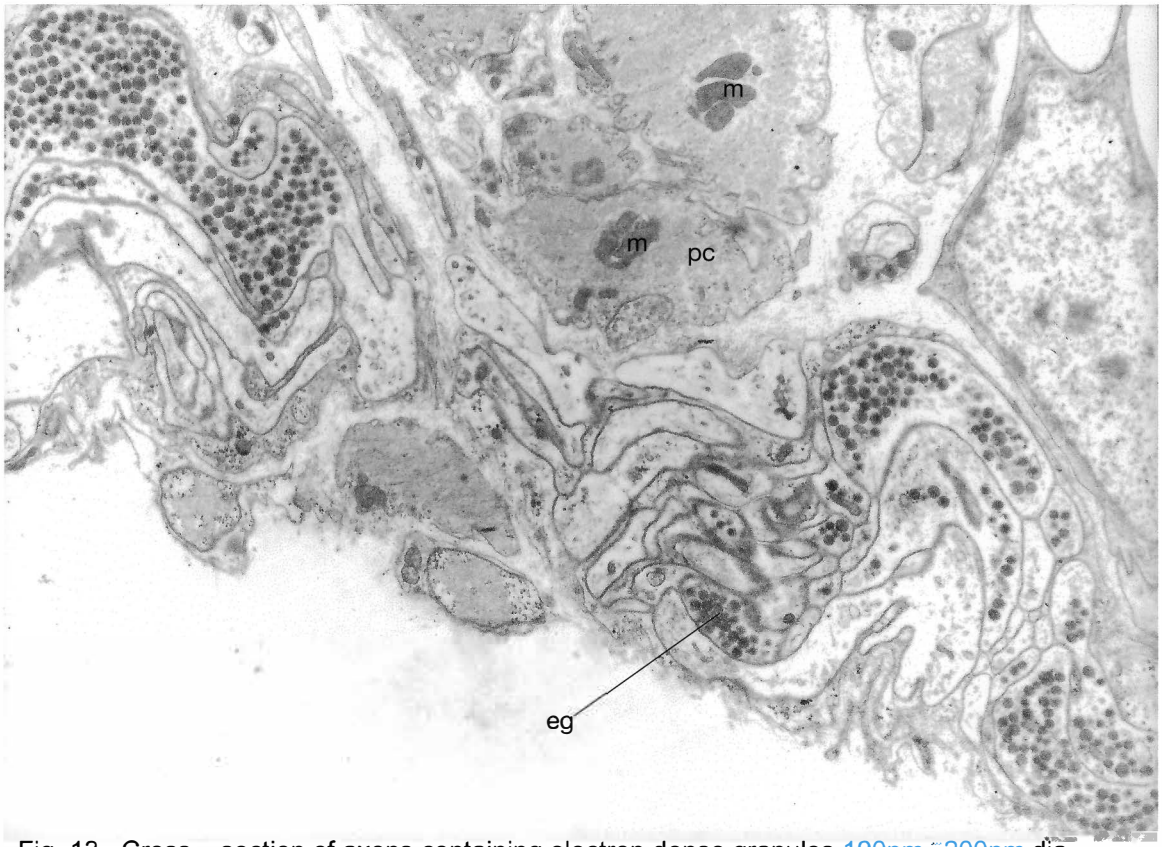


Fig. 13 Cross – section of axons containing electron dense granules 190nm – 200nm dia.. in the epineurium of the cerebral ganglion.

Pore cells, pc; eg, elementary granules; m. mitochondrion.

Acknowledgments

It is with much pleasure that I acknowledge the help and encouragement of Dr. N.W.Runham, who suggested the topic and criticised the manuscript.

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THE ARTERIAL GLAND OF THE SLUG *DEROCERAS RETICULATUM* (PULMONATA, LIMACOIDEA)

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October 1969

SUMMARY

The arterial gland of *Deroceras reticulatum* consists of irregularly shaped masses of opaque whitish tissue situated along the distal portion of the cephalic artery and its branches, especially the posterior pedal artery. The tissue is divided into lobules separated by thick bundles of collagen-like fibres. Each lobule is composed of irregular cells together with intercellular channels; on some case leading directly to the edge of the gland. Intracellular ducts connect with the intercellular channels.

Granules occur within the cells and these appear to be of two types. Each A type granules has an amorphous, moderately homogenous, electron dense content, which normally completely fills its limiting membrane. These granules stain deep blue with toluidine blue. B type granules are less electron dense, their contents have a flocculent appearance and they stain only lightly or moderately with toluidine blue. These granules contain a variable number of irregular spaces. The granules release their contents into the intercellular channels directly or into the intracellular ducts.

Histochemical tests for carbohydrates, certain hydroxysteroid dehydrogenases, calcium, copper and acid phosphatases were all negative. Tests for lipid were only faintly positive. The secretory granules, however stained intensely with bromophenol blue and gave positive reactions to tests for tyrosine and aspartic and glutamic acids. Tests for SH and SS groups were only weakly positive. Analysis for steroids using thin layer chromatography gave negative results.

Electron probe microanalysis revealed an accumulation of copper within the arterial gland tissue, but it was not possible to localise its position within the cells.

Rabbit antiserum to *Helix aspersa* haemocyanin. was prepared and found to cross react with *D. reticulatum* hemocyanin. Immunoelectrophoresis performed using this antiserum and homogenised arterial glands gave negative results.

The arterial gland in *D. reticulatum* contains secretion at all stages of reproductive development. The size of the gland is extremely variable between individuals, but neither size nor histology could be related to reproductive development.

A number of gastropod species were examined for the presence of the gland. Tissue with a similar appearance to the arterial gland of *D. reticulatum* *were found in four: Agriolimax caruanae, Limax flavus, Oxychilis alliarius and Oxychilis cellarius.*

INTRODCTION

During investigation of the central nervous system of *D. reticulatum* a tissue was discovered attached to arteries arising from the cephalic arborescence. It appeared to be glandular and lacked ducts. As the tissue was not only attached to arteries, but also surrounded by the blood of the haemocoel, it was possible that it was an endocrine gland. Because of current interest in gastropod physiology it was decided to investigate this tissue in some detail. It was provisionally named the arterial gland.

MATERIALS AND METHODS

General Microscopy

D. reticulatum were collected locally after nightfall, kept overnight in a closed container together with damp moss or grass and used the next day. Some animals were used from laboratory culture. Animals were killed by injection of Susa's. The whole animal, the entire head region, or part of the posterior pedal artery was then transferred to fresh Susa for 12-24 hours. When appropriate the hermaphrodite gland was also removed and fixed. After two changes of Cellosolve, 12-24 hours each, the material was embedded in ester wax and serial sectioned at 5-10µm. Mercury was removed from sections by treatment with iodine and sodium thiosulphate. Sections were routinely stained in Azan triple stain. 1µm araldite sections of one week old slugs (from culture), and of arterial glands from animals of hermaphrodite gland stages OG (Runham and Laryea, 1969) were also examined.

In addition, serial sections (Susa/azan) were made of the following gastropod species: *Agriolimax caruanae*, *Milax sowerbyi*, *Tandonia budapestensis* (Limacidae); *Arion hortensis*; (Arionidae); *Oxychilis alliarius*, *Oxychilis cellarius* (Zonitidae); *Succinea putris* (Succineidae); *Aplexa hypnorum* (Physidae); *Lymnaea stagnalis* (Lymnaeidae); *Planorbis albus* (Planorbidae). The nerve ring (together with its arterial supply) of *Cepea nemoralis* and *Helix aspersa* (Helicidae), and the posterior pedal artery of *Limax flavus* (Limacidae) were also serially sectioned.

Histochemistry

Animals were usually anaesthetized by being placed in a closed pot on a tray above a small lump of solid carbon dioxide for five to ten minutes (Bailey, 1969). Histochemical investigations were carried out on both fixed and fresh frozen material. Two series of wax sections were made by the alternate serial section technique, of which one was routinely stained in azan, while the appropriate histochemical test was applied to the other. For cryostat work, tissue supported in mouse liver, carboxmethylcellulose, or whole animals, were frozen to metal chucks by immersion in liquid nitrogen. Sections were cut at 8µm - 12µm and attached to clean cover slides by momentary thawing. For the demonstration of hydroxysteroid dehydrogenase activity sections were washed in buffer (5 mins) at room temperature prior to incubation. The incubation medium was the same as that described by Davies et al (1966). Control sections were incubated in media lacking a steroid substrate. Sections of rat liver and adrenal were similarly processed for comparison. The following steroid substrates were used: 3β-hydroxyandrost-5-en-17-one, 5β-pregnan-3α-ol-20-one 17β-hydroxyandrost-4-en-3-one, estradiol 17-(β-d-glucuronide).

Electron Microscopy

The anterior portion of the posterior pedal artery and attached arterial gland was removed from animals anaesthetised with CO_2 and placed in ice cold 6% glutaraldehyde for 3 hours, washed in ice cold buffer overnight and post for 1 hour the next day. After dehydration through a graded series of ethenol or Durcupan solutions the material was embedded in Araldite and sectioned on an L.K.B. Ultratome. Sections were mounted on collodion (2%) coated copper grids and stained with lead citrate and 5% uranyl acetate (Pearse). The sections were studied on an A.E.I. EM 6B electron microscope. Osmium fixed material (ice cold veronal buffered 1% osmium tetroxide, pH 7.3) was similarly treated. Sections stained with a 1% solution of toluidine blue were also viewed by light microscopy.

Haemocyanin

Haemolymph was obtained from *Helix aspersa* by puncturing the shell and mantle epithelium and inverting the shell over a centrifuge tube. The haemolymph was centrifuged to remove shell and tissue debris and then ultracentrifuged for 1 hour at 27, 500 on a Spinco model L ultracentrifuge. After repeated washings the pellet of haemocyanin at the bottom of the tube was re-suspended in saline and centrifuged for an hour (27, 500). The pellet was removed

and centrifuged for another hour (27, 5000). The pellets obtained we washed and pooled.

Preparation of antiserum to *Helix aspersa* haemocyanin.,

The haemocyanin was suspended in saline and mixed with an equal volume of Freund's adjuvant (Difco). Two rabbits received an intramuscular injection in each hind-quarter of 1 ml and the injection was repeated after two weeks. Test bleedings two weeks later gave a strong reaction against freshly prepared *Helix aspersa* haemocyanin and 20 ml of blood was removed from the marginal ear vein of each rabbit. The serum was separated from the cells and clot, and stored at -20º C.

Precipitin Tests

To check for the presence of antibody in the serum and its cross reaction with *D. reticulatum* haemocyanin precipitin tests were carried out for serial dilutions of *Helix aspersa* and *D. reticulatum* haemocyanin's using 0.5 ml of antisera and 0.5 ml of antigen in saline per tube. The tubes were incubated at 37 º C. for 1 hour and stored at 0º C. overnight. Readings were taken the next morning.

Immunoelectrophoresis

Immunoelectrophoresis was performed in 1% Ionagar (Oxoid No.2) made up in veronal acetate butter on microscope slides. After electrophoresis i) *D. reticulatum* haemocyanin and ii) 12 homogenized arterial glands plus *Helix aspersa* antisera was added to wells in the gels then stored in a saturated atmosphere at 37º C. and inspected at intervals over 14 days.

Microprobe analysis

Collodion covered nickel mesh was coated with a layer of carbon. 2 µm thick Araldite sections of *D. reticulatum* hermaphrodite gland and arterial gland (glutaraldehyde fixed) were then mounted on the collodion surface of the mesh, which was then again carbon coated. This material was examined in a JEM Electron Microprobe for copper content. (see Duncumb, 1967) for a review article on this technique).

RESULTS

General Microscopy

The arterial gland of *D. reticulatum* consists of irregularly shaped masses of opaque whitish tissue situated along the distal portion of the cephalic artery and along its branches particularly the posterior pedal artery (Figs 1, 2). It may be present on all these branches near to their origin, but it is most abundant, and only extends for any distance along the posterior pedal artery (Fig 2). Discontinuous masses of the tissue may extend over the length of this vessel, but very little is present on the minor branches which penetrate the foot. Most of the tissue is attached to the lateral margins of the arteries. The size and distribution of the gland is extremely variable between individuals, but individual masses of this tissue may extend for 0.5 mm or more.

A thin connective tissue sheath surrounds the arterial gland. The tissue masses are made up of conglomerations of varying numbers of cells, and in wax embedded material their boundaries are not clearly delineated. Their cell nuclei are well defined, however, with a more or less regular shape; in the main spheroidal ovoid, with finely distributed chromatin. The cell contents appear granular and occur in a bizarre array of shapes and sizes. The outline of many of the granules is suggestive of a crystaline structure, while others are circular in profile. With the azan trichrome stain the granules stain predominantly red and orange; the remainder blue or grey. Neither discharge of granules from the tissue nor evidence of organised ducts was observed in wax embedded material (Fig 3).

Superior fixation was obtained when material was fixed in glutaraldehyde and embedded in Araldite. Using this technique the gland can be seen to be divided into lobules separated by connective tissue. Each lobule is composed of irregular cells with interconnecting intercellular channels. These channels tend to have a transverse orientation and many of them lead directly to the edge of the gland. Intracellular ducts connect with the intercellular channels (Figs 3,4). When stained with toluidine blue the granules within the cells show differential affinity for the dye, and light, moderate, and deeply staining granules may be distinguished. Some granules appear solid, while others have non-staining centres (Fig 5). Serial reconstruction shows many of the granules seen in individual sections to be part of the same irregular mass.

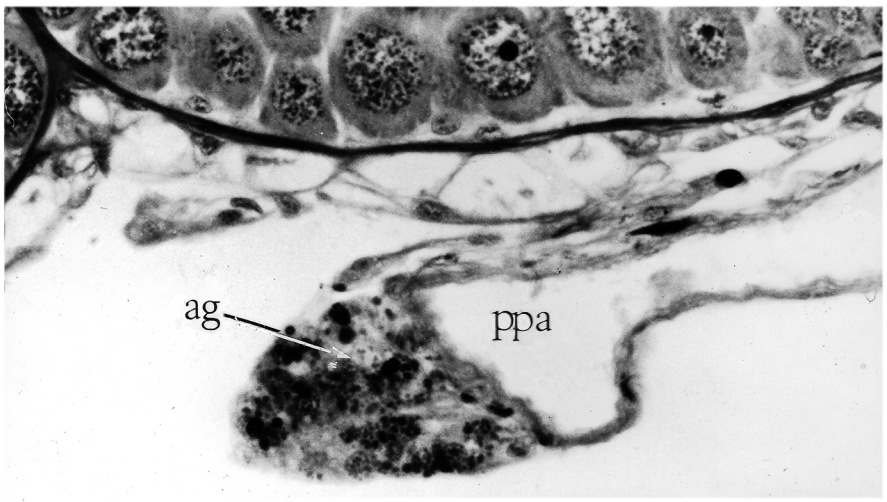


Fig.1 Transverse section of the posterior pedal artery (ppa) and attached arterial gland (ag) containing granules. Fixative: Susa, stain: Azan

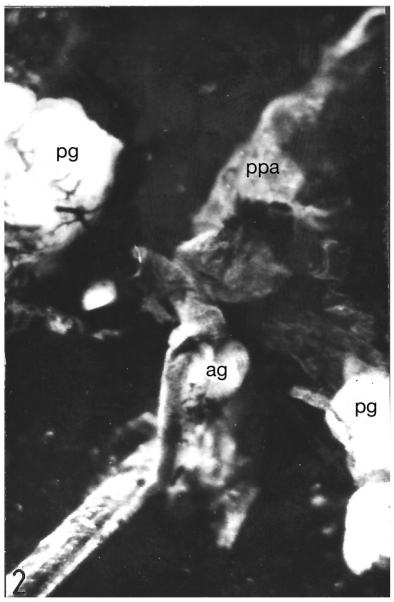


Fig.2 Dissection showing arterial gland tissue (ag) attached to the posterior pedal artery (ppa) of an animal whose arteries have been injected with Indian ink. Separated pedal ganglia (pg).

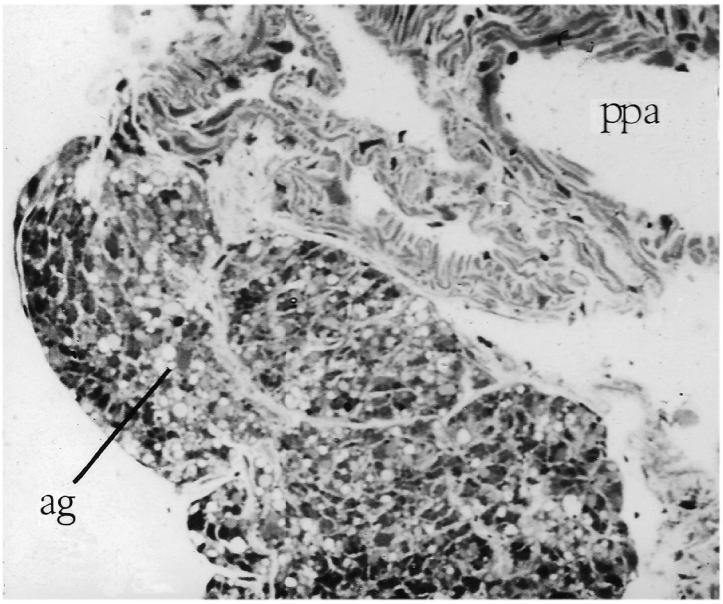


Fig.3 Transverse section of posterior pedal artery, section of arterial gland divided into lobules separated by connective tissue. Fixative: gluteraldehyde, stain: toluidine blue.

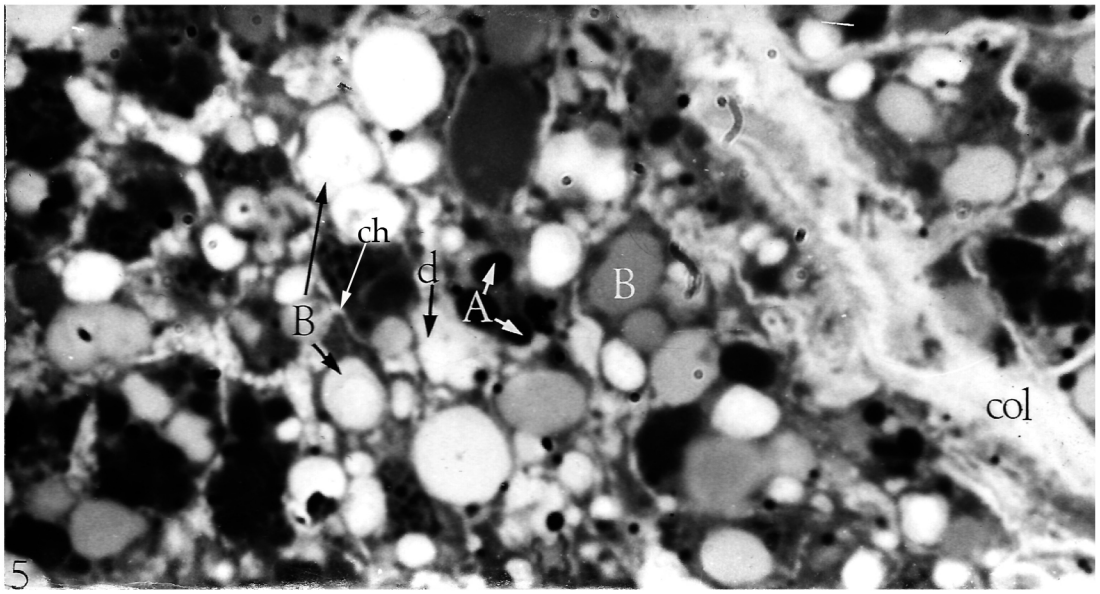


Fig.5 Section of arterial gland showing differential staining of granules (A) and (B), intracellular ducts (d) and intercellular channels (ch). Connective tissue (col) separating lobules of gland. Fixative: gluteraldehyde, stain: toluidine blue.

Ultrastructure

A thin sheath of collagen fibres encapsulates the lobules of the arterial gland and connects it to the artery wall. Individual cells may also be directly connected to the collagenous outer layer of the artery wall .

Many of the arterial gland cells have an extremely irregular outline. Their walls usually show infoldings and innumerable microvilli like processes, which when they follow the long axis of the cell often reach considerable length. Surface irregularities may be such, that they appear like incomplete ducts in section. True ducts exist within the cells, so that the cells often appear to have 'holes' in them. Some of these ducts are in continuity with the small channels which separate most of the cells and which in many cases run to the edge of the gland (Fig 6). These channels are not artifacts, since they contain not only the cell processes, but in some cases interstitial tissue (see below), nerve axons and even secretory products (Fig 10). Collagen fibres do occlude some of these channels. The intercellular channels exhibit a slight tendency to radiate from the centre of attachment of the lobule. Many of these channels interconnect.

Collagen appears to play an important role in the structural integrity of the gland, since it is the major connective tissue element found between the lobules and conventional junctional complexes have not been observed between the cells. The interstitial tissue usually contains particulate matter resembling badly fixed glycogen and is strikingly similar to invertebrate glial tissue (Fig 10). Identical membrane profiles occur in the of the cytoplasm of the arterial gland cells, but since they lack a double wall they cannot be trophospongial like inpushings of the interstitial tissue. The interstital tissue is also found in close association with some of the nerve axons that ramify between the arterial gland cells and between the lobules of the gland and the outer wall of the artery to which they are attached (Fig 10). It must therefore remain a possibility that the interstitial tissue is a form of glial tissue and that an interrelationship exists between this tissue, the nerve axons and the arterial gland cells. Innervation of individual cells by the nerve axons was not observed, and while it is likely that the majority of these axons terminate on or within the artery wall, their function remains obscure. while others are distinctly angular; the majority are irregularly shaped. They have an amorphous moderately homogenous electron dense content which normally completely fills the limiting membrane. These granules stain deep blue with toluidine blue (Fig5).

Plate 2

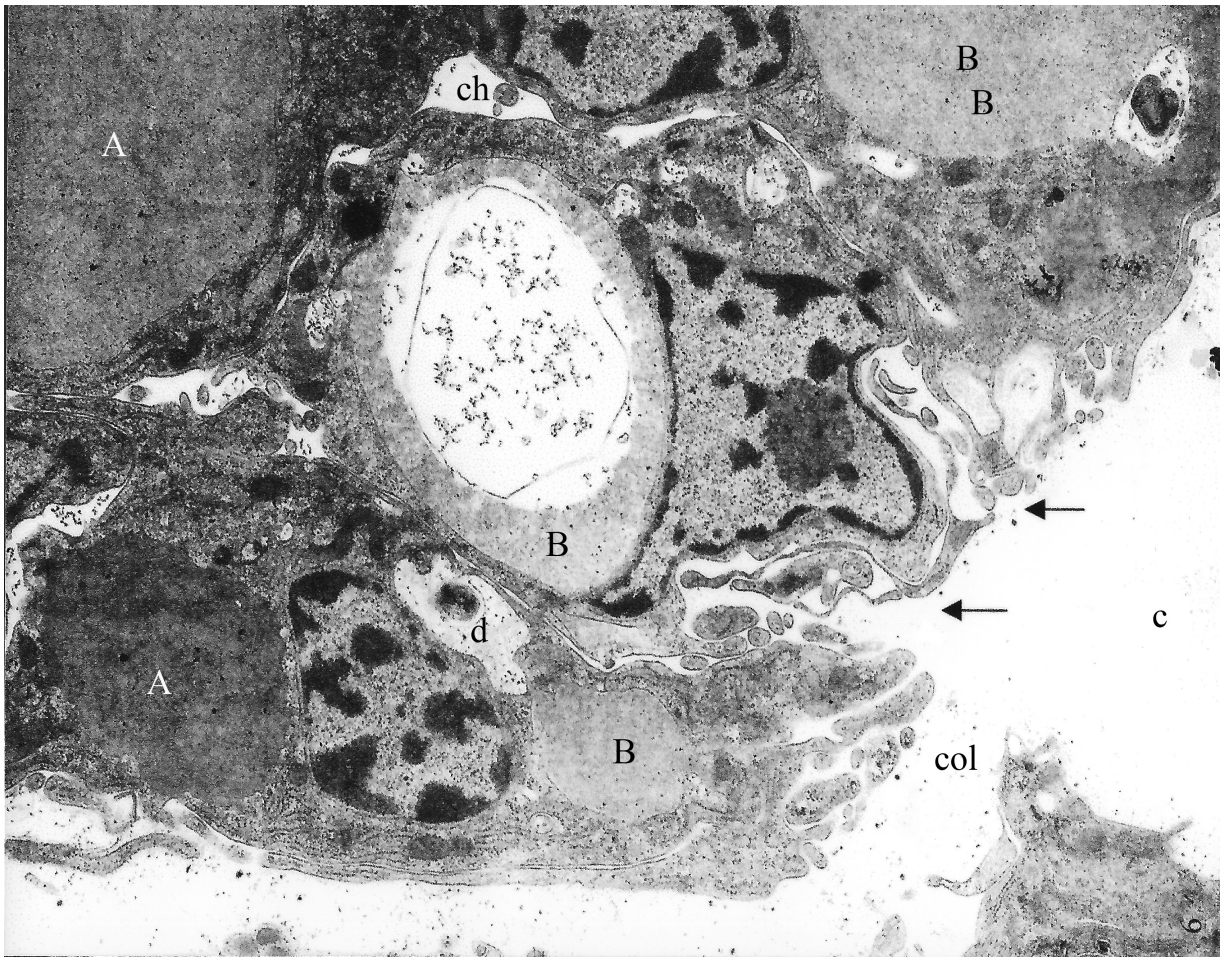
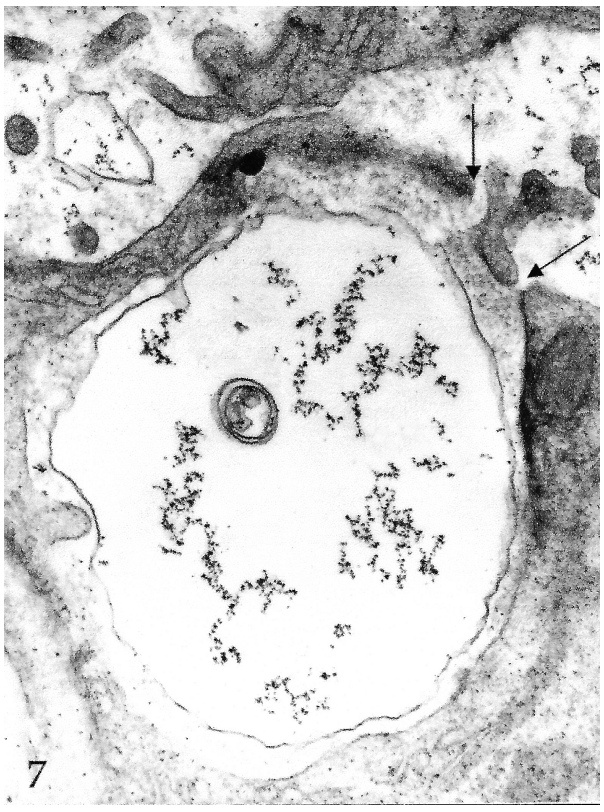


Fig.6. Low power electron micrograph of arterial gland cells bordering a blood capillary (c) showing both type of granule (A) and (B). Note the flocculent nature of the large B type granule surrounding a membrane profile. Intracellular ducts (d) intercellular channels (ch) connective tissue (col) nucleus (n). Channels opening into the capillary (arrows).



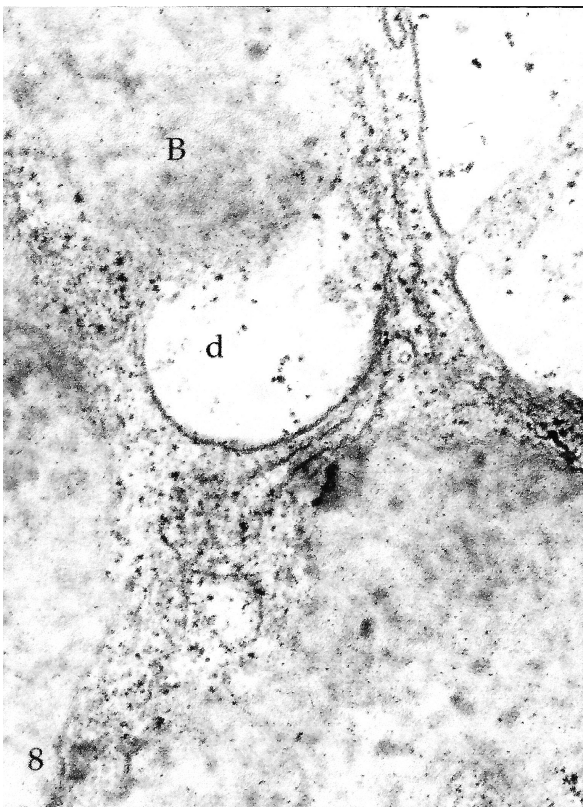


Fig.7. Electron micrograph of B type granule releasing its contents directly into an intercellular channel. Fig.8. Electron micrograph of B type granule releasing its contents into an intracellular duct.

Two main types of granule are present within the cells and both are surrounded by membranes. Type A granules show great variation in shape and size. Many of them are circular in section, while others are distinctly angular; the majority are irregularly shaped. They have an amorphous moderately homogenous electron dense content which normally completely fills the limiting membrane. These granules stain deep blue with toluidine blue (Fig5).

Type B granules are less electron dense than the type A and stain only lightly or moderately with toluidine blue (Figs, 5/6). Although serial reconstruction shows them to be extremely irregular, they tend in section to be circular and infrequently irregular. They contain flocculent material and a variable number of irregular spaces and sometimes membranes.

The differences between the electron density and toluidine blue staining exhibited by the two types of granule may be simply due to a difference in the concentration of their content. While the type A granule always appears to be 'full', the type B granule invariably is not. It is this relative variation in the amount and distribution of the type B granule contents that accounts for the vast array of apparently different granule types that may occur in a particular section (Fig 5). Granules release their contents into the intercellular channels directly (Fig 7) or into the intracellular ducts (Fig 8).

The arterial gland cells do not have a characteristic Golgi complex as a considerable range of form can occur within a single cell. The Golgi complex consists of straight or curved stacks of parallel cisternae. The cisternae are either, short and swollen or elongated with various amounts of swelling. The material within the cisternae is usually electron lucent, but electron dense material does occur. Vesicles present at the extremities of the cisternae reflect their content. Small electron lucent vesicles apparently unassociated with a Golgi complex are also present. In gland cells with a high granular content the Golgi complex is not very pronounced. Rough surfaced endoplasmic reticulum is much more abundant than the smooth form, although both are distributed throughout the cell. The smooth form occurs as a loosely meshed network of branching tubules, while the rough surfaced form is characteristically composed of widely dilated ribosome dotted cisternae, which contain material of low electron density. Polysomes and free ribosomes occur throughout the cell.

Mitochondria are cylindrical and have a homogenous matrix of low electron density and numerous short plate like cristae. Mitochondrial granules are absent. Small multi-vesicular bodies and myelin-like Figures occasionally occur.

Fine Structure of the Posterior Pedal Artery

The posterior pedal artery consists of an inner circularly oriented and an outer longitudinally oriented, layer of muscle fibres embedded in a matrix of collagen. Axons and fibroblasts like cells also occur embedded in the collagenous sheath. Sparsely distributed cells interpreted as fixed amoebocytes occur in parts of the lumen. Collagen fibrils, presumably together with extracellular matrix, border much of the vessel lumen and serves as a basement membrane for the fixed amoebocytes and provides most of the effective blood barrier.

Close opposition of axon profiles and muscle fibres sometimes seen in the artery wall (Fig.9) may represent true myo/neural junctions. Axons containing both electron dense and smaller clear vesicles also occur (Nisbet and Plummer, 1966). Such clear vesicles clustered against an ill-defined membrane (Fig 9) is suggestive of a pre-synaptic membrane, but the nature of pre and post synaptic membranes and the intervening space is unclear. The cell processes of granular cells containing dense granules are frequently associated with axons (Fig 9) (Amoroso et al 1964; Baxter and Nisbet, 1963). Granules of similar size and density are also found in the fibroblasts like cells which are scattered throughout the collagen matrix of the artery wall. Such cells are characterised by invaginations of the plasma membrane and slender processes, which are sometimes found apposed to axon profiles. No ducts or channels link the arterial gland to the lumen of the artery, but many of the intercellular channels which separate most of the cells were found to connect to capillaries. Many of these channels also run to the outer edge of the gland. Collagen fibrils are embedded in the external lamina surrounded in the muscle fibres, and centripetally, presumably together with ground substance, also border much of the vessel lumen. This layer of collagen serves as a basement membrane for the sparse fixed amoebocytes (Fig 14) but does itself provide most of the effective blood barrier.

SUMMARY OF HISTOCHEMICAL REACTIONS

Reaction	Reference	Material demonstrated	Result
periodic acid/schiff (PAS)	Casselman, 1962	glycol or glycolamine groups of polysaccharides	- -
alcian blue	Zugibe et al, 1959	acid groups of polysaccharides	-
mucihaematin	Casselman, 1962	mucin	
azure A metachromasia	Kramer&Windrum,1955	acid groups of polysaccharides	-
bromophenol blue	Mazia et al, 1953	amino groups	++++
morel/sisley	Lillie, 1957	tyrosine	++++
DMAB - nitrite	Adams, 1957	tryptophan	
B+S (alpha-acylamido carboxyl groups)	Barnett & Seligman, 1958	aspartic and glutamic acids	++++
(DDD) dihydroxydinaphthyldisulphide	Pearse, 1960	SH groups	+/-
maleimide / KCN / DDD	Pearse, 1960	SS groups	+/-
Sudan black B / 70% alcohol (SBB)	Casselman, 1962	lipids	+/-
B + A (acid phosphatase)	Barka & Anderson, 1962	acid phosphatase	
silver substitution	Casselman, 1962	calcium	
nuclear fast red	Casselman, 1962	calcium	
rubeanic acid	Pearse, 1960	copper	
o - tolidine - thiocyanate	Pearse, 1960	copper	
3β-hydroxyandrost-5-en-17-one 5β- pregnan-3α-ol-20-one 17β-hydroxyandrost-4-en-3-one estradiol 17-(β-d-glucuronide).	Davies et al, 1966.	hydroxysteroid dehydrogenase	

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The intensity of a positive reaction is represented arbitrarily by the number of + symbols;

+/- represents a weak response; - represents a negative response

TABLE 1.

Histochemistry

The results of the various tests are summarised in Table I.

Carbohydrates: All the tests for carbohydrate and carbohydrate containing substances gave negative results.

Lipids: The absence of free lipid was indicated by a negative reaction to the Sudan black B test. An extremely faint sudanophilia, apparently unrelated to the granules, probably represents mitochondrial phospholipid.

Proteins: The bromophenol blue, general test for proteins gave an intensely positive reaction and the amino acids tyrosine, aspartic and glutamic acids, were identified by the Morel-Sisley test and the Barnett and Seligman test for alphaacylamido carboxyl groups respectively. Protein bound SH and SS groups were demonstratable with the DDD, and Malermide/DDD techniques, but these were only weakly positive.

Acid phosphatase: Both forms of calcium fixed smears and crystal sections gave negative results.

Metals: The tests for calcium and copper both gave negative results.

Steroids: The tests for hydroxysteroid dehydrogenases were negative. Clear vesicles can be found with their membranes apposed to those of blood capillaries (Fig12) which may represent membrane conservation after the release of neurosecretory material.

Electron probe microanalysis

This technique makes use of the fact that X-rays generated in the surface layer of a specimen bombarded by a beam of electrons have a characteristic wavelength depending upon the atomic numbers of the elements responsible. It is thus possible to analyse the X-rays and determine the elements present. By taking a line scan for a particular element, in this case copper, its relative

concentration over the area scanned may be determined.

Fig 11 shows the analysis of arterial gland tissue for copper and is made up of three images superimposed upon each other. 1.An optical image of the arterial wall (M) and arterial gland tissue (AG). 2.A line scan across x------x. 3.Copper count across x------x.No quantitative information was obtained from this analysis of arterial gland tissue, but a positive copper content was recorded although it was not possible to localise its position within the cells. Similar analysis of hermaphrodite gland (control) sections was negative.

Immunoelectrophoresis

As copper and protein were both present within the arterial gland it was decided to test for haemocyanin. A single precipitation was obtained using *D. reticulatum* haemocyan and *Helix aspersa* antisera but no reaction was obtained against the electrophoresed arterial gland proteins.

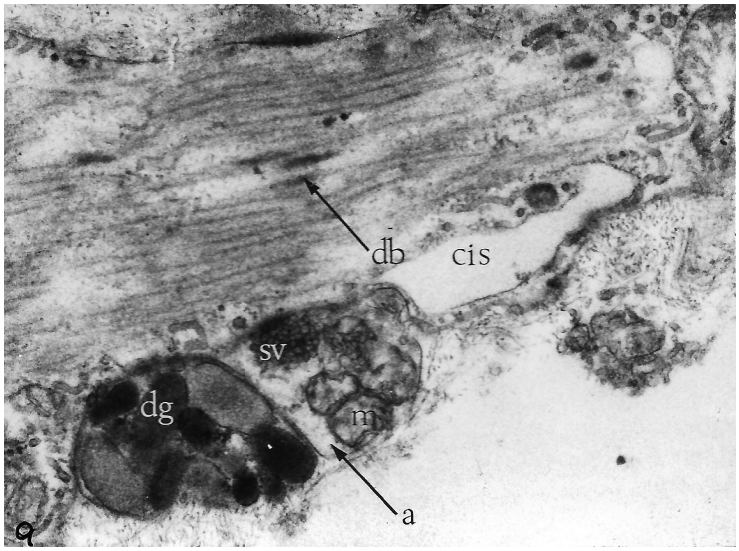
The arterial gland and reproductive development

The arterial gland was found to be present and contain secretion at all stages of reproductive development. Intracellular ducts and intercellular channels were clearly seen in the arterial glands of animals whose hermaphrodite glands were undergoing differentiation (Fig 12), although there was no evidence of granule release. The histology of the arterial gland of animals with a hermaphrodite gland in the late spermatozoon stage was very different (Figs 3,4,5). Enormous variation in the size of the arterial gland occurred within animals at the same reproductive stage, although this was more pronounced in the later developmental stages than the earlier ones. Differences in size were so pronounced that no attempt was made to relate the size of the arterial gland to stages of reproductive developmental. In addition, there was no clear relation between the size of an animal and the size of its arterial gland. The largest glands were, however, found in larger animals. *D. reticulatum* characteristically has a sickly appearance shortly before death. The arterial gland of such an animal (Fig 12) lacks structural integrity. The arterial gland is however very susceptible to changes in pH and ionic concentration and so this histological observation is difficult to interpret.

Gland-like cells associated with the blood vessels of other pulmonates

Of the species examined tissue almost identical to the arterial gland of *D. reticulatum* was only found in *A. caruanae*. Similar tissue was however found in *Limax flavus* and Oxychilis species. In *Limax flavus* this tissue occurs as the inner of two layers of cells attached to the wall of the posterior pedal artery and red and yellow staining granules (azan) occur within the cells. An outer layer of vesicular cells is also present. Vesicular cells occur in some parts of the body of most of the species examined and appear to be identical to the vesicular connective tissue elements described by a number of previous authors (see Laryea, 1969b). In *Oxychilis alliarius* cells similar to arterial gland cells occur attached to the posterior pedal artery and also to a limited extent, to some of the nerves.

PLATE 3



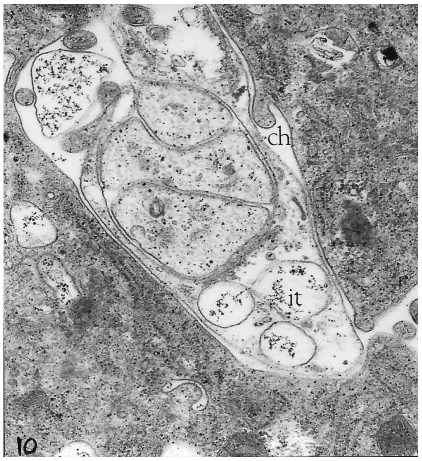


Fig.9 Electron micrograph of muscle in arterial wall showing close opposition of axon (a) and muscle. Note clear vesicles (sv) clustered against a possible presynaptic membrane (small arrows). The dense granules (dg) are probably in the slender process of a fibroblasts. Dense bodies (db) are probably desmosomes between adjacent muscle fibres.
(cis), rough endoplasmic reticulum cisternae; mitochodria, (m).

Fig.10 Nerve axons (a) and interstitaial tissue (it) in an intercellular channel.

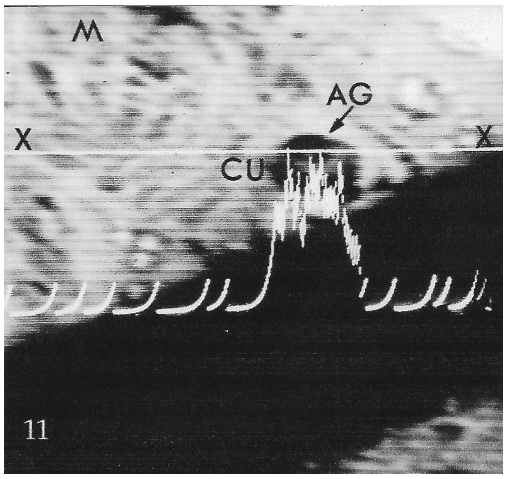
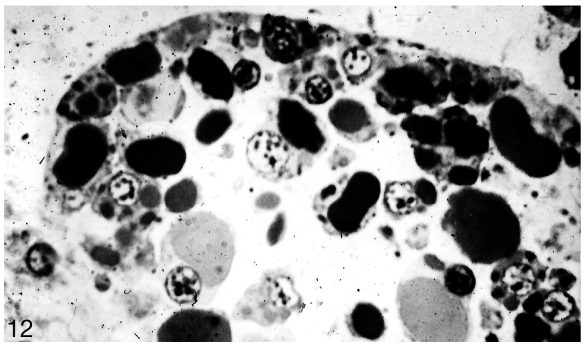


Fig.11 Electron probe microanalysis of arterial gland tissue for copper. The line scan across x------x shows the positive copper count (Cu) when the scan passes through arterial gland tissue (AG).



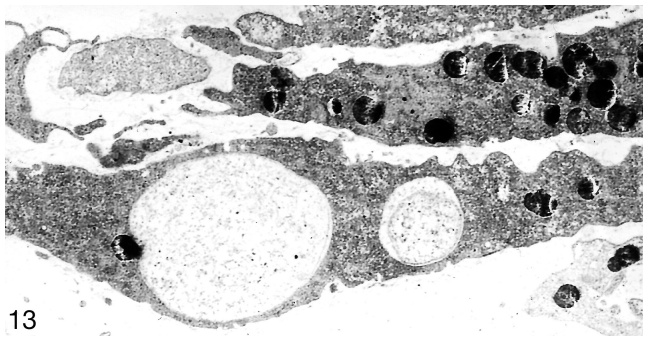


Fig.12 The arterial gland of such a 'sick' *D. reticulatum* showing loss of structural integrity. Fixative: glutaraldehyde, stain: toluidine blue.

Fig.13 Electron micrograph of one type of connective tissue cell from *Lymnaea stagnalis* showing granules (g) similar to the B type granules of *Deroceras reticulatum*. Pigment (p).

Unicellular glands occur in blood vessel walls of *Helix aspersa* (Fernandez, 1966) and in this study similar cells were found in the blood vessel walls of *Cepaea nemoralis* and *Succinea putris*.

The connective tissue membranes of *Lymnaea stagnalis* were briefly examined in the electron microscope at the suggestion of Dr. J. Joosse (Free University, Amsterdam). Some of the 'inclusions' of one type of cell appeared similar to the B type granules of the arterial gland (see Fig 19).

DISCUSSION

Wax sections of susa fixed arterial gland, stained with azan trichrome stain resulted in a tissue full of red, yellow and blue granules in an array of different hues that suggested it might be a gland. The apparent absence of ducts further suggested it might be endocrine. The superior fixation obtained with gluteraldehyde /Araldite/toluidine blue preparations showed lobules of irregular cells with interconnecting intercellular channels leading to intracellular ducts. Such an arrangement didn't rule out an endocrine function, particularly since the gland is only separated from the haemocoel by connective tissue and no major duct was found leading to the lumen of the posterior pedal artery.

The nature of the two types of granule demonstrated by electron microscopy is unclear. Because of the thickness of the sections used for histochemistry, it is not possible to say if the A and B type granules are chemically different. Reconstruction with the light microscope of small B type granules lacking 'holes, clearly showed them to be cytoplasmic structures. Many of the B type granules with 'holes' in them could in fact represent accumulations of moderately electron dense flocculent material within ducts or depressions in the cell wall, in which case inpushings of interstitial tissue could produce the membrane profiles seen within the B type granules in some sections.Since during the earlier reproductive stages only A type granules were present in the arterial glands, the possibility can not be excluded that B type granules with holes in them represent the disgorged contents of A type granules, still present within enlarged intracellular ducts. A combination of autoradiography and serial reconstruction, both at the ultrastuctural level is needed to resolve the nature of the B type granule.

It was not possible to relate the histological results to seasonal climate change or reproductive state and while such changes might be too subtle to be seen with the techniques used, an endocrine role was still a possibility. Classic investigative techniques of extirpation, gland replacement and injections of tissue homogenates were not possible given the position of the arterial gland, and the fact that it is not always present.

The site of haemocyanin production in molluscs is unknown and given the discharge of the granules into the capillaries and a similarity between some granules and flocculent material occasionally found in blood vessels supplying other organs it was decided to test for the presence of copper in the arterial gland using a more precise technique - electron probe microanalysis.

The strongly positive reaction for copper and the presence of proteins indicated by the histochemical tests prompted the immunoelectrophoresis investigation. The negative result was surprising and so the whole process was repeated, with the same result. Because of the difficulty of obtaining large amounts of *D. reticulatum* blood to prepare antisera against *D. reticulatum* haemocyanin, *Helix aspera* blood was used and *Helix aspera* antisera prepared. This was cross-reacted with homogenised arterial glands from *D. reticulatum* and it may be the negative results are a function of inadequate titres.

Another explanation is that the gland produces a precursor or the components of haemocyanain, which are then assembled outside of the gland in the haemolymph. This would fit with the fact that haemocyanin is a very large molecule and any secretions from the arterial gland have, at a minimum, to diffuse through the ground substance that must exists at the confluence of the capillaries and the intercellular channels. The connective tissue sheath attaching the gland to the cephalic arteries would prove an even more effective barrier to diffusion of such a large molecule. Of course the large amount of copper present in the arterial gland may indicate a completely different function; a role in copper metabolism for example. The survey of other pulmonates made in this study was necessarily brief and electron microscopic study is obviously needed. When the function of the arterial gland is known, assay methods for the detection of homologues in other species should not be difficult.

Acknowledgments

It is with much pleasure that I acknowledge the help and encouragement of Dr. N. W. Runham, who also criticised the manuscript.

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STUDIES ON THE MATURATION OF THE REPRODUCTIVE SYSTEM OF *AGRIOLIMAX RETICULATUS* (PULMONATA: LIMACIDAE)

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ABSTRACT

Agriolimax reticulatus Müller were collected in North Wales over a period of 18 months. Breeding, at a maximum in spring and autumn, occurred throughout the year. The reproductive tract of the slugs was dissected and its parts were separated, weighed and sectioned. The maturation stage of the hermaphrodite gland was not closely related to the weight of the animal. The weight of this gland increased to a maximum at the "spermatid stage" and then decreased. Sperm appeared in the hermaphrodite duct and spermatheca at the end of that stage, and were then present until the post-reproductive stage. The maturation of the albumen gland and common duct was closely related to that of the hermaphrodite gland. The glands on the sarcobellum mature at the "spermatozoon stage." These results appear to indicate that the stages in the maturation of the reproductive system are related to the growth phases of the animal, and that both physiological and environmental factors may control this maturation.

INTRODUCTION

The biology of the grey field slug *Agriolimas reticulatus* has been well studied (Quick, 1958; Frömming, 1954; Bett, 1960; Arias & Crowell, 1963; South, 1965). Details of the changes in the reproductive system associated with the breeding cycle are, however, not available for this species. In contrast, detailed descriptions are available for *Arion ater*, reared in the laboratory (Lūsis, 1961) and collected from the field (Smith, 1966), and for the American slug *Philomycus carolinianus* (Kugler, 1965).

As a preliminary to a study of the factors which control the breeding cycle in *Agriolimax reticulatus* a detailed study of the reproductive system was undertaken and is reported below.

MATERIALS AND METHODS

The 2 species *Agriolimax reticulatus* and *A. caruanae* are common in Caernarvonshire and Anglesey, North Wales, particularly on cultivated land. Although these 2 species occur together, they are readily distinguishable by external features.

Slugs were collected after nightfall at weekly intervals, throughout 1965 and thereafter monthly until the end of June, 1966. An area of rough ground at the base of a drystone wall in the college grounds was visited each time. Each sample consisted of the first 10 *Agriolimax reticulatus* discovered. The samples had to be restricted to this size because of the subsequent detailed investigation.

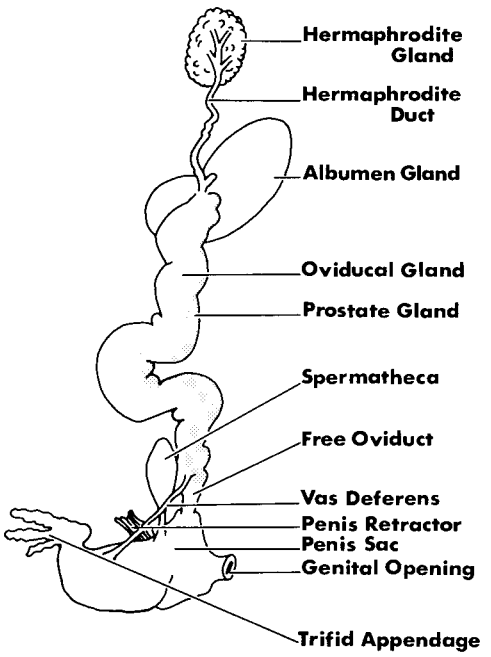

FIG. 1. Diagram of the reproductive tract of *Agriolimax reticulatus* (from Bayne, 1966).

The animals were kept overnight in a closed container together with damp moss or grass. Next morning they were freed from any attached soil by gentle rolling on filter paper and then weighed as quickly as possible to the nearest milligram on a torsion balance.

'Susa' fixative was injected into the slugs to kill them and then the reproductive system was dissected out. The genital tract was subdivided into hermaphrodite gland, hermaphrodite duct, albumen gland, common duct plus free oviduct, spermatheca, and penis together with vas deferens (Fig. 1). These parts were left in fresh fixative for 6-12 hours, then passed through 2 changes of cellusolve for 24 hours each. The pieces were removed one at a time, left for a very short time on filter paper to remove excess moisture and then weighed on a semi-micro balance to the nearest 0.01 mg. The weighings were reproducible, but as no attempt was made to determine the effect of the processing on the weight of the tissue the values obtained were only suitable for comparative purposes. The weight of the whole tract was obtained by summing the weights of its constituent parts.

After weighing, the pieces of reproductive tract were returned to cellusolve and then embedded in ester wax. Sections were cut at 5μ and stained in Azan triple stain.

Of the 706 slugs collected and weighed, 429 were dissected and sectioned while 18 were so small that they were sectioned whole. From the results obtained the means were determined and the fiducial (confidence) limits of these means calculated at the 95% confidence level (Bailey, 1959).

RESULTS

Breeding Season

Since very small animals were collected throughout the year (Fig. 2) it is likely that breeding occurs at all seasons. However, from the numbers of small animals there appeared to be 2 main breeding periods, one in spring and the other in autumn. These findings agree with those obtained by workers in other areas of this country (Bett, 1960).

Reproductive Tract

As with other pulmonates the structure of the reproductive tract is very complicated (Fig. 1) and reflects the complexity of its function. From the histogram (Fig. 3) showing the relation between the weight of the body and of the reproductive tract, it can be seen that there is initially a very slow increase in tract weight followed by a rapid growth phase and then by a further period of slow growth. When the parts of the tract were weighed separately (Fig. 4) considerable differences were found in the types of growth curve. These will be interpreted after the histological descriptions.

Eighteen animals smaller than 80 mg could not be considered here, as it was

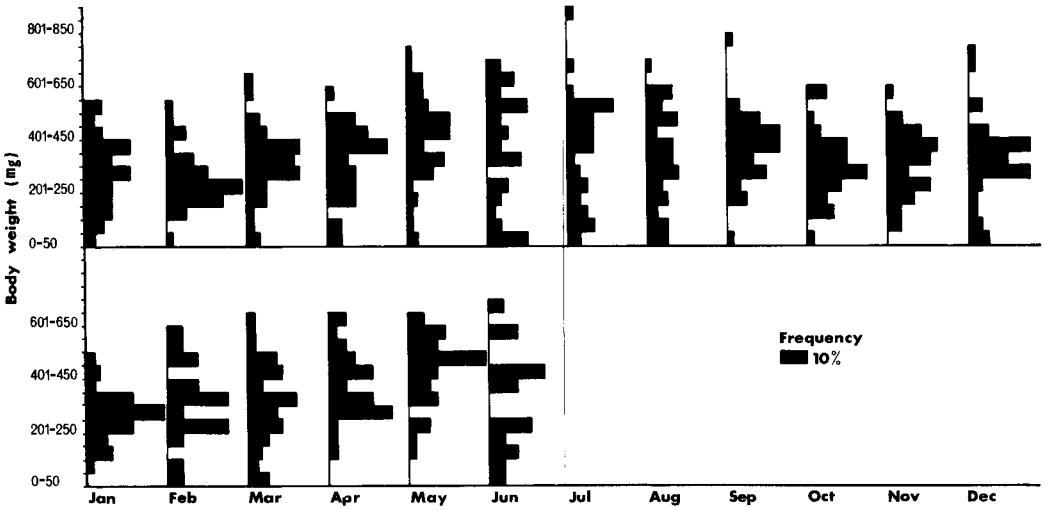

FIG. 2. Percentage frequency of weight groups per month of *Agriolimax reticulatus* collected during 1965 (top) and 1966 (bottom).

difficult to dissect out the reproductive tract cleanly and because of the large error involved in weighing such small organs. The juveniles were, however, sectioned whole in order to study the histology of their genital tracts. These were always continuous, as in older animals, even as early as 4 days after hatching in the laboratory. These findings do not tally with Richter's (1935), who reports that, until 10 days after hatching, the hermaphrodite gland of *Agriolimax* was separate from the rest of the tract.

Hermaphrodite Gland

The cytology of the cells in the hermaphrodite gland has been studied by Gatenby (1918). Richter (1935) carried out a very thorough analysis of the changes in structure of the hermaphrodite gland during maturation. The maturation changes in the gland take place in a continuous orderly sequence but in order to analyse these changes it was convenient to subdivide the development of the gland into a number of stages, i.e. the undifferentiated, spermatocyte, spermatid, early and late spermatozoon, early and late oocyte and post-reproductive stages. While these stages are quite readily distinguished, they do grade into each other. The morphological criteria on which they are based are as follows.

A. Undifferentiated Stage

The gland could not be weighed accurately at this stage. In sections it was seen to be a simple structure containing a solid mass of small undifferentiated cells.

B. Spermatocyte Stage

It is possible to weigh the gland accurately at this stage. Acini have budded out from the main mass of the gland, but it is still filled with a solid mass of cells. Within this mass can now be recognised some or all of the following cell types: spermatogonia (small nuclei with granular chromatin and 1 or occasionally 2 nucleoli), primary spermatocytes (large nuclei with a chromatin network), secondary spermatocytes (small nuclei with a fairly large amount of cytoplasm), oocytes (large nuclei with a very large nucleolus, and a large amount of cytoplasm, often containing yellow staining granules) and nurse cells (very large

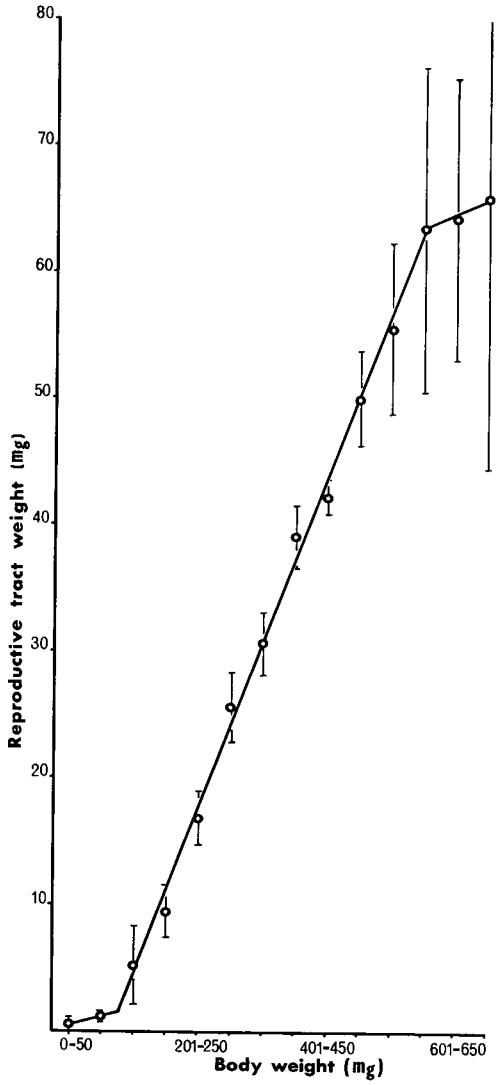

FIG. 3. The average weight of the reproductive tract compared to the body weight. The fiducial limits of the means (95% significance) are shown by the barred lines.

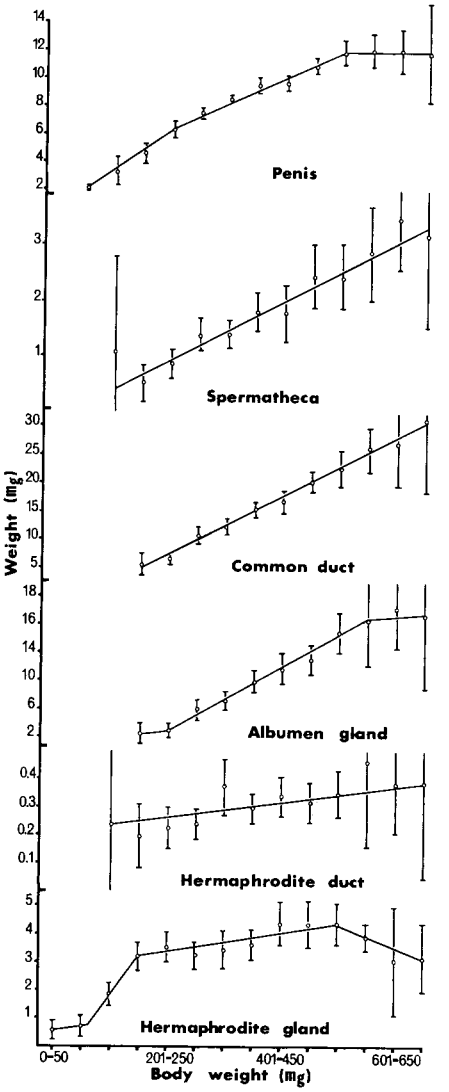

FIG. 4. The average weights of the various organs of the reproductive tract compared to the body weight. The fiducial limits of the means (95% significance) are shown (barred lines).

nuclei with very finely granular chromatin). The latter 2 cell types are usually found attached to the acinar wall.

C. Spermatid Stage

Ducts have appeared in the gland but the acini are still fairly solid. There are now many secondary spermatocytes and spermatids (dark blue to pink staining, evenly stained dense nuclei and sperm tails surrounded by cytoplasm). These 2 cell types tend to be found in groups most frequently towards the centre of the acini.

D. Early Spermatozoon Stage

A lumen is now present in the acinus although it is largely filled with sperm tails. The sperm have characteristically shaped, deep red staining heads and definitive sperm tails. A thick layer of cells in earlier spermatogenesis stages still lines the acinar walls. The sperm tend to be associated in groups related to a nurse cell.

E. Late Spermatozoon Stage

There is a very clear lumen in each acinus and many unattached sperm may be present there. The layer of cells in the earlier spermatogenesis stages is much thinner.

F. Early Oöcyte Stage

The sperm are still present in large numbers but many of the oöcytes are now very large. The oöcytes are covered by a thin layer of cells which forms a follicle. Some very small oöcytes are present, particularly at the narrow neck of each acinus where it opens into a collecting duct. These are possibly the most recently differentiated oöcytes, said by Richter (1935) to be produced in these areas and then to migrate into the base of the acinus.

G. Late Oöcyte Stage

Although still present, the sperm are reduced in number. At least some oöcytes have detached from the wall and are free in the lumen.

H. Post-reproductive Stage

A cuboidal epithelium, not found at any other stage, can be seen to cover at least part of the acinar wall. There is great variation in the numbers of sperm and ova remaining within the acinus.

Little doubt exists as to the sequence of the earlier stages, but some variation in the order of appearance of the "late spermatozoon" and the 2 "oöcyte" stages seems possible. Some slides of late spermatozoon stages revealed an apparent loss of most of their oöcytes. Similarly, at the late oöcyte stage a considerable number of sperm, spermatids, and spermatocytes were still present in some animals, while in others there were few. It is not known whether such variance reflects a variation in the relative amount of sperm and ova developed, or whether egg laying has taken place.

Some slugs, which had parasites in their hermaphrodite gland, will be considered separately (p. 15). It is also possible that a few animals were so very lightly infested that the parasites escaped detection.

When the stages of the hermaphrodite gland and the weights of the animals are compared (Fig. 5) it can be seen that, although there is a pronounced relationship between them, there is considerable variation. Thus late oöcyte stages (G) were found in animals weighing as little as 100 mg while spermatid stages (C) were still to be found in those as large as 350 mg. The relation of the stages of the gland to the time of year was also investigated but no correlation could be found.

From the weight of the gland it can be seen that there is an increase in weight up to the early spermatozoon stage (D, Fig. 6) which is associated with the phase of massive spermatogenesis. As the sperm was voided from the gland, particularly in the oöcyte and post-reproductive stages, so the weight decreased, and this loss was reflected histologically by the shrunken acini.

The uneven growth curve for the hermaphrodite gland, shown in Fig. 4, can now be interpreted in the light of the above findings. The initial slow growth is due to the prevalence of "differentiation" and "spermatocyte" stages in the lower weight groups. Spermatogenesis results in the phase of rapid growth while the decrease in weight found in the largest animals is due to the preponderance of the oöcyte and post-reproductive stages.

The Hermaphrodite Duct

The acini open into small collecting ducts which lead into the hermaphrodite

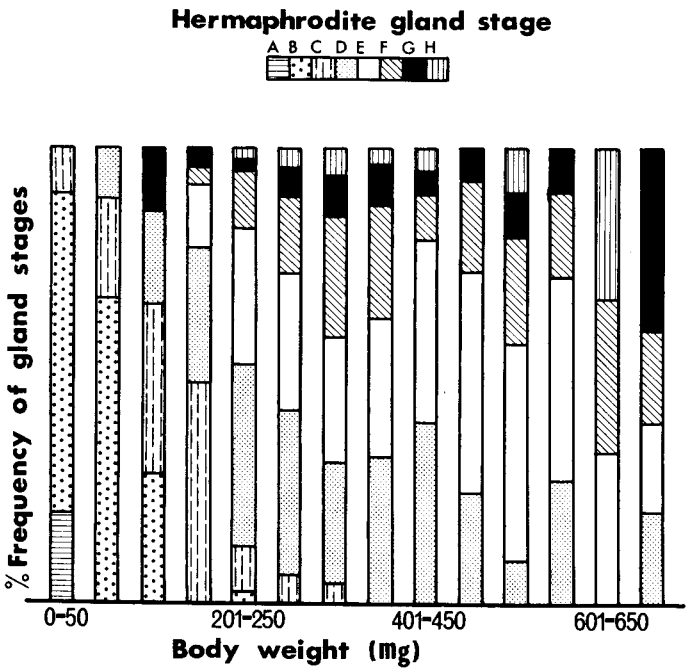

FIG. 5. Percentage frequency of the hermaphrodite gland stages in the different body weight groups. These stages are: A, undifferentiated; B, spermatocyte; C, spermatid; D, early spermatozoon; E, late spermatozoon; F, early oöcyte; G, late oöcyte; H, post-reproductive.

duct. It is a simple tube lined by a ciliated endothelium. Sperm appear in its lumen in the early spermatozoon stage and it is filled with them in subsequent stages, except for the post-reproductive stage. In the latter stage there was some variation: about half of the ducts examined were full, while the others were empty. Due to its small size the hermaphrodite duct was not dissected from the remainder of the reproductive tract in 63 of the smallest animals so that very few of the empty ducts were weighed. As can be seen from Fig. 4 there was considerable variation in the weight of the duct and no significant correlation with the animals' weight could be found.

The Albumen Gland

The hermaphrodite duct opens into the lumen of this gland near the point where it discharges into the upper end of the common duct. The maturation of the albumen gland has been subdivided, for convenience, into stages, but as with the hermaphrodite gland, its development is continuous.

A. Undifferentiated Stage

In very young animals the albumen gland is present as a small hollow diverticulum at the junction of the hermaphrodite duct and the common duct. It enlarges and its walls become folded.

B. Differentiation Stage

Mitoses are very frequent in the epithelium at this stage and tubules extend out from the walls of the diverticulum to form acini. The lining epithelium is columnar.

C. Maturation Stage

The acini are well differentiated and so are the collecting ducts. The cells

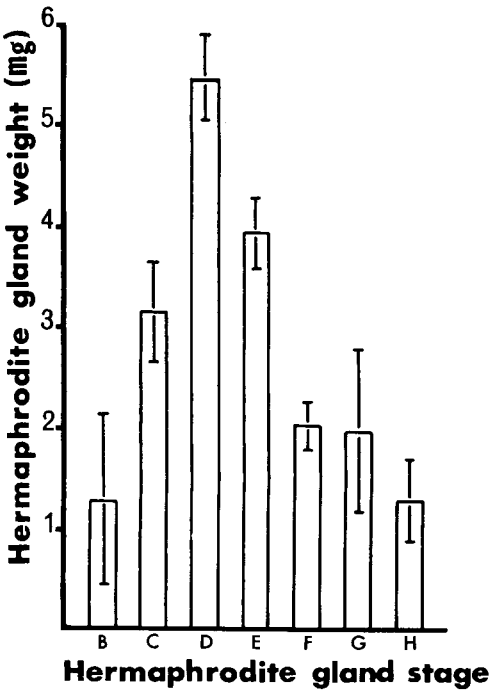

FIG. 6. The average weights of the hermaphrodite gland at its various developmental stages (the same as in Fig. 5). The fiducial limits of the means (95% significance) are shown by the barred lines.

have become more cuboidal but secretory granules are absent.

D. Accumulation Stage

Secretion first appears in the cells as small, red staining granules, but as more granules accumulate, they stain a light blue. The cells gradually fill up and become distended with the secretion.

E. Secretion Stage

The secretion passes from the cells into the lumen of the acini and thence into the collecting ducts. This secretion appears to be released by a breakdown of the apical regions of the cells.

The changes in complexity and size of the albumen gland are clearly reflected in its weight (Fig. 7). Thus the 3 earliest stages are associated with an increase in complexity but only a slight

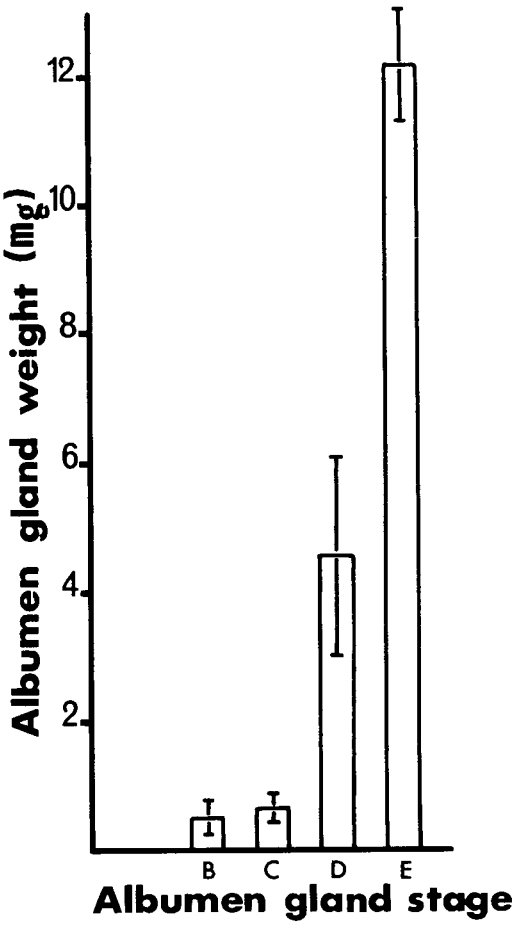

FIG. 7. The average weights of the albumen gland at its various developmental stages. The fiducial limits of the means (95% significance) are shown by the barred lines. The albumen gland stages (for this figure and for Fig. 8) are: A, undifferentiated; B, differentiation; C, maturation; D, accumulation; E, secretion.

increase in size. As the secretion accumulates there is an increase in weight so that, when secretion appears in the lumen, the gland is very large. A similar relation is also shown by a comparison of gland and body weights (Fig. 4). At its maximum size this gland constitutes a large part, up to 6%, of the body weight.

When the stages of the albumen gland

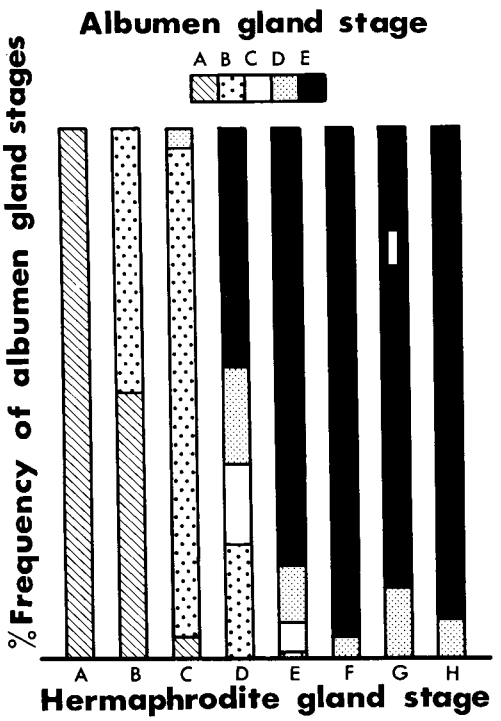

FIG. 8. Percentage frequency of the albumen gland stages at each hermaphrodite gland stage. The hermaphrodite gland stages (A-H) are the same as in Fig. 5, and the albumen gland stages (A-E) as in Fig. 7.

are compared with those of the hermaphrodite gland it can be seen that there is a very close relation between them (Fig. 8). Very few albumen glands were found at the maturation or accumulation stages, so it is possible that these stages are only transitory.

The Common Duct and Free Oviduct

The lumen of the common duct is partially subdivided by lateral folds into male and female ducts. The glands opening into the male duct constitute the prostate gland and those opening into the female duct the oviducal gland. At the lower end of the common duct the female duct continues as the free oviduct while the male duct continues as a completely separate vas deferens. Some details of the structure of this part of the tract and the cytology of the gland cells have been presented by Filhol (1938). Due to the presence and distribution of different glands and their varying rates of differentiation, determination of the changes occurring in the common duct during maturation was extremely difficult. Four stages could, however, be distinguished.

A. Differentiation Stage

Finger-like diverticula are present on the wall of the male side, being more frequent towards the distal part of the duct than in its proximal part. Many cell divisions are visible and the diverticula increase in number and length until the definitive prostate gland is formed. At this stage, however, the cells in the gland appear to lack cilia and secretory granules. The female part of the common duct remains undifferentiated. It is lined by a columnar epithelium and there is a dense underlying stroma in which later develop the oviducal gland cells.

B. Start of Male Secretion

The male duct is lined by a ciliated cuboidal epithelium which is interrupted by the openings of the branched diverticula. The cells of the diverticula are also ciliated. They now contain various types of secretion granules. Around the bases of the diverticula scattered strands of muscle can be found. At least 3 types of glandular cell can be recognised. (1) Situated mainly in the bases of the diverticula, in the upper part of the tract, are flask-shaped gland cells containing large yellow or blue staining granules. Some cells contained only one type of granule, the others a mixture of both. (2) Occurring mainly in the tips of the diverticula are similarly shaped cells containing very fine red staining spheroidal granules. This second type of cell was also reduced in number or absent from the lower part of the tract. (3) Concentrated in the lower part of the tract is a third type of cell in which the secretion, sometimes granular, but usually a large non-granular mass, stains blue. In the upper part of the tract, immediately

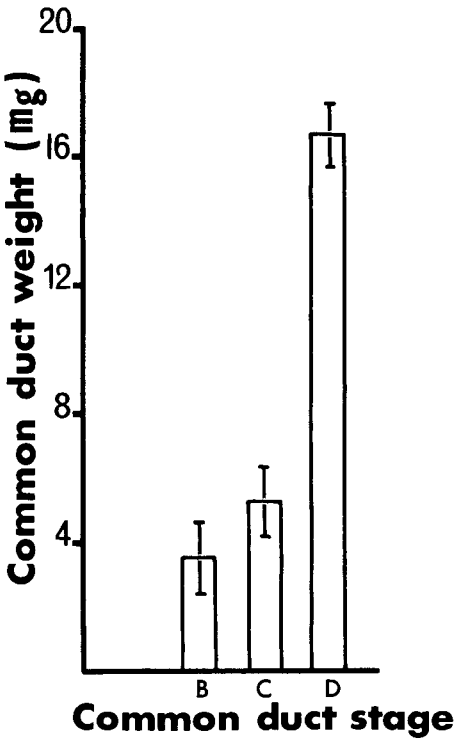

FIG. 9. The average weights of the common duct at its various developmental stages. The fiducial limits of the means (95% significance) are shown by the barred lines. The common duct stages (for this figure and for Fig. 8) are: A, differentiation; B, start of male secretion; C, start of female secretion; D, accumulation of female secretion.

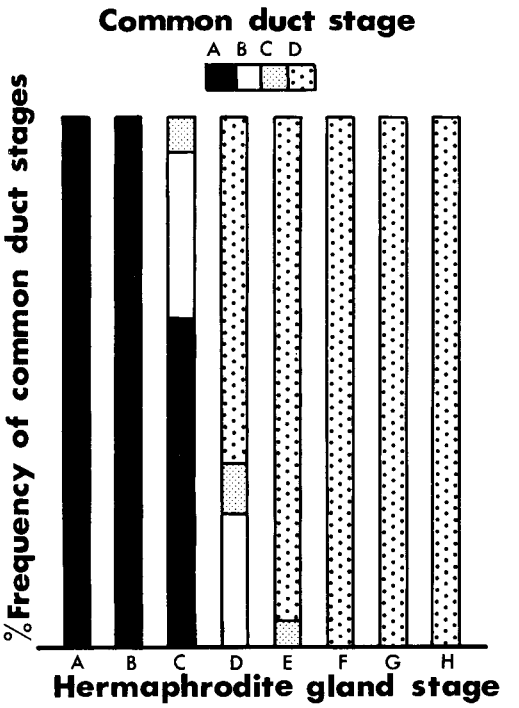

FIG. 10. Percentage frequency of common duct stages at each hermaphrodite gland stage. The hermaphrodite gland stages are the same as in Fig. 5, and the common duct stages as in Fig. 9.

underlying the epithelium of the male duct, there are some cells with a blue secretion which may be homologous with the third type described.

C. Start of Female Secretion

The cells underlying the epithelium of the female part of the common duct have differentiated into the long flask-shaped oviducal gland cells and a blue secretion has appeared in them. A small amount of this secretion is also seen in the lumen. There is a great reduction in the number of these glands towards the lower end of the tract. In contrast to the cuboidal epithelium in the male duct the ciliated epithelium in the female duct is columnar. As secretion accumulates in the cells it stains a lighter blue.

D. Accumulation of Female Secretion

The prostate gland cells are apparently as full of secretion as in the previous stage. The oviducal gland cells are now completely distended with secretion staining a very light blue. As a result of this distension, most of the ciliated epithelium becomes very thin. It is frequently interrupted by the large ducts. When an egg is found in the tract, it can be seen that the oviducal gland secretion forms the multilayered shell. Short strands of muscle are seen at all levels between the oviducal gland cells.

This great expansion of the female gland cells results in a very great increase in the total weight of the tract at

this stage (Fig. 9). As can be seen, however, from Fig. 4, there is a steady growth of the tract throughout the life of the animal. As noted for the albumen gland, a strong correlation with the stages of the hermaphrodite gland was similarly very noticeable for the common duct (Fig. 10). Thus, during the undifferentiated and spermatocyte stages of the hermaphrodite gland, the duct is in its differentiation stage. During the spermatid and early spermatozoon stages of the hermaphrodite gland, the male part of the common duct completes its maturation and the female part starts its maturation. The majority of the animals in the late spermatozoon stage and all those in the later stages have both parts of the genital tract mature.

The histology of the free oviduct did not appear to vary very much. It is a ciliated duct with a surrounding mass of flask cells opening into it.

Spermatheca

This is a simple sac-like organ lined by a ciliated columnar epithelium. There is considerable variation in its weight, but it appears to increase in size as the animal gets larger (Fig. 4). Sperm together with sperm mass material is present in its lumen from the spermatid stage onward. Thus, if it is correct that at copulation the partner's sperm is stored in it for at least a short time, then copulation must have occurred at that stage. In the late oocyte stage, fewer sperm are to be found and considerably fewer at the post-reproductive stage.

Penis and Vas Deferens

The penis is an eversible sac containing the distensible sarcobellum. Its structure is very complex. Few changes are visible during the maturation of the penis, and even in the youngest animals all the adult structures appear to be visible. It enlarges throughout most of the life of the animals (Fig. 4), and cell divisions are found up to the early spermatozoon stage. Gland cells are scarce except in the sarcobellum. In the latter, they are present at the spermatid stage, but secretion does not appear in the cells or in the lumen until the early spermatozoon stage. Over most of the sarcobellum these cells contain a blue secretion, but around its dorsal posterior border there is a band of cells whose secretion dissolved out during preparative treatment. Sperm are found in the lumen of the penis from the end of the early spermatozoon stage.

The trifid penial appendages are interesting structures. Until the early spermatozoon stage they are hollow, but then their epithelium becomes stratified and squamous, shedding cells into the lumen. In some areas the epithelium remains columnar but the cells appear necrotic and breakdown products appear in the lumen. A similar picture is also seen in the epithelium covering the rest of the penis. In some animals the appendages may be completely filled by sloughed cells and cell detritus. In others they may be full of a blue secretion apparently identical to that secreted by the cells of the lower end of the common duct. The breakdown of the epithelium is more frequent in the oöcyte and post-reproductive stages. It is possible that it may be associated with the decreased growth rate of the penis in the largest animals (Fig. 4).

The vas deferens is a simple ciliated and apparently non-glandular duct. In a few cases it contained sloughed cells.

Parasitised Animals

Animals found to contain parasites were excluded from the above study.

The most common parasite found was a protozoan which resembled *Tetrahymena limacis* (Arias & Crowell, 1963). This or a similar parasite has been observed, sometimes in large numbers, swimming in the perivitelline fluid of the eggs and occasionally entrapped in the egg shell (Bayne, in press). Other parasites found were nematodes, a larval trematode and another protozoan. As these parasites were observed in sections only, identification was not possible. In most cases

only a part of the hermaphrodite gland was disorganised so that its developmental stage could be identified from the remainder of the gland. In one animal only was the reproductive tract markedly retarded in its development: the hermaphrodite gland was in an oocyte stage, while the female glands in the tract had not completed their maturation (Stage C). This animal was not very heavily parasitised, but it was the only one in which there was any sign of parasitic castration.

DISCUSSION

The pulmonate reproductive tract is very complicated histologically (Lūsis, 1961; Smith, 1965, 1966; Kugler, 1965) and biochemically (Bayne, 1966, and in press), and it undergoes an elaborate maturation sequence (Lūsis, 1961; Smith, 1966). Because of such complexity one would suspect the existence of well developed controlling mechanisms, perhaps both endocrine and nervous. Our knowledge of such mechanisms is regrettably meagre (Laviolette, 1950a, 1954; Pelluet & Lane, 1961; Pelluet, 1964; Gomot & Guyard, 1964; Guyard & Gomot, 1964). Certainly external factors are also of great importance: the length of time spent in courtship and copulation necessitate suitable climatic conditions, while the sensitivity of the eggs to dessication (Bayne, personal communication) make important the selection of suitable egg laying sites and climatic conditions. Although many authors have stated the importance of such external factors, there is again little evidence of when and how they exert their effect.

Previous detailed work on pulmonate reproductive systems, especially their maturation, has been concerned with annual species. Perhaps the most thoroughly investigated species has been *Arion ater* (Abeloos, 1944; Lūsis, 1961, Smith, 1966) and the importance of internal and external factors has been stressed. *Arion* is normally an annual with a well defined breeding season, whereas *Agriolimax reticulatus* can apparently breed at any time of year, with up to 3 generations in a year. In spite of this difference in breeding cycle, the maturation stages of most of the reproductive tract are similar in both slugs. Thus the hermaphrodite gland, albumen gland, and common duct stages described by Smith (1966) for *Arion ater* are comparable with those described here for *Agriolimax reticulatus*. While Smith was able to subdivide the maturation of the penis and genital atrium of *Arion ater*, we were unable to do this for *Agriolimax reticulatus*. The major differences between the 2 reproductive tracts appear to be due to the greater separation in time of the male and female functions in *Arion ater*. Thus the hermaphrodite gland of *Arion ater* is nearly empty of sperm at the oöcyte stages, and the prostate gland starts to atrophy before the oviducal gland enlarges. This is in great contrast to maturation in *Agriolimax reticulatus*, where although the male system begins to mature first, both male and female systems appear to be functional in the mature animal. Also, copulation takes place some time before oviposition in *Arion ater*, whereas in *Agriolimax reticulatus* mature animals appear to be capable of both oviposition and copulation. Observations in this laboratory have, however, confirmed Luther's (1915) finding that *Agriolimax reticulatus* reared in isolation delay oviposition for a long time, and then lay very few eggs. Therefore, copulation appears to normally precede oviposition in *Agriolimax reticulatus*.

Although breeding did occur throughout the year (Fig. 2), there appeared to be optima in spring and autumn. Possibly the most obvious changes in climate at these times are fairly rapid and extensive temperature changes and high humidity. Carrick (1942) and Arias & Crowell (1963) have clearly shown that high environmental humidity is essential for oviposition in *Agriolimax* and, as we have observed large numbers of animals

in copula at dusk following a heavy shower, humidity may also be important in mating. Other possible evidence for the effect of external factors arises from the observation of variations in the relative quantities of sperm and ova in the spermatozoon, oöcyte and post-reproductive stages. This would imply that the timing and frequency of copulation and oviposition are not dependent on the stage of the hermaphrodite gland in these late stages. That some small animals were found with very mature hermaphrodite glands and some larger animals with immature hermaphrodite glands may imply that environmental factors affect the rate of development of this gland. Such variation could alternatively be due to genetic variation or physiological (Pelluet & Lane, 1961; Pelluet, 1964) or environmental (Bouillon, 1956; Richter, 1935; Rosenwald, 1927; Lūsis, 1966) influences on the relative quantities of the 2 types of gamete.

Laviolette (1950a, 1954), using very elegant experimental techniques, was able to demonstrate conclusively the existence of hormonal factors in the blood of various related arionid and limacid slugs, including *Agriolimax reticulatus*. These hormones apparently emanated from the hermaphrodite gland, and controlled the development of the albumen gland and the common duct but not of the penis. The site of production and nature of the hormone could not be determined. As shown in Figs. 8 and 10 and summarized in Table 1, the developmental stages of the common duct and albumen gland in *Agriolimax reticulatus* were closely related to the stage of the hermaphrodite gland. It is therefore likely that a similar endocrine control exists in this species.

In a very detailed study of growth in several species of Arionidae, Abeloos (1944) observed 3 phases of growth: an initial slow phase, the infantile stage, followed by a rapid phase, the juvenile stage, and lastly a slow one again, the mature stage. He also found that closely related changes took place in the reproductive system, an observation later amplified by Laviolette (1950b). The transition from the infantile to the juvenile stage (pre-puberty) was marked by oogenesis in the hermaphrodite gland and the rapid growth of the juvenile phase was paralleled by massive spermatogenesis. At the end of the juvenile phase the gonad had reached its maximum size. After the transition to the mature stage (puberty) there was marked growth of the reproductive tract. Smith (1966) has also noted that, at one stage in the development of the reproductive system of *Arion ater*, a large number of changes occurred in the tract; that stage would appear to coincide with Laviolette's transitional stage, i.e. at puberty.

In Table 1 our results for the changes observed in the histology of the reproductive tract of *Agriolimax reticulatus* throughout maturation, together with some observation on reproductive behaviour, are correlated to the stages of growth proposed by Abeloos (1944) and Laviolette (1950b) for the annual species of Arionidae. It can be seen that our results easily fit into such a scheme and, while not conclusive, suggest that the stages of growth in *Agriolimax* appear to be similar to those in the Arionidae.

In *Agriolimax reticulatus* there is thus some evidence that reproductive development may be controlled both by internal and by external factors. Experimental work is now needed to isolate the individual factors and to determine their effects.

ACKNOWLEDGMENTS

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TABLE 1. *Agriolimax reticulatus*; Summary of reproductive development

Organ \ Phase of growth	Infantile	Juvenile (Pre-puberty)	Mature (Puberty)	Senile?
Hermaphrodite gland: stage	Undifferentiated	Spermatocyte → Spermatid → Spermatozoa → Oöcyte		Postreproductive
weight	Increasing	Maximum	Decreasing	
duct		Empty	Sperm present	Loss of sperm
Albumen gland	Undifferentiated	Differentiation	Maturation → Secretion	
Common duct	Differentiation →	Male secretion →	Female Secretion	
Spermatheca		Empty	Sperm present	Loss of sperm
Penis		Growth	Maturation of sarcobellum	
			Copulation - - →	
			Oviposition →	

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RÉSUMÉ

ETUDES SUR LA MATURATION DE L'APPAREIL REPRODUCTEUR D'*AGRIOLIMAX RETICULATUS* (PULMONATA: LIMACIDAE)

N. W. Runham et A. A. Laryea

Les *Agriolimax reticulatus* Müller ont été récoltés dans le Nord du Pays de Galles pendant une période de 18 mois. L'élevage a eu lieu tout au long de l'année avec une plus grande activité au printemps et à l'automne. L'appareil reproducteur des limaces a été disséqué et les différentes parties ont été séparément pesées et sectionnées. Le stade de maturation de la glande hermaphrodite n'est pas tout à fait en rapport avec le poids de l'animal. Le poids de cette glande atteint un maximum au "stade des spermatides" et ensuite décroît. Le sperme apparaît dans le canal hermaphrodite et dans la spermathèque à la fin de ce stade et y demeure ensuite jusqu'au stade qui suit la reproduction. La maturation de la glande à albumen et du canal commun est étroitement en rapport avec celui de la glande hermaphrodite. Les glandes du sarcobellum mûrissent au "stade des spermatozoïdes." Ces résultats semblent indiquer que les stades de maturation de l'appareil reproducteur sont en rapport avec les phases de croissance de l'animal et que des facteurs à la fois physiologiques et externes peuvent contrôler cette maturation.

RESUMEN

ESTUDIOS SOBRE EL ESTADO DE MADUREZ DEL SISTEMA REPRODUCTOR DE *AGRIOLIMAX RETICULATUS* (PULMONATA: LIMACIDAE)

N. W. Runham y A. A. Laryea

Agriolimax reticulatus fue colectado en Gales del Norte durante 18 meses. En todo el año se observaron crías que aumentaron al máximo en primavera y otoño. Se hizo la disección de los órganos reproductores y sus partes fueron separadas, pesadas y seccionadas. El peso del animal no demostró relación con el estado de madurez de la glándula hermafrodita; el peso de esta glándula aumenta a un máximo en el "estado espermático" y después se reduce. Al final de ese estado, apareció esperma en el ducto hermafrodítico y continuó presente hasta el estado post-reproductivo. La madurez de la glándula albuminoidea y ducto comun está estrechamente relacionada a aquella de la glándula hermafrodita. Las glándulas sobre el "sarcobellum" maduran en el "estado espermatozoico." Estas conclusiones parecen indicar que los estados de maduración del sistema reproductor se relacionan a las fases de crecimiento del animal y que los factores ambientales pueden controlar la maduración.

АБСТРАКТ

ИЗУЧЕНИЕ СОЗРЕВАНИЯ ПОЛОВОЙ СИСТЕМЫ У *AGRIOLIMAX RETICULATUS* (PULMONATA: LIMACIDAE)

Н. В. РАНХЕМ и А. А. ЛЕРАЙ

Втечение 18 месяцев в северном Уэльсе производились сборы *Agriolimax reticulatus*. Размножение происходило втечение всего года, с максимумами весной и осенью. Половая система улиток отпрепаровывалась, отдельные её части отделялись, взвешивались и делались срезы. Стадия созревания гермафродитной железы оказалась не очень связанной с весом животного. Вес этой железы достигал максимума на стадии "сперматиды", а затем начинал уменьшаться. Сперма появлялась в гермафродитном протоке и в семеприемнике в конце этой стадии и находилась там вплоть до конца периода размножения. Созревание альбуминовой железы и общего протока было тесно связано с созреванием гермафродитной железы. Железы на саркобеллуме созревают на стадии "сперматозоидов".

Всё это, видимо указывает на то, что стадии созревания репродуктивной системы этих моллюсков связаны с фазами их роста; на них могут влиять как физиологические факторы, так и условия среды.

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THE ARTERIAL GLAND OF AGRIOLIMAX RETICULATUS (PULMONATA: LIMACIDAE)

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ABSTRACT

The arterial gland of *Agriolimax reticulatus* consists of irregularly shaped masses of opaque whitish tissue situated discontinuously along the distal portion of the cephalic artery and along its branches, especially the posterior pedal artery. The tissue is divided into lobules with thick bundles of collagen fibres between. Each lobule is composed of irregular cells and intereellular channels, in some cases leading directly to the edge of the gland. Intracellular ducts connect with the intercellular channels.

Granules occur within the cells and these appear to be of two main types. Each **A** type granule has an amorphous, moderately homogenous, electron dense content which normally completely fills its limiting membrane. These granules stain deeply with Toluidine blue. **B** type granules are less electron dense, their contents have a flocculent appearance and they stain only lightly or moderately with Toluidine blue. These granules contain a variable number of irregular spaces.

The granules release their contents into the intereellular channels directly or into the intracellular ducts.

Histochemical tests for carbohydrates, certain hydroxysteroid dehydrogenases, calcium, copper and acid phosphatase were all negative. Tests for lipid were only faintly positive. The secretory granules, however, stained intensely with Bromophenol blue and gave positive reactions to tests for tyrosine and aspartic and glutamic acids. Tests for SS and SH groups were only weakly positive.

Chromatographic analysis for steriods gave negative results.

Microprobe analysis revealed an accumulation of copper within the arterial gland tissue but it was not possible to localise its position within the cells.

As copper and protein were both present within this gland it was decided to test the arterial gland tissue for haemocyanin. Rabbit antiserum to Helix aspersa haemocyanin was prepared and found to cross react with *Agriolimax reticulates* haemocyanin. Immunoelectrophoresis performed using this antiserum and homogenised arterial glands from Agriolimax reticulatus gave negative results.

The arterial gland in *Agriolimax reticulatus* contains secretion at all stages of reproductive development. The size of the gland is extremely variable between individuals but neither size nor histology could be related to reproductive development.

Of a number of gastropod species examined for the presence of the gland, tissue with a similar appearance to the arterial gland of *Agriolimax reticulatus*, when stained with Azan, was found in 4: *A. caruanae, Limax flavus, Oxychilus alliarius, 0. cellarius*.

MALACOLOGIA, 1973, 14: 135-142

PROC. FOURTH EUROP. MALAC. CONGR.

STUDIES OF THE ENDOCRINE CONTROL OF THE REPRODUCTIVE TRACT OF THE GREY FIELD SLUG *AGRIOLIMAX RETICULATUS*

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INTRODUCTION

Pulmonate slugs are protandric hermaphrodites. Many species complete 1 breeding cycle, then die, but some may complete 2 such cycles, e.g., *Milax gagates* (Galangau, 1964). In very young animals the simple sac-like gonad is full of apparently undifferentiated cells. As the animals get older the gonad becomes increasingly lobed, then first oocytes become visible followed by differentiating sperm and nutritive cells. At first the oocytes enlarge slowly while there is very rapid production of large numbers of sperm. When most of the sperm have been shed the ova mature. Reproductive tract maturation is very closely related to this sequence in the gonad. The prostate gland matures preparatory to the male phase of the gonad and functions at copulation. Egg laying is preceded by the maturation of the albumen and oviducal glands. In most species, e.g., *Arion ater* (Lusis, 1961; Smith, 1966), there is a very clear separation of the male and female phases of the cycle, but in *Agriolimax reticulatus* there is often some overlap (Runham & Laryea, 1968).

The relation between the gonad and reproductive tract has been extensively studied by Laviolette (1954). Using various arionid and limacid species with well defined seasonal breeding periods he carried out an extensive series of organ transplants. The gonad or the reproductive tract from a species at one stage of development was transplanted into the body cavity of another species which at that time of year was at a different stage of its reproductive cycle. Laviolette observed that an immature tract transplanted into a 'mature' animal showed a marked enlargement. He deduced, therefore, that there was a hormone in the blood which controlled the maturation of the reproductive tract.

In this study *Agriolimax reticulatus* was used as it will breed all the year round, so all stages of reproductive maturation are available in the one species. It is also usually available in large numbers and can be maintained in the laboratory fairly readily.

MATERIALS AND METHODS

Agriolimax reticulatus were collected from various localities within a 3-mile radius of the Department. Most of the larger animals used for operations were collected from the wild, but as small animals are very difficult to collect laboratory cultures were set up for these. The cultures were maintained in either polystyrene sandwich boxes or polythene washing-up bowls, in both cases filled to a depth of 3-5 cm with sterile soil and having a small aperture covered with gauze in the cover. Animals were usually fed on carrot but also occasionally on lettuce, and cleaned at least twice a week. The only difficulty encountered with the cultures was at the start of the experiments when there was a very high incidence (about 90%) of infection with *Tetrahymena* in locally collected animals. It was found to be impossible to control this parasite, which can be transmitted in the egg, but luckily, for no apparent reason, the incidence of infection in the local population later fell to a very low level.

For all operations the slugs were anaesthetised with carbon dioxide (Bailey, 1969). They were then placed on moist filter paper on the stage of a Zeiss Stereomicroscope III with foot-operated focussing control. Fine forceps, needles and de Wecker iridectomy scissors were the only instruments required for the operations.

a) Sampling the gonad. A small cut was made in the body wall at the point A (Fig. 1); the very deeply pigmented gonad was located and a small piece removed.

b) Castration. The gonad was located as in a); then it was carefully separated from the digestive gland by tearing the connective tissue sheaths and membranes. The main difficulty with this operation is avoiding damage to the overlying rectum particularly when the gonad is pulled out from beneath it. Once the gonad has been separated from the surrounding tissues it is pulled, if possible forwards, then the hermaphrodite duct is cut and the gonad removed. In some cases the gonad had to be removed in 2 pieces because of its size, the region posterior to the rectum and the region anterior to it. Occasionally there are rather small and isolated groups of acini at the anterior edge of the gonad and these were easily left behind. This was always checked at the conclusion of the experiment. Regeneration of the gonad from the cut end of the hermaphrodite duct occurs as in other slugs (Laviolette), but very rarely was there any sign of differentiation by the end of the experiment.

c) Transplants. The transplants (see below) were manipulated under medium and taken up into the end of a trochar needle. A small hole was cut in the body wall at point B (Fig. 1), the trochar inserted and the transplant injected. The body was held against the tip of the trochar when it was removed in case the transplant adhered to the needle.

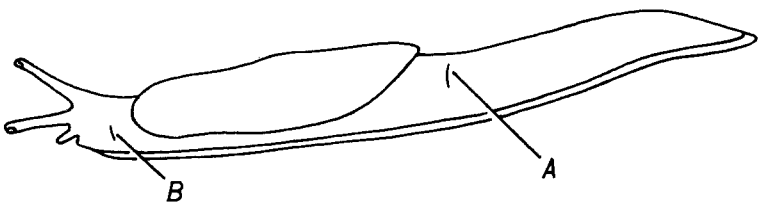

FIG. 1. *Agriolimax reticulatus*. A, position of the incision for removal of the gonad; B, position of the incision for the injection of the transplant.

In none of these operations were any sutures needed in the body wall. After the operation the animals were transferred individually or in small groups to disposable petri dishes lined with moistened filter paper and containing a piece of carrot. Animals were usually wandering around the dish within 30 minutes of operation.

The transplants were obtained from very small animals that had a reproductive tract in the earliest stage of differentiation. After anaesthesia animals were dissected under either Hedon-Fleig saline or organ culture medium (Bailey, 1972). The common duct, in some cases with the albumen gland attached, was removed and cut into 2-7 pieces, one of which was immediately fixed, while the others were transplanted. Transplanting occurred 10-60 minutes after dissection.

At the end of the experiment anaesthetised animals were opened along the length of the body and the transplant searched for in the haemocoel, particularly in the region of the brain and buccal mass. In some cases where the transplant had formed a large swollen cyst it was readily found, but in castrates the very small pieces of tract were exceedingly difficult to discover. The transplant together with the host gonad and sometimes the reproductive tract were fixed.

Tissues were either fixed in susa, washed and dehydrated in cellusolve and embedded in ester wax, or fixed in buffered osmium and embedded in Araldite. Ester wax sections (7 μ) were stained with Azan and 1-3 μ Araldite sections were stained with

toluidine blue. The stages in the maturation of the gonad and reproduction tract have been described elsewhere (Runham & Laryea, 1968).

RESULTS

It was hoped originally to produce quantitative data for the enlargement of the glands and for any histological changes resulting from transplanting. For the following reasons this proved to be impossible. Because of a lack of clear separation of the male and female stages in this species the determination of some stages in the development of the gonad was less accurate than with others. No problems were encountered with the spermatocyte, spermatid, early sperm, late oocyte and post-reproductive stages. The separation of late sperm and early oocyte stages however is dependant on the relative quantities of sperm and oocytes and the size of the latter. In some late sperm stages, with large amounts of sperm present, only a few oocytes were visible - far fewer than normal - probably indicating that in these animals some egg laying had taken place before the more normal loss of the majority of the sperm. Because of variations in the relative proportions of oviducal and prostate gland tissue along the length of the common duct (i.e., oviducal gland predominates at the top of the common duct and prostate gland at the bottom) it was impossible to quantify the changes in the relative proportions of the 2 glands in the small transplants. Frequently the 2 open ends of the transplanted common duct became sealed and secretion by the glands led to the formation of a considerably swollen cyst with greatly distorted glands. A subjective qualitative assessment was therefore developed to assess the changes following transplanting with the above features taken into account.

During normal maturation of the reproductive tract the prostate gland develops first. Diverticulae are formed which enlarge, and then the cubical epithelium becomes underlain by cells which differentiate into a number of different types of secretory cell. Only when secretion has appeared in the prostate does differentiation of the oviducal gland begin. Cells appear beneath the cubical epithelium lining of the oviducal gland which differentiate to become grossly distended with secretion.

Castrated animals were left for a week to recover from the operation and then a piece of common duct from a very young animal was transplanted into the haemocoel. Due to the very small size of these transplants they were only recovered from 6 animals, but in no case was there any increase in size of the common duct after 10 days compared to the controls. The results from 3 series of experiments on normal hosts are given in Tables 1, 2 and 3. When a tract from a very young animal, showing only the earliest stages in the differentiation of the prostate gland, was transplanted into the haemocoel of an animal at a later stage of development and left there for 10 days, rapid transformation of the transplant occurred. In the spermatid stages there was a slight enlargement, while in early sperm and the earliest of the late sperm stages development of the prostate was pronounced (Fig. 2). During the very late sperm stage both the oviducal and prostate glands matured. In the oocyte stages the oviducal gland shows maximum enlargement and secretion while the prostate gland enlarges slightly (Fig. 3). In the post-reproductive stages the oviducal gland alone matured. In normally developing common ducts the oviducal gland matures only after the prostate gland has completed its maturation.

DISCUSSION

As the transplants were left free in the haemocoel, the factors causing the observed changes must be blood-borne, i.e., they are hormones. The results obtained indicate the existance of 2 hormones, one responsible for the maturation of the prostate gland

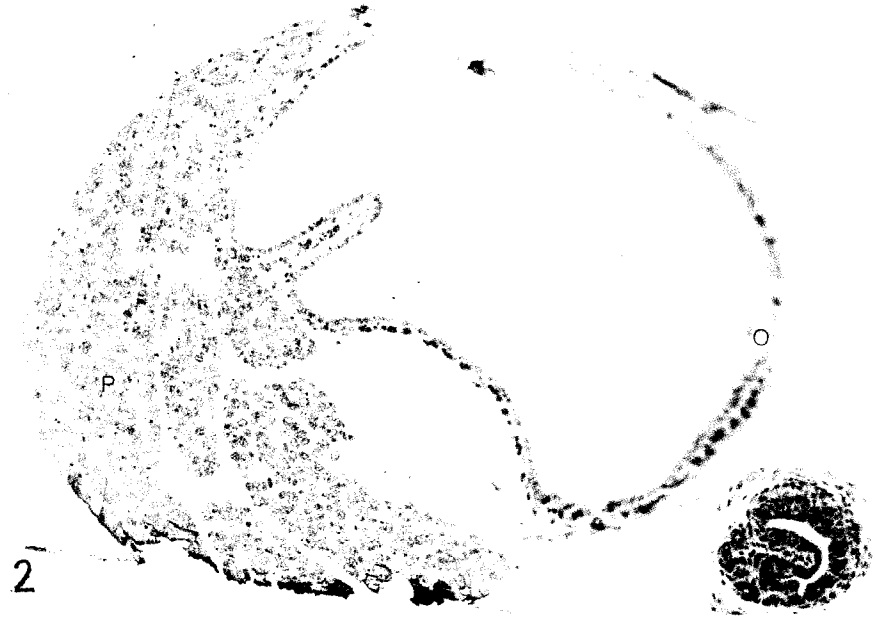

FIG. 2. *Agriolimax reticulatus*. Common duct transplant 10 days after placing in the haemocoel of an early male stage host. Inset, control piece of common duct fixed at the time of transplanting. P, prostate gland; O, oviducal gland.

FIG. 3. *Agriolimax reticulatus*. Common duct transplant 10 days after placing in the haemocoel of an early female stage host. Inset, control piece of common duct fixed at the time of transplanting. P, prostate gland; O, oviducal gland.

TABLE 1. *Agriolimax reticulatus*. Fate of pieces of immature common duct transplanted into the haemocoel of older animals.

Series 1

Host Stage	Prostate Gland		Oviducal Gland		Common Duct	
	% Expansion	Secretion	% Expansion	Secretion	Male Characteristics	Female Characteristics
D	150	-	-	-	+	-
D	300	-	-	-	+	-
D	300	-	500	+	+	+
D	500	-	500	-	++	+-
E	600	-	-	-	++	-
E	1,650	++	-	-	++++	-
E	1,500	+	-	-	++++	-
E	700	++	700	+	+++	+
E	4,000	++	4,000	++	++++	++
E	200	+	2,000	+++	+	+++
F	400	-	?	+++	+	+++
G	4,000	++	4,000	+++	++++	++++
G	1,000	-	1,000	++	++	+++
G	300	+++	1,500	++++	++	++++
G	200	+-	400	+++	+	+++
H	350	++	1,000	++++	++	++++
H	150	++	2,500	++++	+	++++
H	0	++++	?	++++	+	++++
H	0	-	1,000	++++	-	++++

Stages of maturation of the host gonad are:- D early spermatozoon, E late spermatozoon, F early oocyte, G late oocyte, H post-reproductive. The amount of secretion is indicated by the number of + symbols and the absence of secretion by -. The male characteristics of the common duct is a subjective assessment based on the percentage expansion, amount of secretion and the histology of the prostate gland; while the female characteristics is similarly based on the oviducal gland.

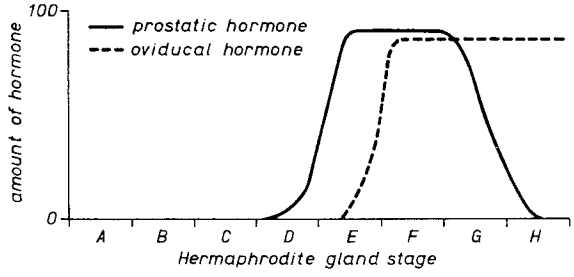

FIG. 4. *Agriolimax reticulatus*. Suggested timing for the secretion of prostatic and oviducal hormones in relation to the stage of development of the hermaphrodite gland. A, undifferentiated; B, spermatocyte; C, spermatid; D, early spermatozoon; E, late spermatozoon; F, early oocyte; G, late oocyte; H, post-reproductive.

TABLE 2. *Agriolimax reticulatus*. Fate of pieces of immature common duct transplanted into the haemocoel of older animals.

Series 2

Host Stage	Prostate Gland		Oviducal Gland		Common Duct	
	% Expansion	Secretion	% Expansion	Secretion	Male Characteristics	Female Characteristics
D	400	-	500	-	+	+
E	0	?	0	0	-	-
E	500	-	400	-	++	-
E	200	+	0	-	++	-
E	800	+	0	-	++	-
E	150	+-	1,000	+-	+	++
E	0	+++	500	+-	++	+
E	400	-	200	-	++	-
E	250	-	?	-	+	-
F	400	-	200	-	+	-
F	300	-	700	+	+	++
G	500	+-	600	++	++	+++
G	150	-	300	?	+	+
G	500	-	500	?	+	+
G	400	-	1,000	++++	+	++++
H	1,000	-	1,000	+++	++	+++
H	700	+	3,000	++++	++	++++
H	0	-	1,000	++++	-	++++
H	0	-	1,600	++++	-	++++

Stages of maturation of the host gonad are:- D early spermatozoon, E late spermatozoon, F early oocyte, G late oocyte, H post-reproductive. The amount of secretion is indicated by the number of + symbols and the absence of secretion by -. The male characteristics of the common duct is a subjective assessment based on the percentage expansion, amount of secretion and the histology of the prostate gland; while the female characteristics is similarly based on the oviducal gland.

and the other for the oviducal gland (Fig. 4). The prostate hormone appears during the late spermatocyte or at the beginning of the spermatid stage and reaches a maximum during the spermatozoan stages. During the late sperm stage, at about the time the amount of prostatic secretion begins to decrease, the oviducal hormone appears and rapidly reaches its maximum. Prostatic hormone appears to be present only in small amounts during the oocyte stages and may be absent from post-reproductive animals.

The processes leading to the maturation of the glands are complex and involve at least the following processes: cell proliferation, with cell migration leading to tissue

TABLE 3. *Agriolimax reticulatus*. Fate of pieces of immature common duct transplanted into the haemocoel of older animals.

Series 3

Host Stage	Prostate Gland		Oviducal Gland		Common Duct	
	% Expansion	Secretion	% Expansion	Secretion	Male Characteristics	Female Characteristics
D	200	-	200	-	+	-
E	300	-	0	-	++	-
E	250	++	250	-	++	-
E	300	++	0	-	++	-
E	400	++	300	+	++	+
E	300	+-	1,000	+	++	++
E	300	+	500	-	++	+
E	300	++	200	++	++	++
E	600	++++	200	-	++++	-
E	300	++++	400	+	++++	++
E	?	?	500	++++	-	++++
E	300	++	400	++++	++	++++
E	400	++	600	++++	++	++++
E	300	+	400	++++	++	++++
E	500	++	500	++++	++	++++
E	300	++	10,000	++++	++	++++
F	150	-	400	-	-	+
F	300	-	600	++++	+	++++
H	0	+	10,000	++++	+	++++
F	150	+	400	++++	++	++++
H	500	+-	1,000	++++	+	++++

Stages of maturation of the host gonad are:- D early spermatozoon, E late spermatozoon, F early oocyte, G late oocyte, H post-reproductive. The amount of secretion is indicated by the number of + symbols and the absence of secretion by -. The male characteristics of the common duct is a subjective assessment based on the percentage expansion, amount of secretion and the histology of the prostate gland; while the female characteristics is similarly based on the oviducal gland.

and organ differentiation, and cell differentiation leading to the formation of secretion by the cells. In the transplants, even in the short period of 10 days, massive enlargement and differentiation both of tissues and cells took place. In some cases it was obvious that differentiation of the cells could occur apparently independently of the other processes. Thus several examples were noted of cell differentiation in the prostate without any apparent increase in the size of the gland compared to the controls; and in addition other examples were found where enormous enlargement of the gland occurred with no, or very little, secretion being formed in the cells. There are several possible explanations for this phenomenon. The effect of the hormone may vary

with its concentration, or formation of secretion is controlled by a different hormone to that controlling organ differentiation. In the case of the prostate gland it is even possible that the oviducal hormone may affect formation of prostatic secretions. Not enough data was however available for an analysis of this problem.

Laviolette (1954) clearly demonstrated that the maturation of reproductive tracts of a variety of limacid and arionid slugs were under hormonal control. This study confirms and extends Laviolette's findings, indicating at least in *Agriolimax reticulatus* that not less than 2 hormones are involved in the maturation of the common duct. The albumen gland was also found by Laviolette to be under hormonal control. In our experiments information on the albumen gland was obtained only in the first series of experiments, and in these enlargement of the gland and the formation of secretion occurred in the latest of the spermatozoan stage and in all the oocyte and post-reproductive stages. This would perhaps indicate that the albumen gland is also influenced by the oviducal hormone.

The source of these hormones is unknown. Laviolette injected extracts of the gonad into various slugs but the reproductive tract did not appear to be affected. Preliminary organ culture experiments (Bailey, 1973) indicate that when the gonad and reproductive tract are cultured in close proximity no maturation changes can be observed in the reproductive tract. However, when the brain, gonad and reproductive tract are cultured close together then maturation changes can be observed in the cells of the reproductive tract. When Laviolette transplanted gonads from mature slugs into castrated immature slugs, maturation of the host reproductive tract resulted. There is therefore tentative evidence that factors are produced by the gonad which cause the brain to produce the prostatic and oviducal hormones.

Further experimental studies are clearly needed to clarify the details of hormonal control of the reproductive tract of slugs.

SUMMARY

An extensive series of organ transplants using the slug *Agriolimax reticulatus* indicate the existence of 2 hormones. When immature common ducts are transplanted into the haemocoel of older animals the changes observed in the transplants clearly reflect the stage in the reproductive maturation of the host. It is concluded that 1 hormone controls differentiation and enlargement of the prostate gland, the 2nd hormone controls the oviducal gland. No changes were observed in common ducts transplanted into the haemocoel of castrated animals. It is suggested that these hormones are produced by the brain.

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