Title Aspects of the Biology of Polar Pycnogonids

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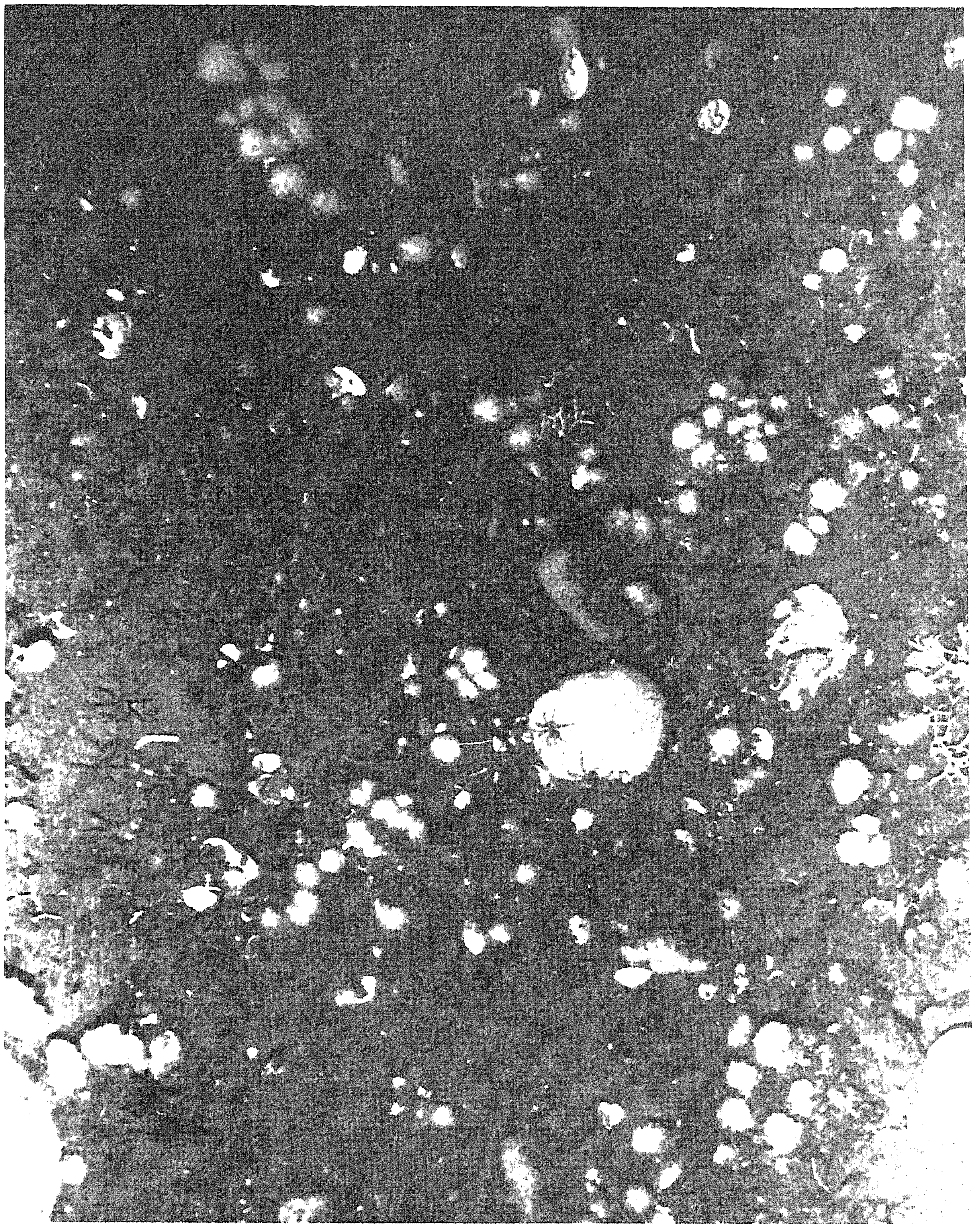

Frontispiece A one metre square area of the Arctic ocean floor, taken at 200 metres depth off Spitzbergen. In the original print at least 30 pycnogonids can be discerned. ( Photograph kindly supplied by Mr. R. Blacker, Lowestoft.-Crown copyright).

# ASPECTS OF THE BIOLOGY OF POLAR PYCNOGONIDS

BY

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SUBMITTED TO THE COUNCIL FOR NATIONAL ACADEMIC AWARDS IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

LUTON COLLEGE OF TECHNOLOGY

OCTOBER 1976.

DECLARATION

This work has not been accepted in substance for any other degree and is not concurrently being submitted in canditature for any other degree.

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## ABSTRACT

The internal morphology of fixed specimens of Antarctic pycnogonids was examined. Theories postulated during the course of these histological studies were then tested and modified by observations on live material and specimens fixed specially for histochemistry on visits both to the Arctic and Antarctic. Live material was also transported back to Britain from these regions and cultured in refrigerated marine aquaria.

The digestive system was studied in considerable detail. It is suggested that digestion is intracellular with gut cells changing their morphology during their lifetime. Embryo cells develop into absorptive cells which at some stage take up a glandular appearance but not a glandular function. There are therefore two gut cell types, 'Embryo' and 'Absorptive/glandular'; this is in disagreement with some previous authors who separate the latter. The role of the gut cell in the light of present day lysosome theory is discussed and a re-interpretation of work by previous authors suggested. It is found that the digestive process is slow and the prey tastes of the species studied, catholic. Furthermore, it is found that some species can survive for long periods without appearing to feed. Suggestions are made as to the significance and mechanisms of these phenomena.

Mass transport in the body cavities is considered and compared with that of *Hydra*, an animal with which previous authors have made comparisons; - their philosophy is questioned. Blood flow, heartbeat and intestine movements are also considered and suggestions for future studies made. The role of blood itself is studied

and a possible clotting system described. Preliminary experiments on blood electrophoresis and chromatography indicate that such techniques may be useful in clarifying some complexities of pycnogonid classification and might provide a means by which future workers in the field might better link nutritional state, mass transport, digestion and external environment conditions.

# ACKNOWLEDGEMENTS.

With such a peripatetic project, the task of distributing acknowledgements is difficult. Though this thesis and its' faults are my own, it has been made possible only by the kindness and co-operation of many people. The omission of any individual from these pages is due to poor memory rather than ungratefulness or failure to recognise their aid.

My main and everlasting thanks must go to my senior supervisor Bill Fry, who introduced me to pycnogonids, and then subtly coaxed work from one whose preferred occupation is 'resting'. To him I respectfully dedicate the better parts of this 'Curate's egg'.

The bulk of the laboratory work was carried out in the Science Department at Luton College of Technology and my thanks must go to Mr. John Howard, the head of that Department, and his entire staff, lecturing and technical, for the happy atmosphere they provided. Messrs.Keay, Froggatt, Witty, Crisp, Zacks, Taylor, Hetherington, Thomas and Ashton all helped me overcome occasional excesses of abstinence and provided stimulating discussion, usually completely unconnected with pycnogonids.

Fieldwork was carried out in conjunction with British Antarctic Survey. Whilst thanking that body as a whole, I must thank in particular its' Director, Dr R.M.Laws and senior marine biologists Dr. Inigo Everson and Mr. Martin White. On Signy Island I must thank Messrs. Hoogesteger, Kellet, Hastings, Collinge and Bissell who dived for specimens. I must also thank the other base members simply for suffering me, I trust their recovery is now complete.

My travel to the Arctic was arranged through M.A.A.F. Lowestoft and my thanks are due here to its' Director for

permitting my trip, and particularly to Mr Reg. Blacker who played a major role in the arranging, was Chief Scientist on my cruise and made the frontispiece plate available.

Neither Arctic nor Antarctic journeys would have been possible without some form of ocean going transport and my thanks are therefore due to the Captains and Crews of R.V. Cirolana and R.R.S. John Biscoe. I quote Captain Sir Edward Belcher in their honour - ' I do conscientiously assert that the greatest credit is due to the fine moral crew which it was my good fortune to command, and return to this country without even the threat of the lash'.

Transmission electron microscopy was carried out using the facilities of the Zoology Department of Oxford University. My thanks are due to Professor J.W.S.Pringle for permitting this and to Mrs. Carol Van Horn for her help. Dr P.J.C. Brunet of that Department kindly made himself available to act as a second supervisor.

My final thanks go to my three typists, Mrs. Linda Jeeves, Mrs. Maggie Norris, and Miss Tanzey Doyle who typed at great pace under trying conditions.

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# CHAPTER 1

# INTRODUCTION

# PROLOGUE

This work contributes to a research programme on polar Pycnogonida instigated at Luton by my supervisor, Mr W. G. Fry. Initially, the specimens available for study had been collected by British Antarctic Survey (B.A.S.) personnel at Signy Island, South Orkneys during the austral summer of 1970/71. The material had been fixed in Bouin's fluid and stored in 70% alcohol. My work started in September 1971 and in December 1972 I was able to collect Arctic pycnogonids when on board R.V.Cirolana on a fisheries cruise to the West Coast of Greenland. For the austral summer of 1973/74 I visited Signy Island and was able to collect Antarctic pycnogonids myself.

The original B.A.S. collected material proved to be an almost pure sample of the species Nymphon australe (Hodgson 1902). Material collected by B.A.S. personnel in the austral summer of 1972/73 had Nymphon orcadense (Hodgson 1908) as the predominant species. This was also the predominant species when I collected there in the summer of 1973/74. The main Arctic collection was of Nymphon hirtipes (Bell 1853).

These species of Nymphonidae (mainly the Antarctic ones) are the main concern of this work. Mention is also made of other polar pycnogonids which were studied when the opportunity arose. These were Ammothea carolinensis (Leach 1814), Decolopoda australis (Eights 1834) and Colossendeis wilsoni (Calman 1915) in the Antarctic and Colossendeis proboscidea (Sabine 1824) in the Arctic. On both Arctic and Antarctic trips very many

specimens were collected and these remain available in Luton for further study.

Work on N. australe and N. hirtipes was almost entirely with preserved material, most of the fresh material work on the Nymphonidae was with N. orcadense. The main emphasis of this thesis is on Antarctic forms.

## INTRODUCTION

'If one were asked to design a Thing from Outer Space, one might do worse than take a pycnogonid as his model.' (Meglitsch 1967). It is sufficient to say that Meglitsch was not the first to notice the eccentric appearance of pycnogonids. More scientifically, they are arthropods, but their relationship to other arthropods is obscure and the subject of continuing debate. Their appearance is spider-like and this has led to their common name of 'Sea-spiders', one which unfortunately causes the non-specialist to immediately classify them as Arachnids. It is suggested that the common name of Sea-spiders is only marginally more useful than the name 'Starfish' of which Hyman (1955) so disapproved. A less confusing common name but which the layman does no find so descriptive is 'Pycnogon'.

Strom and Linnaeus placed the species of pycnogonid they identified with the long-legged harvest spiders in the genus Phalangium. It was Latreille (1804) who first used the term 'Pycnogonides' which means 'crowded knees' (Snodgrass 1952). Other names applied to the group have been 'Podosomata' (Leach 1815), 'Pychnogonides' or 'Crustaces Areiniformes' (Milne-Edwards 1834), 'Crustacea Haustillata' (Johnston 1837) and 'Pantopoda' (Gerstaecker 1863). Some authors (notably Russian and German) still prefer the name Pantopoda for the group. Hedgpeth (1954) divided the Pycnogonida into two orders, Pantopoda for the living forms, Palaeopantopoda for fossils.

Pycnogonids are entirely marine and mostly holobenthic and are found from the littoral zone down to 6,860 metres (Belyaev 1966). Size varies from an adult leg span of 3mm for small littoral species up to 50 cms adult span in some abyssal forms (Hedgpeth 1947). (The largest specimen in the British Museum, Colossendeis colossea Wilson, has a span of 56cm). Although Fry (1964) has suggested that pycnogonids may attain large numbers in various parts of the world and D'Arcy Thompson (1909) stated that they are 'omnipresent in the sea', their main concentration is in the polar regions (see Frontispiece). Hodgson (1907), when referring to N. australe, stated that 'the Headquarters of these animals appears to be in the Southern Seas.'

PYCNOGONIDS AND POLAR EXPLORATION

Research on pycnogonids and polar exploration have long had a special relationship. Strom (1762) was the first to describe and figure a pycnogonid (Pycnogonum littorale). As early as 1766, Konig was collecting Arctic specimens from around the coast of Iceland (Calman 1929). Goodsir, an early British worker on pycnogonids had the unfortunate distinction of perishing with Franklin in his search for the North-West Passage. Goodsir published on British pycnogonids, but it will never be known whether collections of Arctic pycnogonids were lost with him. A contemporary of Goodsir, the American James Eights was, in some ways, more fortunate. He travelled to the Antarctic with Palmer and was the first to describe the ten-legged Antarctic pycnogonid Decolopoda australis

(the usual number of pycnogonid walking legs is eight). Eights' fate was a melancholy one; his paper was either ignored or his description thought to apply to a 'monstrosity'. Hedgpeth (1971) reprinted Eights' original description of Decolopoda australis together with a 'Brief life'. D. australis was rediscovered by Hodgson (1905b), working on material collected by the Scottish National Antarctic Expedition of W. S. Bruce. On the first Discovery Expedition three years earlier, Hodgson himself collected another species of pycnogonid which had ten legs. Fittingly, as a first contribution towards the rehabilitation of the American Eights, Hodgson's ten-legged form was discovered on July 4th (1902). Edward Wilson mentions the event in his diary and at the same time throws some light on the nature of research in the Antarctic and on the degree of seriousness which might profitably be applied to research. "After dinner took the heights of all hands on the mess deck. Not a man on the mess deck measures six feet. Hodgson this evening discovered a strikingly new animal among his various beasts - a Nymphon or Pycnogonid or Sea Spider, with five pair of legs instead of four, a thing hitherto unknown."

With the early twentieth century rush for the South Pole in which Wilson himself was tragically concerned, there was a similar rush to print. Great competition for precedence of names developed as new species of pycnogonid were found. The classification has yet to recover from the chaos this created.

Crapp (1968) attempted to list, (revised to the most recent nomenclature), all species mentioned in the literature to that date. This list totals 563 species. Hedgpeth (1947) divided the living pycnogonids into eight families. These are listed below with the number of species in each (according to Crapp) alongside. Whilst these figures are the most recent available it must be stated that pycnogonid classification is in constant flux, being much subject to the contrasting activities of 'lumpers' and 'splitters'. A thorough revision is needed but the task is awesome and away from the direction that this thesis has taken.

Families of pycnogonids

| Family | Species | |
|---|---|---|
| AMMOTHEIDAE | 140 | |
| COLOSSENDEIDAE | 56 | |
| ENDEIDAE | 9 | |
| PALLENIDAE | 105 | |
| NYMPHONIDAE | 134 | |
| PHOXICHILIDIIDAE | 53 | |
| PYCNOGONIDAE | 28 | |
| TANYSTYLIDAE | 22 ) | TANYSTYLIDAE in Hedgpeth 1947 |
| AUSTRODECIDAE | 16 ) | |

FIGURE 1B Illustrates some of the terminology of Fry and Hedgepeth (1969).

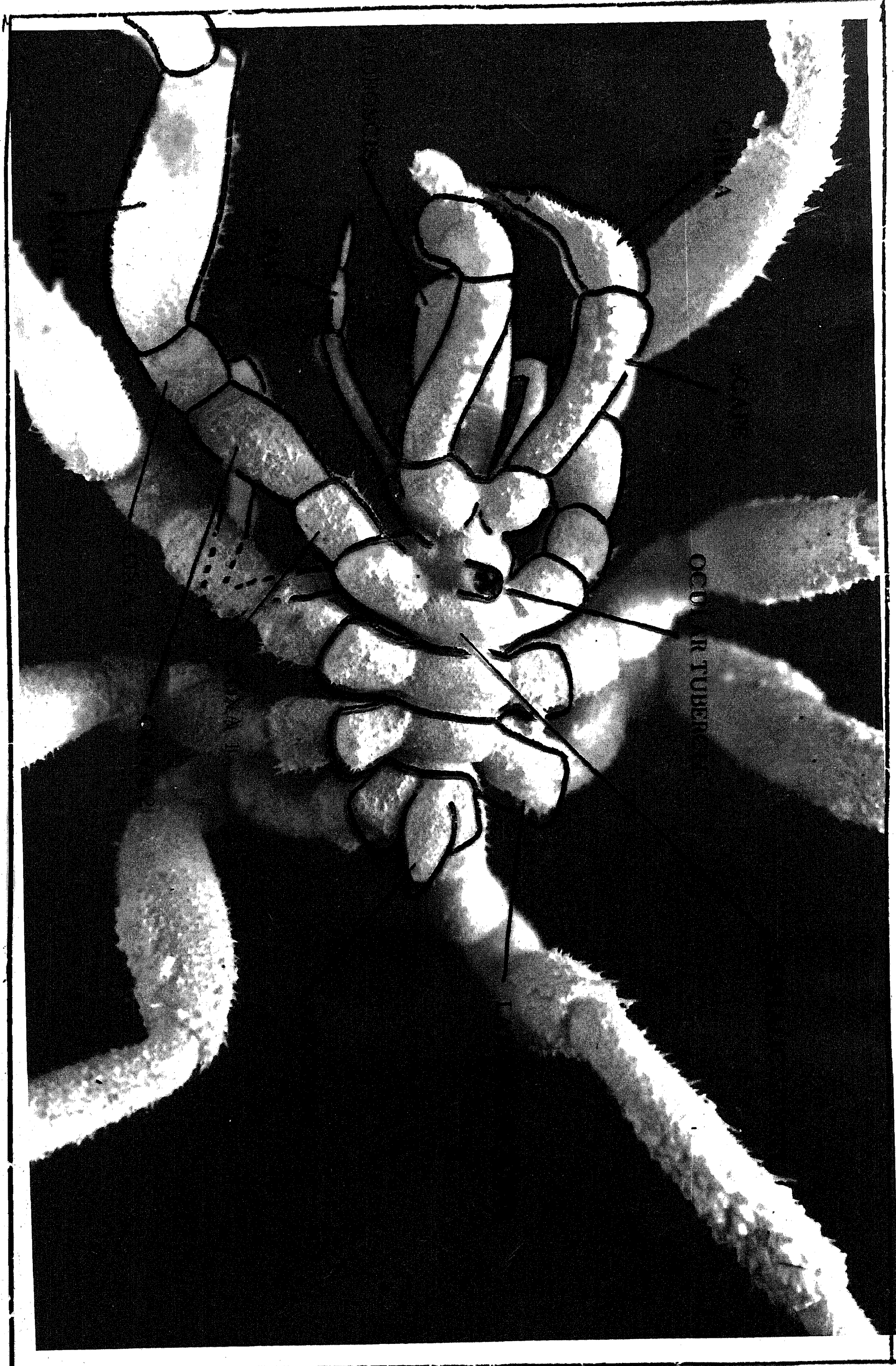


PLATE 1A. *Nymphon orcadense* ( Hodgson 1908 ) Dorsal view of male.

——— = 2 mm

## EXTERNAL MORPHOLOGY

Attempts to describe the external morphology of pycnogonids have been complicated by the fact that 'Naturalists have not always used the same terminology' (Hodgson 1907). This work uses the terms found in Fry and Hedgpeth (1969).

The external morphology of the members of each family differs and the description that follows is intended to apply to the more 'ideal' pycnogonids such as are found amongst the Nymphonidae. Reference is made, however, to those families in which various of the structures are absent or reduced. In this introduction the terms used by Fry and Hedgpeth are in capitals, other terms found in the literature are enclosed within quotation marks. Plate 1A and Figure 1B illustrate the external structure of *Nymphon orcadense*. Table 1C tabulates some, but not all, of the terminology of the literature. Figure 1D repeats Figure 4 p.20 of Fry and Hedgpeth.

BODY:-

Without regard to any of the appendages, the body of pycnogonids is divisible into three regions. At the anterior end is the PROBOSCIS, a characteristic feature of the group. This articulates with the TRUNK ('Cephalothorax') which is joined posteriorly to the ABDOMEN which is little more than a tubercle on which the anus is situated. This reduced abdomen is another group characteristic (cf. other Chelicerates). The

somite of the trunk with which the proboscis articulates is known as the CEPHALIC SOMITE and this gives rise somewhere along its dorsal mid-line to an OCULAR TUBERCLE which may bear EYES. Anteriorly but lateral and dorsal to the articulation with the proboscis, the somite is expanded into paired ANTERIOR CEPHALIC LOBES , each bearing a CHELICERA or 'Cheliphore' or 'Mandible'. Ventral to these lobes and usually slightly ventro-lateral to the articulation with the proboscis are the bases of paired PALPS. The anterior ventral side of the somite bears the bases of paired OVIGERS or 'false legs' or 'ovigerous legs'. These three pairs of appendages, chelicerae, palps and ovigers, all originate anterior to a fourth pair of appendages attached to the cephalic somite, the first pair of WALKING LEGS. These arise on projections known as the LATERAL PROCESSES in the postero-lateral region of the somite.

The cephalic somite is the most complicated of the pycnogonid body and the bulk of classification is based on the presence, absence and modifications of the anterior three appendages it bears. The other somites of the pycnogonid trunk bear only the lateral processes of the remaining walking legs and in some species a ridge or pillar-like DORSAL MEDIAN PROCESS. The last somite of the trunk joins posteriorly (at varying vertical angles to a mid-horizontal axis) with the abdomen. Normally there are three somites carrying walking legs in addition to the cephalic somite (i.e. 4 trunk segments in all, 8 walking legs). In three families, the Nymphonidae, Pycnogonidae and Colossendeidae (Decolopoda and

Dodecolopoda), there are species with one or two extra trunk somites (i.e. 10 and 12 walking legs). Such forms are known only from the Antarctic and American Tropical Regions.

Other names in the literature which are applied to regions of the trunk are 'cephalon', which is that portion of the cephalic somite which is anterior to the first lateral process (Hodgson 1907) and the 'neck'. In Hodgson (op. cit.) the neck is the narrowest part of the cephalic somite between the lateral processes and the more expanded anterior cephalic lobes. In D'Arcy Thompson's writing (1909) it lies between the 'oculiferous tubercle and the proboscis.' In Fry and Hedgpeth (1969) the neck is not mentioned in their idealised diagram, but in Figure 4.p.20 of that work it is the name (neck length) of a measurement between the lateral processes and the articulation of proboscis. This confusion makes it a term to beware.

APPENDAGES:-

The first pair of appendages, the CHELICERAE or 'Cheliphores' or 'Mandibles' form pincer-like claws distally. A chelicera consists of a CHELA (claw) or 'Hand' on a stalk or SCAPE which articulates with the anterior cephalic lobes. The chela in turn consists of a PALM to which is fused an IMMOVEABLE FINGER; a MOVEABLE FINGER articulates with the palm. The two fingers form the pincer and are frequently found armed with teeth or SPINES along their inner edges. Chelicerae are absent or reduced in most adults of the families Ammotheidae, Colossendeidae,

Pycnogonidae and Endeidae.

The second pair of appendages or PALPS vary in number of SEGMENTS between species, a segment being defined by Fry and Hedgpeth as 'that portion of an appendage bounded at one or both ends by a Joint'. Joints in turn are described as of two kinds, ARTICULATIONS (as in the palps) where movement is possible, and SUTURES (as between some trunk segments) where it is not. Palps are absent or reduced in the families Pycnogonidae, Endeidae and Pallenidae.

The third pair of appendages, the OVIGERS, like the palps vary in the number of segments between species. There are usually also sexual differences (except for one species - Marcus 1952 - sexes are separate). Distal segments bear spines and their number and structure (as of those on the chelae) are of taxonomic importance.

The WALKING LEGS are constant in their number of segments though the relative length of segments varies between species. The legs vary in length from equal to that of the body to six or seven times as much. The proximal segment is the FIRST COXA which articulates with a SECOND COXA, the order proceeding to the distal end of the limb then being THIRD COXA, FEMUR, TIBIA1, TIBIA 2, TARSUS, PROPODUS and TERMINAL CLAW. The second coxae possess the genital openings though position and occurrence on all the legs depends upon species and sex. Legs may be smooth or hirsute and this feature and presence or absence of ACCESSORY

CLAWS at the base of the terminal claw are of taxonomic importance.

Features of internal anatomy will be introduced in the appropriate sections.

## GEOGRAPHICAL AND SPECIES INFORMATION

As mentioned in the prologue, pycnogonid specimens were collected from both the Arctic and Antarctic.

### ARCTIC:-

Specimens were collected off the South West coast of Greenland during a survey of the cod fisheries of that area carried out by R.V. CIROLANA (Cruise 8. November 22nd - December 18th 1972). Some 34 trawling stations were made during the cruise. Thirty of these were off West Greenland between Cape Farewell and Godthaab. Three trawls were also made on Faroe Bank and one in the Moray Firth. The shallowest trawl was at 60 metres (Faroe Bank), the deepest 260 metres(Station 41, Banana Bank). Pycnogonids were found at thirteen of the stations. A standard fisheries Granton trawl was used throughout and, where they occurred, the pycnogonids were mixed in with the fish catch. Details of the trawling stations are given in Table 1E.

A detailed examination of the distribution in Arctic waters of the species found is not undertaken as this is part of another project in Luton

which is continuing. Hedgpeth (1963) has given the most recent published review of pycnogonid distribution in the Arctic and the specimens found do not contradict his distribution maps. Snodgrass (1952) takes *N. hirtipes* as his type pycnogonid for description and therefore covers its external morphology in considerable detail. I have no disagreement with his description. Plate 1F shows *N. hirtipes*, Plate 1G shows *Colossendeis proboscidea*. The *C. proboscidea* specimen is interestingly covered with what appears to be mollusc eggs, a phenomenon noted by Hedgpeth (1964) in the Antarctic *Colossendeis megalonyx*. There are no bipolar pycnogonid species (Hedgpeth 1969).

The Arctic specimens were collected and fixed with the intention of comparison with Antarctic forms. One unexpected bonus, however, was their apparent hardiness in surviving in buckets of seawater aboard ship. This fostered an attempt to transport them back to Luton. Refrigerated tanks were hastily built in Luton and specimens were maintained with such success (there were insufficient for physiological and biochemical experiments) that the same transport was attempted for Antarctic forms. The means now exists to study polar pycnogonids in temperate climes

ANTARCTIC:-

As mentioned above, the initial specimens available for study were collected by B.A.S. personnel on Signy Island, South Orkneys and consisted

of an almost pure sample of N. australe (1970/71). In 1971/72 and 1972/73 the samples consisted almost solely of N. orcadense and the author found this species dominant in 1973/74. In the austral summer 1973/74 the author was also able to collect pycnogonids when participating in the Scotia Arc Benthic Survey (S.C.A.B.S.) on R.R.S. John Biscoe off the coast of South Georgia. Station details are given in Table 1H lest they be lost; an analysis of the specimens collected on S.C.A.B.S. has yet to be made.

SIGNY

Signy Island is situated at 60° 43' S and 45° 36' W. It is small, being only about four miles long and less than three miles wide. There is permanent ice cover over a large part of the island, which has a maximum height of 948 feet (Tioga Hill). The coastline is rocky with many indentations. The B.A.S. base is situated on the Eastern side of the island in a cove called Factory Cove (on the site of an old whaling base) between Knife Point and Berntsen Point (see map 1I ). The site of the trawls for 1970 to 1973 are given on map 1J . The depth at which these trawls were made was from 10 to 20 metres. As can be seen from map 1J the collection sites for 1973/74 were slightly different and specimens were collected mainly by diving rather than trawling. This change of locality and collection method could explain the different samples in 1973/74 but it does not explain the differences in previous years.

The ecological relationship of N. australe and N. orcadense is of interest. They are of similar size, appearance and seem to eat the same food. Juveniles and females of the two species are almost impossible to differentiate without sacrifice and microscopical examination. The adult males of the two species can easily be differentiated by the appearance of their ovigers. Thus, the adult male of N. australe has certain portions of its ovigers much inflated and thin walled (Gordon 1932). In the male N. orcadense segment 5 is distinctly clubbed distally (interestingly the Arctic N. hirtipes also possesses this feature in its adult males). Gordon (1932) remarked that N. australe shows affinities with N. orcadense for the palps and chelicerae are very similar in both and there is approximately the same number of denticulate spines on their ovigers.

A general review of the literature cited collections of these two species (which have been known under a variety of synonyms, see Table 1k.i. shows both to be found at variable depths but with N. australe having been collected more often at the greater depths (see Table 1k.ii.)

The keys used for identification in this thesis were those of Hedgpeth (1948) to reach the family level, and those of Gordon (1932 -1944) and Fry and Hedgpeth (1969) to identify species. Neither of Gordon's keys proved very satisfactory in separating specimens of N. orcadense and N. australe other than adult males. The important decision in the use of

both of Gordon's keys was that in N.australe 'auxiliary claws are absent or vestigial', whereas in N.orcadense 'auxiliary claws are well developed'. I have been able to examine the specimens discussed by Gordon (1932 - St 164 E. end of Normanna Strait) housed in the British Museum (Natural History) and found them similar to my own specimens. However I found the statement 'auxiliary claws well developed was open to personal interpretation and this had caused some earlier difficulties.

Ammothea carolinensis was collected at the same sites as N. orcadense and N. australe. Decolopoda australis and Colossendeis wilsoni were collected at greater depths on S.C.A.B.S. and transported live to Signy Island for examination. Decolopoda australis had been caught off Signy at depths of 30 metres or more in previous years ( see also footnote 1, Chapter 2).These species were easily identified using the keys of Fry and Hedgpeth (1969).

## MISCELLANY.

The extensive travelling necessary for this project enforced long periods when the major targets of research could not be pursued. During these periods I felt obliged not only to concentrate on the experiments in hand but also to seize opportunities to make other minor investigations.This has resulted in much more information than can be set down in a logical manner here (eg. several thousand specimens, over a thousand photographs

of pycnogonids, another thousand photographs of Antarctica, between 10,000 and 20,000 sections. What follows for the rest of this chapter is some information which does not fit easily into other chapters, but which may be of interest in an introductory context.

PYCNOGONIDS AS PREY

Chapter 2 includes a lengthy discussion on feeding in pycnogonids and their food substrates. A question which immediately arises in connection with feeding and the place of pycnogonids in the ecosystem is - what feeds on them? The short answer would appear to be nothing.

Reports of their predators usually seem to be based on somewhat circumstantial evidence. Thus, Dearborn (1967) reports finding fragments of pycnogonid in the gut of the isopod Glyptonotus antarctica. However, in his analyses of the gut contents of this animal, the number of findings of pycnogonids is so small that it is difficult to decide whether their uptake was accidental or intended, or an example of necrophagy. The report in Footnote 2, Chapter 2, is based on the absence of specimens which it is thought ought to have been present and the presumption that they must therefore have been eaten. A brief analysis of the stomach contents of fish caught in the same trawls as pycnogonids when on board the R.V.Cirolana yielded no evidence of pycnogonid intake. Personal communication with a number of B.A.S. personnel analysing fish stomach contents also yielded negative answers. In transport of live specimens back to Britain, several pycnogonids were kept in a tank containing young Notothenia rossii. These fish were noted for their appetites

(they were nicknamed 'Antarctic pirhana'). They showed no interest in the pycnogonids, although they appeared to eat almost any other invertebrate that moved.

Of interest are two unrelated personal communications (J. Hoogesteger and M. White) of large colossendids found incorporated as nesting material in the nests of Antarctic terns.

It must be stated that as a large percentage of their body weight is probably composed of cuticle, pycnogonids would not appear to be particularily appetising or digestible meals. On the other hand, little is known about the habits of larval stages of most species after they have left the males ovigers, and a case for total non-predation cannot therefore be made.

OVIGERS

As is seen in Plate 1H and is again seen in Plates 2H of Chapter 2, it is not unusual to find organisms living attached to the cuticles of pycnogonids. Amongst Colossendeis species and Decolopoda australis "shepherd's crook" grooming by the ovigers is a commonly observed behaviour. In the Nymphon species studied here, grooming by the ovigers was not common, indeed for a long time the author thought the ovigers were never used for this purpose.

One interesting observation has been that Colossendeis species seem to undergo a behaviour process which might be called 'displacement' grooming. This was first observed when the author was photographing some aquarium specimens with electronic flash. The more often the flash was set off, so the more frequent the grooming seemed to become. Egg masses have never been observed on the ovigers of Colossendeis species or Decolopoda and failure to observe much grooming in the Nymphon species suggests that in the former the ovigers are essentially grooming appendages while in the Nymphonidae they are primarily egg carrying appendages.

As is seen in Plates 1Q, showing animals with egg masses, the form of the masses and their attachment differs with species. Egg mass attachment, the grooming role of ovigers and peculiarities of structure such as the ballooning of segments in N. australe or the club-like expansion in segments 5 of N. orcadense and N. hirtipes are of considerable interest.

From the evolutionary viewpoint it would be interesting to know whether the ovigers evolved for egg carrying or whether they evolved for grooming and if one type evolved from the other or if the evolutions were independent A further interesting feature is that in the Pycnogonidae and some other forms ovigers are absent, most often in the females.

MOVEMENT

As will be detailed in Chapter 2, polar pycnogonids are slow moving animals. They may generally move over a surface by walking, or they may swim. In the forms which I have observed, swimming has been a rare occurrence. Cole(1901) described crawling and swimming in _Anoplodactylus lentus_ and noted that the legs of crawling animals moved in a similar sequence to those of swimming animals. This observation has been confirmed using cinematographic techniques by Morgan(1971).

I have found that movement in _Nymphon_ species was agitated when they were removed to confined spaces. A small tank was built for photography out of 3" x 2" microscope slides. Attempts to photograph _N. orcadense_ feeding in this were always unsuccessful. Removal from a large tank which probably mimicked the natural environment well, to the small tank in bright light, caused the animal to flex its legs and swim vigorously (in about 3"of water). Feeding animals always dropped their prey and they never seemed to settle to a normal resting stance. When swimming stopped, the animals settled on the bottom and remained in the 'plummeting' posture (with legs

folded dorsally) which has been described several times in the swimming literature (eg, Prell 1910, Morgan 1971). The same phenomenon was noted in Ammothea and Colossendeis specimens in a 'natural condition' aquarium which was situated near a window.

It would seem that the plummeting posture might be associated with light avoidance. Knight Jones, McFadyean and Morgan (1964) have postulated that there is a pressure sense in N. gracile. However, their apparatus consisted of a vessel whose cross-sectional dimensions were similar to my photographic aquarium, into which a strong light was shone. Separation of pressure affected behaviour from light affected behaviour needs careful consideration in view of the above observations. Morgan himself (1964) stated that under 'semi-natural' conditions in an aquarium with stones and hydroids, his animals (N. gracile) rarely left the bottom of the aquarium.

Isaac and Jarvis (1971) have described a phenomenon of ebb-swimming in N. gracile. They claim that their pressure sense causes them to start swimming with the ebb-tide. They are lifted off the bottom and moved out with the current. This prevents them being stranded by the tide. It may be possible that one of N. orcadense or N. australe exhibits similar behaviour with the result that the proportion of each in the same areawill vary - hence the different samples (see above). Obviously, further work in these areas at Signy will have to account for the time of collection, state of tide, and possibly the light regime when samples are taken.

Kellet 1973/74 (B.A.S. report No. ~/1972/H), applied an interesting technique of delayed action underwater cinematography with an apparatus set up under the ice (delicate equipment is less liable to damage by wave action during winter). This was to study Nemerteans, but no doubt it would be equally useful if applied to pycnogonid migrations and behaviour during the day.

Morgan (1964) stated that the limbs of pycnogonids are not specialised for swimming. Whether a behaviour pattern is a natural one is worth consideration before its investigation. Flynn (1928), in reporting four species of pycnogonids from three genera caught in a tow- net, claimed that his report was only the second one of pycnogonids taken in plankton (the first being that of Calman 1923). Ohshima (1933) has reviewed pycnogonids taken with a tow-net. It must be mentioned, however, that the first report he cited, that of Carpenter (1905), was of a tow-net weighted for benthic work. Fage (1932), by using a strong lamp at the surface at night, seems to have been the only person to capture swimming pycnogonids (N. gracile) at will. Hedgpeth (1962) has reported on a bathypelagic specimen and reviewed some previous work on bathypelagic pycnogonids. For the most part, however, the statement that pycnogonids are holobenthic holds true. In my own observations swimming was rare.

TABLE 1 C.

SOME TERMS USED IN PYCNOGONID EXTERNAL MORPHOLOGY

The terms used by Fry and Hedgpeth (1969) are used to head the columns their synonyms are listed below them.

| CHELICERA | CHELA | SCAPE | CEPHALIC SOMITE |
|---|---|---|---|
| cheliphore<br>mandible | claw<br>hand<br>pincers | stalk | cephalon |

| OCULAR TUBERCLE | TRUNK | ABDOMEN | PALP |
|---|---|---|---|
| eye stalk | cephalothorax | | feeler |

| OVIGER | COXA 1 | COXA 2 | COXA 3. |
|---|---|---|---|
| ovigerous leg<br>false legs | — | trochanter 1 | trochanter 2 |

| FEMUR | TIBIA 1 | TIBIA 2 | TIBIA 3. |
|---|---|---|---|
| — | patella | — | — |

| TARSUS | PROPODUS | TERMINAL CLAW | ACCESSORY CLAW |
|---|---|---|---|
| — | — | pretarsus | ungue<br>auxiliary claw |

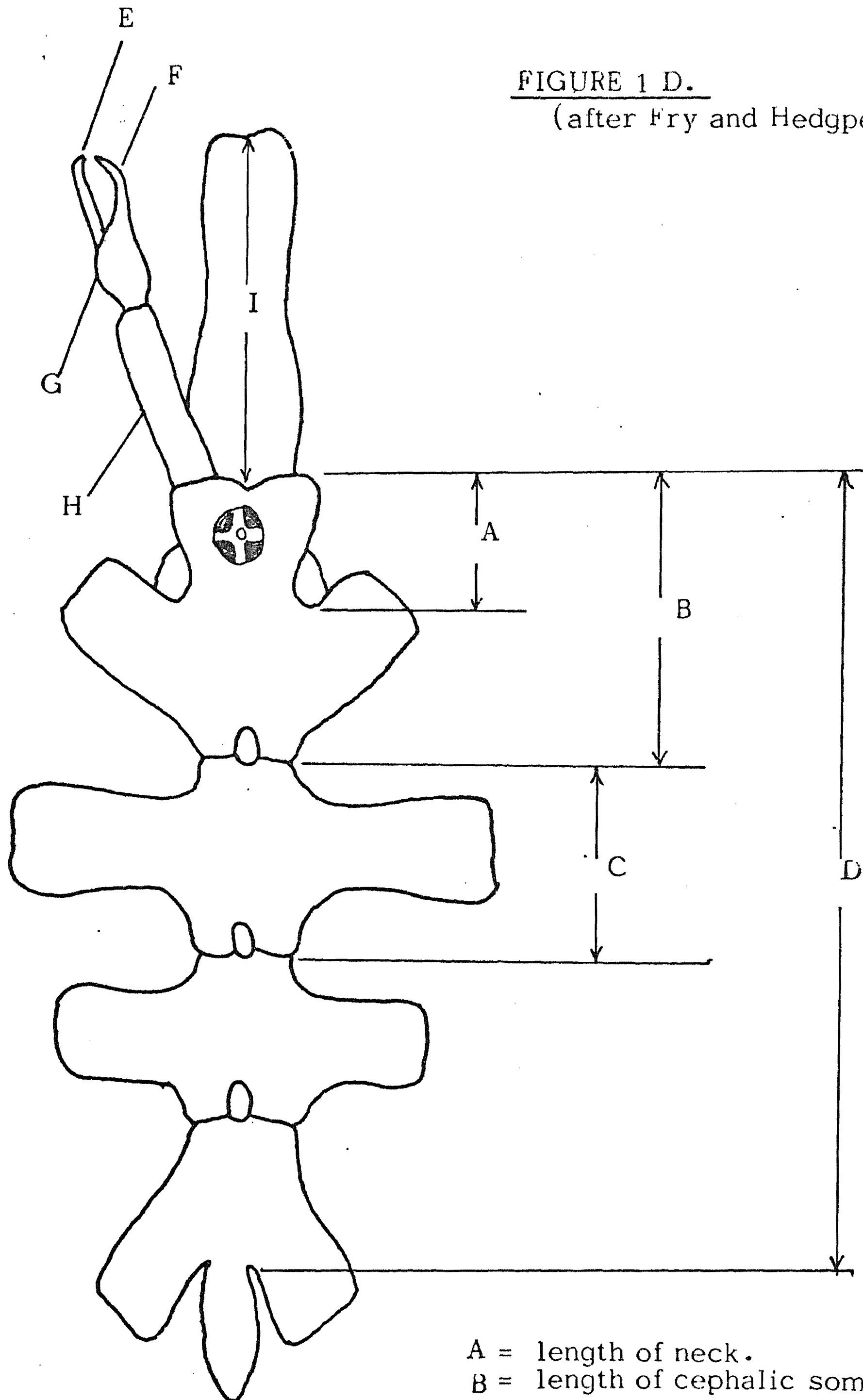



FIGURE 1 D.
(after Fry and Hedgpeth 1969)

A = length of neck.
B = length of cephalic somite.
C = length of 2nd somite.
D = total length of trunk.
E = moveable finger.
F = immoveable finger.
G = palm.
H = scape.
I = length of proboscis.

| Station No. | Date (Dec. 1972) | North | West | Name of Bank | Depth (metres) | Surface temp. °C | Bottom temp. °C | Nymphon spps. | Colossendid spps. | Main benthos in trawl | Main fish in trawl |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 1 | 60°21·5 | 47°10·2' | Malenfjeld | 140 | 0·75 | 4 | + | + | Holothurians | Cod |
| 12 | 1 | " | " | " | " | " | " | + | + | " | " |
| 18 | 2 | 61° 18' | 49°59' | No-name | 130 | 0·75 | 3 | + | + | " | " |
| 19 | 3 | 61° 37' | 50° 34' | No-name | 130 | 1 | 4 | + | - | " | " |
| 24 | 3 | 62°37·5' | 51° 34' | Danas | 130 | 1 | 3 | + | - | Ophuiroids | Long Rough Dabs |
| 25 | 3 | 62° 26' | 51° 19' | Danas | 140 | 1·5 | 4 | + | + | " | Cod |
| 26 | 3 | 62° 29' | 51° 17' | Danas | 88 | 2. | 3 | + | + | " | Long Rough Dabs |

TABLE 1E Gives details of the trawling stations at which the bulk of the Arctic pycnogonid specimens were taken. At other stations specimens of N. hirtipes were taken singly. Details of the Faroes trawls have been omitted.

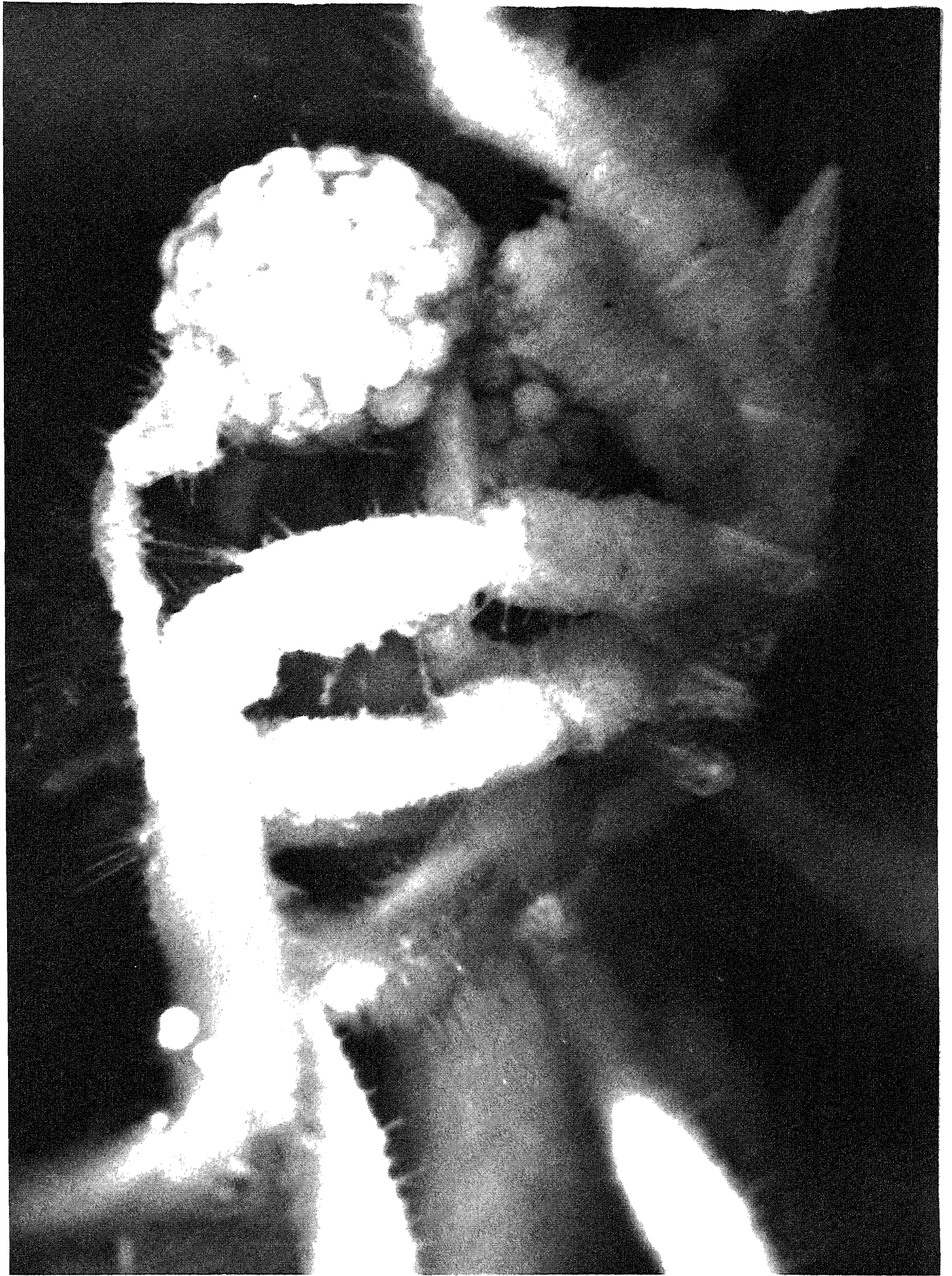


PLATE 1F. Nymphon hirtipes (Bell 1853) male with walking leg missing showing eggs on ovigers.

= 2 mm.

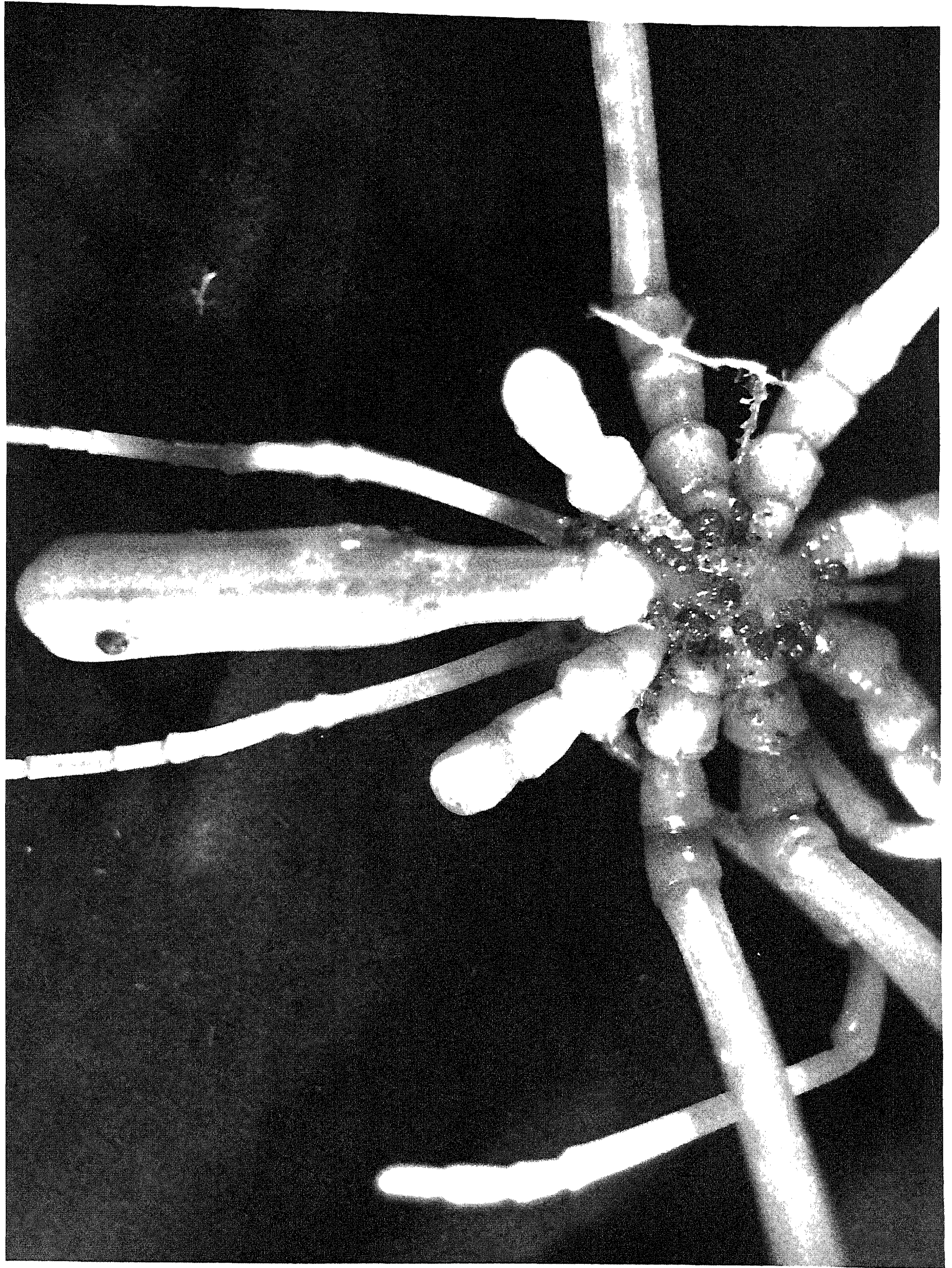
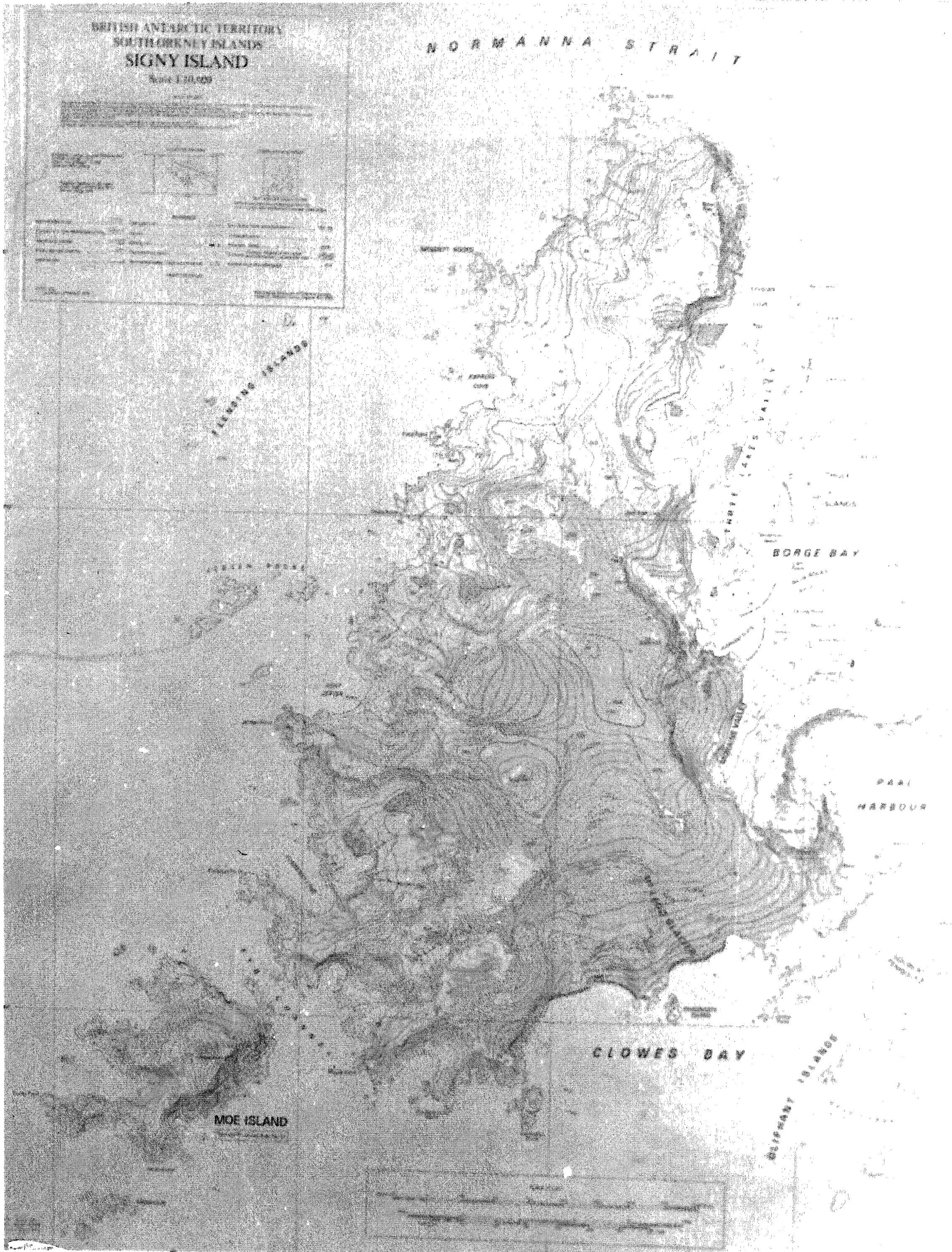


PLATE 1 G. Colossendeis proboscidea (Sabine 1824).
Note the encrusting mollusc eggs and hydroid.

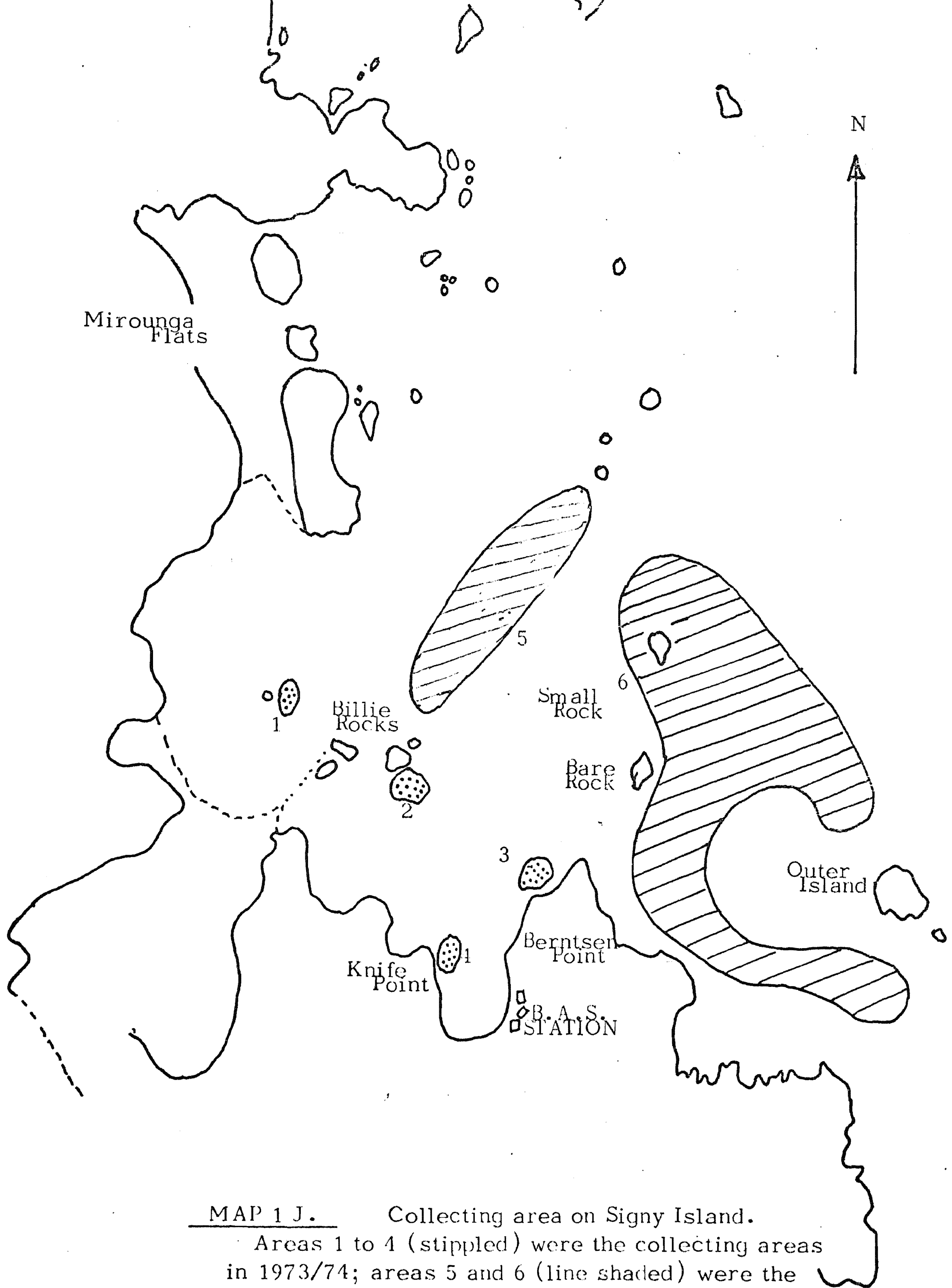
——— = 2·5 cms

TABLE 1 H. S.C.A.B.S. station details 1973/1974

| Station no. | Position (S.Georgia) | Depth | Date (Dec.1973) |
|---|---|---|---|
| 111 | Cape North (145°x 13·2') | 320 | 2 |
| 112 | Nameless Point (222°x 6·9') | 141 | 3 |
| 113 | Cape North (217°x12·9') | 137 | 3 |
| 114 | Welcome Island (172°x 21·1) | 263 | 4 |
| 115 | Black Point (271°x0·25') | 40 | 5 |
| 116 | " " | 100 | 5 |
| 117 | Laurie Point Island (298°x0·47') | 60 | 6 |
| 118 | Ramp Rocks (240°x2·51') | 100 | 7 |
| 119 | Cape North (160°x 17·0') | 113 | 7 |
| 120 | Ramp Rocks (194°x6·00') | 104 | 8 |
| 121 | Ramp Rocks (178°x 11·1') | 238 | 8 |
| 122 | Verdant Island (187°x 24·0') | 274 | 9 |
| 124 | Barff Point (247°x57') | 150 | 10 |




MAP I i Signy Island




MAP 1 J. Collecting area on Signy Island.
Areas 1 to 4 (stippled) were the collecting areas in 1973/74; areas 5 and 6 (line shaded) were the collecting areas in previous years.

TABLE 1 K: Some names used in the literature for N. australe and for N. orcadense. Only the first author to use the name is given.

NYMPHON AUSTRALE :-

Nymphon stylops ......... Bouvier 1911 & 1913

Chaetonymphon australe....Hodgson 1902

C. australe var austrinorium........Hodgson 1902

Chaetonymphon ultioculatum ........Mobius 1902

Nymphon australe var. caecum ..... Gordon 1944

NYMPHON ORCADENSE :-

Chaetonymphon orcadense .......Hodgson 1908

TABLE 1 Kii

| Author | Year | Position or area | Depth (metres) | No. of specimens |
|---|---|---|---|---|
| N·AUSTRALE | | | | |
| Hodgson | 1907 | Cape Adare | 47 | considerable nos. |
| | | off Coulman Island | 183 | 2 |
| | | 78°25'40"S 185°39'6"E | 549 | 1 male (ovig.) |
| | | Winter Quarters(McMurdo) | - | large numbers |
| Calman | 1915 | Cape Adare | 91 | 4 |
| | | 74°25'S 179°3' | 289 | 3 |
| | | Inaccessible Is.(McMurdo) | 406 | 3 |
| | | 77°13'S 164°18'E | 378 | 200 |
| | | 76°56'S 164°12'E | 292 | 20 |
| | | Granite Harbour(McMurdo) | 92 | 11 |
| Flynn | 1928 | 29°44'12"S 31°20'45"E | 84 | 1 male |
| | | 33°50'S 17°59'E | 2,176 | 1 male 1 female |
| Gordon | 1932 | An extensive list of stations (p59&60) around South Georgia, South Shetlands, South Sandwich Islands and the Palmer Archipelago. The range of depths is from 40 to 525 metres | | |
| Gordon | 1944 | 66°48'S 71°42'E | 540 | 2 males |
| | | 66°21'S 58°50'E | 603 | 1 female |
| | | 66°10'S 49°41'E | 300 | 2 |
| | | 66°12'S 49°37'E | 300 | 5 |
| | | 65°48'S 53°16'E | 209 | 1 female |
| | | 65°50'S 54°23'E | 220 | 1 juvenile |
| | | 66°21'S 138°28' | 640 | 1 juvenile |
| | | 67°46'S 67°03'E | 163 | 27 |
| | | 66°45'S 62°03'E | 219 | 17 |
| | | 66°28'S 72°41'E | 1,266 | 2 males 1 juvenile |
| Hedgpeth | 1950 | 65°25'S 101°13'E | 183 | 1 female |
| | | Peter 1 Island | - | 12 |
| Fage | 1952 | La Terre Adelie ! | 40 | 1 female 1 juvenile |
| | | " | 13 | 4 males 2 females. |
| Stock | 1965 | 70°18'S 24°13'E | 255 | 9 |
| Arnaud F. | 1969 | 66°39S 139°55'E | surface to 250 | 370 |

TABLE 1 Kii(continued)

N. ORCADENSE

| Author | Year | Position or area | Depth (metres) | No. of specimens |
|---|---|---|---|---|
| Hodgson | 1908 | Scotia Bay, South Orkneys<br>Burdwood Bank, Falkland Isles | 18<br>102 | Immense nos.<br>1 immature |
| Gordon | 1932 | Paal Harbour, Signy Island<br>Normanna Straits, S. Orkneys | 18 -27<br>24-36 | 18<br>'several' |
| Gordon | 1944 | 67°46'S 67°03'E | 163 | 1 female |

Table 1 K lists the major reports of N.australe and N.orcadense in the literature. Generally it would seem that N.australe has been taken more often at greater depths, but as can be seen, there have not been many reports of N.orcadense.

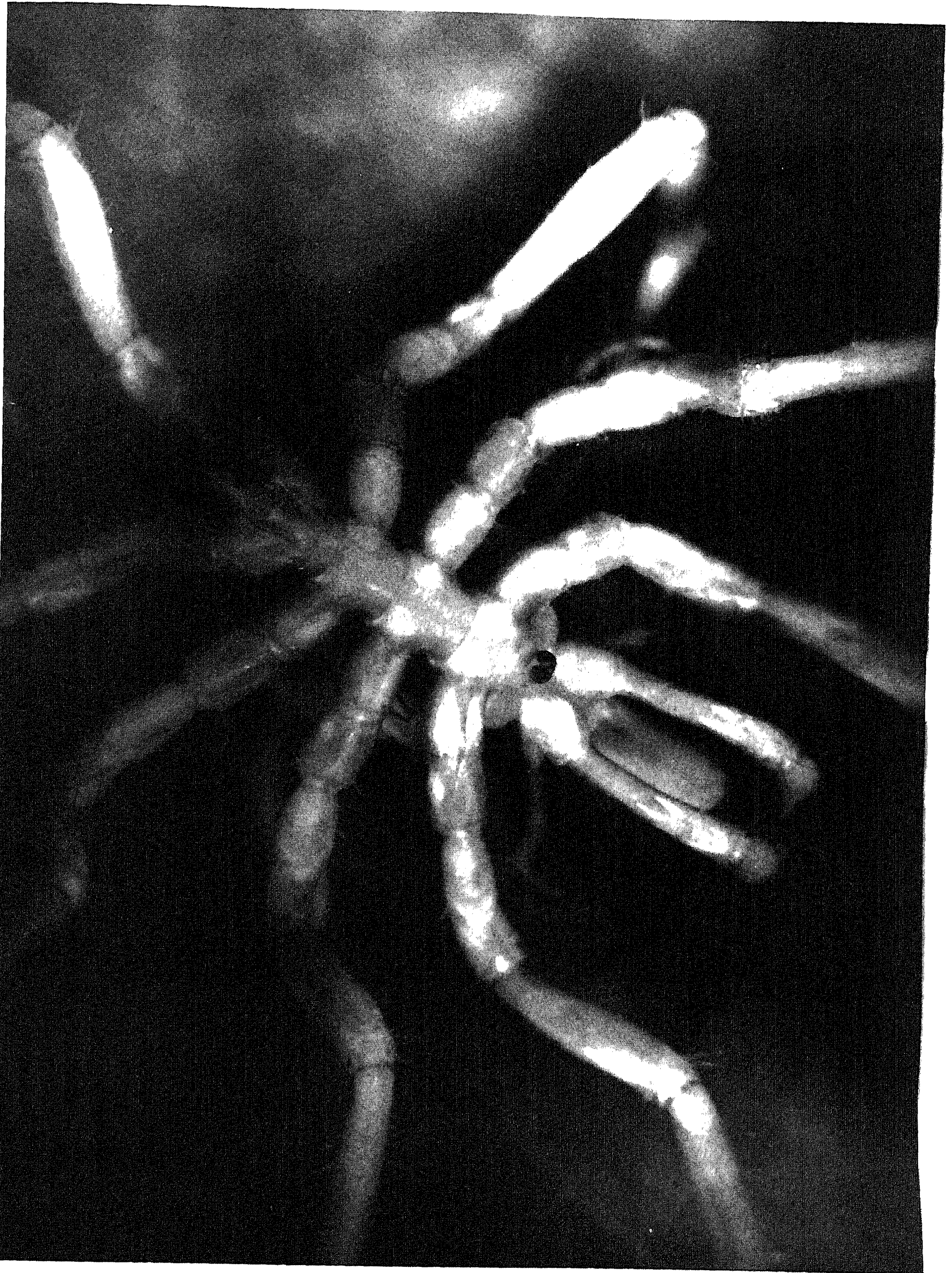


PLATE 1 L  Nymphon australe (Hodgson 1902)  Dorsal view

|———| = 5mm

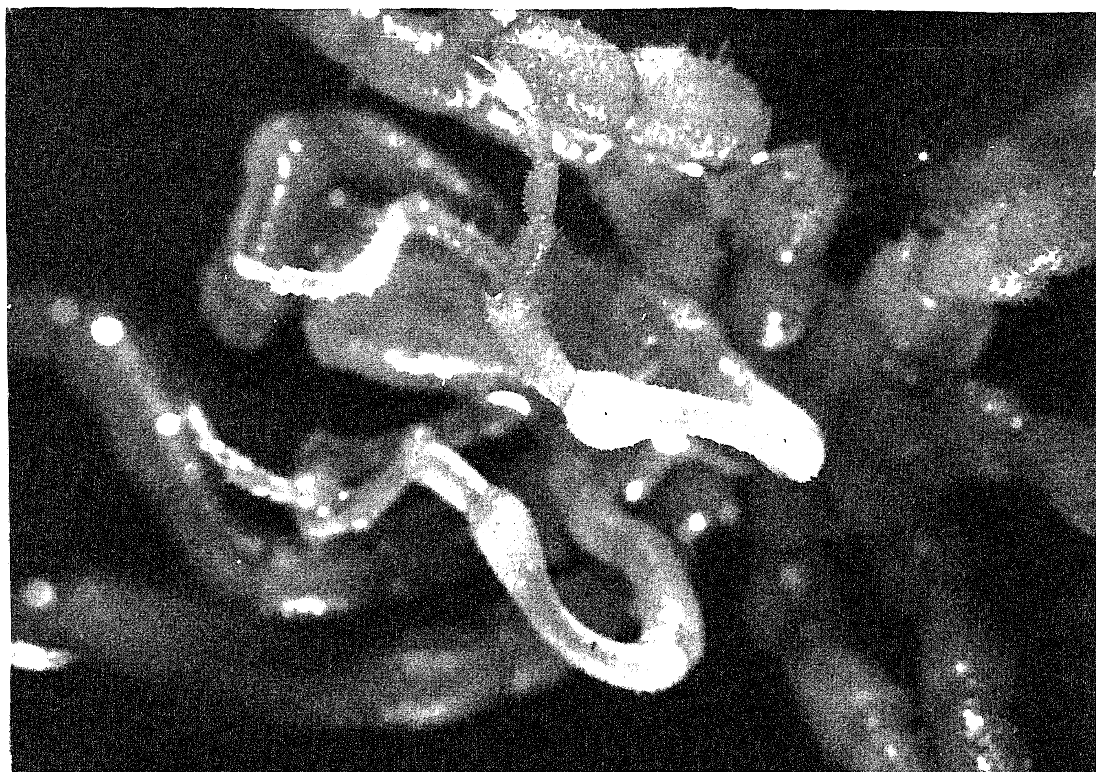
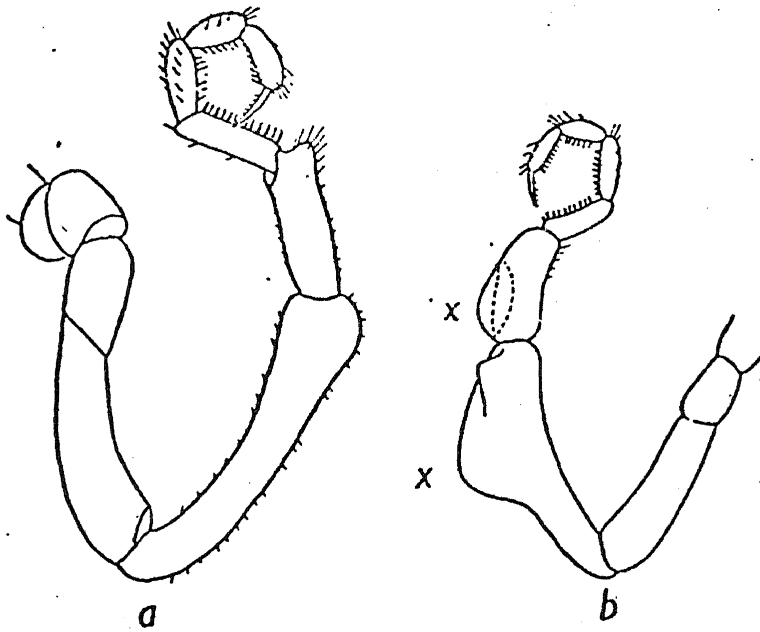


PLATE 1 M.i Male N. orcadense showing distal clubbing of segment 5 of the ovigers.

PLATE 1 M ii· Male N. australe showing 'ballooning' of segments of the ovigers.

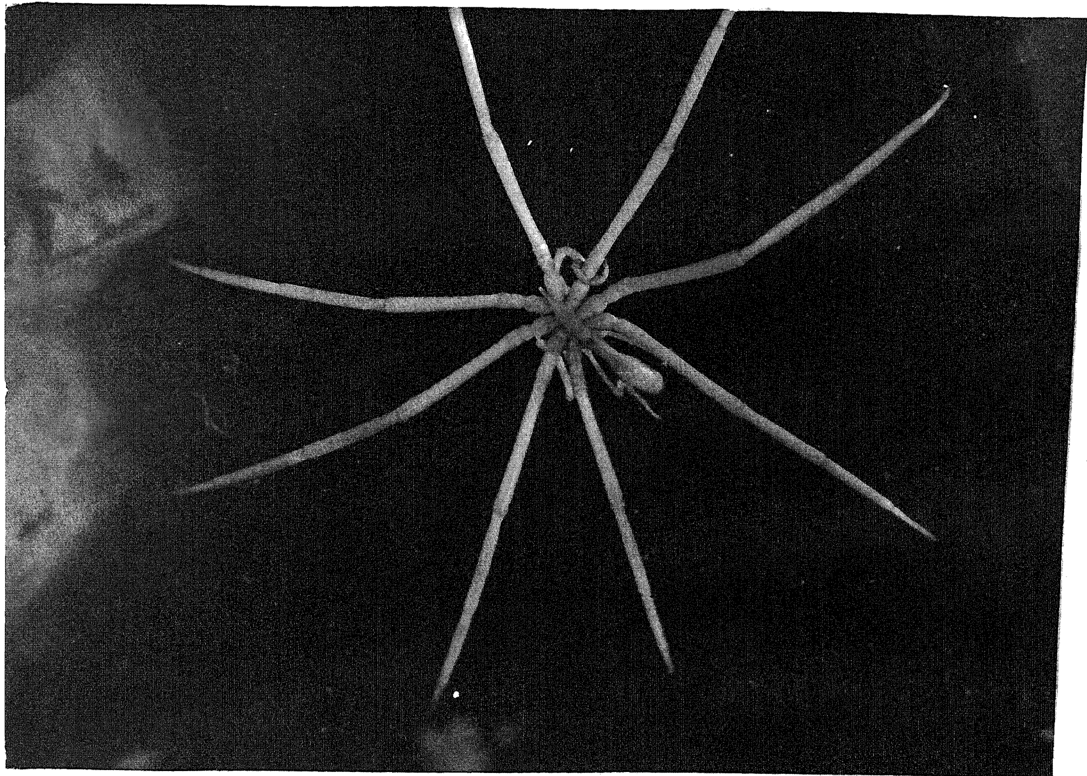



Male oviger of: *a. Nymphon orcadense*, Hodgson: ×17. *b. N. australe*, Hodgson: ×17. Type II *a*; segments 5 and 6 thin-walled and inflated at *x*.

PLATE 1 O. Ammothea carolinensis (Leach 1814)
1 cm

PLATE 1.P.

i)



ii)

iii)

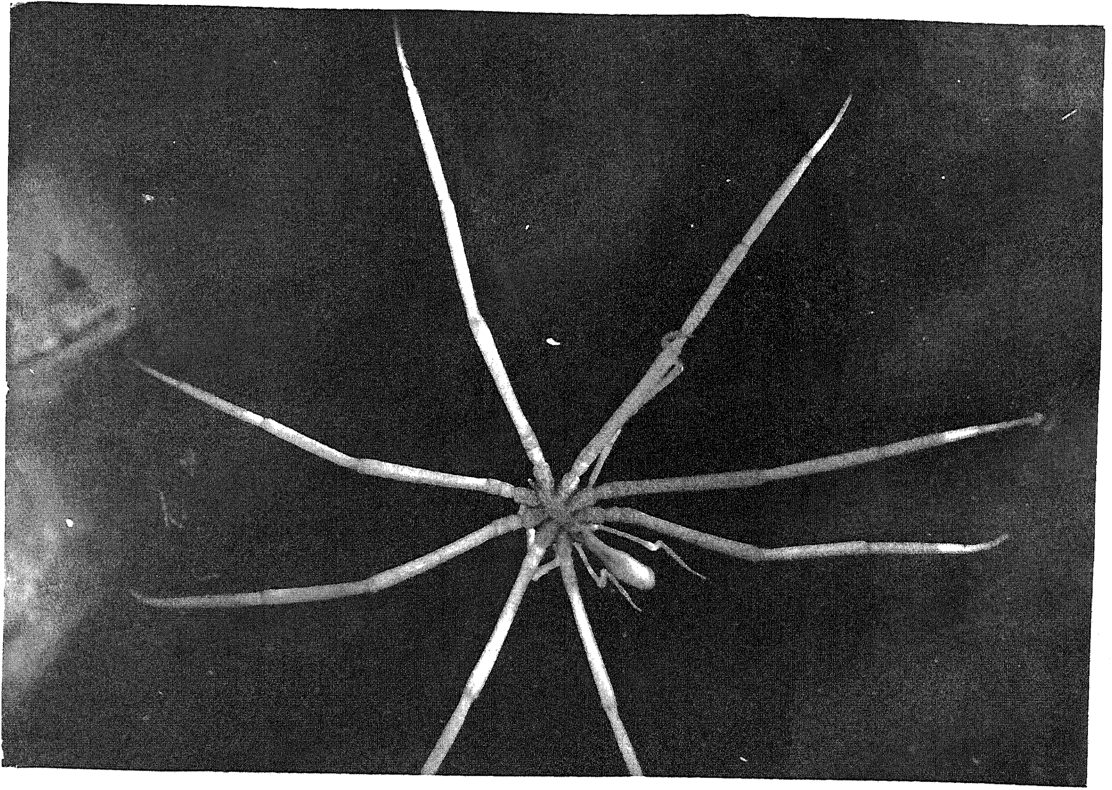
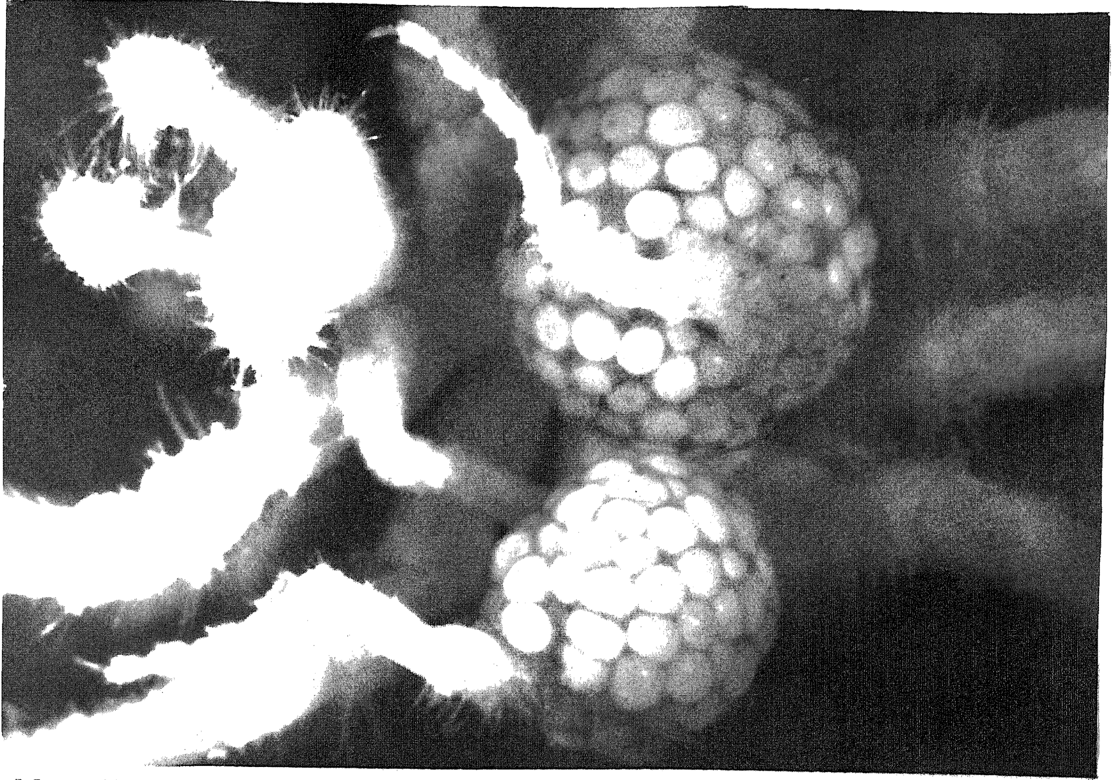
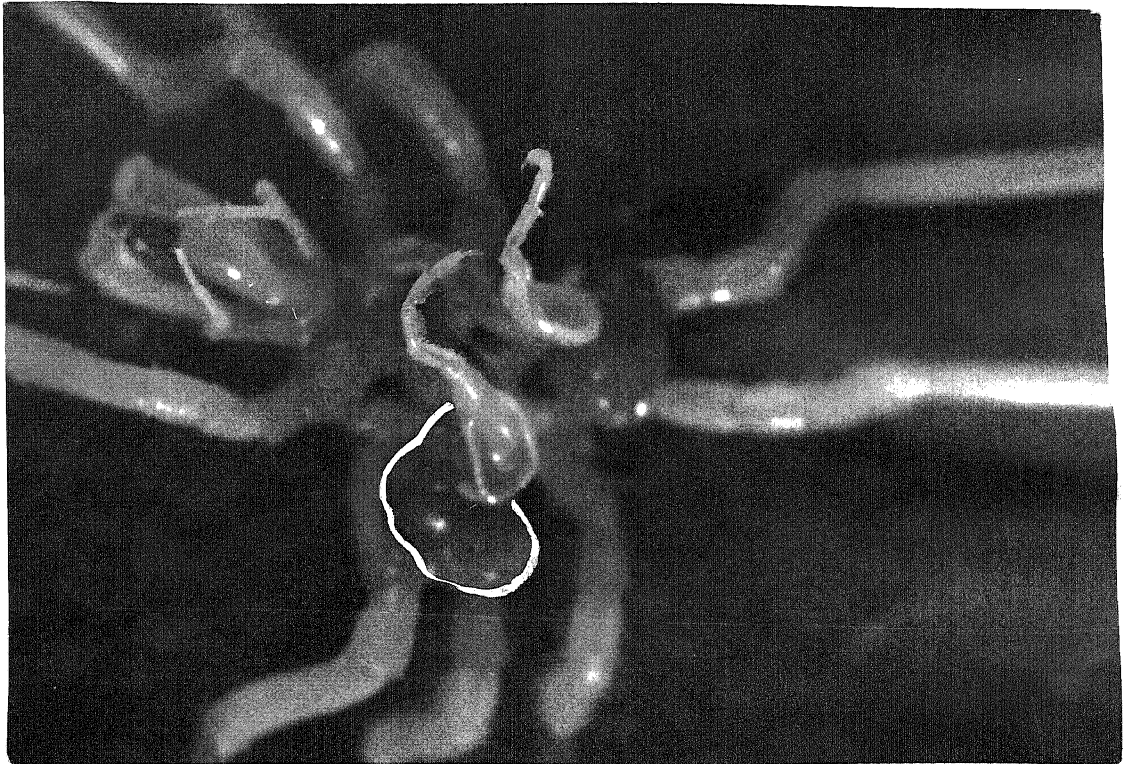


Plate 1 P. shows stages in 'shepherds crook' grooming excited by electronic flash.

PLATE 1 Q.



Male *N. hirtipes* with egg masses.



Male *N. australe* with egg masses (one outlined in white).

# CHAPTER 2

# FEEDING AND RELATED PHENOMENA

## 2.1 INTRODUCTION

The first report of a pycnogonid feeding appears to be that of Konig (1766), who described a species which 'bores through the shells of mussels with its long rostrum and sucks their juices.' Calman (1929) believed Konig's pycnogonid to be Phoxichiliduim femoratum which has a proboscis (rostrum) which appears quite incapable of such a shell drilling feat; Konig's observation was probably incorrect. Ohshima (1927) is generally credited with confirming at least association with bivalve molluscs, but Merton (1906) and Flynn (1918) had previously shown association with molluscs other than bivalves. (Benson and Chivers (1960) summarize much concerning mollusc/pycnogonid associations).

As Konig noted, pycnogonids are slow animals. Without close and prolonged observation it is often difficult to tell whether a pycnogonid is feeding, or merely resting on the substrate. For this reason, though in many associations with other animals it is likely that the pycnogonid is acting as a predator, the number of confirmed reports of feeding is much lower than the reported number of associations. Helfer and Schlottke (1935 pps 198-201) tabulated a 'selection of organisms on which pycnogonids are found, or feed, or both.' Crapp (1968) updated this table and divided it into 'associations' (his table 2.2) and 'established habits in pycnogonids' (his table 3.1). These tables are repeated, slightly modified, by King (1973).

The phyla established as feeding substrates are:- Coelenterates (various authors), Porifera (various), Polyzoa (Fry 1965, Wyer 1972), and Annelids (Arndt 1912, Sanchez 1959, King 1973). As will be seen below, two additional phyla can be added to this list as a result of observations during this work. Of the above, members of the class Hydrozoa seem to be the most often mentioned in the literature.

Other phyla with which pycnogonids are associated and which might therefore be possible food sources are Echinoderms (this includes four of the five living classes:- Crinoidea (Loman 1928), Echinoidea (Loman 1925, Losina-Losinsky 1933, Flynn 1918), Holothurians (Prell 1910, Ohshima 1927) and Asteroidea (Losina-Losinsky 1929)), Ascidians (Dohrn 1881, Helfer 1909) and Fish (Bohm 1879). Wyer (1972) reports detritus feeding in Endeis laevis and Arnaud (1972) found specimens of Nymphon australe on seal meat. King and Wyer (1971) report Achelia longipes feeding on red algae.

Fry(1965) studied the feeding mechanisms of three species and found that there seemed to be morphological adaptations to preferred food materials. In the Nymphonidae studied in this work there seem to be no special adaptations to feeding on one particular species; tastes are catholic.

## 2.2 FEEDING SUBSTRATES IN THE SPECIES STUDIED

Ammothea carolinensis was only observed feeding on anenomes (plate 2B) and seems well adapted for that substrate. Plate 2C (b) is a print taken from an underwater colour slide (kindly provided by Mr. Jan Hoogesteger), showing Nymphon orcadense on a red seaweed. During the austral summer 1973/74 (until early February), Nymphon orcadense was frequently associated with this seaweed which was the favourite habitat of the polychaete shown in plate 2C (a). It was found that when these polychaetes were present in the aquarium, Nymphon orcadense ignored all other food substrates in preference to them. In the holdfast region of the red seaweed, what appeared to be larger versions of this same polychaete were found in tubes. Nymphon orcadense attacked the head end of these tube dwellers, but generally they seemed less available as food than the naked forms.

In February 1974, the naked polychaetes disappeared. It cannot be said with certainty whether this was as the result of a storm during this period which greatly stirred up the benthos in Factory Cove and which may have washed the weed clean, or whether it was due to some natural seasonal migration. From this time, however, it was noted that the Nymphon specimens were caught feeding on other substrates. These substrates were:- dead amphipods (two observations), detritus masses containing small errant polychaetes (several occasions), a spherical mass of animal material (unidentified -

several times), and the small nemertean Tetrastema validum.
I know of no previous reports of pycnogonids feeding on arthropods (amphipods) or Nemerteans.

Nymphon australe was not present in sufficient numbers for similar observations to be made, but it was noted that a starved specimen accepted the polychaete liked by Nymphon orcadense. In preserved samples of Nymphon australe collected during the austral summer of 1970/71 it was noticed that several specimens held small hydroid colonies in their chelicerae as if fixed in the act of feeding. Fry (1965) has reported Nymphon australe on Alyconaria.

On the return journey to Britain, Nymphon orcadense was observed (in tanks aboard ship) feeding on sea anenomes (the same species attacked by Ammothea carolinensis). At the British Antarctic Survey depot at Monkswood (U.K.), specimens fed on a mixture of minced limpet, squid and spratt (bought at local British fishmongers) and whilst in the refrigerated tank at Luton, they fed on the British sea anenome Actinia equina.

Studies of feeding in the arctic Nymphon hirtipes were more difficult. Specimens were caught by picking them from the Cod-end of a commercial Granton trawl and containers in which to maintain any of the benthos on which they may have been feeding were not available. In addition, the large feet of trawlermen often crushed most of the accidental benthos as the fish were sorted and often the 'rubbish' was shovelled overboard before there was time to examine it. Conditions

were not always ideal for careful scientific observation. During the first period of maintenance of polar pycnogonids in refrigerated aquaria (December 1972 until October 1973), Nymphon hirtipes was not observed feeding (see later). In August 1975, Mr. Andrew Hetherington brought back additional specimens of Nymphon hirtipes and in an improved refrigerated aquarium these fed on Actinia equina.

TABLE 2.A.

FOOD SUBSTRATES OF SPECIES INVESTIGATED IN THIS WORK.

Nymphon orcadense

i) Dead amphipods.

ii) Detritus masses containing errant polychaetes,

iii) Detritus mass of unknown animal origin,

iv) Nemertean - Tetrastema validum,

v) Polychaete,

vi) Anenome (Antarctic),

vii) Anenome (British),

viii) Mixture of minced limpet, squid and spratt.

Nymphon australe

i) Polychaete,

ii) Hydroid colony (in fixed material),

iii) Alyconaria (Fry 1965),

iv) Seal meat (Arnaud 1972),

Nymphon hirtipes

i) Anenome (British),

ii) Possibly sponge (see Frontis piece).

Ammothea carolinensis

i) Anenome (Antarctic)

Decolopoda australis

i) Anenome (Antarctic),

ii) See footnotes 1 and 2.

FOOTNOTE 1.

When questioning the divers on Signy Island about any observations they may have made on pycnogonids, I was told that in addition to the types I was studying in the base aquarium (*Nymphon orcadense*, *Nymphon australe* and *Ammothea carolinensis*) and the large *Decolopoda* and *Colossendeis* specimens (which could only be trawled outside safe motor dinghy range of base), there was one small animal about 1 to 2 cms total leg span which was orange coloured and associated with the colonial coelenterate *Myriothela austrogeorgiae*. Collections were not made in the region of these colonies during austral summer 1973/74, but I subsequently received a preserved specimen of the orange form which was later identified as *Decolopoda*.

This must therefore count as a report of association, and perhaps of feeding of *Decolopoda (?) australis* upon *Myriothela glacialis*. Mr. Brian Kellet supplied the specimen.

FOOTNOTE 2.

It was not possible to make feeding observations on *Decolopoda australis* in Britain as the live specimens brought back were lost when the cold room in which their aquarium had been placed developed a fault and heated to 30°C overnight. In cleaning out their tank after this accident a puzzling lack of corpses of specimens of *Nymphon orcadense* was noted (about half the collection of *Nymphon orcadense* had been placed in this tank with the *Decolopoda* and

and *Colossendeis* specimens). No food had been added to this tank and it had been kept for 3 weeks under a bench in the cold room where it was difficult to observe details. As dead pycnogonids remain on the bottom of aquaria for months if not removed, the most logical explanation for the lack of corpses is that they were eaten by the hungry larger forms. *Nymphon*, *Decolopoda* and *Colossendeis* specimens were kept in the same tank in transit to Britain, but soft food (anenomes) was plentiful.

## 2.3 FEEDING BEHAVIOUR

Feeding behaviour depends upon a number of factors, the most important being presence or absence of chelicerae, proboscis shape, and the type of food. (e.g. in *Nymphon australe* which has a varied diet, the method of feeding can vary with the substrate).

Usually when chelicerae are present they are used extensively in feeding behaviour (*Nymphon australe* and *Nymphon orcadense*). *Ammothea carolinensis* which has atrophied adult chelicerae uses different methods. *Decolopoda australis* has chelicerae but they are not longer than the proboscis as is the case with the *Nymphon* species.

The proboscis is essentially a sucking, scraping, sieving organ (Calman 1929), but in some species, whilst retaining these functions it is also adapted to penetrate the tissues of its prey (Fry 1965, Hedgpeth 1971).

### 2.3.1. AMMOTHEA CAROLINENSIS

The series of plates 2B (a to f) shows that *Ammothea carolinensis* (whose proboscis shape is between C' : 1 and D' : 1 of the figure given by Fry and Hedgpeth (1969)) belongs to that group whose proboscis penetrates its prey. Apparently penetration is always through the mouth of the anenome into its digestive cavity; no attempt to penetrate the body wall was observed.

This behaviour suggests the possibility that the ammotheid feeds on the gut contents of the anenome rather than the anenome itself.

(see Calman 1929). It is noted that anenomes always withdraw their tentacles (as shown in the photographs) and indeed seem very sensitive to the approach of Ammothea carolinensis. The chelicerae play no part in the feeding behaviour when approaching the prey, but as can be seen the proboscis is thrust so deeply into the animal (plate 2B(d to f)) that some role within the mouth of the anenome cannot be altogether precluded. A.carolinensis bears palps and presumably these have a sensory role when the animal is approaching its prey (plate 2B(c)). They are not moved around during this process but held rigidly forward, but it was observed that they may enter the anenome mouth with the proboscis and their role within the digestive cavity rests unknown.

### 2.3.2 DECOLOPODA AUSTRALIS

Decolopoda australis was only once observed feeding and this in a somewhat unusual circumstance; aboard ship in a refrigerated aquarium at the Equator. It fed on the same type of anenome enjoyed by Ammothea carolinensis but its approach seemed altogether more brutish. When the observation was made, the meal was already far advanced, a small anenome, pulled off its rock, was in the process of being sucked up into the proboscis of the animal (proboscis shape $D''' : 2 : E'''$ of Fry and Hedgpeth 1969), which was walking round the tank. The meal lasted until all trace of the anenome was lost (about 2 days).

2.3.3. NYMPHON ORCADENSE, NYMPHON AUSTRALE and NYMPHON HIRTIPES.

These species, like Decolopoda australis, have the habit of carrying their prey around with them, differing however in that the prey is not held by the strength of suction of the proboscis, but by both chelicerae. Nymphon orcadense has been observed carrying the same polychaete in its chelicerae for up to six hours intermittently applying the proboscis to the prey. Plates 2D (a to f) show Nymphon orcadense with different prey. Photography of live specimens was found to be extremely difficult (see appendix 3), one factor being that the normally sluggish animals reacted very quickly if disturbed when feeding (e.g. by trying to move the animal nearer the camera, or use of powerful lights), dropping their prey immediately.

Nymphon australe appeared to carry out the same pattern of feeding behaviour as Nymphon orcadense when observed, the prey being gripped in both chelae. Feeding on hydroid colonies was not observed and it is not therefore known if these Nymphons would use the method of tearing off pieces with left and right chelae alternatively as reported for Nymphon leptocheles feeding on Campanularia by Prell (1910), and for Nymphon gracile feeding on Dynamena pumila by King (1973). When feeding on anenomes the chelicerae of Nymphon orcadense and N. australe were used for attachment.

Although showing a preference for attack at the oral end of anenomes, all the Nymphon specimens did not limit their attacks to

this region. They were never seen to put their proboscides (shape B''' or C''' in Fry and Hedgpeth 1969) into the mouths of anenomes as did Ammothea carolinensis, but instead attacked the body wall producing visible damage, an indication that they feed on the tissue of the anenome itself.

## 2.4 DISCUSSION

From the observations on Ammothea carolinensis it is not obvious that the creature east the anenome. Calman (1929) reports an annelid piercing the body wall of a hydroid in order to suck its stomach contents and he indicates that some authors have claimed the same for certain pycnogonids. The fact that the anenome takes avoiding reaction when the ammotheid approaches does not necessarily indicate that it is preyed upon. Pumping out of the stomach contents is obviously not beneficial to an anenome. A more detailed study of the feeding of Ammothea carolinensis is required to clarify the exact nature of its feeding on anenomes. It is imagined that some radioactive label applied to the anenomes might help elucidate the 'anenome or stomach contents' question, but difficulties of technique are foreseen.

The complexities that observations on Ammothea carolinensis introduce serve to re-emphasize the point made earlier that it is difficult to separate feeding from association. Similarly it is difficult to tell when a meal is finished. For example, a specimen of N.orcadense observed carrying its food for 6 hours only ceased

feeding when an attempt was made to remove it to a smaller aquarium for photography. This in turn sheds doubt on the classic technique of studying pycnogonid gut histology by sacrificing animals at feeding and at hourly intervals afterwards (Schlottke 1933). Fry (1965) notes that throughout the nine days of observations his pycnogonids maintained 'full or partially full gut diverticula'. Observations on starved specimens (see later) indicate that it may take as long as two months for the gut to empty. If, as seems to be the case in the antarctic forms studied, feeding and digestion are slow processes, Schlottke's sacrifice time scales will have to be altered considerably for each sacrifice to give stages of digestion different from the previous one. Schlottke draws attention to the difficulty of following the fate of a single food vacuole histologically, since 'after a period of starvation, vacuoles still exist in the endoderm (midgut) in all stages of digestion'. (p. 645).

# SECTION 2. NON-FEEDING

## 2.5 INTRODUCTION

In December 1972 I was able to bring back from the Arctic live specimens of *Nymphon hirtipes*. As mentioned earlier, these had been picked from the cod-end of a Granton trawl and their natural food was not known. The refrigerated aquarium to which they were taken in Luton, incorporated a biological filter (see appendix 4) and there were therefore quantities of detritus on which the animals might feed. At various times, other foods were introduced into the system, but none produced a recognisable feeding response. During the eighteen months that this first batch of animals survived, feeding was not observed.

Attempts to see if Antarctic *Nymphon* species exhibited the same phenomenon were made on Signy Island during the austral summer of 1973/74. Ten specimens of *Nymphon orcadense* were placed in a large Kilner jar filled with seawater which was changed every other day. After two months there had been no fatalities. This Kilner jar was then fitted into the aquarium system on base (seawater was drawn in from Factory Cove and pumped through a succession of aquarium tanks and out again, plate 2E). The flow to the jar was filtered (Fig. 2F), the seawater around Signy Island during the summer usually being rich in diatoms and other phytoplankton. When

I left Signy, this modified apparatus had been in operation for one month. All the animals appeared healthy and the bottom of the jar contained what appeared to be their faeces. Observations on survival after the austral summer 1973/74 were kindly continued by Mr. Neil Tappin of the British Antarctic Survey. He reported in March 1975 that the animals had recently died as the result of an aquarium accident (i.e. not starvation). Nymphon orcadense had therefore survived for 15 months in the apparent absence of any suitable food substrates.

Having presented the phenomenon, the difficulty that remains is to explain it. Unfortunately, it was discovered too late in the project for any further experiments to be performed. The section that follows considers explanations possible but has relevance to many aspects of pycnogonid biology and may indicate directions for further study. The headings considered are:-

Metabolic rate,

Anaerobiosis,

Acclimation,

Uptake of nutrients through the cuticle,

Filter feeding.

2.6 METABOLIC RATE

Antarctic pycnogonids can be described as inactive animals. On Signy Island, base personnel nicknamed them 'Pycnogonkers' ('to gonk' being their eccentric slang for 'sleep'). External inactivity does not, however, necessarily indicate metablic inactivity.

The only published quantitative work related to metabolic rate of pycnogonids is by Douglas, Hedgpeth and Hemmingson (1969) on the oxygen consumption of some large Antarctic specimens. These authors used closed respirometry to make measurements on 14 individuals from 7 species. This unfortunately did not give enough data to make worthwhile comparisons between species, but it was found that the results overall showed a three-fold reduction in oxygen consumption rates over Arctic isopods of equivalent weight.

These findings, when coupled with observations made during this study, indicate a number of possible relationships between feeding behaviour and metabolic rate. One is that the metabolic rate of Antarctic pycnogonids is lower than that in forms from other waters. This is supported by findings that the guts of specimens kept without food take up to two months to empty. Schlottke (1933) gives no indication of the length of time it takes for food to be digested or the gut to be emptied in the temperate forms that he studied. He considers 4 to 8 days to be a starvation period, however. It is assumed from this

that the gut will have emptied and that the digestive process is therefore much quicker in temperate forms. This slow emptying is taken as a reversed reading of Pandian's statement (1975) - 'starvation decreases the metabolic rate;' this being re-read as 'low metabolic rate decreases starvation'. There are dangers in such interpretations, however. Douglas, Hedgpeth and Hemmingson made their comparisons with Arctic isopods, not Arctic or Temperate pycnogonids. Their measurements were not of metabolic rate, but of oxygen consumption.

### 2.6.1. METABOLIC RATE AND OXYGEN CONSUMPTION

Metabolic rate is usually expressed in terms of a 'Q notation', the most common notation being $Q.O_2$ (Q oxygen). This is the rate of oxygen consumption, which is usually expressed as µl of $O_2$/milligram dry weight of tissue/hour. $Q.O_2$ is described as a form of 'indirect calorimetry' (Davson and Eggleton 1962) and it ultimately depends upon the use of oxygen in energy metabolism as an electron acceptor (Gilles 1975) at the end of an electron transport pathway, the 'Respiratory chain' (Lehninger 1975). From this it is deduced that oxygen consumption can only be used as a direct comparison of metabolic rate when the metabolic pathways of the animals to be compared produce the same number of electrons per substrate molecule for the respiratory chain and have no other energy producing pathways. The assumption that must be made is that animals use the Tricarboxylic

acid cycle (T·C·A· or Krebs' cycle) as the final common pathway of all the fue molecules of the cell whether carbohydrates, fats or amino acids. This is a major assumption.

Margulis(1970) postulated that in biochemical evolution the ability to form the molecules called porphyrins came before the development of the Tricarboxylic acid cycle. She shows that the intermediates and enzymes of the TCA cycle must be present for porphyrins to be synthesized and indicates that Krebs' cycle evolved by subsequent linking of the activities of the existent enzymes and intermediates of porphyrin biosynthesis. Krebs himself (1954) does not consider that possession of all the intermediates and enzymes of his cycle is proof that it is in operation.

Lockwood (1968) states that 'at one time' it was thought that tissue respiration in Crustacea was different from that of vertebrates (Rats?) as $C^{14}$ labelled glucose injected into the lobsters, _Panulirus japonica_ and _peniculatus_ did not give $C^{14}$ labelled carbon dioxide (one of the tests for the existence of the Tricarboxylic acid cycle). He neglects to say why thinking has now changed. Gilmour (1961) indicates that in insects a 'pentose phosphate pathway' operates and establishment of the relative importance of this compared with Krebs' pathway in carbohydrate breakdown is difficult.

It is commonly thought that the sole alternative energy producing carbohydrate pathway to the TCA cycle is glycolysis. As long ago as 1955 however, Cohen (a and b) stated that there were at least six different pathways for metabolizing glucose and that 'the time has past when we uncritically ascribe phenomena in carbohydrate metabolism to variations in the Embden-Meyerhoff scheme.' Hochachka, Fields and Mustafa (1973) suggest several alternative pathways for anaerobic carbohydrate metabolism.

Where studies of utilisation of reserves during starvation have been investigated, further light is shed on the differences in metabolic pathways between animals. Thus Nieland and Scheer (1954) found that in the crab *Hemigrapsus nudus* protein stores were first depleted during starvation and carbohydrate reserves were unaffected. In mammals on the other hand, starvation results first in depletion of carbohydrates and in man at least, catabolism of protein for energy is the third and last phase of starvation and 'has an inevitable end' (Lehninger 1975). Barnacles, and the wood-boring isopod *Limnoria* utilise carbohydrates as a first reserve during starvation and this indicates considerable biochemical difference within the same class (Crustacea).

Few animal species have been studied in detail biochemically, and although it may be reasonable to accept that the Tricarboxylic acid cycle is of universal occurrence, this is not proven and there is no reason to believe that it will be of the same importance in

different species or even in the same animal at different stages of its life cycle or in different environments. (A number of intestinal parasites and estuarine invertebrates are facultative or even obligative anaerobes (see e.g. Newell 1970)). Oxygen consumption has been, and will not doubt continue to be, a useful measure of metabolic rate in mammalian physiology. However, the wide biochemical variation which obviously exists amongst invertebrates and the environmentally induced metabolic rate differences occurring in poikilotherms must make oxygen consumption comparisons in such cases of less value.

Hochachka and Somero (1973) found that different enzymes have different activities at the same temperature (varying Q10's). Different metabolic pathways therefore come into operation at different temperature thresholds, (e.g. Hochachka and Somero cite the case of cold acclimated fish in which the pentose phosphate pathway has a greater role in glucose catabolism than does glycolysis).

As Douglas, Hedgpeth and Hemmingson state, the safe conclusion that can be made from their results is that the pycnogonids' $O_2$ consumption is different from Arctic isopods. If there are metabolic affinities between pycnogonids and isopods then it is also likely that Antarctic pycnogonids have reduced metabolic rates.

2.7 ANAEROBIOSIS

Having concluded that it is misleading to compare metabolic rates measured by indirect calorimetry, it seems worthwhile to consider whether Antarctic pycnogonids might be anaerobic. Approached innocently, such a postulate has attractions.

Jones (1972) states that since animal tissues do not contain pyruvic decarboxylase, anaerobic glycolysis cannot yield ethanol and significant amounts of carbon dioxide cannot arise. Under such conditions respiratory gas exchange is in virtual abeyance. One vexing question of pycnogonid morphology has been their lack of obvious respiratory organs, and in most species, lack of a visible respiratory pigment. Although surface area to volume ratio is large, the larger pycnogonids have an increased volume over that calculated for satisfactory gaseous exchange through the surface (Krogh 1941). Anaerobiosis would do away with the need for organs of gaseous exchange, and respiratory pigments, and surface to volume ratios would no longer be so important.

Among the Antarctic *Nymphon* species investigated, there is some circumstantial evidence for anaerobiosis. In a breakdown in the pumping system of the aquarium room on Signy Island, the tanks became anaerobic (smelling strongly of hydrogen sulphide) overnight. This resulted in the deaths of all the marine vertebrates and invertebrates in the tanks with the exception of the pycnogonids (mainly *Nymphon* species), which recovered when placed in tanks of aerated seawater. In the wild (Jan Hoogesteger - personal communication), it appears that

Nymphon orcadense is sometimes found half buried in detritus. This must limit the area for free gaseous interchange and put the animal in what is essentially an anaerobic environment. (Wyer 1972, reports that the 'smaller species are often found amongst debris in rock crevices where the water circulation is restricted and oxygen may not be as readily available').

Other factors contradict the likelihood of an anaerobic metabolism. Thus; Schlottke (1933) stated that the temperate water pycnogonids he studied had to be kept in seawater which was chang twice daily. Isaac and Jarvis (1973) found that Nymphon gracile was very sensitive to anoxia. It has been noted that Nymphon species subjected to anaerobic conditions during aquarium breakdown, became more active (recovered?) when placed in aerated seawater. This suggests that it is more likely that survival was because they were able to build up an 'oxygen debt', that is to say they can be facultative, rather than obligatory, anaerobes.

There is a natural tendency, perhaps because of its low oxygen utilisation, to associate anaerobiosis with low utilisation of reserves. This is fallacious. Carbohydrate anaerobiosis is in fact very wasteful and is usually accomplished at the expense of large amounts of metabolic reserves. (Pandian 1975). Animals such as intestinal parasites and those interstitial estuarine invertebrates which are anaerobes are usually so because nutrient carbohydrates are not a limiting factor. This would appear not to be the case in

pycnogonids, which seem capable of surviving for months without food. Resorption of certain organs as a means of overcoming lack of food is a well known feature in insects and Crustacea (see, for example, Hopkins and King 1964). As indicated, the starving specimens of Nymphon orcadense were left on Signy Island and were not therefore available for examination. Similarly the Arctic nymphons died in Luton whilst the author was in the Antarctic and were not preserved. However, it is suggested that whilst the reproductive system might form a suitable organ for resorption under starvation conditions, the periodic acid schiff test did not reveal riches of carbohydrate in the system of Antarctic forms. Jarvis and King (1972) found that the yolk of temperate forms is a protein-carbohydrate complex, with associated lipid. Though blood lipoproteins are present (see later), their concentration is not high and again one can see no advantage in metabolising lipids into the more wasteful anaerobic pathways.

An additional consideration militating against anaerobiosis comes from Hochachka and Somero (1973), who find that generally there is an increased reliance on aerobic metabolism in the cold acclimated state.

## 2.8 ACCLIMATION

It has been intimated that Antarctic pycnogonids have a lower metabolic rate (exemplified by the slow rate of digestion and emptying of the gut) than have temperate-water forms. This contradicts the

concept of 'temperature acclimation', which holds that animals (poikilotherms) living in cold environments have enzymes and enzyme systems adapted to be at their optimum activity at these temperatures (see e.g. Bullock 1955). In addition, in histochemical tests for gut enzymes in Nymphon orcadense (see later) it was found that reaction optima were at temperatures approaching 37°C rather than the normal environmental temperature (around 0°C).

The imperfection of the Luton refrigeration system has proved useful in giving some accidental acclimations of polar forms to higher temperatures. Thus the Arctic Nymphon hirtipes caught at temperatures below 3°C (see Table 1E) were maintained for well over a year at 6°C (the temperature of the aquarium system could not be lowered to 2°C). These animals exhibited 'non-feeding', but eggs they were carrying hatched and passed through several of the larval stages (plate 2G). The Antarctic Nymphon orcadense was gradually (several days) acclimated to 11°C (as the refrigeration failed to cope with a heatwave in July-August 1975). The animals seemed slightly more active and continued feeding. One of these animals, back at lower temperatures, was still surviving in August 1976.

Whilst not doubting that acclimation occurs in Polar poikilotherms, inculcated heresy fosters the view that the search for acclimating mechanisms stems from pantheism: Nature must be efficient and elegant and able to circumvent any chemical law -

Ramsay (1952) makes comment on this:- "Natural selection is not interested in physiological efficiency for its own sake, but only in so far as it can contribute to the effectiveness of feeding, or escape or reproduction or any other aspect of its biology that brings the animal up against the rub of the environment."

An animal obtaining oxygen by diffusion (as a pycnogonid presumably does) and capable of surviving long periods without seeming to feed (as do polar Nymphon specimens) seems to be at great risk if acclimation occurs.

An interesting recent essay on acclimation is that of Hochachka and Somero (1973 - Chapter 7). They examine the phenomenon by application of chemical kinetics and consideration of the effect of temperature on the weak bonds of the tertiary and quaternary structures of proteins (enzymes). Arrhenius'laws (which are approximations applicable to simple chemical reactions) state that there would normally be a decrease in rate of reaction as temperatures are lowered. Hochachka and Somero believe these laws can be defied (in cold acclimation) by two systems of adaption. In the first, rate compensation is brought about by changes in enzyme concentration. The alternative adaptive system is to possess Isozymes or Allozymes of slightly different tertiary or quaternary structure which will play the major role at lower temperatures. Evidence indicates that Antarctic Nymphonidae possess neither of these systems.

It is interesting to reflect upon the importance of temperature in pycnogonid distribution and whether their success in polar regions is related more to diet and brooding the young rather than any great metabolic adaptation to low temperatures. It is interesting to note that Nymphon orcadense has been collected as far north as the Falkland Islands (Hodgson 1907) and N.australe as far north as the coast of South Africa (Flynn 1928). The temperature of the waters in which these collections were made is not known. (The possibility of dispersal by ship is commented on by Hedgpeth 1947; Hardy 1967 considers the same subject but not in relation to pycnogonids).

## 2.9 UPTAKE OF NUTRIENTS THROUGH THE CUTICLE

The idea of the uptake of nutrients through the integument of aquatic animals is not new. Most authors quote Putter (1909) as being first in the field, but a discussion of the possibility of uptake of organic matter through the integument of deep sea animals is found in Wyville Thomson's writings (1873). The most prolific recent worker on the subject appears to be Stephens (see Stephens G.C. 1972 for a review of amino-acid assimilation). Assimilation through the integument has been reported in the Cnidaria, Platyhelminthes, Rhynchocoela, Ectoprocta, Annelida, Sipunculoidea, Echiuroidea, Mollusca, Echinodermata, Hemichordata and Chordata. There is

only one report of an Arthropod removing organic compounds directly from solution (McWhinnie and Johanneck 1966); interestingly, this is an antarctic species, the crustacean *Euphausia triacantha*. Stephens (op.cit.) disputes this finding by reference to the work of Anderson (see Anderson and Stephens 1969) who reports that the removal of organic compounds directly from solution by arthropods appears to be entirely attributable to micro-organisms on the exoskeleton. The publication of McWhinnie and Johanneck is only a brief note and contains little detail of methods. Anderson and Stephenson the other hand, did not look at Antarctic species. Their tests were for the uptake of amino-acids (those of McWhinnie and Johanneck were for the uptake of acetate and glucose), and exposure of animals to the substrate ($C^{14}$ labelled glycine) was for a maximum of 4 hours.

It is suggested that it is necessary to investigate a spectrum of organic compounds over greater incubation periods to be sure of uptake or non-uptake. While the paper of McWhinnie and Johanneck does not demonstrate its claim convincingly, the nature of the animals tested and the short exposure periods make Anderson and Stephens' work unconvincing disproof. This latter publication makes good emphasis of the difficulties of technique, however, and makes an interesting statement:- 'It is possible to think of the arthropod and its associated epiflora as an ecosystem. Amino-acids

might contribute to the nutrition of the epiflora which could then be cropped periodically and serve as a food source for the arthropod.' Wyer (1972) suggests that debris cleaned off pycnogonids by their ovigers is utilised as food. Certainly pycnogonids can become covered with various forms of life (see Plate 2H).

Preliminary experiments on cuticle uptake were made by keeping Nymphon orcadense inseawater containing the vital dye, neutral red. After several days the animals were sacrificed and fixed. Subsequent sectioning did not show neutral red staining in the cuticle, hypodermis, or the gut. This result cannot be looked upon as final, however, as vital dyes are not always retained by fixation and the chemicals to make Altmann's fixative, recommended for fixation of neutral red vital staining (Baker 1958) were not available on base. Cryostat sections of specimens not previously fixed were too prone to diffusion artefacts to allow a definite interpretation.

Pores have been noted in the cuticle of several species of pycnogonids and are found in all those investigated during this project. Their significance is not known. As mentioned, Nymphon orcadense half buries itself in detritus and should therefore be in an environment rich in dissolved organic substances.

The question of cuticular uptake of nutrients remains unanswered.

## 2.10 FILTER FEEDING

Pycnogonids are essentially filter feeders. Although they may tear off large lumps of prey with their chelicerae, the teeth and setae of the pharynx so grind and filter the food,that what enters the midgut no longer resembles the prey and is particulate. In describing the non-feeding phenomenon it was said that pycnogonids went for long periods without exhibiting their normal feeding behaviour. Sections 2.6 and 2.7 assume that no food is taken in, this section does not.

It is presumed that it is possible for an animal, which normally crushes and filters food in its pharynx, to save the energy required in searching out prey, carrying it or tearing it, and crushing it, by filtering directly an environment rich in particles. Specimens of *Colossendeis proboscidea* from the Arctic were seen opening and closing their mouths goldfish fashion. Hedgpeth (1954) has drawn parallels between the larval proboscis of the archiannelid *Protodrilus*, an apparatus highly specialized for catching microplankton, and the proboscis of pycnogonids. The polychaete worm eaten by *Nymphon orcadense* was not available after February, and although the animals were seen feeding on other prey, these observations were less frequent than were those when polychaetes were available.

It is proposed that the animal may feed part of the year in predatory style, part by remaining inactive and filtering the medium.

Although the starvation experiment on Signy Island incorporated a filtering system for seawater (Fig. 2F) the efficiency of this was probably not great and did not exclude the size of particle that it is imagined would be sieved. Similarly the system in which the Arctic *Nymphon hirtipes* was maintained also contained plenty of particulate matter.

An added attraction of this theory is that there are Antarctic benthic invertebrates in which seasonal change from predatory to filtering behaviour has been noted. Thus the starfish *Odontaster validus* (M.G. White personal communication) spends part of the season aboral side uppermost feeding on bivalves and the rest of the season with oral side uppermost, while using its tube feet in a ciliary role to waft suspended particles to its mouth. It would seem that conversion of *Nymphon orcadense* from predator to filter feeder would require a much less drastic change of behaviour than in *Odontaster.*

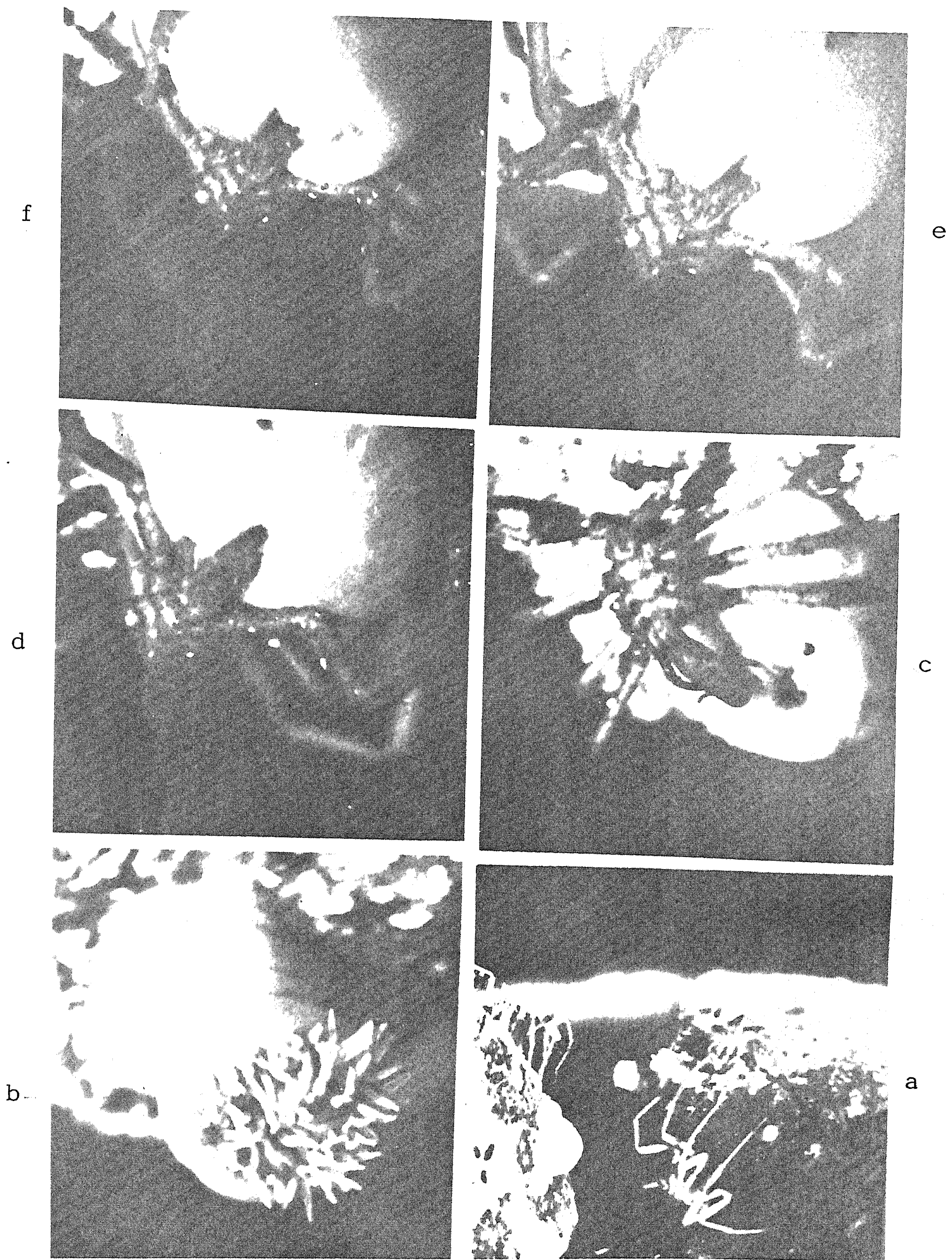



PLATE 2 B. (a to f) Ammothea carolinensis feeding on a Sea anenome.

(this plate has been printed upsidedown by mistake).

PLATE 2 C.a The polychaete preferred as food by *Nymphon orcadense*.

PLATE 2 C.b Print of an underwater colour slide (provided by Jan Hoogesteger) showing Nymphons (probably N.orcadense) on a red seaweed.

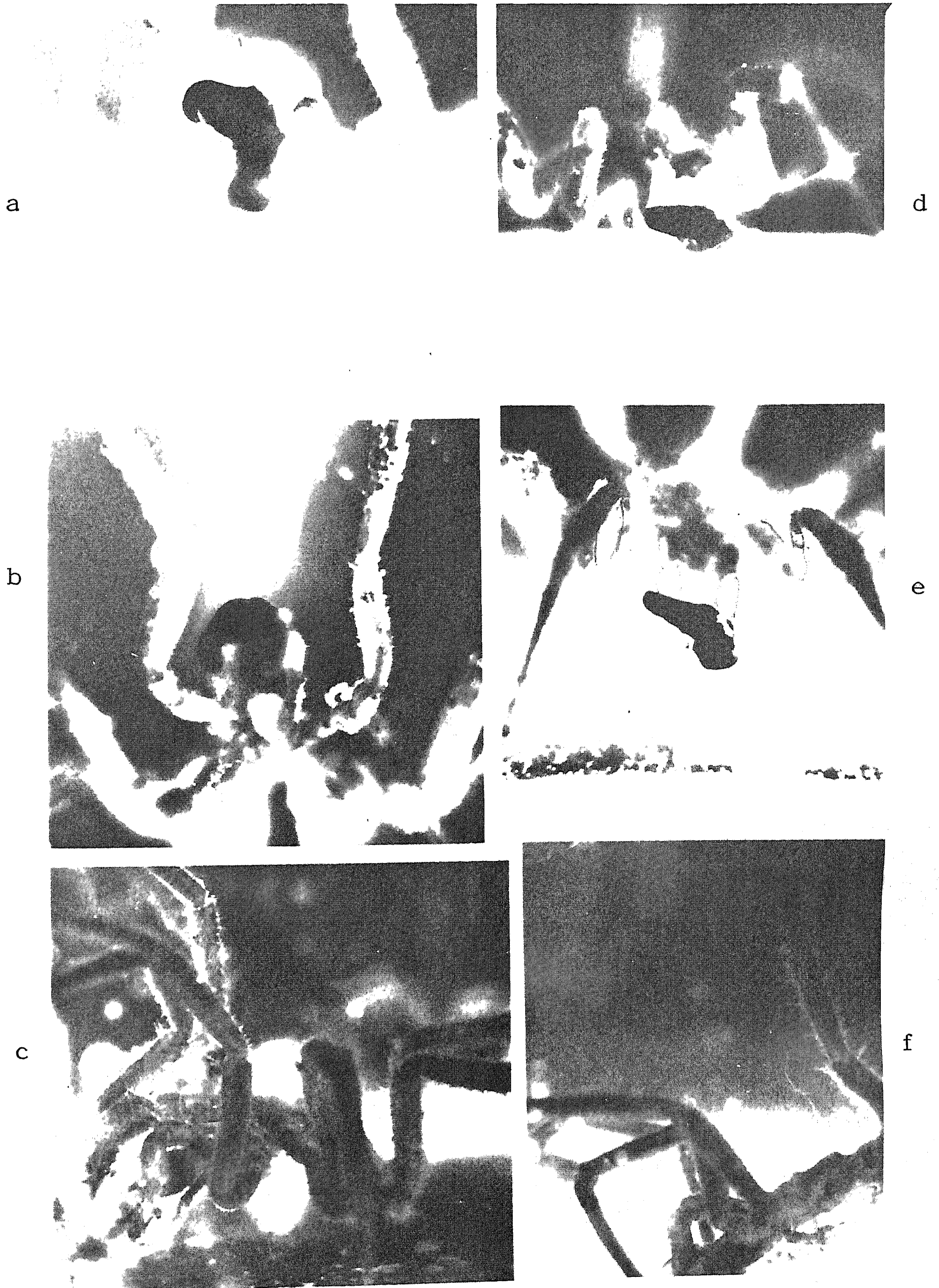



PLATE 2 D. (a to f) Feeding in N. orcadense - the prey is grasped by the chelicerae and the pycnogonid carries it as it feeds

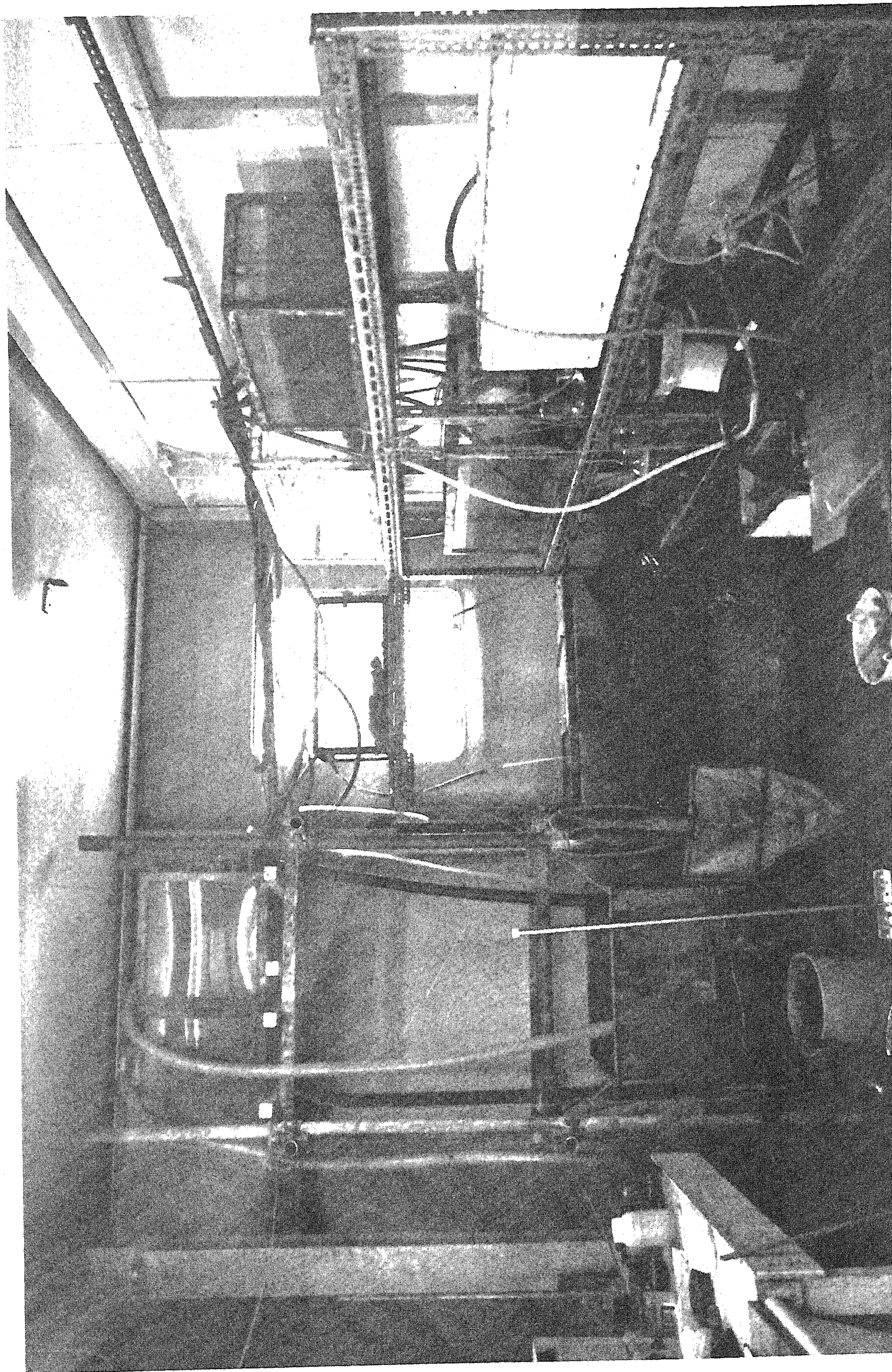


PLATE 2 E. The Aquarium Room, Signy Island.

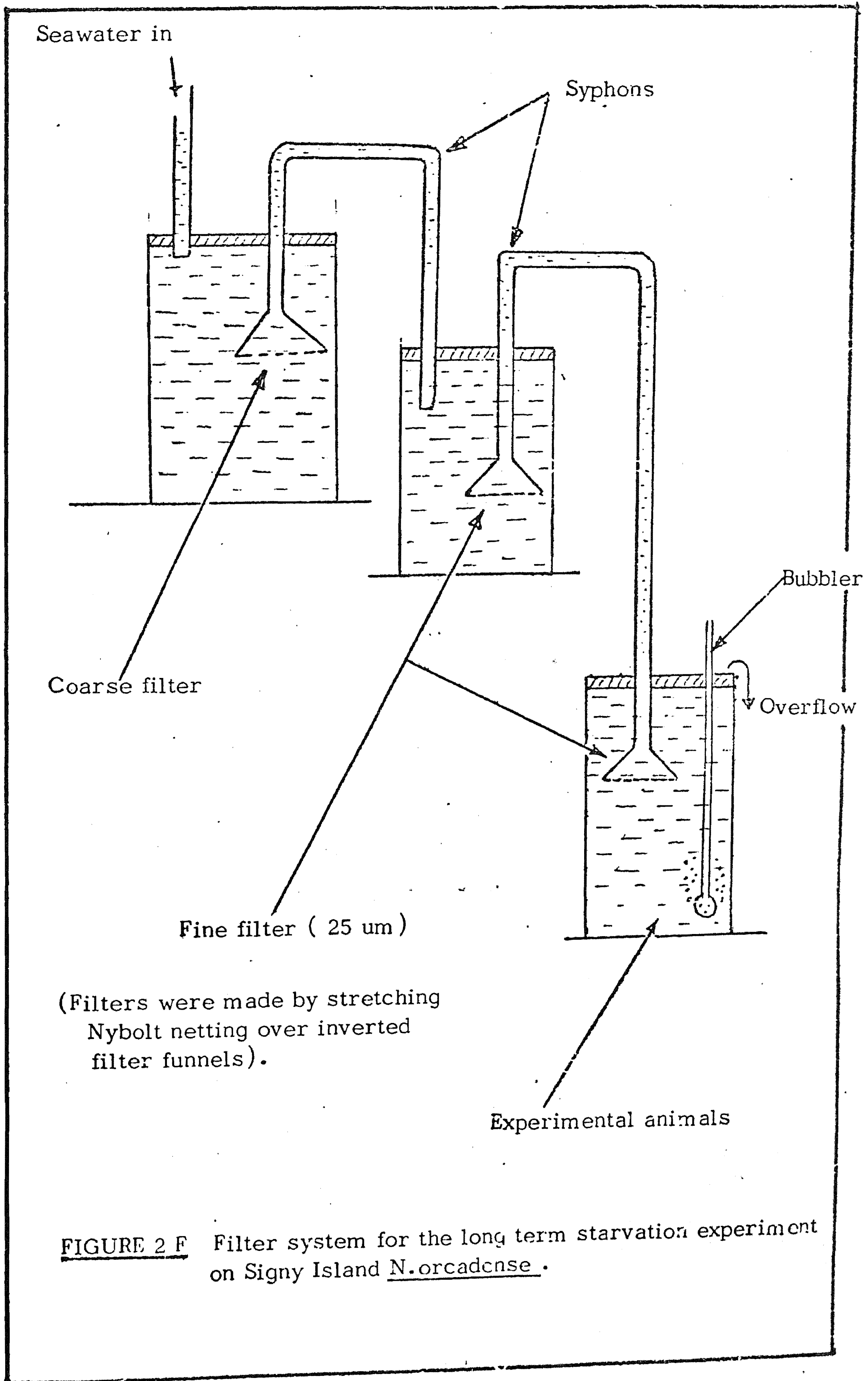
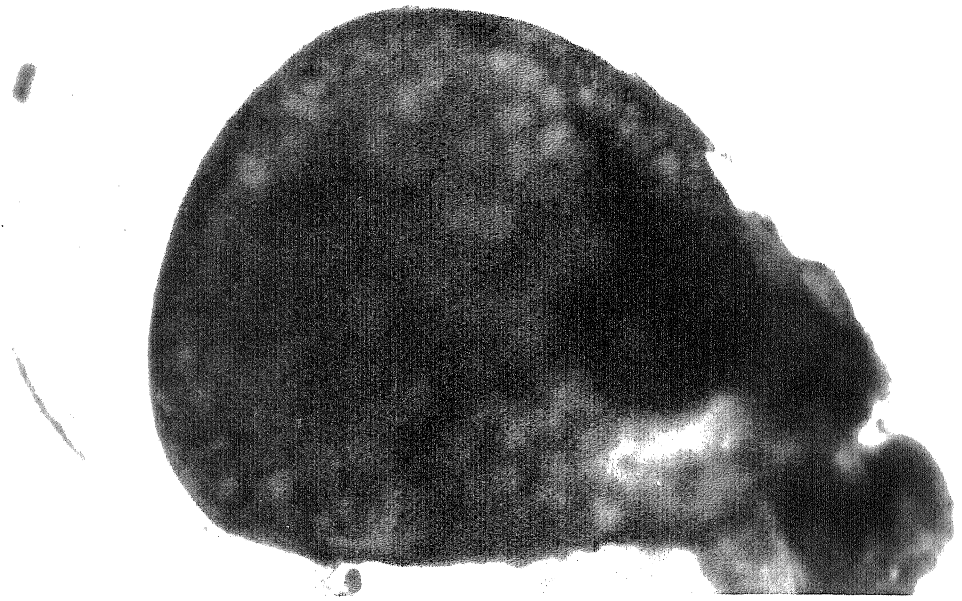



FIGURE 2 F Filter system for the long term starvation experiment on Signy Island N.orcadense .



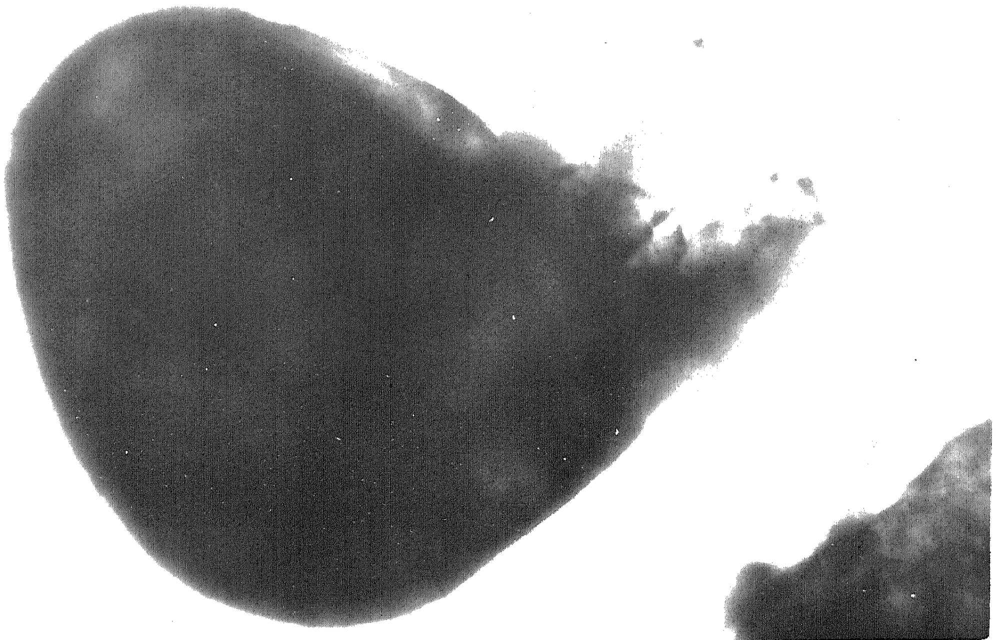
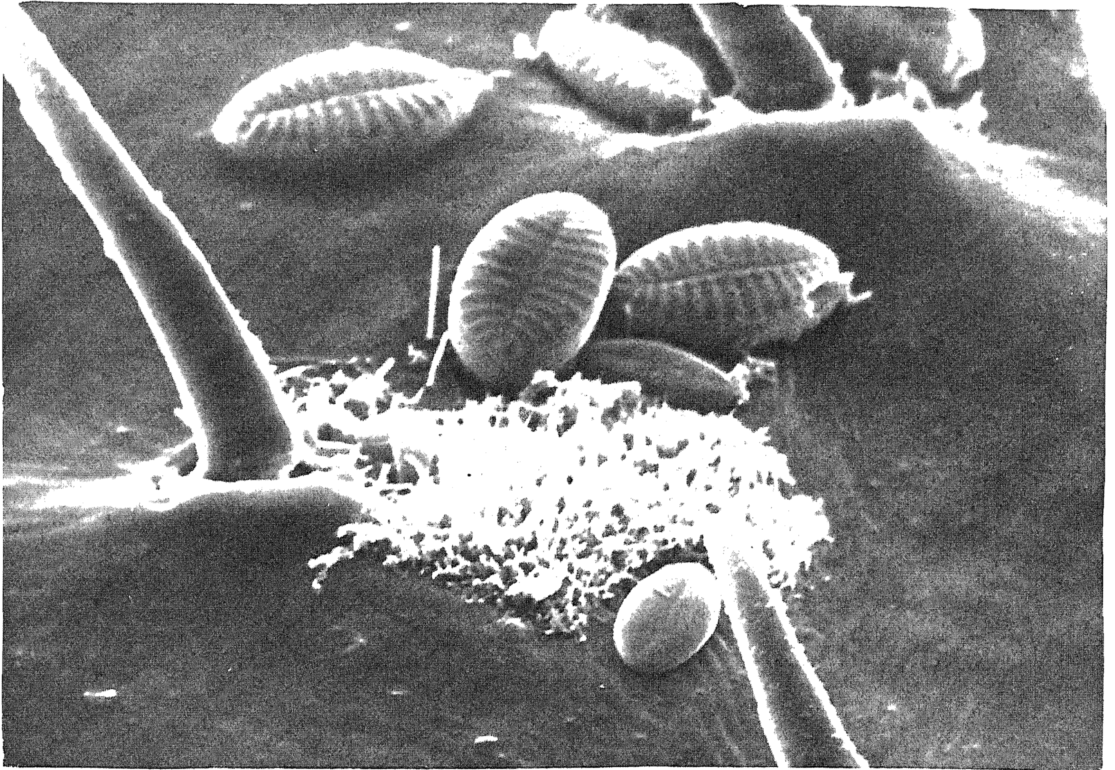
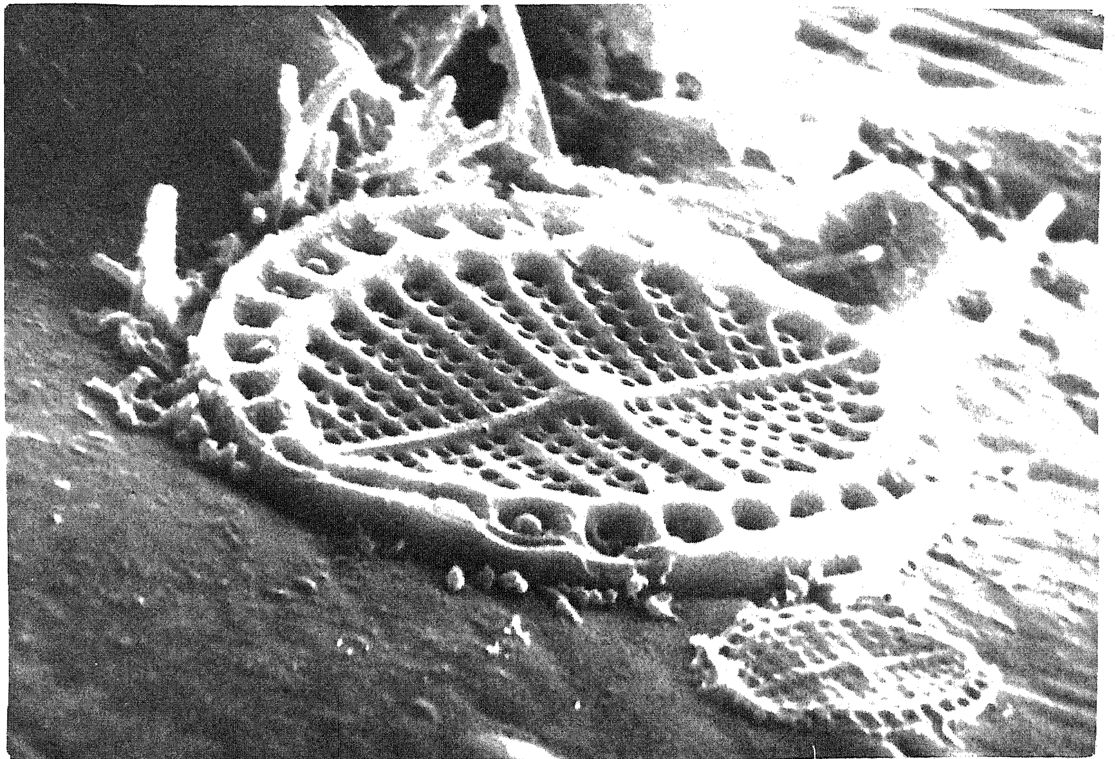


PLATE 2 G. Protonymphons of *N. hirtipes*.

PLATE 2 H. i)

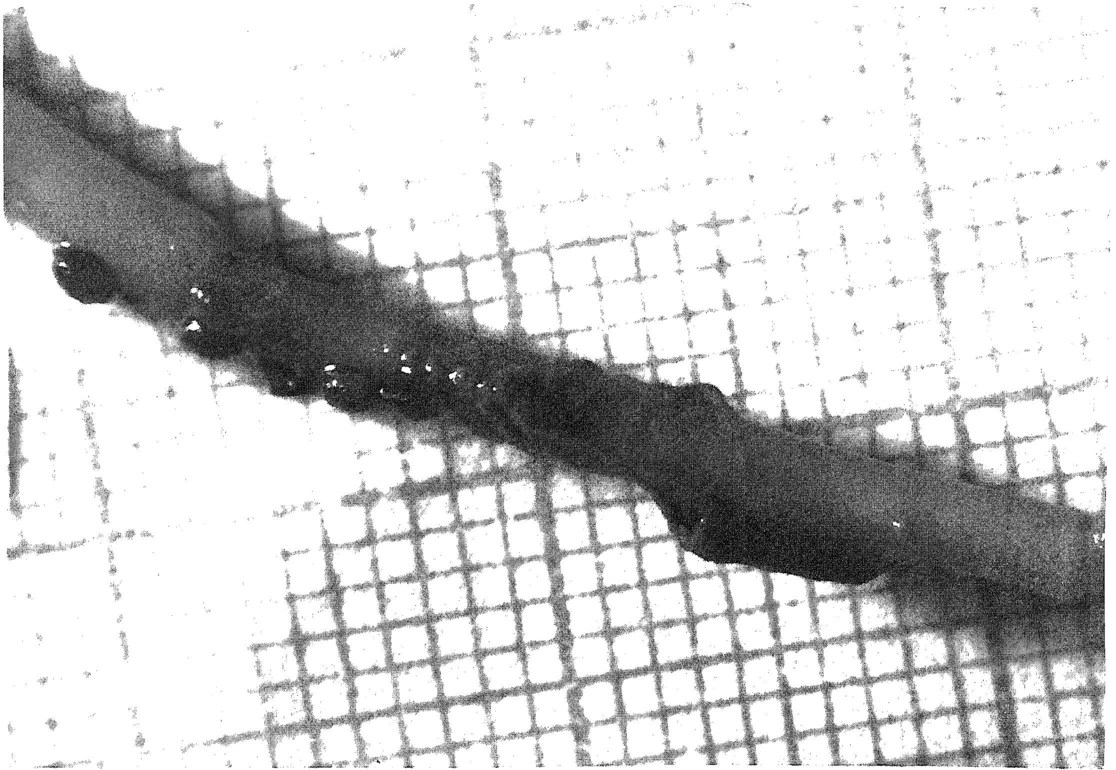


ii)

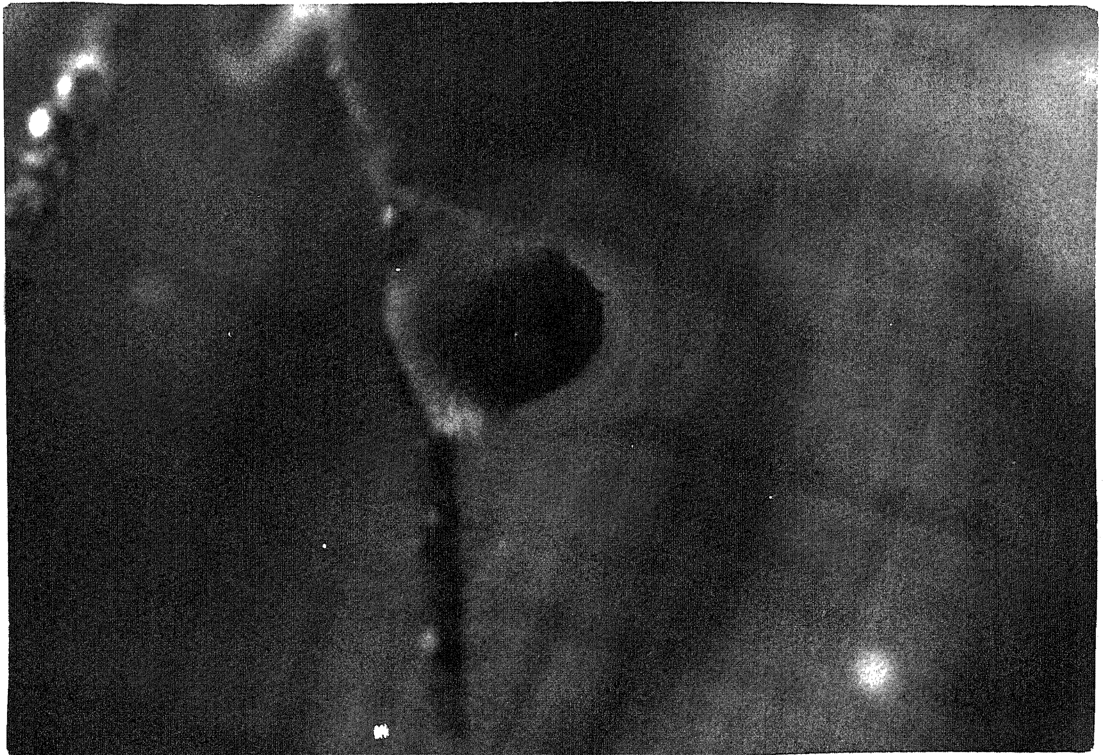


i) and ii) are scanning electron micrographs of the cuticle of *N. orcadense* showing diatoms. i)=x1200
ii) =x4000

iii)



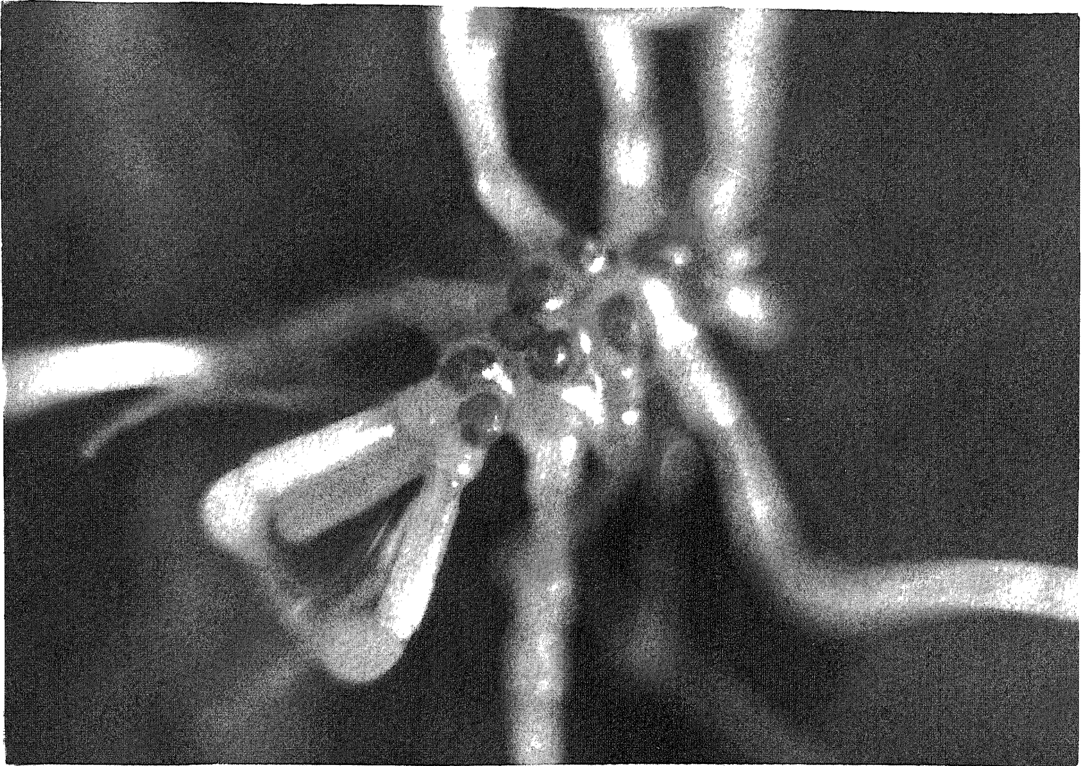
iv)



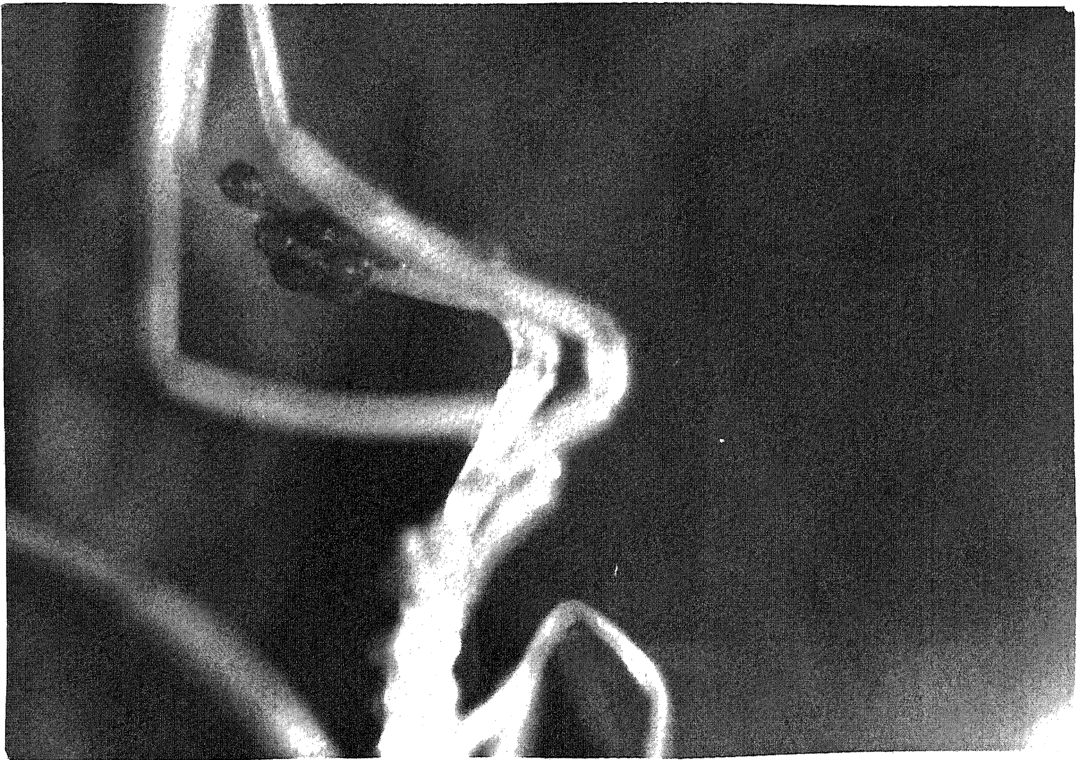
iii) leg of Colossendeis proboscidea with encrusting mollusc eggs.

iv) leg of Arctic Nymphon with encrusting organism.

v)



vi)



Antarctic Nymphon spp. with encrusting mollusc eggs.

# CHAPTER 3

## HISTORY, HYDRA, AND MASS TRANSPORT

## 3.1. INTRODUCTION

The most recent published work concerned solely with the mechanism of digestion in pycnogonids is that of Schlottke (1933), which also summarizes all work to that date. Since then, Sanchez (1959) has examined the structure of the digestive system in certain embryos, and Wyer (1972) has investigated digestion in certain British species. Helfer and Schlottke (1935) contains a section on digestion which is basically Schlottke's paper of 1933 modified and shortened for review purposes. Fage (1949) and Nichol (1967), works which I have seen quoted as original, are review articles and are not reports of research by their authors.

As a scientist is limited by the physical apparatus available to him at the time of his experiments, so he is influenced by the theories and knowledge available to himat that time. Thus, in all probability the apparatus used in the research described in the following chapters did not differ greatly from that used by Schlottke in 1933 (the electron microscope was only available for a short period). However, electron microscopy and other techniques have greatly altered cytological thinking since 1930. 'Le cytoplasme proprement dit se présente, sur le vivant comme une substance colloidale homogène, translucide, optiquement vide a l'ultramicroscope' (Guillermond, Mangenot and Plantifol 1933). The time of such a simple picture of the cytoplasm is passed.

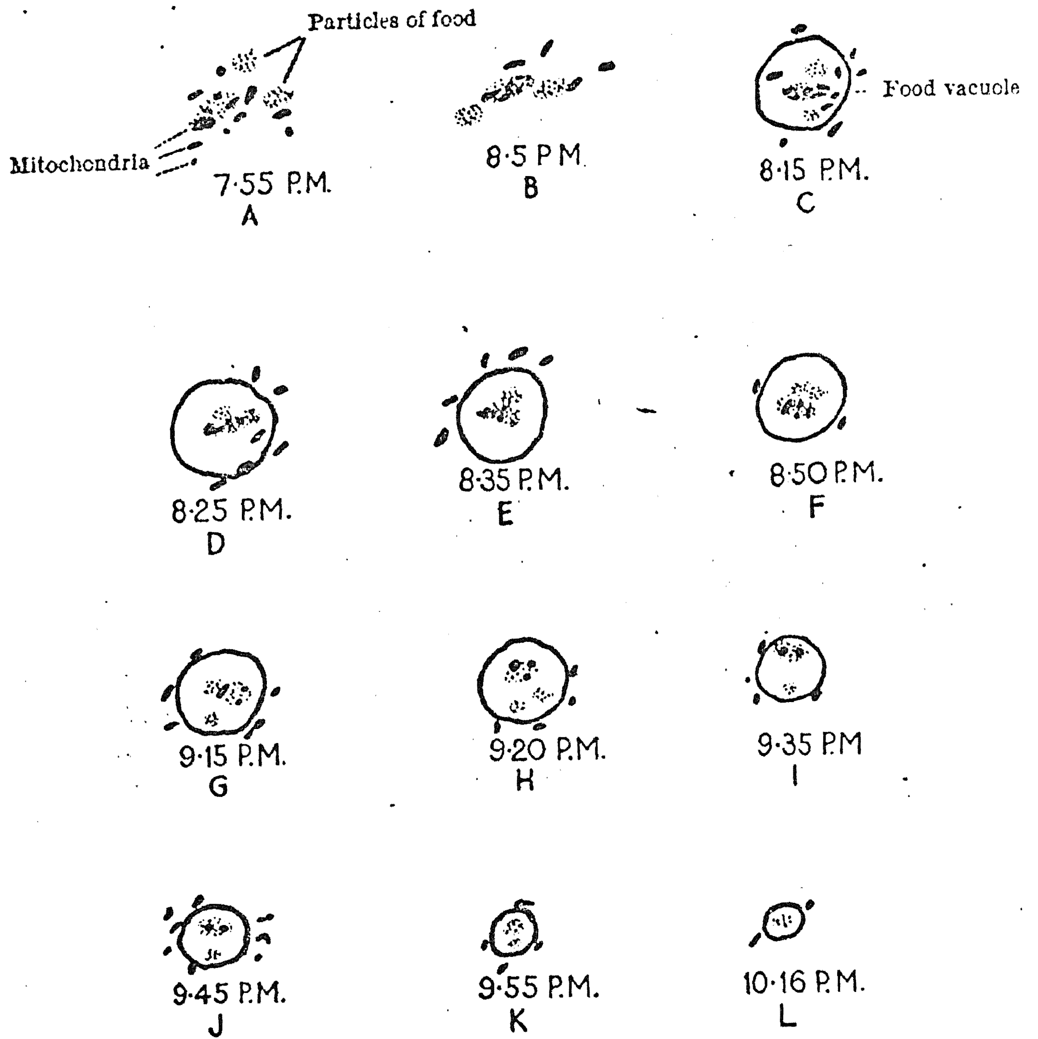
As this work is prejudiced towards present day theories, so Schlottke appears on a number of occasions to be influenced by the theories current in his time. It will be seen how he compared digestion in pycnogonids with that of Hydra and proposed a scheme of nutrient transport based on McConnell's idea of 'endogenous fragmentation' - a scheme which has little mention in current Hydra literature. In describing the problems of nutrient transport he made no reference to the field of mass transport, indeed some of the early work of Krogh on gaseous diffusion had still to be published (e.g. Krogh 1941).

In referring to writings of Beutler (1924), who indicates the presence of enzymes in vacuoles, Schlottke was predating the lysosome concept (see later) by 30 years. Schlottke mentioned phagocytosis, but pinocytosis had only just been discovered (Lewis1931) and was to undergo a period of neglect (see later). On numerous occasions during his histological description of pycnogonid midgut, Schlottke described mitichodria or mitochondrion-like granules. He stated that they are present throughout all stages of digestion in absorption cells and that they are formed at the margin of food vacuoles ( he ascribed no function to these mitochondria). It was not until 1937 that Krebs postulated the Tricarboxylic acid (T·C·A·) cycle and not until 1948-50 that Kennedy and Lehninger discovered that this cycle took place in the mitochondria (Lehninger 1975). The specificity of staining methods for

mitochondria before the time when their true nature had been elucidated is doubted.

When reading Schlottke's paper with cytological theories of his day in mind, the distribution of mitochondria around a food vacuole fits well the theory of Horning (see figure 3A), which gives mitochondria a role in digestion. Schlottke did not mention this theory, but as a digestive physiologist he is almost certain to have been aware of it. Reading with current theories in mind: if some of the digestive 'mitochondria' described by Schlottke are not the specific particles currently defined as mitochondria but are re-classified as primary lysosomes, their distribution would fit into current views of the lysosome concept (see chapter 6).

The preceeding passage is not intended as a slight on Schlottke's work. It merely emphasises that physiological and biochemical scientists in particular tend to be limited by theories current at the time of publication and that it is sometimes necessary to consider their work in its historical context. Thus, for example, to agree (as has one recent author) with Schlottke's belief that digestion of protein in pycnogonids takes place in the cytoplasm, whilst digestion of nucleic acids takes place in vacuoles, is to forget that views on cellular digestion were different in 1933 and to contradict all current theories of the digestive process within cells. (As will be seen later, Rothman (1975) believes that digestive enzymes can occur free within the cyto-plasm, but they are in an inactive form). Conversely if Schlottke was




Digestion of food particles by mitochondria in amoeba. Re-drawn (modified) from Horning

FIGURE 3A:- Scheme of role of mitochondria in intra-cellular digestion due to Horning (taken from Bourne (1951)). This scheme was put forward in the 1920's and is no longer accepted.

right on this point then either much of the lysosome concept is wrong and a complete rethinking of cellular digestion is required, or pycnogonids must have cells and proteins radically different from those known in all other plants and animals.

Schlottke was an Invertebrate physiologist concerned with digestion, rather than a marine biologist or someone exclusively concerned with pycnogonids. His work is quoted at length by Yonge (1937) in his review of digestion in metazoans. Within a short period of the publication of his work on pycnogonid digestion, Schlottke published long articles on the digestion of Hydra (1930), Turbellarians (1933a), Limulus (1934 a, b and 1935), false-scorpions (1933b) and insects (1937 a and b ). This is in addition to his collaboration with Helfer (Helfer and Schlottke 1935).

Yonge (1937 pps 89 and 92) disagreed with Schlottke's view that intracellular digestion is confined to those animals in which the phagocytotic regions of the gut develop from embryonic cells containing yolk, and with his statement that the kind of food and mode of feeding bears no relation to the mode of digestion. Schlottke's generalisation that one type of digestion (extra cellular or intracellular) alone is found in any group was also disputed.

In his 1933 paper on pycnogonid digestion, Schlottke took space to consider digestion in Hydra and a planarian and the distribution of nutrients in Hydra. When stating that the gland cells of the

pycnogonid midgut secrete enzymes into the lumen (P636), he used the similarity in appearance of their large weakly acidophilic globules to those shown by Beutler (1924) to contain enzymes in *Hydra* as the main evidence for secretion. On a number of occasions his text draws parallels between pycnogonids and *Hydra*. Phrases like 'a similar system exists in the resorption endoderm of *Hydra*,' (p643) or (after describing the role of wandering cells in nutrient distribution in *Hydra*) 'a similar process happens in pycnogonids' (p652-653) are common. The comparisons are drawn so strongly that it sometimes appears necessary to know Schlottke's views on *Hydra* before his pycnogonid ones can be fully understood.

The section that follows summarizes the scheme of digestion in pycnogonids but forward by Schlottke (1933). It is believed that his interests made him susceptible to drawing parallels with *Hydra*. It has already been indicated that he held specific views on digestive processes in invertebrates. What then follows is a consideration of digestion in *Hydra*, a brief introduction to mass transport and a consideration of its significance in pycnogonids and coelentrates. The validity of comparisons between these two has to be considered, because Schlottke's views have become so firmly entrenched in the Pycnogonid digestion literature.

## 3.2. SUMMARY OF DIGESTION ACCORDING TO SCHLOTTKE

The midgut epithelium of pycnogonids consists of three types of cell. From lumen towards basement membrane these are:-

ABSORPTION CELLS

GLANDULAR CELLS

TOTIPOTENT EMBRYONIC CELLS

After maceration and filtration by the setae of the pharynx, food enters the midgut via a triangular section valve. No cellular structure is visible in the food pulp entering the midgut and peristalsis moves this pulp along the trunk and into the limb caecae.

Digestion is intracellular but gland cells discharge their weakly acidophilic globules (containing enzymes) into the lumen at the start of food uptake. Because of food intake, the midgut lumen expands and basally situated young absorption cells come into contact with the food. They bulge into the lumen, growing considerably in length and width as food is taken into small vacuoles distally. About $1\frac{1}{2}$ hours after feeding, the number of vacuoles has increased and they seem to be surrounded by mitochondrion-like granules. Their contents precipitate and deposit around the vacuolar walls. At this stage the contents are basophilic. Basophilia gradually extends into the interior of the vacuole and its'contents then become distinctly acidophilic. In the final stage, vacuoles become compacted and weakly basophilic.

One day after feeding the whole epithelium is seen to be crammed with distinct granules, the larger ones being of protein, the smaller ones of fat. Four to eight days after feeding, gland cells are seen to be in an enlarged state and completely surrounded by absorption cells so that no part of the former borders the gut lumen. The height of the epithelium varies with the region of the gut and nutritional state. After a period of starvation, vacuoles in all stages of digestion still exist in epithelial cells. Schlottke noted that it is very difficult to trace the sequence of stages histologically and states : "In order to obtain an exact sequence of the later stages, it is necessary to observe the hind-gut and the formation of faecal cells (kot-balls)".

Faecal cells are divided into two regions, a grey, unstructured region which consists of protein globules which are initially contained in vacuoles. The other region consists of lighter staining (refractile) vacuoles containing chromatin. The two regions, Schlottke claimed, show that digestion of protein and nucleoprotein are separated from each other within the phagocytotic cell. As digestion proceeds the protein globules appear to lose their shape and clump together.

Concomitant with these intracellular changes, changes in the general structure of the epithelium occur. It has already been mentioned that after four to eight days' starvation the gland cells are enlarged and surrounded by absorption cells so that no part of them

borders the gut lumen and that young absorption cells expand and come into contact with the lumen. It has been found that after food intake, a considerable increase in the number of embryonic cells takes place by some form of amitotic division. Used gland cells are reabsorbed. About one day after feeding, the absorption cells start to detach themselves from the epithelium. The cells may either detach singly or, after being formed into larger villi, break off as a clump of cells. Digestion continues within these cells as they circulate in the gut and eventually they re-attach singly to the epithelium (never reaching the basement membrane) so that their products are resorbed. Epidermal cells which contain only waste-products, or little useful material, have less tendency to re-attach and these eventually leave in the faeces. Schlottke's digestive scheme is summarized in Figures 3B and 3C.

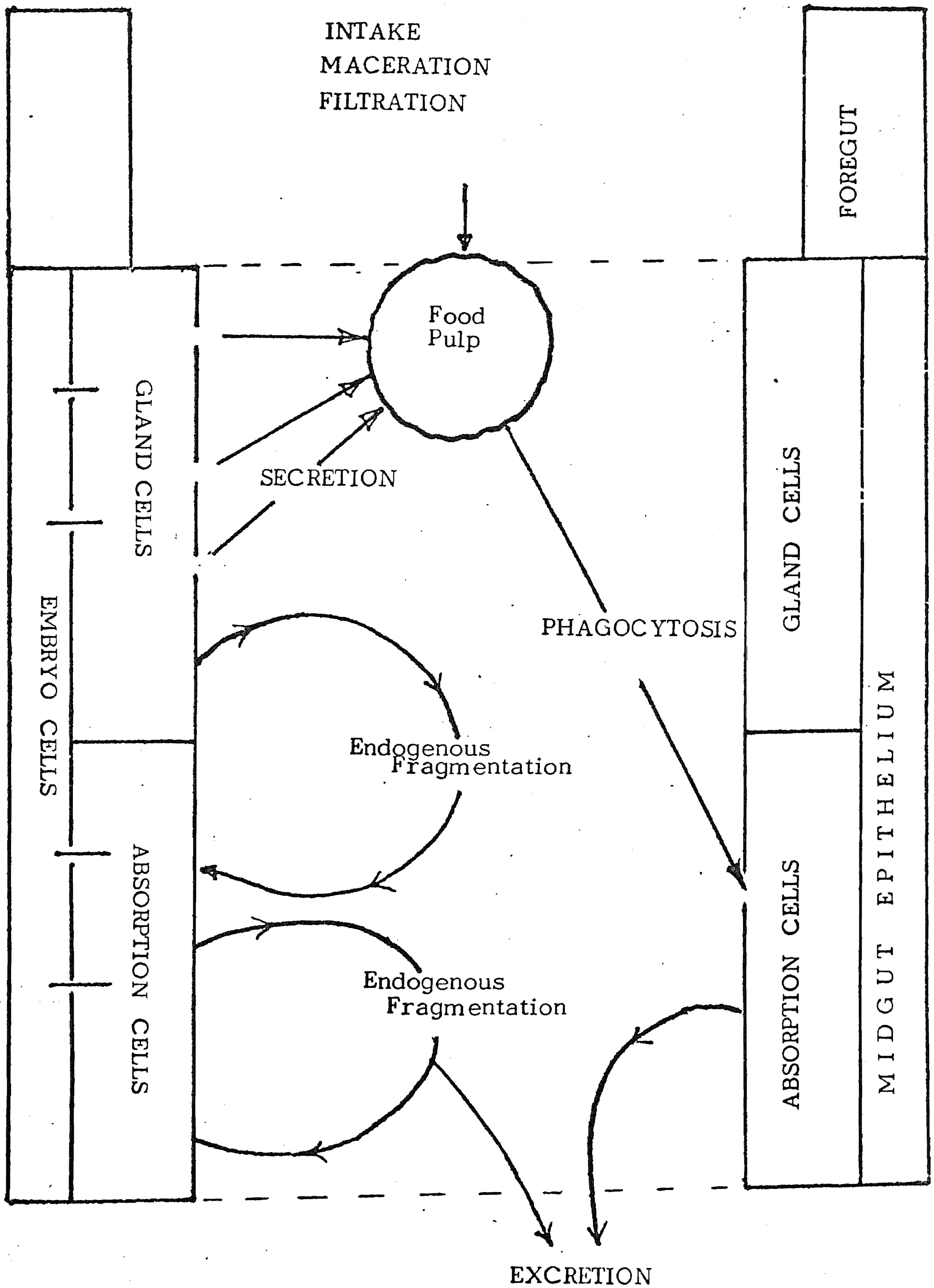
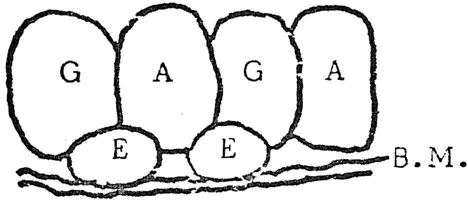
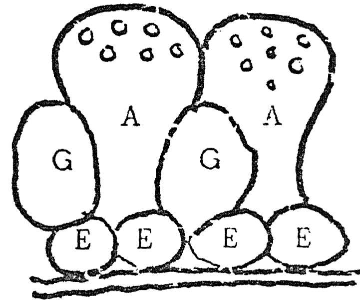



FIGURE 3 B.

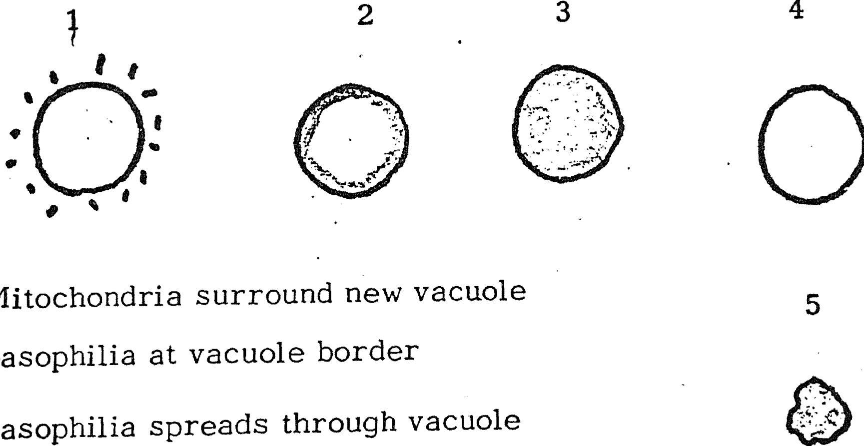
Main events in the pycnogonid gut during digestion according to Schlottke's Scheme.




The number of totipotent embryo cells increases and the absorptive cells expand into the lumen during feeding

FATE OF VACUOLES·




1 Mitochondria surround new vacuole

2 Basophilia at vacuole border

3 Basophilia spreads through vacuole

4 Vacuole becomes acidophilic

5 Vacuole shrinks and is now basophilic

FIGURE 3 C

Some cellular events during Schlottke's Scheme.

## 3.3. FEATURES OF THE DIGESTIVE PROCESS IN HYDRA

Schlottke (1930) stated that, in Hydra, there is no circulatory system and transport of nutrients must be within the epithelium. There are three possible mechanisms for transport:- Firstly, cells loaded with food can wander amongst the other cells. Secondly, fragments of cells break off and are transported to other parts of the body by the action of flagella and are taken up by other endoderm cells. Thirdly, food particles pass from one cell to another. Schlottke rejected the first mechanism, stated that the third may be possible, and noted that the second method was directly observed. Schlottke cited McConnell (1931), who claimed that the second method, which he termed 'endogenous fragmentation' is the most common method of nutrient transport in Hydra. McConnell also saw multicellular villi breaking off into the lumen (as Schlottke describes in pycnogonids) but noted that this may not be a normal process.

McConnell's views on transport in Hydra are indicated at the start of his discussion (it is assumed that Schlottke's thoughts are similar as he agrees with McConnell over both his observations and their interpretation):- "From the standpoint of its Diploblastic nature, Hydra must make cellular adjustments which are peculiar to it. These adjustments are imposed upon Diploblastic organisation because of the lack of mesodermal tissue out of which to elaborate transportation structures. Transportation methods in Hydra therefore involve the

activity of flagella, independent cells and aggregates of cells."

Hyman (1940) dismissed the importance of Diploblastic-Triploblastic difference however:-

"The radiate and bilateral phyla should no longer be distinguished on the basis of the alleged absence of a mesoderm in the former, and should not be offset against each other as diploblastic and triploblastic groups respectively; from a morphological point of view, shorn of phylogenetic theory, no actual difference exists between the construction of a sea anemone and a planarian, as regards general body layers."

Chapman (1958) stated that the polypoid forms of the Coelenterates conform in general terms to the plan of those animals which have longitudinal and circular muscles enclosing a fluid filled cavity. The Diploblastic/Triploblastic condition makes no difference to his physiological consideration of hydrostatics.

Lenhoff (1968) has stressed that in sessile colonial hydroids food has to be distributed over great distances and colonies constantly undergo rhythmic peristaltic contractions. When food is ingested, contraction accelerates and then declines to a resting state after several hours.

Under the heading 'internal transportation', McConnell (1931) stated that although "Diploblastic anatomy does not lend itself to the

development of blood or lymph vessels, the peristalsis of the Hydra, it is true, greatly agitates the fluid contents of the coelenteron ..... all this has been generally known."

Investigation of more recent literature reveals that the digestive process of Hydra has not been elucidated as finally as McConnell and Schlottke believed. Thus, whilst McConnell divided the endoderm into three anatomical regions, Burnett (1959) and subsequent workers accept six distinct regions. In an example already quoted, Schlottke used similarity in appearance of gland cells of the pycnogonid gut and gland cells of the Hydra gastroderm as evidence for the existence of extracellular enzyme packets (globules). Burnett (1959), however, has stated that the function of the gland cells of Hydra is problematical; whilst most sources have assigned to the globules an enzymatic capacity, "it has never been demonstrated experimentally." Burnett concluded that the globules have an enzyme secretory role from the fact that more are present prior to feeding than after. Willier, Hyman and Rifenburgh (1925) worked with Triclad cells of similar morphological appearance to the Hydra gland cells but found their enclosed globules to be protein material. Lentz and Barrnett (1961) have claimed to be the first to publish work on the enzyme histochemistry of Hydra. They found enzymes in the gland cells and agree with the possibility of their extracellular secretion. However, they found a similar stratum of enzymes in the digestive cells to those in the gland cells.

Lenhoff (1968) mentioned some interesting experiments to determine the degree of protein degradation in the lumen (extracellular) compared with the intracellular degradation. Radioactively labelled protein was introduced into the digestive system, digestion allowed to proceed for a time, and then the compartments were fractionated and the amount of radioactivity in the various fractions measured.

Whilst digestion in Hydra seems to have been a popular topic for investigation in the first quarter of this century, physiological and cell biological experiments seem now to be mainly concerned with regeneration and growth. It is here that the radioactive isotope techniques which could give much information on digestion and nutrient diffusion are mainly applied. Regeneration and growth can, however, give information on cell movement which can be of value in this consideration.

## 3.3.1. CELL MOVEMENT IN HYDRA

Tripp (1928) found that there was a constant proliferation of cells in a region just below the hypostome and that these then migrated either towards the tips of the tentacles or down the trunk to the basal disc. Child and Hyman (1919) and Weimer (1928) have found different metabolic rates in different regions of the animal. There are, as indicated above, morphological differences. Lentz and Barrnett (op. cit.) have found enzyme pattern differences. This indicates that cells change as they move from one region to another.

Brien (1949) believed that as new cells were formed they forced older cells to make way for them, so that towards the tentacle tips and basal discs there is a gradation ofprogressively older cells. The tips or the disc are the regions of cell death. Burnett (1959) finds that the above progression only occurs if the Hydra is well fed. During starvation there is a reversal of movement. She suggested that this reversal may be due to the need for redistribution of lipids.

Burnett disagreed with McConnell's views on villi and endogenous fragmentation. She believed that the traditional methylene blue vital stain (used until the advent of radio isotope techniques) should be irregularly distributed if McConnell is correct, a feature she does not find. She also posed the question of whether any cell, other than an interstitial cell, is capable of the morphological and physiological transformations required of it if it is moving between histologically different regions.

Campbell (1967) used radioactively labelled Hydra mosaics and found that cells move as sheets rather than individually. Within the endodermis, for example, glandular and digestive cells move together and there seems to be no individual cell re-arrangement.

### 3.3.2. SUMMARY OF HYDRA DIGESTION

There are six histological regions in *Hydra*, one of these being a growth region which provides cells which move as sheets. There is no evidence for cells wandering back and forth singly and depositing their products. Burnett (1959) seems to be the last to mention McConnell's 'endogenous fragmentation' scheme and she disagreed with it. Some digestion is intracellular and some is extracellular, but the relative importance is difficult to prove. A technique for gauging the importance has been suggested by Lenhoff (1968).

It is generally agreed that *Hydra* and other coelenterate polyps carry out peristalsis, and that this provides a considerable mixing of food. There seems no reason to suppose that the Diploblastic body form presents any more unusual transport problems than Triploblastic forms. At this stage it cannot be said that Schlottke was wrong in drawing comparisons between pycnogonids and *Hydra*, but it can be said that the model of *Hydra* digestion he was using for his comparison was not altogether correct.

## 3.4. AN INTRODUCTION TO MASS TRANSFER

All organisms live in association with a fluid environment, so that any process which involves an interaction between an organism and this environment such as movement, heat exchange or gaseous exchange will be influenced by the behaviour of the fluid (Leyton 1975). Momentum transfer in a fluid is normally considered under the title of Fluid Dynamics, heat exchange comes under the heading of Energy Transport and gaseous exchange or the exchange of any solute comes under the heading of Mass Transport or Transfer (Lih 1975).

There are several types of mass transfer, eg, Diffusion, Active Transport, and Facilitated Transport. Though all these are significant, active transport and facilitated transport will be ignored in this discussion. Diffusion, when coupled with fluid motion due to an external force, is called CONVECTIVE MASS TRANSFER and this, together with some fluid dynamics, will be the main concern of this section.

Liquids are incompressible. They also have a certain viscosity which is due to inter molecular attraction within the liquid and at its interface (i.e. the junction between the liquid and its surrounds). The faster a liquid flows through a tube, the greater the 'fluid friction'.

Fluid friction is similar to 'Shear force' (on a unit area basis) which corresponds to 'Shear stress' (on a unit pressure basis). This is

incorporated into Newton's Law of Viscosity which states that:-

SHEAR STRESS (R) = VISCOCITY ($\eta$) x VELOCITY GRADIENT

Fluids in which viscocity is independent of shear stress are called 'Newtonian' and biological examples are blood serum or plasma. Whole blood is 'Non-Newtonian' in medium sized vessels such as arterioles.

In considering the dynamics of fluids in the guts of pycnogonids or coelenterates, much will depend on the classification of the fluid. If it is Newtonian (eg. full of dissolved substances due to extra-cellular breakdown - no large particles) it will exhibit different properties than if it were Non-Newtonian (eg. containing food boluses as reported in the guts of pycnogonids by Dohrn (1881), Dogiel (1911) or Loman (1907) ). In narrow blood vessels, erythrocytes arrange themselves in more or less single file as the vessel approaches their diameter. The red blood cells move axially, with their faces perpendicular to the direction of flow, creating a phenomenon known as 'Bolus Flow'. In this, eddies occur between the cells.

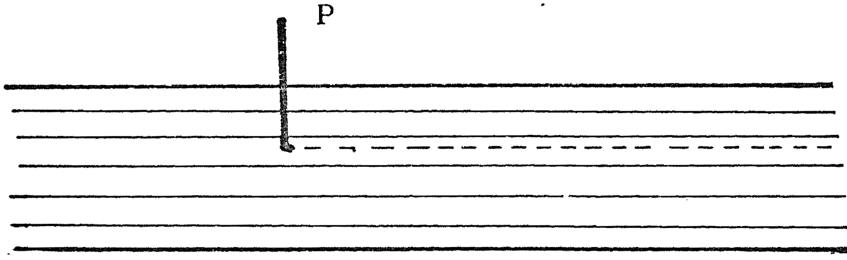
From the equation of Continuity of Incompressible Flow (Blackwood, Kelly and Bell 1963, p.117) it can be deduced that the velocity of a liquid at different points in a tube is inversely proportional to the cross-sectional area of the tube. From this it is found that wherever the velocity of a horizontally moving stream of fluid increases due to

a constriction in the tube, the pressure must decrease. Higher velocity is associated with lower pressure and vice-versa. This will be seen to be important when considering the movement of gut fluids due to peristalsis in the blindly ending pycnogonid midgut caeca. Velocity is also important in the calculation of REYNOLDS NUMBER, a dimensionless number which gives an indication of the type of flow that can occur. Flow is also altered by the diameter of the tube. Thus in the midgut caeca of Nymphon orcadense, with a mean lumen diameter of ca. 100microns, different flow characters may exist when compared with the gut lumen of Colossendeis wilsoni, with a mean diameter of ca. 1,500 microns.

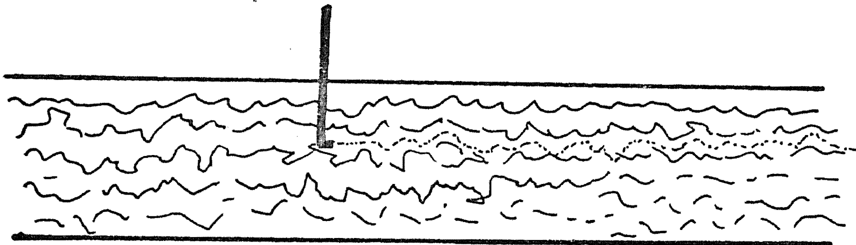
With a slow flow rate and a small diameter tube the type of flow produced is called LAMINAR FLOW (see figure 3D). Friction is greatest at the walls of the vessel so that there is an increasing velocity of fluid towards the centre. Layers of fluid slide over one another forming LAMINAE or layers of flow in two dimensions (in a tube, layers become concentric shells). When the flow rate is above a certain critical level the orderly motion of laminar flow ceases and the fluid becomes TURBULENT; eddies form. As an approximation it is found that when the Reynolds number is above 2,000, turbulent flow occurs.

In laminar flow, mass can only be transported across the

FIGURE 3 D.




Below a critical velocity, liquid particles move on parallel lines in laminar flow. The liquid introduced through pipe P therefore travels in a straight line.



Above the critical velocity, turbulence breaks up the stream.

stream of a fluid by the random thermal movement of its molecules (i.e. by molecular diffusion). This means that the rate of transport of gases, dissolved substances and heat will be relatively low. When turbulent flow occurs, the situation is very different, however, and a very efficient transport system exists - EDDY DIFFUSION, or TURBULENT TRANSPORT. This is what an engineer would advise a 'Creator' of biological systems to aim for.

Leyton (1975) stated that, generally speaking, fully turbulent boundary layers are not developed until at least 50 diameters from the entrance to a tube. In pycnogonids this would mean that in many species turbulent flow would be impossible. Generally it would appear that from the order of sizes of conducting elements and the velocity of flow in biological systems the Reynolds number would be far too low for turbulent flow ever to occur (eg, in a smooth tube 10 millimetres in diameter water would have to flow at a speed of at least 0.2 metres per second for turbulence to be initiated.). However, this treatment is an oversimplification. Reynolds number only holds true as an indicator of turbulence in straight tubes of circular cross-section. If the tube is curved, turbulence occurs wherever there are sharp bends or branches in a circulation system Similarly, local eddies almost always occur where there is an abrupt change in the size of conducting channels.

The description given so far has dealt only with rigid walled tubes. In the case of both pycnogonids and coelenterates it is necessary to consider pulsatile flow in elastic tubes. The effect of distensible walls is to make the size of the vessel dependent on the driving pressure, so altering the relationship between pressure and flow rate. An indication of the complexity involved in describing such a system is shown by a recent example given by Lih (1975, p.205). He attempted to describe a two dimensional model of flow in a renal tubule of fixed diameter in which the main flow is in an axial direction, but part of the water and some solute moves through the walls as flow proceeds. Eight pages of mathematical calculation are then required to derive an approximate expression.

At the start of this section it was indicated that transport phenomena could be grouped roughly under three headings:- Momentum transport (fluid dynamics), Energy or Heat transport and Mass transport. Some common ground connects these subdivisions. Thus, diffusion concepts (Mass transport) can be best explained non-mathematically by analogy with heat conduction. With diffusion, instead of temperature gradient, concentration gradient becomes the main driving force. The rate at which a mass of substance diffuses across an area of a medium being expressed by Fick's First Law.

In thermal analogy, a block of cross-sectional area A and length x is considered. The temperatures at each end, $T_1$ and $T_2$ are different so that $T_1$ is less than $T_2$ and heat will flow in the direction of $T_1$ (Figure3E). It is found that Q, the heat energy flow/unit of time is proportional to:-

i) The cross-sectional area (A), ie $Q \propto A$

ii) The difference in temperature $(T_2 - T_1)$ ie $Q \propto T_2 - T_1$

iii) The reciprocal of the distance between $T_1$ and $T_2$ (i.e. x) ie $Q \propto \frac{1}{x}$

This is written as $Q \propto \frac{A(T_2 - T_1)}{x}$

which is $Q = K \cdot \frac{A(T_2 - T_1)}{x}$ when K is THERMAL CONDUCTIVITY

Thermal conductivity is the heat that flows per unit of time through unit area of a plate of unit thickness; the temperature difference across the plate being $1^{\circ}C$. In diffusion, much the same diagram is used (Figure 3E.b.), but concentration replaces temperature.

Thus:- $T_2 - T_1$ becomes $c_2 - c_1$ or $\delta c$

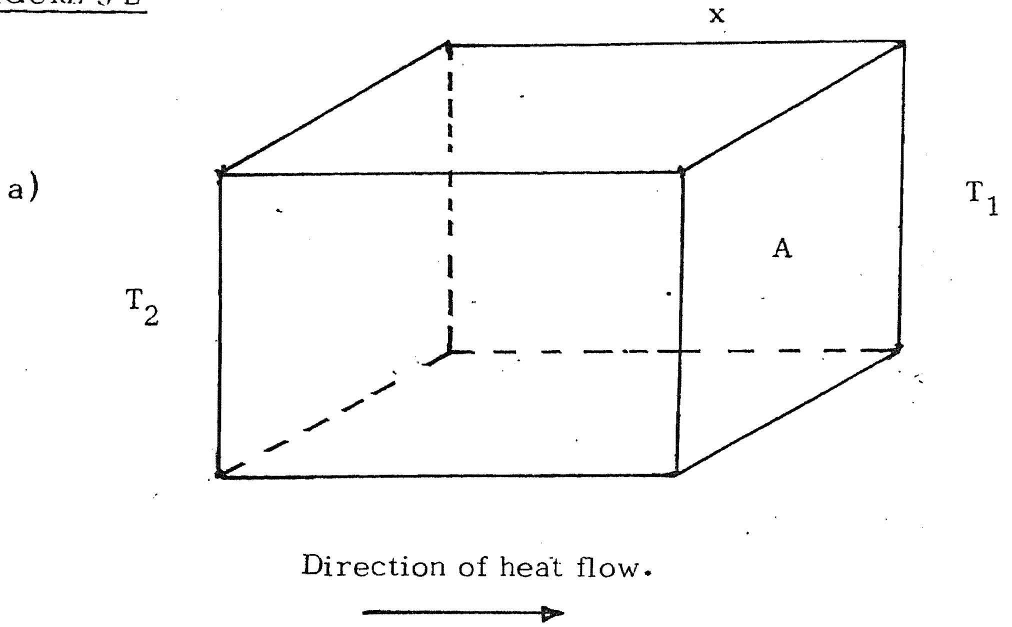
x becomes $\delta x$

Q becomes the number of molecules diffusing per unit of time which is $\frac{\delta n}{\delta t}$

The complete equation thus becomes $\frac{\delta n}{\delta t} = - D \cdot A \cdot \frac{\delta c}{\delta x}$

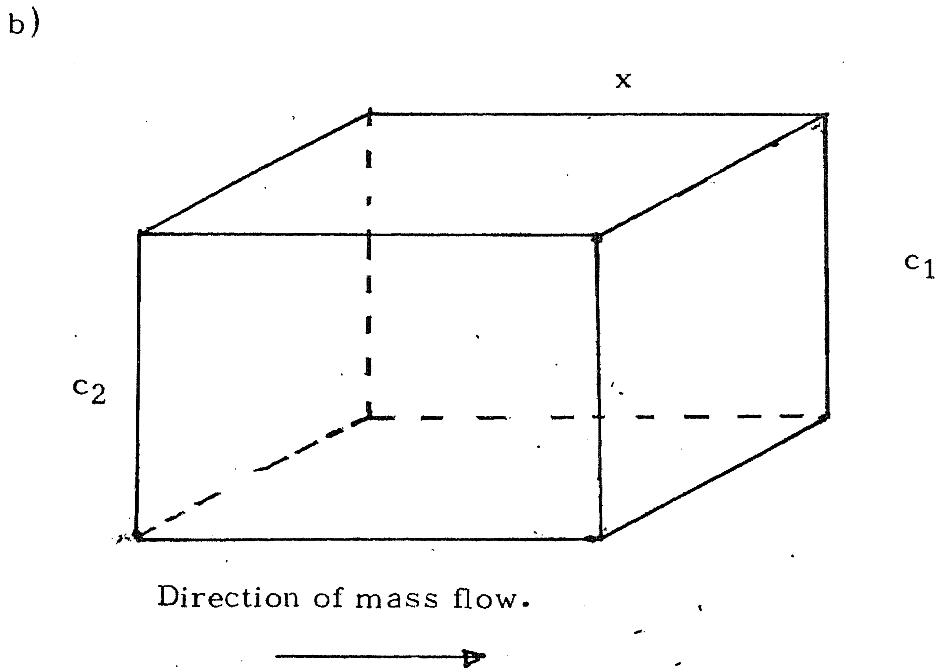
FIGURE 3 E

a)




Direction of heat flow.

b)




Direction of mass flow.

D. corresponds to K, and is known as the DIFFUSION COEFFICIENT or DIFFUSIVITY and is defined as the mass diffusing per unit of time (seconds) across a unit area (square centimetres). The minus sign in the equation indicates that diffusion is in the direction of the lower concentration. The above applies to a STEADY STATE situation in which the concentration of a diffusing substance at any point in the medium does not change with time. In most biological systems, however, utilisation of substances by the tissue means that variations in concentration will occur and a NON-STEADY STATE exists. This makes it necessary to modify the equation above, the false simplicity of the expression being magnified further when it is seen that, as written, it only represents diffusion along one co-ordinate and the actual (three dimensional process) involves three co-ordinates.

After consideration of both flow in tubes and also diffusion, the next stage is to combine the two. Suffice to say it is at this stage that the average biologist "frightened by mathematics at school" (Anon - obituary of Professor Hogben 1975) retires from the scene (the example from Lih (1975) has already been mentioned).

From the previous discussion, however, sufficient is covered to enable a knowledge of the types of measurements required to enable scientific rather than intuitive comparisons of coelenterate and pycnogonid mass transport to be made if the mathematical techniques are

available. It is suggested that these measurements should be as follows:-

(1) Diameter of tube (gut) and of any constrictions in it. In an elastic, contracting living system these measurements will vary with time.

(2) Shape of tube (eg, straight, curved, rough or smooth, circular cross-section). As in (1) this will vary with time.

(3) Velocity of fluid flow.

(4) Pressure gradient across the gut wall.

(5) Type of fluid (whether newtonian or non-newtonian).

(6) Viscosity of fluid.

(7) Concentration of solute under consideration in the gut lumen.

(8) Concentration of solute under consideration in the tissues.

(9) Diffusion coefficient of solute.

(10) Distance over which diffusion occurs.

(11) Change of concentrations with time in a non-steady state system (a knowledge of the animal's metabolic pathways

will probably be required).

(12) Temperature (this will affect viscosity and diffusion rates).

(13) Knowledge of other phenomena which might affect diffusion:- eg, whether there is active transport, facilitated transport, osmotic gradients, Donnan equilibria.

Measurements (1) to (6) inclusive are used to calculate Reynolds number and the flow characteristics of the system; measurements (7) to (9) will be relevant to diffusion calculations.

## 3.5. MASS TRANSFER IN HYDRA AND PYCNOGONIDS

### 3.5.1. HYDRA

Perusal of the list of measurements (above) which have to be made to enable scientific comparisons of mass transport between Hydra and other animals, reveals that few have been measured. This is not as surprising as may at first sight seem. 'Speculation is a pleasant pastime; experimentation is more difficult' (Middleman 1972). Whilst Batham and Pantin (1950) were able to initiate a thorough study of musculature and hydrostatic action in sea-anemones, there does not appear to be any information concerning the function of the fluid/muscle system of the Hydroids. This is no doubt due to the difficulty of measuring hydrostatic pressures in such small animals (Chapman 1958). Chapman and Purdy (1972) claimed to be the first to have made direct measurements of the rates of diffusion of small organic molecules (glucose and glycerine) in coelenterate tissue. Their work was not on Hydra, but on Corymorpha palma, a solitary hydroid which may reach a length of 15 centimetres, have a stalk 0.8 centimetres in diameter and which has some cells which are 200 μm diameter. The size is very much greater than Hydra and this could introduce different problems. This paper is the first application of Fick's law that I have seen in invertebrate physiology.

Burnett (1959) made some interesting observations of the mesoglea of *Hydra* during digestion. She found that it was packed with glycogen three to six hours after feeding and that it returned to normal after eighteen hours. Lenhoff (1968) reported experiments in which $S^{35}$ labelled protein was fed to *Hydra* and found to be retained in the endoderm for 24 hours before being distributed to the ectoderm. Both Burnett's and Lenhoff's findings indicate, indirectly, that the diffusion distance one has to consider may not be from lumen to ectoderm, but a series of step processes. These steps would be from lumen to endoderm, endoderm to mesoglea, and mesoglea to ectoderm. The distance in Fick's formula would, therefore, have to be altered.

The diffusion problems of *Hydra* have not been adequately investigated for even a rough attempt to be made to apply Fick's first law.

### 3.5.2. PYCNOGONIDS

"During the intake of food its uniform distribution is obtained by peristalsis which continues until the food is absorbed. During starvation periods an unequal use of nutrients by different organs occurs and its even distribution would be lost. To rectify this, just as in *Hydra*, absorbed foodstuffs are transported via the endoderm to where they are needed." (Schlottke 1933 p.652). If the rest of the

section from which the above quotation comes is translated, it is seen that Schlottke's implications are that nutrients are transported in the same way as they are in _Hydra_. He had previously listed what he considered to be the three ways _Hydra_ might carry out its transport (see 3.3).

The above statement on uneven utilisation and consequent uneven distribution of nutrients is based on no experimental data. it follows that such a proof would require a detailed knowledge of mass transport and, as with _Hydra_, the necessary measurements for such knowledge have not been made.

## 3.6. PYCNOGONID FLUID DYNAMICS

The midgut of pycnogonids extends through the trunk and into the walking legs, the degree to which the caeca penetrate into these varying with species (see also Chapter 4). Peristalsis has long been noted to occur in pycnogonid gut, but unfortunately its nature has not been clearly observed. This is illustrated by historical reports on pycnogonid circulation. Thus Cole (1910) stated that Johnston (1837), in describing the circulation of body fluids, mistook the beating of the branching intestine for beating blood vessels. De Quatrefages (1845), in stating that blood is agitated back and forth by movement of the legs and also by the muscular movement of the intestine, seems to have been the first to mention intestine movements in pycnogonids by name. Van Beneden (1846) was the first to describe a regular blood circulation but found that due to the "opacity of the intestine" he could not determine whether a heart or dorsal vessel was present. The observational problems of Van Beneden persist, beating hearts and intestines can only be seen when the specimen has a transparent cuticle. Often, opacity varies from one region to another so that it may be difficult to differentiate between a beating heart and a beating intestine although some form of beat is clearly visible.

Wyer (1972) reported a 'trunk midgut' beat of 160 per minute in Nymphon gracile. He stated there are no contractions in the mid-

gut caeca. This trunk midgut beat rate corresponds closely to heart rate figures for Phoxichilidium femoratum of between 120 and 180 beats per minute (Loman 1907) and 172 beats per minute for Endeis spinosa (Cole 1910). I have found that the antarctic species N. orcadense displays a beat in the trunk region (opacity made it difficult to tell whether this was beating heart or intestine) of from 40 to 70 beats per minute and a peristalsis in the caeca of stationary walking legs of between 8 and ten waves per minute (see Table 3F).

Dawson (1934) reported contractions occurring in the midgut caeca of amputated legs of Anoplodactylus lentus and found that these contractions persisted 'for a long time'. I have observed this phenomenon in antarctic species (N. orcadense and N. australe) and Helfer and Schlottke (1935) also reported 'autonomic contractions' of the gut in amputated legs. Schlottke (1933) indicated that peristalsis of the gut 'continues until the food is absorbed'. This might imply that peristalsis does not take place once absorption is complete (i.e. there is no peristalsis during starvation). Such a situation would provide conditions favourable for the uneven distribution of nutrients during starvation as is quoted from Schlottke ( see P 111 ). However, Schlottke made no indication concerning a cessation of peristalsis. Instead he mentioned that, in the last digestion phase, seperate endoderm cells are slowly transported as faeces to the anus by intestine peristalsis. I have found peristalsis to continue in starved specimens, and indeed it appears more obvious in such.

Because of the difficulties of opacity already mentioned and also because of the limited depth of field of high aperture light microscope objective lenses (Wachtel et al. 1969 and see footnote), observation of internal movements in pycnogonids poses a number of unanswered questions:-

a) Are the beats seen in the trunk region heartbeats or intestine beats?

b) Have the heart or intestines any, or sufficient, musculature to achieve the beat rates reported?

c) If the answer to b) is positive, does the trunk intestine beat initiate the heartbeat or vice-versa, or are the intestines and heart independent of one another?

d) If the answer to b) is negative, are the beats achieved solely by contraction of muscles outside the heart and gut (eg, leg muscles)?

e) If the answer to b) is negative, are beats achieved by a combination of heart, intestine and leg musculature?

These questions will be dealt with later. Firstly, however, it may be useful to consider the flow patterns which might be created in model systems based on the pycnogonid gut alone, and then the pycnogonid gut and body cavity combined.

TABLE 3 F.

| Specimen | Comment | Temperature °C | Rate (Beats per minute) |
|---|---|---|---|
| 1 | Trunk beat | 4·7 - 7 · | 58 |
| 2 | Trunk beat | 5·5 -7·8 | 48 |
| 3 | Trunk beat | 7·7 | 50 |
| 4 | Trunk (fresh) | 3·3 | 58 |
| 5 | Trunk (fresh) | 3·3 | 48 |
| 6 | Trunk (fresh) | 3·3 | 50 |
| 4 | $2\frac{1}{2}$hrs. later | 3·3 | 58 |
| 5 | $2\frac{1}{2}$hrs. later | 3·3 | 43 |
| 7 | Trunk (starved) | 4 | 60 |
| | Leg beat | | 10 |

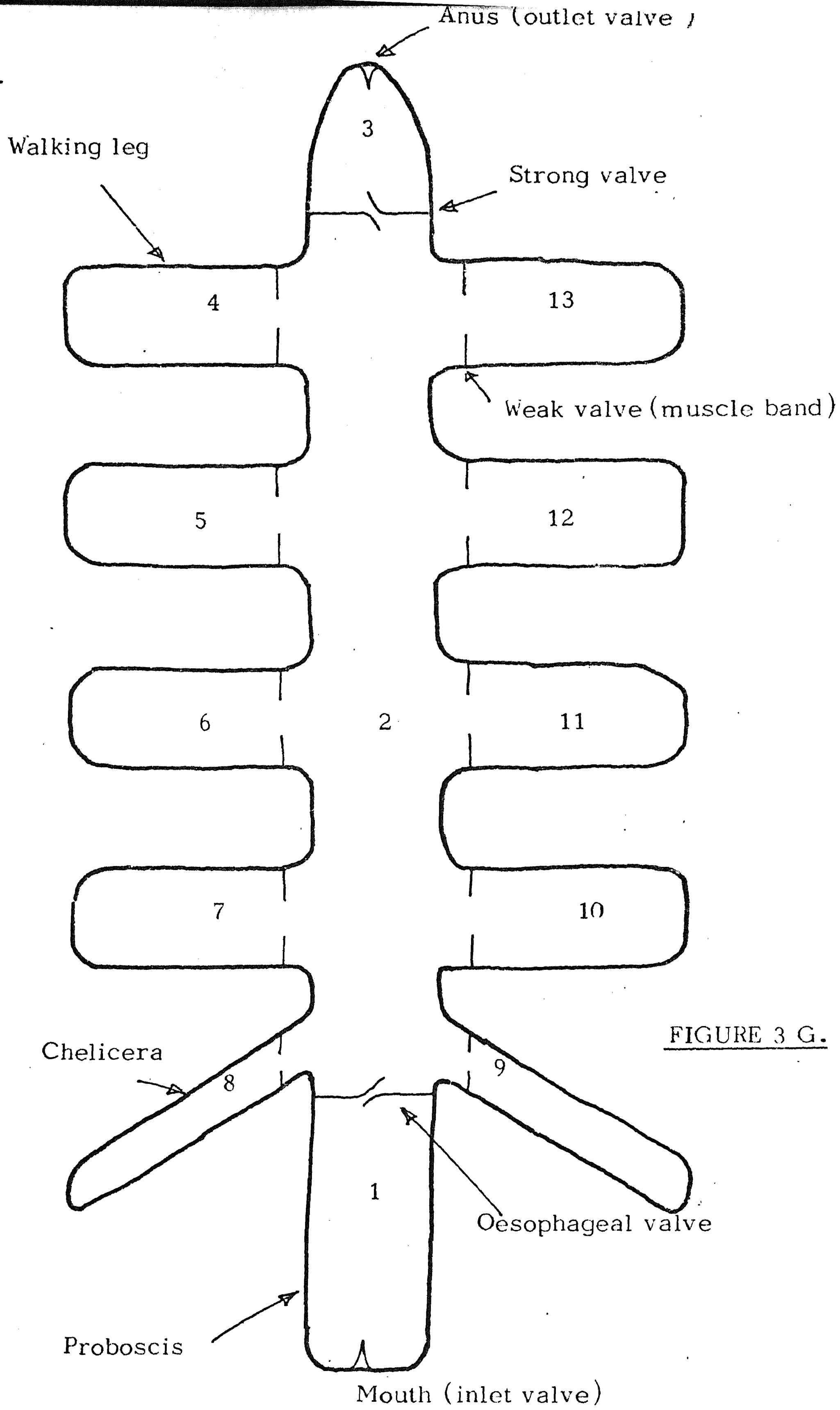



FIGURE 3 G.

### 3.6.1. A MODEL OF PYCNOGONID GUT ALONE

Figure 3G represents a model pycnogonid gut. It can be seen that in the case of an eight legged form with caeca to the chelicerae, it can be divided into 13 compartments (the number of compartments will vary with the number of caeca). In the mid-line, working from anterior to posterior ends, the first compartment, the pharynx, is bounded by the mouth in front and, for the purposes of this discussion, the oesophageal valve behind. The second compartment is the trunk mid-gut bounded by the oesophageal valve in front and the hindgut valve behind. The third midline compartment is the abdomen, or hindgut, bounded by the midgut/hindgut valve and the anus. Laterally each appendage caecum is counted as a compartment.

Whilst the mouth, the tripartite oesophageal and hindgut valves, and the anus, are valves (at least morphologically) capable of closing an orifice, the valves at the open ends of the caeca are not so efficient. I have not so far been able to identify them with certainty in sections, but Helfer and Schlottke report that 'the smooth muscles at the junction between trunk and caeca are especially strong' and that sometimes 'the entrances to the caeca are closed for rather a long time.' Sanchez (1959) reported the existence of a strip of epithelium containing contractile fibres encircling caeca at the level of the second and third coxae and acting as sphincters to them in protonymphon larvae. The division of the midgut caeca into separate compartments certainly

makes theoretical consideration of the model easier.

The sucking action of the pharynx has been indicated by numerous authors. Fry (1965) drew attention to the fact that when the volume of the foregut increases, the hydrostatic pressure of the whole of the body fluids will be increased. For the moment the fact that the gut is in fact a compressible tube suspended within an incompressible one (i.e. the haemocoel and cuticle surround it) will be ignored. This added complication and the alterations it makes will be considered after the simpler model has been discussed.

From the introduction to Mass Transfer (section 3.4.), the following properties of fluid containing tubular systems are known:-

i) Fluids are incompressible.

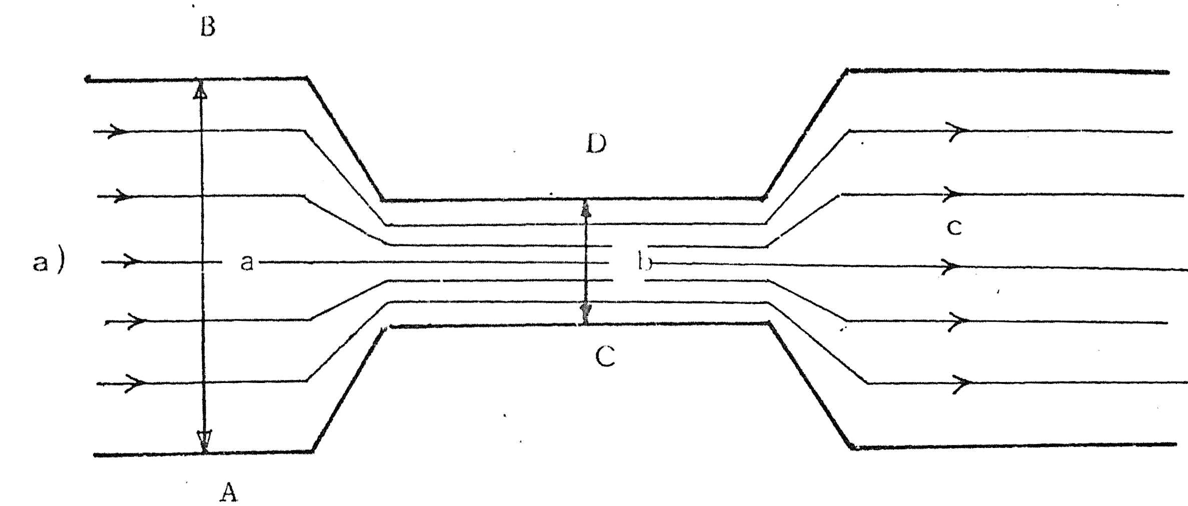
ii) The velocity of a liquid at different points in a tube is inversely proportional to the constriction (area) of the tube.

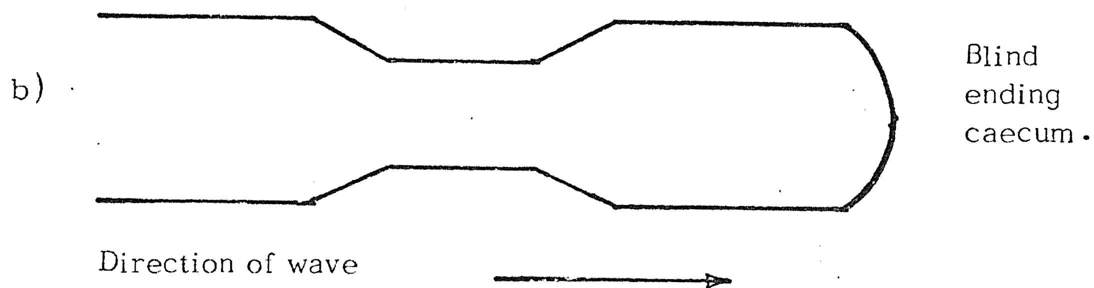
iii) High velocity is associated with low pressure and vice-versa.

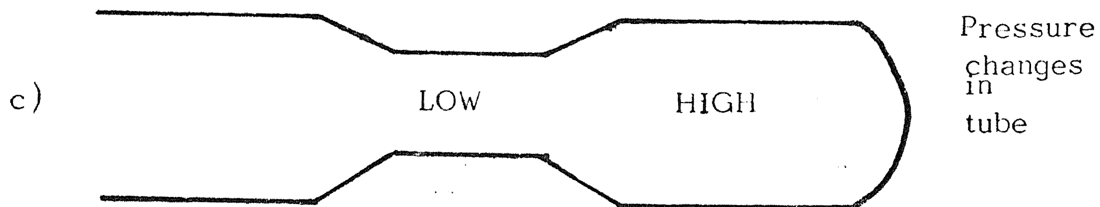
If, for the moment, the laws of viscosity are ignored, a consideration of occurrences in any single compartment (caecum) can be indicated:-

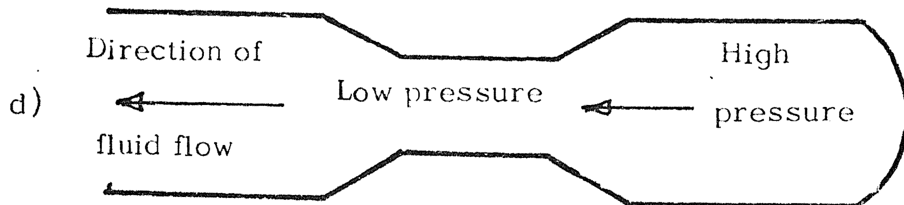
Considering first a tube in which a fluid is flowing and which has

FIGURE 3 H.



a constriction at one point, it can be seen that the velocity of flow is greater in the region of constriction. (Figure 3H a). The line passing from a to b to c in this figure represents a 'streamline', it is not an indication of laminar flow. The same number of streamlines pass through the area of greater cross-section AB as do through the constricted area CD. If a particle carried in the flow is imagined, it will be accelerated when it enters the constricted region as the velocity of the fluid is greater here. In order to accelerate the particle, the force to its rear must be greater than the opposing force in front of it and hence pressure at a must exceed that at b.

In peristalsis the same type of description given above can be applied, the difference being that initially it is the constriction that is moving rather than the fluid. If a blind ending caecum is now taken and a peristaltic wave travelling from its open end towards the blind end is imagined (Figure 3H b), it can be seen that there is a region of low pressure travelling along the tube (Figure 3H c) creating at the same time in the blind end a region of high pressure. Fluid flow will therefore tend to be in the opposite direction to the direction of travel of the peristaltic wave (Figure 3H d). The velocity of flow will be greatest in the region of the constriction and will decrease in the direction away from the blind end.

If a peristaltic wave travels from the blind end towards the body, and if the sphincter at the junction of caecum and trunk is closed, the

reverse of the above will apply.

From the generalisation that turbulent flow does not occur until at least 50 diameters from the entrance of the tube (Leyton 1975), it can be seen that, in N. orcadense, with an approximate caecum lumen diameter of 100 to 200 microns and an approximate length of 2 centimetres, turbulent flow will only be possible in the distal region away from the start of a peristaltic wave. A similar argument applies to C. wilsoni with a lumen diameter of approximately 1,500 microns and a length of 10 centimetres. Turbulence depends upon the velocity of the fluid. The velocity of the peristaltic wave in N. orcadense calculated from my observations. is approximately 20 centimetres per minute. Again, using approximations from Leyton (op. cit.) and substituting the above values, it is found that the Reynolds number is far too low for turbulence to occur. However, the Reynolds number is only fully applicable to straight tubes of circular cross-section and smooth walls (section 3.4.). Pycnogonid gut diverticula are not internally smooth, straight, nor of circular cross-section. Discussion with a flow engineer (P. J. Wason, personal communication) indicates that peristalsis in pycnogonid gut would cause CREEP flow. Velocity is not great enough for turbulence, but neither do roughness and shape permit laminar flow. A slow eddying flow occurs. The eddies produced by this creep flow would be quite adequate for eddy diffusion.

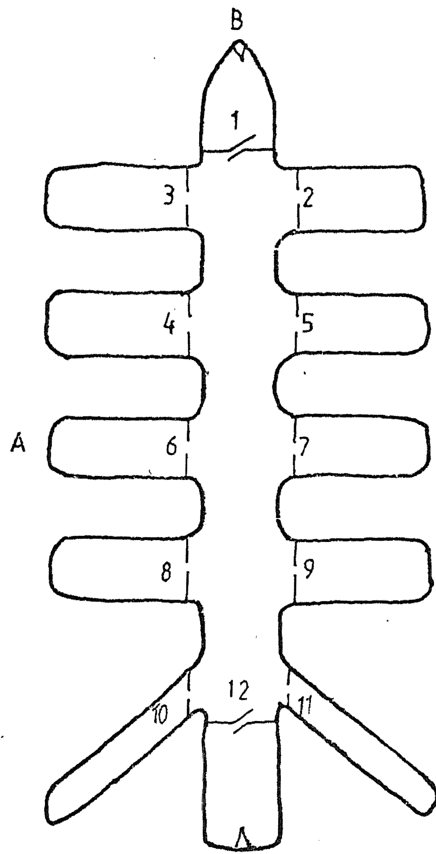



FIGURE 3 I.

Movement of food from the tip of a caecum at A to exit via the anus at B could be brought about as follows:-

A contraction travels towards the tip of the caecum. The valve at 6 opens to enable food to pass out of the caecum. Contractions in the main trunk midgut must now travel towards the proboscis for the food to be moved towards hindgut. Valves 2 to 12 (inclusive must close for food to pass into the hindgut through the open valve 1 and thence to the anus.

If an attempt is made to co-ordinate the compartments of the model so far described, it can be seen that by judicious use of the valves in the system (Figure 31), it is quite possible to move fluid from the tip of one caecum to the tip of another or out through the anus. Creep flow, however, causes a general mixing of the lumen contents and there can never be the situation of undigested food travelling along one side of the lumen whilst waste products travel in the opposite direction along the other side. If the sphincter leading into a caecum is closed (there will probably always be a partial leakage through this) and peristaltic waves travel back and forth along the now isolated compartment, the effect will be analagous to 'Pendular movement' in vertebrate intestines which creates rapid mixing (Hoar 1966). I have noticed such a phenomenon occurring in N. orcadense.

It is postulated that if intracellular digestion is the rule, the system described would be most efficient in the case of intermittent feeding. Food is ingested and circulated in a fully mixed state in the intestine until the lumen is filled only with waste products. The waste products are then voided before the next meal. Continuous feeding would mean that a certain amount of undigested food would be voided with waste products, an inefficient use of food material. Observations on N. orcadense have shown it to feed intermittently and to be capable of going for long periods between meals. In starvation experiments (Chapter 2) it has been found to produce faeces

more than a month after the last meal.

From consideration of Figure 3I, it can be seen that a fairly complex control system for valve operation would be needed to bring about fluid movement from the tip of one caecum to the anus. As previously stated I have not identified the trunk-caecum sphincters and (see Chapter 4) I do not find cell musculature as reported by Schlottke in the midgut epithelium. Morphologically, there does not appear to be sufficient nervous and muscular tissue present to make such a model work. However, as indicated above (p.119), the fact that the gut is really a fluid containing inner tube within a rigid fluid containing outer tube (the haemocoel and cuticle) has been ignored.

### 3.6.2. A MODEL CONSIDERING PYCNOGONID GUT AND HAEMOCOEL

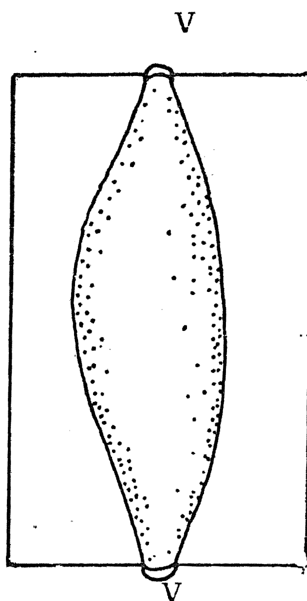
Fry (1965) has noted that the increase in volume of the foregut during feeding increases hydrostatic pressure of the whole of the body fluids. It can be seen that a system with one rigid fluid filled tube surrounding a collapsible fluid filled tube will have particular properties. With the latter tube having outlets to the exterior (mouth and anus) whilst the former has not, opening the anal valve will cause a squeezing of the inner tube (gut) if the whole system is at increased pressure with respect to the environment (Figure 3J). If, at feeding, the hydrostatic pressure of a pycnogonid's body fluids is increased with respect to the environment, then opening the anus at some time

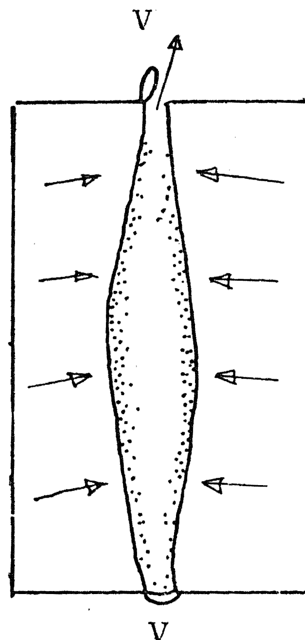
FIGURE 3 J.

Simple model to illustrate theoretical collapsing of pycnogonid gut.

In A, the valves V are closed and the animal is at increased pressure with respect to the external environment.

In B, one valve is opened and pressures equilibrate. As the volume of the animal remains constant (rigid cuticle), the gut must contract.



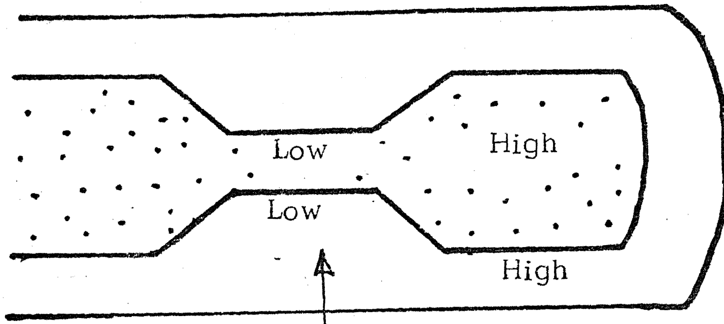
after feeding will cause a squeezing of the gut as pressure is equilibrated, This would reduce the requirement for midgut musculature, and for the complicated co-ordination illustrated in Figure 3I.

If the questions which observations of internal movements in pycnogonids have provoked (p.115) are now considered with this more complex model in mind, the following can be deduced. Question c), (does the intestine beat initiate heartbeat), is now seen to be much involved with momentum transfer. Even if there were no nervous network linking intestine beat with heartbeat, momentum transfer makes it inevitable that one affects the other.

Firstman (1971) appears to be the most recent worker to investigate blood flow in pycnogonids and claimed to describe blood movements differently from any previous investigations. I cannot agree with Firstman's scheme a) on morphological grounds (see Chapter 7), and b) because he made no observations on live animals.

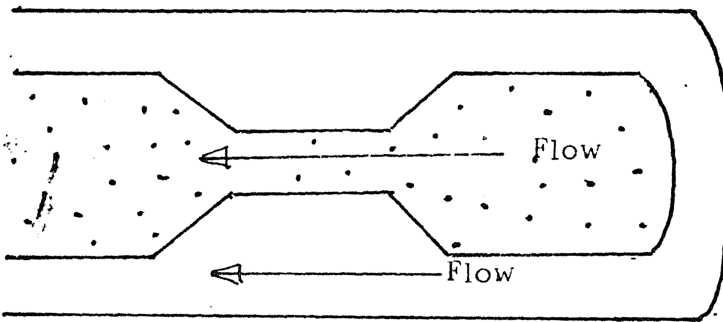
According to Dohrn (1881), blood is driven anteriorly by the heart into the dorsal antimere of the proboscis, runs back along its ventral side and then runs from here laterally into the appendages. Blood flows back to the heart dorsally. Cole (1910) noted that, with his specimens, although on the whole there appeared to be a real circulation from the body out into the legs and back, this was rendered more or less indefinite by the peristaltic contractions of the intestine.

FIGURE 3 K.



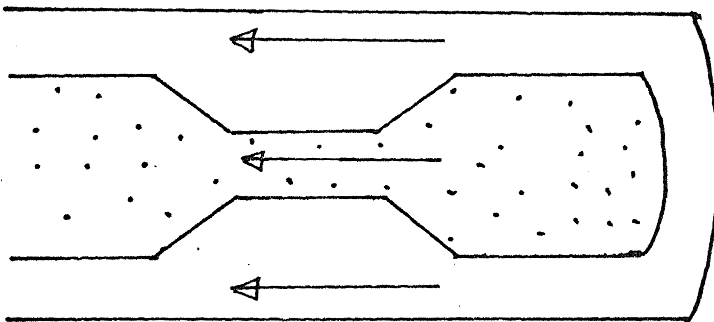

a)

volume increase, pressure decrease.




b)

Direction of contraction wave



c)

These imparted 'a sort of churning to the blood and kept it moving back and forth.' I have noted a similar effect in N. orcadense and this seems not unlike the pendular analogue mentioned earlier (p.124). Movement of corpuscles seemed more closely related to gut movement than heart movement.

If the same consideration as applied in Figure 3H (a to d) is now applied to the more complicated concentric tube model (Figure 3K) and it is taken that fluid movement only occurs in the gut (i.e. the heart is stopped); the following picture emerges.– As a peristaltic constriction passes along the gut, it produces a low pressure area in its lumen. The constriction will also bring about an increase in volume of the particular region which surrounds it (Figure 3K a). This volume increase will in turn cause a pressure decrease. A low pressure region therefore occurs, not only in the lumen, but also around it. The direction of the fluid flow produced in the gut is in the opposite direction to that of the direction of travel of the peristaltic wave. The same direction of flow occurs in both gut and haemocoel (Figure 3K,b). From Figure 3K c it is seen that this flow will occur both dorsally and ventrally in the haemocoel. In theory the horizontal membrane dividing the haemocoel dorso-ventrally will make no difference.

The blood circulation in the leg (outwards ventrally, backwards dorsally) can only be achieved:-

i) If the force of the motion of the blood created by the heart can over-ride the motion created by the intestine.

ii) If gut peristalsis ceases (in N. orcadense the heart beats at - 60 beats per minute, peristaltic waves travel along a caecum 6 to 10 times per minute. There are occasions when the heart is beating when the intestines are not).

iii) If the peristaltic constriction is asymmetrical (the model postulates symmetrical constriction in a tube of circular cross-section). A lopsided constriction travelling spirally along a caecum for example would create different conditions in the haemocoel.

From the consideration in this section it can be seen that little useful information exists on circulation of the body fluids of pycnogonids. Blood and midgut fluid circulations are, because of the construction of the system, intimately linked. More rigorous observation is needed to elucidate this intimacy. Only by measuring heart rates (Carrel and Heathcote (1976) used lasers to measure the heart rates of spiders), gut beat rates and flow rates, will it become possible to calculate phenomena related to mass transport. Only then, too, will it be possible to consider one additional complexity which has been ignored, the role played by the movements of the appendages.

# CHAPTER 4

# DIGESTION II - MORPHOLOGY OF THE DIGESTIVE SYSTEM

## 4.1. INTRODUCTION

The alimentary system of pynogonids is unusual. The mouth is situated at the tip of a large anterior proboscis (see next section) and when closed is Y-shaped, expanding into a triangular shaped aperture when open (Snodgrass 1952). It leads directly into the cavity of a large sac which occupies most of the length of the proboscis. This sac has a triangular to circular cross-section depending upon its dilation and is lined with cuticle. Various authors (eg. Helfer and Schlottke 1935, Sanchez 1959 and King 1973) have called this sac a 'pharynx'. Snodgrass (op. cit.) claimed that it much resembles the 'pharynx' (his quotation marks) of an arachnid but without constrictor muscles. D'Arcy Thompson (1909) calls it an ' oesophageal cavity'. Without wishing to participate in any of the phylogenetic or embryological arguments (see later) which surround proboscis structure, I too will use the term PHARYNX, simply because it is the most common term in the literature. By the same convention, the term 'OESOPHAGUS' is applied to the narrow tube, posterior to the pharynx. Pharynx and oesophagus together comprise the FOREGUT. The oesophagus, like the mouth, has a Y cross-section to its lumen when closed and a triangular to circular one when expanded. In its anterior region are the bases of numerous setae which protrude forwards and form an 'Oyster basket' sieve (Schlottke 1933) at the back of the pharynx. Both pharynx and oesophagus are lined with cuticle which is absent from the posterior region behind the origins of the setae. At some point in the cephalic somite the oeso-

phagus opens via a tripartite valve into the MIDGUT. In N. orcadense and N. australe this is located just posterior to the ocular tubercle. The midgut extends through the rest of the trunk and opens through another valve into the HINDGUT. This valve is similar in gross cross-section to the valve between oesophagus and midgut but differs from the latter in detailed cellular structure. The hindgut opens to the exterior via the anus.

The unusual feature of the midgut is that it gives off caecal diverticula into each of the walking legs. In addition, some species have caeca extending into the chelicerae and some forwards into the proboscis (Figure 4A). There are no reports of diverticula extending into the ovigers or palps. Table 4B (compiled form Helfer and Schlottke 1935, King 1973 and my own observations) shows the distribution and extent of the diverticula in several species. Presently there are insufficient data to draw any taxonomic or evolutionary conclusions from reported differences.

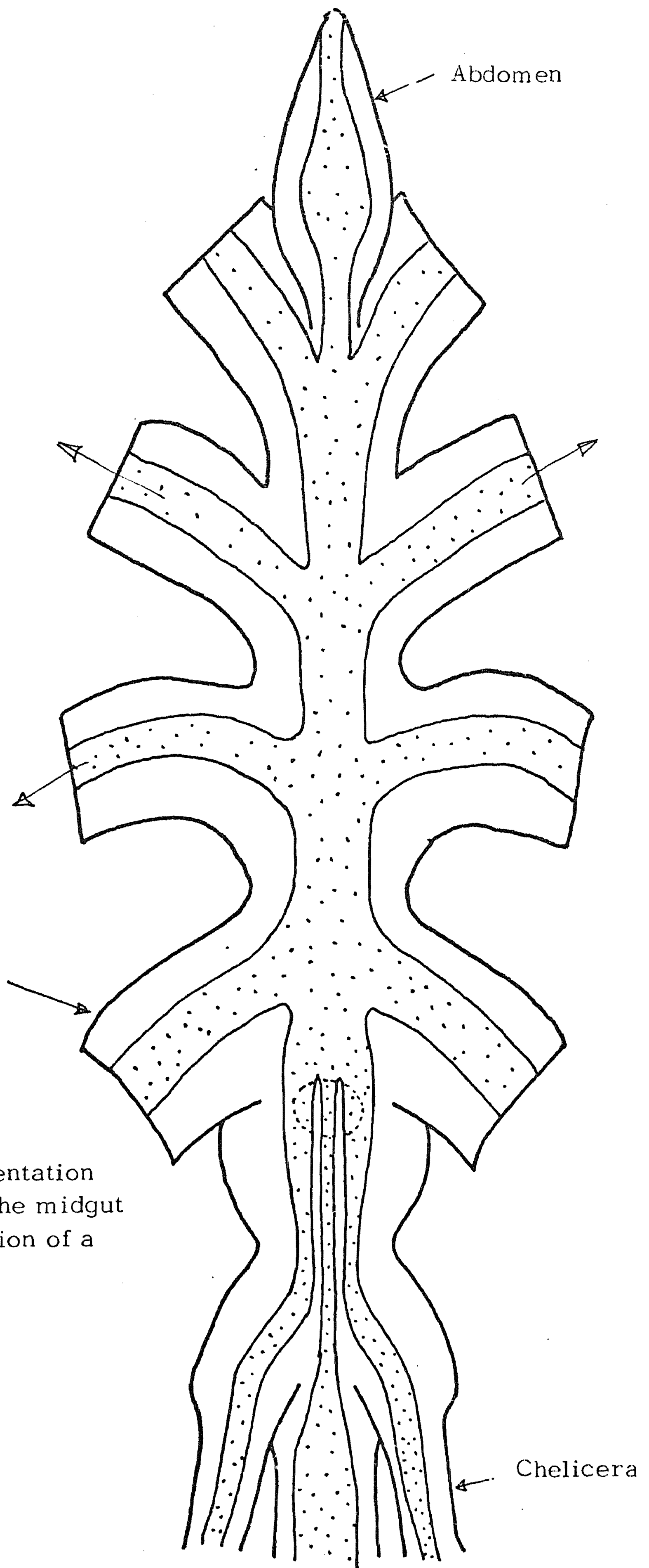



FIGURE 4 A.
Diagrammatic representation of the distribution of the midgut caeca in the trunk region of a typical pycnogonid.

TABLE 4 B.

DISTRIBUTION AND EXTENT OF MIDGUT CAECA IN SOME PYCNOGONIDS.

Caeca extending forwards into the proboscis:-

Colossendeis spps.,
Phoxichilus spps.,
Nymphon brachyrhynchum,
Nymphon gracile,
Phoxichilidium femoratum,
Endeis spinosa,

Extending into second walking leg segment:-

Rhynchothorax mediterraneus,

Extending into the fourth segment of the walking legs:-

Pipetta spps.,

Extending into the sixth segment of the walking legs:-

Nymphopsis,

Phoxichilidium femoratum,

Pycnogonum littorale,

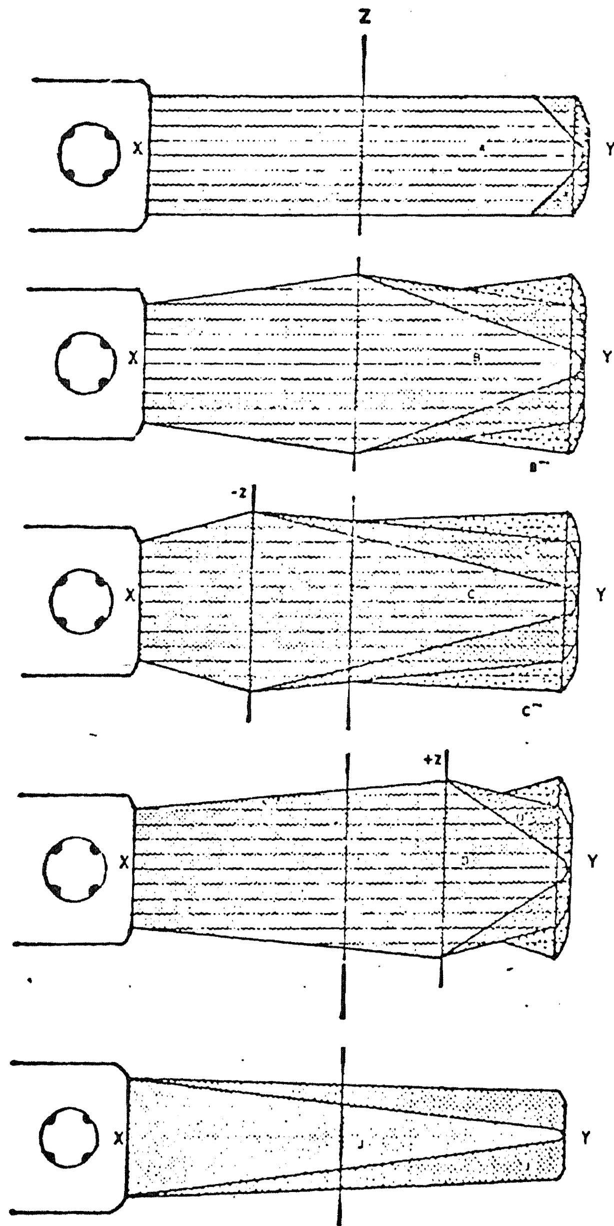
Extending to the end of the propodus:-

Pallenopsis vanhoffeni,
Nymphon gracile,
Achelia echinata,
Nymphon rubrum,
Endeis spinosa,
Nymphon australe,
Nymphon orcadense,
Nymphon hirtipes,
Ammothea carolinensis,
Colossendeis proboscidea,
C. wilsoni,
Decolopoda australis,

## 4.2. THE STRUCTURE OF THE PROBOSCIS

Undoubtedly the most complicated gross structure of the Pycnogonida is the proboscis. The external structure is generally described as 'Cylindrical' but this term covers a multitude of structures and the casual use of descriptions such as 'Clavate','Oval', and 'Spindle shaped' in addition caused Fry andHedgepeth (1969) to define five main types of proboscis shape for taxonomic purposes (Figure 4C).

Longitudinally the proboscis extends from the mouth opening at its distal end (tip) to a ring of flexible cuticle, (arthrodial membrane) where it joins the cephalic segment of the trunk (Figure 4D). In transverse section, the proboscis is tri-radial, consisting of a single dorsal antimere and two ventro-lateral antimeres. Hoek (1881) considered the dorsal antimere to be homologous with the labrum and the ventro-lateral antimeres homologous to the mandibles of insects. Carpenter (1905), Wiren (1918) and Henry (1953) have all attempted homologies between the anterior end of pycnogonids and those of other arthropods. Their work is summarized and critically discussed by Hedgpeth (1954). Sanchez (1959), in embryological studies, found that the dorsal antimere was an outgrowth from a cephalic somite, While the two ventro-lateral antimeres arose from a slightly posterior region.




—The five main types of proboscis shape in the Pycnogonida. The criteria involved are: The presence or absence, and position, of one or two dilations; the relative diameters of the proboscis at its insertion into the cephalic somite and at its distal extremity. X and Y indicate proximal and distal diameters; Z indicates the midpoint of the longitudinal axis. (See text for further details.)

FIGURE 4 C. after Fry and Hedgpeth 1969.

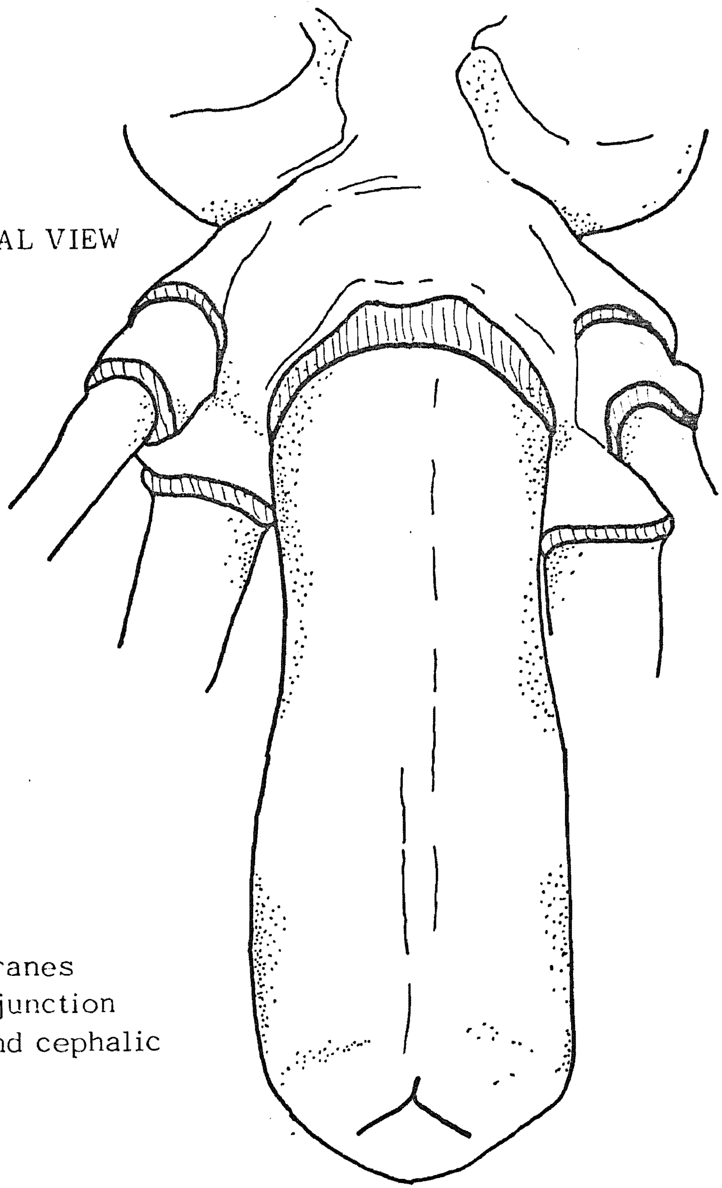
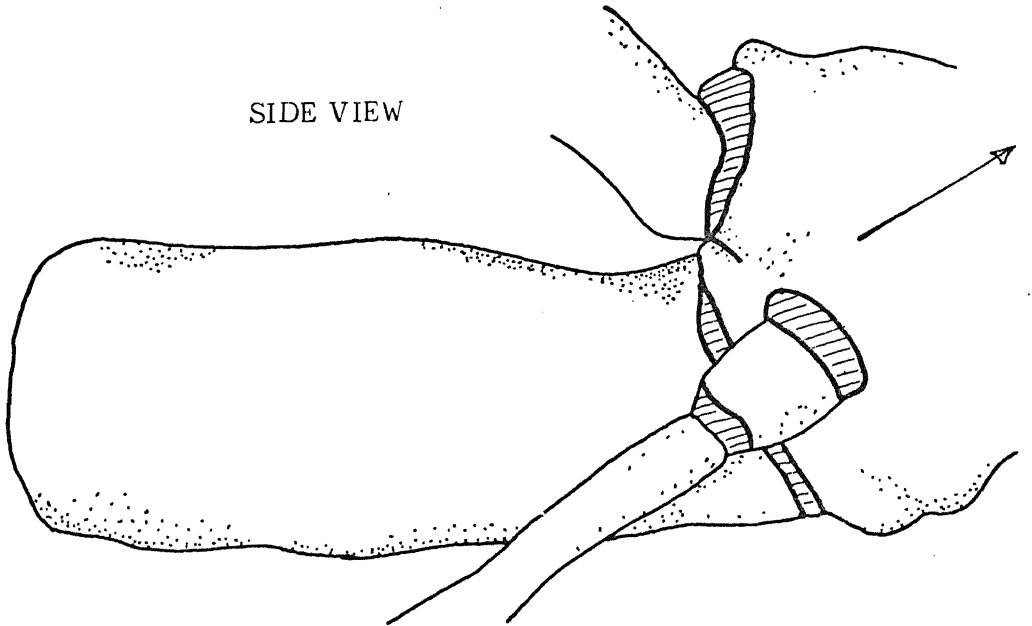



FIGURE 4 D.

Arthrodial membranes (line shaded) at the junction between proboscis and cephalic somite.



Investigations of proboscis function have stimulated less controversy. However, workers have tended to study movements within the proboscis itself (INTRINSIC) as opposed to those mechanisms which move the proboscis with respect to the rest of the animal (EXTRINSIC). Dohrn (1881), Hoek (1881) and Wiren (1918) described intrinsic structures. Fry (1965) seems to have been the first to seriously attempt explanation of both intrinsic and extrinsic structures. Wyer (1972) has also looked at intrinsic and extrinsic structures, but Dencker (1974) seems to have reverted to intrinsic investigations alone.

This work considers both intrinsic and extrinsic mechanisms in N. australe and N. orcadense. Other Nymphonidae whose proboscides have been studied to varying degrees are N. robustum BELL (Hoek 1881), N. brevirostre HODGE and N. mixtum KRÖYER (Wiren 1918), N. gracile (LEACH) and N. rubrum HODGE (Wyer 1972), and N. rubrum HODGE (Dencker 1974).

In N. orcadense and N. australe, the proboscis is straight, appreciably cylindrical and with a very slight dilation about one third of the way from the tip. Its shape corresponds to B''' or C''' of Fry and Hedgpeth's scheme of nomenclature (see Figures 4C and 4D). This contrasts with C' and B' reported by Wyer (1972) for N. gracile and N. rubrum respectively. Internally and longitudinally the proboscis is divided into three fairly distinct regions which are closely

linked to the shape of the foregut lumen. At the distal end is the mouth opening which leads into a LIP REGION. Transverse sections through this region show the lumen to be the truncated Y shape indicated in the previous section. The next region, which extends over about half the length of the proboscis has its lumen expanded and in transverse section this has the shape of an equilateral triangle with its base situated dorsally.* In the proximal half of the proboscis, the lumen is once again restricted to the truncated Yshape of the lip region. Figure 4E shows a simple diagram of the above description.

Prior to Fry's study (1965), intrinsic musculature was named in such a manner as to indicate function, which was usually uncertain, rather than position (see eg, Helfer and Schlottke 1935). Fry devised the scheme (used in this work) whereby in transverse section the bisectors of the angles of the equilateral triangle form RADIAL AXES and the borders of the angles between the radial axes form INTER-RADIAL AXES (Figure 4F).

Although based on a tri-radial symmetry, at no time does the lumen of the foregut show quite such obvious symmetry as that shown by Fry (Figure 4 G) or Dohrn (Figure 4H). <u>N. orcadense</u> and <u>N. australe</u>

* The triangles of pycnogonids are non-euclidian, a perfect gut transverse section revealing a classical hyperbolic triangle of Lovachevsky or Bolya. No doubt D'Arcy Thompson would have approved.

do not possess an interradial groove.

Division has been made into three longitudinal regions (see above) but for further detailed examination it has been found necessary to make further subdivisions. Thus, Figure 4I consists of diagrams and plates indicating a region containing lip musculature (1). Regions 2,3 and 4 are of similar appearance in transverse section, but region 3 has chitinous teeth protruding into the lumen. This has therefore been called the 'Dental region' and regions 2 and 4 thus become 'Pre-dental' and 'Post-dental' respectively. Region 5 shows transition from the large sac-like (equilaterally triangular cross-sectioned) region, the pharynx, to the oesophagus. A cuticular wall surrounded by a thin hypodermis becomes one surrounded with an obvious layer of cells. Region 6 is that in which the setae have their origins. Region 7, the proximal region of the proboscis foregut, stretches from behind the bases of the setae to the junction of foregut and midgut.

### 4.2.1. LIP REGION (REGION 1)

At the tip of each antimere is a chitinous protrusion which is surrounded by arthrodial membrane (blue staining with Mallory's triple stain). Dohrn referred to this protrusion as the 'Lip' and to the arthrodial membrane as the 'Lip support'. In the two Nymphon species under consideration here there are no teeth on the lip sup-

port nor 'brush' to the outside (Figure 4J). However, there is a large tooth on the inside of the lip (Plate 4K).

The most distal transverse sections of the foregut in the lip region show the arthrodial membrane as being thicker interradially. In each antimere is a muscle pair, the bundles being one on either side and close to the interradius (Figure 4L). These muscles will be referred to as the 'Lip interradial muscles'. In these distal sections they are cut obliquely. In more proximal transverse sections the position of the lip interradial muscles becomes more peripheral (i.e. towards the proboscis outer wall) and the angle at which they are cut becomes less oblique and more transverse. The interradial region of the foregut cuticle becomes expanded backwards and outwards into large apophyses. These apophyses are connected to the lip interradial muscles (Figure 4M). Attached to the radial ridges are radial muscles which are cut obliquely. The radial ridges do not appear to be anchored to he outer wall of the foregut by non-staining refractile cuticle as Fry (1965 p.213) reported for Rhynchothorax australis and Pycnogonum stearnsi.

Yet more proximal sections through the lip region show a gradual reduction in the interradial apophysis and in the amount of thickening of the interradial wall of the foregut. The lip interradial muscle bundles become more peripheral and are now cut almost transversely. At the same time, the lumen of the foregut is expanding to the equi-

laterally triangular shape of the next region, region 2.

A large nerve ring is visible in the lip region, with ganglia positioned interradially in each antimere. It was not possible to discern whether there was a difference between dorsal and ventro-lateral ganglia as is claimed by Wiren (op. cit.) for N. brevirostre. The ganglia are much less complex than those that he figured. Longitudianl sections indicate that there is probably an extensive nerve network in the proboscis tip, but the fixation and staining methods applied were not those designed for detailed display of nerve networks.

### 4.2.2. PRE-DENTAL REGION (REGION 2)

Transverse sections show the foregut cuticle to be of even thickness and entirely red staining with Mallory's triple stain in this region. The lip interradial muscle bundles are cut transversely, are reduced and peripheral. To the radial side of each lip interradial bundle, there now arises an additional muscle bundle cut longitudinally in transverse sections. These latter bundles (a pair to each antimere) are the true 'interradial muscles' referred to by Fry (1965). They are present throughout the rest of the proboscis (Figure 4N). There seem to be no connections between the lip interradial muscles and the true interradial muscles. The lip region proper might be said to end when the lip interradial muscles are no longer seen in transverse sections.

Further into region 2 a typical transverse section will show the following features: An equilaterally triangular lumen bounded by cuticle which is red staining with Mallory's triple stain and of uniform thickness. At the radial ridges, radial muscles connect with the outer wall of the proboscis. Three pairs of interradial muscles are present, one pair to each antimere. Each antimere is seen to have a nerve bundle which is positioned interradially in the cavity of the antimere and close to the foregut. A strange, lace-like connective tissue (called 'Bindegewebe' by Dohrn) lies on either side of the radial muscles and forms strands which connect foregut and proboscis wall (Figure 4O). This is present in all transverse sections of the proboscis in other regions.

### 4.2.3. DENTAL AND POST-DENTAL REGIONS

Regions 3 and 4 differ from the typical region 2 transverse section appearance (above) in but few respects. In the most distal sections of region 2 the nerve bundle of each antimere is a single structure. However, within a very short distance of the lip region, the single bundle becomes doubled peripherally along the interradius. The bundle closest to the foregut is of larger diameter and is the one always associated with nerve rings around the foregut when these appear. This division of the nerve bundles corresponds to that found by Wiren (1918) in N. brevirostre. After division of the nerve bundles, transverse sections show small conical chitinous teeth arising from the foregut wall (Figure 4P). This

dental region extends for about the same length of the proboscis as does the lip region. It is present in both Nymphon species considered here, andDencker (1974) has shown a similar region in N. rubrum (Figure 4Q), although he did not label it.

Posterior to this dental region is another region with smooth foregut cuticle (region 4) which is very similar to the proximal part of region 2.

4.2.4. TRANSITION REGION (REGION 5)

As sections are cut at the proximal end of region 4, so the lumen appears increasingly constricted. This constr iction starts inter-radially, and gradually moves towards the interradius (Figure 4R). As the constriction occurs a change in the most radial parts of the cuticle is seen (Figure 4S). The cuticle appears to possess folds, areas staining red with Mallory's triple stain alternating with areas staining blue. There now appears a second region of the foregut with teeth. These are not restricted to the interradial regions as previously but lie all round the foregut wall. They appear smaller than the teeth in the dental region (2).

The nature of the radial folds in the cuticle is not clear. They occur throughout the rest of the length of the proboscis while cuticle is present. It is suggested that they may become unfolded when the foregut is expanded (Figure 4T).

As the constriction of the lumen occurs, so two other features change in transverse sections. Throughout its length thus far, there has been very little tissue surrounding the foregut cuticle. Now, there is an increase in the amount of tissues surrounding the foregut cuticle and as the lumen becomes constricted the surrounding tissue fills the angles between the radii. This tissue is closely packed and of granular appearance (Figure 4U). The other change takes place in the nervous system. Thus far along the proboscis, the distance between the two nerve bundles in each antimere has been the same. Now, however, although the position of the inner bundle remains the same with respect to the outer wall of the proboscis, in the dorsal antimere the smaller outer bundle moves towards the outer wall (i.e. becomes more peripheral). From this region onwards the dorsal antimere can always be identified in single transverse sections because of this feature.

As the region of the arthrodial membrane (which acts as a sleeve-like hinge to the proboscis) is reached, the radial, interradial and circular muscles and the 'Bindegewebe' disappear from transverse sections. No intrinsic muscles connect any part of the proboscis with the cephalic somite and these muscles can therefore have no role in movement of the proboscis with respect to the rest of the animal.

4.2.5. THE EXTRINSIC MUSCULATURE

The problem of elucidating the extrinsic musculature operating the movement of the proboscis of N. orcadense and N. australe has proved perplexing. Longitudinal sections (see appendix 2) can be misleading if they are oblique to the slightest degree. Transverse sections are not always transverse through both proboscis and trunk at the same time. In addition, they are made confusing by the fact that within a relatively short space there is not only the connection of proboscis with trunk, but the origins of the chelicerae, palps and ovigers.

Fry (1965) replaced the terms adductor and abductor used for pairs of extrinsic muscles which had been reported to move the proboscis, by M1 and M2 respectively. This avoided the use of terms which implied non-proven functions. A third extrinsic pair M3, which seemed to have an ancillary function to the M2 muscle was also described. In the three species which Fry described, both M1 and M2 muscles pairs originate in the trunk posterior to the ocular tubercle and connect with the arthrodial membranes anteriorly. In Rhynchothorax australis and in Austrodecus glaciale at least one of these muscle pairs passes through the aperture bordered by the dorsal ganglion, the ventral ganglion and the commisures which connect the two. Thus transverse sections through this region should show muscle in T.S. passing through this aperture alongside the foregut. As can be

seen in Plate 4V, this is not the case in N. orcadense or N. australe. The opening formed by the nervous system has little room for anything other than foregut (oesophagus). As in Pycnogonum stearnsi, Fry's third animal, in which neither the M1 or M2 pairs pass through the aperture described above, one would expect to see muscle cut obliquely transversely on either side of the dorsal ganglion/ventral ganglion/commissure complex. Again (Plate 4W), this is not the case in the twoNymphon species. The extrinsic musculature of N. orcadense and of N. australe is very different from those described for Fry's three species.

It has been found that the only recognisable extrinsic musculature in the above consists of a ventral muscle pair whose position corresponds roughly to the M3 muscles of Rhynchothorax australis. These originate laterally and basally in the trunk anterior to the insertion of the ovigers. Laterally there are thin bands of muscle which just span the arthrodial membrane in an anterior-posterior direction, but little more (Figures 4X, Y and Z). There are no dorsal extrinsic muscles.

Helfer and Schlottke (1935) considered that the arthrodial collar in Nymphonidae is small compared with that of members of other families and that the manoeuverability of the proboscis is therefore reduced. Wyer (1972) who investigated N. gracile and N. rubrum

supported this view.

As mentioned in Chapter 2, the chelicerae are used extensively in the feeding behaviour of both animals. The proboscis is in effect 'handed' the food. It is not used in the same way as the proboscides of Austrodecus glaciale, Pycnogonum stearnsi, or Rhynchothorax australis, or as are those of Ammothea carolinensis and Decolopoda australis. N. orcadense and N. australe could be said to lack musculature for moving the proboscis. It is likely that in most of their feeding situations proboscis movement other than retention of orientation would not be necessary. This may also account for the apparent lack of extrinsic musculature in the figures of Dencker (1974).

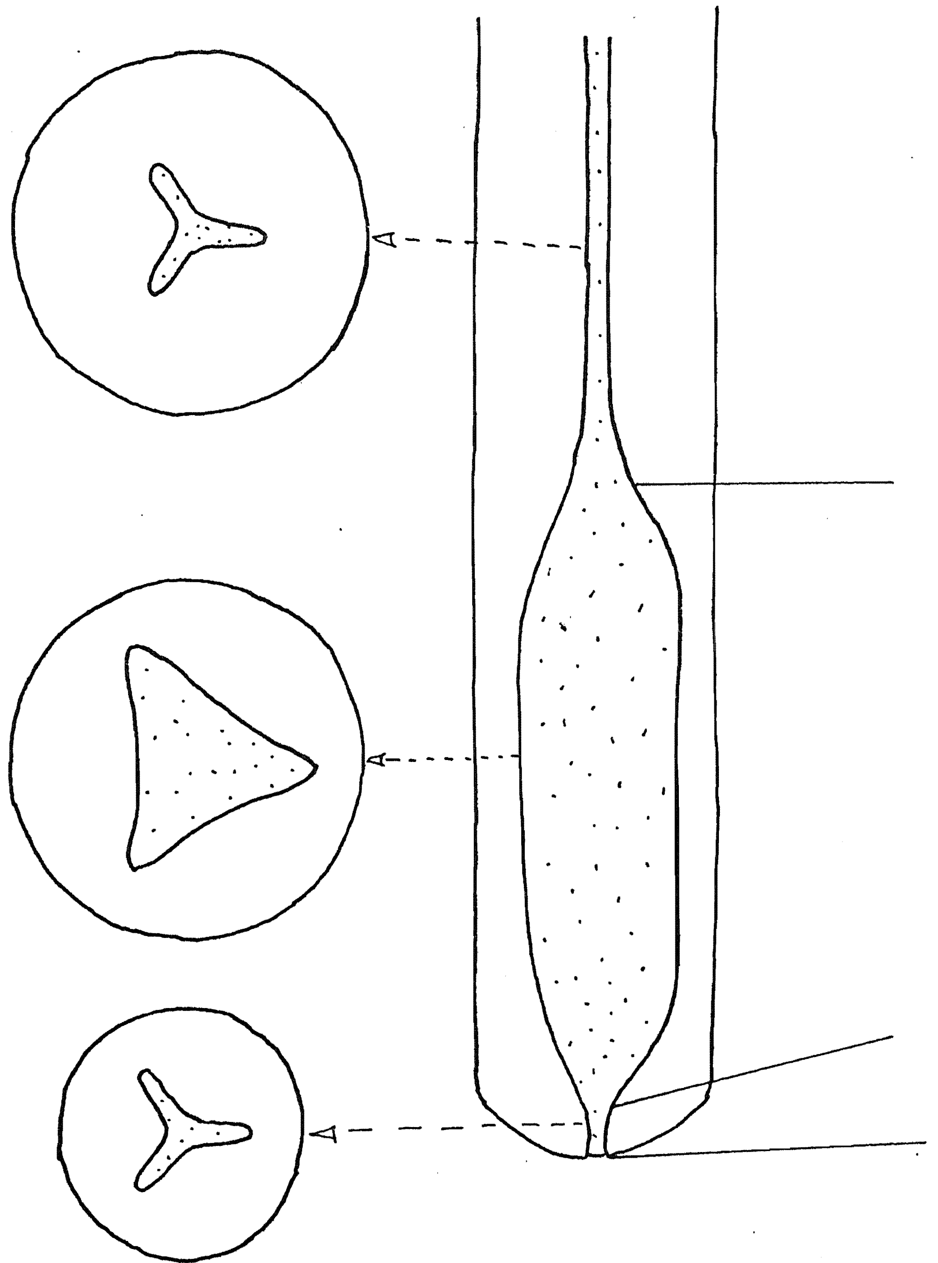


FIGURE 4 E.

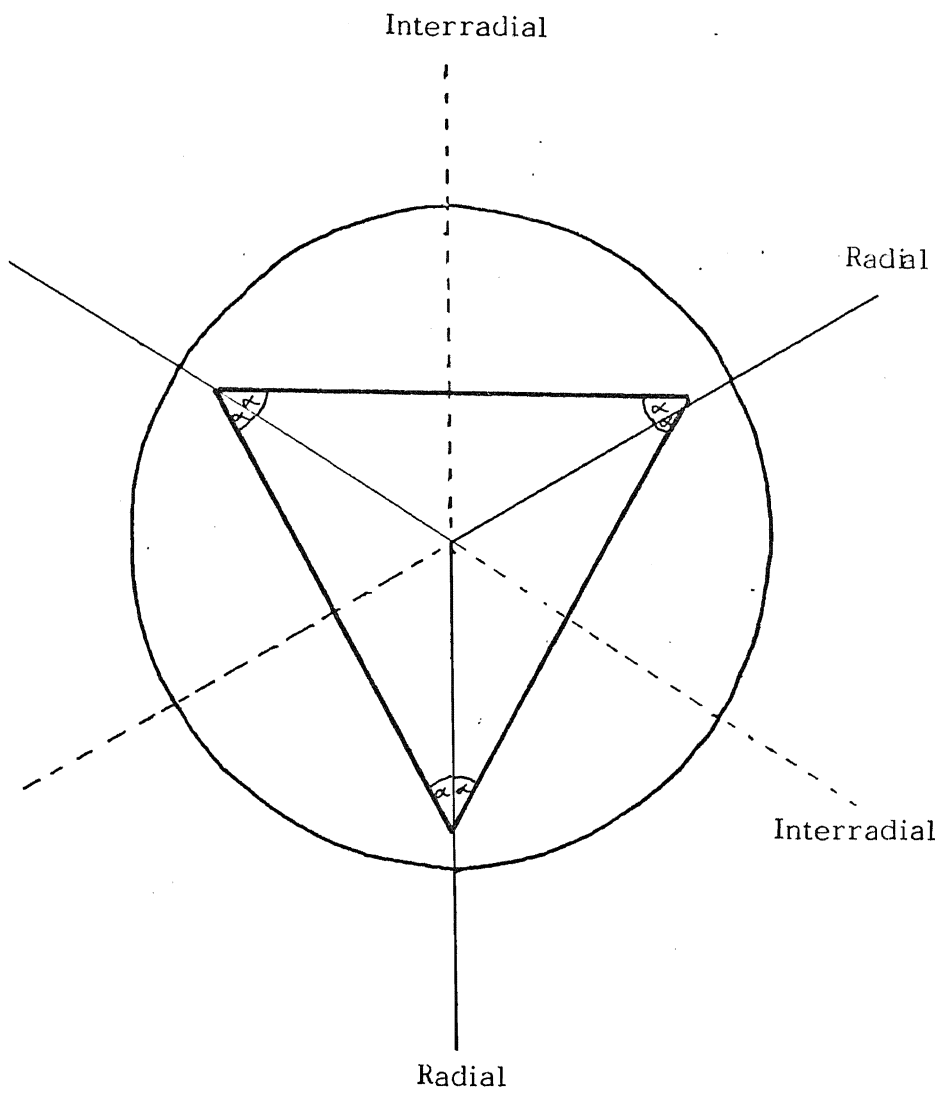



FIGURE 4 F

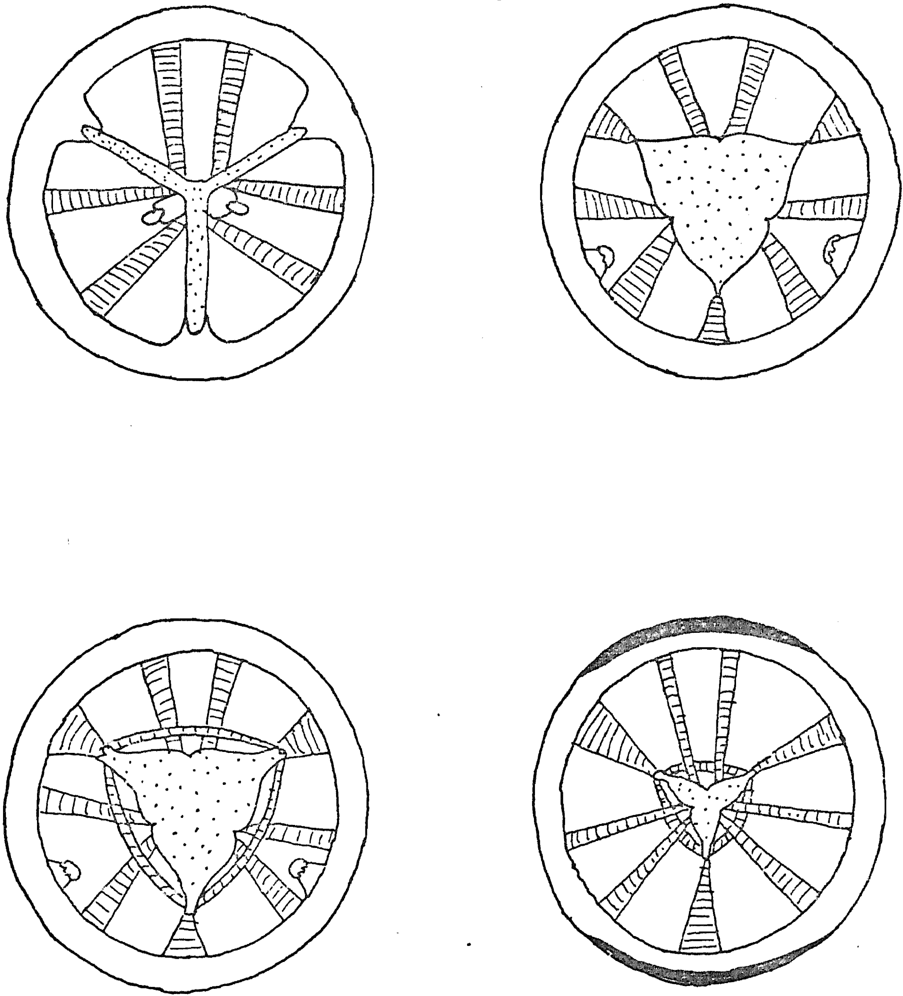
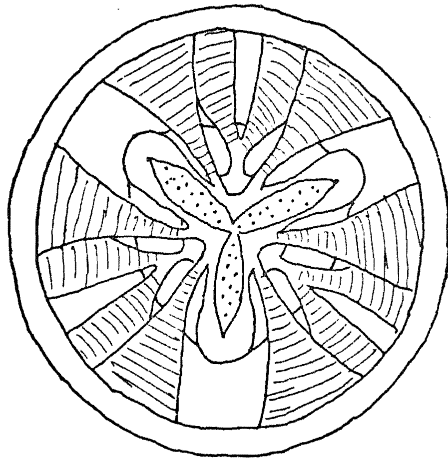


FIGURE 4 G. Redrawn from Fry (1965) and simplified to show the symmetry of the foregut ( stippled). Muscles are line shaded.



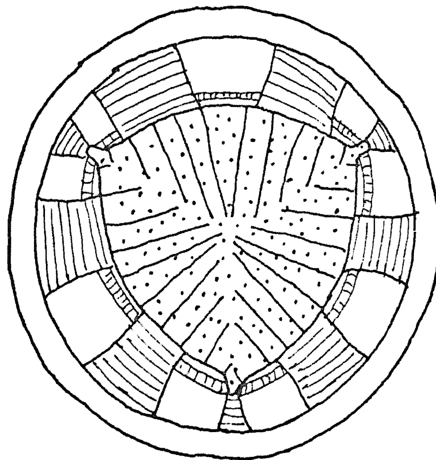


FIGURE 4 H. Redrawn from Dencker (1974) simplified to show the symmetry of the foregut (stippled). Muscles are line shaded.

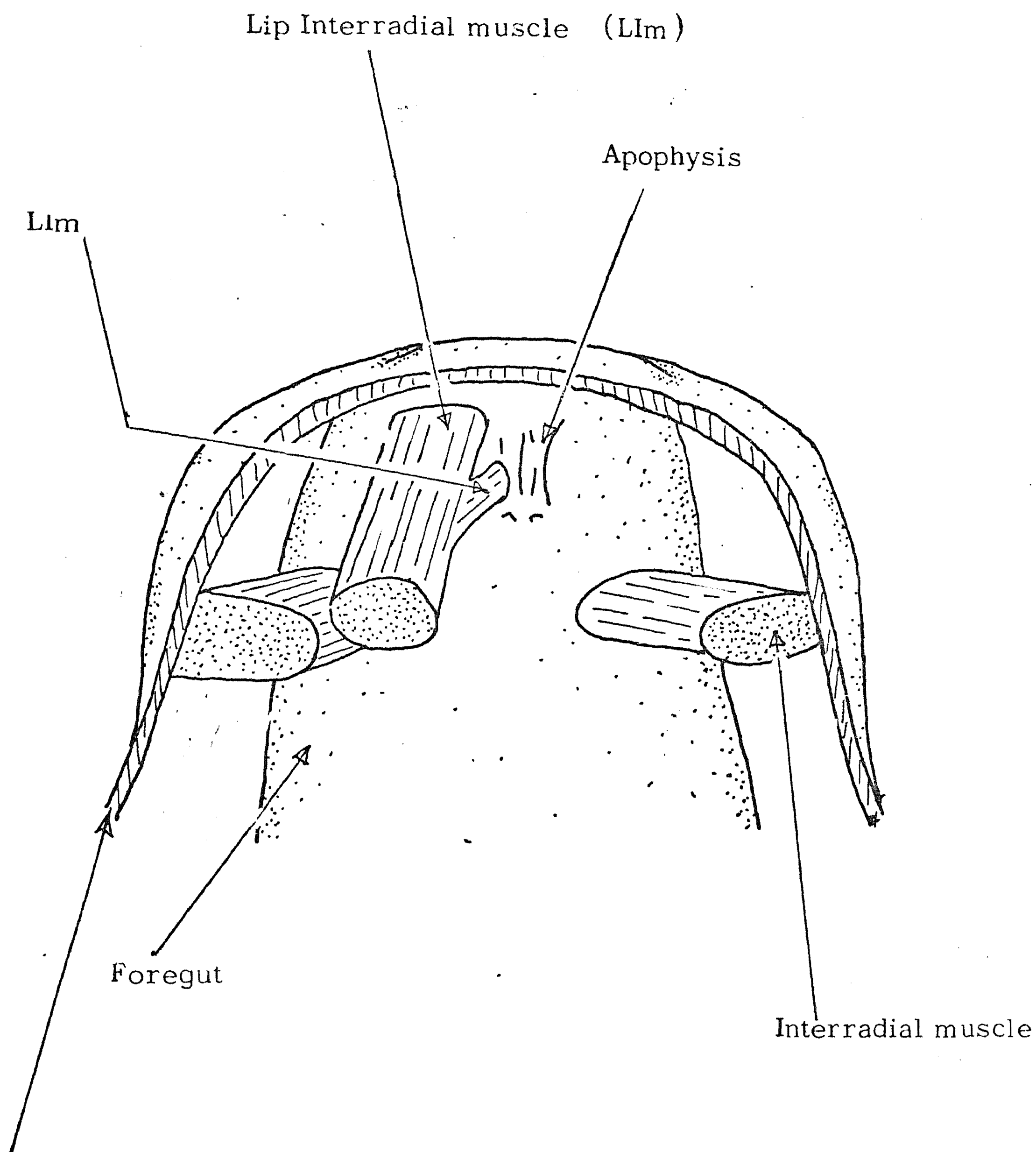



FIGURE 4 1.

FIGURE 4 J.

Lip region L.S.

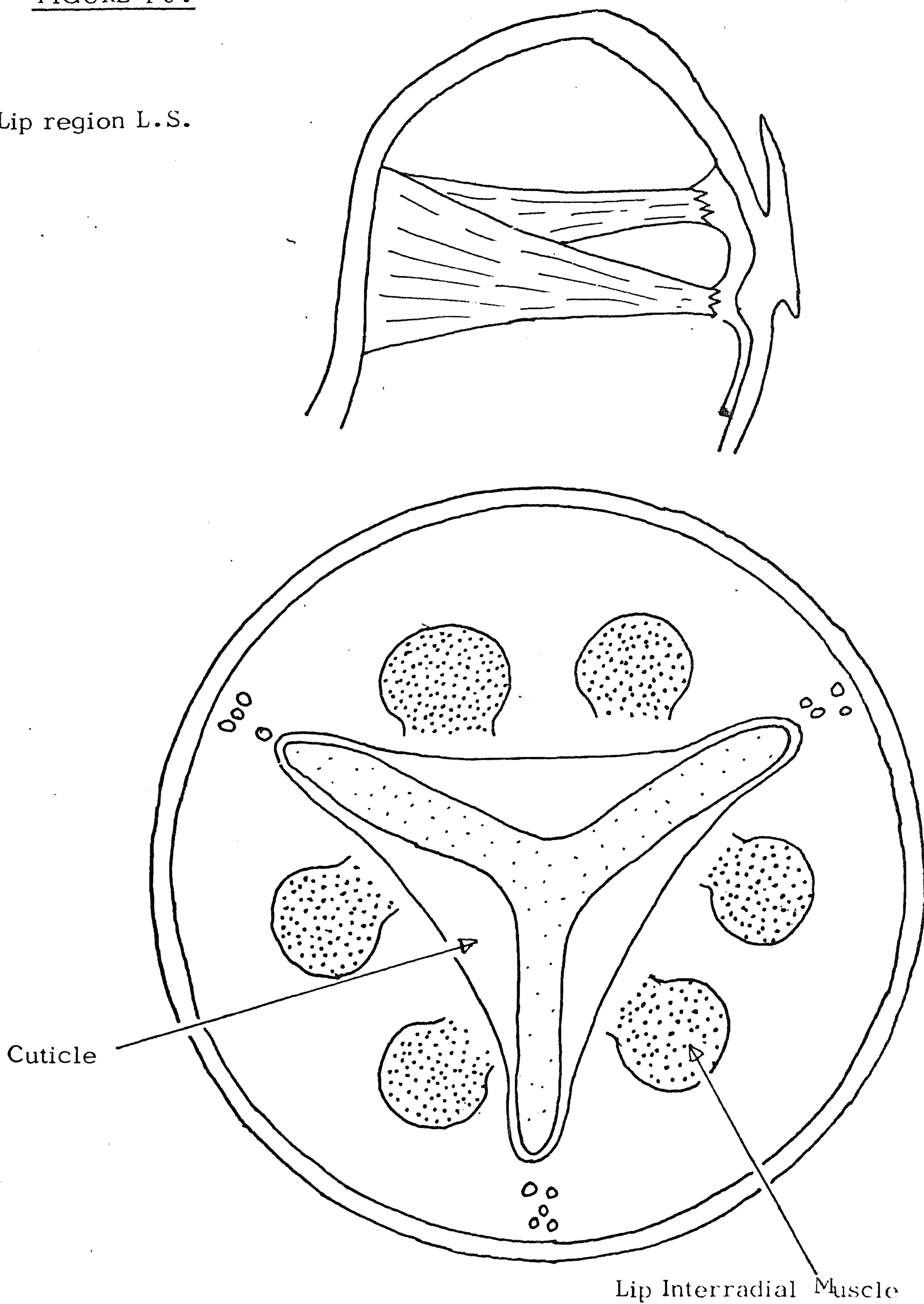
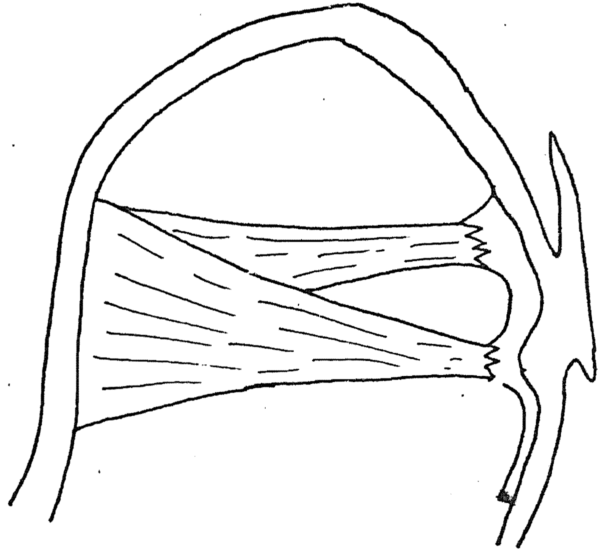


FIGURE 4 J.

Lip region L.S.



Cuticle

Lip Interradial Muscle

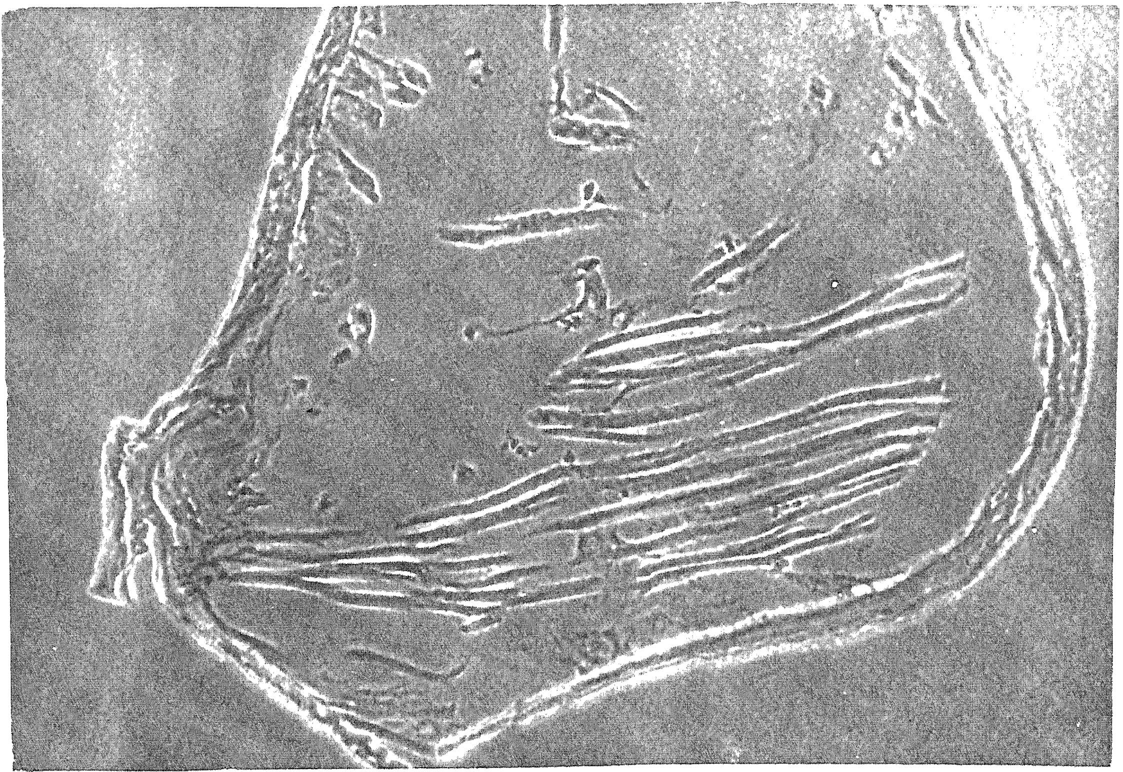


PLATE 4 K. Lip region (L.S.). 50 µm.

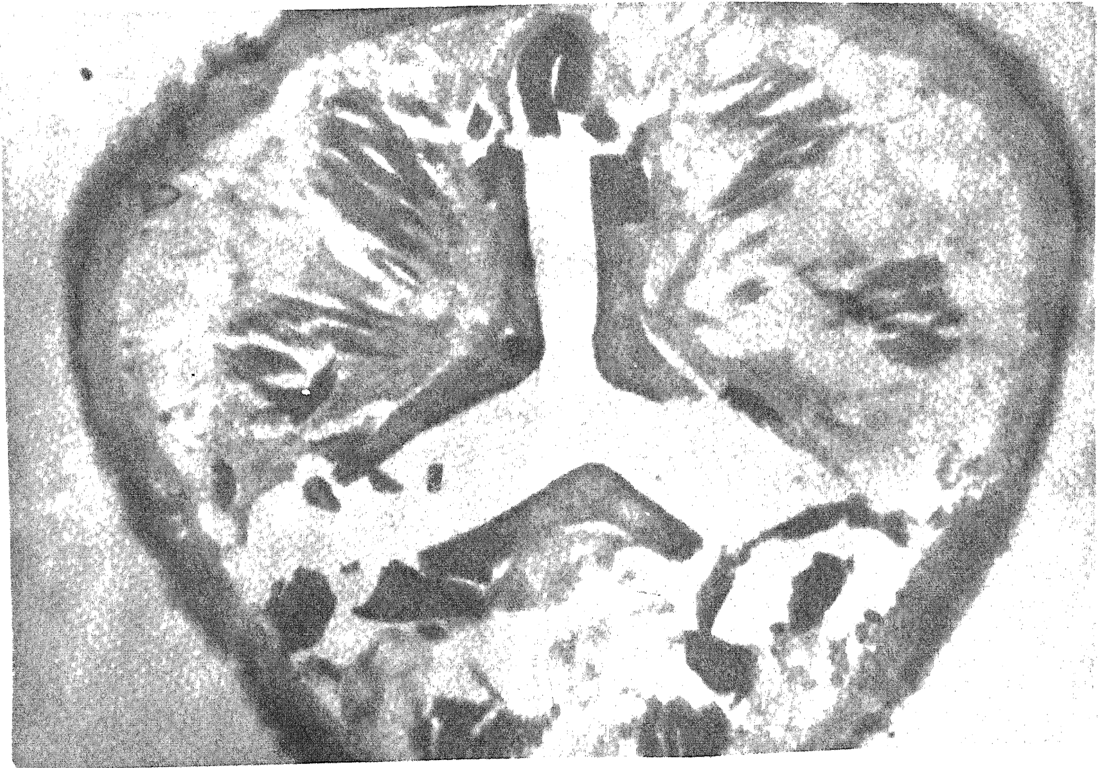


PLATE 4 L Lip region (T.S.). ≃ 100 µm

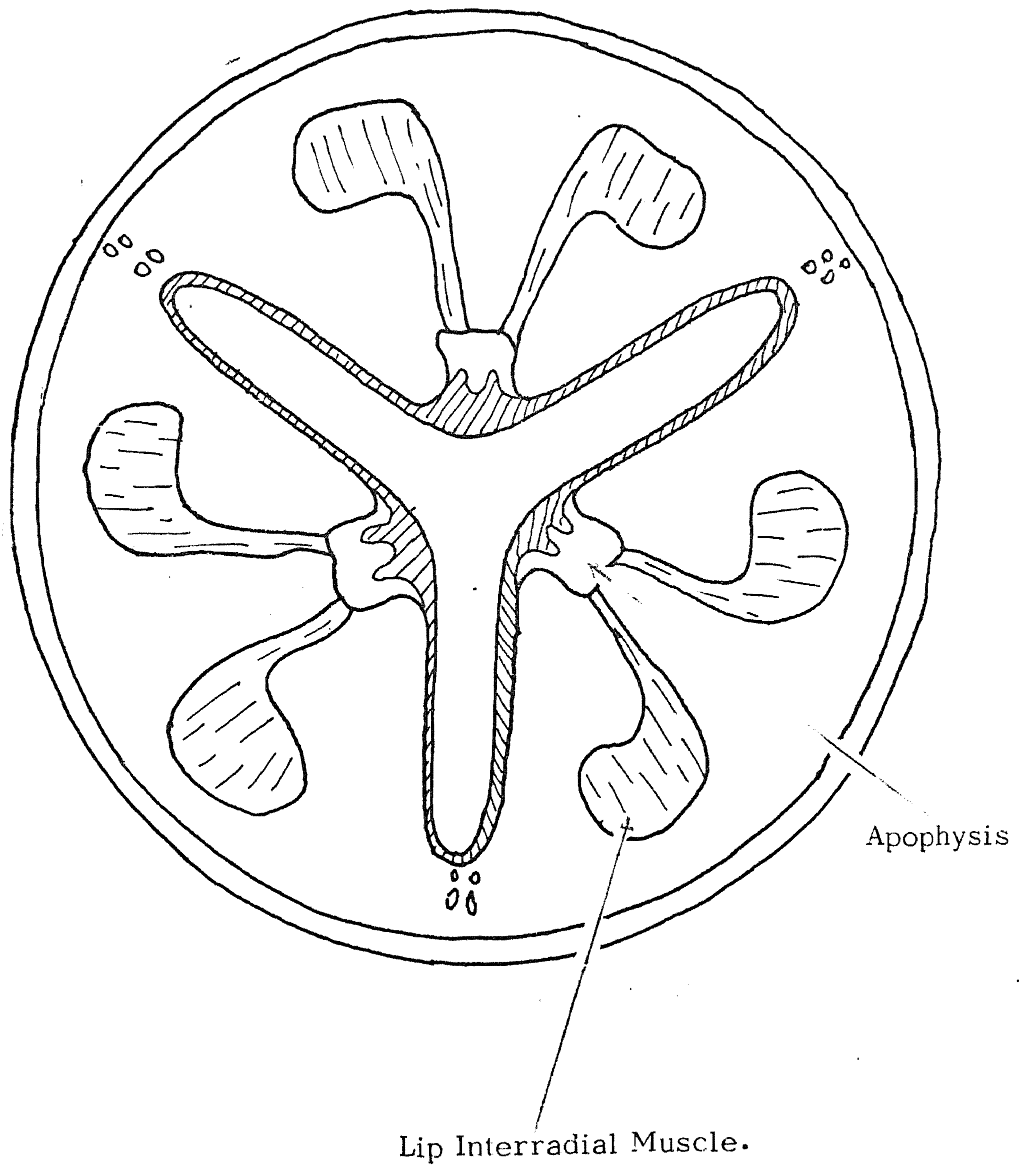
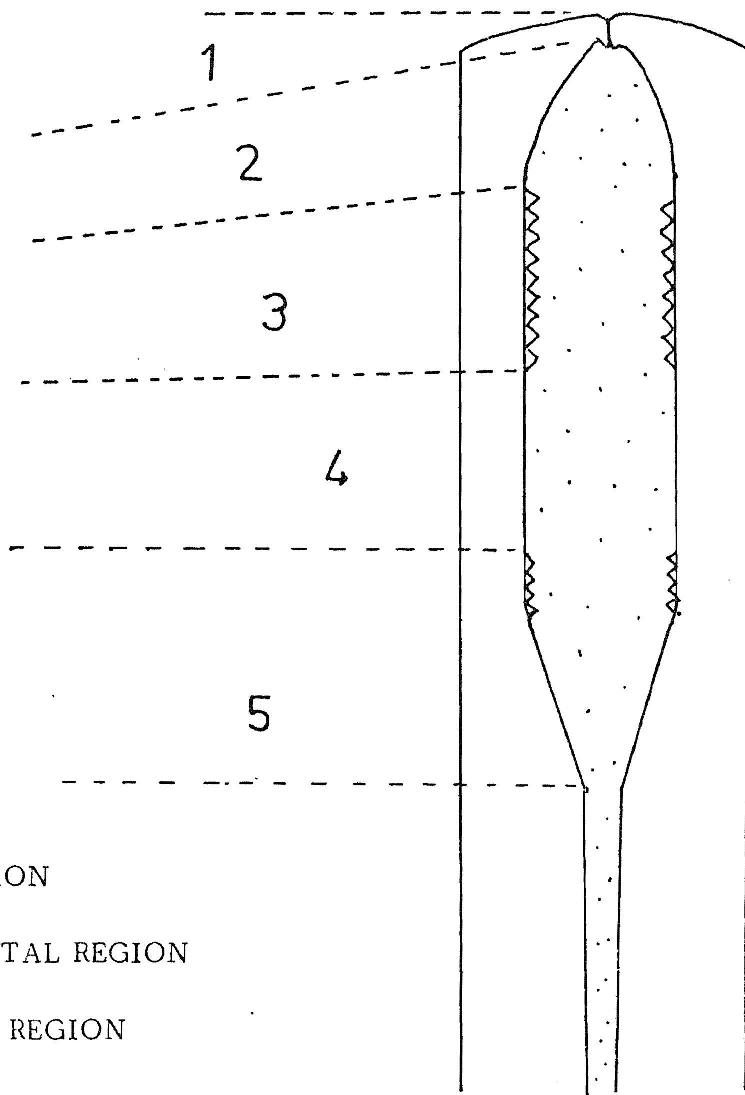



FIGURE 4 M. Lip region.




1 = LIP REGION

2 = PRE DENTAL REGION

3 = DENTAL REGION

4 = POST DENTAL REGION

5 = TRANSITION REGION

FIGURE 4 N.

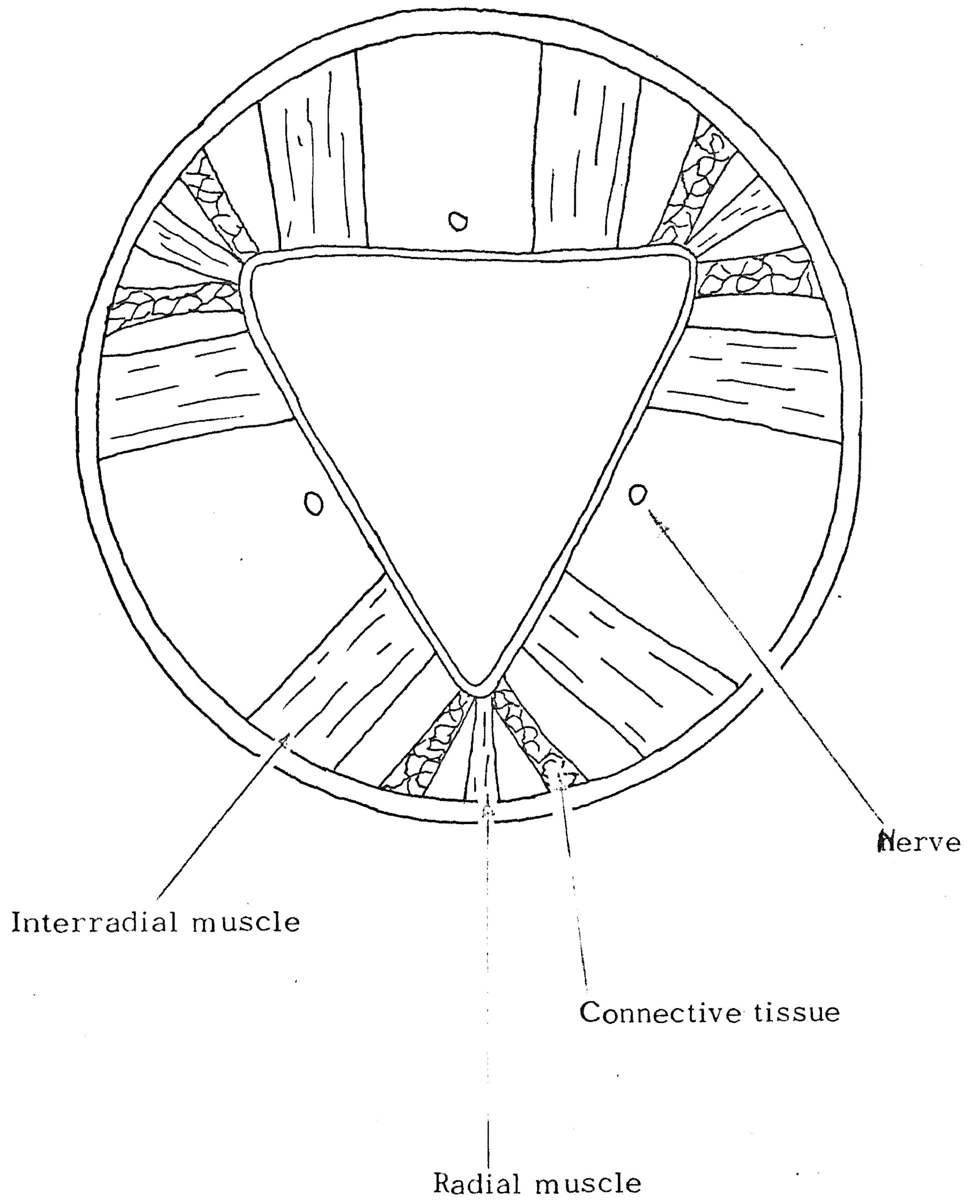



FIGURE 4 O.

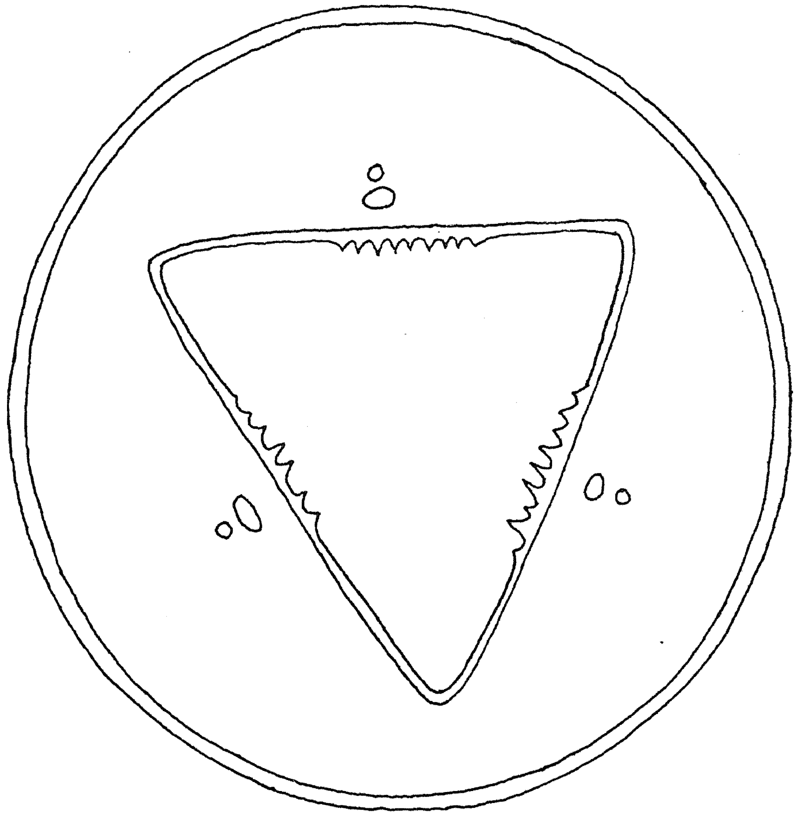


FIGURE 4 P. DENTAL REGION.

Den. = Dental region.

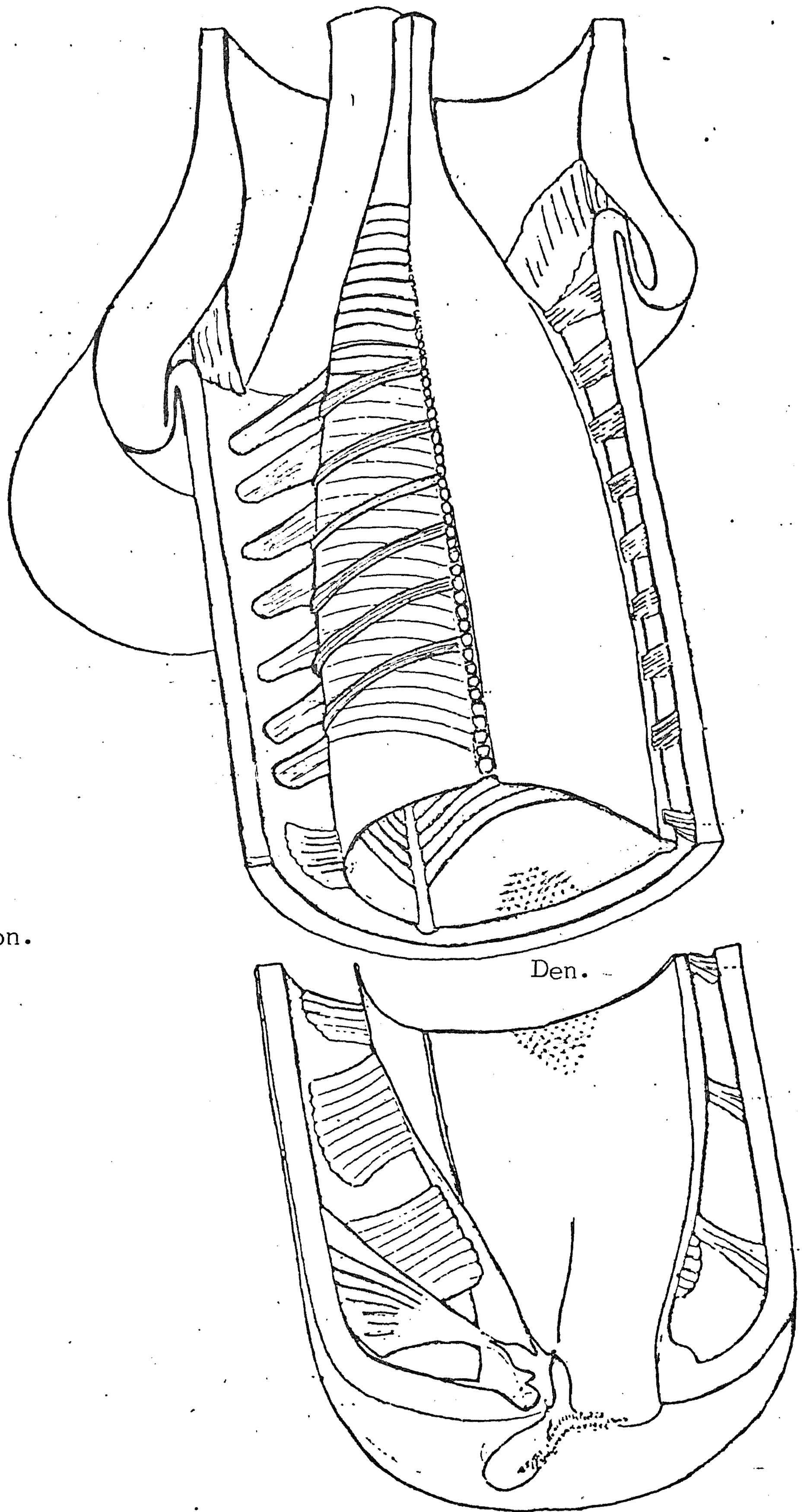
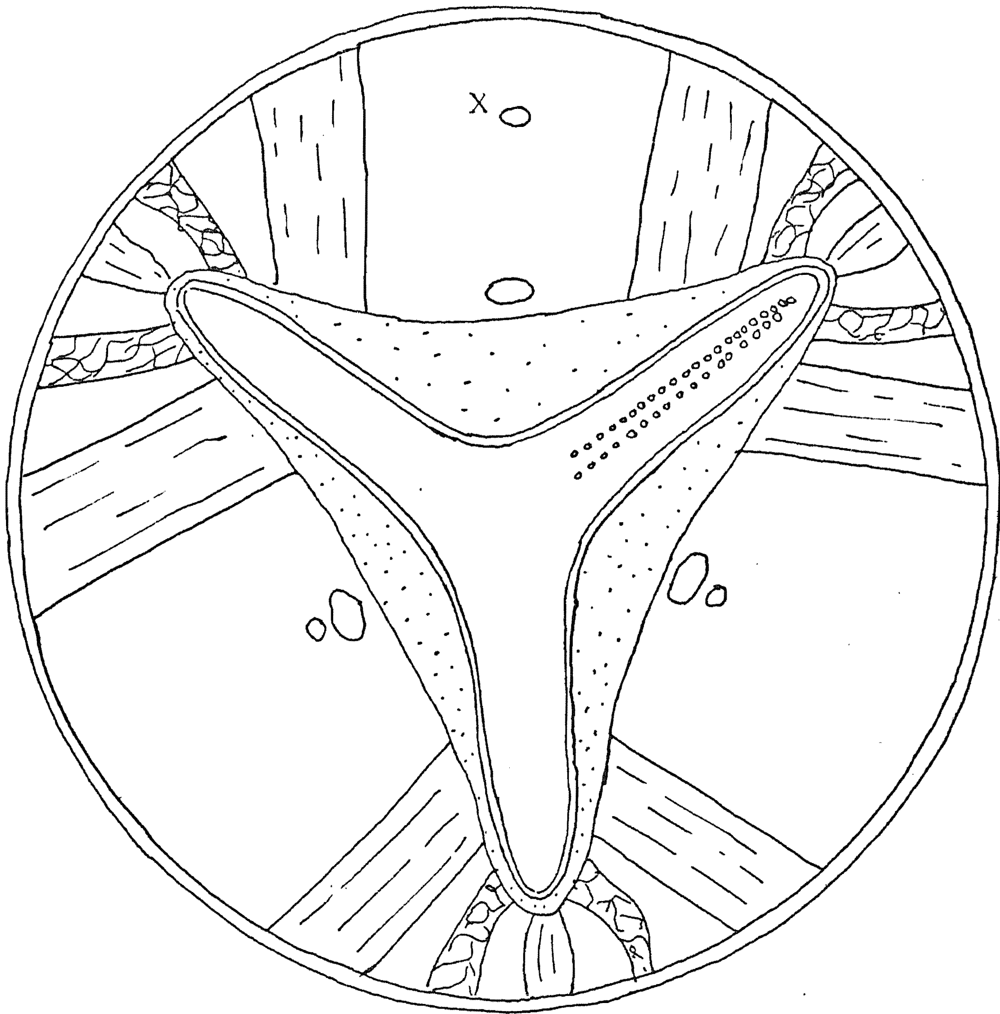



FIGURE 4 Q. From Dencker (1974)

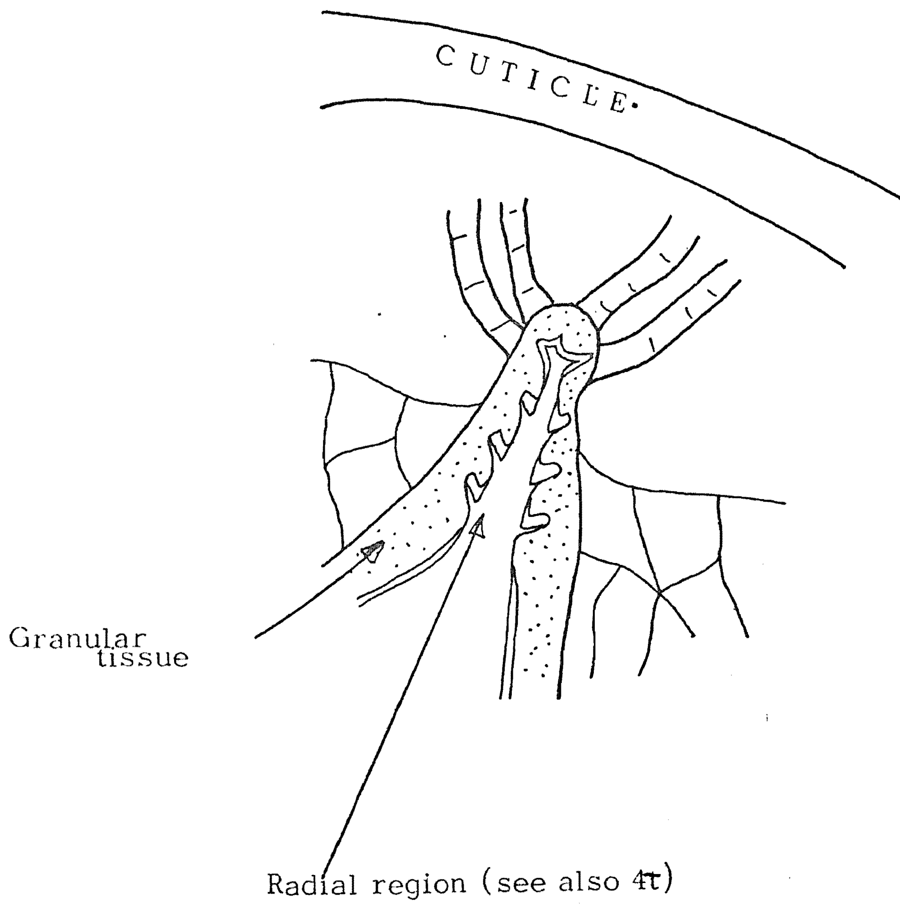
Three dimensional drawing of the proboscis of *Nymphon rubrum*.

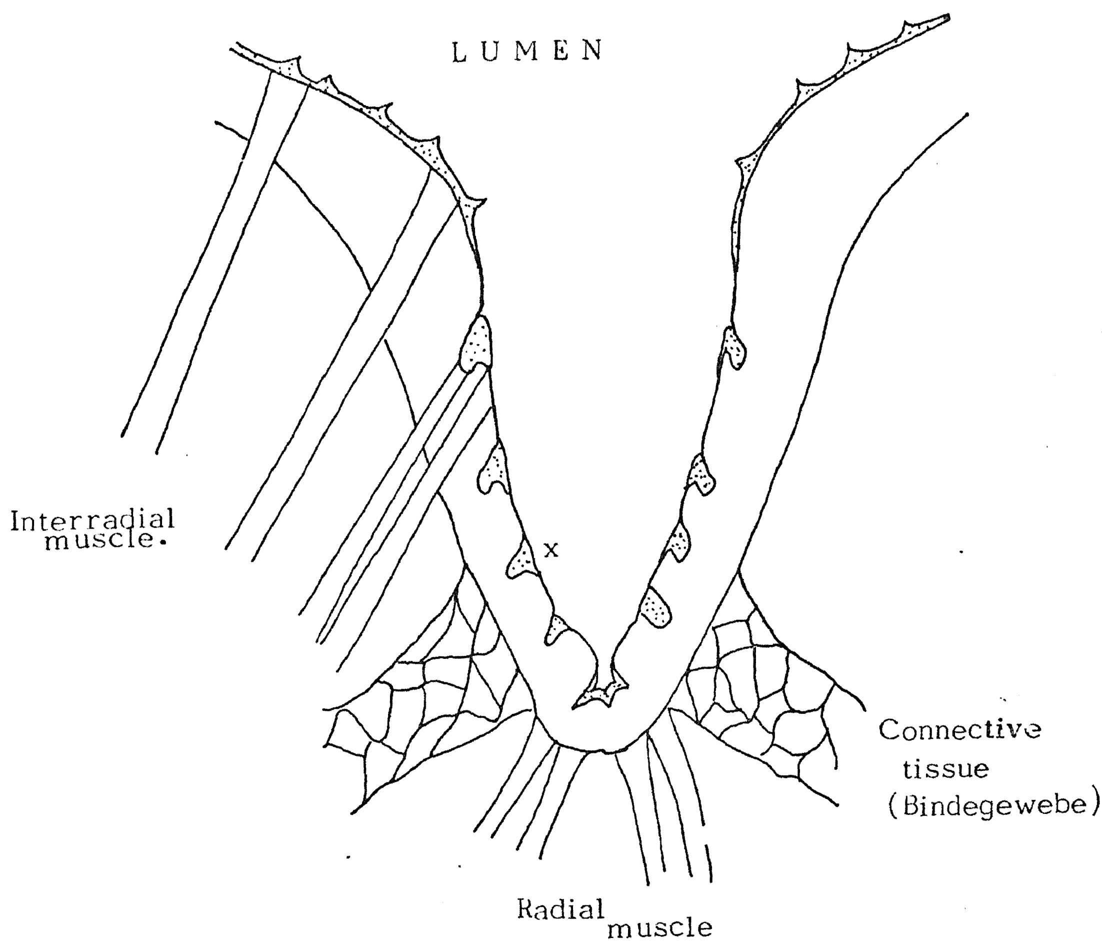



Note position of dorsal peripheral nerve X compared with the peripheral bundles of the other antimeres. Note also the cut ends of setae and the increased amount of granular tissue surrounding the foregut.

FIGURE 4 R.

FIGURE 4 S.





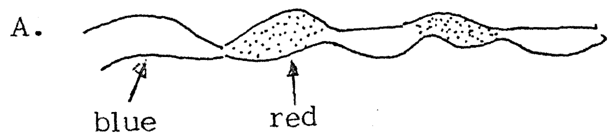



FIGURE 4 T. It is suggested that 'x' above, (regions of cuticle which are red staining with Mallory's triple stain), take up the configuration shown in A. when the gut is expanded, B. when it is relaxed.

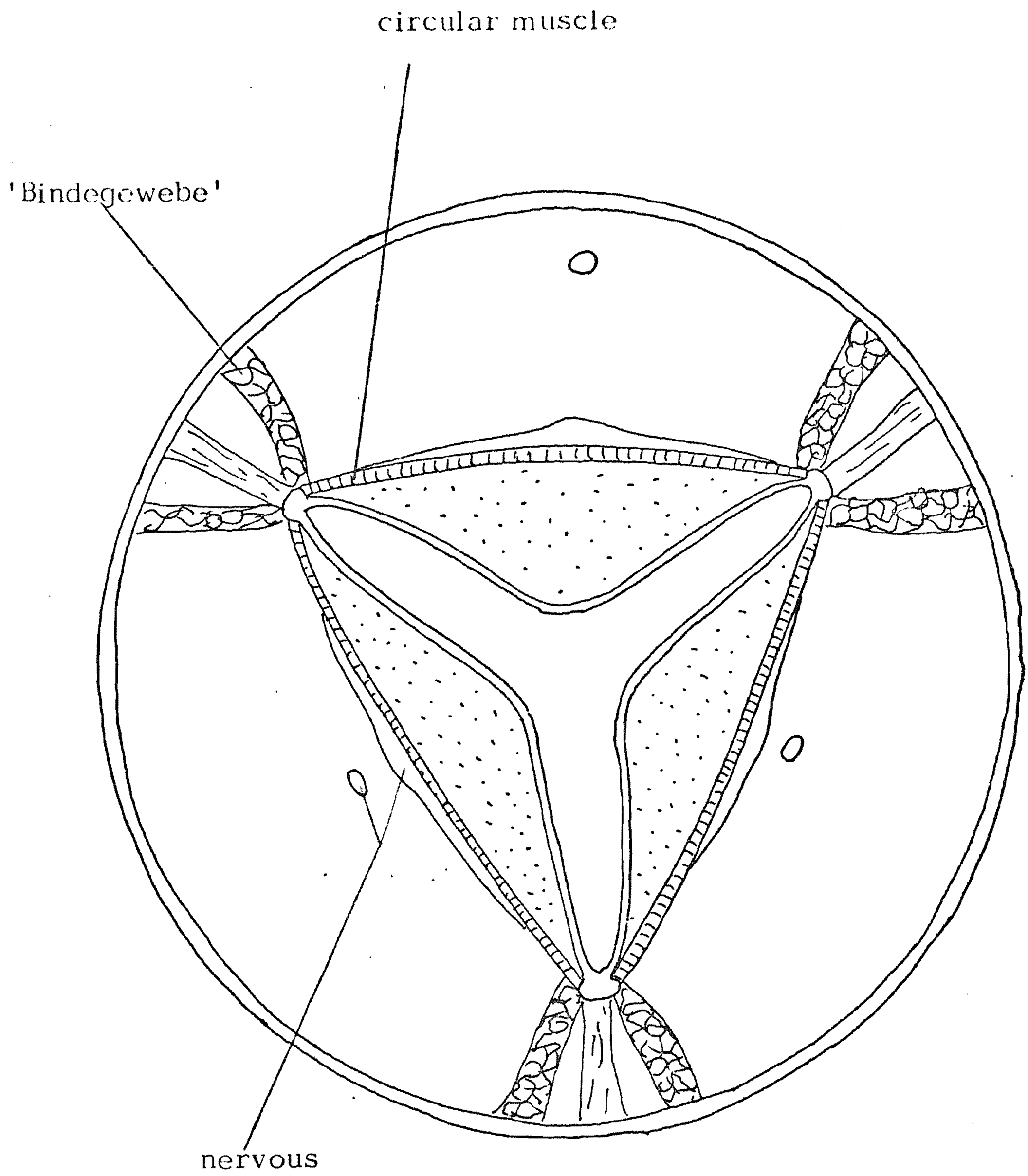



FIGURE 4 U. Transition region.

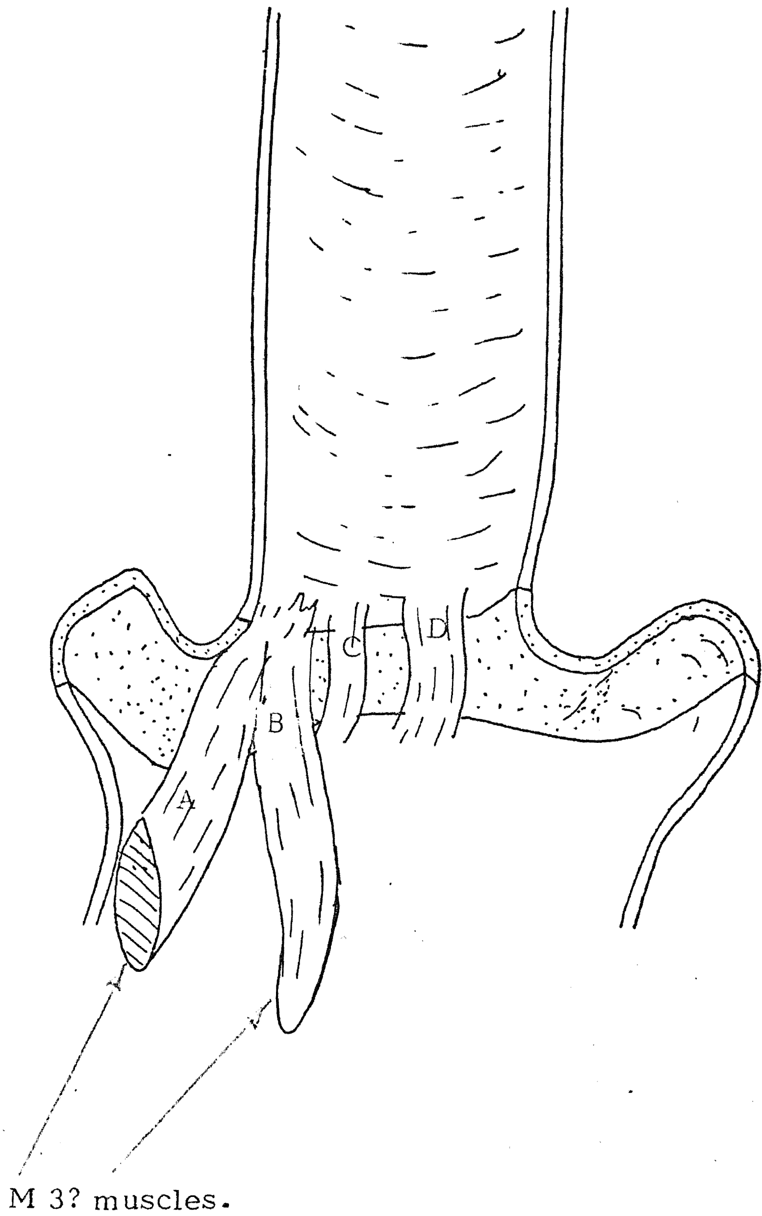



FIGURE 4 XYZ Midsagittal view showing extrinsic muscles at A.B.C. & D.

(Plates 4V &W have been bound overleaf).

└──┘ ≏ 10 μm

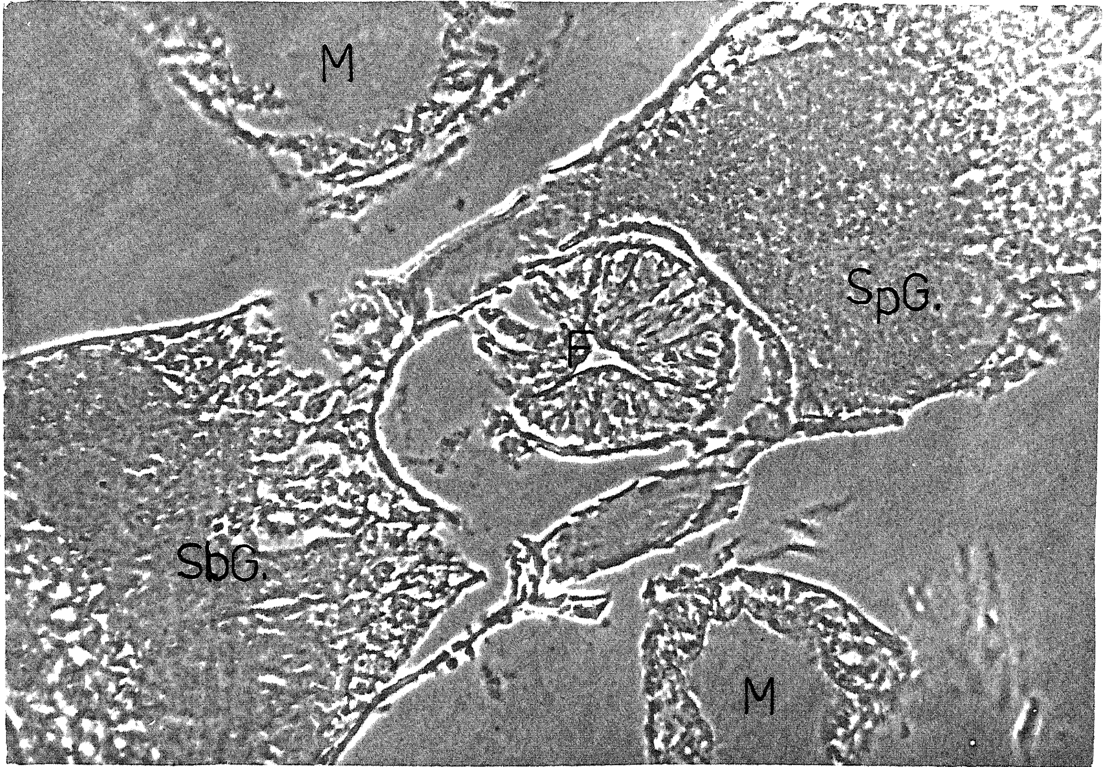



PLATE 4 V. T.S. through foregut (F) where it is surrounded by supra and suboesophageal ganglia (SpG and SbG) and their commissures (C).

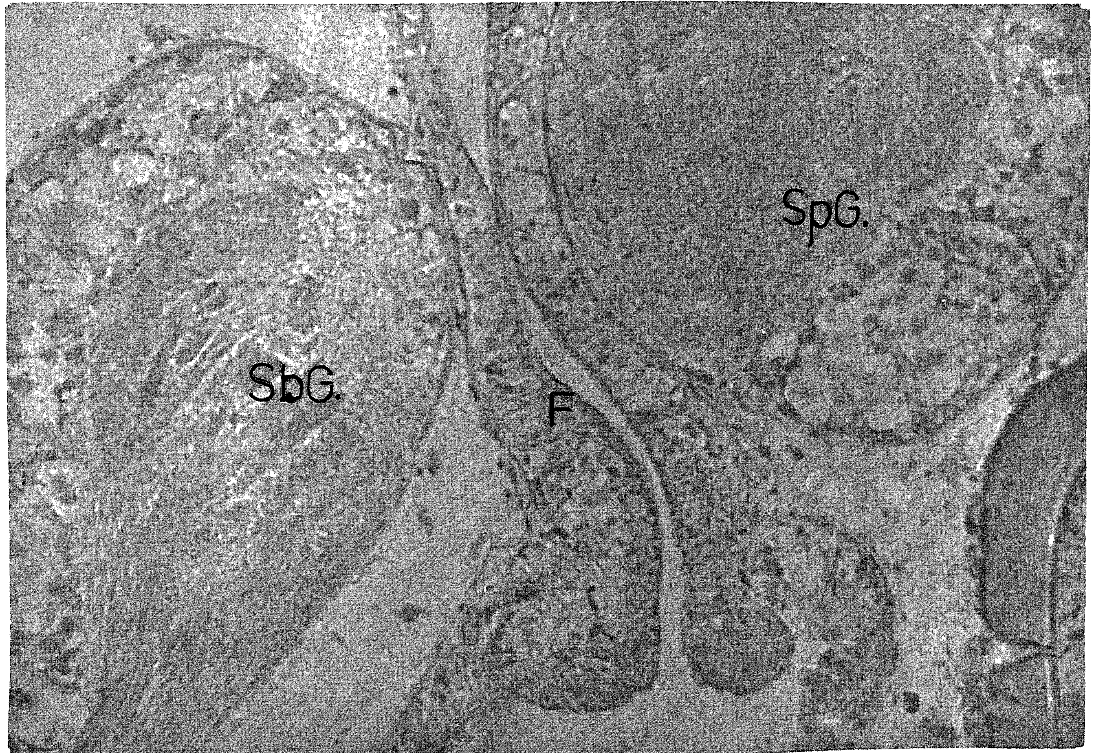
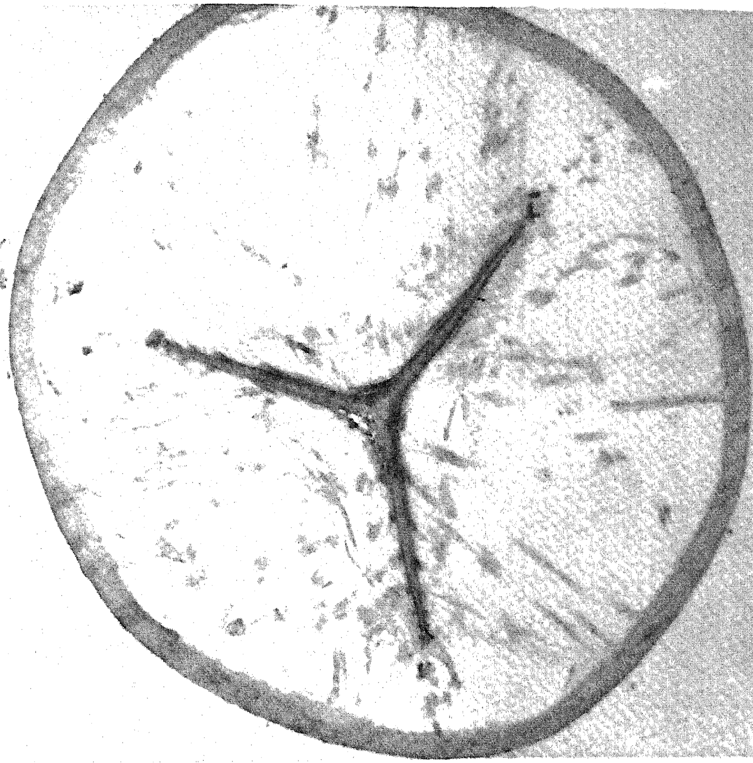


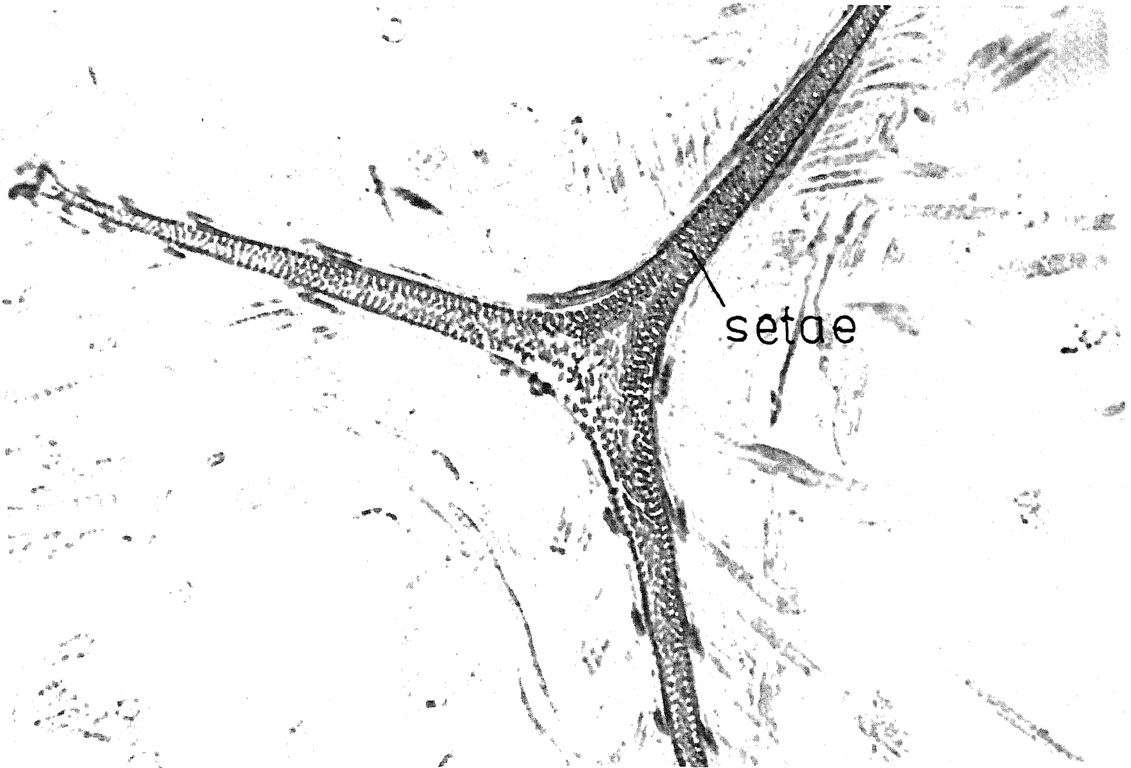

PLATE 4 W. As Plate 4V, but L.S.

└──┘ ≏ 10 μm.

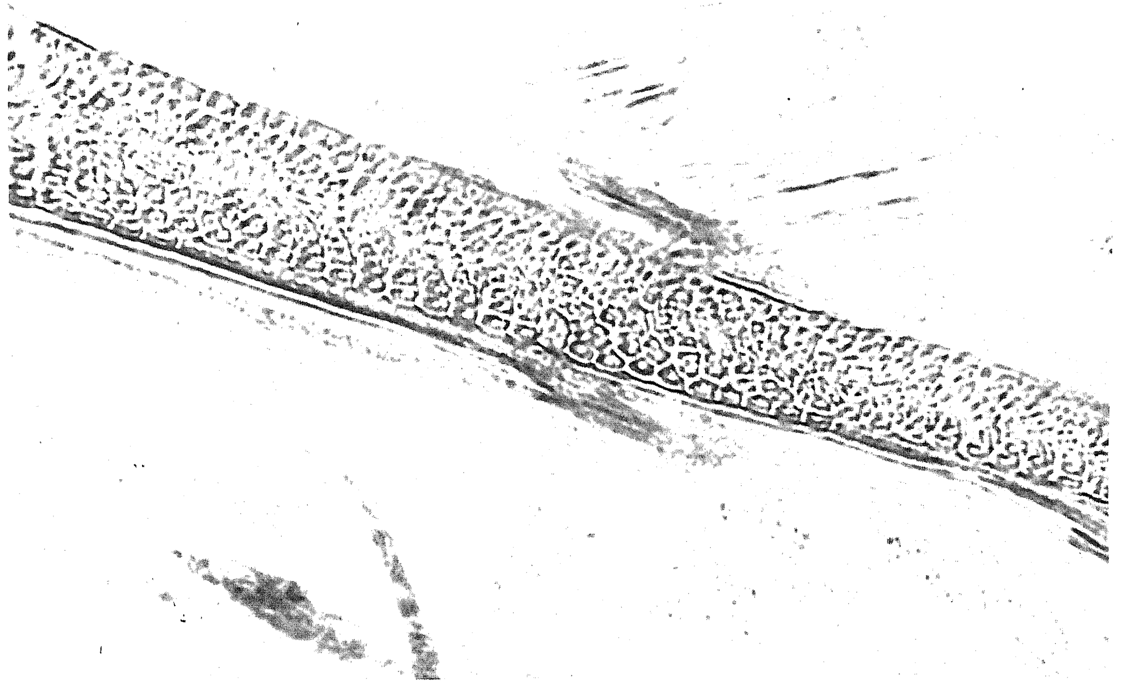
The plates on pages I67 and I68 are of transverse sections through the late Transition region of the proboscis showing setae filling the lumen.



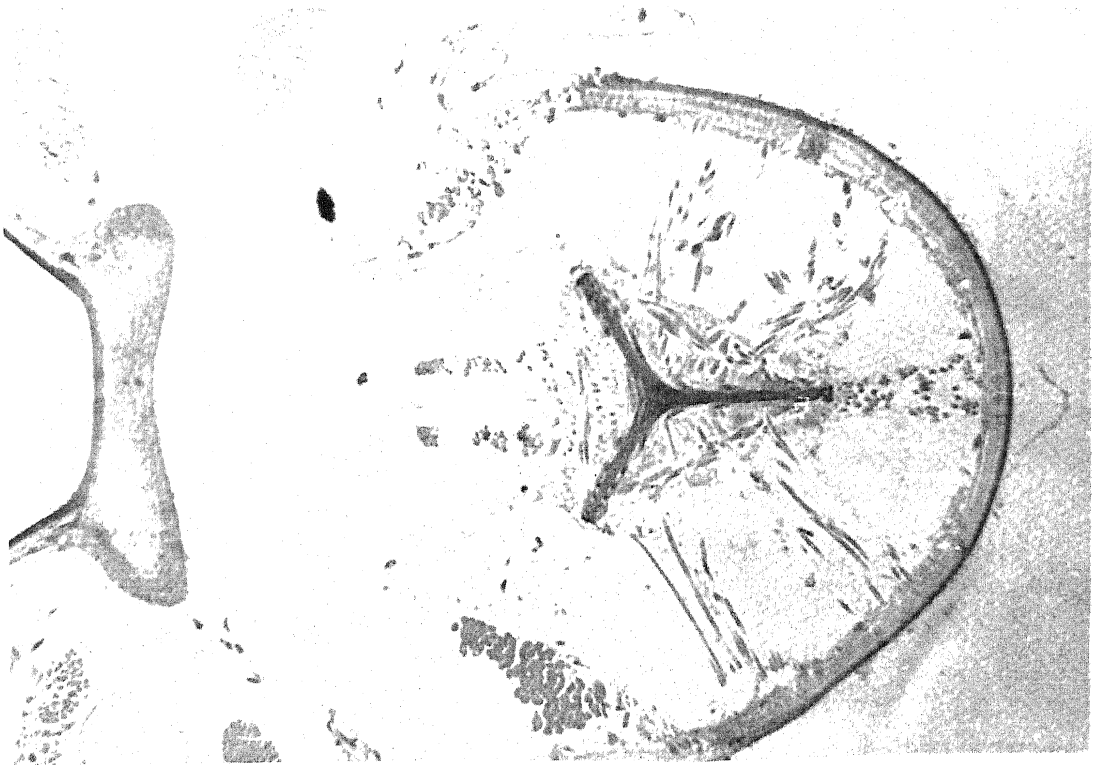
≙ 100 µm




≙ 10 µm



⊢⊣ ≏ 5 μm



⊢⊣ ≏ 200 μm

## 4.3. THE MIDGUT

The histology of the pycnogonid midgut is not as straightforward as preliminary examination might suggest. Its wall is not a simple epithelium, but a stratified one (Ham 1965), although with no arrangement into discrete layers. Historically, the number of cell types found in this epithelium has varied. In the currently most quoted works, those of Schlottke (1933 or Helfer and Schlottke 1935), three cell types are described :- EMBRYO CELLS (EMBRYOZELLEN), GLAND CELLS (DRUZENZELLEN) and ABSORPTION CELLS (RESORPTIONZELLEN). These have already been referred to in Chapter 3.

Sanchez (1959) found that in protonymphon larvae only two types of cell could be distinguished in the midgut:-

a) DIGESTIVE CELLS, whose cytoplasm is crammed with all sorts of vacuoles, nutritive substances and general waste in the form of pigments, and whose nucleus remains large, sometimes with several nucleoli and which is always situated at the base of the cell.

b) Much less numerous SECRETORY CELLS, which have a characteristic appearance. Their spherical nuclei contain a large nucleolus ('un magnifique nucleole') signifying intense activity. The cytoplasm is very dense and strongly acidophilic.

Dohrn (1881) also described two cell types.

I have found it difficult to distinguish between the glandular cells and the absorptive cells described by Schlottke and, like Sanchez and Dohrn, subscribe to an epithelium composed of two cell types. The following classification of the cell types found in N. orcadense and N. australe is based on morphology and does not presume function as do the classifications of Schlottke and Sanchez.

1) SMALL NON-VACUOLATED cells with a distinct nucleus. These are situated near, but not necessarily in contact with, the basement membrane. They are approximately ovoid in shape, typical measurements being 10 to 12 microns long and 5 to 6 microns wide. The nucleus stains obviously and is about 3 microns diameter. The cytoplasm also has a tendency to take up nuclear stains, although always less intensely than the nucleus. The cytoplasm, therefore, has a tendency towards basophilia. These cells never border the gut lumen. It is thought that these cells are the 'Embryo' cells of previous classifications and probably the 'Secretory' cells of Sanchez' classification (though their cytoplasm is not acidophilic).

2) LARGE VACUOLATED CELLS which in some places border the gut lumen. These cells are of variable size, but

always several times the size of the cells mentioned above. Nuclei are not often visible, but there seem to be boundaries between cells and they probably do not therefore form a syncytium. Often the region near the distal border of the cell is granular, has small vacuoles, and takes up cytoplasmic stain. Basally the vacuoles are much larger and do not take up cytoplasmic stain. This basal region has an appearance like that of miniature pancreatic acini. It appears, therefore, as a glandular region, but as it does not take up any of the stains tried in this project and gives only a very mild reaction in tests for acid or alkaline phosphatases, it is unlikely that it is glandular (see autophagic function - Chapter 6) in the sense of secreting for export. Occasionally, these large cells are only granular or only vacuolated. From examination of several thousand sections, it is concluded that these are different stages of the same cell type. It is proposed that this single type of cell constitutes both the Gland and Absorptive cells, so named by Schlottke.

## 4.3.1. THE PRESENCE OR ABSENCE OF MUSCULO-EPITHELIAL CELLS

Schlottke (1933) stated that investigations of the gut wall show that cells at the base of the epithelium have numerous intertwining branches. These form a meshwork whose fibres are fragile and resemble smooth muscle fibres. He stated that in sections they are 'not as clearly recognisable' as the musculo-epithelial cells of Hydra or Ascaris. Dohrn (1881) thought there were muscle fibres on the outside of the gut in Pallene (= Callipallene) but Schlottke considered this to be part of the leg musculature which Dohrn had misinterpreted. In Helfer and Schlottke (1935), Schlottke was more confident in his description of musculo-epithelial cells in pycnogonid midgut, stating that they could be shown in sections and that the basal ends of isolated cells had projections similar in appearance to isolated cells from hydroids.

From wax, methacrylate or frozen sections, thicker than 4 microns and stained with either Mallory's triple stain, Heidenhain's Iron Haematoxylin or by histochemical methods, usually with Mayer's haemalum as the nuclear stain and after fixation in Bouin's fluid or a formaldehyde fixative (10% formal-saline, 10% formal-calcium or 10% neutral buffered formalin), a picture of the gut wall was built up which is not dissimilar to that shown by Schlottke (Figure 4.3.A). There appears to be a fibrous meshwork near the basement membrane (Plates 4.3.B and C) and it is not unreasonable to interpret the cells in this region as

musculo-epithelial.

With material prepared by glutaraldehyde fixation, osmium tetroxide post-fixation, araldite embedding and then sectioned at 1 or 2 microns on an ultramicrotome, quite a different picture emerges.

The fibrous basal meshwork is seen to be entirely due ot the complex interdigitation of cell bases. Muscle fibres are not observed within the cells, even at the extreme limits of resolution of the light microscope. However, there is a fibrous, possibly muscular, region within the basement membrane (Plates 4.3.D and E). These fibres are not present in all regions of the midgut. Plate 4.2.E is of a region where the gut is freely suspended in the haemocoel. Where gut has leg muscle closely applied to it (Plates 4.3.F and G), the fibres are absent. This suggests that an antagonistic relationship could exist between leg muscle and the basement fibres (Figure 4.3.H), but further thin sections are required to be certain of this distribution and to be able to support such a contention with confidence. No thin sections were cut in the trunk region.

As mentioned above (Chapter 3), Sanchez (1959) reported a strip of epithelium containing contractile fibres encircling the gut caeca in the second and third coxa and acting as sphincter to the gut in protonymphons. In the section on electron microscopy which follows later in this chapter, there is evidence of a cell nucleus closely applied to the basement membrane and what appears to be a muscle cell

surrounding the gut. The possibility of the hydrostatic pressure of a pycnogonid's body fluids enabling a relatively delicate muscle system to achieve movement within the gut has been considered (Chapter 3). Plate 4.3.I shows what may be a muscle cell (nervous tissue is an alternative) around the outside of the gut epithelium.

It was found (Plates 4.3.D and E) that the basement membrane occasionally follows a sine wave pattern with the troughs of the waves corresponding to cross striations of the fibres. Plate 4.3.E shows the wave flattened and the period between the striations appears to be increased. There is not at present sufficient morphological evidence to confirm that the basement fibres are muscle fibres, but the apparent increase in the period of the cross striations indicates that the fibres, if not muscle, may be of an elastic nature. The variable period and the change in amplitude of the sine wave membrane suggests a mechanism by which the circumference and thus the diameter of the gut can be increased or decreased by the membrane stretching (low amplitude wave) or relaxing (high amplitude wave) concertina fashion as the fibres change their length (Figure 4.3.J). Interestingly, a somewhat similar sine wave structure is mentioned in Schlottke (1933 - see Figure 4.3.K) which shows the distal end of a gut caecum where the lumen is absent and the gut tapers to a single cell, the whole being surrounded by a sine wave membrane.

In isolated cells (Plates 4.3.L and M) the projections reported in Helfer and Schlottke have not been observed, most cells seeming to round themselves off after a period in vitro. Whatever the mechanisms that bring about gut movement (see Chapter 3), it appears that in the Nymphonidae a musculo-epithelial role is not applicable to midgut epithelial cells.

In plates 4.3.B to 4.3.G. H = Haemocoel

Gu = Gut epithelium

F = 'Fibres' discussed in text

all these plates were taken with the phase contrast microscope.

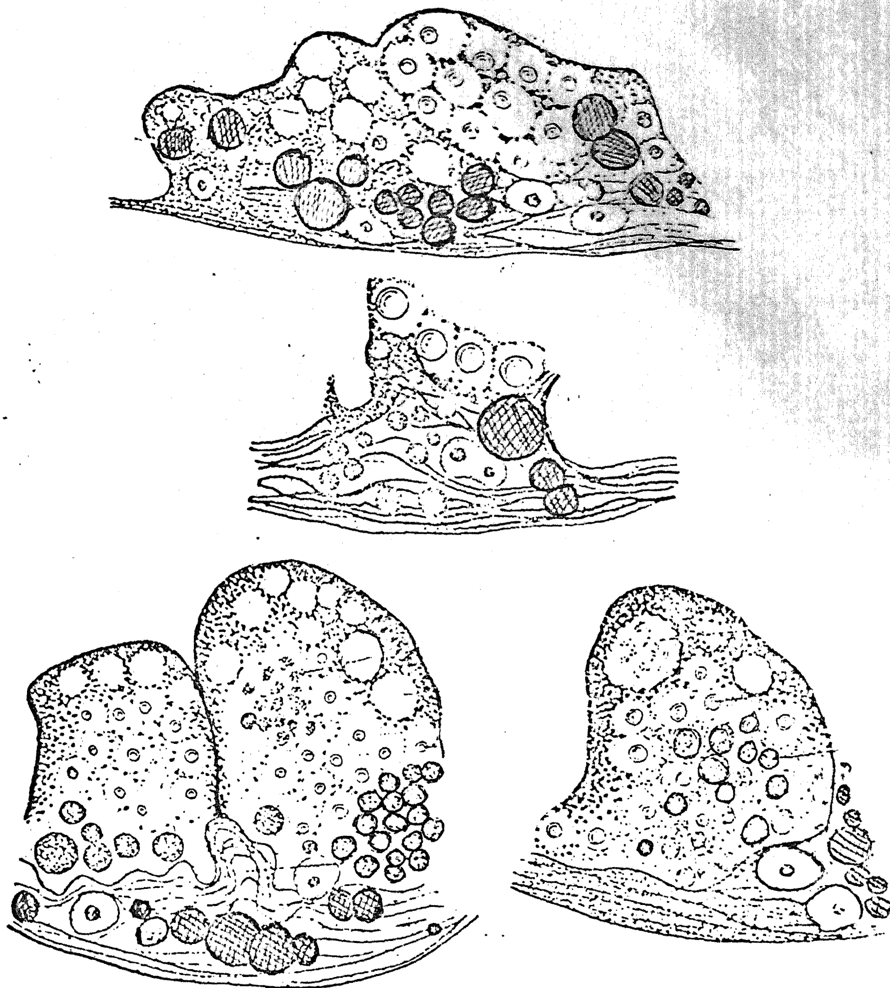


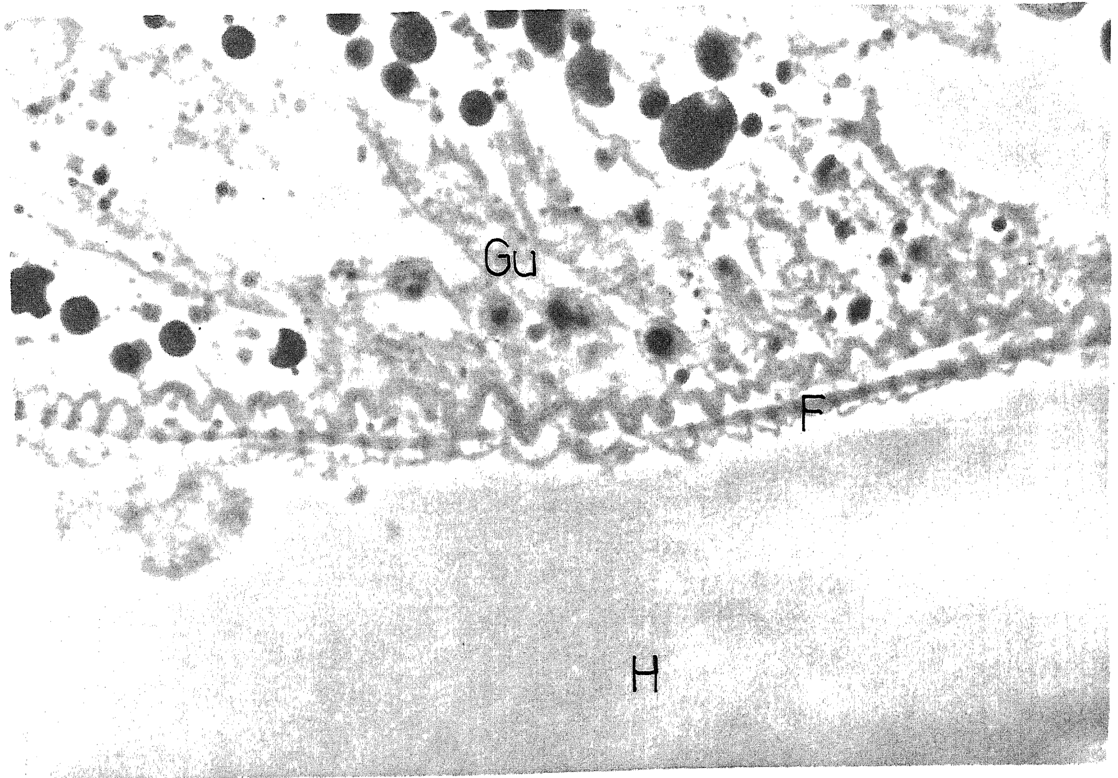
FIGURE 4.3.A From Schlottke I933, showing the fibrous network at the base of the midgut epithelium

PLATE 4.3. B.

⊢⊣ ≏ 15 μm

⊢⊣ ≏ 4 μm.

PLATE 4.3.C.




⊢ ≃ 4µm

PLATE 4.3.D.

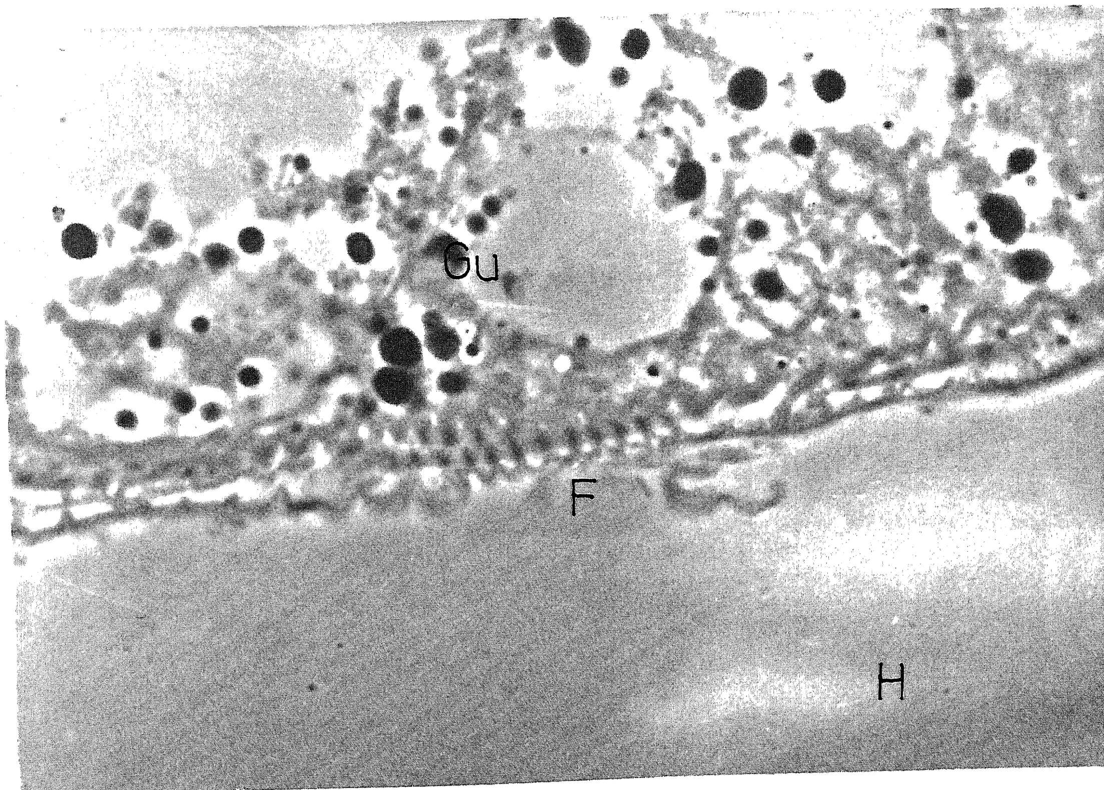



PLATE 4.3.E.

⊢—⊣ ≏ 4 μm

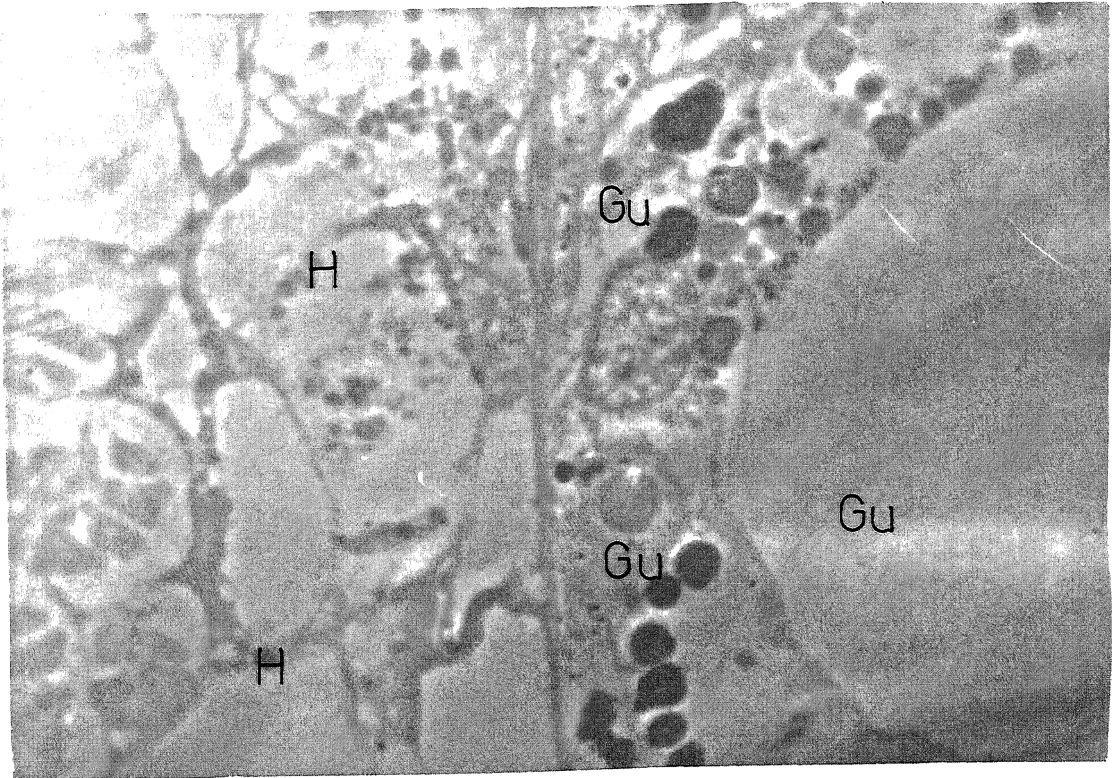



PLATE 4.3.F.

⊢—⊣ ≃ 4 μm

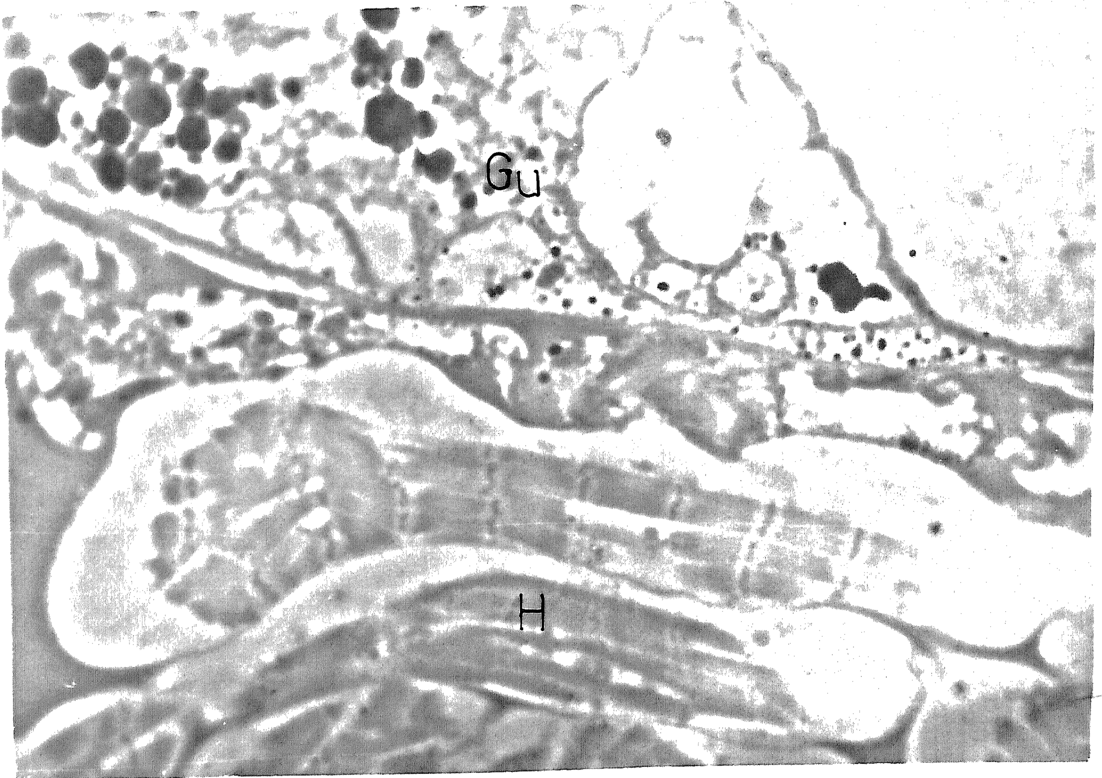
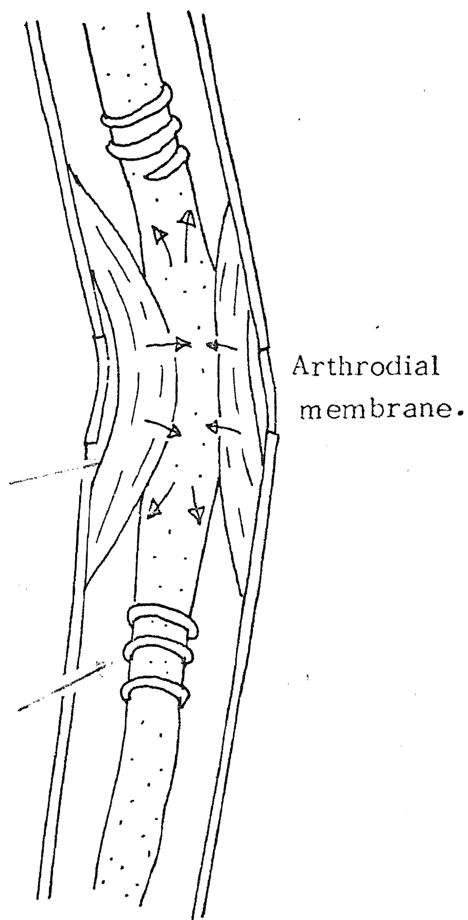



PLATE 4.3.G. ⌊—⌋ ≃ 4 μm

FIGURE 4 .3 . H.

Diagrammatic representation of antagonism between leg muscle and gut muscle.



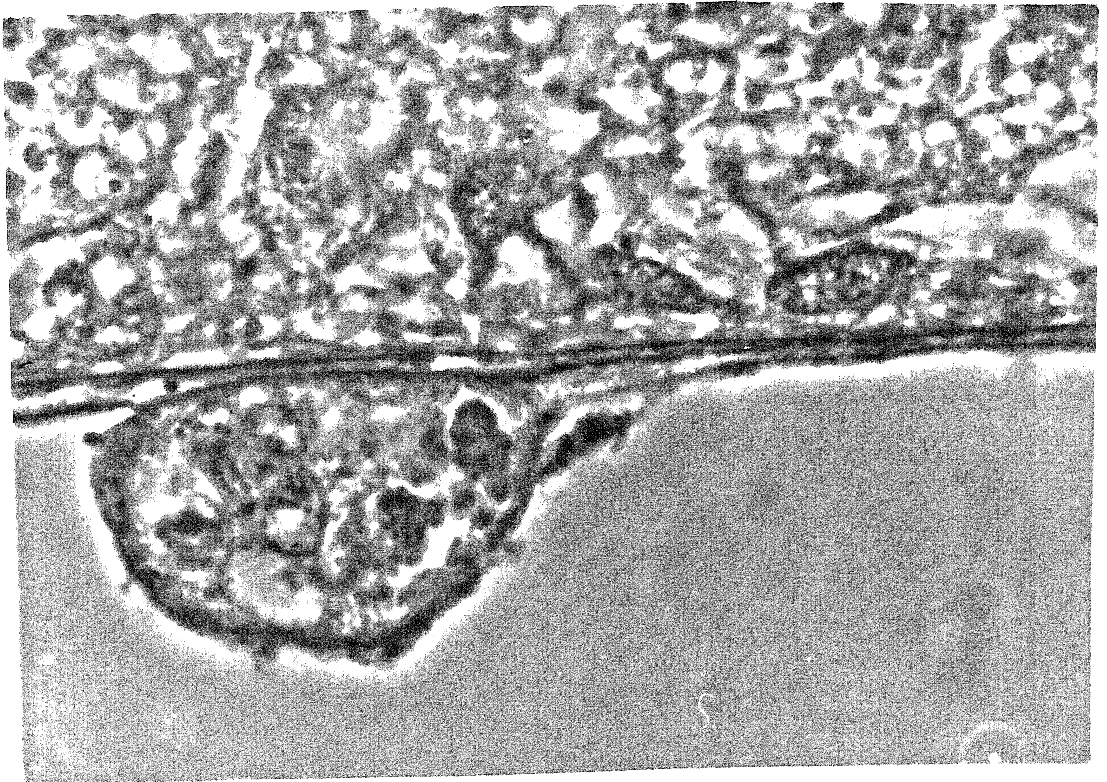
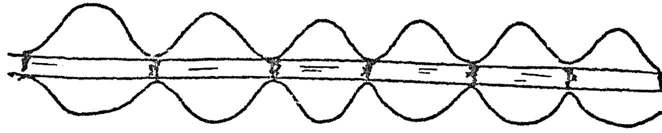


PLATE 4.3.i 'Nerve'or'Muscle' cell attached to the outside of the midgut epithelium.

⟼ ≏ 4µm

FIGURE 4 J.

High amplitude wave (constricted gut ?)



Low amplitude ( expanded gut ?)

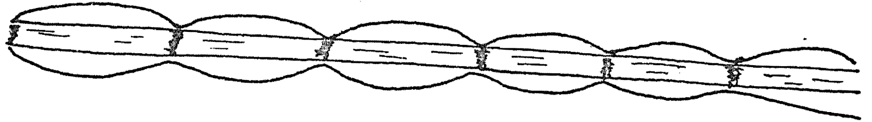
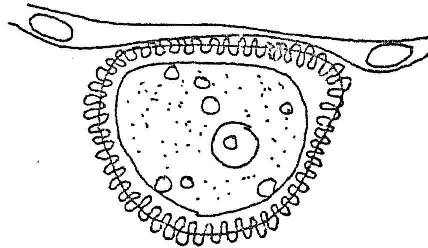


FIGURE 4 K.

Distal end of the gut diverticulum of *Nymphon rubrum* ( Redrawn from Schlottke 1933 p.637).



PLATES 4 L. and 4 M.    Midgut cells in vitro (4K = phase contrast)

⊔ ≃ 6μm.

## 4.4. THE HINDGUT

The midgut opens into the hindgut via a tripartite valve which has the same overall structure as that between foregut and midgut but differs in cellular detail. Like the pharynx, the hindgut proper is of triangular cross-section. The appearance of the hindgut epithelium is very different from that of the midgut, being thinner, and to a certain extent lacking in cellular structure visible with light microscopy (cells are very difficult to identify). It is not vacuolar. It would appear that most work on the hindgut has concentrated on the structure of the midgut/hindgut valve. Schlottke (1933) stated that the valve consists of tall glandular cells containing many protein and fat globules. He ascribed the function of the last line of absorption in the gut to these cells.

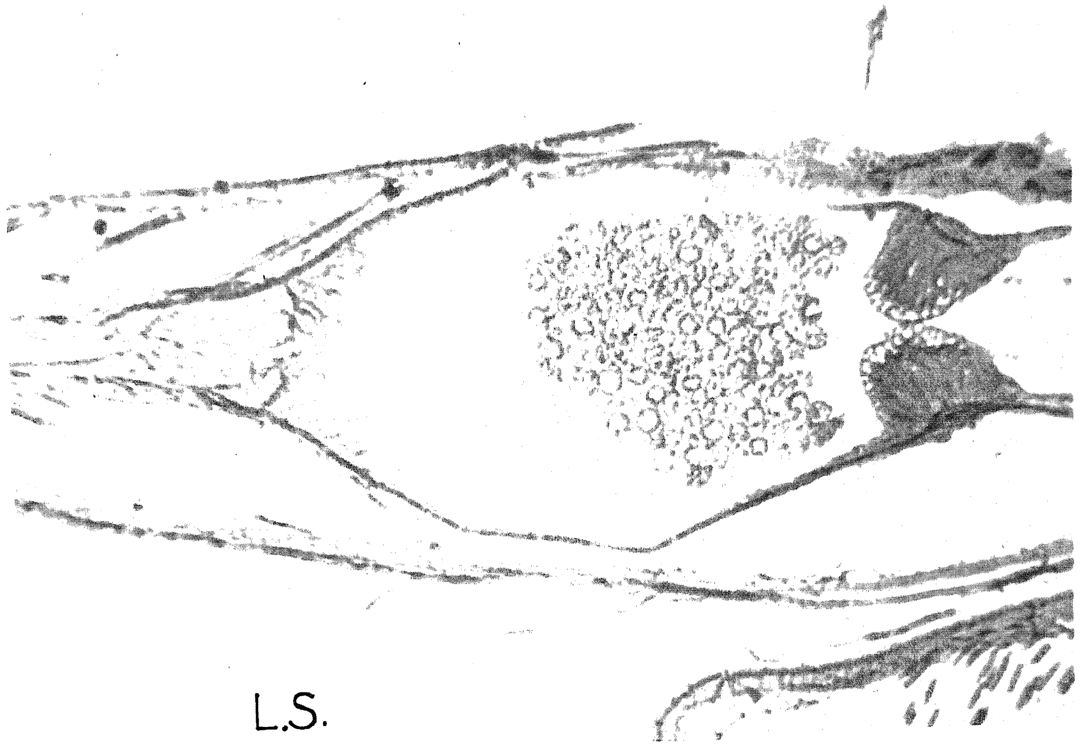
I find that their structure is different from the midgut epithelium cells, their vacuoles appear empty and are totally distal in distribution. They bear no resemblance to the other pycnogonid absorption cells.

Further histological detail of the pycnogonid gut is found in the section on electron microscopy and in the chapter on histochemical analysis (Chapter 5). What remains of this section is a photographic essay in the midgut and its structures. Table 4.3.0 gives an indication of the proportion of cell types in the midgut. Some of the interpretative difficulties are considered in Appendix 2 (Three dimensional recon-

struction), which has been placed elsewhere to aid continuity.

T.S.

⊢ ≃ 100 µm.



L.S.

TABLE 4.3.0. Giving an indication of the proportion of cell types in the pycnogonid midgut (*Nymphon australe* ).

| SECTION NUMBER | EMBRYO | ABSORPTION | 'GLANDULAR' |
|---|---|---|---|
| Na/5/30/I | 30 | 60 | |
| Na/5/3I/I | 49 | 98 | |
| Na/5/49/I | 344 | 678 | |
| Na/35/22/9 | 45 | 85 | |
| Na/35/47/3 | 23 | 6I | |

Compilation of the above table started when decent sections were first obtained. It soon became evident that it was impossible to distinguish Absorption cells from Gland cells with any confidence. Several thousand cells were counted, but as it was calculated that an average adult had in excess of one million midgut cells, and an analysis of comparative numbers of cells in animals in different nutritional states was abandoned. Such an approach might prove valuable for a future worker with a more specific brief.

## 4.5. ELECTRON MICROSCOPY

In July 1974 some Transmission Electron Microscopy (T.E.M.) was carried out in the Zoology Department of Oxford University on material brought back alive from the Antarctic. In January 1976, movement to a new post made a Scanning Electron Microscope (S.E.M.) available.

### 4.5.1. TRANSMISSION ELECTRON MICROSCOPY

Specimens were fixed in cacodylate buffered gluteraldehyde, post-fixed in osmium tetroxide and embedded in araldite (see Appendix 1). Sections were cut on an LKB ultramicrotome, stained with uranyl acetate/lead citrate and viewed on a Hitachi HU 11ES transmission electron microscope. Thicker 1 or 2 micron sections were transferred to glass slides for examination with the phase contrast light microscope.

As is explained in Chapter 5 (p.238), a mechanism exists which causes the cut end of the midgut lumen to close up when a leg is severed. The 1 or 2 micron araldite sections for light microscopy showed the lumen to be occluded (presumably by the above process) and it was not possible therefore to be certain that the thinner sections were not of regions where the gut had not been similarly occluded. It cannot be definitely stated that any of the electron micrographs in this chapter show the lumen/midgut epithelium border.

The most striking feature of the electron microscopy of the pycnogonid midgut is the vacuolar appearance of the tissue. This is shown in Plates 4.5.B and C. The second characteristic feature is the occurrence of bodies shown in Plates 4.5.C, D and E. These were originally termed 'ENIGMOSOMES' but are now thought to be the Residual Bodies or Formed Bodies described in Chapter 6. They also probably constitute a high percentage of the Spherical Bodies (see lipids in Chapter 5) and may be the same as 'Concretion Bodies' reported elsewhere in the literature. The appearance of enigmosomes varies. Sometimes thay consist of concentric rings of electron dense and electron transparent material as in Plates 4.5.D i, ii and 4.5.E i. In other cases the electron opaque and transparent regions are not arranged concentrically and their structure appears more complicated (Plate 4.5.Eii). Enigmosomes are usually found in vacuoles (Plates 4.5.D ii and E) but can occur in the cytoplasm (Plates 4.5.C and D i). Plate 4.5.H is a photograph of a structure which resembles the concentric ringed enigmosome and is taken from Threadgold (1967). Threadgold labels these 'mineralised granules from the Malphigian tubules of Rhodnius found by Wigglesworth and Perry.' He reports similar structures found in the calciferous glands of earthworms. Photographs of similar structures from insect Malphigian tubules due to Wigglesworth and Gupta are reproduced in Smith (1969). In Chapter 6, it is mentioned how Riegel (1966b) drew attention to the work of Wigglesworth and Salpeter (1962) and con-

cluded that his 'formed bodies' are their 'crystallization nuclei'. It is proposed that enigmosomes, formed bodies, crystallization nuclei, mineralised granules and concretion bodies are closely related, if not the same structure in pycnogonid midgut.* (see footnote 1).

It is difficult, if not intellectually dangerous, to ascribe function solely on morphological grounds. The conclusions drawn from the plates presented here are made with the plea that more sections using different fixation techniques as well as parallel electron microscope cytochemistry are required to be certain of their correctness. Much has had to depend upon the somewhat dubious technique of searching the literature for pictures of similar structures (although this seems to be a technique not too frowned upon by electron microscopists). Thus, Plate 4.5.G ii shows an almost classic textbook arrangement of a golgi apparatus with an adjacent lysosome. This is almost perfectly circular, very electron dense and contains a myelin figure. It is likely that throughout these plates the very electron dense bodies are lysosomes. The less electron dense, similarly shaped bodies which show a certain degree of 'chatter' are probably lipid droplets. Plate 4.5.A contains a vacuole which has the classic features of an autophagic vacuole. It is assumed, however, that many other vacuoles in the plates shown are autophagic.

A lack of experience in carrying out electron microscopy on a

variety of animal tissues leads to a certain amount of reflection on the general 'emptiness' of the pycnogonid midgut tissue. Plate 4.5.A in the muscle region, a region in Plate 4.5.C (indicated), Plate 4.5.D i and Plate 4.5.G i show dense cytoplasm. Elsewhere, however, the compact cytoplasm is missing. In Plate 4.5.G ii for example, the golgi apparatus is found in a cell which seems devoid of a dense matrix. The appearance of mitochondria is apparently one criterion the electron microscopist uses to test the adequacy of fixation. Although mitochondria are not numerous, they appear to be properly fixed.*(see footnote) The inculcated heresy mentioned elsewhere provokes the thesis that published electron micrographs are naturally an author's best electron micrographs and therefore atypical. They show areas with densely packed cytoplasm (generally more visually interesting) such as those mentioned above, rather than showing the whole tissue which may have many spaces.

To the statement of vacuolar appearance of pycnogonid midgut cells is added the rider that the frame of reference for comparison purposes is uncertain. Pitt (1975) for example stated that liver mitochondria have a life of about 20 days. Calculations show that if one autophagic vacuole enclosing a single mitochondrion were formed every 15 minutes per liver cell, this could account for the observed rate of mitochondrial turnover. Autophagic vacuoles are not necessarily rare, therefore, when considered over a period. The plates are described individually

as follows:-

PLATE 4.5.A

Plate A consists of two plates joined together which cover a region at the base of the midgut epithelium. The basement membrane is visible as a vertical division of the photograph with leg musculature to its left, gut cells to its right. There are no detectable muscle fibres in the gut cells, and this also applies to Plate 4.5.B.

This contradicts Schlottke's claim of the existence of gut musculo-epithelial cells. None of the midgut cells in any of the electron microscope sections examined possessed muscle fibres (see also section 4.2.). However, in a number of places along the basement membrane it can be seen that there are fibres. In Plate 4.5.B is what appears to be a cell nucleus in the basement membrane region. It is suggested that these features may indicate muscle cells outside the midgut epithelium but closely associated with the basement membrane.

PLATE 4.5.B

As already indicated, Plate B contains a region of basement membrane, a distinct autophagic vacuole and an enigmosome. Close to the autophagic vacuole are lysosomes and a region rich in rough endoplasmic reticulum.

PLATE 4.5.C

Plate C, like the previous two plates is a combination of plates and shows some of the vacuolar complexity of the midgut epithelium. Lysosomes and lipid droplets can be identified, as can rough endoplasmic reticulum. It is assumed that many of the vacuoles are either autophagic or a combination of autophagic and heterophagic vacuoles (ambilysosomes). It is difficult to identify nuclei, a problem already noted with light microscopy. This might indicate a syncytial state of the epithelium or if, as indicated in Chapter 6, there is a general autophagy of absorption cells, the nucleus itself may be reabsorbed in one of these vacuoles.

PLATES 4.5.D and E

Plate 4.5.D shows enigmosomes a) in the cytoplasm, b) within a vacuole. These and the enigmosomes shown in Plate E i are of the alternate electron dense, electron transparent concentric ring form, whilst those shown in E ii are of the more complex form.

PLATE 4.5.F

Plate F i shows two vacuoles, one of which contains material in the process of breakdown. This is shown at a greater magnification in Plate F ii with what appears to be a lysosome about to fuse with a

vacuole. This could be morphological evidence of a primary lysosome increasing the enzyme content of a secondary lysosome (see Chapter 6).

The membrane configurations of Plates F i and ii are of considerable and perplexing interest. The complex does not appearto be of rough endoplasmic reticulum but of unit membrane. Plates F iii and F iv show a similar phenomenon but without the same amount of membrane multiplication. Threadgold (1967 p.109) showed similar structures which he stated represent the process of 'Cytopemphis' or 'Pododcytosis', a form of micropinocytosis. In this, vacuoles cross the cytoplasm intact and are discharged at another cell surface without benefiting the cytoplasm. This process is apparently a particular feature of endothelial cells (Figure 4.5.I). It is difficult to imagine what the pycnogonid gains from such a mechanism. It may be that the morphological resemblance to manifestations of the above process is coincidental.

PLATE 4.5.G

Plate 4.5.G shows two parts of cells which are rich in Golgi figures and appear to be synthesizing. It is probable that both correspond to embryo cells or at least parts of this type of cell.

Footnote 1 After this section had been written, the author attended a meeting of the Royal Microscopical Society (September 15th, 1976) and noted in a paper by George, Pirie, Nott and Mason, a picture of an

'enigmosome' (called an 'inclusion granule' by the authors). Discussion with the authors revealed that they were finishing a review article on the occurrence of these inclusion granules in marine invertebrates. They believe the role of these granules to be particularly important in the excretion of metal ions.

Footnote 2 At the same meeting (above) Millonig delivered a paper which indicated that glutaraldehyde does not fix watery tissues very well. He recommended stronger concentrations and fixation at room (not cooled) temperature.

PLATE 4.5.A. ≏ 4 μm

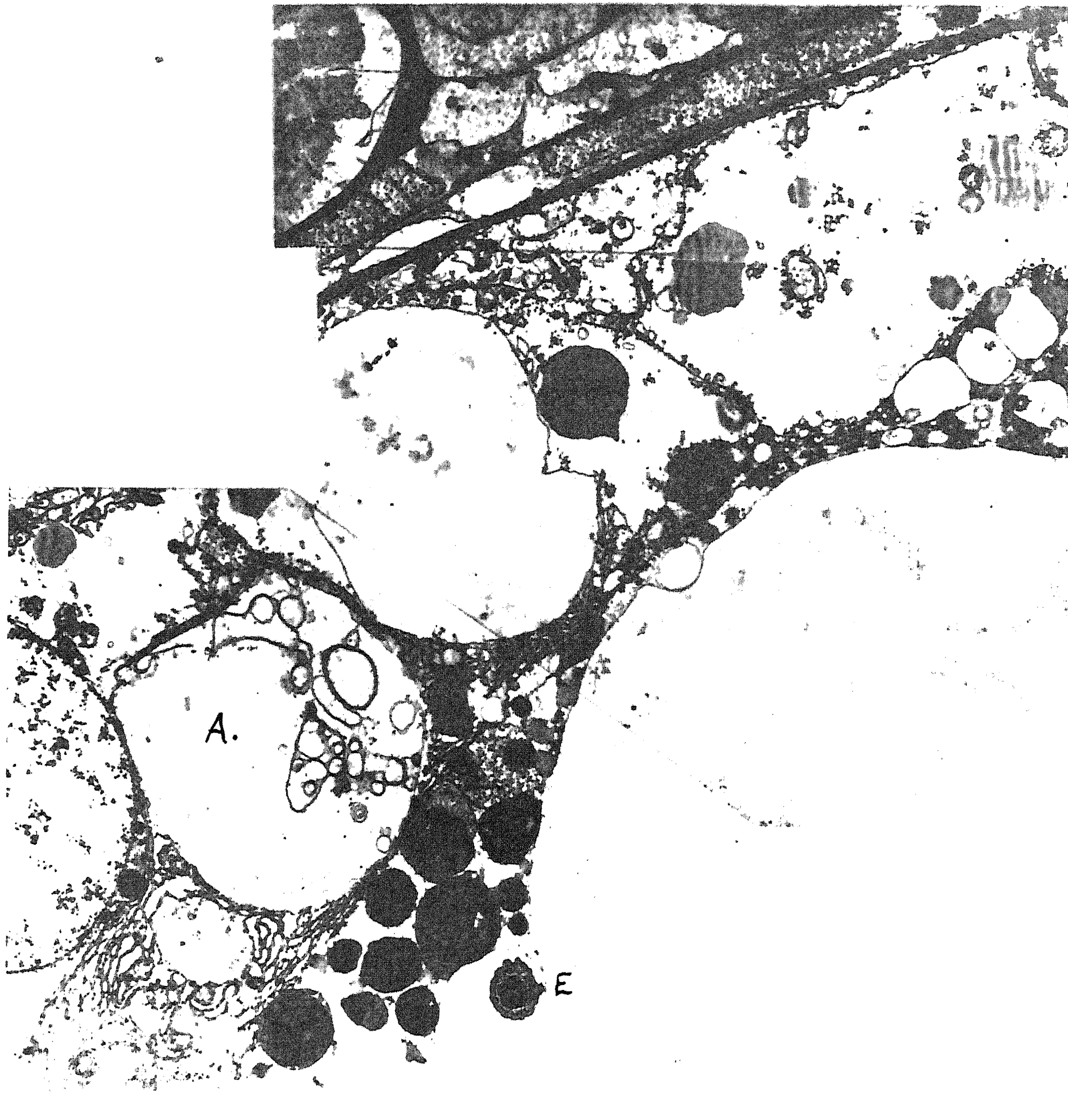



PLATE 4.5.B. ——— ≃ 6μm

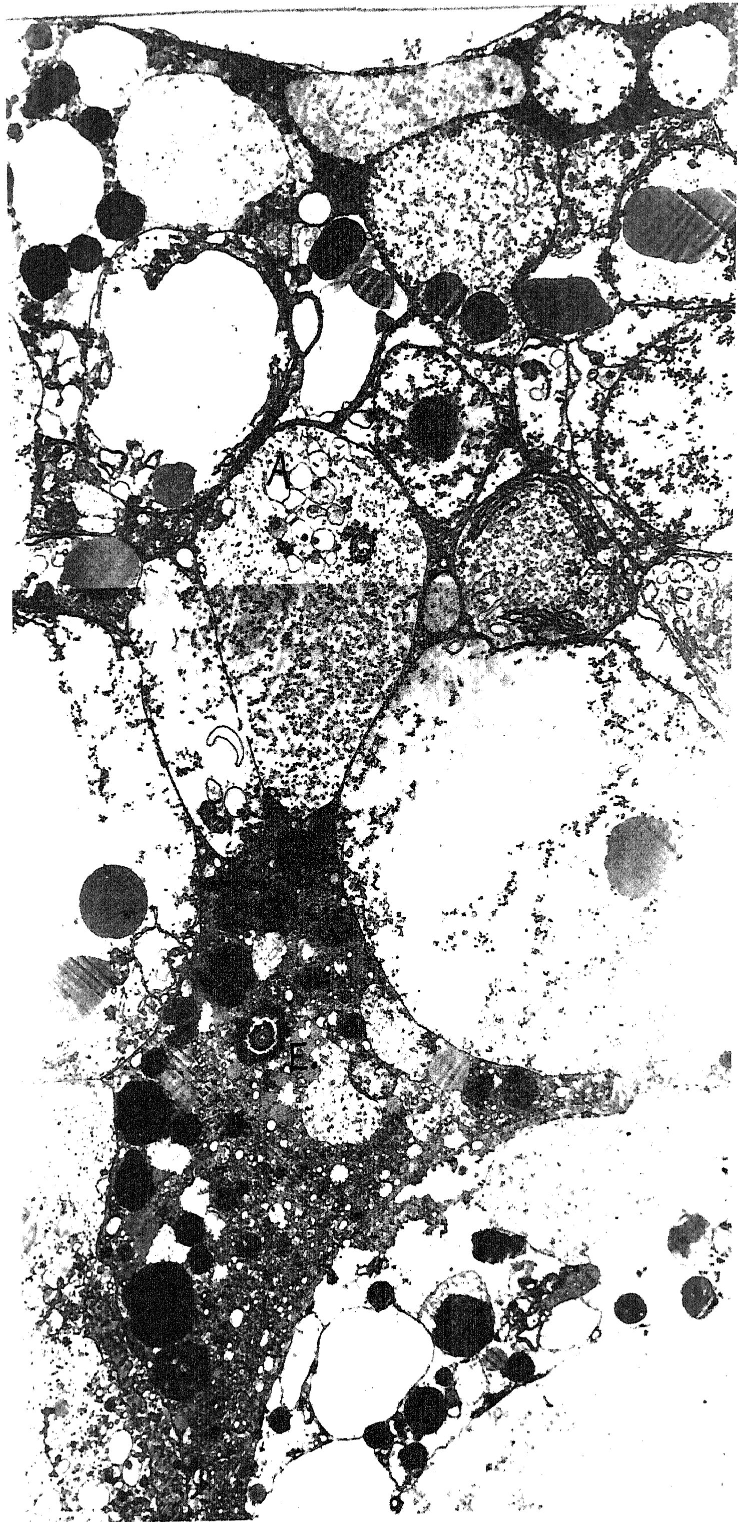
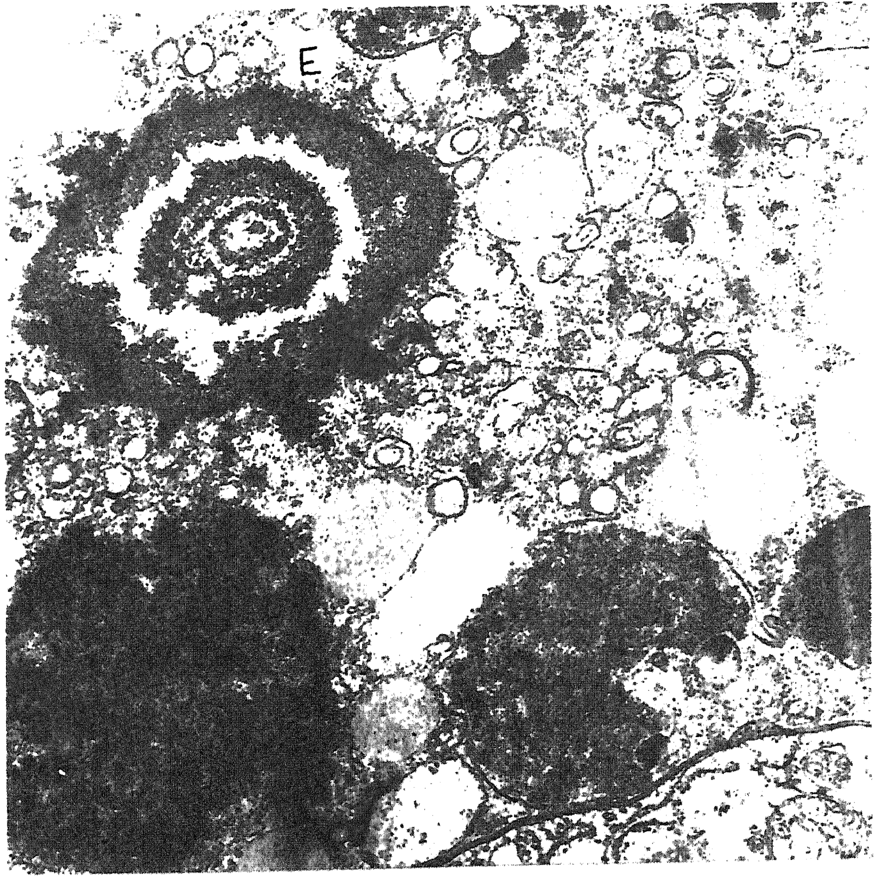



PLATE 4.5.C.

≏ 6μm.

i)




= 1μm

ii)

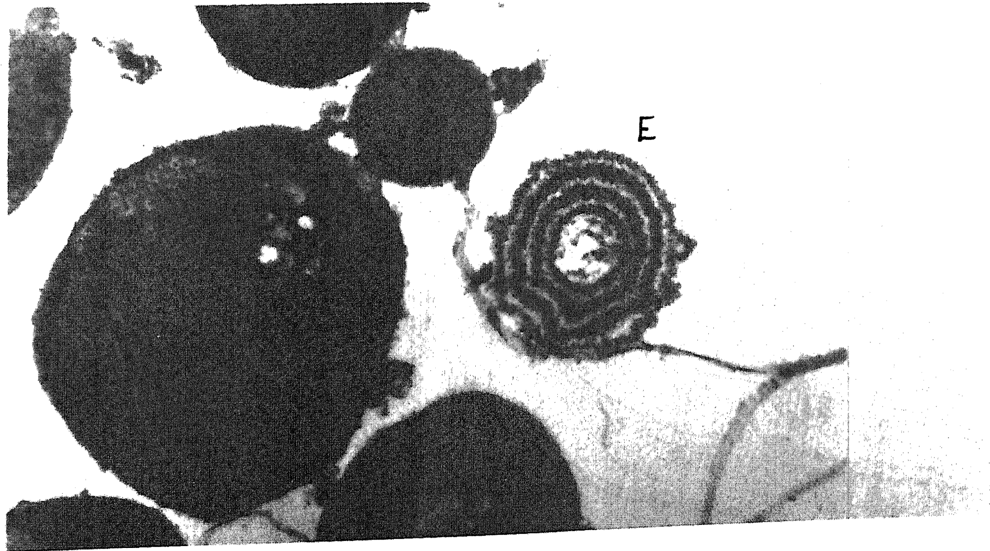
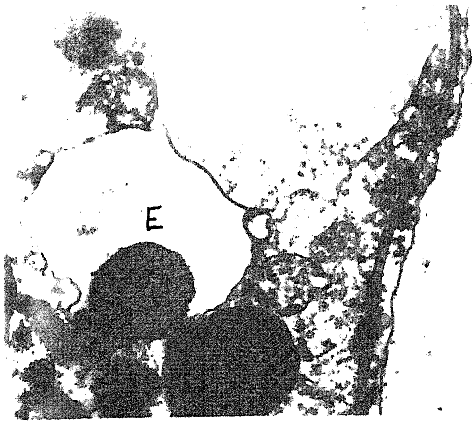



PLATE 4.5.D.

i)




≏ 2μm.

ii)

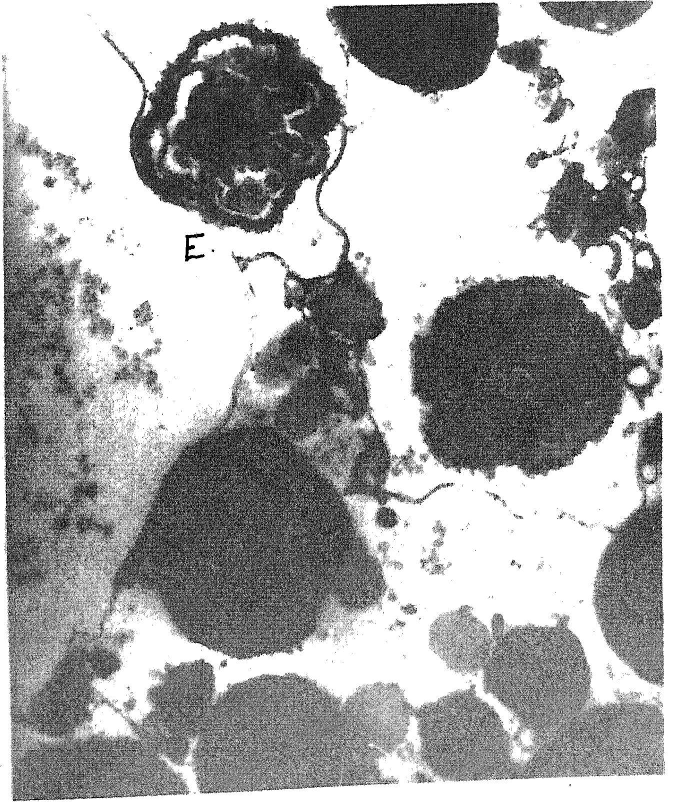
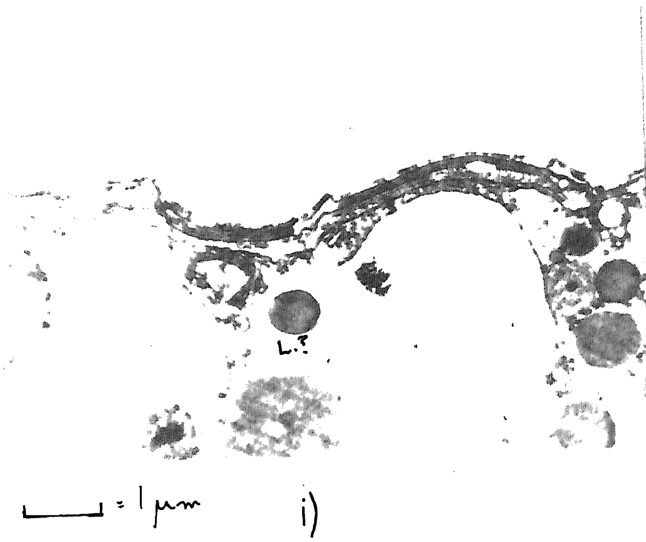
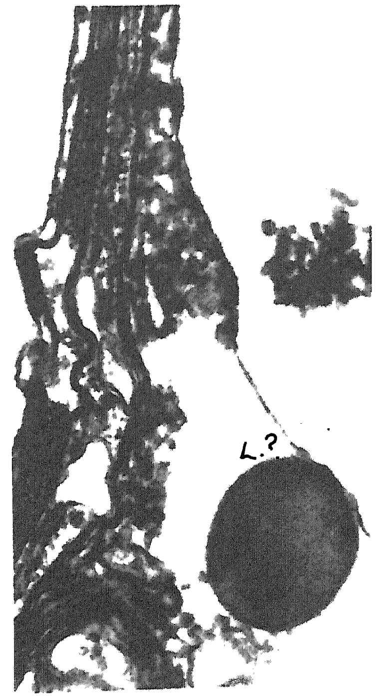



PLATE 4.5.E.

≏ 1μm

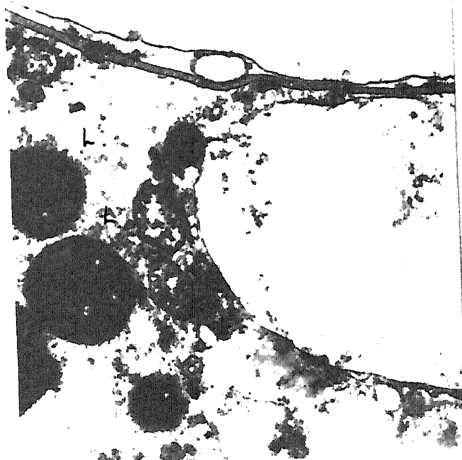



i)

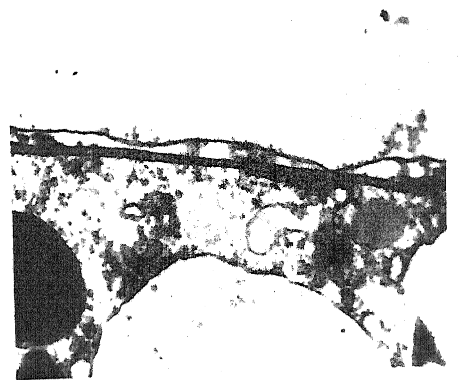



ii)

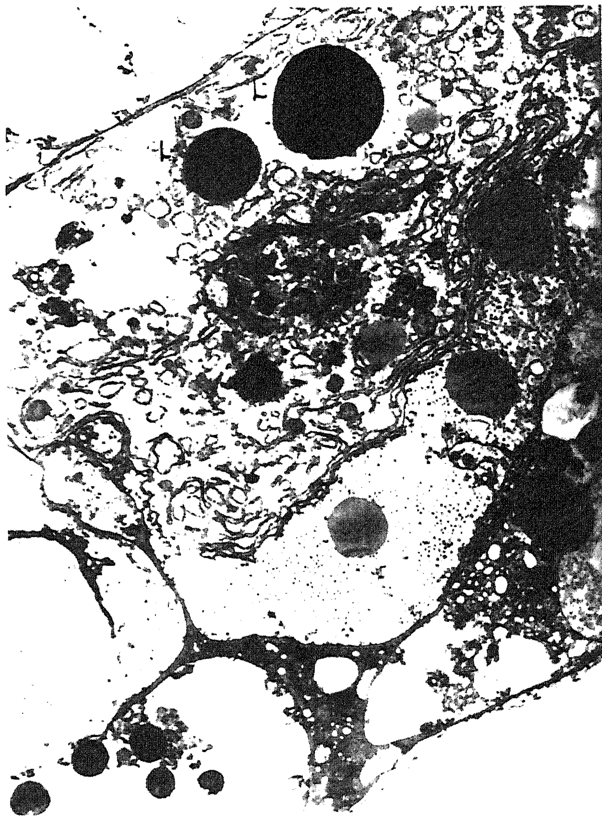
PLATE 4.5.F.




iii)



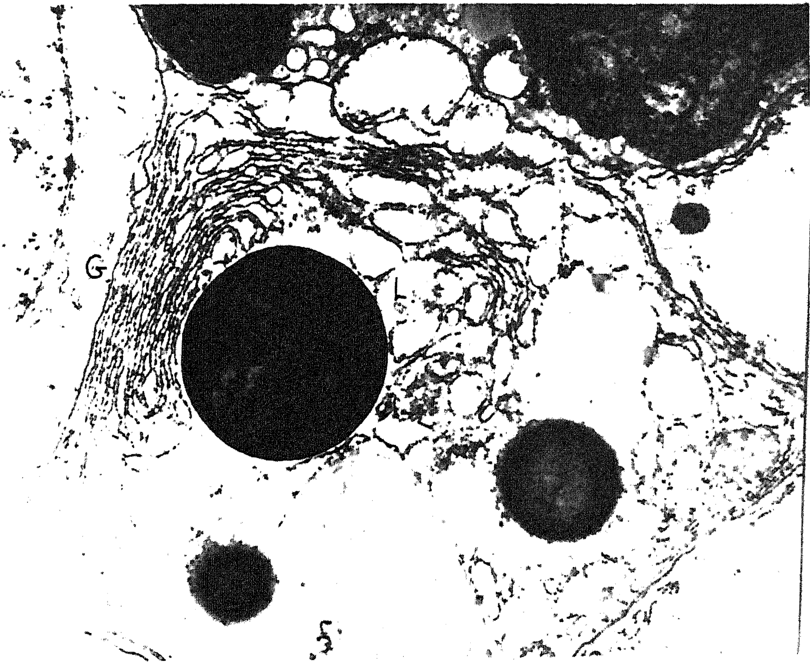

iv)




≃ 2μm.

i)

ii)




≃ 2μm

PLATE 4.5.G.

## 4.5.2. SCANNING ELECTRON MICROSCOPY

The scanning electron micrographs shown in Plates 4.5.H to V will not be discussed in the same detail as the transmission electron micrographs as their interpretation is perhaps less difficult. It is hoped that sufficient information is given in the legends. It would appear that the technique has vast possibilities in digestion studies. In addition, adequate S.E.M. preparations are much more easily obtained than for the transmission electron microscope or the light microscope.

In general, it can be seen that the surface of the hindgut (Plates H to J) is very different from that of the midgut (all other plates). There is also a difference between the surface of the gut of an animal fixed in the process of feeding (Plates 4.5.O to T) and an animal which had been starved for some time (Plates 4.5. K to N). It must be stated, however, that different drying methods were used on each of these specimens. A standardised technique is required before more can be read into these results.

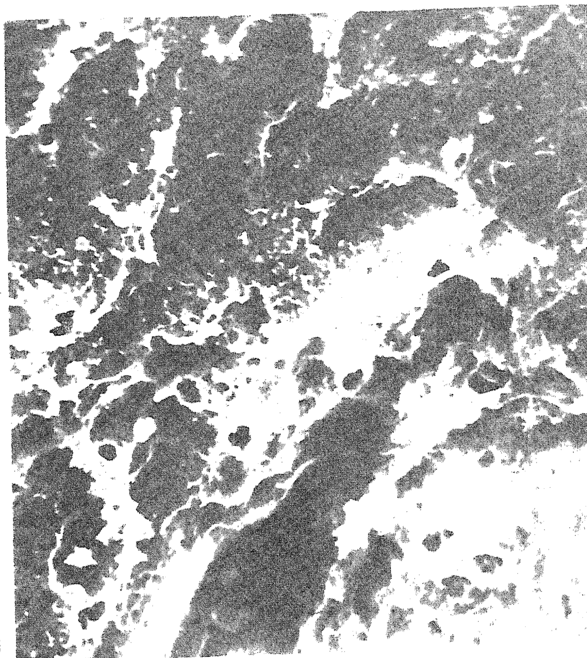
No pseudopodia were seen arising from the midgut cells, and the surfaces of the cells appear relatively smooth. Only a few cells have what appear to be 'ruffle' membranes which are associated with phagocytosis or pinocytosis. These cells have similarities in appearance to macrophages (Carr, Clarke and Salsbury 1968). However, a cautionary

note is suggested as far as technique affecting interpretation is concerned. For example, Alexander, Sanders and Braylan (1976) have shown that the purported difference between Human lymphocyte surface morphology is an artefact of fixation (atypical smooth surfaces are produced in some types).

Plate 4.4.S shows what appears to be a cell in the process of breakdown releasing spherical objects into the lumen. It is thought that this probably corresponds to enigmosome/spherical body release shown elsewhere by light microscopy (see Plate 4.3.C.). These bodies appear like the exotically named 'Zeiotic blebs' (see eg, ROSE, G.G. (1976) ) and are shown free on the epithelium in Plate 4.5.K and particularly in Plate 4. .J. That is, they are found in different specimens fixed and dried by different methods. This indicates that they are not artefacts.

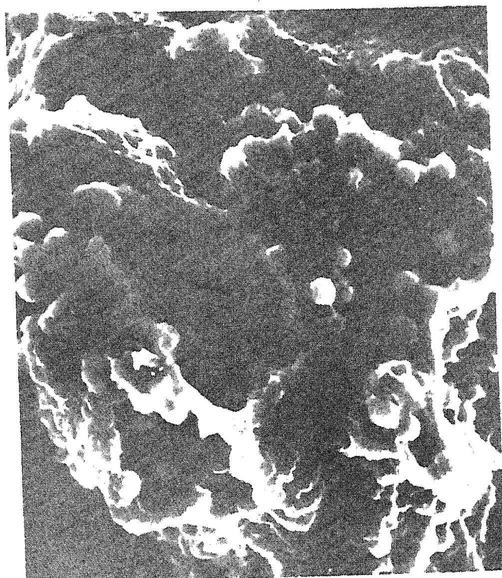
Plates 4.5. O, P, Q, U and V which show the cut surface of midgut epithelial cells indicate them to be vacuolar, supporting findings from other techniques. Plates U and V show a villus as described by Schlottke. These plates are however of a Colossendeis specimen and such large villi have not been observed in the Nymphonidae.

J.



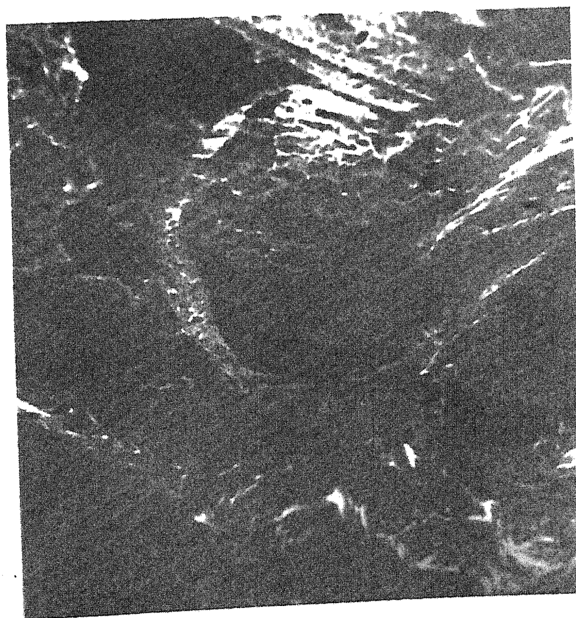
⊢⊣ ≙ 50μm

I.

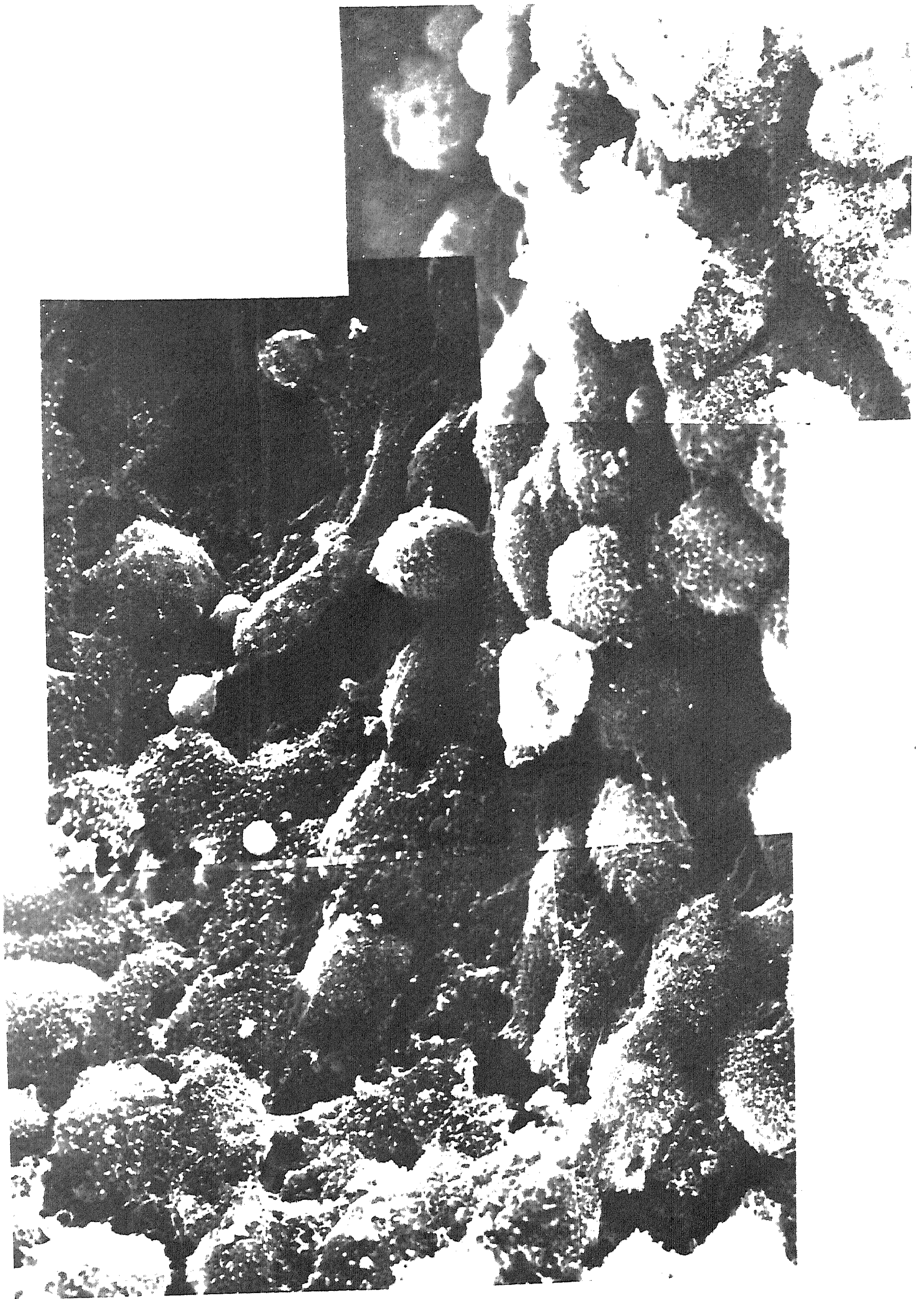


⊢⊣ ≙ 50μm

H.

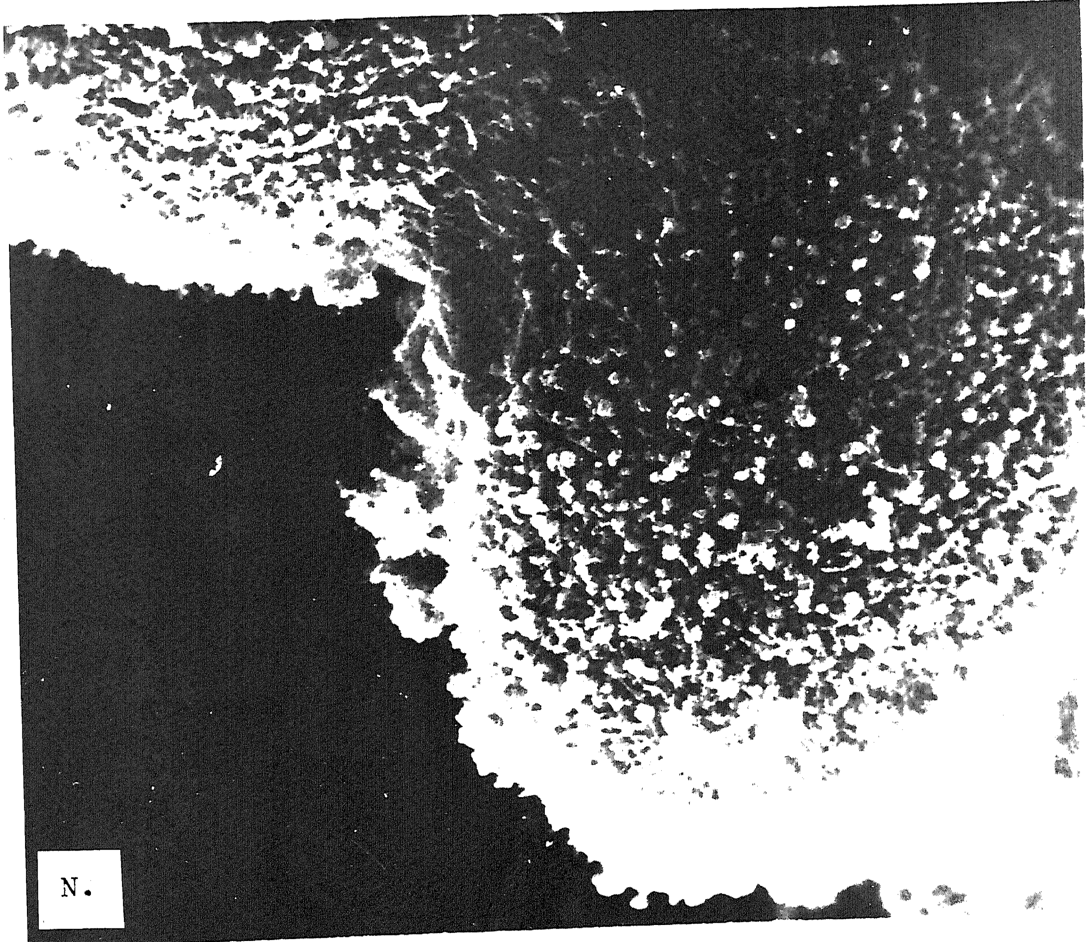


⊢⊣ 200μm.

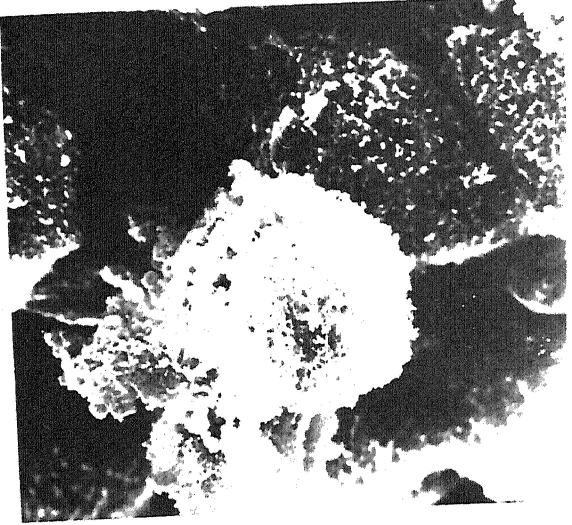


20 μm.

K.




≃ 3 μm.



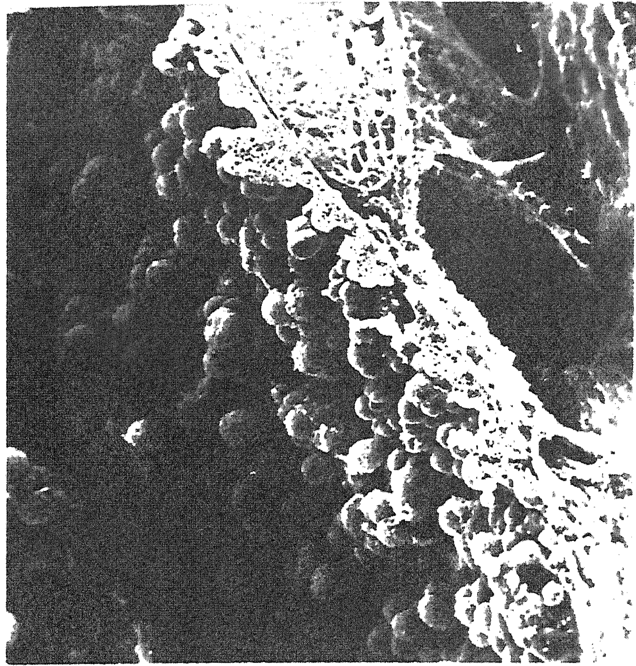
M.

≃ 10 μm

L.

≃ 15 μm.

O.

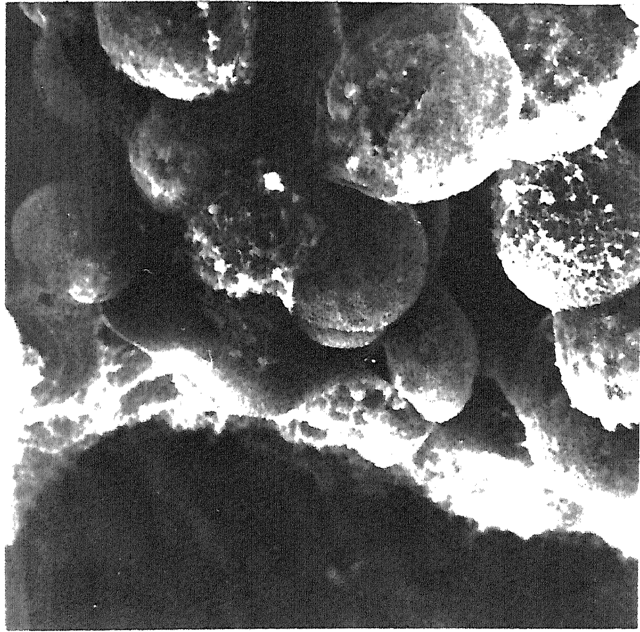


⊢ ≙ 50 μm

P.

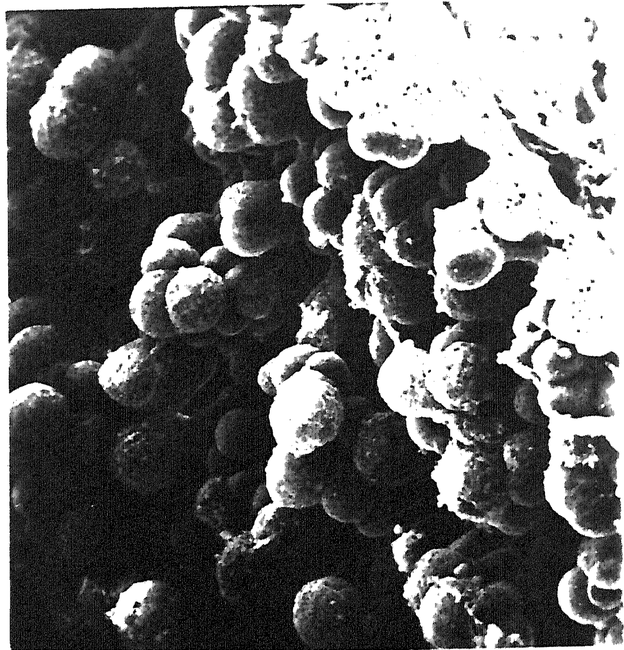
⊢ ≙ 500 μm.

R.



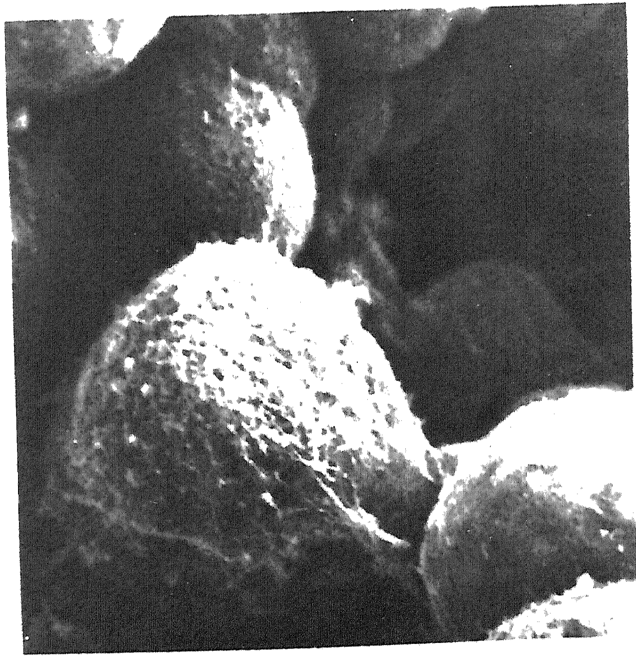
⊢—⊣ = 10 µm

Q.



⊢—⊣ = 20 µm.

S. ⊢—⊣ = 7µm.



T. ⊢—⊣ = 4µm.

# CHAPTER 5

## DIGESTION III - HISTOCHEMICAL AND BIOCHEMIAL ANALYSIS

## 5.1. ACID PHOSPHATASE

Acid phosphatase or phosphomonoesterase II (Casselman 1962) splits monoesters of orthophosphoric acid in an acidic medium (in most tissues the pH optimum is 5·0 to 5·3). Recent studies indicate that there is no single acid phosphatase enzyme as such and tests probably reveal groups of enzymes (Pearse 1968). The importance of acid phosphatase as a lysosomal marker is mentioned in Chapter 6.

Three histochemical tests were used to detect acid phosphatases, the Gomori lead nitrate technique (Carleton 1967), the standard azo-dye coupling technique (Pearse 1968) and the standard napthol AS phosphate method of Burstone (Pearse 1968). Two different methods of section preparation were used, formaldehyde fixed, cryostat frozen sections and cold acetone fixed polyethylene glycol methyl methacrylate sections (see Appendix 1). The Gomori and azo-dye techniques were used on the former sections, the Burstone technique on both types of section. The negative controls used were:- sodium fluoride present as an inhibitor in the incubation medium, sections incubated without substrate and sections which were heated prior to placing in the incubating medium Positive reactions were obtained for midgut, but not for foregut, hindgut, or any other tissue in the pycnogonid.

With Burstone's method and polyethylene glycol methyl methacrylate sections, sections cytologically superior to the frozen sections were

obtained. Unfortunately, the histochemical capabilities of this embedding medium were only discovered late in the project and a range of pycnogonids tested in different nutritive states was only obtained with the other methods in which exact enzyme localisation was difficult.

RESULTS WITH BURSTONE'S METHOD:-

In animals fixed in the process of feeding, distribution within the midgut was lumenad (Plate 5A). Under low magnification, there was a slight difference in staining intensity between anterior and posterior regions of vertical longitudinalsections through the trunk. At the cellular level, distribution appeared to be within the cytoplasm rather than in the large (3umplus) vacuoles of midgut cells (also referred to as 'Spherical bodies' - see else where), but exact localization was difficult (Plate 5B). Some cells, which appeared much like the gland cells described by Schlottke (Figure 5C), were not stained.

RESULTS WITH AZO-DYE AND GOMORI TESTS:-

Ice damage to sections made exact localization of enzymes to lumenad or basalar regions of the midgut difficult. Generally, however there appeared to be little difference between specimens of animals fixed when feeding and animals starved for up to nine days. Again it was sometimes difficult to tell whether the positive reaction was confined to cytoplasm or to the vacuoles. After examination of many sections, it was

decided that distribution was to both cytoplasm and vacuoles (the difficulty of the technique is demonstrated by the fact that there was variation in staining between adjacent sections in the same series incubated on the same slide, in the same medium, at the same time). In starved specimens, where the lumen was not occluded by the sectioning technique, vacuoles as spherical bodies appeared slightly stained.

With none of the three methods was there a detectable release of enzyme into the lumen other than that confined to 'spherical bodies'. The contrast between richly staining midgut and non-staining hindgut and foregut was marked (Plates 5D and 5E). In a specimen which had been cold acetone fixed when feeding (it was dropped with its prey into the fixative), PGMM embedded and stained by Burstone's method, positive staining was noted in the pharynx lumen, particularly in region 1, the lip region. As indicated, the foregut cells gave negative results in tests for acid phosphatase, as in the midgut lumen. This indicates that the acid phosphatase was more likely to be from the prey rather than the pycnogonid predator as it was not present in the foregut cells and it seems highly likely that regurgitation from the midgut would leave at least some evidence of the enzyme in the midgut lumen. A more sophisticated technique is required to be certain of this interpretation. A fluorescent labelled antibody technique might enable the source of the phosphatase to be distinguished.

SUMMARY:-

In summary it would appear that acid phosphatase is distributed distally in cells which adjoin the midgut lumen. Initially, distribution is cytoplasmic but as digestion proceeds it is both cytoplasmic and within vacuoles (spherical bodies). 'Cells' (see Chapter 4) which have a similar morphology to the Gland cells (Schlottke) appear to contain less acid phosphatase than other cells. Acid phosphatase does not appear to be secreted into the foregut or midgut on feeding. Later in the digestive process it may enter the midgut lumen contained in the 'spherical bodies'.

DISCUSSION:-

Wyer (1972) examined acid phosphatase in the guts of some British species using the Gomori method on frozen sections. He found distribution to be particulate and localized in the basal region of midgut cells, especially those swollen with food vacuoles. Whilst agreeing with particulate localization by the Gomori method, I have not found acid phosphatase localized in the basal region of midgut cells, but instead localized distally. I believe frozen sections cause too much damage for unequivocal localization. Wyer also stated that in cells that release enzymes into the gut lumen of insects, acid phosphatase activity is located at the distal end of cells (lumenad). He states that basal distribution means that enzymes cannot be released into the lumen, extra-

cellular digestion is therefore less likely, and phagocytosis must occur. However, Jennings (1962), working on intracellular digestion in triclad Turbellaria, found lumenad distribution of acid phosphatase. It is suggested that acid phosphatase distribution is not a particularly sound criterion for deciding whether digestion is intracellular or extracellular. Wyer also found acid phosphatase in the pharynx lumen, but not in the foregut, hindgut, nor in the midgut lumen. He suggested that extra-oral digestion occurs in pycnogonids ('as in other arachnids'). For reasons already indicated, I believe there is not enough evidence to be certain that this acid phospatase is of pycnogonid origin.

5. 2. ALKALINE PHOSPHATASE

Alkaline phosphatase was detected using the standard azo-dye coupling technique. Staining tended to be faint, and only formaldehyde fixed frozen sections were used. Localization was not specific due to the factors cited in the discussion of frozen sectioning in the previous pages. Generally it seems that distribution is similar to that for acid phosphatase although, as is mentioned in the next chapter, there may be a temporal difference both in distribution and in production related to the production of acid phosphatase.

CARBOHYDRATES

The occurence of carbohydrates did not differ significantly from that reported by Schlottke. Tests (P.A.S.) were never very vivid whether carried out on Bouin's fixed or other material. The vacuoles or granules of the midgut cells were never darkly stained and this, coupled with the failure to detect carbohydrases (see later) might indicate a less important role in pycnogonid metabolism, or at least one more subtle than that indicated by the tests used. As indicated in chapter 2, metabolism of polar animals is complex and carbohydrate utilisation a topic for much deeper study than time and conditions permitted.

(Pages 222 and 223 are omitted due to a mistake in the numbering of pages.)

## 5.3. LIPIDS

Schlottke (1933) did not carry out detailed observations on the occurrence of 'Fat', but did, however, record its presence in pycnogonid midgut. It seems that he used no other method of detection for the presence or absence of fats than black staining by the osmium tetroxide present in his fixatives. Wyer (1972), using the Sudan Black B method of McManus (1946) and the modification for masked lipids (Ackerman 1952), found no lipids in the cells of the midgut. Using the method of McManus (1946) I observed very definite positive results fo the midgut cells, but had negative results with the method of Ackerman (1952) and with the burnt method for bound lipids due to Berenbaum (see all these methods in Pearse 1968).

The terms 'Fat' and 'Lipid' cover a multitude of substances. Casselman (1959) summarized methods of their investigation. It is not considered that the number of tests carried out in this work enables more than the description of positive results as evidence for Sudanophilia (The logistics of Antarctic research necessitated packing, long in advance, all equipment and chemicals needed for the period in Antarctica. Those chemicals required for the extraction methods for the more detailed study of lipids were not included and were not available at the base).

Positive sudanophilia in pycnogonid midgut is most noticeable in the spherical bodies which compose the main mass of the vacuoles.

These are black staining, while a grey staining pervades most of the cytoplasm. The black staining shows two sizes of spherical bodies to be present, larger ones which are mainly distal (lumenad) and smaller ones whose position is mainly more basal (see Plates 5F andG).

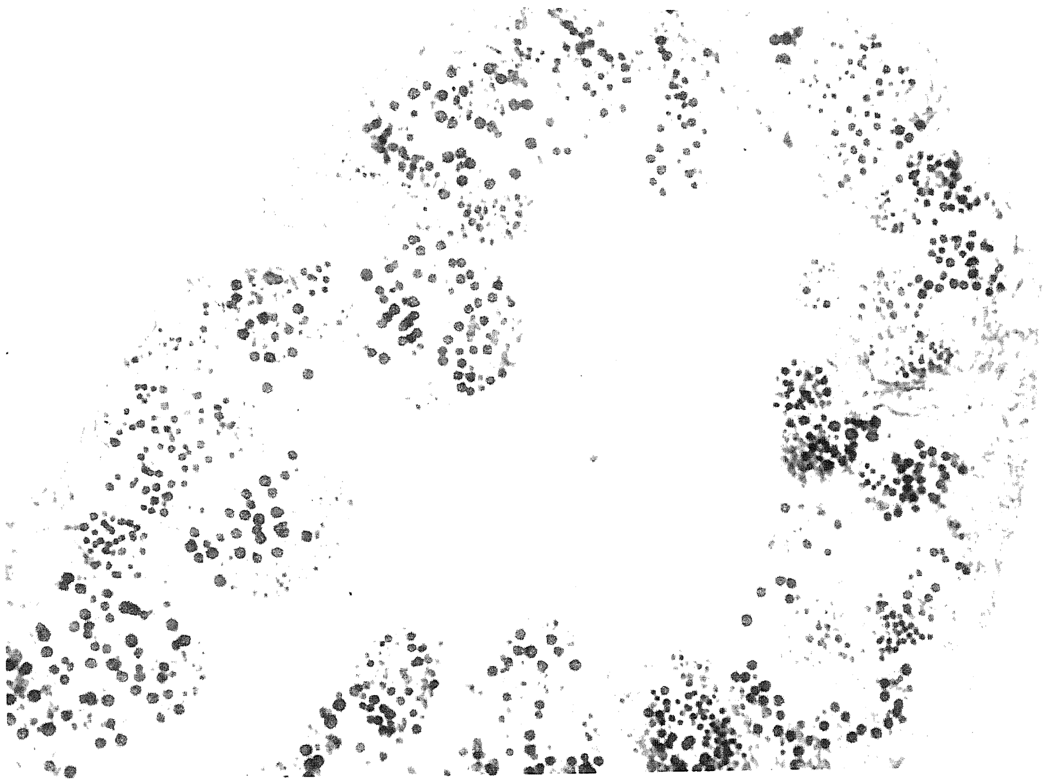


PLATE 5.F. ├──┤ ≏ 15µm.

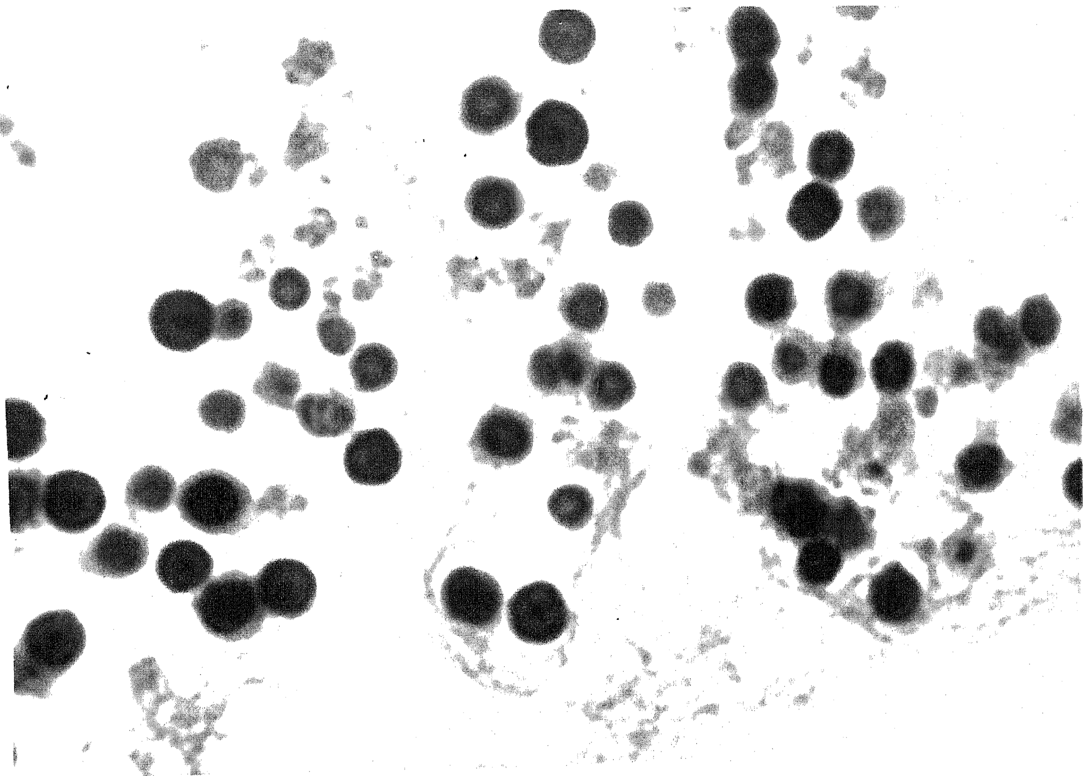


PLATE 5.G. ├──┤ ≏ 4µm.

## 5.4. IN VITRO METHODS

Introducing foreign substances into the gut in order to follow their uptake is no easy task. Attempts to 'force feed' the animal by inserting a fine pipette or syringe into the mouth were thwarted by the animal's ability to prevent these substances passing through the oesophageal valve into the midgut (foreign substances were never detected histologically after sacrifice of force fed animals). In animals as small as N. australe or N. orcadense injection through the cuticle and into the midgut lumen is difficult. To escape these difficulties, gut cells were maintained (but not cultured) in vitro. Three basic types of technique were attempted.

i) Isolation of cells and in vitro vital-staining.

ii) Isolation of cells and investigation of their uptake of fluorochrome labelled protein.

iii) Incubation of gut lengths in horse radish peroxidase medium, followed by fixation and histochemical investigation.

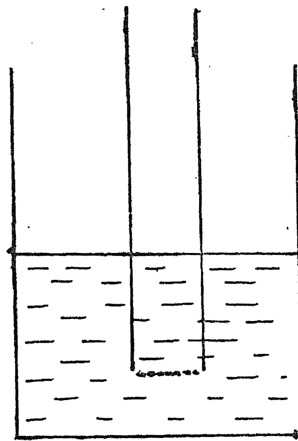
Incubation of gut homogenates with various substrates and a chromatographic study of the products of this incubation was also carried out, but as this method involved neither whole cells, nor histochemical techniques, it is dealt with in a separate section.

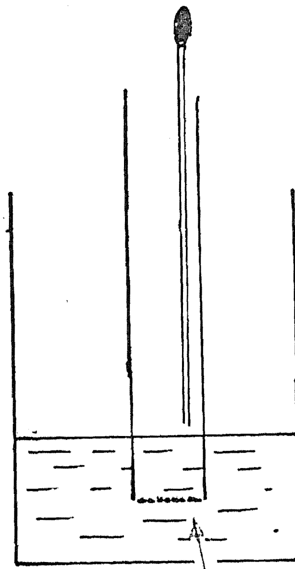
5.4.1. VITAL STAINING

Initial experiments involved attempts to dissect out the gut of N australe in seawater, which was used as the maintenance medium throughout these experiments. At that time, however, I lacked the confidence to differentiate between gut cells and those from other tissues (eg, gonad or blood). Experiments were, therefore, continued on a large specimen of Colossendeis (probably C. wilsoni), where this separation was more certain. A leg was removed, its cuticle slit, the gut eased out, associated gonad and muscle tissue were removed and the cleaned gut placed in a petri dish of seawater cooled on an ice block. The gut was then chopped into small pieces which were transferred to 3% trypsin in seawater (in a test tube) and incubated for 30 minutes at 37°C. The pieces in this incubation tube were then aspirated with a pasteur pipette and transferred to a small beaker of fresh, trypsin-free seawater at room temperature. This was then placed in a refrigerator at 4°C and allowed to stand until needed (between a half and one hour). Whilst the macerates were standing, a tube, closed at one end with a millipore filter, was allowed to settle into the beaker and the filtrate (i.e. seawater without cells) drawn off periodically (Figure 5H). This increased the number of cells per unit volume of the medium (Dobson and Thomas 1964).

Vital dyes were dissolved in absolute Analar ethanol, dropped on coverslips previously prepared for the hanging drop technique (Grimstone

FIGURE 5 H. A 'gentle' method of concentrating cells.




The fluid rises up the inner tube ( whose end is closed by a 'millipore' filter) until the fluid levels are the same. Fluid is then removed from the inner tube with a pasteur pipette. No cells are removed, but the amount of fluid is reduced ( ie the concentration of cells per unit volume of fluid is increased).

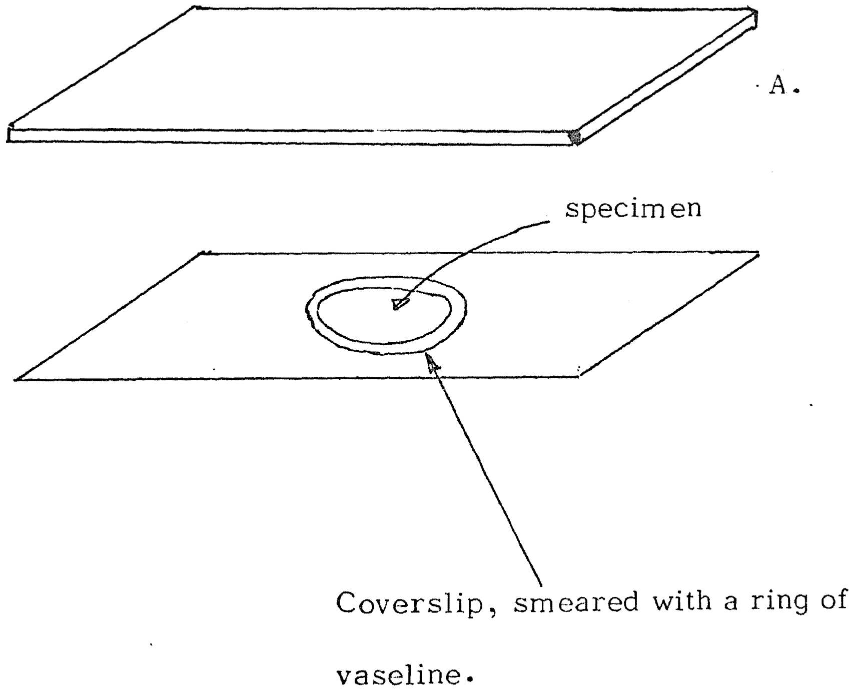
and Skaer 1972) and the alcohol allowed to evaporate. Drops of medium containing gut cells were then introduced to the coverslips and a cavity slide placed over this ( see Figure 5I). The slide with coverslip and hanging drop was then inverted and examined with brightfield optics on a Wild M20 microscope. Observations were made within 10 minutes of contact with the dye and again after four hours. The temperature throughout the observations was room temperature (about 18°C).

In case trypsinization had severely altered the cells or killed them controls were carried out with cells teased out and not subjected to trypsin or incubation at 37°C. There was no significant difference in reactions of these cells to vital dyes. The potentially more harmful method described was used because it was difficult to obtain individual cells by teasing. The binding of the pycnogonid midgut cells in their epithelium appears to be a strong one. Results of incubations with vital dyes are given in Table 5J.

DISCUSSION:-

During the last hundred years the popularity of the application of vital dyes to cellular studies has varied. Their role is discussed at length by Baker (1958). Recently vital dyes have found application in lysosome studies. Gregory (1968) reported considerable morphological resemblance of neutral red granules of mouse exocrine pancreas and the acridine orange particles of Hela cells to lysosomes and lysosomal

FIGURE 5 I.




The cavity of the cavity slide (A), is placed over the vaseline ring of the coverslip. Slide and coverslip are then inverted.

derivatives such as 'autophagic vesicles', 'cytolysosomes', 'residual bodies', or 'phagosomes'. The granules in the cytoplasm of cultured astrocytes and fibroblasts, which stain in life with neutral red, methylene blue, toluidine blue, and Azure B, show acid and alkaline phosphatase and esterase activity. Koenig (1963 and 1965) found that lysosomes are stained in vitro in particulate fractions by neutral red, methylene blue, Azure B and acridine orange. Straus (1967) commented on the close relation between the uptake of vital dyes and the uptake of protein and colloidal materials. Chapman-Andresen (1962) has shown that basic dyes are absorbed and orientated at the surface of amoebae and induce pinocytosis.

The mechanism of ingestion of highly dispersed basic (cationic) dyes such as neutral red or acridine orange is not well understood, and there appears to be a difference between the way that these are taken up and the uptake of acid (anionic)dyes which have a tendency to flocculate into particles of colloidal dimensions (Baker 1958, Gregory 1968, Straus 1967). Gregory (op. cit.) ended her work with the comment:-

"The apparently widely occurring staining of lysosomes by vital dyes may restore vital colouring as a useful technique for light microscopical cytology."

## 5.4.2. UPTAKE OF FLUORESCENT LABELLED PROTEIN

Straus (1967) reported that phagosomes marked by fluorochrome labelled protein may be seen with great clarity. An attempt to demonstrate these in pycnogonid midgut was therefore made using fluorescein thiocyanate labelled immunoglobulin (a fluorescent labelled protein abbreviated to F.I.T.C. for the rest of this discussion). A fluorescence microscope was not available in Antarctica and this experiment was carried out on a single specimen of N. orcadense in Britain.

As trypsinization affects the plasmalemma, it was decided to modify the technique for in vitro srudies. Legs were amputated, cut into small lengths ($\simeq$ 2mm) and placed in the medium (filtered seawater). The gut was then removed from these legs after incubation and gut cells teased out for the hanging drop technique.

Pinocytosis, unlike phagocytosis, is affected by metabolic inhibitors (Johnson 1975). Therefore, the incubation media used consisted of F.I.T.C. at the recommended comcentration of the manufacturer (Behringwerke A.G.) in seawater with controls of F,I.T.C. + seawater + metabolic inhibitors (sodium cyanide $10^{-3}$M, or sodium azide $10^{-3}$M,or 2 deixyglucose $5 \times 10^{-2}$M). Parallel incubations were carried out at $0^{o}$C and $20^{o}$C (room temperature). In addition, an incubation was carried out at both temperatures without the addition of F.I.T.C. to the medium. All incubations were for one hour.

Results were inconclusive. Basically there appeared to be little difference in the fluorescence of cells after incubation at 0°C or 20°C with or without inhibitors in the medium. The controls incubated in sea-water alone exhibited a considerable amount of natural fluorescence. As with the early in vitro experiments carried out in Antarctica, it was noted that teasing was a rather unsatisfactory technique for separating cells from the epithelium and optimum microscope resolution was difficult to obtain.

As an alternative technique the method of Etzler and Branstrator (1974) was attempted. In this technique incubated leg pieces were then frozen, cryostat sectioned, fixed in 95% alcohol and then studied by fluorescence microscopy. There was considerable ice damage and results were again inconclusive for there was equivalent fluorescence in control sections from gut not exposed to F.I.T.C. One interesting observation was considerable natural fluorescence in the epi-cuticle.

Generally, these experiments cannot be claimed to have elucidated the mechanisms of protein uptake, although natural fluorescence of the vacuoles could be an indication of lysosomal activity. These experiments were preliminary, however, and the technique requires further investigation. Negative results need not disprove pinocytosis. Johnson (1975) reported that pinocytosis in amoebae lasts for 30 minutes and is followed by a refractory period of two to three hours before further activity can be induced. In the present experiments incubation was for one hour and

and observations lasted a further two hours. It is possible, therefore, that the material was only ever examined during a refractory period. A further artefact which may have been produced by technique is discussed in the next section.

### 5.4.3. PEROXIDASE/ACID PHOSPHATASE

One method for the investigation of the formation of secondary lysosomes by the fusion of pincytotic vesicles and primary lysosomes is to induce pinocytotic uptake of horse radish peroxidase and carry out histochemical tests for peroxidase and acid phosphatase in the same section (see Chapter 6). Incubation with horse radish peroxidase was carried out following the same scheme as used in the F.I.T.C. experiments (at $0^{o}$C and $20^{o}$C with and without metabolic inhibitors). After three hours incubation the leg lengths were fixed in paraformaldehyde/glutaraldehyde by the method of Karnovsky (Pearse 1972). These pieces were then embedded in P.G.M.M. and the sections produced stained by the D.A.B. method for peroxidase (Pearse 1972) and the napthol AS - BI phosphate method for acid phosphatase of Burstone (Pearse 1968). Results were negative.

These negative results are inconclusive, however, as the embedding process was unavoidably interrupted and the D.A.B./Burstone test is not the usual peroxidase/acid phosphatase test combination. Benzidine, used to test for peroxidase in the method of Straus (1967) could not be obtained.

No further specimens were available and it was not possible, therefore, to repeat the experiment. It is suggested that further experiments might require altering times of incubation and the concentrations of horse radish peroxidase used in the medium.

A phenomenon which might have prevented the incubation medium reaching the midgut epithelial cells (this also applies to the F.I.T.C. experiments and electron microscopy) was noted in the fixed leg lengths. This is best explained by Figure 5K which indicates that when the leg is cut a mechanism exists which causes the cut end of the midgut lumen to be closed off. It can be seen that this would have an obvious advantage to an injured animal in the wild; it is a hindrance to these experiments.

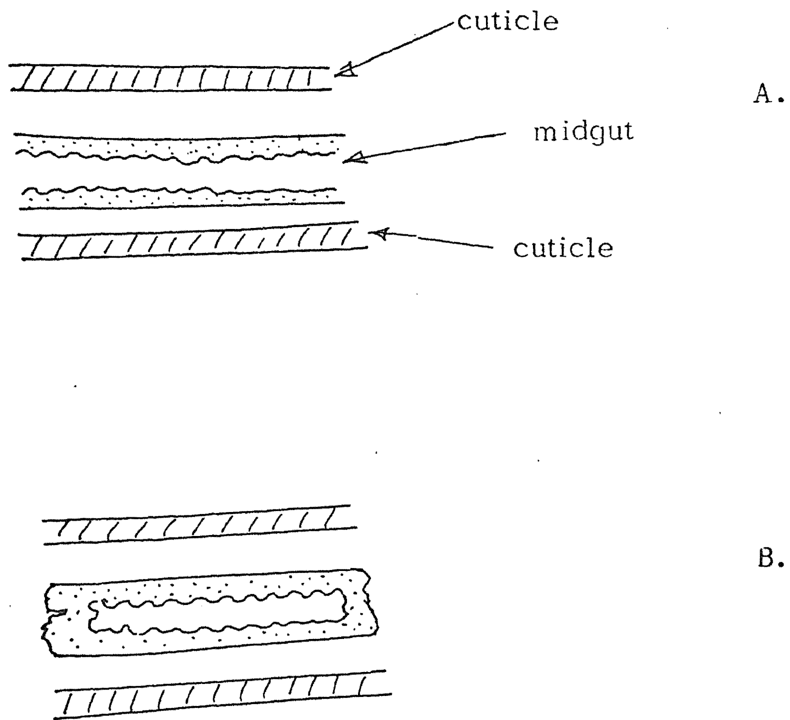



FIGURE 5 K. The effect of cutting live pycnogonid leg into small pieces. A = the ideal situation (for the experiment mentioned in the text).

B = Actual - The cut end of the gut rounds itself off. Sections taken from near the cut end may therefore not be of a region where there is a gut epithelium/gut lumen interface.

## 5.5. DIGESTIVE ENZYMES

The occurrence of acid and alkaline phosphatases has been dealt with above (section 5.1. and 5.2.). Other enzymes of the pycnogonid midgut are dealt with in this section and these are considered under the two methods by which they were studied:-

i) Histochemical techniques.

ii) Incubation of macerations.

### 5.5.1. HISTOCHEMICAL

The histochemical methods used are themselves divided into two main types:-

a) Substrate film techniques.

b) Classical methods - in which a section is cut and incubated with a substrate and dye so that a coloured deposit is left on the section at the site of enzyme action.

#### 5.5.1.1. SUBSTRATE FILM TECHNIQUES

These techniques were selected because on paper their protocol is less complicated than the classical enzyme histochemical methods and were therefore considered more suitable to the then unknown conditions of an Antarctic base laboratory.

The theory of substrate film technique consists essentially of incubating fresh tissue sections in contact with a film of substrate and then testing the film for the presence of unreacted substrate. Where reaction has occurred it is inferred that there has been enzyme activity (Daoust 1965 and 1968). Adams and Tuquin (1961) found that sections containing proteases incubated in contact with exposed and processed panchromatic photographic plates caused digestion of the gelatin of the emulsion layer, the digested site becoming visible following washing out of silver grains loosened by loss of gelatin. The sensitivity of this method depends upon the gelatin layer being completely digested before a positive result is indicated (Dauost 1961). The thickness of this layer is some 20 microns. Fratello claimed that increased sensitivity could be achieved by using unexposed processed colour reversal film. From air to celluloid this film has the following layers in its emulsion:-

| | |
|---|---|
| YELLOW | 5·5 microns |
| COLOURLESS | 2·5 microns |
| MAGENTA | 4·5 microns |
| CYAN | 6 microns |
| COLOURLESS | 1·5 microns |

From this list it can be seen that only 5·5 microns thickness of gelatin (the Yellow layer) has to be digested to give a visible positive result (purple colour) when viewed with transmitted light. A further 7 microns digestion will produce a blue colouration and a total of 18·5 microns

digestion will produce a colourless patch. Fratello claimed that digestion limited to the first coloured layer gave ten times the sensitivity of the panchromatic method. Differences in colour resulting from progressive proteolysis were indicative of enzyme activity in different regions of the section over the same incubation period (Figure 5L). Pearse (1972) has given further substrate film methods for amylases, deoxyribonucleases and ribonucleases.

#### 5.5.1.1.1. Method of Fratello

Initially frozen sections of fixed material were cut and incubated as indicated by Fratello (i.e. 4% formaldehyde-saline for 24 to 48 hours, frozen sections cut and mounted on colour film dampened with 0·15M phosphate buffer at pH 7·6 and incubated at 37°C for 30 minutes in a water saturated atmosphere). Sections revealed no activity. A trypsin solution absorbed on filter paper and used as a substitute section for control also showed no activity. The experiment was repeated using a range of buffers from pH 2 to pH 9, with trypsin and pepsin soaked artificial sections as controls and parallel incubations at 37°C and 0°C (the latter was taken as the environmental temperature of the pycnogonid). Again test and control slides were negative. Baker (1958) stated that there is contradictory evidence as to whether trypsin will digest collagen which has been fixed by formaldehyde. The fixative from the commercially supplied processing kit for the colour film (Ferranicolor CR 50) smelt strongly of formaldehyde. It appears that this chemical is included in all formulae

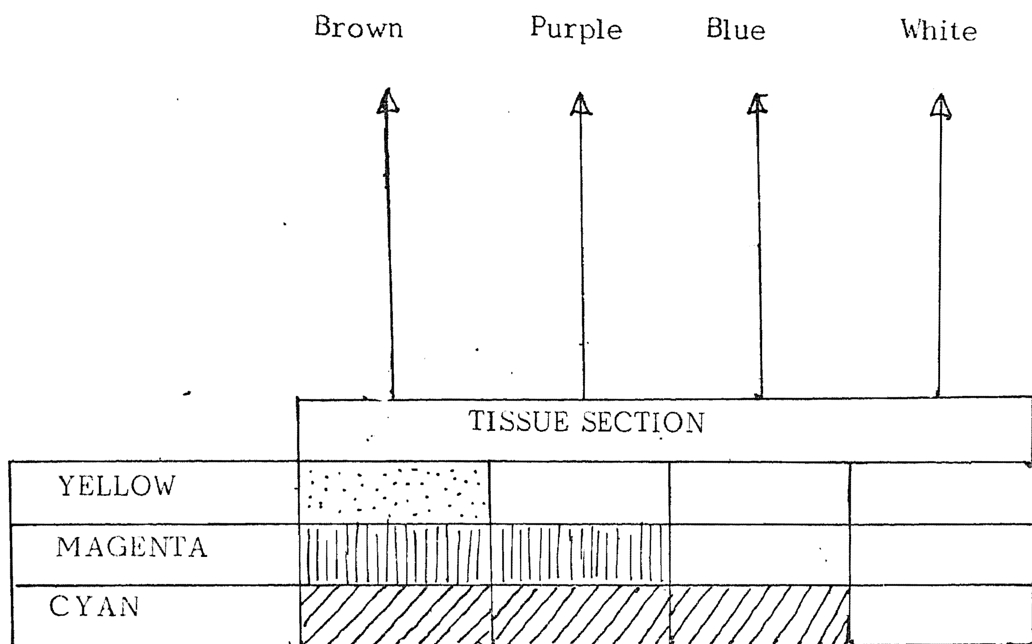



FIGURE 5 L. The effects of progressive digestion of colour reversal film by proteases in tissue sections (from Pearse 1972).

for the processing of commercially available colour film to harden the emulsion (British Journal of Photography Annual 1971, 1972 or 1973). The replacement of the commercial kit fixative with one made up without formaldehyde, produced a film which was digestible. (Processing was tested by exposing at least two frames of a 36 exposure length of 35mm Ferranicolor CR 50 in a camera to check that colour rendition was satisfactory). Film thus treated was used throughout all other experiments. Its results were considered genuine as controls with filter paper (not soaked in enzymes) were negative, whilst pepsin and trypsin artificial sections only produced positives in their normal pH range. Heated sections were negative. Interestingly, it was found that incubations carried out at 0°C had not produced positive results after two weeks. Incubations at 37°C normally required about 4 to 8 hours to produce positive results. This probably indicates low enzyme concentrations and no acclimation to low temperatures (see also Chapter 2).

As indicated by David and Brown (1967) cryostat sections can be histologically adequate, but to obtain cytologically adequate sections with isothermic cryostats (knife and tissue temperature identical) requires lower temperatures (-70°C) than that of most commercially available machines. Elimination of fixation processes meant that sections suffered from great ice damage. The use of polyvinyl pyrollidine as a 'protective' did little to solve this problem. As it was found that formaldehyde fixation had deleterious effects on the sensitivity of the test, most sections

were cut without fixation. They were therefore subject to ice damage and localization was therefore disappointing. Localization was only sufficiently good to indicate that the diffuse digestion produced on the colour film was in the gut region and not produced by muscle, gonad or cuticle.

On return to Britain the experiments were repeated using known trypsin inhibitors and running parallel experiments on a Colossendeis specimen. Liquid nitrogen was used to freeze specimens, but as one must let the specimen heat to the temperature of the cryostat chamber (approximately -20$^{\circ}$C) before sections can be cut, no advantage was gained by using a rapid freezing technique.

From the results of the Antarctic and British experiments it can be seen that N. orcadense exhibits proteolytic activity in its midgut at pH 2 and between pH 5 and 8 (Table 5M). The positive results produced at pH 2 are uncertain, however, and generally speaking pepsin-like proteolytic activity apparently is not found in invertebrates (Prosser and Brown 1961). All experiments (Antarctic and British) show activity in the pH 5 to 8 range and this was also found for the gut of Decolopoda australis.

As has been stated, colour film subjected to treatment with formaldehyde seemed to have an inhibitory effect on the proteases when experiments were carried out in Antarctica. In Britain, a different commercial

TABLE 5 M. PROTEOLYTIC ACTIVITY OF THE PYCNOGONID MIDGUT.

| pH | N. orcadense | Decolopoda australis |
|---|---|---|
| 2 | + | – |
| 3 | – | – |
| 4 | – | – |
| 5 | +++ | + |
| 5·5 | +++ | +++ |
| 6 | +++ | ++ |
| 6·5 | +++ | + |
| 7 | +++ | +++ |
| 7·5 | +++ | – |
| 8 | +++ | – |
| 8·5 | – | – |
| 9 | – | – |
| Inhibited by | Ovomucoid<br>Formaldehyde | $Ca^{++}$<br>Sodium cyanide<br>Soyabean Extract<br>Formaldehyde |

processing kit for the Ferranicolor film was tried ('Steadkit')and this was found to be less inhibitory ('stabilized' in the table - presumably the formaldehyde concentration was lower).

As can be seen in Table 5M, there appears to be a subtle difference between the proteases found in the midgut of N. orcadense and those found in Decolopoda australis. At each pH in N. orcadense the ovomucoid inhibitor of trypsin caused slight inhibition. Also, there seems to be comparatively less inhibition with formaldehyde than in Decolopoda australis. In this species, sodium cyanide, calcium ions and formaldehyde are inhibitory throughout the pH range over which proteolysis was detected. Ovomucoid inhibition only occurred at pH 7. On the available evidence it is suggested that the proteolytic enzyme(s) of N. orcadense is trypsin-like, whilst in Decolopoda australis there is evidence for cathepsin-like enzymes at pH 5·5 to 6·5 and a trypsin-like one at pH 7. These suggestions are only tentative and it is felt that more experiments of a biochemical nature (see later) are required to verify this claim.

#### 5.5.1.1.2. Other Methods

In addition to the substrate film methods for proteases, methods were also tried for amylase, ribonuclease and deoxyribonuclease (Pearse 1972). Results were inconclusive, mainly because there was considerable difficulty in obtaining even, thin films. The method suggested by Pearse appears to be one in which considerable practice is required to obtain the

skill, and the time for this was not available. Daoust (1965) recommends investigation of a method suggested (for electron microscope autoradiography) by Koehler, Mühlethaler and Freywyssling (1963) in which a thin layer is obtained by means of a centrifugal spreading mechanism (an adaption of an ultra centrifuge rotor). I believe attention to Daoust's recommendation is essential before any future work on these tests can be profitable.

5.5.1.2. CLASSICAL ENZYME HISTOCHEMISTRY

Unfortunately, there are not many histochemical techniques for proteases which do not require that the experimenter synthesize his own substrates. Pearse (1972) gives two methods which do not require complicated syntheses. The first is a silver proteinate method for endopeptidases developed by Yamada and Ofuji (1968). The second is a method for 'trypsin-like' enzymes. The former method was attempted, without success in the Antarctic, and repeated, along with the latter method (again both without success) in Britain. A further repeat was not possible owing to the loss of the bulk of the fresh material.

It is likely that the negative result in the silver proteinate method was due to incorrect methodology. The protocol used was that given in Pearse (1972 p.1358). When the original paper was obtained on return to Britain, its protocol was found to be very unlike that given by Pearse. Originals should be consulted wherever possible.

5.5.2. INCUBATIONS

According to Christie and Stoward (1974): "At present histochemical studies carry little weight with those working in more exact disciplines such as biochemistry." To remedy this "it is necessary to show that the results of histochemical experiments are consistent quantitatively with those of other techniques."

With the hope of at least some qualitative correlation with histochemical methods, incubations of midgut macerations on defined substrates followed by chromatographic analysis of the products were carried out using the methods of Evans (1956).

0·1 mls of 1% carbohydrate or protein substrate (of known composition) in 0·2M phosphate buffer at pH 7 was dropped onto a cavity slide and saturated with toluene (which acts as a bactericidal agent). Portions of pycnogonid midgut obtained by amputating a leg, squeezing out its contents and separating muscle and gonads, were then added to this mixture.

A paper chromatogram was then spotted with some of this mixture and developed with n-butanol : pyridine : water in the ratio 3 : 1 : 3 as the solvent. After incubating the reaction mixture (substrate + buffer + toluene + gut) for 12 hours, another chromatogram was spotted and run, and again after 24 hours (incubation was in a water vapour saturated atmosphere - a petri dish with wet filter paper in the bottom). The pro-

duction of a chromatogram with several discrete spots from an incubated reaction mixture was taken as evidence of breakdown of the substrate (unless the control chromatogram showed similar separation). As controls, mixtures in which the gut had been boiled and mixtures without gut were also used. Results for carbohydrate substrate breakdown were negative. For a protein substrate (Casein) results were positive. Table 5N indicates the substrates and the controls used.

The positive result for protein digestion supports the finding of a protease which works at pH 7 in the substrate film technique.

It seems likely that this technique could hold considerable promise in analysis of the digestive enzymes of invertebrates. With carbohydrates for example, it is possible by using known substrates to find out what bonds the carbohydrases present can attack. A negative result for the carbohydrate experiment as obtained here does not necessarily indicate absence of carbohydrases. Paper chromatography has been superseded by much more sensitive chromatographic methods and the sensitivity of detection methods used here is in doubt.

With proteases, by using labelled caesin (azo casein) quantitative estimation of the proteases and information about the kinetics can be found (Evans 1958). It is suggested that dansyl labelled peptides might be used in techniques to differentiate endopeptidases from exopeptidases.

Generally biochemical techniques are more sensitive than histochemical methods (which are usually modifications of the former). The problem of this project was that when fresh material was available (in Antarctica) biochemical apparatus, and - more important - a ready source of biochemical supplies and literature was not available.

In the section on the 'lysosome concept' (Chapter 6) it is mentioned that this was developed mainly from the results of fractionation studies. Fractionation of gut cells and subsequent biochemical analysis of the fractions could prove useful in further investigation of pycnogonid digestion if sufficient material is available. Brown and Whitegeaver (1971) have carried cell fractionation studies on the digestive gland of Limulus polyphemus with results which support intracellular digestion.

# C H A P T E R 6.

## THE LYSOSOME CONCEPT AND PYCNOGONID DIGESTION

## 6.1 INTRODUCTION

Before discussing the digestive process in the pycnogonid midgut, it is necessary to consider some current theories on digestion at the cellular level. These concern enzyme production and secretion and the processes of Endocytosis and Exocytosis in all of which the cell organelle, the LYSOSOME, is of central importance.

## 6.2 THE LYSOSOME CONCEPT

The term 'lysosome' was originally used by DeDuve (1955) to describe a particle found in rat liver homogenates which was characterised by the presence of latent acid hydrolases. These hydrolases are now known to include acid phosphatases, acid deoxyribonucleases, acid ribonuclease and cathespin, (more extensive tests are given by Novikoff 1961, Allison 1974, and Pitt 1975). Lysosomal localization of acid phosphatase has been established more frequently than with any other enzyme (Barrett 1972). Indeed, it is part of the histochemical and biochemical definition of a lysosome that it contains acid phosphatase. However, it is also possible for acid phosphatases to occur outside lysosomes.

The assumption is that populations of lysosomes in all tissues are involved in, and can potentially complete the hydrolysis of all macromolecules found in cells (Pitt 1975). In such circumstances the material to be digested must gain access to the interior of the lysosome without the liberation of hydrolases into the cytoplasm. DeDuve likened

lysosomes to "suicide bags", and it seems that many diseases (e.g. certain types of arthritis, muscular dystrophy, gout, asbestosis, silicosis) are caused when for some reason lysosomal membranes break down and cells start to digest themselves. The process of autolysis which the histologist prevents by use of fixatives is in part due to lysosomal membrane breakdown at death. The question that the presence of such suicide bags within the cell creates is 'how are the enzymes manufactured and enveloped in membrane without self digestion taking place?"

The most widely held current theory of lysosomal enzyme manufacture is due to Palade and his co-workers (see for example Palade 1975). It states that new peptides grow on ribosomes attached to the membrane of the rough endoplasmic reticulum and elongate directly into the cisternal spaces through pores beneath the point of ribosomal attachment. They are thus separated from the cytoplasm of the cell as they are synthesized. Subsequently, it is hypothesized, the completed secretory protein moves out of the endoplasmic reticulum in smooth surfaced vesicles formed as buds from the reticulum network. These vesicles are thought to travel into the golgi region of the cell where, in secretory cells, they empty their contents into larger vesicles called 'Condensing vacuoles'. In non-secretory cells these condensing vacuoles correspond to the 'primary lysosomes'. The above processes are summarized in figure 6A.

RIBOSOMES.

CISTERNAL SPACES.

SMOOTH SURFACED VESICLES.

CONDENSING VACUOLES (GOLGI) PRIMARY LYSOSOMES or ZYMOGEN GRANULES.

LUMEN

EXOCYTOSIS

ENDOCYTOSIS

EXOCYTOSIS

ENDOCYTOSIS

m.R.N.A.

SEQUESTRATION

INTRACELLULAR TRANSFER.

FIGURE 6.A. MODEL FOR PRODUCTION OF LYSOSOMES (ZYMOGEN GRANULES) PALADE'S SCHEME (AFTER ROTHMAN 1975).

1.

LUMEN

secretion granule

2.

LUMEN

FUSION

3.

LUMEN

ELIMINATION OF LAYERS.

4.

FISSION

FIGURE 6.B. DIAGRAMS OF MEMBRANE INTERACTIONS DURING SECRETORY DISCHARGE ( AFTER PALADE 1975).

Rothman (1975) believes that hydrolases can occur in the cytoplasm, i.e. they do not have to enter the cisternae of the endoplasmic reticulum. He drew attention to the fact that the inhibitors to the hydrolases they produce are present in most cells and also to the fact that many hydrolases are produced as the inactive pro-enzyme form and are therefore harmless unless activated. Rothmann also mentions that the argument that biological membranes are impermeable to proteins (i.e. will keep hydrolases in or out), is a derivative of the classical membrane model which proposed that biological membranes contain a oontinuous lipid layer, a view which is no longer accepted by membrane workers. Rothmann proposes an alternative 'equilibrium' transport system which allows for the presence of inactive digestive enzymes within the cytoplasm.

Whatever the method of formation of digestive enzymes, the existance of lysosomes is beyond dispute. Once the stage of the primary lysosome has been reached, the fate of the structure depends on whether it is involved in exocytosis or endocytosis. (See section 6.2.4. for definition).

### 6.2.1 THE LYSOSOME IN ENDOCYTOSIS

In endocytosis, primary lysosomes merge with phagosomes, pinòcytotic or autophagic vacuoles fairly soon after their formation to form the main digestive unit which is known as the 'Secondary lysosome'.

It is presumed that hydrolases within the lysosome are activated at this stage. Repeated fusions may occur between phagosomes, primary lysosomes and secondary lysosomes which may result in the formation of a 'Multivesicular body'. Multivesicular bodies also possess digestive capacity and must rank as secondary lysosomes. Ther e is evidence that secondary lysosomes which are involved in repeated fusions with primary lysosomes, maybe 'topped up' with enzymes from these primary lysosomes. Eventually, secondary lysosomes become depleted of hydrolase activity and become 'Residual bodies'.

There has been a considerable proliferation of the nomenclature concerned with lysosome physiology over the years and, unfortunately, no agreement on standardisation. Figure 6C represents the inter-relationship of cytoplasmic granules as suggested by Gordon, Miller and Bensch (1965). Figure 6D, due to Pitt (1975), shows the inter-relationship of particles and also attempts to standardise names. Table 6E, due to Buvat (1971), li sts the names of the types of vacuoles found in animal cells.

### 6.2.2 THE LYSOSOME IN EXOCYTOSIS

In enzyme secretion - as found, for example, in extracellular digestion, primary lysosomes do not remain within the cell to fuse with phagosomes. In the form of Zymogen granules (which are membrane-bounded) they approach the cell membrane, fuse with it and empty their contents to the exterior (see Figure 6B).

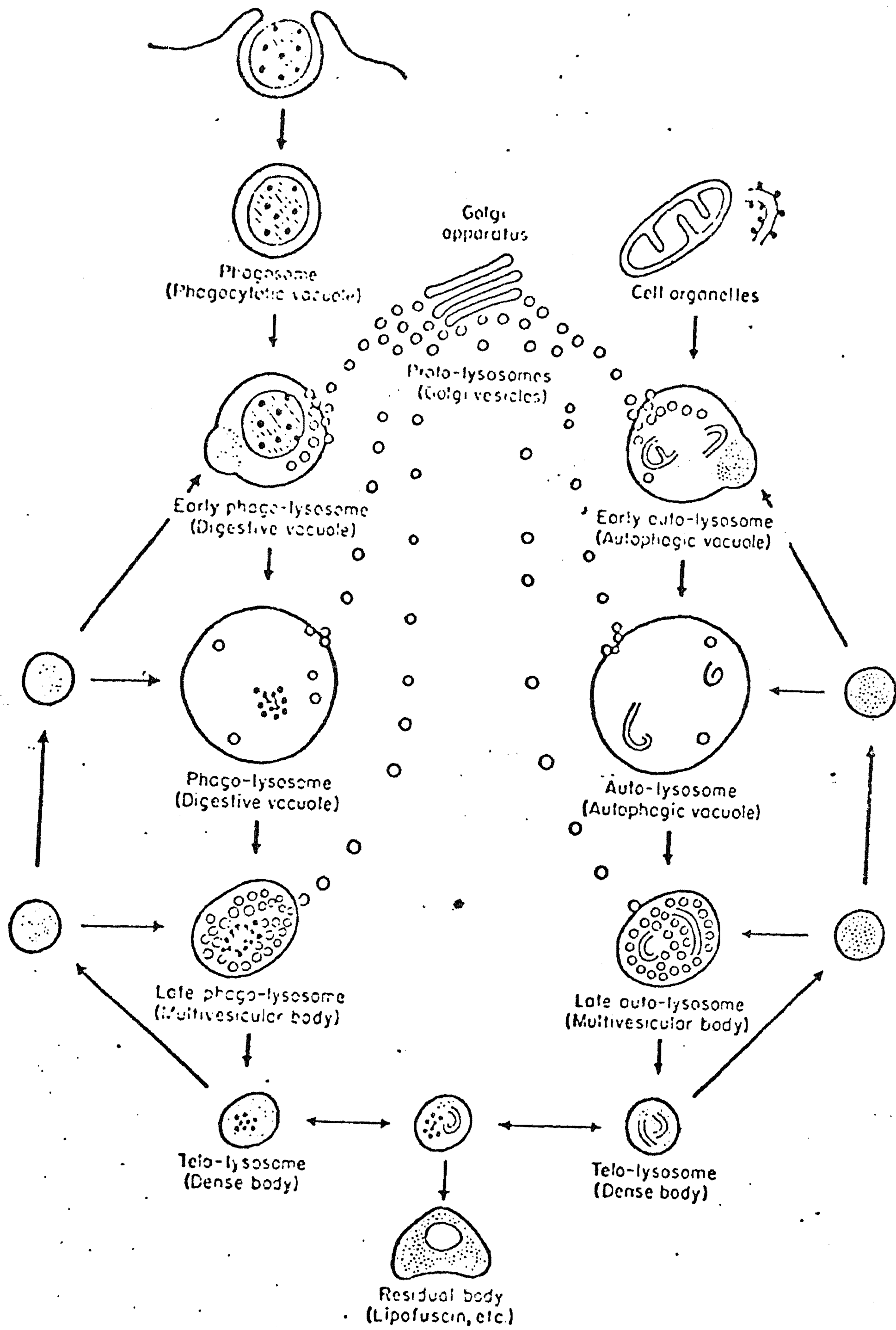



FIGURE 6 C. The relationship of cytoplasmic granules after Gordon, Miller and Bensch (1965).

| Term | Synonym | Characteristics |
|---|---|---|
| *Primary lysosome* | Cytosome; pure lysosome; proto-lysosome; young lysosome | Organelles derived from Golgi and/or ER. Single membrane, contain acid hydrolases |
| *Secondary lysosome* | Phago-lysosome; digestive vacuole; old-lysosome; food vacuole (of protozoa) | Body produced on fusion of a primary lysosome with a vacuole containing material to be digested |
| *Autophagic vacuoles* | Cytolysome; segre-some; cytosegre-some; composite body; secondary lysosome | Single or double membrane. Contain recognizable cyto-plasmic components in various stages of digestion |
| *Multivesicular body* | Late phago-lysosome; secondary lysosome | Usually bounded by a single membrane. Contents vacuolated and may represent a late secondary lysosome |
| *Residual body* | Dense-body; post-lysosome; storage-body; telolysosome | Usually single membrane bounded, may be double. Contains indigestible remains. Often whorled and containing myelin, etc. Low or no hydrolase activity |

FIGURE 6D:- Table of interrelationships of particles concerned with intracellular digestion and some of their synonyms (from Pitt 1975).

TABLE 6 E. THE VACUOLES OF ANIMAL CELLS (after BUVAT 1971).

a) GOLGI VACUOLES - resulting from the vesiculation of Golgi saccules at the distal pole of the dictyosomes.

b) THE VACUOLES OF PHAGOCYTOSIS OR PHAGOSOMES, contain, in an envelope budded from the plasmalemma, nutritional particles for digestion.

c) THE VACUOLES OF PINOCYTOSIS - similar to the preceding ones except that they contain fluid and no solid particles.

d) THE 'PRIMARY' LYSOSOMES with concentrated contents (possibly these are identical with the 'secretion grains' or 'storage granules' described by earlier authors).

e) THE DIGESTIVE VACUOLES resulting from the acquisition of acid hydrolysing enzymes through phagosomes.

f) THE AUTOPHAGIC VACUOLES - these are active in partial cytoplasmic lysis.

g) THE RESIDUAL BODIES result from the two previous types after the processes of digestion have been completed.

h) THE PEROXISOMES - are characterised by their enzymatic contents uniquely composed of oxidases and catalase.

### 6.2.3 THE LYSOSOME AND AUTOPHAGY

In addition to endocytosis and exocytosis, lysosomes have 'autophagic' functions within the cell, wherein they digest portions of the cytoplasm of the cell without causing cell death. Reports in the literature commonly refer to the enclosure of mitochondria within autophagic vacuoles, but in fact most types of cytoplasmic organelle are found within them at some time or other. It would appear that autophagy is a characteristic of normal tissue and possibly plays an important part in the economy of the cell, generally assisting turnover. It also seems that the process is enhanced in cells or tissues which have been deprived of essential nutrients; thus autophagic bodies are reported in starved animals.

### 6.2.4 ENDOCYTOSIS AND EXOCYTOSIS - DEFINITION

Extracellular substances can enter cells in two ways. One way is penetration through the cell membrane via a specific transport mechanism, passive flux or some other process. Alternatively, they can be taken up within invaginations of the plasmalemma which become detached to form vacuoles or microvesicles that move into the cell. This latter process is called 'Endocytosis' and three varieties of endocytosis can be distinguished (Allison and Davies 1974). These are 'phagocytosis' which is uptake of particles greater than 1 micron diameter, and 'pinocytosis' which in turn can be divided

into 'macropinocytosis' (which is intake of fluid droplets 0.3 to 2 µm diameter) and 'micropinocytosis' which is of 70 nm diameter droplets.

Although the occurrence of phagocytosis has been known since the last century, pinocytosis was described by Lewis (1931) only just before the publication of Schlottke's 1933 work on pycnogonid digestion. According to Oberling (1959), pinocytosis was ignored for many years after its discovery. Curtis (1967) has presented the view that all animal cells can do one or other or both of pinocytosis or phagocytosis if sufficiently pursuaded.

Reversed pinocytosis, known as 'Exocytosis' is essentially the secretion process which takes place when enzymes are released extracellularly.

The classic work on endocytosis in amocbae is due to Chapman Andresen (1962). Esteve (1970) has followed the fate of digested material in protozoans and finds that it corresponds to the outline given by Gordon, Miller and Bensch (op.cit.), whose system was composed as a result of work on mammalian cell cultures (figure 6C). Shibko, Caldwell, Sawant and Tappel (1962) have examined the occurrence of the various lysosomal enzymes in a variety of animal phyla and found them to be ubiquitous.

## 6.3 A SCHEME FOR INTRACELLULAR DIGESTION IN PYCNOGONIDS.

The proposed scheme for intracellular digestion in pycnogonids is as follows:- Uptake of nutrients is by macropinocytosis. This is concluded on morphological and vital staining evidence. Large particles of external (i.e. non pycnogonid) origin have not been observed in the midgut lumen of any of the animals investigated although a mush of very minute particles has been observed. In sections, the distal borders of cells do not possess pseudopodia and appear symmetrical. Scanning electron microscopy has not shown pseudopodia nor convincing 'ruffle membranes' which would indicate phagocytosis. Vital dyes taken up by the cells were basic (cationic) ones whose uptake is normally associated with pinocytosis. Vital dyes whose uptake is associated with phagocytosis were not found in cells. Numerous attempts to induce phagocytotic uptake have been unsuccessful. Brush-borders have not been observed in any region of the gut.

Schlottke (1933) reported phagocytotic uptake of food particles by pycnogonids, but a close examination of his paper reveals that he did not observe this process himself and, indeed, his argument in support of the process is illogical. It is based firstly on the report that Dogiel (1913) observed diatoms in the midgut cells of protonymphon larvae in which the pharynx setae had not yet developed.

Confusingly, Schlottke and Helfer and Schlottke (1935) fail to cite and then cite incorrectly Dogiel's paper, and secondly on the following tenet (Schlottke 1933 p. 640):- "The degree of breakdown of substances is important. Proteins must be utilised as amino-acids, polysaccharides as monosaccharides." From this dogma Schlottke argues that, as these are not present in the lumen, so proteins and polysaccharides must be taken into the cells by phagocytosis for further breakdown!

The staining techniques used by Schlottke are not specific for amino acids or monosaccharides (indeed it is doubtful whether it is possible usefully to analyse histochemically the contents of an extracellular cavity). His 'Degree of breakdown' argument therefore has no tenable results to support it. As indicated previously, it is doubtful that Schlottke was aware of pinocytosis.

Personal observations indicate that pinocytotic vesicles formed at the lumen borders of midgut cells gradually move in the direction of the basement membrane. In the region of the lumen boarder the primary lysosomes, which make the cytoplasm appear red with Burstone's naphthol AS phosphate method for acid phosphatase, fuse with the pinocytotic vesicles which then become large sedondary lysosomes. These are the large distal vacuoles of the midgut cells. In movement towards the epithelium basement of the vacuoles, contents become condensed so that eventually 'Residual bodies' are formed.

The 'Enigmosomes' or 'Spherical bodies' (see pps

fall into the category of residual bodies. It seems likely that when residual bodies are formed, autophagy may also occur in the region of the cell in which they are found. This would give rise to vacuolar areas which are morphologically similar to the 'Gland cells' of earlier authors.

It is concluded, therefore, that gland cells are incorrectly named and are in fact regions of formation of autophagic vesicles and residual bodies within absorption cells. Two courses may be open to such regions: either the region may move distally and empty its contents into the lumen, the rest of the cell remaining intact (see figure 6F), or the whole cell may become autophagic and break down into the lumen.

Enigmosomes, residual bodies, or spherical bodies are composed of excretory material, but they also contain acid phosphatase and alkaline phosphatase. By introducing these enzymes into the gut lumen, they contribute towards some extracellular digestion.

In summary, the scheme proposed for intracellular digestion in pycnogonids is :-

a) Pinocytosis.

b) Fusion of primary lysosomes with pinocytotic vesicles.

c) Digestion of the contents of the secondary lysosomes formed in b).

d) Formation of residual bodies ( enigmosomes or spherical bodies) and autophagic vacuoles.

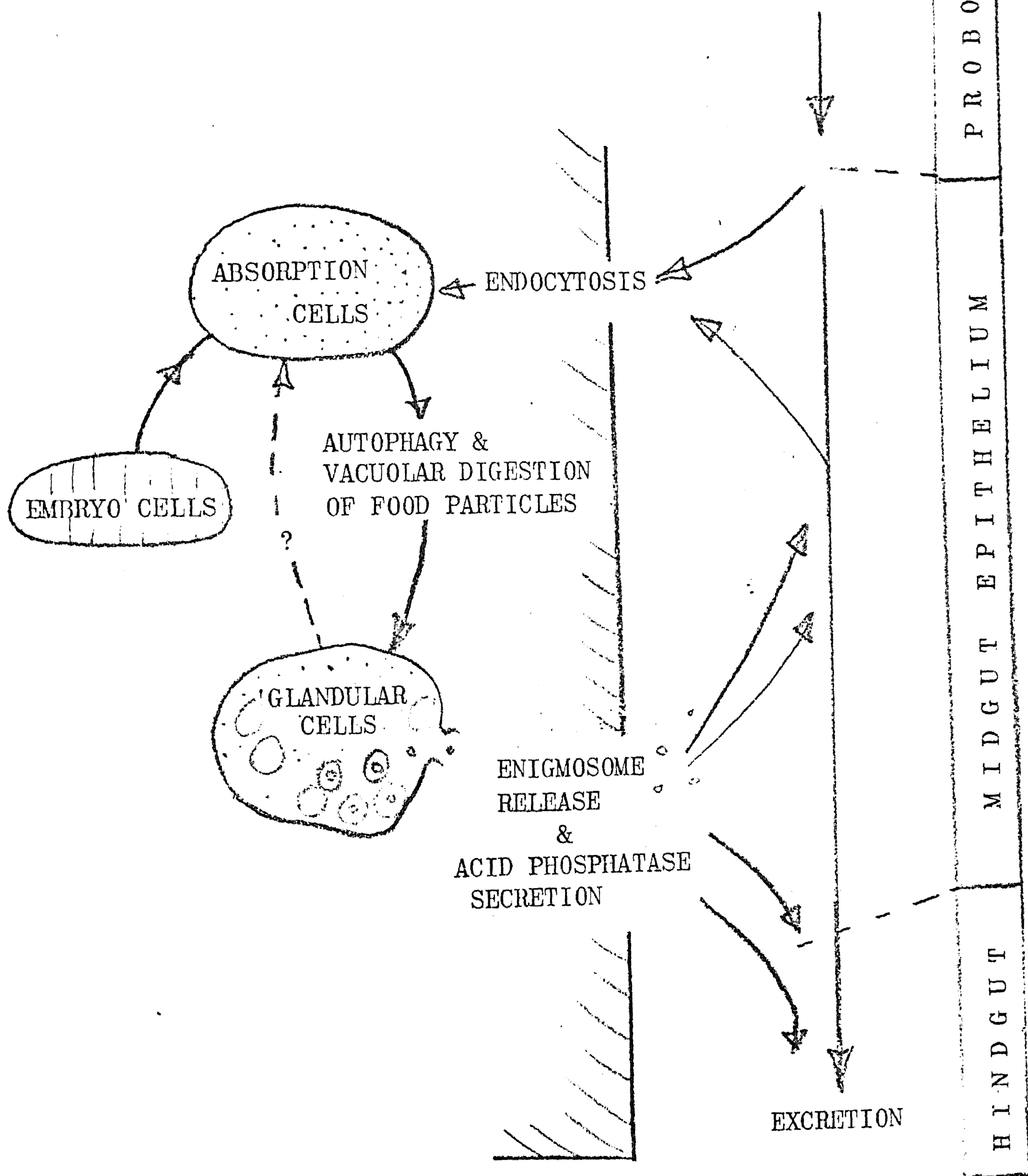



FIGURE 6 F. Proposed scheme for intracellular digestion in pycnogonids.

e) Possible migration and release of residual bodies into the gut lumen.

This scheme is represented by figure 6F.

The release of residual bodies from the cell does not always occur in lysosomal digestion and the finding of enigmosomes in the cytoplasm (plate 4.5.C p 203) as well as in vacuoles (plate 4.5.D p 204) is in keeping with this. Scanning electron micrographs support this scheme. Plates S + T are of the midgut epithelium of an animal which was feeding; the cells are rounded. Plates I & K are of an animal in a different nutritional state. Here the surface is flattened and covered in blebs which I believe correspond to residual body release. The different shapes of the surface epithelium seem to confirm Schlottke's claims on the height of the epithelium, though it is believed that he may not have been justified in his claims with the techniques he used (see appendix 2).

## 6.3.1 A RE-INTERPRETATION OF SOME OF SCHLOTTKE'S FINDINGS.

It is interesting that it is possible to re-interpret most of Schlottke's observations to fit the scheme proposed above:-

At feeding, he notes large, heavily staining vacuoles appearing in the midgut cells which after $1\frac{1}{2}$ hours are surrounded by mitochondrion-like granules. It is suggested that these mitochondrion-like granules are primary lysosomes.

Schlottke stated that 'The contents of the vacuoles become basophil with toluidine blue.' It has been noted that toluidine blue

stains lysosomes vitally. Another observation, basophilia in the vacuoles, could indicate acid content which would be in agreement with the finding of acid phosphatase in vacuoles as well as cytoplasm as digestion proceeds.

Schlottke reported gland cells becoming basal as digestion progressed and if a re-interpretation of gland cells as autophagic regions of cells (or in fact whole cells) is accepted, then this observation is in agreement with the formation of these regions from the basalar movement of vacuoles. He also reported that used gland cells are taken in and broken down by absorption cells. It is suggested that here he was observing the movement of the autophagic regions of cells towards the lumen rather than their uptake after 'wandering' (of which I find no evidence). He noted that at the start of food uptake the gland cells discharge their weakly acidophilic globules into the lumen. This, as well as contradicting his earlier claim that gland cells never border the lumen, suggests that his observation of gland cell resorption could, in fact, be observation of the stage prior to the release of autophagic and residual bodies (enigmosomes) into the lumen. (see figure 6F).

The globules of gland cells Schlottke noted to be weakly acidophilic. This supports the observation that there appear to be a dominance of alkaline phosphatase over acid phosphatase in the spherical bodies (enigmosomes) when they are released into the lumen.

### 6.3.2. PRECEDENTS IN THE LITERATURE

Jennings (1962) has investigated digestion in triclad turbellarians which is mainly intracellular. The scheme he proposed for them is not disimilar to the one proposed here for pycnogonids.

Phagocytosis occurs by the distal borders of the gut cells expanding into the gut lumen and engulfing particles in amoeboid fashion. The resulting food vacuoles are at first quite large and their contents diffuse, but as they pass back into the cell they shrink. As this shrinking - which is probably due to absorption of water, takes place, secretion of acidic juices containing a powerful endopeptidase occurs from the surrounding cytoplasm and as this endopeptidase is secreted into the food vacuole there is a simultaneous appearance of acid phosphatase in the surrounding cytoplasm and to a lesser extent in the vacuolar fluid. The endopeptidase activity gradually decreases and there is a decrease in the amount of acid phosphatase present. The food then passes into a second alkaline phase of digestion. The acid phosphatase activity in the surrounding cytoplasm and vacuolar fluid is replaced by an alkaline phosphatase one. This enzyme seems to play the same role in secretion of alkaline phase enzymes as does the acid phosphatase in the acid phase. One particular endopeptidase, Leucine amino-peptidase, can be detected readily in the food vacuoles (this enzyme was demonstrated but not localized in pycnogonid midgut by Wyer 1972).

The change from acid to alkaline phases of digestion does not necessarily occur at the same time within all food vacuoles of a phagocytotic cell. Only under experimental conditions do all the vacuoles of a cell give a uniform reaction and this situation can only be induced by starving the triclad to clear the phagocytotic cells of all vacuoles. (In pycnogonids capable of surviving long periods of starvation and apparently using autophagy extensively, this task would be difficult). Jennings stated that the products of intracellular digestion are absorbed into the cytoplasm, indigestible remnants remaining in the cell and travelling by cytoplasmic movement back to the free distal margin where they are eventually extruded into the gut lumen.

The basic differences between this scheme for planarians and the proposed scheme for pycnogonids is that in the latter the localization of endopeptidases was not possible and functional gland cells are not found. The gland cells of planarians noted by Jennings are clearly not the later stages of absorption cells.

6.3.2.1 FORMED BODIES

Other reports in the literature having a bearing on pycnogonid digestion concern the nature of the 'Spherical bodies' or 'Enigmosomes'. Thus Riegel (1966a and b) reported finding 'Formed bodies' in the excretory organs of the crayfish, frog and stick insect. He found (1966a) that in the course of formation of urine by the crayfish antennal gland, bodies were secreted and these he called 'Formed bodies'.

These are of lipid character (sudanophilic) contain digestive enzymes, bound potassium, are refractile, yellowish under brightfield illumination and are normally spheroids of less than 10 μm diameter. Photographs of the formed bodies from antennal glands show them to be morphologically very similar to the spherical bodies described in pycnogonids.

Kummel (1964) has presented evidence that vacuoles in the apical region of coelomosac cells of the crayfish are formed by the fusion of tiny vesicles which appear to be produced by the golgi apparatu Riegel regarded these vacuoles as identical with the formed bodies which he collected from the coelomosac. Riegel also considered these spheroids to be structured elements rather than bags of loose accumulated materials (this parallels the enigmosomes). In the work of Wigglesworth and Salpeter (1962) it was seen that small spheres of 0.1 μm and larger were discharged from cells of the upper portion of the malpighian tubule of stick insects. They thought these to be crystallisation nuclei for uric acid (see plate ). Riegel, however, has argued that these are the spheroids he describes as forme bodies. The similarity between the crystallisation nuclei reported by Wigglesworth and certain of the pycnogonid enigmosomes has alread been noted.

6.3.2.2 A NOTE ON THE DISSEMINATION OF INFORMATION

In the writings of both Jennings (1962) and Riegel (1966 a and b it is fascinating to follow a zoological approach to the lysosome

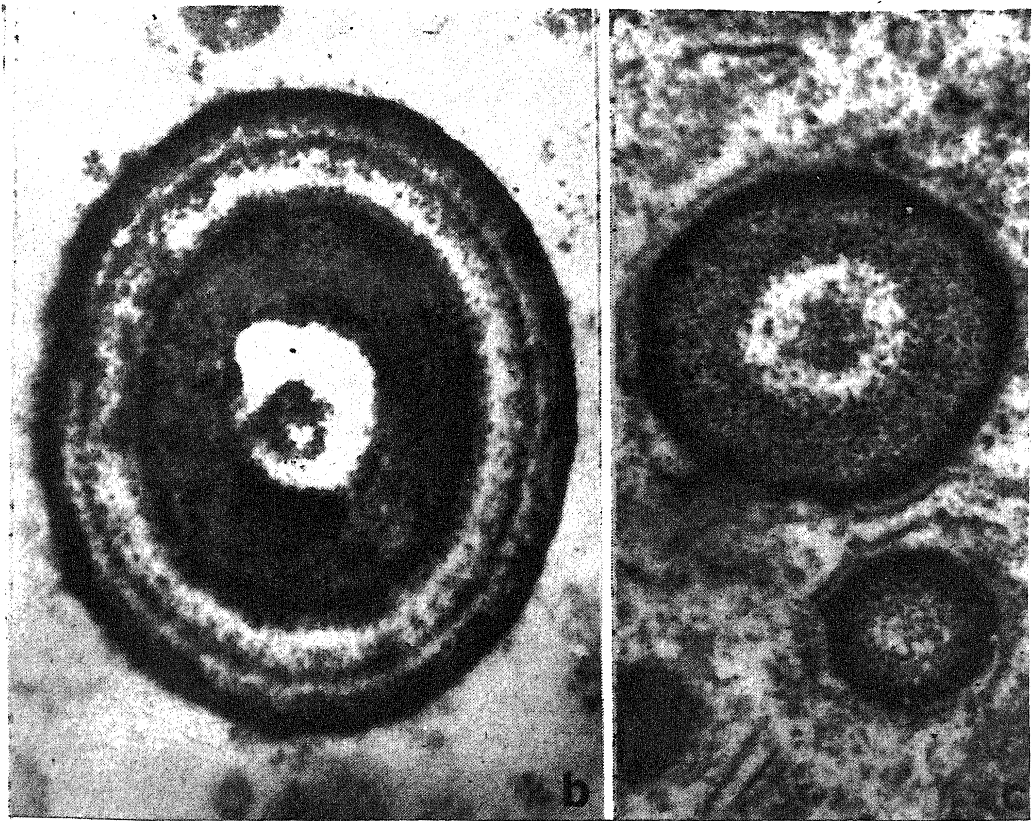



PLATE 6 G. Crystallisation nuclei for uric acid ((Wigglesworth and Perry, from Threadgold (1967)).

concept. Whilst biochemists such as DeDuve and his co-workers and morphologists such as Straus and Palade and co-workers were developing their concept mainly from work on mammalian cells, it can be seen that at the same time similar views were being developed quite independently from work on invertebrates.

Riegel (1966a), for example, comments 'It does not seem to be speculating too wildly to suggest that formed bodies underly a very large number of secretory processes. The production of formed bodies would seem to be a natural corollary to pinocytosis which is to be found in a wide variety of cells.' In 1966b he reported the work of Kummel (1964) who, working on the antennal gland of Cambarus affinis presented evidence that vacuoles in the coelomosac cells are formed by fusion of tiny vesicles in the golgi apparatus. As previously mentioned, Riegel concluded that these vacuoles are the same as formed bodies. Formation from tiny vesicles in the golgi apparatus is in agreement with Palade's scheme for the formation of lysosomes.

Jennings (1962) noted that acid phosphatase is closely linked with the first or endopeptidase stage of digestion and cited Rosenbaum and Rolon (1960) as suggesting that it is associated with food vacuole formation. The classic phago-lysosome experiments of Straus (see e.g. Straus 1967) confirm Rosenbaum and Rolon's suggestion.

It is interesting that neither Jennings nor Riegel seem to have followed up the lysosome concept. Thus Jennings in his book on invertebrate digestion (1972) summarized his 1962 paper without comment. Indeed he made no reference in the rest of the book to lysosomes, any of their related particles (using the nomenclature given in table 6D), nor to DeDuve's nor Palade's work. Riegel, who in fact came very close to starting the lysosome concept in his two 1966 papers, in a later work on formed bodies (1971) is more interested in the role that they might play in ionic balance.

# CHAPTER 7

# BLOOD

7.1. THE CIRCULATORY SYSTEM

Aspects of the circulation and the way in which it may be affected by intestine pulsation have already been considered in Chapter 3. According to Dohrn (1881), blood is driven anteriorly by the heart into the dorsal antimere of the proboscis and then runs back along the ventral side and out laterally into the appendages. Flow back to the trunk occurs from the dorsal region of the appendages. The whole of the pycnogonid body is divided into dorsal and ventral cavities by a horizontal double walled membrane which is here referred to as Dohrn's septum.

Cole (1910) stated that "Although, on the whole, there appeared to be a real circulation from the body out into the legs and back, this was rendered more or less indefinite by the peristaltic contractions of the intestine, which imparted a sort of churning motion to the blood and kept it moving back and forth." This somewhat haphazard movement of blood is characteristic of a fairly primitive circulation. In specialized circulations, flow is directed throughout the system by a series of valves and the pressures are steadily maintained (Hoar 1966). Dohrn (1881) found that there were no vessels apart from the heart. Firstman (1971) described an altogether more complicated system. He claimed that there are two haemocoelic spaces:-

1) A PERIVISCERAL ARTERIAL SINUS

2) A VENOUS HAEMOCOEL which is further subdivided into

ventral venous haemocoels and the pericardial cavity.

According to Firstman, the perivisceral arterial sinus is formed from a membrane which is continuous with the aorta and which envelopes the intestine and central nervous system. It is continous with the double walled Dohrn septum (Figure 7A). Between the two layers of the Dohrn septum there lies a thin arterial blood sinus which is continuous with the rest of the perivisceral blood sinus. Firstman contended that freshly aerated blood is pumped from the heart into the perivisceral arterial sinus where it supplies the gut and the nervous system (Figure 7B). At the distal ends of this sinus, blood passes into the venous haemocoel (i.e. the remaining body cavity), where it is aerated. (vide supra), (Figure 7C).

As can be seen from Plates 7F and G, both the heart and the membranes associated with the body cavity are delicate structures. They are very prone to damage during histological preparation. Firstman's dexterity in displaying these by dissection is remarkable. When membranes are not damaged it is still difficult to define their relationships. Thus Plates 7D and 7E appear to show no perivisceral membrane. Plate 7J, on the other hand, indicates a membrane and sinus, but there are no connections with the nervous system. It would seem that if there is a perivisceral arterial sinus in the Nymphonidae, it does not enclose the nervous system. Firstman's claim of blood flow through this system

is challenged in that sections do not reveal many blood cells closely applied to the gut as one would expect. I have not seen blood cells between the two membranes of the Dohrn septum.

From Firstman's technique section, it does not appear that he examined the circulation of live specimens. Footnote (p.115) has already indicated my views on the limitations of techniques claiming the resolution which would be needed to see cells flowing within the Dohrn membrane in a live animal.

No muscles have been observed in the Dohrn septum during this work. This absence affects Firstman's argument on the evolution of the pycnogonid endosternite. The possibility of the occurrence of muscle fibres within this septum is not precluded, however, but as will be seen the muscular nature of even the heart is difficult to demonstrate histologically.

Helfer and Schlottke (1935) stated that the heart wall is composed of numerous muscle fibres whose nuclei lie in an anterior/posterior line. Hoek (1881) remarked that the fibres are not branched and not striated. Plates 7H and 7I show that, contrary to Hoek, the heart wall possesses true striated muscle. The striations are not easily recognisable in every section, and indeed their demonstration appears to depend on technique. Thus Plates 7H and 7I are of sections stained with a somewhat unusual com-

bined stain for protein, carbohydrates and nucleic acids (a Feulgen/ P.A.S./ Naphthol combination - Ruthman 1970). Striated mescle is also demonstrated with other stains but not as well. Plate 7K gives an indication that muscle occurs in bands rather than as a sheet but information from sections cut in this plane tends to be variable. Plates 7H and 7I were taken using a phase contrast microscope, an apparatus which was not available to workers up to and including Helfer and Schlottke. Most of these plates were taken using oil immersion objectives near the limits of resolution of the light microscope. (It is unlikely, therefore, that such fibres would be detectable under the dissecting microscope).

In his review of arthropod hearts, Krijgsman (1952) concluded that Limulus was the only arthropod in which the heart mechanism was clearly understood. Though Harvey considered work on frogs in his demonstration of the circulation in man, it was not until the use of X-ray cinematography of radio-opaque dyes that Foxon and Rowson (1956) worked out the complete details of circulation in that animal. As was mentioned in Chapter 3, Carrel and Heathcote (1976) have used an exotically named 'cool laser transillumination technique' to clarify heartbeat in spiders. Although there has been much research into the circulation of blood in man, other animals, and particularly invertebrates, have been comparatively little studied.

It is suggested that the use of radio-opaque dyes, and even cold laser transillumination techniques are required to investigate blood flow

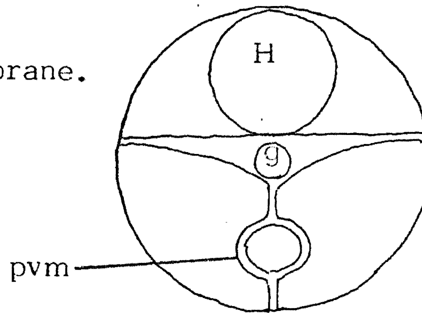
and heart and gut movements in pycnogonids. The complexity of such an investigation requires a thesis in itself.

FIGURE 7 A. Redrawn from Firstman 1971.

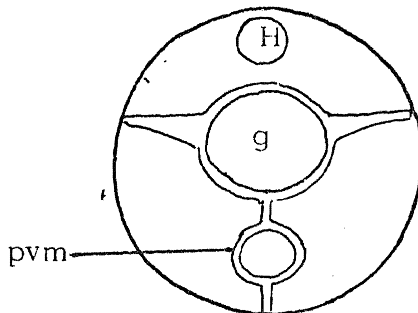
H = heart,

g = gut,

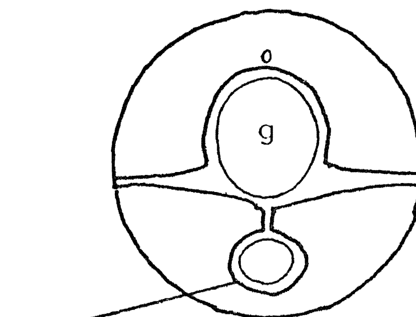
pvm = peri neural vascular membrane.




Colossendeis




Endeis




Pycnogonum

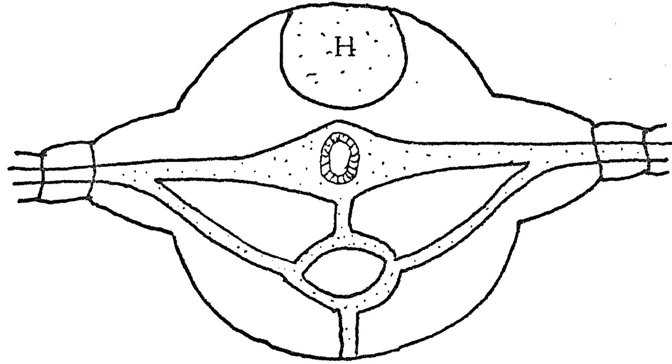



FIGURE 7 B.

Transverse section through a generalised pycnogonid. The heart and arterial system are stippled. (Redrawn from Firstman 1971).

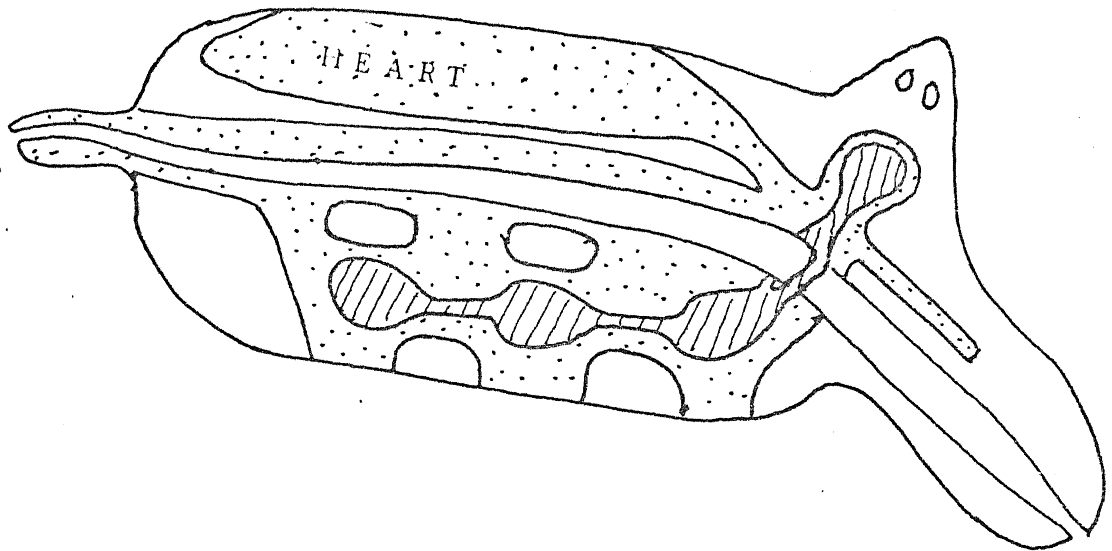



FIGURE 7 C.

Redrawn from Firstman (1971), Midsagittal section of a generalised pycnogonid . The central nervous system is shaded with diagonal lines, the heart and arterial system are stippled. Firstman's blood flow direction lines are omitted, I do not find such an extensive arterial system (see plates).

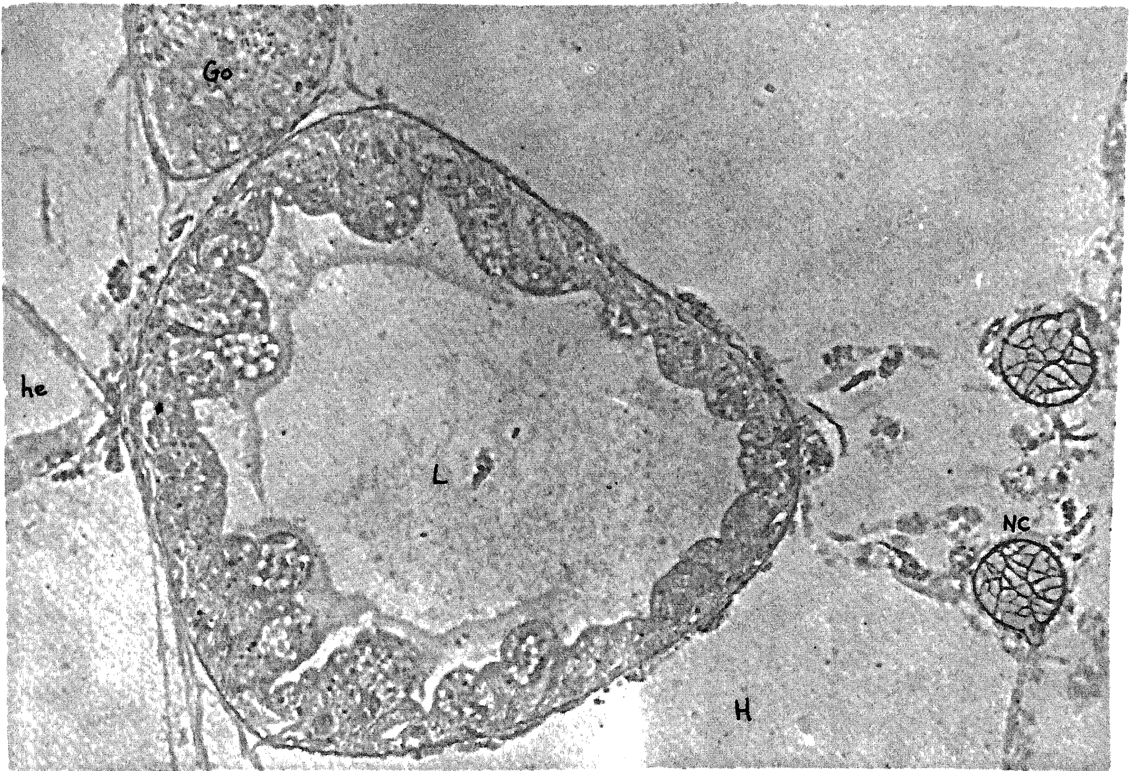
nc = nerve cord

he = heart

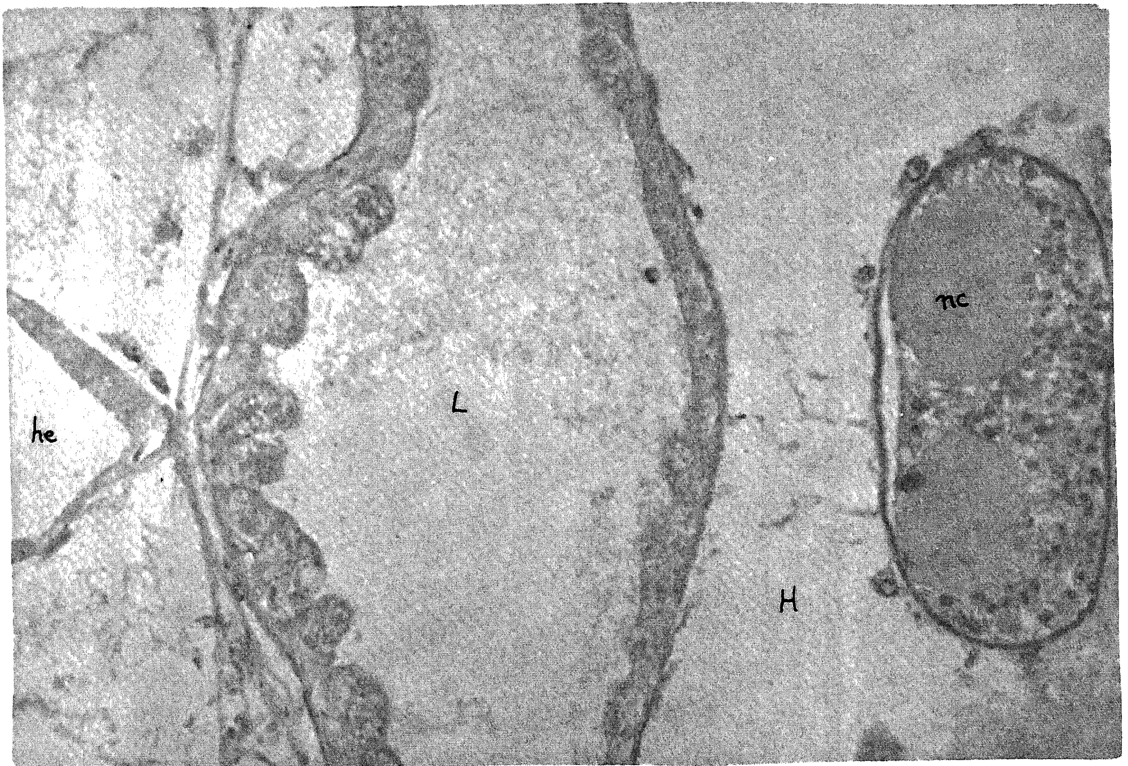
H = haemocoel

L = gut lumen

Go = gonad




PLATES 7 D & E. T.S. through trunk in region of ocular tubercle. Note the absence of a pericardial sinus.



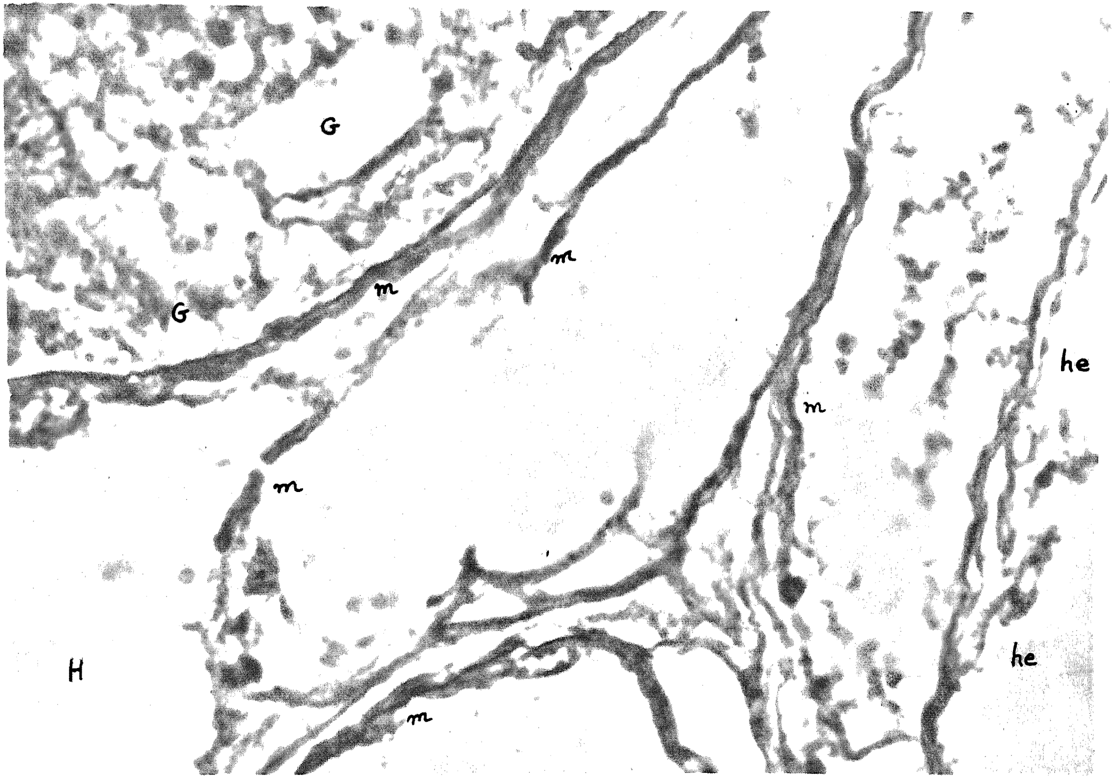

≙ 20μm.

G = gut cells

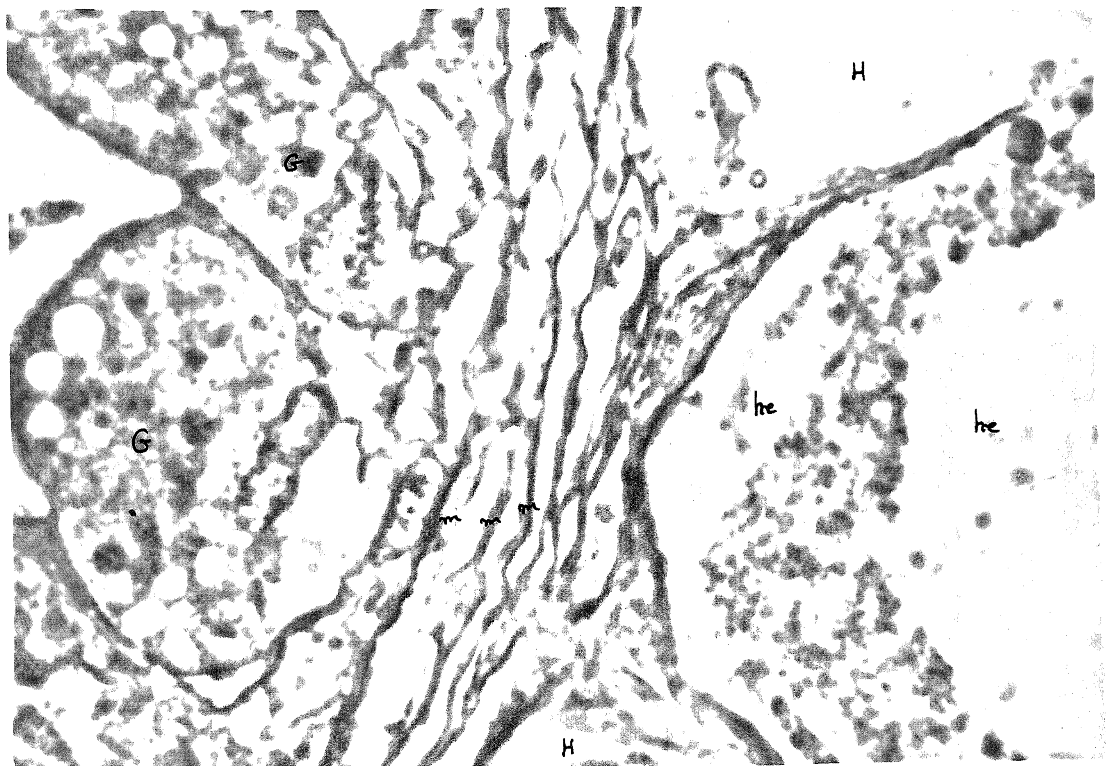
he = heart

m = membranes

H = haemocoel




PLATES 7 F & G. Heart and associated membranes

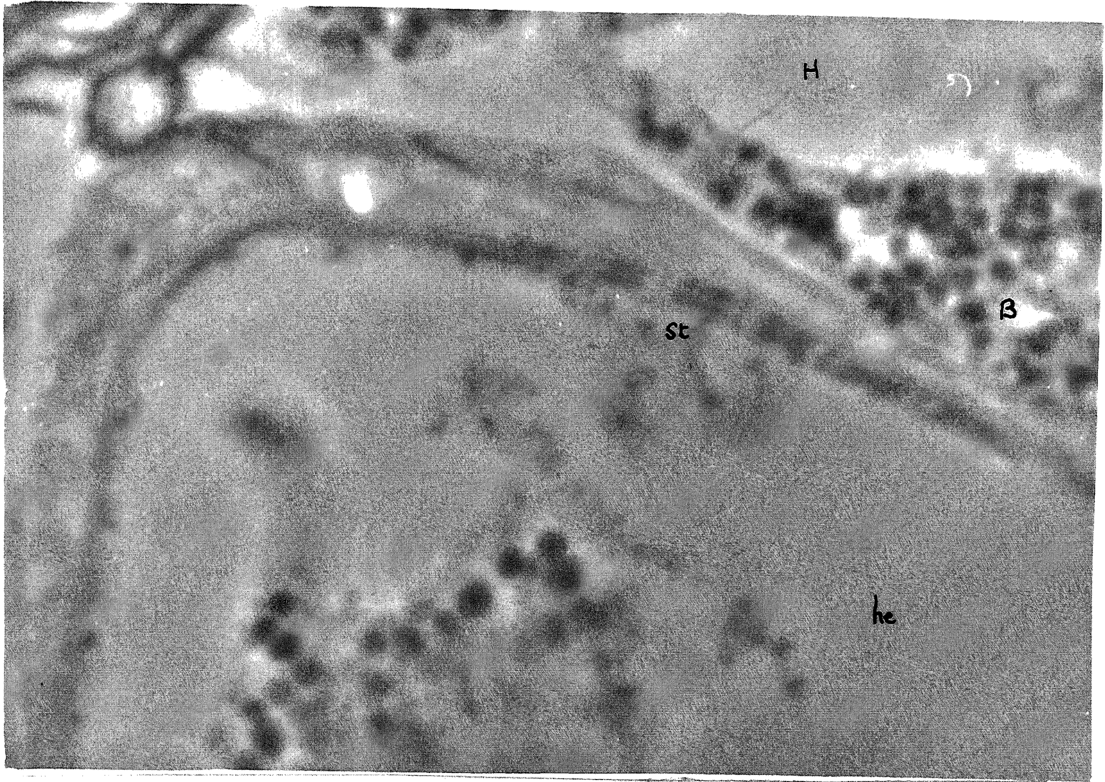


St = striated muscle

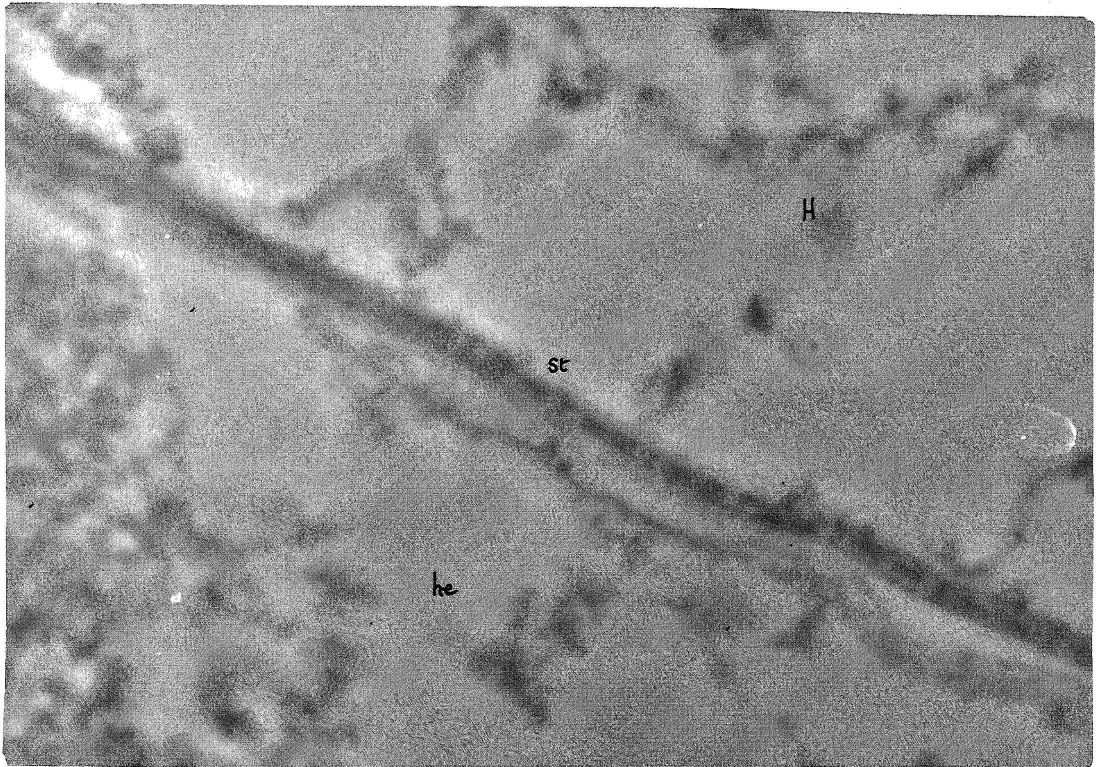
H = haemocoel

he = heart

B = blood cell




PLATES 7 H & I    Striated heart muscle.




≏ 8μm

≃ 30 μm.

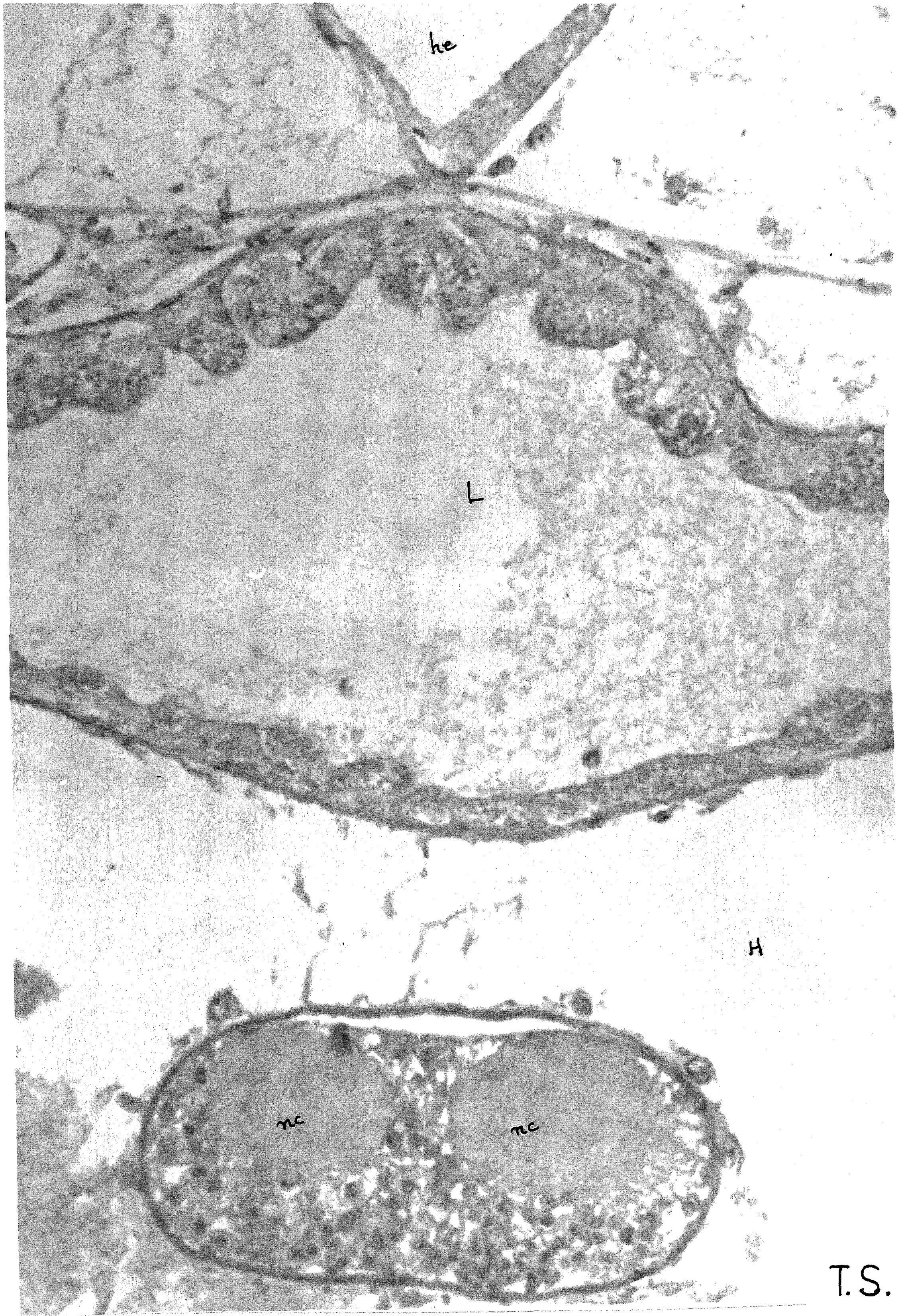



PLATE 7J. Membrane and sinus, but no connection with the nervous system.

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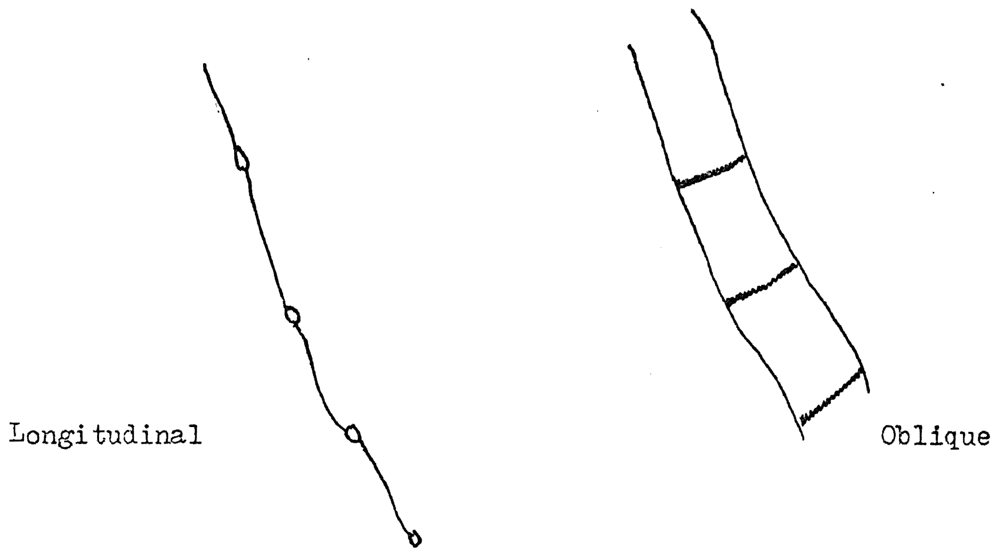



Above = views of heart wall in longitudinal and oblique sections. Swellings in the wall in longitudinal sections show as striations in more oblique ones. It is suggested that each swelling represents a muscle fibre , and that these may be distributed as is shown below.

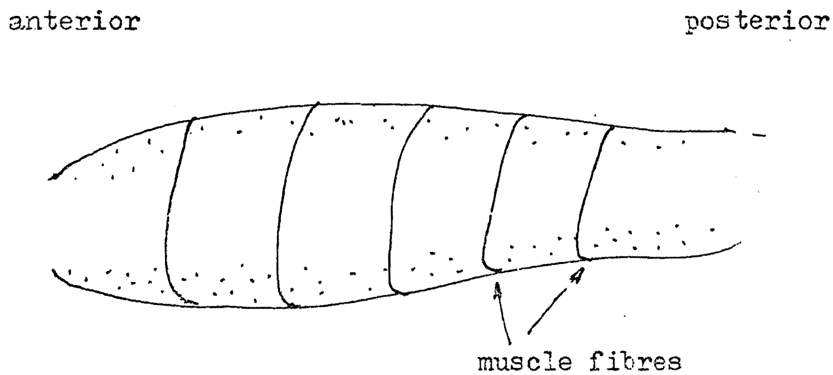



FIGURE 7 K.

## 7.2. BLOOD CELLS

The blood cells of pycnogonids were first closely examined by Dohrn (1879). He found three types of cell. The first type he described as large and balloon-like with a lenticular nucleus and three or four vacuoles. Hoek (1881) termed these blood cells 'Fat cells' whilst Cuenot (1891) called them 'Hematies'. Helfer and Schlottke (1935) considered that this type of cell forms the majority of pycnogonid blood cells. The second type of cell was called an 'Amoebocyte' by Dohrn and has granular cytoplasm. Cuenot (1891) described these as fusiform in shape and about 10 microns long. Loman (1917) found that these did not take up neutral red whilst the balloon cells did. Dohrn described a third type of cell, but Helfer and Schlottke considered that his method of obtaining blood from the cut ends of amputated walking legs may have given rise to preparations contaminated with gut cells and assumed this third type to be such contamination. Dawson (1934) described the blue blood of _Anoplodactylus lentus_ but did not distinguish two types of cell.- His illustrations show shapes which differ from those found by Helfer and Schlottke (1935), Sanchez (1959) or in the present work. Sanchez (1959) found two types of cell. The balloon cell she called 'Leucocytes hyalin', the amoebocytes 'Granulocytes'. Dawson (op. cit.) found the colour of the corpuscles of _Anoplodactylus lentus_ ranged from light pink to dark blue. The cells appeared to highly specialised. He found them to extemely flexible, being folded longitudinally, twisted spirally or folded transversely when subjected to

pressure from the pulsatory intestinal caeca (*Anoplodactylus lentus* is sufficiently small and transparent for the entire animal to be compressed under a small coverslip). He found that no bodies other than the nucleus could be distinguished in fresh cells. In stale preparations the pigment of the cell lost its homogeneity and denser globules of varying size were formed. In sealed coverslip preparations which had stood for several hours, cells developed fine beaded processes at their surfaces. Minute globules separated from their free ends and persisted for some time as free bodies in the plasma. Dawson's results on investigations of the pigment found in these cells were inconclusive. He stated that further work was needed for the chemical nature and functional significance of the cells to be ascertained.

Sanchez (1959), working on cells from *Endeis spinosa*, found that the hyaline cells were formed first during development and were characterised by a pycnotic nucleus, and an irregular shape. Granulocytes appeared later. Under poor conditions, the hyaline cells put out pseudopodia. Vital dyes such as brilliant cresyl blue coloured only one or two vacuoles. The granulocytes, which were much more numerous, were coloured strongly by vital dyes. Sanchez described the role of the blood as multiple. She suggested an excretory role in which the pits in the cuticle, where direct communication fo the exterior is only prevented by a thin hyperdermis, act as regions through which granular cells can rid themselves of phagocytosed excretory material.

Helfer and Schlottke stated that, when circulating the fusiform amoebocytes have no pseudopodia but form them when they collide with objects or when they are released into the surrounding medium if a wound occurs. 'Balloons' seem to be present in greater numbers slightly before ecdysis and in lesser numbers afterwards. Diagrams of the stages of development of these cells are shown as are a similar series for granulocytes (Figure 7b ). The balloons are found mainly in the trunk, particularly in clumps at the bases of the walking legs. Loman (1917) and others seized upon an excretory role for such cells. Helfer and Schlottke's scheme for amoebocyte development shows a general uptake of excretory granules. These amoebocytes are lost either at ecdysis or through the cuticle in the region of the genital pore.

Whilst the appearance of blood cells is apparently uniform in all species examined, there is a variation in size. Thus, for example, there are tiny cells in Anoplodactylus pygmaeus and giant ones in Decolopoda australis.

In the work discussed here, blood was collected from ovigers (to prevent contamination with gut cells) by severing the appendage in the region of segment 6. A micropipette, made by the method of Ramsay and Brown (1955) and which had been wetted with a 10% E.D.T.A. solution which had then been allowed to dry (Baker, Silverton and Lucock 1971), was then inserted into the cut end of the ovigerous leg and suction

applied. It was found that blood flowed (escaping cells were visible) for at least ten minutes if the cut were performed under seawater, whilst clotting was complete within 30 seconds if the wound was made in air. There was little difference in the appearance of cells in their own plasma compared with cells in a plasma and seawater mixture.

Blood cells were examined with a Wild M20 phase contrast microscope, with and without application of vital dyes (applied by the evaporation method quoted in Chapter 5).

N. orcadense

As with previous reports on pycnogonid blood, two cell types were detected, hyaline 'balloons' and granular cells. There seem to be variations in the size and shape of granulocytes and balloons, so that it may be more correct to say that there are two cell groups. It is possible, however, that these variations may reflect different stages in the cell cycle. It is noted that individual cells change their morphology as they are observed in vitro. Hyaline balloons appear very fragile in comparison with the granulocytes. The former were the most numerous cell type in preparations from this species.

In addition to numerical differences, there are also differences in the staining reaction of the cell groups with vital dyes. Brilliant cresyl blue stains hyaline cells intensely, areas of the cell varying from purple

to pink. Granular cells stained blue only after prolonged exposure. As has been noted, hyaline cells are fragile, and as they disintegrated they became pink overall. Granular cells retained their shape.

With acridine orange, the hyaline balloons stained more intensely than the granular cells. Azure B stained both cell types, as did Nile blue. With a Janus green/neutral red mixture the results are summarized as follows:-

These findings are in agreement with Loman (1917), who found that the hyaline cells took up neutral red, whilst the granular cells did not; and in disagreement with Sanchez (1959), who found the granular cells of *Endeis spinosa* were more darkly staining with vital dyes than hyaline cells.

As mentioned above, it has been found that the appearance of the cell changes with time after removal from the animal. It has been found that whilst the structure of the granulocytes remains relatively constant, the behaviour of hyaline cells is very different. Dawson (1934), studying the blood cells of *Anoplodactylus lentus*, found that, as his preparation aged, fine processes were sent out and minute globules separated from the cells as free bodies in the plasma. Eventually there appeared to be complete breakdown of cells. A similar process was seen in the hyaline cells of *N. orcadense*, particularly in the region of foreign particles (eg, Diatoms). Masses were formed similar to those found by Grégoire in his investigations of arthropod clotting mechanisms. Granular cells were never observed blebbing in the same way.

In an attempt to investigate the possibility of different functions of the two cell groups, blood was withdrawn and carmine particles in sea-water were injected into the wound ($\simeq 20 \mu l$ with a Hamilton syringe). After a period ($\simeq 6$ hours) further blood was withdrawn. Carmine particles were observed in the granular cells of the blood, but not in the hyaline cells.

It is proposed that hyaline cells are concerned with blood clotting and the granular cells with phagocytotic activity.

Ammothea carolinensis

Blood was withdrawn from Ammothea carolinensis by the method described above. It was found that granular cells were of a similar appearance to those of N. orcadense, but the large hyaline cells were different. These appeared more like clear droplets than cells, the nuclei which were obvious in the hyaline cells of N. orcadense being absent. These droplet cells stained intensely with Azure B. Granular cells appeared to be in greater numbers, but a haemocytometer for small volumes would have to be designed before any quantitative measurements can be made.

There was no clumping of cells as happened in N. orcadense. Hyaline or droplet cells took up Bismark brown quite readily. This property may be one reason for Hoek (1881) naming hyaline cells 'Fat cells'.

Decolopoda australis

In D. australis, blood cells differed from those of both species described above. Only granular cells were found.

In all three species mentioned, the cells appeared approximately the same size. There was not the progression from small cells to large cells as described by Helfer and Schlottke (Figure 7K). It must be assumed that either the blood cells of Antarctic species are larger than those of temperate forms or that the large cell of Decolopoda austrlalis described by Helfer and Schlottke is an artefact.

Grégoire (1970) has suggested that the reasons for the fragmentary and contradictory results so far obtained on blood coagulation in arthropods are technical. The fixation techniques of bright field microscopy interfere so seriously with the natural behaviour of the haemocytes and the plasma that the true relations between them are obliterated. Use of the phase contrast microscope in investigations of arthropod blood (Grégoire and Florkin 1950) has permitted the elimination of many artefacts. It has been noted that in preserved pycnogonid material (sections of N. orcadense), blood cells are generally poorly preserved and all seem granular, resembling the later diagrams of Helfer and Schlottke. Cells with such large granules were never observed under phase contrast in vitro.

Hardy (1892) discovered a category of very fragile blood cells in crustacea ('explosive cells') which were selectively altered on contact with foreign surfaces. Grégoire (see Grégoire 1970) has found that fragile haemocytes or explosive cells seem to be a peculiarity of arthropods. The role of these fragile cells and that of vertebrate platelets in starting the alterations to the plasma during clotting might be considered

an example of functional convergence in distant zoological groups. Fragile haemocytes represent a high degree of functional specialization and, as indicated, it would seem that these are present in the Nymphonidae in conjunction with granular amoebocytes which have a different specialized role. The different form of the hyaline cells in Ammothea carolinensis and their apparent absence in Decolopoda australis is of considerable interest and worth further study.

Hedgpeth (1947) stated that it is usually contended that the Nymphonidae are the most generalized group of the pycnogonids retaining primitive attributes. The Colossendeidae are intermediate and the other families branch from the tree according to individual fancy or taste. It is interesting to speculate whether a study of blood cells, the proportion of the types and the clotting mechanism in different pycnogonids could help elucidate the relationship between families. The alternative proposal is that differences are concerned more with the adaptation of the animal to its own particular environment.

With N. orcadense it is seen that an inshore benthic animal whose environment is constantly churned up by storms, or scoured by 'bergy bits' (as was noted on Signy Island) is susceptible to damage. It was not unusual to collect N. orcadense in which appendages or parts of appendages were missing, and evidence of regeneration indicated that

damage was not due to the collection process. It is suggested that such damage-susceptible benthic scavengers would require an efficient clotting system to prevent excess loss of blood. Decolopoda australis and Ammothea carolinensis are less ubiquitous and appear to occur associated with one particular anemone or hydrozoan. They occur in deeper water and it is postulated that they are perhaps less prone at greater depths to wave or ice action. No specimens of these species were collected in which there were signs of regeneration of limbs. Comparative blood clotting rates for the three species were not measured, but subjectively it seemed that the 'blood letting' method had a greater effect on A. carolinensis and D. australis. Whilst N. orcadense were feeding and appeared normal 35 minutes after the removal of an ovigerous leg, the other two species seemed 'sick' for some days. It is considered that, because the animals are less frequently damaged in their natural habitat, the blood cells of A. carolinensis and D. australis are more concerned with the ingestion of foreign material (i.e. granulocytes), than clotting (hyaline cells). If an excretory role is also carried out by blood cells it is imagined that in A. carolinensis and N. australe with a greater surface area to volume ratio a greater number of amoebocyte cells would be required for excretion transport (if amoebocytes are the excreting cells).

## BLOOD ELECTROPHORESIS AND CHROMATOGRAPHY

There seems to be considerable generic, inter- and intraspecific variation in quantity and composition of arthropod blood. These fluctuations occur in relation to sexual activity, development, nutritional state, season, infection, parasitism and even with specimens of the same species caught at different localities (eg, Martignoni & Milstead 1964, Marty & Zoltan 1968). Buzatti-Traverso has used paper partition chromatography in a study of some genetic and taxonomic properties of animals. Chromatography and electrophoresis of body fluids is employed routinely in medical diagnosis (eg, Smith 1967). Much can be learned about the state of animals by application of these two techniques.

Chromatography and electrophoresis were carried out with the intention of discovering:-

a) If there were easily identifiable and constant species differences in blood chromatographic or electrophoretic patterns.

b) Whether the methods yielded results which could point a direction for further work (see later).

### CHROMATOGRAPHY

Ascending paper chromatography was carried out in a CHROMABAG tank (essentially a polythene bag stretched over a frame) using Whatman's

No. 1 chromatography paper with n-butanol, glacial acetic acid and water in the ratio 4 : 1 : 1 as the solvent for the first dimension. Iso-propanol, formic acid and water in the ratio 40:2:10 was used as the solvent for the second dimension when two dimensional chromatography was carried out. ( Arx and Neher 1963 ). In the method of blood extraction for examination with the phase contrast microscope, it was possible to extract only a smhll amount of blood from the Nymphon specimen. Chromatography of spots of small amounts of blood did not yield a detectable chromatograph when tested for ninhydrin positive substances (ninhydrin was prepared by the method of Arx and Neher ( 1963). Rather than allow a spot to dry before adding more blood, which would have involved bleeding an animal several times, a method was used in which a spot was made by squeezing out the contents of an oviger (i.e. this was not a pure blood sample but may have been contaminated with muscle).

The method was to remove an oviger from an animal which had been washed briefly in distilled water, blotted gently and placed ventral side uppermost under a Wild M5 stereo microscope. The ovigerous leg was then washed briefly in a jet of distilled water and placed on a piece of fresh 'Parafilm'. The animal meanwhile was replaced in the aquarium (the whole operation took about 30 seconds). As previously mentioned, Nymphon specimens recovered quickly and appeared normal in their behaviour after 15 to 30 minutes. With Ammothea carolinensis and D. australis the operation seemed to require longer recovery. The

oviger was removed from the 'Parafilm' to the origin of the chromatogram where its contents were squeezed out with fine forceps by moving them from tip to cut end. Every effort was made to prevent cross contamination with instruments. These were thoroughly washed between operations on different animals.

Chromatograms were run for about 8 hours in each direction and their results are given in Figures

In all chromatographic experiments with this greater volume of blood/oviger leg contents chromatograms were obtained which showed separation of a number of ninhydrin positive substances (amino acids or peptides having a free - amino group). Qualitatively there appeared little difference between male and female, fed and non-fed specimens of Nymphon orcadense in uni-directional chromatograms.
There appeared to be an extra spot in the chromatograms of N. australe and the chromatograms of A. carolinensis was different from either of these. The chromatograms were repeated several times with the same result. In two way chromatograms again there seemed little qualitative difference between blood from male and female N. orcadense specimens and again their chromatograms were different from those of A.carolinensis and D. australis. It was noted that in A. aarolinensis the chromatogram origin (i.e. oviger contents) turned orange when exposed to air. A similar phenomenon was noted with the gut of C. wilsoni when in vitro

experiments were carried out. This may be evidence of the occurrence of a respiratory pigment.

From the above experiments it was shown that it is quite easy to separate ninhydrin positive blood components of pycnogonids even with the most primitive chromatographic apparatus. There are species differences in patterns, these being small between closely related forms (N. orcadense and N. australe), more obvious between unrelated forms (N. orcadense and A. carolinensis). Such differences may prove useful taxonomically (eg, to distinguish between females of N. orcadense and N. australe morphologically). Sacrifice of the animals, or at least removal of a walking leg, is required for identification using a microscope. Blood letting and chromatography involve a lesser mutilation.

It is suggested that by the more sophisticated techniques of thin layer chromatography, with different solvents and detection methods, and with constant sample volumes, it should be possible to do quantitative analyses. These more sensitive techniques should allow the analyses to be carried out on blood alone (extracted by a pipetting technique) rather than oviger squashes. Clarke (1972) has used thin layer chromatography to examine seasonal variation in Antarctic benthic invertebrates. It would appear that such methods could be used to detect lipids, proteins or carbohydrates changes with season, nutritional state, sex etc. (the fluctuations mentioned at the start of this section). The apparatus required

is not complex or bulky, important considerations on an Antarctic base.

It is further suggested that the fate of radioactively labelled nutrients could be followed by scanning the radioactive distribution in thin layer chromatograms. Analysis of blood samples at different times after radioactive feeding and application of such methods may yield someof the diffusion and transport information discussed in Chapter 3.

ELECTROPHORESIS

Electrophoresis was carried out on cellulose acetate using a Millipore 'phoroslide' apparatus and run according to the makers' instructions for electrophoresis of human serum protein (Millipore Application Manual AM301) for protein analyses and the makers' instructions(Millipore Application Manual AM305) for lipoprotein analysis. In the serum protein experiment the Ponceau S stain was prepared according ti the method of Kohn (1970).

For serum proteins it was found that in both N. orcadense and A. carolinensis there was a separation into two bands with a 1 cm migration in the direction of the cathode. A run (with N. orcadense) for longer than that recommended in the instructions booklet gave a separation into three bands, but the pattern was wavy. Some experimentation was attempted with voltage and ampage of the apparatus and it became evident that it might be possible to improve the resolution of the pycnogonid serum

protein separation by experimentation with buffer, voltage, ampage and time of runs. However, time for this work was minimal and the fact that a separation could be obtained was considered sufficient.

Experiments on blood lipoprotein were carried out on Ammothea carolinensis only. It was found that a quadruple loading of the phoroslide apparatus was required. 'Appreciably more serum must be applied than for ordinary protein staining' (Kohn 1970). A separation into two bands, similar to those for the serum protein was obtained. The quadruple loading required to give a detectable staining reaction indicates that, compared with human blood at least, the quantity of lipoprotein in Ammotheid blood is small. Clinically, more than two loadings indicates a very sick human patient.

## CHAPTER SUMMARY

There are variations in blood between species. This is shown both in variation of cell types in morphology and proportional numbers and in the chromatographic patterns of ninhydrin positive substances.

In the Nymphon species it appears that there are hyaline cells for blood clotting, granular cells for phagocytosis. In Ammothea carolinensis the same system exists, although the hyaline cells are of a different appearance and the proportion of granular cells is higher. In D. australis only granular cells were found and clotting might involve a different mechanism. Electrophoresis shows at least two bands of serum protein and

lipoprotein to be present.

Blood chromatography and electrophoresis may be useful tools (methodology is relatively simple) both taxonomically and in relating blood composition to environmental conditions and intrinsic cycles (eg, reproduction)of the animal.

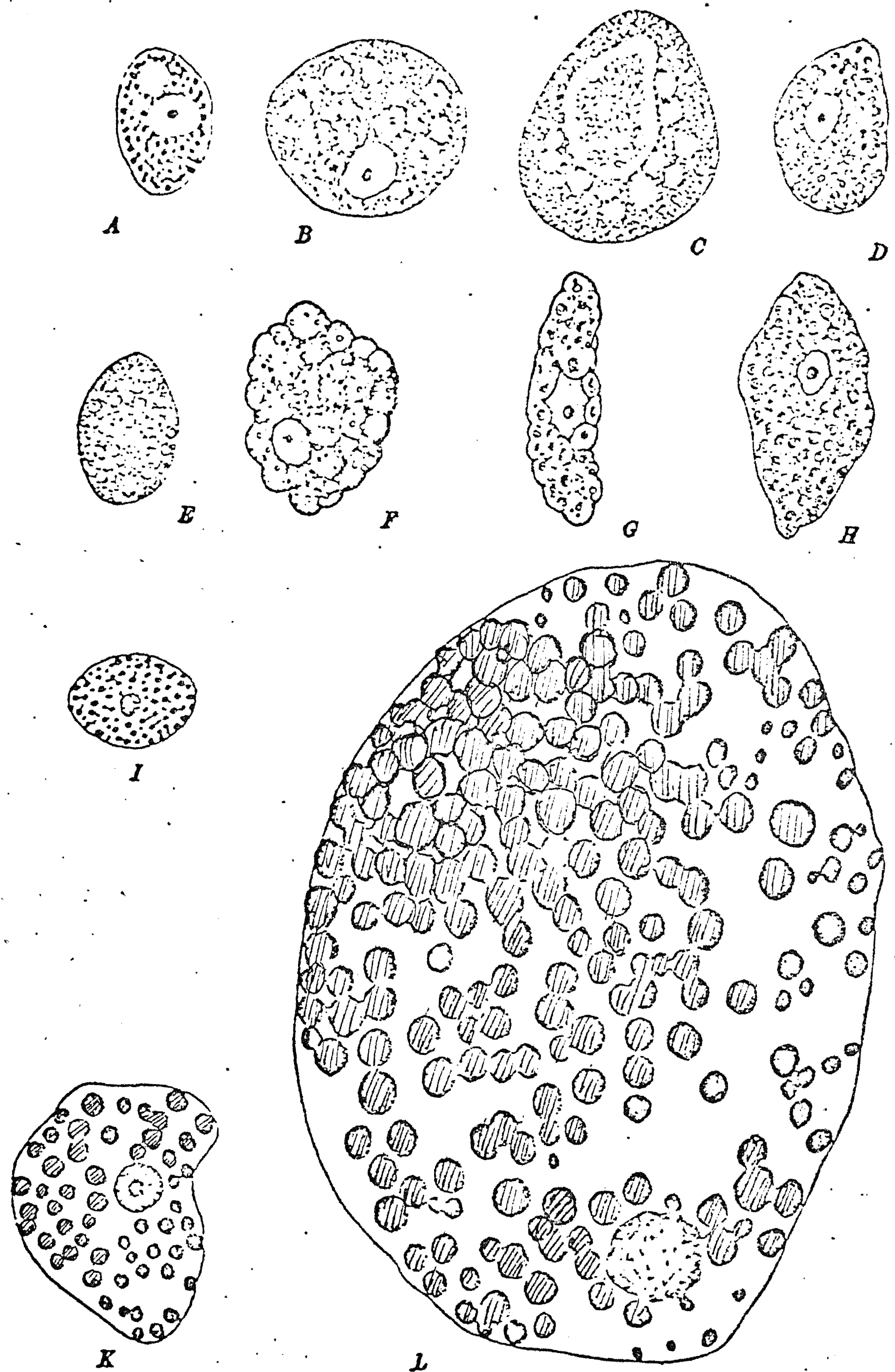
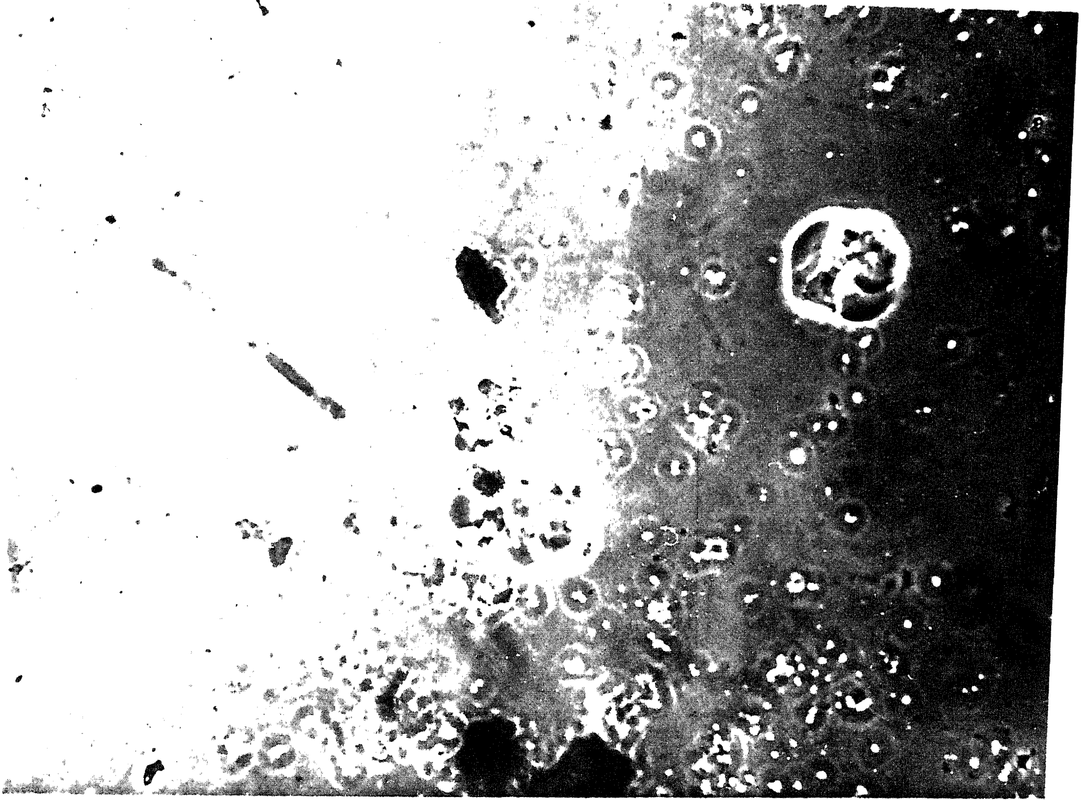
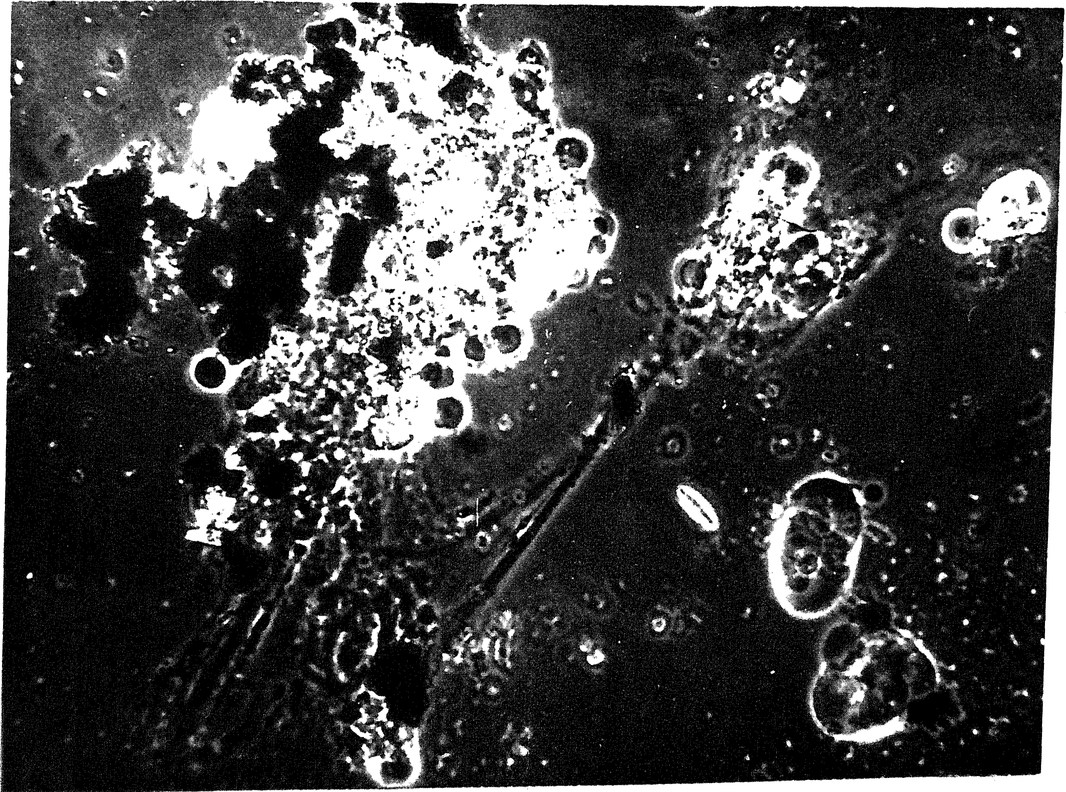
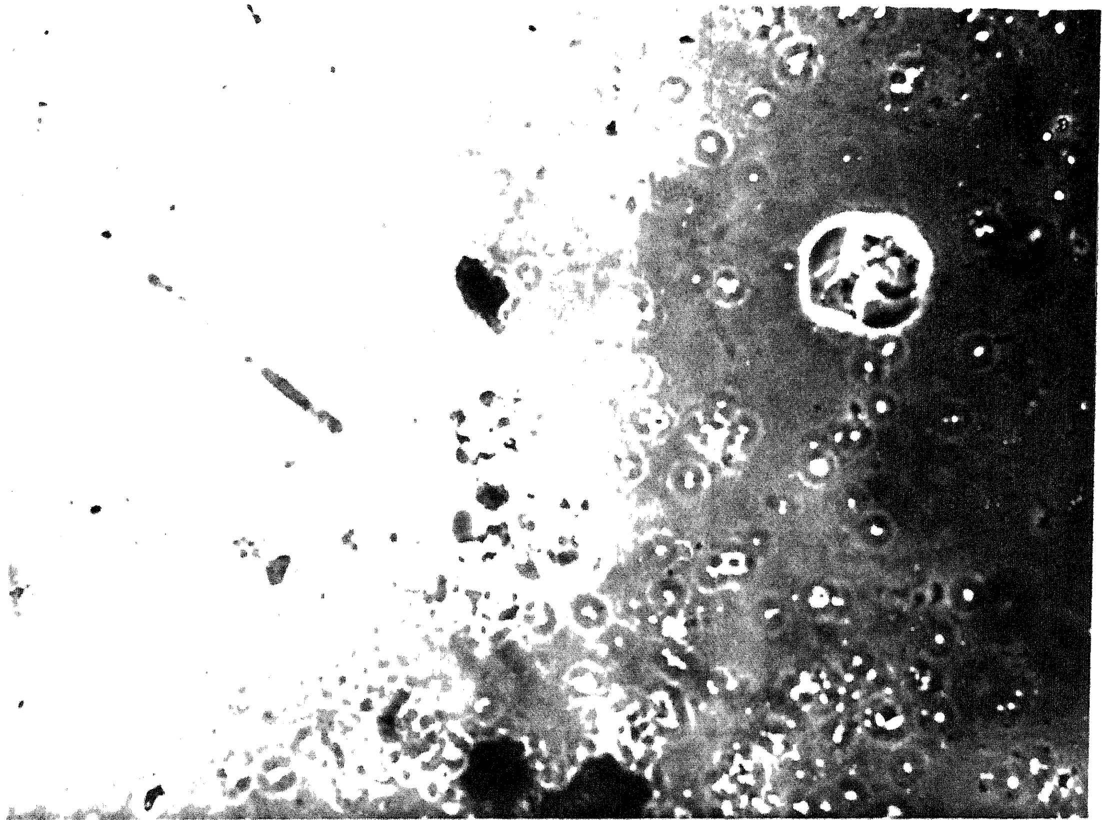


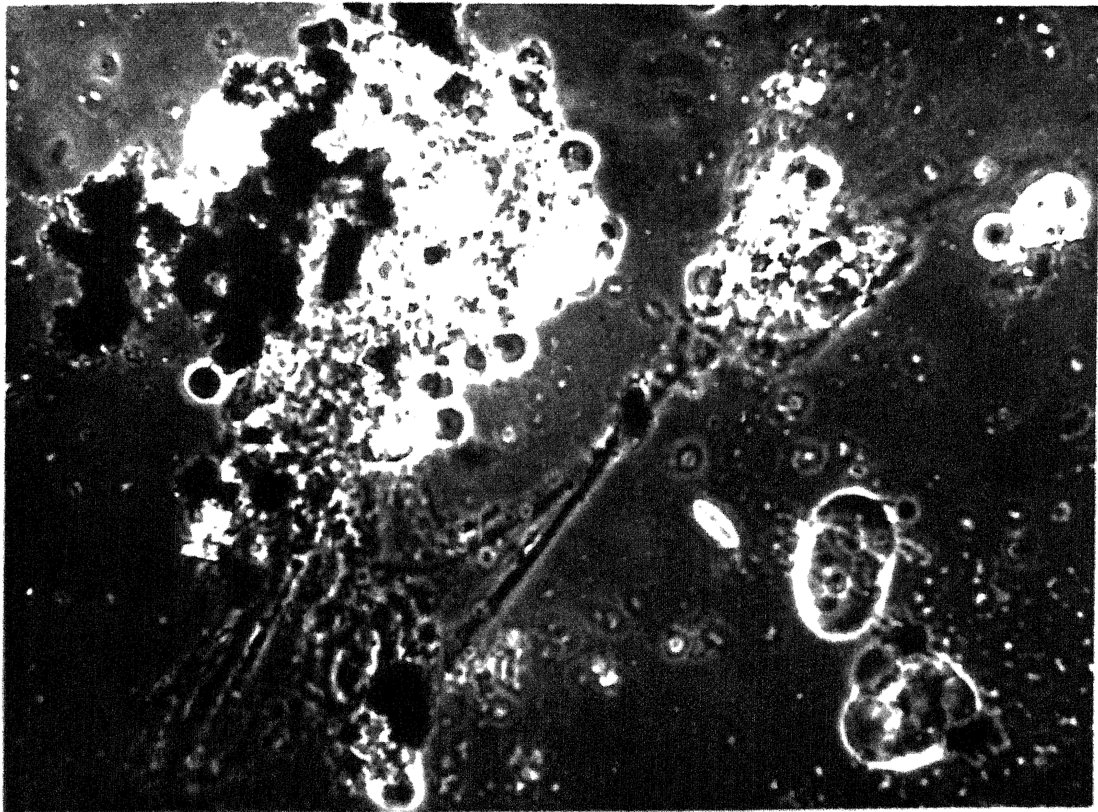

FIGURE 7 K. Progression of size of blood cells from different species as described by Helfer and Schlottke (1935).



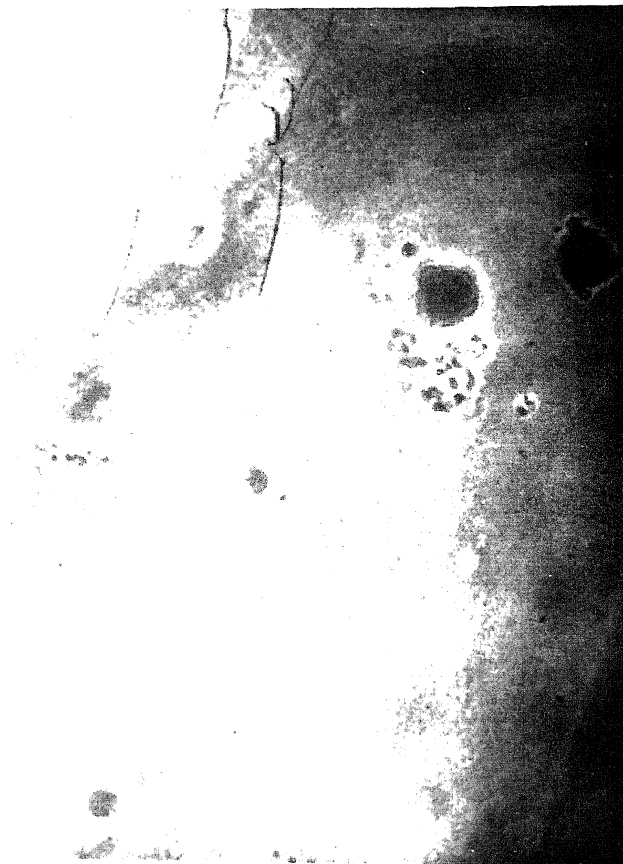


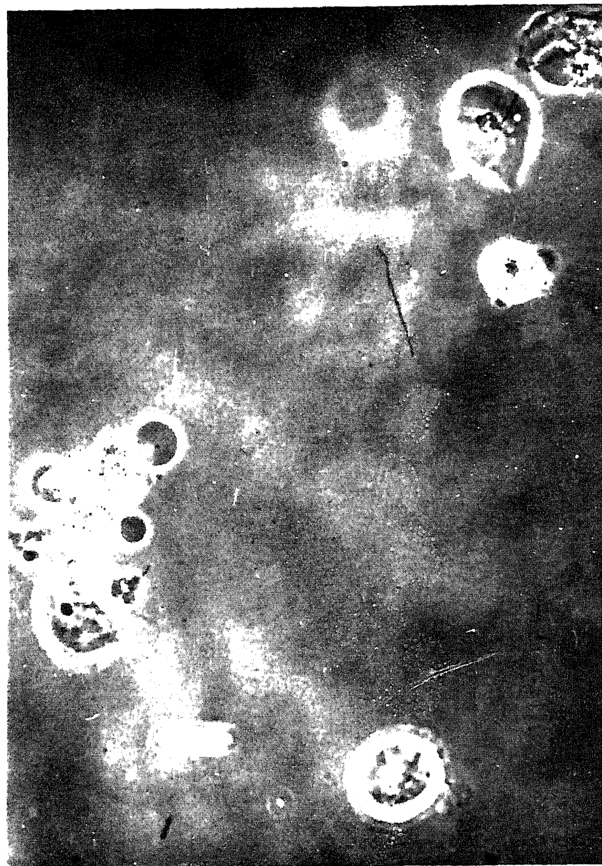
Coagulation islands in *Nymphon orcadense*.

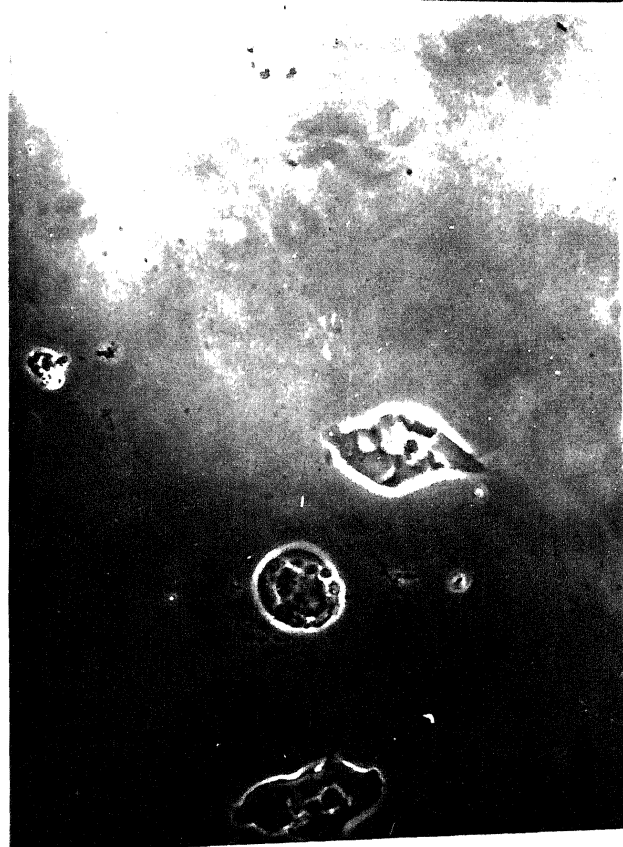


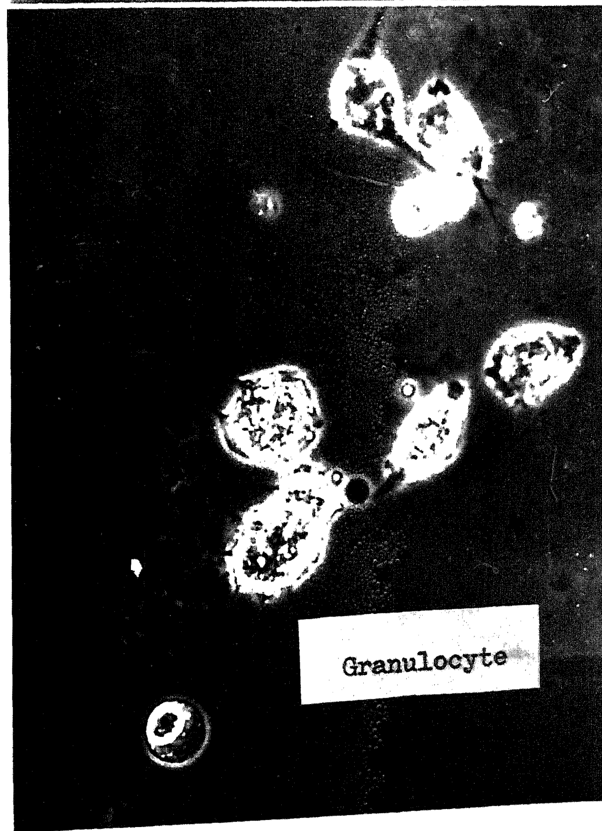


Coagulation islands in *Nymphon orcadense*.

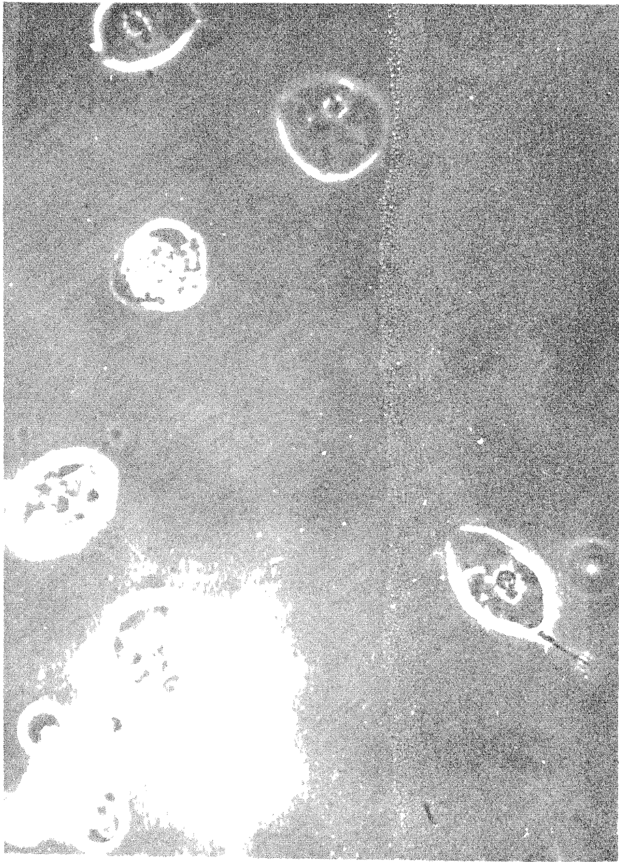


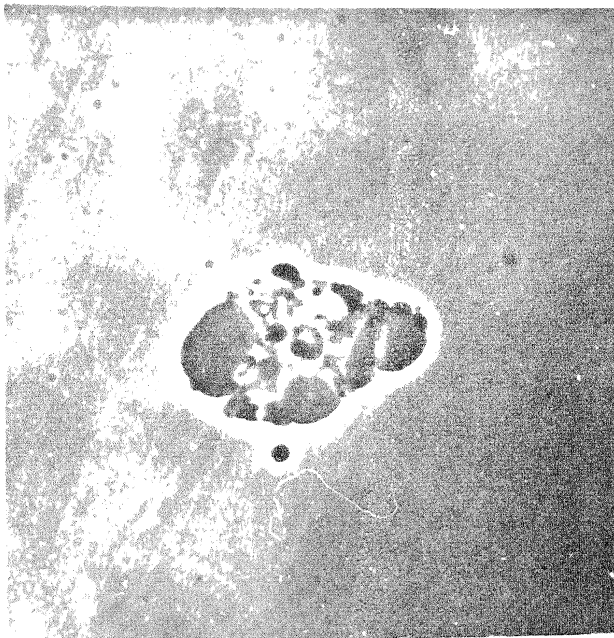


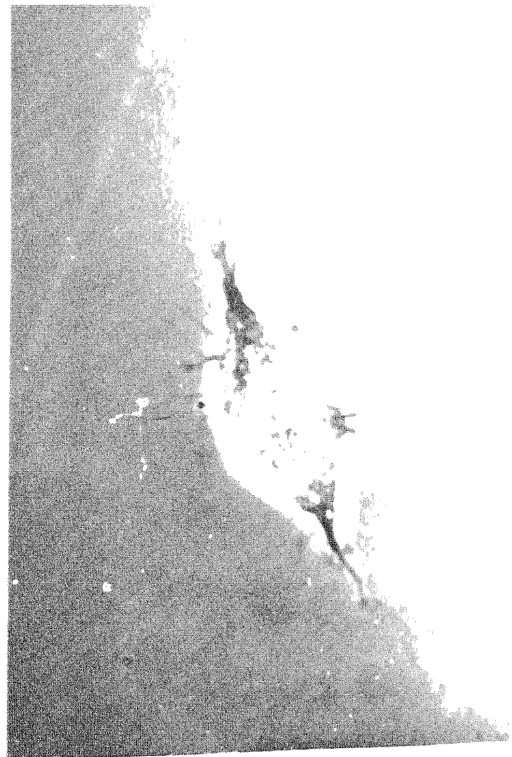


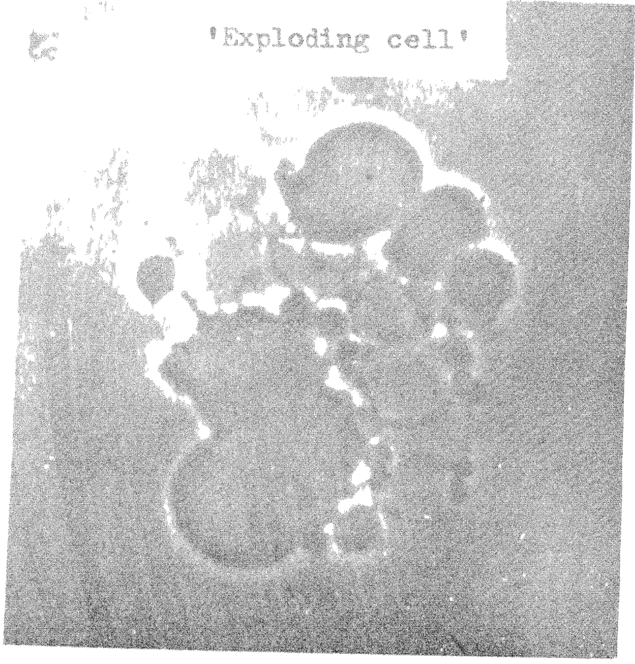

Granulocyte

Nymphon orcadense

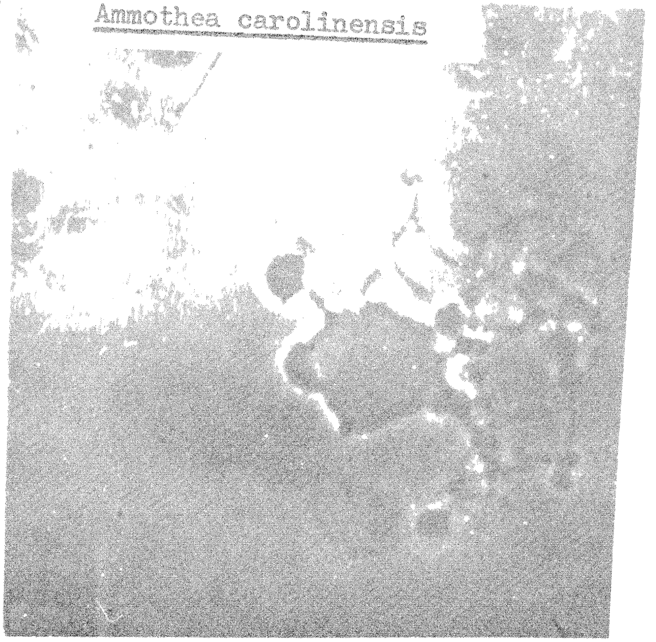




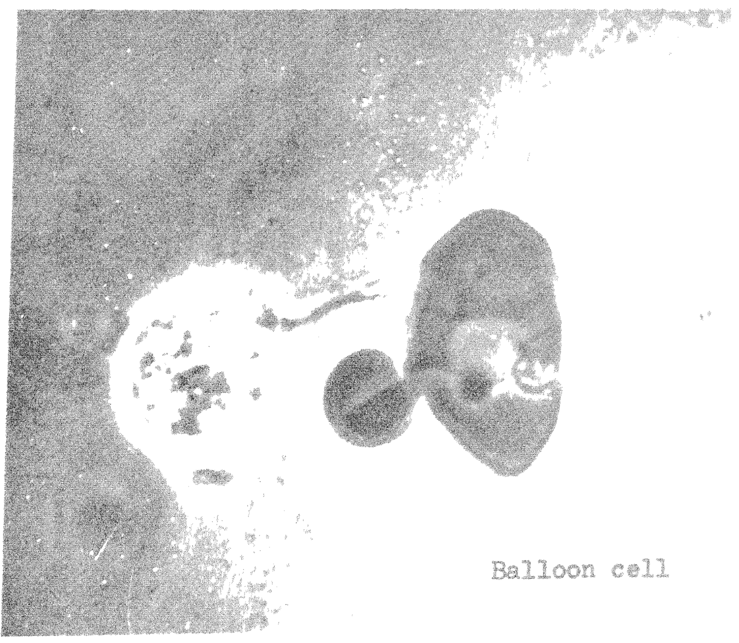




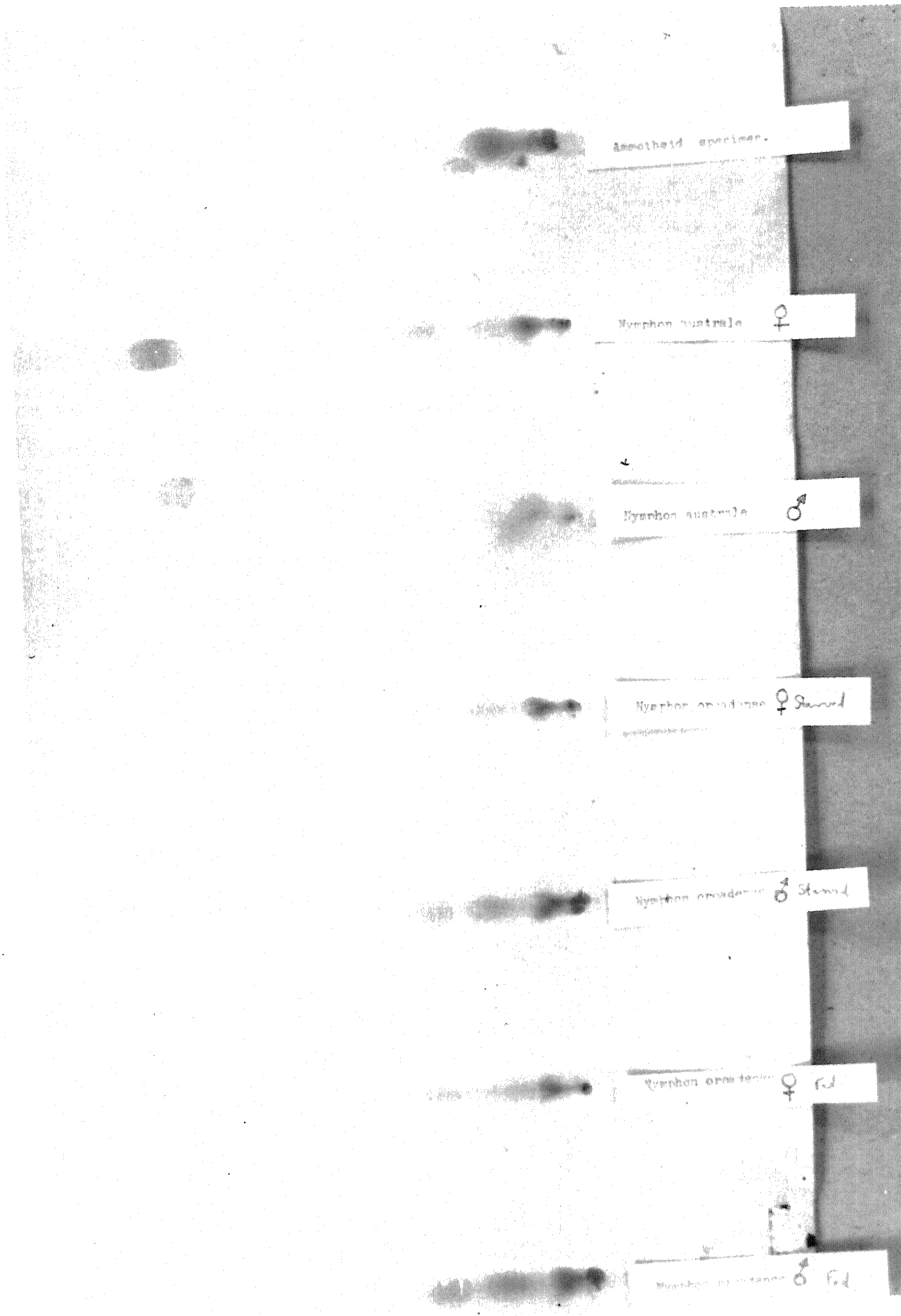
'Exploding cell'



Ammothea carolinensis



Balloon cell




WHOLE BLOOD CHROMATOGRAM (N. orcadense).

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