THE BIOLOGY OF PHASCOLOSOMA MINUTUM KEFERSTEIN

John Loudon Stevens

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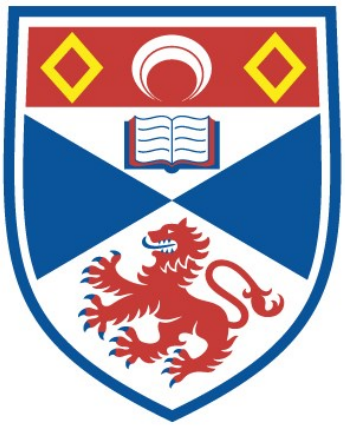
at the
University of St Andrews



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THE BIOLOGY OF *Phascolosoma minutum* KEFERSTEIN

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CERTIFICATE.

I hereby certify that John Loudon Stevens has spent nine terms at Research Work on The Biology of Phascolosoma minutum Keferstein, that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying Thesis in application for the degree of Ph.D.

J.M. Dodd.

1952

MS
1934

DECLARATION.

I hereby declare that the following Thesis is based on the results of experiments carried out by me, that the Thesis is my own composition, and that it has not previously been presented for a higher Degree.

The Research was carried out in the Department of Natural History, St. Salvator's College, St. Andrews.

J.L. Stevens.

PART I. THE ANIMAL IN ITS ENVIRONMENT

1. INTRODUCTION

There exist records of three species of the Sipunculida occurring in St. Andrews Bay. MacIntosh (1875) records all three, *Phascolion strombi*, *Phascolosoma minutum* and *Phascolosoma vulgare*; the last being found only in the stomachs of fish. The famous *Golfingia MacInstoshii* of Lankester (1885) is accounted as *P. vulgare* (Southern, 1913; Stephen, 1934) and Stephen (1934) has a record of one specimen of it from the bay. *P. minutum* is recorded as abundant by MacIntosh (1875) and is the only Sipunculid which can be regarded with certainty as a native of the littoral zone here.

P. minutum has a wide distribution and has been recorded from all over the North Polar regions. (Wesenberg-Lund, 1930; 1932; 1939). It is common around our islands (Southern, 1913; Stephen, 1934) and Heligoland, (Paul, 1909) and in the Baltic. (Théel, 1905). The species has been found by Sluiter (1912) as far South as the Azores; on the Atlantic coast of North America by Gerould (1913), and in the Antarctic by Théel (1911) who records it from the Falkland Islands. In bathymetric range the distribution is also wide and the species has been found from the littoral zone down to the depth of 1290 fathoms reported by Gerould (1913).

The first account of *P. minutum* is due to Forbes (1841) who applied the name *Sipunculus Johnstoni*; however Keferstein (1862) described the species under *Phascolosoma minutum* and in 1865 erected the genus *Petalostoma* to accommodate it because the species is exceptional in a number of features. It was under this last name that a full description of the anatomy and histology of the species was published by Paul in 1909. Southern (1913) held that the rule of priority compelled him to abandon the specific name used by Keferstein in favour of the one first given by Forbes, though he comments on a discrepancy between Forbes's illustration and text: Cuénot (1922) suggested that in view of the discrepancy the better-known name of *Phascolosoma minutum* should stand.

Apart from the question of these synonyms, the great apparent variability of the species has led Théel (1905) to use four other names: *Phascolosoma sabellariae*, *P. anceps*, *P. Sarsii*, *P. improvisum*. Gerould (1913) regarded *P. sabellariae* as synonymous with *P. minutum* but he maintained *P. improvisum* as a separate species. Wesenberg-Lund (1930) unites all Théel's (1905) species except *P. improvisum*, which she holds tentatively to be distinct, and in a later work (Wesenberg-Lund, 1939) she maintains this arrangement.

Whereas Paul (1909) is quite clear that the species is hermaphrodite, Gerould (1913) and Wesenberg-Lund (1930) both found that in the specimens they examined the sexes were separate in all cases; it is worth pointing out however that both workers were examining material which had been preserved for as long as, and sometimes longer than, forty years. The presence of hooks on the proboscis, behind the mouth, in many specimens, but the complete absence of these hooks in others (Wesenberg-Lund, 1930; 1939) is a further point which has given rise to confusion. Other criteria such as the degree of fusion of the two retractor muscles and the distribution of certain glandular bodies within the body wall are said to be most variable. (Wesenberg-Lund, 1930; 1932; 1939. Gerould, 1913).

Paul (1909) deals only with the morphology of P. minutum and others who have noted the species merely make brief reference to where the animals are to be found. Somewhat more information is available for other species. Andrews (1890) gives some details of the habits of Sipunculus (Phascolosoma) Gouldii and Ward (1891) speculates very briefly on the formation of a tube which this species occupies in the sand. Nerve and muscle physiology of the Sipunculida is known through the work of Uexküll (1896, 1903) on Sipunculus nudus and has been related to the habits and

behaviour of the animals: dealing with swimming activity, both Uexküll (1903) and Hérubel (1907) are agreed that swimming occurs in S. nudus, and Uexküll (1903) also quotes Wilson (1900) as recording swimming in P. Gouldii; although Wilson's text is ambiguous his description clearly refers to Echiurus, not Phascolosoma, and Andrews (1890) stated that burrowing is the only means of moving from place to place in P. Gouldii. It may be mentioned that Baltzer (1931) repeats Uexküll's misquotation from Wilson.

Most Sipunculid worms are sand- or mud-living animals feeding on detritus which they take up from the substratum. (Uexküll, 1921, Hérubel, 1907; Cuénot, 1900). Very little attention appears to have been paid to the mechanism by which the food material is obtained and none at all to the food material itself: Yonge (1928) in his review of feeding mechanisms in the invertebrates is unable to present any information on the subject.

There appears to be no published work on the Sipunculida which deals in any detail with the habitat of an individual species or which relates the habits and behaviour of a species to its habitat. The present work is an attempt to present such information for the species P. minutum.

2. MATERIALS.

All the worms used in this study were collected in the littoral zone at St. Andrews.

P. minutum rarely exceeds 6 mm. in length when contracted. It is difficult to maintain a supply of animals in the laboratory because they are readily washed away by circulating water. This difficulty was overcome by suspending the animals, in a glass and fine bolting silk sieve, in an aquarium tank through which sea water circulated; and in this situation the animals lived for up to a month in good condition.

3. HABITAT.

P. minutum is found widespread on the rocky foreshore at St. Andrews in most of the places where fine sand and debris tend to collect, such as the holdfasts of *Laminaria*, or between flakes of laminated rock. Shale is common at St. Andrews but although the species is often present it is not abundant between shale laminae.

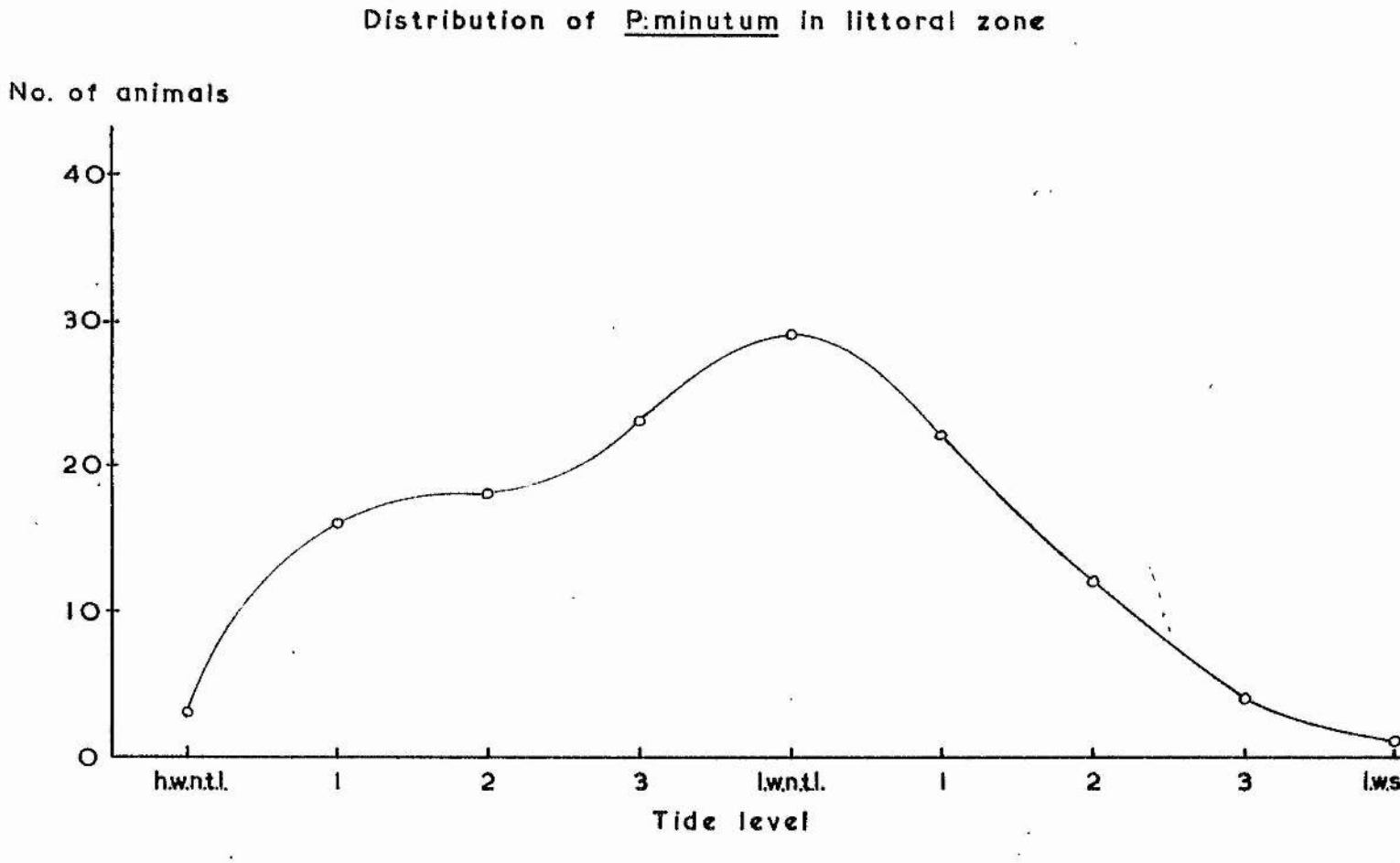
There is a region of the beach at St. Andrews, immediately below the Castle, where a number of gullies traverse the breadth of the littoral zone: each is bounded to the south by a ragged wall of laminated sandstone having a dip of about 30° to the south, whilst on the north side there is the gentle slope of another sandstone outcrop which forms the southern wall of a neighbouring gully (Plate 1.) By erosion the laminae of the southern walls are gradually forced apart and the resulting gaps become filled with detritus. In this location *P. minutum* is found burrowing in great abundance. Of samples taken to determine the distribution of the species between the tide marks, those from stations at low water neap tides level provided the largest number of individuals: an average value for five samples from this level was 29.2 individuals spread over 18 inches of sea frontage, the lowest value being 13 animals, and the highest 55. Since in most cases the animals are

TABLE I.

Distribution of P. minutum in littoral zone.

SAMPLE	Station in littoral zone								
	h.w.n.t.l.	1	2	3	l.w.n.t.l.	1	2	3	l.w.s.t.l.
1	5	14	20	35	27	17	10	3	1
2	0	36	28	27	20	15	17	7	4
3	6	1	16	20	55	32	5	5	0
4	1	12	9	10	13	23	16	2	0
5					31				
Total	12	63	73	92	146	87	48	17	5
Average	3	16	18	23	29	22	12	4	1

TEXT FIG. I



found only along the outer fringe of the rock this represents a very high population density.

Table 1 and Text figure 1. present the distribution of the species in a gully: the greatest density of population is found at or near low water neap tides level, the density steadily decreasing the lower the level of the station. It is noticeable that the gully wall tends to recede the nearer it is to low water spring tides level where erosion due to wave action is more rapid. The gaps between laminae tends to be wider the lower the level of the station on the beach and, as a consequence, the material between the laminae is coarser, wetter, and has less cohesion than the material found at low water neap tides. Above low water neap tides level the population density declines slowly to begin with, but by high water neap tides there is an abrupt decline. Here the absence of wave action results in a failure of the laminae to separate to the extent encountered at lower levels. Moreover organic decay in the fissures is often considerable so that they are unsuitable as a habitat.

The sandstone is composed of grains of quartz and mica ranging in size from 40-120μ in diameter, with an average of about 80μ, and much finely-divided material which I was unable to identify. This material effervesced when treated with hydrochloric acid indicating that it is calcareous.

The material from between the laminae at densely populated levels is composed of similar grains of quartz and mica but with a wider range of size, from 30-190μ, and with a smaller average size of about 40μ. There is much finely-divided material and diatoms are not uncommon, along with empty frustules. Lastly there is a sticky material which holds together the other components: the reactions of this material (Table 2) indicate that it is non-protein but mainly carbohydrate and is probably, therefore, derived from the mucilage secreted by the seaweeds fringing the rock wall. When treated with hydrochloric

Table 2. Reactions of mucilaginous material.

Test	Result.
Sakaguchi reaction for arginine	negative
Biuret test for protein	negative
Ninhydrin test	negative
Millon's reaction for tyrosine	negative
Toluidine blue stain, Lison's method (Glick, 1949)	blue stain (negative)
Periodic acid-Schiff reaction for carbohydrate constituent of mucus (McManus 1946)	red stain (positive)
Isoelectric point (Leob, 1922)	none

and the mucilaginous material becomes green: the whole mud, like the sandstone, effervesces.

The burrow (Plate 2) is a simple cylinder which is lined with mucus produced by the tegumental glands (Part III); it is more or less straight and the head end opens to the outside edge of the fissure in which it is situated, so that the free edge of a flake of rock which has harboured P. minutum is striated radially by the burrows.

4. LIFE HISTORY

Reproduction and development in *P. minutum* are dealt with in detail in Part II. of this work, but, for the purposes of this section an outline of the life-history of the animal is given.

The eggs are spawned into the burrow and develop there. Extensive development takes place within the egg membrane, and at hatching metamorphosis has already started. The newly hatched individual rapidly develops into a juvenile stage which differs externally from the adult only in size and in the possession of several rings of post-oral hooks. This stage can be seen occasionally in the parental burrows at the end of March, three months after the spawning season has ended, but in the majority of cases the burrows are quite free of young animals by this time. Evidently it is during the juvenile stage that distribution occurs.

5. BEHAVIOUR

(a) Movement

Body movements in *P. minutum* are the same in the adult as in the juvenile stage: an animal in a contracted and immobile state begins to move by a contraction of the circular muscles between the posterior end and the anus (which is dorsal and anterior, Plate 3). The constriction of the animal posteriorly forces coelomic fluid to flow towards the head end and has the effect of evaginating the proboscis. As the proboscis emerges its movement imposes upon the animal a backwards thrust which is overcome in an enclosed space by the expanded tail region which acts as an anchor. Forward movement of the anal region is produced by a general contraction of the circular muscles, then follows a wave of contraction of the longitudinal muscles starting at the anal region, which now provides the anchor, and spreading backwards to bring forward the tail region. The structure of the integument (Part III) provides for the considerable deformation of the body wall which this type of movement entails.

The muscular movements of an animal not in an enclosed space, that is, without walls on which the body wall muscles can be braced, are precisely the same as those of animals which are enclosed: the effect which they produce is, however,

different. When the proboscis is evaginated the animal tends to become curved because of the inertia ventrally of the retractor muscles (Plate 3). These muscles encircle the pharynx anteriorly (Plate 4), traverse the coelom unattached, and are inserted posteriorly in the ventral body wall. They are stretched passively during evagination of the proboscis; thus the dorsal side of the animal is on the outside of the body curve, the ventral on the inside, and the animal comes to lie on one or other side. The anus is a convenieht distinguishing mark when making these observations. As the proboscis is being everted the direction of travel always curves more or less ventrally, because of the retractors, but at the same time the proboscis appears to twist through an angle of 90° with reference to the body so that its ventral surface is directed downwards in the normal way. The proboscis then unrolls with its tip pushing against the substratum, so lifting the body curve into the vertical from the horizontal plane. When the proboscis is withdrawn the body falls back or over-balances on to the other side. The dorsal side of the proboscis is not easy to recognise but the dorsal tentacles or the cerebral ganglion when visible act as guides. The animal can move a considerable distance by these uncontrolled falls, but requires an enclosed space for orderly locomotion.

(b) Burrowing.

When discussing burrowing in Arenicola marina, Wells (1948) describes the buccal mass as armed with teeth which are directed backwards when the proboscis is extruded: he shows how repeated extrusion of this apparatus is effective in burrowing. The juvenile stage of P. minutum (Plate 5) is similarly armed with, in this case, post-oral hooks, which are directed backwards when the proboscis is extruded (Plate 6). As the proboscis is extruded the hooks first exert a sideways thrust on any surface to which the proboscis is applied and the thrust becomes progressively backwards as the proboscis is extruded to its full extent (Plate 7, Fig. 2). Finally the tip of the proboscis, fully extruded, becomes inflated. Repeated extrusion of the proboscis escavates a burrow by scraping away particles of the substratum.

Adult animals, above 1 mm. in length contracted, lack post-oral hooks and are unable to form a burrow in any substratum which is compact. The inflation of the tip of the proboscis when fully extruded occurs in both adults (Plate 7, Fig. 1b) and in juveniles (Plate 7, Fig. 2e): it is caused by a local contraction of the circular muscles just behind the region to be expanded, and when burrowing starts it provides an anterior anchor so that the rest of the body can be drawn forwards.

(c) Contact stimulus.

Krumbiegel (1932) drew attention to the fact that many worms, particularly Nemertines, when left free in a dish of sea water tend to form groups in which the individuals are entangled one with another. Krumbiegel (1932) considered that high contact stimulus inhibited movement in such cases and used the term thigmo-taxis to describe it: Fraenkel & Gunn (1940) prefer the term low thigmo-kinesis, but they doubt the conclusion that contact stimulus is concerned in the reaction.

This type of behaviour occurs in P. minutum and the congregation of individuals is brought about as follows:- animals free in sea water move about at random in the manner described earlier (Page 12). Sooner or later two animals will make make contact with each other, probably by the proboscis which will cross above or below the body of the contacted individual; then as the proboscis is withdrawn it will tend to draw the two animals closer together: the curvature of the animals (Page 12) favours this as does the inflation at the tip of the proboscis (Page 13). If there is debris present in the dish it tends to stick to the mucus on the surface of the animals and helps to hold the animals together. If more animals are present their random movements may produce other groups or may enlarge the first one, bringing to it more mucus and debris with additional

cementing effect. In this way compact balls of individuals are formed in which there is no lack of individual activity and, although one animal may become detached from the ball periodically, it is soon caught up again.

High contact stimulus does not inhibit activity or locomotion in P. minutum: a comparison was made between the activity of animals free in a dish of sea water and others which were enclosed between glass plates in sea water. The glass plates had rough surfaces and were irregularly corrugated so that when placed face to face they provided channels through which P. minutum could move when subjected to high contact stimulus. Graph paper was placed under the dishes containing the animals to provide a reference grid and the positions of the animals were plotted periodically. The results (Table 3) show that while the free animals generally covered greater distances in a given time than those which were enclosed, differences in activity were often small and rarely large enough to suggest an inhibitory effect of a high contact stimulus: the differences resulted rather from the relative lack of freedom of the enclosed animals to move in any direction.

In experiments 1c. and 1d., and 3d. and 3e. (Table 3) the results are the sum of tail movements in animals which had reached the edge of the glass plates so that the proboscis, when evaginated, projected from the edge of the plates.

TABLE 3. Activity of free and enclosed animals.

Exp. No.	Distance travelled		Interval between plots mins.	Duration of plotting mins.	Time between initial set up and start of plot. hours	Part of body plotted	Temp. C° ± 1
	enclosed mm.	free mm.					
1. a	113	156	15	90	24	Head end	15
b	151	150	15	180	25½	" "	"
c	18	62	5	35	0	Tail end	"
d	13	18	5	25	16½	" "	"
2. a	73	101	5	45	48	" "	"
b	95	139	5	70	49	" "	"
c	10	40	5	15	72	" "	"
d	31	82	5	65	96	" "	"
e	47	33	5	45	97	" "	"
3. a	78	82	5	50	24	" "	"
b	85	96	5	60	25	" "	"
c	96	59	5	30	26	" "	"
d	12	129	5	60	45	" "	"
e	15	42	5	50	46	" "	"

Animals in such positions remain active and move short distances back and forth but do not change position significantly since forward progress depends upon there being an enclosed space on which the body wall muscles can bear. It is in such positions that adult animals are found on the beach.

(d) Shadow reflex.

Hecht (1924) showed that in Mya arenaria the siphons are withdrawn in response to a change in the intensity of light which is falling upon them, and this type of behaviour is shown also by barnacles (Cole 1929) in which, as in P. minutum, there is no obvious photo-receptor.

The activity of P. minutum was watched, in as near natural conditions as possible, by removing to the laboratory pieces of rock harbouring the animals, great care being taken to prevent disturbance to the burrows. The rock was placed in a pneumatic trough filled with sea water and the animals were observed, when they became active, and emerged from the burrows, through a binoccular microscope mounted horizontally. Illumination was provided by a low power, high intensity lamp (6v. 5A.) focussed on the rock and northern daylight was not excluded. When the lamp was switched off the change in light intensity, which was considerably in excess of the threshold stimulus for Balanus in the same dish, produced no visible reaction in P. minutum.

(e) Light gradient: undirected light.

P. minutum was subjected to a smooth light gradient by means of an optical wedge (Messrs. Ilford Ltd., 8" x 2") set into the top of a box which was otherwise light-tight and had a matt black inner lining. A specimen which had been kept in the dark for at least three hours previously

was enclosed, together with fine sterile sand, between two rectangular plates of glass in a dish of sea water. The dish was placed inside the box. After 24 hours or more when the box was opened the track of the specimen was visible in the sand. The animals showed no tendency, such as Ullyott (1936) has demonstrated in Dendrocoelum lacteum, to arrive at or to stay in any particular region of light intensity, but move apparently at random. Six animals in all were tested in this way.

(f) Directed light.

P. minutum shows no response to a light beam falling on it, either by moving towards the light source or by moving away from it with any regularity.

In view of the above observations, light would appear to have little or no power to regulate the behaviour of P. minutum.

6. FEEDING.

(a) Morphology of the alimentary canal.

In Paul's (1909) description of the anatomy and histology of the alimentary tract in P. minutum, his nomenclature differs slightly from that employed by Andrews (1890) who worked on P. Gouldii. However like Andrews, he described six regions: a pharynx which bears the small and solid tentacles, and which is surrounded by the anterior insertion of the proboscis retractor muscles; an oesophagus; three mid-gut sections, each histologically distinct; and a rectum (Plate 3). The canal is ciliated throughout and the mid-gut regions all bear small blind sacs. In a transverse section of a sac the direction of ciliary beat is circular so that, if a rod were to be thrust into a sac, the rod would rotate about its long axis.

(b) Feeding mechanism.

Intake of food appears to be accomplished by a combination of ciliary and muscular activity. When the proboscis is fully extended there appear from inside the mouth two very small tentacles which point forwards (Plate 8); then the tip of the proboscis becomes inflated (Page 13 and Plate 7, Fig. 1b), so that the mouth is pulled outwards on all sides, and the tentacles come to point laterally exposing

the pharynx (Plate 9). Subsequently the inflation collapses closing together the tentacles and the proboscis is re-invaginated by the retractor muscles. The pharyngeal wall and the inner faces of the tentacles are richly supplied with cilia (Plates 4 & 10) whose beat is towards the oesophagus. When finely divided material such as colloidal graphite, carmine or yeast cells are brought close to the extended tentacles the particles are disturbed only if either in actual contact with the cilia, or all but in contact. When affected by the cilia the particles are swept towards the mouth but only a small proportion of the particles reach it, and the remainder drop away and are lost. Yonge (1935), discussing ciliary feeding mechanisms in Lamellibranchs, states that mucus is invariably associated with the cilia to entangle the food, but in P. minutum no sign of mucous glands or of mucus itself has been found associated with any part of the alimentary tract.

It is evident that ciliary action alone cannot be held responsible for the intake of food and so the possibility of a suctorial method was investigated. The proboscis retractor muscles remain relaxed throughout the evagination of the proboscis and contract only after the inflation of the proboscis tip has collapsed and the tentacles have come together again. However the whole process of retraction

from the position where the tentacles are widespread produces some decrease in pressure, at the end of the proboscis, which is equalised by an inflow of water and suspended matter. The inflow, which increases as the proboscis is withdrawn, can only assist in the intake of food while the tentacles are exposed, i.e. only at the very start of retraction.

Animals have been watched in the natural habitat by the method described earlier (page 17): the anterior end of the animal emerges from the burrow and the proboscis is everted to move slowly amongst the debris which collects along the face of the rock, amongst the shells of barnacles, the tubes of small Polychaetes, Coelenterate colonies and the stipes of small seaweeds all growing there. The proboscis is repeatedly withdrawn and everted, and it moves along the radii of the lower half of a circle drawn with the burrow entrance as centre and the plane of the burrow perpendicular to the plane of the circle. Thus the animals appear to be restricted to gathering food in the lower half only of the area surrounding their burrows, probably because of the body curve imposed by the retractor muscles when the proboscis is everted (page 17). If the proboscis encounters an accumulation of debris it remains more or less fully evaginated in the debris for minutes at a time

with hardly any movements. Presumably at this time food is being taken in but it is not possible to watch the process in fine detail.

(c) Food material.

The material from between the rock laminae where P. minutum burrows are situated has already been described (Page 8). This material corresponds to the débris which collects outside the burrows and on which the animals appear to feed. Examination shows the gut contents to have the same composition as that of the outside débris, except that particles above 30μ in diameter are scarce. However sand grains are occasionally present measuring up to 260μ or more in diameter. There is much finely divided material, diatoms and empty frustules are frequent, and the whole is held together by mucilaginous material. The faecal pellets are cylindrical and compact, being held together by this mucilaginous material for which no iso-electric point could be demonstrated (Page 8).

The material in the alimentary canal, in the oesophagus and in the first mid-gut region is present as small, discontinuous masses loosely held together; there are empty spaces between the masses. The vortices produced by the ciliated sacs in the first mid-gut region can be seen to detach material from the food masses, which material is

then vigorously stirred in the ciliated sacs (Plate 3). In the second and third mid-gut regions the material in the lumen is much more compact than in the first region and the masses are larger, separated only by constrictions of the canal, if at all: the ciliated sacs often contain rods of material revolving with the ciliary beat and when these reach a certain size they leave the sacs and rejoin the main mass within the lumen of the intestine. The rods are not ejected actively from the sacs but seem to become unmanageable to the cilia as they grow and begin to project from the sacs. The rectum is often empty.

(d) Hydrogen ion concentration in the alimentary canal.

Specimens of P. minutum of over 5 mm. contracted length are rare at St. Andrews. For this reason, measurements of the hydrogen ion concentration in the various regions of the alimentary canal are necessarily no more than approximations; particularly since I have been unable to induce the animals to ingest dyed matter.

The methods used were either to place regions of the canal in drops of indicator solution, or to mix them with indicator solution on a white tile and to compare the colour which developed with a standard of known pH. (Yonge, 1935). British Drug Houses, Ltd. indicator solutions were used and gave a pH range from 1.2 to 9.6.

The pharynx and anterior end of the oesophagus are neutral or slightly alkaline in reaction, but the hydrogen ion concentration increases posteriorly in the oesophagus and the first section of the mid-gut has an acid reaction in the neighbourhood pH 4.5-5: thereafter the value decreases in the second mid-gut section and is near neutrality or slightly alkaline in the remaining regions. In life the first mid-gut region is characteristically pale green in colour in contrast to the rest of the alimentary canal, and it is noticeable that this section is frequently divided by local constrictions into sections which, though empty, appear to be distended.

The non-compacted food material and the acid reaction in the first mid-gut section suggest that this is the secretory region of the alimentary canal. The fact that the food material effervesces, and that the mucilage with it becomes green in acid solution, probably accounts for the natural colour of this region of the canal and for its distended appearance.

7. A NOTE ON REGENERATION.

Bülow (1883-4) has described regeneration in *P. vulgare*, showing that after the proboscis had been cut off, and with it the pharynx, oesophagus and cerebral ganglion, complete regeneration was achieved in a matter of from three to five weeks time.

To test whether or not *P. minutum* has similar powers of regeneration the bodies of eight animals were severed anterior to the nephridia and anus. After a month the animals were dissected and three were found to have regenerated the lost parts completely; one animal was lost, and the other four showed less complete regeneration, two lacking only tentacles whilst the mouth was closed in the other two.

It is not uncommon to find in collections of animals from the beach an individual in which the proboscis is extremely short and lightly coloured in contrast to the rest of the body, just as in animals which have regenerated a proboscis in the laboratory. Thus there is evidence that, as might be expected in view of the animals' habits, loss of the proboscis does occur in nature, but that it is readily replaced.

8. DISCUSSION.

P. minutum has a very wide geographical and bathymetric range and it is regarded as a species which shows some variation.

Reports on sex in the species are conflicting: the detailed work of Paul (1909) on fresh material and the present investigation leave no doubt that in the littoral zone the animals are hermaphrodite. However Gerould (1913) and Wesenberg-Lund (1939) working on long-preserved material, but which had come from sub-littoral habitats, found no trace of hermaphroditism. The sub-littoral animals require further investigation particularly because the life history of the littoral form does not seem adapted for wide distribution of the species. It may be that there is a sub-littoral bisexual form having a planktonic stage in its life history; on the other hand the genital products in the animals are the only apparent guide to sex in the animals and for many months in the year only eggs are present (Part II.).

Two other variable characters are probably more apparent than real: the distribution of glandular bodies within the integument has been used as a taxonomic guide, but an apparently irregular distribution (Wesenberg-Lund, 1930) can be produced by an unequal degree of contraction of the

muscles of the body wall (Page 11). Similarly, an apparent variable degree of fusion of the retractor muscles may arise as a consequence of a varying state of contraction of these muscles in preserved animals.

A fourth variable feature is the presence or absence of post-oral hooks in adult animals: they are absent in littoral specimens, with rare exceptions in those from Laminaria, but always present in sub-littoral specimens (Southern, 1931). At St. Andrews they are absent in littoral adults but present in juveniles: Wesenberg-Lund (1930) found hooks present in 90% of specimens from sub-littoral sources. Wesenberg-Lund (1939) regarded the varying appearance of individuals of the species, particularly in regard to hooks, as connected with their adaptability to different bottoms: the point is of interest because the ability of an adult animal to burrow must depend on the presence of hooks and they might have some survival value where the habitat lacks the stability of the laminated sandstone at St. Andrews.

Whatever the method used to excavate a burrow, the burrowing organ must be directed towards the substratum, normally ventrally. Wells (1948) does not consider this point: however in P. minutum the dorso-ventral asymmetry of the body (Page12) ensures the downwards thrust of the proboscis, and the hooks, where present, dislodge the material.

I have demonstrated that high contact stimulus does not have an inhibitory effect on locomotion and the type of evidence on which Krumbiegel (1932) based his description of thigmo-toxis is unacceptable. P. minutum is physiologically trapped in its environment by its normal mode of locomotion and is not governed by such an external stimulus as light, lacking even the escape reflex which is so common in sedentary animals whose mode of life entails the exposure of unprotected parts to predators.

Actual intake of food has not been observed in P. minutum but the evidence available suggests that the ciliated tentacles are important and that they may be aided in taking food by a small decrease in pressure at the mouth as the tentacles are withdrawn. The detritus on which the animals feeds is held together by naturally occurring mucilaginous material and, when disturbed, the débris breaks up into masses which are of a size that could be trapped between the tentacles, and held by the cilia as the tentacles come together and are withdrawn: the food masses which are seen in the oesophagus correspond in size, and the relatively enormous sand grains found occasionally in the intestine of the animal could have been taken in only in this way or by considerable suction, of which there is no evidence. The gut is ciliated throughout and

transport of food can be accomplished to some extent by the cilia. However there is a very poorly developed muscular coat (Paul, 1909) which can alone be responsible for the transport of the largest particles and for the constrictions seen in the gut.

The structure of the ciliated sacs and the activity seen within them in fresh preparations are the same at whatever level of the gut they are found: their cells are richly ciliated and the cytoplasm appears agranular in histological preparations (Paul, 1909). Thus there is no reason to suppose that their function is more than to mix food and digestive enzymes in the secretory region of the intestine and to compact waste matter at a later stage. The secretary region appears to be the first mid-gut section where the epithelial cells are high and, in histological sections, very granular. The borders of these cells in the lumen of the gut are often indistinguishable. (Paul, 1909). The epithelial cells of the second mid-gut section are much lower than those of the first and, in histological preparations, have an agranular cytoplasm which often contains a large vacuole: Paul (1909) regarded these cells as secretory but they correspond more to an absorptive type of cell. Moreover, in this region of the gut rodlets of faecal matter are already being formed in the ciliated sacs.

The mucilaginous material in the food seems to some extent to fulfil the same function as the mucus found in ciliary feeding animals: not only does the mucilage hold food particles together as they are taken in; it also permits the formation of firm, compact faecal pellets where otherwise faecal material might foul the burrow. According to Yonge (1935) the pH values in the intestines of Gastropods and Lamellibranchs can be correlated with the isoelectric point of the mucus in which the food is entangled: the mucus is least viscous at its iso-electric point which corresponds with the acid pH of the secretory region. Thus the food can be readily mixed with the digestive enzymes. In the rectum the pH value is highest and the mucus at its most viscous so that faecal material can be compacted. In R minutum the non-protein mucilage is unlikely to vary in viscosity with varying pH, but this seems to be unimportant since the ciliated sacs appear to be capable both of stirring the food and of consolidating waste material. No doubt the green colouration of the acid region of the gut is caused by the effect of acidity on the mucilaginous material, and the distended appearance correlated with the tendency of the food to effervesce on treatment with acid.

9. SUMMARY.

1. The habitat and distribution of P. minutum in the littoral zone at St. Andrews is described: the animals form burrows in detritus which collects between flakes of laminated rock and they are most abundant at or near low water neap tides level.

2. An outline of the life-history of the species is given.

3. The behaviour of the species is examined and correlated with its habitat: the juvenile is adapted for active burrowing and is the stage during which the species is distributed. The adult is unable to burrow and its mode of locomotion prevents it from leaving the burrow. The animals do not react to light stimuli and do not display low-thigmo-kinesis.

4. The food and feeding mechanisms are examined: the animal feeds on detritus, probably by a combination of muscular and ciliary activity. There is no mucus secretion associated with the alimentary canal but to some extent the functions of mucus are fulfilled by a non-protein mucilage found in the food material. Food transport in the alimentary canal is by ciliary and muscular activity: there is a ciliary mechanism for mixing food and consolidating faecal material.

5. The animals possess the power to regenerate the proboscis which contains the cerebral ganglion, pharynx and oesophagus, and anterior insertion of proboscis retractor muscles.

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PART II. REPRODUCTION AND DEVELOPMENT

1. INTRODUCTION

Reproduction and development in the Sipunculida is known through the work of Hatschek (1883) on *Sipunculus nudus*, and of Gerould (1907) on *Phascolosoma Gouldii* and *Phascolosoma vulgare*. No work of this nature appears to have been carried out on *Phascolosoma minutum*. In view of the fact that this species was found by Paul (1909) to be hermaphrodite and is, according to Cuénot (1922), unique in this respect amongst the Sipunculida, it seemed probable that the reproductive behaviour and embryology of the species would prove to be of some special interest, particularly since self-fertilization must be regarded as a possibility.

There are some obvious difficulties to be encountered in demonstrating self-fertilization in marine animals, not so much in showing that self-fertilization is possible, but rather in establishing that the phenomenon occurs in the animal in its natural habitat. Gerould (1907) found that *P. Gouldii* and *P. vulgare* when ripe spawned spontaneously in laboratory conditions. This has proved not to be the case in *P. minutum* and recourse has had to be made to artificial methods in order to obtain fertilizable eggs. This being the case I propose at this point to give a concise review of the literature dealing with such methods.

While it is possible with some marine animals to obtain mature (fertilizable) eggs in the breeding season by removing eggs from the ovary and allowing them to lie for some time in sea water, this is not always so. Loeb (1902), noticing the maturing effect of sea water on the eggs of Asterias Forbesi, attempted to discover the causes of maturation which "is characterised, morphologically, by the disappearance of the very large, plainly visible nucleus". He showed that free hydroxyl ions and the presence of oxygen are essential to the process: when eggs of A. Forbesi were placed in an isotonic solution of Na Cl. they did not become mature, nor was the addition of potassium or calcium effective. Loeb's (1889-1902) many papers on irritability in living cells and tissues generally, and on ova in particular, stimulated a great deal of interest in this subject: many substances producing not only maturation of eggs, but also artificial parthenogenesis were reported. Wolfsohn (1907) found that not only free hydroxyl ions but also short exposures to weak solutions of benzol and other fat solvents caused the maturation of the eggs of a number of species of the limpet Acmaea, whereas if left in sea water the eggs did not become mature: if the treated eggs were fertilized with sperm they developed to produce active swimming larvae. In 1910 Lillie

discovered that isotonic solutions of certain neutral salts were effective in producing parthenogenesis in the unfertilized (but presumably mature) eggs of Asterias and Arbacia, but that Ca Cl_2 in particular, as well as the chlorides of magnesium and strontium, are toxic.

Most of the later workers have employed isotonic salt solutions and it has become apparent that in different species different agents can cause maturation: Hobson (1928) was able to produce maturation of the eggs of Thalassema neptuni by immersing them in an isotonic solution of Ca Cl_2 alone, whereas the chlorides of sodium, lithium and potassium had no effect. The matured eggs were fertilized and developed; but if left unfertilized only a small percentage showed any cleavage, although the percentage tended to rise the longer the exposure to calcium. Pasteels (1935) worked on the eggs of several Annelids including the Sipunculid, Phascolion, an Echinoderm, and a Mollusc, and again Ca Cl_2 was found to be a most effective stimulating agent, KCl less so, while Na Cl and Mg Cl_2 had no effect. On the other hand Heilbrun & Wilbur (1937) found that while Ca Cl_2 had no effect on Nereis eggs, the germinal vesicle nucleus broke down following on immersion in Na Cl or K. Cl

Lillie (1910) considered that there was an initial period of increased permeability over the surface of the

egg which was responsible for the initiation of cleavage. Increased permeability was produced by an increased concentration of certain salts (e.g. Na Cl) in solution; however, the success of later workers in causing maturation by the use of calcium ions must dispose of this idea, so Heilbrunn (1952) argues, because it is generally recognised that the effect of calcium is to reduce permeability.

We may now turn to the experimental induction of spawning. In 1913 Lillie & Just found that in Nereis limbata, though ripe males will not spawn spontaneously, the presence of a substance secreted by the eggs will induce the shedding of sperm. Later Townsend (1938, 1939) showed that glutathione and extracts of many tissues from many species, all containing glutathione, caused spawning in the male Nereis. Lillie & Just had found earlier that females will respond by spawning in the presence of sperms in the outside medium. Palmer's (1937) work on Arbacia is interesting since she showed that injury of the body tissues will initiate the shedding of genital products; so also will injections of an aqueous extract of tissues obtained from the same species or from such diverse sources as oyster or frog; or injections of the chlorides of calcium and potassium isotonic with Arbacia eggs. This last observation has been put to practical use for sexing Arbacia

(Harvey, 1940) and the British Echinoderms, Echinus esculentes and Psammechinus miliaris (Rothschild, 1951). Orton (1920) came to the conclusion that a temperature stimulus of some kind is the normal trigger inducing sexual activity in marine animals, where other conditions are reasonably constant. Experimentally a sudden rise in temperature does cause spawning in both sexes of the oyster, Ostrea virginica (Galtsoff, 1938, 1940) and in Patina (J.M. Dodd, personal communication).

Recently in this laboratory some success has been obtained by applying certain of these methods to Asterias rubens and Arenicola marina: Sandeman (unpublished) produced spawning in both sexes of Asterias by injecting KCl, aqueous extracts of whole animals, or histamine: Howie (1954) induced spawning in male Arenicola with tissue extracts and obtained suspensions of active sperms. Females of Arenicola were more refractory to such treatment, however, and any eggs shed were unfertilizable.

2. GENITAL PRODUCTS.

(a) Hermaphroditism.

There are in P. minutum two gonads which remain histologically similar throughout the year at St. Andrews. Where these gonads are attached to the body wall (Plate 3) their cells are hardly distinguishable from those of the peritoneum. The farther they are from the attachment the larger are the cells, and at the free end they are about 70μ in diameter and more or less circular in section.

The reproductive cells, both male and female, are shed by the gonads at a very early stage to subsequently mature in the coelom. Whereas recognisable oocytes at various developmental stages are present in the coelom throughout the year, sperm morulae do not appear until the end of August, at the start of the breeding season. From September until December both eggs and sperm can be found together in the coelomic fluid of the worms.

There can be no doubt that the animals are hermaphrodite, and it seems probable that both gonads in an individual produce oocytes in the earlier part of the year and spermatocytes nearer to the spawning season.

(b) Ova.

An egg approaching maturity is ovoid in shape and measures from 280 to 310 μ in length, and from 240 to

260μ in breadth. There is a distinctive vitelline membrane which determines the shape of the egg and which is about 3μ in thickness. This membrane appears at first sight to be perforated by numerous pores (Plate II.). Gerould (1907) and others have noticed a similar membrane around the eggs of other species of Sipunculida and have reported seeing protoplasmic filaments issuing from such pores prior to development. I have never seen similar processes in P. minutum, however. The porous appearance disappears after the outermost layer of the membrane has been removed by tryptic digestion. The outer surface of the membrane can be seen to be patterned with minute dots arranged in hexagonal array: these also disappear after tryptic digestion. Thus it would appear that the dots are on the surface of the membrane only and that they do not represent perforations of the membrane.

As a result of the Sakaguchi test for arginine the isolated membrane takes on the characteristic red colour, and thus it appears to consist at least in part of protein. In Flemming's fixative the osmic acid causes little or no blackening of the membrane which, if it occurred, would indicate the presence of fatty material.

The vitelline membrane of a fresh egg is highly birefringent, with the sign positive with respect to the tangent (Plate 12); the birefringence persists and the

sign remains positive after dehydration and immersion in xylol, but now the birefringence extends all the way across the egg (Plate 13). When the membrane has been digested away completely however, all birefringence in the egg disappears: it therefore cannot be due to cytoplasmic organiztion but rather to the membrane organization alone. From the sign of the birefringence one may conclude that the long axes of the polypeptide chains of the vitelline membrane are extruded within the plane of the membrane, a feature characteristic of protein membranes in general.

There is little or no swelling when the eggs are immersed in distilled water and the membrane is not readily broken.

The number of eggs present in any animal at the start of the spawning season is rarely more than about seventy and there are generally present one or two eggs significantly smaller than the rest but still of considerable size (260μ in length), and also a variable number of very small oocytes. By the middle of October many animals still contain eggs, but twenty to thirty is a more usual number and many of them are undergoing cytolysis (Plate 14 & 15): of six animals examined on 20th October, 1952, all but one contained some "abortive" eggs; one contained twenty eggs of which six showed signs of cytolysis; and another had eleven out of seventeen eggs in that state.

(c) Spermatozoa

An active, mature spermertozoon (Plate 16) is about 50μ in length with a small ovoid head. The head contains a compact nucleus distinguishable after staining by the Feulgen reaction. The head is tipped by an acrosome, and between head and flagellum there is a middle piece. The sperm head shows very little birefringence when viewed in polarized light.

In September, at the beginning of the spawning season sperm morulae and sperm plates are scarce, while loose sperms are very rarely seen. The order of scarcity is such that making a sperm "suspension" is out of the question. In extreme cases no sperm plates are to be seen, although three or four at the most is normal. Rather suddenly, in October at the same time as cytolysing eggs appear in the coelom, both morulae and sperm plates become much more plentiful, and free but inactive sperms are relatively numerous in the coelomic fluid: in one exceptional case an animal had no cytolysing eggs but contained over fifty sperm plates. Ten sperm plates is a more usual number at this time.

Occasionally when an animal was opened and the coelomic contents came into contact with sea water a sperm plate began to break up releasing active sperms, but more

often the plates remained intact. When fertilizations were attempted, it was generally necessary to activate spermatozoa. The method of activation, used by Fuchs (1915) in his work on the physiology of spermatozoa, was to add 0.2 ml. 0.1% NaOH solution to 10 ml. sea water containing coelomic fluid. In many cases sperm plates began to break up within several minutes in such a medium but sperms which were already loose and inactive generally resisted activation, even after three hours in alkaline sea water. When three or four sperm plates were present successful activation yielded a maximum of about a hundred active sperms. It was rarely possible to produce sperm in other than these minute amounts.

3. NATURAL SPAWNING.

The spawning season at St. Andrews is extensive. It begins in the first week in September, while eggs still in the early stages of development may be found on the beach in the burrows well into December. The extent of the season is not reliably indicated by regular counts of the proportion of spent to unspent animals since many animals do not spawn out fully and one having eggs developing in the burrow may also contain a considerable number of coelomic eggs of maximum size. Presumably it is these unspawned eggs which undergo cytolysis and which are

resorbed. Table 1. gives the figures for some collections made between October and February. Evidently by February

Table 1. The proportion of spawned to unspawned animals.

Date	No. of worms collected			Percentage without eggs.
	Total	With eggs	Without eggs	
17.10.52	83	50	33	40
20.10.52	66	44	22	33
23.10.52	37	18	19	50
4.11.52	25	9	16	64
8.11.52	32	13	19	60
19.12.52	26	8	18	69
21.1.53	30	5	25	83
17.2.53	21	8	13	62

many worms still contain eggs, but I have not found early stages of development in the burrows later than December 19th, and the majority of the eggs remaining unspawned after that date undergo cytolysis.

On the beach it is possible to find three neighbouring burrows, in one of which there are developing eggs and the parent worm; in another, a worm full of eggs but none spawned in the burrow itself; and in the third, an animal

without eggs either in the coelom or in the burrow, i.e. one which had probably spawned some time ago. Thus the spawning of one worm does not appear to stimulate a spawning reaction in its immediate neighbours. Frequently animals which appear to have previously spawned eggs contain morulae and sperm plates which may give off spontaneously active spermatozoa.

4. SPAWNING IN THE LABORATORY

Geroud (1907) recorded that in P. Gouldii and in P. vulgare spontaneous spawning occurred in both sexes at night when ripe animals were placed in a dish of clean gently flowing sea water. I have not found this to be the case with P. minutum and the eggs contained by animals kept in such conditions in the laboratory invariably undergo cytolysis within the coelom.

(a) Methods.

Two methods of keeping animals in the laboratory in conditions which approximated to some extent to the natural habitat have been tried out. The first method was to enclose animals, either in isolation or in groups, with fine sterile sand between plates of rough glass immersed in sea water in Petri dishes. The temperature was kept constant at 15° C., ± 1°, and the water was changed every

other day. Fifty-two animals were kept in this way, each specimen for one week within the period 23rd September to 6th November, 1952.

The second method, by which twenty-six animals were each kept for one month over the period 11th September to 10th November, 1953, involved the use of glass tubes 6 mm. in inside diameter and about 15 mm. in length: a specimen was placed in each tube along with, in the first place, mud from the burrows on the beach; but when this was found to decompose rather rapidly cotton wool washed in sea water was used as packing. The glass tubes were pushed half-way through holes in squares of celluloid and were kept singly in small finger bowls (Boveri dishes) containing about 20 ml. sea water, or in groups of three in larger dishes. The water was changed six days out of seven: the temperature was kept constant at 15° C., ± 1°, except after the water had been changed when the dishes were placed in a refrigerator at 1° C. for two hours in the hope that a sudden change of temperature might induce spawning.

The sea water used in both these methods was collected from the open sea, well away from the shore, and brought into the laboratory in a glass carboy. The water was then filtered through Berkfeld kieselguhr cylinders without being allowed to stand in the carboy for more than a day.

Finally the filtered water was stored in sterile glass flasks at a temperature of 15° C. at ± 1°, and used at this temperature.

(b) Results.

None of the animals kept by either of these two methods spawned, although in all cases the animals appeared to be active and in good condition. However on being opened many of the eggs from the specimens of both years were found to be abortive and to show signs of cytolysis.

5. INDUCED SPAWNING.

Small decreases in the hydrogen ion concentration of the water bathing P. minutum failed to produce any noticeable effect, and immersion in sea water containing an excess of calcium, or in an isotonic solution of $CaCl_2$ alone also failed to stimulate a spawning reaction. The treatment had no apparent effect on the contained eggs. Ripe animals left overnight in sea water to which had been added thyroxine, 1 part to 50,000 parts sea water, did not spawn.

The addition of extracts of P. minutum tissue to sea water in varying amounts had no effect in producing spawning, but injections of these extracts into animals did produce some reaction which led to the shedding of eggs and other coelomic contents in some cases.

(a) Methods.

Tissue extracts were made by grinding whole animals containing eggs in a mortar with fine sand and filtered sea water until they formed a thin paste. The supernatant fluid was poured off after centrifuging and stored frozen. Fifty freshly collected animals, of 0.15 gm. total wet weight after surplus water had been removed with filter paper, were ground up in this way and from them 4 ml. of extract were obtained. 0.004 ml. of extract was injected per specimen, and although the punctures so caused were necessarily large vigorous contraction generally sealed the wounds and the injections appeared to be successful. Injections were made also with filtered sea water, with isotonic solution of K Cl and of $CaCl_2$. After injection the animals were placed in filtered sea water, one to a dish, and any coelomic contents spilled from the animals were removed by pipette after a short interval.

(b) Results.

Table II. is a summary of the injection attempts and it shows that while the injection of tissue extracts does cause the shedding of eggs, so also may K Cl solution or plain sea water. The eggs so obtained were of every size and stage of maturity except fully mature and all attempts to fertilize them failed.

Table 2. Summary of injection attempts.

Date	No. of animals injected	Solution injected	No. of animals which shed eggs	No. of eggs shed.
15.10.53	14	Tissue extract	6	85
20.10.53	9	Tissue extract	3	5
- - -	2	Sea Water	0	0
- - -	2	Ca Cl_2	0	0
22.10.53	7	Tissue extract	1	14
- - -	2	Sea water	1	1
- - -	2	K Cl	1	3
23.10.53	7	Tissue extract	1	3
- - -	4	Sea water	1	2
- - -	5	K Cl	1	3
26.10.53	12	K Cl	0	0

There was often evidence of nephridial activity as a result of injection, whether of extracts, K Cl or sea water. An example of this is the protuberance at the nephridiopore (Plate 17), which was often seen whether or not eggs were shed. In one or two cases a stream of coelomic contents, without eggs, was seen to be discharged

through the nephridiopore. In a further case, 16 hours hours after injection with tissue extracts, an animal which had failed to lay eggs was opened and an egg was found protruding from the nephrostome.

On three occasions a section of body wall to which a nephridium was attached was isolated and immersed in tissue extract and its activity compared with that of a nephridium in pure sea water: there was no discernable difference in ciliary activity between the experimentals and the controls, nor was any muscular activity seen.

Whereas in male Arenicola suspensions of active sperms have been obtained after injections of tissue extracts (Howie, 1954), in P. minutum there was never any sperm activity to be seen amongst the eggs and/or coelomic corpuscles shed as a result of injection.

6. NATURAL MATURATION OF OVA.

I have never found eggs in which the germinal vesicle had already broken down in the coelom of P. minutum except where abortive eggs were also present, or when animals had been kept in the laboratory for some time and abortive eggs were to be expected. On such an occasion an animal was found to contain 61 eggs none of which had a visible nucleus, and of them 26 were kept as a control while to the other 35 activated sperm from the same animal were

added. At the same time 71 similar eggs from another specimen were obtained, 33 being kept as a control and 38 used in a fertilization attempt with activated sperm from other animals. No regular development was seen in any of the eggs.

Many attempts have been made to fertilize eggs taken from the coelom but without success. Attempts were therefore made to produce fertilizable eggs by artificial means.

7. ARTIFICIAL MATURATION OF OVA.

(a) Methods.

The eggs to be activated were exposed to a variety of solutions all of which had been reported to cause activation of marine eggs. The eggs were collected into glass and bolting-silk sieves so that the period of exposure to a test solution could be accurately determined; on removal from the solution the eggs were well washed in filtered sea water and examined at intervals thereafter, the disappearance of the germinal vesicle nucleus being employed as a criterion of maturation. When self-fertilization experiments were attempted an animal was washed briefly in distilled water to remove any foreign sperm before the eggs were taken for treatment. After treatment and washing of the eggs active sperm were added to the water by pipette.

All glassware was sterilized in an oven at a temperature exceeding 100° C. for at least one hour.

(b) Results.

Of the various solutions employed to induce maturation of the eggs (Table 3) isotonic Ca Cl_2 alone appeared to be successful.

Table 3. Artificial Maturation of Eggs.

Date	Solution	Duration of exposure	No. of eggs exposed	No. of eggs affected in 24 hours
14.11.51	0.1% NaOH, 0.1 ml./20 ml. sea water	1 hour	37	0
"	" " " " / " " "	2 hours	39	0
6.10.52	" " 0.4 ml./ " " "	18 hours	33	1
"	0.56M NaCl + 1 drop satd. $NaHCO_3$/ 200 ml. sea water	6 mins.	86	2
"	0.56M KCl + 1 drop satd. $NaHCO_3$/ 200 ml. sea water	6 mins.	52	0
17.10.52	0.3M $CaCl_2$	4 mins.	83	37
30.9.52	Filtered sea water	24 hours	136	1
29.9.53	Thyroxine, 1/50,000 parts sea water	18 hours	29	0
11.11.53	Benzol, 2 drops/30 ml. sea water	2½ mins.	20	0
"	" " " " " " "	5 mins.	24	0
10.10.52	0.3M $CaCl_2$	1 min.	102	44
"	" "	2 mins.	114	0
"	" "	3 mins.	69	69
"	" "	4 mins.	54	48
9.11.52	0.3M $CaCl_2$ 50%, sea water 50%	52 mins.	80	10
"	" " " " " "	54 mins.	90	8

Artificial fertilisations were attempted using eggs in which the nucleus disappeared subsequent to treatment for four minutes. It was noticed that both control lots and lots to which sperm had been added showed apparent cell division (Plate 19); thereafter an exposure of three minutes to $CaCl_2$ solution was used and the control eggs then generally failed to show any activity beyond the breakdown of the nucleus. Table 4. gives the results of five experiments in which a limited amount of success was obtained in producing development in eggs by this method. The experiment of 26.10.53 shows that exposure for four minutes may appear to

Table 4. Effect of exposure to isotonic $CaCl_2$ solution

1 Date	2 Exposure to $CaCl_2$ Time in minutes	3 Total eggs used	4 No. of eggs affected	5 No. of eggs with sperm	6 No. of eggs in control	7 No. of eggs cleaving in 5.	8 No. of eggs cleaving in 6.
26.9.52	4	99	86	40	43	2	1
26.10.53	4	168	167	Nil	167	Nil	20
13.10.52	3	46	29	16	13	1	0
9.10.52	3	69	69	32	37	1	0
30.10.53	3 +	46	31	20	11	3	0

produce parthenogenesis in P. minutum eggs. Plate 18. is a photograph of a section of one of these eggs, however, and it shows that cell division was highly abnormal.

In the experiment of 26.9.52 one egg from the lot to which sperm were added developed and hatched in eight days. It survived for a further twenty-four days but failed to free itself completely from the vitelline membrane and had an abnormal growth of tissue at the mouth opening. The two eggs from the experiments of 9.10.52 and 13.10.52 both developed and one, though active and apparently regular within the membrane, failed to hatch; the other hatched on the seventh day and was quite regular.

The experiment of 30.10.53 was an attempt at self-fertilization and three eggs, all from one animal, showed some activity: one failed after one abnormal division; the second achieved the four cell stage; the third appeared to divide in a normal manner but failed to hatch nor did it ever become active within the vitelline membrane.

The visible effect of $CaCl_2$ upon the eggs, apart from causing the disappearance of the germinal vesicle nucleus, is to cause local shrinkage of the cytoplasm from the vitelline membrane, particularly near the narrower end: the effect is more pronounced the longer the exposure to salt solution and the membrane itself may bulge outwards opposite the inshrinking of the cytoplasm.

Apparently exposure of eggs to 0.3M $CaCl_2$ for three minutes causes changes which render the eggs fertilizable, and fertilization some development may occur even when the

eggs and sperm are from the same animal. A longer exposure to the salt causes cytolysis.

8. DEVELOPMENT

(a) Cleavage.

Observations on early cleavage were made using some eggs which had been collected from a burrow prior to the extrusion of their polar bodies: eighteen eggs collected at low tide about noon and examined two hours later were found to show no nucleus but were undivided. By 5 p.m. both polar bodies had been extruded in all the eggs at the active pole, and by 7 p.m. first cleavage was completed by a furrow originating at the active pole near the polar bodies and proceeding towards the narrow end, resulting in two very unequal blastomeres (Plate 20a) lying side by side. By 7.30 p.m. the second cleavage had commenced, again from the active pole but in a plane at right angles to the first cleavage furrow, and resulted in three blastomeres of roughly equal size and a fourth which was much larger (Plate 20b). Third cleavage began at about 10.30 p.m. with furrows running across the length of the egg; the division of the large fourth blastomere is retarded in comparison with the other three blastomeres and is again unequal (Plate 20c). At this stage it is already impossible

to see all the blastomeres in one egg and further cleavages have not been followed.

Development proceeds with overgrowth from the active pole of the larger, presumptive endoderm cells by small cells, leading to epibolic invagination (Plate 20d).

(b) Differentiation of the tissues.

Differentiation of the tissues (Plate 21) proceeds with the formation at the active pole of an apical group of very small cells at whose centre can be made out four larger cells. Around the border of the apical group appear the relatively enormous prototrochal cells. The ring of prototrochal cells is interrupted ventrally where the stomodeum forms. Posterior to the prototroch the surface of the embryo is covered with a layer of epidermal cells which now give rise to the internal fibrillar layers of the cuticle (Part III.). This can be seen using polarized light as a delicate cross-hatching at the surface of the epidermis though it is only weakly birefringent in sectioned material. At this stage the retractor muscles also appear as a pair of lateral strands (Plate 22) stretching from the marginal cells of the apical group to an insertion posterior to the stomodeum.

There is thus a trochophore larva but this larva never shows signs of cilia on the prototrochal cells, nor flagella

associated with the apical cells. No pigment spots appear, nor is there a postoral band of ciliated cells. Although the prototrochal cells are obvious enough there is no postoral circlet of cells distinguished as a mesotroch.

(c) Metamorphosis.

How long the embryo takes to reach the larval stage and for how long that stage lasts is uncertain. However, eggs which developed in the laboratory from the start hatched in six days at a stage when metamorphosis had already begun (Plate 24). For at least some hours prior to the rupture of the vitelline membrane the embryo within is active, pushing forward the apical region and prototroch, both of which become invaginated by the activity of the retractor muscles. The prototrochal cells are eventually resorbed into the general economy of the animal and the apical cells contribute to the cerebral ganglion. No proctodeum is visible in unhatched larvae nor can one be seen in the stage immediately subsequent to hatching when the mouth is perforated but the gut ends blindly.

Sections of newly hatched individuals show that the retractor muscles, which first appeared in the embryo as one pair, are now arranged as three pairs, of which one is dorsal, one ventral and the third lateral (Plate 23). When metamorphosis is complete, however, two pairs have disappeared leaving the ventral pair alone.

(d) Juvenile stage.

A juvenile stage (Plate 25) develops slowly as the larva elongates and the anterior end becomes invaginated, while the anus forms dorsally in the region between the rudiments of the nephridia. The juvenile possesses hooks (Plate 6 & 20e & 25) which develop on the proboscis behind the mouth and which are arranged in several rings (Plate 7, fig. 2). The hooks are composed of fibrillar inner cuticle which is secreted by the syncytial epidermis (Part III.) (Plate 33). Initially the juvenile has no outer, non-fibrillar, cuticle but this soon begins to form, after the appearance of tegumental glands in the body wall, and it covers the inner cuticle except on the proboscis. Those regions of the epidermis which lie immediately below each hook (Plate 20e) are thickened so that the cytoplasm projects into the bases of the hooks. Within each thickened region there is a nucleus which is larger than the ordinary epidermal nuclei. These special regions of the epidermis appear to be concerned in the formation of the hooks.

On hatching, the animals are opaque with yolk which is only gradually used up. They can live without feeding in the laboratory for at least ten weeks after hatching from the egg. Attempts to feed juveniles by adding to the water suspensions of the green alga, Chlorella; the diatom, Nitschia; or very fine liver powder all failed.

Particles of a carmine suspension were not taken in, but when a suspension of colloidal graphite was used traces of this were noticed in the gut of four juveniles: the graphite remained as a trace only until the animals died some seven weeks later.

The whole course of development takes place within the parental burrow where the eggs (Plate 2) are held in a group about the adult by mucus, presumably the product of the tegumental glands (Part III.). Subsequent to hatching dispersal begins as the juvenile stage becomes active. As late as March occasional juveniles may still be found within the parental burrows.

9. DISCUSSION

Without observations on natural spawning of eggs and sperm in P. minutum it is quite impossible to prove or disprove the occurrence of self-fertilization, either as a possibility or as the normal process. Since fertilizable eggs have not been obtained without artificial stimulation no information has been gained about the natural degree of self-fertility or self-sterility of the eggs.

There is some slight evidence that self-fertilization can occur although this is far from conclusive. The fact that it is rare to find a spawned egg on the beach which

is not developing suggests that the means of ensuring fertilization must be efficient. It is clear that in an extended breeding season the number of individuals spawning simultaneously must be small and these widely separated. This is confirmed by direct observation of spawned eggs, though not of sperms. The maintenance of a supply of sperm in the water adjacent to the burrows, available to fertilize eggs at any time during the spawning season, would seem to be out of the question, particularly in view of the limited amount of sperm to be found at any time within P. minutum. It must also be borne in mind that the eggs which are spawned remain in the burrows, well away from currents of water likely to be bearing sperm. All of these considerations suggest that self-fertilization is indeed practised.

Gerould (1907) states that the eggs of P. Gouldii and P. vulgare reach first meiotic metaphase in the coelom before being spawned and remain in that condition after spawning unless fertilized, so that presumably, they are fertilizable when in the coelom. Concerning Phascolion, Pasteels (1935) is not so specific but states that eggs from the coelom display varying stages of maturity and that from 5 to 30%, exceptionally 59%, mature spontaneously in sea water. In P. minutum on occasion eggs from the coelom have been found to have no visible nucleus, but they have always been associated with abortive eggs or

else have occurred where abortive eggs were to be expected. At all opportunities fertilizations were attempted with such eggs but always unsuccessfully: it thus appears that these eggs also were abortive. I have found only one account of comparable abortive eggs in the literature. Orton (1933) reported that the protandrous hermaphrodite Ostrea edulis retains many eggs at spawning time and that these are shed later as a false spawn from which there is no development. He offers no explanation of this phenomenon. In P. minutum, if the eggs become fertilizable within the coelom and self-fertilization is the rule, abortive eggs may conceivably result from the limited amount of sperm available at any one time. Although sperm is often more abundant when abortive eggs are present it may well have become available in quantity too late to fertilize all the eggs which were mature. The gonads appear to produce oocytes in the earlier part of the year, and spermatocytes nearer to the spawning season. This probably accounts for the fact that only in late October and in November do the majority of spermatocytes develop into spermatozoa, in which event only late maturing eggs are likely to be fertilized.

There is a communication by Kobayashi and Yoshida (1951), unfortunately not available for study in this country, on the production of rythmical movements in

isolated nephridia of Sipunculids using tissue extracts; and Sharrer (1953) has confirmed several earlier reports that there is a substance extractable from the nephridia and other organs of Sipunculids having a like effect on isolated nephridia. While isolated nephridia of P. minutum do not seem to react, injection of tissue extracts does produce nephridial activity leading to the shedding of eggs and other coelomic contents.

In view of the work of Lillie & Just (1913) and of Townsend (1938, 1939) it seems reasonable to suppose that in P. minutum it is the direct effect of a substance, diffusing from eggs in the appropriate state for spawning, that stimulates the activity of the nephridia. This view is supported by the fact that only developing eggs are found in the burrows; that is, that only eggs which will develop are spawned and those which will not develop are not spawned. Townsend (1939) considered that glutathione was important in the spawning reaction of the male Nereis, and she claimed that marine eggs (unspecified) contain glutathione; but this work does not seem to have been followed up.

The results of the experiments on the fertilization of eggs activated by isotonic salt solution indicate that it may be possible to stimulate development artificially,

even development after self-fertilization. Although one may argue that it is no indication of normal behaviour if an egg, matured artificially, can be fertilized by sperm from the same animal, any evidence of the possibility of self-fertilization in P. minutum is of interest. Further work will be necessary before this question can be settled.

The spermatozoa of P. minutum are not particularly striking objects but the ova, by reason of their large size and prominent vitelline membranes, are of interest. Their size is evidently correlated with the lengthy period of development during which growth without active feeding takes place. The vitelline membrane appears to have a protective function. It is composed chiefly of protein in which the polypeptide chains lie in the plane of the membrane. There is little or no swelling when the egg is immersed in distilled water and it does not burst, so the vitelline membrane is evidently inextensible and strong. This may be correlated with the lengthy period through which the eggs pass free in the coelom: here they are exposed to the constant agitation and circulation of the body fluids, which are kept in motion by groups of ciliated cells attached to the outside of the alimentary canal and to the peritoneum.

Gerould (1907) mentions the porous appearance of the

vitelline membrane of the eggs of both P. Gouldii and P. vulgare, and that it is in fact porous is supported by his description of protoplasmic filaments issuing from the membrane. He shows that in the larval stages the vitelline membrane persists until metamorphosis and that the cilia of the prototroch, mesotroch and the flagella of the apical organ traverse the membrane, presumably through the pores, and that these cilia are effective in locomotion. In P. minutum development of the egg, so far as the early stages have been followed, corresponds well with development in the species which Gerould (1907) studied. The trochophore stage is very similar also, and is well-developed; however the prototroch cells are never ciliated nor do flagella develop as an apical organ, and pores through the vitelline membrane are not present. Pigment spots are wanting in P. minutum but are present as a pair in the trochophores of P. Gouldii and P. vulgare, and these larvae also display positive phototaxis (Gerould, 1907). P. minutum appears to have lost an active, pelagic stage from its life history. This may be correlated with the special habitat of the species, since, if there were a pelagic larva in the life history of this animal, the larva would require a very elaborate behaviour pattern to find a suitable settling place, such as is described in Part I., in which to complete its life cycle after

metamorphosis. The absence of an active trochophore must reduce the efficiency with which the species is distributed and must also reduce the opportunities for outbreeding.

Paul (1909) noted in P. minutum the lack of the free ciliated bodies, or "urns", and a vascular system connected to hollow tentacles which are found in most other Sipunculids. From these and the features of development discussed above P. minutum appears to be a highly specialised, rather than a primitive Sipunculid.

10. SUMMARY.

P. minutum is hermaphrodite. The eggs and spermatozoa are described, and the significance of "abortive" eggs is discussed.

The breeding season at St. Andrews is from September to December. P. minutum will not spawn readily in the laboratory and attempts to induce spawning by the injection of tissue extracts have been only partly successful.

Artificial fertilizations have been attempted but artificial maturation of the eggs is a prerequisite and few successful fertilizations have been obtained.

Evidence in favour of self-fertilization has been examined.

Some developmental stages have been described. Early

divisions of the eggs correspond with those of P. Gouldii and P. vulgare. There is a trochophore larva which has lost the power of locomotion. After metamorphosis there is a juvenile form which is the actively burrowing stage in the life history.

The eggs are shed into the parental burrow and are retained there until the juvenile stage.

The presence of a trochophore larva which plays no active role in the life history suggests that P. minutum is a specialised rather than a primitive Sipunculid.

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PART III. THE STRUCTURE AND PROPERTIES OF THE BODY WALL.

1. INTRODUCTION.

The structure of the body wall in Sipunculida is well-known through the work of Andrews (1890), Ward (1891) and Paul (1909). Certain features have received special attention, particularly the cuticle and certain epidermal organs. Whereas Paul (1909) described the cuticle in P. minutum as chitin-like (chitinartig), Andrews (1890) working on P. Gouldii, Ward (1891) on S. nudus and Cuénot (1900) on P. vulgare all recognised the non-chitinous nature of the cuticle by its solubility in boiling potassium hydroxide. Paul (1909) also drew attention to a lattice of fibres within the cuticle; fibres running in two directions and intersecting at an angle of about 120°. The epidermal organs have been described in detail in P. Gouldii by Nickerson (1901) and she was able to assign a sensory function to some, while others were glandular.

The work now to be described was carried out in order to establish the nature and properties of the cuticle in P. minutum; and also to establish whether or not the epidermal organs possess any sensory function which would be important in relation to behaviour in this animal.

While the cuticle in Sipunculids has attracted little attention since Paul's (1909) observations, a similar type

of structure has been observed in the earthworm (Cerfontaine, 1890; Stephenson, 1930) and this latter has been described in detail by Reed & Rudall (1948) who used electron microscopy. Later Rudall (1951) showed that the cuticle gives a collagen type X-ray diffraction picture, although the fibres of which the cuticle is composed do not show the banded structure (Reed & Rudall, 1948) typical of the collagen fibres of vertebrates (Randall et al., 1952). In Aphrodite (Polychaeta) the fibrillar structure is also present and the fibrils are of a protein belonging to the collagen group according to Picken (Picken, Pryor & Swann, 1947).

The problems associated with the production of such oriented fibrils have been discussed by Picken, Pryor & Swann (1947) and by Reed & Rudall (1948).

Randall and his co-workers (1952) have isolated collagen fibres from the connective tissue of many vertebrates as well as from the skeleton of Echinoderms and from body wall muscle of Annelids. All these fibres show a fine cross-banded structure which has been regarded as typical. However Randall et al. also found that in Mytilus the byssus threads, which give a normal high angle X-ray diffraction pattern for collagen, do not show the typical banded structure.

Although relatively little is known about the synthesis of fibrous protein in animal cells, there is some evidence

that the enzymes acid and alkaline phosphatase play a part in these processes. Thus Fell & Danielli (1943) have reported that in the rat there is a high concentration of alkaline phosphatase at the site of small, healing, skin lesions. In the chick Moog (1944) has found both acid and alkaline phosphatase activity during tissue differentiation and, later in development, alkaline phosphatase activity in the sites of bone deposition. Similar findings have been reported by Mazia et al. (1948) in the development of Arbacia, and by Yao (1950) in the development of Drosophila: Yao suggests that, judging from the wide diversity of the taxonomic levels of the animals so far studied, high phosphatase activity in embryonic development may be a universal phenomenon. Danielli (1953), reviewing the subject of alkaline phosphatase in relation to protein formation generally, quotes a number of examples of the association, but he also mentions an unpublished report by C.H. Brown, who found no alkaline phosphatase activity associated with any stage in the production of the byssus thread in Mytilus.

Jackson (1954), working in association with Randall, has been concerned with the secretion of extra-cellular collagen fibres in vertebrates and has shown by electron microscopy possible intra-cellular stages in the formation of such fibres.

The chemistry of structural protein formation in invertebrates has received much attention in recent years and the work on insects alone has been extensive (Pryor, 1940). Brown (1950a) has attempted to correlate the type of supporting or protective skeleton possessed by diverse animals with characteristics of their environment and she has reviewed the methods available for determining the types of chemical forces stabilizing those skeletal materials which are mainly protein. Quinone tanning, establishing covalent bonds, appears to be widely used in the Animal Kingdom, in contrast to the vegetable tannins which form electrovalent links (Brown, 1950a). Evidence of quinone tanning has been reported in Thalassicola (Radiolaria) and in the chaetae of the Polychaete, Aphrodite (Brown, 1950b); the byssus threads and periostracum of Mytilus are also quinone-tanned (Brown, 1950b). The egg cases of Dendrocoelum (Nurse, 1950) and of Fasciola (Stephenson, 1947); the cuticle and hooks of Cestodes (Cruez, 1948); the cuticle of Ascaris (Chitwood, 1936) and of Limulus (Lafon, 1943); earthworm chaetae (Dennel, 1952), and the cuticle in insects (Pryor, 1940) all give evidence of phenolic (quinone) tanning.

2. STRUCTURE OF THE BODY WALL

(a) Gross anatomy.

The body wall (Plate 26) comprises, from outside to inside, a stout cuticle covering a very low epidermis, a layer of muscles arranged circularly and a further layer of muscles arranged longitudinally. In a contracted animal the cuticle is about 20μ in thickness, the epidermis about 5μ, and the muscular layers each 25 to 30μ. The longitudinal muscles are separated from the coelom by a thin peritoneum. The epidermis is interrupted fairly regularly by the epidermal or tegumental organs, whose pores penetrate the cuticle and open to the exterior.

(b) Structure of the cuticle.

The cuticle is conspicuous in sections of the whole animal, being comparable in thickness to either of the muscular layers. For the greater part of its width it is laminated, only the outer covering being structureless when examined in transverse section using an ordinary light microscope. I have found that the cuticle can be separated from the underlying epithelium by stripping and that the cuticle itself can be readily separated into two layers, particularly if it is first dried at room temperature for a short time and then wetted again. The outer layer of cuticle so obtained varies in colour from brown to pale

yellow, the colour depending upon thickness, which varies from animal to animal to some extent, and from posterior to anterior in any one animal. It is particularly thin on the proboscis and thickest at the posterior end. When looked at in plan view the outer layer appears to be broken up into areas, roughly rectangular or square, by a system of furrows which are both longitudinal and circular with reference to the whole animal. The longitudinal furrows are more or less continuous, while the circular ones tend to be discontinuous and less well-marked. Using a polarizing microscope, the only birefringent material of the outer cuticle appears to be in the furrows (Plate 27) and this birefringence persists after the cuticle has been dehydrated and immersed in xylol. This indicates that the birefringence is either intrinsic (caused by molecular orientation) or caused by strain which has oriented material above the molecular level. When compared with striated muscle the birefringence is found to be of the opposite sign and negative with respect to the long axes of the longitudinal furrows, but positive with respect to the long axes of the circular furrows: this indicates an orientation of material in the furrows, along the circular furrows but across the longitudinal ones. The material bounded by the furrows is hyaline, but many of the areas are pierced by a hole through which projects a papilla of inner cuticle which bears the pore of a tegumental gland.

Preparations of outer cuticle fixed in 0.2% osmic acid and teased out with fine forceps have been examined by means of an electron microscope. The preparations were mounted on copper grids with supporting films of Formvar and were shadowed in vacuo with palladium-gold. No well-defined structure (Plate 28) was seen at a magnification of 5000 times and it is thus difficult to account for the oriented material of the furrows. A possible explanation is, however, suggested in the discussion on the function of the body wall (Page 92).

The inner cuticle is composed of the striated material described by Paul (1909). Fresh preparations of whole cuticle examined in plan view by means of a polarizing microscope (Plate 29a & b) show areas of intense birefringence at the external openings of the pores of the tegumental organs, indicated by the appearance of Maltese crosses. With compensation the Maltese crosses change so that there are two dark and two light quadrants (Plate 27) instead of four of each (Plate 29a & b). The explanation for this is that the birefringence is a property of the fibrils of which the cuticle is composed, and these fibrils are deflected from the two directions noticed by Paul (1909) to run round the pores of the tegumental glands: there is, effectively, a circle of fibrils around each pore. Because

of this it is possible to find the sign of the birefringence since in the quadrants (Plate 29b) all fibrils run in the same direction; whereas away from the pores the fibrils cross each other so that it is impossible to say when those running in one direction are at extinction: this is illustrated in Plate 30. The sign of the birefringence is positive with respect to the long axes of the fibrils; imbibition with xylol indicated that the birefringence is either intrinsic or caused by strain.

An examination of fibrillar cuticle by the electron microscope was made using, in the first place, the same teasing technique as that already used for studying the outer cuticle (Plate 31 (Page 78)). Later thin sections were used and provided the better method. It can be seen from a section (Plate 32) that the cuticle is built up from unidirectional sheets of fibrils each of which has a diameter of the order 500-600Å, and that the fibrils are without a cross-banded structure. There is no evidence of an interfibrillar matrix or cement such as Reed & Rudall (1948) found in the earthworm.

(c) Chemistry of the cuticle.

I have applied a number of common protein tests to both layers of the cuticle (Table 1), many of them giving positive results, so that, besides indicating that the

cuticle is composed largely of protein, a number of amino acids have been identified as present in one or other cuticular layer. The periodic acid-Schiff reaction (McManus, 1946) applied to sectioned material indicated the presence of polysaccharide in both layers of cuticle,

Table 1. Protein tests applied to cuticle.

Test	Result		Indication
	Inner cuticle	Outer cuticle	
Biuret test	+	+	Protein
Arginine test	+	+	Arginine
Millon's reaction	-	-	Tyrosine
Xanthoproteic test	+	+	Phenylalamine, tyrosine, or tryptophane
Ehrlich's aldehyde test	-	+	Tryptophane
Cystine test	+	+	Cystine
Thiol test	-	-	Cysteine

but tests for chitin (Table 2) gave negative results. Tests for mucus showed a thin layer over the whole external surface of the cuticle.

Table 2. Chitin tests applied to cuticle.

Test	Cuticle of *Crangon vulgaris*	*P. minutum*		Authority
		Inner cuticle	Outer cuticle	
HCl. conc.	Dissolves	No solution after 24 hours	No solution after 24 hours	Yonge, 1932
Hot satd. KOH.	No solution	Dissolves	Dissolves	Yonge, 1932 & Smyth, 1950.

Table 3. Iso-electric point of cuticle.

pH	$Fe(CN)_6''''$		Basic Fuchsin	
	Inner cuticle	Outer cuticle	Inner cuticle	Outer cuticle
2.4	Blue	Blue	Colourless	Colourless
2.6	Blue	Blue	Colourless	Colourless
2.8	Pale blue	Blue	Colourless	Colourless
3.0	Palest blue	Pale blue	Colourless	Pink tinge
3.2	Colourless	Colourless	Pink tinge	Pale pink
3.4	Colourless	Colourless	Pale pink	Pink
3.6	Colourless	Colourless	Pink	Pink

The iso-electric points of both layers of cuticle (Table 3) have been determined by the method devised by Loeb (1922): a series of dishes is set out, each dish containing 20 ml. of buffer solution of varying pH to provide a wide range. Five drops of 0.1% potassium ferrocyanide ($K_4Fe(CN)_6$) and a small portion of cuticle are added to each dish; after two hours the pieces of cuticle are washed in distilled water and placed in very dilute ferric chloride ($FeCl_3$). The protein of the cuticle, in conditions more acid than its iso-electric point, dissociates as an acid and combines with the ferrocyanide ion ($Fe(CN)_6^{''''}$); ferrocyanide reacts with ferric chloride to give a blue colour. Similarly on the alkaline side of the iso-electric point basic fuchsin stains the protein red.

There appears to be little or no difference in the iso-electric points of the two layers of the cuticle and in both dissociation of the protein is at a minimum between pH 3.0 and pH 3.2.

The series of solubility tests suggested by Brown (1950a) as a method for determining the types of chemical bonds stabilizing structural proteins in animals has been employed for both inner and outer layers of the cuticle (Table 4), except that sodium sulphide has been substituted for neutralised thioglycollic acid; both these reagents are effective in splitting disulphide groups according to Goddard & Machaelis (1934).

Table 4. Solubility tests applied to cuticle.

Reagent	Type of bond broken	Layer of cuticle	Immediate reaction	Reaction after 24 hours
Boiling aq. dest.	Van der Waals (weak bonds)	Inner & outer	None	None
0.2N HCl	Eledtrovalent	" " "	"	"
0.2N NaOH	"	" " "	"	Slight swelling
6M Urea	"	" " "	"	Some swelling
Formamide	"	" " "	"	None
2M $CaCl_2$	"	" " "	"	Some swelling
Satd. lithium thiocyanate	"	" " "	Swelling	Much swelling
Satd. calcium thiocyanate	"	" " "	None	Some swelling
0.5M sodium sulphide	Covalent (-S-S-)	" " "	Swelling & colour change	Outer cuticle jelly-like; fibrils very swollen; green-blue colour, both layers.
1% sodium hypochlorite	Aromatic tanning (-SH or -S-S-)	" " "	Rapid swelling with effervescence	Decolourised: both layers in incomplete solution

The results of these tests show that the forces stabilizing the cuticle are of the strongest and that aromatic tanning of both layers of the cuticle is probable. Amongst the protein tests (Table 1) the thiol test failed to indicate -SH groups (thiol group), but the cystine test was positive and confirms the -S-S- (disulphide) linkage which is indicated by the disruption of both cuticular layers and the colour change produced by sodium sulphide solution.

(d) Structure of the epidermis.

The illustrations to Paul's (1909) work indicate that he regarded the epidermis as composed of many separate cells. The epidermis is a very thin layer of tissue (Plate 26), about 5μ in thickness at its maximum, and the nuclei have a diameter of 5μ or slightly less, so that where they occur the epidermis is slightly thickened to accommodate them: in transverse sections, 10μ or more in thickness, the undulant surface of the epidermis caused by the nuclei produces the appearance of incomplete divisions between the cells. I have been unable to see true cell boundaries. In fixed animals, when the cuticle is stripped off, the epidermis remains attached to the underlying musculature and it is possible then, by carefully peeling off muscle fibres, to make a preparation consisting

of epidermis supported by a thin layer of circular muscles and, occasionally, completely to free small regions of the epidermis from muscle. Such a preparation is shown in plan view in Plate 33 and it appears that the cytoplasm of the epidermis is continuous between nuclei. Attempts have been made to impregnate possible cell boundaries using von Recklinghausen's silver method but, although there was some impregnation at the boundaries between the cells of other tissues, all attempts with the epidermis failed. The epidermis therefore appears to be syncitial.

In view of the close association of the epidermis with the fibrillar layers of the cuticle it seems likely that the epidermis is responsible for the production of the cuticle. Epidermal cytoplasm is very finely granular and when the tissue, mounted in water after fixation in aqueous Bouin's fluid, is examined using a polarizing microscope there can be seen a very weak birefringence evenly spread throughout the epidermis. In a tangential section of P. minutum fixed in Zenker's (acetic) fluid and stained with haemalum and eosin the birefringence is much increased (Plate 34) and is clearly caused by material which is arranged in a linear pattern reflecting directly the arrangement of the fibrils in the cuticle. Fixation in a mixture of formalin and basic lead acetate was found

later to show this structure particularly well (Plate 35). It has not been possible to establish the sign of the birefringence in relation to any axis, as was done with the fibrillar cuticle (Page 79). Although the epidermis is raised in papillae round the pores of the tegumental glands, as is the fibrillar cuticle (Plate 38), I have not been able to make a preparation in which the epidermis could be examined at the papillae.

(e) Chemistry of the epidermis.

The cytoplasm of the epidermis is not stained when sections of the animal are treated according to Lison's (Glick, 1949) method for polysaccharide esters of sulpuric acid, nor when sections are subjected to the periodic acid - Schiff reaction of McManus (1946). After treatment with Lison's modification of the chromaffin reaction (Glick, 1949) a faint light-brown colouration can be seen in the epidermis which was fixed in potassium iodate-formol. After fixation in formol-Muller's fluid and exposure to 3% potassium iodate for 5 hours the reaction is improved and positive, indicating a tissue phenol.

The epidermis of late larval stages, juvenile animals and adults was tested for alkaline phosphatase activity by the Gomori-Takamatsu method as modified by Danielli (1953) using alcoholic fixation: both incubated sections

and unincubated control sections show a black deposit, normally indicative of enzyme activity, after the treatment. The black deposit is not prevented in control sections by prior treatment with citrate buffer solution which would remove any calcium in the tissue likely to interfere with the reaction. Omission of the calcium nitrate bath which follows incubation also fails to prevent the formation of the deposit. No deposit forms when the sections are immersed in the final ammonium sulphide bath if the prior step of cobalt nitrate treatment is omitted.

No conclusion can be drawn about the activity of alkaline phosphatase within the epidermis: the test in this case appears to be inappropriate because of a substance within the tissue, other than the enzyme or calcium, which apparently reacts with cobalt nitrate.

(f) Tegumental glands.

Nickerson (1901), employing the methylene blue intra vitam method, found four types of epidermal organs in P. Gouldii; of these two were glandular and two non-glandular, probably sensory. In the sensory organs there are bipolar sensory cells whose peripheral processes, explanded below the cuticle, give off hair-like fibres which pass through the cuticle to the exterior. Although she found difficulty in fixing these external fibres Nickerson was able to see

them in living tissue.

In P. minutum Paul (1909) found comparable organs difficult to study because of their small size, but he was of the opinion that they were all similar and glandular. Each organ is a more or less round body with a narrow pore canal which penetrates through the cuticle. Within the body there is often a fine-grained mass probably to be regarded as secretion, and although ten or more nuclei can be seen in the centre of the body no cell borders can be traced.

I have found that the distribution of the glands is regular over the surface of the body except at the tip of the proboscis near the mouth where they are absent. There is no histological difference between the organs (Plate 36) and I have been unable to find any sensory structures associated with them when nerve staining techniques such as the methylene blue vital staining and Holmes' silver impregnation methods were used. In sections stained with Heidenhain's Haematoxylin a darkly staining filament is often noticeable lying within the pore canal and occasionally projecting slightly from it. When living tissue, cut from the body wall, is examined many fine projections from the cuticle are visible but these do not project from the pores and are merely débris adhering to the animal.

Several other staining methods have been used (Table 5) for the demonstration of mucus secretion and, although the important periodic acid-Schiff reaction failed after both aqueous (Zenker) and alcoholic (Carnoy) fixation,

Table 5. Tegumental glands, tests for mucus.

Stain	Result
Methylene blue (vital)	Metachromasia in glands
Toluidine blue (Lison's method)	" " "
Mucicarmine	Red colour in glands
Periodic acid-Schiff reaction:-	
Aqueous fixation;	No staining in glands
Alcoholic fixation.	" " " "

there is a strong indication that the tegumental glands are concerned with mucus secretion. After staining a large portion of body wall with methylene blue the material within the majority, but not all, of the glands exhibits metachromasia. This variability may result from the glands being at different stages of secretion.

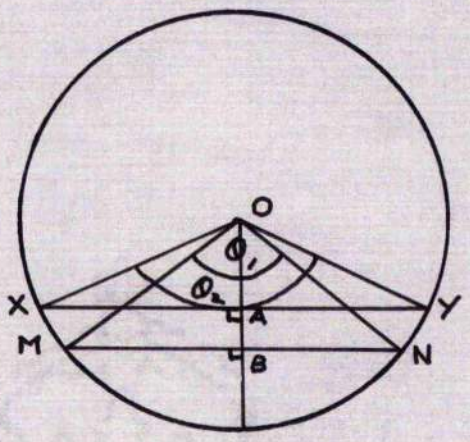
3. DISCUSSION.

The value of the angle at which the submicroscopic fibrils of the inner cuticular layer cross one another varies as the animal contracts or elongates; it varies from a value of about 80° for the angles facing front and rear when the animal is relaxed, to a value of about 110° when contracted. In this respect the fibrils are similar to the argentophil fibres of the body wall musculature in the Nemertine, Amphiporus lactifloreus, studied by Cowey (1952). Cowey predicted mathematically the size of the angle at which the fibres would cross at any given contraction or extension of the animal and he showed that predicted values corresponded closely to observed values.

In P. minutum it is possible to measure the angle at which the fibrils cross in animals fixed at a given contraction or extension by direct observation under a microscope with a stage revolving through 360° and graduated in degrees. In living animals the ratio of the length contracted to the length relaxed can be obtained by measuring the distance between the posterior end of the animal and the anus when the animal is stimulated to contract fully and again when the worm has been narcotised using $MgCl_2$, 0.3M solution. If the angles at which the fibrils cross in these two states are known it is possible to check

mathematically whether or not there is a direct relationship between the angle of cross of the fibrils and the length of the animal using the relationships $^{OB}/_{OM} = \cos \frac{1}{2} \emptyset$, and, since OX = OM, $^{OA}/_{OM} = \cos \frac{1}{2} \emptyset_2$. These are derived from text figure I., where

Text figure I.



$\emptyset_1$ and $_2$ equals the angle at which fibrils cross;
OB is the length, from tail to anus, relaxed;
OA is the length, from tail to anus, contracted.
I have calculated that in the contracted state the value of $\emptyset$ should be of the order 132°- 140°, whereas by direct observation the value is only 110°; however an explanation of the discrepancy is afforded by study of a section of the body wall in a contracted specimen (Plate 38): the cuticle is thrown into folds and thus allows a contraction beyond the limit up to which the fibrillar "trellis" can close. The furrows of the non-fibrillar cuticle are most obvious in a fully contracted worm and must result from

the change of surface area imposed upon the outer cuticle by the fibrillar layer. The furrows appear to be lines of weakness in the outer cuticle thus it seems likely that the birefringence of the cuticle at the furrows is the result of strain. From the sign of the birefringence the strain appears to be across the longitudinal furrows, but along the circular furrows; that is, in one direction only instead of, as might have been expected, in the two directions, one at right angles to the circular furrows, and one at right angles to the longitudinal furrows. The explanation of this may be that the greatest stress on the cuticle occurs when a region of the body of P. minutum has been expanded to form an anchor during locomotion (Part I., page 11): any strain in the cuticle as a result of this stress would be across the longitudinal furrows and might also affect the circular furrows, though to a lesser degree.

It seems not unreasonable to suppose that fibrils which are oriented and continuous across the surface of an animal might be produced more readily by a tissue in which the cytoplasm is also continuous, that is, by a syncytium, rather than by a tissue composed of discrete cells. However in the earthworm the epidermis is composed of very high epithelial cells which are nevertheless

capable of producing lengthy fibrils (Reed & Rudall, 1948). That the epidermis in P. minutum is syncytial is therefore unlikely to be related to the function of secreting cuticular fibrils. The significance of the syncytium is to be seen perhaps in the extent to which the body wall can be stretched (Plate 37) when the epidermis and its nuclei become attenuated to a degree which might be disruptive to a columnar epithelium.

Cowey (1952) does not consider this problem and I have no information on how the epithelia of Nemertines withstand the deformation to which they must be subjected. In the earthworm the problem does not arise because, as is clear from Gray's & Lissmann's (1938) description of movement, there is no considerable change in the surface area of the body wall but rather an arching of the wall of a segment as the animal contracts: Reed & Rudall (1948) found an interfibrillar matrix in the earthworm's cuticle which must restrict angle changes. In the earthworm the cuticle is very thin in comparison with that of Phascolosoma. The fibrillar structure is important in locomotion in Phascolosoma but not so in the earthworm. Gerould (1907) found evidence of a transitory metamerism in the nerve cord and mesoblastic bands in the trochophore of P. Gouldii immediately before metamorphosis. He suggests that these

are either vestiges of a completer metamerism which may have existed in the ancestors of the Sipunculids, or perhaps the incipient tendencies of a somewhat primitive organism. The fibrillar cuticle in the earthworm may be a vestige of a cuticle, similar to that of Phascolosoma, which may have been important in locomotion in unsegmented ancestors of earthworms. This argument favours the view that Phascolosoma is a primitive organism.

The close association of the epidermis with the cuticle and the presence of material within the epidermis oriented as the cuticular fibrils are oriented, together with the evidence of phenolic tanning in the cuticle and the presence of a phenol in the epidermis, suggest that the cuticular fibrils are secreted by the epidermis and are to some extent preformed within the epidermis. The granules of the epidermis do not appear to be similar to the carbohydrate fraction of the fibrils because the periodic acid-Schiff reaction, which stains the cuticle strongly, does not produce a marked colouration of the epidermis. The same statement holds for tests with Lison's method for polysaccharide sulphate compounds. Protein tests would be of little value in this connection. The failure of the test for alkaline phosphatase activity in the epidermis of P. minutum and in the byssus gland of

Mytilus (Brown: quoted by Danielli, 1953) is a coincidence, particularly since the protein produced by both tissues is tanned. Polyphenols are known to have an affinity for cations such as Ca^{++} and Co^{++}; furthermore cobalt is notorious in forming complexes with other substances. I therefore thought that the presence of a polyphenol as a precursor of the quinone tanning agent might well interfere with the test for enzyme activity because immersion in calcium nitrate solution and then cobalt nitrate solution are steps necessary in the demonstration of alkaline phosphatase activity. However Miss C.H. Brown, in a personal communication, has assured me that the problem which I encountered in P. minutum epidermis does not occur with Mytilus byssus gland.

There is evidence that most if not all of the epidermal organs secrete mucus, but there is no evidence to suggest that they possess any sensory function either from histological investigation or from the behaviour (Part I.) in the animal itself. In development (Part II.) the appearance of the glands coincides with the appearance of the non-fibrillar layer of the cuticle, and this, along with the metachromasia of the outside of the cuticle after staining in methylene blue and toluidine blue suggests that the glands are responsible for the production of this layer of the cuticle, which may be tanned mucus.

4. SUMMARY.

The cuticle in P. minutum consists of two distinct layers: the outer protective layer exhibits little structure, and the inner layer is composed of crossed fibrils each having a diameter of the order 500 to 600Å.

The cuticle as a whole consists chiefly of tanned protein, but it also possesses a carbohydrate fraction.

The cuticle is to the animal a tough protective covering which is at the same time flexible and extensible, so that the worm may extend and contract its body within wide limits during locomotion and feeding.

The epidermis is syncytial and has a finely granular cytoplasm in which the granules reflect directly the arrangement of the cuticular fibrils.

The epidermis almost certainly secretes the fibrillar cuticle which is to some extent preformed in the cytoplasm.

It is suggested that the syncytial nature of the epidermis allows it to be reversibly deformed during movement of the animal.

The tegumental glands secrete a mucus which appears to form the outer cuticle.

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PART IV. PERMEABILITY OF THE CUTICLE.

1. INTRODUCTION

Adolph (1936), working on Phascolosoma Gouldii, came to the conclusion that the animal behaved as an osmometer when immersed in either hyper- or hypotonic sea water, losing or gaining weight by the loss or uptake of water. He regarded the integument of the animal as semipermeable despite the earlier work of Bethe (1934) who showed that in animals representing the phyla Mollusca, Annelida, Arthropoda and Echinodermata, the body walls are permeable to both sea water and all the ions of sea water. Bethe's experiments have suffered some criticism but his general conclusions are now generally accepted (Krogh, 1939; Webb, 1940).

Adolph (1936) also found that the gain in weight by P. Gouldii in hypotonic sea water was more rapid than its loss when the animal was returned once more to pure sea water; therefore he described the body wall as "differentially permeable". Krogh (1939) pointed out that Adolph's claim may be true but that his experiments, which did not exclude the alimentary canal or the nephridia, cannot be regarded as conclusive.

In this section I have been concerned with the possibility that a differential permeability may be experimentally

produced in the cuticle in P. minutum: in view of the structure of the cuticle I considered it possible that the cuticular fibrils (Part III.) may become hydrated and swollen as the body fluids are diluted by the uptake of water when a specimen is placed in diluted sea water. Swollen cuticular fibrils might then form a greater barrier to the loss of water when the animal is returned to pure sea water.

2. VOLUME CHANGES IN LIVING P. minutum.

I have attempted to measure the rate of volume change in P. minutum when immersed in hypotonic sea water and also when returned to pure sea water, in order to test whether or not the evidence on which Adolph based his claim also holds for P. minutum. Weight changes in this small species were found to be more difficult to measure than volume changes.

A volumometer, the design of which is due to Gnanamuthu (1952), was used and the displacement in sea water of four specimens together at the same time was measured:-

(a) in 100% sea water;

(b) at intervale after a period in sea water diluted to 70% by the addition of distilled water;

(c) at intervals after return from diluted sea water to 100% sea water.

Three sets of four animals each were measured in this way and the results (Table 1) show that there may be an increase in volume of up to 36% when P. minutum is immersed in 70% sea water. The results also show that the animals swelled to the maximum degree within one hour, but that in no case did they return to their original volume within one hour and a half of their return to 100% sea water. The largest animals displayed the largest percentage increase in volume in hypotonic sea water and the smallest animals the lowest percentage increase.

All the animals used remained active throughout these experiments. Earlier experiments on volume change in P. minutum had shown that the animals will live for at least a week in the laboratory after immersion in 70% sea water for six hours.

Table 1. Volume changes in living animals.

A.

Sea water strength	100%			Transferred to 70%				Transferred to 100%						
Time	a.m. 10.15	10.30	11.15	11.30	11.45	p.m. 12.5	1.30	1.45	2.45	3.00	3.15	3.30	3.45	4.00
Volume in arbitrary units	2.70	2.55	3.85	3.55	3.50	3.40	3.15	3.10	2.55	2.50	2.70	2.50	2.50	2.80
Mean	2.63			3.58			%age increase 36.							

B.

Sea water strength	100%				Transferred to 70%					Transferred to 100%						
Time	a.m. 9.45	10.00	10.15	10.30	11.15	11.30	11.45	12.00	p.m. 1.30	1.45	2.45	3.00	3.15	3.30	4.00	4.15
Volume in arbitrary units	2.50	2.35	2.35	2.30	3.05	3.40	3.35	3.30	3.00	3.20	3.25	3.30	2.95	3.00	2.55	2.75
Mean	2.38						3.22		%age increase 35.							

C.

Sea water strength	100%			Transferred to 70%					Transferred to 100%	
Time	2.45	3.00	4.15	4.30	5.15	5.30	7.15	7.30	9.15	9.45
Volume in arbitrary units	1.75	1.75	2.35	2.10	2.30	2.00	2.10	2.40	2.35	2.25
Mean	1.75				2.21		%age increase 26.			

3. PERMEABILITY OF THE CUTICLE.

The permeability of the cuticle to chloride ions was determined in the following manner. The posterior two thirds of P. minutum was severed from the anterior part. The posterior part was stripped of internal tissues: this provides a small pouch (Phascolos; Gr. = leathern pouch) which, since in this animal the anus is anterior, is unperforated except for the pores of the tegumental glands. The bag was then slipped over the end of a pipette and tied in position with cotton thread in such a way (Plate 39, fig. 1) that the outer cuticle is always in contact with the thread in order to prevent damage to the fine fibrils, and to ensure that the seal is similar whether the outer or the inner layer of cuticle is outermost during an experiment. The same cuticle was always used in two consecutive experiments, allowing the ions to diffuse from inner to outer cuticle in one, and from outer to inner in the other experiment; the order in which these directions were tested was regularly alternated.

In series 1. of tests the tissues were macerated in running tap water overnight and then the cuticle soaked in 1M NaCl solution before use. The pipette, filled to a mark with 1M NaC. solution, was fitted inside a glass

jar containing 25 ml. distilled water so that the level of the solution in the pipette coincided with the level of the water (Plate 39, fig. 2). After six hours the pipette was raised from the water and washed down with distilled water; it was then removed altogether and the cuticle stored in 1M NaCl solution overnight. The amount of chloride which had diffused into the distilled water was determined by titration with 1% silver nitrate ($AgNO_3$) solution.

In Series 2 the cuticle, during preparation and when stored overnight, was kept in a sea water which had been sterilized by filtration through Berkfeld kieselguhr cylinders. All sea water used in the experiments was sterilized in this way. The solutions used were sea water in the pipette, and sea water diluted to 70% with distilled water in the jar. The pipette was suspended from a small electric motor and passed through a closely-fitting glass sleeve into the jar of 70% sea water (Plate 39, fig. 3). During experiments the pipette was vigorously revolved about its long axis to ensure mixing both inside and outside. The amount of diffusion of chloride after 24 hours was determined by titration with 4% $AgNO_3$ solution as follows:- total Cl' in pipette filled to mark with pure sea water, less total Cl' in pipette after experiment.

In Series 3 the method was similar to Series 2 except

that the 70% sea water used was reduced in chloride content by replacement with nitrate so as to increase the rate of chloride diffusion.

To reduce the chloride content in sea water 19 gm. $AgNO_3$ were dissolved in 85.7 ml. distilled water and added gradually, and with stirring, to 200 ml. pure sea water. After the precipitate of silver chloride had settled two drops of 5% potassium chromate (K_2CtO_4) were added as an indicator to ensure no excess of $AgNO_3$. Then the supernatant fluid was filtered off.

The experiments lasted 8 hours and the amount of chloride which had diffused into the jar was determined by titration with 1% $AgNO_3$.

Series 4 differed from Series 3 in that the solution in the jar was isotonic with pure sea water but was reduced in chloride content as follows:- 19 gm. $AgNO_3$ ware added to 200 ml. pure sea water, very slowly and with continuous agitation. After the precipitate had formed the procedure was as in Series 3.

Table 2. Series 1.

Permeability of cuticle in milligram ions chloride/6 hours.

Right Way			Outside in		
Experiment	Control	Result	Experiment	Control	Result
0.5371	0.1669	0.3702	0.9126	0.1669	0.7457
0.8448	0.1564	0.6884	0.8812	0.1669	0.7143
0.1153	0.1564	0.9966	1.9610	0.1460	1.8150
0.6624	0.1669	0.4955	1.3560	0.1669	1.1891
0.7092	0.1669	0.5423	1.2620	0.1669	1.0951
0.5944	0.1460	0.4484	0.8032	0.1825	0.6207
0.8553	0.1669	0.6884	1.0380	0.1669	0.8711
0.8604	0.1564	0.7040	1.2510	0.1877	1.0633
0.5944	0.1669	0.4275	1.8570	0.1669	1.6901
0.5841	0.1356	0.4485	0.6674	0.1669	0.5005
Average		0.58098			1.03049
Sums of sqs. of dev. from mean		0.322838			1.736429
Standard deviation		± 0.1894			± 0.4392
Standard error		± 0.05989			± 0.1389

Standard error of difference 0.1512 ∴ "t" = 2.9729.

Value of "t" at probability level 0.01 and 18 degrees of freedom = 2.878.

Table 3. Series 2.

Permeability of cuticle in milligram ions chloride/24 hours.

Right way			Outside in		
Experiment	Control	Result	Experiment	Control	Result
5.299	6.676	1.377	4.590	6.676	2.086
5.925		0.750	6.299		0.377
5.008		1.668	6.091		0.585
5.340		1.336	4.840		1.836
5.674		1.002	5.632		1.044
Average		1.222			1.186

From the data above "t" = 1.151. "t", for 8 degrees of freedom and probability level 0.3, = 1.108.

Table 4. Series 3.

Permeability of cuticle in milligram ions chloride/8 hours

Right way			Outside in		
Cl' in jar			Cl' in jar		
at start	at end	Result	at start	at end	Result
17.52	24.31	6.79	17.52	23.78	6.26
17.52	25.24	7.72	17.52	23.99	6.47
17.52	26.70	9.18	17.52	26.17	8.65
15.12	23.15	8.03	15.12	23.46	8.34
15.12	20.33	5.21	15.12	20.85	5.73
Average		7.386			7.090

From the data above "t" = 0.3731. "t", for 8 degrees of freedom and probability level 0.5, = 0.706.

Table 5. Series.

Permeability of cuticle in milligram ions chloride/8 hours.

Right way			Outside in		
Cl' in jar		Result	Cl' in jar		Result
at start	at end		at start	at end	
32.22	38.18	5.96	32.22	38.80	6.58
32.22	39.43	7.21	32.22	40.26	8.04
29.00	37.76	8.76	29.00	37.76	8.76
29.00	37.96	8.96	29.00	36.93	7.93
29.00	37.76	8.76	29.00	37.35	8.35
Average		7.93			7.93

From the data above "t" = 0. "t", for 8 degrees of freedom and probability level 0.5., = 0.706.

Statistically, if the value of "t" calculated from two sets of figures exceeds a value of "t" calculated for appropriate degrees of freedom and a given level of probability, then the chance that the two sets of figures are different is of the given level of probability. Thus in Series 1. the permeability of the cuticle is greater in the outside-to-inside sense than in the inside-to-outside sense.

In Series 2, 3 and 4 no difference between the rates of diffusion of ions has been demonstrated.

4. DISCUSSION.

The method used in Series 1. was based on Yonge's (1936) method for measuring the permeability of the cuticle in the lobster, Homarus vulgaris: Yonge's results indicated a differential permeability. Webb (1940), however, in a further examination of the problem, criticised Yonge's (1936) method which makes no attempt to provide physiological conditions. Webb (1940), working on Carcinus maenas, repeated Yonge's experiments under conditions which he described as approximately physiological and found no trace of irreciprocal or differential permeability. Koizumi (1935), working on the Holothurian, Caudina, used solutions which were isotonic with sea water but were ionically unbalanced, lacking particularly calcium which is of first importance in the permeability of living cells and tissues, but he found no trace of differential permeability.

The results of the Series 1. experiments in this work correspond with the volume changes in living animals when transferred from 100% sea water to 70% and back again, and appear to indicate that, as with P. Gouldii (Adolph, 1936), water gain when placed in hypotonic water is more rapid than water loss when returned to pure sea water. However the Series 1. experiments must themselves

be regarded as unphysiological: the results which they give are probably typical of this type of double membrane under the conditions of an experiment in which there is a dehydrating influence on one side of the membrane and a dehydrating one on the other side. Homarus cuticle is also composed of two distinct layers, one of chitin and the other of protein (Yonge, 1932).

The experimental Series 2. and 3. were designed to test the permeability of the cuticle with salt solutions as close to normal as possible. The results do not suggest a differential permeability but the extent to which they vary casts considerable doubt on their value. Series 4. provides some check on this variation: since it persists in Series 4., where the solution within the pipette was isotonic with the solution outside it, it would appear that the method is not sufficiently sensitive to reveal any differences if they do exist. Webb (1940) did not publish the actual results for his experiments on Carcinus cuticle and later, in a personal communication, he indicated that he had been unable to devise an experimental method which was completely satisfactory.

5. SUMMARY.

Volume changes in P. minutum indicate that it swells in hypotonic sea water, but returns to original volume when re-immersed in 100% sea water.

Increase in volume when placed in hypotonic sea water is more rapid than the decrease when returned to 100% sea water.

The cuticle has been shown to be differentially permeable when tested under unphysiological conditions. An attempt to test the permeability of the cuticle under salt conditions as close to normal as possible has been unsuccessful.

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YONGE, C.M. (1932) On the Nature and Permeability of Chitin I. Proc. Roy. Soc. B. 111, 298-329.

(1936) On the Nature and Permeability of Chitin II. Proc. Roy.Soc. B. 120, 15-41.

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Plate 1

The southern wall of a gully on the beach at St. Andrews. P.minutum is to be found between the rock laminae.

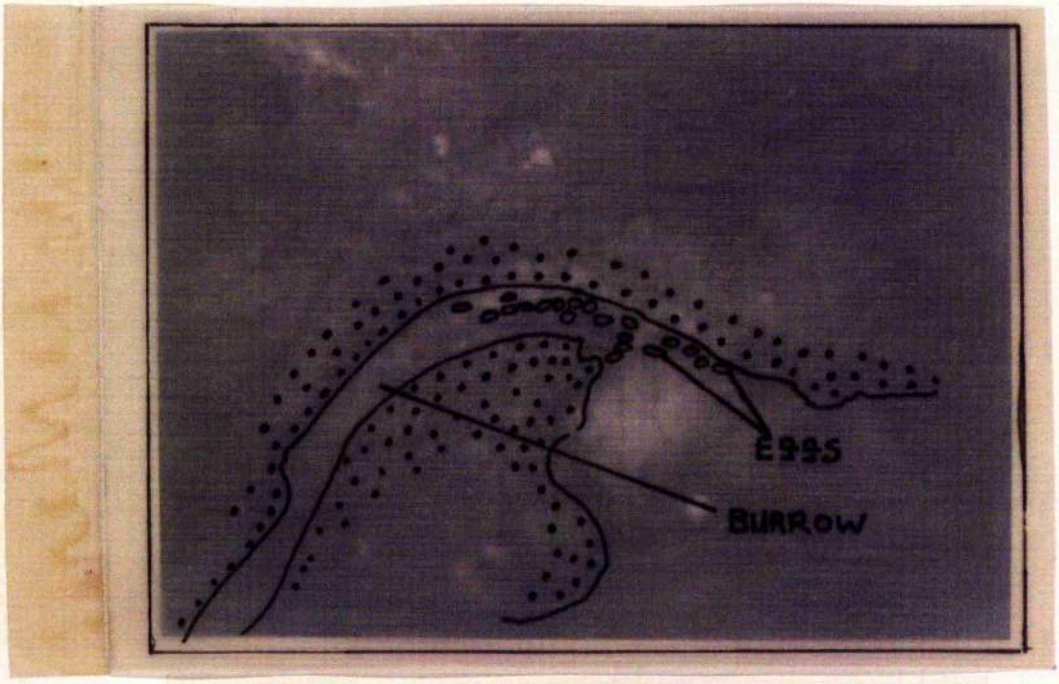


Plate 2

Burrow of P.minutum, containing eggs at the head end. (Magn. x 7)

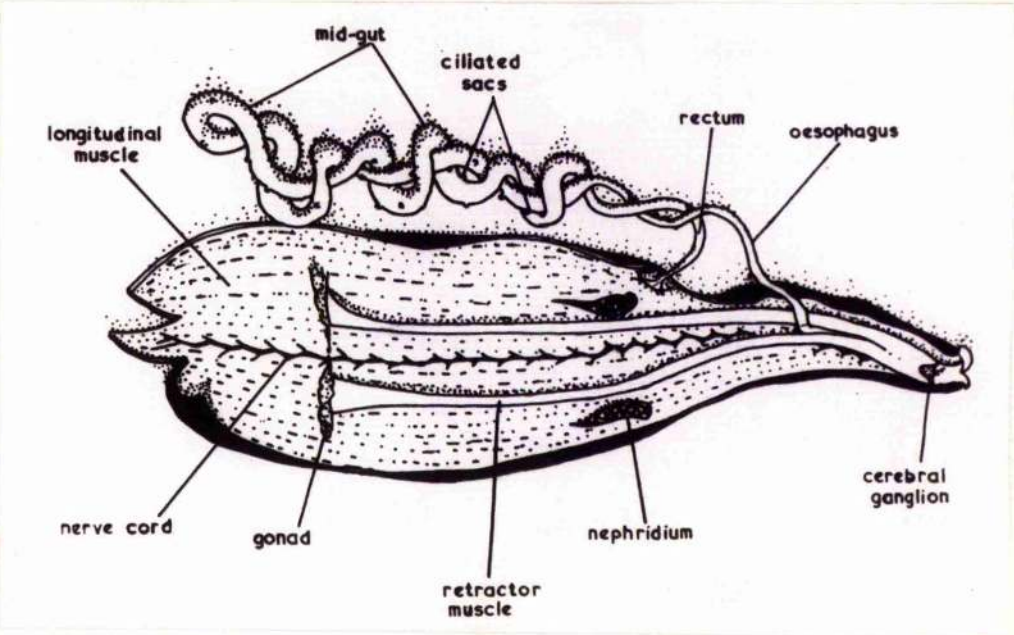


Plate 3.

Diagram of a dissection of P. minutum. (Magn. about x 7.)

Plate 4.

Transverse section of P. minutum in the region of the cerebral ganglion. The photograph shows a commissure given off by the ganglion itself lying above the pharynx, and both enclosed by right and left retractor muscles. Stained with Heidenhain's haematoxylin. (Magn. x 270)

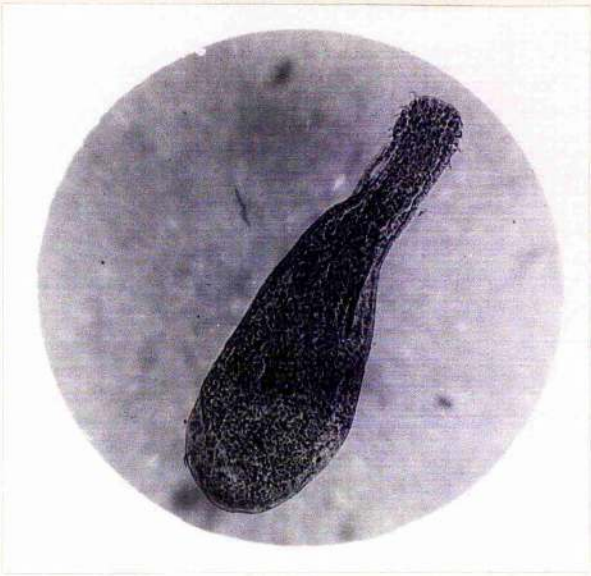


Plate 5.

Juvenile of P. minutum, stained with borax carmine. (Magn. x 100)

Plate 6

Juvenile hooks in P. minutum.
Fresh preparation. (Magn. x 450).

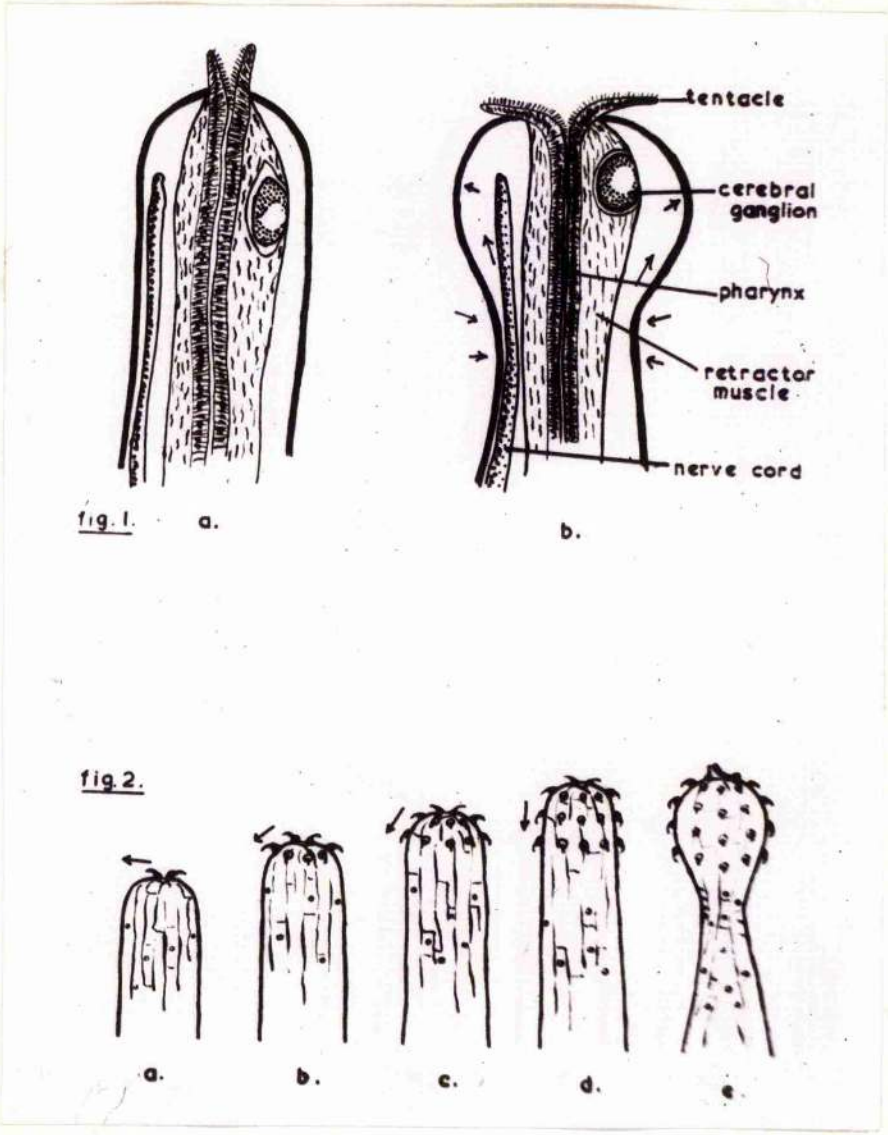


Plate 7.

Fig.1. P. minutum. a. Tip of extended proboscis, unexpanded.

b. The same, expanded.

Fig.2. Juvenile of P. minutum.

a - e. Stages in the protrusion of the proboscis to show the direction of movement of the hooks.

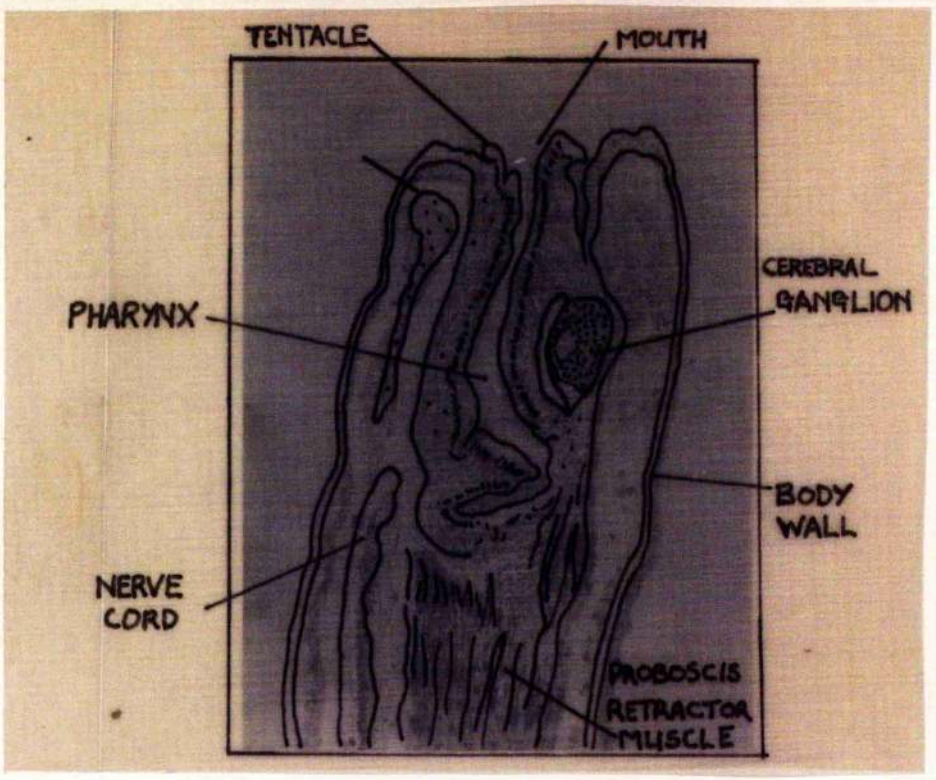


Plate 8.

P. minutum. Longitudinal section of tip of extruded proboscis, unexpanded. Stained with Heidenhain's haematoxylin. (Magn. x 100)

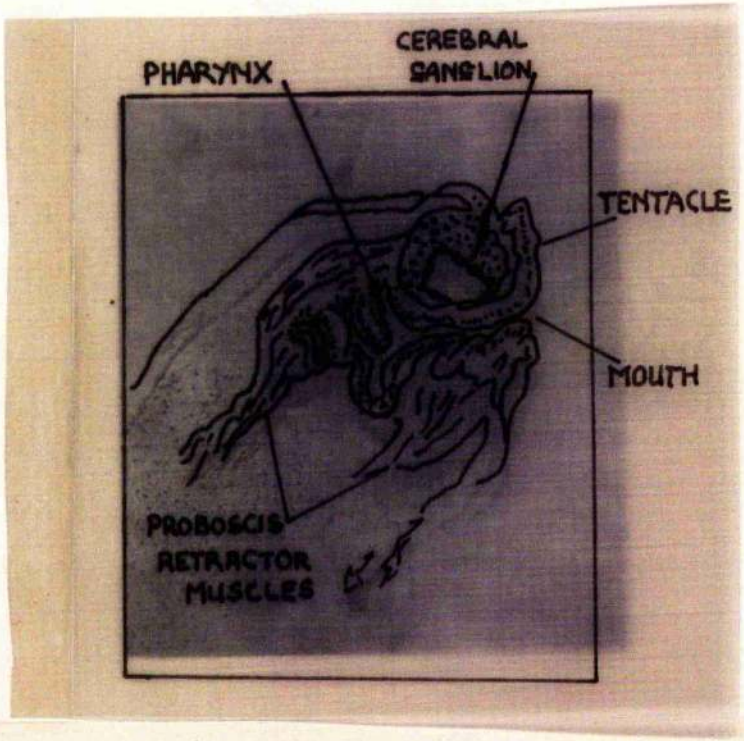


Plate 9

P. minutum. Longitudinal section of tip of extruded proboscis, expanded. Stained with haemalum and eosin. (Magn. x 100)

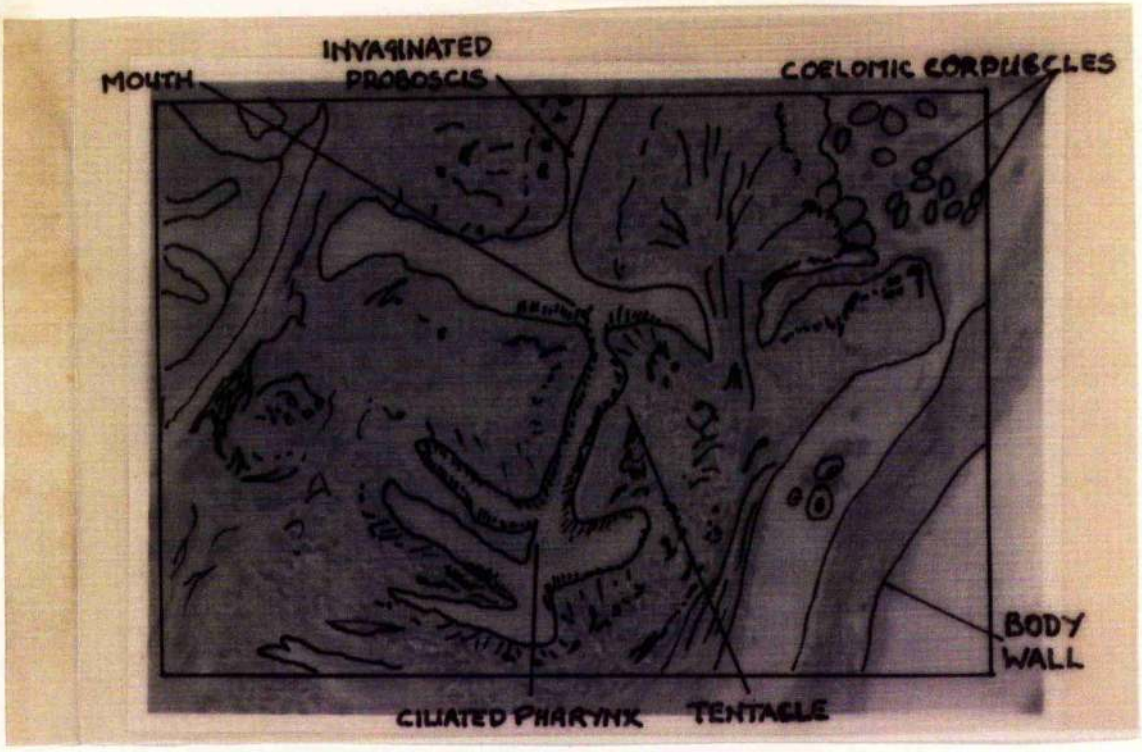


Plate 10.

P. minutum. Longitudinal section of tip of proboscis, invaginated. Stained with Heidenhain's haematoxylin. (Magn. x 270)

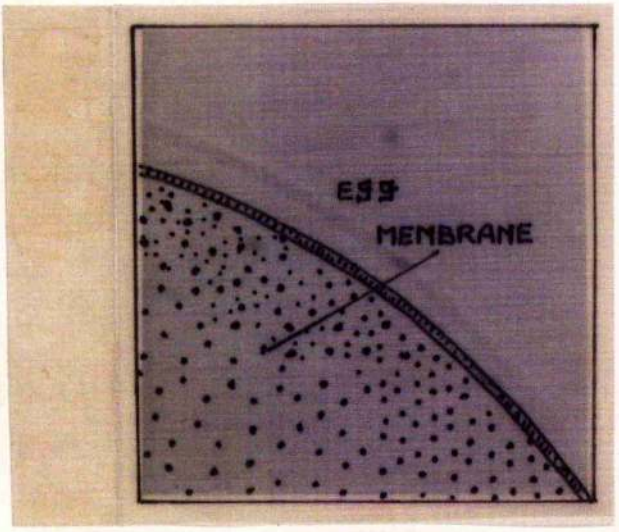


Plate 11.

Vitelline membrane in P. minutum. Fresh preparation. (Magn. x 900).

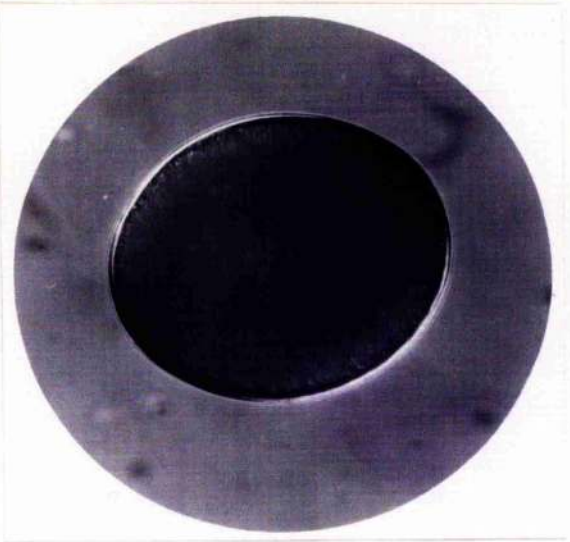


Plate 12.

Egg of P. minutum. Fresh preparation under polarizing microscope with positive compensation. (Magn. x 240)

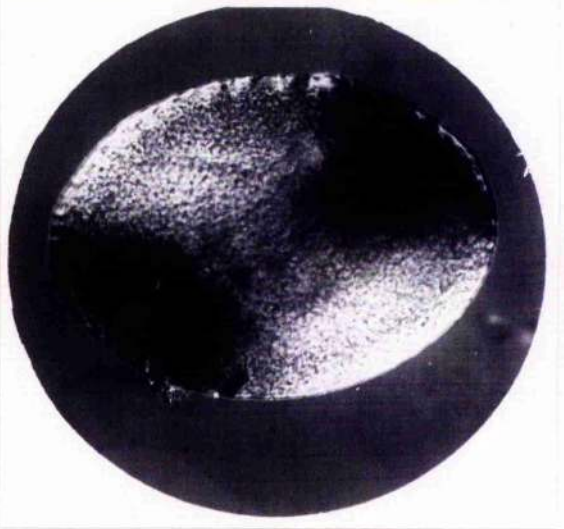


Plate 13.

Egg of P. minutum. Preparation in xylol under polarizing microscope with positive compensation. (Magn. x 240)

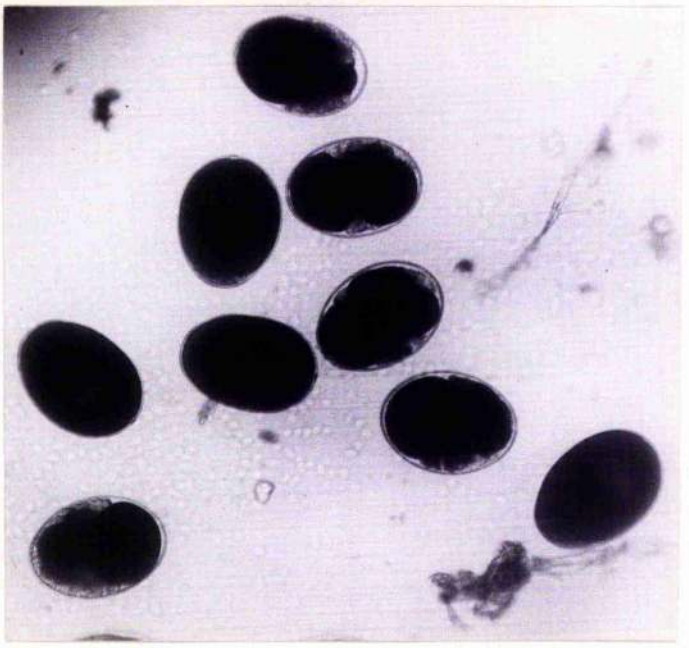


Plate 14.

P. minutum. Abortive eggs from the coelom. Fresh preparation. (Magn. x 60)

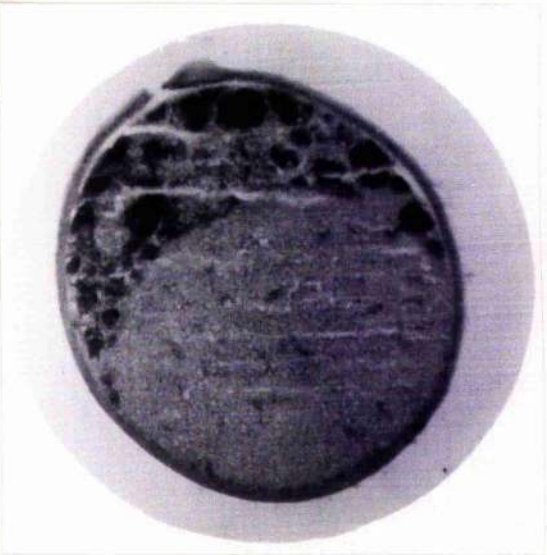


Plate 15.

P. minutum. Section of abortive egg from the coelom. Stained with haemalum and eosin. (Magn. x 270)

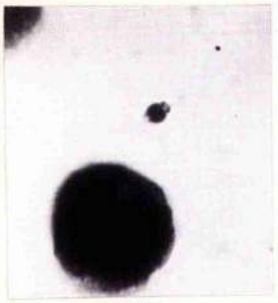


Plate 16.

Spermatozoon of P. minutum. Stained with Heidenhain's haematoxylin. (Magn. x 900)

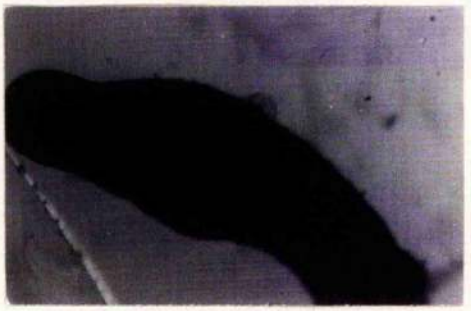


Plate 17.

P. minutum after injection with tissue extracts, to show "blister" at nephridiopore. (Magn. x 20)

Plate 18.

P. minutum. Section of egg which had been immersed in 0.3 M. Ca Cl_2 for 4 minutes. Stained with haemalum and eosin. (Magn. x 270)

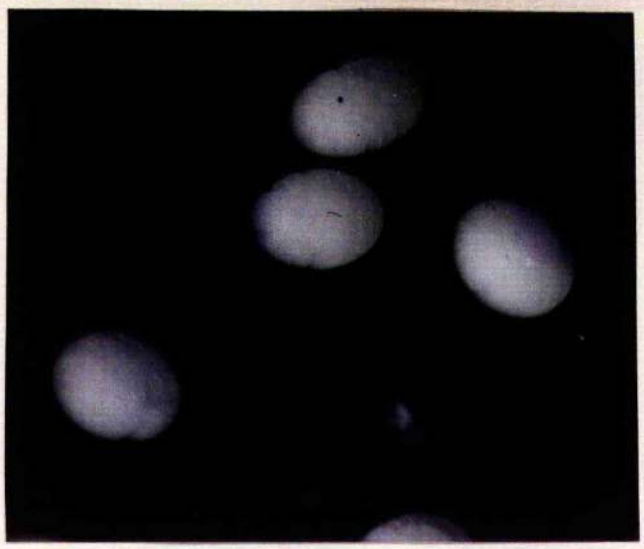


Plate 19.

P. minutum. Eggs which had been immersed in $0.3M\ CaCl_2$ for 4 minutes. Fresh preparation. (Magn. x 60)

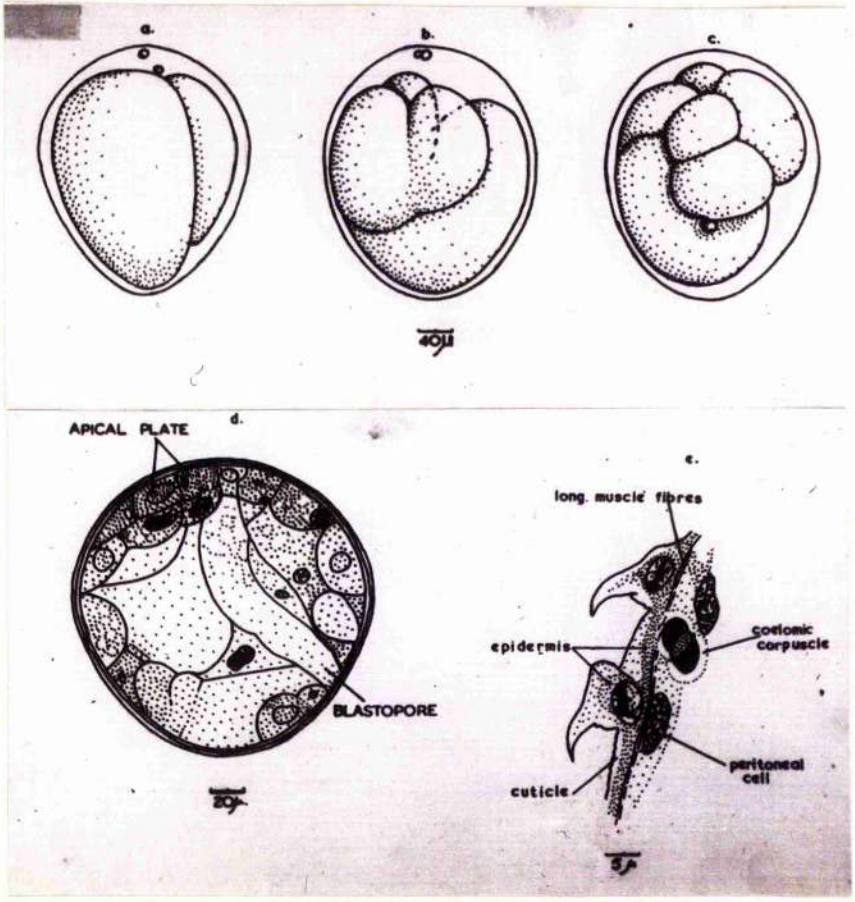


Plate 20.

P. minutum. Stages in development.

a. Two cell stage. b. Four cell stage. c. Eight cell stage.
d. Gastrulation. e. Juvenile hooks.

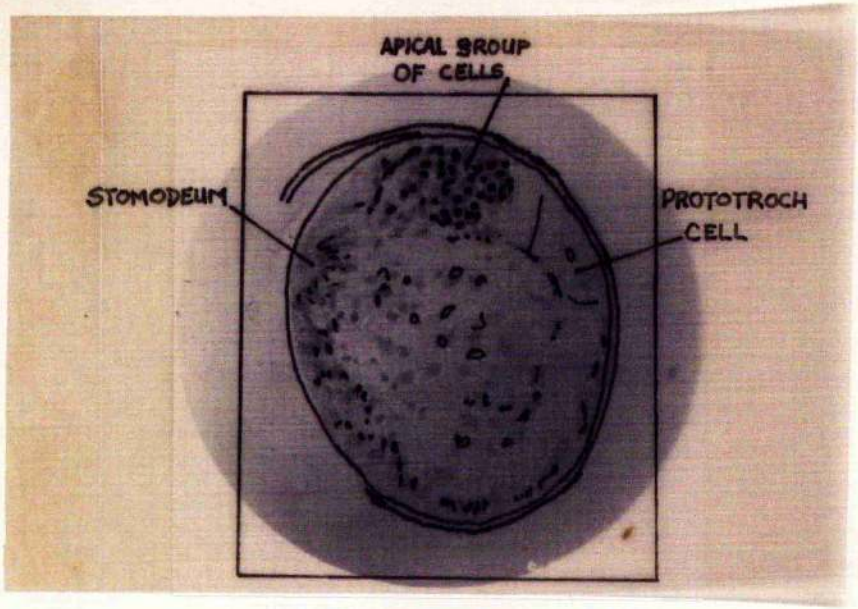


Plate 21.

P. minutum. Longitudinal section through trochophore larva. Stained with Ehrluch's haematoxylin and eosin. (Magn. x 270).

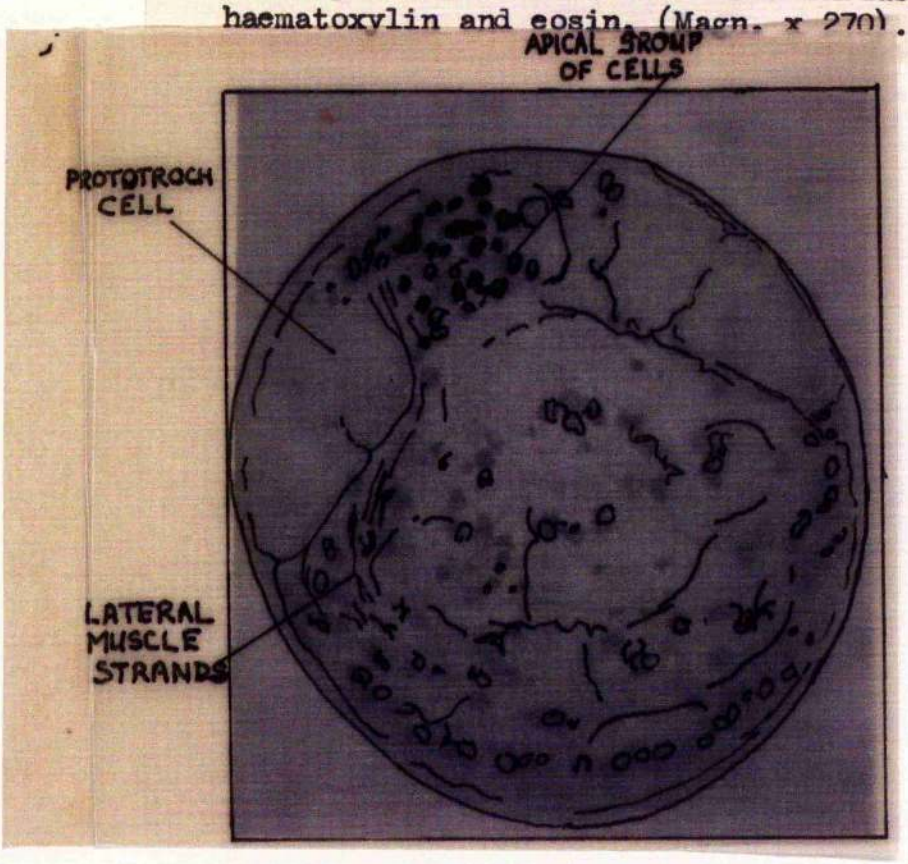


Plate 22.

P. minutum. Longitudinal section through trochophore larva. Stained with haemalum. (Magn. x 450)

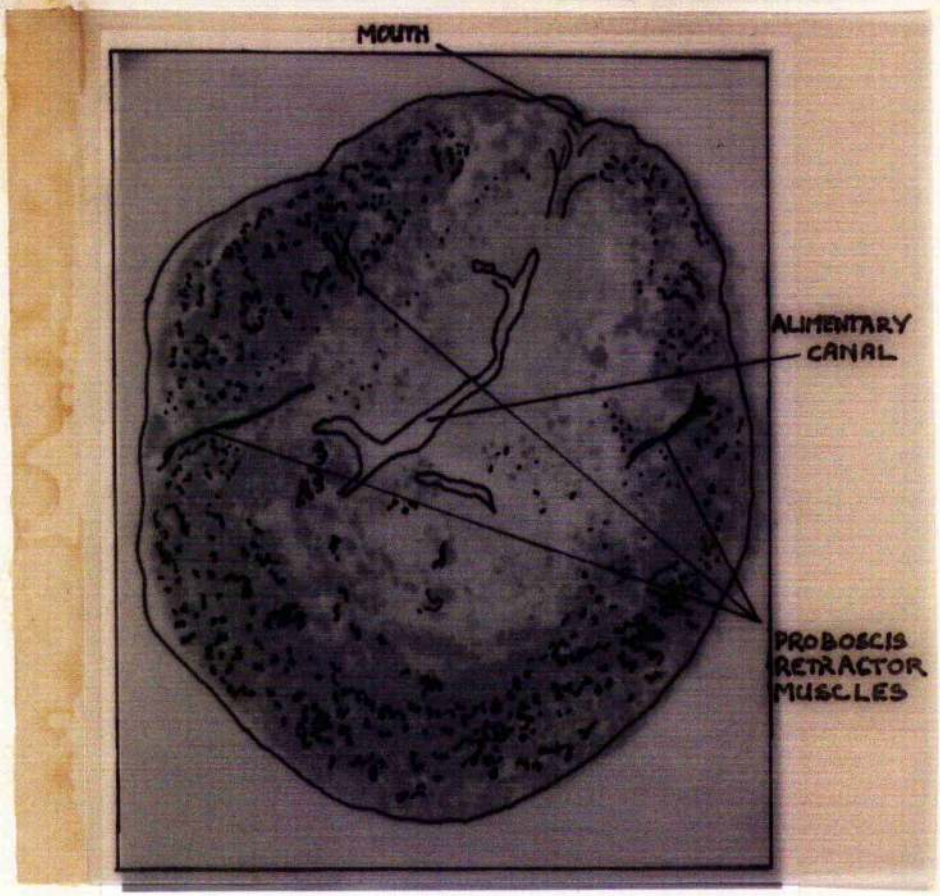


Plate 23

P. minutum. Longitudinal section through early juvenile stage. Stained with Heidenhain's haematoxylin and eosin. (Magn. x 450)

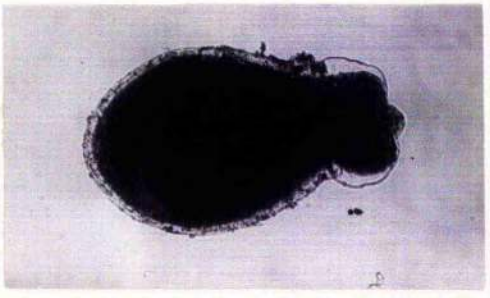


Plate 24.

P. minutum. Early juvenile stage. Fresh preparation. (Magn. x 60)

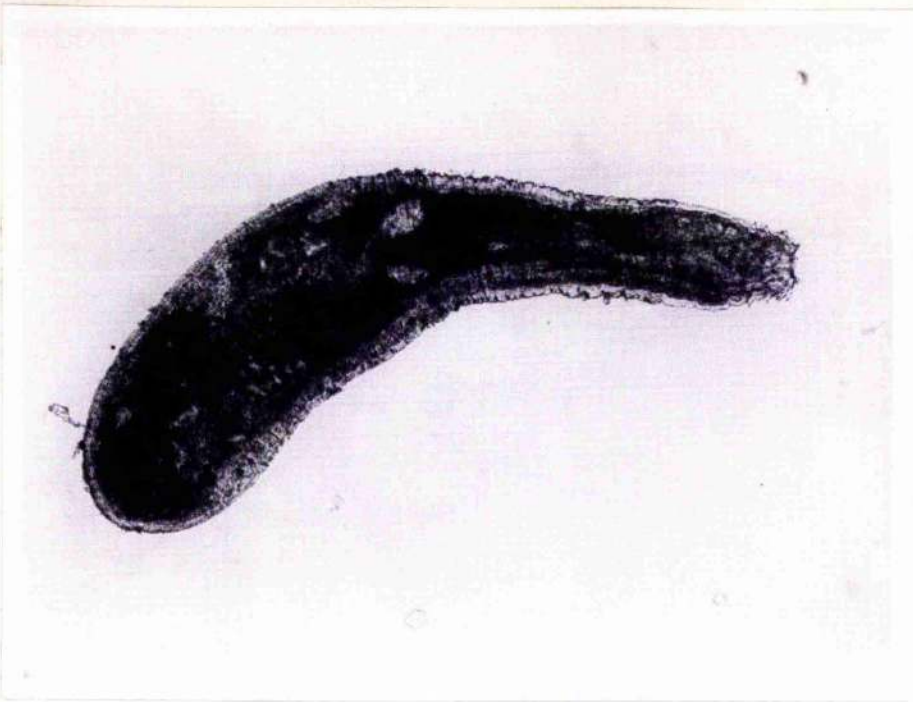


Plate 25

P. minutum. Late juvenile stage.
Fresh preparation. (Magn. x 60)

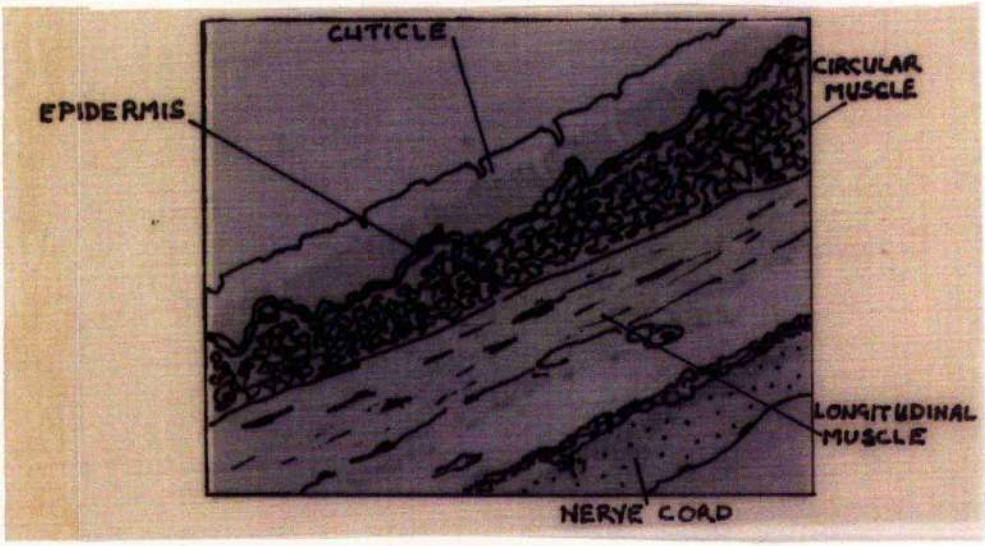


Plate 26.

Longitudinal section of body wall in P. minutum, contracted. Stained with haemalum. (Magn. x 270)

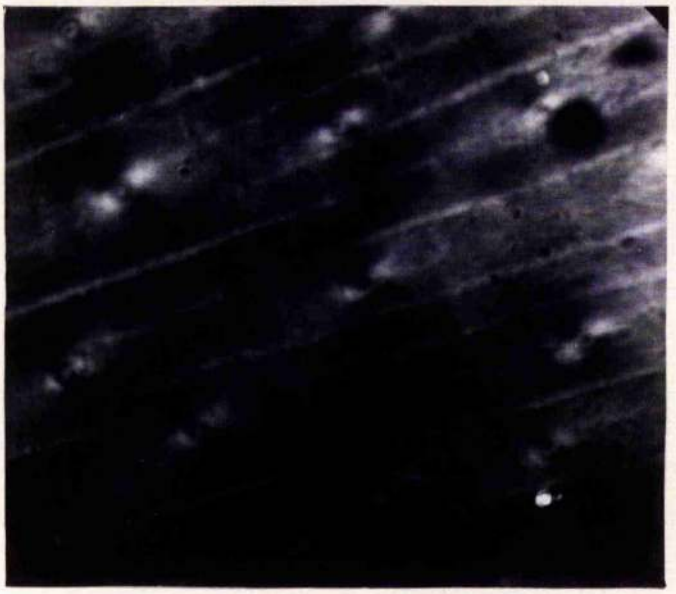


Plate 27.

Outer cuticle of P. minutum under polarizing microscope with negative compensation. Fresh preparation. (Magn. x 240)

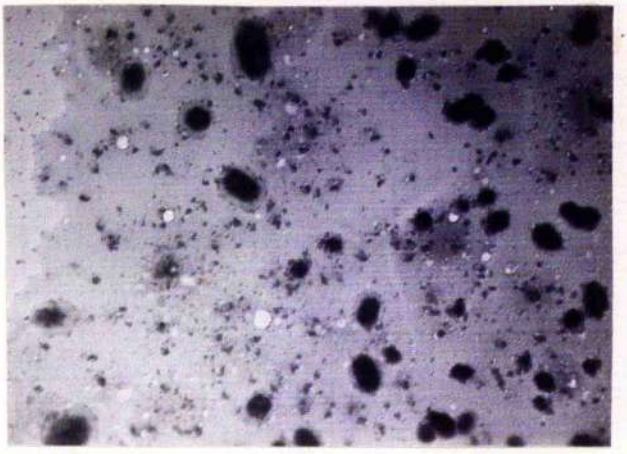


Plate 28.

Electron micrograph of teased preparation of outer cuticle of P. minutum. Cuticle fixed in 0.2% osmic acid. Preparation supported by Formvar film on copper grid and shadowed with palladium-gold. (Magn. x 5,000)

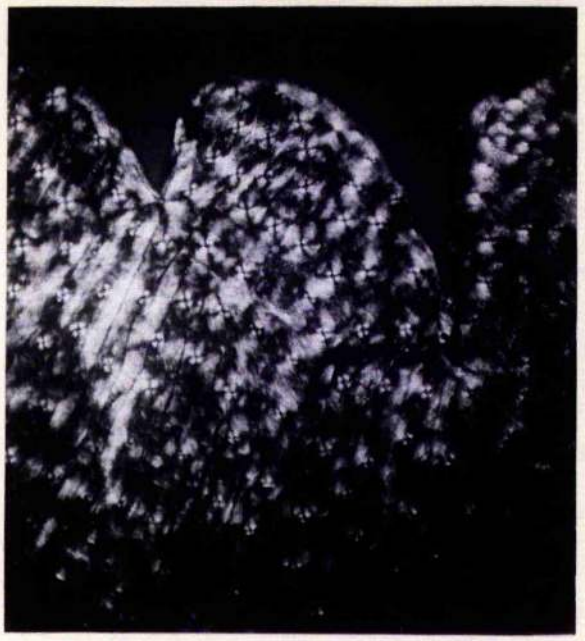


Plate 29 a.

Whole cuticle of P. minutum under polarizing microscope, no compensation. Fresh preparation. (Magn. x 60)

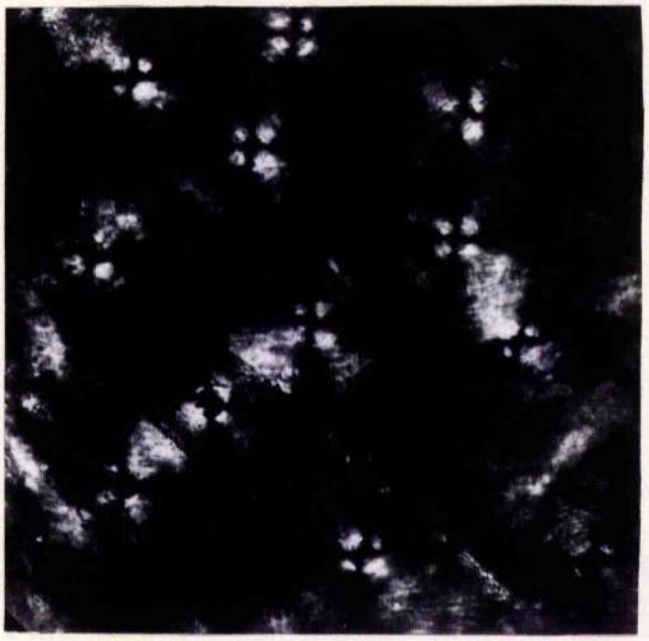


Plate 29 b

Whole cuticle of P. minutum under polarizing microscope, no compensation. Fresh preparation. (Magn. x 240)

Plate 30.

Fibrillar (inner) cuticle of P. minutum under polarizing microscope with negative compensation. Fresh preparation. (Magn. x 240)

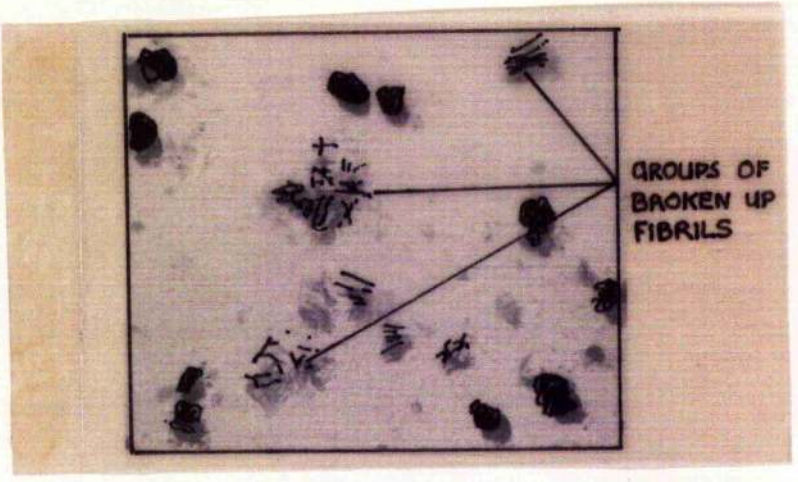


Plate 31.

Electron micrograph of teased preparation of fibrillar (inner) cuticle of P. minutum. Cuticle fixed in 0.2% osmic acid. Preparation supported by Formvar film on copper grid and shadowed with palladium-gold. (Magn. x 5,000).

Plate 32.

Electron micrograph of thin section (0.1 μ) of fibrillar cuticle of P. minutum. Cuticle fixed in 0.2% osmic acid. Section supported by Formvar film on copper grid and shadowed with palladium-gold. (Magn. x 7,200).

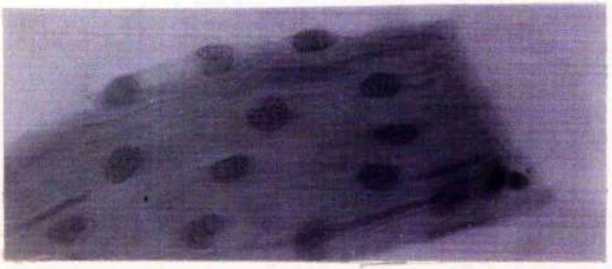


Plate 33.

P. minutum. Epidermis, supported by circular muscle, in plan view. Stained with haemalum and eosin. (Magn. x 540)

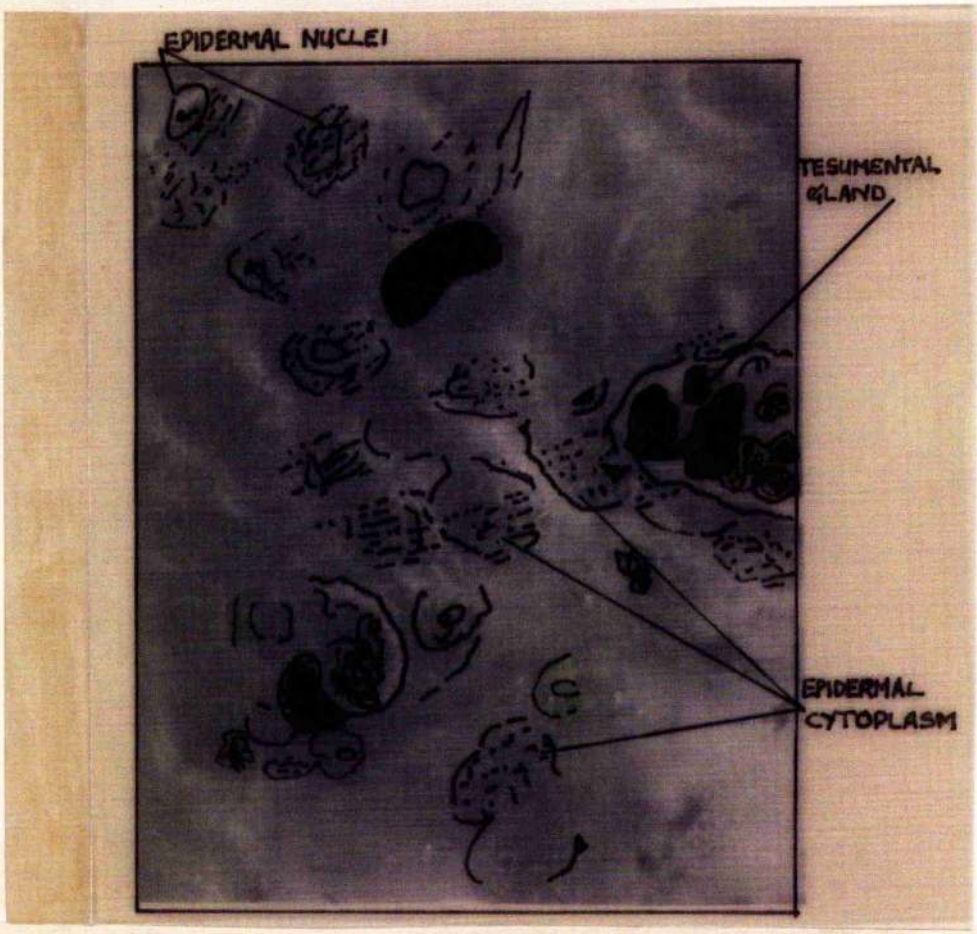


Plate 34.

P. minutum. Tangential section of body wall to show epidermis in plan view under polarizing microscope. Stained with Ehrlich's haematoxylin and eosin. (Magn. x 950)

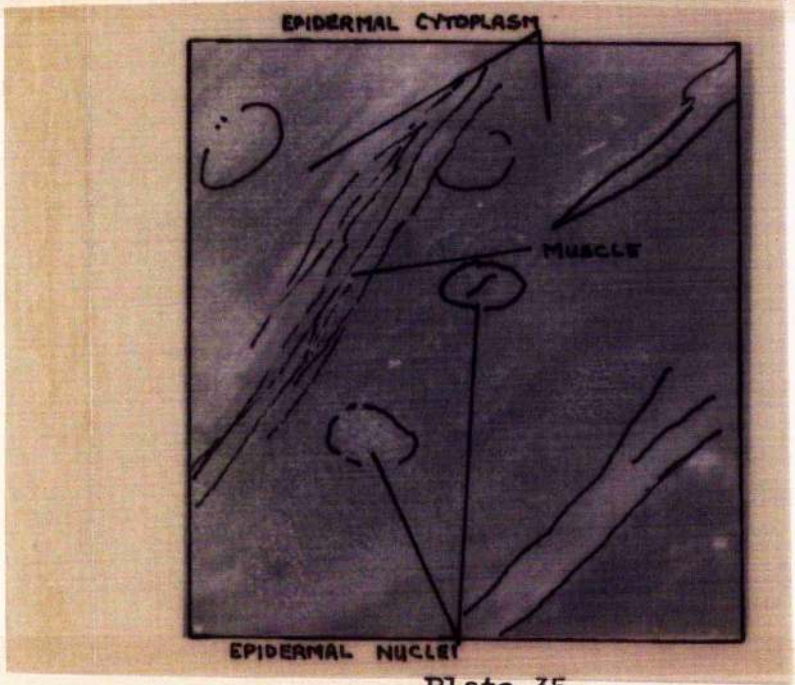
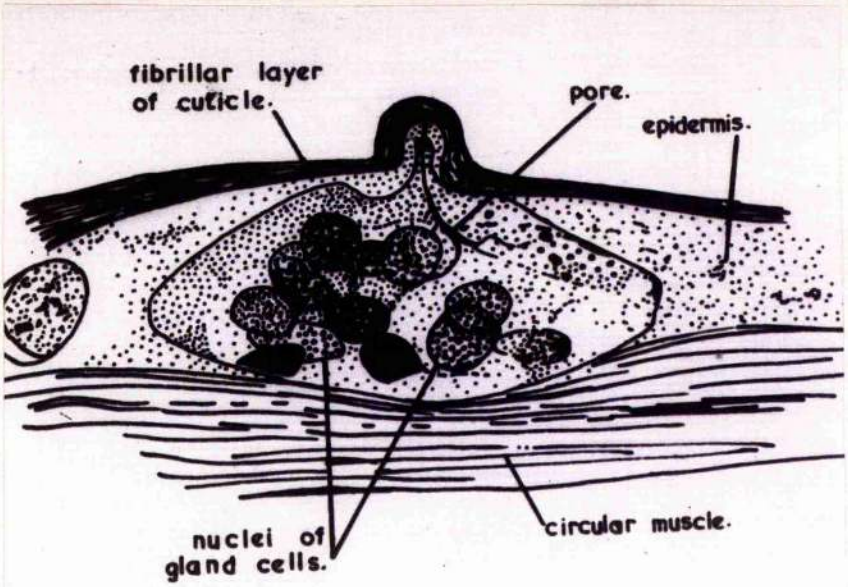


Plate 35.

P. minutum. Tangential section of body wall to show epidermis in plan view under polarizing microscope. Fixation: formol-basic lead acetate. Unstained. (Magn. x 950)



3μ.

Plate 36.

P. minutum. Diagram of transverse section of the body wall to show a tegumental gland.

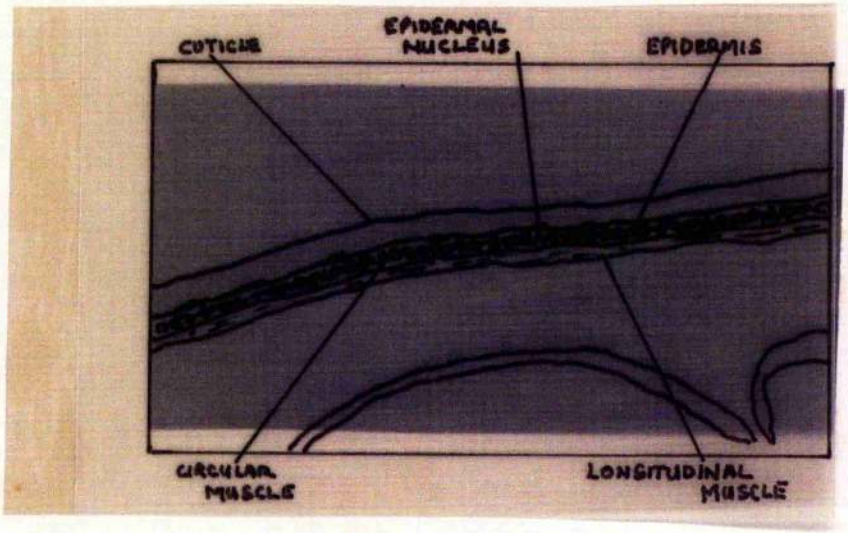


Plate 37.

P. minutum. Longitudinal section of body wall, extended. This plate and plate 26 show different regions of the same longitudinal section of body wall. Stained with haemalum. (Magn. x 270)

Plate 38.

P. minutum. Longitudinal section of body wall, strongly contracted. Stained with Heidenhain's haematoxylin and eosin. (Magn. x 450)

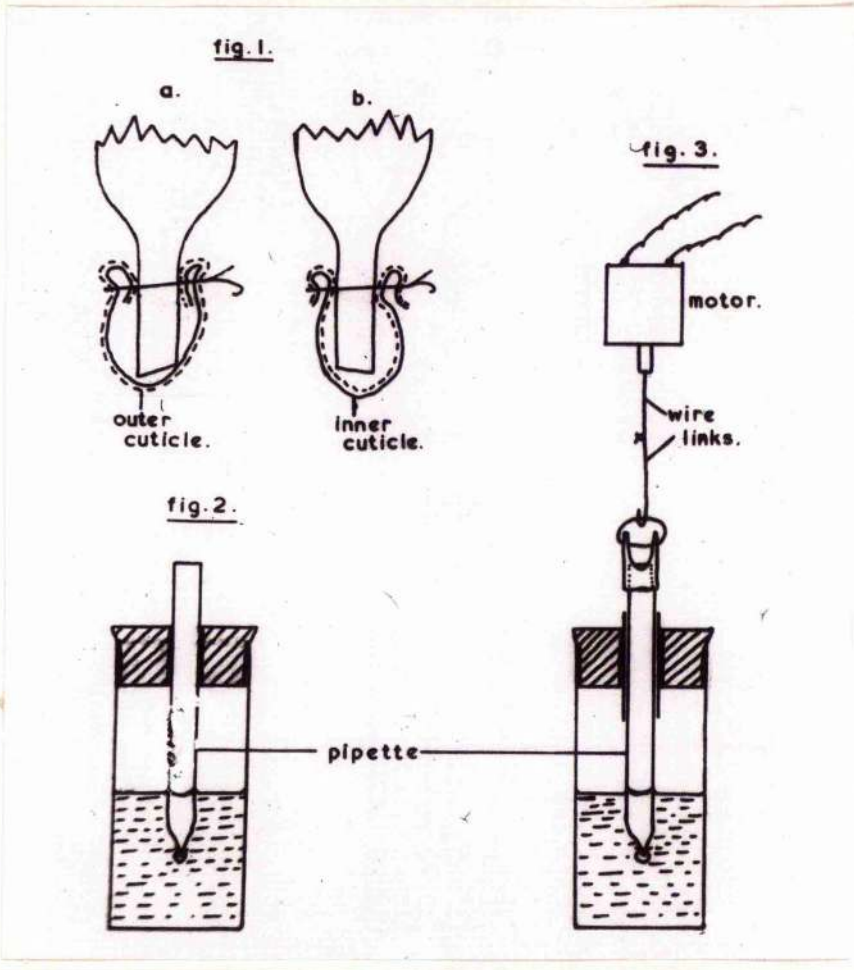


Plate 39.

Fig. 1. a. Cuticle on pipette in "right way" position.
b. Cuticle on pipette in "inside out" position.

Fig. 2. Arrangement of pipette, cuticle and jar in Series 1. of permeability tests.

Fig. 3. Arrangement of pipette, cuticle, jar and motor in Series 2, 3 and 4 of permeability tests.