DOCTOR OF PHILOSOPHY

Hormones in Cirripedes

Clare, Anthony

Award date:
1984

Awarding institution:
Bangor University

HORMONES IN CIRRIPEDES

A Thesis

submitted to the University of Wales

by

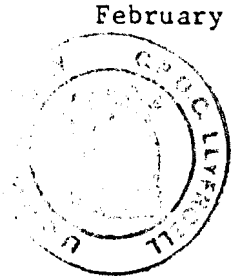
Anthony Simon Clare B.Sc. (C.N.A.A.),
M.Sc. (Wales), M.I.Biol.

in candidature for the degree of

Philosophiae Doctor

N.E.R.C. Unit of Marine Invertebrate Biology
Marine Science Laboratories
University College of North Wales
Bangor Gwynedd

February 1984



CONTENTS

ABBREVIATIONS USED IN FIGURES

The following list does not include abbreviations used in chapter one, the definitions of which are given in the appropriate figure legend.

A. Mu.	adductor muscle
A. Mu. N.	adductor muscle motor nerve
A.N.	antennular nerve
Ax.	axon
Ax. T.	axon tract
B.L.	basal membrane
C.	commissure
C.1	first cirrus
Ca. P.	carinal plate
C.B.	cell body
Cl.	suboesophageal cleft
C.N. 1-6	cirral nerves 1-6
Co.	collagen fibres
C.O.C.	circumoesophageal connective
C.P.	cell process
D.	dendrite
D.C.V.	dense-core vesicle
De.	desmosome
D.T.	dendrite terminal
E.-D.G.	electron-dense granule
E.L.V.	electron-lucent vesicle
E.M.	egg mass
E.R.	endoplasmic reticulum
Fi.	fibril
G.	granules
Ga.	glia
G.B.	Golgi body
G.C.	glial cell
Gl.	gliosome
Gn.	glycogen
G.P.	glial process
G.S.N.	great splanchnic nerve

H.	haemolymph
I.O.G.	infraoesophageal ganglion
L.	lobe
Li.	lipid
L.O.	lateral ocellus
L.P.N.	lateral photoreceptor nerve
Ly.	lysosome
M.	mitochondrion
Ma.	mantle
M.C.	mantle cavity
M.-D.N.	mid-dorsal nerve
Ml. B.	multilamellar body
M.N.	mandibular nerve
M.O.	median ocellus
Mt.	microtubules
Mu.	muscle
Mv.	microvilli
Mv. B.	multivesicular body
N.	nucleus
Ne.	neurone
Nl.	neurilemma
Np.	neuropile
N.P.	nuclear pore
N.S.C.	neurosecretory cell
N.S.G.	neurosecretory granule
Nt.	neurotubules
Nu.	nucleolus
O.	ovary
Oe.	oesophagus
O.G.	oviducal gland
O.N.	ocellar nerve
Oo.	oocyte
Ov.	oviduct

P.	pseudopodium
Pa.	parenchyma cells
P.C. or Ph.C.	photoreceptor cell
Pe.	perilemma
Pr.	prosoma
P.V.	pinocytotic vesicles
R.	free ribosomes
R.B.	residual body
R.E.R.	rough endoplasmic reticulum
Rh.	rhabdomere
R.P.	rostral plate
S.	space
S.E.R.	smooth endoplasmic reticulum
Sh.	sheath
S.O.G.	supraoesophageal ganglion
S.P.	scutal plate
S.S.N.	suprasplanchnic nerve
S.V.	synaptic vesicles
T.	tracheole
T.P.	tergal plate
V.	vacuole
V.N.	ventral nerve

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my supervisor Dr. Graham Walker for the invaluable help he has provided during the course of this study. I would also like to thank Professor D.J. Crisp, under whose directorship the study was carried out, for his liberal advice and stimulating discussions. Dr. D.L. Holland helped considerably with the hatching substance work and John East provided expert technical assistance over the same period. Also in connection with this work I thank Dr. P.A. Gabbott, both for advice and for procuring financial assistance, Dr. R. Kelly of the M.R.C. Reproductive Biology Unit for preliminary G.L.C./M.S. analysis, Dr. K.H. Gibson and his colleagues at I.C.I. Pharmaceuticals Division, Macclesfield, for their generous gift of prostaglandin standards, for performing the G.L.C./M.S. analyses and for writing the G.L.C./M.S. section of chapter 8, Roche Products Ltd., Welwyn Garden City for the kind gift of the drugs benserazide HCl and tetrabenazine and finally Dr. Anne Whittle and Ian Cotton for much useful discussion.

Thanks are also due to Sheila Walker who did some of the sectioning of material for T.E.M., Mr. Andrew Barber performed the iontophoretic injections of Lucifer Yellow CH and Dr. W.W. Stewart kindly supplied a sample of this fluorescent dye before it came on to the market. Dr. A.B. Yule aided with the use of strain gauges in chapter 5, Jamie Whillis carried out the bioassay of barnacle seminal plasma on his plaice gut preparation and Dr. D.J. Grove provided samples of tetrodotoxin and curare. The egg measurements in chapter 6 were made by a visiting Manchester undergraduate Mr. D.T.J. Littlewood. Collections of Balanus hameri were made from the R.V. Prince Madoc and I thank the captain and

crew for their cooperation.

The excellent standard of typing throughout the thesis owes itself to Mrs. D. Clapton, Mrs. J. Forrest, Mrs. E.B. Kinghorn and Mrs. C. Weills

Part of this work was carried out whilst in tenure of a N.E.R.C. Studentship and the remainder whilst holding a N.E.R.C. Unit honorarium and a research assistantship to Dr. G. Walker.

SUMMARY

A comprehensive review has been made of crustacean hormones together with a summary of invertebrate prostaglandin studies. The review aims to provide a background to the study of cirripede hormones.

The morphology of the central nervous system of Balanus balanoides and B.hameri is described and briefly compared to that of Conchoderma auritum, thereby providing a framework for the neurosecretory studies which follow.

The phenomenon of neurosecretion, especially pertaining to the supraoesophageal ganglion, is dealt with in detail for B.hameri, and also discussed for B.hameri and C.auritum. 4 neurosecretory cell types have been recognized in B.hameri and their secretory product has been matched tentatively with elementary granule types present in the neuropile and nerves of the supraoesophageal ganglion. The origin of one type of elementary granule remains unknown. Sites at which exocytosis of granules occur include the neuropile and the nerve terminals beyond the median ocellus. The latter constitute the first known example of a neurohaemal area in the Entomostraca.

Neurosecretory cells are also present in the median ocellus and the ultrastructure of this photoreceptive organ together with its relation to the supraoesophageal ganglion are described.

Attempts to artificially induce egg laying in barnacles have failed. However, evidence is presented to support the contention of Barnes et al. (1977) that the stimulus to egg laying is derived from some factor present in the seminal plasma.

The embryology of B.balanoides and the effect of 20-hydroxyecdysone on this process have been studied. There is no obvious effect from hormone concentrations of up to 1 µg/ml until egg stage 9, but beyond this stage

the eggs undergo progressive cytolysis. Probable reasons for this effect are discussed.

The hatching substance of B.balanoides has been extracted and partially purified. The compound is a prostaglandin and is released by adult tissues in response to feeding. The prostaglandin acts by inducing the release of dopamine within the embryo, which in turn stimulates embryonic muscle.

CHAPTER 1

Hormones in Crustacea : a review

SECTION I

Neuropeptides in Crustacea

A. The endocrine organs

i) The neurosecretory system of the eyestalk

a) The sinus gland

The sinus gland, first discovered by Hanström (1933), has subsequently been the subject of intensive research. Its gross morphology is now well documented (for reviews see Hanström, 1947; Knowles & Carlisle, 1956; Carlisle & Knowles, 1959; Welsh, 1961; Bullock & Horridge, 1965; Highnam & Hill, 1977) and will not be elaborated on here. The secretory material of the sinus gland, which can be visualized by histochemical means (cf. Knowles & Carlisle, 1956), was for many years believed to be produced *in situ*. However, the development of methods designed to selectively ablate the sinus gland demonstrated that the hormones were in fact produced outside the gland (Knowles, 1951; Enami, 1951; Passano, 1951a, b; Bliss & Welsh, 1952).

Much attention has been given to the number of neurosecretory granule types present in the sinus gland of representatives of the Malacostraca and the Entomostraca (Table 1). Electron microscopy has revealed that the types of granules range in number from two to a maximum of five. The obvious inference to be drawn from these observations is that each granule type corresponds to a different hormone complex. However, such a theory needs much further study to be confirmed, particularly since differences in number have been reported within the same species (see Meusy, 1968 and Smith, 1974 for *Carcinus maenas*).

It is evident from the above studies that the number of granule types is fewer than the reported number of effects attributed to eyestalk hormones. The possibility therefore arises that a granule type may give rise to more than one hormone. Alternatively a hormone may have more

Table 1. Number of neurosecretory granule types within the sinus gland of some decapods.

Species	Number of granules	Reference
Cardisoma carnifex	6	Weatherby, 1981
Palaemon serratus	5	Strolenberg et al., 1977a
Astacus leptodactylus	5	Strolenberg et al., 1977b
Callinectes sapidus	5	Andrews et al., 1971
Procambarus clarkii	5	Bunt & Ashby, 1967
Uca pugnax	5	Silverthorn, 1975
Carcinus maenas	5	Smith, 1974
Gammarus oceanicus	5	Brodie & Halcrow, 1977
Pachygrapsus marmoratus	4	Bressac, 1976
Palaemon pancidens	4	Hisano, 1976
Carcinus maenas	3	Meusy, 1968
Porcellio dilatutus	3	Martin, 1972
Cambarellus shufeldti	2	Fingerman & Aoto, 1959
Gecarcinus lateralis	2	Hodge & Chapman, 1958
Gecarcinus lateralis	2	Weitzman, 1969
Orconectes nais	2	Shivers, 1967
Squilla mantis	2	Knowles, 1959

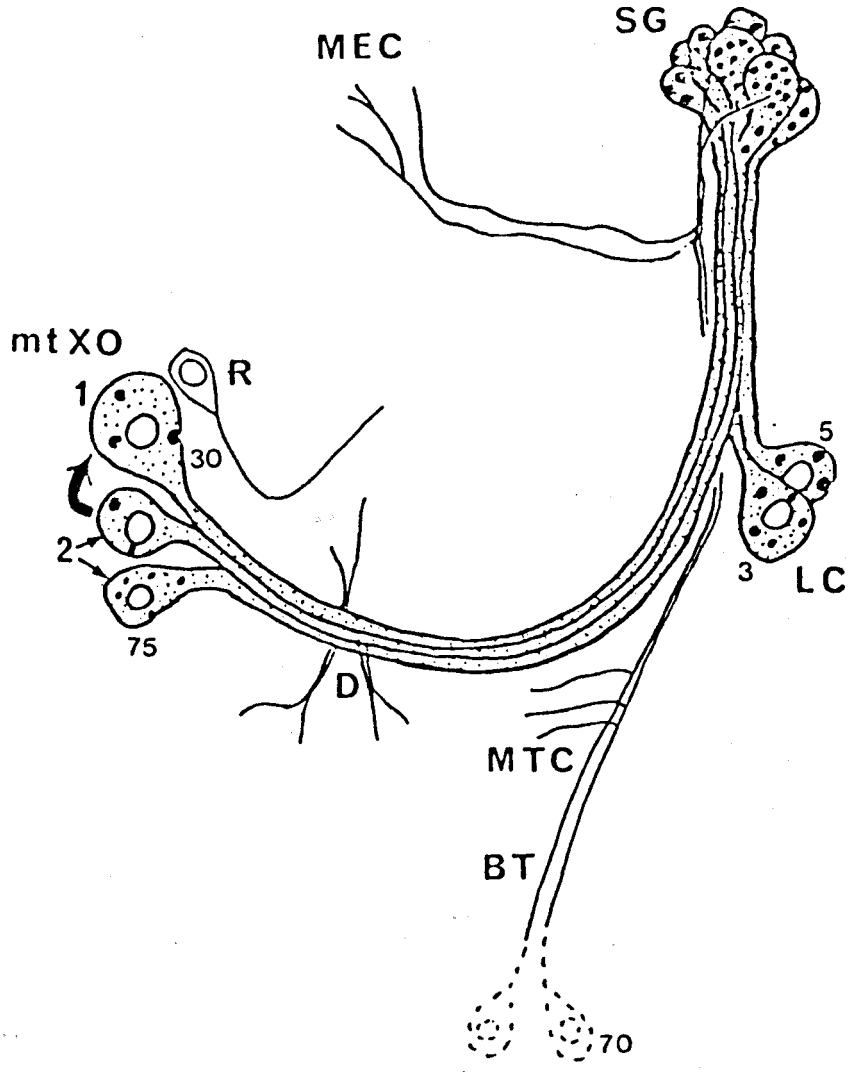
than one physiological effect (Kleinholz & Keller, 1979).

b) The X-organ

Axons terminating in the sinus gland originate from a group of neurosecretory cell bodies, termed the X-organ, which is located in the medulla terminalis ganglion (Passano, 1951a, b; Enami, 1951). Unfortunately, due to indiscriminate use of the name X-organ in the early literature, several different structures have been grouped under the same term. The problem was clarified to some extent by Knowles & Carlisle (1956), although these workers maintained that the neurosecretory terminals within the sinus gland were derived from multiple sources. Recently Andrew et al. (1978), using cobalt iontophoresis and transmission electron microscopy, gained a more simplified view of the neurosecretory system of the decapod (crayfish) eyestalk (Fig. 1). These workers obtained no evidence to suggest that the neurosecretory axons entered the sinus gland from any region other than the medulla terminalis X-organ (m.t.Xo). The only other axons to enter the sinus gland probably originated from the brain and were not neurosecretory; such axons are likely to represent those which stained with methylene blue of earlier reports (Bliss & Welsh, 1952; Bliss et al., 1954). Cobalt backfilling of these axons from the brain showed that collaterals branched from them to enter all three eyestalk ganglia, but were most prominent in the medulla externa ganglion. This ganglion is very near to neural elements involved in photoreception and so may offer some link between light reception and the release of sinus gland hormones.

Andrew et al. (1978) determined that a sinus gland is supplied by approximately 115 neurosecretory axons. Jaros (1978) has shown that not all of these axons have their cell bodies in the m.t.Xo. Eight cell

Fig. 1. Schematic diagram of the crayfish eyestalk neurosecretory system. Brain tract axons (BT) are not actively neurosecreting; dashed lines represent their hypothetical cell bodies in the brain. Types 1 and 2 cells, containing neurosecretory vesicles, comprise the medulla terminalis X organ-sinus gland (mt Xo-SG) complex. Type 1 can be recruited from a type 2 pool (solid arrow).
D, dendrites; LC, lateral cells; MEC, medulla externa collaterals; MTC, medulla terminalis collaterals; R, regular neurone. Small numbers are approximate cell body counts. After Andrew et al. 1978; Andrew & Saleuddin, 1979.


MEC
SG
mtXO
1
R
30
2
75
D
5
3
LC
MTC
BT
70

bodies on the lateral side of the medulla terminalis ganglion also produce neurosecretory vesicles. Axons from these cells form a tract which runs to the sinus gland (Fig. 1). It is therefore apparent that the crayfish m.t.Xo. constitutes approximately 90 - 95% of those cells which synthesize the neurosecretory product stored in the sinus gland (Andrew & Saleuddin, 1979). It has also been suggested that what were originally thought to be two separate cell types of the m.t.Xo. are in fact different secretory stages of the same cell; cell type 2 representing an immature and less active form of cell type 1,

ii) The brain and thoracic ganglia

Information on neurosecretory cells in the brain and thoracic ganglia of Crustacea is fragmentary and not all of the sub-classes have been studied. Selected references to some of these studies are presented in Table 2. The Malacostraca have received most attention and consequently the distribution of neurosecretory cells in the central nervous system (C.N.S.) of this sub-class is best characterized. However, most of the work has been performed at the light microscope level, using stains which are not specific for neurosecretory products, and so is equivocal. Furthermore, until recently the sinus gland was thought to be a neurohaemal area for the release of neurosecretory products from the brain and thoracic ganglia; this is now known not to be the case (see above) and so one of the prerequisites to the labelling of a cell as being neurosecretory, namely to be able to demonstrate that the products are released to the circulatory system (Scharrer, 1969) has not been satisfied in the majority of studies. There is therefore a need for light microscopial studies to be coupled with ultrastructural studies to establish that the 'neurosecretory' perikarya contain elementary granules. Mapping the axons of these cells to release sites, by use of cobalt iontophoresis (Pitman

Table 2. Selected neurosecretory studies on the ganglia of various crustaceans.

BRANCHIOPODA		
ANOSTRACA:	Artemia salina	Lochhead & Resner, 1958; Baid & Ramaswain, 1965; Hentschel, 1965; Kulakorskii, 1973; van den Bosch de Aguilar, 1976.
	Eubranchipus sp.	Lochhead & Resner, 1958.
	Streptocephalus sp.	Menon, 1962.
	Chirocephalus sp.	Hentschel, 1965; Lake, 1969b; 1970; 1971.
NOTOSTRACA:	Triops cancriformis	Mosconi-Bernardini & Pettenazza, 1966.
DIPLOSTRACA:	Daphnia sp.	Sterba, 1957; Angel, 1967; Halcrow, 1969; Bohm & Parker, 1968; Wolff & Guldner, 1970; van den Bosch de Aguilar, 1969; 1972.
	Simocephalus vetulus	Sterba, 1957.
	Podon intermedius	van den Bosch de Aguilar, 1971.
OSTRACODA		
PODOCOPA:	Cyprideis littoralis	Weygoldt, 1961.
	Cypris puberba	
COPEPODA	Calanus finmarchicus	}Carlisle & Pitman, 1961.
	Calanus helbgolandicus	
CALANOIDA	Heleodiaptomus sp.	Govindarajulu, 1968.
BRANCHIURA		
ARGULOIDA:	Argulus sp.	Madsen, 1963; Hentschel, 1969; van den Bosch de Aguilar 1973a.
MALACOSTRACA		
NEBALIACEA:	Nebalia geoffroyi	Duveau, 1961.
ISOPODA:	Rocinela sp.	Amar, 1953.
	Idotea japonica	Miyawaki, 1958.
	Ammadillidium vulgare	Matsumoto, 1959.
	Tectipes japonicus	Oguro, 1960a; 1960b.
	Argeia pugettensis	Oguro, 1961.
	Anilocra physodes	Juchault & Legrand, 1965.
	Athelges japonicus	Oguro, 1961.
	Porcellio dilatatus	Martin, 1972; 1974.
	Cyathura carinata	Chataigner, 1976.
	Sphaeroma serratum	Chataigner et al., 1978.
	Ligia oceanica	Juchault & Kouigan, 1975.
DECAPODA:		
Natantia	Crangon sp.	Clausen & Palz, 1959.
	Penaeus sp.	}Knowles &Carlisle, 1956
	Lysmata sp.	
Reptantia	Sesarma sp.	Enami, 1951.
	Gecarcinus lateralis	Bliss & Welsh, 1952
	Various spp.	Bliss et al., 1954.
	Eriocheir japonicus	Matsumoto, 1954.

Paratelphusa hydrodromous	Parameswaran, 1956.
Orconectes virilis	Durand, 1956.
Pachyrapsus crassipes	Inoue, 1957.
Various spp.	Matsumoto, 1958.
Cambarellus shufeldti	Fingerman & Aoto, 1959.
Astacus leptodactylus	Konok, 1960.

et al., 1972) and Lucifer Yellow (Stewart, 1978), is also desirable.

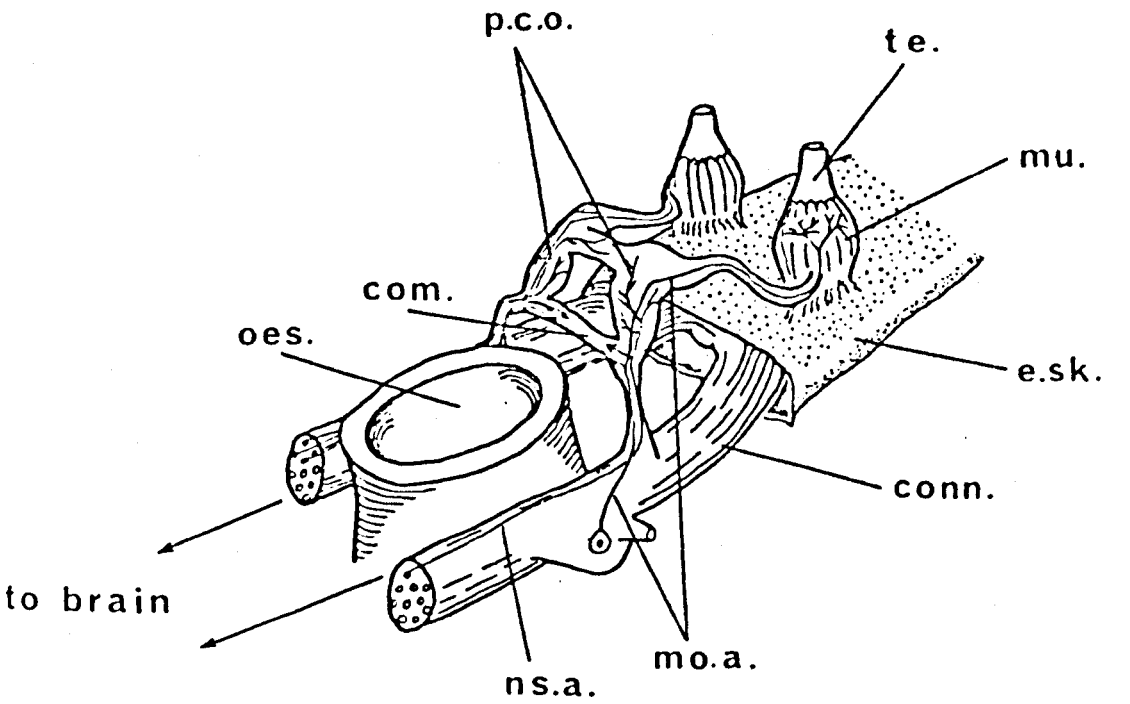
iii) The post-commissure organs

In 1940 Brown & Ederstrom demonstrated that the circum-oesophageal connectives of the shrimp, Crangon (= Crago), are a highly potent source of chromatophorotrophins (chromactivating hormones). In later experiments (Brown, 1946) it was found that the greatest activity lay not in the connectives, but in the post-oesophageal commissure and especially near the post-commissure organs (Fig. 2). These organs have been described as neurohaemal areas by Knowles (1951; 1953) and although the nerve cell bodies associated with the post-commissure axons are unknown, they are believed to be in the tritocerebrum (Knowles, 1959). In addition to being important in the hormonal control of colour change, the post-commissure organs have been implicated in the control of heart rate of the shrimp Paratya compressa (Hara, 1952a; 1952b).

iv) The pericardial organs

The pericardial organs are neurohaemal areas which store and release neurosecretory material(s) which affects the frequency and amplitude of the heartbeat (Alexandrowicz & Carlisle, 1953). The pericardial organs are neuropile-like meshworks, surrounded by loose connective tissue, which either hang freely in the pericardial cavity, or are in the form of plexuses in the membranes lining the cavity. Elementary neurosecretory granules, of approximately 120 mm. diameter, have been observed in the nerves which make up the pericardial organs of Cancer borealis (Bela-marich & Terwilliger, 1966). In the lobster, neurosecretory granules of between 100 and 200 mm. diameter, have been equated with octopamine, a metabolite of tyrosine (Evans et al., 1976b). Octopamine had been shown in an earlier study to be concentrated at two sites in the lobster

Fig. 2. A semi-diagrammatic view of the post-commissure organs of Leander serratus to show their relation to nearby structures. Each connective contains four neurosecretory axons which run to the post-commissure organs; two motor axons have also been seen traversing each commissure organ. In this figure, for the sake of clarity, only one neurosecretory axon and one motor axon are shown in the left connective and left post-commissure organ. ns.a., neurosecretory axon; mo.a., motor axon; conn., circum-oesophageal connective; com., commissure; p.c.o., post-commissure organs; oes., oesophagus; mu., muscle; te., tendon; e.sk., endophragmal skeleton. After Carlisle & Knowles, 1959.


p.c.o.
te.
mu.
com.
oes.
e.sk.
conn.
to brain
mo.a.
ns.a.

nervous system, in the proximal region of the second thoracic nerve root and in the pericardial organ. Nerve cell bodies located in the vicinity of the thoracic nerve root have large varicosities in the same region where profiles containing neurosecretory granules were also observed. Although the axonal endings have not been traced directly back to the nerve cell bodies, the latter contain granules of similar size to those in the endings. It is suggested that the root cell neurones are neurosecretory cells which release octopamine at two sites : one near the cell bodies into haemolymph on route to the gills, the other at the pericardial organs, near the distal ends of the roots, into haemolymph which has just passed through the gills on its way to the pericardial sinus (Evans et al., 1976a). Since octopamine is released into the haemolymph, all organs are potential sites of action for the compound. Indeed, it is thought that octopamine may regulate carbohydrate metabolism in the insect nervous system (Robertson & Steele, 1974).

An extract from the pericardial organs has also been shown to cause an increase in the frequency of scaphognathite beating of the crab, Carcinus maenas, and the crayfish, Orconectes sp. There is, therefore, a potential mechanism in some Crustacea for coordinating heart rate and gill ventilation. The active factor is thought to be a peptide, although there is no direct evidence to substantiate this claim (Berlind, 1976).

v) Neurohaemal areas of the lower Crustacea

The above discussion has been confined to describing the neurosecretory cells and neurohaemal areas of the Malacostraca. In the Entomostraca, neurosecretory cells have been described in the C.N.S. (see Table 2). However, to date, no conclusive evidence has been forwarded to suggest that they possess neurohaemal areas. Those workers that have

described neurohaemal areas have based their arguments solely on light microscopical observations. Therefore, it is not known if the stained product is neurosecretory. Furthermore, release of the product has not been demonstrated, and perikarya serving as a source of the product have not been found. Moreover, the removal of the 'endocrine structures' of the eyestalk of Artemia caused no perceptible change in the physiology of the animal (Lochhead & Resner, 1958; Hentschel, 1967). Thus, it is evident that studies on the neurosecretory systems of the Entomostraca have advanced little since neurosecretion was first shown to be present by Sterba (1957).

B. The endocrine control of metabolism

i) Moulting

A brief account of the crustacean moult cycle is given in section 3 of this chapter.

It is now almost eighty years since Zeleny (1905) found that eyestalk removal in Crustacea leads to precocious moulting. This was later confirmed by Meguvar (1912), but then forgotten until, among others, Brown & Cunningham (1939) obtained the same results using a crayfish (Cambarus spp.). As the effect was not a response to injury (Smith, 1940), it was assumed that moulting was under the control of an eyestalk hormone. An alternative explanation was provided by Kleinholz and Bourquin (1941). They argued that the eyestalk contains a sizeable portion of the tissues of the C.N.S., so its removal is tantamount to the removal of the higher inhibitory centres in a decerebrate vertebrate. Therefore, it is evident that adequate proof had not been obtained to conclude that the eyestalks control moulting. The removal of the eyestalks of the crayfish, Cambarus, during the winter anecdysis was

more conclusive in this respect (Kyer, 1942; Scudamore, 1942, 1947). It was found that the development of the gastroliths was induced - a sign of proecdysis - and a moult occurred after about three weeks. Furthermore, the development of the gastroliths and subsequent moult could be delayed if sinus glands were implanted into the eyestalk-ablated crayfish. The critical experiment of selective removal of the sinus glands was performed by Bliss (1951; 1953a) and Passano (1951a, 1953b); they demonstrated that there was no resultant effect on moulting. Instead the m.t.Xo. was found to be the source of the moult-inhibiting hormone (M.I.H.) since removal of this organ mimicked the effect of eyestalk ablation. Moreover, when an m.t.Xo. and sinus gland were implanted together, with the connection between them intact, then precocious moulting was inhibited; moulting was only partially inhibited if the neurosecretory complexes were implanted separately, or together with their connections severed. Thus, it is apparent that M.I.H. produced in the cells of the m.t.Xo., is transported to the sinus gland for storage and release. The Y-organ ablation experiments of Echalier (1954, 1955) pointed towards this organ as being the target tissue for M.I.H. and this has since been confirmed (cf. Passano 1960; Gersch, 1976; Highnam & Hill, 1977). M.I.H. inactivates the Y-organ and so prevents the release of ecdysone. It is not yet known how the Y-organ is inactivated.

The removal of eyestalks from some Crustacea normally results in a shortened intermoult period. However, in several instances delayed proecdysis results (Sochasky, 1973; Highnam & Hill, 1977). The latter effect can be explained if it is assumed that eyestalks contain a moult-stimulating hormone (M.S.H.). Such a hormone has been found in the eyestalks of Lysmata seticaudata (Carlisle & Dohrn (1953) and in the eyestalks and supraoesophageal ganglia of Faxonella clypeata (Mobberly, 1963) and Caridina weber: (Nagabhushanam & Chinnayya, 1972). In those Crustacea

that possess a sensory pore X-organ (s.p.Xo.), in addition to the sinus gland the m.t.Xo. produces M.S.H., as do certain neurosecretory cells in the supraoesophageal ganglia. The removal of the eyestalks, therefore, does not remove all the M.S.H. Whether proecdysis is accelerated or delayed in these animals will then depend on the prevailing titre of M.I.H. In the Brachyura and Astacura the s.p.Xo. has been lost and the supraoesophageal ganglia are the sole source of M.I.H. Thus, eyestalk ablation in these groups will only remove the source of M.I.H., whilst M.S.H. from the brain will cause ecdysone production and release from the Y-organs, thereby shortening the intermoult period. Erri Babu et. al. (1979) support the view that M.S.H. is produced in neurosecretory cells of the brain of Brachyura. They contend, however, that M.I.H. acts indirectly on the Y-organs by inducing the neurosecretory cells to produce M.S.H. Their arguments are based on histological observations on the neurosecretory cells of eyestalk-ablated animals and so suffer from certain inadequacies.. Namely, i) light microscopy is not the ideal way to identify neurosecretory cells (see above); ii) the removal of eyestalks is a drastic way in which to eliminate M.I.H. Not only are the animals deprived of their sense of sight, but a considerable portion of the C.N.S. is removed, including the m.t.Xo. The sinus glands are also removed and these contain several metabolic hormones in addition to M.I.H. (see below). It is apparent, therefore, that the eyestalk-ablated animals would be under stress; iii) stress itself is known to stimulate the release of neurosecretory material, so it is not surprising that Erri Babu and co-workers noted that changes occurred in four of the five types of neurosecretory cell under study. It was not known which of these types produced M.S.H. Finally, no sham-operated controls were used. It is clear, therefore, that the conclusions drawn by Erri Babu et al., (1979) should be viewed with some reservation. It is also clear that the evidence

for the existence of M.S.H. is somewhat equivocal. Furthermore, the dual control of the Y-organ by M.I.H. and M.S.H. seems to be energetically expensive.

The hormonal control of moulting has recently been studied using larvae of the crab Rhithropanopeus harrisii (Freeman & Costlow, 1980). previous studies on these larvae (Costlow, 1966a) and other larvae of crustacean species (Costlow, 1963; Hubschman, 1963; Little, 1969; Costlow, 1966b) produced variable results with regard to eyestalk extirpation. Freeman & Costlow (ibid), noted, however, that the moult cycle of eyestalk ablated larvae was shortened. It is possible, therefore, that a moult-inhibiting hormone is present in the larval eyestalk as is the case for the adult.

ii) Sclerotization of cuticle

The hardening and darkening of the newly formed arthropod cuticle is termed sclerotization (Fraenkel & Hsiao, 1963). In insects the whole process is controlled by the hormone bursicon (Fraenkel & Hsiao, 1962; Cottrell, 1962a, b, c, d). This hormone is a protein with a molecular weight of approximately 40,000 (Fraenkel, et al., 1966); it is produced by neurosecretory cells in the brain (Fraenkel & Hsiao, 1963) and is probably released from the posterior half of the fused thoracico abdominal ganglion (Mills et al., 1965; Vincent, 1972). The known actions of bursicon are fourfold. First, it is necessary for the thickening of the endocuticle (Fogal & Fraenkel, 1969) and initiates endocuticle deposition (Vincent, 1971). Secondly, bursicon renders the membrane of certain haemocytes permeable to tyrosine (Post, 1972). This amino acid is converted to N-acetyldopamine (NADA), the principle cuticle hardening agent (Karlson et al., 1962), within the haemocytes. The latter contain the enzymes which convert tyrosine to 3,4-dihydroxyphenylalanine (DOPA)

and dopamine (see Post & Jong, 1973). Bursicon is thought to potentiate the activity of tyrosine hydroxylase, the enzyme which converts tyrosine to DOPA (Seligman et al., 1969; Seligman & Doy, 1972). Finally, since DOPA is the common precursor of both NADA and melanin (Seligman, 1980), it is obvious that bursicon also controls melanization (darkening) of the cuticle.

It should be noted that an alternative mechanism of cuticle sclerotization has been proposed by Vincent & Hillerton (1979). Instead of quinones cross-linking with cuticular proteins (Hackman, 1974; Brunet, 1980), there is thought to be a controlled dehydration of the cuticle and this is achieved by quinones squeezing the water out by selectively occupying strongly hydrated groups.

Tanning of the cuticle of crustaceans is also under hormonal control. Fingerman & Yamamato (1964) working on the dwarf crayfish, Cambarellus shufeldti, concluded that a substance from the eyestalk enhances tanning in this species. It was postulated that the moult inhibiting hormone may be the hormone controlling sclerotization. However, the possibility that an additional hormone, from outside the eyestalk, controls tanning was not dismissed. It has since been suggested, in the light of the work on insects, that the moult inhibiting hormone and the tanning hormone may be separate substances. It is proposed that the Crustacean equivalent of bursicon increases the permeability of the hypodermis during early proecdysis, allowing it to take up tanning agents (Vacca & Fingerman, 1975b). The presence of bursicon or a similar hormone in crustaceans is yet to be demonstrated conclusively.

iii) Hyperglycemia

The hyperglycemic hormone (H.G.H.) of the crustacean eyestalk was first reported by Abramowitz et al. (1944). It has since been the

subject of a series of reviews (Keller, 1974; Kleinholz, 1976; Keller, 1976; Kleinholz, 1978; Keller & Sedlemeier, 1978; Kleinholz & Keller, 1979). H.G.H. is a polypeptide with a molecular weight of approximately 7000 daltons. The actual molecular weight has been found to vary with the species studied, Table 3.

Table 3. Hyperglycemic hormone from various crustaceans

Species	Molecular weight (daltons)	Reference
Cancer magister	6700	Kleinholz et al., 1967
Pandalus jordani	6400	Keller, 1968
Orconectes limosus	7400	Kleinholz & Keller, 1973
	6812 +	Keller, 1981
Carcinus maenas	6726	Keller & Wunderer, 1978

Sinus glands have been shown to be a better source of pure H.G.H. than whole eyestalks. For example, Keller & Wunderer (1978) prepared approximately 280 µg. of pure H.G.H. from about 1000 sinus glands of C. maenas. This material was used to obtain the amino acid composition of H.G.H. From such studies evidence has been gained to support earlier contentions of interspecific differences in H.G.H. (Keller, 1969; Kleinholz & Keller, 1973). It is now clear that the amino acid sequences of pure H.G.H. differ between species (Table 4) (Keller & Sedlmeier, 1978; Keller, 1981). It is therefore surprising to find that eyestalk extracts of the freshwater crab, Oziotelphuse (Paratelphusa) Senex senex and the marine prawn, Penaeus monodon, cause hyperglycemia in the scorpion, Heterometrus fulvipes (Reddy & Ramamurthi, 1980).

Table 4. The amino acid composition of H.G.H. a - values for Carcinus taken from Keller & Wunderer, 1978. b - aspartic acid or asparagine, glutamic acid or glutamine. After Keller & Sedlmeier, 1978.

amino acid	number of residues	
	Carcinus	Orconectes
Asx[b]	9	8
Thr	2	2
Ser	4	3
Glx[b]	5	6
Pro	1	1
Gly	1	2
Ala	4	3
Half-Cys	4	4
Val	4	5
Met	3	1
Ile	1	3
Leu	5	5
Tyr	4	5
Phe	2	3
Trp	1	n.d.
His	1	0
Lys	2	3
Arg	4	4
total:	57	58-?

However, crude extracts were used in the latter study and so there is the possibility that some fraction(s) other than a hyperglycemic hormone was responsible for the hyperglycemic response. For example, the tests were performed on whole scorpions so that some fraction from the crustacean extract may have caused the release of an endogenous hyperglycemic hormone.

The mode of action of H.G.H. is not entirely clear. It is presumed that crustacean H.G.H. elevates blood sugar levels (mainly glucose) through glycogenolysis (Kleinholz & Keller, 1979). Keller & Andrew (1973) have determined that in response to an injection of H.G.H., glycogen is depleted from abdominal muscle, integument, gonads and gills, but not from the hepatopancreas (see also Telford, 1975). These effects are thought to be mediated through two enzyme systems, namely, phosphorylase and glycogen synthetase (uridine-diphosphate-glucose glycogen transglucosylase or UDPG-GT). Phosphorylase has been shown to be activated by crude eyestalk extracts both in vivo (Keller, 1965) and in vitro (Bauchau et al., 1968). An inhibitor of UDPG-GT is also present in eyestalk extracts (Wang & Siheer, 1963; Keller, 1967). The reactions which result in hyperglycemia are preceded by a transitory rise in the cyclic nucleotides, cAMP and CGMP (Keller & Sedlmeier, 1978). The exact rôle of these nucleotides is not known at present, but they are thought to act as secondary messengers which influence the aforementioned enzymes (Sedlmeier, 1982). In this respect, a comparison with the vertebrate system of regulating glycogen synthetase and phosphorylase is pertinent. For example, liver phosphorylase a is stimulated by cAMP (Stalmans et al., 1974). In other words, an increased state of phosphorylation (in the form of cAMP) leads to an increase in glycogenolysis. The converse, a decrease in the synthesis of glycogen also follows, since phosphorylase a inhibits glycogen synthetase a. Therefore, a rise in the level of cAMP indirectly inhibits the synthesis of

glycogen (see Hers, 1976).

The sites at which H.G.H. is produced and stored in the eyestalk of the crayfish, Astacus leptodactylus, have recently been studied with immunocytochemistry (Gorgels-Kallen & Van Herp, 1981). H.G.H. was found to be produced in a group of neurosecretory cells of the MTGX (medulla terminalis X-organ). The hormone is transported in the form of granules along a tract to the sinus gland where it constitutes 10-20% of the total sinus gland protein. Interestingly, the diameter of the granules increases with passage to the sinus gland (Table 5). This may reflect an 'ageing'

Table 5. Quantitative data on the H.G.H. - containing granules in the medulla terminalis X-organ, the tract and the sinus gland of the crayfish, Astacus leptodactylus (after Gorgels-Kallen & Van Herp, 1981).

	Number of measured granules	Mean diameter (nm.)	S.E.
Perikarya	160	130	2.1
Tract	245	139	1.7
Sinus gland	4500	143	0.3

process of the hormone, as discussed in chapter 4. The release of the hormone is by exocytosis. This process and the resulting hyperglycemia, can be promoted by injecting either serotonin or cAMP (Strolenberg & Van Herp, 1977; Strolenberg, 1979; Van Herp & Strolenberg, 1980). Whether the release of H.G.H. is mediated by either of these compounds has yet to be elucidated.

iv) Lipid synthesis

Lipid is the main organic reserve of the crustacean hepatopancreas (Bollenbacher et al., 1972). The amount of lipid stored in this organ varies with the stage of the moult cycle (Paul & Sharpe, 1916; Renaud, 1949; Travis, 1955; O'Connor & Gilbert, 1969), increasing to a maximum during premoult and gradually decreasing after ecdysis. The rise in lipid levels is effected by lipid synthesis in the hepatopancreas, which appears to be under the control of an eyestalk hormone. O'Connor & Gilbert (1968) have determined that the removal of the eyestalks from Procambarus sp. results in an almost immediate increase in the rate of lipid synthesis as measured by the amount of ^{14}C-1-sodium acetate incorporated into fatty acids. Utilizing the same methods, the increase in lipid synthesis was shown not to be mediated by 20-hydroxyecdysone; the amount of radio-labelled acetate which was incorporated into newly synthesized lipid in the crayfish hepatopancreas after incubation with the hormone, did not differ significantly from that incorporated by controls. Similarly, the removal of the Y-organs from crabs of the species Pachygrapsus crassipes, prior to eyestalk removal, did not elevate lipid synthesis above that of eyestalk-ablated animals with their Y-organs intact (Bollenbacher et al., 1972). Thus the changes in lipid synthesis during early proecdysis are not mediated by 20-hydroxyecdysone, but instead are probably controlled by an eyestalk neurosecretory hormone.

v) Protein synthesis

The rate of protein synthesis and the ribosomal RNA content of the midgut gland, epithelium and somatic muscle of the land crab Gecarcinus lateralis, were measured throughout the moult cycle by Skinner (1966). In all tissues there was an increase in both of these parameters during

the premoult period. Such effects may be controlled by an eyestalk hormone(s). Fingerman et al., (1967) have shown that the removal of eyestalks from the crayfish, Procambarus clarkii, markedly reduced the synthesis of amylase by the hepatopancreas. Furthermore, RNA content was found to decrease to zero during a 7 day post-operative period. RNA reappeared when eyestalk extracts were injected into the crayfish. Similarly the endoplasmic reticulum of the hepatopancreatic cells showed degenerative changes following eyestalk removal, which further indicates an effect on protein synthesis.

vi) Hydromineral regulation

Scudamore (1947) was the first to provide evidence that a hormone(s) from the eyestalk of crustaceans controls water uptake. He noted that the removal of the eyestalk or sinus gland from the crayfish, Procambarus clarkii, resulted in a net increase in body weight due to an increased water content of the tissues. This increase in weight could be prevented by implanting a sinus gland. Similar results were reported by Carlisle (1956) for the crab Carcinus maenas. In addition it was noted that the eyestalk factor controlling water uptake was distinct from the moult inhibiting hormone of Carcinus, since sinus gland extracts prepared from this species, at any time during the moult cycle, reduced water uptake in normal and eyestalkless crabs.

Gecarcinus lateralis is a land crab and as such faces different problems in controlling the water content of its body than those encountered by marine, estuarine or freshwater forms. This is reflected in the control of water content during proecdysis; water is obtained in advance of this stage of the moult cycle (Bliss, 1968). The increase in size at ecdysis in this species is effected by a movement of water, from the haemolymph

into the gut and its diverticula. This redistribution of water may be controlled by a hormone present in the thoracic ganglia, for implanting the latter into eyestalk-ablated Gecarcinus, several days prior to ecdysis, caused an increase in the water content of the gut (Bliss et al., 1966, Bliss, 1968).

Removal of the eyestalks from the fresh water crayfish, Procambarus clarkii, caused both an influx of water and an increased flow of urine (Kamemoto, et al., 1966). The result was a reduced concentration of blood chloride ions, an effect which could be reversed by injecting brain or eyestalk extracts. An ATPase of the antennal gland of P. clarkii, which is thought to be involved in sodium reabsorption to produce highly hypotonic urine, is also affected by eyestalk removal. The activity of the enzyme was reduced in eyestalk-ablated animals, but could be restored by injecting an eyestalk extract. Conversely, the injection of a brain extract reduced the activity of the enzyme. The ATPase may, therefore, be regulated by two factors which act antagonistically (Kamemoto & Tullis, 1972).

Brain extracts, which were partly purified by column chromatography, have been prepared from a range of crustaceans and assayed on P. clarkii. In this way, a factor which raised the level of salts in the blood and increased sodium influx was demonstrated in extracts of freshwater decapods, terrestrial decapods associated with fresh water, but not of marine decapods (Kamemoto & Tullis, 1972; Tullis & Kamemoto, 1974). Not only do these experiments demonstrate that the effect of the brain hormone(s) is dependent on the environment in which it operates, but also that the hormone(s) is not species specific.

Tullis & Kamemoto (1974) have further shown that water influx may be controlled independently from sodium movement. Two active factors

were extracted from the C.N.S. of the estuarine crab, Thalamita crenata. When assayed on Procambarus or Thalamita the factors caused opposite effects on water movement; an acetone-soluble fraction increased water influx, whilst a water-soluble, low molecular weight substance decreased water influx.

Aside from the sinus glands, another neurohaemal area has been implicated in osmoregulation. Extracts of the pericardial organs of crayfish contained a factor(s) which increased sodium influx in addition to increasing the chloride concentration of the blood (unpublished, cited by Kamemoto, 1976).

Although the gut wall has commonly been regarded as the major surface over which water influx occurs (Drach, 1939; Groghan, 1958; Passano, 1960; Mykles, 1980), evidence has recently been presented which shows that the anus is also an important site for water uptake, at least for the crayfish, P. clarkii (Muramoto, 1978; 1979). Moreover, the influx of water in this species is controlled by a factor in the eyestalks. Water influx in eyestalk-ligated animals was found to be reduced compared to controls. This effect could be reversed by injecting eyestalk extracts. Conversely the injection of eyestalk extracts into intact crayfish caused an increase in weight of the animals due to a greater water content (Muramoto, 1981).

vii) Respiratory metabolism

20-hydroxyecdysone is claimed to have an important effect on respiratory metabolism (see Highnam & Hill, 1977). In Uca pugilator, however, the eyestalks appear to contain hormones which control respiratory metabolism independent of 20-hydroxycedysone. Silverthorn (1973) found that

the removal of eyestalks from warm-acclimatized crabs caused their oxygen consumption to be raised by 117%. Similarly the oxygen consumption of cold-acclimatized crabs increased by 47%. Since cold-acclimatized crabs do not moult, 20-hydroxyecdysone was assumed not to be involved. More recently, Vasantha et al., (1979) have studied the control of respiratory metabolism in the crab Barytelphusa guerini. Their results show that a hormone is present in the eyestalk which accelerates respiratory metabolism. Since the crabs used by these workers were maintained under one temperature regime only, it is not surprising that an eyestalk hormone which depresses respiratory metabolism was not noted. However, the latter was present in the brain and thoracic ganglia of B. guerini, although its effect became apparent only after eyestalk removal. There is evidence to suggest that the sinus glands store the eyestalk hormone. Extracts of sinus glands from cold-acclimatized crabs increased oxygen consumption when injected into warm-acclimatized crabs and vice versa. Thus, it is apparent that two eyestalk hormones may operate to control respiratory metabolism (Silverthorn, 1975).

viii) Neuronal Activity

Although expressed on the basis of limited evidence there are indications that the crustacean eyestalk contains a factor which depresses the responsiveness of sensory structures. This substance has been termed the neurodepressing hormone (N.D.H.) (see Aréchiga & Huberman, 1980). N.D.H. acts to depress the responsiveness of sensory and motor neurones (Aréchiga et al., 1973; 1974a, 1974b). Preliminary studies on the biochemical properties of the hormone indicate that it is a peptide of low molecular weight (Aréchiga et al., 1974b). More recently (Huberman et al., 1979) N.D.H. has been shown to have a molecular weight of approximately 1200, and its amino acid composition has been elucidated.

N.D.H. is not confined to the eyestalks of Procambarus, for there is also some synthetic activity in the supraoesophageal and thoracic ganglia (Aréchiga et al., 1979). Most activity, however, originates from the X-organ-sinus gland complex; two thirds of the N.D.H. being stored in the sinus gland (Aréchiga et al., 1977). Although there are multiple sources of the hormone, results obtained with electrophoresis indicate that N.D.H. from the eyestalks is the same molecule as that found in the remainder of the nervous system (Aréchiga & Huberman, 1980).

Crustaceans exhibit a behavioural phase of low activity during the day (Aréchiga and Huberman, 1980). It has been postulated that N.D.H. causes this reduced activity as a result of its action on sensory and motor neurones. The theory receives support from measurements of the N.D.H. over 24 hour periods. The amount of N.D.H. in Procambarus was found to be much higher during the day than at night (unpublished work cited by Aréchiga & Huberman, 1980).

C. The endocrine control of reproduction

i) Gonad-inhibiting hormone

Panouse (1943) showed that the removal of the eyestalks from Palaemon (= leander) serratus caused precocious vitellogenesis in the ovary. A similar effect has since been demonstrated in several species of both the Reptantia and the Natantia (cf. Adiyodi & Adiyodi, 1970; Quackenbush & Herrnkind, 1981). The factor which acts on the ovary has been termed the gonad-inhibiting hormone (G.I.H.) by Adiyodi & Adiyodi (1970) which replaced the earlier name of ovary-inhibiting hormone, because the male reproductive organs and androgenic gland are also responsive to the eyestalk factor (Démeusy, 1953; 1967a, b; Ōtsu, 1961, 1963; Gomez, 1965). The accelerated development of the ovary in eyestalk-ablated animals is

prevented either completely, as in Palaemon serratus (Panouse, 1943, 1947) and Lysmata seticaudata (Carlisle, 1953a), or incompletely, as for Scylla serrata (Rangneker & Deshmukh, 1968), Uca pugilator (Brown & Jones, 1949), Rithropanopeus harrisii (Bomirski & Klek, 1974) and Panulirus argus (Quackenbush & Herrnkind, 1981) by implanting a sinus gland. This suggests that G.I.H. is stored in the sinus gland. The incomplete inhibition of gonad development may be accounted for if the stored product within the sinus glands is depleted resulting in a low titre of G.I.H. (Quackenbush & Herrnkind, 1981). It is assumed that the cells of the m.t.Xo. are the source of G.I.H. (see Matsumoto, 1958 and Section Ii)b)). Indeed, cytological changes in the cells of the X-organ, which were correlated with gonad development, have been reported for the shrimp, Pandalus gracilis (Kessleri) (Aoto & Nishida, 1956) and the freshwater crab, Potamon dehaani (Hanaoka & Ōtsu, 1957). The titre of G.I.H. has also been found to fluctuate during the reproductive cycle of crustaceans. For example, eyestalk removal from Potamon dehaani in which vitellogenesis was well under way, did not appreciably accelerate ovarian development, suggesting that during this phase the endogenous titre of G.I.H. is low (Ōtsu, 1963). An alternative explanation is that the ovaries vary in their response to G.I.H. according to their phase of development. Bomirski & Klęk-Kawinska (1974) have postulated that the response of the ovaries of anecdysic species to G.I.H. is different from that of diecdysic species. The crab, Rhithropanopens harrisii, (an anecdysic species) and the prawn Crangon crangon (a diecdysic species) both exhibit three phases of ovarian development : (1) slow vitellogenesis; (2) rapid vitellogenesis and (3) a resting phase. G.I.H. has been shown to cause the phase of slow vitellogenesis in R. harrisii and the resting phase of C. crangon.

G.I.H. has recently been partially purified from the eyestalks of Cancer magister and tested for activity using the Crangon assay of Klęk-kawinska & Bomirski (1975). The hormone was found to be neither sex- nor

species-specific (see also Quackenbush & Herrnkind, 1981) and both thermostable and dialyzable. G.I.H. also has a molecular weight estimate of approximately 2000 which was arrived at using a Sephadex G.25 column.

ii) Gonad-stimulating hormone

The most commonly held belief is that G.I.H. is released into the circulation and acts on the central nervous system to suppress the production and release of a gonad-stimulating hormone (G.S.H.).

The existence of G.S.H. was first indicated from the results of eyestalk ablation experiments in which decapods responded with precocious vitellogenesis. Since the operation serves to remove both the gonad-inhibitory and moult-inhibitory principles, an alternative pathway was postulated to explain why proecdysis was not also initiated. A gonad-stimulatory factor (i.e. G.S.H.) has been shown to occur in both the brain and thoracic ganglia of the crabs Potamon dehaani (Ōtsu, 1963) and Paratelphusa hydrodromous (Gomez, 1965). It is conceivable that G.S.H. or a secretory product from the gonads could act to supress the Y-organs.

iii) Ovarian hormones

The control of the secondary sex characteristics of female amphipods and isopods has been reviewed by Charniaux-Cotton (1960a). The female hormone (F.H.) responsible for such control is believed to be produced by the ovaries. However, the evidence to suggest that F.H. exists in decapods is incomplete. This is a result of the difficulty experienced in attempting to completely remove the ovary. Such an operation can be more readily achieved in amphipods and isopods (Adiyodi & Adiyodi, 1970). There are two lines of evidence which suggest that the ovary of decapods controls temporary sex characters. First Reinhard (1950) reported that

vitellogenesis was inhibited in female Callinectes sapidus which had been sacculinized. Concomitant with this inhibition was the disappearance of those sex characters which are involved with incubating the eggs, namely the pleopods. Secondly, in female Orchestia the brood pouch is formed by the oostegites. These structures are a permanent secondary sex character. At the moult prior to egg-laying, the short juvenile setae of the oostegites are replaced by long ovigerous setae, which are temporary sex characters. It has been found that implants of ovaries from young or maturing females into males from which the androgenic glands had been removed, caused the latter to develop oostegites. Furthermore, the ovigerous setae of females were replaced by juvenile setae following ovariectomy. These ovigerous setae were found to redevelop when an ovary was implanted into an ovariectomized female. Therefore, both the permanent and the temporary secondary female sex characters are controlled by ovarian hormones (Charniaux-Cotton, 1952).

iv) Androgenic hormone

The androgenic gland was discovered by Charniaux-Cotton (1954) in males of the amphipod, Orchestia gammarellus. The gland has since been described in males of nearly all of the Malacostraca (see Charniaux-Cotton 1960a, 1962 and Charniaux-Cotton et al., 1966). Each gland lies near the end of a vas deferens, between the muscles of the coxopodites of each pair of walking legs. The fine structure of the gland has been described in the crabs Pachygrapsus (King, 1964) and Carcinus (Meusy, 1965). It has been noted that the ultrastructural features of the gland resemble those of cells involved with protein synthesis and secretion, although no secretory granules were observed. Katakura et al., (1975) partially purified the adrogenic hormone from the isopod Armadillidium vulgare. Its molecular weight was found to be within the range of 15000-17000 daltons. Using proteolytic digestion they confirmed that the

hormone is indeed a protein.

The function of the androgenic gland and its secretion has been gained mainly from removal/implantation experiments. When the androgenic glands are removed from young male Orchestia, the primary germ cells of the testis produce oocytes instead of spermatocytes. It would appear, therefore, that the androgenic hormone is necessary for the normal development of the testis. The converse situation, of ovaries being transformed into testes, was achieved by transplanting androgenic glands into female Orchestia. As a result the primary germ cells were found to give rise to spermatocytes which developed through to viable spermatozoa (Charniaux-Cotton, 1958). Extracts of androgenic glands injected into female Orchestia were also capable of masculinizing their ovaries, as was the blood of a male Carcinus.

The secondary sexual characteristics of the Malacostraca also appear to be controlled by the androgenic gland. For example, Charniaux-Cotton (1957) has shown that the feminine form of the gnathopod of Orchestia could be masculinized by androgenic gland implants. Furthermore, the masculinized females behaved as males.

It is clear from the above reports that the androgenic hormone is neither sex- nor species-specific.

SECTION II

Neuropeptides in cirripedes

Barnes & Gonor (1958a, b) were the first to report the presence of neurosecretory product in certain cirripedes, namely, Pollicipes polymerus, Balanus glandula, B. hesperius laevidomus, B. nubilus, B. rostratus and Chthamalus dalli. Their histological and histochemical studies centred on P. polymerus. The central nervous system (C.N.S.) of this species did not show any blue-white refractile material characteristic of neurosecretory product. However, when sectioned and appropriately stained, the supra- and suboesophageal ganglia were found to contain two types of neurosecretory neurone. The first type was 30-45 µm. in diameter and contained an oval nucleus 12-15 µm. in diameter. The cytoplasm was basophilic and the nucleoplasm acidophilic. Arising from the perinuclear Nissl zone were parldehyde-fuchsin-positive 'neurosecretory granules', which could also be demonstrated by the periodic acid - Schiff (PAS) method. The second type of neurosecretory neurone was approximately 20µm in diameter and exhibited extensive vacuolation. The spherical nucleus, of 6-8 µm. in diameter, was acidophilic and concentrated around it were the neurosecretory granules. There was no structure associated with the 'eye' (presumably the median photoreceptor) which was neurosecretory in nature in either P. polymerus or in the balanids.

Barnes & Gonor (ibid) were able to follow a cycle of secretory activity in the large neurosecretory cell-type. Furthermore, they postulated two modes of release for the secretory product; one via axon transport to an unknown storage-release organ and the other by way of the periphery of the cells.

More recent histological reports (McGregor, 1967a, b; van den Bosch de Aguilar, 1973, 1976, 1979) at the light microscope level, have added little to the initial reports of Barnes & Gonor (1958a, b). Neurosecretion as a general phenomenon in cirripedes has been confirmed by extending the list of species studied (Table 6). Unlike Barnes & Gonor (1958a), McGregor (1967a) was able to demonstrate the presence of white refractile neurones

Table 6. A list of barnacles in which neurosecretory cells have been reported.

Sub class	Species	Reference
Thoracica	Pollicipes polymerus	
	Balanus glandula	
	B. hesperius laevidomus	Barnes & Gonor, 1958a, b.
	B. nubilus	
	B. rostratus	
	Chthamalus dalli	
	C. stellatus	
	Verruca stroemia	
	Elminius modestus	
	B. crenatus	McGregor, 1967a, b.
	B. perforatus	
	B. spongicola	
	B. hameri	
	B. balanoides	
	B. perforatus	van den Bosch de Aguilar 1973a, b, 1976, 1979.
Rhizocephala	Sacculina carcini	

in C. stellatus. However, these neurones did not occur in a constant position within the C.N.S. When stained with paraldehyde fuchsin, a reproducible pattern of one cell in each lobe of the supraoesophageal ganglion was obtained. Unfortunately, no indication was given of the position of these cells within the ganglion.

A search for a seasonal pattern to neurosecretory activity also proved unsuccessful. Since an obvious prerequisite to such studies is the ability to identify, from animal to animal, a cell or group of cells of constant position, this result is not surprising.

An association of vacuoles with the neurosecretory cells has been noted in all the light microscope studies. Those described by van den Bosch de Aguilar (1979) usually appeared empty, but sometimes contents could be stained by PAS or Bests Carmine giving an homogenous and slightly acidophilic appearance. Otherwise the contents took the form of granules or of highly acidophilic particles which were compared with the multi-lamellar bodies observed in the ultrastructural studies of Bohm & Parker (1968) and Lake (1971). Van den Bosch de Aguilar (ibid) noted an absence of neurosecretory elements in the protocerebrum of Balanus species. This feature was related to a lack of development of the cortex of this region of the supraoesophageal ganglion, for in Balanus species it is the deutocerebral and tritocerebral centres that are directly dependent on the median photoreceptor (Millechia & Gwilliam, 1972). The neurosecretory elements, at the level of the deutocerebrum and tritocerebrum were found to be separated into either individual perikarya or small groups of perikarya. In turn, the ventral nerve cord showed a tendency towards the concentration of neurosecretory elements.

Contrary to the results of light microscopy, the ultrastructural studies of Fahrenbach (1965) revealed axons in the median optic nerve containing dense-core granules, which from his micrographs are of the

order of 140 nm. in diameter. This size of granule is at the upper extreme of the range for catecholamine granules (50-140 nm), but within the range for polypeptide granules (100-300 nm)(Scharrer, 1969). Fahrenbach was therefore correct in interpreting these granules as being neurosecretory.

Further evidence for the presence of neuropeptides in cirripedes has come from physiological studies. Sandeen & Costlow (1961) extracted a substance from the nervous systems of Balanus eburneus, Chelonibia patula and Lepas sp., which dispersed the black pigment of the fiddler crab Uca pugilator. This substance was both heat stable and inactivated by trypsin. Costlow (1963) further investigated the activity of the chromatophorotropin of B. eburneus and showed it to be cyclic and furthermore the cycle occurred within one intermoult period of the barnacle. There is no evidence to show that the chromatophorotropin has a capacity to regulate the moult cycle of barnacles, or indeed has any rôle, particularly in view of the fact that barnacles do not possess chromatophores!

The control of moulting in cirripedes is thought to be analogous with the process in the Malacostraca, in so far as the same hormones responsible for such control have similar actions within both 'groups'. The actual moulting hormone (M.H.) will be discussed in a later section (SECTION IV). A moult-inhibiting hormone (M.I.H.), such as that produced by the neurosecretory cells of the X-organ of the Malacostraca, and whose action is to inhibit the production of M.H., has been extracted from the C.N.S. of B. improvisus (Davis & Costlow, 1974). These workers demonstrated that moult-inhibiting hormone (M.I.H.) from Uca pugilator inhibited the onset of ecdysis when injected into stage Do, but not stage C B. improvisus. Similarly, extracts of stage C B. improvisus increased the time to ecdysis when injected into stage Do animals. However, extracts of stage Do B. improvisus had no effect on stage C animals. These results were explained

in terms of a high titer of M.I.H. being present in stage C animals, but a low titer in stage Do animals. Thus the injection into stage C animals of M.I.H. was merely adding to an already sufficient titre. In stage Do animals, the M.I.H. could be temporarily increased to near stage C levels, thereby inhibiting the onset of ecdysis.

Cheung (1973) proposed that M.I.H. is produced by type A neurosecretory cells in the suboesophageal ganglion of barnacles. It was found that at 0 to 24 hours postmoult the number of these neurosecretory cells is highest and coincides with M.I.H. activity in the suboesophageal ganglion. Suboesophageal ganglia derived from barnacles at more advanced moult stages (24-48 hrs, 48-72 hrs and 72-96 hours postmoult) and implanted into recipient barnacles, did not prolong the intermoult period of the latter. Instead the intermoult period was shortened by 15%. Since sham operations also resulted in a similarly shortened intermoult period, the effect was regarded to have been due to the operation. It is also possible, and the two theories are not mutually exclusive, that the supraoesophageal ganglia contained a moult-stimulating hormone (M.S.H.). Cheung (ibid), thus, proposed that M.I.H. and M.S.H. act in concert on an "ecdysial gland" to control moulting; the response of the gland depending on the prevailing balance between the two hormones.

Kauri (1962) studied the frontal filaments and nauplius eye of the larvae of various Balanus species. The filaments were found to receive no nervous innervation and so could not be regarded as frontal organs. In a later publication, Kauri (1966) proposed that the frontal filament base of cirripede larvae is a sensory papilla X-organ. More recently, Walker (1974) reinvestigated the structure of the frontal filament complex of barnacle larvae at the ultrastructural level. No evidence was found to support the filament base as being a neurosecretory structure. On the contrary, the fine structure of the frontal filament complex was more in line with it having a "pressure and/or orientation sense". Unfortunately,

the concept of a cirripede X-organ is now firmly implanted in the literature (cf. van den Bosch de Aguilar, 1979). It should be emphasized, therefore, that the evidence on which the presence of the X-organ is based is highly ambiguous.

SECTION III

Ecdysteroids in Crustacea

Ecdysteroids are a group of steroid hormones that control physiological processes involved in growth, differentiation and morphogenesis in arthropods (Koolman, 1982). Since Gabe (1953) originally hypothesized that the crustacean Y-organ is analogous to "la glande de la mue" (prothoracic gland) of insects (Carlisle & Knowles, 1959) its involvement in the initiation of moulting in crustaceans has been demonstrated repeatedly, as reviewed by Kleinholz & Keller (1979).

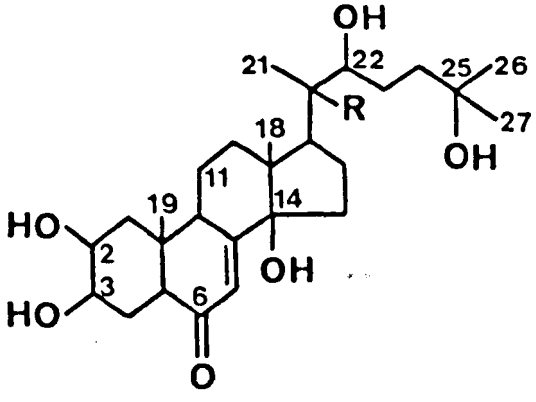
Ecdysone, the moulting hormone was originally isolated from pupae of the silk worm Bombyx mori (Butenandt & Karlson, 1954). Another active compound, 20-hydroxyecdysone, was later isolated by Karlson (1955, 1956). The structure of ecdysone was elucidated by Karlson et al., (1963) from 250 mg. of sample isolated from approximately 4 tons (fresh weight) of Bombyx pupae. A 2 mg. sample of 20-hydroxyecdysone isolated from 1 ton of frozen crayfish waste, served for the determination of the structure of this compound (Hampshire & Horn, 1966). The structure of the two ecdysteroids is illustrated in Fig. 3. Both compounds possess the same carbon skeleton as cholesterol and differ from each other merely by the presence of one extra hydroxyl group in 20-hydroxyecdysone, as the name implies.

The site of synthesis of ecdysone in insects has been firmly established, from in vitro experiments, as being the prothoracic gland or its equivalent ring gland (Chino, et al., 1974; Hoffman, et al., 1974; King et al., 1974; King & Marks, 1974; Bollenbacher, et al., 1976). Since, as previously mentioned, the crustacean Y-organ resembles in both morphology and function, the prothoracic gland of insects, the possible biosynthesis of ecdysone by the former was examined. The first indications that the Y-organs were indeed the site of synthesis of ecdysone came from chromatographic experiments (Carlisle & Connick, 1973 on Orconectes propinquus; Chang, et al., 1976 on Pachygrapsus crassipes; Spaziani

Fig. 3. Chemical structure of ecdysone and 20-hydroxyecdysone.

Ecdysone – R= H

20-Hydroxyecdysone – R=OH


OH
21
22
25
26
R
27
18
OH
11
19
14
HO
2
OH
3
6
HO
O

& Kater, 1973 on Hemigrapsus and Willig & Keller, 1976 on Orconectes limosus). The evidence, however, was equivocal, being based on the product of the Y-organs having chromatographic properties similar to those of ecdysone. The first unequivocal evidence was obtained by Chang & O'Connor (1977). These workers cultured the Y-organs of Pachygrapsus crassipes and Cancer antennarius. The Y-organ secretory product from the culture medium was purified by chromatography and analyzed by mass spectrometry. The spectrum obtained was identical to that of authentic ecdysone. Moreover, ecdysone was the only moulting hormone secreted by the Y-organs of these crab species.

20-hydroxyecdysone is generally considered to be the true arthropod moulting hormone rather than ecdysone. This contention is based on several lines of evidence. First, 20-hydroxyecdysone is more active than ecdysone in promoting ecdysis (Rao et al., 1972; cf. Karlson & Koolman, 1976). Secondly, extractions of ecdysteroids yield more 20-hydroxyecdysone than ecdysone (Chang et al., 1976; Gagosian et al., 1974; Gagosian & Bourbonniere, 1976; Galbraith et al., 1968 and Horn, 1971). Furthermore, ecdysone is not stored by the Y-gland, but is released into the haemolymph (Kleinholz & Keller, 1976) and subsequently hydroxylated to 20-hydroxyecdysone (King & Siddall, 1969; Highnam & Hill, 1979). However, since ecdysone is the substance produced and released by the Y-organ, it can be argued (Karlson & Koolman, 1976) that ecdysone is the true moulting hormone and this is converted to the active molecule (20-hydroxyecdysone) by the target tissue(s).

Cholesterol is the presumed C27 precursor to ecdysteroids for arthropods generally (c.f. Thompson, et al., 1972; Spaziani & Kater, 1973). In common with insects (Thompson et al., 1972), crustaceans do not possess the mechanism for de novo biosynthesis of sterols (Morris & Culkin, 1977). Therefore, in order to synthesize ecdysterones along with the other sterols

necessary for normal physiology, crustaceans must obtain a dietary source of sterols. Carnivorous crustaceans may be able to obtain all the cholesterol they require from their diet. On the other hand, since cholesterol is not very abundant in algae, herbivorous crustaceans would require a metabolic pathway for converting C28 and C29 algal sterols into cholesterol. Such a pathway appears to be present in crustaceans (Kanazawa, et al., 1971; Teshima, 1971a, b; Teshima & Kanazawa, 1971a, b; 1972). Furthermore, Teshima & Kanazawa (1973) demonstrated the ability of a penaiid prawn to convert desmosterol to cholesterol and they further postulated that the bioconversion of C28 and C29 sterols in crustaceans probably follows the same route as that found in insects (Fig. 4) (Svoboda & Robbins, 1968; Thompson et al., 1972).

The next step, the conversion of cholesterol to ecdysone, is not completely known, although it is believed to proceed through a series of hydroxylation reactions (Bollenbacher et al., 1978). The evidence for the biosynthetic scheme of events has been derived mainly from in vivo studies on insects (Gilbert et al., 1977; Sakurai et al., 1977), although in vitro studies have been employed. The most recent of these studies (Hetru et al., 1982) utilized the ovaries of adult Locusta migratoria and resulted in the proposed scheme of ecdysone biosynthesis illustrated in figure 5.

At present there is no evidence that the above scheme is applicable to crustaceans. However, the synthesis of cholesterol and the conversion of ecdysone to 20-hydroxyecdysone (see below) appear to be similar processes in both insects and crustaceans. Logically, therefore, the biosynthesis of ecdysone by these two arthropod classes can be assumed to be the same. A mass spectroscopic study of the steroids from the Y-organs of crustaceans would be illuminating in this sense, for the presence of intermediates from the above scheme of ecdysone synthesis would go some way towards

confirming the mutuality of the biosynthetic pathway in insects and crustaceans.

Horn et al., (1966) were the first to suggest that ecdysone is the precursor to 20-hydroxyecdysone. Evidence has since been gained to support this contention. King & Siddall (1969) have shown that Crangon nigricauda and Uca pugilator were able to convert injected ^{3}H ecdysone into a labelled compound which was identified as 20-hydroxyecdysone on the basis of its TLC characteristics. Similar results were obtained by monitoring the label incorporated from ^{14}C-cholesterol into the ecdysteroids from the Y-organs of Hemigrapsus nudis (Spaziani & Kater, 1973) and Orconectes limosus (Willig & Keller, 1976). Although again the labelled hormones were identified on the basis of their chromatographic characteristics and so the conclusions drawn from these experiments remained tentative. The results obtained by Gagosian et al., (1974) are more convincing. (4-^{14}C) cholesterol was injected into the blood sinus of female premoult Homarus americanus. After 16 hours the muscle and viscera were extracted for ecdysteroids and the extracts purified by TLC. Three radioactive spots were obtained, one of which co-chromatographed with 20-hydroxyecdysone. Furthermore, acetylation of this product gave 4 radioactive products which co-chromatographed with 4 products obtained when authentic 20-hydroxyecdysone was acetylated. By the use of GC/MS, these workers verified that 20-hydroxyecdysone was present in extracts of freshly moulted female lobsters, and to a concentration of 3 µg per 500g female lobster (wet weight).

More recently it has been shown that crustacean tissues can convert ecdysone to 20-hydroxyecdysone in vitro. Chang et al., (1976) examined this ability in several tissues of the crab Pachygrapsus crassipes. The testes were found to have the greatest capacity to convert ^{3}H ecdysone to 20-hydroxyecdysone. The hepatopancreas and muscle also displayed a capacity for such conversion, but to a lesser extent. In contrast,

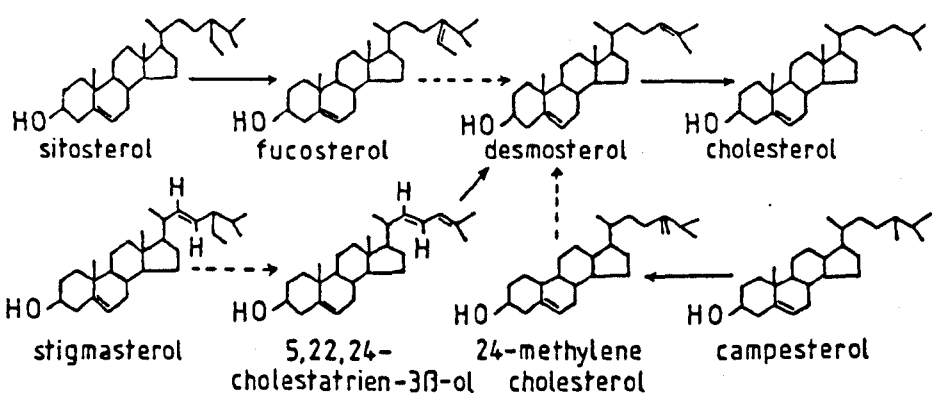
Fig. 4. Conversion of sitosterol, stigmasterol and campesterol to cholesterol in insects.

(After Thompson et al., 1972)

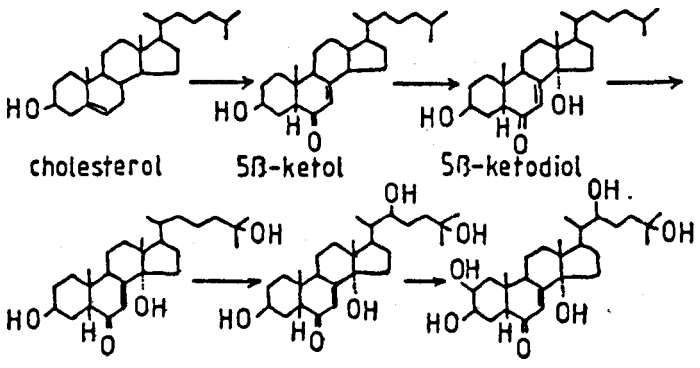
Fig. 5. Possible pathway for ecdysone biosynthesis in insects.

(After Hetru et al., 1982)

4.


HO
sitosterol
HO
fucosterol
HO
desmosterol
HO
cholesterol
H
H
HO
stigmasterol
H
H
HO
5,22,24-
cholestatrien-3ß-ol
H
HO
24-methylene
cholesterol
HO
campesterol

5.


HO
cholesterol
HO
H
O
5ß-ketol
HO
H
O
OH
5ß-ketodiol
OH
OH
HO
OH
H
O
2,22-dideoxyecdysone
OH
OH
HO
OH
H
O
2-deoxyecdysone
OH
OH
OH
HO
OH
H
O
ecdysone

the ovaries, embryos, Y-organs and sperm did not convert the radio-labelled ecdysone.

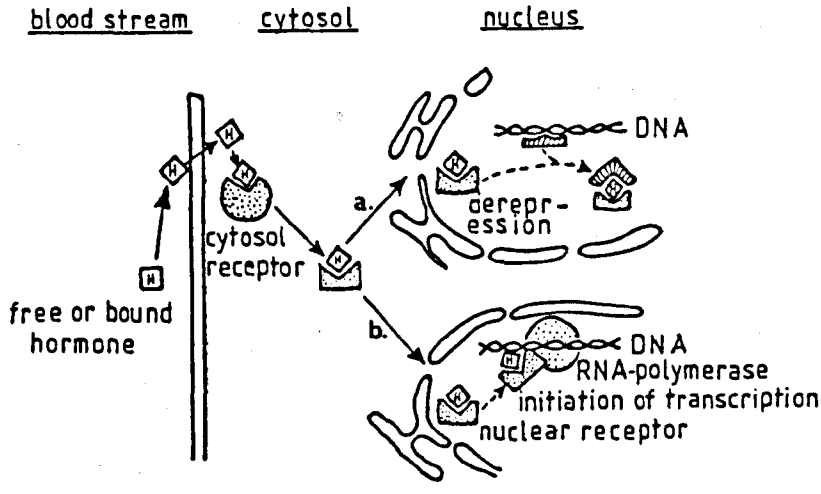
Chang et al. (1976) also reported an important feature of the circulating ecdysteroids, namely, they are not bound to carrier proteins. This has previously been suggested for insects (Chino et al., 1970), although somewhat equivocal evidence to the contrary has been presented (Emmerich, 1970a, b), and has since been confirmed for crustaceans by Kuppert et al., (1978). If ecdysteroids do circulate in the unbound state then this would be contrary to the situation for steroid hormones in mammals. In mammals, liposoluble steroids are transported by carrier proteins. These plasma proteins, such as the high affinity glycoproteins and low affinity albumin, taken together can constitute a binding capacity of between 85 and 98% of the steroids present. (Westphal, 1971; Giorgi, 1980). There is considerable evidence to support a permeability barrier to bound steroids at the membrane level (cf. Giorgi, 1980). Here may be the reason for ecdysteroids circulating in the free state in arthropods, whereas the converse is true of mammalian steroids. Mammalian steroid hormones such as oestrogen and progesterone are involved in the control of long term events, in this case pregnancy. A high level of hormone is maintained in the circulation and regulation of cellular hormone levels is exerted at the plasma membrane. This degree of control is presumably unnecessary in terms of the comparatively short term events in which ecdysteroids are involved. For example the ecdysteroid titre during the moulting process of crustaceans remain low except for a peak just prior to ecdysis (see below). Since there is no barrier to the transport of free steroids into animal cells, the control of cellular ecdysteroid levels in arthropods occurs at the sites of production and metabolism of the ecdysteroid precursors. From the above discussion it would be supposed that ecdysteroids enter target cell rapidly from the circulation. The results of Kuppert et al., (1978a) support this view. Uptake of

radiolabelled ecdysteroids by tissues of the crayfish Orconectes limosus maintained at 20-22°C was found to be maximal at approximately half an hour following injection. It is worth noting that both ecdysone and 20-hydroxyecdysone are bound by the tissues of Orconectes. Since 60-70% of the injected (^{3}H) ecdysone was rapidly excreted by the lobster, a feature not noted for (^{3}H) 20-hydroxyecdysone, it is not surprising that the former was bound to a lesser extent. Of equal importance is the site of uptake of the ecdysteroids. The study conclusively demonstrated that different organs do not display the same capacity to bind ecdysteroids. The hypodermis bound 20-hydroxyecdysone to the greatest extent and the male gonads were also important in this respect. In contrast, (^{3}H)-ecdysone was bound most effectively by the male gonads (c.f. Chang et al., 1976); the binding capacity of the hypodermis being slightly less. That both hormones are bound by tissues tends to support the views of Karlson & Koolman (1976), namely, that ecdysone is not merely a precursor of 20-hydroxyecdysone, but that both compounds are hormones with different biological functions.

Kuppert et al., (1978b) extended the above study to see if the binding of the ecdysteroids was by specific cytoplasmic receptors. In vitro assays were performed and these utilized a range of tissues from O. limosus. It was found that most of the binding occurred in the cytosol. The binding was specific, of high affinity and the binding sites could be fully occupied by both hormones and thereby fitted the criteria for mammalian steroid receptors. Furthermore, the binding molecules were proteins and the association between the ecdysteroids and receptors was rapid being maximal between 10 and 20 min at 5 to 32°C. Here again the crustacean steroid receptors have properties analogous to their mammalian counterparts. In the mammalian system the target tissues contain protein receptors which specifically bind the steroid hormones. It is thought (Karlson & Koolman, 1976) that the steroid hormone-protein complex acts

Fig. 6. Alternative models (a, b) for the control of transcription within a cell.

(Modified from Karlson et al., 1976)


blood stream
cytosol
nucleus
DNA
a.
derepr-
ession
cytosol
receptor
free or bound
hormone
b.
DNA
RNA-polymerase
initiation of transcription
nuclear receptor

at the level of the chromatin in the cell nucleus and not the steroid hormone itself. The mechanism of action of the complex is not fully understood but RNA synthesis appears to be stimulated (Karlson & Peters, 1965) and this synthesis is biased towards the production of mRNA (cf. Sekeris, 1974). Two models have been proposed to explain how transcription is induced (Karlson et al., 1976) and these are illustrated in Fig. 6. In the first model, (a) transcription is blocked by a repressor protein which covers the site of initiation (c.f. Burdon, 1976; promotor region) of transcription of the DNA template. In the other model (b), the steroid hormone-protein complex binds to the promotor region of the DNA and possibly to RNA polymerase, thereby initiating transcription.

Nuclear receptors have been demonstrated in crustacean tissues (Kuppert et al., 1978; Spindler-Barth, et al., 1980; Londershausen & Spindler, 1981; Kuppert & Spindler, 1982). The hypodermis of the crayfish, Orconectes limosus, has been used to obtain isolated nuclei (Spindler-Barth, et al., 1980). The binding sites within these nuclei had a high affinity to (^{3}H)-ecdysteroids. Enzymatic digestion experiments indicated that the nuclear receptor(s) was a protein and furthermore, since a reduction of binding results from a partial reduction in the DNA content of the nuclei, the receptor was bound to the chromatin (Kuppert & Spindler, 1982). There are several lines of evidence to suggest that ecdysone and 20-hydroxyecdysone are bound by different receptors. It was found, using (^{3}H)-ecdysone as the radio ligand, that ecdysone was better able to compete for binding sites with the radio ligand than was 20-hydroxyecdysone. The reverse was true when (^{3}H)-20-hydroxyecdysone was used as the radio ligand (Kuppert & Spindler, 1982). In addition, Spindler-Barth et al., (1980) found that when O. limosus was injected with (^{3}H)-ecdysone, they were able to extract both (^{3}H)-ecdysone and (^{3}H)-20-hydroxyecdysone from the nuclei.

More recently the cytoplasmic and nuclear ecdysteroid receptors

from the crayfish hypodermis have been characterized and compared (Londershausen & Spindler, 1981; Kuppert & Spindler, 1982; Londershausen et al., 1982). The two are thought to be identical or at least very similar. Both have a molecular mass of 70,000 Da as determined by sodium dodecyl sulphate-gel electrophoresis. The ligand specificity of the two receptor groups was also very similar, as were their dissociation constants (K_D). Furthermore, dodecyl sulphate-electrophoresis and isoelectric focussing have demonstrated that the ecdysteroids are covalently bound to the receptors. The sulphydryl groups of the receptor proteins are important in this respect.

Having discussed the biosynthesis and mode of action of ecdysteroids the remainder of this section will relate the rôles of these hormones in the control of crustacean physiological events.

The control of the moult cycle of crustaceans has received most attention and consequently is the best understood of these processes wherein ecdysteroids exert their effect. Before addressing this problem, a brief description of the moult cycle is necessary (see Table 7 for summary). Most studies on crustacean moulting have employed the alphabetical subdivisions of the moult cycle as described by Drach (1939) for decapods or slightly modified versions of these (see Passano, 1960; Stevenson, et.al., 1968). The term premoult or proecdysis (stage D in the terminology of Drach) refers to that period in which the crustacean prepares for moulting. During this period the hypodermal cells and hepatopancreas are activated. The hypodermal cells separate from the cuticle (apolysis, Jenkin & Hinton, 1966), divide and start to secrete the new exoskeleton (D_2). At the same time calcium is resorped from the old exoskeleton (D_3) resulting in an increase in the level of calcium salts in the body glands. Storage of this excess calcium can occur in the hepatopancreas to be re-utilized as a partial source for the new

Table 7. The brachyuran moult cycle.+

Stage	Characteristics.
A1	Continued water absorption and initial mineralization.
A2	Exocuticle mineralization.
B1	Endocuticle secretion begins.
B2	Active endocuticle formation, chelae hard; tissue growth begins.
C1	Main tissue growth.
C2	Tissue growth continues.
C3	Completion of exoskeleton; membranous layer formed.
C4	"Intermoult"; major accumulation of organic reserves.
or	
C4T	Terminal stage in certain species; no further growth.
D0	Hypodermal and hepatopancreas activation.
D1	Epicuticle formed and spine formation begins.
D2	Exocuticle secretion begins.
D3	Major portion of skeletal resorption.
D4	Ecdysial sutures open.
E	Rapid water uptake and exuvation.

+ after Passano (1960).

exoskeleton (c.f. Gibson & Barker, 1979). The end of premoult (D4) is marked by the splitting of the old cuticle. Ecdysis (stage E) is a short period during which exuvation occurs and there is a rapid uptake of water. Postmoult or metecdysis (stages A, B and C1-C3) begins with the continued uptake of water and the initial mineralization of the new exoskeleton (A1). Stage A2 is the main period of mineralization, following which the endocuticle begins to be secreted (B1). Feeding which has not occurred since stage D2, recommences in stage B2. This stage also sees active endocuticle formation and the start of tissue growth. The bulk of tissue growth occurs during stage C1 and is completed in C2. Postmoult ends (stage C3) when the exoskeleton is fully formed. Stage C4, the intermoult period, is the longest period of the moult cycle. Feeding continues and those metabolites which are in excess of immediate requirements are stored in the hepatopancreas. The animal thereby prepares itself for the next moult.

As mentioned in section I, the consequence of a low moult-inhibiting hormone titre in Crustacea is to allow the Y-organs to secrete their moulting hormone, ecdysone. There appears to be general agreement in the literature that, in the Crustacea, the titre of circulating ecdysteroids is very low during stage C. Secretion of ecdysone results in an increasing hormone titre at stage Do which becomes maximal at stage D2 (see Willig & Keller, 1973; Hoarau & Hirn, 1978). During stage D3 the hormone titre falls rapidly to attain its lowest values after ecdysis (Hoarau & Hirn, 1978). It appears, therefore, that with regard to ecdysteroids, the moult cycle can be divided into two phases. The preecdysial phase, which is dependent on moulting hormone(s) and the postecdysial phase, which is independent of the hormone(s).

The most detailed study of the events occurring in the crustacean hypodermis during the moult cycle has been described by Stevenson (1972). Working on the crayfish Orconectes obscurus and O. sanborni he noted a

dramatic drop in DNA content of the cells at stage Do and a rise at stage A. The protein content of the hypodermis per unit weight DNA was also measured and this too was found to be lowest at stage Do. Mitosis occurs at stages Do and D1 (Tchernigovtzeff, 1959) as does cell enlargement (Travis, 1955; Dennell, 1960; Skinner, 1962; Stevenson et al., 1968) and it might reasonably be expected that there would be a concomitant increase in the DNA and protein content of the cells. The discrepancy can be explained in terms of absorption of water by the cells. The drop in DNA content may, therefore, not be real, but instead is the result of a dilution effect. The reported cell enlargement without an increase in protein content of the cells can also be explained by water absorption. Hypodermal protein synthesis, on the other hand, did not mirror protein content, since it did not vary significantly during postmoult. The catabolism of protein must, therefore, increase as the postmoult cycle progresses. Protein synthesis increased during premoult, in agreement with Skinner (1965) and McWhinnie & Mohrherr (1970). The haemolymph protein concentration of Crustaceans (c.f. Chaix et al., 1981) also rises during premoult and reaches a maximum value just before ecdysis. This increase in protein concentration exactly follows the ecdysteroid titre (Fig. 7) as is the case for the hypodermal concentrations.

The rise in ecdysteroid titre during premoult also matches the increase in chitin synthesis (Hornung & Stevenson, 1971; Gwinn & Stevenson, 1973a) and the increased production of RNA needed for such chitin synthesis (Keller & Adelung, 1970; Stevenson & Tung, 1971). Chitin, one of the main constituents of the new cuticle (Richards, 1951), is synthesized from glucose (Fig. 8). Using ^{14}C-glucose, Hornung & Stevenson (1971) were, thereby, able to monitor the changes in the rate of chitin biosynthesis during the moulting cycle. It was found that the rate of

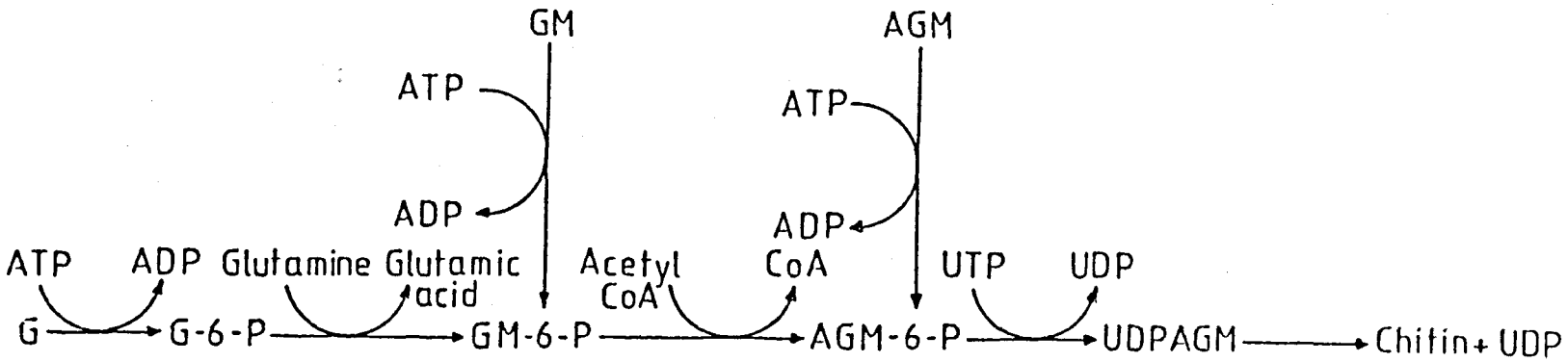
Fig. 7. Ecdysone titre (—●—) and protein concentration (-●-) in haemolymph during the moult cycle of Acanthonyx lunulatus. Vertical bars represent standard deviation calculated from 5-20 spider crabs at each moult stage. 1 mm on the abscissa represents one day of the moult cycle.

(After Chaix et al., 1981)

Ecdysone titre (p mole/100µl)
30
40
60
AB C2 C3 C4 D0 D1 D'1 D''1 D2 D3
40
80
140
protein (mg/ml)

Fig. 8. The pathway of chitin biosynthesis. The point of entry of acetylglucosamine is theoretical. G = glucose, Gm = glucosamine, AGM = acetyl-glucosamine, -6-P = 6-phosphate.

(After Stevenson, 1972)


GM
AGM
ATP
ATP
ADP
ADP
ATP
ADP
Glutamine
Glutamic acid
Acetyl CoA
CoA
UTP
UDP
G
G-6-P
GM-6-P
AGM-6-P
UDPAGM
Chitin + UDP

synthesis increased during late premoult (D3-E) to reach a peak at stage B. These results were to a certain extent in accord with the timing of cuticle synthesis. The latter begins at stage D2, (see above) and further, the cuticle is known to thicken fastest at stage B (Stevenson unpublished, cited by Stevenson, 1972). The discrepancy between the start of cuticle synthesis (D2) and that of chitin synthesis (D3) could be explained in terms of an alternate pathway to chitin synthesis. Acetylglucosamine (AGM) (see Fig. 8) was used as the precursor to chitin significantly earlier in premoult than was glucose. Furthermore, AGM could be utilized more efficiently than glucose. Thus, it is possible that during premoult, AGM derived from the breakdown of the old cuticle, is used in preference to glucose (Stevenson, 1972).

Actinomycin D is an RNA synthesis inhibitor. This compound was utilized by Stevenson & Tung (1971) to show that RNA synthesis is necessary for chitin biosynthesis, only when the latter is increasing in rate. It is noteworthy that the period during which the rate of chitin synthesis is increasing coincides with the period of increasing titre of moulting hormone (see above). Moreover, as has already been discussed, the action of ecdysterone is mediated by mRNA synthesis within the cell nucleus. A possible relationship between chitin biosynthesis and moulting hormone is therefore clear. Ecdysterone could initiate the transcription process resulting in the enzyme(s) necessary for the conversion of glucose to chitin. Indeed, Wittig & Stevenson (1974) demonstrated that ecdysterone significantly increased the activity of UDP-acetylglucosamine pyrophosphorylase; the enzyme which catalyzes the conversion of N-acetylglucosamine-1-phosphate and UTP to UDP-N-acetylglucosamine. More recently Armstrong & Stevenson (1979) looked at the effect of 20-hydroxyecdysone on ^{14}C-N-acetylglucosamine incorporation into chitin. The hormone was found to significantly increase the rate of chitin synthesis during the

moulting stages C3 to D1 and especially during the early premoult stages. In other words, 20-hydroxyecdysone was most effective "when both the endogenous hormone titre and the rate of chitin synthesis were low". There is good evidence, therefore, to suggest that 20-hydroxyecdysone directly activates the chitin biosynthetic pathway. However, the hormone seems to act specifically, since not all the enzymes of the pathway change activity with the moult cycle. Hexokinase is an example of such an enzyme (Gwinn & Stevenson, 1973b).

During late premoult in Crustacea, calcium is mobilized from the old cuticle and is in part stored for the calcification of the new cuticle (see above). There seems to be general agreement in the literature that the actual mobilization of calcium is not controlled by 20-hydroxyecdysone (Adelung, 1969; McWhinnie et al., 1972; Steel, 1977). Instead, 20-hydroxyecdysone may influence the reabsorption of the organic matrix of the cuticle and thereby its associated calcium content (McWhinnie et al., 1972; Highnam & Hill, 1977). The storage of calcium in such reserves as gastroliths, granules or sternal plates is probably controlled by the moulting hormone, but its subsequent mobilization is not (Steel, 1977).

The hepatopancreas of Crustacea is an organ of diverse function. In many ways it is analogous to the fat body of insects and the liver of vertebrates. McWhinnie et al. (1972) have reviewed the evidence implicating the hepatopancreas as a probable primary or secondary site of moulting hormone action. For example, the rate at which labelled leucine was incorporated in vitro into hepatopancreatic protein of Orconectes virilis increased during early premoult and decreased at late premoult (Gorell & Gilbert, 1971). Furthermore, injected 20-hydroxyecdysone was found to increase, in vitro, the amount of leucine incorporated into protein and uridine into RNA by the hepatopancreas (Gorell & Gilbert, 1969). Further evidence to implicate the hepatopancreas as a site of hormone action has been

provided by Gorrel et al., (1972b). A series of in vivo and in vitro experiments by these workers utilizing Sephadex chromatography and sucrose density gradient ultra-centrifugation revealed the presence of two proteins in the cytosol fraction of the hepatopancreas that bound label. Most important was the finding that labelled metabolite bound to these proteins was not ecdysone nor any ecdysone-like compounds described at that time. Two possible molecular structures were proposed by Gorell et al., (1972a, 1972b) to account for the ecdysone metabolite complexed to the receptor proteins. It was suggested that the complex may be the active form of the moulting hormone (Gorell & Gilbert, 1972b).

Recent evidence has been obtained to suggest that the crustacean larval moult is also under the control of an ecdysteroid. The control of moulting in the first and third stage larvae of the crab, Cancer anthonyi were investigated by McConaugha (1977; 1979). Zoea were exposed to varying concentrations of 20-hydroxyecdysone (0.1-0.4 µg/ml). Only those larvae in which the Y-organs were activated and were secreting responded to exogenous ecdysone, albeit in an abnormal way. McConaugha (1979) later identified the Y-organs of C. anthonyi at all six larval stages. The organs showed increasing complexity as larval development proceeded and, furthermore, cyclic activity of the glands was noted which corresponds with the larval moulting cycle.

McConaugha (1977, 1979) was unable to demonstrate a shortening of the moult cycle of crab larvae and this was attributed to an insufficient amount of hormone entering the larvae to raise the titre above early pre-moult levels. Experiments on the first and fourth stage zoea of Rhithropanopeus harrisii utilized higher (1 & 5 µg/ml) 20-hydroxyecdysone concentrations (McConaugha & Costlow, 1981). Consequently a significant decrease in the duration of the moult cycle of both larval stages was observed. Moreover, the larval response to the hormone was dose dependent.

The most conclusive evidence of ecdysteroid control of the moulting cycle was obtained by Chang & Bruce (1981). These workers measured the ecdysteroid titres of the first three larval stages of the lobster, Homarus americanus, throughout the moult cycle, by means of radio-immunoassay. At each stage a peak concentration of approximately 2.6 x 10^{-6}M was obtained and this peak occurred about midway through each stage. On the basis of chromatographic evidence, 20-hydroxyecdysone could account solely for the peak in hormone titre. This finding is consistent with the isolation of 20-hydroxyecdysone as the only moulting hormone from adult H. americanus (Gagosian et al., 1974).

There is controversy in the literature regarding the relationship between ecdysis and copulation in crustaceans. Generally speaking, two different views are held. According to Dunham & Skinner-Jacobs (1978) who worked on the lobster, Homarus americanus, mating can occur quite frequently involving females at least three weeks past moulting. This result contradicted the previously held view that mating in the American lobster is restricted to a 24 hour period immediately following the female moult (Templeman, 1934). The results of Templeman are upheld by most workers in the field, but the most unequivocal evidence of a link between a moult and subsequent mating has been presented by Atema et al., (1979). These workers, who stated that the studies of Dunham & Skinner-Jacobs (1978) were "statistically significant but biologically artificial", used large aquaria to observe lobsters under conditions designed to closely resemble those experienced by the animals in the natural environment. As a result, pair bonding was found to occur as much as seven days before moulting and such bonding appeared to involve both behavioural displays and a sex pheromone. Mating took place at approximately one half hour following the female moult.

The dose relationship between ecdysis and mating was the basis for

investigating the possibility that 20-hydroxyecdysone could have a dual function. In addition to being the crustacean moulting hormone it might also act as a sex pheromone and elicit a copulatory response in the male. This theory was first propounded by Kittredge & Takahashi (1972) and the evidence upon which it was based came largely from their earlier work on male crabs (Kittredge et al., 1971). In these experiments, 20-hydroxyecdysone was judged to elicit courtship displays in the males. However, these views were premature and the criticisms raised by Dunham (1978) are valid. It is possible that the responses observed may have been agonistic and/or feeding related.

Atema & Gagosian (1973) tested the 20-hydroxyecdysone hypothesis using male H. americanus. It was found that neither this ecdysteroid nor any of the other ecdysteroids tested (inokosterone, ponasterone A and cyasterone) could induce courtship behaviour. Subsequent experiments using metabolites of 20-hydroxyecdysone, or structurally similar compounds, gave the same result. It appears, therefore, that neither 20-hydroxy-ecdysone or its metabolites function as the sex pheromone in crustaceans.†

Ecdysteroids appear to be necessary for both oogonial (Arvy et al., 1954; Demeusy, 1962) and spermatogonial proliferation (Arvy et al., 1956) in crustaceans. In contrast, vitellogenesis and spermiogenesis, which do not require mitoses, can proceed in certain crustaceans in the absence of moulting hormone (Charniaux-Cotton & Kleinholz, 1964). For example, the Y-organs of Carcinus maenas regress after the pubertal moult (terminal moult). Such crabs are, however, capable of ovulating and carrying embryos (Arvy et al., 1954; Passano, 1960). It is pertinent in this

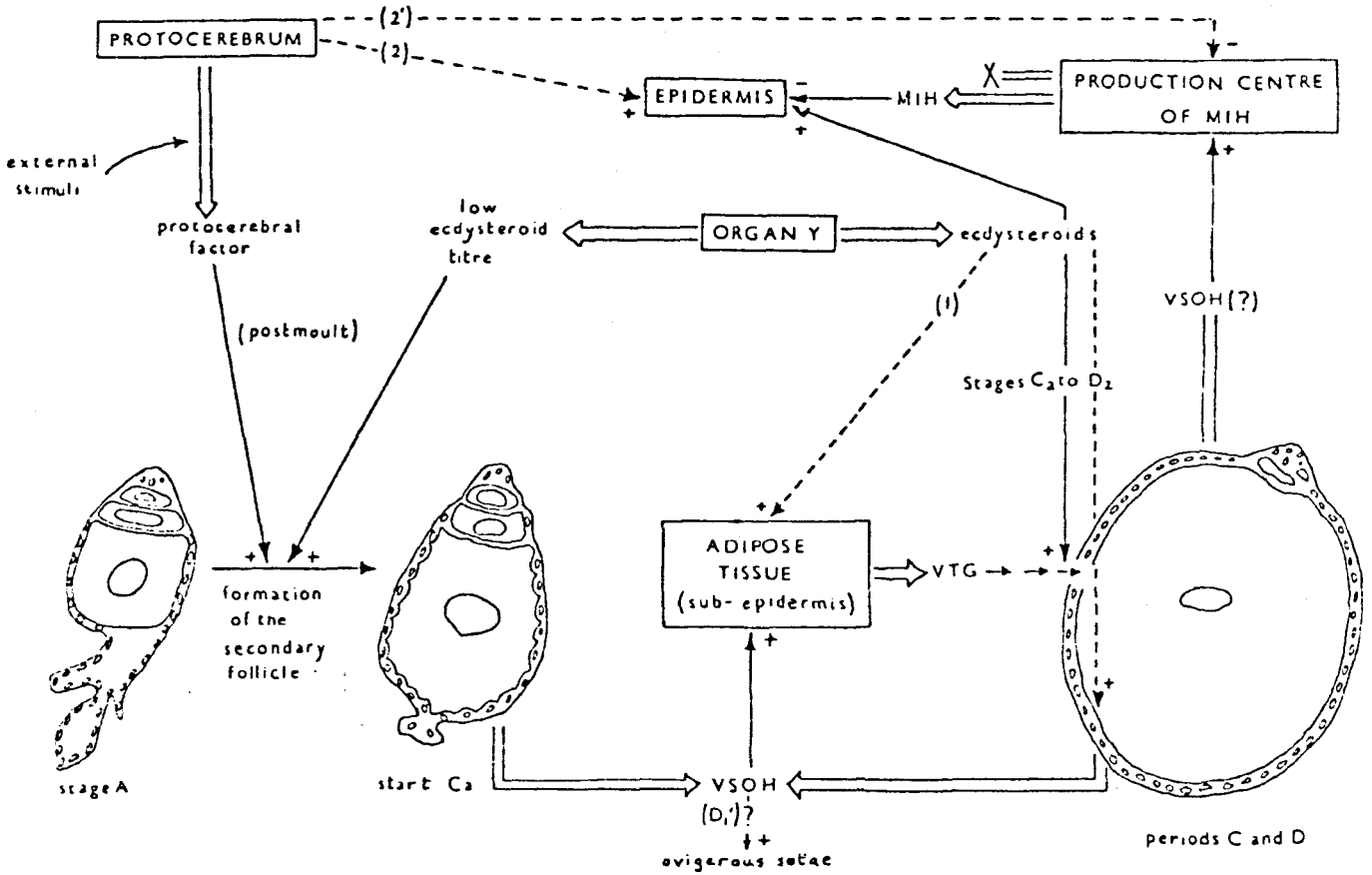
---

†The exact nature of the crustacean sex pheromone has not been elucidated to date, but it is believed to comprise some component of urine released from the female antennal glands (c.f. Lyes, 1979; Bauer, 1979; Gleeson, 1980).

respect, that ecdysteroids have been detected in the ovary of C. maenas (Lachaise & Hoffman, 1977; Lachaise, 1981) as they also have in the spider crab Acanthenyx lunulatus (Chaix & DeReggi, 1982). In the latter, the titres of ecdysone and 20-hydroxyecdysone have been found to show two peaks during oogenesis. One at the beginning of vitellogenesis and another as the oocytes reach maturity. At the same time, the ecdysteroids are absent from the haemolymph. The ecdysteroid titre of the ovary of C. maenas during oogenesis showed similarities to that of A. limulatus. The titre increased during vitellogenesis, whereas the blood titre remained low at less than one tenth of the minimal concentration found in the ovaries (Lachaise & Hoffman, 1977).

Of those crustaceans in which vitellogenesis requires ecdysteroids, the Isopoda and Amphipoda have received most attention. In the amphipod, Orchestia gammarellus, the maturation of the ovaries is closely associated with the moult cycle. Mathieu-Capderou (1980), by manipulating the timing of ecdysis, was able to demonstrate that the two events remained simultaneous when ecdysis was advanced or delayed. Moreover, the ovary did not mature when ecdysis was blocked by cauterizing the Y-organ. The ovaries of O. gammarellus have, in fact, been shown to contain ecdysteroids throughout their development. The ecdysteroid titre (as measured by radioimmunoassay) was found to vary little during stage D_1 (20-30 pg/ovary), but increased during the final period of vitellogenesis in D_2 (70 pg/ovary - this represents 3% of the total ecdysteroids). This increase was in synchrony with the peak in the ecdysteroid titre of the tissues and circulation (Blanchet et al., 1979). Since the ovary of O. gammarellus is dependent on ecdysteroids from the Y-organ for vitellogenesis, it would appear that the ovarian ecdysteroid titre is insufficient to fulfil this rôle, unlike that of C. maenas and A. limulatus. In this recent paper of Blanchet-Tournier (1982) a mechanism is proposed whereby the processes of moulting and vitellogenesis in O. gammarellus are hormonally coordinated (Fig. 9).

Fig. 9. Schematic diagram illustrating interactions between the organs controlling the moult and the vitellogenic ovary in the mature female of Orchestia gammarellus. ⟹ production; ⟶ stimulation (+) or inhibition (-); ------> hypothetical stimulation (+) or inhibition (-). V.S.O.H., vitellogenin stimulating ovarian hormone; VTG, vitellogenin; M.I.H., moult inhibiting hormone. Stages A, Ca ..... stages of the intermoult. After Blanchet-Tournier, M.-F. 1982.


PROTOCEREBRUM
(2')
(2)
EPIDERMIS
MIH
PRODUCTION CENTRE OF MIH
external stimuli
protocerebral factor
low ecdysteroid titre
ORGAN Y
ecdysteroids
(1)
VSOH (?)
(postmoult)
Stages C2 to D2
ADIPOSE TISSUE (sub- epidermis)
VTG
formation of the secondary follicle
stage A
start C2
VSOH (D1')?
ovigerous setae
periods C and D

Secondary vitellogenesis occurs after folliculogenesis in post moult under the control of a protocerebral hormone and a weak ecdysteroid titre. The ovary then produces vitellogenin stimulating ovarian hormone (V.S.O.H.), possibly under the control of the Y-organs, which stimulates the adipose tissue to produce the protein, vitellogenin. Vitellogenesis continues during stages C and D of the moult cycle and requires a continuous source of vitellogenin and the capture of this protein by the oocytes; vitellogenin enters the oocytes under the control of ecdysteroids from the Y-organ. The ovary, by releasing V.S.O.H., also stimulates the production of moult-inhibiting hormone; moult-inhibiting hormone, which is antagonistic to moulting hormone at the level of the hypodermis, lengthens the time to ecdysis.

In the female isopod, Porcellio dilatatus, the Y-organs are indispensable for the maintenance of vitellogenesis. Secondary vitellogenesis is interrupted by the removal of the Y-organs and can be re-established by injecting ecdysone (Besse & Maissiat, 1971; Besse, 1976). The effect of the lowered ecdysteroid titre is to reduce vitellogenin release into the haemolymph; 20-hydroxyecdysone stimulates this release (Souty et al., 1982) from the hepatopancreas (Picaud & Souty, 1980).

SECTION IV

Ecdysteroids in cirripedes

The Cirripedia conform with what is apparently the rule for Arthropoda in their inability to synthesize essential sterols. Desmosterol, the precursor of cholesterol, was isolated from the barnacle Balanus glandula by Fagerlund & Idler (1957). This sterol comprised 34.2% of the total sterols of the barnacle. Cholesterol (59.8%) comprised the bulk of the remaining sterols, as is the case for crustaceans in general. Whitney (1970) ratified that desmosterol is an important sterol in cirripedes; he found that desmosterol constituted 42% of the total sterols of B. nubilus tissues. Prior to 1970 demosterol had not been reported in marine invertebrates other than barnacles. Whitney (1970) injected (1-^{14}C) acetate and (2-^{14}C) mevalonate into the coelomic space of B. nubilus and found that the label was not incorporated into the squalene or sterol fractions of this barnacle. B. nubilus is, therefore, unable to synthesize sterols and a dietary source is required. The high levels of desmosterol found in barnacles is not a common feature of Crustacea and may reflect differences in diet. Larvae of the crab, Callinectes sapidus, for example, do not survive beyond the second zoeal stage when reared on a purely algal diet (Hopkins, 1944; Sandoz & Rogers, 1944). This result coupled with the fact that cholesterol comprises almost the entire sterol fraction of crabs suggests that sterols are obtained from an animal source. In contrast, barnacle larvae can be reared solely on a microalgal diet. They may, therefore, have a metabolic pathway, in common with herbivorous crustaceans, for converting C28 and C29 algal sterols into cholesterol (Morris & Culkin, 1977). Desmosterol is the intermediate sterol in this bioconversion (Thompson et al., 1972).

Although there is no direct evidence to date, it is reasonable to assume that the biosynthesis of moulting hormones (ecdysteroids) in barnacles conforms to that outlined in the previous section for the Crustacea. Tighe-Ford (1977) reports a personal communication from

Carlisle concerning what appears to be the first indication that ecdysteroids are present in barnacles. By injecting extracts of adult Elminius modestus into Y-organ-ablated Carcinus maenas, Carlisle was able to induce a resumption of moulting by the crabs. A similar response was obtained with ecdysteroid extracts from other arthropods (Carlisle, 1965). Ecdysteroids have since been implicated in the control of several physiological processes in barnacles. Understandably, the hormonal control of the moulting cycle of barnacles has received most attention. Tighe-Ford & Vaile (1972) using a micro-injection technique previously developed for barnacles (Tighe-Ford, 1968) were able to demonstrate that 20-hydroxyecdysone increased the moulting activity of adult B. balanoides. 2 µl doses of 0.02 and 0.2 µg 20-hydroxyecdysone/animal were employed, the latter being injected as a single dose in one group of animals and sequentially over 4 days (0.05 µg per day) in another. Two control groups were used. One group received 2 µl sterile sea water per day for 4 days, the other group remained untreated. All the injection regimes resulted in an acceleration of moulting compared to the untreated controls. Three cycles of activity were observed for the sequentially injected groups. The response to the single injection of 0.2 µg 20-hydroxyecdysone was more rapid, but only two cycles of accelerated activity were noted. The differences in response between the singly and sequentially injected groups were explained in terms of the former being an unnaturally high hormone content within the animals and the initially slower, but similar response to sequential injections reflected the physiological action of the hormone. It is noteworthy that the sea water injected barnacles exhibited an increase in moulting activity compared to the untreated group. This response was apparently due to the wounding of the animal (Tighe-Ford & Vaile, 1972; Tighe-Ford, 1974). Additional consequences of the wounding were the presence of dense wound tissue around the drill holes and calcification which extended from the shell plate and developed as a layer around

the wound tissue. Apparently, increased moulting activity and calcification are the healing response to wounding. Since calcification in barnacles appears to be correlated with the moult cycle (Bocquet-Védrine, 1965), it is plausible that the two processes are under similar endocrine control (Tighe-Ford & Vaile, 1972).

Tighe-Ford (1974) reported further evidence for the involvement of 20-hydroxyecdysone in the moulting cycle of B. balanoides. Using comparatively low doses of hormone, he established that the injected dose of 20-hydroxyecdysone required to successfully induce moulting lay in the range 0.005-0.01 µg/animal, i.e. a possible minimum of 0.05 µg/g, assuming a tissue wet weight of 0.1g. Moreover, an accidental dose of 0.0125 µg/g resulted in some stimulation of moulting. Such results indicate that the sensitivity of B. balanoides to 20-hydroxyecdysone equates with that reported for other arthropods (see previous section). Tighe-Ford (ibid) also assayed extracts, supplied by Bebbington (see Bebbington & Morgan, 1977 for extraction procedure), of 150 Kg of adult B. balanoides for moulting hormone activity. The test animals received 2 µl injections of extract solutions containing 2.5 "barnacle equivalents". All the extracts tested were found to stimulate moulting in relation to the control animals and so provided evidence of the presence of moult-stimulating substance(s) in barnacles. The nature of these extracts was later determined by Bebbington & Morgan (1977). These workers extracted 1500 Kg of adult B. balanoides (with slight contamination by Elminius modestus) for ecdysteroids. Two ecdysteroids were identified and quantified from the extract, namely, 20-hydroxyecdysone (1.4 mg) and ecdysone (8 µg). This constituted the first report of the presence of ecdysone in a crustacean.

The mode of action of ecdysteroids in the moulting and reproductive processes of barnacles has been studied using in vitro culture techniques. The successful maintenance of isolated appendages (Cheung, 1973; Cheung & Nigrelli, 1974) and explants of barnacle tissue (Fyhn, et al., 1977;

Freeman & Costlow, 1979; Freeman, 1980) in culture media for several days has been instrumental in this approach. By employing such methods, a substance(s) has been shown to be present in the body fluids of Balanus eburneus with moult-stimulating properties (Cheung, 1973). The moulting hormone was not, however, present in a sufficient titre to stimulate ecdysis throughout the moult cycle. Fluid from barnacles undergoing ecdysis and from 24-48 hours post-moult animals did not induce a moulting response in isolated appendages after 11 days of culture. However, there were signs of moulting when appendages were cultured for 5-6 days in fluid from barnacles at 48-72 and 72-96 hours post-moult. The separation of the old cuticle from the hypodermal cells was noted, together with the formation of a thin layer of new cuticle. Cheung & Nigrelli (1974) using the same bioassay technique, obtained a moulting response using sea water solutions of ecdysterone. Although the levels of hormone used were physiologically very high (1 mg/20 ml), the results do indicate that 20-hydroxyecdysone is present in the body fluids of B. eburneus and furthermore, the titre of the hormone varies during the moulting cycle.

Freeman & Costlow (1979) demonstrated that the response of mantle tissue hypodermis to 20-hydroxyecdysone was dose- and time-dependent. Tissue explants from intermoult B. amphitrite, when cultured in 5-20 µg/ml 20-hydroxyecdysone, induced apolysis well within the 24 hour period required for the transition from intermoult to premoult in vivo. Moreover, ecdysone (10-20 µg/ml) was equally effective in promoting this response. Freeman (1980) extended the above study to demonstrate that chitinolytic activity in mantle hypodermis was stimulated by 20-hydroxyecdysone. Chitinolysis was measured in terms of release of ^{3}H-n-acetylglucosamine from the cuticle, which is effected by chitinase. It is not known if 20-hydroxyecdysone acts by stimulating the synthesis and secretion of chitinase or activates the previously formed enzyme.

Fyhn et al. (1977) employed similar in vitro culture techniques to elucidate the effect of 20-hydroxyecdysone on barnacle vitellogenesis. Pieces of mantle tissue from B. amphitrite and B. eburneus, comprising mantle parenchyma, ovarioles and cement glands were cultured in media containing 20-hydroxyecdysone at concentrations of 0.0001, 0.001, 0.01 and 1 µg/ml. The explants were incubated for 72 hr and after such time it was found that there was a decrease in the amount of yolk droplets of mature oocytes with increasing concentration of hormone. Oogonia were not affected by 20-hydroxyecdysone as was the case for the other tissues cultured. The tentative conclusion drawn from this experiment was that 20-hydroxyecdysone may have a gonad-inhibiting function in cirripedes.

The effect of 20-hydroxyecdysone on moulting and the development of ovary is significant in the light of the early literature on the control of these events. It is well established that constant illumination will inhibit the development of barnacle ovary (Barnes & Stone, 1972 & personal observation). Since constant illumination is also found to stimulate moulting in B. balanoides during winter/reproductive anecdysis (Tighe-Ford, 1974), it is possible that such a light regime results in either an increase in the moulting hormone titre of barnacles or a decrease in the level of moult inhibiting hormone or both. It should be noted that Barnes (1952), Costlow & Bookhout (1956) and Crisp & Patel (1960) were unable to show any effects of light upon moulting. However, as Tighe-Ford (1974) explained, these studies were performed on barnacles which were already moulting naturally.

The first moult after the period of anecdysis in B. balanoides is characterized by a cast which contains all the tissues of the penis (Crisp & Patel, 1958; 1960). 20-hydroxyecdysone has a marked effect on the nature of this moult (Tighe-Ford, 1974). The injection of 0.2 µg of 20-hydroxyecdysone into B. balanoides in reproductive anecdysis resulted

in a significant number of the exuviae possessing only the cuticle of the penis or only a portion of the penis tissue. The penis degenerates from the tip down and a transverse layer of cuticle delimits this breakdown and also marks the point of "abscission". In order for cuticle formation to occur at this point, there is a prerequisite that hypodermal cells migrate from the periphery of the penis. It is postulated that 20-hydroxyecdysone interferes with this process. Thus, the injection of 20-hydroxyecdysone before tissue degeneration has occurred, could stimulate hypodermal cells to produce new cuticle along the length of the penis, leading to the absence of tissue in the first exuviae. The partial loss of tissues at the first moult, on the other hand, may be explained in terms of the injection of 20-hydroxyecdysone into animals in which the tissues of the penis are already degenerating. In such cases ecdysterone may induce hypodermal cells to migrate beyond the limits of degeneration.

In cirripedes, copulation is accompanied by a moult (Barnes & Barnes, 1956; Crisp & Patel, 1958; Patel & Crisp, 1961), as appears to be the case in the majority of other Crustacea (see previous section). Contrary to the suggestion of Barnes & Barnes (1956), a moult does not occur after fertilization, but there is instead a period of anecdysis (see above). Moreover, in B. balanoides at least, there is some evidence that a moult precedes copulation (Patel & Crisp, 1961), for individuals which had recently moulted were better able to act as 'females' than those that had moulted more than a week previously. This relationship between breeding and moulting, together with reports that moulting hormones act as sex pheromones in crustacea (Kittredge et al., 1971; Kittredge & Takahashi, 1972) led Tighe-Ford (1974) to investigate if 20-hydroxyecdysone acts as a sex pheromone in B. balanoides. Individual animals were transferred to dishes containing 20 ml of sea water or 2.5×10^{-5}M 20-hydroxyecdysone in sea water (1 mg/100 ml), or to a flowing sea water system to which hormone

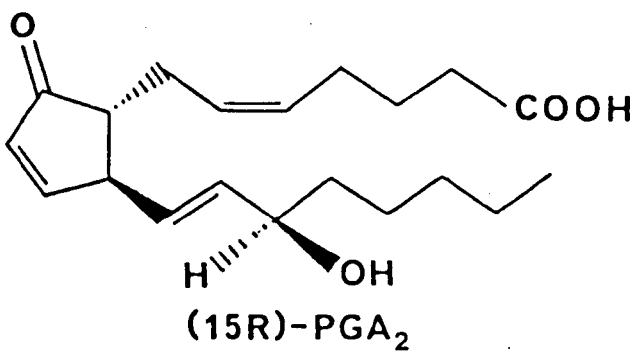
oncentrations ranging from about 10^{-5}M to an "infinite dilution" were administered. Activities of barnacles were followed for up to 2 hours, but in neither case was an effect of the hormone observed. Albeit that there is evidence of a sex pheromone in barnacles (Clegg, 1955; Walker, 1980), 20-hydroxyecdysone does not fulfil this rôle.

Based on the fact that liberation of young in barnacles is accompanied by a moult (Patel & Crisp, 1961), Tighe-Ford (1974) tested the effect of 20-hydroxyecdysone on gravid B. balanoides, to see if the hormone could be equated with the hatching substance of this species (Crisp, 1956; Barnes, 1957; Crisp & Spencer, 1958; Crisp, 1969). The result, however, proved negative and, unless the concentration of 20-hydroxyecdysone used (Ca. 2×10^{-5}M) was inappropriate, it can be assumed that 20-hydroxyecdysone is not the hatching substance.

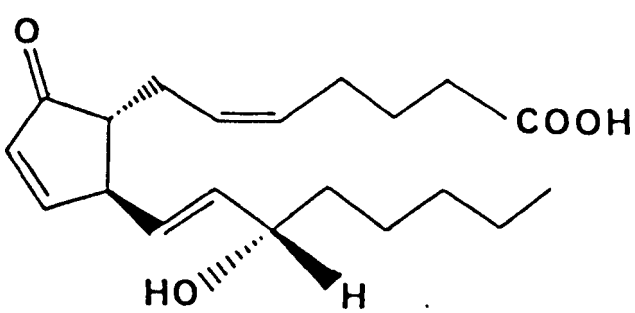
SECTION V

Prostaglandins in invertebrates

In 1969, Weinheimer & Spraggins reported for the first time, the presence of prostaglandins (PG's) in an invertebrate. The animal of notoriety, which later as a "major medical marine resource" had a symposium devoted to it (Bayer & Weinheimer, 1974), was the gorgonian Plexaura homomalla. Since that date evidence has accumulated for the presence of PG's in a number of invertebrate phyla (Table 8). Morse et al., (1978) made an extensive survey of the presence of the enzyme prostaglandin synthetase (a term used for a collection of enzymes) in corals from the Caribbean and Pacific. Apart from confirming the finding that P. homomalla is an exceptionally rich source of PG synthetase, Morse et al. (ibid) identified several other coelenterates with comparable biosynthetic activity. P. homomalla reportedly has prostaglandins of both the 15S and 15R configuration, i.e. the prostaglandins are epimeric at carbon 15, e.g.



(15R)-PGA_2



(15S)-PGA_2
(mammalian)

Table 8. Prostaglandin studies in invertebrates

	Species	Reference
COELENTERATA		
ANTHOZOA	*Plexaura homomalla*	Weinheimer & Spraggins 1969; Light & Samuelsson, 1972; Schneider, *et al.* 1972; Corey *et al.* 1973, 1975; Cao *et al.* 1975; Nomura & Ogata, 1976.
	Anthopleura midori	Nomura & Ogata, 1976.
	Anthoplexaura sp.	Christ & Van Dorp, 1972.
	Numerous spp.	Morse *et al.* 1978.
HYDROZOA	Numerous spp.	Morse *et al.* 1978.
MOLLUSCA	*Mytilus* spp.	Christ & Van Dorp,1972; Nomura & Ogata, 1976; Morse *et al.* 1977; 1978; Nomura *et al.* 1979.
	Haliotus spp. *Hinnites giganteus* *Crassostrea rhizophora*	Morse *et al.* 1977; 1978.
	Crassostrea gigas	Ono *et al.* 1982.
	Ligumia subrostrata	Graves & Dietz, 1979.
	Modiolus demissus	Freas, 1978; Freas & Grollman, 1980.
	Nordotis gigantea	Tanaka, 1978.
	Patinopecten yessoenis *Meretrix lusoria*	Nomura *et al.* 1979.
ECHINODERMATA	*Strongylocentrotus* spp. *Lytechinus* spp.	Morse unpublished
ANNELIDA	*Lumbricus* sp.	Christ & Van Dorp, 1972.
ARTHROPODA		
CRUSTACEA	*Homarus* sp.	Christ & Van Dorp, 1972.
	Cambarus clarkii *Portunus trituberculatus*	Nomura & Ogata, 1976.
	Euphausia superba	Mezykowski & Ignatowska-Switalska, 1981.
INSECTA	*Boophilus microplus*	Dickinson *et al.* 1976; Higgs *et al.* 1976.
	Acheta domesticus	Destephano *et al.* 1974, 1976; Destephano & Brady, 1977; Worthington *et al.* 1981.
	Hyalomma anatolicum excavatum	Shemesh *et al.* 1979.
	Bombyx mori	Setty & Ramaiah, 1979; 1980; 1982.
	Teleogryllus commodus	Loher, 1979; Loher *et al.* 1981; Stanley-Samuelson & Loher, 1983; Ganjian *et al.* 1981.
	Gryllus spp.	Worthington *et al.* 1981.
	Trichoplusia ni	Hagan & Brady, 1982.

Weinheimer & Spraggins (1969) found only (15R) - PGA_2 in their hexane extract of coral from the Caribbean. Also from the Caribbean, Corey et al., (1973) reported exclusively (15S) PGA_2 from their ethyl acetate extracts. Only one study reports the simultaneous extraction of both epimers from the same coral sample. Using P. homomalla from the Florida region and extracting with chloroform-methanol, Light & Samuelsson (1972) confirmed the findings of Weinheimer & Spraggins (1969), but in addition noted other (15R)- prostaglandins, notably (15R)- PGE_2, and small amounts of (15S)- prostaglandins. Assuming these differences in product are not a reflection of the various extraction procedures, and the various workers correctly identified the spp. used, we are left with the intriguing possibility that the same corals from different regions synthesize different prostaglandins.

Coral 15-epi-PGA_2 is unable to lower dog blood pressure unlike PGA_2 itself (Weinheimer & Spraggins, 1969). Surprisingly, the (15S) PGA_2 from P. homomalla may not be truly analogous with its mammalian counterpart. Corey, et al., (1973; 1975) have found that coral and mammalian (15S)-PGA_2 are synthesized via disparate pathways. Furthermore, the coral PG synthetase is not subject to inhibition by indomethacin and 5, 8, 11, 14- eicosatetraynoic acid, which are potent mammalian PG synthetase inhibitors.

The PG synthetases of the abalone Haliotus rufescens (Morse, et al., 1977) and the cricket Acheta domesticus (Destephano, et al., 1976) can be equated with that of P. homomalla since indomethacin is ineffective in blocking their action. However, indomethacin, which inhibits cyclooxygenase (an enzyme which catalyzes the first step in the conversion of arachidonic acid to the prostaglandin endoperoxides, PGG_2 and PGH_2) in mammalian systems (Vane, 1971), also blocks the action of the cyclooxygenase of Modiolus demissus (Freas & Grollman, 1980) and Bombyx mori (Yamajah Setty & Ramaiah, 1979). This apparent inconsistency of

action of indomethacin on invertebrate PG synthetases may be explained in terms of dissimilar drug levels used between the studies. For example, Corey et al., (1973) and Destephano et al., (1976) used indomethacin at a concentration of 2.5×10^{-5}M and 10^{-4}M respectively, whereas Freas & Grollman (1980) and Yamajah Setty & Ramaiah (1979) used the higher concentrations of 10^{-3}M and approximately 2.8×10^{-3}M respectively. Alternatively invertebrates may be capable of synthesizing PG's from arachidonic acid by more than one route.

Although it is now 13 years since Weinheimer and Spraggins presented their findings, there is still no known function for prostaglandins in P. homomalla. The situation for invertebrates in general is little better and reflects two fundamental problems in the research to date. First, most workers have merely been concerned in confirming the ubiquity of prostaglandins in invertebrates. Secondly, having gained evidence for potential rôles for specific prostaglandin compounds, the final step of extracting the known compound from the animal and testing its effect, has not been made.

Perhaps the best characterized rôle for prostaglandins in invertebrates is their involvement in the egg laying of the cricket Teleogryllus commodus (Loher et al., 1981). During mating the male transfers a spermatophore to the female's spermatheca. The spermatophore contains a PG-synthesizing complex which is in intimate association with the spermatozoa. The spermatheca of the female contains arachidonic acid, the precursor to 2-series prostaglandins. Mated females are thus able to synthesize prostaglandins. PGE_2 (tentatively identified by high pressure liquid chromatography) has been demonstrated in the spermothecae of such females, whereas there is a virtual absence of this prostaglandin in the spermathecae of virgins and in spermatophores. Furthermore, nanogram quantities of PGE_2, when injected into the oviduct of crickets, elicit egg release comparable with that observed following mating. A

similar study by Destephano & Brady (1977) also implicated prostaglandins in the oviposition of the cricket Acheta domesticus. By way of slight contrast to the situation in crickets, Setty & Remaiah (1980) have shown that oviposition is triggered in the female silkmoth Bombyx mori by the direct transfer of prostaglandins from the male during copulation, rather than by transferring the enzymes responsible for prostaglandin biosynthesis. The direct transfer of prostaglandins is analogous to the process in mammals, and in particular humans, where the semen contains at least 13 PG's (Embrey, 1975).

The cultured reproductive organs of the bovine tick Hyalomma anatolicum excavatum also contain prostaglandins, namely PGF and PGE_2 (Shemesh et al., 1971), but their function is uncertain. Their presence in the salivary glands, however, supports the contention of Tatchell & Moorhouse (1964) that pharmacologically active compounds are present in the saliva and that these cause an increase in flow of tissue fluids from the bovine capillaries. The probable agent responsible for the dilation and increased permeability of skin capillaries is PGE_2 (Dickinson et al., 1976).

There is evidence of two rôles for prostaglandins in molluscs to date. Morse et al., (1976, 1977) reported that hydrogen peroxide is able to induce spawning in gravid male and female abalones, Haliotus rufescens, and in male and female mussels Mytilus californianus. These observations were extended to include H. corrugata, H. fulgens, M. edulis, the purple-hinged rock scallop Hinnites giganteus and the mangrove oyster Crassostrea rhizophora (Morse et al., 1978) Hydrogen peroxide is thought to act via the stimulation of prostaglandin cycl - oxygenase. This enzyme catalyzes the first step in the conversion of arachidonic acid to endoperoxides. Aspirin which is known to inhibit the cyclooxygenase (Vane, 1971) also inhibits H_2O_2 indirection of spawning in the abalone. Furthermore, Morse and coworkers (1977) found

that prostaglandins E and F (unfractionated isomeric mixtures) were occasionally effective in inducing spawning in both male and female abalones.

The remaining proposed function for prostaglandins in molluscs is their involvement in the acclimation of animals to hyposmotic stress. Freas & Grollman (1980) demonstrated PGE_2 in the gill tissue of Modiolus demissus. When gill sections from different animals were incubated in hyposmotic sea water (25% water soln.), there was an increase in released prostaglandins (mainly PGE_2) from the tissue. The same stimulus also resulted in an increase in the synthesis of prostaglandins within the tissue. Similarly, ionic stimuli affected the release of prostaglandins from gill tissue. Magnesium-free, but not sodium-free, sea water was found to increase the quantity of prostaglandins released. Increasing either ion concentration did not have a significant affect on prostaglandin release compared to sea water controls. It should be noted that the observed increase in PG synthesis and release in hyposmotic saline could not be equated with reduced magnesium concentrations.

In freshwater bivalves the body fluids are hyperosmotic to the surrounding water with sodium being the principal cation. Graves & Dietz (1979) have extracted prostaglandin-like material from the blood of the freshwater mussel Ligumia subrostrata with T.L.C. characteristics indicative of PGE_2 and arachidonic acid. Both these compounds have been shown to affect sodium transport in this animal, by depressing sodium influx.

CHAPTER 2

The morphology and histochemistry of the central nervous system of the barnacles, *Balanus balanoides* (L.) and *Balanus hameri* (Ascanius) with some comparative observations on *Conchoderma auritum* (L.).

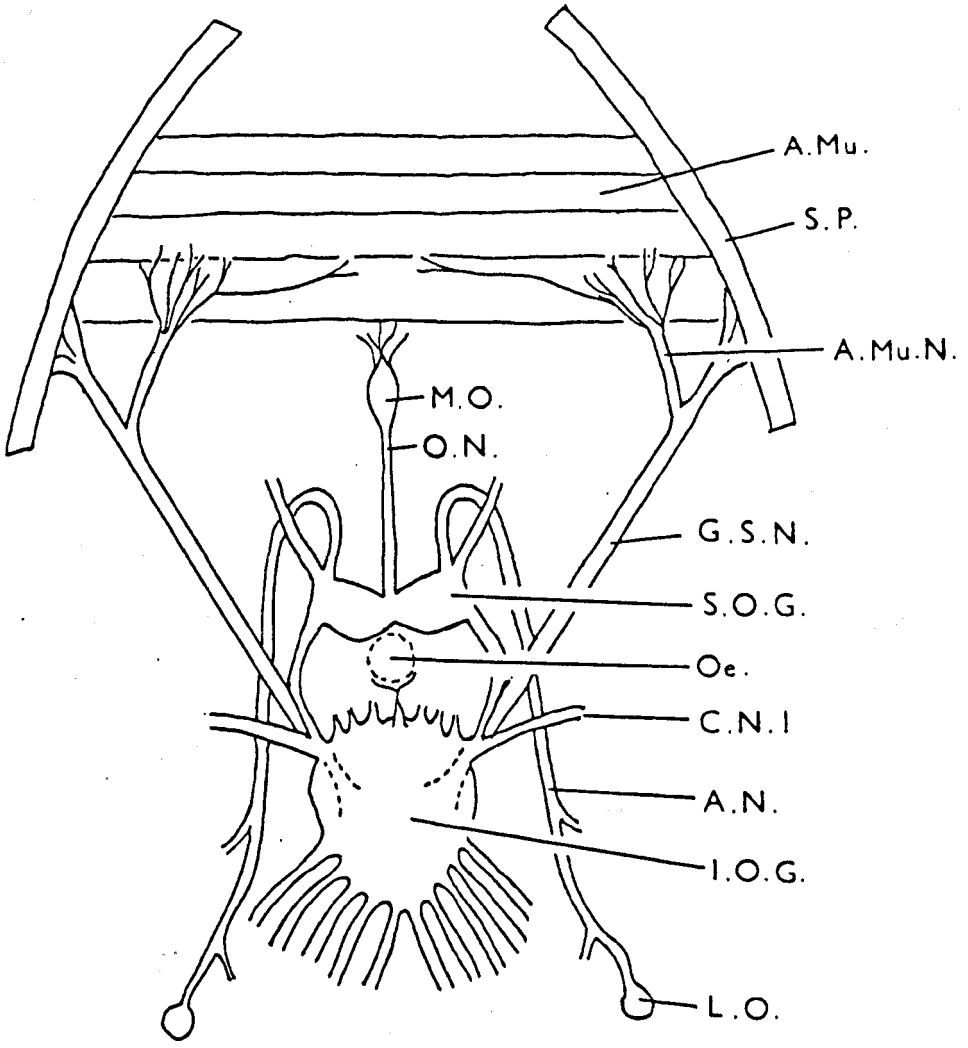
CHAPTER 2

The morphology and histochemistry of the central nervous system of the barnacles, Balanus balanoides (L.) and Balanus hameri (Ascanius) with some comparative observations on Conchoderma auritum (L.).

INTRODUCTION

There are very few reports in the literature regarding the detailed organisation of the nervous system of barnacles. Most of those which have appeared (e.g. Darwin, 1854; Gruvel, 1905; Sewell, 1926; Cornwall, 1936, 1953; Kruger, 1940; Batham, 1944; Cannon, 1947; Gwilliam, 1963, 1965, 1976; Stubbings, 1975) were concerned with gross anatomy. Only recently has the internal organisation of the ganglia been examined. The initial descriptions came from studies of the shadow reflex of barnacles (e.g. Millechia & Gwilliam, 1972; Lantz & Millechia, 1975; Gwilliam, 1976; Hudspeth & Stuart, 1977; Hudspeth et al., 1977), but were restricted to the sensory interneurones involved in this response. More comprehensive descriptions are those of Allison (1981) who studied the motorneurones of the infraoesophageal ganglion of B. hameri and Gwilliam and Cole (1979) who described the morphology of the central nervous system of B. cariosus. Some of the techniques used to study B. cariosus have also been employed in the present study and so the results of the two can be compared directly. The supraoesophageal ganglion of barnacles, in contrast to its counterpart in decapods (Bullock & Horridge, 1965), is a small and unspecialized structure (Fig. 1). Although involved in integrating sensory information, the input arriving at the ganglion is low. There are neither compound eyes nor antennae in the adult barnacle, thus obviating the need, respectively, for well developed optic lobes or a deutocerebrum. Indeed, Bullock and Horridge (1965) have reported that the protocerebrum of barnacles is very small,

Fig. 1. Generalized diagram of the balanoid central nervous system. After Gwilliam 1965.


A.Mu.
S.P.
A.Mu.N.
M.O.
O.N.
G.S.N.
S.O.G.
Oe.
C.N.I
A.N.
I.O.G.
L.O.

with only 2 to 3 nerves running to the median and lateral photoreceptors and the deutocerebrum is absent. The tritocerebrum comprises the largest part of the supraoesophageal ganglion.

The circumoesophageal connectives join the supraoesophageal ganglion to the infraoesophageal (or ventral) ganglion. The latter is variable in form, from the five ladder-like ganglia of Lepas and other lepadomorphs (Darwin, 1851), to the single ventral mass of balanomorph cirripedes. This degree of concentration is uncharacteristic of the lower Crustacea and closely resembles the situation in the Brachyura (see Bullock & Horridge, 1965).

The present study was undertaken to provide a detailed anatomy of the nervous system of B. balanoides and B. hameri into which studies on neurosecretion (see Chapters 3 and 4) could be integrated. To this end, the cobalt backfilling technique has been employed to provide the position of the cell bodies and their axon pathways. The histology, including the ultrastructure, and histochemistry of the nervous system are also described.

MATERIALS AND METHODS

Collection and maintenance of animals

Specimens of Balanus balanoides were collected on rocks from the shore beneath the Menai Suspension Bridge between October 1979 and February 1980, during July 1980 and from September 1980 to January 1981. B. hameri were dredged up, attached to the horse mussel Modiolus modiolus, off Langness Point, Isle of Man, during November 1979 and March, July and November, 1980. Conchoderma auritum were scraped from the hull of a Shell tanker whilst moored at the oil terminal at Amlwch, Anglesey on 13 November, 1980.

B. hameri were maintained in large tanks with a through-flow of sea water, in a cool basement. The animals obtained their food from that available in the sea water supply.

B. balanoides and C. auritum were dissected and/or fixed as soon as they were brought into the laboratory.

Method of dissecting ganglia:

The method of exposing the central nervous system of both B. balanoides and B. hameri was essentially the same and so a generalized procedure will be described.

The opercular plates, with the body of the barnacle attached, were removed. The specimen was then placed, opercular plates down, in a wax-lined petri dish filled with sea water. The tergal and scutal plates were prized apart, thus tearing the adductor muscle of the scuta, and pinned in this position. The cirri were then pulled away from the scuta and pinned in position so that the entire ventral surface (see White, 1978,

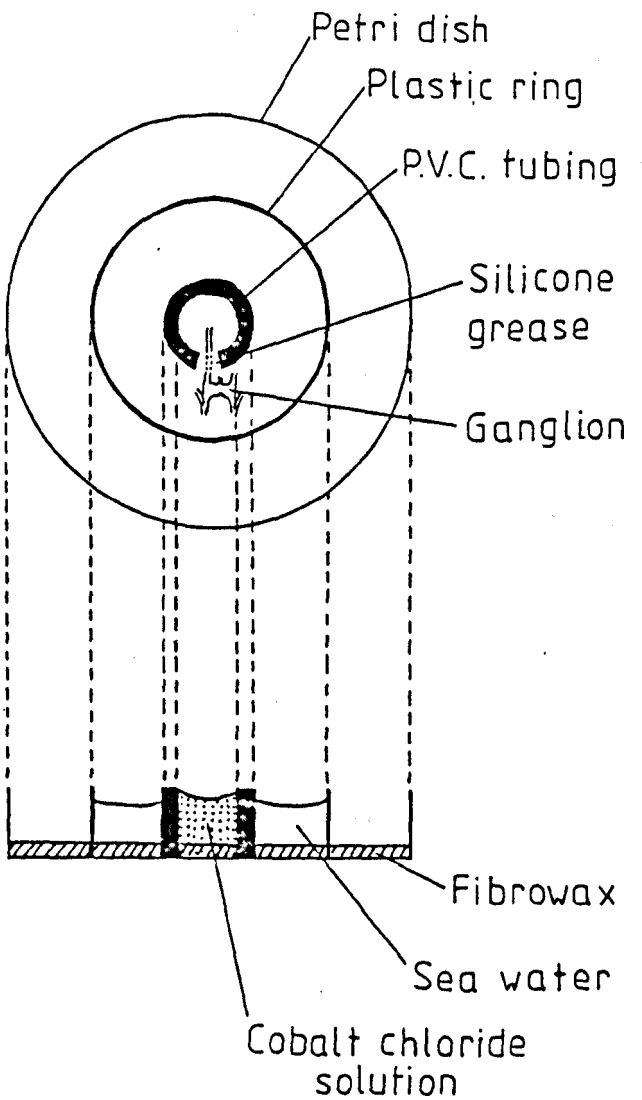
p. 15 for orientation nomenclature) of the body was exposed. To reveal the supraoesophageal ganglion, the oral cone was carefully removed and an incision made in the mid-line of the cuticle at this point and extended anteriorly to the scutal adductor muscle. The ganglion is positioned just anterior to the oral cone.

The infraoesophageal ganglion is a more readily accessible structure, being large and positioned just below the cuticle, extending from the anterior margin of the first pair of cirri to that of the fourth pair.

Cobalt-backfilling of nerves to the ganglia:

A conventional cobalt chloride procedure was utilized (see Strausfeld & Obermayer, 1976) but with slight modification to achieve good backfills for B. hameri, the animals used in this part of the study. B. hameri, owing to the comparatively large size of its ganglia and nerves, proved to be a better experimental animal than the more readily available B. balanoides. The experimental chamber used to backfill neurones is illustrated in Fig. 2. The ganglia, with a long length of the appropriate nerve to be backfilled, was excised from the animal and floated in the outer well of the chamber which contained sea water. The nerve was draped over a high vacuum silicone grease barrier (BDH), with the cut end of the nerve projecting into the inner well and the V-shaped wedge in the PVC tubing was then completely filled with grease. The inner well was then filled with a 6% (0.25M) aqueous solution of cobalt chloride, to which 0.13 mg bovine serum albumin (BSA)/ml had been added (Strausfeld & Obermayer, 1976). The free end of the submerged nerve was then re-cut and cobalt ions were allowed to diffuse up the axons for 4-20 hours and exceptionally up to 40 hours, at 4°C. Following this period ganglia were rinsed in fresh sea water and the cobalt ions precipitated by addition of concentrated ammonium

Fig. 2. Diagram of a cobalt backfill chamber.


Petri dish
Plastic ring
P.V.C. tubing
Silicone grease
Ganglion
Fibrowax
Sea water
Cobalt chloride solution

sulphide - a seemingly harsh treatment but one which barnacle ganglia, with their thick neural sheath (see below) are well able to withstand. The ganglia were again rinsed in sea water and pinned out in a small, wax-lined receptacle where they were dehydrated through a series of alcohols and cleared in methyl salicylate (BDH). None of the preperations were block intensified (Pitman, 1979). Ganglia were mounted on glass slides in DPX and viewed under a Leitz Dialux microscope. Drawings were made with the aid of a camera lucida attachment. The preparations were also photographed using either a Leitz Orthoplan photomicroscope or a Wild M5 stereomicroscope with a MPS 15/11 Semiphotomat in trinocular assembly.

Light microscopy:

Bodies of all the barnacle species were fixed in Carnoy's, Stieve's, Susa's, Zenker's and Bouin/sea water fixatives. Specimens of B. hameri and C. auritum had the tips of their cirri cut off to aid penetration of the fixative. After fixation the bodies were dehydrated and cleared in Cellosolve (2-ethoxyethanol), then placed in toluene prior to embedding in Fibrowax (Raymond A. Lamb). Routinely, 7-8 μm serial sections were cut, either in the longitudinal or the transverse plane, and stained with Heidenhain's Azan.

In a separate study, which aimed to display the neuronal perikarya and their relative locations, whole mounts of the supra- and infraoesophageal ganglia of B. hameri were stained with methyl green-pyronin G using a technique slightly modified to that described by Johansen and Frederiksen (1980). Ganglia, from which the sheath had not been removed, were fixed in 2.5% gluteraldehyde in 0.2M sodium cacodylate (pH 7.4) at 4°C for 24 hr. After washing in distilled water the ganglia were stained for 30 min in methyl green-pyronin G, differentiated in 96% ethanol and dehydrated in

3 changes of absolute ethanol over 1½ hr. The tissues were cleared in methyl salicylate for 10 mins prior to mounting in Depex.

A range of histochemical tests were applied to wax sections of B. hameri ganglia (see Table 1). In addition the presence of lipid was assessed in fresh frozen sections (cryostat) using the sudan black B technique (Pearse, 1968).

The photographic techniques used were as described previously.

Transmission electron microscopy:

Ganglia were dissected out as described earlier and fixed according to one of the following procedures:-

a) (i) 2.5% Gluteraldehyde in 0.2M sodium cacodylate buffer at either pH 7.0, 7.4 or 7.8 for 1-2 hr.

(ii) Wash in buffer ½-8 hr.

(iii) Post-fixed in 2% osmium tetroxide in Na cacodylate buffer at the same pH as the pre-fixative for ½ hr.

b) (i) and (ii) as for a) at pH 7.4

(iii) Post-fixed in 2% osmium tetroxide in veronal buffer (Palade, 1952) at pH 7.4 for ½ hr.

c) as for a), but U.V.-irradiated filtered sea water was substituted for the cacodylate buffer.

Following fixation, which was carried out either over ice or at room temperature, the tissues were washed in their respective buffer for 1 hr, and either dehydrated through a series of ethanols, transferred to propylene oxide and embedded in Spurr resin (Spurr, 1969), or dehydrated with 1,4-dioxane and embedded in Polybed 812 (Shearer & Hunsicker, 1980). Thin sections were cut with glass knives and double stained with uranyl

TABLE 1 Histochemical tests carried out on wax sections

Test	Significance	References
i) General		
Heidenhain's azan		GRAY (1954)
ii) Proteins and amino acids		
Mercury-bromphenol blue	Proteins	PEARSE (1968)
Sakaguchi reaction	Arginine	"
Diazotization coupling	Tyrosine	"
p-Dimethylaminobenzaldehyde (D.M.A.B.)	Tryptophan	"
2'2'-Dihydroxy-6,6'-di-naphthyl disulphide (D.D.D.)	-SH groups alone	"
D.D.D. (thioglycollate reduction)	-SS and SH groups	"
D.D.D. (iodoacetate block)	-SS groups alone	"
iii) Carbohydrates		
Periodic Acid-Schiff (PAS)	General carbohydrate	"
Bests carmine	Glycogen	"
Alcian blue	Mucopolysaccharides	"
Alcian blue pH 1.0	Sulphated acid mucopoly-saccharides	"
Alcian blue pH 2.5	Acid mucopolysaccharides	"
iv) Metachromasia		
Toluidine blue	Acid mucopolysaccharides	KRAMER & WINDRUM (1955)

acetate and lead citrate (Pease, 1964) and examined in either a GEC-AEI Corinth 275 electron microscope or a Philips 301 electron microscope operating at 40-80 kV. Thick (1 μm) sections were stained with toluidine blue (1% in a 1% aqueous borax solution) for examination with the light microscope.

Sections of the suboesophageal region of the infraoesophageal ganglion of B. hameri were treated with the thiosemicarbazide-silver proteinate method for carbohydrates, as described by Lewis and Knight (1977). The sections were mounted on gold grids prior to examination in the transmission electron microscope.

Scanning electron microscopy:

Isolated infraoesophageal ganglia of B. hameri were frozen in liquid nitrogen and freeze fractured in the transverse plane using a backed razor blade. The material was fixed in 2.5% gluteraldehyde in sodium cacodylate buffer (pH 7.8) for 2 hr over ice, then dehydrated in ethanol and transferred to acetone before being critical point dried. The ganglia were then mounted on aluminium stubs using double-sided Sellotape, sputter-coated with gold and viewed in a Cambridge Stereoscan Mark 2a operating at 10 kV.

RESULTS

Gross morphology

The supraoesophageal ganglion

The central nervous system of both Balanus species (Figs. 3 and 4) is essentially similar to that of the generalized balanoid system illustrated in Figure 1. The supraeosophageal ganglion is a small bilobed

Fig. 3. Central nervous system of Balanus hameri. Scale bar, 1 mm After Allison, 1980.

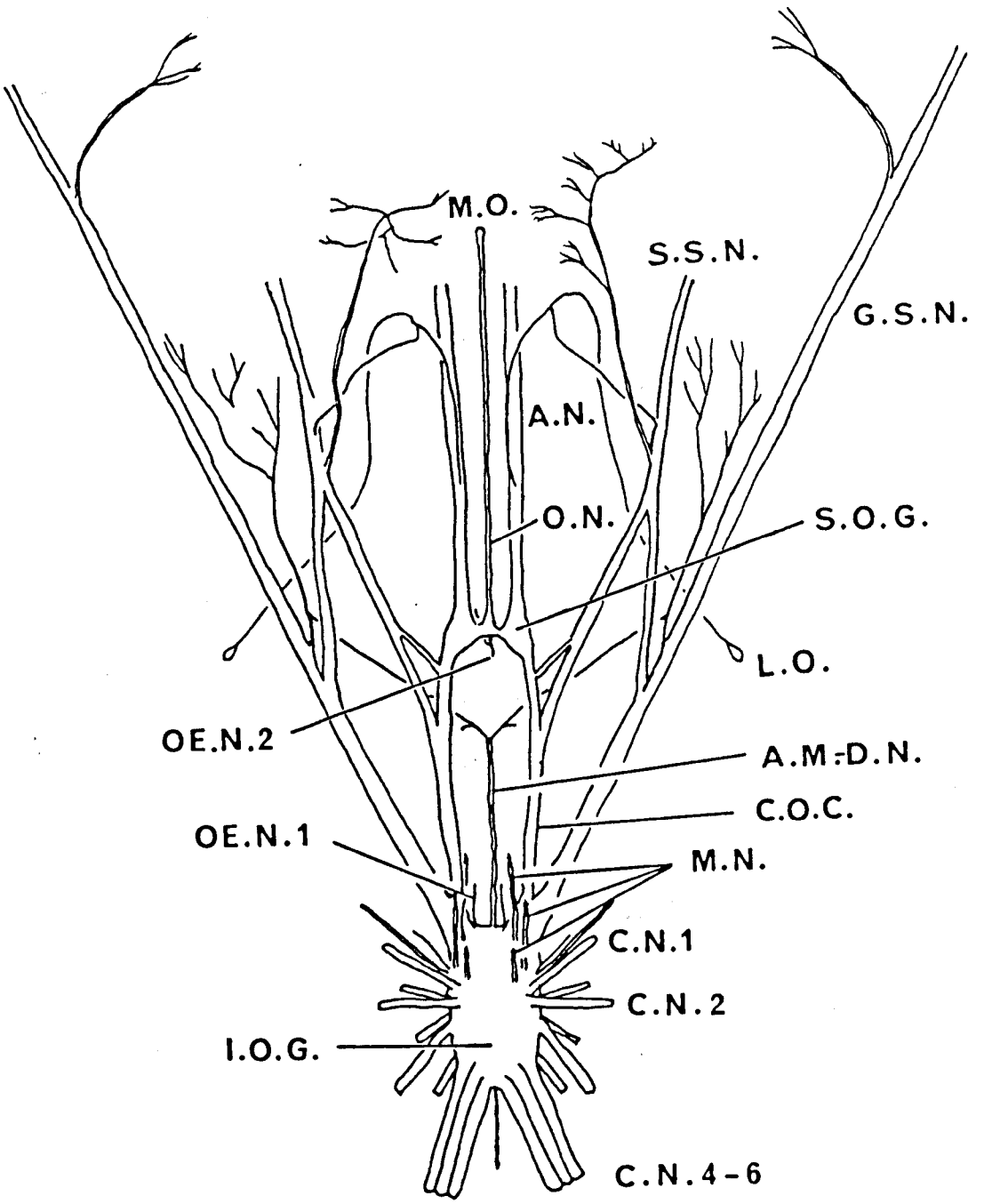
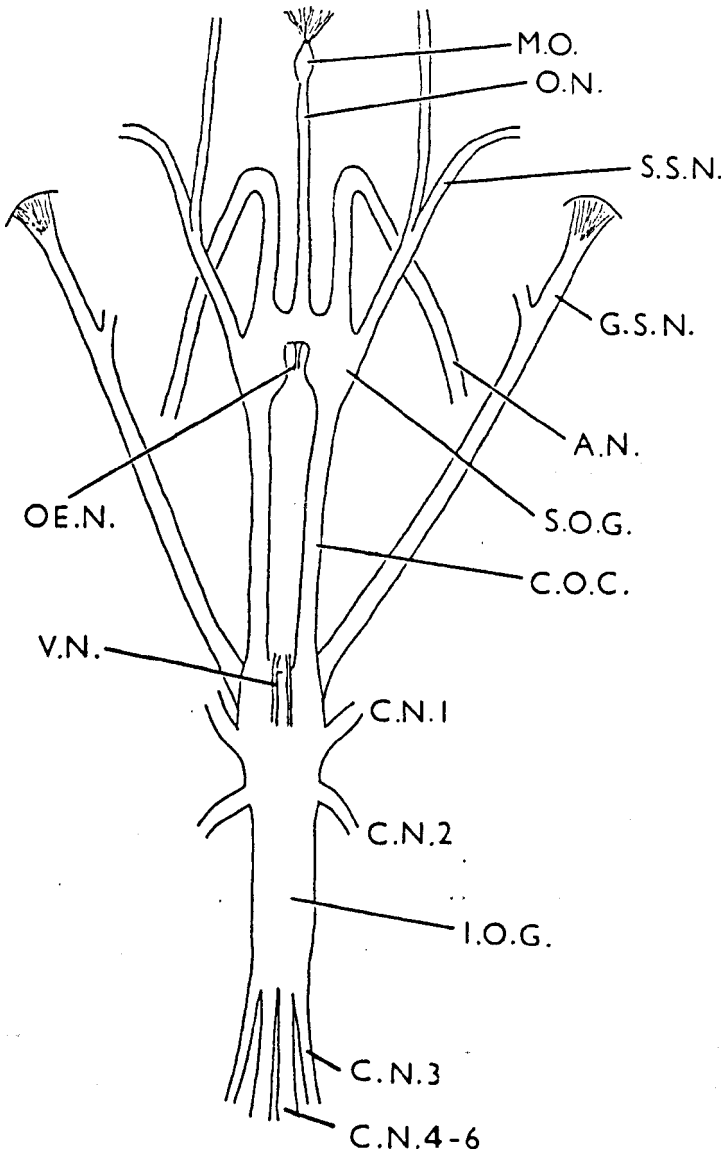

M.O.
S.S.N.
G.S.N.
A.N.
O.N.
S.O.G.
L.O.
OE.N.2
A.M.-D.N.
C.O.C.
OE.N.1
M.N.
C.N.1
C.N.2
I.O.G.
C.N.4-6

Fig. 4. Camera lucida drawing of the central nervous system of Balanus balanoides. Scale bar, 1 mm.


M.O.
O.N.
S.S.N.
G.S.N.
A.N.
OE.N.
S.O.G.
C.O.C.
V.N.
C.N.1
C.N.2
I.O.G.
C.N.3
C.N.4-6

structure, each lobe measuring 260 μm in width by 310 μm in length for B. balanoides and 360 μm in width by 440 μm in length for B. hameri. It is joined to the infraoesophageal ganglion by the circumoesophageal connectives. Arising at the lateral margin of these nerves, at any place between the mid-point of the circumoesophageal connective and the lateral margin of the supraoesophageal ganglion, is a nerve which joins a branch from the great splanchnic nerve (see below) to form the suprasplanchnic nerve. This nerve innervates the mantle (c.f. Gwilliam, 1963, Fig. 1). From the antero-lateral edge of each lobe a large nerve runs directly anterior for some distance before turning dorsally to run adjacent to the oviducts. These nerves are the antennular nerves which innervate the rostral scutum depressor muscles and the intrinsic body musculature (Gwilliam & Cole, 1979). Darwin (1854) proposed that the barnacle antennular nerves correspond to the nerves running to the antennules of other crustaceans. The antennular nerves also contain axons from the lateral photoreceptors. The point at which the photoreceptor axons become distinct from the antennular nerves is highly variable and they may even arise from the anterior margin of the supraoesophageal ganglion. A lateral photoreceptor nerve also contains a branch of the suprasplanchnic nerve arising just anterior to the junction between the branch from the great splanchnic and the suprasplanchnic nerve.

Arising from the anterio-medial edge of the commissure, which joins the two lobes of the supraoesophageal ganglion, is the median ocellar nerve. This nerve extends anteriorly to the median photoreceptor (see Chapter 4). It runs adjacent to a small muscle, also noted by Fahrenbach (1965), connecting the oesophagus with the mantle, just anterior to the photoreceptor. Finally, on the posterio-dorsal margin of the supra-oesophageal commissure, there arises a small unpaired nerve which supplies the oesophagus; the oesophageal nerve (OE.N2 of Allison, 1981).

The infraoesophageal ganglion

As previously described, in the lepadomorph barnacles there can be as many as five thoracic ganglia. A more advanced state is shown by the balanomorphs, where the thoracic and abdominal ganglia tend to be concentrated as a single mass, the infraoesophageal ganglion, which represents the complete fusion of the suboesophageal ganglion and paired thoracic ganglia. The infraoesophageal ganglion exists in this form in both B. balanoides and B. hameri. Indeed, in outward appearance, there is no evidence of segmentation.

The circumoesophageal connectives arise from the anterio-ventral margin of the infraoesophageal ganglion. The great splanchnic nerves, as the name implies, are the largest nerves of the nervous system. They arise at a point directly below the origin of the connectives on the dorsal surface of the ganglion. The physiological experiments of Gwilliam and co-workers have established that these nerves supply much of the anterior musculature, the lining of the mantle cavity, and the following extrinsic muscles: the rostral scutal depressors, the tergal depressors and the scutal adductor muscle (c.f. Gwilliam & Cole, 1979). They also send a motor supply to the anterior prosomal muscles and the attrahens.

Originating in the mid-line, on the dorsal surface of the ganglion, is the anterior mid dorsal nerve. In B. hameri this nerve has been shown to contain motor axons supplying the transverse musculature of the thorax and it also innervates the outer fibres of the attrahens (Allison, 1981).

The nerves from the infraoesophageal ganglion supplying the oesophagus, the mouthparts and the cirri have been described adequately by Allison (1981) for B. hameri and there is no need for further elaboration. The corresponding nerves in B. balanoides are very similar. The only marked difference is that cirral nerve 3 arises at the posterior margin of

the ganglion with cirral nerves 4 to 6, whereas in B. hameri it originates in a more anterior position, at the lateral margin of the ganglion. In this respect the infraoesophageal ganglion of B. balanoides resembles more closely that of B. cariosus (c.f. Gwilliam & Cole, 1979). Also, the paracirral nerves of B. balanoides were not evident and it is assumed that they branch from the cirral nerves at some distance from the ganglion.

Neurone anatomy

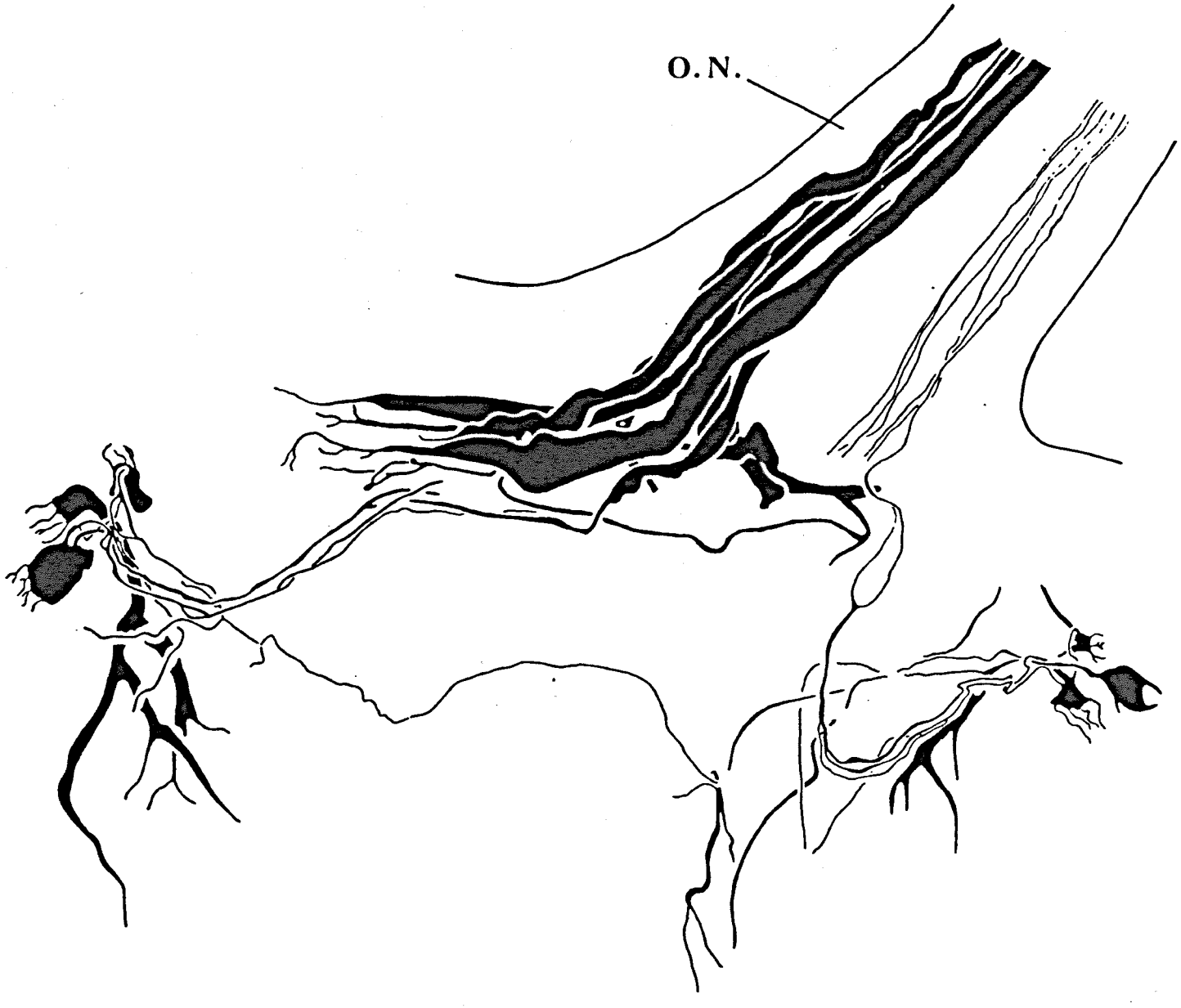
The results of successful backfills of nerves to the supraoesophageal ganglion and the anterior region of the infraoesophageal ganglion (i.e. the suboesophageal region) are presented below, commencing with the nerves which are most anterior in position and proceeding posteriorly. Nerves to the mouthparts were not filled as the neurones innervating them have been dealt with in detail by Allison (1981). In addition, the nerves to the fused thoracic region of the infraoesophageal ganglion were not filled, since these probably contain processes of motor neurones alone (Gwilliam & Cole, 1979). Replicate backfills were made of all the nerves and when nerves were paired, each one was backfilled to check that they were bilaterally symmetrical.

The supraeosophageal ganglion

1. Median ocellar nerve

Seven large sensory axons, from the photoreceptor cells of the median photoreceptor, were backfilled in the ocellar nerve (Figs. 5 and 6). These axons ended blindly in the supraoesophageal commissure, but adjacent to axons from two groups of cells, one on either side of the commissure, and may indeed have formed synaptic contacts with them. The three perikarya each send a fine axon towards the median photoreceptor

Fig. 6. Camera lucida drawing of a backfill similar to that shown in Figure 5.


O.N.

(Fig. 7). Axons from the perikarya also project posteriorly into the neuropile of the tritocerebrum. Gwilliam and Cole (1979) believed these cells to be motorneurones supplying axons to the small muscle which runs adjacent to the median ocellar nerve. Evidence will be presented in later chapters (Chapters 3 and 4) to demonstrate that these cells are in fact neurosecretory.

2. Antennular nerves

Between 25 and 29 large cell bodies were backfilled via the antennular nerve (Figs. 8 and 9). Only those neurones which could be clearly distinguished were recorded; this was quite difficult, because the whole area of the tritocerebrum was darkly stained. The numbers given above may, therefore, be an underestimate. The majority of the cell bodies are spherical and confined to the ipsilateral lobe of the ganglion. Two neurones were, however, noted in the commissure. They were much smaller (47 μm and 63 μm in length) than most of the cells comprising the main group (38-94 μm in diameter) and were elongate in shape. Ramifications of their axons were probably in synaptic contact with processes from the main cell group. A single cell was also backfilled in the contralateral lobe. Axons from this cell extended through the commissure to the main body of cells. A single axon also ran down the contralateral circumeosophageal connective as described by Gwilliam and Cole (1979).

3. Lateral photoreceptor nerves

Two axons were backfilled in this nerve, only one of which could be traced into the supraoesophageal ganglion (Figs. 10 and 11). The axon ended blindly in the contralateral circumoesophageal connective and is probably sensory. Branches from the axon were observed at two

Fig. 9. B.hameri : camera lucida drawing of a preparation similar to that shown in figure 8. ○, dorsal; ○, midline; ○, ventral - applies to all backfill figures.

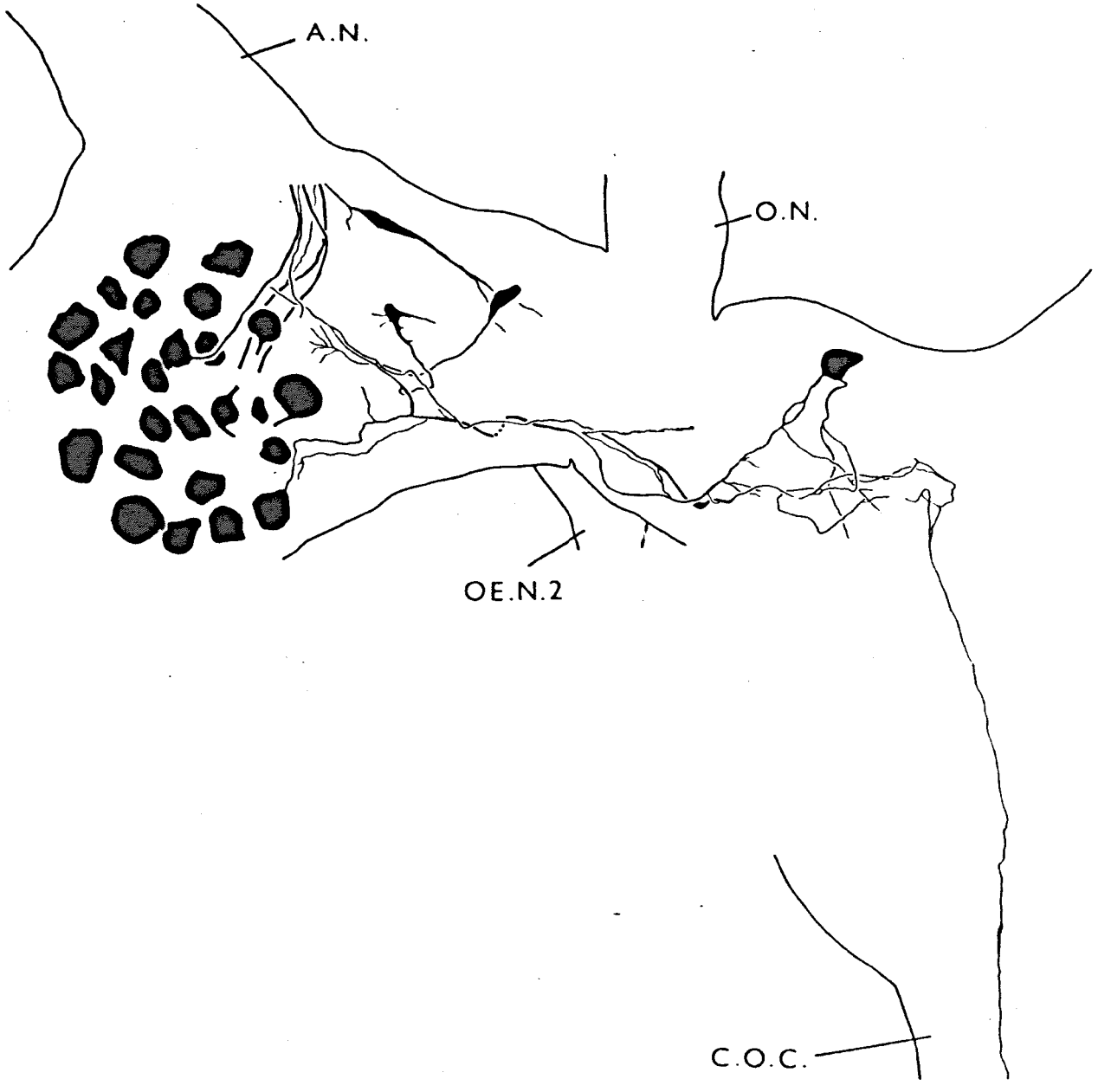
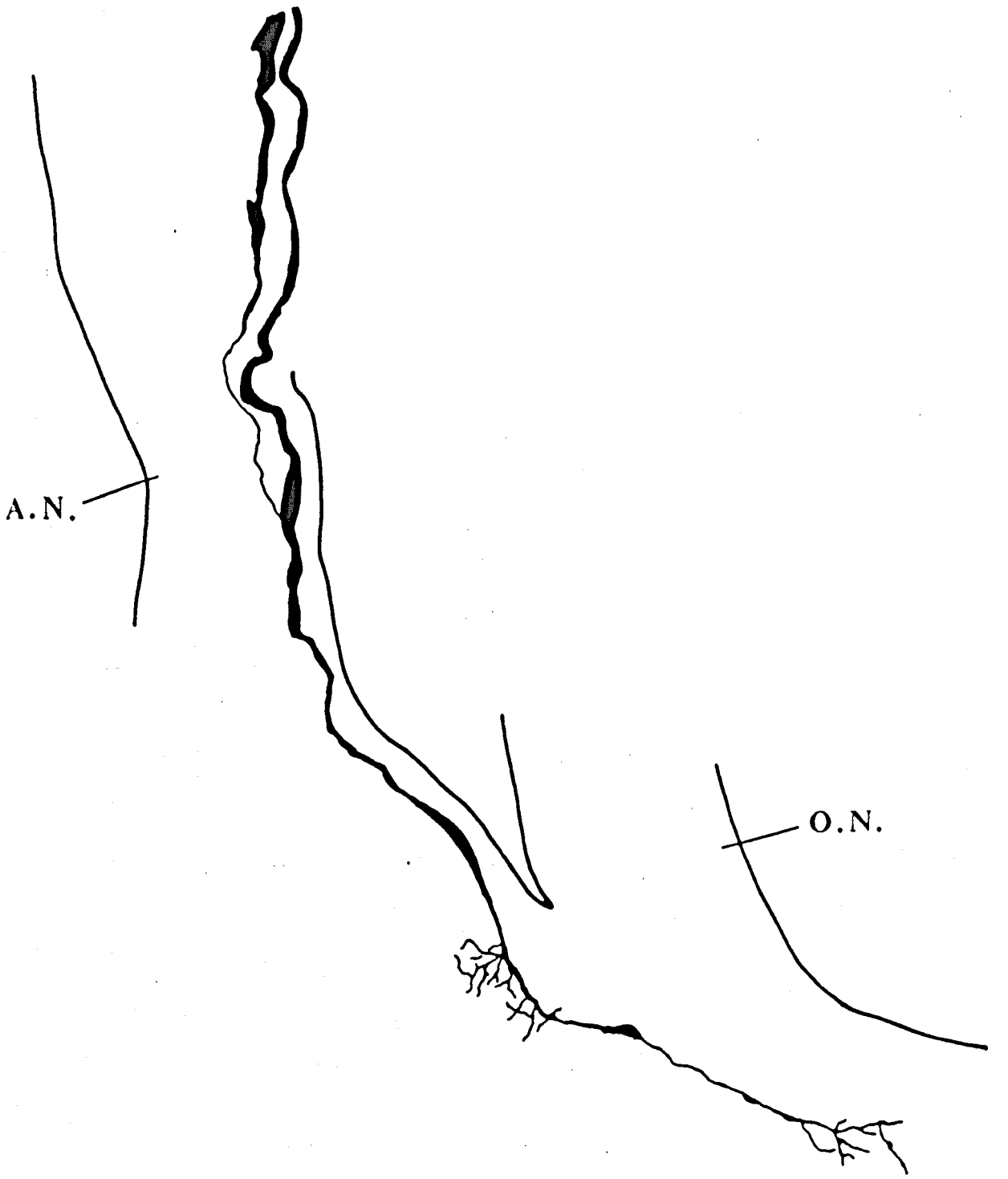

A.N.
O.N.
OE.N.2
C.O.C.

Fig. 11. Camera lucida diagram of figure 10.


A.N.
O.N.

points along its length, namely on either side of the median ocellar nerve. This is the region in which processes from the median photoreceptor cells also end (see above).

4. Suprasplanchnic nerves

The majority of the backfilled axons passed directly to the infraoesophageal ganglion via the circumoesophageal connective (Figs. 12 and 13). Only one axon was observed to pass into the supraoesophageal ganglion. It entered via the connective between the suprasplanchnic nerve and the ganglion to pass across the commissure to a cell body on the contralateral side. A branch from the axon also passed down the contralateral circumoesophageal connective to the infraoesophageal ganglion.

5. Circumoesophageal connectives

Between 50-60 cell bodies were backfilled via the connective (Figs. 14 and 15). These were distributed in small groups, mainly on the ipsilateral side, but a small group also occurred in the contralateral lobe, midway between the bases of the antennular nerve and the median ocellar nerves. A similar group of cells occurred in a symmetrical position in the ipsilateral lobe. The remaining groups were distributed near the base of the antennular nerve and the circumoesophageal connective. A bundle of approximately nine axons leaves the ipsilateral nerve via the ipsilateral antennular nerve. Two processes also leave the ipsilateral lobe via the oesophageal nerve.

It should be noted that the oesophageal nerve itself was not backfilled. It was not possible to dissect free a sufficient length of this nerve to use in the technique described in the present study.

Fig. 13. Camera lucida diagram of figure 12.

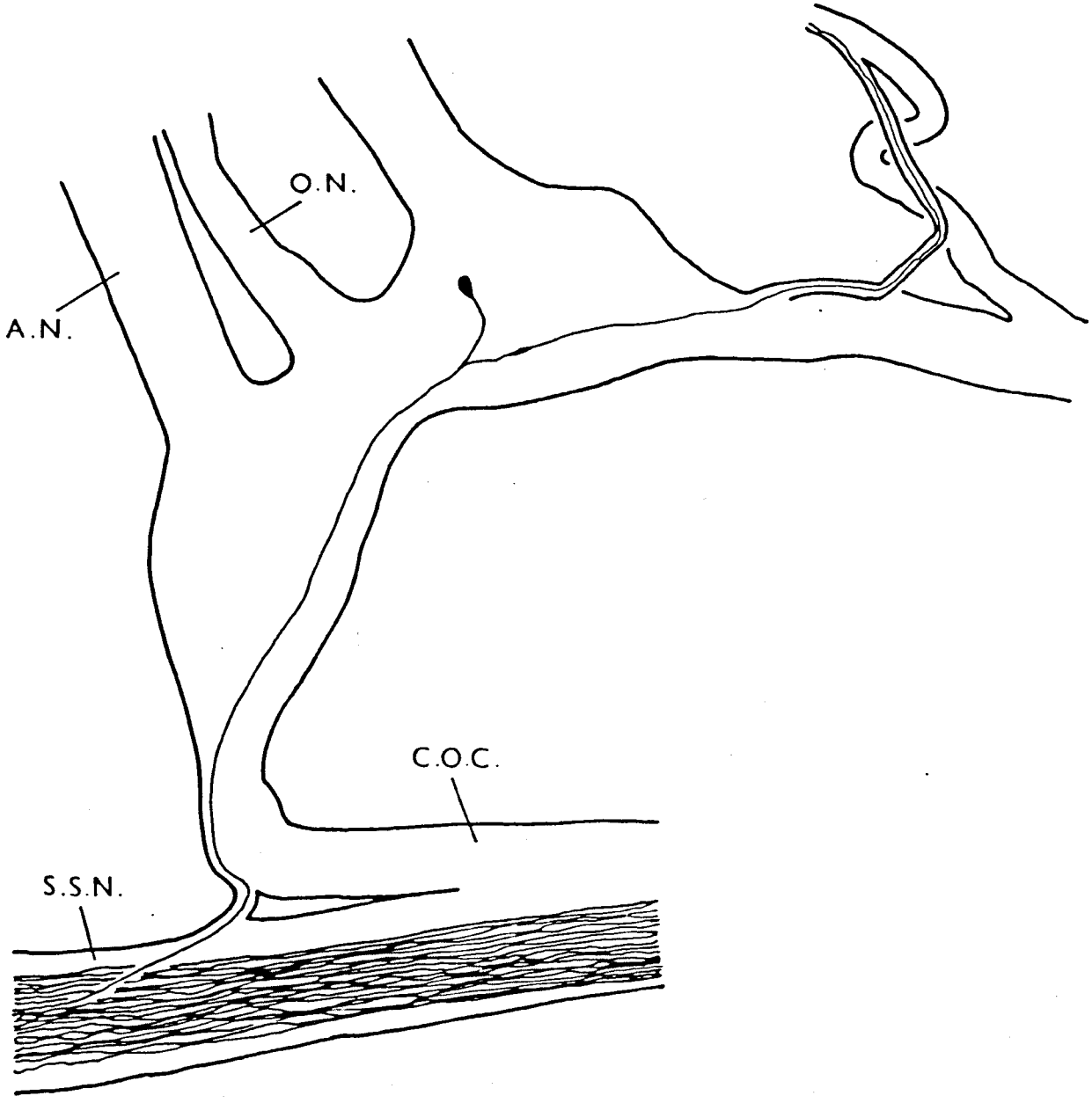
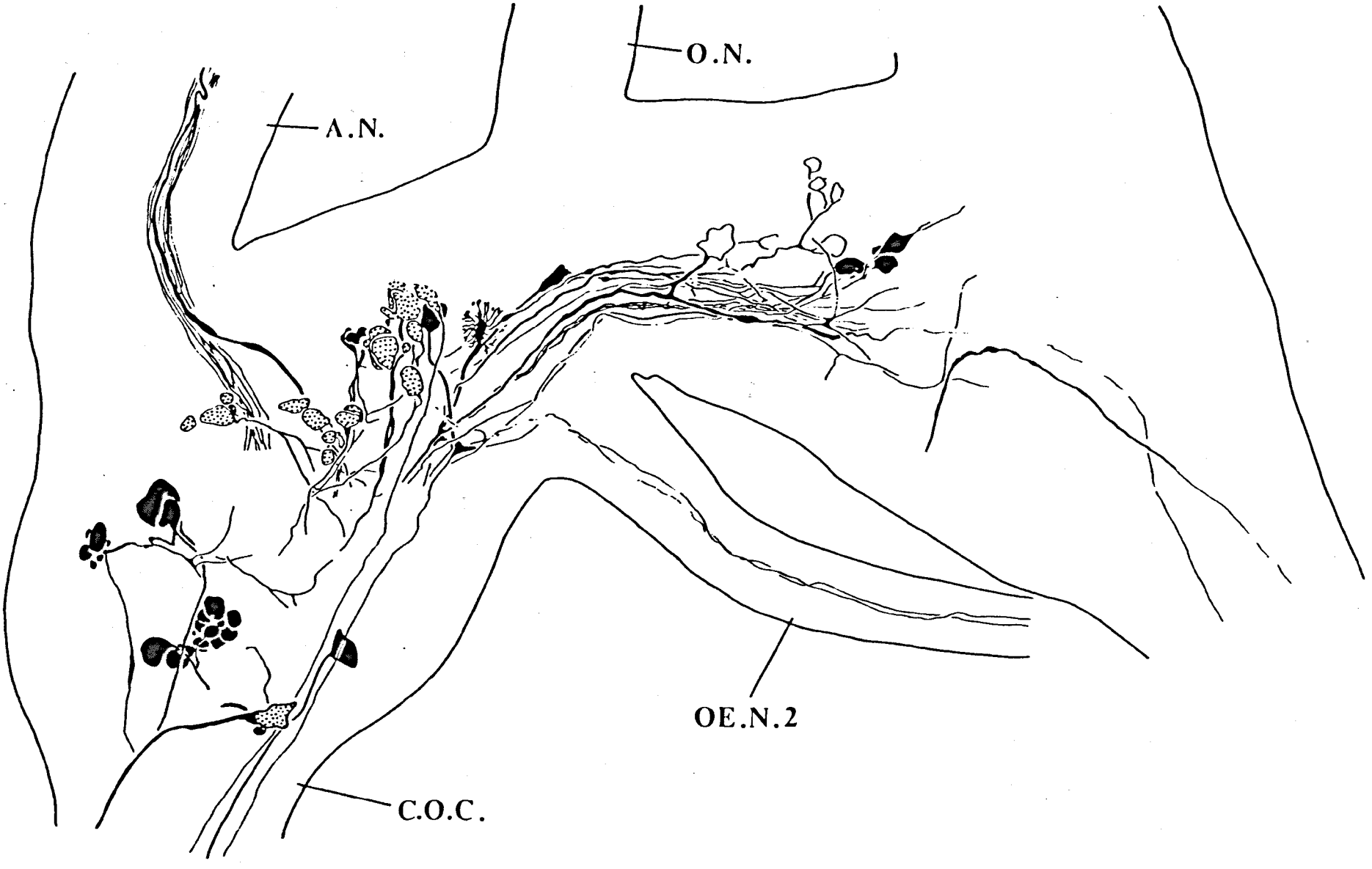

O.N.
A.N.
C.O.C.
S.S.N.

Fig. 15. Camera lucida drawing of figure 14.


O.N.
A.N.
OE.N.2
C.O.C.

The infraoesophageal ganglion

1. Circumoesophageal connectives

When backfilled distal to the branch to the suprasplanchnic nerve, a large number of cell bodies were backfilled - both ipsi- and contra-lateral , and along the entire length of the ganglion (Figs. 16 and 17). Indeed, more cells were filled on the contra- than the ipsilateral side, small groups of the former being associated with cirral nerves 1-3, whereas the latter were confined to the base of cirral nerve 1. Processes from the contralateral neurones crossed at three main points along the length of the ganglion, although several axons did traverse between these points.

Finally, there was a group of cell bodies associated with the base of the contralateral circumoesophageal connective. Two axons from these cells joined a tract which crossed the ganglion, transversely, at its anterior margin. Cell bodies positioned anterior to cirral nerve 1 tended to contribute to this tract, whereas the remaining axons ran in a tract on the ipsilateral side, which was roughly parallel to the longitudinal axis of the ganglion.

When the circumoesophageal nerve was filled proximal to the branch to the suprasplanchnic nerve, it also contained those axons of the ipsi-lateral suprasplanchnic nerve which bypassed the supraoesophageal ganglion (Figs. 18 and 19). Therefore, by comparing Figures 17 and 19 we can determine which somata send their processes to the suprasplanchnic nerve. By doing this, it can be seen that there were very few somata additional to those illustrated in Figure 17. The most noteable of those which could be distinguished were a group of large cell bodies (approx. 75 μm in diameter) which were mainly confined to the anterior region of the ganglion, on the ipsi-lateral side. There were also a few cell bodies

Fig. 17. Camera lucida drawing of figure 16.

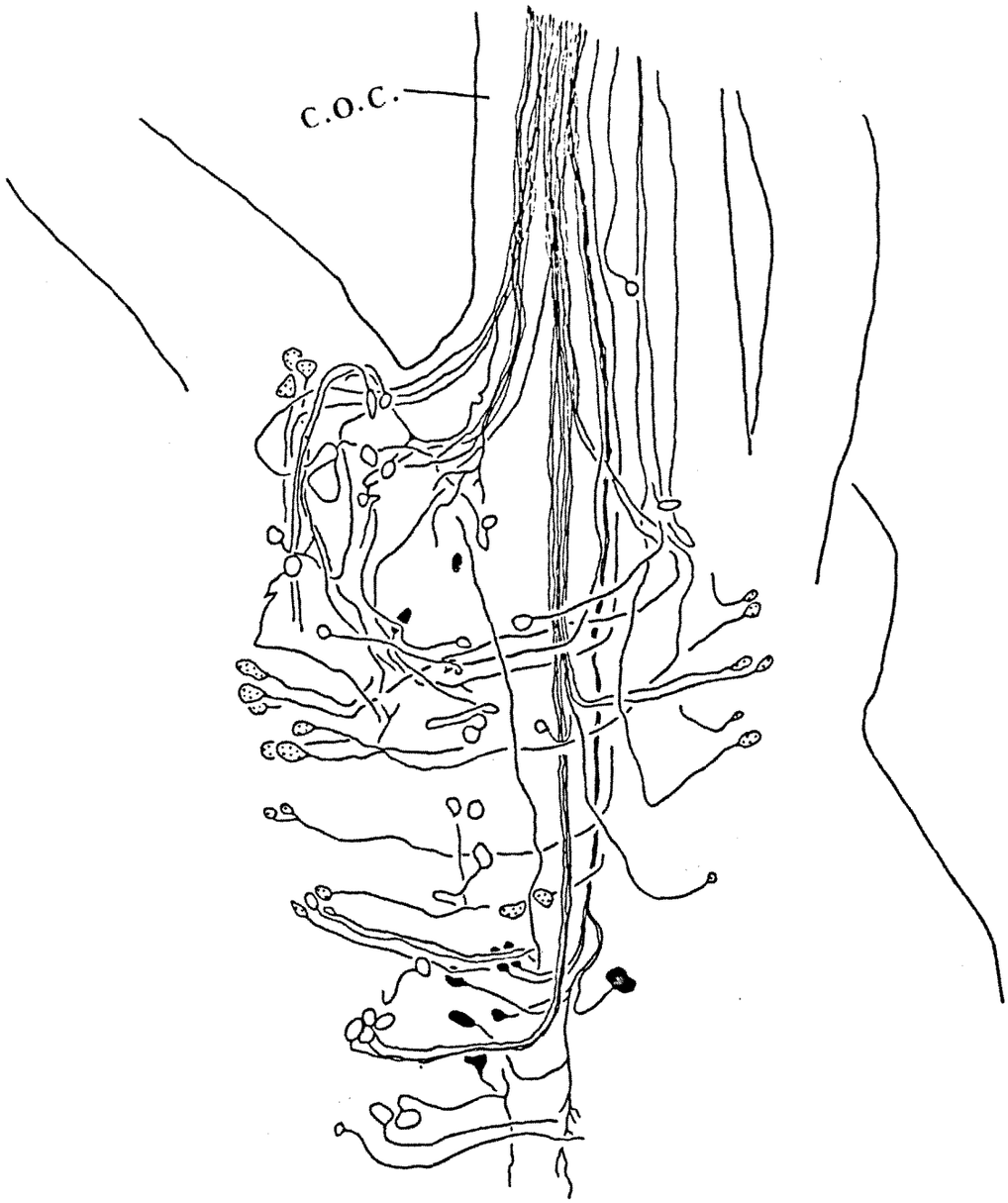
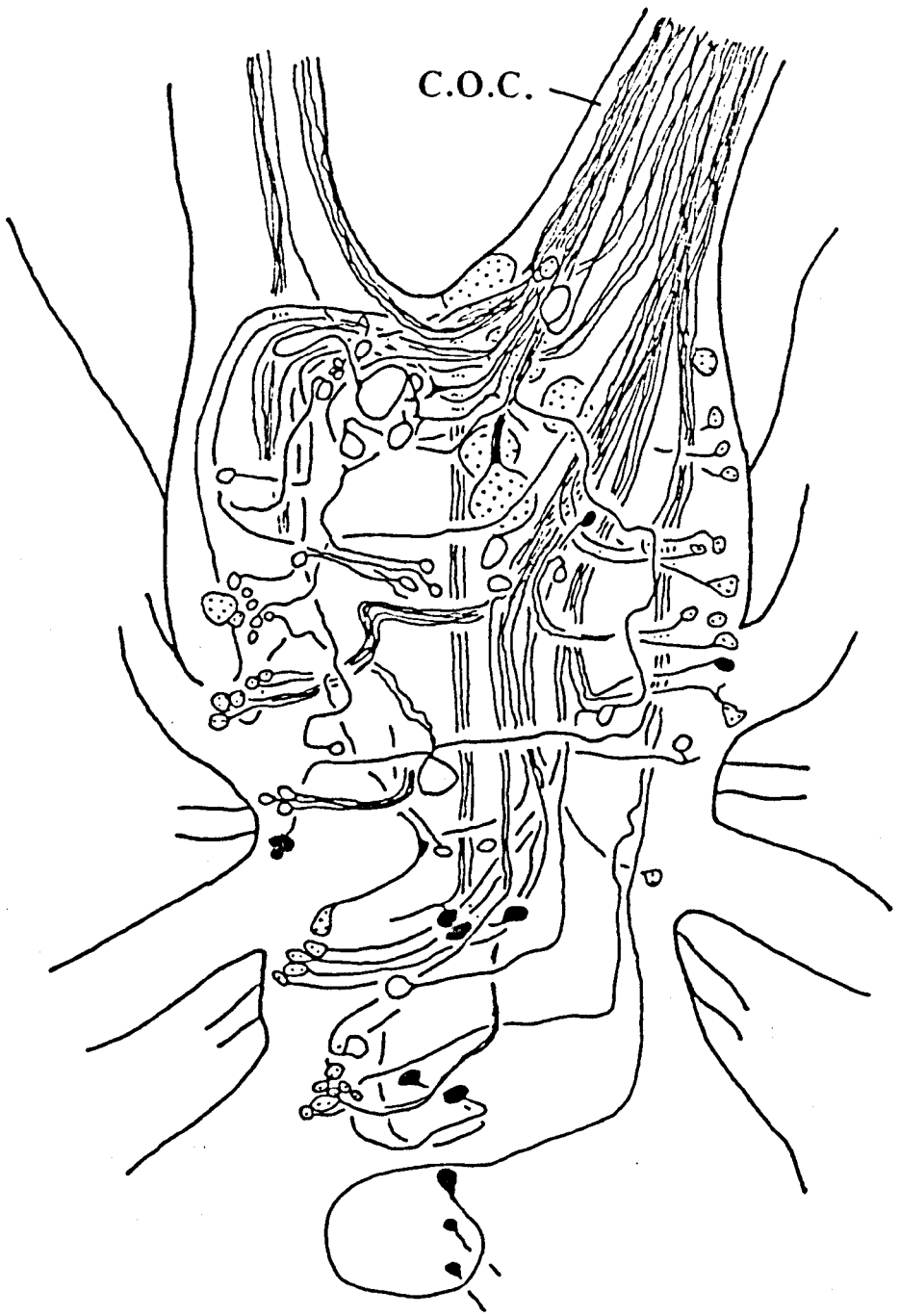

C.O.C.

Fig. 19. Camera lucida drawing of figure 18.


C.O.C.

associated with the ipsilateral cirral nerves and a large ventrally placed neurone, at the level of cirral nerve 1, near to the midline.

Two small groups of axons extended up to the contralateral circumoesophageal connective. It is not known if they passed to the suprasplanchnic nerve or to the supraoesophageal ganglion. One of the tracts appeared to pass directly from the backfilled connective. The origin of the other group was difficult to trace, but one axon originated from a small ventrally placed neurone, at the level of cirral nerve 1.

2. Mid-dorsal nerve

Although only eight cell bodies were backfilled (Fig. 20) there were many blind-ending axons only a few of which could be traced. The latter may have originated from peripheral sensory cells. Those cell bodies which were backfilled occurred in the mid-line of the ganglion, on both the ipsi- and contralateral sides. Some of these cells were bilaterally symmetrical.

3. Great splanchnic nerves

Backfills of the great splanchnic nerve, cut distally to the branch joining the suprasplanchnic nerve, revealed surprisingly few cell bodies when the size of the nerve is considered (Fig. 21). The vast majority of the axons ended blindly in the neuropile at the base of the filled nerve and may be sensory axons from cells in the mantle lining (c.f. Gwilliam & Cole, 1979). Neurones were filled on both the ipsi- and contralateral side of the ganglion. Three large neurones were also backfilled in the midline.

If the great splanchnic nerve was backfilled proximal to the branch to the suprasplanchnic nerve (Figs. 22 and 23) then those cell bodies

Fig. 20. B.hameri : camera lucida drawing of a cobalt backfill of the infraoesophageal ganglion via the mid-dorsal nerve. Scale bar, 100 µm.

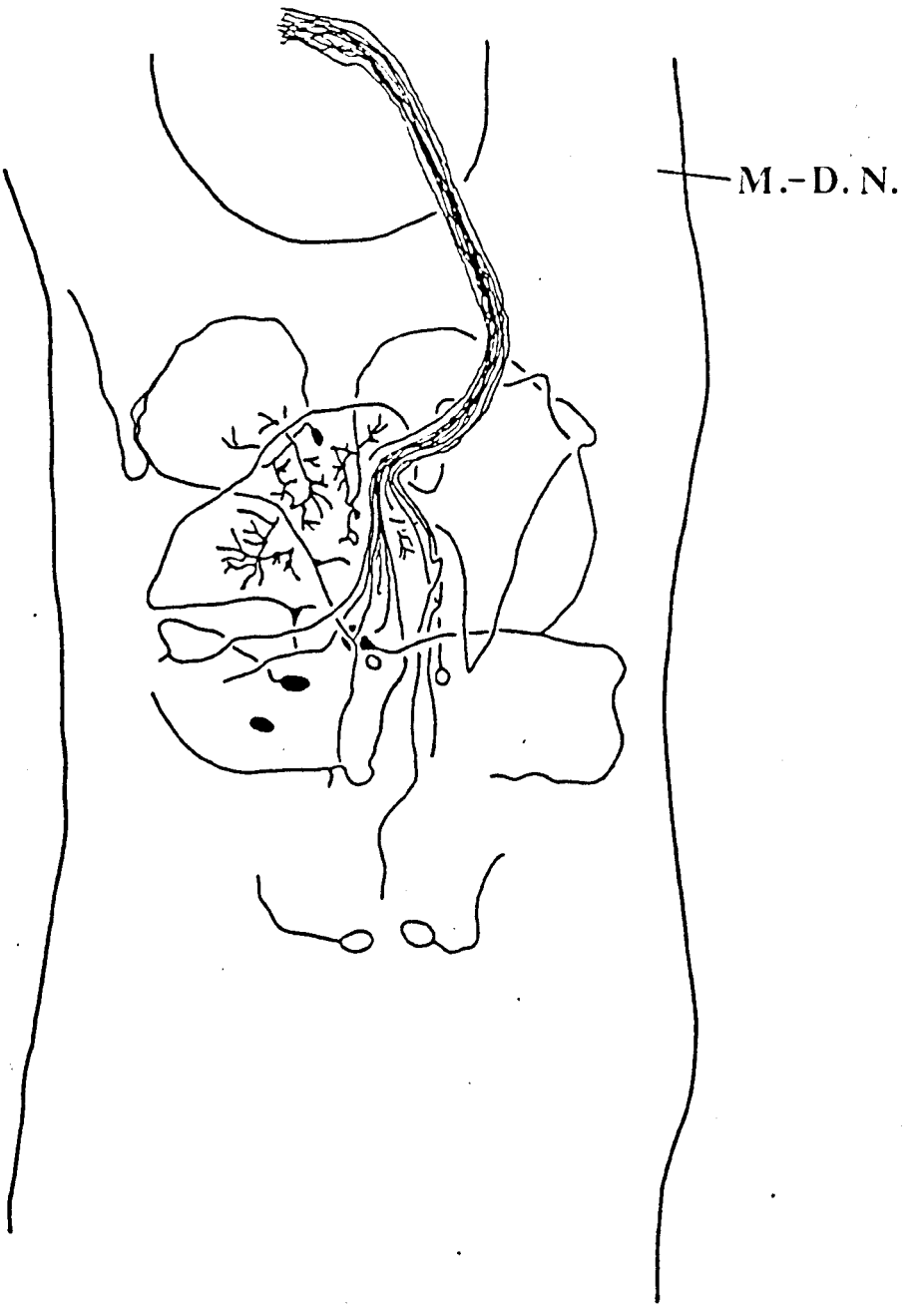

M.-D. N.

Fig. 21. B.hameri : camera lucida drawing of a cobalt backfill of the infraoesophageal ganglion via the great splanchnic nerve cut distal to the branch to the suprasplanchnic nerve. Scale bar 100 µm.

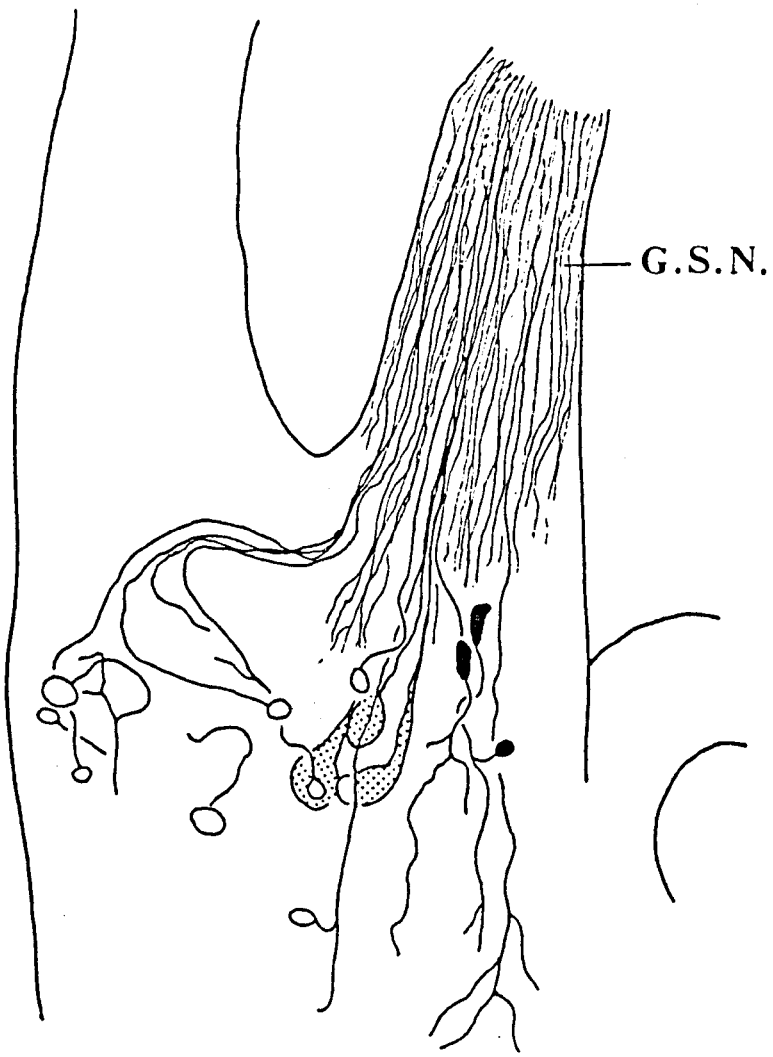
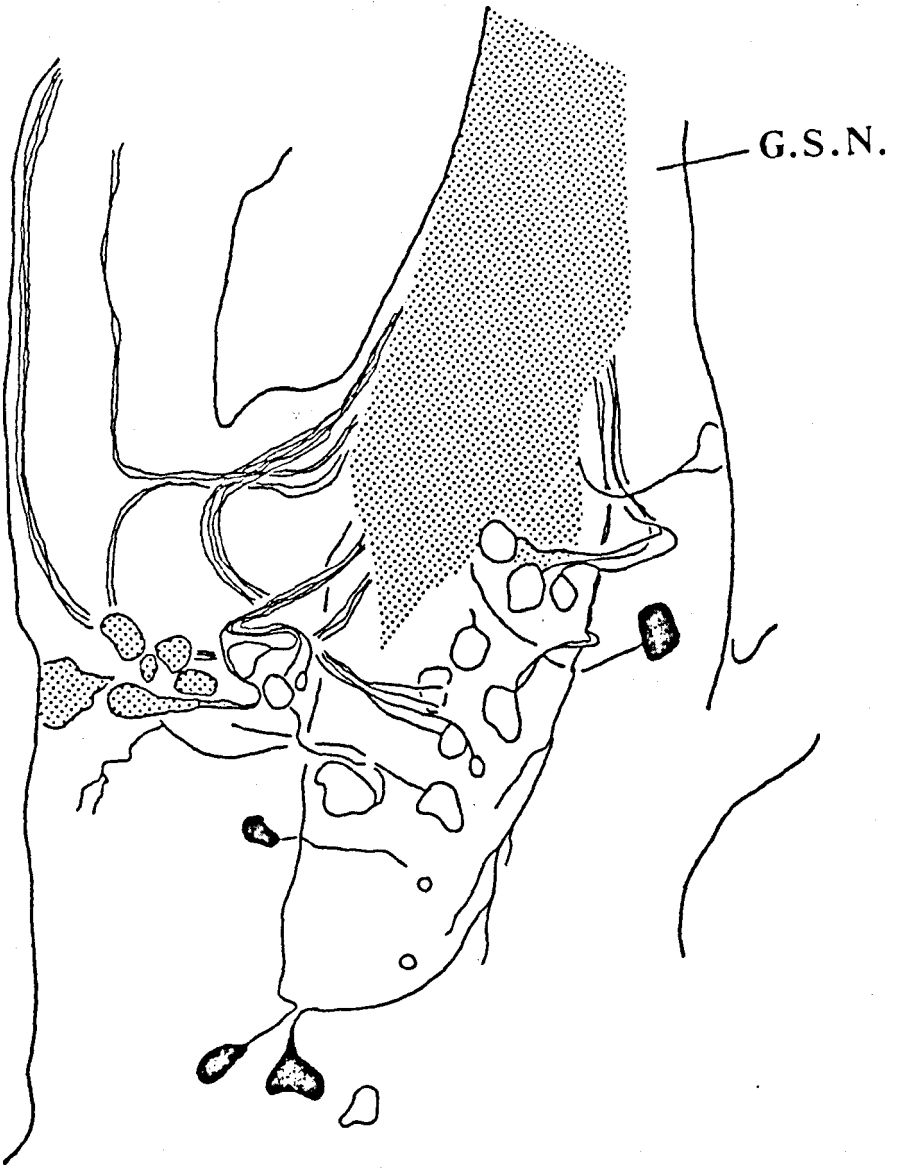

G.S.N.

Fig. 23. Camera lucida drawing of figure 22.


G.S.N.

which had processes in the latter could also be distinguished. In fact, few additional cell bodies were filled. Most notable of these were (i) several cells clustered around the base of the back filled nerve, (ii) several ventrally and dorsally placed cells in the midline, and (iii) a group of cells close to the lateral margin of the contralateral side of the ganglion near the base of the contralateral great splanchnic nerve. Some of the neurones from this latter group may be analogous to the motoneurones of the adductor and oral retractor muscles, described by Allison (1981).

Many more 'sensory' axons were backfilled when the suprasplanchnic nerve was included. They ended blindly in the neuropile at the base of the filled nerve. Two groups of axons also entered the contralateral splanchnic nerve. One group, containing two to three axons, passed into this nerve directly from the filled nerve. The origin of the three axons of the remaining group were less discernible, since they passed under a group of somata. Allison (personal communication) has suggested that they originate from neurones which have axonal projections in both the contra- and ipsilateral splanchnic nerves.

Light microscopy

Supraoesophageal ganglion

The supraoesophageal ganglion of both *Balanus* spp. examined was very similar in structure and remarkable in its simplicity (Fig. 24). A large proportion of the ganglion was occupied by large (20-25 μm diameter in *B. balanoides*; 48-65 μm diameter in *B. hameri*), monopolar cell bodies of the tritocerebrum, many of which had processes in the antennular nerves. The perikarya were spherical with a large (11-12 μm diameter in *B. balanoides*; 16-19.5 μm diameter in *B. hameri*), centrally

placed nucleus, which normally contained one nucleolus, but occasionally two (Fig. 25). Figure 26 illustrates diagrammatically the variable appearance of the perikarya which may be indicative of a cycle of secretory activity. Stage I was characterised by an homogenous, finely granular cytoplasm, which stained pale pink with azan. The nucleus had a similar granular appearance, but stained orange with azan. The cytoplasm of cells in Stage II of the cycle were more coarsely granular, as was the chromatin of the nucleus. Stage III cells were notable for their perinuclear Nissl zone which stained red with azan. This region extended into the axon hillock in Stage IV cells and was presumably indicative of a secretory phase. The nature of this secretion will be explored further below.

When histochemical staining methods were applied to sections of these cells the results were disappointing, in that the cytoplasm did not stain intensely with any of the stains (Table 2). Weakly positive reactions were obtained for proteins, arginine, acid mucopolysaccharides and lipid. A more positive reaction was obtained for glycogen with Best's Carmine although the PAS method gave a negative result for carbohydrate.

The perikarya lay subjacent to the sheath, as a single layer which covered the entire ventral surface of the tritocerebrum and extended laterally on either side of each lobe. Beneath this layer were several layers of glial cells (Fig. 27), the cell bodies of which contained a sparse cytoplasm, but a prominent, spherical to oval nucleus (approximately 7 μm in diameter), which stained orange with azan. Interspersed between these cells were inclusions which stained a deep red with azan and are equivalent to the gliosomes described by Pipa (1961). It is probable that such inclusions represent nutrition for the central nervous system.

Fig. 26. Diagram to illustrate a proposed cycle of secretory activity within the ventral perikarya of B.hameri and B.balanoides.

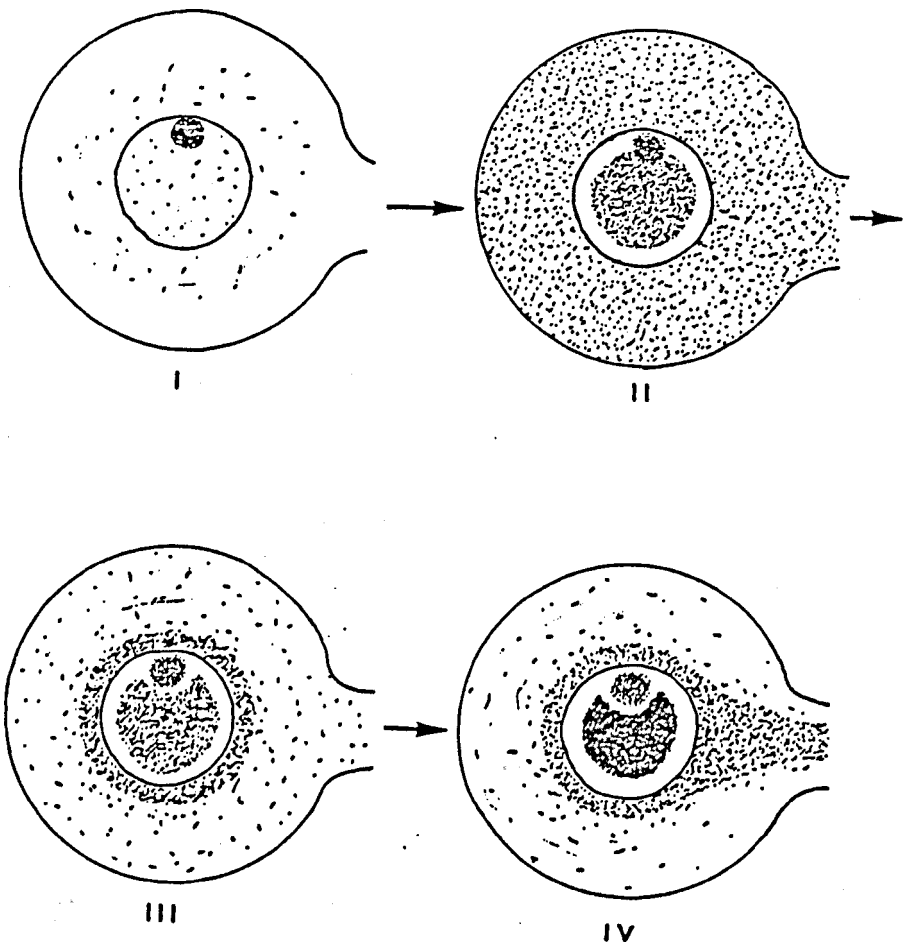

I
II
III
IV

TABLE 2 Histochemical characteristics of various elements of the barnacle ganglion

HISTOCHEMICAL TEST	SHEATH	NEURONES		NEUROPILE	GLIOSOMES
		CYTOPLASM	NUCLEUS		
Azan	blue	pale red	orange	pale purple	orange-purple
Mercury bromphenol blue	++	+	+++	+	+++
Sakaguchi reaction	+++	+	++	+	++
Diazotization-coupling	-	-	-	+	+++
D.M.A.B. nitrite	-	-	-	-	+++
D.D.D. (SS + SH)	-	-	-	+	-
P.A.S.	++	-	-	+	-
Best's carmine	+++	++	+	+	-
Alcian blue	+	-	-	-	-
Alcian blue pH 1.0	+	-	-	-	-
Alcian blue pH 2.5	+	+	-	-	-
Toluidine blue	+	-	+	+	-
Sudan black	-	+	-	+	-

- negative reaction; + weakly positive; ++ positive; +++ strongly positive

The gliosomes showed a highly positive reaction when stained for protein and specific amino acids. Interestingly, when ganglia were fixed in Stieve's solution and stained with azan the gliosomes were highly variable in colour, ranging at the extremes from orange to purple (Table 3). It would appear, therefore, that the gliosomes differed in their protein composition and this may reflect various stages of maturation and/or breakdown. Both the glial cells and gliosomes became more numerous around the periphery of the neuropile (Fig. 28), forming a layer which, in B. hameri, measured 20 to 25 μm in width.

Also underlying the ventral cell bodies were a group of much smaller cell bodies. In wax sections they were not readily resolved from the glial cells, but the greater detail made possible with 1 μm resin sections enabled their distinction (Fig. 29). They measured approximately 25 μm in diameter in both species, and had a centrally placed spherical nucleus of 8 μm diameter. The cytoplasm of the cells in B. hameri was more homogenous than that of B. balanoides. The latter contained large areas of pale blue cytoplasm when stained with toluidine blue, interspersed with regions which stained a dark blue, the overall impression being of a patchy cytoplasm.

The neuropile of the tritocerebrum, on the basis of axon configuration, can be described as unstructured diffuse, in the terminology of Maynard (1962). It is a very extensive region and thereby emphasizes the large input of sensory information to the tritocerebrum - in particular from the mantle cavity. The neuropile is traversed at several points by axon tracts extending between the antennular nerves and the circumoesophageal connectives (Fig. 30), and from cell bodies in the commissure and contralateral lobe.

In the protocerebrum, a second group of smaller cells were positioned

TABLE 3 Staining reaction of elements of the ganglion of barnacles using various fixatives

FIXATIVE	PERIKARYA			GLIAL CELLS			GLIOSOMES	NEUROPILE
	CYTOPLASM	NUCLEUS	NUCLEOLUS	CYTOPLASM	NUCLEUS	NUCLEOLUS		
BOUIN'S	Pale Pink	Orange	Bright Orange-Red	Pink/Purple	Orange	Red	Deep Red	Pale Mauve
STIEVE	Pale Brown	Orange/ Brown	Red	Pale Brown	Orange	Red	Orange and Green	Pale Grey/ Brown
CARNOY	Blue/Red	Red/Orange	Orange	Orange	Orange	Red/Orange	Bright Orange-Orange/Red	Blue/Red
ZENKER	Orange	Blue	Red	-	-	-	Orange/Purple	Red/Blue
SUSA S	Yellow/Orange	Orange	Bright Red	Pale Purple	Orange	Red	Orange-Red/Brown	Pale Blue/Pink
I	Blue/Red	Red	Bright Red	Red/Blue	Red	-	Yellow-Red	Blue/Purple

S - supraoesophageal ganglion; I - infraoesophageal ganglion

medially, in the commissure, near to the base of the antennular nerve. Processes from these cells supplied a small neuropile which was distinct from that of the tritocerebrum. In whole mounts of ganglia which had been stained with aqueous methylene blue, this region stained dark blue (Fig. 24). Three cells (approximately 40 μm in diameter in B. hameri) from this group, in each lobe, are equivalent to those described from the results of cobalt backfills, as having processes in the median ocellar nerve (Fig. 31). Also present in this region were large (35 μm diameter) discrete 'vacuoles' containing a highly granular material (Fig. 32). The granules appeared to be of two types, since they stained either an intense orange or red with azan. No such 'vacuoles' were observed in 1 μm resin sections. The possibility that they represent storage areas for neurosecretory material will be discussed in Chapter 3.

The remaining cell bodies of the protocerebrum were small (16-23 μm in diameter in B. hameri) with a highly vacuolated cytoplasm and a nucleus of between 9 and 11.5 μm in diameter. They became more numerous towards the dorsal surface of each lobe (Fig. 33). Similar cells have been noted in B. perforatus by van den Bosch de Aguilar (1979), who claimed that they represented neurosecretory cells which had released their neurosecretory product.

Also on the dorsal surface of the ganglion, closely apposed to the above group of cells, but underlying the neuropile of the tritocerebrum, and at the lateral margin of each lobe, was a single, large (55 μm diameter) unipolar perikaryon. It appeared identical to the large perikarya which cover the ventral surface of the ganglion. The axon from this cell could not be traced from the ganglion, but was observed to enter the commissure between the two lobes.

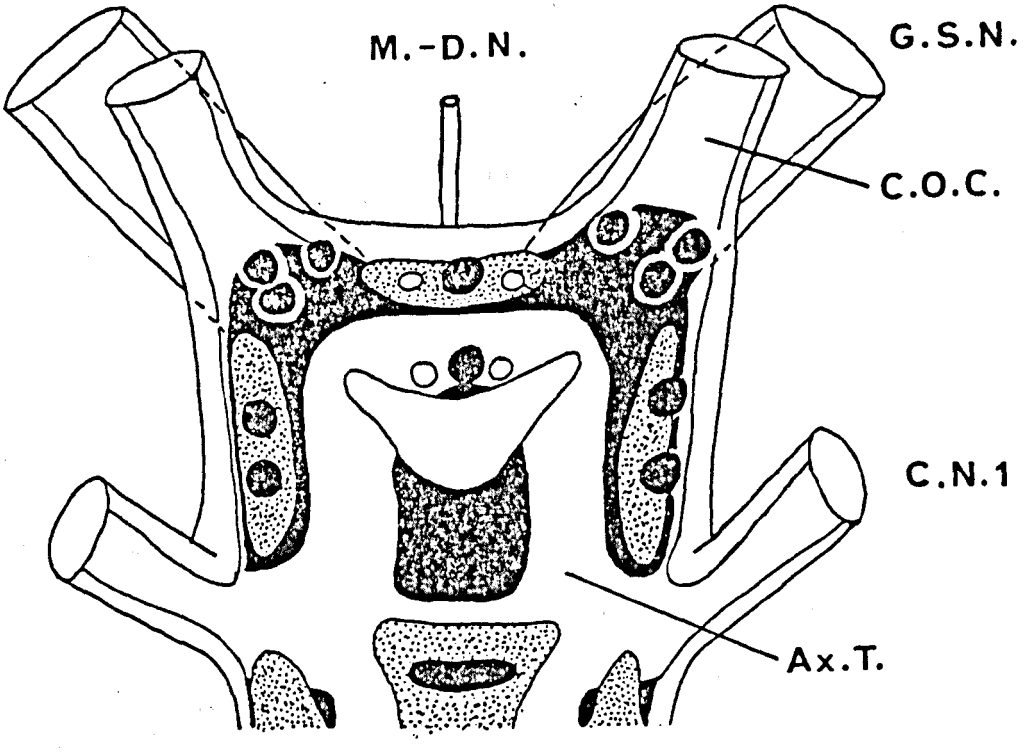
A thick sheath, or perineurium, enveloped the entire supraoesophageal ganglion (Fig. 34). This sheath could be resolved into two distinct layers. The inner layer, termed the perilemma, was composed of specialized glial cells which could be distinguished from the deeper glial cells by staining differences (c.f. Table 2). This feature has also been noted in other Crustacea (Bullock & Horridge, 1965) and in insects (Hess, 1958; Wigglesworth, 1959). The outer layer of the sheath, the neurilemma or neural lamella (Hoyle, 1953; Hess, 1958) was a thin (1-2 μm) noncellular layer, which, in arthropods, is thought to be secreted by cells of the perilemma (Bullock & Horridge, 1965). This layer stained a deeper blue with azan than did the perilemma.

Histochemical studies of the perilemma (Table 1) showed it to be a storage site for carbohydrate (P.A.S.) and in particular, glycogen (Fig. 35, and see below for ultrastructure). The glial cell processes also gave a positive reaction for protein and a strongly positive reaction for arginine. Lipid was never observed in the sheath.

Infraoesophageal ganglion: suboesophageal region

The suboesophageal region to be described is that which extends from the anterior margin of the ganglion to the level of cirral nerve 1. The major cell groups of this region are illustrated diagramatically in Figure 36. Four perikarya (approximately 23 μm in diameter in B. hameri) were positioned, symmetrically, either side of the tract to the mid-dorsal nerve. Two of these cells are illustrated in Figure 37. The cytoplasm was coarsely granular and stained red with azan. The chromatin of the nucleus (12.6 μm in diameter), which stained a darker red, had apparently shrunk away from the nuclear membrane, giving a halo effect. A similar phenomenon has been noted in Pollicipes polymerus (Barnes & Gonor, 1958)

Fig. 36. B.hameri : diagram to show the position of the major cell groups within the suboesophageal region of the infraoesophageal ganglion. ○ , individual ventral perikarya; ⬭ , ventral cell groups; ⬭ , medial cell groups; ⬭ , dorsal cell groups; ● , large dorsal perikarya.


M.-D.N.
G.S.N.
C.O.C.
C.N.1
Ax.T.

regardless of the fixative used, and in Munida iris (Bursey, 1979). In the latter case, the perikarya with this type of nucleus were termed halo cells, and the phenomenon was ascribed to a loss of material during histological processing rather than to shrinkage.

Just posterior to the above perikarya, extending from the neuropile of the circumoesophageal connectives toward the midline, were a group of small (10-25 μm in diameter in B. hameri) cell bodies which appeared to be of two types. Figure 38 shows the predominant smaller type. The cytoplasm of these perikarya, which stained pink with azan, was sparse and highly vacuolated. The spherical nucleus, 8 μm in diameter, contained granular chromatin and a single nucleolus. The second, larger perikaryon type (Fig. 39) had a more extensive cytoplasm which was finely granular and also stained pink with a slightly darker perinuclear region. The periphery of the cell was vacuolated. The nucleus was spherical, 12.5 μm in diameter and stained more densely than that of the previous cell type. Only one nucleolus was present.

Several groups of cells occupied a medial position in the ganglion. At the anterior margin a group extended between the tracts of the circumoesophageal connectives and was limited anteriorly and posteriorly by the sheath and a transverse tract respectively. At the lateral margins of the ganglion similar groups of cells extended between the nerve roots of the circumoesophageal connectives and cirral nerve 1, and between cirral nerves 1 and 2. Finally, in the midline, a group extended posteriorly from the transverse tract between the first pair of cirral nerves. The majority of the cells occupying these groups were the same as the two cell types described from the ventral surface. However, a third cell type was present. These cell bodies were between 15 and 25 μm in diameter. The cytoplasm of the cell was granular and stained

a dark red with azan. The nucleus, with a mean diameter of 11 μm, was spherical and centrally situated in the cell. The dense chromatin stained orange/red with azan and one or two nucleoli were observed. The possible neurosecretory nature of these cells will be discussed in Chapter 3.

Similarly, between the medially placed groups and the dorsal surface of the ganglion, the majority of the cells could be ascribed to one of the above cell types. There were, however, in addition, several large, symmetrically placed perikarya which were similar in appearance to the large ventral perikarya of the supraoesophageal ganglion. Evidence from cobalt backfills (see Fig. 19) suggests that some of these cell bodies send processes to the mantle via the suprasplanchnic nerve. Therefore, these cells are probably sensory neurones. The boundaries of the groups of cells on the dorsal side of the ganglion were delineated by the tracts which were very extensive in this half of the ganglion (Fig. 40).

The neuropile of the suboesophageal ganglion was divided into two lobes, which were positioned by dorso-ventrally projecting glial processes (Fig. 41). Either side of this cleft, at the central core of neuropile, was a region which differed strikingly in its staining characteristics from the rest of the neuropile. The region stained a dark red with azan and was positive for proteins (bromphenol blue) and polysaccharides (P.A.S.).

The supraoesophageal ganglion of Conchoderma auritum

The basic features of this ganglion, the neurone types and their distribution, are essentially the same as those already described for B. hameri and B. balanoides. The large perikarya of the ventral group (Fig. 42) measured approximately 35 μm in diameter and their large (14 μm in diameter), spherical, centrally placed nucleus had a single

nucleolus. The cytoplasm of some of these cells was highly vacuolated (Fig. 43).

Underlying this layer and closely apposed to the neuropile of the tritocerebrum were small unipolar perikarya.

Similar sized cells, but more elongate in shape (Fig. 44) occupied a position between the ocellar nerve and the antennular nerves. Some of these cells had darkly staining cytoplasm and may have been neurosecretory (see Chapter 3).

There was a large group of small, irregularly spherical perikarya on the dorsal side of each lobe. They measured 17 μm in diameter with a centrally placed spherical nucleus of 8 μm diameter.

The most striking difference between this ganglion and that of the Balanidae was the virtual absence of glial cells, with the exception of those contributing to the sheath. This condition is reminiscent of that found in the coelenterates and would appear to be a primitive characteristic (see Bullock & Horridge, 1965). Furthermore, gliosomes were absent from the ganglion. This is a feature which has been noted in some insects, for example, Rhodnius (Wigglesworth, 1959) and Oncopeltus (Johanssen, 1957).

Also absent from Conchoderma were the 'speckled spaces' of the protocerebrum. Large vacuoles were present, however, at the lateral margins of the ganglion, and it is possible that the contents had leached out during fixation.

Electron microscopy

Supraoesophageal ganglion:

Only the supraoesophageal ganglion of B. hameri was examined in

detail in the electron microscope. Although sections of B. balanoides were examined, the differences observed between the two species were merely a matter of size and do not warrant a separate description.

(i) Ventral perikarya of the tritocerebrum

Two types of perikarya could be identified on the basis of differences in the size of dense cored vesicles (d.c.v.'s) within their cytoplasm. These cells have been designated as types A and B (Figs. 45 and 46). Type A contained d.c.v.'s with a mean diameter of 77 nm; those of Type B measured 86 nm. The two vesicle types contained a core of material of similar electron density. Between this core and the limiting membrane was an electron-lucent zone - the halo - which is a characteristic of monoamines. In other respects the two cell types were very similar. Both contained a well developed endoplasmic reticulum of the smooth and rough type, and this was most prominent in the perinuclear zone (Fig. 47). This region corresponds to the Nissl zone as described from light microscope observations. Free ribosomes were also abundant in the cytoplasm. They tended to occur in clumps, which gave the cytoplasm a patchy appearance (Fig. 48). Mitochondria occurred throughout the cytoplasm, but were more prominent around the nucleus and near to the plasma membrane of the cell. They measured 0.4 μm in diameter and their cristae were of the transverse septate sype. Closely associated with the mitochondria, around the nucleus, were Golgi bodies (Figs. 49 and 50). In figure 50 vesicles, with electron-lucent contents, can clearly be seen budding from the rough endoplasmic reticulum. The vesicles carry the transcribed molecules for assembly and packaging within the Golgi sacs. The latter contained electron-dense material in those cells which were in a synthetic phase. Only 4-6 sacs were present in a Golgi complex, an unusually low number for an invertebrate, but not exceptional, since the neurones and epi-

thelial cells of the limpet, Patella vulgata, similarly have only 4-6 sacs (Threadgold, 1967). The d.c.v.'s budded off from the lateral margins of these sacs.

At the periphery of the perikarya and penetrating into the cytoplasm were many glial processes (Fig. 51), which contained cytoplasm of the surrounding glial cells (see below) and numerous vesicular inclusions. These processes correspond to the trophospongium described by other workers (Dehorne, 1935; Malhotra, 1957; Hess, 1958; Wigglesworth, 1959; Bullock, 1961). They are thought to pass nutrients to the neurones and to provide mechanical support (see Bullock & Horridge, 1965).

One of the small cell bodies which underlie type A and B cells is illustrated in figure 52. This cell type will be referred to, hereafter, as type C. The chromatin of the nucleus was in the form of electron-dense patches. In the cytoplasm d.c.v.'s of moderate electron density were present, but they were widely separated. They measured 86 nm in diameter and possessed a wide halo around their core. The most prominent features of the cell body were the rough endoplasmic reticulum, free ribosomes (Fig. 53) and large numbers of mitochondria, suggesting an actively synthesizing cell. Other cytosomes included membrane whorls and multivesicular bodies. Although the cell body was enveloped by a thin (70-200 nm in diameter) glial process, there was no invagination of the glial cytoplasm into the neurones.

Normally positioned beneath the large perikarya, but occasionally found subjacent to the sheath, was a fourth type of cell, type D (Figs. 54 and 55). Type D cells measured 15-20 μm in diameter and were characterized by large areas of cytoplasm of moderate electron density (c.f. cells of dorsal group). These areas were mainly confined to projections of the cell body (Fig. 56) and contained particulate material. The

individual particles measured 35 nm in diameter and may correspond to β particles of glycogen (c.f. Revel, 1964). A large number of mitochondria and a well developed system of rough endoplasmic reticulum occupied the periphery of the cell projections, as indeed they did within the cell body. Additional features of some of the projections were vacuoles and lysosomes. The latter were similar to those described as residual bodies by Threadgold (1967). They were also present in the cytoplasm of the cell body. Golgi bodies were situated close to the nucleus with small dense cored vesicles (diameter 103 nm) arising from their lamellae. The nucleus of the cell body in figure 55 is particularly interesting, since it illustrates that the 'halo' effect observed at the light microscope level with conventional fixatives (e.g. Bouin's) is in fact real. The chromatin of the nucleus is concentrated in a large central region leaving a band of nucleoplasm subjacent to the nuclear membrane, containing comparatively little chromatin. The latter region appeared empty in wax sections.

(ii) Perikarya of the protocerebral cell group:

One of the perikarya, positioned between the ocellar nerve and the antennular nerve, is shown in figure 57. The nucleus of this cell type was large and much of it was occupied by a central region of moderately electron-dense chromatin. Around this region and subjacent to the nuclear membrane was a narrow zone relatively free of chromatin. Many Golgi bodies were present, particularly in the perinuclear zone. Dense core vesicles were also present, but too few were observed to give a reliable mean value of their size. They were, however, all smaller than 100 μm and possessed a halo around their core and so they were probably neurotransmitters. The cytoplasm was packed with mitochondria and there was

also much rough and smooth endoplasmic reticulum and free ribosomes. Few lysosomes were observed. Projecting into the cell cytoplasm was a well developed trophospongium (Fig. 58). The depth to which the glial processes projected varied between cells of this type; some extended very close to the nucleus.

A second type of protocerebral cell is shown in figure 59. The perikarya of this type were much smaller than those of the previous cell type. The nucleus which was small, spherical and centrally placed within the cell body had a small, central area of moderately electron-dense chromatin. The nucleolus was positioned at the periphery of the nucleus in a zone containing small electron-dense patches of chromatin. The cytoplasm contained a well developed system of rough endoplasmic reticulum which was mainly confined to the perinuclear zone. Free ribosomes and lysosomes were abundant and occurred throughout the cytoplasm. There was, however, little smooth endoplasmic reticulum. Golgi bodies and the dense-cored vesicles arising from them were also few in number. The former occurred in the perinuclear zone. The dense-cored vesicles were again too few to give a reliable estimate of size, but appeared to be neurotransmitters.

A third type of cell was also found in the protocerebral region and will be discussed in Chapters 3 and 4.

(iii) Perikarya of the dorsal cell group:

Apart from the large dorsal perikarya described in the light microscope section, only one type of neurone was found in the dorsal cell group (Fig. 60). This appeared very similar to the type D cell bodies which underly the large ventral perikarya of the protocerebrum. At the centre of the cell body was a spherical nucleus. It contained a large

central area of chromatin of moderate electron density and around this was a band of nucleoplasm containing small, electron-dense clumps of chromatin. There were many profiles of smooth endoplasmic reticulum and free ribosomes in the cytoplasm, particularly in the perinuclear zone. Lysosomes, including residual bodies, were also abundant. The latter were of varying electron density and the majority appeared to contain membrane whorls (Fig. 61) which also occurred freely in the cytoplasm. A few dense-cored granules were present in the cytoplasm. The granules had a mean diameter of 78 nm and a prominent electron-lucent zone between the core and the limiting membrane.

(iv) The neuropile:

No specialized regions of neuropile, such as glomeruli or stratified (c.f. Bullock & Horridge, 1965) were observed at the light microscope level and the ultrastructural observations have corroberated its simplicity relative to other mobile crustaceans. The neuropile which forms the core of the tritocerebral lobes is the main region in the supraoesophageal ganglion. Within this region several types of dense-cored granule were observed. The large types, i.e. those larger than 100 nm, will be discussed in Chapters 3 and 4, since they were deemed likely to be peptidergic. Regarding the smaller granules, four types could be resolved strictly on the basis of granule size, with the possibility of a further two types existing through differences in the electron density of their granule core. The different types are illustrated in figures 62-66. They have been designated letters signifying the cell type from which they are likely to have originated; i.e. type a granules are similar to the granules found in type A perikarya. Granules of type e and f could not be equated with any definite cell type. There are two important

points to note regarding type b granules. First, although this granule type is present in type B neurones, the granules could equally be derived from the dorsal neurones, because the dense-cored granules in these two cell types are indistinguishable. It is perhaps for this reason that, of those granules in the neuropile which measure less than 100 nm in diameter, type b granules predominate. Secondly, exocytosis of type b granules has been observed in the neuropile (Fig. 67). The granules were not released at synapses and this fact suggests that they could be small neurosecretory granules.

Electron-lucent vesicles (ca. 40 nm) were occasionally observed in the same axons as the dense-cored granules (Fig. 68) and this feature is probably indicative of synaptic transmission. Axo-axonal synapses were in fact frequently observed within the neuropile (Fig. 69). Characteristically, clusters of clear vesicles were apposed to the presynaptic membrane and this was separated from the postsynaptic membrane by a synaptic cleft which was about 280 nm wide and contained electron-dense material. The membranes of the axons were thickened in this region. This axon type is probably equivalent to that described as "simple contact en passant" by Bullock and Horridge (1965) and is the dominant type in the invertebrate neuropile.

A further type of nerve fibre common to the neuropile contained no granules or vesicles (Fig. 70). These axons were normally much larger in cross-section than the granule-containing types. Their most obvious feature was the evenly distributed neurotubules of about 40 nm in diameter. Interconnecting the tubules was a complex system of neurofilaments (Fig. 70, inset). Occasional mitochondria and profiles of smooth endoplasmic reticulum were also observed in these axons. Similar nerve processes have been noted, by Bohm and Parker (1968, figure 2), in the supraoesophageal ganglion of Daphnia schlødleri.

The only difference between the two is the smaller diameter of the neurotubules in Daphnia (ca. 26 nm).

(v) Glial cells:

Figure 7 illustrates two glial cells typical of those observed in the central nervous system. The nuclei were irregular spheres containing patches of electron-dense chromatin. The latter were more pronounced in the region subjacent to the nuclear membrane.

The soma and axons of most of the nerves were enveloped in thin glial processes. Some of these processes penetrated the perikarya forming the trophospongium, as described above, but were also found to project into the axoplasm (Fig. 72). A similar phenomenon has been noted in the axons of Helix (Schlote, 1957) and Aplysia (Batham, 1961). Gliosomes were occasionally noted in the cytoplasm of the glial processes around the axon (Fig. 73). However, the most prominent feature was the microtubules which ran parallel to the longitudinal axis of the axons (Fig. 74). The microtubules presumably act as a cytoskeleton within the nerve and help to maintain its integrity. The plasma membrane of the glial cell was separated from that of the neurone/axon by an intercellular space of about 20 nm width. The membranes of the glial cell processes were interconnected along their length by numerous desmosomes (Fig. 75).

Although glial cells at all levels of the ganglion contained gliosomes, these cytoplasmic inclusions were most evident in the glial cell rind surrounding the neuropile (Fig. 28). Gliosomes of varying electron density were observed (Fig. 76), thus supporting the light microscope evidence for their heterogeneity. The origin of the gliosomes is still a matter of conjecture. Golgi bodies were present in the glial

cytoplasm (Fig. 77) but 'precursor granules' were never seen to arise from their lamellae. However, electron-dense granules of varying size were observed in the cytoplasm (Fig. 78) and these may give rise to mature gliosomes.

Other inclusions commonly found in the glial cytoplasm were mitochondria, small electron-lucent vesicles, vacuoles and rough endoplasmic reticulum. Glial fibrils, such as those observed in insect (Pipa, 1961) and molluscan (Wendelaar Bonga, 1970; Nolte et al., 1976) glial cells, were not observed in the barnacle supraoesophageal ganglion, although cells of this type were present in the infraoesophageal ganglion (see below).

(vi) The perineurium:

The entire nervous system is separated from the surrounding haemolymph by a perineurium of varying thickness. Figure 79 is representative of much of the covering of the supraoesophageal ganglion. The inner perilemma was formed from glial cell processes similar to those which enveloped the nerves (see above). The perilemma has a 'spongy' appearance when viewed in the scanning electron microscope (Fig. 80). The outer neurilemma was a more complex structure. Glial cytoplasm, containing oval to elongate nuclei, mitochondria and glycogen rosettes, was interspersed between a meshwork of collagen fibrils (Fig. 79, inset).

The exception to the above was that part of the sheath which extended over perikarya of the ventral and in particular the protocerebral cell group. Here the sheath appeared to lack a perilemma and was composed solely of a thin neurilemma cotaining collagen fibrils (Fig. 81).

In the outermost layer of the perineurium occasional discontinuities in the neurilemma could be observed. These pores (Fig. 82) opened into channels which ran between the perilemma cells. The channels could not be traced over long distances and so their destination remains uncertain, but they can certainly be considered to be extensions of the circulatory system.

Occasionally, axons containing small (<100 nm) dense-core vesicles were present in the sheath (Fig. 83). Barber (1982) also demonstrated monoamine-containing varicosities in the neural sheath of Philine aperta. The axon terminals which are thought to contain serotonin (5-HT) may have a secretory rôle.

Infraoesophageal ganglion

The infraoesophageal ganglion was not examined in detail. Only those aspects of the suboesophageal region studied by light microscopy and which eluded interpretation were re-investigated using the transmission electron microscope. These were the thick sheath and its polysaccharide content, the central region of the neuropile, in either lobe of the ganglion, which stained positively with P.A.S. and bromphenol blue, and the nature of the 'cleft' which partitioned the two lobes of the ganglion.

(i) Silver proteinate test for glycogen in the perineurium:

Figure 84 is a transverse section of the perineurium of the suboesophageal ganglion of B. hameri stained with silver proteinate. The compartments containing glycogen, with the characteristic α rosette (Fig. 84, inset), are clearly defined from the rest of the sheath, which takes up little or no stain.

(ii) The suboesophageal 'cleft':

At the ultrastructure level the 'cleft' was found to be composed of inpushings of glial cell processes, which contained vesicles, lysosomes, microtubules and glycogen (Fig. 85). The latter occurred as both β and α particles (Fig. 86). Their identity was confirmed with the silver proteinate test (Fig. 87). Also in the cleft were structures which resembled the tracheoles of the cockroach, Periplaneta americana, figured by Hess (1958) (Fig. 88).

(iii) The neuropile:

On either side of the cleft were numerous axons which, in general, ran along the longitudinal axis of the ganglion (Fig. 89). Several types could be identified on the basis of their content (Fig. 90). The most common contained dense-cored granules and microvesicles, which may explain the positive result obtained with bromphenol blue. Many also contained glycogen (Fig. 91), thereby explaining the positive P.A.S. reaction of this region.

DISCUSSION

The dearth of information on the internal anatomy of the central nervous system of cirripedes necessitated such a study before the more specialized topic of neurosecretion (Chapters 3 and 4) could be tackled.

Previously, our knowledge of the anatomy of cirripede ganglia was largely reliant on the morphological study of Gwilliam and Cole (1979) which utilized the cobalt backfilling technique. Since their needs were for neurone maps to use in neurophysiological experiments, many important aspects of the ganglia were not examined. As a result, some of the questions posed by Gwilliam and Cole (ibid) which could not be answered on the basis of their results can now be addressed. It is also possible

to compare the results of the two studies.

The neurone maps of B. cariosus (= Semibalanus cariosus), in general, correlate well with those presented for B. hameri. However, differences in detail do exist, the main ones being as follows:-

(i) Backfills of the median ocellar nerve of B.cariosus revealed two cell bodies in each lobe, with a single axon extending from them into the nerve. Gwilliam and Cole believed these cell bodies to be motor neurones, innervating the muscle which accompanies the ocellar nerve. Backfills of this nerve in B. hameri revealed two groups of cells, each containing three cell bodies, which contributed a total of six thin processes at one side of the nerve. It is believed that these cells are, in fact, neurosecretory (see Chapters 3 and 4).

(ii) In addition to the large perikarya of the tritocerebrum, which were filled via the antennary nerve of both barnacle species, three cells from the protocerebrum of B. hameri were filled, one of which occurred in the contralateral lobe. It is possible that these cells do not exist in B. cariosus, but it is equally possible that the filling times employed by Gwilliam and Cole (1979) were too short to reveal them.

(iii) A single cell was filled in the contralateral lobe of the supra-oesophageal ganglion by backfilling the suprasplanchnic nerve of B. hameri, whereas in B. cariosus three perikarya were filled.

(iv) Backfills of the circumoesophageal connective toward the supra-oesophageal ganglion filled many more neurones in B. hameri than in B. cariosus. It is readily apparent in both cases, however, that many of the cells of the protocerebral group (and in B. hameri the dorsal group also) send processes to the infraoesophageal ganglion. Since axons of both the median and the lateral photoreceptor cells end blindly in the protocerebral region, one or more of the cells filled via the

connective may represent the interneurones which are thought to be responsible for conveying information from the photoreceptors through the circumoesophageal connectives (c.f. Millecchia & Gwilliam, 1972; Lantz & Millecchia, 1975).

(v) More cells were filled in the infraoesophageal ganglion *via* the circumoesophageal connective in *B. hameri* than in *B. cariosus* (figures 4C and 5A combined of Gwilliam and Cole, 1979). The general pattern, though, of a greater number of cells being backfilled in the ipsilateral side and most of these being associated with the cirral nerves, was true of both species.

(vi) Backfills *via* the mid-dorsal nerve resulted in a larger number of cells filled, with greater symmetry, in *B. cariosus* than was the case in *B. hameri*. The small diameter of the nerve (ca. 6.5 μm) together with the very fine nature of the axons which it contains (Fig. 92), make this a difficult nerve to backfill. It is distinctly possible that when the nerve of *B. hameri* was cut prior to backfilling, some of the axons were pinched, thus preventing the diffusion of cobalt along them. This would obviously give an underestimate of the number of perikarya with processes in the nerve. The backfill figured by Gwilliam and Cole may thus represent a more complete picture.

(vii) Many more cells were backfilled *via* the great splanchnic nerve in *B. hameri* than in *B. cariosus*. The distribution of the backfilled perikarya in the two species is also different. Allison (1981) also backfilled this nerve in *B. hameri* and obtained similar results to those of the present study (compare Fig. 32 of Allison with figures 21 and 23 combined of the present study). It is unlikely, therefore, that the backfills *via* the splanchnic nerve were artifactual in *B. hameri*.

It is pertinent, with reference to the differences between the two

studies, that a report describing artifacts observed in cobalt backfilling techniques has recently been published (Hackney & Altman, 1982). "Parallel filling" - a term used to describe the process whereby neurones are filled with cobalt, even though they do not have a process in the backfilled nerve (Altman & Tyrer, 1980) - is risked under the following extreme conditions of backfilling:- (i) high cobalt chloride concentrations (0.4M and above), (ii) cutting nerves other than the one to be backfilled, (iii) applying a current across the preparation, (iv) amplifying cobalt deposits with the silver intensification procedure (Tyrer & Bell, 1974) and (v) long filling times (24-48 hrs.). In the present study, only point (ii) was transgressed. Furthermore, Hackney and Altman (1982) used a filling time of 18 hr. at 4°C. This length of time was rarely used to backfill B. hameri nerves. It can be concluded that the backfill results presented for B. hameri are unlikely to be artifactual. The study of Gwilliam and Cole (1979), however, transgressed all the conditions, other than applying a current across the preparation. It is therefore possible that their results are not a true representation of the neurone morphology of B. cariosus.

Six neurone types, excluding those which appeared to be peptidergic, have been shown to be present in the supraoesophageal ganglion of B. hameri. Two of these types can be recognized in reports figuring 'neurosecretory cells'. Barnes and Gonor (1958) described two neurosecretory cell types in the ganglia of Pollicipes polymerus. The larger type can be equated with type A and B cells of B. hameri. The smaller type, with their highly 'vacuolated' cytoplasm, are similar in both description and position to type D cells. Van den Bosch de Aguilar (1976) also described a cell type ("P") in the supraoesophageal ganglion of B. perforatus and B. balanoides which could be equivalent to type D of

the present study. The type "G" and "A" cells described by van den Bosch de Aguilar (ibid) on the "ventral" (= dorsal in this study) surface of the suboesophageal ganglion are similar to type A and B cells of B. balanoides and B. hameri. It is pertinent that van den Bosch de Aguilar (ibid) distinguished type "G" from type "A" cells on the basis of histochemical tests, since type A and B cells of the present study have been shown to contain dense-cored granules of different types. The fact that both van den Bosch de Aguilar (1979) and Barnes and Gonor (1958) described these large motorneurones (c.f. Gwilliam and Cole, 1979) as being neurosecretory on the basis of their staining characteristics when viewed at the light microscope level, emphasizes the inadequacy of this technique for neurosecretory work (see Chapter 3).

The internal anatomy of the supraoesophageal ganglia of the three species of barnacle studied was very similar and illustrates well the phenomenon of convergent evolution. Of course C. auritum is a lepadomorph barnacle with segmented thoracic ganglia - a primitive characteristic - but essentially its neurophysiology cannot be envisaged to be remarkably different from that of the balanomorphs. The similarity in neurone anatomy of the two groups is, therefore, not surprising. The apparent lack of gliosomes in the supraoesophageal ganglion of C. auritum, however, may not be a normal condition. It must be remembered that this is a barnacle which will have been in temperate waters for some time prior to collection. Consequently, the animals used in this study may have been in a malnourished state. Since gliosomes are assumed to be a source of nutrients for the neurones, then an undernourished animal may well be depleted of gliosomes.

During the metamorphosis of the cypris larva to the adult barnacle,

the compound eyes and the antennules of the former degenerate. Their integrative regions in the supraoesophageal ganglion, namely the optic lobes and the deutocerebrum, are also lost. Moreover, certain neurones in the central nervous system degenerate (Walley, 1965). The result is a supraoesophageal ganglion which is much simplified compared to that normally encountered in the Crustacea (c.f. Bullock & Horridge, 1965). There does, however, appear to be some compensation in the adult for these losses. The tritocerebrum has come to occupy a major portion of the supraoesophageal ganglion, presumably reflecting an important rôle for the mantle lining in terms of chemoreception. A similar situation has been reported to occur in terrestrial isopods and amphipods (Graber, 1933; Madsen, 1960). Attached to the adaptation to the terrestrial mode of life is a reduction in the antennules and the olfactory lobes and an increase in the importance of the antennae as sensory structures. As a result, the neuropile of the tritocerebrum enlarges and the glomeruli cells become well developed. The latter do not occur in barnacles as has been noted, albeit indirectly, by Barnes and Gonor (1958).

Barnes and Gonor (1958) also noted that each lobe of the suboesophageal ganglion contained a large centrally located area of neuropile, which stained intensely with paraldehyde fuchsin (a neurosecretory stain - see Chapter 3). The neuropile was found to be composed of small axons. This region has also been noted in B. balanoides and B. hameri. It stained positively with bromphenol blue and P.A.S. Furthermore, the presence of many fine axons in this region has been confirmed. They have been shown to contain dense-cored granules of varying dimensions, but within the size range commonly accepted as representing neurotransmitters.

The perineurium, together with the 'cleft' of the suboeosophageal ganglion, has been shown to contain large stores of glycogen. Similarly,

the perineurium cells of the cockroach Periplaneta americana were found to be full of glycogen (Wigglesworth, 1960). Wigglesworth (ibid) has proposed that glycogen is transferred from the sheath to the neurones by way of the glial cells and in particular the trophospongium. Although this route is possible, the actual presence of glycogen within the glial cytoplasm of the perineurium has yet to be shown. Glycogen was never observed in the trophospongium of barnacle neurones, although small amounts were occasionally observed in the neuronal and glial cytoplasm. A trophic rôle for the glia has recently received support from the findings of Viancour et al. (1981). Lucifer yellow CH dye injected into the giant axons of the crayfish, Procambarus clarkii was found to rapidly enter the adaxonal sheath glia. It has been suggested that this transfer of dye represents a pathway for the exchange of intercellular molecules between axons and glia.

Fig. 5. B.hameri : cobalt backfill of the supraoesophageal ganglia via the ocellar nerve. Scale bar, 100 µm.

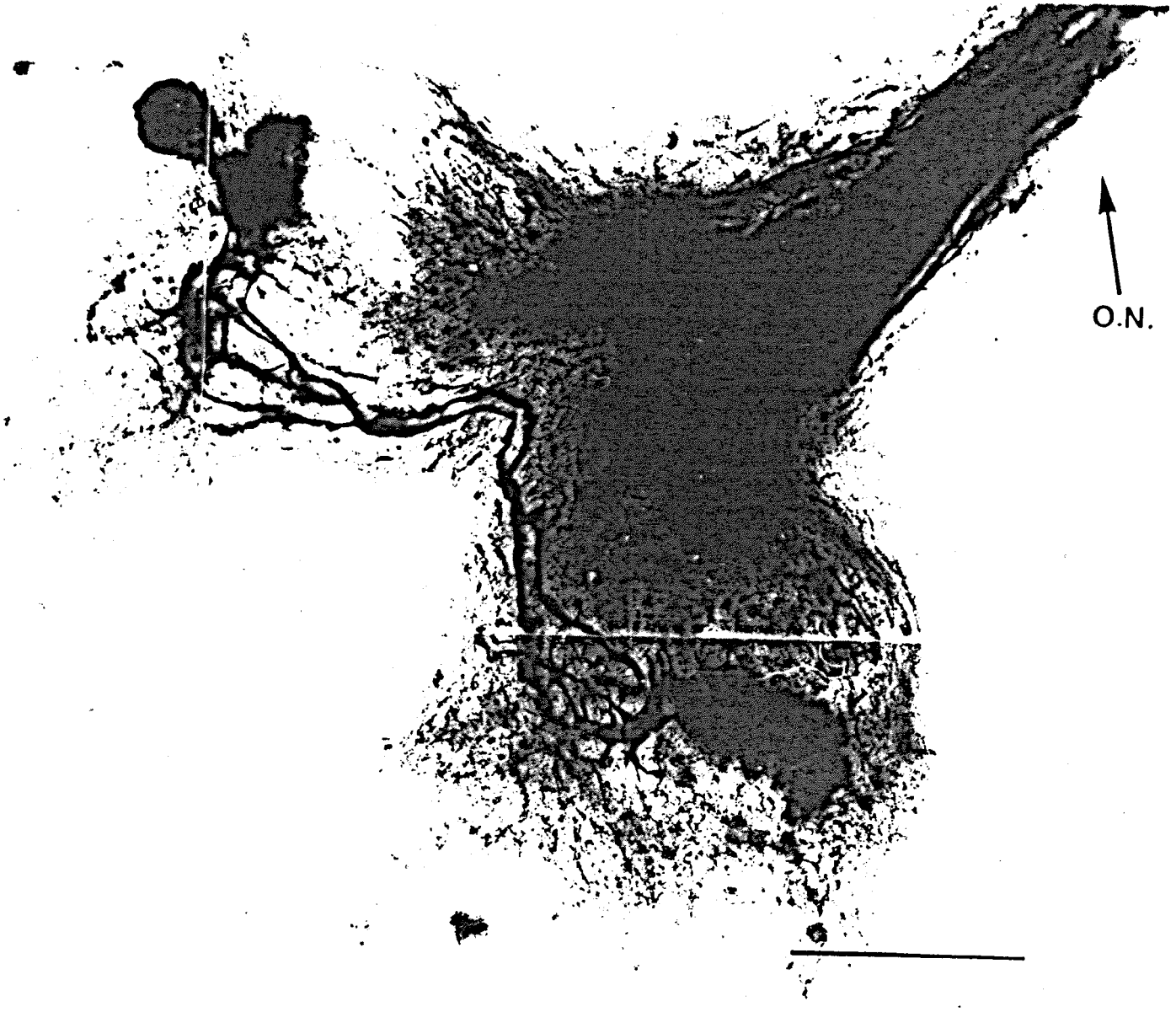
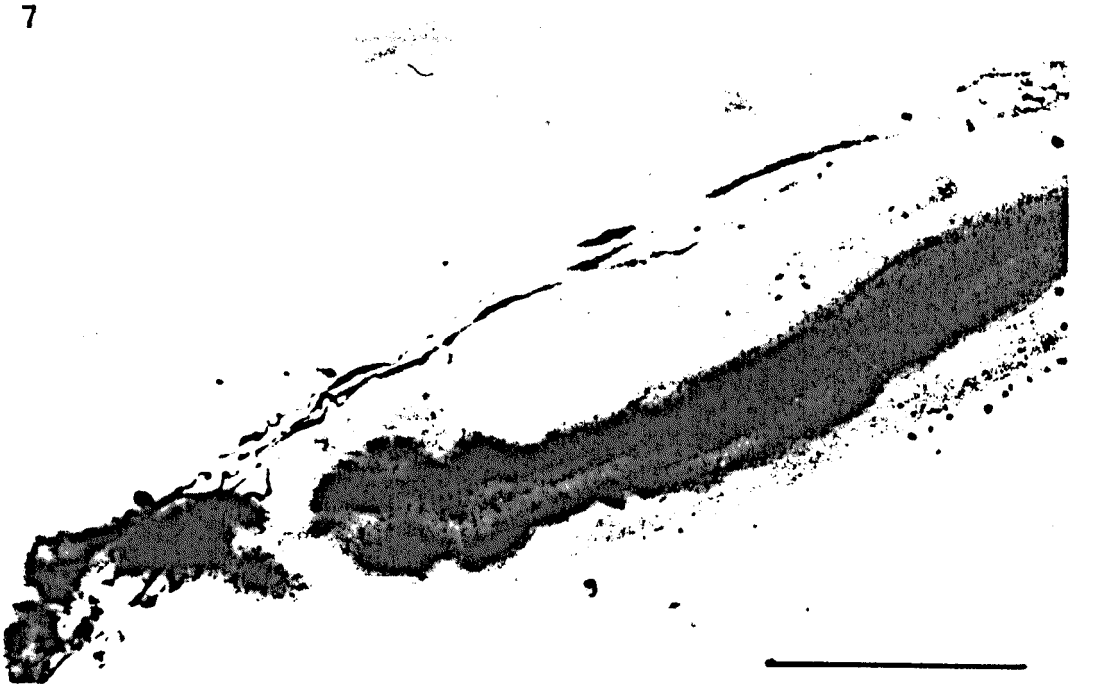

O.N.

Fig. 7. B.hameri : cobalt backfill of the supraoesophgeal ganglion via the ocellar nerve showing the fine axons which extend from neurones within the ganglion. Scale bar, 100 µm.

Fig. 8. B.hameri : cobalt backfill of the supraoesophageal ganglion via the antennular nerve. Scale bar, 100 µm.

7



8

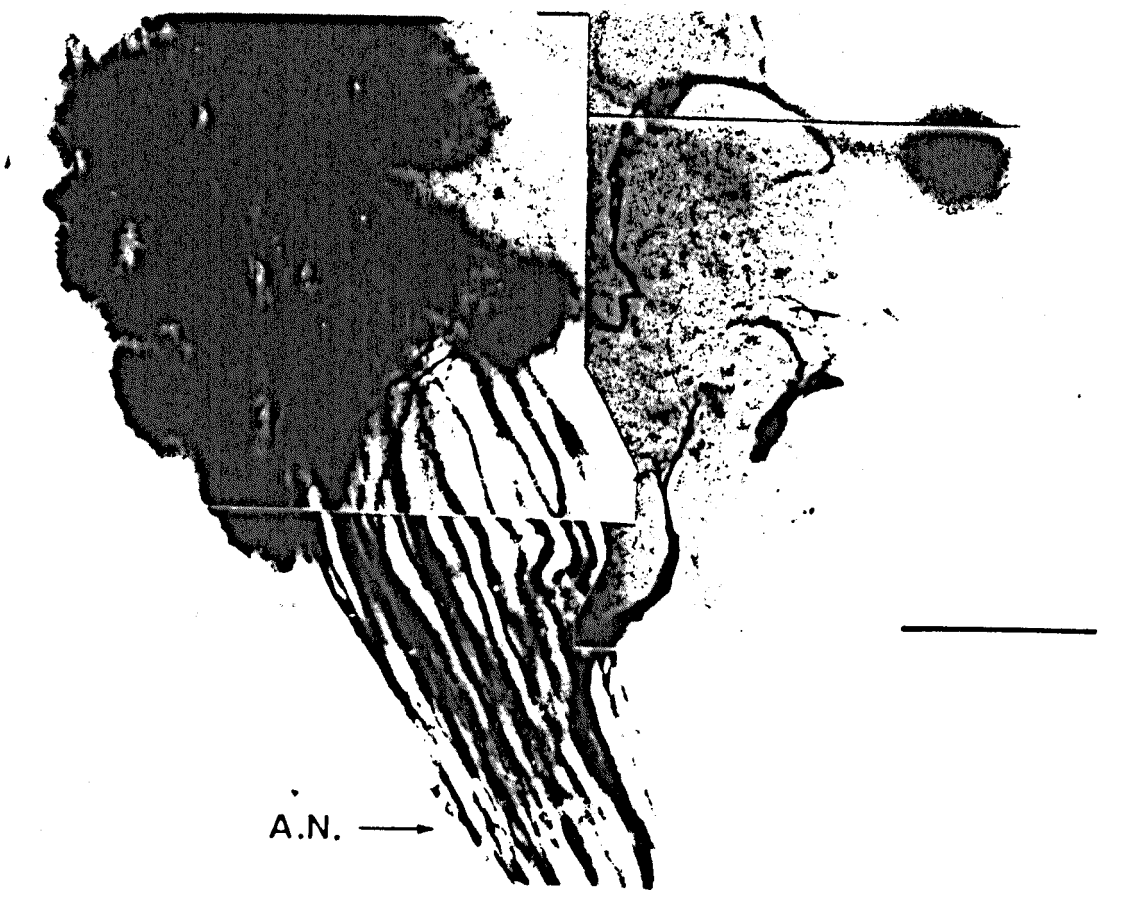
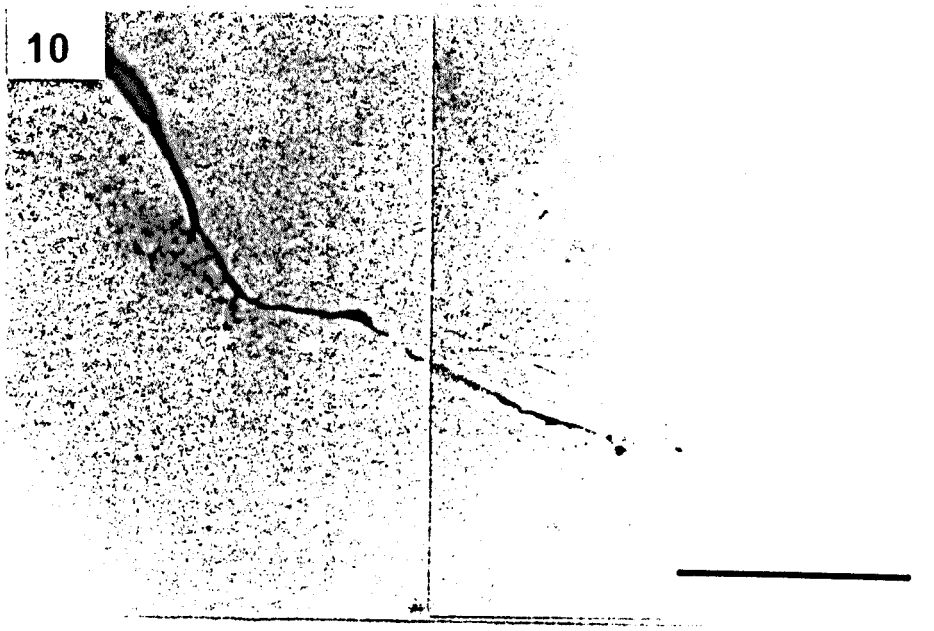


Fig. 10. B. hameri: cobalt backfill of the supraoesophageal ganglion via the lateral photoreceptor nerve. Scale bar, 100 μm.

Fig. 12. B. hameri: cobalt backfill of the supraoesophageal ganglion via the suprasplanchnic nerve. Scale bar, 100 μm.


10

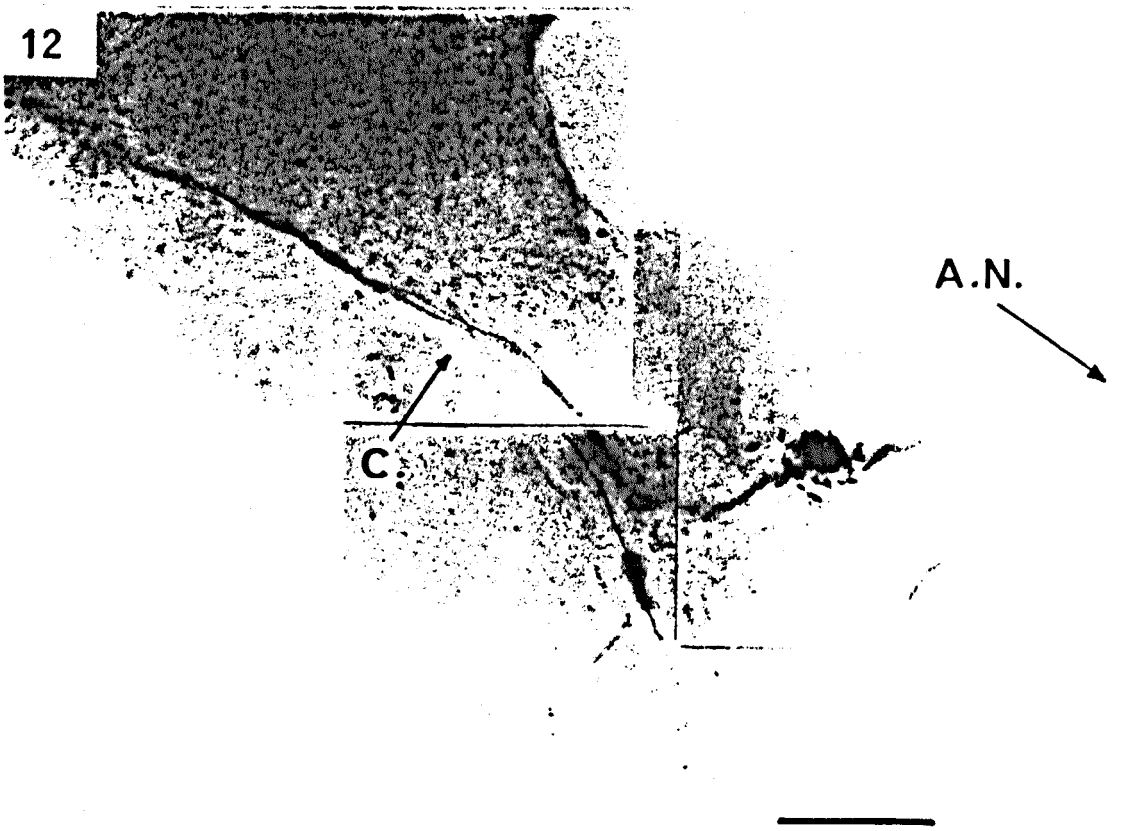
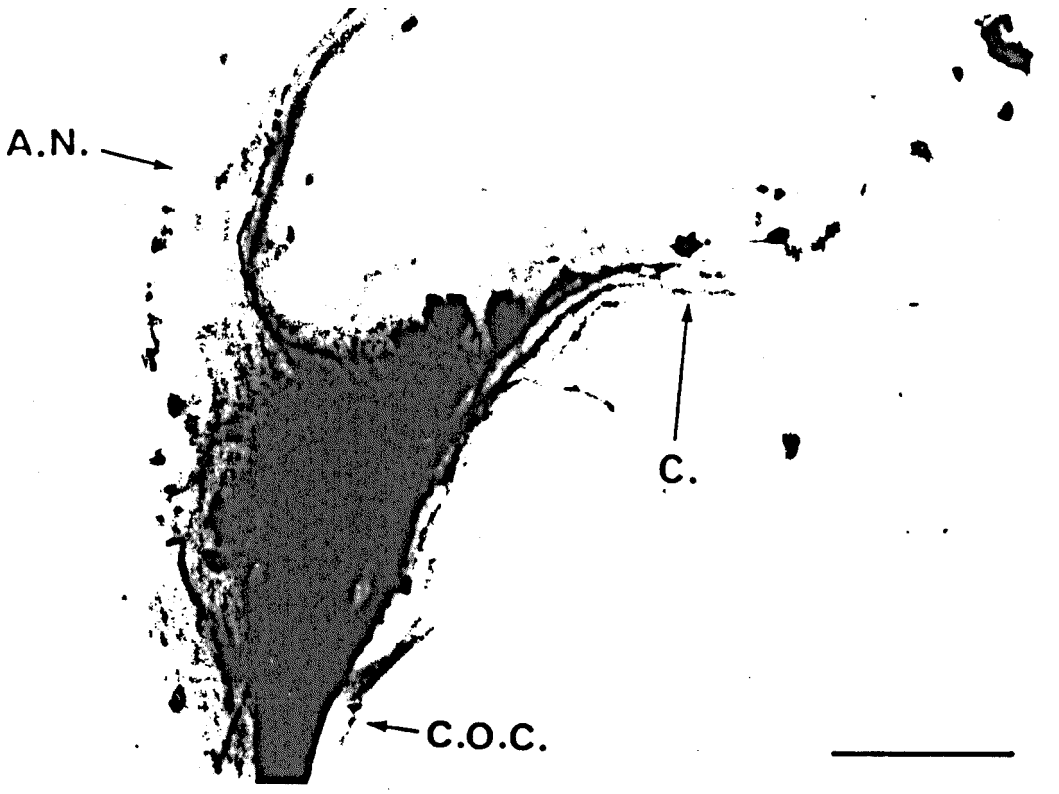

12
A.N.
C

Fig. 14. B.hameri : cobalt backfill of the supraoesophageal ganglion via the circumoesophageal connective. Scale bar, 100 μm.

Fig. 16. B.hameri : cobalt backfill of the infraoesophageal ganglion via the circumoesophageal connective cut distal to the branch to the suprasplanchnic nerve. Scale bar, 200 μm.

14

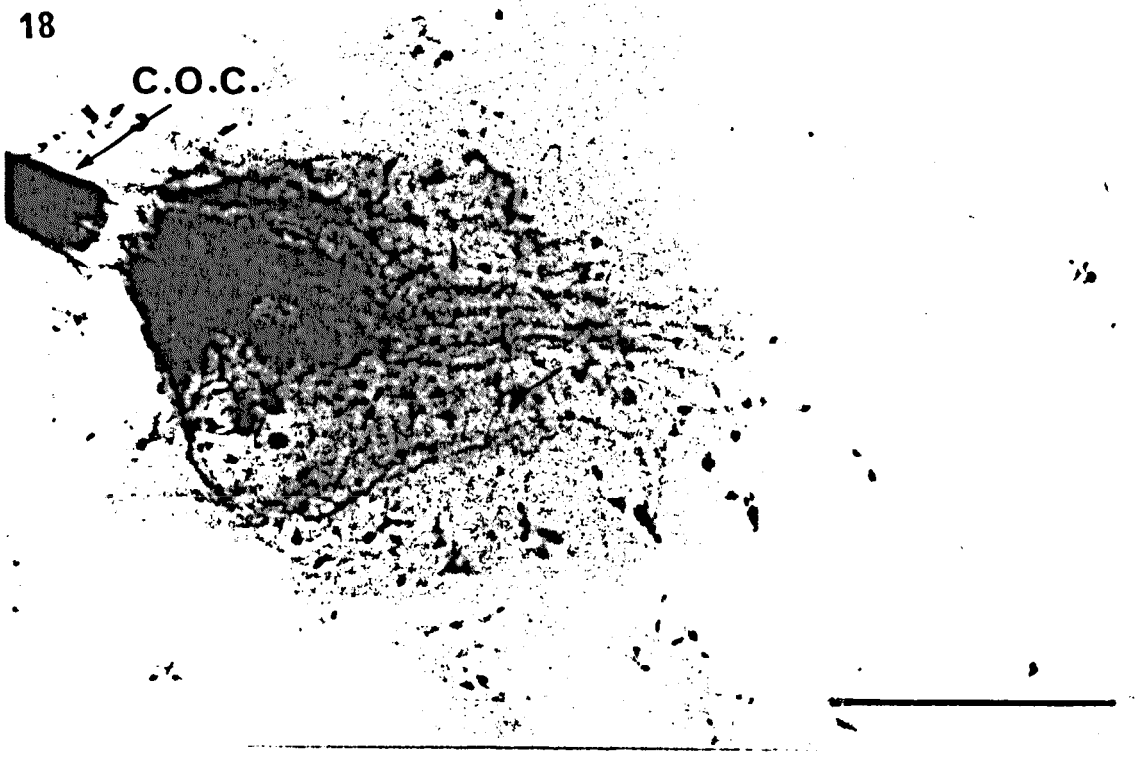


16

Fig. 18. B.hameri : cobalt backfill of the infraoesophageal ganglion via the circumoesophageal connective cut proximal to the branch to the suprasplanchnic nerve. Scale bar, 800 µm.

Fig. 22. B.hameri : cobalt backfill of the infraoesophageal ganglion via the great splanchnic nerve cut proximal to the branch to the suprasplanchnic nerve.

18



22

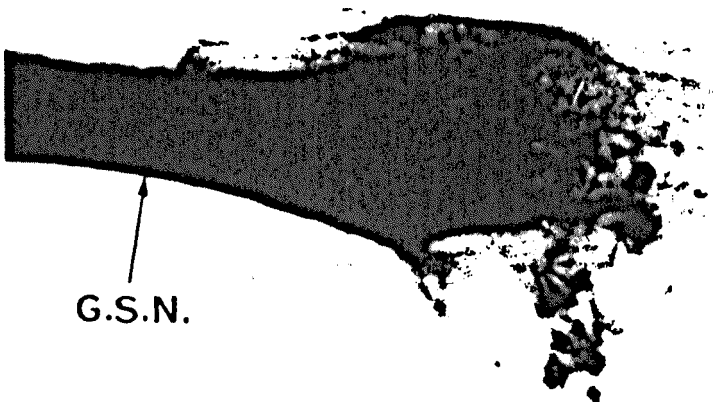


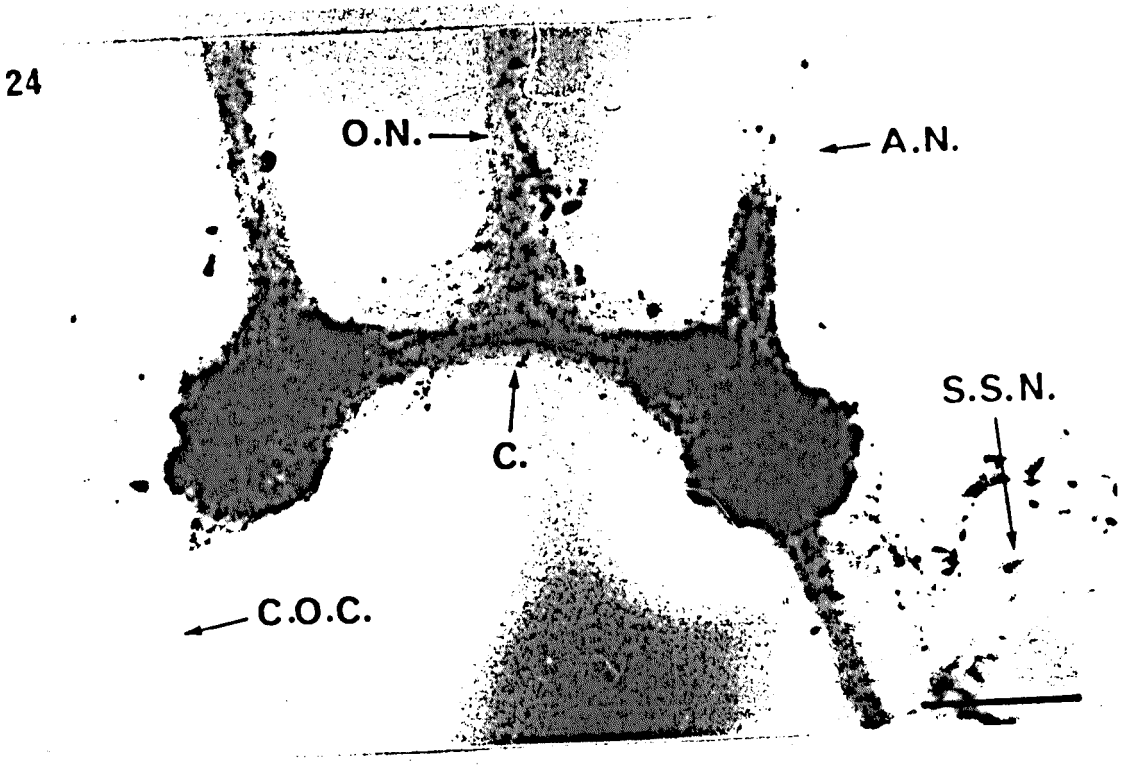
Fig. 24. B. hameri: whole mount of the supraoesophageal ganglion stained with aqueous methylene blue. The large spherical neurones of the tritocerebrum and the more darkly staining neurones of the protocerebrum are clearly visible. Scale bar, 1 mm.

Fig. 25. B. hameri: light micrograph of a wax section of the supraoesophageal ganglion showing one of the large perikarya of the tritocerebrum. Scale bar, 25 μm.

Fig. 27. B. hameri: light micrograph of a 1 μm resin section through a group of glial cells. Scale bar, 25 μm.

Fig. 28. B. hameri: 1 μm resin section through the supra-oesophageal ganglion showing the layer of glial cells (arrow) around the central neuropile. Scale bar, 100 μm.

Fig. 29. B. balanoides: 1 μm resin section through the trito-cerebrum showing one of the small perikarya (arrow) which underlie the large ventral perikarya. Scale bar, 50 μm.


24
O.N.
A.N.
S.S.N.
C.
C.O.C.


25
N.
Sh.
27
N.

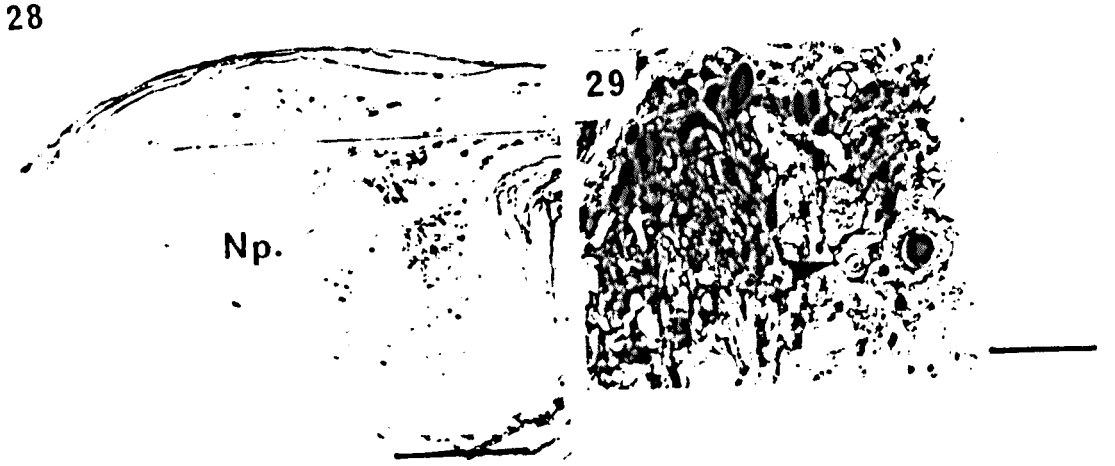

28
Np.
29

Fig. 30. B. hameri: wax section showing the axon tracts (arrow) that extend between the antennular nerves and circumoesophageal connectives. Scale bar, 100 μm.

Fig. 31. B. hameri: 1 μm resin section through the supraoesophageal ganglion showing one of the neurones of the protocerebral group (arrow). Note that the axon extends into the commissure. Scale bar, 100 μm.

Fig. 32. B. hameri: wax section of the protocerebral region. Three large vacuoles of granular content (termed "speckled spaces") are positioned either side of a neurone. Scale bar, 25 μm.

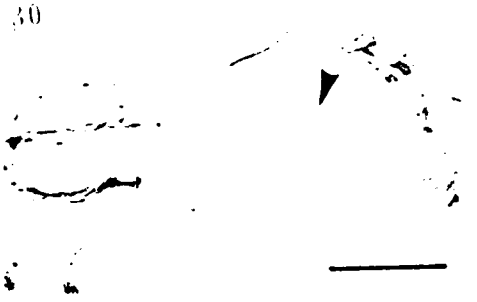
Fig. 33. B. hameri: wax section through the dorsal side of the protocerebrum. The small neurones have a highly vaculated cytoplasm. Scale bar, 50 μm.

Fig. 34. B. hameri: wax section through a region of the sheath which envelops the supraoesophageal ganglion. Scale bar, 25 μm.

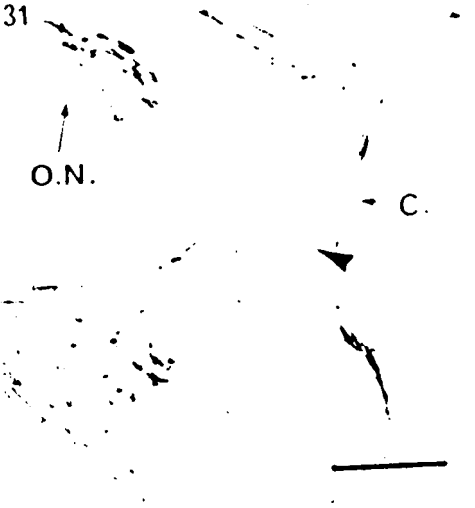
Fig. 35. B. hameri: wax section of the perilemma stained with Best's carmine. Large aggregates of glycogen are present in the cell processes. Scale bar, 25 μm.

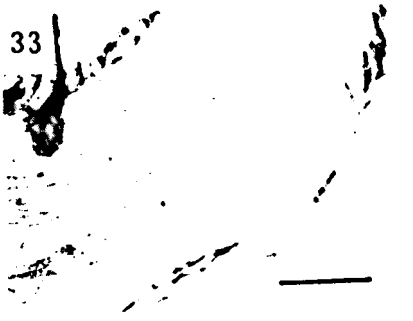
Fig. 37. B. hameri: wax section through the suboesophageal region of the infraoesophageal ganglion showing two of the four neurones which are positioned either side of the tract to the mid dorsal nerve (arrow). Scale bar, 100 μm.

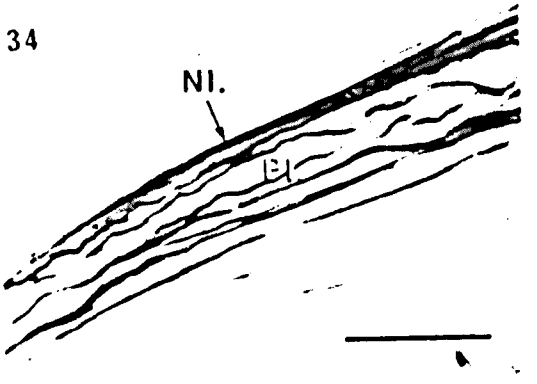
Fig. 38. B. hameri: wax section of a group of small neurones in the suboesophageal region of the infraoesophageal ganglion. Note the highly vacuolated cytoplasm. Scale bar, 25 μm.

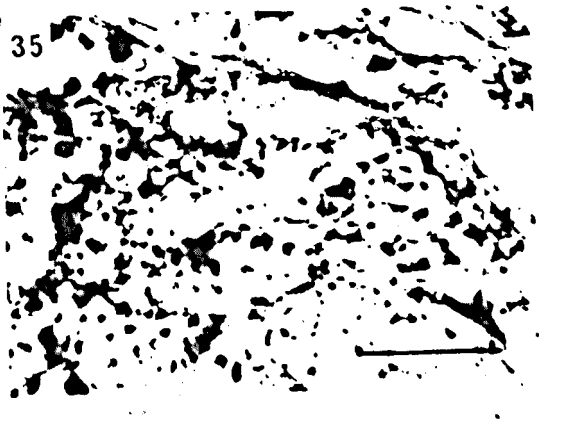


30









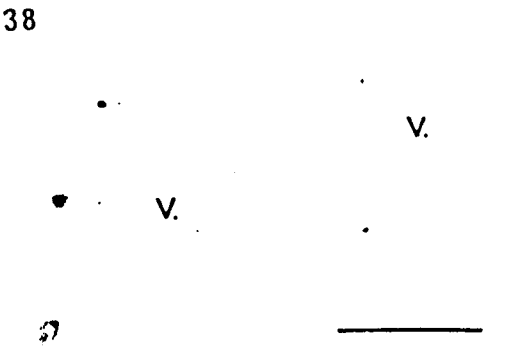


Fig. 39. B.hameri : wax section of the infraoesophageal ganglion showing two of the large type of neurones present in the suboesophageal region. The chromatin is clumped within each nucleus producing a halo beneath the nuclear membrane. Scale bar, 25 μm.

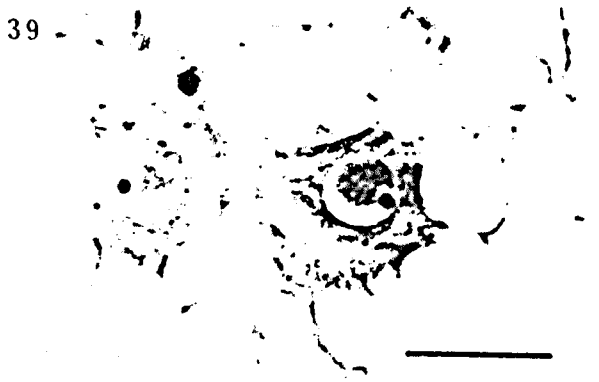
Fig. 40. B.hameri : longitudinal wax section through the dorsal side of the infraoesophageal ganglion showing the axon tracts prominent in this region. Perikarya are positioned in between the tracts. Scale bar, 200 μm.

Fig. 41. B.hameri : longitudinal wax section of the suboesophageal region showing the darkly staining neuropile (arrows) either side of the central cleft. Scale bar, 100 μm.

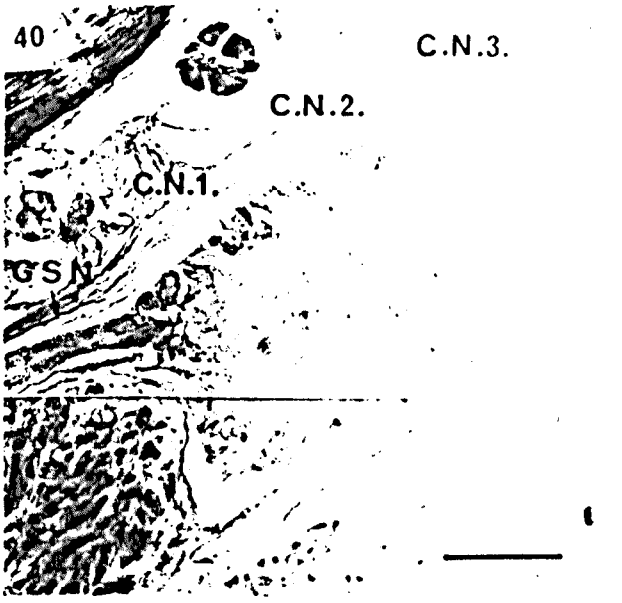
Fig. 42. C.auritum : wax section of the supraoesophageal ganglion showing some of the large perikarya from the ventral group of neurones. Scale bar, 50 μm.

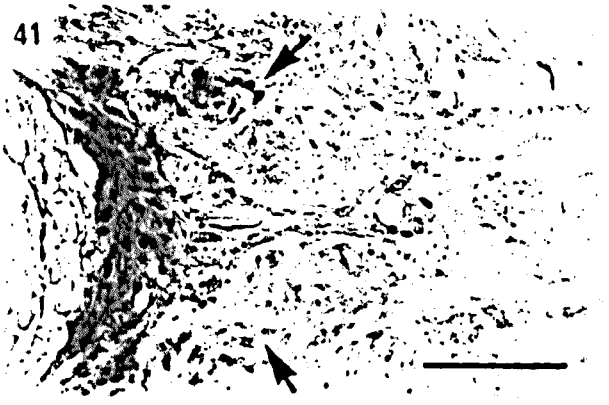
Fig. 43. C.auritum : wax section through the ventral region of the supraoesophageal ganglion. One of the perikarya contains a large vacuole. Scale bar, 50 μm.

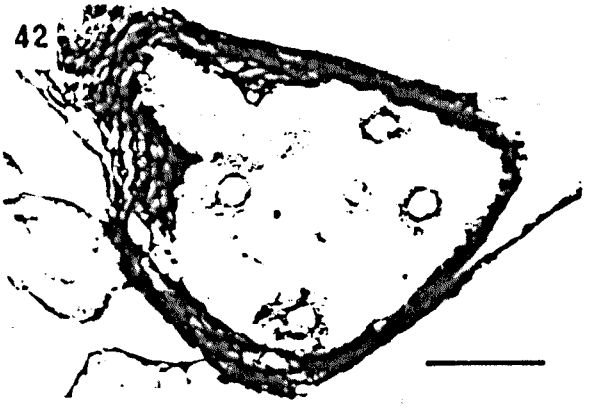
Fig. 44. C.auritum : wax section through the protocerebrum revealing a group of neurones at the base of the antennular nerve. Scale bar, 50 μm.

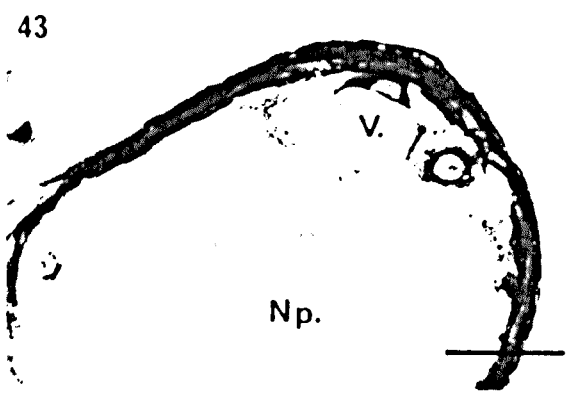


39









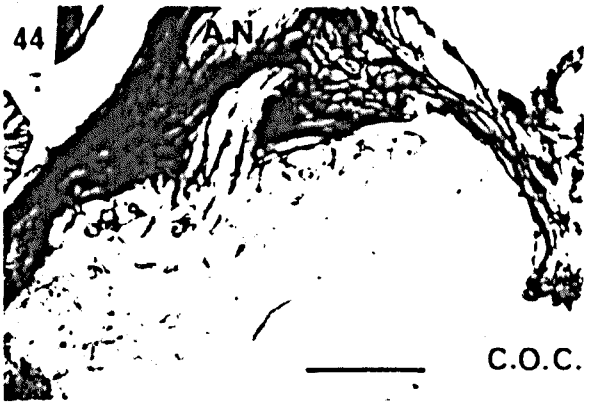


Fig. 45. B.hameri : electron micrograph of a type A perikaryon. Scale bar, 10 μm.

Fig. 46. B.hameri : electronmicrograph of a type B perikaryon. Scale bar, 15 μm.

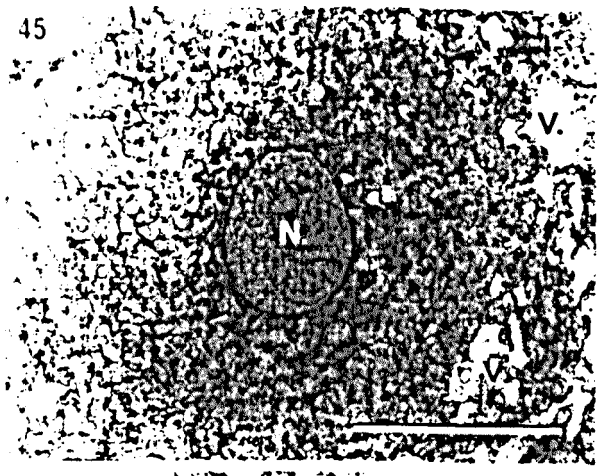
Fig. 47. B.hameri : electron micrograph of a region of cytoplasm from a type A neurone. Note the rough endoplasmic reticulum and large number of Golgi bodies. Dense-cored vesicles are indicated by arrows. Scale bar, 1.5 μm.

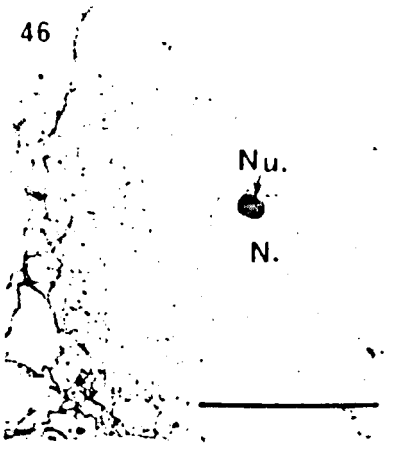
Fig. 48. B.hameri : electron micrograph of an area of cytoplasm in a type B neurone. Clumps of ribosomes are a characteristic feature of the cytoplasm. Scale bar, 2 μm.

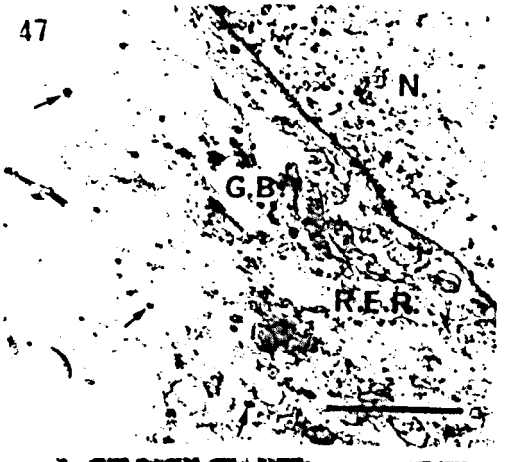
Fig. 49. B.hameri : electron micrograph of a typical Golgi body present in the cytoplasm of type A and B neurones. A dense-cored granule is apparently budding from the lateral margin of a Golgi sac (arrow). Scale bar, 1 μm.

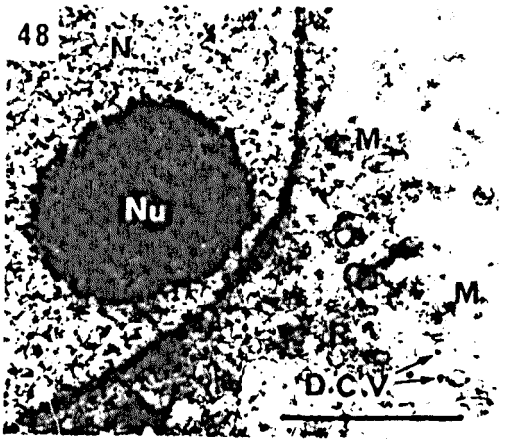
Fig. 50. B.hameri : electron micrograph showing electron-lucent vesicles budding from the endoplasmic reticulum (arrows) and passing to the Golgi sacs. Scale bar, 1 μm.

Fig. 51. B.hameri : electron micrograph showing an area of trophospongium within the cytoplasm of type A and B neurones. Scale bar, 2 μm.


45
V.
N.


46
Nu.
N.


47
N.
G.B.
R.E.R.


48
N.
Nu
M.
M.
D.C.V.


49


50

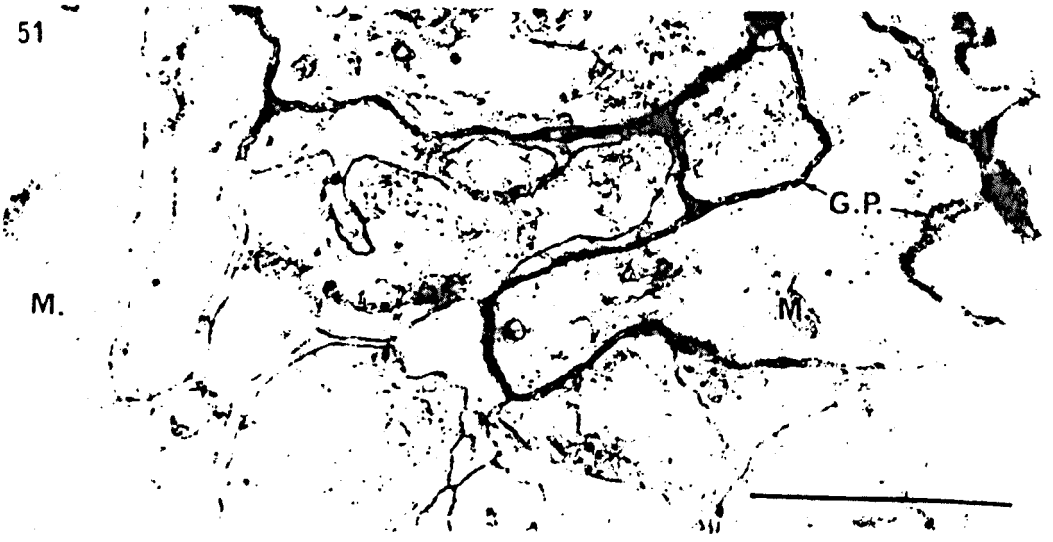

51
G.P.
M.
M.

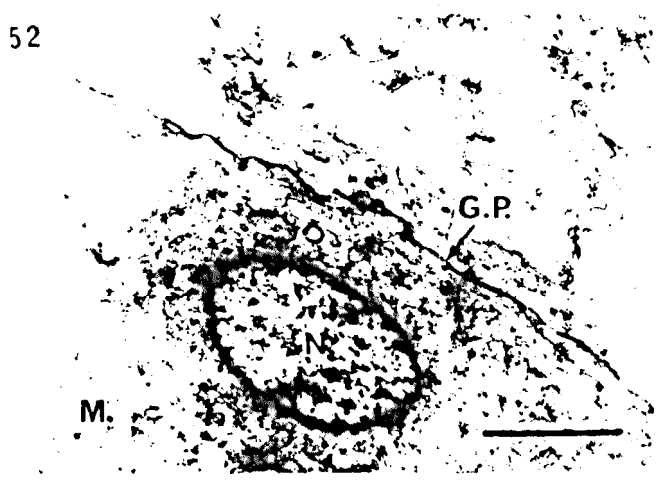
Fig. 52. B.hameri : electron micrograph of a type C perikaryon. Scale bar, 2 μm.

Fig. 53. B.hameri : electron micrograph showing an area of cytoplasm in a type C neurone. Scale bar, 1 μm.

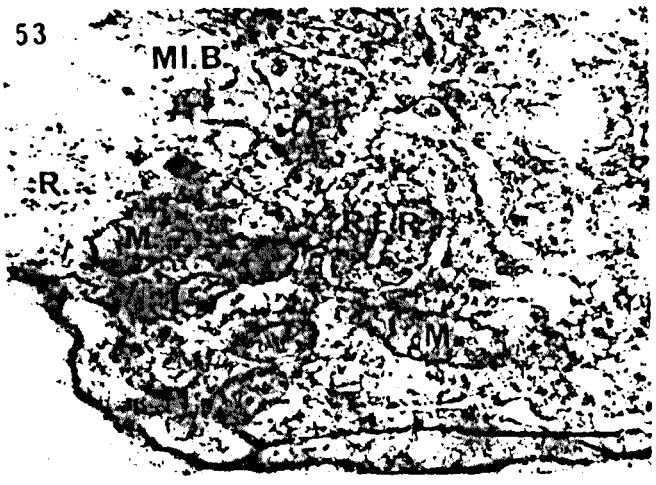
Fig. 54. B.hameri : electron micrograph of an elongate type D neurone showing the large areas of cytoplasm of moderate electron density situated at the periphery of the perikaryon. Scale bar, 5 μm.

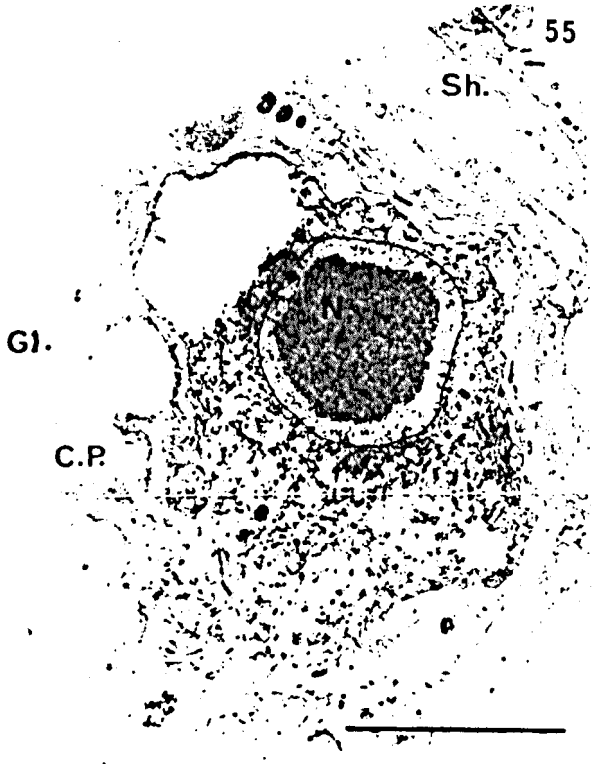
Fig. 55. B.hameri : electron micrograph of another type D neurone positioned subjacent to the sheath and markedly different in shape to that shown in the preceding figure. Scale bar, 10 μm.

Fig. 56. B.hameri : electron micrograph showing a cell process arising from a type D neurone. The moderately electron dense granules may be glycogen. Scale bar, 2 μm.


52
G.P.
N
M.


54
V.
V.
R.B.
N


53
MI.B.
R.
M
R.E.R
M


55
Sh.
N
Gl.
C.P.


56
R.E.R.
M.

Fig. 57. B.hameri : electron micrograph showing part of a perikaryon from the protocerebral cell group. A few dense-cored vesicles (arrowed) are visible. Scale bar, 2 μm.

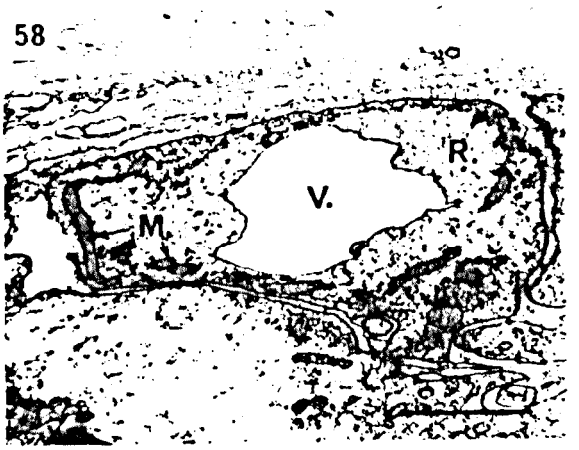
Fig. 58. B.hameri : electron micrograph showing a region of trophospongium in a protocerebral cell. Scale bar, 2 μm.

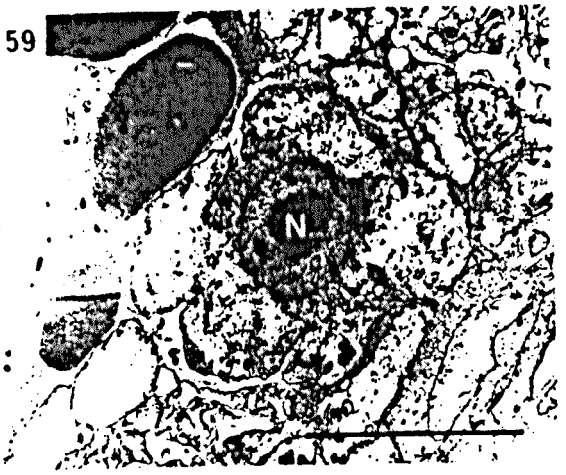
Fig. 59. B.hameri : electron micrograph of a small neurone type within the protocerebral cell group. Scale bar, 10 μm.

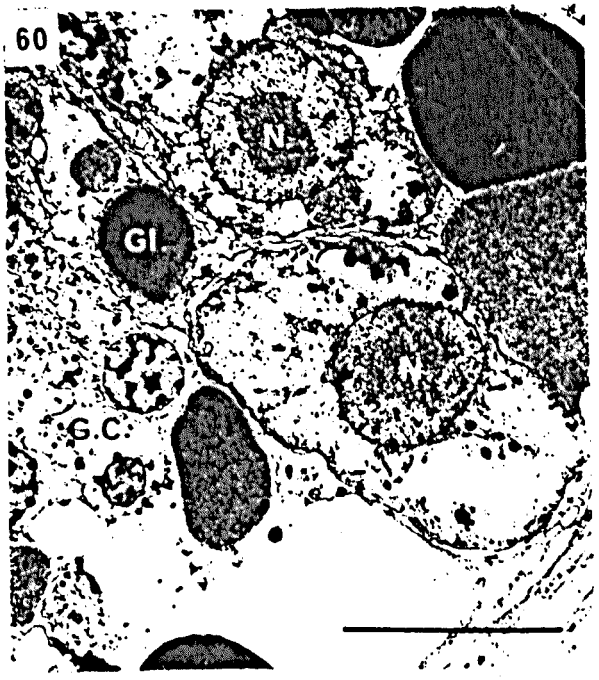
Fig. 60. B.hameri : electron micrograph of two cells of the dorsal cell group. Superficially they appear similar to type D neurones and the small neurones of the protocerebral cell group. Scale bar, 10 μm.

Fig. 61. B.hameri : electron micrograph showing a region of cytoplasm of a neurone from the dorsal group. Scale bar, 1 μm.


57
R.B.


58
V.
R.
M.


59
N.


60
N.
Gl.
G.C.


61
D.C.V.
M.
MI.B.

Fig. 62. B.hameri : type a and b dense-core vesicles. Scale bar, 1 μm.

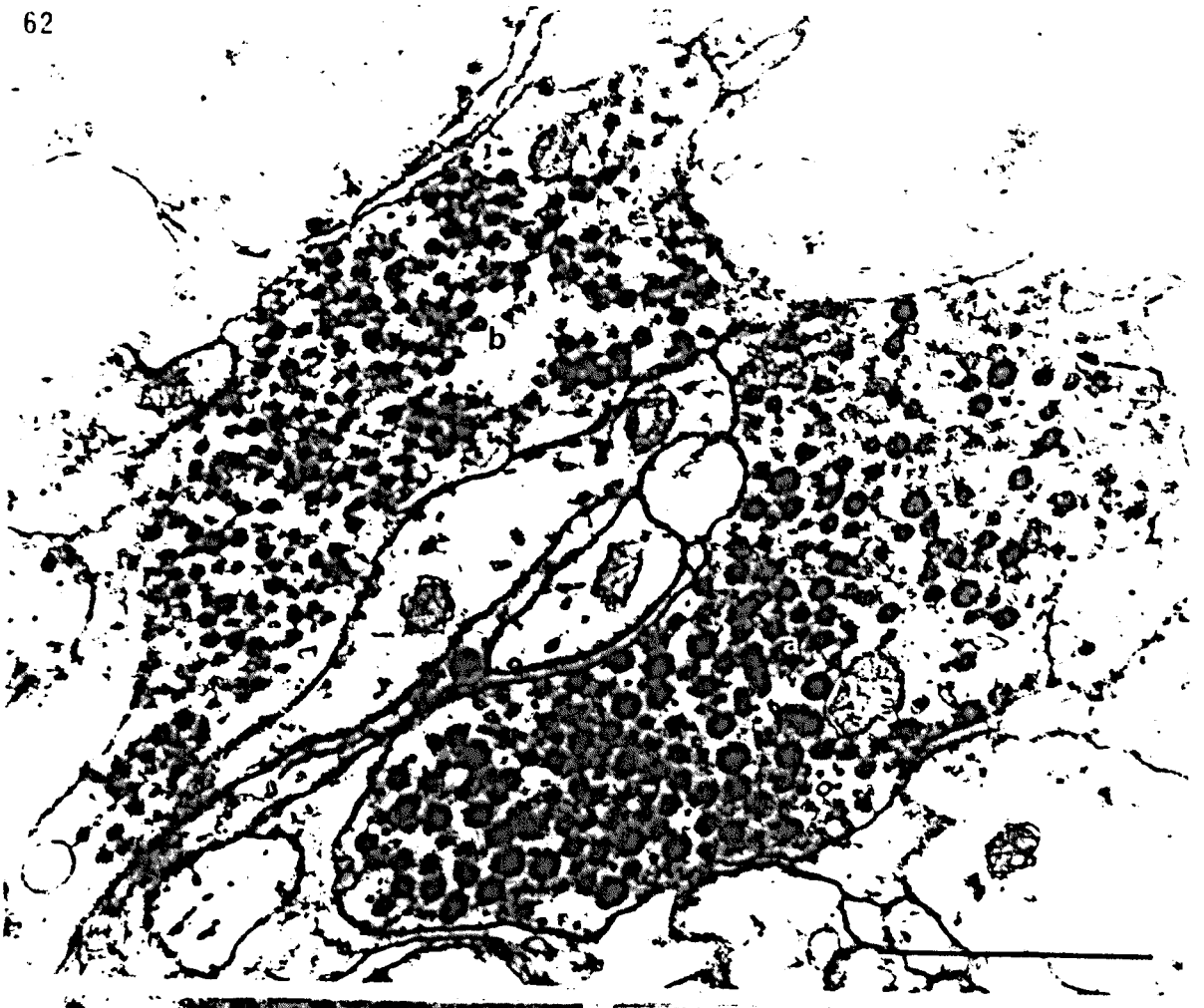
Fig. 63. B.hameri : type c dense-core vesicles. Scale bar, 0.5 μm.

Fig. 64. B.hameri : type d dense-core vesicles.

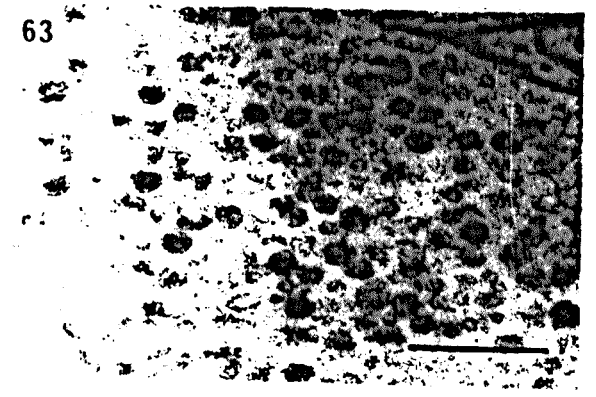
Fig. 65. B.hameri : type e dense-core vesicles.

Fig. 66. B.hameri : type f dense-core vesicles.

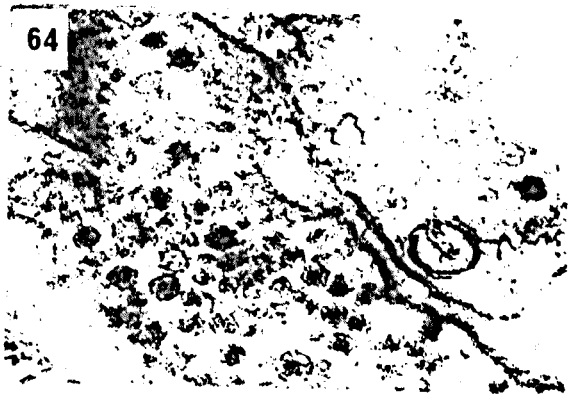
62



63



64



65

66

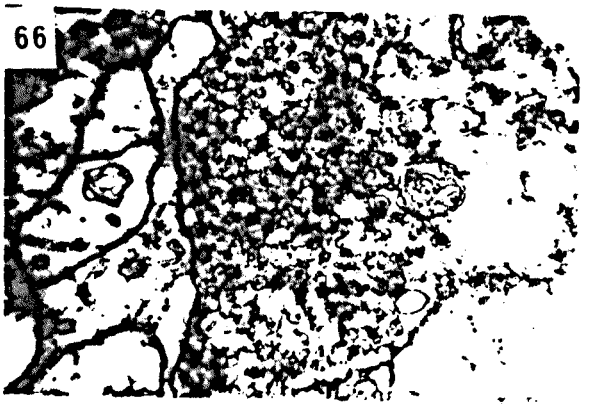


Fig. 67. B.hameri : electron micrograph of part of the neuropile within the supraoesophageal ganglion showing exocytotic profiles (arrows) involving type b granules. Scale bar, 0.5 μm.

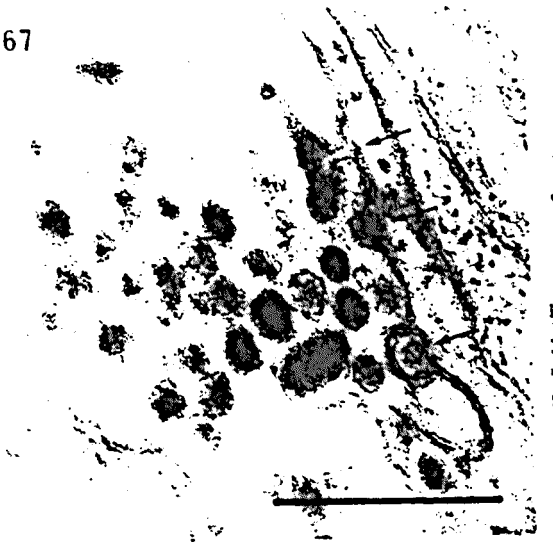
Fig. 68. B.hameri : electron micrograph of two axons containing numerous synaptic vesicles. Dense-cored vesicles are also present. Scale bar, 0.5 μm.

Fig. 69. B.hameri : electron micrograph of a synapse within the neuropile of the supraoesophageal ganglion. Exocytosis of one of the dense-cored granules can be clearly seen (arrow). Scale bar, 1 μm.

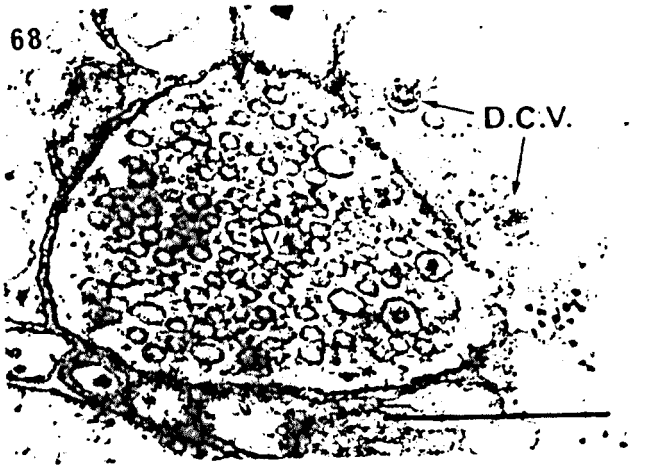
Fig. 70. B.hameri : electron micrograph of an axon type which is devoid of any granular or vesicular inclusions. Note the numerous neurotubules (arrows) which are linked by cross-bridges. Inset : higher power of the neurotubules and the neurofilaments which link them (arrows) (x67,000). Scale bar, 1 μm.

Fig. 71. B.hameri : electron micrograph showing two glial cells within the supraoesophageal ganglion. Scale bar, 5 μm.

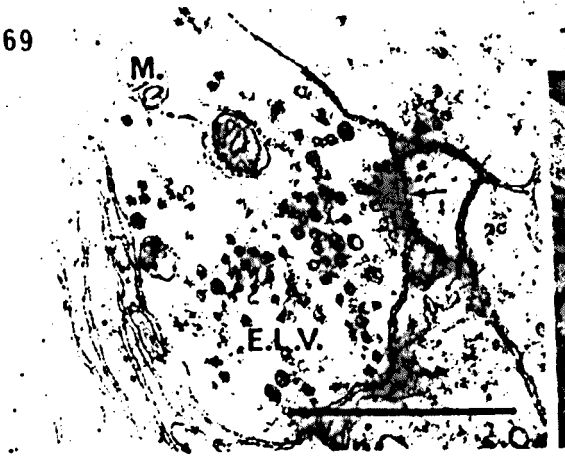
67



68



69



70

71

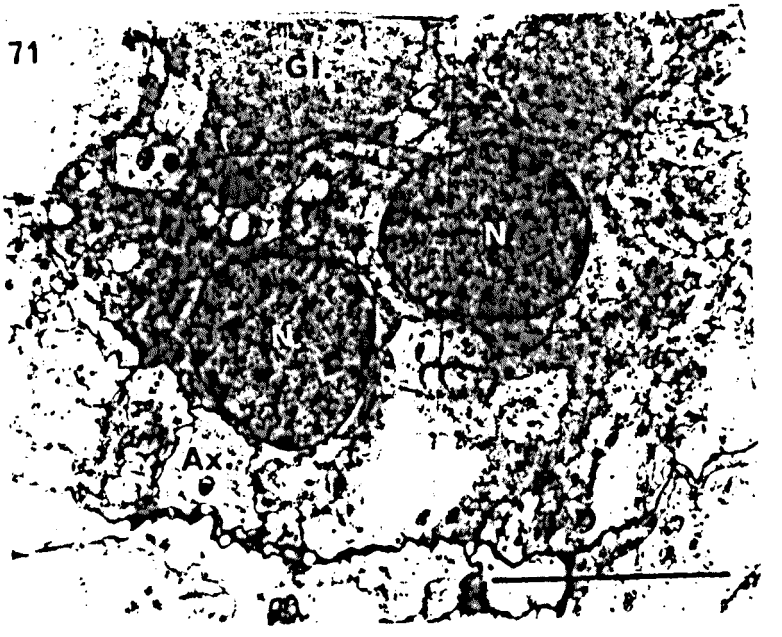


Fig. 72. B.hameri : electron micrograph of part of an antennular nerve. The large axon is penetrated by an inpushing of one of surrounding glial processes (arrow). Scale bar, 5 μm.

Fig. 73. B.hameri : electron micrograph of a glial process surrounding an axon and containing a gliosome. Scale bar, 0.5 μm.

Fig. 74. B.hameri : electron micrograph showing the microtubules running in the longitudinal axis of the glial processes. Scale bar, 0.2 μm.

Fig. 75. B.hameri : electron micrograph of a desmosome joining together two glial processes. Note the striations within the intracellular space of the desmosome. Scale bar, 3 μm.

Fig. 76. B.hameri : electron micrograph of a region of neuropile within the supraoesophageal ganglion showing gliosomes of differing electron density. Scale bar, 10 μm.

Fig. 77. B.hameri : electron micrograph showing the perinuclear zone of a glial cell. * indicates the swollen cisternae of the endoplasmic reticulum. Scale bar, 0.5 μm.

Fig. 78. B.hameri : electron micrograph showing a glial cell, the cytoplasm of which contains many electron-dense granules. Scale bar, 2 μm.

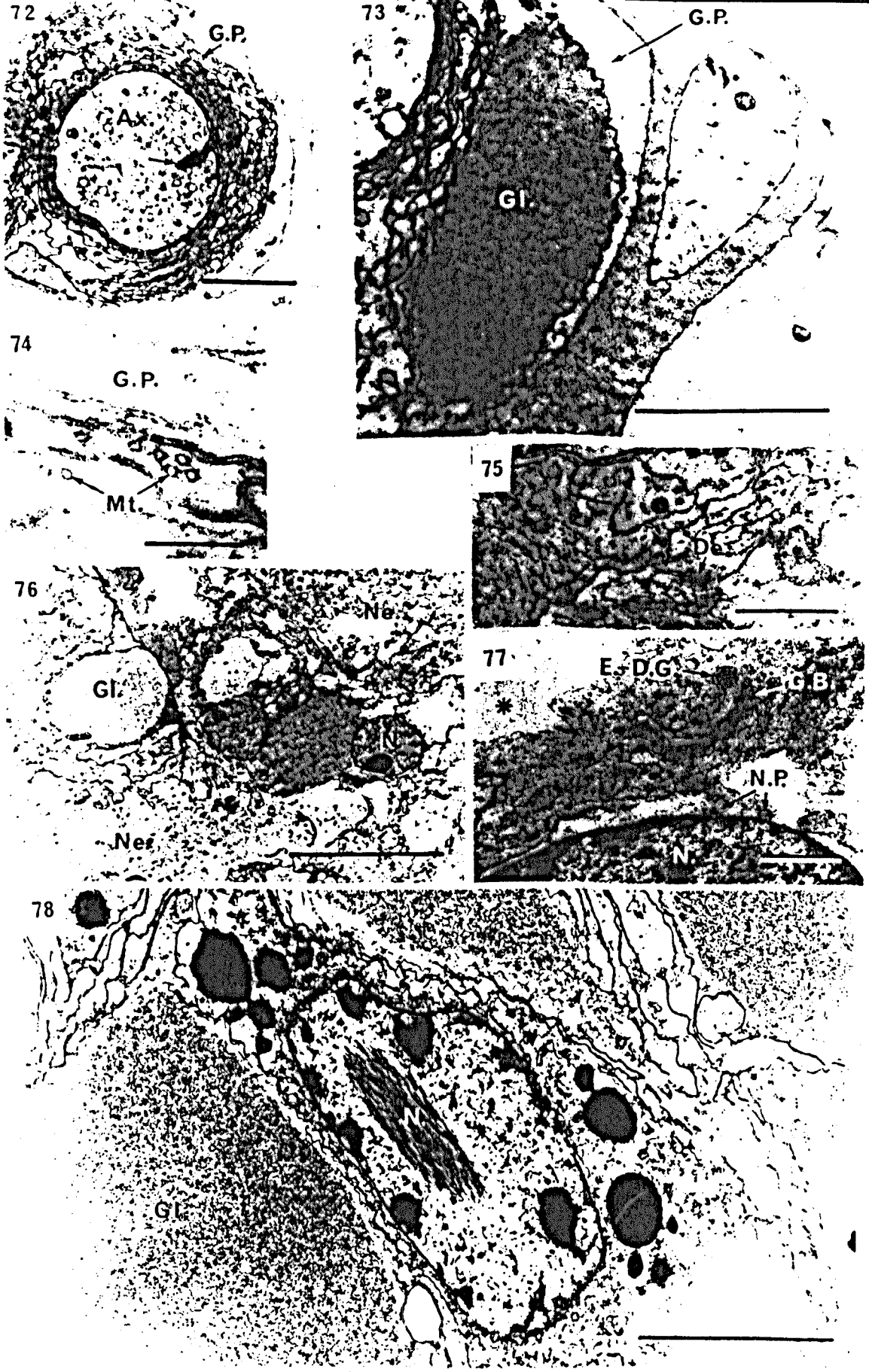

72
G.P.
Ax
73
G.P.
Gl.
74
G.P.
Mt.
75
76
Ne.
Gl.
Ner.
77
E.D.G.
G.B.
N.P.
N.
78
Gl.

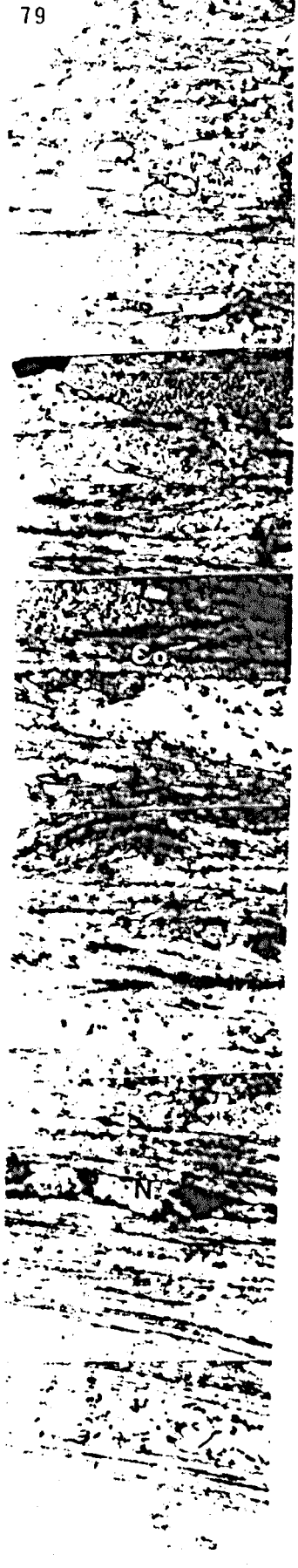
Fig. 79. B.hameri : electron micrograph of a section of the sheath surrounding the supraoesophageal ganglion. Scale bar, 1.5 μm.

Fig. 80. B.hameri : scanning electron micrograph of a transverse section through the infraoesophageal ganglion revealing the multi-layered sheath. Scale bar, 20 μm.

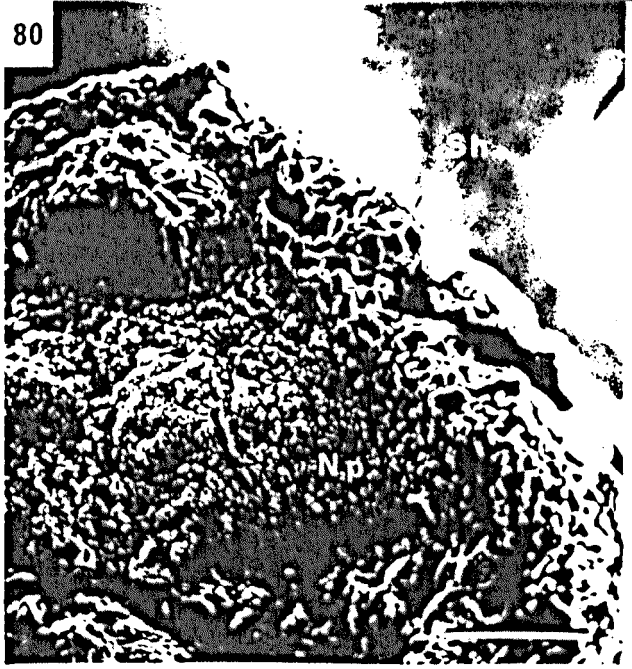
Fig. 81. B.hameri : electron micrograph of the sheath covering the protocerebrum. The former is composed solely of a neurilemma; no perilemma being present. Scale bar, 2.5 μm.

Fig. 82. B.hameri : electron micrograph showing a pore (arrow) within the sheath of the supraoesophageal ganglion. Scale bar, 2 μm.

79



80



81

82

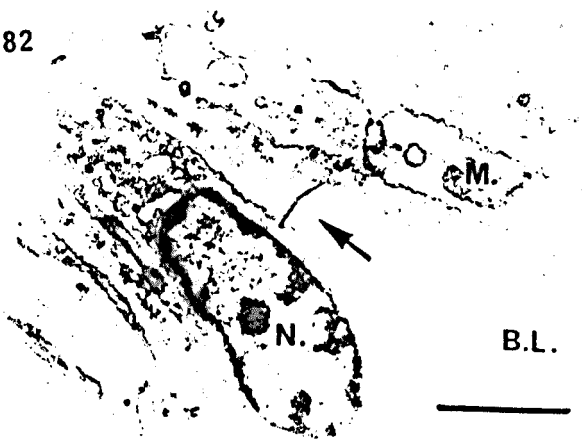


Fig. 83. B.hameri : electron micrograph of a small axon within the sheath of the supraoesophageal ganglion containing many dense-cored vesicles. Scale bar, 0.5 μm.

Fig. 84. B.hameri : electron micrograph showing a transverse section through the sheath of the infraoesophageal ganglion stained with silver proteinate for glycogen. The compartments of the sheath can be seen to be full of electron-dense glycogen. Inset, x 25,000. Scale bar, 3 μm.

Fig. 85. B.hameri : electron micrograph of a transverse section through the suboesophageal cleft showing the composition of the glial inpushings. Scale bar, 1 μm.

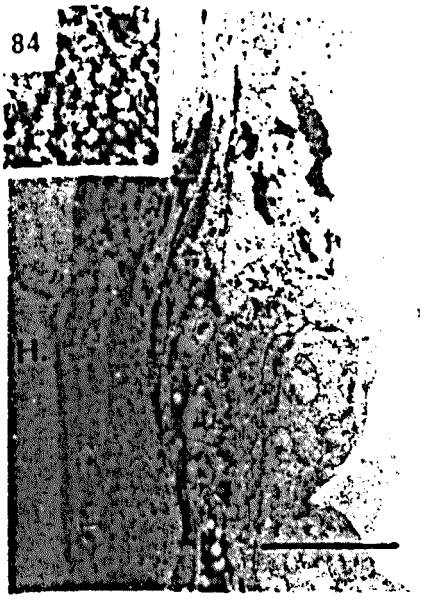
Fig. 86. B.hameri : a high power electron micrograph showing the glycogen content of the glial processes. Both α and β glycogen particles are present. Scale bar, 0.2 μm.

Fig. 87. B.hameri : electron micrograph of a transverse section through the suboesophageal cleft stained with silver proteinate. Scale bar, 3 μm.

Fig. 88. B.hameri : electron micrograph of a transverse section through the suboesophageal cleft showing a large aggregate of glycogen and what appears to be a tracheole. Scale bar, 2 μm.

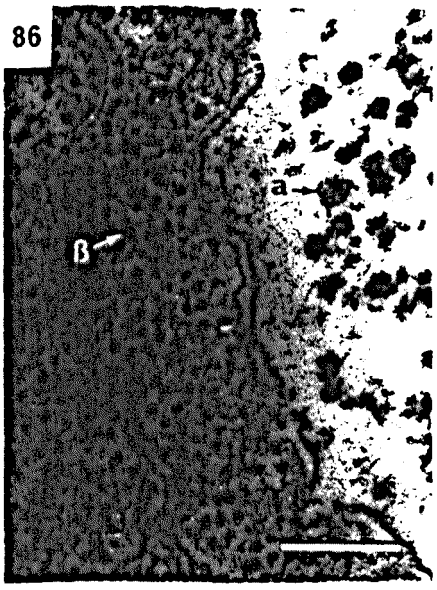
83

84



85

86



87

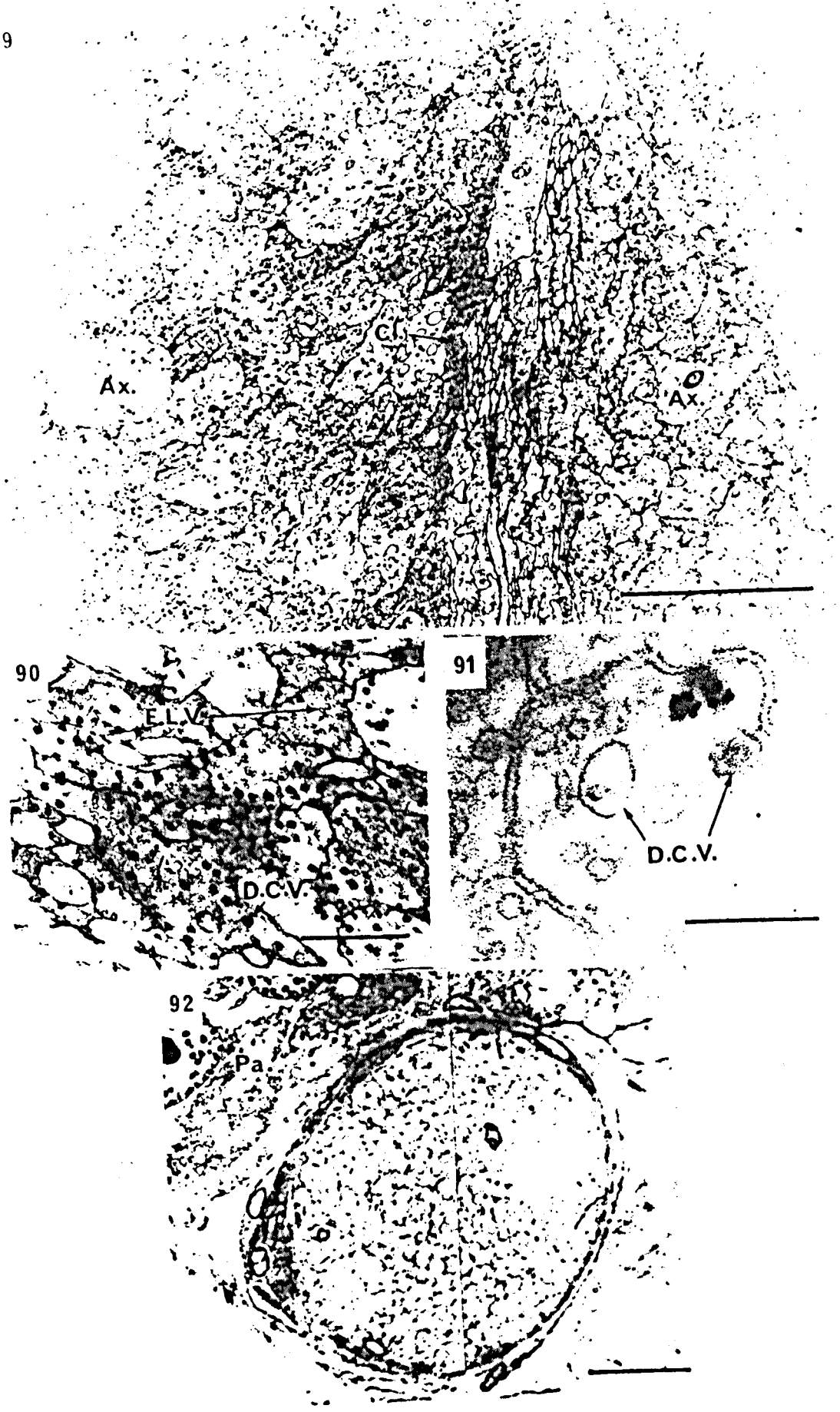
88

Fig. 89. B.hameri : electron micrograph of a transverse section through the suboesophageal ganglion showing the many small axons which extend in the longitudinal axis of the ganglion on either side of the cleft. Scale bar, 5 μm.

Fig. 90. B.hameri : electron micrograph showing the content of the axons either side of the suboesophageal cleft. Scale bar, 1 μm.

Fig. 91. B.hameri : electron micrograph of one of the small axons in the vicinity of the suboesophageal cleft stained for glycogen by the silver proteinate method. Scale bar, 0.5 μm.

Fig. 92. B.hameri : 1 μm resin section cut transversely through the anterior mid dorsal nerve revealing the many small axons which this nerves contains. Scale bar, 20 μm.


Ax.
Cr.
Ax.
90
E.L.V.
D.C.V.
91
D.C.V.
92
Pa

CHAPTER 3

Neurosecretion in *Balanus balanoides* (L.) and *Balanus hameri* (Ascanius) with some comparative observations on *Conchoderma auritum* (L.)

CHAPTER 3

Neurosecretion in Balanus balanoides (L.) and Balanus hameri (Ascanius) with some comparative observations on Conchoderma auritum (L.)

INTRODUCTION

Several studies have been made on neurosecretion in cirripedes and these have already been reviewed in Chapter 1. The main conclusion to be derived from these studies is that neurosecretory cells probably exist, but there are no specialized areas or organs for the release of the neurosecretory product. What physiological functions the neurosecretory products control remain largely speculative, although some evidence has been presented to suggest a rôle in moulting (Davis & Costlow, 1974).

In the words of Mortlock (1968), who studied neurosecretion in barnacle larvae, "any study on a neuroendocrine mechanism should aim at answering the following questions. (1) Where is the neurosecretion produced? (2) How is it transmitted and released? (3) Is it stored or not? (4) What extrinsic and intrinsic factors control the mechanism?" The present study, in conjunction with that presented in Chapter 4, attempts to answer these questions and so provide a firm basis for the further study of control mechanisms in barnacles and also for electrophysiological and biochemical investigations.

MATERIALS AND METHODS

Barnacles were collected and maintained as described in Chapter 2.

Fixation was carried out between 9 a.m. and 1 p.m. For the staining techniques which employed whole animals, either sea water Bouin's or a

modified Bouin's solution (Ewen, 1962) were employed. Serial sections of Fibrowax embedded material were cut at 7-10 μm thickness. The following methods were used to stain the neurosecretory product for light microscopy: Gomori's chrome-alum haematoxylin (CAH) and chrome-alum haematoxylin-phloxin (CAHP) after Bargmann (1949), paraldehyde fuchsin (PAF) after Ewen (1962) and alcian blue/alcian yellow (AB/AY) after Wendelaar Bonga (1970).

Additional methods used the isolated central nervous system of B. hameri which was dissected from the animal as described in Chapter 2. When these preparations were viewed under bright white light (from a Volpi 250 H fibre optic system) and against a dark background, cell bodies were frequently observed with a blue-white refractile appearance. The position of these cell bodies was noted. Some ganglia were fixed in the modified sea water Bouin's solution (Ewen, 1962) and a PAF technique was applied to the intact ganglia (Dogra & Tandan, 1964). Whole mounts, in DPX, were prepared following the removal of the ganglion sheath and clearing of the tissues in xylene. Ganglia, either whole or as fresh frozen sections, were vital stained with acridine orange by the method of Beattie (1971). The preparations were viewed exactly 2 minutes from the time of removal of stain (Anne Griffiths, pers. comm.), in blue light, using a Leitz Orthoplan photomicroscope, an ultra high pressure mercury lamp as light source, with a BG 12 excitation filter and a K530 suppression filter.

Isolated ganglia were fixed for transmission electron microscopy in 2.5% gluteraldehyde in either 0.2M sodium cacodylate buffer or sea water and post-fixed in 2% osmium tetroxide, as described in Chapter 2. Thick 1 μm sections for light microscopy were stained with 1% toluidine blue in a 1% aqueous borax solution, on a hot plate. Silver to gold

sections were mounted on copper grids and double stained with uranyl acetate and lead citrate. The sections were veiwed in a Philips 301 electron microscope operating at 40 to 80 kV.

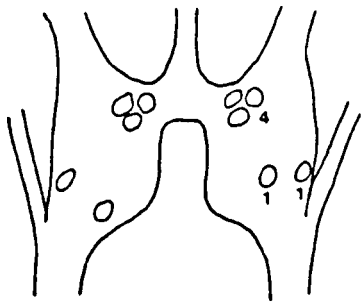
The morphology of an identified group of neurosecretory cells (see below) was studied using the fluorescent dye Lucifer Yellow CH. Following treatment of the ganglion with 0.1% pronase (Barber, 1983) cells were iontophoretically injected with a 3% aqueous solution of the dye, for about 15 mins., by applying a continuous current of approximately -10nA through the electrode. The preparation was then stored in sea water at 4°C for 1 hr. to allow further diffusion of the dye. Following this period, the ganglion was dissected free of the animal and mounted on a cavity slide in sea water. The marked cells were observed in blue light with a Leitz Dialux 20 fluorescent microscope.

RESULTS

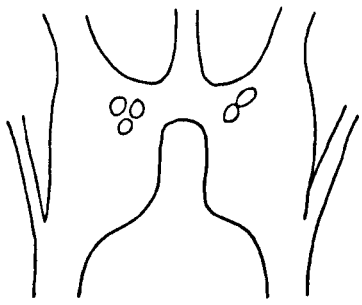
Light microscopy of neurosecretory cells

A record was kept of the position of white, opaque cell bodies in the supraoesophageal ganglion of B. balanoides prior to fixation of the ganglia for electron microscopy. Although the numbers of ganglia examined were insufficient to be able to link with any certainty the numbers of cell bodies to physiological events, the records (Fig. 1) are noteworthy in that they do show a trend in neurosecretory activity over the period of September 1980 to, and including, February 1981. In B. balanoides this period of the year encompasses several key events in the reproductive cycle, namely, the final stages of vitellogenesis in September and October, cross-fertilization and egg laying in November and brooding of the eggs until March-April, when the larvae are liberated.

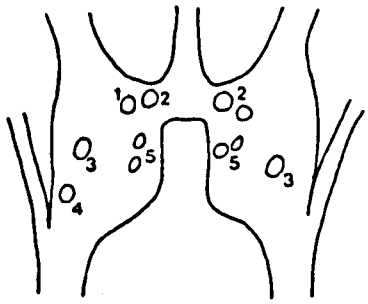
Fig. 1. Diagrammatic representation of the varying positions of opaque white perikarya within the supraoesophageal ganglion of B.hameri over a period of approximately 5½ months. The numbers adjacent to the perikarya indicate the degree to which they reflect white light and are on an arbitrary scale of 1-5.



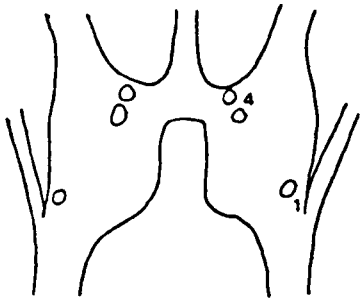
14.9.80



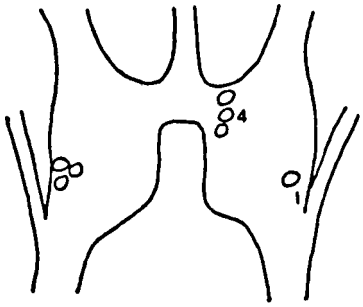
2.10.80



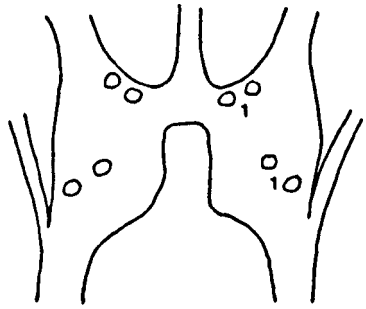
23.10.80



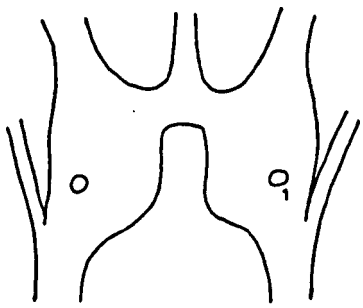
18.11.80



25.11.80



2.12.80



26.2.81

It can be seen from figure 1 that the white cell bodies are most numerous in September and October. Furthermore, the group of cells in the circumoesophageal commissure were consistently present during these months. Following egg laying, the cell bodies are less numerous and when present contain relatively little refractile material.

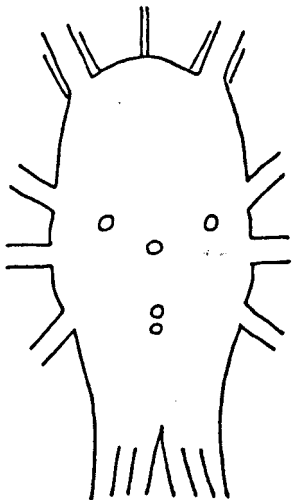
The position of white refractile cell bodies is even more variable in the infraoesophageal ganglion of B. balanoides (Fig. 2).

White refractile cell bodies were also noted in the ganglia of B. hameri, B. crenatus and Conchoderma auritum.

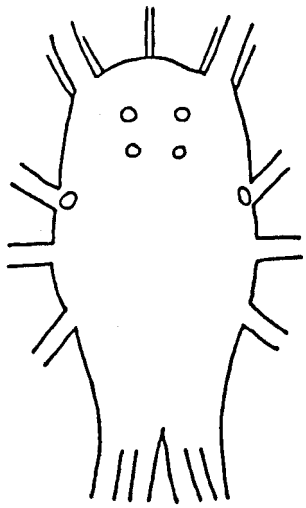
Attempts were made to inject Lucifer Yellow into the white cell bodies in the commissure of the supraoesophageal ganglion of B. hameri. This species was used in preference to B. balanoides because the cell bodies of the former are much larger (see Chapter 2) and consequently are easier to inject. Fig. 3 shows a cell which has been successfully injected with the dye. The cell body has an axon which extends into the median ocellar nerve and towards the median ocellus. Clearly, this cell and the small group in the commissure to which it belongs is equivalent to those cell bodies which were revealed by cobalt-backfilling the median ocellar nerve (c.f. Fig. 5, Chapter 2).

Vital staining of the supraoesophageal ganglion of B. hameri with acridine orange gave results which, in terms of staining, were in good agreement with those of Beattie (1971), who worked on nervous tissue from the cockroach, Periplaneta americana. Excellent differentiation was obtained between the 'ordinary' nerve cells and the presumed neurosecretory cells. The cytoplasm of the former fluoresced green as did their axons. Nuclei of nerve cells, glial cells and sheath cells all

Fig. 2. Diagram showing the positions of opaque white perikarya in the infraoesophageal ganglion of B.hameri in September and February.



4.9.80



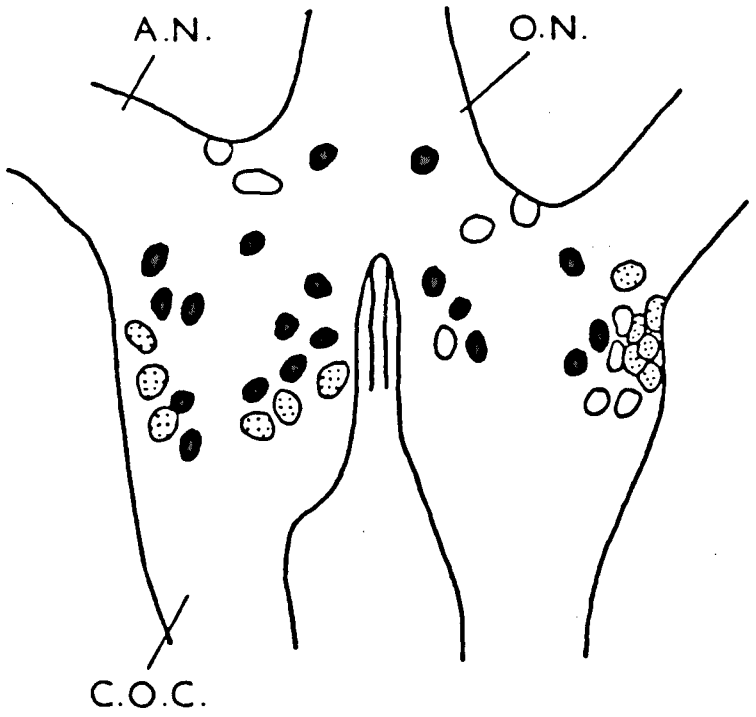
26.2.81

had a green fluorescence. One reaction, not noted by Beattie (ibid), was that of the gliosomes which fluoresced either green yellow or not at all. Neurosecretory material fluoresced red. Not only cell bodies were revealed in whole mounts of the ganglia; it was also possible to determine which nerves contained neurosecretory product. Moreover, individual axons could occasionally be resolved. Serial, fresh frozen sections, when stained with acridine orange revealed cell bodies of comparable distribution to those found in whole mounts. Figure 4 illustrates the position of the neurosecretory cells based on a reconstruction from serial sections. 20 cell bodies occur in each lobe of the ganglion. Most of these cell bodies occupy a medial or dorsal position. Included in those on the ventral surface are two cells in each lobe which correspond in position to the cell bodies with axons in the median ocellar nerve (see above). Indeed, red fluorescent processes were observed in the median ocellar nerve. Red fluorescent processes were also observed in the following nerves: antennular, lateral ocellar, oesophageal, circumoeosophageal and in the connective between the suprasplanchnic nerve and the ganglion. The axons of the circumoesophageal connectives were in fact partitioned into two discrete bundles. The group which ran between the infraoesophageal and the supraoesophageal ganglia fluoresced red, whereas those axons which extended along the suprasplanchnic nerve had a green fluorescence. The latter probably constitute axons of motor neurones and/or sensory cells.

Very few cell bodies stained intensely with the so-called neurosecretory stains CAH, CAHP, PAF and AB/AY. This statement applies to both the supra- and infraoesophageal ganglia of each of the three species of barnacle studied.

Fig. 4. B.hameri : diagram to illustrate the positions of acridine orange-positive cells in the supraoesophageal ganglion.

○ = ventral perikarya ○ = medial perikarya ● = dorsal perikarya


A.N.
O.N.
C.O.C.

The CAH and CAHP techniques stain more cell bodies than do the other staining techniques. The results for both the Balanus species were very similar using these Gomori stains. Many of the large ventral perikarya of the supraoesophageal ganglion possess a deeply staining perinuclear zone (Fig. 5) which is occasionally found to extend into the axon hillock. The majority of the small cell bodies underlying the ventral group of cells gave a negative reaction to the Gomori stains. Those few which did react positively were widely spaced throughout the tritocerebrum (Fig. 6). Most of the protocerebral cells, but especially the small vacuolate type, also give a negative or a weak reaction. However, the cytoplasm of most of the large cell bodies has an affinity for CAH and several cell bodies in each lobe stain intensely (Fig. 7). The cytoplasm of several cells within the dorsal cell group reacts moderately to CAH (Fig. 8). The "speckled spaces", described in Chapter 2, do not stain with CAH, but they are phloxinophilic (Fig. 9).

Few cell bodies react positively to CAH in the supraoesophageal ganglion of mature C. auritum compared to the Balanus species. This supports the finding that there were fewer opaque white cell bodies in this species. None of the large ventral cell bodies react positively. However, intense CAH staining material is present in the neuropile of the tritocerebrum (Fig. 10). A single small, positively stained cell body is symmetrically placed within each lobe. The perikarya of this cell contains a large vacuole which is devoid of CAH stain (Fig. 11). Within the protocerebrum of each lobe there is also one cell body which is strongly stained by CAH (Fig. 12). The axon of this cell extends posteriorly, so it is not equivalent to the positive cells of the Balanus species, with axons in the median ocellar nerve. Nevertheless, CAH-positive material is present within the median ocellar nerve of

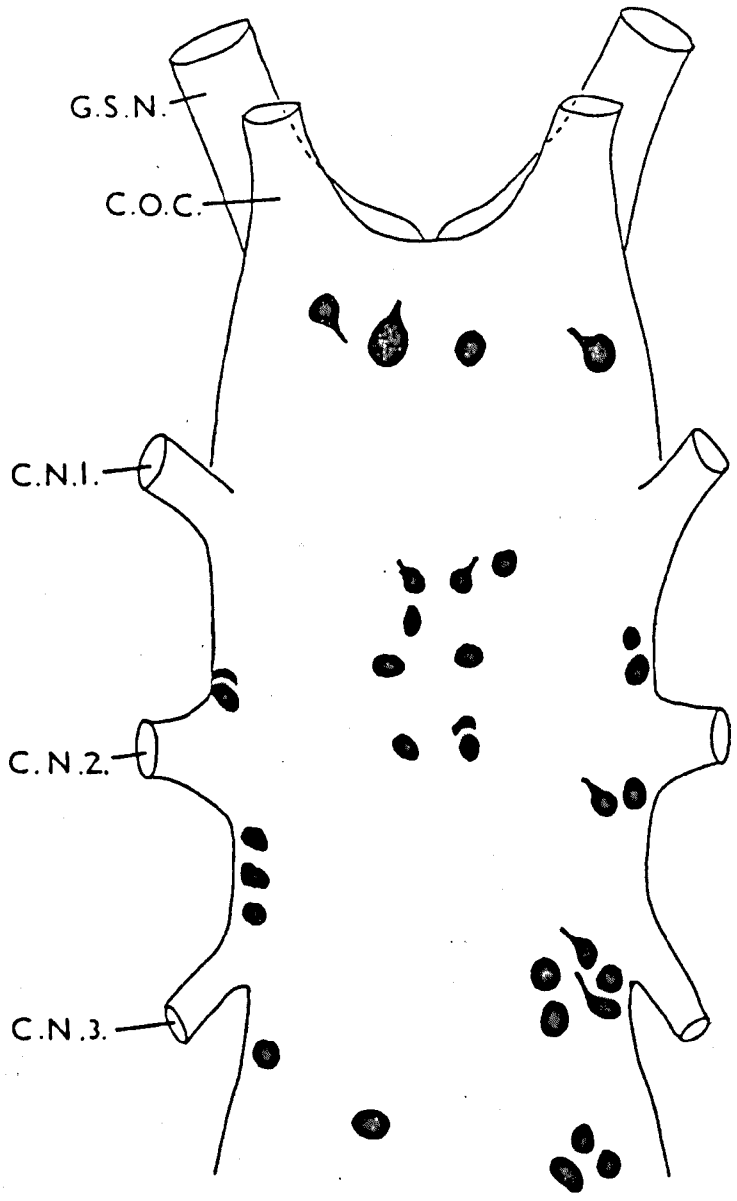
C. auritum (Fig. 13). The dorsal cell group of each lobe also contains a single, strongly stained cell (Fig. 14).

The Gomori stains were not applied to sections of the infraoesophageal ganglia. However, whole mounts of the infraoesophageal ganglion of B. hameri, stained with PAF, were prepared according to the technique of Dogra and Tandan (1964). Figure 15 illustrates the location of the PAF-positive cell bodies in this ganglion. It is readily apparent that their distribution does not resemble that of the opaque, white cell bodies (Fig. 2). Sections of the infraoesophageal ganglion of B. hameri, prepared at the same time of year (January) and stained with PAF, did not reveal any strongly stained cell bodies. The most notable feature of the ganglion is the central core of neuropile in each lobe of the suboeosophageal region which is strongly stained by PAF (Fig. 16). An identical feature was noted in the suboesophageal ganglion of Pollicipes polymerus by Barnes and Gonor (1958). It has already been noted (in Chapter 2) that this region of the ganglion of B. hameri is composed of numerous small axons containing small (<100 nm) dense core vesicles, which are probably neurotransmitters. This suggests that PAF is not specific for neurosecretory product.

None of the nerves extending from the infraoesophageal ganglion of B. hameri contain PAF-positive material. However, the sheath of the circumoesophageal connectives stains intensely, especially proximal to the ganglion (Fig. 17).

PAF did not reveal any strongly positive cells within the supraoesophageal ganglion of either B. hameri or B. balanoides. Furthermore, PAF did not stain the perinucelar zone of the large ventral cell bodies. This difference between the staining properties of CAH and PAF has also been noted in vertebrates (Streefkerk, 1967) and in the pulmonate mollusc

Fig. 15. B.hameri : diagram illustrating the position of P.A.F. - positive cells within a whole mount of the infraoesophageal ganglion.


G.S.N.
C.O.C.
C.N.1.
C.N.2.
C.N.3.

Succinea putris (Cook, 1966). 2-3 bodies within the protocerebral cell group stain moderately with PAF (Fig. 18). Positive material is also present in the supraoesophageal commissure of B. balanoides.

The AB/AY technique has been used most effectively in Lymnaea stagnalis to reveal tinctorial differences in the neurosecretory product of various cell types (Wendelaar Bonga, 1970; Swindale & Benjamin, 1976; Benjamin et al., 1980). Although the technique has also been used in the Entomostraca (Lake, 1970), it has not previously been applied to sections of barnacle ganglia. The results of the present study using AB/AY were disappointing. All the cell bodies of the tritocerebrum of the Balanus species and of the entire supraoesophageal ganglion of C. auritum fall into the category of Light Yellow Cells, as described by Wendelaar Bonga (1970). However, cell bodies are present in the protocerebral cell group of both Balanus species, which have a greater affinity for alcian yellow and can be described as Yellow Cells.

The phloxinophilic "speckled spaces" of the supraoesophageal ganglion are not stained with either alcian blue or alcian yellow.

No cell bodies within the infraoesophageal ganglia of the Balanus species have a marked affinity for AB/AY. However, the central area of neuropile in the suboesophageal region which stains with PAF (see above) also stains deep yellow with AB/AY.

It is interesting to note that none of the cell bodies in any of the barnacle ganglia examined have an affinity for alcian blue. In theory, disulphide (S-S) bonds and sulphydryl (-SH) groups, in cystine and cysteine, have an affinity for alcian blue at pH 1.0 (Peute & van de Kramer, 1967). Thus the neurones of barnacle ganglia contain little or

none of these amino acids. This contention receives support from histochemical experiments (Chapter 2). Barnacle neurones were found to give only weak reactions for S-S bonds and -SH groups. Similarly, McGregor (1967b) using performic acid-victoria blue, concluded that the neurosecretory product of barnacles does not contain sulphydryl groups.

Ultrastructure of the neurosecretory cells

Owing to the extremely low number of presumed neurosecretory cells which could be identified at the light microscope level, it was impossible to identify with any certainty the corresponding neurosecretory cells at the ultrastructure level. The study has been aided by a staining property of the neurosecretory product in 1 μm resin sections, namely the deep blue reaction of the product to toluidine blue. This property has been particularly useful in identifying storage regions where there is a concentration of neurosecretory product, for example, in axon dilations (Fig. 19). Neurosecretory perikarya, however, stained only marginally more intensely than did "ordinary neurones" and this weak reaction is perhaps a reflection of a small number of neurosecretory granules (see below).

The ultrastructure within the supraoesophageal ganglia of B. hameri and B. balanoides has been examined. Altogether three types of neurosecretory cells were identified, on the basis of neurosecretory granule size in the ganglion of B. hameri. Two of these cell types belong to the ventral group of cells and are positioned subjacent to the sheath. Figure 20 shows the perikarya of a cell type (type G) containing neurosecretory granules of mean diameter 141 nm†. The granules have an electron-dense core which is surrounded by a narrow

† All neurosecretory granule diameters are corrected values using the method of Froesch (1973).

electron-lucent zone (Fig. 21). Very few neurosecretory granules are present in the perikarya. Although the perikaryon illustrated is elongate, measuring 26 μm by 6 μm, spherical perikarya also occur. There is a narrow region of rough endoplasmic reticulum surrounding the spherical nucleus; free ribosomes and smooth endoplasmic reticulum occur throughout the cytoplasm. A few small mitochondria are also present, mainly at the peripheral margins of the cell. Golgi bodies are scarce; their cisternae contain material of the same electron density as the neurosecretory granule core. Residual bodies are a prominent feature of some of the cells, whereas in others they are scarce or even absent. β-particles of glycogen are also present in the cytoplasm.

The second neurosecretory cell type (Fig. 22) of the ventral group, type H, contains electron dense neurosecretory granules with a mean diameter of 107 nm. Figure 23 illustrates some of the salient features of the cytoplasm. Free ribosomes occur as clumps and give the cytoplasm a patchy appearance. Small profiles of smooth endoplasmic reticulum, mitochondria, and β-particles of glycogen are evenly distributed in the cytoplasm. Glial processes penetrate deep into the cytoplasm forming a trophospongium.

It is apparent that many features of type H cells are similar to type D cells (see Chapter 2). For example, both cell types occur in the same region of the supraoeosophageal ganglion, they both contain dense-core granules of similar size and both possess a well developed trophospongium. These cells cannot, therefore, be distinguished at the light microscope level. However, one important difference between the two cell types does exist, namely the dense core granules of type D cells have a prominent halo about their core, whereas granules in type H cells do not (Fig. 24).

A third possible neurosecretory cell type occurring in the ventral cell group of the tritocerebrum has previously been described in Chapter 2. Although type B cells contain dense-core granules which are less than 100 nm in diameter, they may be neurosecretory, because exocytosis of the same granule type was observed in the neuropile. It should also be noted that some of the neurones in the dorsal cell group may also be neurosecretory for the same reasons.

Only one neurosecretory cell type has been identified in the protocerebrum of B. hameri. This type, hereafter referred to as type I, contains electron-dense granules with a narrow electron-lucent zone and a mean diameter of 123.5 nm. The neurosecretory granules are evenly distributed throughout the cytoplasm (Fig. 25). The spherical nucleus of the cell illustrated in figure 24 is unusual, because it is surrounded by several rows of what appear to be tubules. Although this arrangement is reminiscent of the "manchette" of spermatozoa, where microtubules are involved in the shaping of the nucleus during spermatogenesis (Dustin, 1978), the diameter of the profiles is too large (ca. 70 nm) to be microtubules (c.f. Dustin, 1978, page 310). Instead the spherical profiles probably represent cross-sections of nuclear pores, as noted by Peel et al. (1973) in the mother cells of the alga, Corrallina officinalis. The few Golgi bodies that are present in the cytoplasm are widely separated. Electron-dense material is often observed within the cisternae (Fig. 26). Mitochondria and free ribosomes are abundant especially near the nucleus.

Since the axons from type I cells run into the supraoesophageal commissure, it is believed that this cell type is equivalent to the white perikarya which have axons in the median ocellar nerve (see light microscope section).

Contrary to the situation in B. hameri, no neurosecretory cells have been observed in the protocerebrum of B. balanoides. Two neurosecretory cell types have, however, been noted in the tritocerebrum of this species.

The first type (Fig. 27) is about 15 μm in diameter and contains neurosecretory granules, of mean diameter 120 nm, which have a narrow electron-lucent zone separating the electron-dense core from the limiting membrane. The few Golgi bodies that are present give rise to neurosecretory granules at their lateral margins (Fig. 28). The cytoplasm also contains many mitochondria and a well developed system of rough endoplasmic reticulum. The peripheral region of the cell contains fewer organelles, but is instead highly vacuolated. These vacuoles are associated with a trophospongium which penetrates the cell to a depth of 2.5 μm.

The second neurosecretory cell type (Fig. 29) is about 14 μm in diameter and contains neurosecretory granules which, although similar in appearance to those of the previous cell type, are considerably larger, having a mean diameter of 155 nm. Large vacuoles, of electron-lucent content, are an obvious feature of the cytoplasm and neurosecretory granules tend to be associated with them. Golgi bodies are comparatively numerous and their lamellae characteristically contain much material of the same electron density as the contents of the neurosecretory granules (Fig. 30). The rough endoplasmic reticulum occurs as clumps in the cytoplasm. Free ribosomes are abundant. The cytoplasm also contains a few lysosomes. Deep infoldings of glial tissue are present at the cell periphery forming a trophospongium and many mitochondria are associated with this region (Fig. 31).

Four types of dense-core granule, with diameters exceeding 100 nm,

have been observed in the neuropile of the supraoesophageal ganglion in B. hameri (Figs. 32-35). In accord with the granule classification introduced in Chapter 2, the various granule types have been designated letters to indicate the cell type from which they are thought to originate. Type j does not correspond to any cell type so far observed in the central nervous system of B. hameri. There may be very few of the corresponding cell type, in which case they would be difficult to locate or alternatively type j granules may originate from neurones in the infraoesophageal ganglion.

Evidence of exocytosis within the neuropile has been observed for granule types g, h and i. Although no omega profiles have been observed in type g terminals, other notable indications of exocytosis do occur. First, material of the same electron density as the core of the neurosecretory granules has been observed in the intercellular space (Fig. 36). Moreover, cytoplasmic bridges in the form of regularly distributed densities are present between granules which are near to the plasmalemma (Fig. 37). Such bridges have also been noted between chromaffin granules and the plasmalemma (Aunis et al., 1979) and vacuoles and the plasmalemma (Nordmann & Morris, 1980) and are thought to be involved in exocytosis and membrane retrieval respectively. Omega profiles of type h granules have been seen (Fig. 38). Another important feature of these granules, which may be related to hormone release, is the presence of "pseudpodia" extending from the granule membrane (Figs. 32 & 38). "Pseudopodia" are not a feature of any other granule type in B. hameri and so are unlikely to be a fixation artefact. This view is supported by the fact that "pseudopodia" have been noted by other workers (Castel, 1977; Scharrer & Wurzelmann, 1977, 1978; Gustafsson et al., 1981) who suggested that they are involved in granule release. Gustafsson et al. (ibid) have in

fact observed "pseudopodia", from neurosecretory granules, penetrating the plasmalemma. Such an occurrence has not been observed in B. hameri. It is more likely that the "pseudopodia" of type h granules represent a weakening of the granule membrane prior to release. This effect will be discussed in more detail in Chapter 4.

Figure 39 illustrates exocytosis of type i granules. It will be noted that these terminals contain granules with cores of variable electron density. This change in the granule contents, or "ageing" process, is a characteristic feature of both vertebrate and invertebrate neurosecretory granules and occurs during axonal transport (see Gainer et al., 1977; Andrew & Salueddin, 1979; Minnen & Reichelt, 1980; Nordmann & Labouesse, 1981). The ageing process does not normally occur within the perikarya and it is for this reason that the neurosecretory granules observed in Type I cells are homogeneous in appearance.

It has not been possible to trace directly the pathways of neurosecretory axons within the central nervous system of B. hameri. However, an alternative method is to match the granules observed in nerves to neurosecretory cell types and hence arrive at tentative pathways for the neurosecretory product. Figure 40 is a region of the antennular nerve containing a neurosecretory axon. The granules in this axon correspond both in size (mean diameter 106.5 nm) and appearance to type h granules. Several other axons containing this granule type were present in the antennular nerve, as well as axons containing neurotransmitter granules.

Similarly, the oesophageal nerve (Fig. 41) contains three axons bearing type a dense-core granules (mean diameter 96 nm). It is not known, at this stage, whether type a granules have a hormonal or a neurotransmitter function.

Figure 42 is a region of the connective between the supraoesophageal ganglion and the suprasplanchnic nerve; it contains both type j and type i neurosecretory axons (mean diameter of granules are 114 and 128 nm respectively). Moreover, evidence of exocytosis of type j granules, in the form of omega profiles and vacuoles, has been observed (Fig. 43).

Four types of dense-core granule occur in the neuropile of the supraoesophageal ganglion of B. balanoides (Figs. 44-46). Each type has been designated a Roman numeral, i.e. I - IV. Types I (mean diameter 116 nm) and II (mean diameter 140 nm) are of similar dimension to B. hameri granule types j and g respectively. Types III (mean diameter 180 nm) and IV (mean diameter 238 nm) are much larger than any granule type encountered in the neuropile of B. hameri. The release sites of the B. balanoides neurosecretory granules have not been elucidated.

In barnacles, not only the process of release of neurosecretory material is synonymous with that of other phyla; so too are the mechanisms of transport, storage and breakdown.

Neurosecretory granules are commonly found to be transported along axons in the form of granule trains which run parallel to neurotubules (Fig. 47). The granules are held together by cross-bridges between the granule membrane (Fig. 48). Similar cross-bridges have been observed between the neurosecretory granules and neurotubules (Fig. 49). It has been postulated that such bridges, or microfilaments, propel both neurosecretory and neurotransmitter granules along neurotubules (Smith, 1971; Maddrell & Nordmann, 1979). However, this is just one of several theories to account for axonal transport of neurosecretory granules (c.f. Grafstein & Forman, 1980). Nevertheless, neurotubules are indispensable to whatever mode of transport operates, as has been demonstrated

using colchicine (Loh et al., 1975).

Only small amounts of neurosecretory material are evident in either the perikarya or the axon terminals (see also Chapter 4) of barnacle neurosecretory cells. In contrast, preterminal axon dilations occur which contain large numbers of neurosecretory granules (Fig. 50). These dilations correspond to the regions of axons which stain intensely with toluidine blue (c.f. Fig. 19). Similar dilations of neurosecretory axons have been noted in vertebrates (Heap et al., 1975) and insects (Steel, 1977). The granules in dilations are not part of the pool of hormone which is released following membrane depolarization. Instead they represent the storage of "older" granules which have not been released (Dreifuss, 1975). Since lysosomes are also associated with the dilations, such regions of the axon may be sites of granule breakdown (Fig. 51). Lysosomal breakdown of neurosecretory granules is also a feature of Herring bodies (axon dilations) in the posterior lobe of the hypophysis of rats (Boudier et al., 1979).

The regions of the supraoeosphageal ganglion, termed "speckled spaces" have been found to be intensely phloxinophilic. Hence there was a possibility that such regions represented either the storage or a release site of neurosecretory product. Such possibilities were dispelled when the regions were examined ultrastructurally. In fact they are large vacuoles which contain a granular matrix of moderate electron density (Fig. 52). The content is reminiscent of haemolymph protein which has been precipitated during fixation (see Chapter 4). Thus, these areas may constitute blood spaces.

DISCUSSION

Throughout Chapter 2 and the present chapter it has been assumed that granules which have a broad electron-lucent zone about the granule core and which have a diameter less than 100 nm are likely to be neurotransmitter granules. Similarly, granules larger than 100 nm, without a prominent halo, are probably neurosecretory granules. These assumptions have been based on the granule descriptions of many workers (Knowles, 1965; Iverson, 1967; Scharrer, 1969; Mason & Bern, 1977). However, such descriptions are subjective and prone to error. For example, it has been noted in the present study that type b granules, which have a mean diameter of 82.5 nm, undergo exocytosis into the extracellular milieu of the neuropile. In addition, type a granules which have a mean diameter of 96 nm, but no distinct halo, could be small neurosecretory granules. The author is well aware that in order to label a granule type as being neurosecretory, its release into the extracellular medium must be established (see Berlind, 1977 for discussion). Such release has been observed for granule types a, g. h. i and j of B. hameri. The remaining granule types of this species which exceed 100 nm diameter and the four types found in B. balanoides can only be described, at this stage, as being possible neurosecretory granules.

Exocytosis of neurosecretory granules within the neuropile is not a novel observation. For example, such exocytosis has been observed within the neuropile of the cerebral ganglia of several species of polychaetes and oligochaetes (Golding, personal communication). Perhaps the compounds which these granules contain have rôles as neuromodulators. Indeed, there is strong evidence that a neurodepressing hormone exists in the central nervous system of crustaceans (Aréchiga & Huberman, 1980).

In general the results obtained with neurosecretory stains, at the

light microscope level, were in good agreement with the ultrastructural observations. The poor staining of perikarya appears to be due to the low numbers of neurosecretory granules. Where granules do occur in large numbers, i.e. in axon dilations, the stains have an affinity for these regions. The anomalies which exist between the results of the two methods, for example, the positive reaction to acridine orange by some cells of the dorsal group and the staining by CAH of type A and B cells, have indirectly been explained, because type b granules, and perhaps also type a granules, are neurosecretory.

Various types of neurosecretory granules from species of molluscs (Wendelaar Bonga, 1970; Minnen & Sokolove, 1981) insects (Girardie & Girardie, 1972; Steel & Morris, 1975) and a crustacean (Lake, 1969) contain sulphur-rich protein. By analogy with vertebrates, the granules may contain a carrier protein which is similar, if not identical to neurophysin. On the basis of the staining reactions of barnacle neurosecretory cells to alcian blue/alcian yellow, such a carrier protein may be absent, or more likely, markedly different from neurophysin. Alternatively, the barnacle cells may contain insufficient amounts of neurosecretory product, to show a reaction to alcian blue. This dilemma could be resolved either by X-ray microanalysis of the barnacle neurosecretory product or by investigating the incorporation of 35 S-cysteine into the product.

The neurosecretory axon dilations observed in barnacles are analogous to those present in vertebrates (Seyama et al., 1980) and insects (Steel, 1977). Furthermore, Steel (ibid), who noted that the storage of neurosecretory granules in Megoura viciae occurs in dilations and not in cell bodies or their terminals in known neurohaemal sites, is an adaptation to long distance transport of neurosecretion. In barnacles, storage of

neurosecretory granules is also a feature of the axons and so may relate to long distance transport of neurosecretion. It is pertinent that no neurohaemal sites have been found in barnacles to date. One possible explanation is that such sites are far removed from the central ganglia.

Fig. 3. B.hameri : whole mount of a Lucifer yellow preparation. An opaque white perikaryon in the protocerebrum has been injected with the fluorescent dye which has diffused along the axon from this cell and entered the median ocellar nerve. Scale bar, 200 μm.

Fig. 5. B.hameri : light micrograph of one of the large ventral perikarya within the supraoesophageal ganglion showing the intensely stained perinuclear zone. C.A.H. Scale bar, 25 μm.

Fig. 6. B.hameri : light micrograph of a small intensely stained cell underlying one of the ventral perikarya of the tritocerebrum. C.A.H. Scale bar, 100 μm.

Fig. 7. B.hameri : light micrograph showing a group of Gomori-positive cells within the protocerebrum. C.A.H. Scale bar, 100 μm.

Fig. 8. B.hameri : light micrograph of a group of dorsal neurones in the supraoesophageal ganglion which have a moderate affinity for C.A.H. Scale bar, 100 μm.

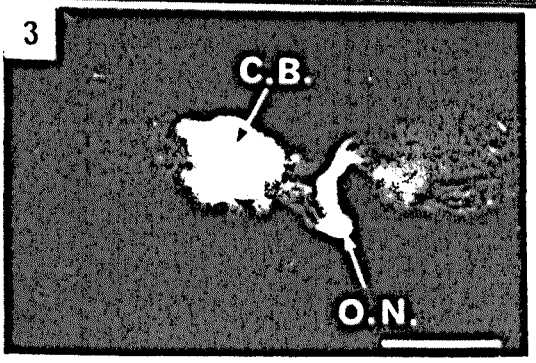
Fig. 9. B.hameri : light micrograph of a "speckled space" (arrowed) within the protocerebrum. C.A.H.P. Scale bar, 25 μm.

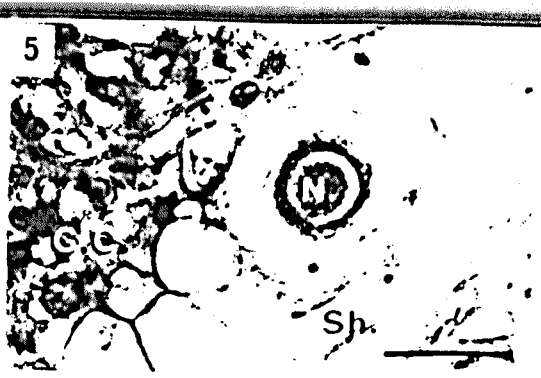
Fig.10. C.auritum : light micrograph showing Gomori-positive material within the neuropile of the supraoesophageal ganglion (arrows). C.A.H. Scale bar, 100 μm.

Fig.11. C.auritum : light micrograph of a transverse section through the supraoesophageal ganglion showing a vacuolated cell, the cytoplasm of which is positively stained. C.A.H. Scale bar, 100 μm.

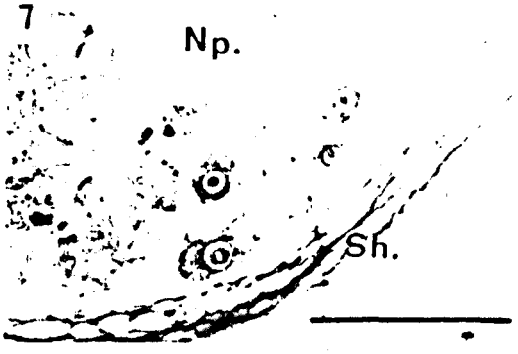
Fig.12. C.auritum : light micrograph of a Gomori-positive neurone within the protocerebrum. C.A.H. Scale bar, 100 μm.

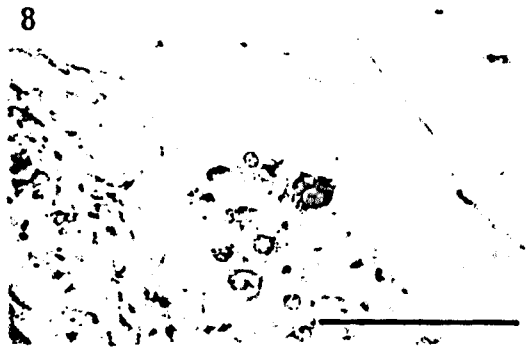
Fig. 13. C. auritum: Light micrograph of a transverse section through the median ocellar nerve containing material which is positively stained (arrows). Scale bar, 100 μm.


3
C.B.
O.N.

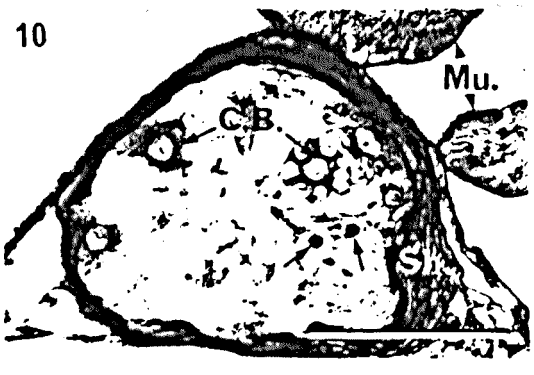

5
N.
Sh.

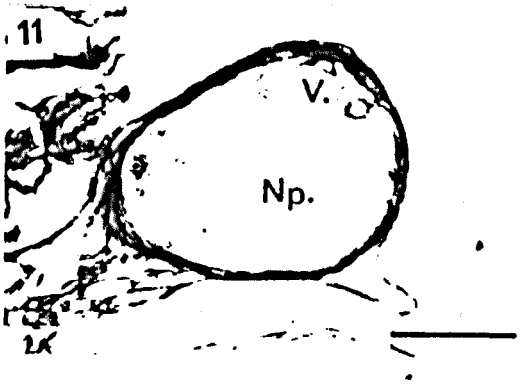

6

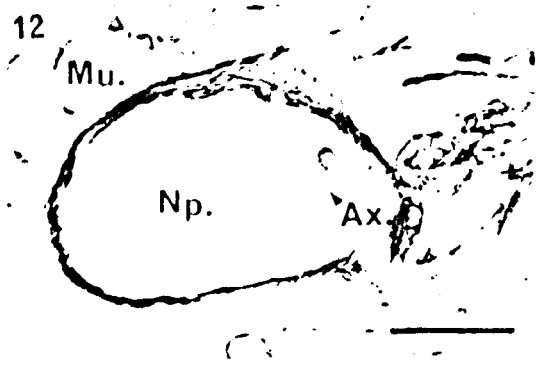

7
Np.
Sh.


8


9
Sh.
Np.


10
Mu.
C.B.
S.


11
V.
Np.


12
Mu.
Np.
Ax.


13
S.O.G.

Fig. 14. C.auritum : light micrograph of a positively stained neurone (arrow) within the dorsal cell group of the supraoesophageal ganglion. C.A.H. Scale bar, 100 μm.

Fig. 16. B.hameri : light micrograph of a transverse section through the suboesophageal ganglion showing the two areas of positively stained material (arrowed) either side of the suboesophageal cleft. P.A.F. Scale bar, 100 μm.

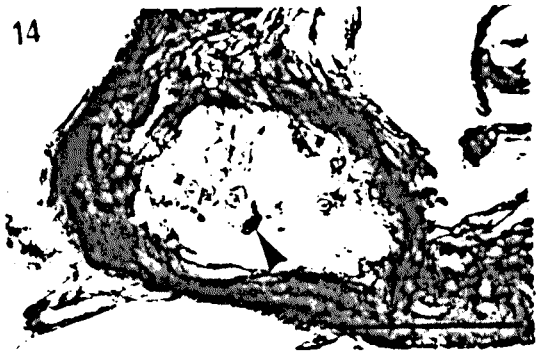
Fig. 17. B.hameri : light micrograph of a transverse section through one of the circumoesophageal connectives showing the intensely stained sheath. P.A.F. Scale bar, 100 μm.

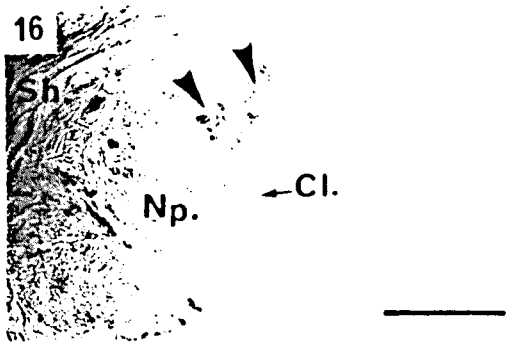
Fig. 18. B.hameri : light micrograph of a moderately stained neurone (arrow) within the protocerebral cell group. P.A.F. Scale bar, 100 μm.

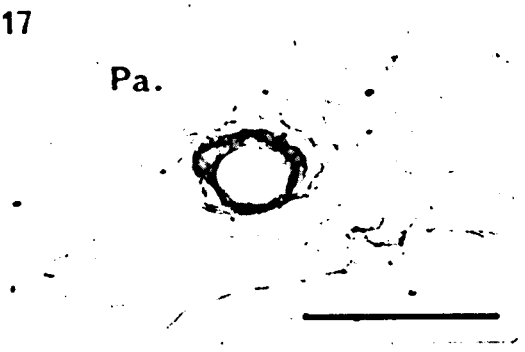
Fig. 19. B.hameri : light micrograph of a 1 μm resin section of the protocerebrum. Intensely stained material can be seen in axons (arrows) entering the commissure. Scale bar, 25 μm.

Fig. 20. B.hameri : electron micrograph showing a type G perikaryon. Scale bar, 5 μm.

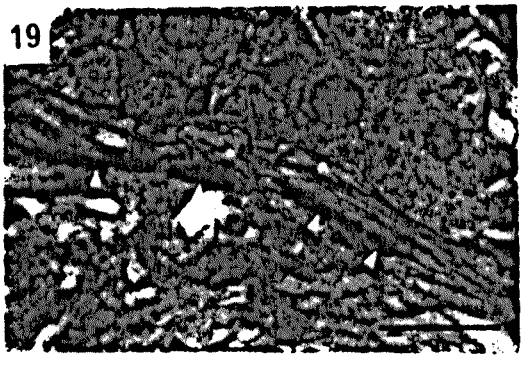
Fig. 21. B.hameri : electron micrograph of an area of cytoplasm within a type G neurone. An electron-dense granule can be seen budding from a Golgi body (arrow). Scale bar, 1 μm.


14


16
Sh
Np.
Cl.


17
Pa.


18


19


21
R.E.R.
D.C.V.
N
S.E.R.

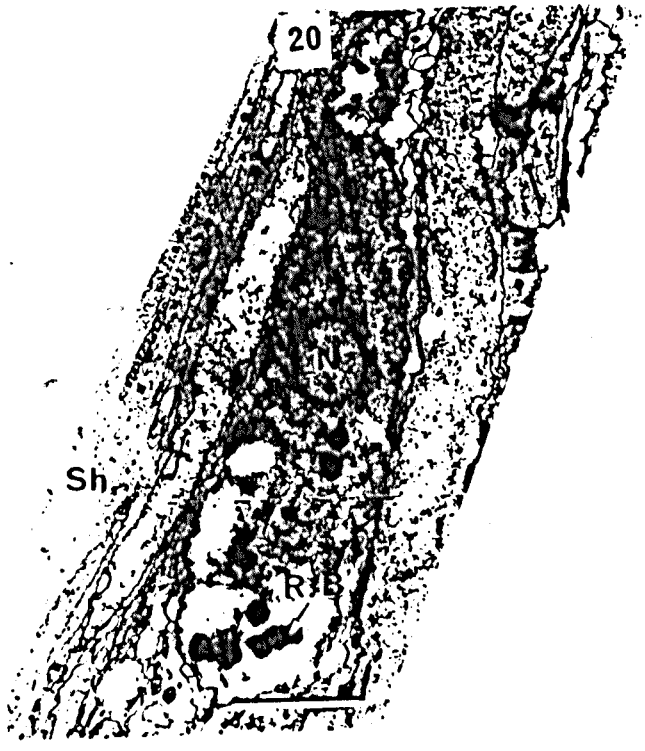

20
N
Sh
R.E.R.

Fig. 22. B.hameri : electron micrograph of a type H perikaryon within the tritocerebrum and underlying the sheath. Scale bar, 5 μm.

Fig. 23. B.hameri : electron micrograph of an area of cytoplasm within a type H neurone. Scale bar, 2 μm.

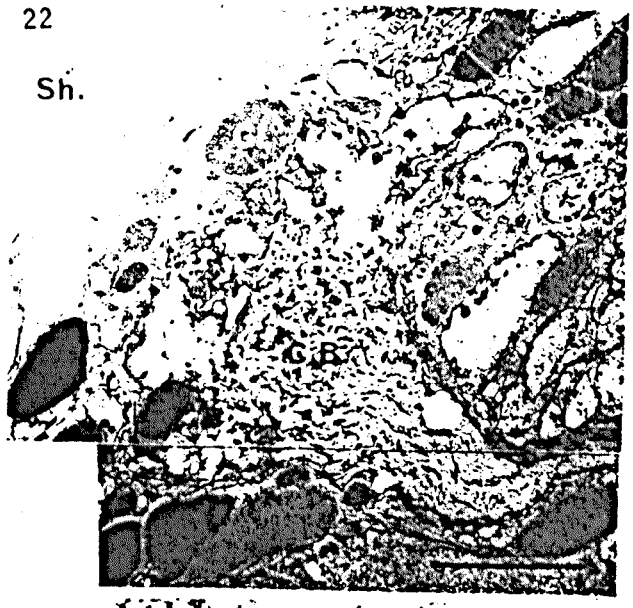
Fig. 24. B.hameri : electron micrograph of type h dense-core granules. One of the granules is in the process of budding from a Golgi sac (arrow). Note that there is very little space between the limiting membrane of the granules and their electron-dense core. Scale bar, 1 μm.

Fig. 25. B.hameri : electron micrograph of a type I neurosecretory cell within the protocerebrum. Scale bar, 4 μm.

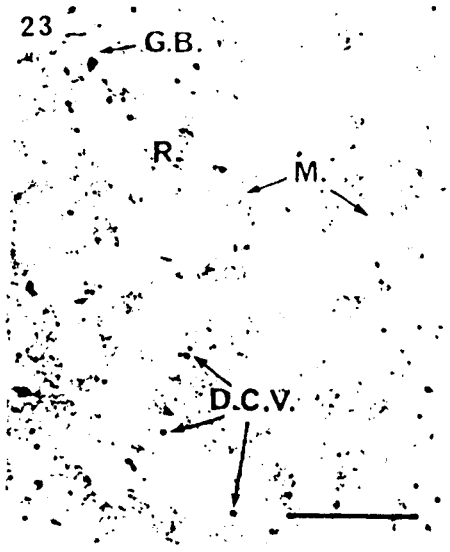
Fig. 26. B.hameri : electron micrograph of a Golgi body within a type I neurone. The Golgi cisternae contain electron-dense material similar to that within the dense core vesicles. Scale bar, 2 μm.

Fig. 27. B.balanoides : electron micrograph of a neurosecretory cell within the tritocerebrum. Scale bar, 3 μm.

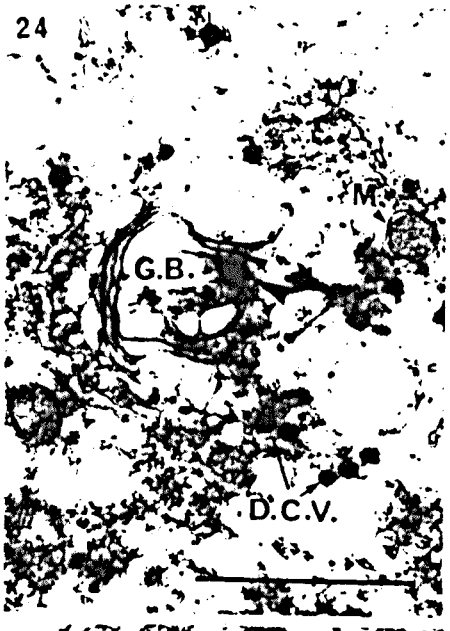
22



23

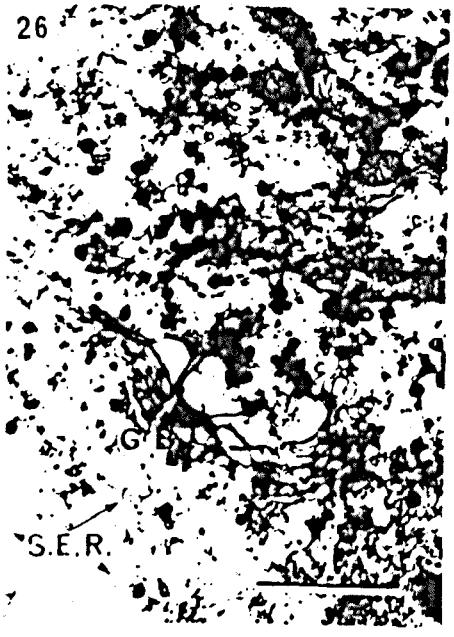


24



25

26



27

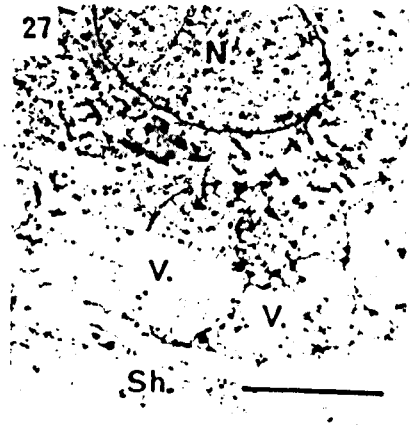


Fig. 28. B.balanoides : electron micrograph showing a Golgi body, the cisternae of which contain electron-dense material. Scale bar, 1.5 μm.

Fig. 29. B.balanoides : electron micrograph of a neurosecretory cell within the tritocerebrum, indistinguishable from that shown in figure 27 apart from the larger size of the dense-core vesicles within the former. Scale bar, 10 μm.

Fig. 30. B.balanoides : higher power of the cell cytoplasm showing rough endoplasmic reticulum and a Golgi body giving rise to dense-core vesicles. Scale bar, 1 μm.

Fig. 31. B.balanoides : electron micrograph showing an area of trophospongium at the periphery of the cell. Note the concentration of mitochondria. Scale bar, 1 μm.

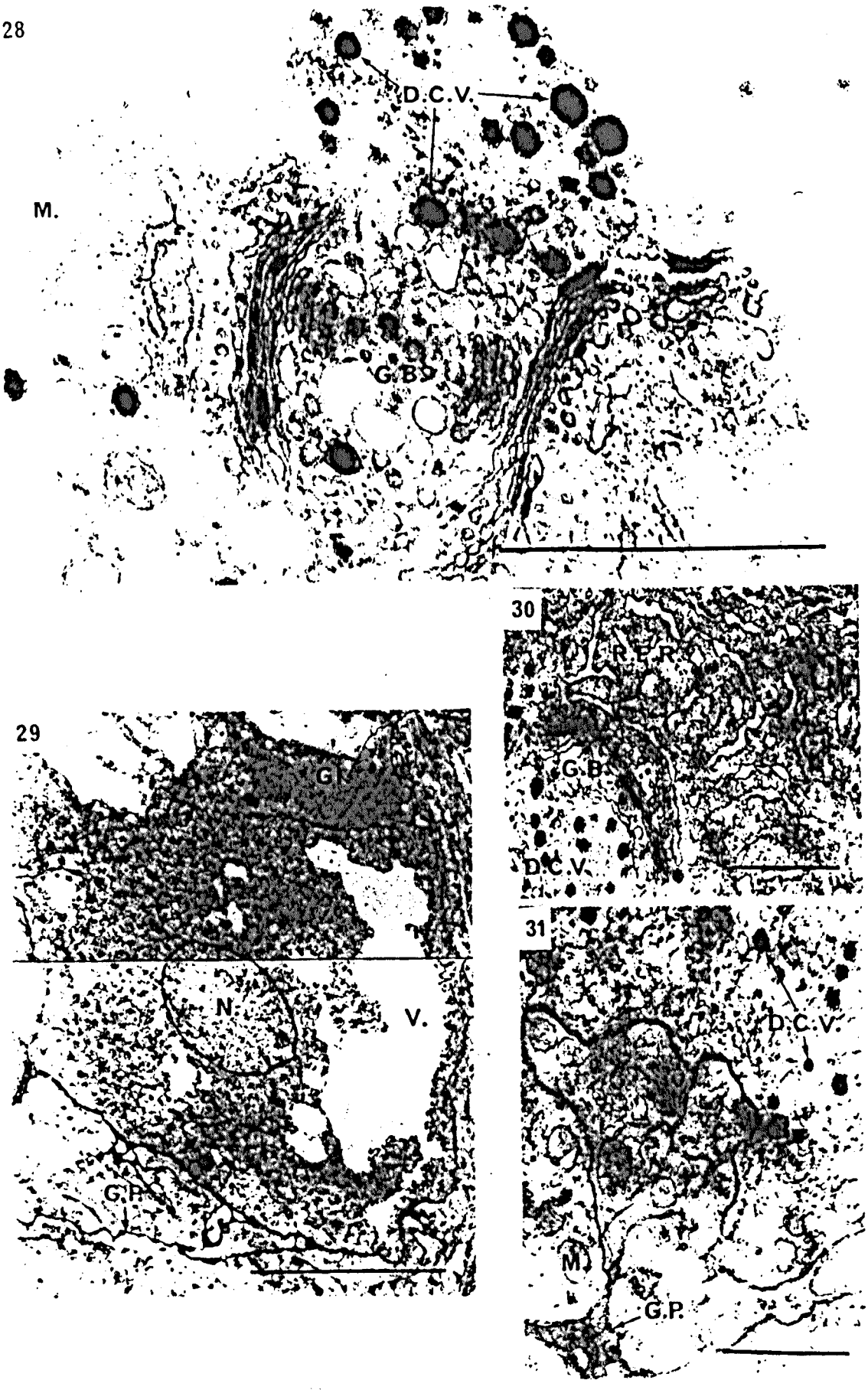

28
D.C.V.
M.
G.B.
30
R.
G.B.
D.C.V.
29
G.
N.
V.
G.P.
31
D.C.V.
M.
G.P.

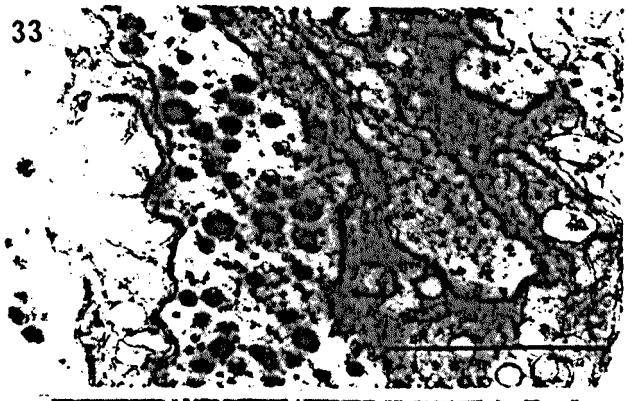
Fig. 32. B.hameri : type g dense-core vesicles. Scale bar, 1 μm.

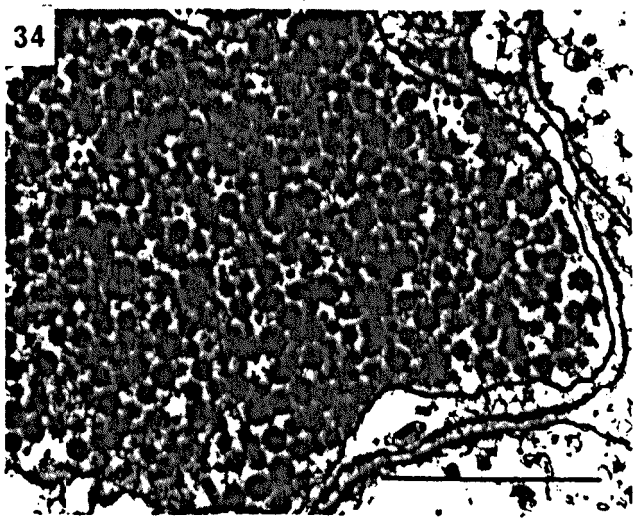
Fig. 33. B.hameri : type h dense-core vesicles. Scale bar, 1 μm.

Fig. 34. B.hameri : type i dense-core vesicles. Scale bar, 1 μm.

Fig. 35. B.hameri : type j dense-core vesicles. Scale bar, 1 μm.


32


33


34

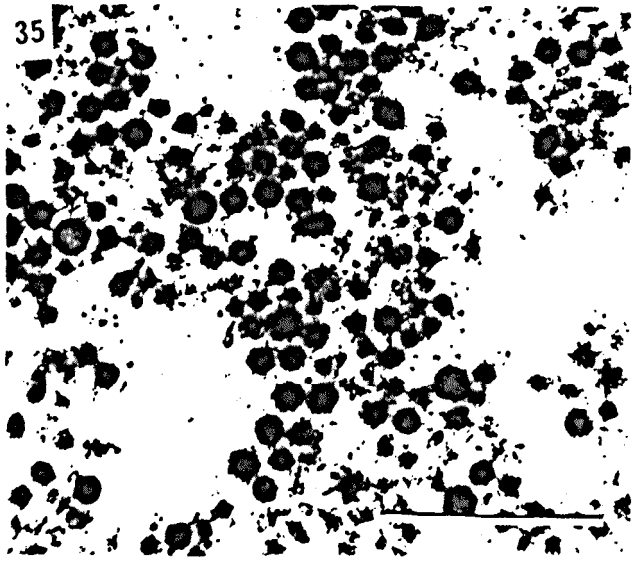
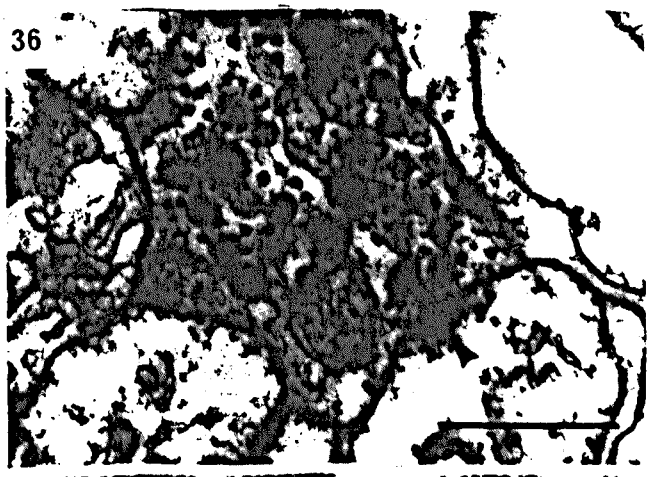

35

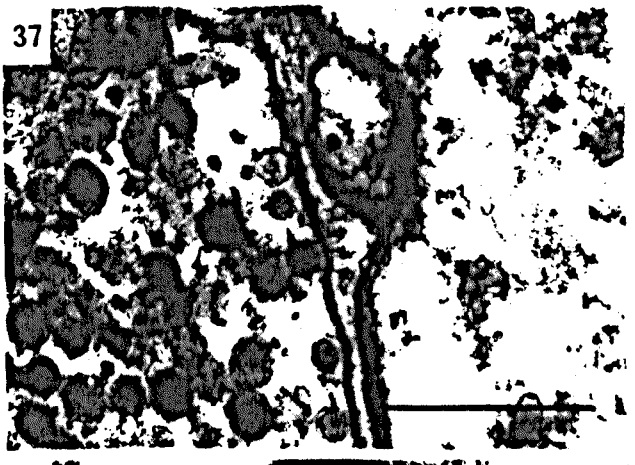
Fig. 36. B.hameri : electron micrograph of an axon within the neuropile of the supraoesophageal ganglion containing type g granules. Note that material of the same electron density as the granules is present in the intercellular space (arrow). Scale bar, 0.5 μm.

Fig. 37. B.hameri : electron micrograph of type g granules. Note the presence of electron-dense connections (arrowed) between one of the granules and the plasmalemma. Scale bar, 0.5 μm.

Fig. 38. B.hameri : electron micrograph of an omega profile (large arrow) in the plasmalemma of a type h axon terminal. Note also the presence of a pseudopodium on one of the granules (small arrow). Scale bar, 0.5 μm.

Fig. 39. B.hameri : electron micrograph showing exocytosis of a type i dense-core granule (arrow). Scale bar, 1 μm.


36


37


38

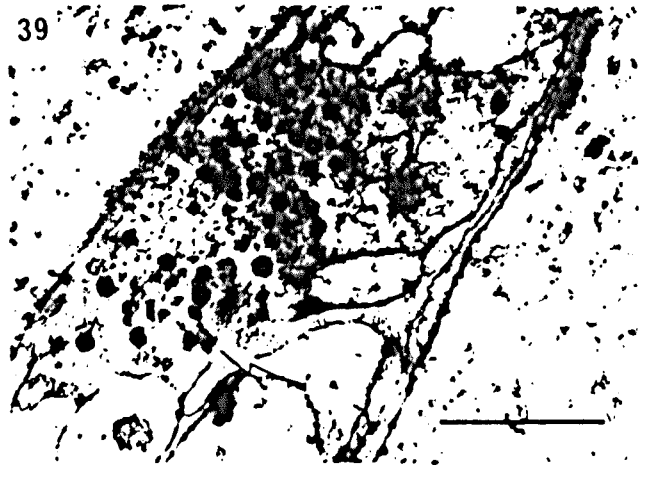
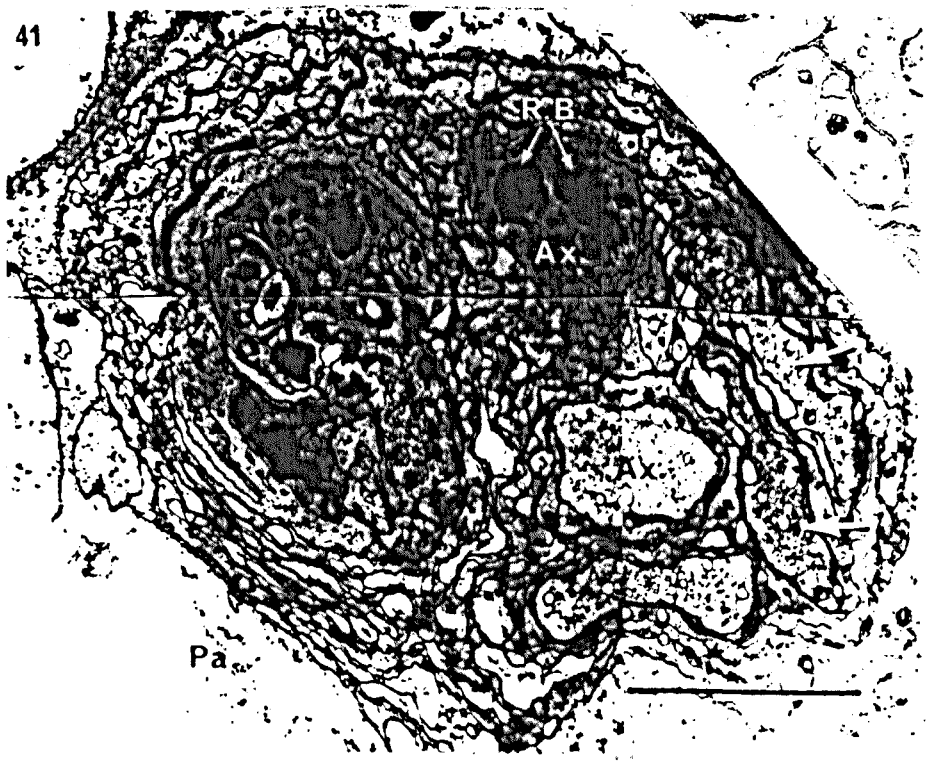

39

Fig. 40. B.hameri : electron micrograph of part of the antennular nerve showing a small axon containing type h dense-core vesicles. Scale bar, 5 μm.

Fig. 41. B.hameri : electron micrograph showing a transverse section of the oesophageal nerve. Two axons containing neurosecretory granules are visible (arrows). A third axon, also containing neurosecretory granules, but not visible in the plane of this section, is shown in the inset. Scale bar, 5 μm.

Fig. 42. B.hameri : electron micrograph of a transverse section through the connective between the suprasplanchnic nerve and the supra-oesophageal ganglion showing axons containing two types of dense-core vesicle. Scale bar, 2 μm.

Fig. 43. B.hameri : electron micrograph of an axon in the connective showing a possible omega profile involving type j granules (arrow). Scale bar, 0.5 μm.



42

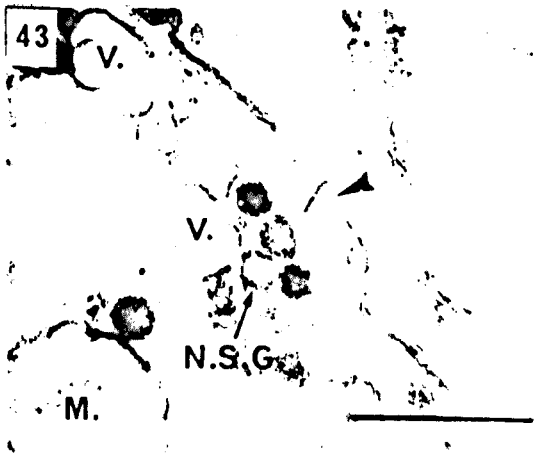
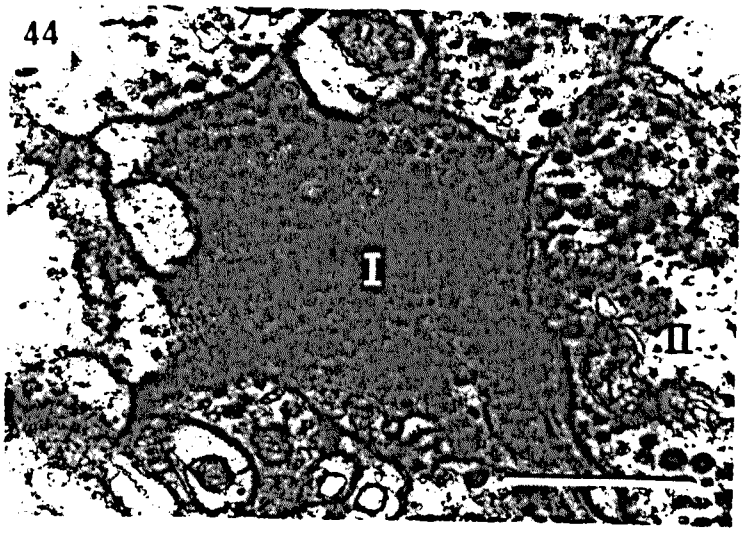


Fig. 44. B.balanoides : Type I and II dense-core vesicles. Scale bar, 1 μm.

Fig. 45. B.balanoides : type III dense-core vesicles. Scale bar, 1 μm.

Fig. 46. B.balanoides : type IV dense-core vesicles. Scale bar, 1 μm.

44



45

46

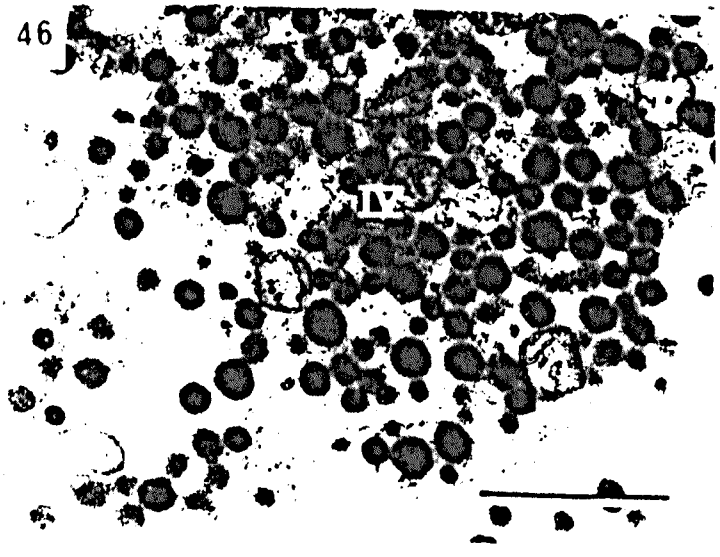


Fig. 47. B.hameri : electron micrograph showing a longitudinal section of an axon. Dense-core vesicles occur as a chain adjacent to a microtubule. Scale bar, 0.5 μm.

Fig. 48. B.hameri : electron micrograph showing cross-bridges (arrowed) between neurosecretory granules. Scale bar, 0.3 μm.

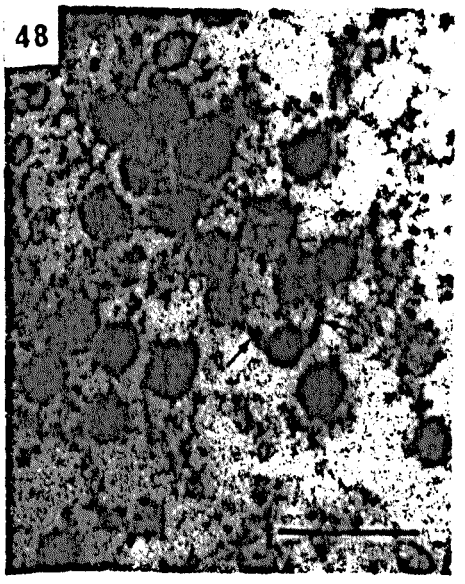
Fig. 49. B.hameri : electron micrograph showing cross-bridges (arrowed) between a neurosecretory granule and a microtubule. Scale bar, 0.3 μm.

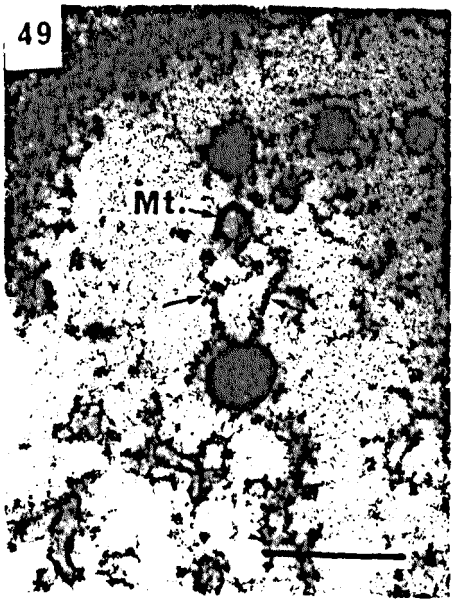
Fig. 50. B.hameri : electron micrograph showing a preterminal axon dilation containing a large number of neurosecretory granules. Scale bar, 2 μm.

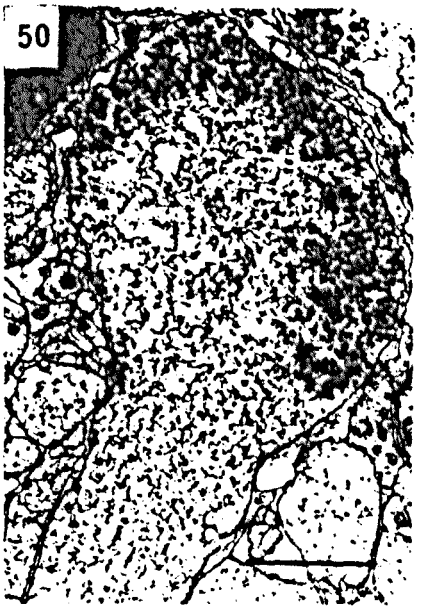
Fig. 51. B.hameri : electron micrograph of a lysosome within an axon dilation. Dense-core vesicles can be seen within the lysosome (arrows). Scale bar, 0.5 μm.

Fig. 52. B.hameri : electron micrograph showing the granular content of the phloxinophilic "speckled spaces" of the supraoesophageal ganglion. Scale bar, 3 μm.


47
M
DCV


48


49
Mt.


50


51


52

CHAPTER 4

A study of the median ocellus of *Balanus hameri* (Ascanius) with especial reference to neurosecretion

CHAPTER 4

A study of the median ocellus of Balanus hameri (Ascanius) with especial reference to neurosecretion

INTRODUCTION

The cypris larva of barnacles possesses a pair of laterally placed compound eyes and a median nauplius eye which is tripartite in structure. Concomitant with the metamorphosis to the adult is the loss of the compound eyes and the separation of the nauplius eye into its three constitutent parts. The two dorso-lateral components of the nauplius eye come to lie under the rostral shell plates whereas the ventral component forms the median ocellus, which underlies the mantle flaps of the scutal plates at the posterior margin of the adductor muscle.

The past 15 years have witnessed an intense research effort on the photoreceptors of barnacles and in particular those of adults (see Table 1). These cells offer an ideal model for the study of the process involved in the 'propagation' of visual signals from the photoreceptive membrances, via the axon and presynaptic terminals, to the postsynaptic cells (Hudspeth & Stuart, 1977). The simple ocelli lie peripherally at some distance from the supraoesophageal ganglion and so possess long axons. Commonly, the cell bodies of the photoreceptor are large and few in number. For instance, the median photoreceptor of the giant Pacific barnacle, Balanus nubilus, contains only four, (Ross & Stuart, 1976).

The receptors of the ocelli respond to light by slowly depolarizing so that the amplitude of the receptor potential is dependent on the intensity of the stimulus, i.e. the greater the intensity of light, the larger the response (Millechia & Gwilliam, 1972). Due to the high

TABLE 1. Studies on barnacle ocelli

Species	Topic	Reference
Balanus sp.	shadow reflex	Coldstream 1836
B.rugosus	morphology	Leidy 1848
Lepas anatifera	morphology	Darwin 1851
Balanus spp.	morphology	Darwin 1854
Pollicipes polymerus	morphology	Nussbaum 1890
L.anatifera	morphology	Gruvel 1893
B.eburneus	shadow reflex	Fales 1928
Balanus sp.	shadow reflex	von Buddenbrock 1930
B.balanoides, *B.crenatus*, *B.improvisus*	nauplius eye morphology	Kauri 1962
Balanus spp.	nauplius eye morphology	Elofsson 1963
B.cariosus, *B.eburneus*, *L.anatifera*, *P.polymerus*	shadow reflex	Gwilliam 1963
Balanus sp.	nauplius eye ultrastructure	Fahrenbach 1964
B.cariosus, *B.eburneus*, *B.tintinnabulum*	shadow reflex	Gwilliam 1965
B.amphitrite, *B.cariosus*	ultrastructure	Fahrenbach 1965
B.balanoides	ultrastructure	Aldous 1968
B.balanoides	nauplius/cyrid morphology	Walley 1969
B.amphitrite, *B.eburneus*	electrophysiology	Brown *et al*. 1970;1971
B.cariosus, *B.nubilus*	physiology and anatomy	Gwilliam & Bradbury 1971
B.eburneus	electrophysiology	Koike *et al*. 1971
B.cariosus	electrophysiology	Millechia & Gwilliam 1972
B.eburneus	electrophysiology	Shaw 1972
B.nubilus	electrophysiology	Stuart *et al*. 1974
B.amphitrite, *B.eburneus*	electrophysiology	Brown & Carter-Cornwall 1975
Balanus sp.	pharmacology	Lantz & Millechia 1975

TABLE 1. (cont'd)

Species	Topic	Reference
B.nubilus	electrophysiology	Stuart & Poo 1975
B.eburneus	electrophysiology	Brown & Ottoson 1976
B.cariosus, B.hameri	shadow reflex	Gwilliam 1976
B.eburneus	ultrastructure	Krebs & Schaten 1976
B.nubilus	electrophysiology	Ozawa et al. 1976
B.nubilus	electrophysiology	Ross & Stuart 1976
B.amphitrite, B.eburneus	electrophysiology	Brown & Saunders 1977
B.eburneus	electrophysiology	Dahl et al. 1977
B.amphitrite	electrophysiology	Hanami & Shaw 1977
B.nubilus	electrophysiology and morphology	Hudspeth & Stuart 1977
B.nubilus	electrophysiology	Hudspeth et al. 1977
B.eburneus	electrophysiology	Lantz et al. 1977
B.nubilus	electrophysiology	Ozawa et al. 1977
B.eburneus	electrophysiology	Saunders & Brown 1977
B.amphitrite	visual response	Atzmon et al. 1978
B.amphitrite	electrophysiology	Corson 1978
B.eburneus	sensitivity	Lantz & Mauro 1978
B.amphitrite, B.eburneus	visual pigment	Minke & Kirschfeld 1978
B.eburneus	light adaptation	Strong & Lisman 1978
B.eburneus	electrophysiology	Stuart & Oertel 1978
B.eburneus	pigment diffusion	Almagor et al. 1979
B.nubilus	electrophysiology	Edgington & Stuart 1979; 1981
B.nubilus	electrophysiology	Oertel & Stuart 1981
B.balanoides	nauplius eye ultrastructure	Ong & Walker
Elminius modestus	ultrastructure	unpublished

resistivity of the photoreceptor membrane (Hudspeth et al., 1977), the light-induced voltage changes spread passively along the axon to the supraoesophageal ganglion (Millechia & Gwilliam, 1972). Millechia & Gwilliam (ibid) identified possible interneurones in this ganglion on the basis of potential changes during the shadow reflex. No more than two neurones were found in each preparation. They are positioned within the commissure of the supraoesophageal ganglion, in the vicinity of the photoreceptor axon terminals (Millechia & Gwilliam, 1972; Hudspeth & Stuart, 1977). During illumination the photoreceptor terminals are thought to release an inhibitory transmitter, gamma-amino butyric acid, (GABA) (Lantz & Millechia, 1975), which causes the postsynaptic neurones to become hyperpolarized. When the median ocellus is shadowed, the release of GABA stops and the postsynaptic cells depolarize. The resultant burst of impulses travel along the circumoesophalgeal connectives to the infraoesophageal ganglion where they 'affect' the motor neurones controlling those muscles involved in the shadow reflex. Gwilliam (1970) has established that this 'affect' involves both excitatory and inhibitory mechanisms on two groups of "burster" neurones. One group, effecting withdrawal of the animal into the shell and closure of the opercular plates, is excited by the reduced light intensity, the other controlling extension, is inhibited.

When the extent of the research interest on the adult photoreceptors is considered it is surprising that relatively few studies have addressed the fine structure of these receptors. The most detailed ultrastructural study is that of Fahrenbach (1965). He examined the median photoreceptor of B.cariosus and the lateral photoreceptors of B.amphitrite. They contained, respectively, seven and three photoreceptor cell bodies which possessed many branching dendrites, each capped distally by a rhabdomere. A large axon, enveloped by glial cells, extended from the cell bodies to the supraoesophageal ganglion.

A particularly interesting feature, was the presence of a number of small axons, within the median ocellar nerve of B.cariosus, some of which contained neurosecretory granules, which reportedly extended to and from the supraoesophageal ganglion. The origin or possible function of these granules was not examined. It was, in part, for this reason that the ultrastructure of the median photoreceptor of B.hameri was investigated. Furthermore, this species is sublittoral and so provides the opportunity to compare its photoreceptor with that of B.cariosus, an intertidal barnacle. In this respect, it is pertinent that the shadow reflex of B.balanoides, another intertidal barnacle,is better developed than that of B.hameri (personal observation).

MATERIALS AND METHODS

The animals used in this study were collected and maintained as described in chapter 2. The photoreceptors examined were processed during the months of November and December 1980, following a collection of animals on November 15. One group were processed on arrival in the laboratory. Dissections were normally carried out between 9 a.m. and 1 p.m.

Having prepared an animal for dissection, as described in chapter 2, the median photoreceptor was located by making an incision in the cuticle, anterior to the oral cone and extending this cut to the adductor muscle. The photoreceptor occupied a position just posterior to this muscle and when exposed the sea water was drained from the dissecting dish and the preparation flooded with fixative. After 5 min. in fixative, the photoreceptor was dissected free from the surrounding tissues and placed into fresh fixative.

The fixatives used were the trialdehyde fixative of Lake (1973), which has been used successfully to fix the nauplius eye of barnacles (Ong and Walker, unpublished) and 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.4) with 1% osmium tetroxide in cacodylate buffer as a post-fixative. The procedure is more fully described in chapter 2. Fixation was carried out either over ice, or at room temperature.

The preparation and staining of 1 µm and ultrathin sections were as described in chapter 2.

Cobalt backfills of the median ocellar nerve, either towards the supraoesophageal ganglion or towards the median photoreceptor were carried out as described in chapter 2.

RESULTS

The median photoreceptor of an adult B.hameri of rostro-carinal diameter 1.8 cm. measures 0.78 mm in length by 0.39 mm in width. This photoreceptor is light-orange to orange in colour and so is readily distinguished from the surrounding white to pale yellow parenchyma tissue. In fresh tissue, examined under the binocular microscope, the photoreceptor has a bilobed appearance and this is emphasized when it is stained with aqueous methylene blue (Fig. 1). Accompanying the median ocellar nerve for its entire length is a small striated muscle (Fig. 2) which by-passes the median photoreceptor and inserts amongst the parenchyma tissue at the posterior margin of the adductor muscle. Hudspeth & Stuart (1977) have suggested that this muscle serves to take up slack in the ocellar nerve when the body of a barnacle is withdrawn into the mantle cavity.

The ganglionic masses of the photoreceptor are encapsulated by a perineurium, which is about 80 µm thick at its widest point. It is continuous with the perineurium of the ocellar nerve and the supra-oesophageal ganglion. The two constituents of the latter, namely the

perilemma and the fibrous neurilemma, can also be distinguished around the ocellus. The perilemma (Fig. 3) is composed of several layers of flattened, elongate cells of about 4 μm width. These cells were described as "capsular fibroblasts" by Fahrenbach (1965). The most prominent feature of their cytoplasm is the numerous microtubules which extend along the long axis of the cell. Other organelles, including mitochondria and endoplasmic reticulum, are sparse. The nuclei, which are normally elongate, measure between 7 and 8 μm in length. Rarely, lobulate nuclei are observed (Fig. 4).

Surrounding the fibroblasts are many layers of fibrils which are probably composed of collagen (see chapter 2). The outer layer, or neurilemma, is composed solely of these fibrils (Fig. 5) and is about 1.4 μm thick.

The ocellus is embedded in an area of loosely arranged parenchyma cells (the "endothelial cells" of Fahrenbach, 1965), some of which are closely apposed to the neurilemma (Fig. 6). Their oval nucleus contains patches of electron-dense chromatin which are most prominent around the periphery. In freshly collected specimens, parenchyma cells are full of lipid (Fig. 7).

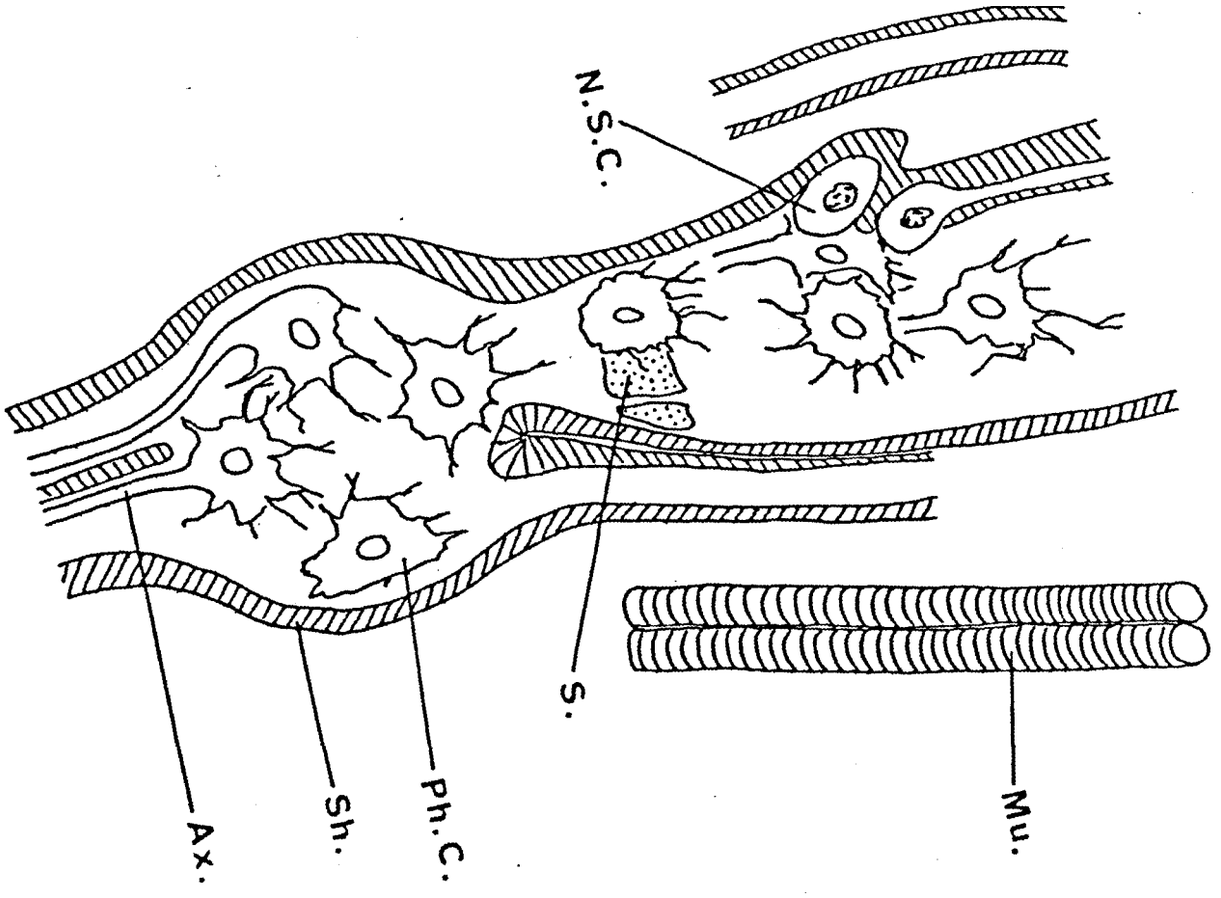
However, in those animals which were sacrificed in late November or December, the parenchyma cells were virtually devoid of lipid. The cytoplasm contains few additional inclusions (Fig. 8); a small amount of rough endoplasmic reticulum, a scattering of microtubules and a few small (ca. 0.5 μm diameter) mitochondria are present. Therefore, the bulk of the cytoplasm appears electron-lucent. Between the parenchyma cells and the haemolymph is a thin (ca. 46.5 nm) basal lamina which appears to be fenestrated (Fig. 9). In some regions it becomes highly convuluted.

The parenchyma cells do not constitute a barrier between the ocellus and the haemolymph for spaces between the cells effectively form channels through which the haemolymph is able to percolate (Fig. 10). The

haemolymph becomes precipitated when fixed and appears to be composed of two fractions (Fig. 11). This may reflect varying degrees of clotting. Numerous haemolymph cells are present and they are commonly found adhering to the neurilemma or to the parenchyma cells. The most prominent type, also noted by Fahrenbach (1965), has a spherical nucleus, of between 2.5 and 4.0 μm in diameter and a single large nucleolus. The cytoplasm contains a moderate amount of rough endoplasmic reticulum, microtubules, a few lysosomes, mitochondria and occasionally a few small (178 nm), electron-dense granules. Golgi bodies have not been observed. The cell possesses a single pseudopodium, which has been observed to reach lengths of 11 μm (Fig. 12). This haemolymph cell type is most closely related to the hyaline cells of crustaceans (cf. Bauchau, 1981).

Most of the ocellus is occupied by photoreceptor cell bodies and neuroglia. Using 1 μm serial sections the ocellus has been found to contain 10 (neuronal) cell bodies (Fig. 13), which is a greater number than has been reported for other Balanus species - 4 in B.nubilus and 7 in B.cariosus. Eight of the cell bodies belong to photoreceptor cells and are divided equally between the two compartments of the ocellus. The cell bodies are large (ca. 50-60 μm in diameter) and contain a centrally placed, spherical nucleus (ca. 14 um diameter) which has a single nucleolus. The chromatin of the nucleus is homogenous and moderately electron-dense (Fig. 14). The cytoplasm (Fig. 15) contains well developed endoplasmic reticulum, mainly of the rough type. The mitochondria, which are of the transverse septate type, reach lengths of 2.3 μm, though normally they are much shorter than this (ca. 1 μm) and have a diameter of about 350 nm. Golgi bodies were rarely observed, but when present they were situated in the perinuclear zone. Electron-lucent vesicles, 70 nm in diameter bud off from the lateral margins of the lamellae (Fig. 16). However in some cells dense-cored granules of diameter ca. 111.5 nm have been observed.

Fig. 13. Schematic diagram of the median ocellus of B.hameri, illustrating the cell types and their relative positions. Not to scale.


N.S.C.
S.
Ax.
Sh.
Ph.C.
Mu.

Most conspicuous are the many residual bodies present throughout the cytoplasm (Fig. 17). Large aggregates of glycogen, in the form of rosettes (α particles), are also prominent, and are frequently present within the axon hillock (Fig. 18).

The perikarya of the photoreceptor cells give rise to several dendrites which branch repeatedly (Fig. 19). The processes are distinguished from the surrounding glial tissue by virtue of their electron-dense cytoplasm (Fig. 20). Neurotubules are abundant and are in close association with various inclusions in transit to and from the dendrite terminal. They include electron-lucent vesicles, mitrochondria and lysosomes. Each process ends in a bulbous terminal which is capped by a rhabdomere (Fig. 21). The rhabdomeres are formed mostly by simple finger-like projections (microvilli) of the dendrite terminals. The microvilli, which measure between 2.0 and 2.5 μm in length by about 68 nm in diameter, are roughly hexagonal in cross-section (Fig. 22) and are tightly packed in a honeycomb-like arrangement to form the rhabdomere.

The cytoskeleton within a microvillus as described by Blest et al., (1982) is not well preserved by the fixatives employed in the present study. The microvilli are, however, not totally devoid of content. What appear to be filaments, have been observed in cross-sections of microvilli, but not in longitudinal sections.

Microvilli are normally envisaged as constituting simple finger-like projections of the apical plasma membrane of a cell so greatly enlarging the surface area (see for example Threadgold, 1967). This model is based on observations of intestinal cells. However, it has recently become apparent that the microvilli of intestinal cells and rhabdomeres are not formed by the same process. Stowe (1980) has demonstrated that in the crab, Leptograpsus variegatus, microvilli are assembled from sheets of "doublet ER"

although exactly how the new membrane is incorporated into the rhabdomere remains abstruse. A further complication is that when micrographs of barnacle rhabdomeres were examined, it became clear that their structure was more complex than first realized.

Figure 23 illustrates a microvillus which has bifurcated, but most retain the simple configuration. More frequently, microvilli are found to be bent through 180°, (Fig. 24). This bending explains some of the curious features noted in the basal region of the rhabdomeres. In particular, microvilli are observed to be both open and closed to the cytoplasm of the dendrite terminal, and this occurs in a random manner (Fig. 25). The phenomonon can be explained if it is assumed that each microvillus is bent through 180° at least once (Fig. 26).

The plasma membrane of the microvilli is frequently seen to invaginate into the cytoplasm of the dendrite terminal. The pinocytosed membrane may account for some or all of the profusion of vesicular inclusions within the terminal (Fig. 25). Larger, vacuoles (ca. 70-140 nm) with double membranes are also a prominent feature, (Fig. 27). Their origin is uncertain, but similar electron-lucent structures are occasionally found to be either fusing with, or budding from, the basal region of the microvilli (Fig. 28). Characteristically, the dendrite terminals contain many mitochondria, whilst lysosomes have only occasionally been noted. The latter occur as residual bodies (Fig. 29) identical to those present in the photoreceptor perikaryon.

The apical region of the rhabdomere appears to be more stable in terms of membrane turnover. In longitudinal section, the microvilli are found to end blindly and are separated from the plasma membrane of the glial cell (or mesaxon; see Fahrenbach, 1965) by an intracellular space of ca. 130 nm. (Fig. 30). Microvilli which are continuous with the

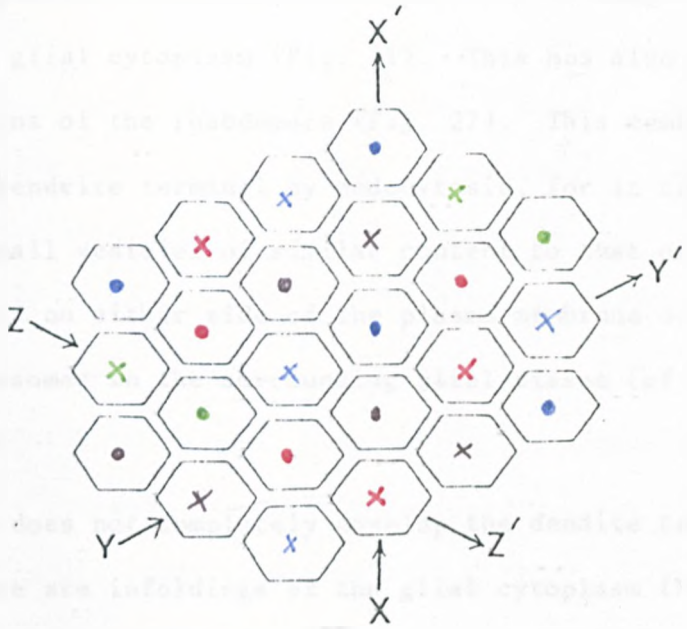
Fig. 26. A. Diagrammatic transverse section through a small area of rhabdomere viewed from the apical end. It is assumed that each microvillus bends through 180° once and that the direction of the bends is random.

B. Based on the above assmptions it is possible to extrapolate and construct longitudinal sections of microvilli which can explain any of the profiles observed in electron micrographs of the basal region of rhabdomeres.

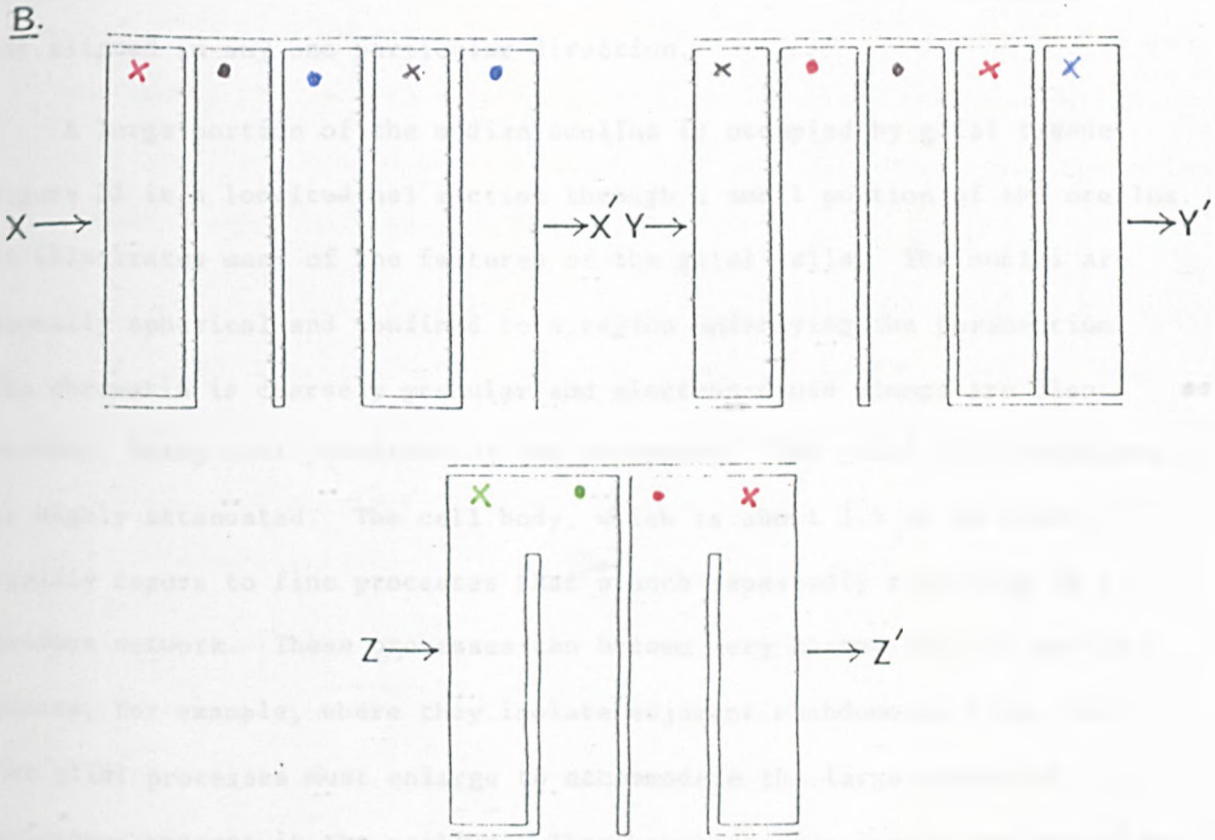
•, basal-apical direction

X, apical-basal direction

A.



B.



cytoplasm of the glial tissue are observed infrequently (cf. Figs. 21 and 23). A mechanism for membrane recycling, in addition to the pinocytosis observed in the basal region, has been noted at the apical end of the rhabdomere. Small portions of microvilli appear to be sloughed off the rhabdomere into the glial cytoplasm (Fig. 31). This has also been noted at the lateral margins of the rhabdomere (Fig. 27). This membrane may be returned to the dendrite terminal by endocytosis, for it can be seen in figure 27 that small vesicles of similar content to that of the rhabdomeres are found on either side of the plasma membrane of the terminal. The presence of lysosomes in the surrounding glial tissue (cf. Fig. 24) has also been noted.

The rhabdomere does not completely envelop the dendite terminal. At certain points, there are infoldings of the glial cytoplasm (Fig. 24) as noted by Fahrenbach (1965). It is also apparent that the microvilli are not aligned in any one particular direction.

A large portion of the median ocellus is occupied by glial tissue. Figure 32 is a longitudinal section through a small portion of the ocellus. It illustrates many of the features of the glial cells. The nuclei are normally spherical and confined to a region underlying the perineurium. The chromatin is coarsely granular and electron-dense clumps are also present, being most prominent at the periphery. The glial cell cytoplasm is highly attenuated. The cell body, which is about 3.5 μm in width, rapidly tapers to fine processes that branch repeatedly resulting in a profuse network. These processes can become very narrow (ca. 14 nm) in places, for example, where they isolate adjacent rhabdomeres (Fig. 33). The glial processes must enlarge to accommodate the large number of gliosomes present in the ocellus. These proteinaeous inclusions are of an homogenous and finely granular nature. Most often they are moderately electron-dense, but a range of densities have been observed (Fig. 34) as

was the case for inclusions in the supra- and infra-oesophageal ganglia (cf. chapter 2).

A large amount of rough endoplasmic reticulum, of an unusual type, is present in the perinuclear zone of the glial cells (Fig. 35) and also in the glial processes. The ribosomes lie on profiles with an homogenous, finely granular and moderately electron-dense content. It is pertinent that this content is very similar to that of the gliosomes. The profiles, in cross-section, measure a maximal diameter of 0.4 μm.

The most prominent feature of the glial cell cytoplasm is the presence of numerous microtubules. They occur in bundles and extend over long distances in the longitudinal axis of the ocellus (see Fig. 32). Occasionally they are found to insert onto gliosomes (Fig. 36). The microtubules, when viewed in cross-section, are seen to have an electron-lucent core of 20 nm diameter. The outer diameter measures 26.5 nm. The outer surface of the microtubules is covered by flocculent material which appears to 'bind' each bundle together (Fig. 37). It is assumed that the microtubules play a cytoskeletal role.

The glial processes are joined together intermittently by desmosomes (Fig. 38). Tonofilaments, which are normally found to radiate from the attachment plaque of desmosomes (cf. Threadgold, 1967), are absent. Instead, microtubules are found on either side of the desmosome as noted by Fahrenbach (1965).

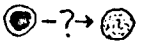
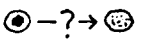
The remaining cell type has not previously been noted in the ocellus of barnacles. Two projections arise from the anterior compartment of the median ocellus of B.hameri. Each projection contains one cell body, neither of which would appear to have a role in photoreception, since they do not possess rhabdomeres. The anterior-most perikaryon has an axon which extends anteriorly. This together with the fact that one of the photoreceptor cell

bodies from the posterior compartment sends an axon beyond the ocellus (see Fig. 13) explains why cobalt backfills of the ocellus, via the median ocellar nerve, fill only eight cell bodies (Figs. 39 and 40). This technique clearly reveals one of the projections, confluent with the anterior compartment, containing a solitary cell body. It is interesting that the axon from this cell body branches in the posterior compartment of the ocellus and appears to make contact with the axon hillock of one of the photoreceptor cell bodies therein.

The fine structure of the other solitary cell body (i.e. the one whose axon extends anteriorly) is particularly noteworthy (Fig. 41). The perikaryon is elongate, measuring 25 μm in diameter at its widest point. It is thus considerably smaller than the photoreceptor cell bodies. The nucleus is proportionately small and in places convuluted in outline which is indicative of an active cell in terms of secretion synthesis. Golgi bodies are abundant and their lamellae, which are full of an electron-dense material, give rise to dense-cored granules with a mean diameter of 115.5 nm (Fig. 42). Other constitutents of the cytoplasm are rough endoplasmic reticulum, mitrochondria, electron-lucent vesicles, multivesicular bodies (Fig. 45) and membrane whorls (Fig. 44). In addition there are large patches of moderately electron-dense cytoplasm (Figs. 41 and 42) of unknown content.

The axon of this cell contributes to a region beyond the ocellus which is rich in axons and their terminals (Fig. 45). These axons, with their content of dense-cored granules, have prevously been noted by Fahrenbach (1965) and Hudspeth & Stuart (1977a). However, the morphology of the terminals beyond the ocellus was not described. Six granule types have been identified in the axons running parallel to the ocellus and their terminals, anterior to the ocellus, in B.hameri. This statement is based on differences in size and content of the granules (Table 2). The various granule types are shown in figures 46 to 51. Six granules, randomly

TABLE 2. Size and morphology of dense-core vesicles present in the axons and terminals of the median ocellar nerve.

Granule type	Morphology	Mean diameter (nm)	n	S.D.	S.E.
1		75.6	30	7.6	± 1.2
2		93.7	18	6.6	± 1.9
3		124.6	24	9.0	± 2.1
4		128.6	138	11.7	± 0.9
5		108.4	66	3.1	± 0.4
6		146.6	18	3.7	± 0.9

selected from each axon or terminal, were measured. The total number of granules measured (n, in Table 2) is, therefore, a reflection of their abundance. Clearly the axons which contain granule types 4 and 5 are most numerous. It will also be noted from Table 2 that numbers 4 and 5 each represent two granule forms, distinguished by the appearance of their contents. In axons containing type 4 granules, the most numerous of these granules have a flocculent content of moderate electron density and little or no halo. The less numerous type of granule is smaller, has an electron-dense content and a distinct halo. The two granule types, collectivley designated as type 5, are similar both in size and number. They do, however, display the same differences in content described for type 4 granules. Granules which can only be described as having an intermediate appearance are also present in both types of axon (see Figs. 49 and 50).

An interesting feature of the granules with a flocculent content and which is most noticeable in axons of type 4 granules, is that their limiting membranes are occasionally observed to be ruptured (Fig. 58). This may indicate a weakening of the granule membrane prior to the release of hormones.

Beyond the median ocellus, several features have been noted within the axon terminals which are indicative of granule release (see below). First, spherical microvesicles of approximately 61 nm diameter are frequently observed, either interspersed between the neurosecretory granules or in clusters (Fig. 53). The microvesicles are sometimes found to group around a neurosecretory granule which is closely apposed to the plasma membrane of the terminal (Fig. 54). Secondly, vacuoles of both spherical and irregular outline are also prominent (Fig. 55). They are normally of comparable magnitude to the neurosecretory granules, but vacuoles of up to ca. 300 nm diameter have been noted. Finally, and most conclusive, omega profiles have been observed, albeit rarely (Fig. 56). Such profiles

represent release of the granule content into the extracellular milieu by exocytosis.

The axon terminals are separated from the surrounding haemolymph sinus by thin glial processes. In some regions this glial investment is only one process thick. Such an investment is unlikely to present an effective barrier to the diffusion of released hormone into the surrounding haemolymph. Therefore, it is proposed that a region beyond the median ocellus is a neurohaemal area.

DISCUSSION

Fahrenbach (1965) used 20 different fixing fluids in an attempt to obtain good preservation of the ocellar tissue of B.cariosus and B.amphitrite. None of these fixatives was found to be entirely satisfactory. The best solution employed was hypertonic, but produced artefacts indicative of hypotonicity. Both fixation procedures employed in the present study produced acceptable results as judged by the well preserved mitochondria and the narrow perinuclear space. Since the glutaraldehyde fixative is easier to prepare than the trialdehyde fixative, the former was used routinely. This fixative has an osmolarity of approximately 660 m-osmole/kg. and hence is highly hypotonic and yet it does not yield swollen mitochondria. The fixative had originally been used to fix inorganic granules in the hepatopancreas of Carcinus maenas (Clare, 1980; Hopkin, 1980). It maintained the granules within the tissue better than did more hypertonic fixatives. This feature has since been confirmed in Pomatocerus (Cotton, personal communication). Presumably the fixative rapidly penetrates both the tissues and the inorganic granules. This phenomena may be more important than the toxicity of the solution in preserving ocellar tissue. Against this argument, however, are the results of Hudspeth & Stuart

(1979) on B.nubilus; they obtained well preserved tissue by using a fixative of 1050 m-osmole/kg.

Median ocelli of B.hameri were fixed over a period of two months following their collection. It is interesting, in view of the above discussion, that those ocelli which were fixed within 12 hrs. of arrival in the laboratory were well preserved. Fixation of ocelli at subsequent times produced progressively inferior results. Since some of the barnacles used by Fahrenbach (1965) had not been fed for a prolonged period, they may have been in a degenerative state. This may well explain the discrepencies in fixation between his study and the present one.

The photoreceptor cells of B.hameri are very similar to those described previously in B.cariosus (Fahrenbach, 1965) and B.nubilus (Hudspeth & Stuart, 1977). There are no rhabdomes since neighbouring photoreceptor cells are isolated from one another by glial cells. Those rhabdomeres that are fused originate from the same receptor cell. This absence of coupling between receptor cells of the median ocellus differs from the situation in the lateral ocelli of B.eburneus, where the receptor cells are coupled (Brown et al., 1971; Shaw, 1972) and may reflect a functional difference between the median and lateral ocelli, (Hudspeth & Stuart, 1977). The lack of contact between photoreceptor cells is not only evident at the level of the rhabdomere. No synapses have been observed between receptor cell bodies or their dendrites, nor have axo-axonal synapses been seen. This, lack of coupling agrees with the findings for the other balanomorph species examined (cf. Fahrenbach, 1965; Hudspeth & Stuart, 1977).

It is well documented that the ocelli of barnacles are only receptive to a reduction in the amount of light, hence the shadow response. Barnacles cannot see, and this lack of visual acuity is manifest in the anatomy of the ocelli. For example, the rhabdomere microvilli extend in

a multi-directional manner. As discussed by Meyer-Rochow (1981), the microvilli need to be aligned in one direction if they are to perceive the plane of polarized light. Moreover, balanomorph receptor cells do not possess a large number of microvilli. Fahrenbach (1965) estimated that each receptor cell of B.cariosus bears 1-2 x 10^5 microvilli. He compared this number with the Apis retinula cell which has about 10^6 microvilli (cf. Goldsmith, 1962). B.hameri, despite possessing a greater number of receptor cells than B.cariosus, has a comparable total number of microvilli. It has been estimated, from micrographs of rhabdomeres, that each dendrite terminal has 2,500 microvilli. Assuming that each dendrite gives rise to 6 terminals through branching and that 20 dendrites arise from the perikaryon of each receptor cell, we arrive at a total figure of 3 x 10^5 microvilli per receptor cell. Since there are 8 receptor cells in the median ocellus of B.hameri, this gives a total figure of 24 x 10^5 microvilli. There are a total of 7-14 x 10^5 microvilli in B.cariosus (Fahrenbach, 1965). However, since the overall dimensions of the microvilli of B.hameri are approximately twice those in B.cariosus, the total area available to receive light is that much greater.

These comparative measurements do not, therefore, explain the observation of Gwilliam (1976) that the shadow response of B.hameri is less well developed than that of B.cariosus. An alternative explanation may lie in the comparative lengths of the photoreceptor cell axons. The axons of B.cariosus measure 2.5 - 3.5 mm (Fahrenbach, 1965), whereas those of B.hameri measure 3.5 - 4.0 mm. Therefore, if B.hameri is to respond to diminished light as rapidly as B.cariosus, the resultant voltage change in the axon must spread more quickly. One way to increase the speed of voltage conduction by an axon is to increase its diameter. Consequently the axons of B.hameri may be expected to have a greater diameter than those of B.cariosus. However, this is not the case. They are roughly equal

in size, with the result that B.hameri has a slower shadow response than B.cariosus.

It may also be postulated that B.hameri will be less perceptive to changes in light intensity than B.cariosus, although no such study has yet been performed. The extent to which the voltage change decays along an axon is a function of its length constant (λ) and is dependent on two properties of the axon, the membrane resistance (rm) and the internal resistance of the axon (ri). The relationship between these factors has been expressed by Tasaki (1959) in the equation :

$$\lambda = \sqrt{\frac{rm}{ri}}$$

It can be seen from this equation that λ increases with increasing axon diameter. Thus, a stimulus-induced voltage change which is just large enough to elicit a postsynaptic response in B.cariosus may have decayed to beyond the point where it does so in B.hameri.

Less than 0.1% of the light incident at the surface penetrates to the depth from which B.hameri were dredged, i.e. 20 fathoms (cf. Figure 20 of Friedrich, 1973). Thus, it is apparent that the change in light intensity caused by an object passing over a barnacle at this depth will not be as pronounced as that produced by the same event occurring intertidally. It is, therefore, tempting to assume that the shadow response is relatively unimportant in B.hameri, even though other features of the ocellus testify against this assumption. It has already been mentioned that the microvillar area of B.hameri is large in comparison to that of B.cariosus and is presumably an adaptation to capturing as many light photons as possible. Two additional adaptations are the orange colour of the ocellus and the almost total absence of black screening pigment.

The same adaptations have been noted in the eye of the amphipod, Orchomene sp. cf. O.rossi by Meyer-Rochow (1981). The specimens studied were caught from 597 m below sea level and 238 m below the 420 m thick Ross Ice Shelf in Antartica. Meyer-Rochow (ibid) argued that in such an environment the eye of Orchomene sp. cf. O.rossi should be occupied by "reddish visual and not black screening pigment", if it is to increase its sensitivity to photons. It is believed that the eye of Orchomene sp. cf. O.rossi is responsive to light in the wavelength range 490-530 nm. This blue-green part of the spectrum penetrates water to a greater depth than other wavelengths (Friedrich, 1973). The prediction would, therefore, be that B.hameri will also have a spectral sensitivity peak between 490-530 nm.

In view of the above discussion, we should consider the possible deleterious effect of surface light on the median ocellus of B.hameri. For example, at 20 fathoms, B.hameri will not have experienced the ultra violet part of the spectrum. Meyer-Rochow (1981; 1982) has in fact noted sunlight-induced changes in the ultrastructure of the rhabdomes of amphipods and isopods. Such changes do not necessarily occur immediately. Meyer-Rochow & Tiang (1982) found that the greatest ultrastructural damage did not occur to the retinular cells of the lobster, Jasus edwardsii, until at least 24 hr following a 2 hr exposure to sunlight. Consequently, it is pertinent that no ultrastructural damage was noted in the median ocellus of B.hameri within 24 hr of collection (see above). With time and longer exposure to light intensities greater than ambient levels, the tissues were less well preserved. Since personal observations made on the shadow response of B.hameri were not made in the first few hours following collection an inaccurate assessment of the response may have been deduced.

A daily cycle of rhabdomere membrane turnover has been described in many anthropods including chelicerates (Miller & Cawthon, 1974, Behrens, 1974; Blest, 1978; Blest & Day, 1978), crustaceans (Debaisieux,

1944; Itaya, 1976; Nässel & Waterman, 1979; Eguchi & Waterman, 1979; Hafner et al., 1980; Stowe, 1980) and insects (Williams & Blest, 1980; Horridge et al., 1981). The ocelli of barnacles have not been analysed over a 24 hr period and so it is not known whether their microvilli undergo cyclic breakdown and renewal. However, it is clear that membrane which has been bleached following exposure to light must be renewed at some stage. In a perpetually dim environment, such as that experienced by B.hameri, the breakdown and renewal of receptor membrane need only be gradual.

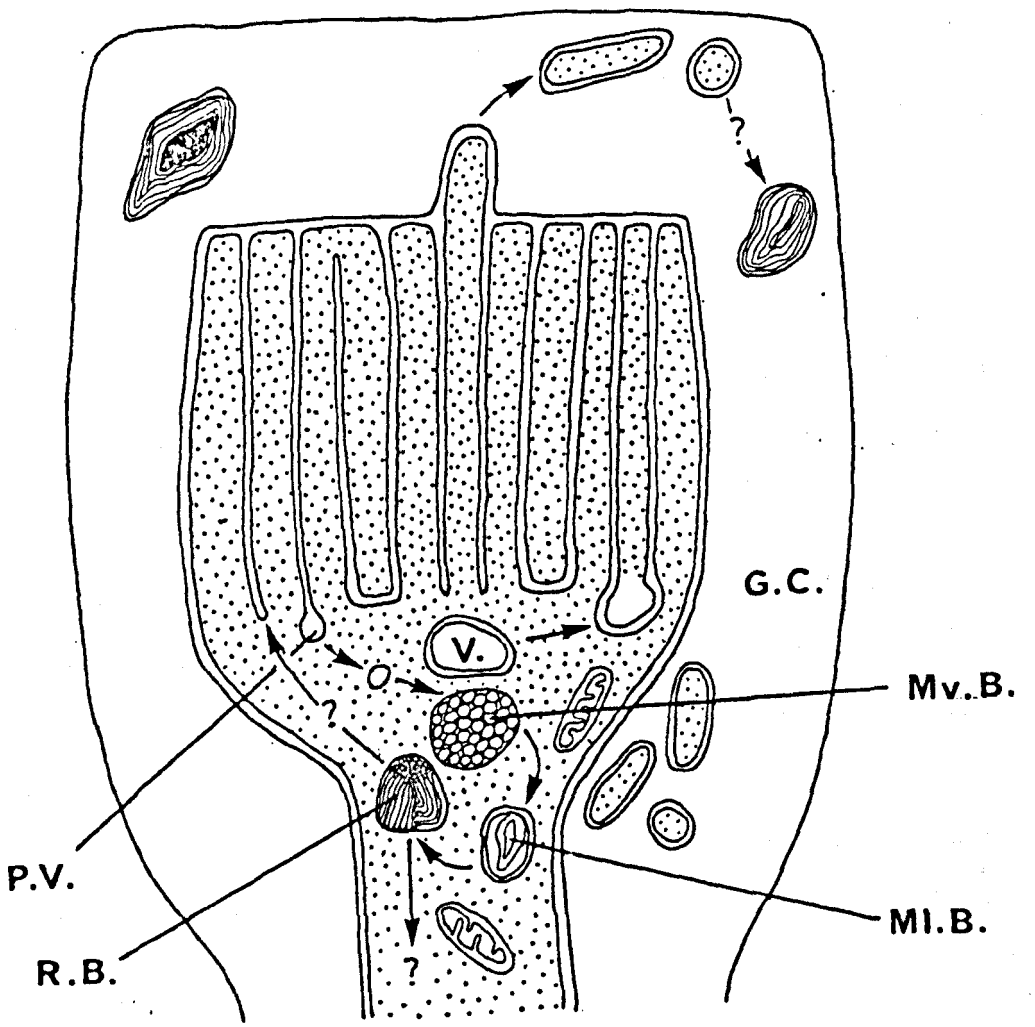
Evidence of membrane turnover, comparable to that found in other arthropods, has been observed in B.hameri. The removal of the photoreceptive membrane of arthropods occurs in two ways. Endocytosis of the plasma membrane at the base of the microvilli has been noted most frequently (Holtzman & Mercurio, 1980). However, in some arthropods the shedding of membrane to the extracellular space is a more important method (Blest & Day, 1978; Blest & Maples, 1979; Williams & Blest, 1980; Blest & Price, 1981). Evidence has been obtained to suggest that this shed membrane is retrieved by the receptor cell, either directly (Williams & Blest, 1980; Blest et al., 1982) or via glial cells (Blest & Maples, 1979). The result of both methods is that retrieved membrane, in the form of multivesicular bodies, appears in the receptor cell cytoplasm. It is generally accepted that the multivesicular bodies are degraded to multilamellar bodies. The latter are then lysed, as in the crab, Leptograpsus variegatus (Blest et al., 1980), or transformed into dense or residual bodies as in the spider Dinopis (Blest et al., 1978) and tipulid flies (Blest et al., 1982). Residual bodies, where they occur, are thought to be the terminal organelle in membrane degradation (Blest et al., 1978).

Multivesicular bodies, multilamellar bodies and residual bodies have all been noted in the cytoplasm of B.hameri photoreceptor cells. However, they do not occur in the profusion normally associated with a circadian cycle of membrane turnover, as would be expected if such turnover was continuous. The membrane which constitutes these structures is retrieved from the rhabdomere by one of two methods. At the basal end of the microvilli, membrane is pinocytosed, as in other arthropods. However, at the apical end there is evidence of a novel method of retrieval. Microvilli which extend into the surrounding glial cytoplasm are abscised. Moreover, not only is the membrane of the microvilli shed, but also their contents. Microvillar membrane has not been observed to be shed to the extracellular space. The microvilli may be degraded in the glial cytoplasm, since lysosomes have been observed which are similar in appearance to those in the receptor cytoplasm.

The large vacuoles with double membranes present in the dendrite terminals may be involved in rhabdomere microvilli assembly. Similar structures have been observed in the eyes of Limulus (Behrens & Krebs, 1976), Palaemonetes (Itaya, 1976) and Leptograpsus (Stowe, 1980). They have been termed 'concentric ellipsoids' by Itaya (1976) and 'doublet ER' by Stowe (1980). Such structures have also been found in continuity with the bases of the microvilli (Itaya, 1976; Eguchi & Waterman, 1976; Blest et al., 1978) as has been observed in B.hameri. It is suggested that this fusion represents the addition of new membrane to the rhabdomere. Figure 57 summarizes diagramatically the possible methods of membrane recyling in B.hameri.

Six distinct types of dense-cored granule have been observed in the small axons which bypass the median ocellus and in the axon terminals beyond this ganglion. The granules can be matched on the basis of size with those

Fig. 57. Diagram to summarize the hypothetical events associated with photoreceptor membrane turnover in the rhabdomeres of B.hameri. Membrane is shed from the microvilli by two routes : microvilli protruding from the apical region of the rhabdomere are sloughed off into the glial cytoplasm and degraded by lysosomes. In addition membrane is retrieved by pinocytosis at the basal region of the rhabdomere and this membrane is degraded through multivesicular bodies and in turn, multilamellar bodies and residual bodies. The latter are probably transported to the perikaryon of the photoreceptor cell, but may also be recycled and incorporated into the rhabdomere. Membrane may be added to the rhabdomere in the form of double-membraned vesicles.


?
G.C.
V.
Mv.B.
P.V.
MI.B.
R.B.
?

occurring in the supra- and infraoesophageal ganglia, but not all have a similar morphology. Types 3 and 5 beyond the ocellus have no counterpart in the rest of the C.N.S. This dissimilarity may reflect the phenomenon of granule ageing. Such a process involves a modification of the granule content during axonal transport (Gainer et al., 1977; Andrew & Saleuddin, 1979; Nordmann & Labouesse, 1981).

It is proposed that type i granules of the supraoesophageal ganglion (cf. chapter 3) be equated with type 4 granules beyond the ocellus. Type I neurones are believed to represent the white perikarya observed in the protocerebrum of B.hameri which have axons in the median ocellar nerve. The presence of similar elementary granules beyond the ocellus supports this theory. Moreover, it is to be expected that type 4 granules should be the most numerous, since the largest 'neurosecretory' axons in the median ocellar nerve originate from the white perikarya.

There is also a close correlation between type g granules of the supraoesophageal ganglion and type 6 granules at the median ocellus. Although the remaining granule types can be matched on the basis of size, with elementary granules in the supra- and infraoesophageal ganglia, the similarity does not extend to morphology. However, this does not necessarily mean that there are additional granule types in the vicinity of the median ocellus. Account must be taken of granule ageing.

The two neurone types present in the median ocellus contain very similar elementary granules. Both have a mean diameter slightly greater than 100 nm and so they may be neurosecretory, although it should be remembered that barnacle photoreceptor cells are thought to contain GABA (Lantz & Millechia, 1975). It will be remembered also that GABA acts on postsynaptic neurones in the commissure of the supraoesophageal ganglion. It is possible that the white perikarya observed in the commissure of B.hameri are

equivalent to these postsynaptic cells. They could thus be fulfilling a role as interneurones in the shadow reflex pathway in addition to their role as neurosecretory cells which release their secretory product in the neurohaemal area beyond the median ocellus.

The existence of a neurohaemal area in B.hameri is the first such observation for the Entomostraca. It is tempting to speculate that the supraoesophageal/median ocellar complex of B.hameri is homologous to the X-organ/sinus gland complex of the Malacostraca. There are in fact some striking similarities between the two. First, both systems are in close association with photoreceptive structures. Secondly, the neurosecretory product is transported in axon tracts for some distance from its site of production to be released at axon terminals which are in close association with the haemolymph. The axon terminals of both neurohaemal areas appear to contain six types of elementary granule (c.f. Weatherby, 1981). The terminals are also characterized by large vacuoles and a sparse populations of microvesicles. In B.hameri these microvesicles have a corrected mean diameter of approximately 61 nm which is very close to the 57 nm estimated for the microvesicles in the sinus gland terminals of Carcinus maenas (Nordmann & Morris, 1980). As Nordmann and Morris pointed out, the existence of only a sparse microvesicle population is an unusual feature of neurohaemal areas and contrasts markedly with the situation in mammalian and insect neurosecretory systems. In C.maenas and indeed in general (Morris and Nordmann, 1982) it is the vacuoles and not the microvesicles which are believed to be involved in membrane retrieval following exocytosis. There is no reason to believe that this is also not the case in B.hameri, although conformation will have to await the results of marker studies.

Fig. 1. Whole mount of the median ocellus stained with methylene blue. Scale bar, 100 μm.

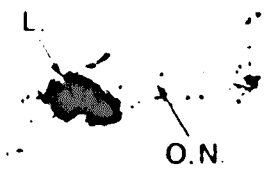
Fig. 2. Light micrograph of a 1 μm longitudinal section of the median ocellus and the surrounding parenchyma cells showing the striated muscle which runs parallel to the ocellar nerve and ocellus. Scale bar, 200 μm.

Fig. 3. Electron micrograph of a region of the perilemma. Note the elongate nuclei. Scale bar, 2.5 μm.

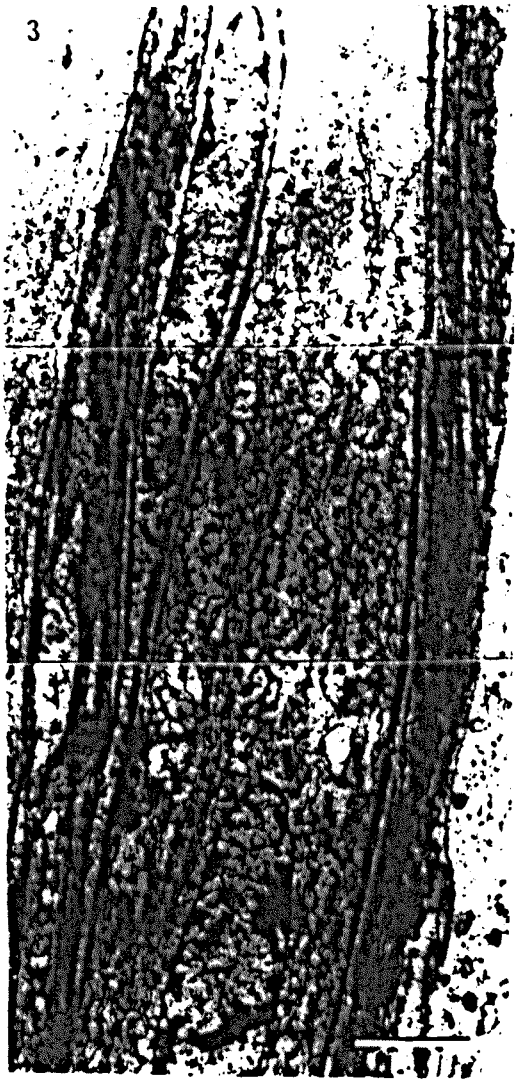
Fig. 4. Electron micrograph showing a lobulate nucleus with the perilemma. Scale bar, 2.5 μm.

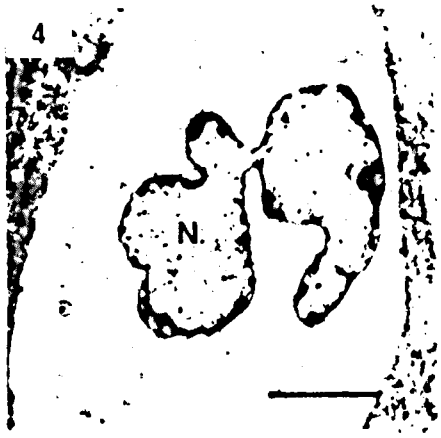
Fig. 5. Electron micrograph of the outer layer of the neurilemma showing the collagenous fibrils. Scale bar, 2 μm.

Fig. 6. Electron micrograph of parenchyma cells enveloping the median ocellus. Scale bar, 10 μm.


1
L
O.N


2
M.O.
Mu
Pa


3


4
N.


5
Fi.


6
NT.
NT.
H.

Fig. 7. Electron micrograph of parenchyma cells around the median ocellus showing their lipid content. Scale bar, 5 μm.

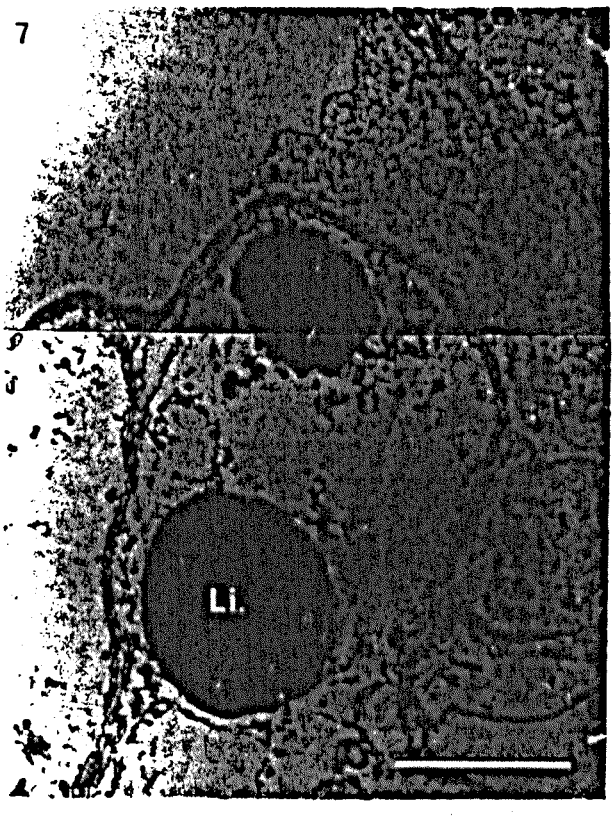
Fig. 8. Electron micrograph of a parenchyma cell from an animal fixed in December. Note the absence of lipid. Scale bar, 2 μm.

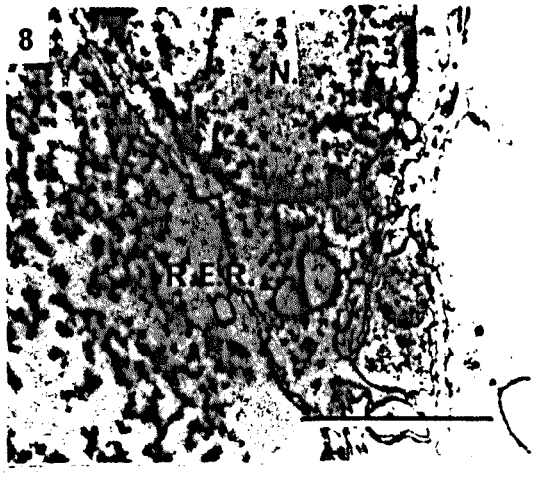
Fig. 9. Electron micrograph showing the basal lamina. Note the fenestrations (arrows). Scale bar, 0.5 μm.

Fig.10. Electron micrograph showing the channels (arrow) between the parenchyma cells which are filled with haemolymph. Scale bar, 5 μm.

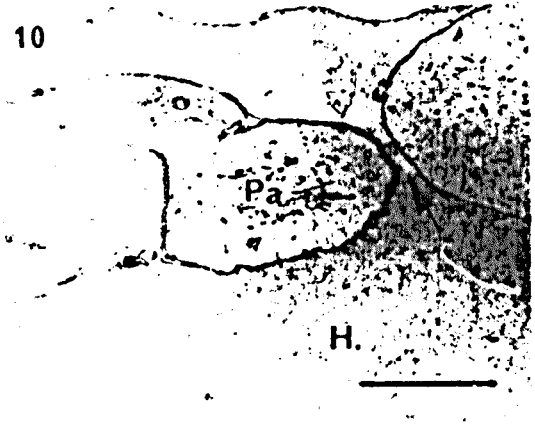
Fig.11. Electron micrograph of precipitated haemolymph. Note the heterogenous nature of the haemolymph. Scale bar, 5 μm.

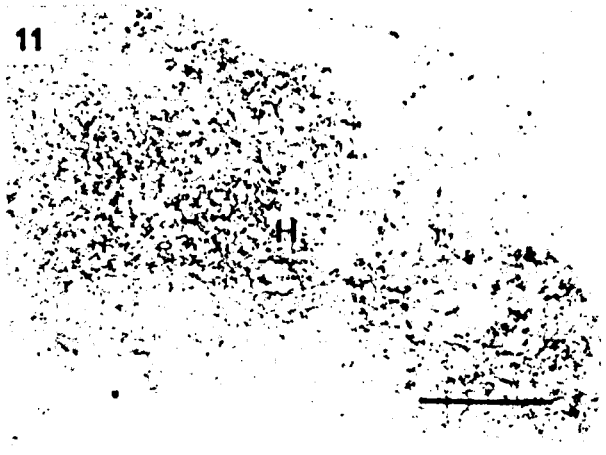
Fig.12. Electron micrograph of an haemolymph cell. Scale bar, 2 μm.


7
Li.


8
N.
R.E.R.


9
B.L.


10
Pa.
H.


11
H.

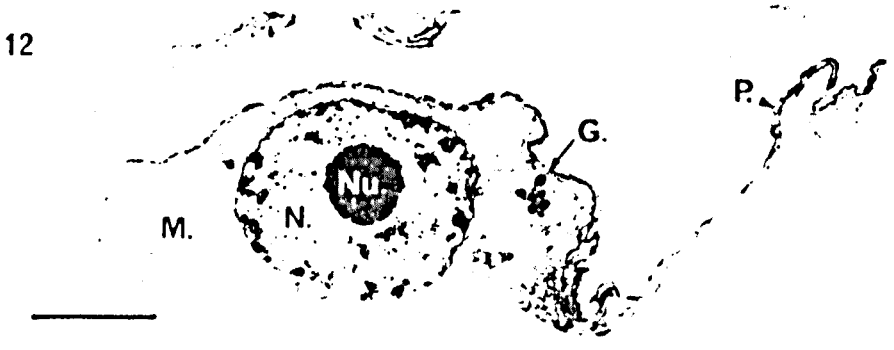

12
P.
G.
Nu.
N.
M.

Fig. 14. Electron micrograph of a region of the nucleus of a photoreceptor cell. Scale bar, 2 μm.

Fig. 15. Electron micrograph of a region of cytoplasm within a photoreceptor cell. Scale bar, 2 μm.

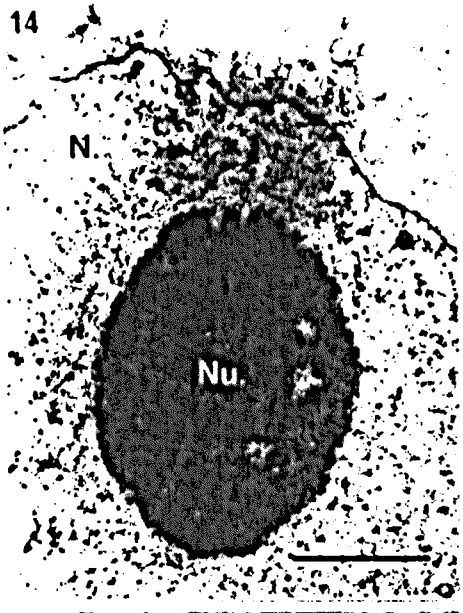
Fig. 16. Electron micrograph showing a Golgi body in the perinuclear zone with electron-lucent vesicles in close association. Scale bar, 0.5 μm.

Fig. 17. Electron micrograph of a residual body in the cytoplasm of a photoreceptor cell. Scale bar, 1 μm.

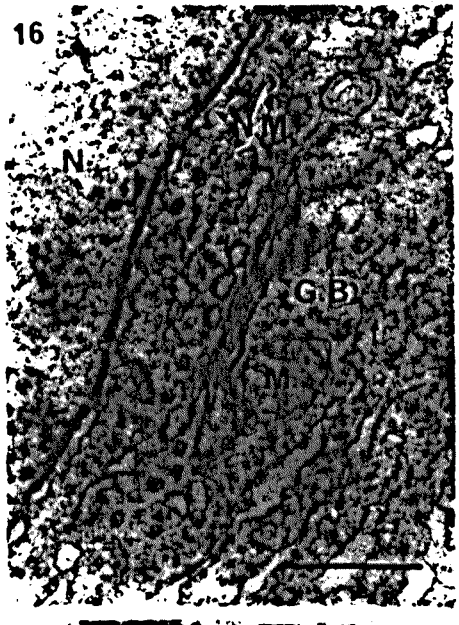
Fig. 18. Electron micrograph of a glycogen aggregate in the axon hillock of a photoreceptor cell. Scale bar, 1 μm.

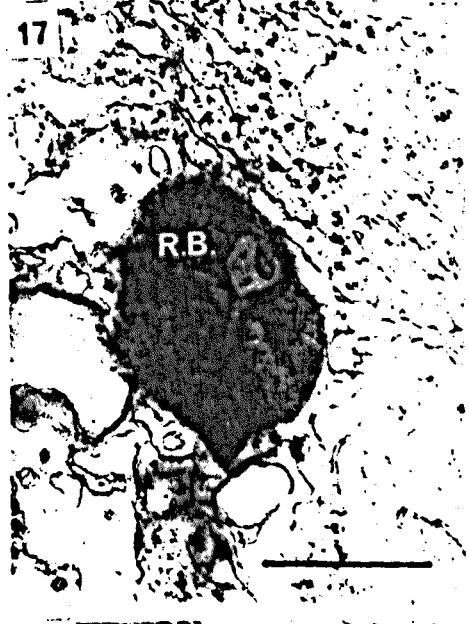
Inset : high power micrograph showing the α-particles of glycogen which make up the aggregate (x54,000).

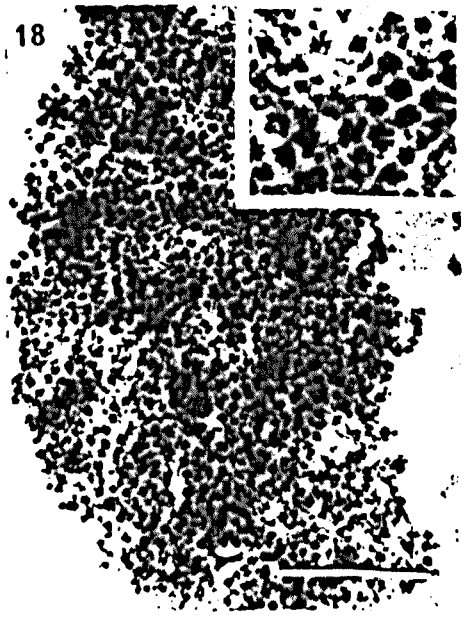
Fig. 20. Electron micrograph of a longitudinal section of a photoreceptor dendrite. Scale bar, 0.5 μm.


14
N.
Nu.


15
N.


16
N
M
G.B


17
R.B


18

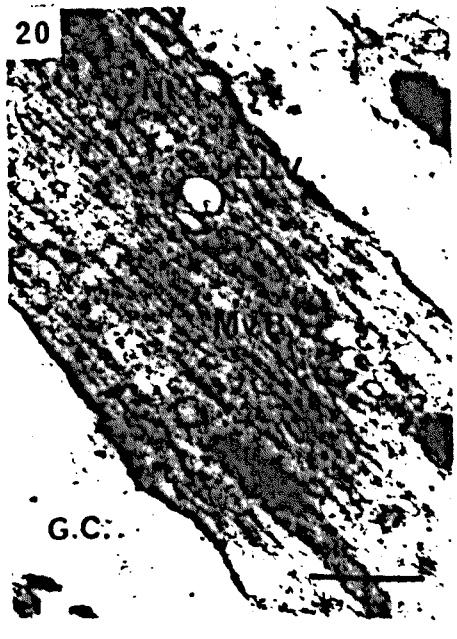

20
M.B
G.C.

Fig. 19. Composite electron micrograph showing several dendrites of a photoreceptor cell. The dendrite terminals are capped by rhabdomeres. Scale bar, 10 μm.

19

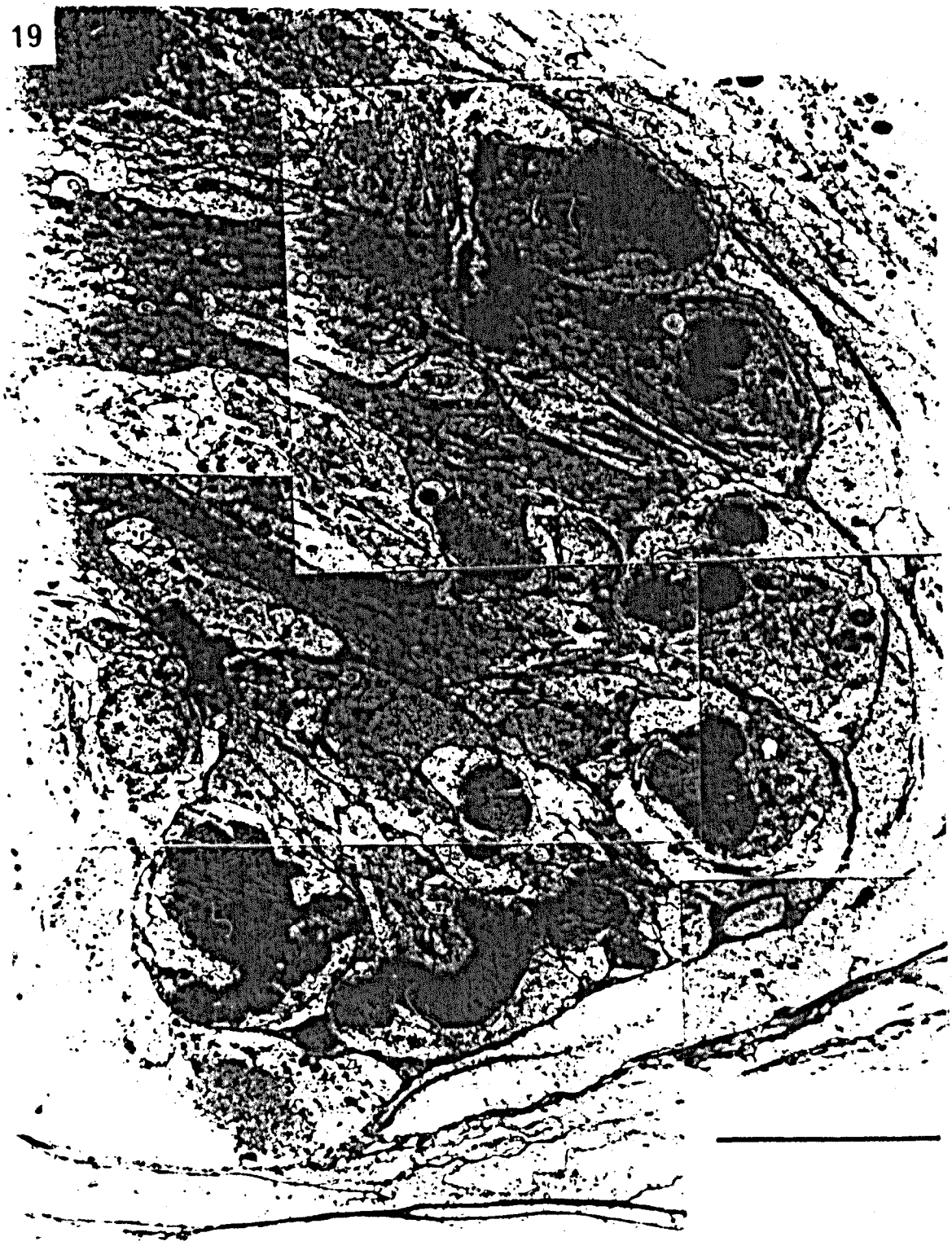


Fig. 21. Electron micrograph showing a longitudinal section of a rhabdomere. Scale bar, 1 μm.

Fig. 22. Electron micrograph of a transverse section of an area of rhabdomere, showing the 'honeycomb' arrangement of the microvilli. The cytoskeleton of the microvilli is just visible (arrow). Scale bar, 1 μm.

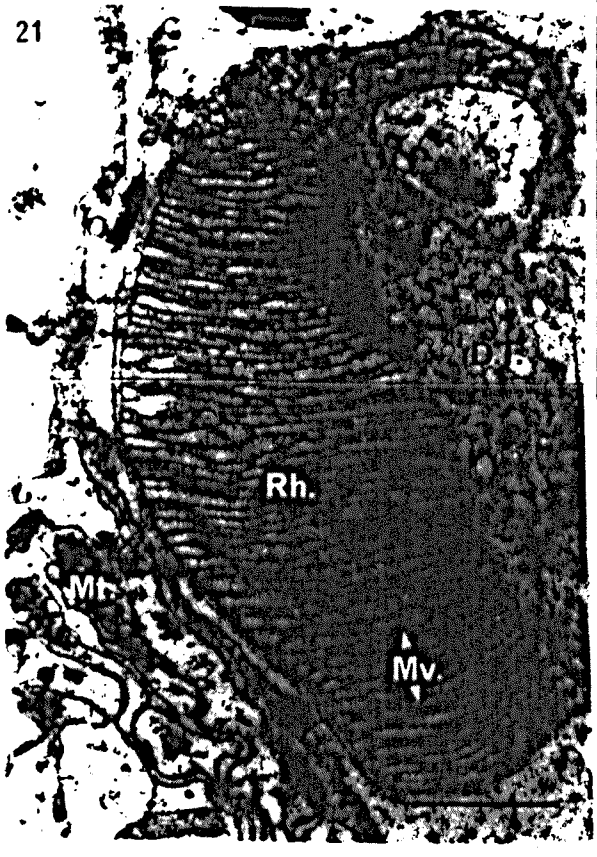
Fig. 23. Electron micrograph showing a microvillus which has bifurcated (arrow). Scale bar, 1 μm.

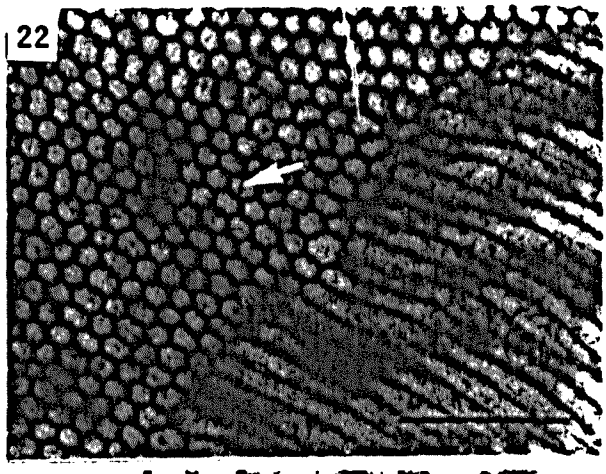
Fig. 24. Electron micrograph of a section through the proximal region of a dendrite terminal. 180° bends are visible at the apical end of the microvilli (arrows). Scale bar, 2 μm.

Fig. 25. Electron micrograph of the basal region of a rhabdomere showing both closed and open-ended microvilli. Note the profusion of vesicular elements in the dendrite terminal. Scale bar, 2 μm.

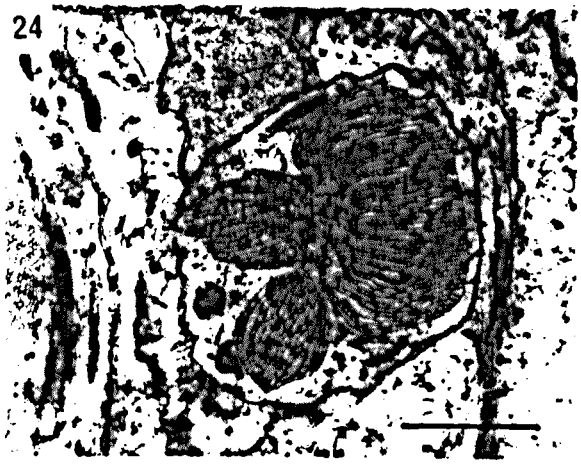
Fig. 27. Electron micrograph of the basal region of a rhabdomere showing large vacuoles within the dendrite terminal. Note also that portions of microvilli are being sloughed off into the glial cytoplasm (arrow). Scale bar, 1 μm.

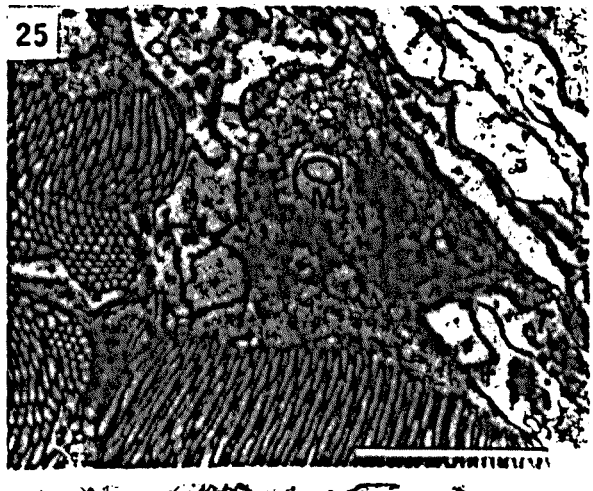
Fig. 28. Electron micrograph of the basal region of a rhabdomere showing electron-lucent vacuoles either fusing with or budding from the microvilli. Scale bar, 0.5 μm.

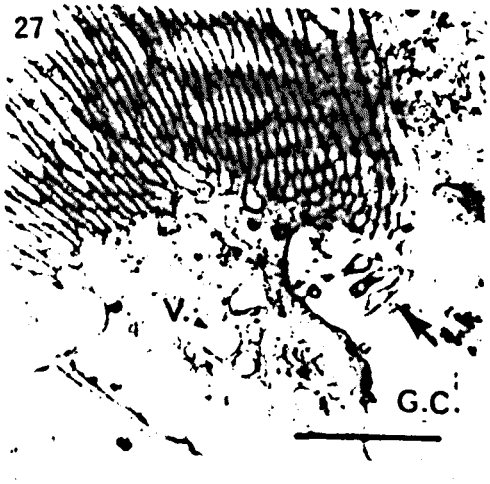

21
Rh.
Mt.
Mv.


22


23


24


25


27
V.
G.C.


28
V.

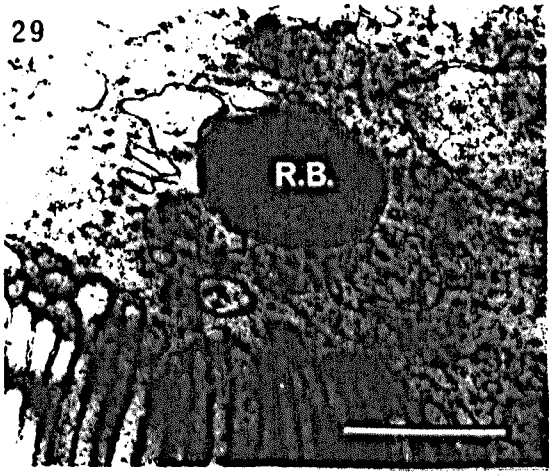
Fig. 29. Electron micrograph of a residual body in the dendrite terminal of a photoreceptor cell. Scale bar, 0.7 μm. .

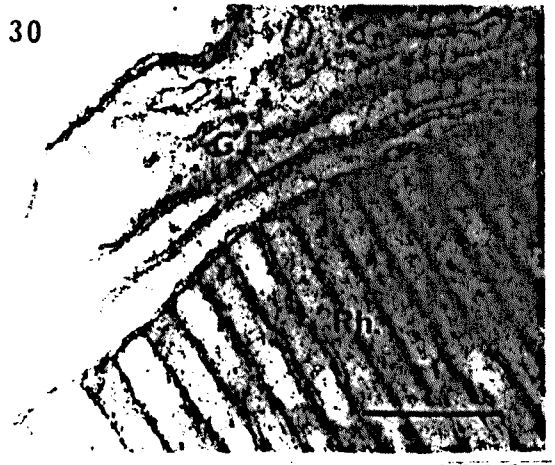
Fig. 30. Electron micrograph of a longitudinal section through the apical region of a rhabdomere. Scale bar, 0.3 μm.

Fig. 31. Electron micrograph of a longitudinal section of a rhabdomere showing portions of microvilli protruding and being sloughed off into the glial cytoplasm (arrow). Scale bar, 1 μm.

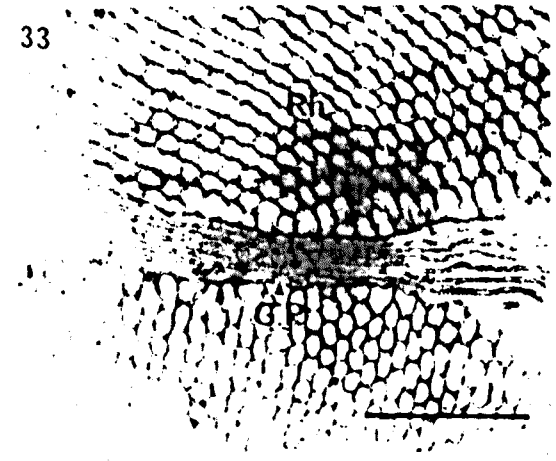
Fig. 32. Montage of a longitudinal section through a typical region of the median ocellus. Scale bar, 5 μm.

Fig. 33. Electron micrograph showing narrow glial processes separating adjacent rhabdomeres. Scale bar, 0.7 μm.


29
R.B.


30
G.
Rh.


31
D.T.
Rh.


33
Rh.
G.P.


32
Rh.
Gl.
G.C.
Mt.

Fig. 34. Electron micrograph showing gliosomes of varying electron density. Scale bar, 5 μm.

Fig. 35. Electron micrograph of a transverse section through a glial cell. Scale bar, 2 μm.

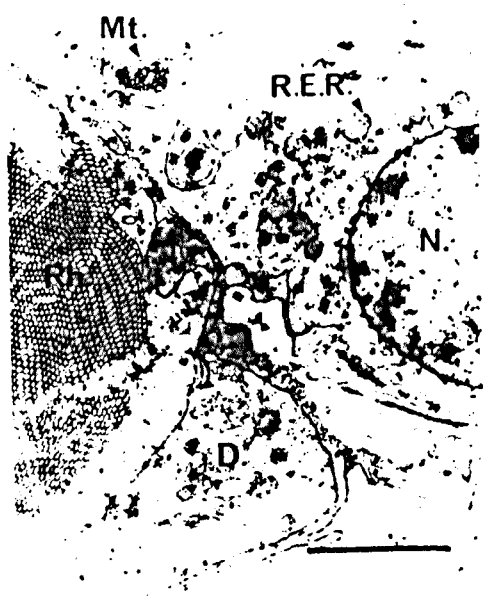
Fig. 36. Electron micrograph of a tangential section through glial tissue showing microtubules apparently inserting onto a gliosome (arrow). Scale bar, 5 μm.

Fig. 37. Electron micrograph of a transverse section through a bundle of microtubules showing flocculent material which coats each microtubule. Scale bar, 0.3 μm.

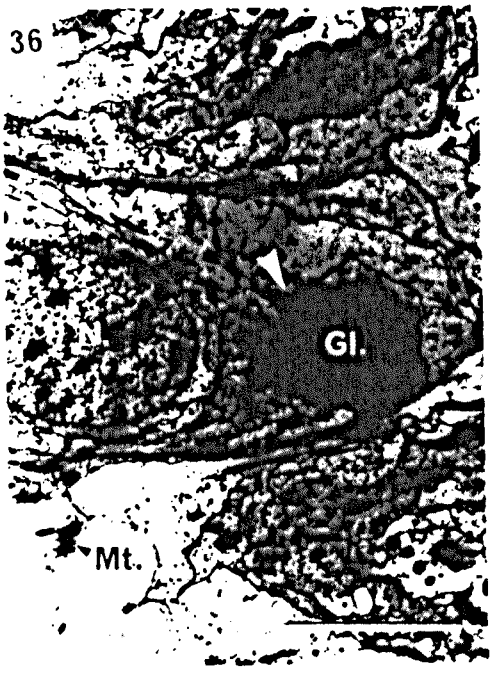
Fig. 38. Electron micrograph of a desmosome joining adjacent glial processes. Scale bar, 0.3 μm.

34

35



36



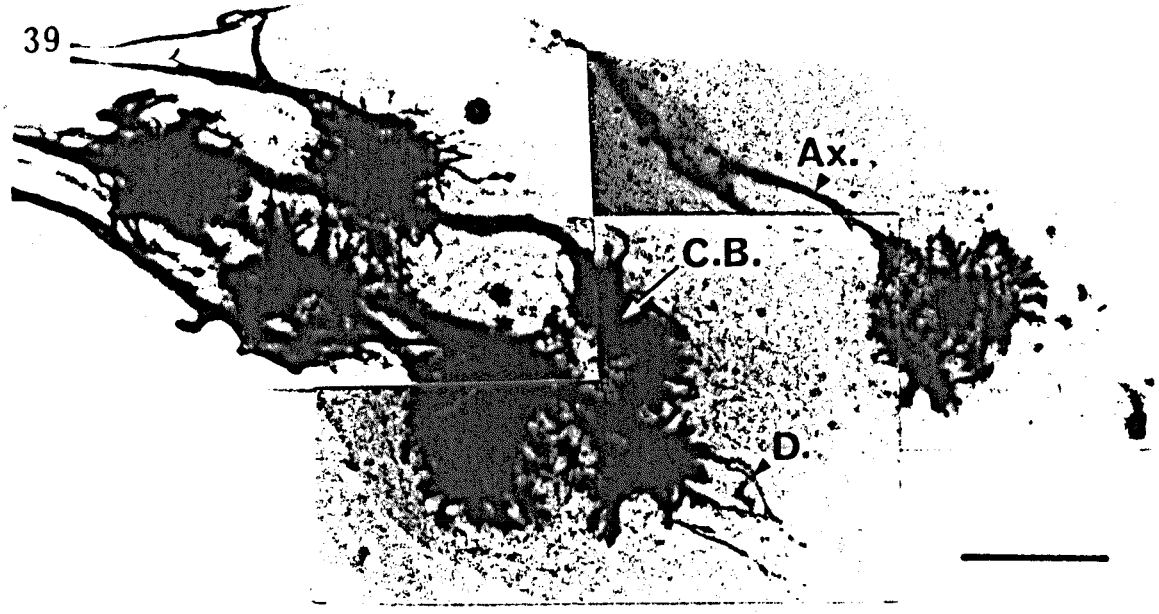
37

38

Fig. 39. Light micrograph of a cobalt backfill of the median ocellus filled via the median ocellar nerve. Scale bar, 100 μm.

Fig. 40. Camera lucida drawing of the same preparation shown in figure 39.

39



40

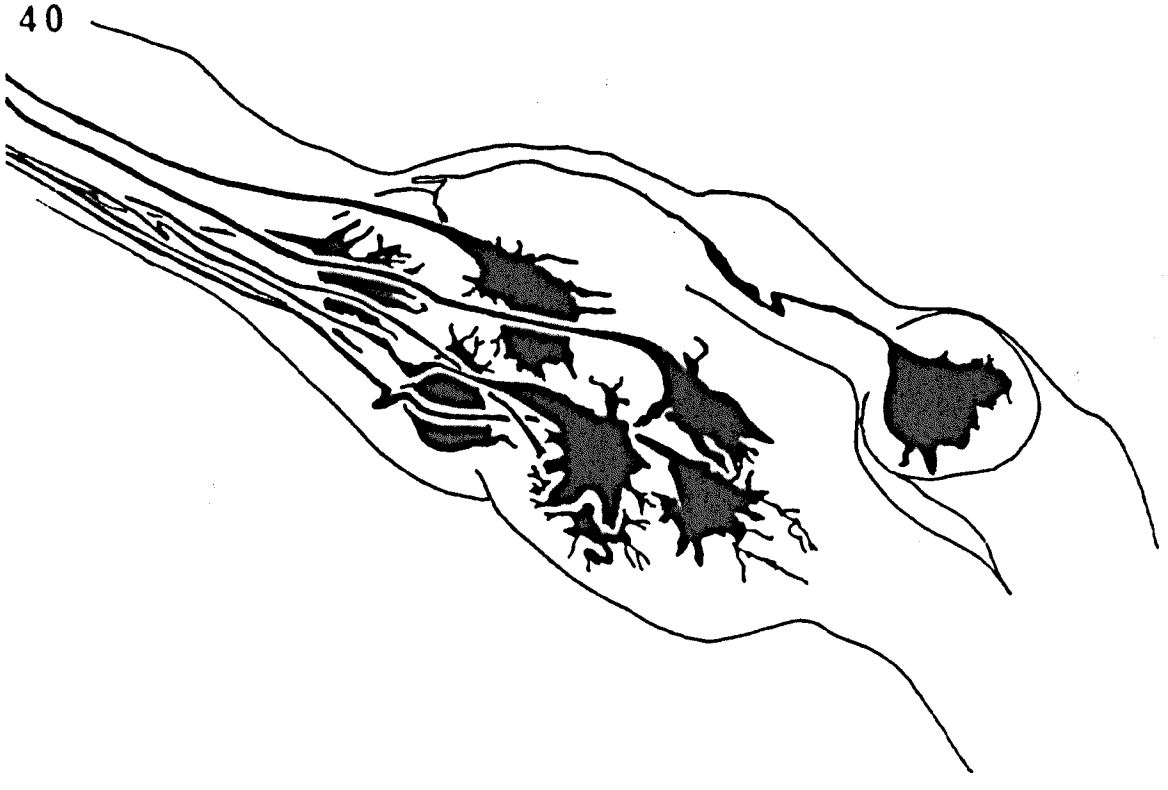


Fig. 41. Electron micrograph of a longitudinal section through a neurosecretory cell in the median ocellus. Scale bar, 10 μm.

41

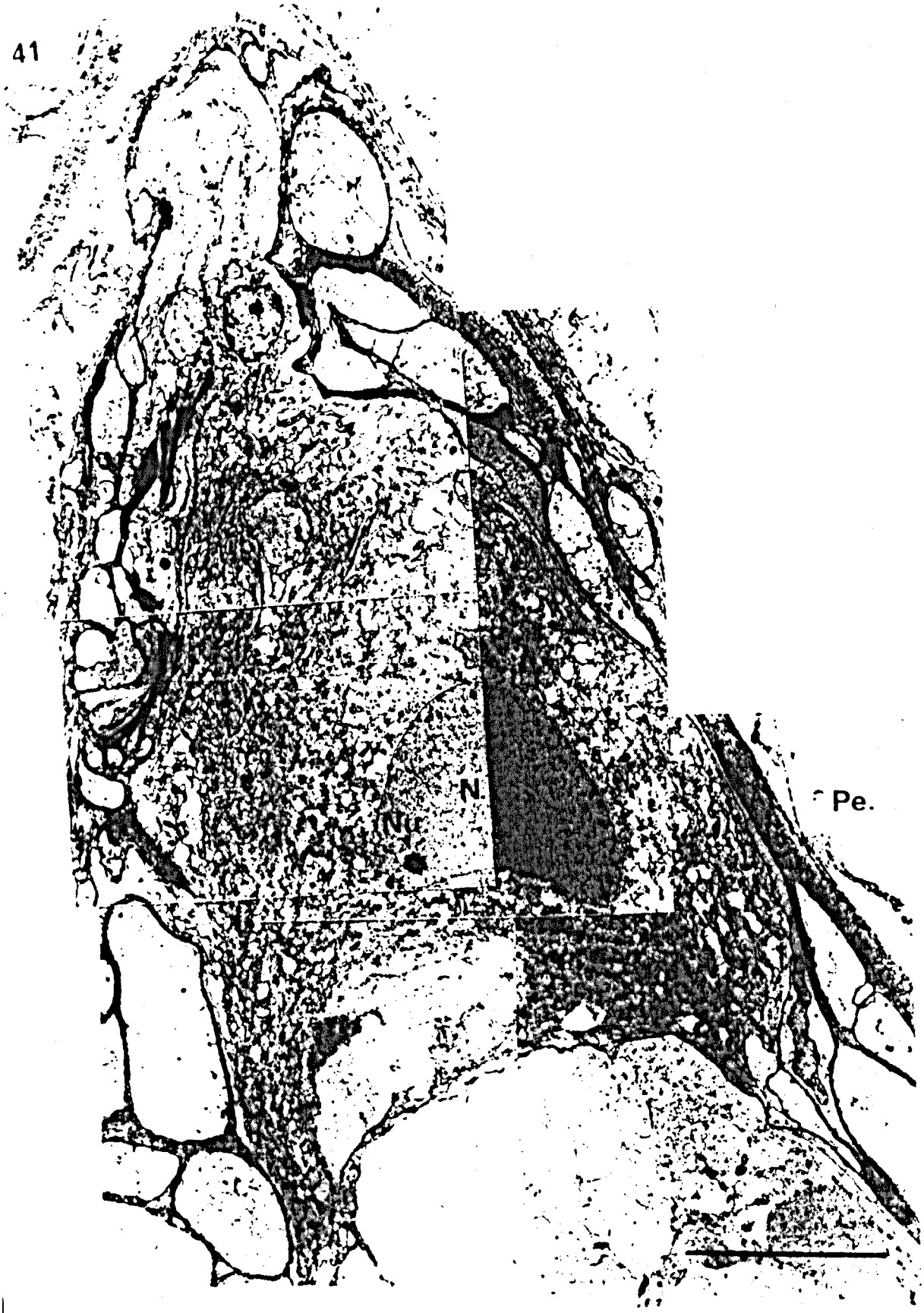
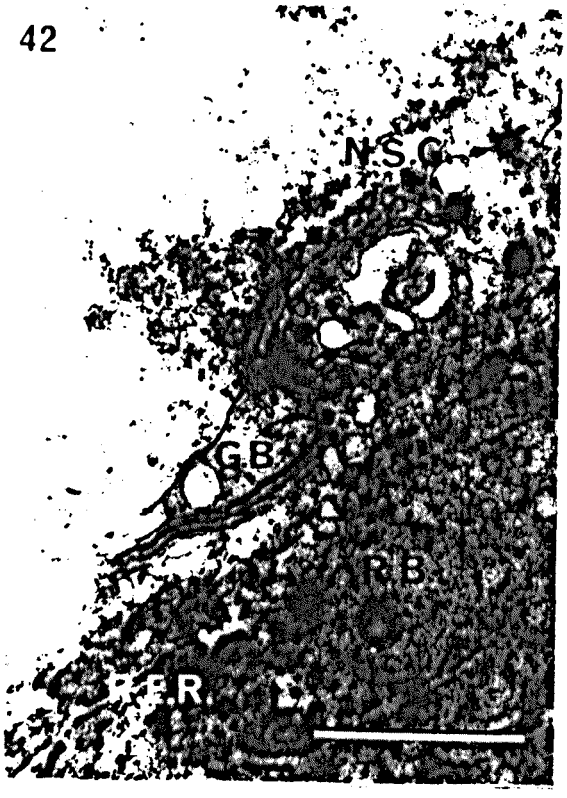


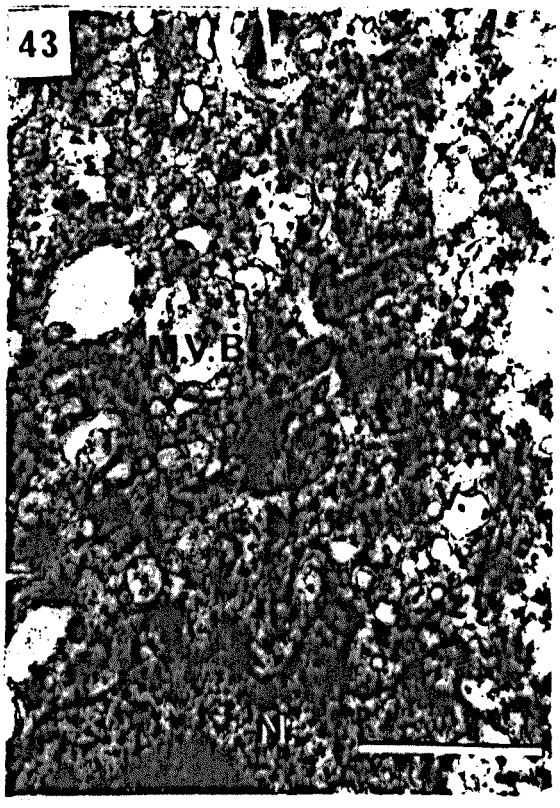
Fig. 42. Electron micrograph of an area of the perikaryon of the ocellar neurosecretory cell showing a Golgi body and elementary granules arising from its lamellae. Scale bar, 1 μm.

Fig. 43. Electron micrograph of the perinuclear zone of the neurosecretory cell. Scale bar, 1 μm.

Fig. 44. Electron micrograph showing membrane whorls in the cytoplasm of the neurosecretory cell. Scale bar, 1 μm.

Fig. 45. Dark field light micrograph of a whole mount of the median ocellus showing the nerves which extend beyond the ocellus. Scale bar, 400 μm.


42
N.S.C.
G.B.
R.B.
P.P.R.

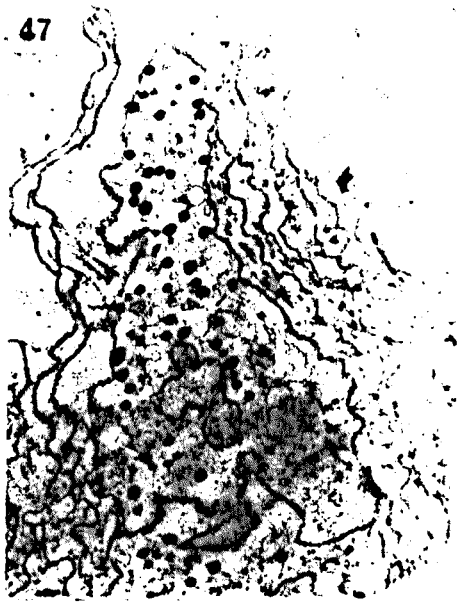

43
M.V.B.
N.


44


45

Figs. 46-51. Electron micrographs of the six types of dense-core vesicle found in the axons running parallel to the median ocellus and in their terminals beyond the ocellus. Scale bar, 1 μm.


46


47


48


49


50

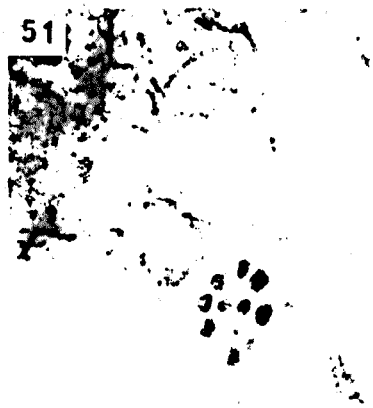

51

Fig. 52. Electron micrograph illustrating a feature frequently noted in both type 4 and 5 axons, namely, the rupture of the limiting membranes of the granules of flocculent content. Scale bar, 1 μm.

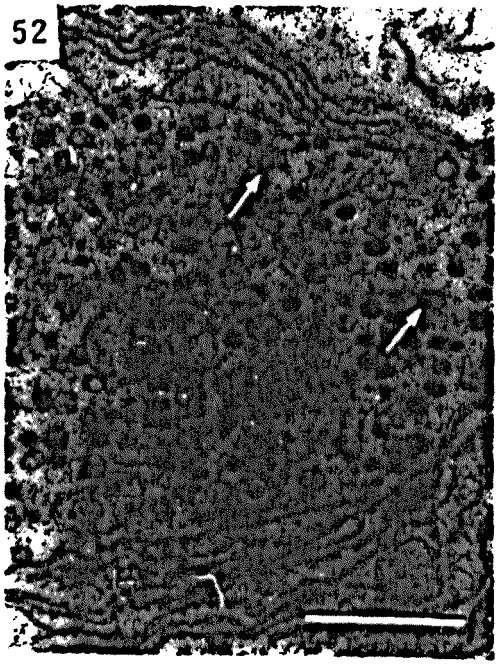
Fig. 53. Electron micrograph showing an axon which contains many microvesicles, both interspersed between the dense-core vesicles (arrow) and also in clusters (double arrow). Scale bar, 1 μm.

Fig. 54. Electron micrograph showing microvesicles clustered around a dense-core vesicle which is in close association with the plasmalemma of the axon (arrow). Scale bar, 0.7 μm.

Fig. 55. Electron micrograph of a small axon containing both dense-core vesicles and large electron-lucent vesicles. Scale bar, 0.5 μm.

Fig. 56. Electron micrograph showing exocytosis of a neurosecretory granule (arrow). Scale bar, 0.7 μm.

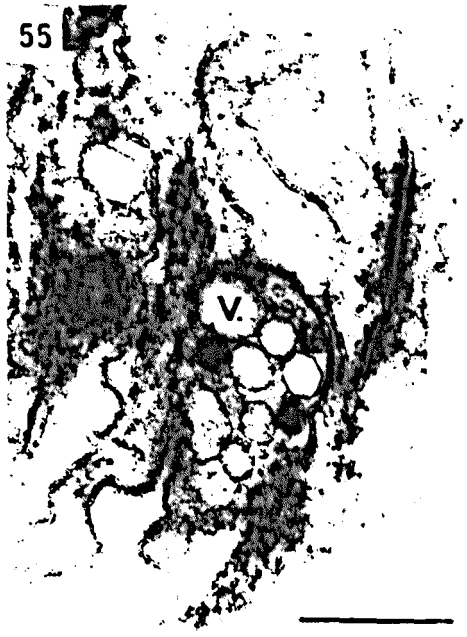
52



53

54

55



56

CHAPTER 5

Studies on egg laying and its control in *Balanus balanoides* (L.) and *Balanus hameri* (Ascanius)

CHAPTER 5

Studies on egg laying and its control in Balanus balanoides (L.) and Balanus hameri (Ascanius)

INTRODUCTION

Detailed descriptions of the copulatory process of certain balanomorph barnacles have been presented by Clegg (1955), Barnes & Barnes (1956), Barnes et al. (1977) and Walker (1980). It is clear that for Balanus balanoides, at least, cross-fertilization is obligatory and there is good evidence to suggest that this is true for B.hameri and B.balanus, also (Walker, unpublished).

Soon after copulation, the exact time varies with the species, the acting female ovulates. Eggs pass along each oviduct (figure 1) and into an oviducal gland where they enter an elastic sac. This ovisac gradually distends with eggs and is forced out into the mantle cavity (figure 2). In B.balanoides, oviducal gland fluid is displaced by the eggs and activates the immotile sperm in the mantle cavity; B.hameri sperm do not require an activating factor. Each egg mass remains attached to the neck of the oviducal gland until fully formed, whereupon, both are released and manouvered under the body of the barnacle and moulded to the contours of the mantle cavity (Walley 1965; Walley et al. 1971; Walker 1980).

It is clear from the above description that the events leading up to egg laying and also those ensuing, are well defined. What is not understood is the 'trigger' to egg laying. One theory, propounded by Barnes et al. (1977), is that the seminal plasma contains a factor which induces egg laying by stimulating muscles to contract.

Fig. 1. Diagrammatic cross-section of a generalized balanoid barnacle prior to egg laying.

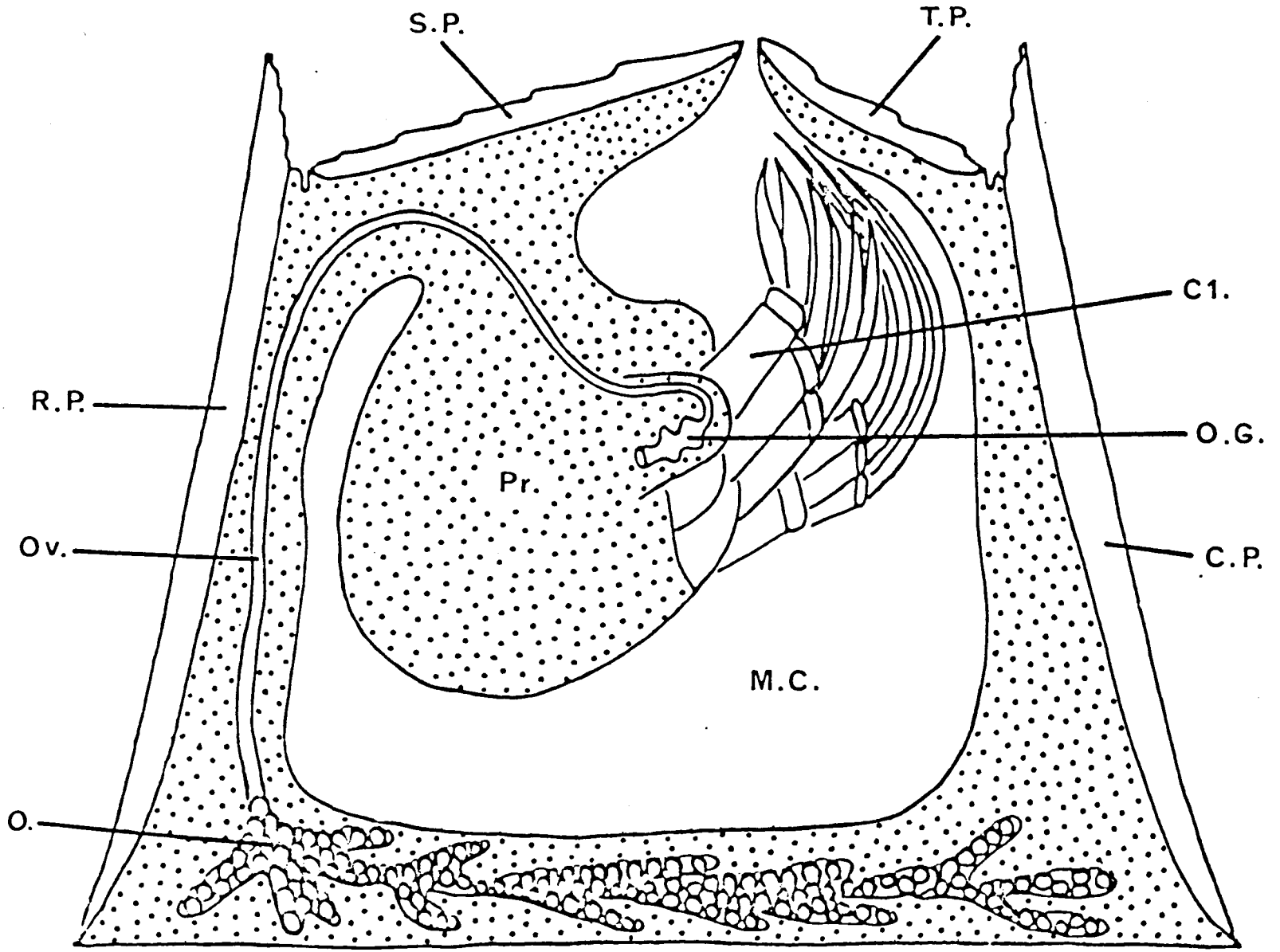
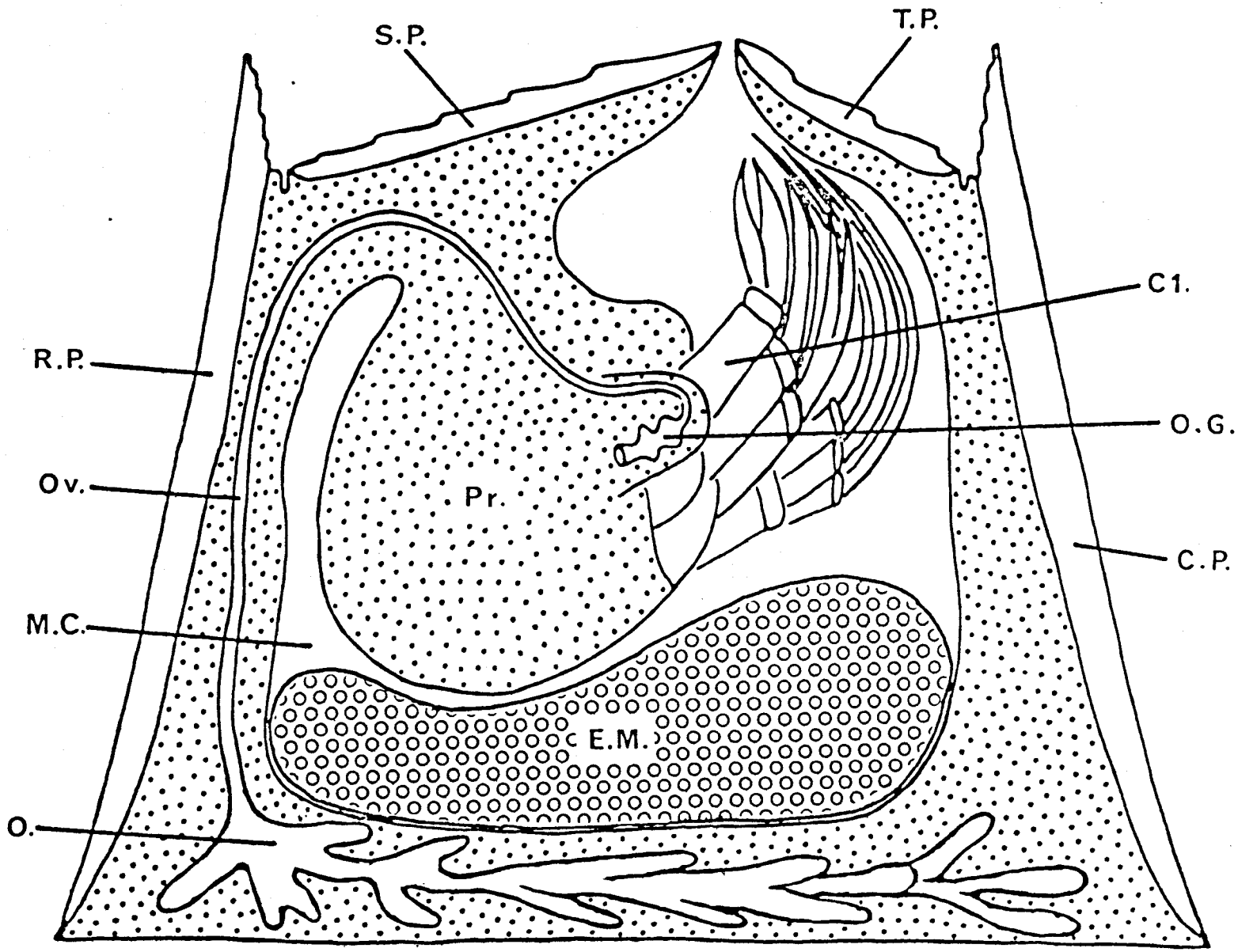

S.P.
T.P.
C1.
R.P.
O.G.
Pr.
Ov.
C.P.
M.C.
O.

Fig. 2. Diagrammatic cross-section of a generalized balanoid barnacle after egg laying showing the position of the paired egg mass within the mantle cavity.


S.P.
T.P.
C1.
R.P.
O.G.
Ov.
Pr.
C.P.
M.C.
E.M.
O.

The present study examines the egg laying process and the stimulatory properties of barnacle seminal plasma. Two species have been employed for this purpose, namely, Balanus balanoides and B.hameri.

1. Cross-fertilization in barnacles

The overriding difficulty to the study of copulation and ensuing egg laying in balanomorphs is their enclosure within calcareous plates and their permanent attachment to a substrate. Therefore, under natural circumstances it is not possible to study egg laying without dissection of the barnacle. For this reason previous studies have relied primarily on external observations (see Barnes & Barnes 1956; Barnes et al. 1977; Walker 1980). This problem can be overcome, at least for those barnacles with a membranous base, by allowing such barnacles to settle and grow on glass panels. Such a method has been employed for B.balanoides in the present study.

Spat attached to glass panels were kept on a raft moored in the Kenai Strait. Growth under such conditions is extremely rapid and barnacles become sexually mature within their first year (Walker & Foster 1979). The development of the male and female gonads and at a later date the egg masses, can readily be observed through the glass in such specimens.

Glass panels with attached B.balanoides were used for three experiments. First, to follow the behaviour of barnacles prior to copulation and egg laying. Secondly, to study the egg laying process, and finally to examine the effect of isolation of individual barnacles on egg laying. The final experiment was run in conjunction with an isolation experiment which employed barnacles attached to the mussel, Mytilus edulis.

i) Behaviour of barnacles prior to copulation

The ovigerous moult of crustaceans is generally considered to accompany and probably precede copulation. B.balanoides has been shown to conform to this pattern (Patel & Crisp 1961). Seemingly conflicting evidence was presented by Barnes & Barnes (1956), but it should be remembered that although shedding of the cuticle may occur after copulation, this does not obviate ecdysis (i.e. the separation of the old cuticle from the underlying hypodermis) occurring beforehand.

In view of these results, it was considered likely that 'acting females', whose behaviour facilitates the acceptance of the penis of 'acting males', are individuals which have recently moulted. To examine this theory a detailed record was kept of the behaviour of a number of B.balanoides (on glass panels) prior to copulation. Recent copulation was assessed either by the presence of freshly extruded sperm masses on the opercular valves of the 'female' and/or newly deposited egg masses within the mantle cavity of the female.

Table 1 is a record of the behaviour of 38 individuals maintained in running sea water for approximately one month. The most obvious feature of the data is that not all the barnacles laid their eggs; indeed 71% failed to do so. A small number of these barnacles later died, but most appeared healthy at the end of the experimental period. This situation is markedly different from that observed for populations of B.balanoides, on the raft or shore, where in mid-December individuals which have not produced egg masses are only rarely observed. It must be assumed, therefore, that the barnacles used in this study were unrepresentative of field populations. Presumably the transition from the raft to the laboratory upset the physiology of the barnacles in some way. Nevertheless 29% of the barnacles did lay their eggs.

TABLE 1. Record of the behaviour of 38 individual specimens of B.balanoides over a period of 26 days.

	NOVEMBER 1982													DECEMBER 1982													
	19	20	21	22	23	24	25	26	27	28	29	30	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1			M		♀													D								
2				M																						
3		M																								
4								M	♀																	
5					♂		♂	♂	♂																D	
6					♂			♀	E																	
7																		M							D	
8				M																						
9				M																						
10										M																
11									M		♂	♂					I									
12					♂											E										
13							M	♀		♂	♂															
14							♂	♂																	D	
15								♂				M														
16								M											E							
17					M						♀	I				D										
18	M									M	♂	I														
19							M				♂															
20		M																								M
21																									E	
22	M										M															
23							M																			
24											M	♀	♂													
25										M			I													
26							M		E																	
27							♂		♂							D										
28					M																I					E
29									M	I																
30					M		E																			
31							M		E																	
32									M		E															
33							E																			
34											M															
35							M																			
36			M																							
37							♂																			
38																E										

E = egg layed, I = inseminated, M = moulted, D = dead

It is pertinent, with regard to the theory proposed above, that 67% of the barnacles which either laid eggs, were inseminated, or were acting females, had moulted within the preceding week. Significantly, Crisp & Patel (1958) found that individuals which had moulted within the previous week were better able to act as females.

In view of these findings an attempt was made to see if egg laying could be induced artificially by injecting barnacle seminal plasma, as well as other substances, into the mantle cavities of receptive females (see section 2i)

ii) The egg laying process

The initial stages of the egg laying process have as yet not been observed in B.balanoides. Thus it is not known how the barnacle initiates the movement of eggs from the ovary into the oviducts. However, a few individuals have been observed immediately before and after egg laying commences.

Prior to egg laying the eggs are loose within the ovarian tubules, as noted by Barnes et al. (1977). In some individuals eggs have been found to lie free within the central duct of the ovary which is continuous with the oviduct proper. The movements of these eggs, coincident with the movements of the parent barnacle whilst its opercular plates are closed, may point toward events occurring within the barnacle at egg laying.

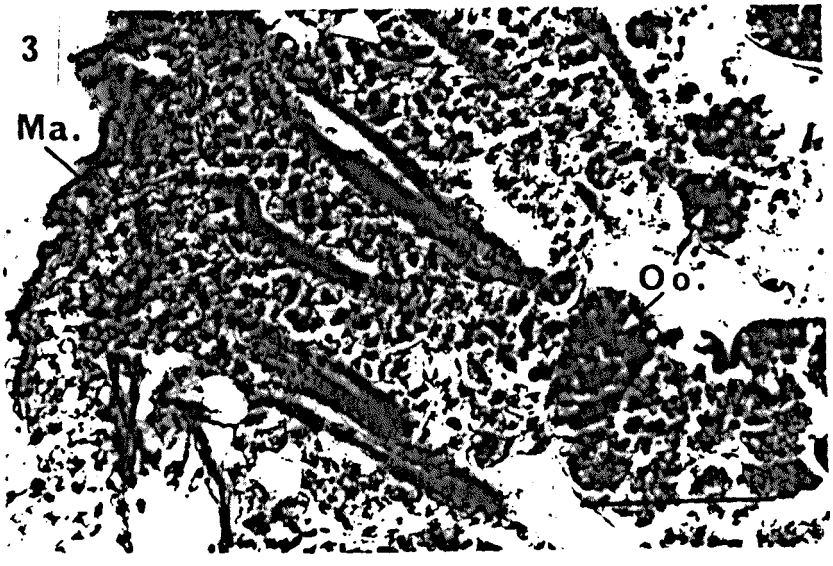
By inverting a glass panel in a tray of sea water and placing a mirror underneath a barnacle it is possible to watch movements of the opercular,plates together with movements of the eggs and the body of the barnacle within the mantle cavity.

It was found that when the opercular plates were raised and the body of the barnacle was pulled up towards the plates, so enlarging the mantle cavity, then the eggs moved towards the oviducts. The reverse action, namely, the depression of the opercular plates in conjunction with the body coming into contact with the base of the mantle, greatly reduces the volume of the mantle cavity and results in the movement of eggs away from the oviduct and in some instances out of the central duct and back into the tubules. It is probable that such egg movements result from changes in the internal pressure of the mantle cavity. The enlargement of the cavity, due to the upward movements of the plates and body, will decrease the pressure within the mantle cavity relative to that in the ovary, so that eggs will tend to be 'siphoned' into the former.

It is significant that during egg laying the valves of the female are shut tight (Walker 1980), but remain raised, presumably resulting in a low mantly cavity pressure. The violent rocking of the opercular plates immediately after insemination may also bring about movement of eggs into the oviducts.

Barnes et al. (1977) observed that the eggs of B.balanus are layed in regular pulses, suggesting muscular involvement. Two groups of muscles were considered to be operating. Muscle fibres which extend between the basal membrane and the hypodermis of the inner mantle and thus pass between the ovarian tubules are shown in figure 3. Contraction of these muscles may help to force eggs into the oviduct. In addition, the oviduct itself has circular muscle fibures which may operate in a peristaltic fashion (Barnes et al. 1977), although no such muscular action has been observed in B.balanoides. Indeed in this barnacle a constant stream of eggs pass up the oviducts; no regular peristalsis has been observed.

Fig. 3. B.hameri: Cross section through the ovary showing the muscles which extend between the basal membrane and the hypodermis of the inner mantle. Scale bar, 100 μm.


3
Ma.
Oo.

It has been proposed by Barnes et al. (1977) that the seminal plasma may induce egg laying in the acting female. This is an attractive argument primarily for two reasons. First, it would explain why such a large volume of seminal plasma, containing an excess of spermatozoa, needs to be inseminated before egg laying occurs; presumably a certain threshold of the active substance needs to be attained. Secondly, comparable methods to stimulate egg laying are employed by other invertebrates. For example, in the abalone, Haliotus rufescens, male spawn causes synchronous spawning in the female (and vice versa) (Morse et al. 1977). Also egg laying in the cricket, Teleogryllus commodus, is induced by a compound whose precursor is derived from the male spermatophore (Loher et al. 1981).

Interestingly both the active compounds in the preceding two examples appear to be prostaglandins. The occurrence of prostaglandins in seminal fluid is not unique to invertebrates. Indeed this group of compounds was first discovered in human semen (Kurzrok & Lieb 1930; von Euler 1934; 1935; Goldblatt 1933). Of the 13 or so prostaglandins present, prostaglandins of the E-series are known to cause uterine contractions (Karim & Hillier 1975). The possibility that prostaglandins may be present in barnacle seminal plasma should be borne in mind particularly considering the fact that prostaglandins have already been shown to be involved in the larval release of this sub-class (Clare et al. 1982).

An attempt has been made in the laboratory to induce egg laying in acting females of B.hameri and B.balanoides. In so doing, various approaches have been adopted.

2. The stimulus to egg laying

i) Artificial insemination of barnacles

The suggestion of Barnes et al (1977) that the barnacle seminal plasma contains a factor which stimulates egg laying has been tested by introducing

seminal plasma directly into the mantle cavity of acting females.

Seminal vesicles were dissected from sexually mature adults, homogenized in sea water and the mixture centrifuged at 9980 g for 2 min. The supernatant was used for 'injection'. This extract (at 4 seminal vesicle equivalents, i.e. from 2 animals) was introduced into the mantle cavity of the acting female, either by using a Pasteur pipette which had been drawn out to a fine point, or a syringe with a number 17 hypodermic needle attached. Both B.balanoides and B.hameri were treated in this way during the months of November and January respectively, when copulation and egg laying occur naturally. However, in neither case was egg laying induced.

A major problem faced during these experiments and which may accout for the negative result, was the inability to introduce a large sample of seminal plasma into the recipients mantle cavity. As has been mentioned earlier, the opercular plates of the acting female are held high during cross ferilization so maximizing the volume of the mantle cavity. Such behaviour does not accompany artificial insemination. In fact the opercular valves both close tight around the needle and are pulled downwards, thus greatly reducing the size of the mantle cavity and consequently the quantity of seminal plasma which can be introduced. Nevertheless, the small amounts which have been introduced into both B.balanoides and B.hameri have not been without effect, as will be discussed in the next section.

ii) In vivo bioassay systems

a) B.balanoides

A large specimen of B.balanoides, attached to a small stone, was placed in a continuous-flow perspex chamber (cf. Yule 1983) (flow rate, 300 ml/min; sea water temperature, 12.5°C). A length of nylon surgical thread was

attached at one end to one of the rostral plates with cyanoacrylate adhesive (Loctite Superglue-3) and at the other end it was linked to the isometric lever of a kymograph. An air stream was directed over the container in a rostral-carinal direction.

Figure 4 shows a trace depicting opercular movements of the barnacle over a ca. 12 min. period, during which time the barnacle was not disturbed. This trace shows the pumping beat (cf. Crisp & Southward 1961).

Figure 5 was obtained following the delivery of 0.1 ml sea water into the mantle cavity of the barnacle. It can be seen that the barnacle, after initially closing its opercular plates, quickly returns to the pumping beat indicating that such physical disturbance has little effect. At intervals during the pumping beat the opercular plates were closed and pulled downwards.

Figure 6 shows the trace obtained following the introduction of 0.1 ml of seminal plasma, obtained from the seminal vesicles and after centrifugation at 9980 g (see section 2i) Prolonged periods of closure of the opercular valves resulted from this 'injection' and these closures were interspersed by rockings of the opercular valves. A second artificial insemination, 21 minutes after the first, produced an even more dramatic effect (figure 7). The periods of closure were prolonged, up to 11 min. and 12 sec. in one case, and were again interspersed with rockings of the opercular valves.

34 min. after the second insemination the inseminated sperm was extruded from the mantle cavity. Interestingly, this occurred after the longest period of valve closure (i.e. 11 min. 12 sec.) and was accompanied by violent rockings of the opercular plates (figure 7). Such behaviour has also been shown to follow cross-fertilization in B.balanoides. Barnes et al. (1977) found that expulsion of inseminated sperm occurred 10 to 30 min. after the final insemination and during this

Fig. 4. B.balanoides: kymograph trace depicting movements of the opercular valves during pumping beat.

Fig. 5. B.balanoides: kymograph trace of opercular valve movements following the introduction of 0.1 ml. sea water into the mantle cavity (arrow). The solid lines indicate periods during which the opercular plates were closed and pulled downwards.

Fig. 6. B.balanoides: kymograph trace of opercular valve movements following the introduction of 0.1 ml. seminal fluid into the mantle cavity (arrow).

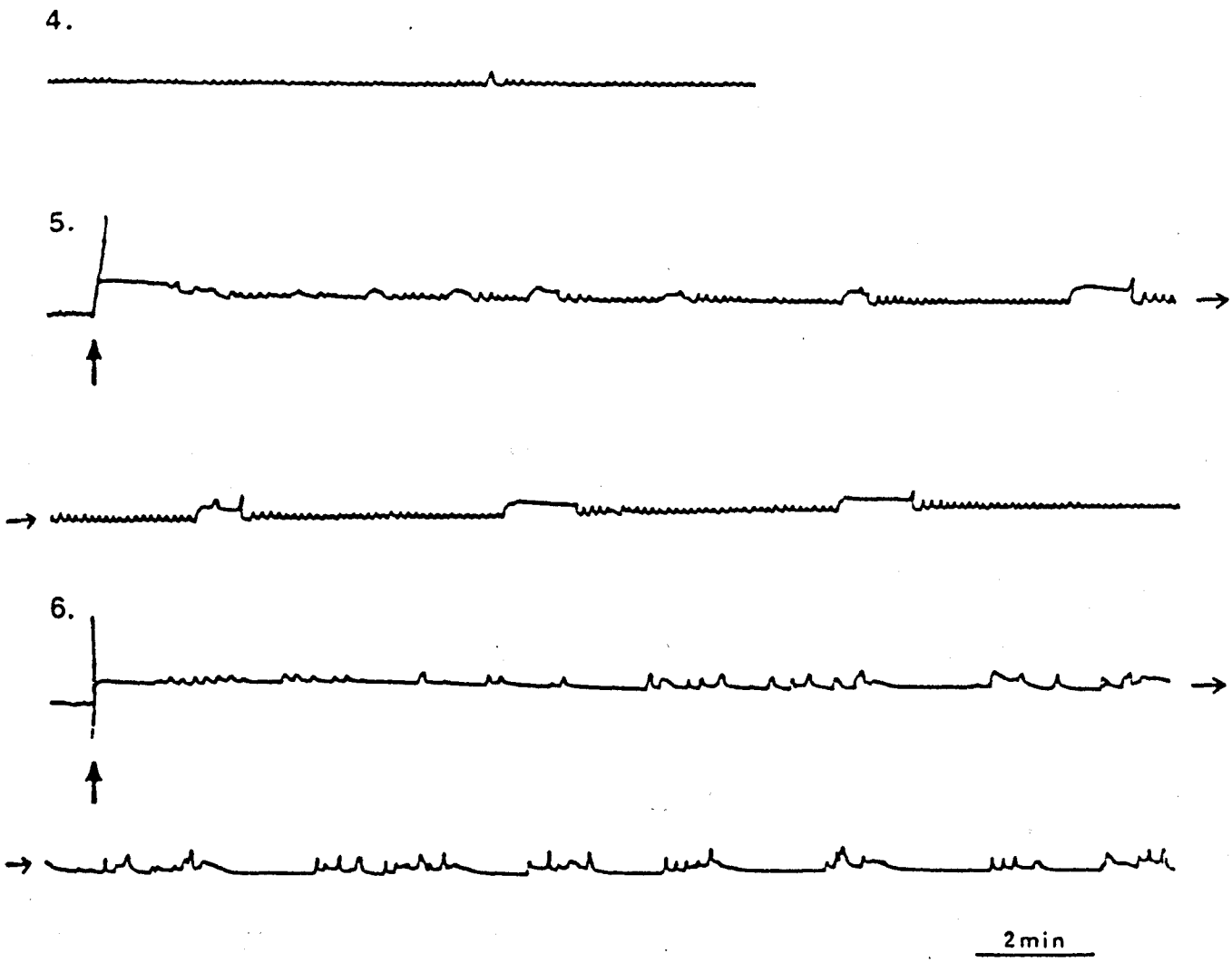
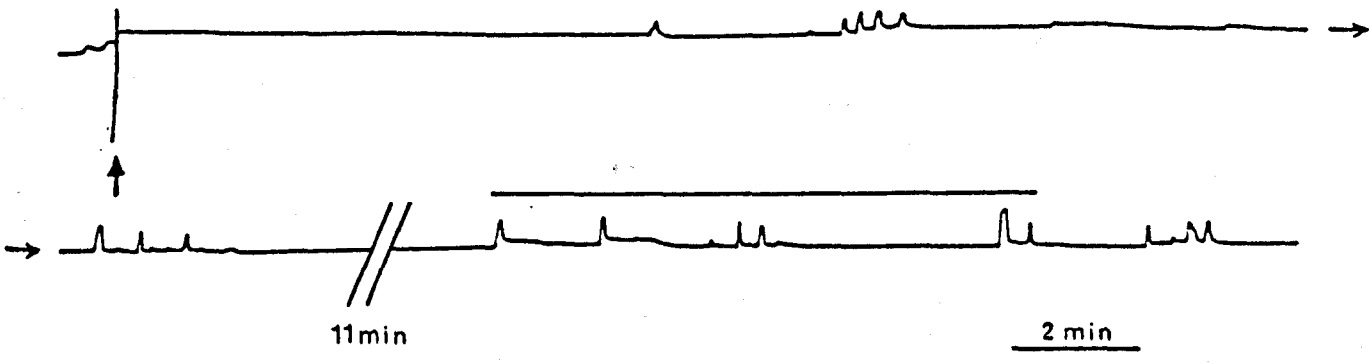

4.
5.
6.
2min

Fig. 7. B.balanoides: kymograph trace obtained following the introduction of a second 0.1 ml. sample of seminal plasma (arrow), 21 minutes after the first (shown in figure 6). The solid line indicates the duration of a period of opercular valve closure.

7.



period the opercular valves were shut tight. Similar observations were made by Walker (1980), who found that jelled sperm masses were extruded 16 min. after the final insemination.

However, in the present study, egg laying did not follow the sequence of events described above. After a further 18½ min. period, punctuated by rockings of the opercular plates, the barnacles returned to a regular pumping beat.

b) B.hameri

Two methods have been devised to overcome the problem of intoducing a large volume of seminal plasma into a barnacle. They involve either, (i) drilling a hole through the carinal or rostral plates with a dental drill and fine bit with the subsequent injection of plasma through the hole or (ii) removing the upper portion of the carinal shell plate. The latter method has been employed successfully in the present study. It has the advantage both of exposing the mantle cavity, thus facilitating the introduction of seminal plasma, and also exposing the tergal depressor muscle. This large muscle is the principle muscle involved in moving the operculum; the lateral and rostral scutal depressors relax only slightly during such action (Stubbings 1975).

B.hameri was used as the experimental animal, because its large size means that the amplitude of muscular contractions are correspondingly large, thereby increasing the sensitivity of the assay and also because a large volume of seminal plasma can be obtained from the seminal vesicles of a single animal; which is perhaps the reason why a single insemination is sufficient in 'induce' egg laying in this species (see Walker 1980).

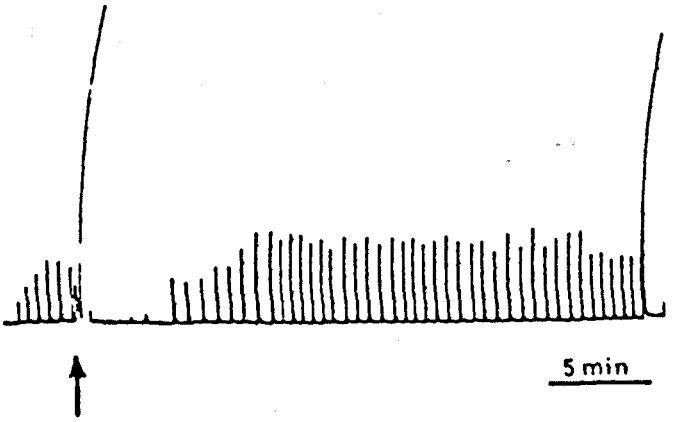
Having removed the upper portion of the carinal shell plate with bone clippers, surgical thread was tied around the exposed tergal depressor muscle, just below its point of attachment to the tergal plates, and

Fig. 8. B.hameri: kymograph trace showing movements of the tergal depressor muscle during pumping beat.

Fig. 9. B.hameri: kymograph trace showing movements of the tergal depressor muscle following the introduction of 0.2 ml. sea water into the mantle cavity (arrow).

8.

9.



linked to the isotonic lever of a kymograph. The barnacle was maintained in a perspex container (see above) with a through flow of sea water (320 ml/min) which was interrupted prior to the application of solutions to the preparation. The preparation was thoroughly flushed with sea water after each application of test solution.

Figure 8 is a trace depicting the movements of the tergal depressor muscle during the pumping beat. This particular barnacle was pumping at a rate of 22 beats/min. The large spikes (arrowed on the trace) indicate lateral rocking of the opercular plates.

When 0.2 ml. of sea water was introduced into the mantle cavity of the barnacle, adjacent to the exposed muscle, the response shown in figure 9 was obtained. The initial large spike resulted from the introduction of the water sample. However, the barnacle quickly returned to the pumping beat.

In contrast, the introduction of 0.2 ml. of B.hameri seminal plasma into the preparation resulted in a comparatively long term effect (figure 10). The barnacle took ca. 3.7 min. to return to the pumping beat and this period was punctuated by several rocks of the opercular plates. Similar behaviour was observed following the artificial insemination of B.balanoides, but in this latter case a much longer period expired before the barnacle resumed the pumping beat. The much shorter period observed for B.hameri is presumably due to the mantle cavity of this preparation being open to the sea water so that any introduced solution would gradually diffuse out of the mantle cavity.

c) Crab heart

The heart of a female Carcinus maenas was exposed by removing the carapace and its beats recorded using an isometric strain gauge. Prior to the application of test solutions the heart was beating at a rate of

Fig. 10. *B.hameri*: kymograph trace showing the effect of the introduction of 0.2 ml. seminal plasma into the mantle cavity (arrow) on movements of the tergal depressor muscle.

10.

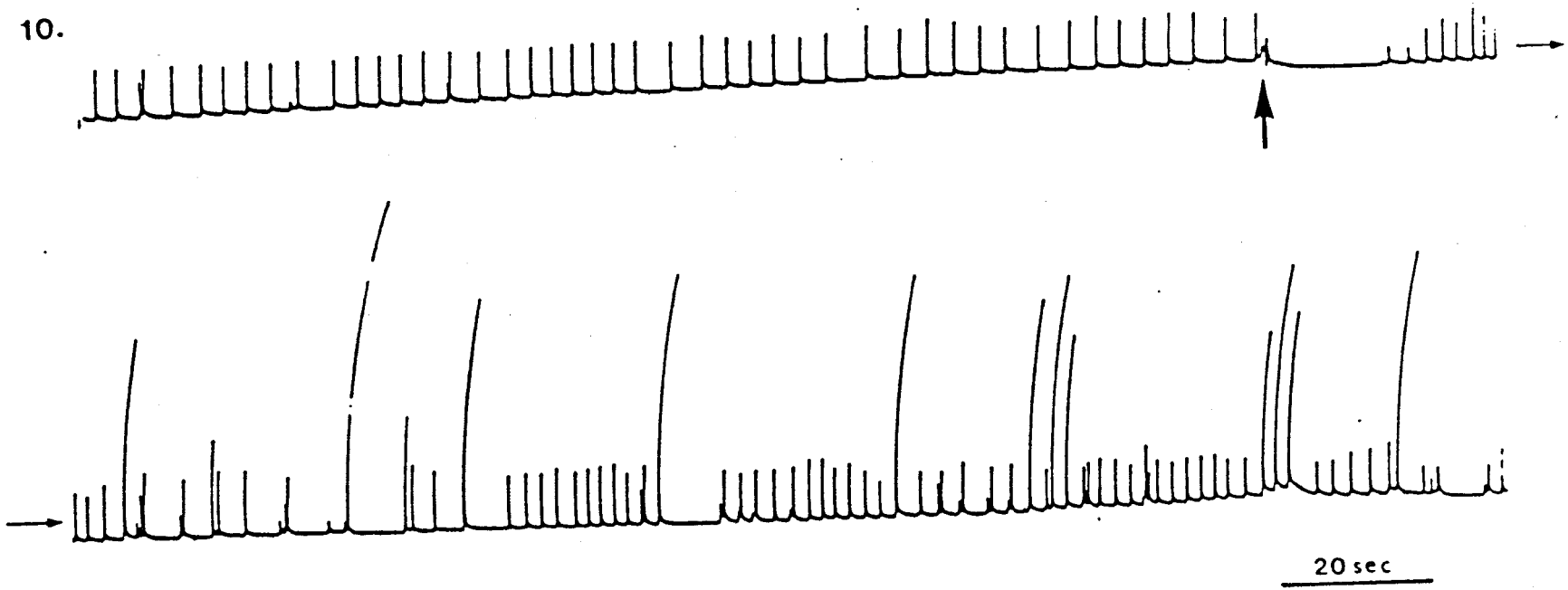
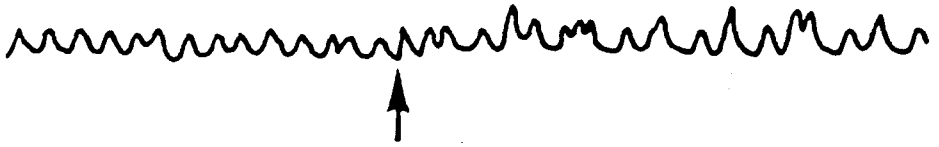


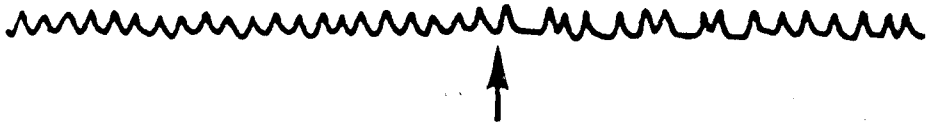
Fig. 11. C.maenas: effect of applying 0.5 ml. B.hameri seminal plasma (arrow) to the heart on its beat.

Fig. 12. C.maenas: effect on the heart beat resulting from the application of 10 µl 10^{-5}M acetylcholine to the heart (arrow).

11.



12.



10 sec

45 beats $min.^{-1}$. Figure 11 is a trace obtained following the application of ca. 0.5 ml. B.hameri seminal plasma to such a preparation. It can be seen that there was little effect on the amplitude of the heart beat, but the beat frequency decreased to 33 beats $min.^{-1}$. The heart rate returned to normal when the pericardial cavity was flushed out with sea water.

This effect of seminal plasma was similar to that elicited by 10 µl of 10^{-5} M acetylcholine, although in this case the amplitude, as well as the frequency of heart beat, was altered (figure 12).

iii) In vitro bioassay systems

a) tergum depressor muscle

Barnes et al. (1977) demonstrated that the seminal plasma of B.balanus is able to promote contractions of isolated retractor muscles of this species. Accordingly, the effect of B.hameri seminal plasma on the isolated tergal depressor muscle of B.hameri was examined. This particular muscle was selected for reasons discussed in the previous section.

The muscle was dissected from the barnacle with its point of insertion intact. Specimens of B.hameri attached to Modiolus modiolus were used to facilitate this operation. One end of the muscle was attached to an isometric strain gauge, the other to an extensible unit (figure 13). The muscle was immersed in 120 ml. of barnacle Ringer (Hoyle & Smith 1963) which was bubbled with air. In order to restrict diffusion of the test solution into this large volume of Ringer a short length of 1.5 cm glass tubing was placed over the muscle.

Contrary to the results of Barnes et al. (1977), no detectable contraction occurred when seminal plasma (approximately 1 ml.) was applied to the isolated muscle (figure 14).

Fig. 13. Diagram of the apparatus used to assay solutions on the isolated tergal depressor muscle of B.hameri.

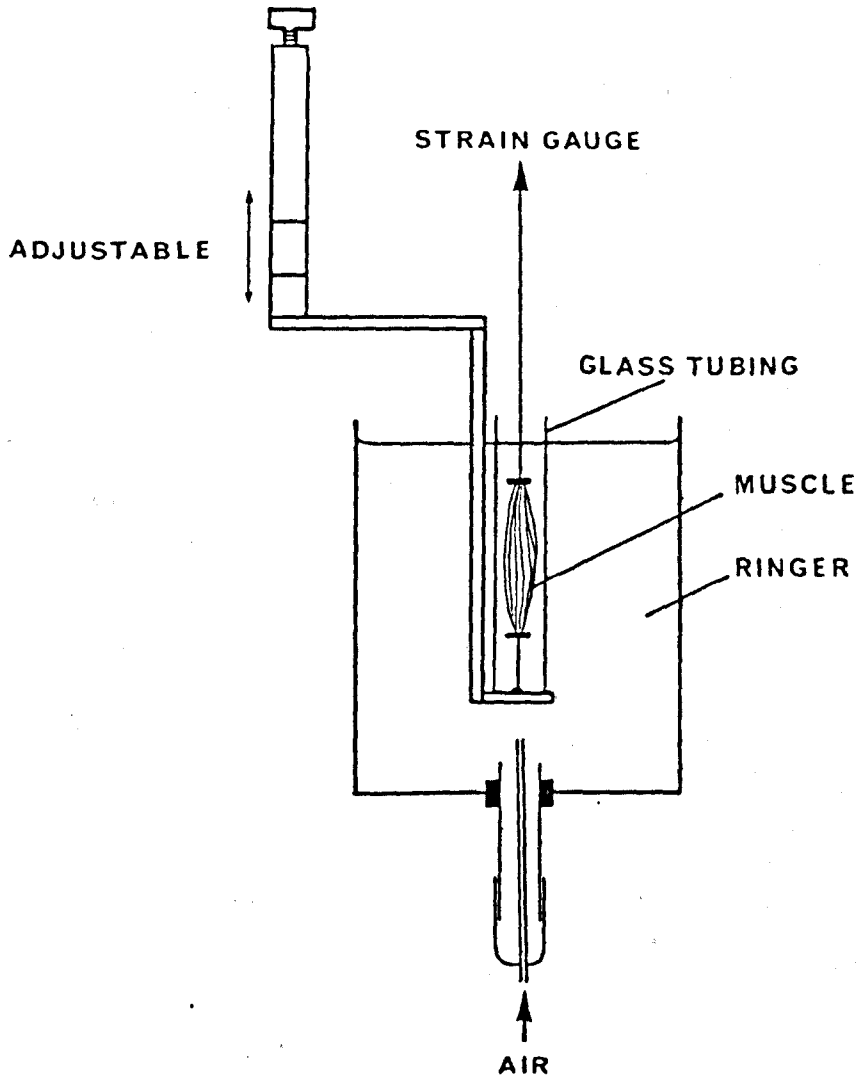

STRAIN GAUGE
ADJUSTABLE
GLASS TUBING
MUSCLE
RINGER
AIR

Fig. 14. B.hameri: trace showing that no measurable effect results from the application of ca. 1 ml. B.hameri seminal plasma (arrow) to an isolated tergal scutum depressor muscle.

14.

b) plaice intestine

B.balanoides seminal plasma was bioassayed on in vitro plaice intestine, Trendelenburg preparation (cf. Whillis 1982).

When 1.2 ml. of seminal plasma was applied to such a preparation a massive contraction resulted (see Whillis, plate 10, trace 5ii).

It is perhaps significant that a very similar result was obtained when 10^{-12}M PGE_2 was bioassayed (see Whillis, plate 10, trace 5i). However, lower concentrations of this drug, namely 10^{-16}M had no detectable effect on fish intestinal motility.

3. Isolation of prestaglandins from barnacle seminal plasma

In view of the evidence, albeit limited, that a prostaglandin(s) may be responsible for the smooth muscle stimulating properties of barnacle seminal plasma an attempt has been made to isolate such compounds from the seminal plasma of B.balanoides.

Seminal plasma from the seminal vesicles of 10 large animals was extracted specifically for prostaglandins by the method of Salmon & Karim (1976). The final ethyl acetate fraction was evaporated to dryness under nitrogen and the residue re-dissolved in chloroform/methanol (2:1). The entire sample was applied as a single spot to a 10 x 20 cm MERCK pre-coated silica gel $60F_{254}$ thin layer chromatography (T.L.C.) plate. The plate was developed in the solvent system : petroleum ether/diethylether/glacial acetic acid(85:15:1) for ca. 40 min. during which time the solvent front migrated to a distance of ca. 18.5 cm.

It had previously been established that prostaglandins do not migrate in such a solvent system (see Chapters 7 and 8) and so the origin of this plate was scraped off and eluted three times with methanol. The combined eluates were evaporated to dryness under a stream of nitrogen

and redissolved in chloroform/methanol (2:1) prior to being applied to a second T.L.C. plate. This plate was developed in a solvent system which has been termed system II in chapters 7 and 8 and comprised chloroform/methanol/glacial acetic acid/water (90:9:1:0.65). The plate was developed for ca. 60 mins. and to a distance of ca. 17.5 cm.

Several bands were observed on this plate when viewed under short wavelength ultraviolet light and their Rf (x100) values are presented in Table 2. By comparing these values with those obtained for hatching substance (Table 5, Chapter 7) it is apparent that fewer bands are resolved from the seminal plasma extract. It is reasonable to claim, therefore, that the seminal plasma sample was not significantly contaminated by hatching substance. However, it is noteworthy that each of the extracts gave rise to two bands sharing similar RF (x100) values, namely, 19.5 and 43.9 in the seminal plasma extract and 20.0 and 44.1 in the whole body extract. This latter band is active in hatching stage 12 eggs of B.balanoides (see Chapter 7).

It would appear, therefore, that there may be at least 4 prostaglandin (or related) compounds in the seminal plasma of B.balanoides. Whether any of these compounds is able to induce contractions of barnacle muscles is as yet uncertain.

Discussion

The experiments described in this chapter lend support to the proposal of Barnes et al. (1977) that barnacle seminal plasma contains a muscle stimulant. Although the nature of this factor remains unknown there is some evidence that the seminal plasma of B.balanoides contains several prostaglandins which complies with its ability to stimulate vertebrate smooth muscle.

TABLE 2. Rf (x100) values of bands separated from a prostaglandin extract of B.balanoides seminal plasma when developed on a T.L.C. plate in the solvent system chloroform/methanol/glacial acetic acid/ water (90:9:1:0.65) and visualized under short wavelength U.V. light.

Rf (x100)
19.5
43.9
50.4
72.5

Barnes (1962) found that the seminal plasma of B.balanus contains 21 µg/ml ascorbic acid and postulated two roles for this compound. First, since considerable quantities of cystine were also present, ascorbic acid may serve to protect sulphydryl enzymes. Secondly, ascorbic acid may induce penis activity such as that found in B.amphitrite by Collier et al. (1956). However, in view of the likely presence of prostaglandins in barnacle seminal plasma there is a further explanation for the role of ascorbic acid. For, Polgar & Taylor (1980) have shown that ascorbic acid increases prostaglandin synthesis in human lung fibroblasts in vitro, thus, suggesting that ascorbic acid may be similarly involved in the biosynthesis of prostaglandins in barnacle seminal vesicles. Ascorbic acid acts via hydrogen peroxide and so the mechanism of action is synonomous to that which induces spawning in the abalone (cf. Morse et al. 1977).

Accepting the above arguements, the factor does not appear to cause egg laying directly in either B.balanoides or B.hameri. There are two obvious reasons for this negative result. First, the barnacle which has been artificially inseminated may not be an 'acting female'. Although an animal may be exhibiting typical female behaviour, and such specimens were used whenever possible in the present study, there is no guarantee that a penis would subsequently be accepted under normal circumstances.

Secondly, an egg laying hormone may instead be derived from the central nervous system. This does not necessarily obviate the involvement of the seminal plasma, since the egg laying hormone may be released in response to insemination. In support of this arguement is the observation that the content of certain neurosecretory cells in the supraoesophageal ganglion of B.balanoides appears to be markedly depleted following egg laying (cf. Chapter 3).

Homogenates of barnacle ganglia have in fact been injected into barnacles, but without any apparent effect. Nevertheless, recent work on the spawning

hormone of the trochid, Gibbula umbilicalis, has indicated that the hormone is present in the cerebral ganglia for a restricted period only (Clare, unpublished). It is thus possible that ganglion extracts were bioassayed at the wrong time in the present study. Therefore, it would be desirable if such experiments were repeated over a protracted period centreing on the time of natural cross-fertilization.

CHAPTER 6

A preliminary note on the effect of 20-hydroxyecdysone on the development of *Balanus balanoides* (L.) embryos *in vitro*.

CHAPTER 6

A preliminary note on the effect of 20-hydroxyecdysone on the development of Balanus balanoides (L.) embryos in vitro.

INTRODUCTION

Ecdysteroids, which have been extracted from adult barnacle tissue (Bebbington & Morgan, 1977) are involved in controlling the moult cycle (Cheung, 1973; Cheung & Nigrelli, 1974; Freeman & Costlow, 1979; Freeman, 1980). Fyhn et al. (1977) have suggested that 20-hydroxyecdysone also acts as a gonad inhibiting hormone in barnacles, although this rôle has since been questioned (Adiyodi, 1978).

Barnacle eggs are brooded in the mantle cavity which is continuous with the external environment. It is unlikely, therefore, that the developing embryos are subject to fluctuations in parental ecdysteroids. However, it has recently been demonstrated that the embryos of Carcinus maenas themselves contain ecdysteroids, and that the titre of ponasterone A, the predominant ecdysteroid, fluctuates throughout embryonic development (Lachaise & Hoffman, 1982). It is not known if ecdysteroids are present in barnacle embryos. However, since stage I nauplii of B. balanoides moult within a few hours of hatching, it may reasonably be expected that at least the later embryonic stages have some capacity for ecdysteroid synthesis.

The present study examines the effect of 20-hydroxyecdysone on the embryonic development of B. balanoides.

MATERIALS AND METHODS

Adult Balanus balanoides were collected, attached to rocks, from the shore beneath the Menai Suspension Bridge during November. The animals were maintained in the laboratory in running sea water and examined on a regular basis for freshly extruded sperm masses. The presence of a jelled sperm mass on the opercular plates indicates that cross-fertilization has recently occurred (c.f. Walker, 1980). Barnacles with such sperm masses were carefully removed from the rocks and examined for recently laid eggs. Only egg masses with stage I embryos were used in this study and the egg stage was identified from the description of Lucas (1980). Egg masses from two individuals were placed in glass petri dishes containing a solution of pronase AS (Sigma) at a concentration of 0.11 mg/ml ultraviolet (U.V.)-irradiated, filtered sea water. The solution also contained streptomycin (0.05 g/l) and penicillin G (0.03 g/l) (both obtained from Glaxo). Pronase digests the glycoprotein matrix which binds the eggs and so the eggs become separated. Solutions of 20-hydroxyecdysone (Sigma) were prepared in U.V.-irradiated, filtered sea water, containing streptomycin and penicillin G, to the concentrations of 1.0 µg/ml, 0.01 µg/ml and 0.001 µg/ml. U.V.-irradiated, filtered sea water, containing streptomycin and penicillin G, served as the control. Batches of separated eggs were washed twice in one of the above solutions before being placed in 9 x 2.5 cm glass tubes containing 20 ml of the appropriate ecdysteroid solution. The tubes were plugged with cotton wool. Eggs from each adult barnacle were treated separately and the experiment was performed in triplicate. The eggs were kept continuously agitated by placing the tubes into a waterbath containing a shaker unit operating at 140 strokes/min. The temperature of the water bath was thermostatically maintained

between 6° and 10°C.

Culturing of the eggs began on the 28th November, 1980. The experimental solutions were replaced with freshly prepared solutions on the 22nd December, 1980 and the 6th January, 1981. On the latter occasion the solutions contained twice the previously described concentrations of streptomycin and penicillin G, i.e., 0.01 g/l and 0.6 g/l respectively. The experiment was terminated with the appearance of swimming stage I nauplii within the control solutions.

During the experimental period, the eggs were examined on approximately a weekly basis; at these times ten eggs were removed from each tube and examined on a slide with a Leitz Orthoplan photomicroscope. The stage of development of each egg was noted and a photographic record made. It was, therefore, possible to calculate the dimensions of eggs at each developmental stage.

17 x 11 cm photographic enlargements were used to calculate the volume of eggs and embryos. Both were divided into a series of discs of radius, r (μm) and height, h (μm). Each disc was treated as a cylinder and its volume was calculated using the formula:

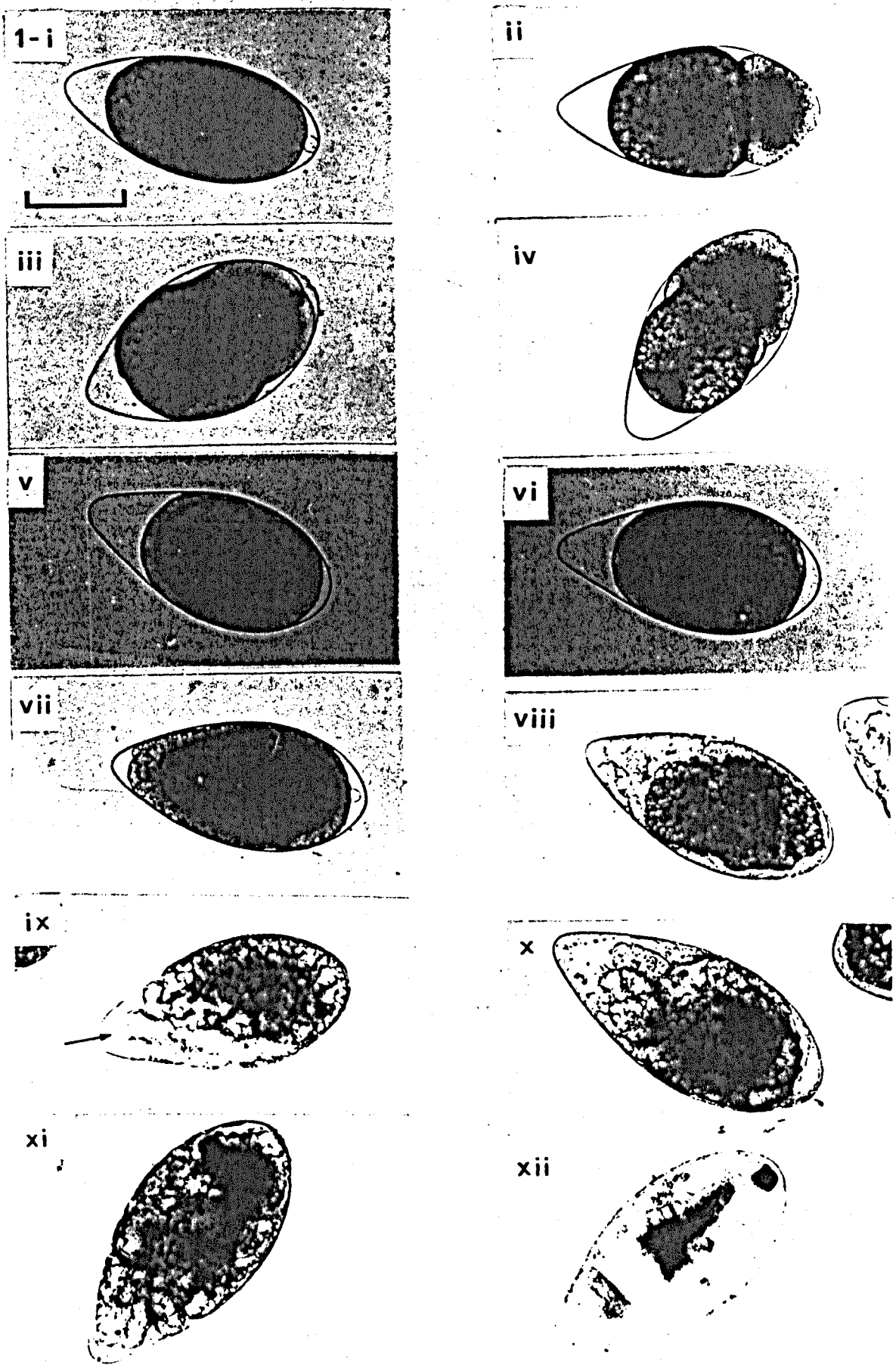
$$\text{Volume of a cylinder} = \pi r^2 h$$

The calculated volumes are, therefore, overestimates, since the outer egg membrane is a curved surface.

RESULTS AND DISCUSSION

Figure 1 illustrates the 12 stages of embryonic development of B. balanoides in the sea water control solutions. The development is essentially the same as that noted by other workers for B. balanoides

Fig. 1. Developmental stages of B.balanoides eggs cultured in vitro. Scale bar, 100 μm.


1-i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii

(Crisp, 1959; Lucas, 1980) and also for B. balanus (Crisp, 1954), although dimension differences do exist between the eggs studied by Lucas (1980) and those of the present study. Table 1 compares the mean length of each egg stage for the two studies. Clearly, the mean length measurements of Lucas (ibid) are consistently greater at each stage of development. Furthermore, the present study determined that there is only a single decrease in the mean length of eggs and this occurs between stages 3 and 4, whereas Lucas (1980) noted four such decreases, at stages 2, 4, 8 and 9. Finally, Lucas (ibid) observed that eggs swell substantially at stage 9 and yet recorded a decrease in mean length at this stage.

Volume changes throughout development, for both the eggs and the embryos within, are presented in figures 2 and 3 respectively. There is a large rise in both the volume of the embryo and the egg at stage 2, which is consistent with the division of the undifferentiated yolk into the anterior micromere (blastoderm) and posterior macromere (yolk). At stage 4 there is a large decrease in volume of both the egg and embryo. This volume drop might be the result of yolk being utilized as an energy source or converted to carbohydrate which takes up less volume, weight for weight. However, Lucas (1980) detected little change in either the lipid or protein content of the eggs between stages 3 and 4. Only small changes in volume occur until stage 7. Between this stage and stage 8 the volume of the embryo increases more than that of the egg and the ratio between the two volumes approaches unity (Fig. 4). Lucas (1980) also noted that the embryo swells at stage 8 to occupy all the space within the egg. The substantial swelling of the egg as a whole between stages 8 and 9, descibed by Lucas (ibid), was not noted in the present study. However, such an increase did occur between stages 9 and

TABLE 1. Comparative values for mean length measurements of *B.balanoides* eggs.

Stage	Present study mean length (um) ± S.E.	Lucas (1980) mean length (um) ± S.E.
1	229.26 ± 18.10	271.04 ± 22
2	257.14 ± 21.60	260.75 ± 18
3	267.20 ± 27.63	264.13 ± 17
4	250.86 ± 23.95	262.30 ± 24
5	251.43 ± 16.16	268.80 ± 24
6	253.14 ± 10.98	285.60 ± 19
7	253.60 ± 9.23	289.89 ± 21
8	257.14 ± 13.55	287.49 ± 18
9	259.20 ± 14.54	279.80 ± 17
10	282.86 ± 25.43	284.44 ± 20
11	293.14 ± 25.89	295.72 ± 22
12	295.77 ± 14.53	313.30 ± 30

Fig. 2. Changes in the volume of B.balanoides eggs during development.

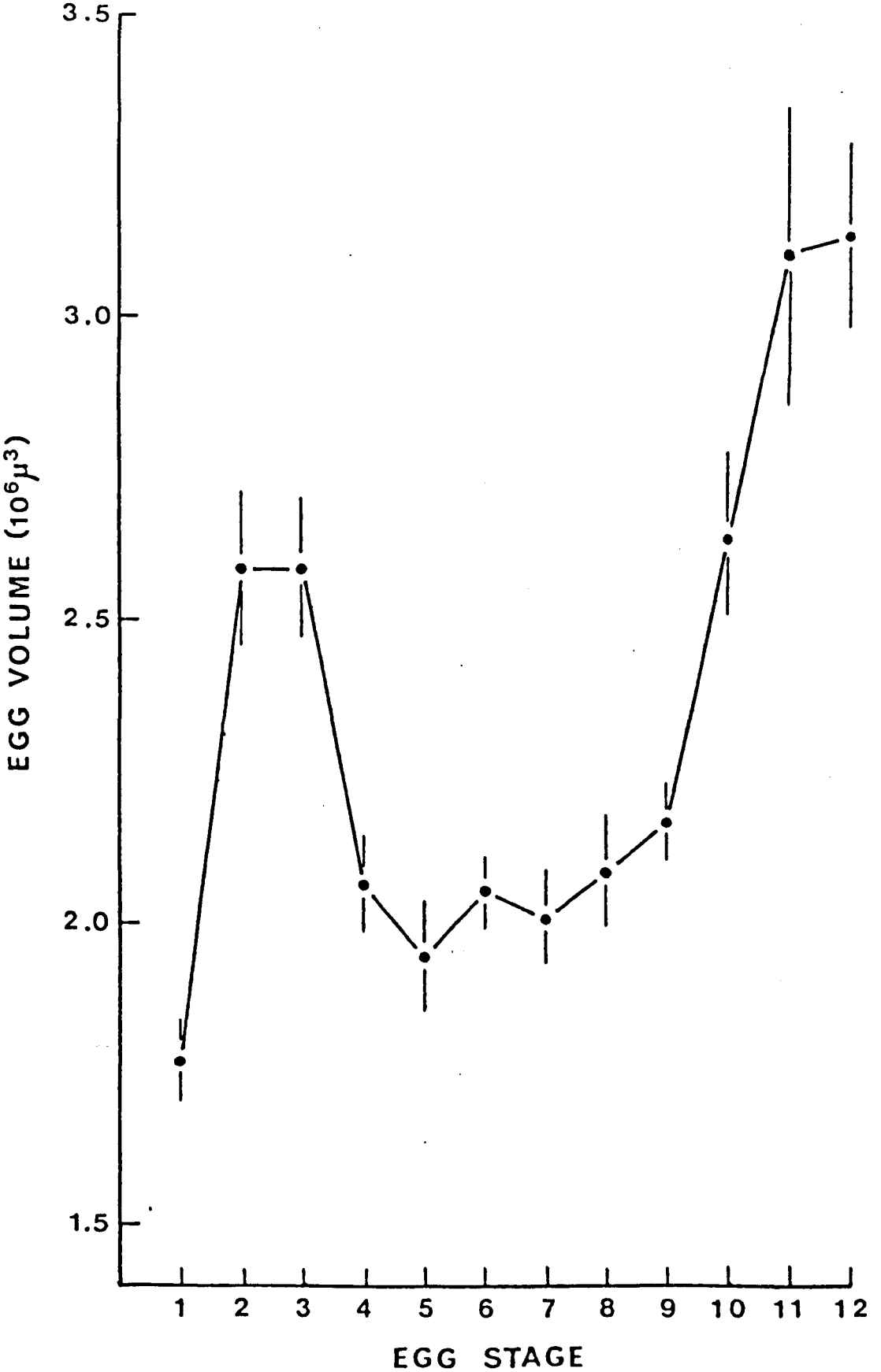

3.5
3.0
2.5
2.0
1.5
EGG VOLUME ($10^6\mu^3$)
1
2
3
4
5
6
7
8
9
10
11
12
EGG STAGE

Fig. 3. Changes in the volume of B.balanoides embryos during development.

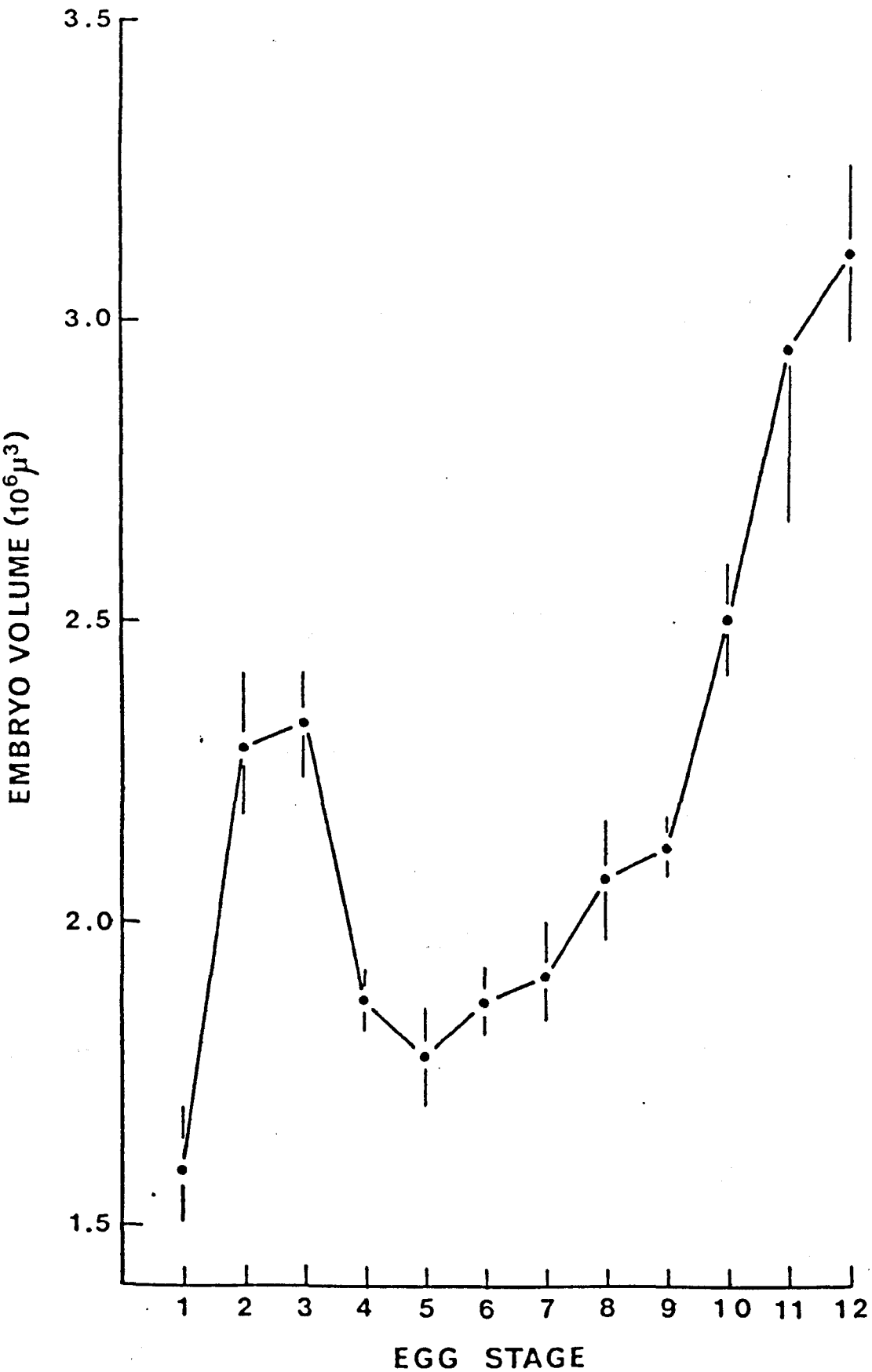
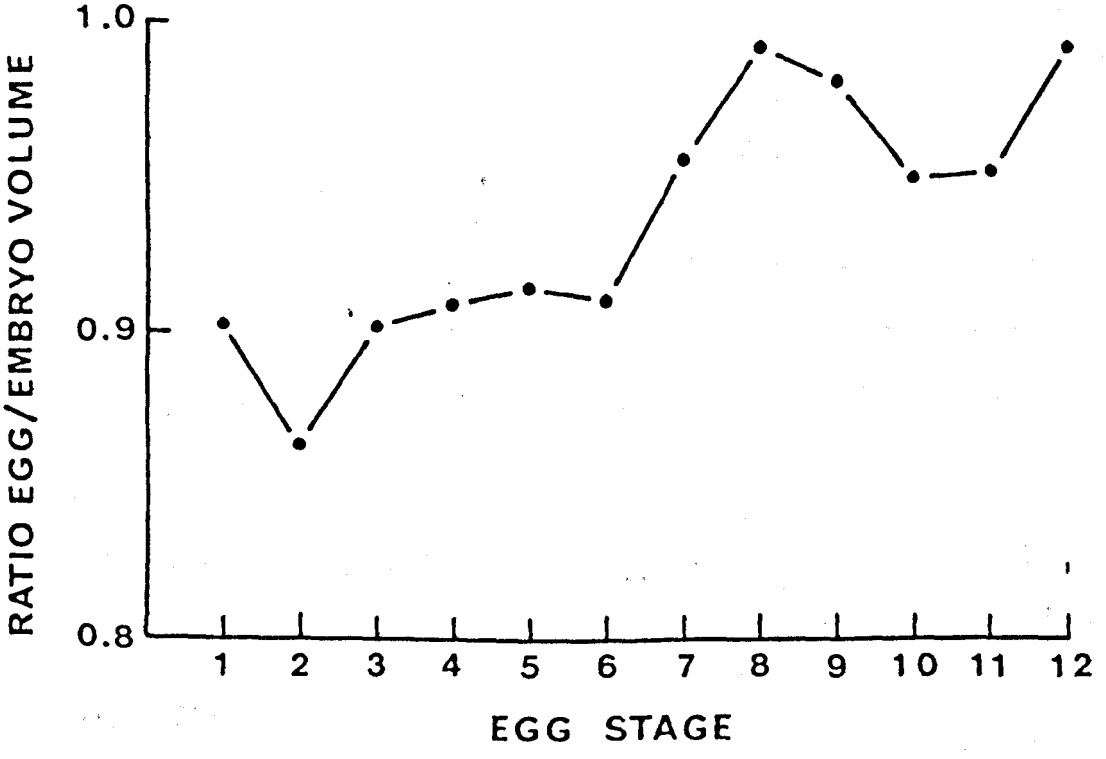

3.5
3.0
2.5
2.0
1.5
EMBRYO VOLUME ($10^6\mu^3$)
1
2
3
4
5
6
7
8
9
10
11
12
EGG STAGE

Fig. 4. Ratio of egg to embryo volume at each developmental stage.


1.0
0.9
0.8
RATIO EGG/EMBRYO VOLUME
1
2
3
4
5
6
7
8
9
10
11
12
EGG STAGE

10. Presumably the volume increase results from an intake of water. The accompanying decrease in the embryo to egg ratio supports this view (Fig. 4). It is pertinent in this respect that setae appear at stage 10. Water may, therefore, be inbibed to expand the exoskeleton.

At the same time as the eggs were increasing in overall size, the embryo to egg volume ratio was decreasing (Fig. 4). Small increases in size occurred between stages 10 and 12. The embryo to egg volume ratio also increased to near unity during this period.

Egg development in 20-hydroxyecdysone solutions did not differ from that of the controls until they reached stages 8 or 9 (Table 2) after which they underwent progressive cytolysis (Fig. 5). Actually, the dimensions of the cytolyzed eggs (Table 3) indicate that development proceeds to stage 9 before cytolysis commences. Owing to the small number of eggs examined, no difference of effect could be detected between the three concentrations of hormone used. Interestingly, cytolyzed eggs were not subject to fungal attack, a feature noted by Crisp (1959). However, Crisp (ibid) maintained eggs in sea water which was changed daily, but did not use either antibiotics or fungicides as did the present study and the latter is probably the reason for the discrepancy.

It is also interesting that 20-hydroxyecdysone should have a deleterious effect on embryo development at stage 9 and not on earlier stages, since several important events occur between stages 9 and 10. First, there is the appearance of an additional egg membrane (Fig. 1). Significantly, ecdysteroids have been implicated in the control of deposition of an embryonic envelope in C. maenas (Lachaise & Hoffman, 1982). If it is assumed that the deposition of the egg membrane in B. balanoides is similarly under the control of an ecdysteroid, then the

TABLE 2. *B.balanoides* egg development (mean stage values) in 20-hydroxyecdysone solutions and sea water

Date	Control						20 - Hydroxyecdysone concentration (µg/ml)																	
							0.001						0.01						1.0					
	Egg mass A			Egg mass B			Egg mass A			Egg mass B			Egg mass A			Egg mass B			Egg mass A			Egg mass B		
	Tube no.			Tube no.			Tube no.			Tube no.			Tube no.			Tube no.			Tube no.			Tube no.		
	1	2	3	13	14	15	4	5	6	16	17	18	7	8	9	19	20	21	10	11	12	22	23	24
2.12.80	6	-	-	-	-	-	6	-	-	-	-	-	7	-	-	-	-	-	7	-	-	-	-	-
3.12.80	-	-	-	7	-	-	-	-	-	7	-	-	-	-	-	7	-	-	-	-	-	7	-	-
6.12.80	-	8	-	-	-	-	-	8	-	-	-	-	-	8	-	-	-	-	-	8	-	-	-	-
11.12.80	-	-	-	-	7	-	-	-	-	-	7	-	-	-	-	-	7	-	-	-	-	-	7	-
22.12.80	-	-	8	-	-	9	-	-	9	-	-	8	-	-	9	-	-	A	-	-	9	-	-	8
12. 1.81	F	F	F	12	-	-	-	-	-	A	-	-	-	-	-	8	-	-	-	-	-	9	-	-
14. 1.81				-	7	-				-	A	-				-	A	-				-	A	-
19. 1.81				12	F	-				A	-	-				A	-	-				11*	-	-
23.1.81				-		11				-	-	A				-	-	A				-	-	A
28. 1.81				12		-				-	-	-				A	-	-				12*	-	-
5.2.81				-		N				-	-	-				-	-	-				-	-	-
6.2.81				N		-				-	-	-				-	-	-				11*	-	-

N - nauplii, F - fungus, A - abnormal, * > 40% abnormal

Fig. 5. *B. balanoides* egg at an advanced stage of cytolysis. Scale bar, 100 μm.

5

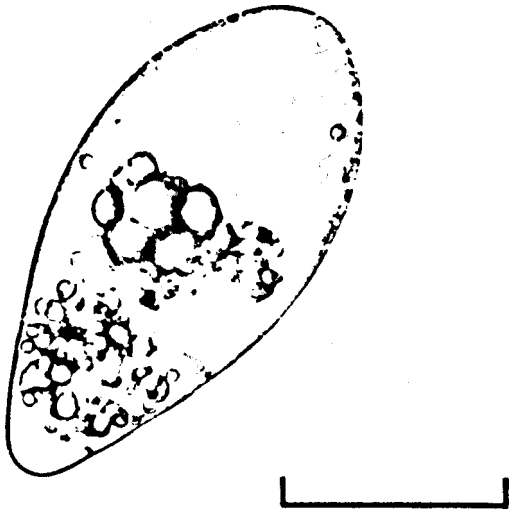


TABLE 3. Size of cytolyzed eggs in the control and 20-hydroxyecdysone test solutions

20-hydroxyecdysone concentration (µg/ml)	Mean length (µm)	Standard Error
Control	267.23	± 11.56
0.001	276.26	± 9.60
0.01	268.38	± 4.54
1.0	270.55	± 6.72

presence of additional hormone or a high titre of the wrong ecdysteroid could interfere with the process. Secondly, there is a large increase in egg volume which has been suggested to be the result of a net water influx. Although water absorption in crustaceans begins immediately after ecdysis and thus soon after a peak in the ecdysteroid titre (see Chapter 1, section III), it is not known if the two events are interrelated. There is thus no evidence to support the possibility that 20-hydroxy-ecdysone may affect water uptake by barnacle eggs. Thirdly, setae, and it follows also cuticle, develop. Deposition of cuticule in barnacles, as well as in arthropods generally, is known to be controlled by ecdysteroids (see Chapter 1, sections III and IV). Furthermore, it has been suggested that the deposition of embryonic cuticle in C. maenas is controlled by ecdysteroids (Lachaise & Hoffman, 1982). Hence, if the same hormonal control operates in barnacle embryos, then the presence of additional hormone in the media and/or the presence of a high titre of hormone too early could affect cuticle deposition. Finally, Lucas (1980) noted that embryos at stage 9 switch from using mainly carbohydrate as an energy source to using lipid.

Clearly the above described period of development is critical to the embryos of B. balanoides. Evidence has also been presented to tentatively suggest that two of the events known to occur during this period of development are sensitive to ecdysteroids and it cannot be ruled out that other events are so controlled. Nevertheless, although it is evident that 20-hydroxyecdysone has an affect on barnacle embryology, this does not mean that the embryos themselves possess the hormone. A fuller understanding of the rôle 20-hydroxyecdysone plays in the development of B. balanoides eggs must await results of the titre of this hormone throughout development.

CHAPTER 7

Barnacle egg hatching : a novel rôle for a prostaglandin-like compound.

CHAPTER 7

Barnacle egg hatching : a novel rôle for a prostaglandin-like compound.

INTRODUCTION

In N. Wales Balanus balanoides lays its eggs in November. This process involves the passage of eggs from the ovary along the two oviducts; each oviduct terminates in an oviducal gland. As the eggs pass into a gland they enter an elastic sac (the ovisac) secreted by the gland. Each ovisac gradually distends with eggs and is eventually forced out through the oviducal gland opening into the mantle cavity, but remains attached within the gland until the egg mass is fully formed. The egg masses are then released and become moulded to the shape of the mantle cavity where they lie until the following March (see Barnes & Barnes, 1956; Walley, 1965; Walker, 1980). Although embryos are capable of hatching at Menai Bridge in early February (personal observation), they are retained until the time of the Spring algal bloom (Barnes, 1955; Crisp, 1956; Crisp & Spencer, 1958; Crisp, 1962; Rzepishevsky, 1962). Barnes (1957) proposed that the parent barnacles concentrate a substance, derived from diatoms, which acts in the form of one of their excretory metabolites and causes the eggs to hatch. Although this theory would have accounted for the synchrony between the Spring diatom bloom and liberation of nauplii, it is incorrect. Crisp (1956) had previously shown that barnacles which were deprived of food did not liberate their larvae; liberation took place in response to feeding, whether on plant or animal food. Moreover, sea water extracts of unfed barnacles were just as effective in causing eggs to hatch as were extracts of fed animals. The factor which stimulated the eggs to hatch was termed the "barnacle hatching substance" (Crisp &

Spencer, 1958). Hatching substance was investigated in detail by Crisp and Spencer (ibid) and subsequently by Crisp (1969). It was concluded that hatching substance is not present as such in the living adult barnacle tissues. Instead a precursor is stored, mainly in the hypodermis, which can be artificially converted to the active substance in the first few minutes after the tissues are macerated. Hatching substance was found to pass through dialysis tubing, was heat stable, stable in alkali, but acid labile. The lability of the compound at low pH was explained in terms of lactone or lactam formation. Moreover, hatching substance is non-specific in that it is produced by a variety of thoracican barnacles.

Although Barnes and Barnes (1959) claimed that 5-hydroxytryptamine (5-HT) was an effective hatching substance in B. nubilus, it was later shown by Crisp (1969) to be ineffective in hatching the eggs of B. balanoides. Other physiologically active amines-tryptamine, histamine and tyramine were also discounted. Hatching substance acts directly on the fully developed embryo stimulating muscle (Barnes, 1955). It is not yet known whether this action affects smooth or striated muscle or both. Crisp (1969), however, showed that hatching substance is unable to stimulate vertebrate smooth muscle.

The present study is a further investigation on the hatching substance of B. balanoides. A method is described to extract and partially purify the substance, and its probable nature and mode of action are discussed.

The process of egg-hatching

Barnes (1955) described the hatching process of barnacles as being purely mechanical. This has been confirmed in the present study from numerous observations of eggs from a variety of barnacles and from a

ciné film of the hatching process of B. balanoides. The first sign of hatching is a loss of sheen on the outer surface of the egg case. A slight depression appears on the egg case just posterior to the nauplius eye of the embryo. This is closely followed by perceptible movements of the embryo itself. Most noticeable is an antero-dorsal movement of the gut. In addition, spasmodic beats of the limbs are made against the egg case. The movements become progressively more violent and ultimately result in the rupture of the egg case at the anterior end, the larva swimming clear.

Bioassay of hatching substance

The bioassay employed was a modified version of that described by Crisp and Spencer (1958). For each assay the two egg masses from an individual were carefully removed intact. After washing for a minimum of 1 hr in running sea water, one egg mass was placed in a watch glass containing test solution and the other in an ultra violet (U.V.)-irradiated filtered sea water control. After 15 minutes, the time required for the effect of hatching substance to go to completion, a lethal quantity of 2.5% gluteraldehyde in sea water was added and the number of stage I nauplii and unhatched eggs in each solution were counted using a Bogorov tray. On occasion egg masses were used which contained immature eggs at their centre, these eggs were not included in the counts; only stage 12 eggs (see Crisp, 1954, 1959) were counted. Although stage 11 eggs possess a nauplius eye and apparently well developed limbs, they must be deficient in some respect, for they are incapable of hatching.

Extraction of hatching substance

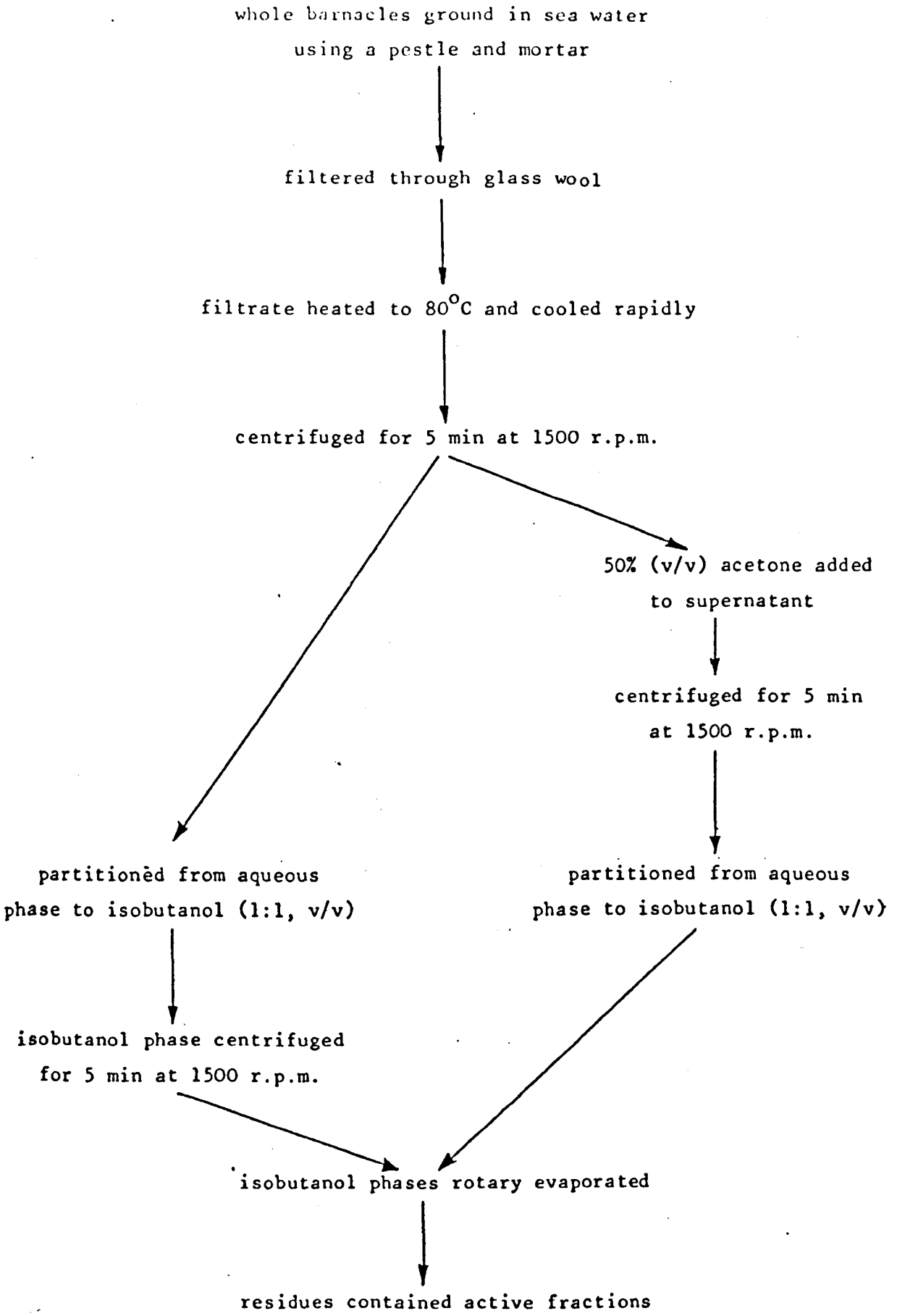
Whole adult B. balanoides were scraped off rocks taken from the

shore beneath the Menai Suspension Bridge on the 27th March, 1981, a time when the majority of specimens contained fully developed egg masses. A majority of the barnacles were deep frozen. Hatching substance was extracted immediately from the remainder by a technique slightly modified from that described by Crisp and Spencer (1958). The barnacles were first ground in 100 ml of U.V.-irradiated filtered sea water using a mortar and pestle and left to stand for 1 hr at room temperature with intermittent stirring. The tissue and shell debris were removed by filtering through polyester fibre (Beckfoot Mill, Silsden). The filtrate obtained was then heated to 80°C giving a large amount of precipitated material and cooled rapidly. Following centrifugation at 1500 rpm for 5 min, the resultant supernatant was divided into two fractions (fractions I and II). Subsequent treatment of the two fractions differed only in that to fraction II an equal volume of acetone was added. A considerable amount of material was further precipitated by this method and was removed by centrifugation (1500 rpm; 5 min). The supernatant thus obtained, and fraction I, were partitioned separately against an equal volume of butan-1-ol. The aqueous phase was discarded whilst the organic solvent phase was centrifuged (1500 rpm; 5 min) and divided into three equal volumes, which were subsequently evaporated to dryness at 50°C in a Büchi rotary evaporator. The three residues of both fractions I and II were then dissolved in one of the following: (a) methanol (b) 50% aqueous ethanol (c) U.V.-irradiated filtered sea water. Fractions Ic and IIc were used for bioassay whilst the solvent fractions were stored in the deep freeze. A summary of the extraction procedure for fraction II is presented in Figure 1.

The purification of hatching substance

Having obtained the extracts they were spotted onto thin layer

Fig. 1. Flow diagram of the butanol-extraction procedure used to obtain a concentrated extract of the hatching substance of *Balanus balanoides*.


whole barnacles ground in sea water
using a pestle and mortar
filtered through glass wool
filtrate heated to 80°C and cooled rapidly
centrifuged for 5 min at 1500 r.p.m.
50% (v/v) acetone added
to supernatant
centrifuged for 5 min
at 1500 r.p.m.
partitionèd from aqueous
phase to isobutanol (1:1, v/v)
partitioned from aqueous
phase to isobutanol (1:1, v/v)
isobutanol phase centrifuged
for 5 min at 1500 r.p.m.
isobutanol phases rotary evaporated
residues contained active fractions

chromatography (T.L.C.) plates (10 x 20 cm or 20 x 20 cm MERCK pre-coated silica gel 60F 254) alongside lipid standards. The plates were developed in a running solvent system composed of petroleum ether (40°-60°C)/diethyl ether/glacial acetic acid (85:15:1) (solvent system I) in closed tanks which had been equilibrated for 30 min. Each plate was developed for ca. 45 min during which time the solvent front migrated ca. 18.5 cm. Following a run, the plates were air dried and the separated bands detected, either by exposure to iodine vapour, or by viewing under short wavelength (approximately 255 nm) and long wavelength (approximately 310-390 nm) U.V. light, to detect U.V.-absorbing and U.V.-fluorescing bands respectively. Each band was then scraped off the plate and eluted three times in methanol (Bygdeman et al., 1969). Following evaporation of the methanol under nitrogen, the resulting residues were dissolved in U.V.-irradiated filtered sea water and bioassayed. All the hatching activity was found in the eluates of the origin; none of the other bands promoted egg hatch. Furthermore, the hatch obtained when the origin eluates were bioassayed was complete (Table 1).

In order to further purify the hatching substance another running solvent system was employed which was known to separate the neutral from the polar lipids. This solvent system (system II) comprised chloroform/methanol/glacial acetic acid/water (90:9:1:0.65); the origin eluted from a system I plate was developed in this system for ca. 60 min giving a solvent front migrating distance of ca. 17.5 cm. The number of bands separated out on system II plates was slightly variable, but the position of the bands was not. A typical set of Rf (x 100) values is given in Table 2. By eluting and bioassaying these bands as described above, a discrete region eliciting hatching activity was noted. The region contained four U.V.-absorbing bands, one of which also fluoresced, and

TABLE 1. The activity of various extracts, as measured by their ability to promote barnacle egg hatching.

Material assayed	Hatch response	
Sea water extracted whole barnacles (hatching substance)	+++	
Butanol-extracted hatching substance		
(1) origin of plate run in System I	+++	(+2077)
(2) major band of plate run in System II	++	(+ 318)
(3) active bands (2) of plate run in System III	++	(+ 439)*
Sea water-extracted Synton 706	+++	(+ 879)
Ethylacetate-extracted Synton 706		
(1) active region of plate run in System II	++	(+ 657)
(2) active band of plate in System III	++	(+ 381)

+++ complete hatch

++ significant hatch

NB: The numbers in parentheses indicate the difference in number of eggs hatched in the test solution from that of the control.

* Numbers of eggs hatched by the two bands are combined

TABLE 2. Rf values (x100) of bands derived from the origin of a system I plate when developed in solvent system II.

Rf (x100)
0.0
9.9
13.0
15.7
22.9
34.6
39.6
41.8
44.1
52.7
56.5
79.4

was delineated by Rf (x 100) values of 36 and 45. This region was eluted and developed in a third solvent system, chloroform/methanol/ 28% ammonium hydroxide (75:25:4 v/v). Solvent system III was designed to further separate the bands of the 'active' region on system II plates. Plates were developed in system III for ca. 1 hr 15 min during which time the solvent front migrated ca. 17.5 cm. Characteristically, seven bands could be resolved on system III plates, two of which were active in the bioassay (see Table 3) and had Rf (x 100) values of ca. 18 and 21; neither of these bands U.V.-fluoresced. Clearly, there was an apparent "loss" of two active bands on development in solvent system III. Since the 'active' bands observed on system II plates ran close together, it is possible that there were in fact only two 'active' bands; the apparent activity of the other two being the likely result of contamination.

Bioassay of pharmacologically active lipids

It was evident from the earlier work of Crisp and Spencer (1958), although not stated by these workers, that hatching substance was a pharmacologically active lipid. The results presented above support this view. Vogt (1963) assayed the pharmacological activity of a number of phospholipids and glycolipids on isolated strips of rabbit duodenum. Lysophosphatidic acid was found to be the most active phospholipid tested. Accordingly, lysophosphatidic acid was compared using T.L.C. developed in solvent system II against hatching substance which had been eluted from a system I plate. The lysophosphatidic acid stock solution was resolved into five bands as detected by U.V. absorbance one of which co-ran with the hatching substance region. This band was scraped off the plate and eluted in methanol, then evaporated to dryness under nitrogen and dissolved in U.V.-irradiated filtered sea water. The latter served as the test solution in the bioassay. Similarly, the lysophosphatidic

TABLE 3. Rf values (x100) of bands derived from the 'active' region of a system II plate when developed in solvent system III

Rf (x100)
8.9
17.7
20.8
23.1
47.1

acid stock solution itself was bioassayed. Neither solution promoted egg hatch.

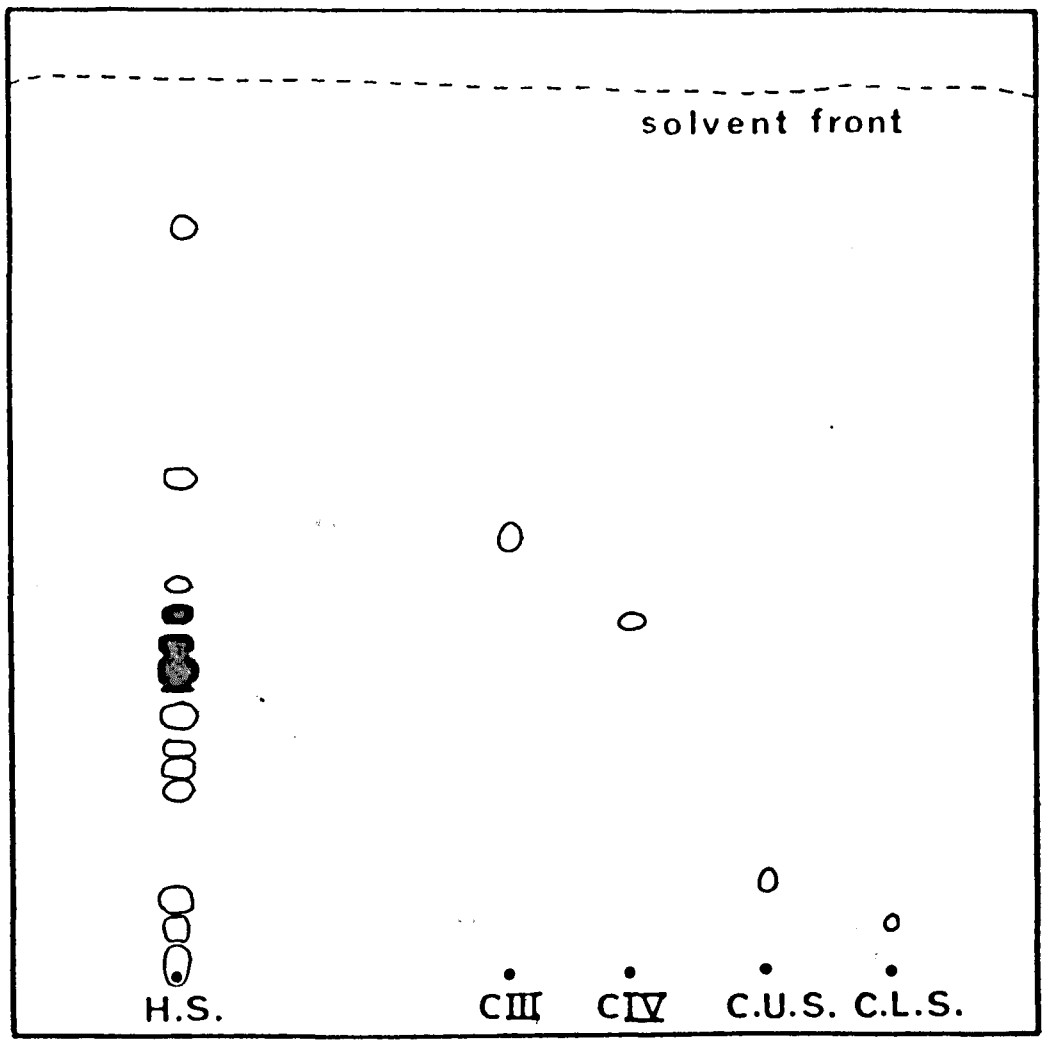
Cerebrosides were also found by Vogt (1963) to be pharmacologically active. Type I and II cerebrosides (Sigma) were, therefore, developed in solvent system II against an extract of hatching substance. In addition type II and IV ceramides were developed on the same plate. Figure 2 clearly shows that the cerebrosides did not migrate on the plate as far as the region which includes hatching substance. The ceramides, on the other hand, are less polar and migrated close to hatching substance. This is particularly true of ceramide type IV. However, ceramide type IV was inactive in the barnacle bioassay.

Influence of moult on the liberation of nauplii

A typical female decapod crustacean broods her eggs externally. The eggs are attached to the pleopods with a cement secreted by glands (Barnes, 1963). There is an obvious danger, therefore, that the eggs could be lost if the ovigerous female should moult. Thus in some crustacea the intermoult period is extended whilst the young are attached, with moulting occurring soon after the eggs have hatched (Patel & Crisp, 1961).

In cirripedes the eggs are brooded outside the body, but within the mantle cavity. The eggs of balanomorphs lie freely, but are moulded to the contours of the mantle cavity. Most lepadomorphs on the other hand possess structures termed ovigerous fraena, which are extensions of the inner mantle. The tips of the fraena possess setae over which a proteinaceous material is secreted and which serves to adhere the egg masses to the fraena (Walker, 1983). Crisp (1956) observed that moulting

Fig. 2. Comparison of the mobilities of hatching substance with certain pharmacologically active lipids. The T.L.C. plate was developed in the solvent system chloroform/methanol/glacial acetic acid/water (90 : 9 : 1 : 0.65) and the bands visualized with iodine vapour. The solid bands are those, which when eluted, are active in promoting egg hatch. H.S., hatching substance; CIII, ceramide type III; CIV, ceramide type IV; C.M.S., cerebroside type II (upper spot of the cerebroside doublet); C.L.S., cerebroside type I (lower spot of the cerebroside doudlet).


solvent front
H.S.
CIII
CIV
C.U.S.
C.L.S.

can occur in gravid B. balanoides. However, this barnacle seldom moults during the winter months. It does resume moulting sometime between late December and February after a period of anecdysis (cf. Crisp, 1957). The moulting rate slowly increases in February and March, as the eggs mature, to reach its normal rate in May. Most important to the present study was the finding of Patel (1959) and Patel and Crisp (1961) that the liberation of nauplii was accompanied by the moult. It was then suggested that the process of moulting and liberation are linked (Patel & Crisp, 1961). Tighe-Ford (1974) took this idea further and tested the arthropod moulting hormone, 20-hydroxyecdysone, for hatching substance activity on gravid B. balanoides (see Chapter 1, section 4). The results, however, were negative.

Tighe-Ford's experiment was modified in the present study so that instead of injecting the adult barnacle with moulting hormone a solution was applied directly to an isolated egg mass as described in the bioassay procedure (see above). Ecdysone and 20-hydroxyecdysone were prepared to a concentration of 4 µg/ml in 5% ethanol in U.V.-irradiated filtered sea water and filtered sea water respectively. The solutions were serially diluted and assayed. 20-hydroxyecdysone was found to be inactive in the bioassay, thus supporting the results of Tighe-Ford (1974). In contrast, the solutions of ecdysone stimulated the eggs to hatch, but so also did the aqueous ethanol controls. When subsequently tested, aqueous suspensions of ecdysone were inactive. It therefore appears that neither of the moulting hormones are the hatching substance. Ethanol is also not the hatching substance. Although 5% ethanol promoted a significant hatch (ca. 25-50%) the process did not compare with that promoted by true hatching substance. Hatching not only took much longer to commence (20-35 min) but was a more gradual process.

Following ecdysis the new cuticle is tanned (see Chapter 1, section 1). It was thought possible, therefore, that tyrosine or one of its metabolites (c.f. Chapter 1, section 1A) may be involved in the hatching process. Significantly, Crisp and Spencer (1958) showed that the enrichment of a barnacle macerate with 10^{-3}M tyrosine enhanced the hatching activity of such extracts.

The present study has confirmed that tyrosine is itself not the hatching substance. A 10^{-3}M sea water solution of tyrosine was inactive in the bioassay as was a derivative of tyrosine, 3,4-dihydroxyphenylalanine (DOPA) at the same concentration.

Another tyrosine derivative, dopamine (as the hydrochloride) was initially tested at a concentration of ca. 5.3×10^{-5}M and was found to mimic exactly the effects of hatching substance. The range of concentrations over which dopamine is effective was determined by assaying solutions which had been prepared by serially diluting a 10^{-2}M stock solution (Table 4). Eggs were found to hatch in dopamine solutions between the concentrations of 10^{-2}M and 9.75×10^{-6}M. However, the top two concentrations used were toxic to the hatched nauplii. Furthermore, below the concentration of 1.56×10^{-4}M, dopamine became progressively less effective in inducing eggs to hatch, until at the concentration of 4.88×10^{-6}M no hatching was observed.

As previously mentioned, hatching substance was partially characterized by Crisp and Spencer (1958). In order to ascertain if dopamine was the hatching substance some of the tests employed by Crisp and Spencer were repeated with solutions of dopamine. First a 5×10^{-3}M solution of dopamine was acidified to pH 2.6 with INHCl. After a period of 15 min at this pH the solution was adjusted to pH 8.0 with INNaOH and then bioassayed. The control and the test solutions were made equimolar by

TABLE 4. Activity of serially diluted solutions of dopamine on isolated egg masses

concentration of dopamine (M)	activity	hatch	behaviour of hatched nauplii
1.0×10^{-2}	+	complete	abnormal
5.0×10^{-3}	+	complete	abnormal
2.5×10^{-3}	+	complete	normal
1.25×10^{-3}	+	complete	normal
6.25×10^{-4}	+	complete	normal
3.13×10^{-4}	+	complete	normal
1.56×10^{-4}	+	complete	normal
7.81×10^{-5}	+	incomplete	normal
3.91×10^{-5}	+	incomplete	normal
1.95×10^{-5}	+	incomplete	normal
9.75×10^{-6}	+	incomplete	normal
4.88×10^{-6}	-	•	•

adding sodium chloride to the former. In contrast to acid-treated hatching substance, solutions of dopamine which had been so treated retained their activity in the bioassay. Secondly, dopamine in solution was found to be heat stable. In common with hatching substance dopamine displayed no loss of activity when a solution (2.5×10^{-3}M) was heated to 80°C and quickly cooled. According to Crisp and Spencer (1958) the activity of hatching substance is lost after heating in sea water at 100°C for 100 min. Similar treatment of a dopamine solution, however, did not destroy its activity. This result contradicts the results of Berlind (1976) who reported that the muscle stimulating activity of dopamine was destroyed by boiling for 3 min. It is clear, therefore, that dopamine is not the true hatching substance, but it may play a rôle in the hatching process or in the production of hatching substance. These possibilities are assessed in Chapter 8.

N-acetyldopamine, a metabolite of dopamine and the compound necessary for the tanning of arthropod cuticle was bioassayed as a 10^{-3}M sea water solution. The compound did not stimulate eggs to hatch.

The rôle of prostaglandins in the hatching process of Balanus balanoides

Another group of pharmacologically active lipids mentioned by Vogt (1963) are the prostaglandins. These substituted cyclopentance C20 acids (Bergström, 1967) are potent vertebrate smooth muscle stimulators but also have a range of other biological actions (see Hammarström, 1982). The sytematic studies of Christ and Van Dorp (1972) and Nomura and Ogata (1976) suggest that prostaglandins are ubiquitous to the animal kingdom. However, little is known of the rôle of these compounds in the lower phyla.

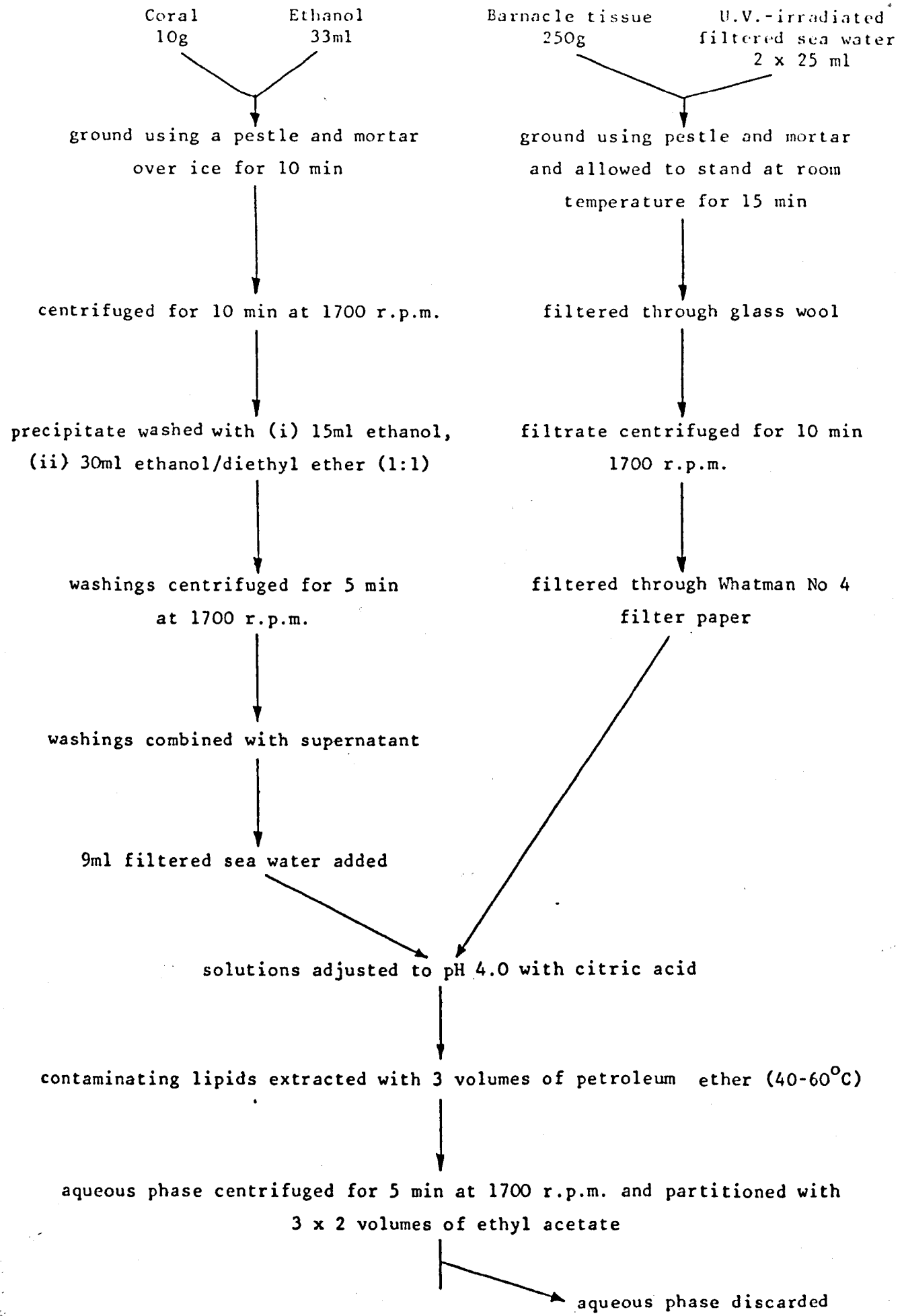
The major marine invertebrate source of prostaglandins is the gorgonian coral, Plexaura homomalla (Weinheimer & Spraggins, 1969). When a crude sample of dried cortex from this coral, known commercially as 'Synton 706' (P-L biochemicals Ltd., Northampton), was extracted with sea water and bioassayed, it promoted hatching of barnacle eggs in a manner identical to that displayed by hatching substance (Table 1).

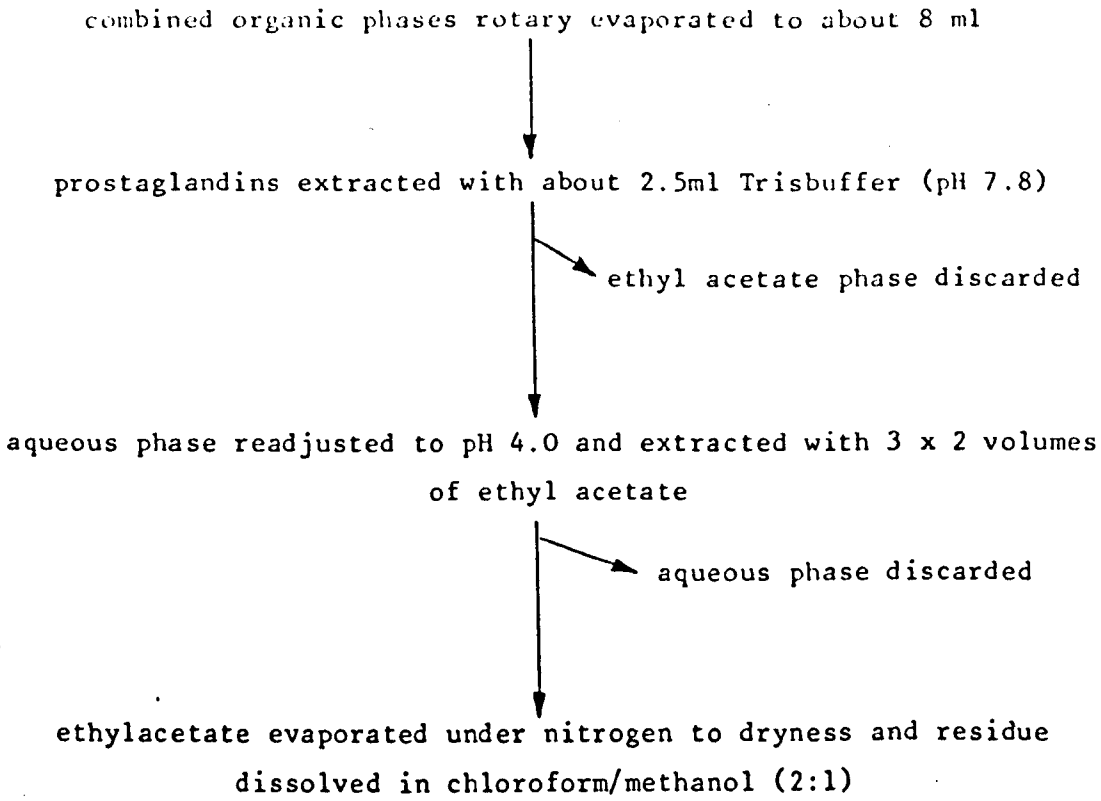
A prostaglandin fraction was extracted from P. homomalla and from whole B. balanoides, the former using the method of Salmon and Karim (1976), the latter by a slightly modified procedure (Fig. 3). The final ethyl acetate phase obtained from both extracts was a colourless solution. It was evaporated to dryness under a stream of nitrogen and redissolved in sea water prior to bioassay. The extracts from the coral were active although not to the same extent as the sea water extracted material (Table 1), as was the case for barnacle extracts.

Thin layer chromatography was then employed to separate and concentrate these fractions. The same three solvent systems which have been previously described were used for this purpose.

When the barnacle and coral extracts were developed in solvent system I, all the hatching activity was located at the origins. Elution followed by development in solvent system II did not give consistent results for any of the extracts, in terms of the number of U.V. (short wavelength) bands obtained. This may have been due to variation in the amounts of extracts used in each run. The coral extract exhibited a minimum of twelve U.V.-absorbing bands with an "active" region (Rf (x 100) values 35-43) containing four of these bands; one of these also fluoresced under long wavelength U.V. light.

Fig. 3. Flow diagram of the ethyl acetate extraction procedure used to obtain a prostaglandin fraction from tissues of *B.balanoides* and *Plexaura homomalla*.


Coral
10g
Ethanol
33ml
Barnacle tissue
250g
U.V.-irradiated
filtered sea water
2 x 25 ml
ground using a pestle and mortar over ice for 10 min
ground using pestle and mortar and allowed to stand at room temperature for 15 min
centrifuged for 10 min at 1700 r.p.m.
filtered through glass wool
precipitate washed with (i) 15ml ethanol, (ii) 30ml ethanol/diethyl ether (1:1)
filtrate centrifuged for 10 min 1700 r.p.m.
washings centrifuged for 5 min at 1700 r.p.m.
filtered through Whatman No 4 filter paper
washings combined with supernatant
9ml filtered sea water added
solutions adjusted to pH 4.0 with citric acid
contaminating lipids extracted with 3 volumes of petroleum ether (40-60°C)
aqueous phase centrifuged for 5 min at 1700 r.p.m. and partitioned with 3 x 2 volumes of ethyl acetate
aqueous phase discarded


combined organic phases rotary evaporated to about 8 ml
prostaglandins extracted with about 2.5ml Trisbuffer (pH 7.8)
ethyl acetate phase discarded
aqueous phase readjusted to pH 4.0 and extracted with 3 x 2 volumes of ethyl acetate
aqueous phase discarded
ethylacetate evaporated under nitrogen to dryness and residue dissolved in chloroform/methanol (2:1)

The barnacle prostaglandin extract gave T.L.C. results similar to those following butanol extraction. Although there were more U.V.-absorbing bands, most of those observed shared similar Rf values to those obtained after butanol extraction (Table 5). As for the coral extract, there were four U.V.-absorbing bands corresponding to the "active" region of the barnacle extract, one of which also U.V.-fluoresced. The region was delineated by the Rf (x 100) values of 35 and 43.

Following the development of the coral extract in solvent system III, the bulk of activity was restricted to a single band with the Rf (x 100) value of ca. 18. In addition, a band of Rf (x 100) value ca. 24 showed slight activity.

The barnacle extract, however, exhibited two very active bands with Rf (x 100) values of ca. 18 and 21. It is significant that the coral active band co-ran with one of the barnacle active bands (see Chapter 8). This, together with the fact that egg hatch is promoted by a prostaglandin fraction from both B. balanoides and P. homomalla is good evidence to suggest that hatching substance is a prostaglandin or a related compound. Unfortunately, comprehensive data of the extractable prostaglandins from this gorgonian, together with their T.L.C. characteristics, are not available. Thus the identification of the coral active fraction is not possible at present and must await the results of gas liquid chromatography (G.L.C.) and mass spectroscopy (M.S.) analysis.

Preliminary results have been obtained by GLC/MS to indicate that barnacle hatching substance is a prostaglandin with a PGF-like structure. The active fraction which was analyzed was that of a butanol extract eluted from a system III plate.

TABLE 5. Rf values (x100) of ethyl acetate-extracted 'hatching substance' developed in solvent system II

Rf (x100)
8.4
12.9
15.3
20.0
26.6
29.4
31.3
36.3
38.0
39.7
42.4
75.6

Acid lability of sea water extracted 'Synton 706'

Evidence has been presented above to suggest that a coral fraction - henceforth called 'coral active fraction' - has hatching substance-like properties and indeed may be the same compound. To further test this hypothesis, its stability to acid was tested.

'Synton 706' was dissolved in U.V.-irradiated, filtered sea water to a concentration of 25 mg/ml. The solution was adjusted to pH 2.75 by adding INHCl and held at this level for 15 min. The pH was then re-adjusted to 7.95 with INNaOH and the solution stored at 4°C for 2 hr. prior to its bioassay. The control solution employed was one in which chloride ions had been added in the form of sodium chloride to achieve the same final concentration as for the test solution. Extracts treated in this manner were not active in inducing eggs to hatch. Moreover, this inactivation was irreversible since activity was not restored if an extract was re-adjusted to an alkaline pH. It is, therefore, clear that hatching substance and the coral active fraction are acid labile, so providing further evidence that they are the same or closely related compounds.

Effect of aspirin and indomethacin on the formation of hatching substance

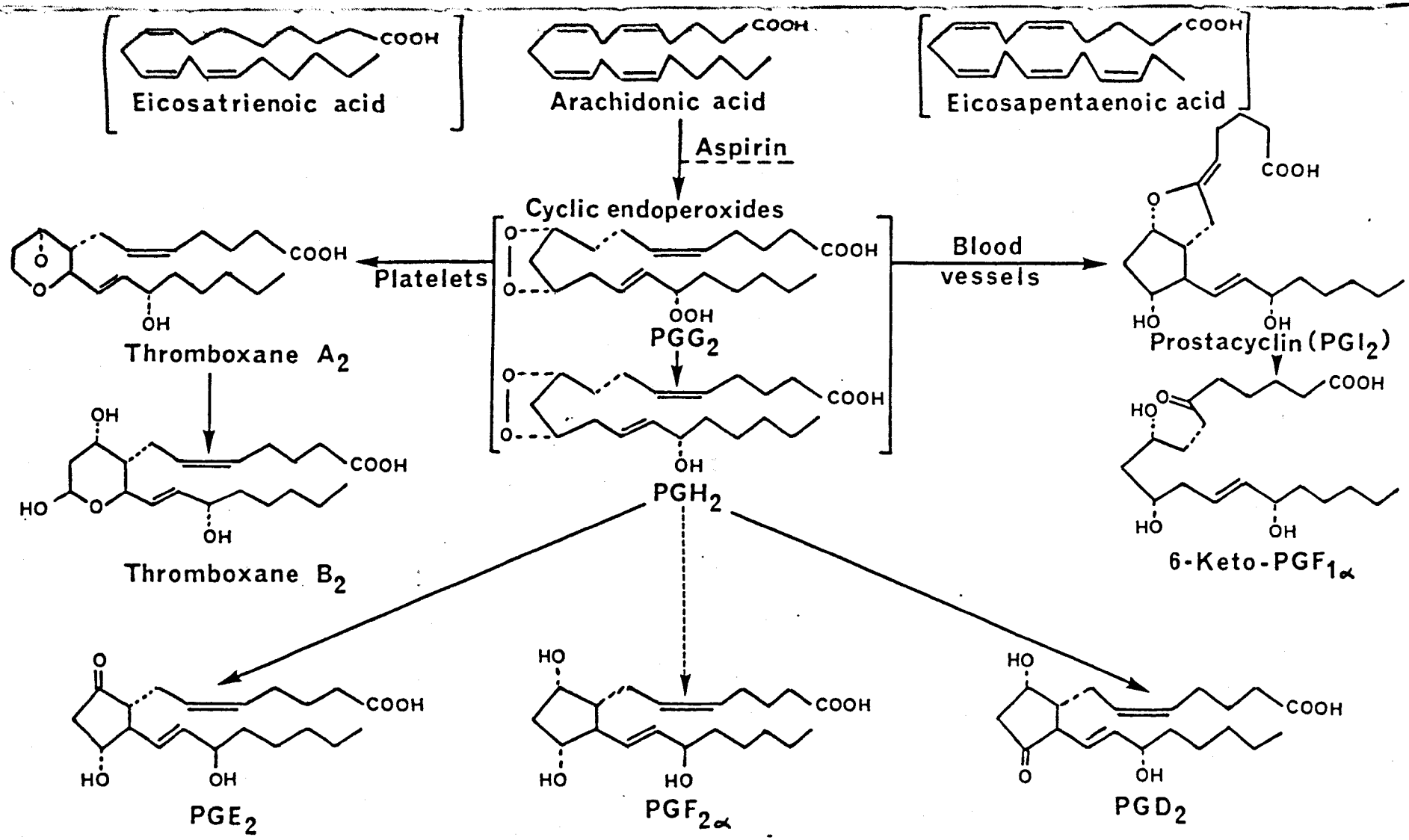
Prostaglandins are synthesized by the cell from long-chain poly-unsaturated carboxylic acids (Newton & Roberts, 1982). The synthesis of the 2-series prostaglandins from arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid) is the best known of these synthetic pathways, although the biosynthesis of the 1-series and 3-series prostaglandins from dihomo-8-linolenic acid and 5, 8, 11, 14, 17-eicosapentaenoic acid respectively is believed to be by analogous pathways (Gibson, 1982).

Arachidonic acid is stored to a large extent in the phospholipids of membranes in the form of various esters. Apparently, the phospholipid lecithin (phosphatidylcholine) is the major source of free arachidonic acid in mammalian systems. Phospholipase A_2 enzymes catalyze the release of arachidonic acid from the 2-position of lecithin and these enzymes are released in response to a range of physical and chemical stimuli (Gibson, 1982). The free arachidonic acid can then enter one of several pathways, depending on the tissue type, which culminate in a variety of eicosanoids (term introduced by Corey et al., 1980) (see Fig. 4). The first step in the pathway to the primary prostaglandins is the oxidation of arachidonic acid to the prostaglandin endoperoxides (PGG_2 and PGH_2). This conversion is metabolized by the fatty acid cyclo-oxygenase which is associated with the microsomal fraction of most animal cells (Vane et al., 1982).

The non-steroidal anti-inflammatory drugs, aspirin and indomethacin were reported simultaneously to irreversibly inhibit cyclooxygenase (Vane, 1971; Ferreira et al., 1971; Smith & Willis, 1971) and thus the biosynthesis of prostaglandins. In order to obtain further evidence that hatching subtance is a prostaglandin the ability of aspirin and indomethacin to inhibit its formation was assessed.

A range of concentrations of soluble aspirin BP (content of tablets - acetylsalicylic acid, in fine powder, 300 mg; anhydrous citric acid, 30 mg; calcium carbonate, 100 mg; saccharin sodium, 3 mg - British Pharmacopoeia), namely 0.45mM, 1.0mM, 5.0mM and 10.0mM, were prepared in U.V.-irradiated filtered sea water. Two bodies of adult B. balanoides were homogenized in each concentration and left to stand at room temperature for 15 min. Similar control barnacle extracts were prepared, without

Fig. 4. Biosynthetic pathways of several eicosanoids. Note the leukotrienes are omitted from this scheme.


Eicosatrienoic acid
Arachidonic acid
Eicosapentaenoic acid
Aspirin
Cyclic endoperoxides
Platelets
Blood vessels
Thromboxane A_2
Thromboxane B_2
PGG_2
PGH_2
Prostacyclin (PGI_2)
6-Keto-$PGF_{1\alpha}$
PGE_2
$PGF_{2\alpha}$
PGD_2

aspirin, in filtered sea water. All solutions were tested for hatching activity in the bioassay system.

As Table 6 shows, the 5 and 10mM aspirin solutions were inhibitory. It should be noted that the few larvae which did hatch in these concentrations became moribund within the 15 min test period. This small hatch, together with the fact that egg masses which had been bioassayed in the aspirin solutions, when well washed in U.V.-irradiated, filtered sea water were capable of hatching when hatching substance was added, shows the inhibitory effect of aspirin is not due to the embryos being incapacitated or killed.

The possibility remained, however, that the above result was not due to an inhibitory effect of aspirin on the formation of hatching substance, but instead was because aspirin destroyed hatching substance after it had formed. To test this possibility extracts were prepared as described above, but aspirin was added 15 min after tissue maceration, by which time hatching substance would have been formed. Such solutions displayed no loss in activity when bioassayed, thus demonstrating that aspirin does not destroy or modify hatching substance itself. Another very important point shown by this experiment is that hatching is not due to the activation of intramural synthesis of prostaglandins by the embryo.

In view of the instability of hatching substance below pH 3 (Crisp & Spencer, 1958) the pH of aspirin solutions was measured and in all cases was found to be well above this level (e.g. pH 4.85 for a 5mM solution). It is clear therefore that aspirin would have had no effect on the activity of hatching substance on the basis of pH alone.

The inhibition of hatching substance formation by aspirin may appear,

TABLE 6. Effect of aspirin on the biosynthesis of hatching substance

aspirin concentration mM	hatch	hatch in control
0.45	+++	+++
1.0	+++	+++
5.0	+	+++
10.0	-	+++

+++ Complete hatch

\+ Negligible hatch

\- No hatch

therefore, as unequivocal evidence that hatching substance is a prostaglandin. However, the possibility that other enzyme systems could also be affected by aspirin must not be ignored.

The soluble aspirin used in the above experiments contained 300 mg of acetylsalicylic acid per 433 mg tablet. The actual concentration of acetylsalycilate which actively inhibits hatching substance formation is not 5mM, therefore, but 11mM. However, when aspirin is added to the barnacle homogenate, most will be bound by protein (c.f. Smith & Dawkins, 1971). In man, for a total plasma salicylate concentration of 5mM, only 30% is in the free form. Consequently, aspirin available for binding to the cyclooxygenase will be much less than 11mM. In addition much of the acetylsalicylate administered is rapidly hydrolyzed. Since acetylation of proteins, other than cyclooxygenase, by acetylsalicylate occurs over hours rather than minutes, the 15 minute incubation period used in the present study was probably too short for the function of other proteins to be affected.

The concentration range over which acetylsalicylate inhibits prostaglandin synthesis has been reported to be as large as 35 μM to 15mM; the effective concentration varies with the enzyme preparation used (Flower, 1974). The effective concentration of acetylsalicylate used in the present study was within this range. This result was achieved using a crude tissue homogenate, but microsomal preparations of tissue homogenates have been used in most other studies. This is because the synthetases are located in this fraction and also because such preparations have a high specific activity (Flower, 1974). Thus the values reported in the literature can be expected to be lower than those obtained for the barnacle preparation.

It is reasonable to propose, therefore, that at the levels of acetylsalicylate and with the incubation time used in the present study, enzyme systems other than cyclo-oxygenase are unlikely to have been affected to any significant extent.

A 10mM stock solution of indomethacin (Sigma) was prepared in U.V.-irradiated filtered sea water. Samples of this stock solution were diluted to 5mM and 1mM with filtered sea water and all the solutions tested for their ability to inhibit the formation of hatching substance. 2 ml of each solution were used as the homogenizing medium for two whole B. balanoides which had been removed from their substrate immediately prior to being used. After the animals had been ground up, the homogenates were left to stand at room temperature for 15 min prior to bioassay. Each bioassay employed a sea water control against which the hatch within the test solution was compared. Eggs were found to be fully capable of hatching in all three concentrations of indomethacin. However, both the 5mM and the 10mM solutions were toxic to the hatched nauplii. Nauplii in the 10mM solution became moribund immediately after they had hatched. The 5mM solution was slightly less toxic requiring several minutes to affect the nauplii. The hatch observed in a 5mM solution appeared to be roughly equivalent to that normally elicited by a hatching substance solution. To test this the hatching substance prepared in the 5mM solution was assayed against hatching substance prepared without indomethacin and the number of eggs hatched in each solution counted. It was found that the hatches were indeed similar, a representative set of figures being a 70.4% hatch in the test solution and a 80.5% hatch in the control solution.

The inability of indomethacin to inhibit prostaglandin biosynthesis in barnacles is in marked contrast to the situation in vertebrates where,

with the exception of rat skin homogenates (McDonald-Gibson, 1972) indomethacin has been reported to be more potent than aspirin in inhibiting cyclo-oxygenase (Vane, 1971; Flower, 1974; Vane et al., 1982). In view of the recent report by Curry et al. (1982) regarding the stability of indomethacin solutions, it should be noted that the solutions used in the present study were freshly prepared by adding sea water (pH 7.85) to the indomethacin powder. In this way the pitfalls outlined by Curry et al. (ibid) have been avoided and it is highly likely that the indomethacin solutions used were stable. It would appear, therefore, that the inactivity of indomethacin in the bioassay is a property of the cyclo-oxygenase which catalyzes the biosynthesis of hatching substance.

Comparable results have been reported for other invertebrate systems. The prostaglandin synthetases of Haliotus rufescens (Morse et al., 1977), Plexaura homomalla (Corey et al., 1973, 1975) and Acheta domesticus (Destephano et al., 1976) were all unaffected by indomethacin. A notable exception is the prostaglandin synthetase of Modiolus demissus which was inhibited by a 1mM indomethacin solution (Freas & Grollman, 1980). A possible explanation for these discrepancies is that the sensitivity of cyclo-oxygenase to indomethacin varies from organism to organism. Indeed in mammalian systems there are differences in the effects of non-steroidal anti-inflammatory drugs from tissue to tissue together with a variable sensitivity of cyclo-oxygenase to inhibition by acetylsalicylate in different tissues. As a result Ferreira and Vane (1974) proposed that the enzyme exists in multiple molecular forms or isozymes. The variable effect of indomethacin in invertebrates may be yet another example of this phenomenon.

Identification of the barnacle and coral active fractions by T.L.C.

Having obtained evidence to suggest that barnacle hatching substance

and the coral active fraction are prostaglandins, a more specific identification of these fractions was sought using T.L.C.

A sample of the prostaglandin fraction, obtained from barnacle tissues, was run on a silica gel plate in the solvent system, chloroform/methanol/glacial acetic acid (90:5:5) (system CI of Daniels, 1976). The developed plate was air dried then heated to 120°C and immediately dipped into a 1% solution of antimony pentachloride in carbon tetrachloride-ethylene chloride (6:1) to visualize the bands (Shaw & Ramwell, 1969). The prostaglandin fraction was well resolved by this solvent system. Table 7 shows the mobility of the various bands separated by system CI. Several of the bands had Rf (x 100) values very close to those published for primary prostaglandins (c.f. Daniels, 1976). Most notable of these are the bands with Rf's (x 100) of 16 and 53. $PGF_{1\alpha}$ and $PGF_{2\alpha}$ both have an Rf (x 100) of 16 and PGA_1, PGA_2, PGB_1 and PGB_2 all have an Rf (x 100) of 55. Only three of the extract bands gave colours, on staining, which could be associated with those obtained for prostaglandins. The band with an Rf (x 100) of 7 stained yellow which is associated with PGB compounds and the bands with Rf's (x 100) of 20 and 21 both stained red, this colour being associated with PGF compounds. The staining and mobility results are, therefore, incompatible with regard to primary prostaglandins.

The active region of prostaglandin-extracted coral, eluted from a plate run in solvent system II was applied to a re-activated, ferric chloride impregnated, silica gel plate and developed in the solvent system, ethyl acetate/acetic acid/hexane (30:1:19) (Wickramasinghe & Shaw, 1973). In order to detect the bands, the plate was sprayed with a 10% solution of dodeca-molybdophosphoric acid in ethanol, followed by heating the plate in an oven at 100°C (Green & Samuelsson, 1964). This method resolved the coral active region into six bands. All of these bands

TABLE 7. Rf values (x100) of bands derived from an ethylacetate-extracted barnacle sample

Rf (x100)	Published Rf (x100) values[a]	Compound
7	10	$PGF_{1\beta}$, $PGF_{2\beta}$
16	16	$PGF_{1\alpha}$, $PGF_{2\alpha}$
20		
21		
26	31	PGE_1, PGE_2
48	47	$MePGE_1$, $MePGE_2$
53	55	PGA_1, PGA_2, PGB_1, PGB_2
75		

a - Daniels (1976)

were dark blue in colour, which is the characteristic colour reaction of prostaglandins (Wickramasinghe & Shaw, 1973). Two of the bands, with Rf (x 100) values of 12 and 36 (Table 8) have mobilities comparable to those published by Wickramasinghe and Shaw (ibid) for prostaglandins developed in the same T.L.C. system. PGE_1 and PGB_1 have Rf (x 100) values of 10 and 38 respectively.

The single active band from a coral extract developed in solvent system III was eluted from a plate and applied to an impregnated Whatman K5 T.L.C. plate. Impregnation involved dipping the plate for 10 sec in a 10% silver nitrate solution in water/methanol (1:1). The plate was then oven dried at 110°C for 15 min and protected from light during storage. The coral fraction was run against several primary prostaglandins, as markers, in the solvent system chloroform/methanol/glacial acetic acid (90:5:5) for 52 min and to a solvent front distance of 17.7 cm. The spots were detected by spraying with a 10% (w/v) solution of phosphomolybdic acid in ethanol and heating in an oven at 165°C for 30 min. Figure 5 shows the mobilities in this system, together with the mobility of the active coral fraction. It is clear that the coral fraction runs close to $PGF_{2\alpha}$. Since the coral active fraction co-runs with one of the two active bands of barnacle hatching substance it can be presumed that the latter would also have a mobility similar to $PGF_{2\alpha}$.

Identity of the hatching substance

From all the evidence presented above it can be concluded that the barnacle hatching substance is either a prostglandin or prostaglandin-like compound. The exact identity of this compound is, however, still a matter of some conjecture. Preliminary GC/MS data has indicated a compound which is PGF-like in structure. Comparative T.L.C. has supported this data, since the active coral fraction co-ran with $PGF_{2\alpha}$. A comparison

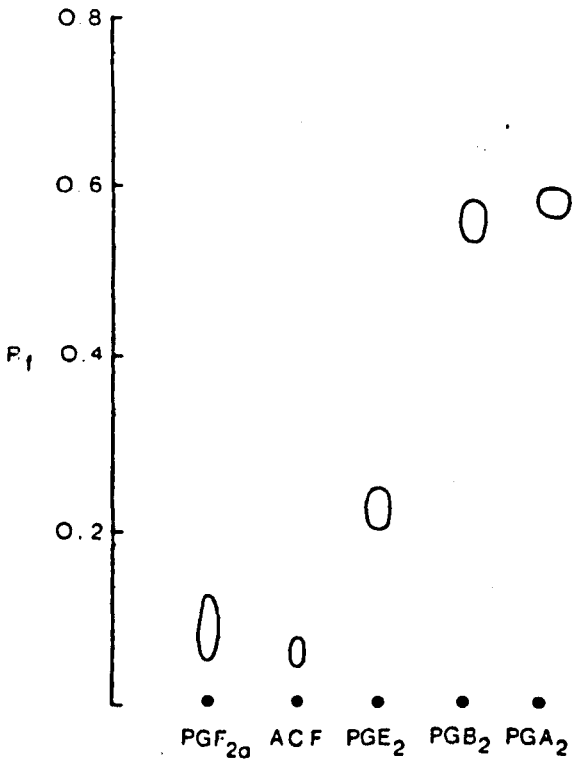
TABLE 8. Rf values (x100) of bands derived from an ethylacetate-extracted coral sample

Rf (x100)	Published Rf (x100) values [b]	Compound
0		
12[a]	10	PGE_1
36	38	PGB_1
70		
79		
90		

a - major band

b - Wickramasinghe & Shaw (1973)

Fig. 5. Thin-layer chromatogram of the coral "active" fraction (C.A.F.) which has been developed against prostaglandin standards on a silver nitrate-impregnated, Whatman K5 T.L.C. plate in the solvent system, chloroform/methanol glacial acetic acid (90 : 5 : 5).


0.8
0.6
0.4
0.2
P_1
$PGF_{2\alpha}$
ACF
PGE_2
PGB_2
PGA_2

of the stability of hatching substance (Crisp & Spencer, 1958) and prostaglandins of the F-series (Karim et al., 1968) in solutions of various pH is also illuminating. Hatching substance, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ all exhibit a loss in smooth muscle stimulating activity if held in solution below pH 3. Furthermore, solutions of these compounds are all stable between pH 5 and 11. Certain other properties, though, are incompatible with hatching substance being an authentic compound of the PGF series. For example, PGF compounds do not absorb U.V. light, whereas hatching substance does. Further, there is little loss in smooth muscle stimulating activity of $PGF_{1\alpha}$ and no loss in activity of $PGF_{2\alpha}$ when heated at 110°C and 10 lb. sq. $inch^{-1}$ for 1 hr (Karim et al., 1968); the activity of hatching substance is destroyed by prolonged heating at 100°C and so presumably would also be destroyed by autoclaving.

It is clear that dopamine and hatching substance are not the same compound. Dopamine has been reported to stimulate the biosynthesis of mammalian prostaglandins, most notably $PGF_{2\alpha}$ (Wolfe et al., 1976a, 1976b), as has its precursor DOPA (Ramwell & Shaw, 1966). Wolfe and co-workers have postulated that biosynthesis is stimulated via the donation by dopamine of hydrogen atoms to the endoperoxide intermediates PGG_2 or PGH_2 or both.

However, in barnacles, dopamine may directly cause embryonic muscle to be stimulated prior to hatching; hatching substance could thereby exert its effect by stimulating the release of dopamine, as a neurotransmitter, in the embryo.

It is now established that prostaglandins are present in a wide range of invertebrates (see Chapter 1, section 5). Furthermore, possible physiological rôles for these compounds have been reported. For example,

the initiation of spawning in Haliotus rufescens (Morse et al., 1977), and acclimation of Modiolus demissus to hypoosmotic stress (Freas & Grollman, 1980) and the control of egg laying in Teleogryllus commodus (Loher et al., 1981). In none of these cases, however, was the active fraction isolated and tested. The present study represents the first isolation of a prostaglandin-like substance from invertebrate tissue with an established function.

CHAPTER 8

Further studies on the control of the hatching process in *Balanus balanoides*(L.)

CHAPTER 8

Further studies on the control of the hatching process in Balanus balanoides (L.)

INTRODUCTION

Several findings have recently been presented (Clare et al., 1982; Chapter 7) regarding the hatching substance of B. balanoides. First, hatching substance is a prostaglandin-like compound; it is released by the parent and not by the egg, in response to feeding. Secondly, a fraction from the cortex of the coral, Plexaura homomalla, mimics the effect of hatching substance. Significantly, this coral fraction co-migrates with hatching substance on a thin-layer chromatography plate. Furthermore, dopamine may be involved in the hatching process, since sea water solutions of the drug promote egg hatch.

The present report presents conclusive evidence that hatching substance is a prostaglandin. Moreover, a tentative molecular structure for the coral active fraction, is given. In addition, the metabolic pathway leading to hatching substance is examined.

Evidence is also presented to show that dopamine is in fact involved in the egg hatching process. Dopamine is released by the embryo consequent to the secretion of hatching substance by the parent.

1. Incubation of (1-^{14}C) arachidonic acid with a barnacle homogenate

Arachidonic acid is the precursor to 2-series prostaglandins and is stored in the form of various esters. For example, the phosphatidyl esters of membrane phospholipids are an important source of arachidonic acid (Gibson, 1982).

Since the prostaglandins of invertebrates found to act as hormones have mainly been of the 2-series (e.g. Loher et al., 1981; Graves & Dietz, 1982) and most important, the main prostaglandin from the coral P. homomalla is of the 2-series (Weinheimer & Spraggins, 1969), it was considered possible that arachidonic acid is the precursor of hatching substance. The following radiotracer experiments were performed to examine this possibility.

1μ Ci (1-^{14}C) arachidonic acid (Amersham International Ltd.) was suspended in 1 ml 0.05M Tris/1M NaCl, titrated to pH 8.0 with 1NHCl (Corey et al., 1973). 25g of a March sample of whole Balanus balanoides, previously stored frozen for 3 months, were allowed to thaw out. The sample was ground using a mortar and pestle after the (1-^{14}C) arachidonic acid solution, made up to 25 ml with buffer, was added. The incubation mixture stood at room temperature for 1hr before being filtered through polyester fibre. Later experiments employed only a 15 min incubation period. The biosynthetic process was quenched by adding an equal volume of acetone. Acetone had the effect of precipitating proteins, as noted in the previous chapter. The filtrate was then centrifuged for 10 min at 1600 r.p.m. and the acetone removed in a rotary evaporator. An equal volume of butanol was then added to the aqueous fraction and the mixture separated in a partitioning funnel. The butanol phase, containing hatching substance (Crisp & Spencer, 1958) was rotary evaporated to dryness. 1 ml of chloroform ('AnalaR') was added to the sample and then evaporated to about 100 μl under nitrogen. The sample was spotted on a thin layer chromatography (T.L.C.) plate (10 x 20 cm or 20 x 20 cm MERCK pre-coated silica gel 60F 254) in solvent system I (see Chapter 7). The developed plate was scanned for radioactivity on a Berthold thin-layer scanner II incorporating a 2π-counter and an autochron-XY-recorder. A servoscribe

RE 511.20 potentiometric recorder (Camlab, Cambridge) was attached to obtain radio-chromatograms.

Figure 1 shows a radiochromatogram of a plate developed in solvent system I. Clearly, little activity has migrated from the origin. This is consistent with the fact that hatching substance does not migrate from the origin in this particular solvent system (Clare et al., 1982).

The origin from the system I plate was scraped off and eluted 3 times with methanol ('AnalaR'). The methanol was evaporated off under nitrogen and the residue dissolved in chloroform. The latter was applied to a silica gel-T.L.C. plate and developed in solvent system II (see Chapter 7). Figure 2 is a stroke diagram derived from such a system II plate. The radioactivity has migrated from the origin and is concentrated in two discrete regions. The plate was also examined under short wavelength ultraviolet (U.V.) light and the Rf values of the bands calculated (Table 1). It was found that the upper radioactive region contained 4 bands with Rf (x 100) values very similar to those obtained for those bands which when eluted from a system II plate are active in hatching barnacle eggs (c.f. Chapter 7, Clare et al., 1982). In other words, the label from (1-^{14}C) arachidonic acid is incorporated into hatching substance. It should be noted at this point that (1-^{14}C) arachidonic acid has an Rf (x 100) value of 55 when developed in solvent system II. Arachidonic acid is thus metabolized in the barnacle homogenate.

Finally, the upper labelled region was scraped from the plate and eluted with methanol. The methanol was evaporated to dryness and the residue dissolved in chloroform prior to applying it to a T.L.C. plate which was then developed in solvent system III (see Chapter 7). Figure 3 shows a stroke diagram of such a system III plate. The radioactivity is confined to a discrete region on the plate. When the plate was

Fig. 1. Radiochromatogram of a T.L.C. plate upon which a barnacle homogenate, which has been incubated with ($1^{-14}C$) arachidonic acid and butanol-extracted, has been developed in solvent system I.

ORIGIN

Fig. 2. Radiochromatogram of a T.L.C. system II plate upon which the origin from an arachidonic acid radiolabelled system I plate had been developed.

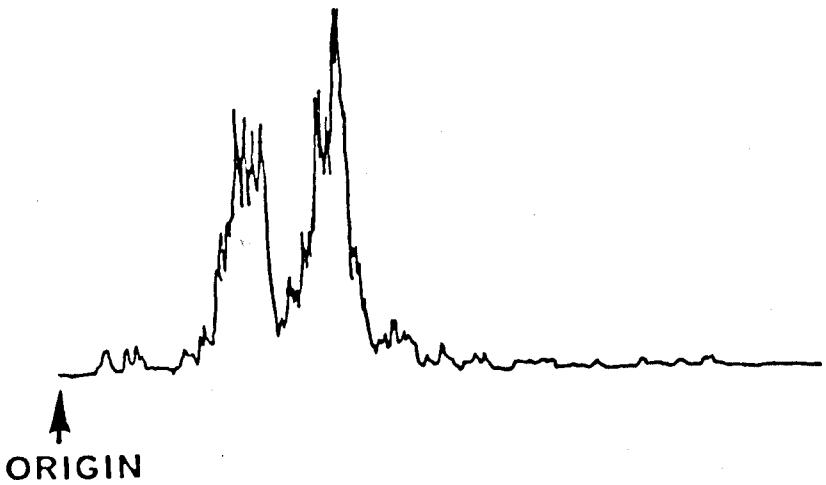
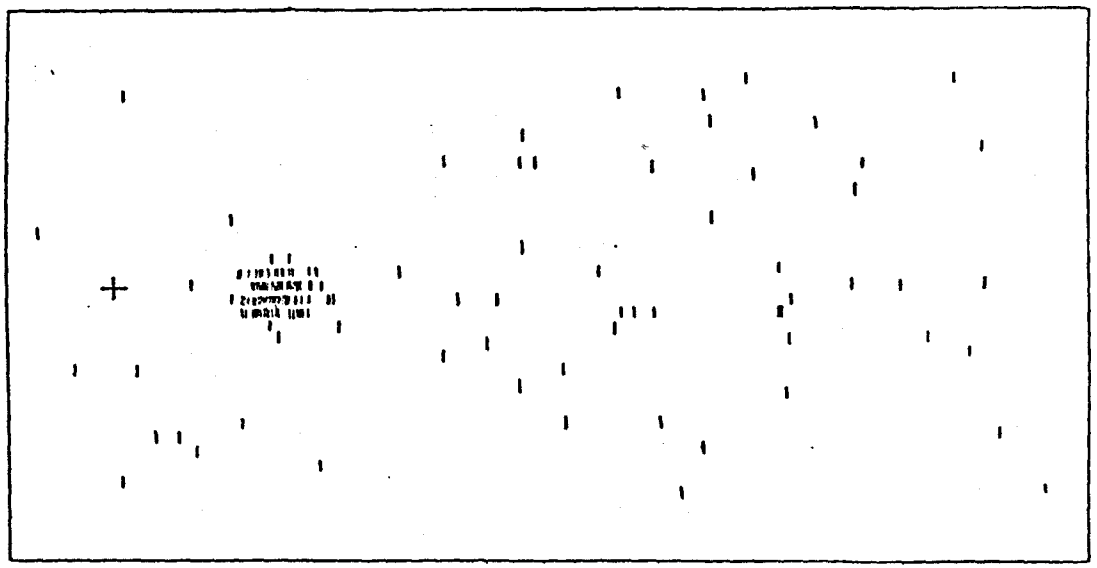

ORIGIN

TABLE 1. Rf (x100) values derived from an aqueous extract of B.balanoides spiked with (1- ^{14}C) arachidonic acid which has been butanol-extracted and developed in T.L.C. system II. The bands within the boxed area are those bands which were eluted and developed on a system III T.L.C. plate.

Rf (x100)
3.4
7.6
11.0
15.3
20.8
24.3
29.9
33.0
37.3
40.6
43.3
48.0
50.8
53.4
56.0
76.7

Fig. 3. Radiochromatogram of a T.L.C. system III plate upon which the hatching substance region of a system II plate has been developed.



examined under short wavelength U.V. light, 9 U.V.-absorbing bands were observed (Table 2). The band shown in figure 3 has an Rf (x 100) value of 17.05, which corresponds with one of these U.V.-absorbing bands (Table 2). However, the radioactive region encompasses three U.V.-absorbing bands with Rf (x 100) values of 14.35, 16.90 and 18.89. Significantly, barnacle hatching substance occurs as two bands on a system III plate. Moreover, these bands run in the same region of the plate as that labelled by (1-^{14}C) arachidonic acid (c.f. Chapter 7). Thus it would appear that the label from (1-^{14}C) arachidonic acid does transfer to hatching substance.

2. Incubation of (2-^{14}C) eicosa-8, 11, 14-trienoic acid with a barnacle homogenate

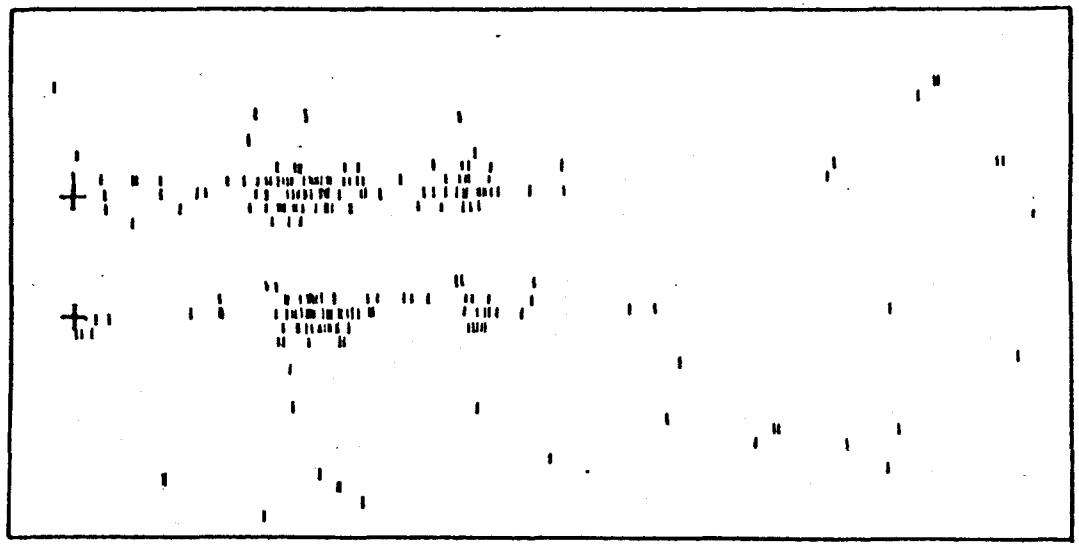
In mammalian systems eicosatrienoic acids (20:3ω6) are the precursors to 1-series prostaglandins. However, these metabolites are of minor importance since eicosatrienoic acids are rapidly converted into arachidonic acid (Wolfe, 1982). Significantly the prostaglandin synthetase of P. homomalla can also utilize eicosa-8, 11, 14-trienoic acid as a substrate (Corey et al., 1973). Accordingly the possibility was examined that radioactive eicosatrienoic acid could label hatching substance.

25g B. balanoides were homogenized in 25 ml Tris buffer (pH 8.0) containing 1u Ci (2-^{14}C) eicosa-8, 11, 14-trienoic acid. The mixture was incubated at room temperature for 15 min. Hatching substance was extracted from the homogenate as described previously for the(1-^{14}C) arachidonic acid experiment. When the extract was developed on a T.L.C. plate in solvent system I all the radioactivity was found at the origin. The origin was eluted in methanol and developed in solvent system II. Figure 4 is a stroke diagram of a system II plate. Two radioactive regions

TABLE 2. Rf (x100) values of bands derived from the upper radioactive region of a system II T.L.C. plate when developed in solvent system III.

Rf (x100)
8.0
10.9
14.3
16.9
18.9
21.6
42.1
58.7
85.7

Fig. 4. Radiochromatogram of a system II T.L.C. plate upon which the origin from a system I plate has been developed. In this case a barnacle homogenate was incubated with (2- ^{14}C) eicosa -8, 11, 14 - trienoic acid prior to butanol-extraction and thin layer chromatography.



occur on the plate as was the case with (1-^{14}C) arachidonic acid. The similarity extends to the mobility of the radioactive regions (c.f. Fig. 2). The lower region did not contain any U.V.-absorbing bands, but the upper region contained two, with Rf (x 100) values of 43.08 and 45.76. It will be noted that these values are greater than those obtained for hatching substance. A region, bounded by the Rf (x 100) values of 38.14 and 53.39, was scraped off the plate and eluted with methanol. When applied to a T.L.C. plate and developed in solvent system III, six U.V.-absorbing bands were obtained. The Rf (x 100) values of these bands are given in Table 3. A low level of radioactivity was concentrated in a region of Rf (x 100) 17.23 (Fig. 5). This radioactive region contained only one U.V.-absorbing band with an Rf (x 100) of 17.09. This value is very close to that obtained for one of the hatching substance bands (c.f. Chapter 7).

Therefore, it would appear that barnacle tissues, as well as mammalian tissues, are able to transform eicosa-8, 11, 14-trienoic acid into arachidonic acid and in turn use arachidonic acid for the synthesis of prostaglandins.

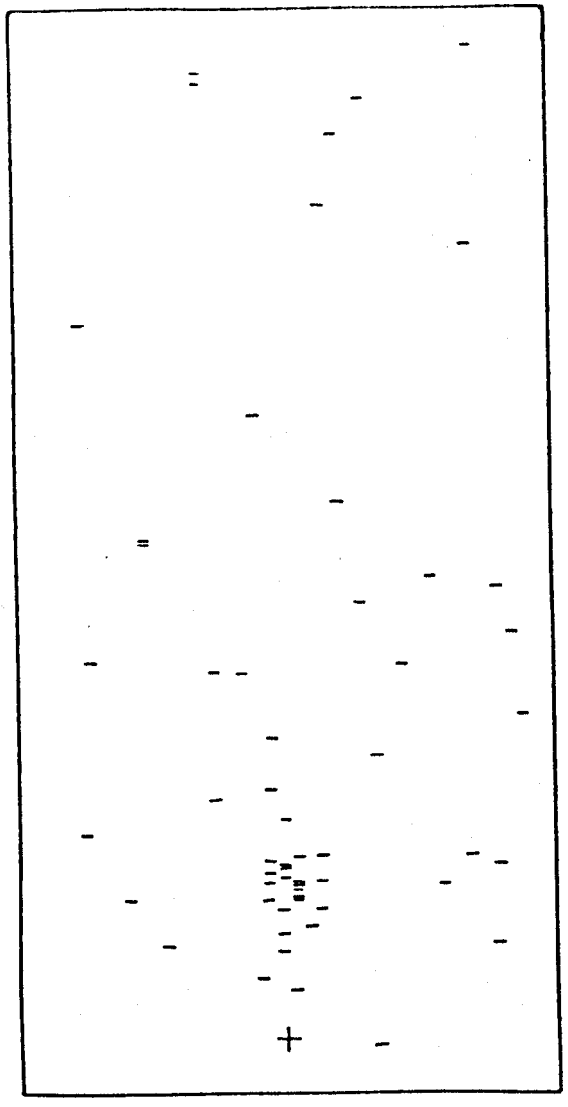
3. Incubation of (1-^{14}C) oleic acid with a barnacle homogenate

The possibility remained that both (1-^{14}C) arachidonic acid and (2-^{14}C) eicosa-8, 11, 14-trienoic acid were broken down in the barnacle homogenate to acetate units (C_2). The radioactive label may then have been incorporated into hatching substance by the synthesis of the latter from labelled acetate. Oleic acid is an ω-9 unsaturated fatty acid. As there can be no interconversions between omega series, the ^{14}C label from oleic acid should not be incorporated into 2-series prostaglandins, unless the oleic acid is broken down into smaller carbon units, for

TABLE 3. Rf (x100) values of bands separated from an eluate of a system II plate when developed in solvent system III. The radioactive origin of a system I plate had been developed on the system II plate, and this in turn had been derived from an aqueous homogenate of B.balanoides which had been incubated with (2- ^{14}C) eicosa -8,11,14-trienoic acid .

Rf (x100)
9.2
17.1
27.7
44.8
74.9
86.2

Fig. 5. Radiochromatogram of a system III plate upon which the upper (2- ^{14}C) - labelled region of the plate shown in figure 4 has been developed.



example acetate, and these units are reassembled into the prostaglandins.

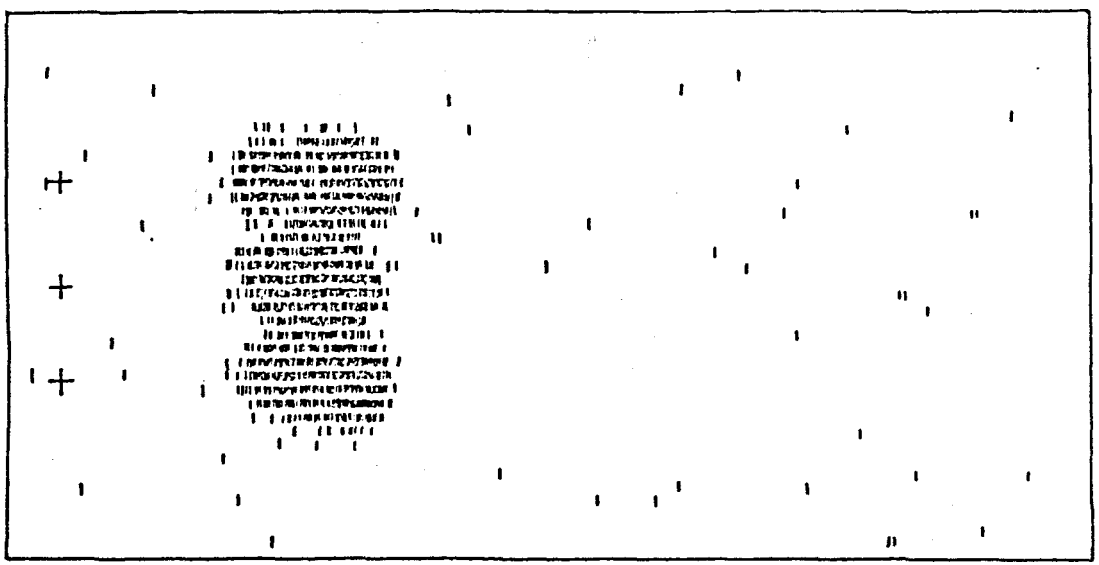
In order to check that hatching substance was not radiolabelled by the above disparate pathway, 1μ Ci (1-^{14}C) oleic acid was incubated for 15 min in a homogenate of 15g B. balanoides in 25 ml Tris buffer (see above) at room temperature. Hatching substance was extracted as described above. The resulting extract was spotted on a T.L.C. plate and developed in solvent system I.

Figure 6 is a stroke diagram of a solvent system I plate showing the regions of radioactivity. Significantly no radioactivity was detected on the origin. Hatching substance is, therefore, not labelled by (1-^{14}C) oleic acid and it can be said that the labelling of hatching substance by (1-^{14}C) arachidonic acid and (2-^{14}C) eicosa-8, 11, 14-trienoic acid is not via the breakdown of the labelled molecule.

It is also worth noting that the oleic acid migrates from the origin in solvent system I, thus demonstrating that it is a less polar compound than hatching substance.

Unfortunately, at the time when the oleic acid control experiment was performed, it was not realized that oleic acid could inhibit the conversion of arachidonic acid into prostaglandins (c.f. Pace-Asciak & Wolfe, 1968) and hence inhibit the formation of hatching substance. In mammalian tissues the concentration of oleic acid required to inhibit prostaglandin synthetase is within the range 1.8-5.0 mM (Flower, 1974). The concentration of oleic acid which was incubated with barnacle tissue was 0.7 pm. It is, thus, extremely unlikely that the barnacle prostaglandin synthetase was inhibited by such a low concentration of oleic acid. Nevertheless, such inhibition has not previously been investigated in invertebrates and it is entirely feasible that invertebrate prostaglandin

Fig. 6. Radiochromatogram of a T.L.C. plate upon which a barnacle homogenate, which has been incubated with (1- ^{14}C) oleic acid and butanol-extracted, has been developed in solvent system I.



synthetase is more sensitive than its mammalian counterpart. This possibility was therefore investigated.

A 5g sample of B. balanoides, prised from their substrate was placed into 5 ml U.V.-irradiated filtered sea water containing 1 µg of cold oleic acid. The barnacles were ground using a mortar and pestle and incubated with oleic acid for 1 hr at room temperature. Following this period, the solution was centrifuged at 9980 g for 2 min and the supernatant bioassayed against a sea water control. The extract was found to be active resulting in the complete hatch of an egg mass within 15 min. Evidently 0.7 ρm oleic acid does not inhibit the synthesis of hatching substance.

4. Affect of aspirin on the incorporation of (1-^{14}C) arachidonic acid into hatching substance

It was noted in Chapter 7 that aspirin inhibits the formation of hatching substance. Since aspirin inhibits the cyclo-oxygenase which catalyzes the conversion of arachidonic acid into the endoperoxides, PGG_2 and PGH_2, aspirin should prevent the incorporation of arachidonic acid into hatching substance.

25g of whole B. balanoides were ground in Tris/HCl buffer containing 10mM soluble aspirin (BP) and 1µ Ci (1-^{14}C) arachidonic acid. A 1 hr incubation period was allowed. After this period, hatching substance was extracted with butanol as described previously. The extract was spotted on a T.L.C. plate and developed in solvent system I. A stroke diagram of such a plate is shown in Figure 7. It will be noted that the radioactive label is confined to the origin, as was found for the (1-^{14}C) arachidonic incubation without aspirin. The origin was scraped off and

Fig. 7. Radiochromatogram of a system II T.L.C. plate upon which a butanol extract of a barnacle homogenate has been developed. The homogenate was incubated with (1- ^{14}C) arachidonic acid together with 10mM soluble aspirin.

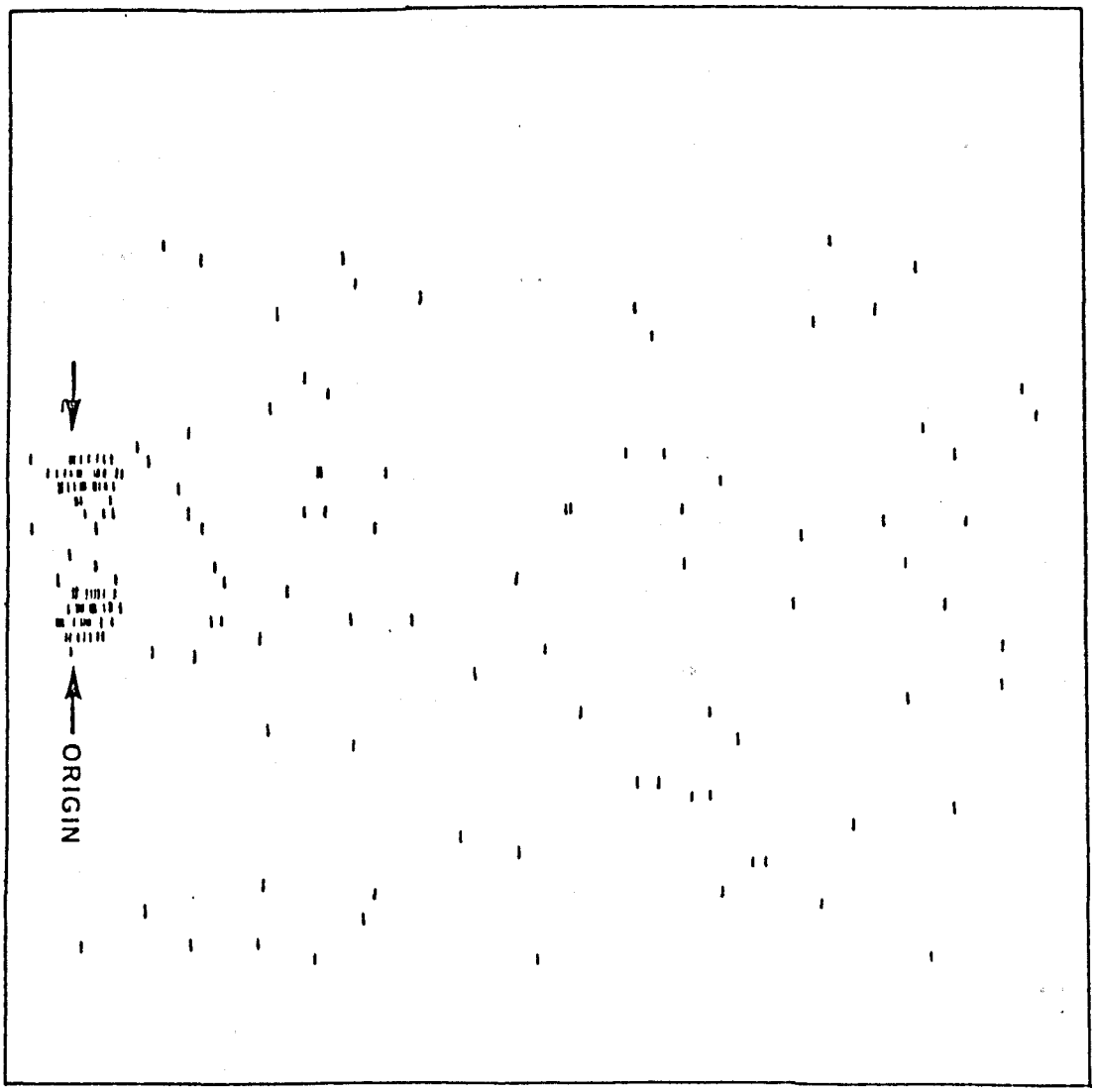
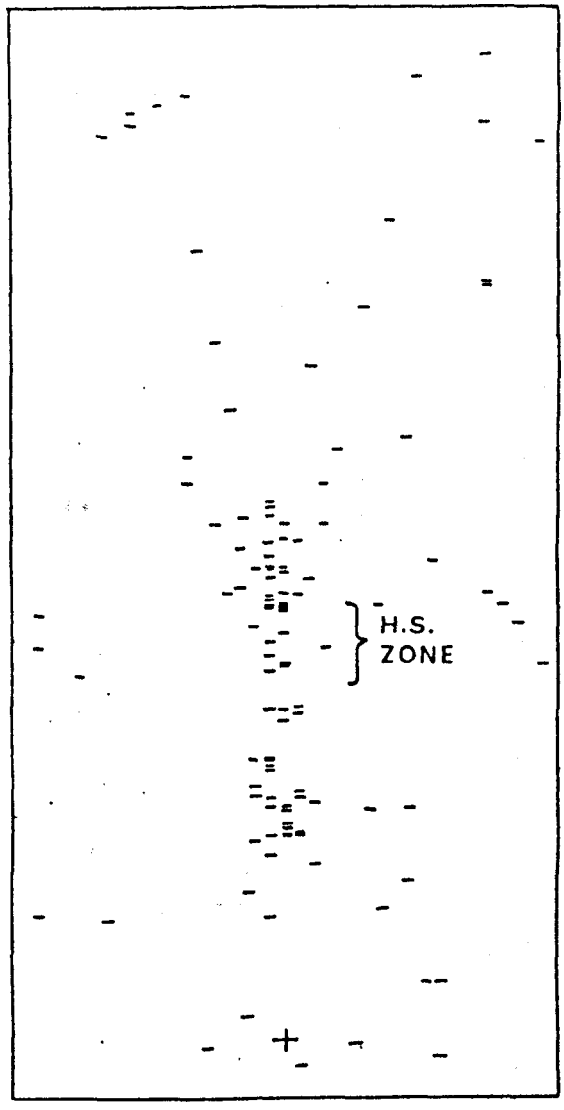

ORIGIN

Fig. 8. Radiochromatogram of a system II T.L.C. plate upon which the origin of the plate shown in figure 7 has been developed.


H.S.
ZONE

eluted with methanol. When this extract was developed on a T.L.C. plate in solvent system II it was seen that aspirin did have an effect on hatching susbtance biosynthesis. Figure 8 is a stroke diagram of a system II plate and shows that the radioactive label is not concentrated in any specific region. Moreover, the area of the plate which normally contains hatching substance displayed little radioactivity. Thus, it would appear that aspirin does inhibit the synthesis of hatching substance from arachidonic acid.

5. Bioassay of hatching substance from B. balanoides on a monthly basis

It became clear during the course of the present investigation that extracts of B. balanoides prepared in late February were less active in promoting stage 12 eggs to hatch than extracts prepared in April. Crisp (1969) claimed that hatching substance was present all year round. The variation above and the lack of data to support Crisp's claim prompted a closer analysis of the presence of hatching substance throughout the year.

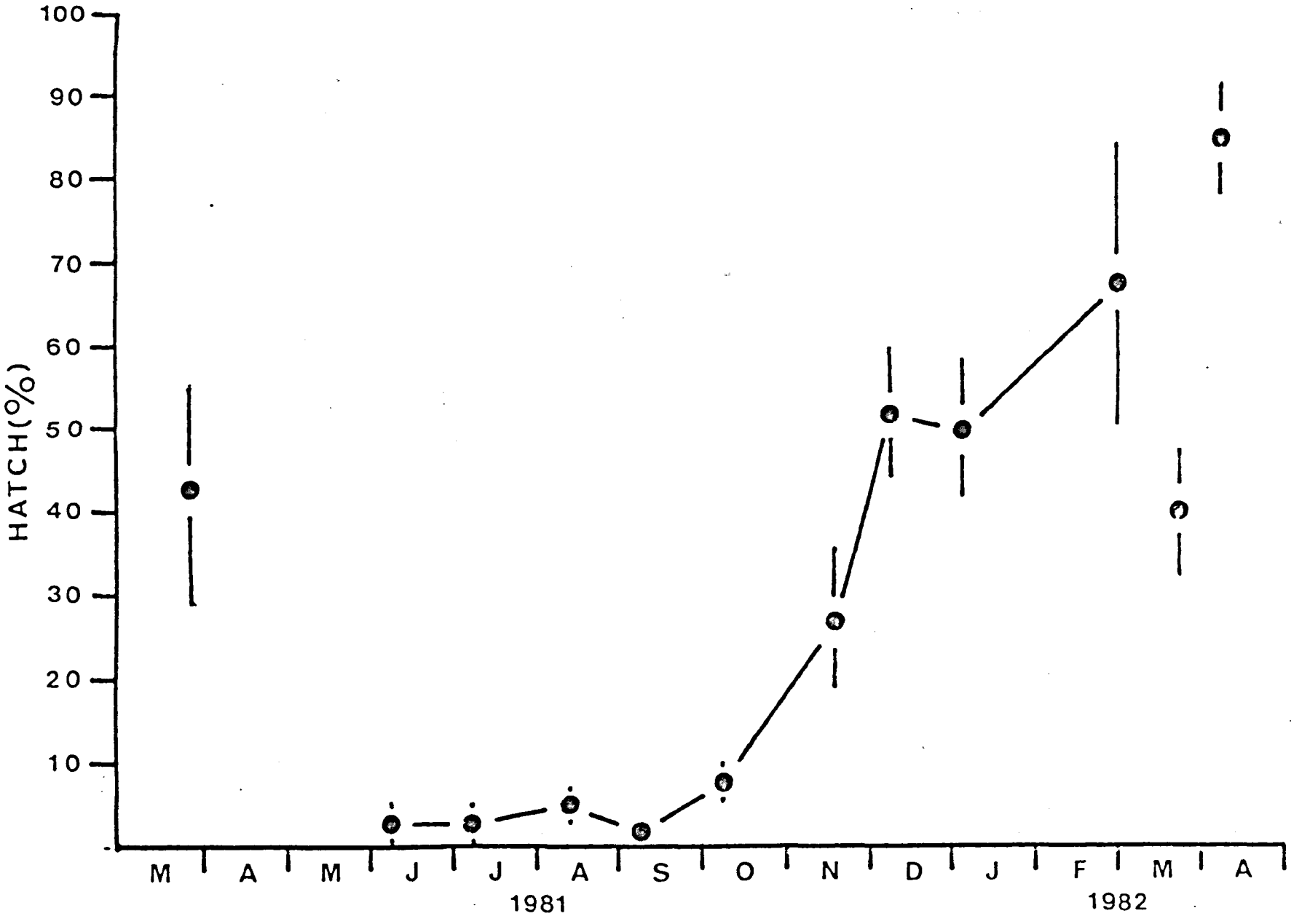
B. balanoides were collected attached to rocks from the shore beneath the Menai Suspension Bridge during the period March 1981 to April 1982. On arrival in the laboratory, specimens were prised from the rocks and immediately frozen. The specimens were stored at -20^{o}C until March 1982 when their homogenates were bioassayed. However, the March and April 1982 samples were bioassayed in early May 1982 and April 1982 respectively. For the bioassay, 1g of a barnacle sample (whole barnacles) was ground in 1 ml U.V.-irradiated filtered sea water using a pestle and mortar. The sample was allowed to stand at room temperature for 15 min and then centrifuged at 9980 g for 2 min. The

resulting supernatant was bioassayed as described in Chapter 7. The samples from each month were bioassayed three times. After each bioassay the number of eggs hatched in both the sea water control and test solutions was counted with the aid of a Bogorov tray so that the percentage hatch could be calculated.

Figure 9 shows the activity of samples over the experimental period in terms of percentage hatch. Clearly, between June and October hatching substance activity is very low. This period is coincident with the development of the new ovary. In a separate experiment, performed in June 1982, the effect of ovarian tissue on the synthesis of hatching substance was examined. The ovaries from 10 B. balanoides were removed and homogenized with 2 whole B. balanoides (from a March 1982 sample) in 2 ml U.V.-irradiated filtered sea water. The homogenate was allowed to stand at room temperature for 15 min after which it was centrifuged at 9980 g for 2 min. The supernatant was assayed on an egg mass obtained from a Robin Hood's Bay B. balanoides; barnacles from Robin Hood's Bay were found to liberate their larvae about one month later than their Menai Bridge counterparts. The control solution used was prepared from 2g March B. balanoides in 2 ml U.V.-irradiated filtered sea water and was found to be active. The test solution, however, displayed little hatching substance activity. Although the result is preliminary it suggests that the ovary of B. balanoides contains some factor which inhibits the formation of hatching substance.

Activity of the barnacle extract begins to rise in October and continues to rise through to the following April. The mean percentage hatch obtained for March 1982 samples is believed to be an underestimate. There is in fact good evidence to support this statement. The March 1982

Fig. 9. The hatching substance activity exhibited by homogenates of whole B.balanoides sampled from the shore on a monthly basis.


HATCH(%)
100
90
80
70
60
50
40
30
20
10
M
A
M
J
J
A
S
O
N
D
J
F
M
A
1981
1982

samples were bioassayed on egg masses from Robin Hood's Bay animals in May 1982. A repeat bioassay of the April 1982 samples on the egg masses from the same animals gave a mean percentage hatch (51.0±14.3) which was much lower than that obtained in April (84.1±6.5). Thus it is possible that the egg masses used in May contained a number of non-viable eggs.

It is important to note that barnacles which were stored at -20°C for one year still contained hatching substance. Of course there is the possibility that hatching substance which was formed prior to storage was being measured, but this is believed to be unlikely. It will be remembered that barnacles which had been stored frozen for 2 months were still able to synthesize hatching substance from (1-^{14}C) arachidonic acid (see above). Hence the enzyme complex which catalyzes the biosynthesis of hatching substance appears to be stable at -20°C for prolonged periods.

6. Effect of prolonged starvation on the hatching activity of B. balanoides extracts

Crisp and Spencer (1958) noted that gravid B. balanoides do not liberate their young if they have been starved for a prolonged period. Intriguingly, it was also noted that extracts prepared from starved barnacles were slightly more active in causing eggs to hatch from isolated egg masses than extracts prepared from well-fed barnacles. Unfortunately, the length of time over which the barnacles were starved was not noted.

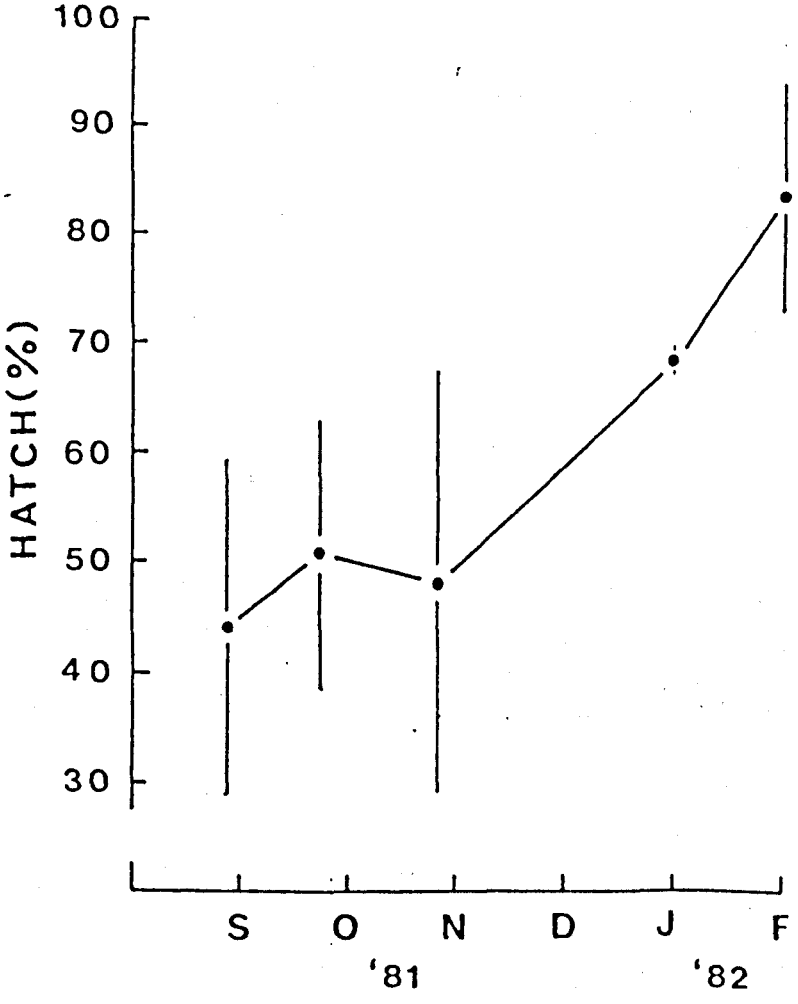
The effect of starvation on the capacity of B. balanoides tissues to produce hatching substance has been examined in the present study. Small rocks bearing many adult B. balanoides were collected from the

shore beneath the Menai Suspension Bridge on 27 August 1981. They were brought into the laboratory and cleaned thoroughly. The barnacles were placed in buckets containing U.V.-irradiated filtered sea water which was well aerated and the temperature thermostatically maintained at 12°C. It was important during the first week of maintenance that the barnacles were removed from the water for several hours every day and allowed to dry. This treatment prevents fungal infection. During the subsequent starvation period the sea water was changed three times each week and the barnacles were exposed to the air for several hours on each occasion. The B. balanoides so maintained appeared healthy after a six month period of starvation. Once a month during this period, samples of the barnacles were prised from the rocks and stored at -20°C. The supply of barnacles was exhausted in February 1982. The samples were bioassayed for hatching substance activity in March 1982, as described in the previous section and the results expressed as a mean percentage hatch (Fig. 10).

The rise in hatching activity of shore barnacle extracts from November onwards (Fig. 9) was also shown by extracts of the starved samples. The most striking feature of the data, however, is that the activity of the extracts from the starved barnacles was higher than that of the fed barnacles at each month. Furthermore, this apparent increase in activity of the starved samples was evident in animals which had been kept without food for only one month.

It is possible that the low levels of activity found in the extracts from fed barnacles between June and October 1981 may be the effect of some dietary inhibition. Animals which have been starved will thus have had any dietary inhibition removed.

Fig. 10. The hatching substance activity exhibited by homogenates of B.balanoides which have been deprived of food for a period of 1 (August samples) to 6 (February samples) months.


100
90
80
70
60
50
40
30
HATCH(%)
S
O
N
D
J
F
'81
'82

7. Titre of prostaglandin precursors in barnacle tissues throughout the year

In order to obtain a more detailed insight into why the activity of samples, in terms of ability to promote egg hatch, should fluctuate throughout the year, it was decided to measure the titre of probable precursors to hatching substance. The same samples which were extracted for hatching substance and bioassayed,were extracted for fatty acid esters for analysis by gas liquid chromatography (G.L.C.).

The first step was to extract each sample for total lipid and this was done by the method of Folch et al. (1957). The lipid extracts were then transesterified for G.L.C. by placing a drop of each extract into a 5 ml reactivial followed by 3 ml of 14% boron trifluoride in methanol/ dichloromethane (2:1). The mixture was flushed with nitrogen and mixed thoroughly before being heated for 1 hr at 100°C. After the sample had cooled, 2 vol petroleum ether (30-40°C) and 1 vol of water were added. The mixture was shaken and the two phases allowed to separate out. The top ether phase, containing the lipids, was pipetted off. This extraction was carried out twice. The ether phases were combined and the ether evaporated off under nitrogen. The lipid residue was taken up in 100 µl chloroform for T.L.C. The sample and a methyl ester standard were spotted on a silica gel G T.L.C. plate and developed in a continuous elution tank with the solvent system, petroleum ether/diethyl ether (95:5) for 75 min. The standard methyl ester was visualized with iodine vapour and the area of plate corresponding to the standard band was scraped off and eluted with chloroform/methanol (2:1). The supernatant was evaporated to dryness under nitrogen and the residue taken up into 10 µl hexane.

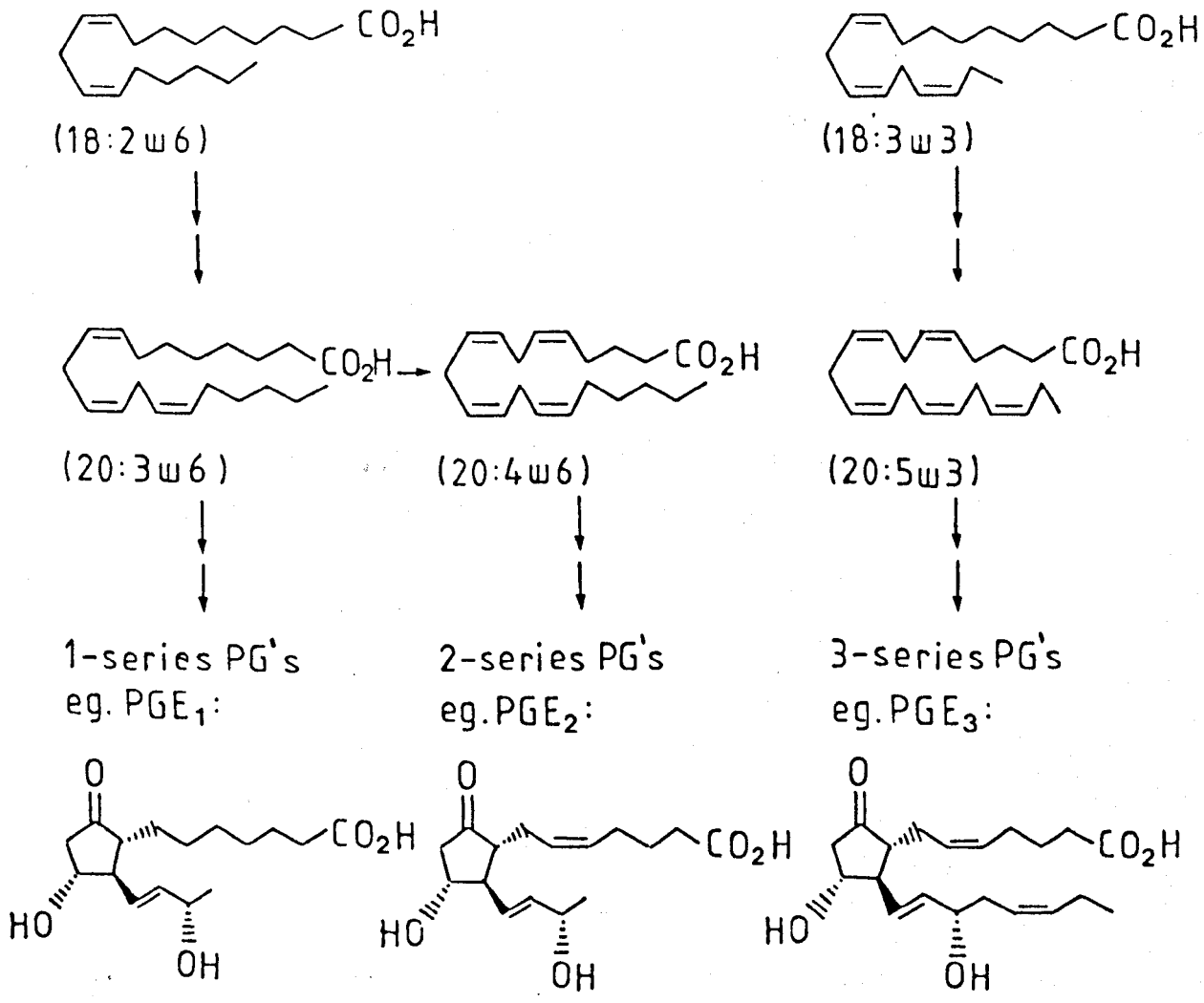
A 1 µl aliquot of the sample was injected 'on column' into a Carlo

Erba high resolution gas chromatograph equipped with a SP1000 glass capillary column and a FID detector. Hydrogen served as the carrier gas. The temperature programme, controlled by a LT programmer model 430, was: a cold trap at 50°C for 10 min, after which the oven temperature was raised to 99.9°C for 1 min and then to 140°C for 5 min. The oven temperature was raised to 200°C at the rate of 3°C a minute and was held at this temperature for 35 min.

Peaks were identified from their relative retention times and by comparison with runs of authentic standards. Quantitative estimation of peak areas was done using an electronic integrator and results were expressed as the percentage of each component peak of the total fatty acid spectrum (c.f. Waldock, 1979).

As previously noted, dihomo - linoleic acid (20:3ω6), arachidonic acid (20:4ω6) and 5, 8, 11, 14, 17-eicosapentaenoic acid (20:5ω3) are the precursors to 1-, 2- and 3-series prostaglandins respectively (Fig. 11). 20:3ω6 was only detected in trace amounts (i.e. less than 0.2%) in both the neutral and phospholipid fractions of B. balanoides. Therefore, although it is able to serve as a precursor to hatching substance in vitro, it is unlikely to do so in vivo. 20:4ω6 was present in both the neutral lipid and phospholipid fractions, although it was present at a higher level (about 1.5-2%) in the latter. This disproportionate distribution of 20:4ω6 was to be expected since this polyunsaturated fatty acid (PUFA) is normally associated with membranes in mammals (Gibson, 1982). It is interesting that Worthington et al. (1981) found comparable levels of 20:4ω6 in the reproductive tract of the male house cricket, Acheta domesticus and field cricket, Gryllus spp. It was suggested by these workers that at such low levels the major function of 20:4ω6 may be as a precursor to

Fig. 11. Biosynthetic pathway for the production of 1, 2 and 3-series prostaglandins from essential fatty acids.


CO2H
(18:2ω6)
CO2H
(18:3ω3)
CO2H
CO2H
CO2H
(20:3ω6)
(20:4ω6)
(20:5ω3)
1-series PG's
eg. PGE1:
2-series PG's
eg. PGE2:
3-series PG's
eg. PGE3:
O
HO
OH
CO2H

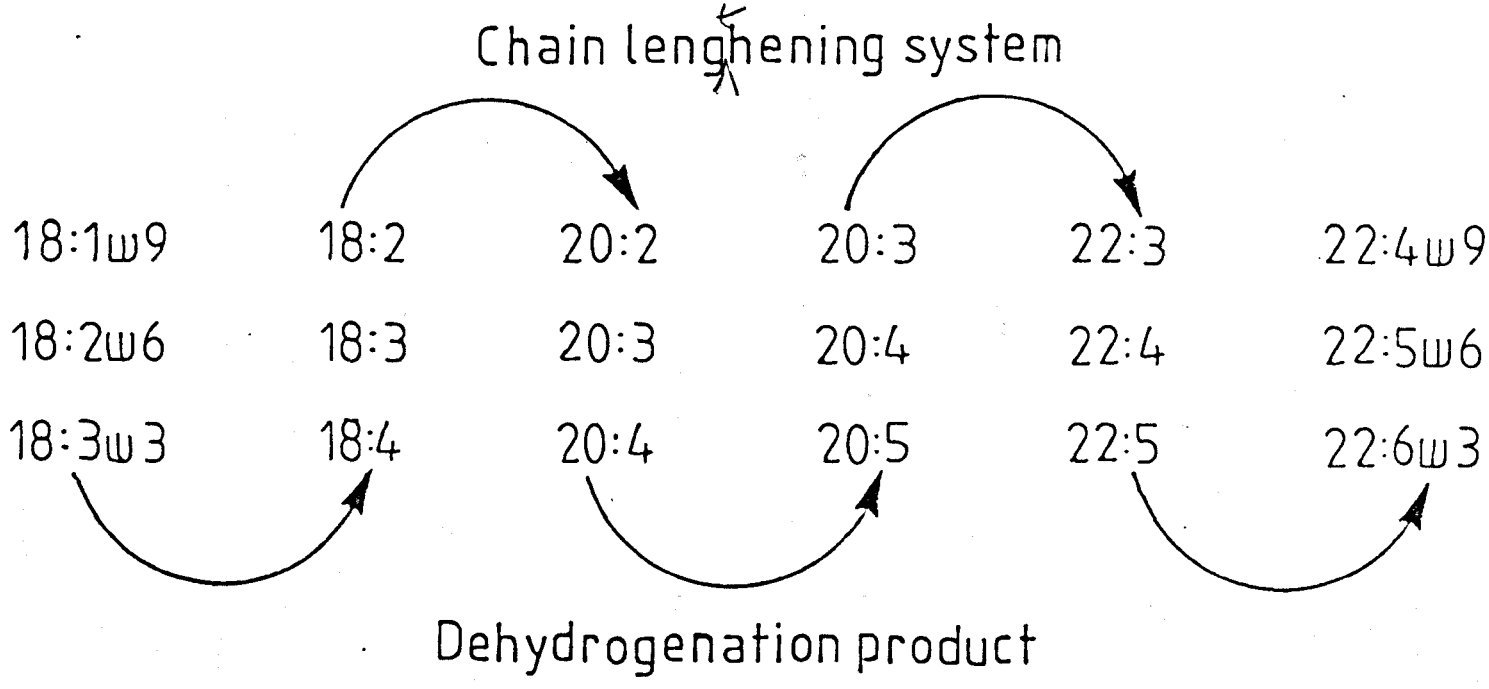
prostaglandins rather than as a structural component of membranes.

20:5ω3 was present at very high levels in both the neutral and the phospholipids, in fact it was the major fatty acid for most of the year. 20:5 is ubiquitous to marine animals (Morris & Culkin, 1976) and it is thought to originate from the phytoplankton, where 20:5 is generally considered to be the typical PUFA (Ackman et al., 1968; Chuecas & Riley, 1969; DeMort et al., 1972). Furthermore, it has been suggested by Ackman et al., (1968) that 20:5 is characteristically deposited in the lipids of filter feeders. On the other hand, Culkin and Morris (1970) found that 22:6 occurred in greater amounts than 20:5 in two species of tunicate filter feeders, the reverse of that found in the present study.

Only small seasonal changes could be discerned for 20:4ω6, as exemplified by a slight increase in amount in both the neutral and the phospholipids during the winter months. It is remotely possible that such a small increase could account for the marked increase in the hatching substance activity of the samples observed over the same period of the year (see above).

Seasonal changes in the levels of 20:5ω3 in both the neutral and phospholipids were more pronounced. In the phospholipid fraction, there was a large increase between November and December after which the level stayed about the same through to the following April. The increase in the neutral lipid fraction was more gradual and may be accounted for by a gradual storage of lipid derived from the barnacles' food. It is interesting that α-linolenic acid (18:3ω3), a precursor to 20:5ω3, and 22:6ω3, a dehydrogenation product of 20:5ω3 (Fig. 12) show similar increases during the winter and early spring. The increase in 20:5ω3 accounts,

Fig. 12. Graphic representation of the involvement of polyunsaturated fatty acids of three families in the same metabolic route. After Holman, 1966.


Chain lengthening system
18:1w9
18:2w6
18:3w3
18:2
18:3
18:4
20:2
20:3
20:4
20:3
20:4
20:5
22:3
22:4
22:5
22:4w9
22:5w6
22:6w3
Dehydrogenation product

to a large extent, for the simultaneous increase in PUFA's of the neutral lipids (Table 4) and the phospholipids (Table 5). Increase in PUFA's were counterbalanced mainly by changes in MUFA's (monounsaturated fatty acids) and to a lesser extent SFA's (saturated fatty acids), as noted by Gardner and Riley (1972). Interestingly, a winter increase in PUFA's was not noted by Gardner and Riley (ibid); on the contrary, they noted a decrease in the PUFA's from January onwards. Such increases have, however, been noted by Cook and Gabbott (1972) and Tooke (1982).

The increase in the phospholipid PUFA's is probably related to cold-tolerance. As noted by Cook and Gabbott (1972), polyunsaturated lipids have lower melting points than saturated lipids. Hence, membranes containing lipids with a high degree of unsaturation have a greater tendency to remain fluid at low temperatures.

8. Effect of starvation on the composition of fatty acids of the neutral and phospholipids

The samples previously analyzed for hatching substance activity were analyzed for fatty acid composition as outlined in the proceeding section.

Compared to the shore samples, a more pronounced increase was observed in the arachidonic acid (20:4ω6) of the phospholipids between September and March. When compared on a month for month basis, however, the relative percentage composition of 20:4ω6 in the starved samples was generally lower. Hence it is not possible to explain the markedly higher hatching substance activity of the starved animals solely on the basis of their 20:4ω6 content.

TABLE 4 Percentage composition of SFA, MUFA and PUFA in B. balanoides neutral lipids.

Fatty Acid Group	% COMPOSITION DATE 6/81	7/81	8/81	9/81	10/81	11/81	12/81	1/82	2/82	3/82	4/82
SFA	16.8	18.3	18.1	17.5	17.5	17.9	18.1	19.0	18.2	18.3	17.7
MUFA	35.8	40.1	35.2	35.0	35.5	37.2	36.7	42.5	37.0	33.9	34.2
PUFA	31.8	30.5	30.0	34.7	36.3	39.1	41.4	28.3	40.6	42.6	43.1

TABLE 5 Percentage composition of SFA, MUFA and PUFA in B. balanoides phospholipids.

Fatty Acid Group	% COMPOSITION										
	DATE										
	6/81	7/81	8/81	9/91	10/81	11/81	12/81	1/82	2/82	3/82	4/82
SFA	22.8	25.0	28.2	23.6	25.3	24.5	21.8	27.8	16.6	21.3	21.3
MUFA	33.6	37.8	39.1	33.0	36.3	40.5	34.0	37.2	32.9	30.0	27.3
PUFA	32.8	30.2	28.9	36.1	30.0	28.3	39.7	31.0	40.0	43.2	45.1

Similar increases in composition were noted for 5, 8, 11, 14, 17-eicosapentaenoic acid (20:5ω3) in the phospholipids. Notably there was little change in the content of this fatty acid in the neutral lipids. This point is important. 20:5ω3 competes very effectively with 20:4ω6 for metabolism by cyclooxygenase (McGiff, 1981) and so would lead to a decrease in the biosynthesis of 2-series prostaglandins. Assuming hatching substance is a 2-series prostaglandin (and there is some evidence that it is) then the biosynthesis of this compound would benefit from a low titre of 20:5ω3. Therefore, the increase in the hatching substance activity of the starved samples over the shore samples may, to some extent, reflect differences in the level of 20:5ω3 in the neutral lipid fractions.

The degree of unsaturation of the neutral and phospholipids was yet again a reflection of their content of 20:5ω3. Since there was no discernible seasonal change in the 20:5ω3 content of the neutral lipids, likewise there was no overall change in the degree of unsaturation (Table 6). The phospholipids, however, exhibited a marked increase in the degree of unsaturation between September and March (Table 7).

The absence of any increase in unsaturation of the neutral lipids suggests that it is these lipids and not the phospholipids which are mobilized as food reserves. Previous studies on marine invertebrates have also shown that the phospholipid fatty acid composition is less variable than that of the neutral lipid fractions (c.f. Tooke (1982) for discussion).

Molecular structure of the coral active fraction

A sample of the coral active fraction (C.A.F.) extracted and purified

TABLE 6 Percentage composition of SFA, MUFA and PUFA in the neutral lipids of starved B. balanoides.

Degree of unsaturation	% COMPOSITION				
	DATE				
	9/81	10/81	11/81	2/82	3/82
SFA	18.1	18.6	17.2	16.7	17.3
MUFA	35.8	39.6	38.4	38.1	37.0
PUFA	37.8	33.0	36.5	36.3	37.9

TABLE 7 Percentage composition of SFA, MUFA and PUFA in the phospholipids of starved B. balanoides.

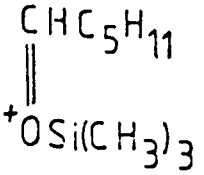
Degree of unsaturation	% COMPOSITION				
	DATE				
	9/81	10/81	11/81	2/82	3/82
SFA	30.3	27.5	26.7	23.4	22.7
MUFA	40.5	40.8	38.1	38.2	34.7
PUFA	23.3	28.5	30.9	35.3	41.0

as described in Chapter 7 was analyzed by gas liquid chromatography and mass spectrometry (G.L.C./M.S.).

The sample was derivatized for GLC by two procedures. The first procedure involved methylating the compound with diazomethane in ether/methanol. The methylated residue was then methoxymated with a 2% solution of methoxyamine. HCl in pyridine. After removal of the pyridine the methoxymated residue was silylated using N,O-bis-trimethylsilyl-trifluoroacetamide (B.S.T.F.A., Sigma). The derivatized sample (designated as C.A.F.1) was injected into a capillary SE 52 column with a solid sample injector. The temperature programme used was 4 min at 170°C followed by heating the oven to 240°C at the rate of 10°C/min.

The second derivatization procedure differed from the above only in that the methoxymation step was omitted. The derivative so obtained was termed C.A.F.2. The G.L.C. temperature programme used for this derivative was 8 min at 170°C, followed by a 10°C/min rise in temperature to 240°C.

Figure 13 shows the G.L.C. profile of the methylated, trimethylsilylated sample, C.A.F.1. The trace shows major peaks at scan numbers 262, 267 and 275. The first of these, scan 262, is probably prostaglandin A (Fig. 14); the latter two are clearly closely related isomers (I and II) as they gave virtually identical mass spectra (Figs. 15 and 16 respectively). The relevant ion in the spectra is m/z 408, but a weak ion at m/z 493 in scan 275 corresponds to the usual loss of CH_3 from $OS{:}(CH_3)_3$ derivatives. The molecular ion at m/z 173 corresponds to:



and m/z 336 its loss in a McLafferty rearrangement, i.e.

Fig. 13. Gas liquid chromatogram of the methylated, trimethylsilylated coral active fraction (C.A.F. 1).

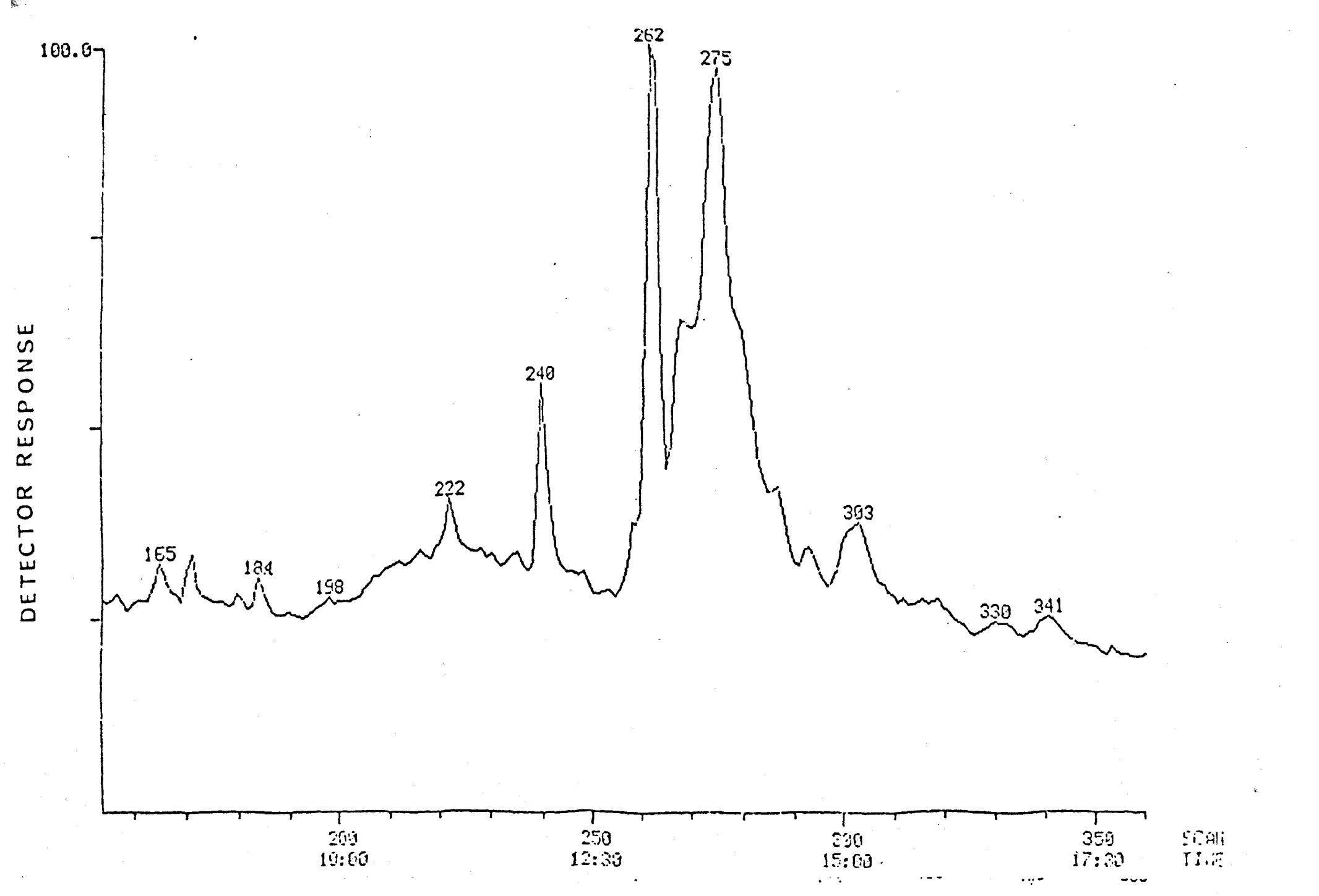

100.0
DETECTOR RESPONSE
262
275
240
222
165
184
198
303
330
341
200
10:00
250
12:30
300
15:00
350
17:30

Fig. 14. Mass spectrum of scan 262 of C.A.F. 1, tentatively identified as prostaglandin A.

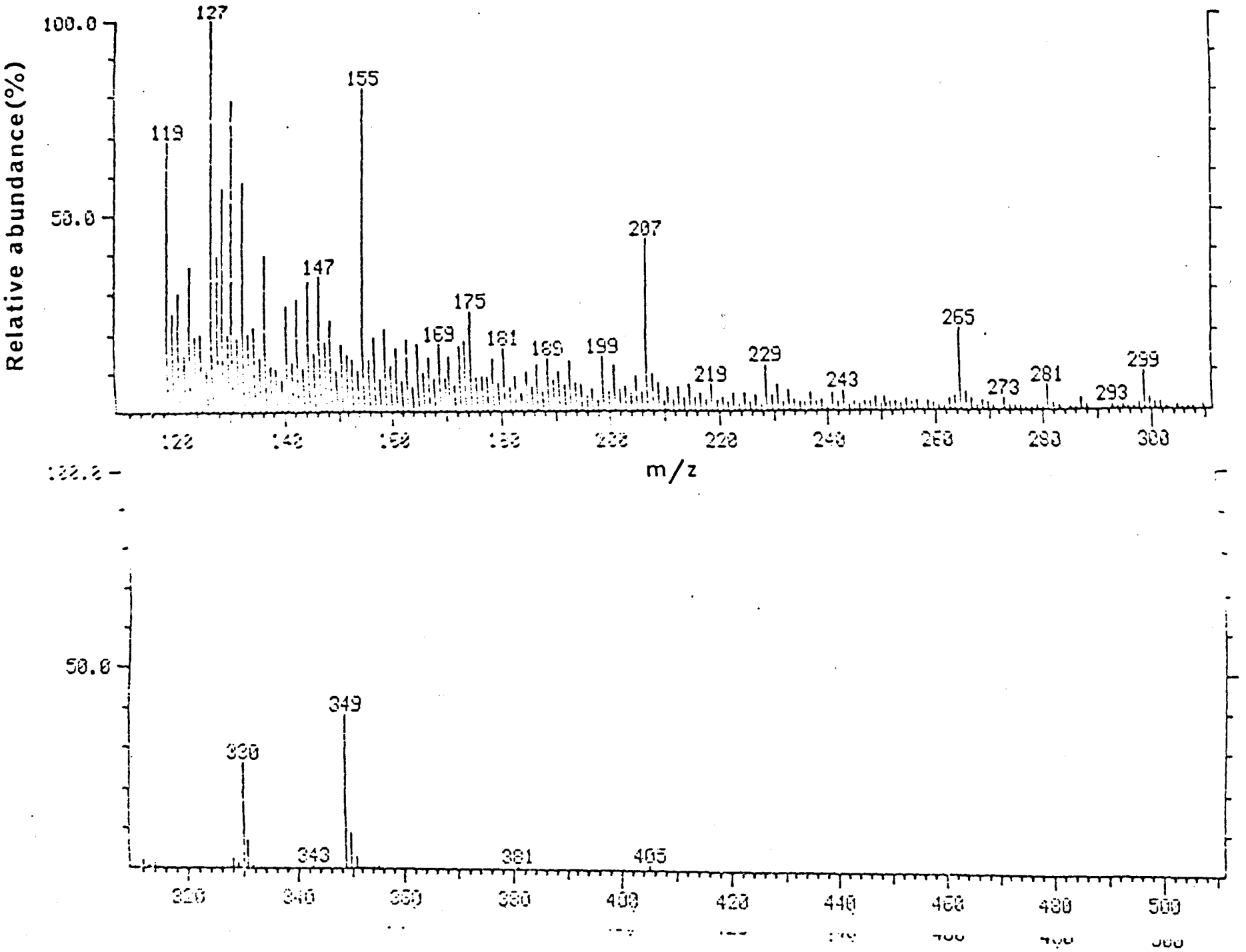

Relative abundance(%)
100.0
50.0
127
155
119
147
207
175
169
181
189
199
219
229
243
265
273
281
293
299
m/z
50.0
349
330
343
381
405

Fig. 15. Mass spectrum of scan 267 of C.A.F. 1.

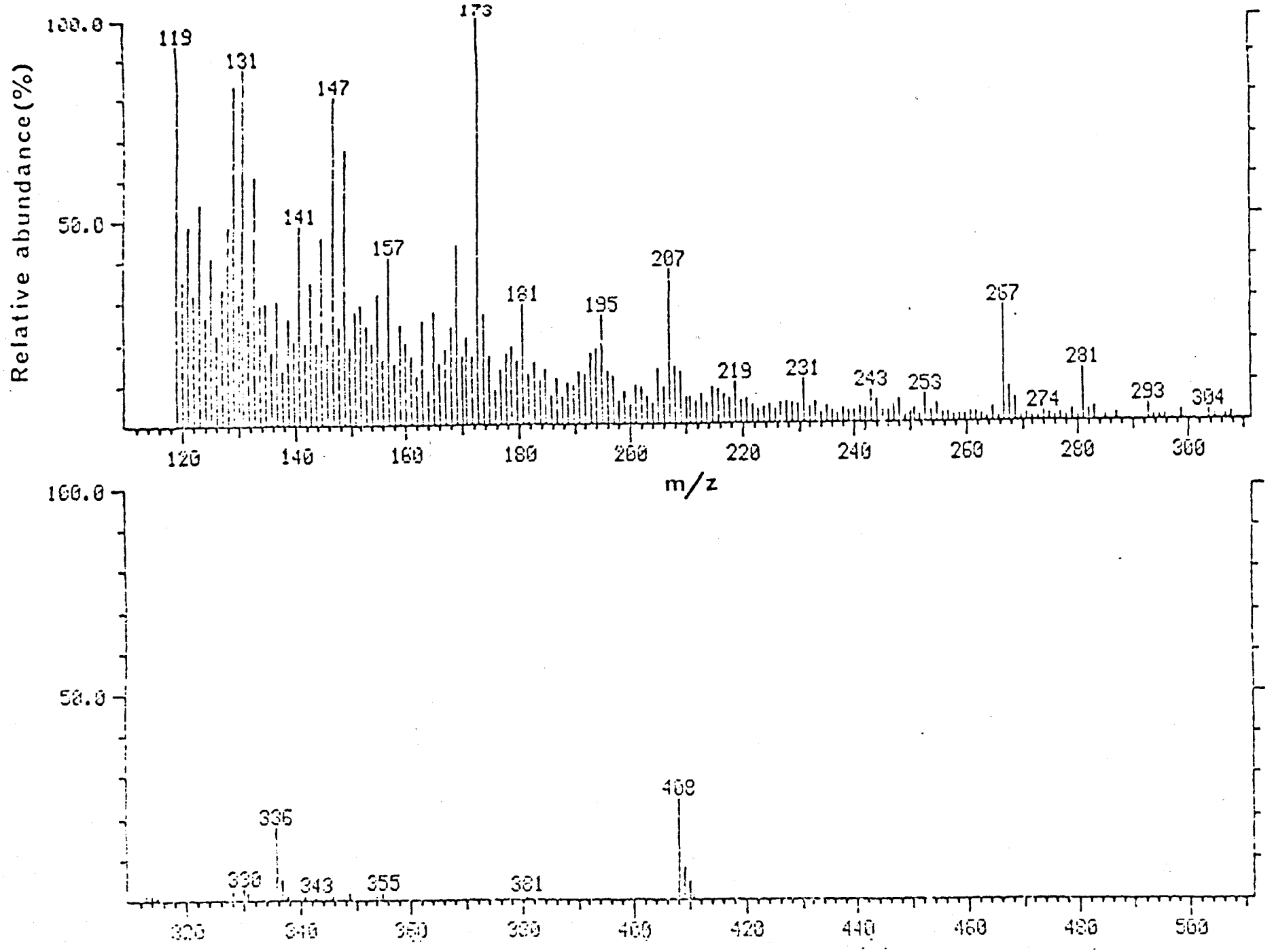
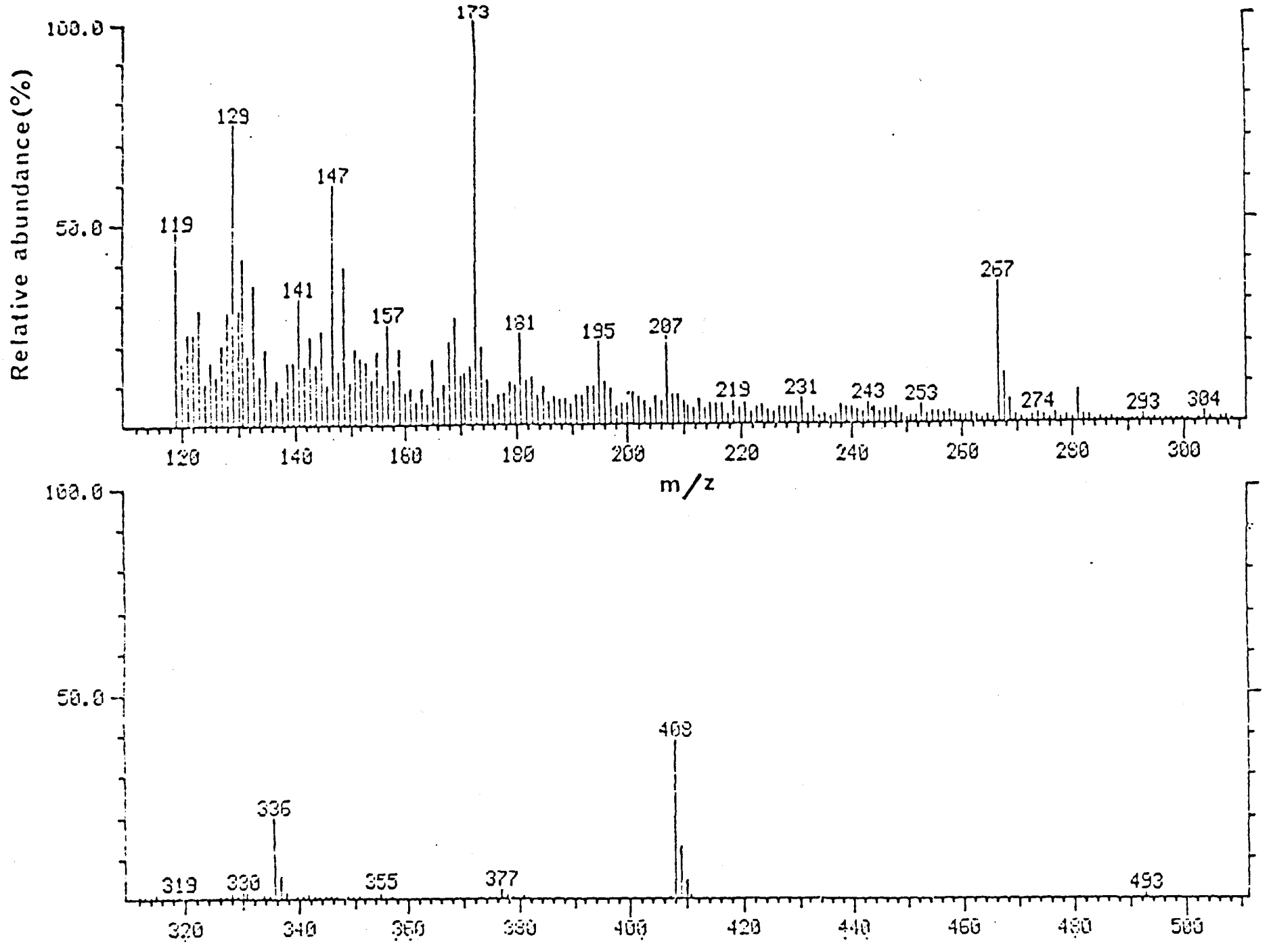
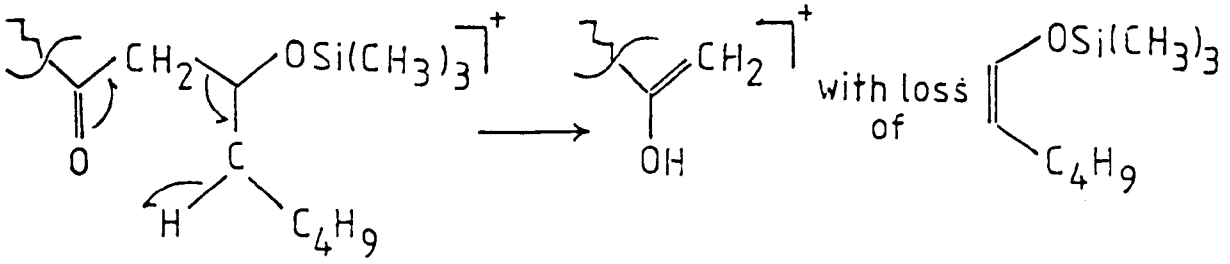
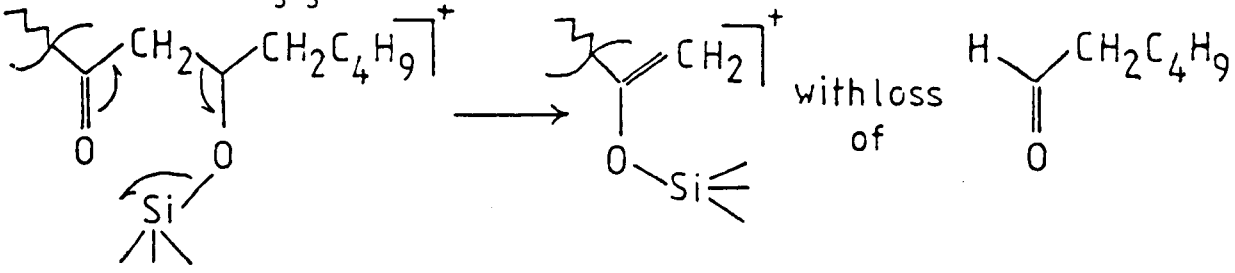

Relative abundance(%)
100.0
50.0
119
131
147
173
141
157
181
195
207
219
231
243
253
257
274
281
293
304
120
140
160
180
200
220
240
260
280
300
m/z
100.0
50.0
335
330
343
355
381
408
300
320
340
360
380
400
420
440
460
480
500

Fig. 16. Mass spectrum of scan 275 of C.A.F. 1. Note the similarity to the trace shown in figure 15.

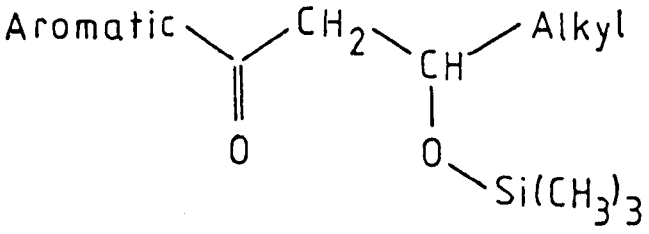

100.0
50.0
Relative abundance(%)
173
129
147
119
141
157
181
195
207
267
219
231
243
253
274
293
304
120
140
160
180
200
220
240
260
280
300
m/z
100.0
50.0
408
336
319
330
355
377
493
320
340
360
380
400
420
440
460
480
500



The molecular ion m/z 408 corresponds to the same fragmentation with transfer of $Si(CH_3)_3$ instead of hydrogen:



It has been confirmed that this migration does occur by examining compounds of structure:



The ion at m/z 267 corresponds to the loss of CH_2 - CH = CH - $(CH_2)_3$-CO_2.Me from the ion at m/z 408.

Unfortunately the methoxymation reaction did not go to completion and most of the spectra are complex, because they consist of unresolved components. However, it is possible to find a true, methylated, methoxymated trimethylsilylated derivatives corresponding to C.A.F.2 at scan 228 (Fig. 17). The spectrum shows a molecular ion at m/z 537 and losses of CH_3 (m/z 522), OCH_3 (m/z 506) and $(CH_3)_3SiOH$ + OCH_3 (m/z 416) confirm this. Loss of $C_4H_9CH_2CHO$ to give m/z 437 and C_4H_9C = $COSi(CH_3)_3$ to give m/z 365 are the same reactions as in the methoxymated compounds. Both these ions then lose OMe. Again m/z 173 is:

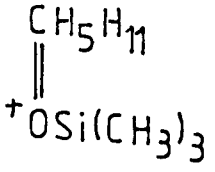
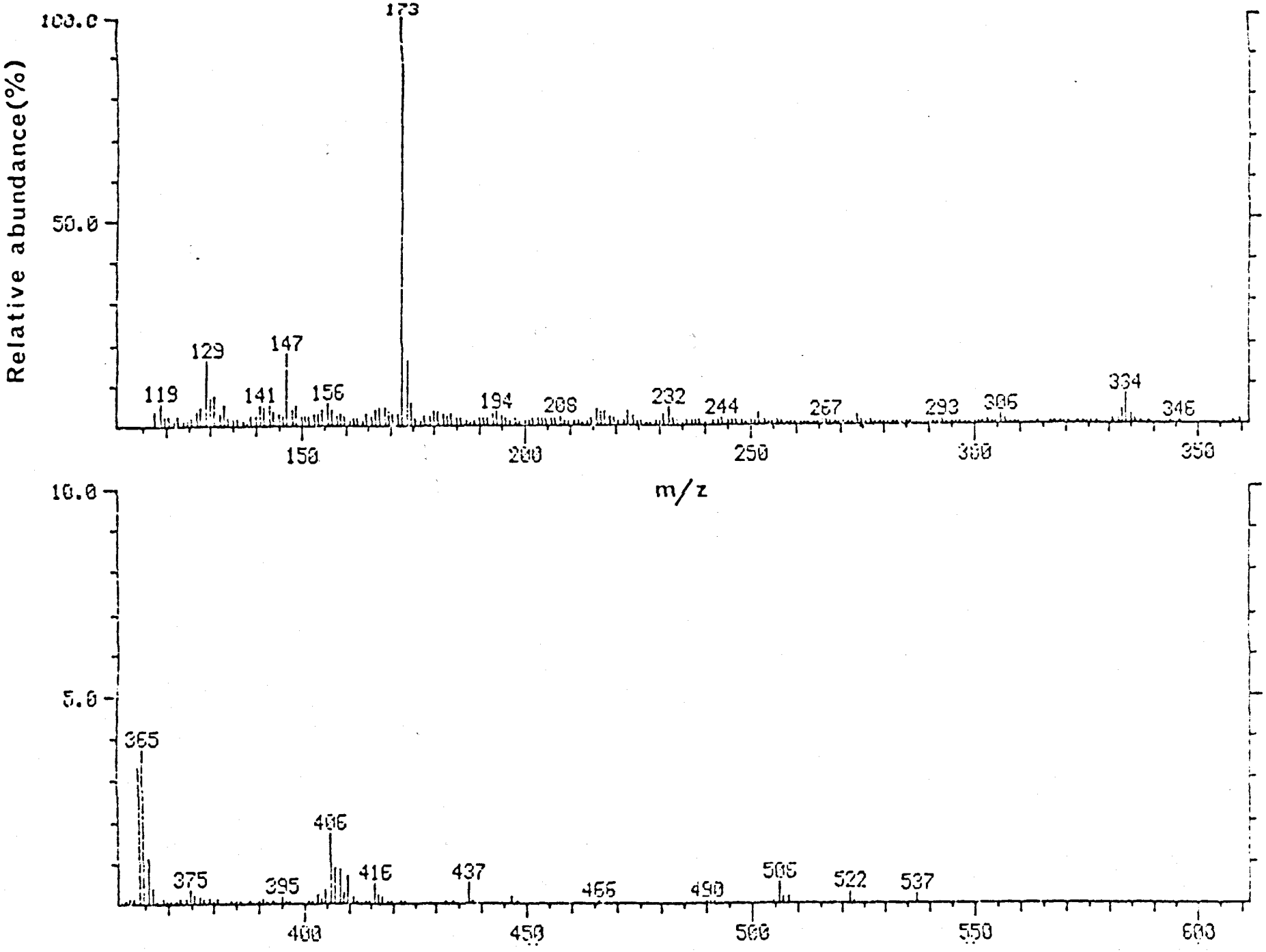


Fig. 17. Mass spectrum of scan 228 of the methylated, methoxymated trimethylsilylated derivative of the coral active fraction (C.A.F. 2).


Relative abundance(%)
100.0
50.0
10.0
5.0
173
129
147
119
141
156
194
208
232
244
257
293
305
334
346
365
375
395
406
416
437
466
490
505
522
537
150
200
250
300
350
400
450
500
550
600
m/z

Essentially these fragmentations prove the structure of the side chains. The isomerism of the ring and the difference between the isomers cannot be derived from the mass spectra, so the evidence to date gives the tentative structure shown in Figure 18.

7. Activity of extracts prepared from other cirripedes and from Hemioniscus balani

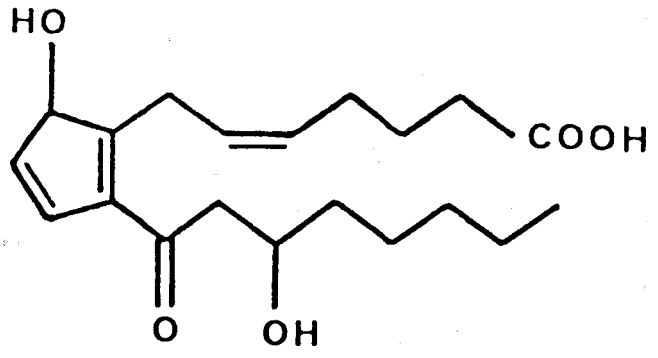
Crisp & Spencer (1958) prepared extracts from several species of cirripedes and tested them for hatching activity on the eggs of B.balanoides. Their results are presented in Table 8.

The present study aimed in part to confirm and to extend the observations of Crisp & Spencer (ibid). To this end, extracts from several barnacle species were prepared and where possible tested on egg masses of B.balanoides and/or egg masses of the donor species. The results are presented in Table 9.

Where the results of the present study can be compared with those of Crisp and Spencer (1958), it is clear that there are no discrepancies.

Two additional Balanus species have been examined in the present study. B.hameri is found to possess hatching substance. This result is particularly interesting, because the eggs of this species are capable of hatching in response to a rise in temperature alone (personal observation). This response is similar to that observed for Elminius modestus. Consequently such egg masses are unreliable for the bioassay of hatching substance. In this respect it is pertinent that sea water extracts of B.perforatus, which are inactive on the eggs of B.balanoides, were active on the eggs of E.modestus. This latter result must be viewed with some caution.

Fig. 18. Tentative molecular structure of the coral active fraction.


HO
COOH
O
OH

If, as seems probable, B.perforatus does not possess a hatching substance (and the only way to resolve the ambiguity is to bioassay extracts on B.perforatus egg masses), then this would be the only Balanus species so far tested which does not. Although Crisp and Spencer (1958) obtained the same result, they did not remark on why B.perforatus should not possess hatching substance. It may be that warm water species do not require a hatching substance since there is no need to synchronize the liberation of their young with phytoplankton blooms. In conflict with this theory is the finding that B.amphitrite has a hatching substance (Crisp & Spencer, ibid). However, these workers did not bioassay extracts of B.amphitrite on egg masses from the same species. Since Crisp (1969) held the view that hatching substance was present throughout the year, there would be no need to do so. It is now obvious, though, that hatching substance is capable of being synthesized by the tissues of B.balanoides for a restricted period only (see above). Hence Crisp and Spencer (1958) may have bioassayed B.amphitrite at the wrong time of year. This point also applies to Chthamalus stellatus and Verruca stroemia, and also Sacculina carcini in the present study. Clearly these species need to be re-examined before any theory regarding the specificity of hatching substance can be erected.

Although an attempt has been made to collate data for those barnacles available (Tables 8, 9) regarding the presence of hatching substance, they are not sufficient to allow any conclusions to be reached, concerning the specificity or geographical distribution, other than to say that the 'boreoarctic' species require hatching substance.

8. The role of dopamine in the hatching process

Sea water solutions of dopamine between the concentration range of 1.17×10^{-5}M and 3.0×10^{-3}M are capable of promoting viable embryos to hatch as nauplii from barnacle eggs (Clare et al., 1982; Chapter 7).

TABLE 8 Activity of extracts of different barnacle species. After Crisp and Spencer (1958).

test species	donor species used to prepare extract: *Balanus balanoides*	*B. crenatus*	*B. balanus*	*B. amphitrite*	*B. perforatus*	*Elminius modestus*	*Chthamalus stellatus*	*Verruca stroemia*
Balanus balanoides	+	+	+	+	-	+	+	-
B. crenatus	+	+	+	.	.	+	.	.
B. balanus	+	+	+	.	.	.	.	.
B. amphitrite	+	.	.	.	.	.	.	.
B. perforatus	+	.	.	.	.	+	.	.
Elminius modestus	+	.	+	.	.	+	.	.

TABLE 9 Activity of extracts of different barnacle species and the barnacle parasite Hemioniscus balani.

test species	donor species used to prepare extract: Balanus balanoides (Menai Bridge)	B. balanoides (Alaska)	B. hameri	B. perforatus	Elminius modestus	Lepas anatifera	Trypetesa lampas	Sacculina carcini	Hemioniscus balani (barnacle parasite)
Balanus balanoides (Menai Bridge)	+	+	+	-	.	.	-	-	-
B. balanoides (Alaska)	+	+	.	.	.	.	.	.	.
B. hameri	+	.	+	.	.	.	.	.	.
B. perforatus	+	.	.	.	.	.	.	.	.
Elminius modestus	.	.	.	+	+	.	.	.	.
Lepas anatifera	-	.	.	.	.	-	.	.	.

It has been postulated that the rôle of dopamine is to act as a neurotransmitter within the embryo; the barnacle hatching substance may stimulate the release of this neurotransmitter, which then stimulates embryonic muscle (c.f. Chapter 7). This hypothesis has been investigated using a DOPA decarboxylase inhibitor and dopamine antagonists and agonists:

(i) Effect of benserazide on the hatching process

The conversion of 3,4-dihydroxyphenyl alanine (DOPA) into dopamine is catalyzed by the enzyme DOPA decarboxylase. Benserazide (Fig. 19A) is a competitive inhibitor of this enzyme (Bowman & Rand, 1980).

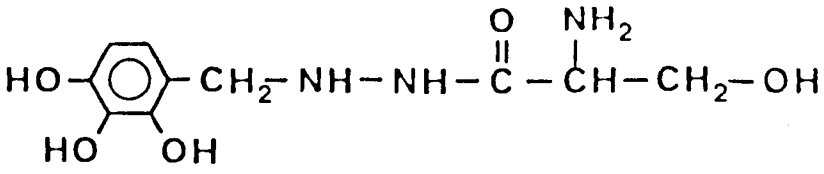
Benzerazide HCl (Roche, Welwyn Garden City) was dissolved in U.V.-irradiated filtered sea water to a concentration of 2.1×10^{-3}M. An egg mass was immersed in this solution for 15 min prior to bioassay. The other egg mass, from the same barnacle, was placed in sea water for 15 min and served as the control. Hatching substance was prepared by grinding whole B.balanoides in 1 ml U.V.-irradiated filtered sea water using a mortar and pestle. The extract was centrifuged at 9980 g for 2 min. 500 µl of the supernatant was then added to both the test and the control egg masses. This experiment was performed three times and on each occasion egg masses in both the test and the control solutions hatched completely.

The fact that benserazide did not have an effect on the hatching process suggests that if dopamine is involved, then it is stored dopamine and not newly synthesized dopamine which is released in response to hatching substance.

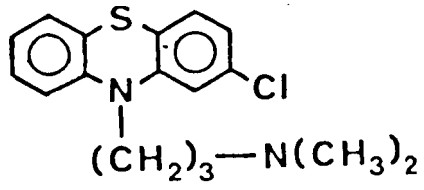
(ii) Effect of chlorpromazine on the hatching process

Chlorpromazine (Fig. 19B) is an antipsychotic drug of the phenothiazine class (Müller et al., 1981). The drug is a dopamine antagonist and acts by blocking dopamine receptors.

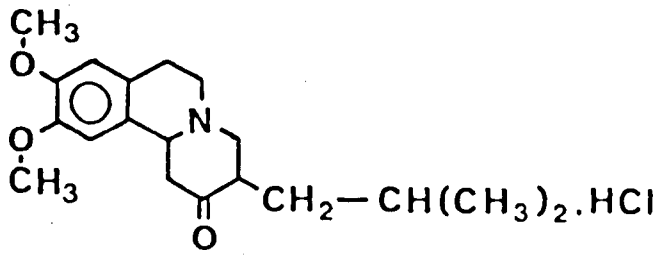
Fig. 19. Molecular structures of the drugs used in this study to investigate the role of dopamine in the hatching process.



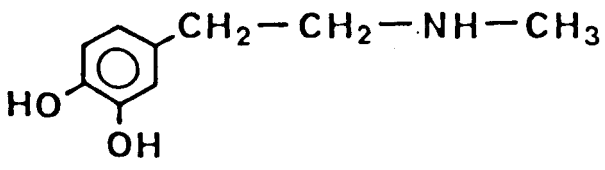
A. Benserazide



B. Chlorpromazine



C. Tetrabenazine



D. Epinine

A 10^{-3}M stock solution of chlorpromazine (Sigma) was freshly prepared in U.V.-irradiated filtered sea water. One of a pair of egg masses from a barnacle was incubated in the drug solution prior to bioassay with hatching substance. The remaining egg mass, in sea water, served as the control. A range of concentrations of chlorpromazine was tested and the results are presented in Table 10. Chlorpromazine at the concentrations of 10^{-3}M and 5×10^{-4}M prevented eggs from hatching after hatching substance had been added to them. The lower concentrations of 10^{-5}M and 10^{-6}M were partly effective. It is important to note the same eggs were capable of hatching after being washed for several hours in running sea water, although the nauplii soon became moribund. It is pertinent in this respect that chlorpromazine also inhibits many enzymes at high concentration.

(iii) Effect of tetrabenazine on the hatching process

Tetrabenazine (Fig. 19C), a synthetic benzoquinolizine derivative, is reserpine-like in its action (Quinn et al., 1959). However, in mammals it differs from reserpine because it does not deplete peripheral stores of catecholamines. Instead the depleting action is confined to the central nervous system where dopamine and also serotonin and noradrenaline are effectively removed.

Tetrabenazine (trade name NITOMAN, Ro 01-9569, Roche, Welwyn Garden City) was dissolved in ethanol and then diluted with U.V.-irradiated, filtered sea water to the required concentration. The solution was then heated to 37°C, to re-dissolve any of the drug which came out of solution, and rapidly cooled to the ambient sea water temperature.

Two concentrations of tetrabenazine, namely 10^{-4}M and 10^{-6}M, were tested for their effect on the hatching process. Egg masses were immersed in the drug solution for 30 min after which a crude extract of hatching substance was added. The larvae which had hatched after a 15 min test

TABLE 10. Effect of chlorpromazine on hatching substance-induced egg hatch

Concentration (M)	Percentage hatch		Exposure time to drug (min)
	Test	Control	
10^{-3}	0	98.1	60
5×10^{-4}	0	93.5	30
10^{-5}	39.4	65.8	30
10^{-6}	66.2	95.3	45

period were counted and the numbers compared with those which had hatched in a sea water control to which hatching substance had also been added.

Tetrabenazine at the concentration of 10^{-4}M was completely effective in preventing hatching substance from inducing eggs to hatch. 10^{-6}M tetrabenazine, on the other hand, was only partly effective in this respect; approximately 75% of the eggs did not hatch at this drug concentration.

It is tempting to extrapolate from the vertebrate situation and assume that tetrabenazine acts on the central nervous system of barnacle embryos, implying that peripheral stores of dopamine are not important to the hatching process.

(iv) Effect of epinine on the hatching process

Epinine (Fig. 19D) is a putative dopamine receptor agonist with a potency comparable to that of dopamine on crustacean foregut preparations (Lingle, 1981). Similarly, epinine is as potent as dopamine on the peripheral DA_1 (a subtype of dopamine receptor) receptors of mammals (Goldberg & Kohli, 1981).

Solutions of epinine(Sigma) were prepared from a 10^{-3}M stock solution in U.V.-irradiated, filtered sea water. Egg masses in epinine solutions were bioassayed against control masses in sea water. The results are presented in Table 11.

Clearly epinine does promote barnacle eggs to hatch and it does so in the same concentration range as dopamine. However, dopamine is more potent than epinine. for example, dopamine at a concentration of 10^{-4}M in sea water results in a complete hatch of an egg mass (c.f. Chapter 7).

TABLE 11 Potency in terms of % hatch of various epinine solutions versus sea water controls.

	Concentration of epinine (M)		
	10^{-3}	10^{-4}	10^{-5}
Test	63.9	16.8	3.2
Control	0	0	0.2

(v) Effect of ethanol on the hatching process

Whilst investigating the effect of ecdysone on the hatching process (c.f. Chapter 7) it became apparent that the 5% ethanol solution in which it was dissolved was itself active in promoting eggs to hatch. The potency of ethanol, however, in comparison to hatching substance, is low. Embryos did not start to respond to ethanol until about 30 min after it was administered, whereas hatching substance is effective within 5 min.

This effect of ethanol is pertinent with respect to the rôle of dopamine in the hatching process, because ethanol is known to interact with the dopaminergic system of mammals (Bustos et al., 1981; Barbaccia et al., 1982). In fact the acute administration of ethanol enhances the release of dopamine from the rat striatum (Barbaccia et al., 1980). Thus it is possible that ethanol causes the release of dopamine from tissues of the barnacle embryo, which in turn causes it to hatch.

(vi) Effect of 2-phenylethylamine together with 3, 4-dihydroxyphenylalanine on the hatching process of B.balanoides

It has been established that the effect of parental hatching substance is not mediated by the release of prostaglandin(s) within the embryo (c.f. Chapter 7). However, Crisp and Spencer (1958) have shown that egg masses do contain hatching substance, although much less than was obtained from shells and the hypodermis lining the shell. Secondly, eggs are capable of hatching when cultured in vitro (Crisp, 1959; Chapter 6). Under these conditions it is obvious that parental hatching substance is not essential to the hatching process.

It is possible that dopamine causes barnacle eggs to hatch by stimulating biosynthesis and the release of a prostaglandin from the embryo. The enhancement of prostaglandin biosynthesis by large amounts of catecholamines and serotonin is well documented (Leslie, 1976; Wolfe et al., 1976;

Schaefer et al., 1978). Further, such biosynthesis is only stimulated by catecholamine analogues which are substrates for the enzyme monoamine oxidase (Schaefer et al., 1978). Interestingly, monoamine oxidase degrades catecholamines and at the same time forms hydrogen peroxide (Tipton, 1968; Sinet et al., 1980) which in turn is able to activate prostaglandin synthetase in invertebrates (Morse et al., 1977) as well as vertebrates (Smith & Lands, 1972; Panganamala et al., 1974; Polgar & Taylor, 1980).

2-Phenylethylamine (PEA) is a catecholamine analogue and a substrate of monoamine oxidase (Neff & Yang, 1974), yet it is unable to increase $PGF_{2\alpha}$ production in the particulate fraction of rat brain homogenates (Seregi et al., 1982). However, when PEA is administered with any of the 3, 4-dihydroxyphenyl (catechol) derivatives or metabolites of catecholamines it does enhance $PGF_{2\alpha}$ formation (Seregi et al., 1982).

3, 4-Dihydroxyphenylalanine (DOPA) (Sigma) is the catechol derivative which, in combination with PEA (Sigma) was bioassayed in the present study. Sea water solutions of the drugs were tested at two concentrations, namely 10^{-3}M PEA with 10^{-3}M DOPA and 2.5×10^{-4}M PEA with 2.5×10^{-4}M DOPA. The drugs were bioassayed for only a 15 min period (Chapter 7), an additional control was employed in which DOPA, without PEA, was tested in the 1 hr bioassay.

Table 12 shows that the solutions containing both PEA and DOPA were active, but so too was DOPA alone. It would appear that the 1 hr test period is sufficient for DOPA to be converted to dopamine by the embryo and so lead to egg hatch. Presumably the 15 min test period previously employed (Chapter 7) is insufficient in this respect.

Although still preliminary it is desirable to draw together the results of the dopamine experiments and postulate a rôle for this compound. It is true to say that dopamine is involved in the hatching process, but at what stage its effect is exerted remains unknown. Since hatching substance,

TABLE 12. Influence of PEA + DOPA versus DOPA alone on egg masses in terms of mean percentage hatch

Drug	Concentration (M)	n	Percentage hatch Test	Control
DOPA	2.5×10^{-4}	3	46.4	0.4
PEA + DOPA	10^{-3}	1	6.2	0
	2.5×10^{-4}	4	24.1	0.7

when added to embryos with their dopamine receptors blocked, does not elicit egg hatch, it would seem that dopamine is released by the embryo in response to hatching substance. Furthermore, evidence has been obtained to suggest that central stores of dopamine are released. First, the DOPA decarboxylase inhibitor, benserazide, did not prevent the hatching process. Secondly, the hatch caused by DOPA took much longer than the 5 min period required for either dopamine or hatching substance. Finally, tetrabenazine which is considered to deplete central stores of dopamine only, prevented egg hatch in response to hatching substance.

In mammals prostaglandins of the E-series have been implicated in the control of neurotransmission (Hedqvist, 1970; Gripenberg et al., 1976). Of particular interest to the present study was the finding of Bergstrom et al. (1973) that PGE_2 can reduce the release of dopamine from nerve terminals of the C.N.S. Hatching substance has the opposite effect, but of course does not belong to the E-series of prostaglandins. It would be interesting to see what effect, if any, hatching substance has on mammalian nerve terminals.

General Conclusions

The hatching substance of Balanus balanoides is a prostaglandin; it is produced by the tissues of the adult. The ability of tissues to synthesize hatching substance is greatly reduced between June and October, but between early November and the following April there is a large increase in biosynthetic capacity, which may be the result of the removal of some, as yet uncharacterized, inhibition. Following the resumption of feeding in the Spring and probably coincident with a moult, hatching substance is secreted into the mantle cavity of the adult. This compound stimulates the release of dopamine within the embryo which induces vigorous muscular movement and results in the hatch of the first stage nauplius. Since

hatching substance is not secreted by starved individuals, but only by those which have been feeding, its release with subsequent liberation of nauplii into food-rich waters is of obvious survival value, particularly in the boreo-arctic region, where the Spring algal bloom is of short duration.

APPENDIX

Levels of phospholipids and neutral lipids in the tissues of whole, adult *B.balanoides*

Levels of phospholipids in the tissues of whole adult *B.balanoides* (shore samples)
as expressed as a percentage composition of the free fatty acids

	DATE COLLECTED										
Fatty Acid	8.6.81	8.7.81	14.8.81	4.9.81	9.10.81	19.11.81	9.12.81	4.1.82	1.2.82	2.3.82	9.4.82
16:0	19.0	21.0	24.2	19.5	21.2	21.0	19.0	23.0	13.4	17.6	18.2
16;1w9	T	0.9	0.3	0.4	0.6	1.8	T	T	T	T	T
16:1w7	9.1	12.7	12.7	8.9	8.1	8.6	7.8	7.3	5.3	5.0	4.7
18:0	3.8	4.0	4.0	4.1	4.1	3,5	2.8	4.8	3.2	3.7	3.1
18:1w9+w11	11.5	11.9	12.7	10.8	11.7	11.9	11.3	11.3	10.8	10.2	8.3
18:1w7	7.6	6.9	8.3	7.5	7.6	11.4	8.8	10.2	9.3	8.9	9.0
18:2w6	0.7	0.6	0.5	0.5	0.5	0.6	0.5	0.4	0.7	0.9	1.1
18:3w3	0.6	0.7	0.5	0.7	0.5	0.6	0.5	0.4	1.0	0.8	2.2
18:4w3	2.0	2.5	1.7	1.6	1.1	0.9	1.3	1.0	1.4	0.7	1.3
20:1w9,w11	2.6	2.1	2.2	2.4	2.5	2.3	2.5	3.2	2.7	2.6	2.3
20:1w7,w5	1.8	1.5	1.6	1.4	1.9	2.3	2.2	2.5	2.1	1.8	1.7
20;2w6	0.2	T	T	T	T	T	T	T	0.2	0.3	0.3
20:3w6	T	T	T	T	T	T	T	T	T	T	T
20:4w6	1.67	1.29	1.91	1.46	1.39	1.35	1.83	1.44	1.90	1.86	1.64
20:5w3	22.6	21.5	20.5	25.5	20.9	19.6	27.8	24.5	28.6	27.8	28.0
22:1w11	T	T	T	T	T	T	T	T	T	T	T
22:1w9	T	T	T	T	T	T	T	T	T	T	T
22:1w7	1.0	1.8	1.3	1.2	2.7	2.2	1.4	2.7	2.1	1.2	1.3
22:5w3	T	T	T	T	T	T	T	T	T	T	T
22:6w3	5.0	3.6	3.8	6.3	5.6	5.2	7.8	3.3	6.2	10.8	10.6
24:1w9	T	T	T	0.4	1.2	T	T	T	0.6	0.5	T

Levels of neutral lipids in the tissues of whole adult B.balanoides (shore samples) as expressed as a percentage composition of the free fatty acids

	DATE COLLECTED										
Fatty Acid	8.6.81	8.7.81	14.8.81	4.9.81	9.10.81	19.11.81	9.12.81	4.1.82	1.2.82	2.3.82	9.4.82
16:0	14.8	16.4	16.2	15.6	15.6	16.2	16.5	19.8	16.8	16.2	15.4
16:1w9	-	-	-	-	-	-	-	-	-	-	T
16:1w7	18.9	21.5	15.4	18.8	16.9	15.3	14.5	22.3	14.0	11.1	13.9
18:0	2.0	1.9	1.9	1.9	1.9	1.7	1.6	2.2	1.4	2.1	2.3
18:1w9+w11	8.8	10.3	10.9	8.7	9.3	11.1	10.3	9.8	10.6	11.2	8.9
18:1w7	5.3	5.3	6.2	5.4	6.3	7.9	8.6	7.6	8.7	7.7	7.6
18:2w6	0.6	0.7	0.8	0.8	0.9	1.0	0.7	0.7	1.2	1.7	1.3
18:3w6	0.3	0.3	0.3	0.4	0.4	0.3	T	0.3	0.3	0.3	0.3
18:3w3	0.8	0.9	0.7	0.9	0.7	0.8	T	0.7	1.1	1.0	1.1
18:4w3	6.2	6.5	5.0	5.9	4.5	4.2	4.1	3.9	4.6	2.6	3.9
20:1w9,w11	1.4	1.5	1.6	1.3	1.6	1.7	1.9	1.5	2.1	2.2	2.1
20:1w7,w5	0.9	1.5	1.1	0.8	1.1	1.2	1.4	1.1	1.6	1.4	1.7
20:2w6	T	T	T	T	T	T	T	T	T	T	T
20:3w6	T	T	T	T	T	T	T	T	T	T	T
20:4w6	0.97	0.59	0.69	0.94	0.90	1.28	1.10	0.70	1.75	1.37	1.26
20:5w3	18.2	17.2	17.0	19.9	22.4	24.3	26.0	17.6	27.9	28.3	25.9
22:1w11	T	T	T	T	T	T	T	T	T	T	T
22:1w9	T	T	T	T	T	T	T	T	T	T	T
22:1w7	0.5	T	T	T	0.3	T	T	0.2	T	0.3	T
22:5w3	T	T	T	T	T	T	T	T	T	T	T
22:6w3	4.7	4.3	5.5	5.9	6.5	7.2	9.2	4.4	3.7	7.0	9.3
24:1w9	T	T	T	T	T	T	T	T	T	T	T

Levels of phospholipids in the tissues of whole adult B.balanoides (starved samples) as expressed as a percentage composition of the free fatty acids

	Approximate period of starvation (months)				
Fatty Acid	1 28.9.81	2 26.10.81	3 27.11.81	4 5.2.82	5 4.3.82
16:0	25.5	23.0	22.1	19.5	18.7
16:1w9	T	T	T	T	T
16:1w7	12.4	10.8	8.3	7.3	5.1
18:0	4.8	4.5	4.6	3.9	4.0
18:1w9,w11	11.2	11.8	11.2	12.1	9.7
18:1w7	9.7	13.6	11.8	13.0	12.4
18:2w6	0.4	0.5	0.4	0.5	0.4
18:3w3	0.5	0.3	0.3	0.6	0.4
18:4w3	1.2	1.2	0.9	1.3	1.0
20:1w9,w11	3.0	2.7	2.6	3.0	3.1
20:1w7,w5	1.9	1.9	2.6	2.0	3.3
20:2w6	T	T	T	T	T
20:3w6	T	T	T	T	T
20:4w6	1.03	1.19	1.29	1.66	1.78
20:5w3	15.5	20.6	22.2	28.0	29.3
22:1w11	T	T	T	T	T
22:1w9	T	T	T	T	T
22:1w7	2.3	T	1.6	0.8	T
22:5w3	T	T	T	T	T
22:6w3	4.7	4.7	5.8	3.2	8.1
24:1w9	T	T	T	T	T

Levels of neutral lipids in the tissues of whole adult *B.balnoides* (starved samples) as expressed as a percentage composition of the free fatty acids

	Approximate period of starvation (months)				
Fatty Acid	1 28.9.81	2 26.10.81	3 27.11.81	4 5.2.82	5 4.3.82
16:0	16.4	16.9	15.7	15.4	16.0
16:1w9	-	-	-	T	T
16:1w7	16.5	20.8	19.9	20.3	19.0
18:0	1.7	1.7	1.5	1.3	1.3
18:1w9,w11	9.4	9.6	10.0	11.8	8.5
18:1w7	7.1	6.7	6.3	3.8	7.2
18:2w6	0.7	0.6	0.7	0.6	0.7
18:3w6	0.4	0.3	0.4	0.3	0.4
18:3w3	0.8	0.7	0.8	0.6	0.7
18:4w3	5.5	5.6	5.7	6.1	5.5
20:1w9,w11	1.6	1.4	1.3	1.2	1.3
20:1w7,w5	1.2	1.1	0.9	1.0	1.0
20:2w6	T	T	T	T	T
20:3w6	T	T	T	T	T
20:4w6	0.73	1.14	1.34	0.81	1.05
20:5w3	23.0	20.0	22.0	22.4	23.3
22:1w11	T	T	T	T	T
22:1w9	T	T	T	T	T
22:1w7	T	T	T	T	T
22:5w3	T	T	T	T	T
22:6w3	6.7	4.7	5.6	5.5	6.2
24:1w9	T	T	T	T	-

REFERENCES

ABRAMOWITZ, A.A., HISAW, F.L., & PAPANDREA, D.N., 1944. The occurrence of a diabetogenic factor in the eyestalks of crustaceans. Biol. Bull., 86 : 1-5.

ACKMAN, R.G., EATON, C.A., & HOOPER, S.N., 1968. Lipids of the fin whale (Balaeonoptera physalus) from North Atlantic waters. IV. Fin whale milk. Can. J. Biochem., 46 : 197-203.

ADELUNG, D., 1969. Die Ausschüttung und Funktion von Hautungshormon wahrend eines zwischenhautungs - Interalls bei der Strandskrabbe, Carcinus maenas L. Z. Naturforsch., 24B : 1447-1455.

ADIYODI, K.G., & ADIYODI, R.G., 1970. Endocrine control of reproduction in decapod Crustacea. Biol. Rev., 45 : 121-165.

ADIYODI, R.G., 1978. Endocrine control of ovarian function in crustaceans. In. P.J. Gaillard & H.H. Boer (Eds.). Comparative Endocrinology : Amsterdam : Elsevier/North Holland Biomedical Press. pp. 25-28.

ALDOUS, M.J., 1968. A study of the fine structure of the nauplius eye. M.Sc. Thesis, University of Wales.

ALEXANDROWICZ, J.S., & CARLISLE, D.B., 1953. Some experiments on the function of the pericardial organs in Crustaceans. J. mar. biol. Ass. U.K., 32 : 175-192.

ALLISON, P., 1981. Aspects of the neurobiology of Balanus hameri. Ph.D. Thesis, University of Wales.

ALMAGOR, E., HILLMAN, P., & MINKE, B., 1979. Upper limit on translational diffusion of visual pigment in unfixed barnacle receptors. Biophys. Struct. Mechanism, 5 : 243-248.

ALTMAN, J.S., & TYRER, N.M., 1980. Filling selected neurons with cobalt through cut axons. In N.S. Strausfield & T.A. Miller (Eds.). Experimental Entomology : Neuroanatomical Techniques. New York : Springer-Verlag. pp. 37-407.

AMAR, R., 1953. Sur l'existence de cellules neurosécrétrices dans le cerreau de Rocinela (Crustacea : Isopoda). Bull. Soc. zool. Fr., 78 : 171-173.

ANDREW, R.D., ORCHARD, I., & SALEUDDIN, A.S.M., 1978. Structural re-evaluation of the neurosecretory system in the crayfish eyestalk. Cell Tissue Res., 190 : 235-246.

ANDREW, R.D., & SALEUDDIN, A.S.M., 1979. Two-dimensional gel electrophoresis of neurosecretory polypeptides in Crustacean eyestalk. J. comp. Physiol., 134B : 303-313.

ANDREWS, P.M., COPELAND, D.E., & FINGERMAN, M. 1971. Ultrastructural study of the neurosecretory granules in the sinus gland of the blue crab, Callinectes sapidus. Z. Zellforsch., 113 : 461-471.

AOTO, T., & NISHIDA, H., 1956. Effect of removal of the eyestalks on growth and maturation of the oocytes in a hermaphroditic prawn, Pandalus kessleri. J. Fac. Sci. Hokkaido Univ. Ser. 6: Zool. 12 : 412-424.

ARÉCHIGA, H., CABRERA-PERALTA, C., & HUBERMAN, A., 1979. Functional characterization of the neuro-depressing hormone in the crayfish. J. Neurobiol., 10 : 409-422.

ARÉCHIGA., H., FUENTES, B., & BARRERA, B., 1973. Circadian rhythm of responsiveness in the visual system of the crayfish. In J. Salanki (Ed.). Neurobiology of Invertebrates : 403-421. Budapest : Publishing House of the Hungarian Academy of Science.

ARÉCHIGA, H., FUENTES-PARDO, B., & BARRERA-MERA, B., 1974a. Influence of retinal shielding pigments on light sensitivity in the crayfish. Acta physiol. latinoam., 24 : 601-611.

ARÉCHIGA, H., & HUBERMAN, A., 1980. Hormonal modulation of circadian behaviour in crustaceans. Front. Hormone Res., 6 : 16-34.

ARÉCHIGA, H., HUBERMAN, A., & MARTINEZ-PALAMO, A. 1977. Release of a neuro-depressing hormone from the crustacean sinus gland. Brain Res., 128 : 93-108.

ARECHIGA, H., HUBERMAN, A., & NAYLOR, E., 1974b. Hormonal modulation of circadian neural activity in Carcinus maenas (L.). Proc. R. Soc. Lond., 187 : 93-108.

ARMSTRONG, P.W., & STEVENSON, P.J. 1979. The effect of ecdysterone on ^{14}C-N-acetylglucosamine incorporation into chitin in the crayfish Orconectes obscurus during the molt cycle. Comp. Biochem. Physiol., 63B : 63-65.

ARVY, L., ECHALIER, G. & GABE, M., 1954. Modifications de la gonade de Carcinides maenas L. après ablation bilatérale de de l'organe Y. C.r. hebd. Séanc. Acad. Sci. Paris, 239 : 1853-1855.

ARVY, L., ECHALIER, G., & GABE, M., 1956. Organe Y et gonade chez Carcinides maenas L. Ann. sci. nat. Zool. biol. animale, 18: 263-267.

ATEMA, J., & GAGOSIAN, R.B., 1973. Behavioural responses of male lobsters to ecdysones. Mar. Behav. Physiol., 2 : 15-20.

ATEMA, J., JACOBSON, S., KARNOFSKY., E., OLESZKO-SZUTS, S., & STEIN, L., 1979. Pair formation in the lobster Homarus americanus : behavioural development, pheromones and mating. Mar. Behav. Physiol., 6: 277-296.

ATZMON, Z., HILLMAN, P., & HOCHSTEIN, S., 1978. Visual response in barnacle photoreceptors is not initiated by transitions to and from metarhodopsin. Nature, Lond., 274 : 74-75.

AUNIS, D., HESKETH, J.E., & DEVILLIERS, G., 1979. Freeze-fracture study of the chromaffin cell during exocytosis: evidence for connections between the plasma membrane and secretory granules and for movements of plasma membrane-associated particles. Cell Tissue Res., 197 : 433-441.

BABU, D.E., SHYAMA SUNDARI, K., & RAO, K.H. 1979. Effect of bilateral ablation of eyestalks on the brain and moulting in the crab Menippe rumphii Fabricus. Indian J. exp. Biol., 17 : 1394-1396.

BAID, I.C., & RAMASWAMI, 1965. Neurosecretory cells in Artemia salina L. Experientia, 21 : 528-529.

BARBACCIA, M.L., BOSIO, A., LUCCHI, L., SPANO, P.F., & TRABUCCHI, M., 1982. Neuronal mechanisms regulating ethanol effects on the dopaminergic system. Life Sciences, 30 : 2163-2170.

BARBACCIA, M.L., REGGIANI, A., SPANO, P.F., & TRABUCCHI, M., 1980. Ethanol effects on dopaminergic function : modulation of the endogenous opioid system. Pharmac. Biochem. Behav., 13 : 303-306.

BARBER, A., 1982. Monoamine-containing varicosities in the neural sheath of a gastropod mollusc demonstrated by glyoxylic acid histofluorescence. Cell Tissue Res., 226 : 267-273.

BARBER, A., 1983. Properties of the serotonergic cerebral ganglion neurons of the gastropod mollusc, Philine aperta. Comp. Biochem. Physiol., 76C : 135-149.

BARGMANN, W., 1949. Uber die neurosekretonische Verknüpfung von Hypothalamus und Neurohypophyse. Klin. Wschr., (1949) : 617-622.

BARNES, H., 1952. The effect of light on the growth rate of two barnacles Balanus balanoides and Balanus crenatus Brug. under conditions of total submergence. Oikos, 4 : 104-112.

BARNES, H., 1955. The hatching process of some barnacles. Oikos, 6 : 114-123.

BARNES, H., 1957. Processes of restoration and synchronization in marine ecology. The spring diatom increase and the "spawning" of the common barnacle Balanus balanoides (L.). Année biol., 33 : 67-85.

BARNES, H., 1962. The composition of the seminal plasma of Balanus balanoides. J. exp. Biol., 39 : 345-351.

BARNES, H., & BARNES, M., 1956. The formation of the egg mass in Balanus balanoides (L.). Arch. Soc. zool.-bot. fenn. Vanamo, 11 : 11-16.

BARNES, H., & BARNES, M., 1959. Note on the stimulation of cirripede nauplii. Oikos, 10 : 19-23.

BARNES, H., BARNES, M., & KLEPAL, W., 1977. Studies on the reproduction of cirripedes. I. Introduction : copulation, release of oocytes and formation of the egg lamellae. J. exp. mar. Biol. Ecol., 27 : 195-218.

BARNES, H., & GONOR, J.J., 1958a. Neurosecretory cells in some cirripedes. Nature, Lond., 181 : 194.

BARNES, H., & GONOR, J.J., 1958b. Neurosecretory cells in the cirripede Polycipes polymerus J.S. Sowerby. J. mar. Res., 17 : 81-102.

BARNES, H., & STONE, R.L., 1972. Suppression of penis development in Balanus balanoides (L.). J. exp. mar. Biol. Ecol., 9 : 303-309.

BARNES, R.D., 1963. Invertebrate Zoology. Ist. ed. Philadelphia : W.B. Saunders Company.

BATHAM, E.J., 1944. Pollicipes spinosus Quoy and Gaimard. I. Notes on biology and anatomy of adult barnacle. Trans. R. Soc. N.Z., 74 : 359-374.

BATHAM, E.J., 1961. Infoldings of nerve fibre membranes in the opisthobranch mollusc Aplysia californica. J. biophys. biochem. Cytol., 9 : 490-492.

BAUCHAU, A.G., 1981. Chapter 12. Crustaceans. In N. A. Ratcliffe & A.F. Rowley (Eds.). Invertebrate Blood Cells. London : Academic Press.

BAUCHAU, A.G., MENGEOT, J.C., & OLIVIER, M.A., 1968. Action de la sérotonine et de l'hormone diabétogène des Crustacés sur la phosphorylase musculaire. Gen. comp. Endocr., 11 : 132-138.

BAUER, R.T., 1979. Sex attraction and recognition in the caridean shrimp Heptacarpus paludicola Holmes (Decapoda : Hippolytidae). Mar. Behav. Physiol., 6 : 157-174.

BAYER, F.M., & WEINHEIMER, A.J., 1974. Prostaglandins from Plexaura homomalla : ecology, utilization and conservation of a major medical marine source. Florida : University of Miami Press. 165pp.

BEATTIE, T.M., 1971. Vital staining of neurosecretory material with acridine orange in the insect Periplaneta americana. Experientia, 27 : 110-111.

BEBBINGTON, P.M., & MORGAN, E.D., 1977. Detection and identification of molting hormone ecdysones in the barnacle Balanus balanoides. Comp. Biochem. Physiol., 56B : 77-79.

BEHRENS, M.E., 1974. Photomechanical changes in the ommatidia of the Limulus lateral eye during light and dark adaptation. J. comp. Physiol., 89 : 45-57.

BEHRENS, M., & KREBS, W., 1976. The effect of light-dark adaptation on the ultrastructure of Limulus lateral eye retinula cells. J. comp. Physiol., 107 : 77-96.

BELAMARICH, F.A., & TERWILLIGER, R.C., 1966. Isolation and identification of cardioexcitor hormone from the pericardial organs of Cancer borealis. Am. Zool., 6 : 1010-106.

BENJAMIN, P.R., SLADE, C.T., & SOFFE, S.R., 1980. The morphology of neurosecretory neurones in the pond snail, Lymnaea stagnalis, by the injection of Procion Yellow and horseradish peroxidase. Phil. Trans. R. Soc. Lond., 290B : 449-478.

BERGSTROM, S., 1967. Prostaglandins : members of a new hormonal system. Science, N.Y., 157 : 382-391.

BERGSTROM, S., FARNEBO, L-O., & FUXE, K., 1973. Effects of prostaglandin E_2 on central and peripheral catecholamine neurons. Eur. J. Pharmacol., 21 : 362-368.

BERLIND, A., 1976. Neurohemal organ extracts affect the ventilation oscillator in crustaceans. J. exp. Zool., 195 : 165-170.

BERLIND, A., 1977. Cellular dynamics on invertebrate neurosecretory systems. Int. Rev. Cytol., 49 : 171 -251.

BESSE, G., 1976. Contribution à l'étude expérimentale de la physiologie sexuelle femelle chez les Crustacés Isopodes terrestres. These Doct. Etat. Poitiers, CNRS AO 13 017 : 261pp.

BESSE, G., & MAISSIAY, J., 1971. Action de la glande de mue sur la vitellogenèse du Crustacé Isopode Porcellio dilatatus (Brandt). C. r. hebd. Séanc. Acad. Sci. Paris, 273D : 1975-1978.

BLANCHET, M.F., PORCHERON, P., & DRAY, F., 1979. Variations du taux des ecdystéroïdes au cours des cycles de mue et de vitellogènese chez le Crustacé Amphipode, Orchestia gammarellus. Int. J. invertebr. Reprod., 1 : 133-139.

BLANCHET-TOURNIER, M.-F., 1982. Some aspects of hormonal interactions between molting and vitellogenesis in Orchestia gammarellus (Pallas) (Crustacea, Amphipoda). Reproduction, nutrition, développement, 22 : 325-344.

BLEST, A.D., 1978. The rapid synthesis and destruction of phtoreceptor membrane by a dinopid spider : a daily cycle. Proc. R. Soc. Lond., 200B : 463-483.

BLEST, A.D., & DAY, W.A., 1978. The rhabdomere organization of some nocturnal pisaurid spiders in light and darkness. Phil. Trans. R. Soc. Lond., 283B : 1-13.

BLEST, A.D., KAO, L., & POWELL, K., 1978. Photoreceptor membrane in the spider Dinopis : the fate of rhabdomere products. Cell Tissue Res., 195 : 425-444.

BLEST, A.D., & MAPLES, J., 1979. Exocytotic shedding and glial uptake of photoreceptor membrane by a salticid spider. Proc. R. Soc. Lond., 204B : 105-112.

BLEST, A.D., & PRICE, D.G., 1981. A new mechanism for transitory, local endocytosis in photoreceptors of a spider, Dinopis. Cell Tissue Res., 217 : 267- 282.

BLEST, A.D., STOWE, S., EDDEY, W., & WILLIAMS, D.S., 1982. The local depletion of a microvillar cytoskeleton from photoreceptors of tipulid flies during membrane turnover. Proc. R. Soc. Lond., 215B : 469-479.

BLEST, A.D., STOWE, S., & PRICE, G.D., 1980. The sources of acid hydrolases for photoreceptor membrane degradation in a grapsid crab. Cell Tissue Res., 205 : 229-244.

BLISS, D.E., 1951. Metabolic effects of sinus gland or eyestalk removal in the land crab, Gecarcinus lateralis. Anat. Rec., 111 : 502-503.

BLISS, D.E., 1953. Neurosecretion and crab metabolism. Anat. Rec., 117 : 599.

BLISS, D.E., 1956. Neurosecretion and the control of growth in a decapod crustacean. In K.G. Wingstrand (Ed). Bertil Hanström, Zoological Papers in Honour of his Sixty-Fifth Birthday, November 20th, 1956. Lund : Zoological Institute. pp. 56-75.

BLISS, D.E., 1968. Transititon from water to land in decapod crustaceans. Am. Zool., 8 : 355-392.

BLISS, D.E., & BOYER, J.R., 1974. Environmental regulation of growth in the decapod crustacean, Gecarcinus lateralis. Gen. comp. Endocr., 4 : 15-41.

BLISS, D.E., DURAND, J.B., & WELSH, J.H., 1954. Neurosecretory systems in decapod Crustacea. Z. Zellforsch., 39 : 520-536.

BLISS, D.E., WANG, S.M.E., & MARTINEZ, E.A., 1966. Water balance in the land crab Gecarcinus lateralis during the intermolt cycle. Am. Zool., 6 : 197-212.

BLISS, D.E., & WELSH, J.H., 1952. The neurosecretory system of brachyuran Crustacea. Biol. Bull., 103 : 157-169.

BOCQUET-VÉDRINE, J., 1965. Étude du tégument et de la mue chez le cirripède operculé Elminius modestus Darwin. Trav. Stn biol. Roscoff (N.S.)., : 16 : 30-75.

BOHM, M.K., & PARKER, R.A., 1968. The fine structure of daphnid supraoesophageal and optic ganglia and its possible functional significance. J. Morph., 126 : 373-393.

BOLLENBACHER, W.E., BORST, D.W., & O'CONNOR, J.D., 1972. Endocrine regulation of lipid synthesis in decapod crustaceans. Am. Zool., 12 : 381-384.

BOLLENBACHER, W.E., GOODMAN, W., VEDECKIS, W.V., & GILBERT, L.I., 1976. The in vitro synthesis and secretion of α -ecdysone by the ring glands of the fly, Sarcophaga bullata. Steroids, 27 : 309-324.

BOLLENBACHER, W.E., & O'CONNOR, J.D., 1973. Production of an ecdysone by crustacean Y-organs in vitro. Am. Zool., 13 : 1274.

BOLLENBACHER, W.E., SMITH, S.L., & GILBERT, L.I., 1978. The biosynthesis of α - and β - ecdysone : in vivo , in vitro and cell free approaches. In P.J. Gaillard & H.H. Boer (Eds.). Comparative Endocrinology : 491-494. Amsterdam : Elsevier/North Holland Biomedical Press.

BOMIRSKI, A., & KLEK, E., 1974. Action of eyestalks on the ovary in Rhithropanopeus harrisii and Crangon crangon (Crustacea : Decapoda). Mar. Biol., 24 : 329-337.

BOUDIER, J.A., BOUDIER, J.L., MASSACRIER, A., CAU, P., & PICARD, D., 1979. Structural and functional aspects of lysosomes in the neurosecretory neurons. Biologie cell., 36 : 185-192.

BOWMAN, W.C., & RAND, M.J., 1980. Textbook of Pharmacology. 2nd ed. Oxford : Blackwell Scientific Publications.

BRESSAC, C., 1976. Étude ultrastructurale de la glande du sinus du crabe Pachygrapsus marmoratus. C. r. hebd. Séanc. Acad. Sci. Paris, 282D : 1529-1531.

BRODIE, D.A., & HALCROW, K., 1977. The ultrastructure of the sinus gland of Gammarus oceanicus (Crustacea : Amphipoda). Cell Tissue Res., 182 : 557-564.

BROWN, F.A., 1946. The source and activity of Crago-darkening hormone (CDH). Physiol. Zoöl., 19 : 215-223.

BROWN, F.A., & CUNNINGHAM, O., 1939. Influence of the sinus gland of crustaceans on normal viability and ecdysis. Biol. Bull., 77 : 104-114.

BROWN, F.A., & EDERSTROM, H.E., 1940. Dual control of certain black chromatophores of Crago. J. exp. Zool., 86 : 53-69.

BROWN, F.A., & JONES, G.M., 1949. Ovarian inhibition by a sinus-gland principle in the fiddler crab. Biol. Bull., 91 : 228-232.

BROWN, H.M., & CARTER CORNWALL, M., 1975. Ionic mechanism of a quasi-stable depolarization in barnacle photoreceptor following red light. J. Physiol., Lond., 248 : 579-593.

BROWN, H.M., HAGIWARA, S., KOIKE, H., & MEECH, R.M. 1970. Membrane properties of a barnacle photoreceptor examined by the voltage clamp technique. J. Physiol.,Lond., 208 : 385-413.

BROWN, H.M., HAGIWARA, S., KOIKE, H., & MEECH, R.W., 1971. Electrical characteristics of a barnacle photoreceptor. Fedn Proc., 30: 69-78.

BROWN, H.M., & OTTOSON, D., 1976. Dual role for potassium in Balanus photoreceptor : antagonist of calcium and suppression of light-induced current. J. Physiol. Lond., 257 : 355-378.

BROWN, H.M., & SAUNDERS, J.H., 1977. Cation and anion sequences in dark-adapted Balanus photoreceptor. J. gen. Physiol., 70 : 531-543.

BRUNET, P.C.J., 1980. The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. Insect Biochem., 10 : 467-500.

BULLOCK, T.H., 1961. On the anatomy of the giant neurons of the visceral ganglion of Aplysia. In E. Florey (Ed.). Nervous Inhibition. Oxford : Pergamon Press.

BULLOCK, T.H., & HORRIDGE, G.A., 1965. Structure and function in the nervous systems of invertebrates. Vols. 1 & 2. San Fransisco : W.H. Freeman.

BUNT, A.H., & ASHBY, E.A., 1967. Ultrastructure of the sinus gland of the crayfish, Procambarus clarkii. Gen. comp. Endocr., 9 : 334-342.

BURDON, R.H., 1976. RNA Biosynthesis. London : Chapman & Hall. 61 pp.

BURSEY, C.R., 1979. Microanatomy of the supraoesophageal ganglion of Munida iris (Decapoda, Galatheidae). Trans. Am. microsc. Soc., 98 : 369-385.

BUSTOS, G., LIBEROWA, J.L., & GYSLING, K., 1981. Regulation of transmitter synthesis and release in mesolimbic dopaminergic nerve terminals. Effect of ethanol. Biochem. Pharmacol., 30 : 2157-2164.

BUTENANDT, A., & KARLSON, P., 1954. Uber die Isotierung eines Metamorphose-Hormons der Insekten in Kristellisierter Form. Z. Naturf., 96 : 389-391.

BYGDEMAN, M,., SVANBORG, K., & SAMUELSSON, B., 1969. A method for determination of prostaglandins in human seminal fluid. Clinica chim. Acta, 26 : 373-379.

CANNON, H.G., 1947. On the anatomy of the pedunculate barnacle Lithotrya. Phil. Trans. R. Soc. Lond., 233B : 89-136.

CAO, C.M.M., CEPERO, E.M., & RODRIGUEZ, I.R., 1975. Identification of prostaglandins in several species of Cuban Gorgoniae. Chem. Abstr., 15540 p.

CARLISLE, D.B., 1953. Studies on Lysmata seticaudata Risso (Crustacea : Decapoda). IV. On the site of origin of the moult-accelerating principle - experimental evidence. Pubbl. Staz. zool. Napoli, 24 : 285-292.

CARLISLE, D.B., 1956. On the hormonal control of water balance in Carcinus. Pubbl. Staz. zool. Napoli, 27 : 227- 231.

CARLISLE, D.B., 1959. On the sexual biology of Pandalus borealis. II. The termination of the male phase. J. mar. biol. Ass. U.K., 38 : 481-492.

CARLISLE, D.B., 1965. The effects of crustacean and locust ecdysones on moulting and proecdysis in juvenile shore crabs, Carcinus maenas. Gen. comp. Endocr., 5 : 366-372.

CARLISLE, D.B., & CONNICK, R.O., 1973. Crustecdysone (20-hydroxy-ecdysone) : site of storage in the crayfish Orconectes propinquus. Can. J. Zool., 51 : 417-420.

CARLISLE, D.B., & DOHRN, P.F.R., 1953. Studies on Lysmata seticaudata Risso (Crustacea Decapoda). II Experimental evidence for a growth and moult-accelerating factor obtainable from eyestalks. Pubbl. Staz. zool. Napoli, 24 : 69-83.

CARLISLE, D.B., & KNOWLES, F.G.W., 1959. Endocrine control in crustaceans. Cambridge : University Press.

CARLISLE, D.B., & PITMAN, W.J., 1961. Diapause, neurosecretion and hormones in Copepoda. Nature, Lond., 190 : 827-828.

CASTEL, M., 1977. Pseudopodia formation by neurosecretory granules. Cell Tissue Res., 175 : 483-497.

CHAIX, J-C., MARVALDI, J., & SECCHI, J., 1981. Variations of ecdysone titers and hemolymph major proteins during the molt cycle of the spider crab Acanthonyx lunulatus. Comp. Biochem. Physiol., 69B : 709-714.

CHAIX, J-C., & REGGI, M. de., 1982. Ecdysteroid levels during ovarian development and embryogenesis in the spider crab Acanthonyx lunulatus. Gen. comp. Endocr., 47 : 7-14.

CHANG, E.S., & BRUCE, M.J., 1981. Ecdysteroid titers of larval lobsters. Comp. Biochem. Physiol., 70A : 239-241.

CHANG, E.S., & O'CONNOR, J.D., 1977. The secretion of α- ecdysone by crab Y -organs in vitro. Proc. natn Acad. Sci. U.S.A., 74 : 615-618.

CHANG, E.S., SAGE, B.A., & O'CONNOR, J.D., 1976. The qualitative and quantitative determinations of ecdysones in tissues of the crab, Pachygrapsus crassipes, following molt induction. Gen. comp. Endocr., 30 : 21-33.

CHARNIAUX-COTTON, H., 1952. Castration chirurgicale chez un Crustacé Amphipods (Orchestia gammarella) et déterminisme des caractères sexuel secondaires. Premier résultats. C. r. hebd. Séanc. Acad. Sci. Paris, 234 : 2570-2572.

CHARNIAUX-COTTON, H., 1954. Découverte chez un Crustacé amphipode (Orchestia gammerella) d'une gland endocrine responsible de la differenciation de caractères sexuel primairs et secondaires mâle. C. r. hebd. Séanc.Acad. Sci. Paris, 239 : 780-782.

CHARNIAUX-COTTON, H., 1957. Croissance régénération et determinisme endocrinien des caractères sexuels d'Orchestia gammarella (Pallas) Crustacé Amphipode. Ann. sci. nat. Zool. biol. animale, 19 : 411-459.

CHARNIAUX-COTTON, H., 1958. Contrôle de la différenciation du sexe et de la reproduction chez les Crustacés supérieurs. Bull. Soc. zool. Fr., 83 : 314-336.

CHARNIAUX-COTTON, H., 1960. Sex determination. In T.H. Waterman (Ed.). The Physiology of Crustacea Vol. 1. London : Academic Press. pp. 411-447.

CHARNIAUX-COTTON, H., 1962. Androgenic gland of crustaceans. Gen. comp. Endocr., (Suppl.). 1 : 241-247.

CHARNIAUX-COTTON, H., & KLEINHOLZ, L.H., 1964. Hormones in invertebrates other than insects. In G. Pincus, K.V. Thimann & E.B. Astwood (Eds.). The Hormones Vol. 4. New York :Academic Press. pp. 135-198.

CHARNIAUX-COTTON, H., ZERBIB, C., & MEUSY, J.J., 1966. Monographie de la glande androgène des Crustacés supérieurs. Crustaceana, 10 : 113-136.

CHATAIGNER, J.P., 1976. Contribution à l'étude des centres neurosécréteurs céphaliques chez les Crustacés Isopodes Cyathura carinata Kr. (Anthuridé) et Sphaeroma serratum Fr. Flabellifère. Thèse Doct. 3ème Cycle, Poitiers.

CHATAIGNER, J.P., MARTIN, G., & JUCHAULT, P., 1978. Etude histologique, cytologique et éxperimentale des centres neurosécréteurs céphaliques du Flabellifère Sphaeroma serratum Fr. (Crustacé Isopode). Gen. comp. Endocr., 35 : 52-69.

CHEUNG, P.J., 1973. Cyclic neurosecretion and its relation to molting in acorn barnacle Balanus eburneus Gould. Ph.D. Thesis, University of New York. 96pp.

CHEUNG, P.J., & NIGRELLI, R.F., 1974. Ecdysterone-induced molting in isolated penis and appendages of barnacle, Balanus eburneus Gould, kept in sterilized sea water. Am. Zool., 14 : 1266.

CHEUNG, T.S., 1966. The development of egg membranes and egg attachment in the shore crab, Carcinus maenas, and some related decapods. J. mar. biol. Ass. U.K., 46 : 373-400.

CHINO, H., GILBERT, L.I., SIDDALL, J.B., & HAFFERL, W., 1970. Studies on ecdysone transport in insect haemolyph. J. Insect Physiol., 16 : 2033-2040.

CHINO, H., SUKURAI, S., OHTAKI, T., IKEKAWA, N., MIYAZAKI, H., ISHIBASHI, M., & ABUKI, H., 1974. Biosynthesis of α - ecdysone by prothoracic glands in vitro. Science, N.Y., 183 : 529-530.

CHRIST, E.J., & van DORP, D.A., 1972. Comparative aspects of prostaglandin biosynthesis in animal tissues. Biochem. biophys. Acta, 270 : 537-554.

CHUECAS, L., & RILEY, J.P., 1969. Component fatty acids of the total lipids of some marine phytoplankton. J. mar. biol. Ass. U.K., 49 : 97-116.

CLARE, A.S., 1980. The organic matrix of calcium phosphate granules from the hepatopancreas of Carcinus maenas (L.). M.Sc. Thesis, University of Wales.

CLARE, A.S., WALKER, G., HOLLAND, D.L., & CRISP, D.J., 1982. Barnacle egg hatching : a novel rôle for a prostaglandin-like compound. Mar. Biol. Lett., 3 : 113-120.

CLAUSE, D-M., & PALZ, F-E., 1959. Das Probleme der Sekretion im Zentralenervensystem der Crustacean. Zool. Anz., 22: (Suppl.). : 92-101.

CLEGG, D.J., 1955. Reproduction in Cirripedia with special reference to Balanus balanoides. M.Sc. Thesis, University of Wales.

COLDSTREAM, J., 1836. In R.B. Todd (Ed.). Cyclopedia of Anatomy and Physiology. London : Sherwood, Gilbert & Piper.

COLLIER, A., RAY, S., & WILSON, W.B., 1956. Some effects of specific organic compounds on marine organisms. Science N.Y., 124 : 220.

COOK, H., 1966. Morphology and histology of the central nervous system of Succinea putris (L.). Arch néerl. Zool., 17 : 1-72.

COOK, P.A., & GABBOT, P.A., 1972. Seasonal changes in the biochemical composition of the adult barnacle, Balanus balanoides, and the possible relationships between biochemical composition and cold-tolerance. J. mar. biol. Ass. U.K., 52 : 805-825.

COREY, E.J., ENSLEY, H.E., HAMBERG, M., & SAMUELSSON, B., 1975. Disparate pathways of prostaglandin biosynthesis in coral and mammalian systems. J. Chem. Soc. Chem. Commun., 1176: 277-278.

COREY, E.J., NIWA, H., FALCK, J.R., MIOSKOWSKI, C., ARAI, Y., & MARFAT, A., 1980. Recent studies on the chemical synthesis of eicosanoids. In B. Samuelsson,P.W. Ramwell & R. Paoletti. (Eds). Advances in Prostaglandin and Thromboxane Research Vol. 6. New York : Raven Press. pp. 19-25.

COREY, E.J., WASHBURN, W.N., & CHEN, J.C., 1973. Studies on the prostaglandin A_2 synthetase complex from Plexaura homomalla. J. Am. chem. Soc., 95 : 2054-2055.

CORNWALL, I.E., 1936. On the nervous system of four British Columbian barnacles (one new species). J. biol. Bd Can., 1 : 469-475.

CORNWALL, I.E., 1953. The central nervous system of barnacles. (Cirripedia). J. Fish. Res. Bd Can., 10 : 76-84.

CORSON, D.W., 1978. On dantrolene sodium and differences in the responses of two invertebrate photoreceptors to calcium-free seawater. Diss. Abstr. 39B : 95.

COSTLOW, J.D., 1963. The effect of eyestalk extirpation on metamorphosis of megalops of the blue crab, Callinectes sapidus Rathbun. Gen. comp. Endocr., 3 : 120-130.

COSTLOW, J.D., 1966a. The effect of eyestalk extirpation on larval development of the mud crab, Rhithropanopeus harrissi (Gould). Gen. comp. Endocr., 7 : 255-274.

COSTLOW, J.D., 1966b. The effect of eyestalk extirpation on larval development of the crab, Sesarma reticulatum Say. In H. Barnes (Ed.). Some Contemporary Studies in Marine Science. New York : Appleton-Century-Crofts. pp. 209-224.

COSTLOW, J.D., & BOOKHOUT, C.G., 1956. Molting and shell growth in Balanus amphitrite niveus. Biol. Bull., 110 : 107-116.

COTTRELL, C.B., 1962a. General observations on the imaginal ecdysis of blowflies. Trans. R. ent. Soc. Lond., 114 : 317-333.

COTTRELL, C.B., 1962b. The imaginal ecdysis of blowflies. Detection of the blood-borne darkening factor and determination of some of its properties. J. exp. Biol., 39 : 418-430.

COTTRELL, C.B., 1962c. The imaginal ecdysis of blowflies. Observations on the hydrostatic mechanism involved in digging and expansion. J. exp. Biol., 39 : 431-448.

COTRELL, C.B., 1962d. The imaginal ecysis of blowflies. Evidence for a change in the mechanical properties of the cuticle at expansion. J. exp. Biol., 39 : 449-458.

CRISP, D.J., 1954. The breeding of Balanus porcatus (da Costa) in the Irish Sea. J. mar. biol. Ass. U.K., 33: 473-494.

CRISP, D.J., 1956. A substance promoting hatching and liberation of yourg in cirripedes. Nature, Lond., 178 : 263.

CRISP, D.J., 1957. Effect of low temperature on the breeding of marine animals. Nature, Lond., 179 : 1138-1139.

CRISP, D.J., 1959. The rate of development of Balanus balanoides (L.). embryos in vitro. J. Anim. Ecol., 28 : 119-132.

CRISP, D.J., 1962. Release of larvae by barnacles in response to the available food supply. Anim. Behav., 10 : 382-383.

CRISP, D.J., 1969. Studies of barnacle hatching substance. Comp. Biochem. Physiol., 30 : 1037-1048.

CRISP, D.J., & PATEL, B.S., 1958. Relation between breeding and ecdysis in cirripedes. Nature, Lond., 181 : 1078-1079.

CRISP, D.J., & PATEL, B.S., 1960. The moulting cycle of Balanus balanoides (L.). Biol. Bull., 118 : 31-47.

CRISP, D.J., & SOUTHWARD, A.J., 1961. Different types of cirral activity of barnacles. Phil. Trans. R. Soc. Lond., 243B : 271-308.

CRISP, D.J., & SPENCER, C.,1958. The control of the hatching process in barnacles. Proc. R. Soc. Lond., 149B : 278-299.

CULKIN, F., & MORRIS, R.J., 1970. The fatty acid composition of two marine filter-feeders in relation to a phytoplankton diet. Deep Sea Res., 17 : 861-865.

CURRY, S.H., BROWN, E.A, KUCK, H., & CASSIN, S., 1982. Preparation and stability of indomethacin solutions. Can. J. Physiol. Pharmacol., 60 : 988-992.

DAHL, R.D., KRISCHER, C.C., MEISSEN, R., & RITTINGHAUS, K.F. 1977. Early receptor potentials and comparison of the electrical properties of median and lateral phtoreceptors of the barnacle. Vision Res., 17 : 519-525.

DANIELS, E.G., 1976. Chromatography of prostaglandins. In G.V. Marinetti (Ed.). Lipid Chromatographic Analysis. 2nd ed. New York : Marcel Dekker Inc., pp. 611-662.

DARWIN, C., 1851. A monograph of the sub-class Cirripedia. Vol. 1. London : Ray Society.

DARWIN, C., 1854. A monograph of the sub-class Cirripedia Vol. 2. London : Ray Society.

DAVIS, C.W., & COSTLOW, J.D., 1974. Evidence for a molt-inhibiting hormone in the barnacle Balanus improvisus Crustacea Cirripedia. J. comp. Physiol., 93 : 85-92.

DEBAISIEUX, P. 1944. Les yeux des Crustacés. Cellule, 50 : 9-122.

DEHORNE, A., 1935. Sure le trophosponge des cellules nerveuses géantes de Lanice conchylega Pallas. C. r. hebd. Séanc. Acad. Sci. Paris, 120 : 1188-1190.

DEMEUSY, N., 1953. Effets de l'ablation des pedoncules oculaires sur le développement de l'appareil génital mâle de Carcinus maenas Pennant. C. r. hebd. Séanc. Acad. Sci. Paris, 236 : 974-975.

DEMEUSY, N., 1962. Role de la glande de mue dans l'évolution ovarienne du crabe Carcinus maenas Linné. Cah. biol. mar., 3 : 37-56.

DEMEUSY, N., 1967a. Crossiance relative d'un caractère sexuels externe male chez la Décapode Brachyoure Carcinus maenas L. C. r. hebd. Séanc. Acad. Sci. Paris, 265 : 568-570.

DEMEUSY, N., 1967b. Modalities d'action du contrôle inhibiteur pedonculaire exercé sur les caractères sexuels externes mâles du Décapode Brachyoure Carcinus maenas L. C. r. hebd. Seanc. Acad. Sci. Paris, 265 : 628-630.

DE MORT, C.L., LOWRY, R., TINSLEU, I., & PHINNEY, H.K., 1972. The biochemical analysis of some estuarine phytoplankton species. I. Fatty acid composition. J. Phyool., 8 : 211-216.

DENNELL, R., 1960. Integument and exoskeleton. In T.H. Waterman (Ed.). The Physiology of Crustacea Vol. I. New York : Academic Press. pp. 449-472.

DESTEPHANO, D.B., & BRADY, U.E., 1977. Prostaglandin and prostaglandin synthetase in the cricket, Acheta domesticus. J. Insect Physiol., 23 : 905-911.

DESTEPHANO, D.B., BRADY, U.E., & WOODALL, L.B., 1976. Partial characterization of prostaglandin synthetase in the reproductive tract of the male house cricket, Acheta domesticus. Prostaglandins, 11 : 261-273.

DICKINSON, R.G., O'HAGEN, J.E., SCHOTZ, M., BINNINGTON, K.C., & HEGARTH, M.P., 1976. Prostaglandin in the saliva of the cattle tick Boophilus microplus. Aust. J. exp. Biol. med. Sci., 54 : 475-486.

DOGRA, G.S., & TANDON, B.K., 1964. Adapatation of certain histological techniques for in situ demonstration of the neurosecretory endocrine system of insect and other animals. Q. J. microsc. Sci., 105 : 455-466.

DRACH, P., 1939. Mue et cycle d'intermue chez les crustacés décapodes. Annls Inst. océanogr. Monaco, 19 : 103-391.

DREIFUSS, J.J., 1975. A review on neurosecretory granules : their contents and mechanisms of release. Ann. N.Y. Acad. Sci., 248 : 184-201.

DUNHAM, P.J., 1978. Sex pheromones in Crustacea. Biol. Rev., 53 : 555-583.

DUNHAM, P.J., & SKINNER-JACOBS, D., 1978. Intermolt mating in the lobster (Homarus americanus). Mar. Behav. Physiol., 5 : 209-214.

DURAND, J.B., 1956. Neurosecretory cell types and their secretory activity in the crayfish. Biol. Bull., 111 : 62-76.

DUSTIN, P., 1978. Microtubules. Berlin : Springer-Verlag. p. 310.

DUVEAU, J., 1961. Données morphologiques sur la voie neuro-sécrétrice orotocéphalique de Nebalia geoffroyi Leach. Bull. Soc. zool. Fr., 86 : 51-58.

ECHALIER, G., 1954. Recherches expérimentales sur le rôle de "l'organ Y" dans la mue de Carcinus maenas (L.). (Crustacé Decapode. C. r. hebd. Séanc. Acad. Sci. Paris, 238 : 523-525.

ECHALIER, G., 1955. Rôle de l'organe Y dans le déterminisme de la mue de Carcinides (Carcinus) maenas L. (Crustacé Decapode) : expériences d'implantation. C. r. hebd. Séanc. Acad. Sci. Paris, 240 : 1581-1583.

EDGINGTON, D.R., & STUART, A.E., 1979. Calcium channels in the high resistivity axonal membrane of photoreceptors of the giant barnacle. J. Physiol. Lond., 294 : 433-445.

EDGINGTON, D.R., & STUART, A.E., 1981. Properties of tetraethylammonium ion-resistant K^+ channels in the photoreceptor membrane of the giant barnacle. J. gen. Physiol., 77 : 629-646.

EGUCHI, E., & WATERMAN, T.H., 1976. Freeze-etch and histochemical evidence for cycling in crayfish photoreceptor membranes. Cell Tissue Res., 169 : 419-434.

ELOFSON, R., 1963. The nauplius eyes and frontal organs in Decapoda (Crustacea). Sarsia, 12 : 1-68.

EMBREY, M.P., 1975. The Prostaglandins in Human Reproduction. Edinburgh : Churchill-Liyingstone.

EMMERICH, H., 1970a. Ecdysonbindende proteinfraktionen in den speicheldrüsen von Drosphila hydei. Z. vergl.Physiol., 68 : 385-402.

EMMERICH, H., 1970b. Uber die hämolymphproteine von Pyrrhocoris apterus und über die binding von ecdyson durch hämolymph proteine. J. Insect Physiol., 16 : 725-747.

ENAMI, M., 1951. The sources and activities of two chromatophorotropic hormones in crabs of the genus Sesarma II. Histology of incretory elements. Biol. Bull., 101 : 241-258.

EULER, U.S. von 1934. Zur Kenntnis der pharmakologischen Wirkungen von Nativesekreten und Extrakten männlichen accessorischer Geschlechtsdrüsen. Arch. exp. Path. Pharmak., 175 : 78-84.

EULER, U.S. von 1935. Uber die spezifische blutdrucksenkende Substanz des menschlichen Prostataund Samen blasensekretes. Klin. Wschr., 14 : 1182-1183.

EVANS, P.D., KRAVITZ, E.A., & TALAMO, B.R., 1976a. Octopamine release at two points along lobster nerve trunks. J. Physiol., Lond., 262 : 71-89.

EVANS, P.D., KRAVITZ, E.A., TALAMO, B.R., & WALLACE, B.G., 1976b. The association of octopamine with specific neurones along lobster nerve trunks. J. Physiol., Lond., 262 : 51-70.

EWEN, A.B., 1962. An improved aldehyde fuschin staining technique for neurosecretory products in insects. Trans. Am. microsc. Soc., 81 : 94-96.

FAGERLUND, U.H.M., & IDLER, D.R., 1957. Marine sterols. IV. 24-dehydrocholesterol : isolation from a barnacle and synthesis by the Whittig reaction. J. Am. chem. Soc., 79 : 6473-6475.

FAHRENBACH, W.H., 1964. The fine structure of a nauplius eye. Z. Zellforsch., 62 : 182-197.

FAHRENBACH, W.H., 1965. The micromorphology of some simple photoreceptors. Z. Zellforsch., 66 : 233-254.

FALES, D.E., 1928. The light receptive organs of certain barnacles. Biol. Bull., 54 : 534-547.

FERREIRA, S.H., MONCADA, S., & VANE, J.R., 1971. Indomethacin and aspirin abolish prostaglandin release from the spleen. Nature, Lond., 231 : 237-239.

FERREIRA, S.H., & VANE, J.R., 1974. New aspects of the mode of action of non-steroid anti-inflammatory drugs. Ann. Rev. Pharmacol., 14 : 57-73.

FINGERMAN, M., & AOTO, T., 1959. The neurosecretory system of the dwarf crayfish Cambarellus shufeldti, revealed by electron and light-microscopy. Trans. Am. microsc. Soc., 78 : 305-317.

FINGERMAN, M., DOMINIEZAK, T., MIYAWAKI, M., & OGURO, C., 1967. Neuroendocrine control of the hepatopancreas in the crayfish Procambarus clarkii. Physiol. Zool., 40 : 23.-30.

FINGERMAN, M., & YAMAMOTO, Y., 1964. Endocrine control of tanning in the crayfish exosekeleton. Sci. Life, 44 : 3625.

FLOWER, R.J., 1974. Drugs which inhibit prostaglandin biosynthesis. Pharmac. Rev., 26 : 33-67.

FOGAL, W., & FRAENKEL, G., 1969. The rôle of bursicon in melanization and endocuticle formation in the adult fleshfly Sarcophaga bullata. J. Insect Physiol., 15 : 1235-1247.

FOLCH, J., LEES, M., & SLOANE-STANLEY, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. biol. Chem., 226 : 497-509.

FRAENKEL, G., & HSIAO, C., 1962. Hormonal and nervous control of tanning in the fly. Science, N.Y., 138 : 27-29.

FRAENKEL, G., & HSIAO, C., 1963. Tanning in the adult fly : a new function of neurosecretion in the brain. Science, N.Y., 141 : 1057-1058.

FRAENKEL, G., HSIAO, C., & SELIGMAN, M., 1966. Properties of bursicon : an insect protein hormone that controls cuticular tanning. Science N.Y., 151 : 91-93.

FREAS, W., 1978. Prostaglandins in a marine bivalve, Modiolus demissus. Ph.D. Dissertation, University of Maryland.

FREAS, W., & GROLLMAN, S., 1980. Ionic and osmotic influences on prostaglandin release from the gill tissue of a marine bivalve, Modiolus demissus. J. exp. Biol., 84 : 169-185.

FREEMAN, J.A., 1980. Hormonal control of chitinolytic activity in the integument of Balanus amphitrite in vitro. Comp. Biochem. Physiol., 65 : 13-17.

FREEMAN, J.A., & COSTLOW, J.D., 1979. Hormonal control of apolysis in barnacle mantle tissue epidermis in vitro. J. exp. Zool., 210 : 333-345.

FREEMAN, J.A., & COSTLOW, J.D., 1980. The molt cycle and its hormonal control in Rhithropanopeus harisii larvae. Devl.Biol., 74 : 479-485.

FRIEDRICH, H., 1969. Marine biology : an introduction to its problems and results. London : Sidgwick & Jackson. 474pp.

FROESCH, D., 1973. A simple method to estimate the true diameter of synaptic vesicles. J. Microsc., 98 : 85-89.

FYHN, U.E.H., FYHN, H.J., & COSTLOW, J.D., 1977. Cirriped vitellogenesis effect of ecdysterone in vitro. Gen. comp. Endocr., 32 : 266-271.

GABE, M., 1953. Sur quelques applications de la coloration par la fuchsine-paraldehyde. Bull. microsc. Appl., 3 : 153-162.

GAGOSIAN, R.B., & BOURBONNIERE, R.A., 1976. Isolation and purification of the molting hormones from the American lobster (Homarus americanus). Comp. Biochem. Physiol., 53B : 155-161.

GAGOSIAN, R.B., BOURBONNIERE, R.A., SMITH, W.B., COUCH, E.F., BLANTON, C., & NOVAK, W., 1974. Lobster molting hormones : isolation and biosynthesis of ecdysterone. Experientia, 30 : 723-724.

GAINER, H., LOH, Y.P., & SARNE, Y., 1977. Biosynthesis of neuronal peptides. In H. Gainer (Ed.). Peptides in neurobiology. New York : Plenum Press. pp. 183-219.

GAINER, H., SARNE, Y., & BROWNSTEIN, M.J., 1977. Neurophysin biosynthesis : conversion of putative precursor during axonal transport. Science, N.Y., 195 : 1354-1356.

GALBRAITH, M.N., HORN, D.H.S., & MIDDLETON, E.J., 1968. Structure of deoxycrustecdysone, a second crustacean moulting hormone. Chem. Commun, 83-85.

GANJIAN, I., LOHER, W., & KUBO, I., 1981. Determination of prostaglandin E_2 in the cricket, Teleogryllus commodus, by reverse phase high performance liquid chromatography. J. Chromatogr., 216 : 380-384.

GARDNER, D., & RILEY, J.P., 1972. Seasonal variations in the component fatty acid distributors of the lipids of Balanus balanoides. J. mar. biol. Ass. U.K., 52 : 839-845.

GERSCH, M., 1976. Biochemistry including mode of cellular action of neurohormones in arthropods. Colloques int. Cent. natn. Rech. scient., 251 : 281-305.

GIBSON, K.H., 1982. Biosynthesis and metabolism of prostaglandins and thromboxanes. In R.F. Newton & S.M. Roberts (Eds.). Prostaglandins and Thromboxanes. London : Butterworth Scientific. pp. 8-18.

GIBSON, R., & BARKER, P.L., 1979. The decapod hepatopancreas. Oceanogr. mar. Biol., 17 : 285-346.

GILBERT, L.I., GOODMAN, W., & BOLLENBACHER, W.E., 1977. Biochemistry of regulatory lipids and sterols in insects. In T.H. Goodwin (Ed.). International Review of Biochemistry. Biochemistry of Lipids II. 14 : 1-50.

GILGAN, M.W., & IDLER, D.R., 1967. The conversion of androstenedione to testerone by some lobster (Homarus americanus Milne Edwards) tissues. Gen. comp. Endocr., 9 : 319-324.

GIORGI, E.P., 1980. The transport of steroid hormones into animal cells. Int. Rev. Cytol., 65 : 49-115.

GIRARDIE, J., & GIRARDIE, A., 1972. Evolution de la radioactivité des cellules neurosécrétrices de la pars intercerebralis chez Locusta migratoria migratoroides (Insecte Orthoptère) apres injection de cystéine S^{35}. Étude autoradiographique aux microscopes optique et électronique. Z. Zellforsch., 128 : 212-226.

GLEESON, R.A., 1980. Pheromone communication in the reproductive behaviour of the blue crab, Callinectes sapidus. Mar. Behav. Physiol., 7 : 119-134.

GOLDBERG, L.I., & KOHLI, J.D., 1981. Agonists and antagonists of peripheral pre- and post-synaptic dopamine receptors : clinical implications. In G.L. Gessa & G.U. Corsini (Eds.). Apomorphine and Other Dopaminomimetics Vol. I. Basic Pharmacology. New York : Raven Press.

GOLDBLATT, M.W., 1933. A depressor substance in seminal fluid. J. Soc. chem. Ind. Lond., 52 : 1056-1057.

GOLDSMITH, T.H., 1962. Fine structure of the retinulae in the compound eye of the honey bee. J. cell Biol., 14 : 489-494.

GOMEZ, R., 1965. Acceleration of development of the gonads by implantation of brain in the crab Paratelphus hydrodromus. Naturwissenschaften, 52 : 216.

GORELL, T.A., & GILBERT, L.I., 1969. Stimulation of protein and RNA synthesis in the crayfish hepatopancreas by crustecdysone. Gen. comp. Endocr., 13 : 308-310.

GORELL, T.A., & GILBERT, L.I., 1971. Protein and nucleic acid synthesis during the crayfish moult cycle. Z. vergl. Physiol., 73 : 345-356.

GORELL, T.A., GILBERT, L.I., & SIDDALL, J.S., 1972a. Binding proteins for an ecdysone metabolite in the crustacean hepatopancreas. Proc. natn.Acad. Sci. U.S.A., 69 : 812-815.

GORELL, T.A., GILBERT, L.I., & SIDDALL, J.B., 1972b. Studies on hormone target issues. Am. Zool., 12 : 347-356.

GORGELS-KALLEN, J.L., & VAN HERP, F., 1981. Localization of crustacean hyperglycemic hormone (CHH) in the X-organ sinus gland complex in the eyestalk of the crayfish, Astacus leptodactylus (Nordmann, 1842). J. Morphol., 170 : 347-355.

GOVINDARAJULU, P., 1968. Neurosecretion and neurosecretory cells in a calanoid copepod Heleodiaptomus sp. Curr. Sci. India, 37 : 672-673.

GRABER, K., 1933. Über die Gehirne der Amphipoden und Isopoden. Z. Morph. Ökol. Tiere, 26 :

GRAFSTEIN, B., & FORMAN, D.S., 1980. Intracellular transport in neurons. Physiol. Rev., 60 : 1167-1283.

GRAVES, S.Y., & DIETZ, T.H., 1979. Prostaglandin E_2 inhibition of sodium transport in the freshwater mussel. J. exp. Zool., 210 : 195-201.

GRAVES, S.Y., & DIETZ, T.H., 1982. Cyclic AMP stimulation and prostaglandin inhibition of Na transport in freshwater mussels. Comp. Biochem. Physiol., 71A : 65-70.

GRAY, P., 1954. The Microtomists' Formulary and Guide. London : Constable & co. 794pp.

GREEN, K., & SAMUELSSON, B., 1964. Prostaglandins and related factors : XIX. Thin layer chromatography of prostaglandins. J. Lipid Res., 5 : 117-120.

GRIPENBERG, J., JANSSON, S-E., HEINANEN, V., HEINONEN, E., HYVARINEN, J., & TOLPPANEN, E-M., 1976. Effect of prostaglandin E_1 on neuromuscular transmission in the rat. Br. J. Pharmac. Chemother., 57 : 387-393.

GROGHAN, P.C., 1958. The mechanism of osmoregulation in Artemia salina (L.) : the physiology of the gut. J. exp. Biol., 35 : 243-249.

GRUVEL, A., 1893. Contribution a l'étude des cirrhipèdes. Archs.Zool. exp. gen., 3 : 401-610.

GRUVEL, A., 1905. Monographie des Cirrhipedes ou Thecostraces. Paris.

GUSTASSON, M.K.S., & WIKGREN, M.C., 1981. Release of neurosecretory material by protrusions of bounding membranes extending through the axolemma, in Diphyllobothrium dendriticum (Cestoda). Cell Tissue Res., 220 : 473-479.

GWILLIAM, G.F., 1962. Electrophysiological concomitants of the shadow reflex in certain barnacles. Biol. Bull., 123 : 498.

GWILLIAM, G.F., 1963. The mechanism of the shadow reflex in Cirripedia. I. Electrical activity in the supraesophageal ganglion and ocellar nerve. Biol. Bull., 125 : 470-485.

GWILLIAM, G.F., 1965. The mechanism of the shadow reflex in Cirripedia. II. Photoreceptor cell response, second order response, and motor cell output. Biol. Bull., 129 : 244-256.

GWILLIAM, G.F., 1976. The mechanism of the shadow reflex in Cirripedia. III. Rhythmical patterned activity in central neurons and its modulation by shadows. Biol. Bull., 151 : 141-160.

GWILLIAM, G.F., & BRADBURY, J.C., 1971. Activity patterns in the isolated central nervous system of the barnacle and their relation to behaviour. Biol. Bull., 141 : 502-513.

GWILLIAM, G.F., & COLE, E.S., 1979. The morphology of the central nervous system of the barnacle, Semibalanus cariosus. (Pallas). J. Morph., 159 : 297-310.

GWINN, J.F., & STEVENSON, J.R., 1973a. Role of acetylglucosamine in chitin synthesis in crayfish. I. Correlation of ^{14}C-acetylglucosamine incorporation with stages of the molting cycle. Comp. Biochem. Physiol., 45B : 769-776.

GWINN, J.F., & STEVENSON, J.R., 1973b. Role of acetyylglucosamine in chitin synthesis in crayfish. II. Enzymes in the epidermis for incorporation of acetylglucosamine into UDP-acetylglucosamine. Comp. Biochem. Physiol., 45B : 777-785.

HACKMAN, R.H., 1974. Chemistry of insect cuticle. In M. Rockstein (Ed.). The Physiology of Insecta. Vol. 6. pp. 216-270.

HACKNEY, C.M., & ALTMAN, J.S., 1982. Cobalt mapping of the nervous system : how to avoid artifacts. J. Neurobiol., 13 : 403- 411.

HAFNER, G.S., HAMMOND-SOLTIS, G., & TOVARSKI, T., 1980. Diurnal changes of lysosome-related bodies in the crayfish photoreceptor cells. Cell Tissue Res., 206 : 319-332.

HAGAN, D.V., & BRADY, U.E., 1982. Prostaglandins in the cabbage looper, Trichoplusia ni. J. Insect Physiol., 28 : 761-765.

HALCROW, K., 1969. Sites of presumed neurosecretory activity in Daphnia magna Straus. Can. J. Zool., 47 : 575-577.

HAMMARSTRÖM, S., 1982. Biosynthesis and biological actions of prostaglandins and thromboxanes. Archs Biochem. Biophys., 214 : 431-445.

HAMPSHIRE, F., & HORN, D.H.S., 1966. Structure of crustecdysone, a crustacean moulting hormone. Chem. Commun., 37-38.

HANANI, M., & SHAW, C., 1977. A potassium contribution to the response of the barnacle photoreceptor. J. Physiol. Lond., 270 : 151-163.

HANSTROM, B., 1933. Neue Untersuchungen über Sinnesorgane und Nervensystem der Crustaceen II. Zool. Jb. Anat. Ontog. Tiere, 56 : 387-520.

HANSTROM, B., 1947. The brain, the sense organs, and the incretory organs of the head in the Crustacea Malacostraca. Kungl. Fysiogr. Sällsk. Handl. N.F., 58 : 1-44.

HARA, J., 1952a. On the hormones regulating the frequency of the heart beat in the shrimp Paratya compressa. Annotnes zool.jap., 25 : 162-171.

HARA, J., 1952b. On the effects of extracts of the grapspid crab, Sesarma picta, and of the head of the pill bug, Armadillidium vulgare, upon the heartbeat of the shrimp, Paratya compressa. Annotnes zool. jap., 25 : 411-414.

HEAP, P.F., JONES, C.W., MORRIS, J.F., & PICKERING, B.T., 1975. Movement of neurosecretory product through the anatomical compartments of the neural lobe of the pituitary gland. Cell Tissue Res., 156 : 483-497.

HEDQVIST, P., 1970. Studies on the effect of prostaglandins E_1 and E_2 on the sympathetic neuromuscular transmission in some animal tissues. Acta physiol. scand., 79 : Suppl. 345 : 1-40.

HENTSCHEL, E., 1965. Neurosekretion und Neurohämalorgan bei Chirocephalus grubei Dybowsci und Artemia salina Leach (Anostraca, Crustacea). Z. wiss. Zool., 171 : 44-79.

HENTSCHEL, E., 1967. Experimentelle Untersuchungen zum Häutungsgeschehen geschlechtsreifer Artemien (Artemia salina Leach, Anostraca, Crustacea). Zool. Jb., 73 : 336-342.

HENTSCHEL, E., 1969. Das neurosekretorische System von Argulus foliaceus L. (Branchiura, Crustacea). Z. wiss. Zool., 180 : 46-53.

HERS, H.G., 1976. The control of glycogen metabolism in the liver. Ann. Rev. Biochem., 45 : 167-189.

HESS, A., 1958. The fine structure of nerve cells and fibres, neuroglia, and sheaths of the ganglion chain in the cockroach (Periplaneta americana). J. biophys. biochem. Cytol., 4 : 731-742.

HETRU, C.C., KAPPLER, C., HOFFMANN, J.A., NEARN, R., BANG, L., & HORN, H.S., 1982. The biosythetic pathway of ecdysone : studies with vitellogenic ovaries of Locusta migratoria (Orthoptera). Mol. Cell Endocr., 26 : 51-80.

HIGGS, G.A., VANE, J.R., HART, R.J., POTTER, C., & WILSON, R.G., 1976. Prostaglandins in the saliva of the cattle tick, Boophilus microplus (Canestrini) (Acarina, Ixodidae). Bull. ent. Res., 66 : 665-670.

HIGHNAM, K.C., & HILL, L., 1977. The Comparative Endocrinology of the Invertebrates. 2nd ed. London : Edward Arnold. 357pp.

HISANO, S., 1976. The ultrastructure of the sinus gland of the freshwater prawn, Palaemon paucidens. J. Fac. Sci. Hokkaido Univ. Ser. 6 :20 : 167-174.

HOARAU, F., & HIRN, M., 1978. Evolution du taux des ecdysteroïdes. au cours du cycle de mue chez Hellevia brevicornis Ebner (Isopode terreste), C. r. hebd. Seanc. Acad. Sci. Paris, 286 : 1443-1446.

HODGE, M., & CHAPMAN, G.B., 1958. Some observations on the fine structure of the sinus gland of the land crab, Gecarcinus lateralis. J. biophys. biochem. Cytol., 4 : 571-574.

HOLMAN, R.T., 1966. In G. Blix (Ed.). Polyunsaturated Fatty Acids as Nutrients. Symp. Swedish Nutrition Foundation IV. pp. 31-42.

HOLTZMAN, E., & MERCURIO, A.M., 1980. Membrane circulation in neurons and photoreceptors : some unresolved issues. Int. Rev. Cytol., 67 : 1-57.

HOPKIN, S.P., 1980. The stucture and function of the hepato-pancreas of the shore crab Carcinus maenas (L.) : a study by electron microscopy and X-ray microanalysis. Ph.D. Thesis, University of Wales.

HOPKINS, S.H., 1944. The external morphology of the third and fourth zoeal stages of the blue crab, Callinectes sapidus Rathbun. Biol. Bull., 87 : 145-152.

HORN, D.H.S., 1971. The ecdysones. In M. Jaconson & D.J. Crosby (Eds.). Naturally Occurring Insecticides. New York : Dekker pp. 333-459.

HORN, D.H.S., MIDDLETON, E.J., & WUNDERLICH, J.A., 1966. Identity of the moulting hormones of insects and crustaceans. Chem. Commun, 339-341.

HORNUNG, D.E., & STEVENSON, J.R., 1971. Changes in the rate of chitin synthesis during the crayfish molting cycle. Comp. Biochem. Physiol., 40B : 341-346.

HORRIDGE, G.A., & BLEST, A.D., 1980. The compound eye. In M. Locke & D. Smith (Eds.). Insect biology in the future. New York : Academic Press. pp. 705-733.

HORRIDGE, G.A., DUNIEL, J., & MARČELJA, L., 1981. A 24-hour cycle in single locust and mantis photoreceptors. J. exp. Biol., 91 : 307-322.

HOYLE, G., 1953. Potassium ions and insect nerve muscle. J. exp. Biol., 30 : 121-135.

HOYLE, G., & SMYTH, T., 1963. Giant muscle fibers in a barnacle Balanus nubilis Darwin. Science, N.Y., 139 : 49-50.

HUBERMAN, A., ARECHIGA, H., CIMET, A., ROSA, J. de la & ARAMBURO, C., 1979. Isolation and purification of a neurodepressing hormone from the eyestalk of Procambarus bouvieri (Ortmann). Eur. J. Biochem., 99 : 203-208.

HUBSCHMAN, J.H., 1963. Development and function of neurosecretory sites in the eyestalks of larval Palaemonetes (Decapoda : Natantia). Biol. Bull., 125 : 96-113.

HUBSCHMAN, J.H., & ARMSTRONG, P.W., 1972. Influence of ecdysterones on molting in Palaemonetes. Gen. comp. Endocr., 18 : 435-438.

HUDSPETH, M.M.P., POO, M.M., & STUART, A.E., 1977. Passive signal propagation and membrane properties in median photoreceptors of the giant barnacle. J. Physiol. Lond., 272 : 25-43.

HUDSPETH, M.M.P., & STUART, A.E., 1977. Morphology and responses to light of the somata axons and terminal regions of individual photoreceptors of the giant barnacle. J. Physiol. Lond., 272 : 1-23.

INOUE, H., 1957. On the neurosecretory cells of Pachygrapsus crassipes. Mem. Gakingei Fac. Akita Univ. nat. Sci., 7 : 84-92.

ITAYA, S.K., 1976. Rhabdom changes in the shrimp, Palaemonetes. Cell Tissue Res., 166 : 265-273.

IVERSON, L.L., 1967. The uptake and storage of noradrenaline in sympathetic nerves. Cambridge : University Press.

JAROS, P.P., 1978. Tracing of neurosecretory neurons in crayfish optic ganglia by cobalt iontophoresis. Cell Tissue Res., 194 : 297-302.

JEGLA, T.C., 1967. Annual cycles in cave organisms, especially crayfish. Am. Zool., 7 : 808-809.

JENKIN, P.M., & HINTON, H.E., 1966. Apolysis in arthropod moulting cycles. Nature, Lond., 211 : 871.

JOHANSEN, J., & FREDERIKSEN, K., 1980. A simple and rapid whole-moult staining method with methyl green-pyronin G applied to a molluscan brain preparation. Stain Technol., 54 : 305-308.

JOHANSSON, A.S., 1957. Neurosecretion and metamorphosis in the milkweed bug, Oncopeltus fasciatus (Dallas). Experientia, 13 : 410-411.

JUCHAULT, P., & KOUIGAN, S., 1975. Contribution à l'étude des systèmes de neurosécrétion céphalique chez l'Oniscoïde Ligia oceanica L. (Crustacé Isopode) : les centres neurosécréteurs protocérébraux et le plexus nerveux latéral. Bull. Soc. zool. Fr., 100 : 459-467.

JUCHAULT, P., & LEGRAND, J.J., 1965. Contribution à l'étude des systèmes neurosécrétion d'Anilocra physodes L. (Crustacé Isopode Cymothoïdé). C. r. hebd. Séanc. Acad. Sci. Paris, 260 : 1491-1494.

KAMEMOTO, F.I., 1976. Neuroendocrinology of osmoregulation in decapod crustacea. Am. Zool., 16 : 141-150.

KAMEMOTO, F.I., KATO, K.H., & TUCKER, L.E., 1966. Neurosecretion and salt and water balance in the Annelida and Crustacea. Am. Zool., 6 : 213-219.

KAMEMOTO, F.I., & TULLIS, R.E., 1972. Hydromineral regulation in decapod Crustacea. Gen. comp. Endocr., Suppl. 3 : 299-307.

KANAZAWA, A., TANAKA, N., TESHIMA, S., & KASHIWADA, K., 1971. Dietary value for ayu Plecoglossus altivelis of rotifer Brachiomus plicatilis cultured with baker's yeast, Saccharomyces cerevisiae supplemented with cuttlefish liver oil. Bull. Jap. Soc. scient. Fish., 37 : 1015-1019.

KARIM, S.M.M., DEVLIN, J., & HILLIER, K., 1968. The stability of dilute solutions of prostaglandins E_1, E_2, F_1 and F_2. Eur. J. Pharmac., 4 : 416-420.

KARIM, S.M.M., & HILLER, K., 1975. In S.M.M. Karim (Ed.). Prostaglandins and Reproduction. Lancaster : Medical and Technical Publications. p. 23.

KARLSON, P., 1955.

3rd Int. Congr. Biochemistry, Brussels, Commun 12 :3.

KARLSON, P., 1956. Chemische Untersuchungen über die Metamorphosehormone der Insekten. Ann. sci. nat. biol-animale, 18 : 125-138.

KARLSON, P., DOENECKE, D., & SEKERIS, C.E., 1976. Intracellular mechanisms of hormone action. In M. Florkin & E.H. Stotz (Eds.). Comprehensive Biochemistry. 25 : 1-63.

KARLSON, P., HOFFMEISTER, H., HOPPE, W., & HUBER, R., 1963. Zur Chemie des Ecdysons. Justus Liebigs Annln Chem., 662 : 1-20.

KARLSON, P., & KOOLMAN, J., 1976. Ecdysone : mode of action, inactivation and elimination. Colloques int. Cent. natn. Rech. scient., 251 : 325-341.

KARLSON, P., & PETERS, G., 1965. Zum Wirkungsmechanisms der Hormone. IV. Der Einfluss des Ecdysons auf den nucleinsaeurestoffwechel von Calliphora larven. Gen. comp. Endocr., 5 : 252-259.

KARLSON, P., SEKERIS, C.E., & SEKERI, K.E., 1962. Zum Tyrosinstoffwechsel der Insekten. VI. Identifizierung von N-Acetyl-1,3, 4-dehydroxy - β- Phenäthylanin (N-Acetyl-dopamine) als Tyrosinmetabolit. Hoppe-Seyler's Z. physiol. Chem., 327 : 86-94.

KATAKURA, Y., FUGIMAKI, Y., & UNNO, K., 1975. Partial purification and characterization of androgenic gland hormone from the isopod crustacean Armadillidium vulgare. Annotnes zool. Jap., 48 : 203-209.

KAURI, T., 1962. On the frontal filaments and nauplius eye in Balanus. Crustaceana, 4 : 131-142.

KAURI, T., 1966. On the sensory papilla X-organ in cirriped larvae. Crustaceana, 11 : 115-122.

KELLER, R., 1965. Uber eine hormonale Kontrolle des Polysaccharidstoffwechsels beim Flusskrebs Cambarus affinis Say. Z. vergl. Physiol., 51 : 49-59.

KELLER, R., 1967. Uber eine hormonale Regulation der Glykogensynthese beim Flusskrebs, Orconectes limosus Rafinesque (Cambarus affinis Say). Zool. Anz. 30 Suppl. : 272-279.

KELLER, R., 1968. Uber Versuche zur Characterisierung des diabetogenen Augenstielhormons des Flusskrebses Orconectes limosus. Verh. dt. zool. Ges., 628-635.

KELLER, R., 1969. Untersuchungen zur Artspezifität eines Crustaceenhormons. Z. vergl. Physiol., 63 : 137-145.

KELLER, R., 1974. Stoffwechselregulation durch Neurohormone bei Crustaceen. Fortschr. Zool., 22 : 34-54.

KELLER, R., 1976. Electrophoretic analysis of neurosecretory substances from the sinus gland of decapod Crustacea. Colloques int. Cent. natn. Rech. scient., 251 : 247-254.

KELLER, R., 1981. Purification and amino acid composition of the hyperglycemic neurohormone from the sinus gland of Orconectes limosus and comparison with the hormone from Carcinus maenas. J. comp. Physiol., 141B : 445-450.

KELLER, R., & ADELUNG, D., 1970. Verglsichende morphogische und physiologische Untersuchungen des Integumentgeswebs und des Häutubgshormongehaltes beim Flusskrebs Orconectes limosus während eines Häutungszyklus. Wilhem Roux Arch. EntwMech. Org., 164 : 209-221.

KELLER, R., & ANDREW, E.M., 1973. The site of action of crustacean hyperglycemic hormone. Gen. comp. Endocr., 20 : 572-578.

KELLER, R., & SEDLMEIER, D., 1978. Hyperglycemic hormones in crustaceans. In P.J. Gaillard & H.H. Boer (Eds.). Comparative Endocrinology. Amsterdam : Elsevier/North Holland Biomedical Press. pp. 437-440.

KELLER, R., & WUNDERER, G., 1978. Purification and amino acid composition of the neurosecretory hyperglycemic hormone from the sinus gland of the shore crab, Carcinus maenas. Gen. comp. Endocr., 34 : 328-335.

KING, D.S., 1964. Fine structure of the androgenic gland of the crab, Pachygrapsus crassipes. Gen. comp. Endocr., 4 : 533-544.

KING, D.S., BOLLENBACHER, W.E., BORST, D.W., VEDECKIS, W.V., O'CONNOR, J.D., ITTYCHERIAH, P.I., & GILBERT, L.I., 1974. The secretion of α -ecdysone by the prothoracic glands of Manduca sexta in vitro. Proc. natn. Acad. Sci. U.S.A., 71 : 793-796.

KING, D.S., & MARKS, E.P., 1974. The secretion and metabolism of α-ecdysone by cockroach (Leucophaea maderae) tissues in vitro. Life Sci., 15 : 147-154.

KING, D.S., & SIDDALL, J.B., 1969. Conversion of α-ecdysone to β-ecdysone by crustaceans and insects. Nature, Lond., 221 : 955-956.

KITTREDGE, J.S., & TAKAHASHI, F.T., 1972. The evolution of sex pheromone communication in the Arthropoda. J. theoret. Biol., 35 : 467-471.

KITTREDGE, J.S., TERRY, M., & TAKAHASHI, F.T., 1971. Sex pheromone activity of the moulting hormone, crustecdysone, on male crabs (Pachygrapsus crassipes, Cancer antennarius, Cancer anthonyi). Fish. Bull. U.S., 69 : 337-343.

KLEINHOLZ, L.H., 1976. Crustacean neurosecretory hormones and physiological specificity. Am. Zool., 16 : 151-166.

KLEINHOLZ, L.H., 1978. Crustacean neurosecretory hormones and their specificity. In P.J. Gaillard & H.H. Boer (Eds.). Comparative Endocrinology. Amsterdam : Elsevier/North Holland Biomedical Press. pp. 397-400.

KLEINHOLZ, L.H., & BOURQUIN, E., 1941. Effects of eyestalk removal on decapod crustaceans. Proc. natn. Acad. Sci. U.S.A., 27 : 145-149.

KLEINHOLZ, L.H., & KELLER, R., 1973. Comparative studies in crustacean neurosecretory hyperglycemic hormones. I. The initial study. Gen. comp. Endocr., 21 : 554-564.

KLEINHOLZ, L.H., & KELLER, R., 1979. Endocrine regulation in Crustacea. In E.J.W. Barrington (Ed.). Hormones and Evolution Vol. 1. New York : Academic Press. pp. 159-213.

KLEINHOLZ, L.H., KIMBALL, F., & McGARVEY, M., 1967. Initial characterization and separation of hyperglycemic (diabetogenic) hormone from the crustacean eyestalk. Gen. comp. Endocr., 8 : 75-81.

KLEK-KAWINSKA, E., & BOMIRSKI, A., 1975. Ovary-inhibiting activity in the shrimp (Crangon crangon) eyestalks during the annual reproductive cycle. Gen. comp. Endocr., 25 : 9-13.

KNOWLES, F.G.W., 1951. Hormone production within the central nervous system of a crustacean. Nature, Lond., 167 : 564.

KNOWLES, F.G.W., 1853. Endocrine activity in the crustacean nervous system. Proc. R. Soc. Lond., 141B : 248-267.

KNOWLES, F.G.W., 1959. The control of pigmentary effectors. In A. Gorbman (Ed.). Comparative Endocrinology. New York : Wiley. pp. 223-232.

KNOWLES, F.G.W., 1965. Neuroendocrine correlations at the level of ultrastructure. Archs Anat. micros. Morph. exp., 54 : 343-357.

KNOWLES, F.G.W., & CARLISLE, D.B., 1956. Endocrine control in the Crustacea. Biol. Rev., 31 : 396-473.

KOIKE, H., BROWN, H.M., & HAGIWARA, S., 1971. Hyperpolarization of a barnacle photoreceptor membrane following illumination. J. gen. Physiol., 57 : 723-737.

KONOK, I., 1960. Studies on the neurosecretory activity of the brain in the freshwater crustacean Astacus leptodactylus Escholz (Decapoda). Annls. Inst. biol. hung. Acad. Sci., 27 : 15-28.

KOOLMAN, J., 1982. Ecdysone metabolism. Insect Biochem., 12 : 225-250.

KRAMER, H., & WINDRUM, G.M., 1955. The metachromatic staining reaction. J. Histochem. Cytochem., 3 : 227-237.

KREBS, W., & SCHATEN, B., 1976. The lateral photoreceptor of the barnacle, Balanus eburneus. Quantitative morphology and fine structure. Cell Tissue Res., 168 : 193-207.

KRUGER, D., 1940. Cirripedia. In Breuns's Tierreich. Bd 5, Abt. 1, Bch. 3, Teil 3, pp. 1-560. Leipzig : Akademische Verlagsges.

KULAKOVSKII, E.E., 1973. On the "neurohaemal organ" in low-organized Crustacea. Proc. Acad. Sci. U.S.S.R., 209 : 246-248.

KUPPERT, P., BUCHLER, M., & SPLINDER, K-D., 1978a. Distribution and transport of moulting hormones in the crayfish Orconectes limosus. Z. Naturf., 33C : 437-441.

KUPPERT, P., DAIG, K., LONDFRSHAUSEN, M., & SPLINDLER, K-D., 1981. Binding of ecdysreroids in crayfishes. In F. Sehnal, A. Zabza, J.J. Menn & B. Cymbrowski (Eds). Regulation of Insect Development and Behaviour Part II. pp. 663-672.

KUPPERT, P.G., & SPINDLER, K-D., 1982. Characterization of nuclear ecdysteroid receptor from crayfish integument. J. Steroid Biochem., 17 : 205-210.

KUPPERT, P., WILHEM, S., & SPLINDER, K-D., 1978b. Demonstration of cytoplasmic receptors for the molting hormones in crayfish. J. comp. Physiol., 128 : 95-100.

KURUP, N.G., 1963. Crustacean hormones. J. Anim. Morph. Physiol., 10 : 113-149.

KURZROK, R., & LIEB, C.C., 1930. Biochemical studies of human semen on the human uterus. Proc. Soc. exp. Biol. Med., 28 : 268-272.

KYER, D.L., 1942. The influence of the sinus glands on gastrolith formation in the crayfish. Biol. Bull., 82 : 68-78.

LACHAISE, F., 1981. Recherches sur les ecdysteroides au cours de la reproduction et du developpement-embryonnaire de Carcinus maenas (L.) (Crustacé Décapode). Thesis, Université Pierre et Marie Curie, Paris.

LACHAISE, F., & HOFFMANN, J.A., 1977. Ecdysone et développement ovarien chez un Décapode, Carcinus maenas. C. r. hebd. Seanc. Acad. Sci. Paris, 285D : 701-704.

LAKE, P.S., 1969a. Changes in the histochemical nature of neurosecretory materials during axonal transport in the crab, Paragrapsus gaimardii. Experientia, 25 : 1314.

LAKE, P.S., 1969b. Neurosecretion in Chirocephalus diaphanus Prevost (Anostraca). I. Anatomy and cytology of the neurosecretory system. Crustaceana, 16 : 273-287.

LAKE, P.S., 1970. Histochemical studies of the neurosecretory system of Chirocephalus diaphanus Prevost (Crustacea : Anostraca). Gen. comp. Endocr., 14 : 1-14.

LAKE, P.S., 1971. Ultrastructural observations of the protocerebrum of Chirocephalus diaphanus Prevost (Branchiopoda, Anostraca), with particular reference to neurosecretion. Crustaceana, 21 : 41-51.

LAKE, P.S., 1973. Trialdehyde fixation of crustacean tissue for electron microscopy. Crustaceana, 24 : 244-246.

LANTZ, R.C., & MAURO, A., 1978. Alteration of sensitivity and time scale in invertebrate photoreceptors exposed to anoxia, dinitrophenol and carbon dioxide. J. gen. Physiol., 72 : 219-231.

LANTZ, R.C., & MILLECCHIA, R.J., 1975. Identification of Gamma-Aminobutyric Acid as a neurotransmitter in the barnacle shadow reflex pathway. Neurosci. Abstr., 1 : 567.

LANTZ, R.C., WONG, F., & MAURO, A., 1977. Liability of the prolonged depolarizing after potential in Balanus photoreceptors. J. gen. Physiol., 70 : 441-452.

LEIDY, J., 1848. On the existence of the eye in the perfect Cirrhipoda. Proc. Acad. nat. Sci. Philadelphia, 4 : 1-2.

LESLIE, C.A., 1976. Prostoglandin biosynthesis and metabolism in rat brain slices. Res. Commn. Chem. Pathol. Pharmac., 14 : 455-469.

LEWIS, P.R., & KNIGHT, D.P., 1977. Staining methods for sectioned material. In A.M. Glauert (ed.). Practical Methods in Electron Microscopy. Amsterdam : Elsevier/ North Holland Biomedical Press.

LIGHT, R.J., & SAMUELSSON, B., 1972. Identification of prostaglandins in the gorgonian, Plexaura homomalla. Eur. J. Biochem., 28 : 232-240.

LINGLE, C., 1981. The modulatory action of dopamine on crustacean neuromuscular preparations. J. exp. Biol., 94 : 285-299.

LITTLE, G., 1969. The larval development of the shrimp, Palemon macrodactylus Rathbun, reared in the laboratory and the effect of eyestalk extirpation on development. Crustaceana, 17 : 69-87.

LLOYD, A.J., & YONGE, C.M., 1940. Correlation between egg-carrying setae and cement glands in decapod crustacea. Nature, Lond., 146 : 334.

LOCHHEAD, J.H., & RESNER, R., 1958. Functions of the eyes and neurosecretion in Crustacea Anastraca. Proc. Int. Congr. Zool., 15 : 397-399.

LOH, Y.P., SARNE, Y., & GAINER, H., 1975. Heterogeneity of proteins synthesized, stored and released by the bag cells of Aplysia californica. J. comp. Physiol., 100 : 283-295.

LOHER, W., 1979. The influence of prostaglandin E_2 on oviposition in Teleogryllus commodus. Ent. exp. appl., 25 : 107-119.

LOHER, W., GANJIAN, I., KUBO, I., STANLEY SAMUELSON, D., & TOBE, S.S., 1981. Prostaglandins: their role in egg laying of the cricket Teleogryllus commodus. Proc. natn. Acad. Sci. U.S.A., 78 : 7835-7838.

LONDERSHAUSEN, M., KUPPERT, P., & SPINDLER, K-D., 1981. Characterization of cytoplasmic ecdysteroid receptors in the hypodermis of the crayfish Orconectes limosus. Mol. cell. Endocr., 24 : 253-265.

LONDERSHAUSEN, M., KUPPERT, P., & SPINDLER, K-D., 1982. Ecdysteroid receptors : a comparison of cytoplasmic and nuclear receptors from crayfish hypodermis. Hoppe-Seyler's Z. physiol. Chem. 363 : 797-802.

LYES, M.C., 1979. The reproductive behaviour of Gammarus duebeni (Lilljeborg), and the inhibitory effect of a surface active agent. Mar. Behav. Physiol., 6 : 47-56.

McCONAUGHA, J.R., 1977. The effects of exogenous 20-hydroxyecdysone on first and third stage zoea of the crab, Cancer anthonyi. Am. Zool., 17 : 899.

McCONAUGHA, J.R., 1979. The effects of 20-hydroxyecdysone on survival and development of first and third stage Cancer anthonyi larvae. Gen. comp. Endocr., 37 : 421-427.

McCONAUGHA, J.R., & COSTLOW, J.D., 1981. Ecdysone regulation of larval crustacean molting. Comp. Biochem. Physiol., 68 : 91-93.

McDONALD-GIBSON, W., 1972. Prostaglandin biosynthesis by skin and effects of anti-inflammatory drugs. J. invest. Derm., 58 : 258.

McGIFF, J.C., 1981. Prostaglandins, prostacyclin and thromboxanes. Ann. Rev. Pharmacol. Toxicol., 21 : 479-509.

McGREGOR, D.B., 1967a. The digestive and neurosecretory systems of barnacles. Ph.D. Thesis, University of London.

McGREGOR, D.B., 1967b. The neurosecretory cells of barnacles. J. exp. mar. Biol. Ecol., 1 : 154-167.

McWHINNIE, M.A., KIRCHENBERG, R.J., URBANSKI, R.J., & SCHWARZ, J.E. 1972. Crustecdysone mediated changes in crayfish. Am. Zool., 12 : 357-372.

McWHINNIE, M.A., & MOHRHERR, C.J., 1970. Influence of eyestalk factors, intermolt cycle and season upon ^{14}C leucine incorporation into protein in the crayfish (Orconectes virilis). Comp. Biochem. Physiol., 34 : 415-437.

MADDRELL, S.H.P., & NORDMANN, J.J., 1979. Neurosecretion. Glasgow : Blackie. 173pp.

MADSEN, N., 1960. The brain of the amphipod Orchestia platensis Krøyer. Crustaceana, 1 : 173-178.

MADSEN, N., 1963. The anatomy of Argulus foliaceus Linné with notes on Argulus coregoni Thorell and Argulus africanus Thiele. Part I. Integument, central nervous system, sense organs, praeoral spine, and digestive organs. Lunds Univ. Arssk., Avd. 2 Bd. 59, 13 : 1-32.

MALHOTRA, S.K., 1957. Cytoplasmic inclusions of the neurones of gastropods. Q. J. microsc. Sci., 98 : 65-77.

MARTIN, G., 1972. Contribution à l'étude ultrastructurale de la gland du sinus de l'oniscoide Porcellio dilatatus Brandt. C. r. hebd. Séanc. Acad. Sci. Paris, 275 : 839-842.

MARTIN, G., 1974. Structure et ultrastructure des systèmes de neurosécrétion protocérébraux des Crustacés Isopodes terrestres. Données expérimentales sur leur fonctionnement. J. Physiol., Paris, 68 : 4B.

MASON, C.A., & BERN, H.A., 1977. Cellular biology of the neurosecretory neuron. In Handbook of Physiology. Section 1. The Nervous System. Vol. 1 Cellular Biology of Neurons Part 1. Maryland : American Physiological Society. pp. 651-689.

MATHIEU-CAPDEROU, C., 1980. Relation between the oocyte maturation and the exuvation in the crustacean Amphipoda Orchestia gammarellus. (Pallas). C. r. hebd. Séanc. Acad. Sci. Paris, 290D : 1495-1498.

MATSUMOTO, K., 1954. Neurosecretion in the thoracic ganglion of the crab Ericheir japonicus. Biol. Bull., 106 : 60-68.

MATSUMOTO, K., 1958. Morphological studies on the neurosecretion in crabs. Biol. J. Okayama Univ., 4 : 103-176.

MATSUMOTO, K., 1959. Neurosecretory cells of an isopod, Armadillidium vulgare Latreille. Biol. J. Okayama Univ., 5 : 43-50.

MAYNARD, D.M., 1962. Organization of neuropile. Am. Zool., 2 : 79-96.

MEGUSAR, F., 1912. Experimente über den Farbwechsel der Crustaceen. (I. Gelasimus. II.Potamobius, III.Palaemontes. IV. Palemon). Wilhem Roux Arch. EntwMech. Org., 33 : 462-665.

MENON, M., 1962. Neurosecretory system of Streptocephalus sp. (Anostraca : Branchiopoda). In H. Heller & R.B. Clark (Eds.). Neurosecretion. London : Academic Press. pp. 411-416.

MEUSY, J.J., 1965. Modification ultrastructurales des glandes androgènes de Carcinus maenas L. (Crustacé Décapode) consecutives de l'ablation des pedoncules oculaires. C. r. hebd. Séanc. Acad. Sci. Paris, 260 : 5901-5904.

MEUSY, J.J., 1968. Précisions nouvelles sur l'ultrastructure de la glande du sinus d'un Crustacé Décapode Brachyoure, Carcinus maenas L. Bull. Soc. zool. Fr., 93 : 291-299.

MEYER-ROCHOW, V.B., 1981. The eye of Orchomene sp. c.f. O. rossi, an amphipod living under the Ross Ice Shelf (Antarctica). Proc. R. Soc. Lond., 212B : 93-111.

MEYER-ROCHOW, V.B., 1982. The divided eye of the isopod Glyptonotus antarcticus : effects of unilateral dark adaptation and temperature elevation. Proc. R. Soc. Lond., 215B : 451-467.

MEYER-ROCHOW, V.B., & TIANG, K.M., 1982. Comparison between temperature-induced changes and effects caused by dark/light adaptation in the eyes of two species of Antarctic crustaceans. Cell Tissue Res., 221 : 625-632.

MEZYKOWSKI, T., & IGNATOWSKA-SWITALSKA, H., 1981. High levels of prostaglandins $PGF_{2\alpha}$ and PGE_2 in Antarctic krill, Euphausia superba Dana. Meeresforsch. 29 : 64-66.

MILLECCHIA, R., & GWILLIAM, G.F., 1972. Photoreception in a barnacle : Electrophysiology of the shadow reflex pathway in Balanus cariosus. Science, N.Y., 177 : 438-441.

MILLER, W.H., & CAWTHON, D.F., 1974. Pigment granule movement in Limulus photoreceptors. Invest. opthalmol. vis. Sci., 13 : 401-405.

MILLS, R.R., MATHUR, R.B., & GUERRA, A.A., 1965. Studies on the hormonal control of tanning in the American cockroach, I. Release of an activation factor from the terminal abdominal ganglion. J. Insect Physiol., 11 : 1047-1053.

MILLS, R.R., MATHUR, R.B., & GUERRA, A., 1968. Correlation of phenoloxidase activity with ecdysis and tanning hormone release in the American cockroach. J. Insect Physiol., 14 : 603-611.

MINKE, B., & KIRSCHFELD, K., 1978. Microspectrophotometric evidence for two photointerconvertible states of visual pigment in the barnacle lateral eye. J. gen. Physiol., 71 : 37-45.

MINNEN, J. van & REICHELT, D., 1980. Effects of photoperiod on the activity of neurosecretory cells in the lateral lobes of the cerebral ganglia of the pond snail Lymnaea stagnalis with particular reference to the canopy cell. Proc. K. ned. Akad. Wet., 83C : 15-24.

MINNEN, J. van & SOKOLOVE, P.G., 1981. Neurosecretory cells in the central nervous system of the giant garden slug, Limax maximus. J. Neurobiol., 12 : 297-301.

MIYAWAKI, M., 1958. On the neurosecretory system of the isopod, Idotea japonica. Annotnes zool. Jap., 31 : 216-221.

MOBBERLY, W.C., 1963. Hormonal and environmental regulation of the molting cycle of the crayfish, Faxonella clypeata. Tulane Stud. Zool., 11 : 79-96.

MORRIS, J.F., & NORDMANN, J.J., 1982. Membrane retrieval by vacuoles after exocytosis in the neural lobe of Brattleboro rats. Neuroscience, 7 : 1631-1639.

MORRIS, R.F., & CULKIN, F., 1976. Marine lipids : analytical techniques and fatty acid ester analysis. Oceanogr. mar. Biol. Ann. Rev., 14 : 391-433.

MORRIS, R.F., & CULKIN, F., 1977. Marine lipids : sterols. Oceanogr. mar. Biol. Ann. Rev., 15 : 73-102.

MORSE, D.E., DUNCAN, H., HOOKER, N., & MORSE, A., 1976. An inexpensive chemical method for the control and synchronous induction of spawning and reproduction in molluscan species important as protein-rich food resources. F.A.O. Fish. Rept. 200 : 291-300.

MORSE, D.E., DUNCAN, H., HOOKER, N., & MORSE, A., 1977. Hydrogen peroxide induces spawning in mollusks, with activation of prostaglandin endoperoxide synthetase. Science, N.Y., 196 : 298-300.

MORSE, D.E., KAYNE, M., TIDYMAN, M., & ANDERSON, S., 1978. Capacity for biosynthesis of prostaglandin-related compounds : distribution and properties of the rate-limiting enzyme in hydrocorals, gorgonians, and other coelenterates of the Carribean and Pacific. Biol. Bull., 154 : 440-452.

MORTLOCK, A.M., 1968. Studies on possible mechanisms for the control of molting and metamorphosis in barnacle larvae. In Biodeterioration of materials : microbiological and allied aspects. New York : Elsevier Publishing Co. pp. 639-657.

MOSCONI-BERNARDINI, P., & PETTENAZZA, D., 1966. Il sistema neuro-secretorio di Triops cancriformis Bosc. Boll. Zool., 33 : 293-300.

MÜLLER, H., MRONGOVIUS, R., & SEYBERTH, H.W., 1981. Improved sample preparation for the quantitative mass spectrometric determination of prostaglandins in biological samples. J. Chromatogr., 226 : 450-454.

MURAMOTO, A., 1978. Relationship between anal uptake of water and anal rhythm in the crayfish. Comp. Biochem. Physiol., 61A : 685-688.

MURAMOTO, A., 1979. Effect of defaecation, animal size and temperature on the anal rhythm in the crayfish. Comp. Biochem. Physiol., 62A : 509-518.

MURAMOTO, A., 1981. Effects of eyestalk extracts and ecdysterone on water intake through the anus of the crayfish. Comp. Biochem. Physiol., 69A : 197-203.

MYKLES, D.L., 1980. The method of fluid absorption at ecdysis in the American lobster, Homarus americanus. J. exp. Biol., 84 : 89-101.

NAGABHUSHANAM, R., & CHINNAYYA, B., 1972. Neurosecretory control of moulting in the prawn, Caridina weberi. Marathwada Univ. J. Sci. Sect. B. Biol. Sci., 11 : 67-77.

NASSEL, D.R., & WATERMAN, T.H., 1979. Massive diurnally modulated photoreceptor membrane turnover in crab light and dark adaptation. J. comp. Physiol., 131A : 205-216.

NEFF, N.H., & YANG, H-Y. T., 1974. Another look at the monoamine oxidases and the monoamine oxidase inhibitor drugs. Life Sci., 14 : 2061-2074.

NEWTON, R.F., & ROBERTS, S.M., 1982. History, nomenclature and potential uses of prostaglandins and thromboxanes in the clinic. In R.F. Newton & S.M. Roberts (Eds.). Prostaglandins and Thromboxanes. London : Butterworth Scientific. pp. 1-7.

NOLTE, A., REINECKE, M., KUHLMANN, D., SPECKMANN, E-J, & SCHULZE, H., 1976. Glial cells of the snail brain some remarks on morphology and function. Neurobiology of Invertebrates. Gastropoda Brain. Tihany. pp. 123-138.

NOMURA, T., & OGATA, H., 1976. Distribution of prostaglandins in the animal kingdom. Biochem. biophys. Acta, 431 : 127-131.

NOMURA, T., OGATA, H., & LUCAS, A., 1979. Mise en evidence de prostaglandines chez des mollusques bivalves. Revue int. Océanogr. méd., 53-54 : 87-93.

NORDMANN, J.J., & LABOUESSE, J., 1981. Neurosecretory granules : evidence for an aging process within the neurohypophysis. Science, N.Y., 211 : 595-597.

NORDMANN, J.J., & MORRIS, J.F., 1980. Depletion of neurosecretory granules and membrane retrieval in the sinus gland of the crab. Cell Tissue Res., 205 : 31-42.

NUSSBAUM, M., 1890. Anatomische Studien an Californischen Cirripedien. Bonn : Max Cohen und Sohn.

O'CONNOR, J.D., & GILBERT, L.I., 1968. Aspects of lipid metabolism in crustaceans. Am. Zool., 8 : 529-539.

O'CONNOR, J.D., & GILBERT, L.I., 1969. Alterations in lipid metabolism associated with premoult activity in a land crab and fresh water crayfish. Comp. Biochem. Physiol., 29 : 889-904.

OERTEL, D., & STUART, A.E., 1981. Transformation of signals by interneurones in the barnacles visual pathway. J. Physiol., Lond., 311 : 127-146.

OGURO, C., 1960a. On the neurosecretory system in the cephalic region of the isopod, Tecticeps japonicus. Endocrinology Jap., 7 : 137-145.

OGURO, C., 1960b. Effects of castration on the neurosecretory system in the cephalic region of the isopod, Tecticeps japonicus. I. Effects on the sinus gland of male animals. Annotnes zool. Jap., 33 : 37-41.

OGURO, C., 1961. On the neurosecretory system of two parasitic isopods, Argeia pugettensis Dana and Athelges japonicus Shiino. Annotnes zool. Jap., 34 : 43.

ONO, K., OSADA, M., MATSUTANI, T., MORI, K., & NOMURA, T., 1982. Gonadal prostaglandin $F_{2\alpha}$ profile during sexual maturation in the oyster, Crassostrea gigas Thunberg. Mar. Biol. Lett., 3 : 223-230.

ŌTSU, T., 1961. The accelerating influence of eyestalk ablation on spermatogenesis and on development of the male genital ducts in young Potamon deharrni. Bull. Yamagata Univ., Ser. Nat. Sci., 5 : 379-383.

ŌTSU, T., 1963. Bihormonal control of sexual cycle in the fresh water crab Potamon dehaani. Embryologia, 8 : 1-20.

OZAWA, S., HAGIWARA, S., & NICOLAYSEN, K., 1977. Neural organization of shadow reflex in a giant barnacle, Balanus nubilus. J. Neurophysiol., 40 : 982-995.

OZAWA, S., HAGIWARA, S., NICOLAYSEN, K., & STUART, A.E., 1976. Signal transmission from photoreceptor to ganglion cells in the visual system of the giant barnacle. Cold Spring Harb. Symp. quant. Biol., 40 : 563-570.

PACE-ASCIAK, C., & WOLFE, L.S., 1968. Inhibition of prostaglandin synthesis by oleic, linoleic and linolenic acids. Biochim. biophys. Acta, 152 : 784-787.

PANGANAMALA, R.V., SHARM, H.M., SPREECHER, H., GEER, J.C., & CORNWELL, D.G., 1974. A suggested role for hydrogen peroxide in the biosynthesis of prostaglandins. Prostaglandins, 10 : 3-11.

PANOUSE, J.B., 1943. Influence de l'ablation du pedoncule oculaire sur la croissance de l'ovaire chez la crevette Leander serratus. C. r. hebd. Séanc. Acad. Sci. Paris, 217 : 553-555.

PANOUSE, J.B., 1947. La glande du sinus et la maturation des produits génitaux chez les crevettes. Bull. biol. Fr. Belg. Suppl. 33 : 160-163.

PARAMESWARAN, R., 1956. Neurosecretory cells of the central nervous system of the crab, Paratelphusa hydrodromous. Q. J. microsc. Sci., 97 : 75-82.

PASSANO, L.M., 1951a. The X-organ, a neurosecretory gland controlling molting in crabs. Anat. Rec., 112 : 460.

PASSANO, L.M., 1951b. The X-organ sinus gland neurosecretory system in crabs. Anat. Rec., 111 : 502.

PASSANO, L.M., 1953. Neurosecretory control of moulting in crabs by the X-organ sinus gland complex. Physiologia comp. Oecol., 3 : 155-189.

PASSANO, L.M., 1960. Molting and its control. In T.H. Waterman (Ed.). The Physiology of Crustacea Vol. I. New York : Academic Press. pp. 473-536.

PATEL, B.S., 1959. The influence of temperature on the reproduction and moulting of Lepas anatifera L. under laboratory conditions. J. mar. biol. Ass. U.K., 38 : 589-597.

PATEL, B., & CRISP, D.J., 1961. Relation between the breeding and moulting cycles in cirripedes. Crustaceana, 2 : 89-107.

PAUL, J.H., & SHARPE, J.S., 1916. Studies on Ca metabolism. I. The deposition of lime salts in the integument of decapod Crustacea. J. Physiol., Lond., 50 : 183-192.

PEARSE, A.G.E., 1968. Histochemistry : theoretical and applied. Vol. I. London : Churchill Livingstone.

PEASE, D.C., 1964. Histological technique for electron microscopy. 2nd ed. New York : Academic Press.

PEEL, M.C., LUCAS, I.A.N., DUCKETT, J.G., & GREENWOOD, A.D., 1973. Studies of sporogenesis in the Rhodophyta I. An association of the nuclei with endoplasmic reticulum in post-meiotic tetraspore mother cells of Corrallina officinalis L. Z. Zellforsch., 147 : 59-74.

PEUTE, J. & KAMER, J.C. van de., 1967. On the histochemical differences of aldehydefuchsin positive material in the fibres of the hypothalamo-hypophyseal tract of Rana temporaria. Z. Zellforsch., 83 : 441-448.

PICAUD, J-L., & SOUTY, C., 1980. Demonstration immunohistochimique de la présence de vitellogénine dans le tissu adipeux et l'hépatopancreas du Crustacé Isopode Oniscoide Porcellio dilatatus (Brandt). C. r. hebd. Séanc. Acad. Sci. Paris, 290 : 123-125.

PIPA, R.L., 1961. Studies on the hexapod nervous system. III. Histology and histochemistry of cockroach neuroglia. J. comp. Neurol., 116 : 15-26.

PITMAN, R.M., 1979. Block intensification of neurones stained with cobalt sulphide : a method for destaining and enhanced contrast. J. exp. Biol., 78 : 295-297.

PITMAN, R.M., TWEEDLE, C.D., & COHEN, M.J., 1972. Branching of central neurones. Intracellular cobalt injection for light and electron microscopy. Science, N.Y., 176 : 412-414.

POLGAR, P., & TAYLOR, L., 1980. Stimulation of prostaglandin synthesis by ascorbic acid via hydrogen peroxide formation. Prostaglandins, 19 : 693-700.

POST, L.C., 1972. Bursicon : its effects on tyrosine permeation into insect haemocytes. Biochim. biophys. Acta, 290 : 424-428.

POST, L.C., & DE JONG, B.J., 1973. Bursicon and the metabolism of tyrosine in the moulting cycle of Pieris larvae. J. Insect Physiol., 19 : 1541-1546.

QUACKENBUSH, L.S., & HERRNKIND, W.F., 1981. Regulation of molt and gonadal development in the spring lobster, Panulirus argus (Crustacea : Palinuridae) : effect of eyestalk ablation. Comp. Biochem. Physiol., 69A : 523-527.

QUINN, G.P., SHORE, P.A., & BRODIE, B.B., 1959. Biochemical and pharmacological studies of RO 1-9569 (tetrabenzine), a non-indole tranquilizing agent with reserpine-like effects. J. Pharmac. exp. Ther., 127 : 103-109.

RAMWELL, P., & SHAW, J.E., 1966. Prostaglandin release from tissues by drug, nerve and hormone stimulation. In Proc. 2nd Nobel Symp., pp. 283-292.

RANGNEKER, P.V., & DESHMUKH, R.D., 1968. Effect of eyestalk removal on the ovarian growth of the marine crab, Scylla serrata (Forskal). J. Anim. Morph. Physiol., 15 : 116-126.

RAO, K.R., FINGERMAN, M., & HAYS, C., 1972. Comparison of the abilities of α-ecdysone and 20-hydroxyecdysone to induce precocious proecdysis and ecdysis in the fiddler crab Uca pugilator. Z. vergl. Physiol., 76 : 270-284.

REDDY, P.S., & RAMAMURTH, R., 1980. Effect of crustacean eye-stalk extracts on carbohydrate levels in the South Indian scorpion Heterometrus fulvipes (Koch). Experientia, 36 : 318-319.

REINHARD, E.G., 1950. An analysis of the effect of a sacculinid parasite on the external morphology of Callinectes sapidus Rathbun. Biol. Bull., 98 : 277-288.

RENAUD, L., 1949. Le cycle des réserves organiques chez les Crustacés Décapodes. Annls Inst. océanogr., Monaco, 24 : 259-357.

REVEL, J.P., 1964. Electron microscopy of glycogen. J. Histochem. Cytochem., 12 : 104-114.

RICHARDS, A., 1951. The Integument of Arthropods. Minneapolis : University of Minnesota Press. 411pp.

ROBERSTON, H.E., & STEELE, J.C., 1974. Octopamine in the insect central nervous system : distribution, biosynthesis and possible physiological role. J. Physiol., Lond., 237 : 34P-35P.

ROSS, W.N., & STUART, A.E., 1976. Regenerative activity in the presynaptic terminal region of the barnacle photoreceptor. Biol. Bull., 151 : 427-428.

RZEPHISHEVSKY, I.K., 1962, Conditions of the mass liberation of the nauplii of the common barnacle, Balanus balanoides (L.), in the Eastern Murman. Int. Revue ges. Hydrobiol. Hydrogr., 47 : 471-479.

SAKURAI, S., IKEKANA, N., OHTAKI, T., & CHINO, H., 1977. 3β-Hydroxy-5α cholestan-6-one : a possible precursor of α-ecdysone biosynthesis. Science N.Y., 198 : 627-628.

SALMON, J.A., & KARIM, S.M.M., 1976. Methods for analysis of prostaglandins. In S.M.M. Karim Ed. . Prostaglandins : Chemical and Biochemical Aspects. Baltimore : University Park Press. pp. 25-87.

SANDEEN, M.I., & COSTLOW, J.D., 1961. The presence of decapod-pigment-activating substances in the central nervous system of representative Cirripedia. Biol. Bull., 120 : 192-205.

SANDOZ, M., & ROGERS, R., 1944. The effect of environmental factors on hatching, moulting, and survival of zoea larvae of the blue crab, Callinectes sapidus Rathbun. Ecology, 25 : 216-228.

SAROJINI, S., 1963. Comparison of the effects of adrogenic hormone and testerone propionate on the female Ocypod crab. Curr. Sci., 32 : 411-412.

SAUNDERS, J.H., & BROWN, H.M., 1977. Liquid and solid-state Cl-sensitive microelectrodes. Characteristics and application to intracellular Cl activity in Balanus photoreceptor. J. gen. Physiol., 70 : 507-530.

SCHAEFER, A., KOMLÓS, M., & SEREGI, A., 1978. Effect of biogenic amines and psychotropic drugs on endogenous prostaglandin biosynthesis in the rat brain homogenates. Biochem. Pharmacol., 27 : 213-218.

SCHARRER, B., 1969. Neurohumors and neurohormones : definitions and terminology. J. neuro-visceral Relations, Suppl. 9 : 1-20.

SCHARRER, B., & WURZELMANN, S., 1977. Neurosecretion. XVI Protrusions of bounding membranes of neurosecretory granules. Cell Tissue Res., 184 : 79-85.

SCHARRER, B., & WURSELMANN, S., 1978. Neurosecretion XVII. Experimentally induced release of neurosecretory material by exocytosis in the insect Leucophaea maderae. Cell Tissue Res., 190 : 173-180.

SCHLOTE, F.W., 1957. Submikroskopische Morphologie von Gastropodennerven. Z. Zellforsch., 45 : 543-568.

SCHNEIDER, W.P., HAMILTON, R.D., & RHULAND, L.E., 1972. Occurrence of esters (15S) - prostaglandin A_2 and E_2 in coral. J. Am. chem. Soc., 94 : 2122-2123.

SCUDAMORE, H.H., 1942. Hormonal regulation of molting and some related phenomena in the crayfish Cambarus immunis. Anat. Rec., 84 : 514-515.

SCUDAMORE, H.H., 1947. The influence of the sinus gland upon molting and associated changes in the crayfish. Physiol. Zoöl., 20 : 187-208.

SEDLMEIER, D., 1982. The mode of action of the crustacean neurosecretory hyperglycemic hormone (CHH). II. Involvement of glycogen synthase. Gen. comp. Endocr., 47 : 426-432.

SEHERIS, C.E., 1974. In N.J. Burdette (Ed.). Invertebrate Endocrinology and Hormonal Heterophylly. New York : Springer-Verlag. pp. 55-86.

SELIGMAN, I.M., 1980. Bursicon. In T.A. Miller (Ed.). Neurohormonal Techniques in Insects. New York : Springer-Verlag. 137-153.

SELIGMAN, I.M., & DOY, F.A., 1972. Studies on cyclic AMP mediation of hormonally induced cytolysis of the alary hypodermal cells and of hormonally controlled DOPA synthesis in Lucilia cuprina. Israel J. Entomol., 7 : 129-142.

SELIGMAN, I.M., FRIEDMAN, S., & FRAENKEL, G., 1969. Bursicon mediation of tyrosine hydroxylation during tanning of the adult cuticle in the fly, Sarcophaga bullata. J. Insect Physiol., 15 : 553-562.

SEREGI, A., SERFŐZŐ, P., ZSUZSANNA, M., & SCHAEFER, A., 1982. On the mechanism of the involvement of monoamine oxidase in catecholamine-stimulated prostaglandin biosynthesis in particulate fraction of rat brain homogenates : role of hydrogen peroxide. J. Neurochem., 38 : 20-27.

SETTY, B.N.Y., & RAMAIAH, T.R., 1979. Isolation and identification of prostaglandins from the reproductive organs of male silkmoth, Bombyx mori L. Insect Biochem., 9 : 613-617.

SETTY, B.N.Y., & RAMAIAH, T.R., 1980. Effect of prostaglandins and inhibitors of prostaglandin biosynthesis on oviposition in the silkmoth Bombyx mori. Indian J. exp. Biol., 18 : 539-541.

SETTY, B.N.Y., & RAMAIAH, T.R., 1982. Effect of PGE_1 on lipid mobilization from pupal fat bodies of the silkworm Bombyx mori. Indian J. Biochem. Biophys., 19 : 115-118.

SEWELL, R.B.S., 1926. A study of Lithotrya nicobarica Reinhardt. Rec. Indian Mus., 28 : 269.

SEYAMA, S., PEARL, G.S., & TAKEI, Y., 1980. Ultrastuctural study of the human neurohypophysis. I. Neurosecretory axons and their dilations in the pars nervosa. Cell Tissue Res., 205 : 253-271.

SHAW, S.R., 1972. Decremental conduction of the visual signal in barnacle lateral eye. J. Physiol., Lond., 220 : 145-175.

SHEARER, T.P., & HUNSICKER, L.G., 1980. A rapid method for embedding tissues for electron microscopy using 1,4 - Dioxane and Polybed 812. J. Histochem. Cytochem., 28 : 465-467.

SHEMESH, M., HADANI, A., SHKLAR, A., SHORE, L.S., & MELEGUIR, F., 1979. Prostaglandins in the salivary glands and reproductive organs of Hyatomma anatolicum excavatum Koch (Acari : Ixodidae). Bull. ent. Res., 69 : 381-385.

SHIVERS, R.R., 1967. Fine structure of crayfish optic ganglia. Univ. Kansas Sci. Bull., 47 : 677-733.

SILVERTHORN, S.U., 1973. Respiration in eyestalkless Uca (Crustacea : Decapoda) acclimated to two temperatures. Comp. Biochem. Physiol., 45A : 417-420.

SILVERTHORN, S.U., 1975. Hormonal involvement in thermal acclimation in the fiddler crab Uca pugilator (Bosc). I. Effect of eyestalk extracts on whole animal respiration. Comp. Biochem. Physiol., 50A : 281-283.

SINET, P.M., HEIKKILA, R.E., & COHEN, G., 1980. Hydrogen peroxide production by rat brain in vivo. J. Neurochem., 34 : 1421-1428.

SKINNER, D.M., 1962. The structure and metabolism of a crustacean integumentary tissue during a molt cycle. Biol. Bull., 123 : 635-647.

SKINNER, D.M., 1965. Amino acid incorporation into protein during the molt cycle of the land crab, Gecarcinus lateralis. J. exp. Zool., 160 : 225-234.

SKINNER, D.M., 1966. Molecular changes associated with the growth of crustacean tissues. Am. Zool., 6 : 235-242.

SMITH, D.S., 1971. On the significance of cross-bridges between microtubules and synaptic vesicles. Phil. Trans. R. Soc. Lond., 261B : 395-405.

SMITH, G., 1974. The ultrastructure of the Organ of Bellonci of Carcinus maenas (Crustacea : Decapoda). Cell Tissue Res., 115 : 127-134.

SMITH, J.B., & WILLIS, A.L., 1971. Aspirin selectivity inhibits prostaglandin production in human platelets. Nature, Lond., 231 : 235-237.

SMITH, M.J.H., & DAWKINS, P.D., 1971. Salicylate and enzymes. J. Pharm. Pharmacol., 23 : 729-744.

SMITH, R.I., 1940. Studies on the effects of eyestalk removal upon young crayfish (Cambarus clarkii) Girard. Biol. Bull., 79 : 145-152.

SMITH, W.L., & LANDS, W.E.M., 1972. Oxygenation of polyunsaturated fatty acids during prostaglandin biosynthesis by sheep vesicular gland. Biochemistry, 11 : 3276-3285.

SOCHASKY, J.B., 1973. Failure to accelerate molting following eyestalk ablation in decapod crustaceans : a review of the literature. Tech. Rept. Fish. Res. Bd Can., 431.

SOUTY, C., BESSE, G., & PICAUD, J-L., 1982. Stimulation par l'ecdysone du taux hémolymphatique de la vitellogénine chez le Crustacé Isopode Porcellio dilatatus Brandt. C. r. hebd. Séanc. Acad. Sci. Paris, 294 : 1057-1059.

SPAZIANI, E., & KATER, S.B., 1973. Uptake and turnover of cholesterol - ^{14}C in Y-organs of the crab Hemigrapsus as a function of the molt cycle. Gen. comp. Endocr., 20 : 534-549.

SPINDLER, K-D., KELLER, R., & O'CONNOR, J.D., 1980. Role of ecdysteroids in the crustacean molting cycle. In J.A. Hoffmann (Ed.). Progress in Ecdysone Research. Amsterdam : Elsevier/North Holland Biomedical Press. pp. 247-280.

SPLINDLER-BARTH, M., BASSEMIR, U., KUPPERT, P., & SPLINDLER, K-D., 1980. Isolation of nuclei from crayfish tissues and demonstration of nuclear ecdysteroid receptors. Z. Naturforsch., 36C : 326-332.

SPURR, A.R., 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. ultrastruct. Res., 26 : 31-45.

STALMAN, W., LALOUX, M., & HERS, H.G., 1974. The interaction of liver phosphorylase a with glucose and AMP. Eur. J. Biochem., 415-427.

STANLEY-SAMUELSON, D.W., & LOHER, W., 1983. Arachidonic acid and other long-chain polyunsaturated fatty acids in spermatophores and spermathecae of Teleogryllus commodus : significance in prostaglandin-mediated reproductive behaviour. J. Insect Physiol., 29 : 41-45.

STEEL, C.G.H., 1977. The neurosecretory system of the aphid Megoura viciae with reference to unusual features associated with long distance transport of neurosecretion. Gen. comp. Endocr., 31 : 307-322.

STEEL, C.G.H., & MORRIS, G.P., 1975. Evidence from X-ray micro-analysis of sulphur rich protein in insect neurosecretion : a new approach to the estimation of neurosecretory activity. Gen. comp. Endocr., 26 : 517-524.

STERBA, G., 1957. Die neurosekretorischen Zellgruppen einiger Cladoceren (Daphnia pulex und magna, Simocephalus vetulus). Zool. Jb. (Anat.), 76 : 303-310.

STEVENSON, J.R., 1972. Changing activities of the crustacean epidermis during the molting cycle. Am. Zool., 12 : 373-380.

STEVENSON, J.R., GUCKERT, R.H., & COHEN, J.D., 1968. Lack of correlation of some proecdysial growth and developmental processes in the crayfish. Biol. Bull., 134 : 160-175.

STEVENSON, J.R., & TUNG, D.A., 1971. Inhibition by actinomycin D of the initiation of chitin biosynthesis in the crayfish. Comp. Biochem. Physiol., 39B : 559-567.

STEWART, W.W., 1978. Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracer. Cell, 14 : 741-759.

STOWE, S., 1980. Rapid synthesis of photoreceptor membrane and assembly of new microvilli in a crab at dusk. Cell Tissue Res., 211 : 419-440.

STRAUSFELD, N.J., & OBERMAYER, M., 1976. Resolution of intra-neuronal and transynaptic migration of cobalt in the insect visual and central nervous systems. J. comp. Physiol., 110 : 1-12.

STREEFKERK, J.G., 1967. Functional changes in the morphological appearance of the hypothalamo-hypophyseal neurosecretory and catecholaminergic neural system, and in the adenohypophysis of the rat. A light, fluorescence and electron microscope study. Amsterdam : Van Soest.

STROLENBERG, G.E.C.M., 1979. Functional aspects of the sinus gland in the neurosecretory system of the crayfish Astacus leptodactylus: an ultrastructural approach. Thesis, Catholic University Nijmegen, The Netherlands. 140 pp.

STROLENBERG, G.E.C.M., BELLON-HUMBERT, C., VAN HERP, F., & VAN WORMHOUDT, A., 1977a. Etude ultrastructurale des granules de neurosécrétion dans la glande du sinus de Palaemon serratus. C. r. hebd. Séanc. Acad. Sci. Paris, 284 : 123-125.

STROLENBERG, G.E.C.M., VAN HELDEN, H.P.M., & VAN HERP, F., 1977b. The ultrastructure of the sinus gland of the crayfish Astacus leptodactylus (Nordmann). Cell Tissue Res., 180 : 203-210.

STROELENBERG, G.E.C.M., & VAN HERP, F., 1977. Mise en évidence du phénomène d'exocytose dans la glande du sinus d'Astacus leptodactylus (Nordmann) sous l'influence d'injections de sérotonine. C. r. hebd. Séanc. Acad. Sci. Paris, 284D : 57-60.

STRONG, J., & LISMAN, J., 1978. Initiation of light adaptation in barnacle photoreceptors. Science, N.Y., 200 : 1485-1487.

STUART, A.E., NICOLAYSEN, K., & HUDSPETH, A.J., 1974. Physiology and anatomy of cells in the visual system of the giant barnacle. Assoc. Res. Vision Ophthalmol. p. 14.

STUART, A.E., & OERTEL, D., 1978. Neuronal properties underlying processing of visual information in the barnacle. Nature, Lond., 275 : 207-290.

STUART, A.E., & POO, M.M., 1975. Decremental signal conduction in the median photoreceptor of the giant barnacle. Assoc. Res. Vision Ophthalmol., p. 28.

STUBBINGS, H.G., 1975. Balanus balanoides. L.M.B.C. Mem., 37.

SVOBODA, J.A., & ROBBINS, W.E., 1968. Desmosterol as a common intermediate in the conversion of a number of C28 and C29 plant sterols to chlosterol by the tobacco hornworm. Experientia, 24 : 1131-1132.

SWINDALE, N.V., & BENJAMIN, P.R., 1976. The anatomy of neuro-secretory neurones in the pond snail Lymnaea stagnalis (L.). Phil. Trans. R. Soc. Lond., 274 : 169-202.

TANAKA, Y., 1978. Spawning induction of the abalone Nordotis gigantea, by chemical control with hydrogen peroxide. Bull. Tokai. Reg. Fish. Res. Lab., 96 : 93-101.

TASAKI, I., 1959. Conduction of the nerve impulse. In J. Field H.W. Magoun & V.E. Hall (Eds.). Handbook of Physiology. Vol. I. Washington : American Physiological Society.

TATCHELL, R.J., & MOOREHOUSE, D.E., 1968. The feeding processes of the cattle tick Boophilus microplus (Canestrini). Part II. The sequence of host tissue changes. Parasitology, 58 : 441-459.

TCHERNIGOVTZEFF, C., 1959. Croissance épithéliale et cycle d' intermue chez Leander serratus (Pennant). C. r. hebd. Séanc. Acad. Sci. Paris, 248 : 600-602.

TELFORD, M., 1975. Blood glucose in crayfish III. The source of glucose and role of the eyestalk factor in hyperglycemia of Cambarus robustus. Comp. Biochem. Physiol., 51B : 69-73.

TEMPLEMAN, W., 1934. Mating in the American lobster. Contr. Can. Biol. Fish., 8 : 423-458.

TESHIMA, S., 1971a. In vivo transformation of ergosterol to cholesterol in crab, Portunus trituberculatus. Bull. Jap. Soc. scient. Fish., 37 : 671-674.

TESHIMA, S., 1971b. Bioconversion of β-sitosterol and 24-methylchlosterol to cholesterol in marine Crustacea. Comp. Biochem. Physiol., 39B : 815-822.

TESHIMA, S., & KANAZAWA, A., 1971a. Bioconversion of the dietary ergosterol to cholesterol in Artemia salina. Comp. Biochem. Physiol., 38B : 603-607.

TESHIMA, S., & KANAZAWA, A., 1971b. Utilization and biosynthesis of sterols in Artemia salina. Bull. Jap. Soc. scient. Fish., 37 : 720-723.

TESHIMA, S., & KANAZAWA, A., 1972. Bioconversion of the dietary brassicasterol to cholesterol in Artemia salina. Bull. Jap. Soc. scient. Fish., 38 : 1305-1310.

TESHIMA, S., & KANAZAWA, A., 1973. In vivo bioconversion of β-sitosterol to cholesterol in the crab, Portunus trituberculatus. Mem. Fac. Fish. Kagoshima Univ., 21 : 91-95.

THOMPSON, M.J., SVOBODA, J.A., KAPLANIS, J.N., & ROBBINS, W.E., 1972. Metabolic pathways of steroids in insects. Proc. R. Soc. Lond., 180B : 203-221.

THREADGOLD, L.T., 1967. The ultrastructure of the animal cell. Oxford : Pergamon Press. 313pp.

TIGHE-FORD, D.J., 1968. A micro-injection technique for barnacles. Crustaceana, 15 : 225-228.

TIGHE-FORD, D.J., 1974. A study of hormone action in barnacles. Ph.D. Thesis, C.N.A.A. London.

TIGHE-FORD, D.J., 1977. Effects of juvenile hormone analogues on larval metamorphosis in the barnacle Elminius modestus Darwin (Crustacea : Cirripedia). J. exp. mar. Biol. Ecol., 26 : 163-176.

TIGHE-FORD, D.J., & VAILE, D.C., 1972. The action of crustecdysone on the cirripede Balanus balanoides. J. exp. mar. Biol. Ecol., 9 : 19-28.

TIPTON, K.F., 1968. The prosthetic groups of pig brain mitochondrial monoamine oxidase. Biochim. biophys. Acta, 159 : 451-459.

TOOKE, N.E., 1982. The role of phospholipids in the cold tolerance of barnacles. Ph.D. Thesis, University of Wales.

TRAVIS, D.F., 1955. The molting cycle of the spiny lobster, Panulirus argus Latreille. II. Pre-ecdysial histological and histochemical changes in the hepatopancreas and integumental tissues. Biol. Bull., 108 : 88-112.

TULLIS, R.E., & KAMEMOTO, F.I., 1974. Separation and biological effects of CNS factors affecting water balance in the decapod crustacean Thalamita crenata. Gen. comp. Endocr., 23 : 19-28.

TYRER, N.M., & BELL, E.M., 1974. The intensification of cobalt-filled neurone profiles using a modification of Timm's sulphide-silver method. Brain Res., 73 : 151-155.

VACCA, L.L., & FINGERMAN, M., 1975. The mechanism of tanning in the fiddler crab, Uca pugilator. II. The cyclic appearance of tanning agents and attached carrier proteins in the blood during the moulting cycle. Comp. Biochem. Physiol., 51B : 483-488.

van den BOSCH de AGUILAR, Ph., 1969. Nouvelles données morphologiques et hypothèses sur le rôle du système neurosécréteur chez Daphnia pulex (Crustacea : Cladocera). Annls Soc. r. zool. Belg., 99 : 27-44.

van den BOSCH de AGUILAR, Ph., 1972. Les caractéristiques tinctoriales des cellules neurosécrétrices chez Daphnia pulex (Crustacea, Cladocera). Gen. comp. Endocr., 18 : 140-145.

van den BOSCH de AGUILAR, Ph., 1973a. Le système neurosécréteur de l'Argulus foliaceus (L.). Crustaceana, 23 : 123-132.

van den BOSCH de AGUILAR, Ph., 1973b. Le système neurosécreteur de Balanus perforatus and B. balanoides. 7th Conf. European Comparative Endocrinologists, Budapest, Hungary, Abstract 9.

van den BOSCH de AGUILAR, Ph., 1976. Etude histochemique du système neurosécréteur de Balanus perforatus et B. balanoides (Crustacea : Cirripedia). Gen. comp. Endocr., 30 : 228-230.

van den BOSCH de AGUILAR, Ph., 1979. Neurosecretion in the Entomostraca crustaceans. La Cellule, 73 : 29-48.

VANE, J.R., 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature, Lond., 231 : 232-235.

VANE, J.R., FLOWER, R.J., & SALMON, J.A., 1982. Inhibitors of arachidonic acid metabolism with especial reference to the aspirin-like drugs. In Prostaglandins and Cancer : First Int. Conf. : New York : Alan R. Liss. pp. 21-45.

VAN HERP, F., & STROLENBERG, G.E.C.M., 1980. Functional aspects of the neurosecretory system in the eyestalk of the crayfish Astacus leptodactylus with special reference to the hyperglycemic hormone. Gen. comp. Endocr., 40 :364.

VAN HERP, F., & van BUGGENUM, H.J.M., 1979. Immunocytochemical localization of hyperglycemic hormone (HGH) in the neurosecretory system of the eyestalk of the crayfish Astacus leptodactylus. Experientia, 35 : 1527-1528.

VASANTHA, N., GANGOTRI, M.S., & VENKATACHARI, S.A.T., 1979. Neuroendocrine control of respiratory metabolism in the freshwater crab, Barytelphusa guerni Milne Edwards. Indian J. exp. Biol., 17 : 974-976.

VIANCOUR, T.A., BITTNER, G.D., & BALLINGER, M.L., 1981. Selective transfer of Lucifer Yellow CH from axoplasm to adaxonal glia. Nature, Lond., 293 : 65-67.

VINCENT, J.F.V., 1971. The effects of bursicon on cuticular properties in Locusta migratoria migratorioides. J. Insect Physiol., 17 : 625-636.

VINCENT, J.F.V., 1972. The dynamics of release and the possible identity of bursicon in Locusta migratoria migratorioides. J. Insect Physiol., 18 : 757-780.

VINCENT, J.F.V., & HILLERTON, J.E., 1979. The tanning of insect cuticle - a critical review and a revised mechanism. J. Insect Physiol., 25 : 653-658.

VOGT, W., 1963. Pharmacologically active acidic phospolipids and glycolipids. Biochem. Pharmac., 12 : 415-420.

VON BUDDENBROCK, W., 1930. Untersuchungen über den Schattenreflex. Z. vergl. Physiol., 13 : 164-213.

WALDOCK, M.J., 1979. The fatty acid composition of triacylglycerols of oysters, mussels and barnacles. Ph.D. Thesis, University of Wales.

WALKER, G., 1974. The fine structure of the frontal filament complex of barnacle larvae(Crustacea : Cirripedia). Cell Tissue Res., 152 :449-465.

WALKER, G., 1980. A study of the oviducal glands and ovisacs of Balanus balanoides (L.), together with comparative observations on the ovisacs of Balanus hameri (Ascarius) and the reproductive biology of the two species. Phil. Trans. R. Soc. Lond., 291B : 147-162.

WALKER, G., 1983. A study of the ovigerous fraena of barnacles. Proc. R. Soc. Lond., 218B : 425-442.

WALKER, G., & FOSTER, P., 1979. Seasonal variation of zinc in the barnacle Balanus balanoides (L.) maintained on a raft in the Menai Strait. Mar. Environ. Res., 2 : 209-221.

WALLEY, L.J., 1965. The development and function of the oviducal gland in Balanus balanoides. J. mar. biol.Ass. U.K., 45 : 115-128.

WALLEY, L.J., 1969. Studies on the larval structure and metamorphosis of Balanus balanoides (L.). Phil. Trans. R. Soc. Lond., 256B : 237-280.

WALLEY, L.J., WHITE, F., & BRANDER, K.M., 1971. Sperm activation and fertilization in Balanus balanoides. J. mar. biol. Ass. U.K., 51 : 489-494.

WANG, D.H., & SCHEER, B.T., 1963. UDPG- glycogen transglucosylase and a natural inhibitor in crustacean tissues. Comp. Biochem. Physiol., 9 : 263-274.

WEATHERBY, T.M., 1981. Ultrastructural study of the sinus gland of the crab, Cardisoma carnifex. Cell Tissue Res., 220 : 293-312.

WEINHEIMER, A.J., & SPRAGGINS, R.L., 1969. The occurrence of two new prostaglandin derivatives (15 epi-PGA_2 and its acetate methyl ester) in the gorgonian Plexaura homomalla. Tetrahedron Lett., 59 : 5185-5188.

WEITZMAN, M., 1969. Ultrastructural study on the release of neurosecretory material from the sinus gland of the land crab, Gecarcinus lateralis. Z. Zellforsch., 94 : 147-154.

WELSH, J.H., 1961. Neurohumors and neurosecretion. In T.H. Waterman (Ed.). The Physiology of Crustacea Vol. II. New York : Academic Press pp: 281-311.

WENDELAAR BONGA, S.E., 1970. Ultrastructure and histochemistry of neurosecretory cells and neurohaemal areas in the pond snail Lymnaea stagnalis (L.). Z. Zellforsch., 108 : 190-224.

WESTPHAL, U., 1971. Steroid-Protein Interactions. Berlin : Springer-Verlag. 567pp.

WEYGOLDT, P., 1961. Zur Kenntnis der Sekretion im Zentral-nervensystem der Ostrakoden Cyprideis littoralis (G.S.Brady) (Podocopa Cytheridae) und Cypris puberba (O.F.M.) (Podocopa Cytheridae). Neurosekretion und Sekretzellen im Perineurium. Zool. Anz., 166 : 69-79.

WHILLIS, J.H., 1982. Studies on intestinal co-ordination in the plaice, Pleuronectes platessa L. M.Sc. Thesis, University of Wales.

WHITE, K.N., 1978. Excretion in cirripedes. Ph.D. Thesis, University of Wales.

WHITNEY, J.O., 1970. Absence of sterol biosynthesis in the blue crab, Callinectes sapidus Rathbun and in the barnacle, Balanus nubilus Darwin. J. exp. mar. Biol. Ecol., 4 : 229-237.

WICKRAMASINGHE, J.A.F., & SHAW, S.R., 1973. Separation of prostaglandins A, B and C by thin-layer chromatography on ferric chloride impregnated silica gel. Prostaglandins, 4 : 903-914.

WIGGLESWORTH, V.B., 1959. The histology of the nervous system of an insect, Rhodnius prolixus (Hemiptera). Q. J. microsc. Sci., 100 : 299-314.

WIGGLESWORTH, V.B., 1960. The nutrition of the central nervous system in the cockroach Periplaneta americana L. The role of perineurium and glial cells in the mobilization of reserves. J. exp. Biol., 37 : 500-512.

WILLIAMS, D.S., & BLEST, A.D., 1980. Extracellular shedding of photoreceptor membrane in the open rhabdom of a tipulid fly. Cell Tissue Res., 205 : 423-438.

WILLIG, A., & KELLER, R., 1973. Molting hormone content, cuticle growth and gastrolith growth in the molt cycle of the crayfish Orconectes limosus. J. comp. Physiol., 86 : 377-388.

WILLIG, A., & KELLER, R., 1976. Biosynthesis of α- and β-ecdysone by the crayfish Orconectes limosus in vivo and by its Y-organs in vitro. Experientia, 32 : 936-937.

WOLFE, L.S., 1982. Eicosanoids : prostaglandins, thromboxanes, leukotrienes, and other derivatives of carbon-20 unsaturated fatty acids. J. Neurochem., 38 : 1-14.

WOLFE, L.S., PAPPIUS, H.M., & MARION, J., 1976a. The biosynthesis of prostaglandins by brain tissue in vitro. In B. Samuelsson & R. Paoletti (Eds.). Advances in Prostaglandin and Thromboxane Research Vol. I. New York : Raven pp. 345-355.

WOLFE, L.S., ROSTOROWSKI, K., & PAPPIUS, H.M., 1976b. The endogenous biosynthesis of prostaglandins by brain tissue in vitro. Can.J. Biochem., 54 : 629-640.

WOLFF, J.R., & GULDNER, F.H., 1970. Uber die Ultrastruktur des Nervus opticus und des Ganglion opticum von Daphnia pulex. Z. Zellforsch., 103 : 526-543.

WORTHINGTON, R.E., BRADY, U.E., THEAN, J.E., & WILSON, D.M., 1981. Arachidomic acid : occurrence in the reproductive tract of the male house cricket (Acheta domesticus) and field cricket (Gryllus spp). Lipids, 16 : 79-81.

YONGE, C.M., 1955. Egg attachment in Crangon vulgaris and other Caridea. Proc. R. Soc. Edinb., 65B : 369-400.

YULE, A.B., 1982. The application of new techniques to the study of planktonic organisms. Ph.D. Thesis, University of Wales.

ZELENY, C., 1905. Compensatory regulation. J. exp. Zool., 2 : 1-101.