THE HISTOPHYSIOLOGY OF GROWTH AND REPRODUCTION IN *HYMENOLEPIS DIMINUTA* (RUDOLPHI, 1819)

by

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CLAIM OF ORIGINALITY AND CONTRIBUTION TO KNOWLEDGE

A detailed study of the development of Hymenolepis diminuta in the rat was made using histological and histochemical methods.

The structure of both male and female reproductive systems of this tapeworm are described in detail.

The physiology of the male reproductive system, including the process of sperm transfer, was examined in detail for the first time. The histochemical nature of the secretion elaborated by the gland cells of the external seminal vesicle (the so-called prostate glands of trematodes and cestodes) is reported for the first time in a parasitic platyhelminth.

The study of the female system included the first detailed histochemical analysis of the ovary, the vitelline gland and Mehlis' gland of a cyclophyllidean cestode.

The anatomy of the scolex is described in detail; and the mode of attachment of the scolex to the intestine of the rat was examined as well. Neurosecretory cells, showing a cycle of secretory activity, were observed within the rostellum of the scolex; this is the first description of neurosecretory cells in a cestode.

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CHAPTER I

AN INTRODUCTION

Hymenolepis diminuta is a tapeworm which inhabits the small intestine of rats. It was first discovered by Olfers in Rio de Janeiro, and later described, from incomplete specimens, as a new species by Rudolphi in 1819 (references from Joyeaux, 1920). A similar, but more exact description of the worm including its characteristic features was provided by Dujardin in 1843. Grassi and Rovelli in 1888 were the first workers to show that certain insects were the intermediate hosts in the life cycle of H. diminuta (references in Joyeaux, 1920). Subsequently it was demonstrated that several arthropods could have this role, and for a recent list of the arthropod intermediate hosts of Hymenolepis diminuta one could consult the reports of Joyeaux (1920) and Oldham (1931). Zschokke (1889) made the first detailed study of the anatomy of the adult worm, and to date it remains, as far as the writer is aware, the only such published investigation.

Hymenolepis diminuta is cosmopolitan in distribution and its life cycle is easily duplicated in the laboratory. The adult worms are readily maintained in

rats, and the larvae, called cysticercoids, in the flour beetle Tribolium confusum. Because of the ease in maintaining this tapeworm in the laboratory it is used extensively in investigations on cestode biochemistry, physiology, metabolism, fine structure and development. Despite the extensive use of this tapeworm in many laboratories, there still remain many gaps in our knowledge regarding its developmental physiology in the definitive host. An appreciable body of literature is available regarding development in tapeworms, especially in H. diminuta, and a large proportion of this work is occupied by investigations in growth. This is not surprising since tapeworms like H. diminuta, because of their large size and bulk, provide sufficient material for biochemical analysis. Despite the advances made within recent years, with the extensive use of electron microscopy and the refining of in vitro cultivation techniques, we still lack much information regarding the physiology of development in tapeworms. For example we are still largely ignorant of the mechanisms which operate in initiating and controlling growth and maturation in tapeworms, or many details pertaining to their reproductive physiology, although there is much information available concerning the rate of growth or the nutritional and other requirements for growth in

these animals. Studies of development of H. diminuta in the definitive host, using precise cytological techniques, have been somewhat ignored. The present investigation was undertaken with this in mind, and this dissertation, it is hoped, will increase our knowledge and understanding of the developmental biology of Hymenolepis diminuta.

The life cycle of H. diminuta, including brief morphological details, is as follows. The adult lives in the small intestine of rats. It has a small unarmed scolex bearing 4 well-developed suckers by which it fastens itself to the intestinal wall. The rest of the body consists of a short unsegmented region called the neck, followed by a long strobila consisting of several hundreds of segments or proglottids. Each proglottid eventually possesses the full complement of reproductive structures. When mature, the proglottids start producing eggs which are eventually passed with the faeces of the host. A flour beetle becomes infected with the parasite by ingesting viable eggs. The egg contains a 6-hooked larva or hexacanth surrounded by a set of envelopes. The hexacanth is liberated from the surrounding envelopes within the gut of the insect. It rapidly penetrates the gut wall and enters the body cavity where, in a period of a week to 10 days, it grows into a second larva called the cysticercoid. The cysticercoid consists of a

scolex suspended in a cavity and enclosed by a complex of membranes. A small channel serves as a communication between the central cavity and the exterior. On entering the stomach of a rat the cysticercoids become activated and the scolex begins to emerge from the protective housing through the channel. This process is completed in the small intestine and the whole event is referred to as excystation. The young worm soon attaches itself to the intestinal wall and then enters a period of rapid growth. A so-called germinative region, occurring posterior to the scolex, produces proglottids which are so oriented that the oldest formed proglottid is furthest away from its point of origin, and the newest formed nearest it. The resultant tape-like body thus shows a gradation in developmental stages from very immature segments to those in which development is at its maximum. When mature, the proglottids start producing eggs which will eventually serve as the source for the next generation of worms.

In H. diminuta the duration of the period between excystation and the appearance of gravid proglottids is about 16-17 days. Within this period, the parasite has grown from a length of less than 1 mm to as much as 50-60 cm. The spectacular increase in length and mass during development has greatly influenced the methods of

studying the developmental physiology of H. diminuta. Much of the work has therefore been an estimation of growth as measured by changes in length, weight, chemical composition, proglottid number and gross morphology over a period of time. Variables in the host-parasite relationship have been introduced so that more information concerning growth rates and growth requirements could be obtained. Such variables are produced by introducing qualitative changes in the host diet, using hosts of different sizes, sexes, species and even genera, or using metabolically altered hosts such as gonadectomised rats or "bileless" rats. The environment of the parasite could be further altered or modified by introducing a second parasitic species along with the parasite in question, or by raising it simultaneously with other individuals of the same species in different population sizes. Studies on the growth of H. diminuta on the lines mentioned above have been made by several workers. Their observations have been summarised by Roberts (1961) and in more detail by Read and Simmons (1963).

Goodchild and Harrison (1961) studied the growth of H. diminuta during the first 5 days in the final host. In vitro observations on excystation showed that the time taken for activation, and the subsequent escape

of the young worm from its surrounding envelopes could be as little as 3 minutes. Young worms emerged in the small intestine about 3-4 hr after infection. The newly emerged worm is very small and the excretory ducts are visible in it. A large cavity is present posteriorly and this becomes occluded within the first 24 hr. The scolex has four suckers and "within the scolex there is a remarkable sparcity of cells. Scattered loosely among the permanent organs of the scolex are mesenchyme-like cells" (Goodchild and Harrison, 1961). They noted the presence of 2 major types of cells in the strobila of 24-48 hr old worms; rounded cells beneath the "cuticula" and elongate spindle-shaped cells in the medullary region. Segmentation and differentiation of the reproductive anlagen were observed to begin 4 days later. The growth rate of the worms, as measured in length and area, was exponential in character. After an initial lag it increased to a maximum on the 3rd day with slight declines in rate observed on the 4th and 5th days. They also found that during the 1st 3 days, no significant size differences were detectable in worms obtained from male and female rats, and that large doses (100 or more cysticercoids per rat) had no effect on the size of the worms; that is the crowding effect was not operating at this stage. Roberts (1961) investigated

the growth of H. diminuta in the definitve host during the period after excystation until patency. Growth was described using measurements of length, proglottid number, changes in the major chemical constituents and morphological changes. By studying growth in worms from populations of different intensities, using the same criteria, he was able to observe the effect of crowding on the developmental physiology of the parasite. It was observed that the first 7 days constituted the period of most rapid growth and that this rate was unaffected by population density. The maturation rate was independent of the rate of growth as measured in length, weight and rate of proglottid formation. Population density did not affect the length of the prepatent period which was 16-17 days in infective doses of 1, 50 or 100 cysticercoids per rat. Crowding, however, did affect the number of proglottids produced and "apparently has a direct effect on the germinative region" (Roberts, 1961). He also studied morphological development and provided a general outline of development correlated with time. These observations were made on material prepared as whole mounts stained in Semichon's acetic carmine, prior to which the worms were relaxed in distilled water and fixed. This technique was adequate for his purpose, but is obviously limited in the quality and quantity of

information it could provide regarding developmental events. Thus a more careful study using more precise histological techniques will amplify the observations of Roberts on the morphological development of H. diminuta in the rat.

Development includes growth, as manifested by an increase in length and mass, as well as maturation processes. Bell and Smyth (1958) and Smyth (1959) have provided a generalised scheme of development in a pseudophyllidean cestode Diphyllobothrium dendriticum in the rat. Using this as a guide, a similar scheme was constructed for H. diminuta, and it is shown below.

Cysticercoid -- ingested by rat

priming and activation of cysticercoid in the stomach

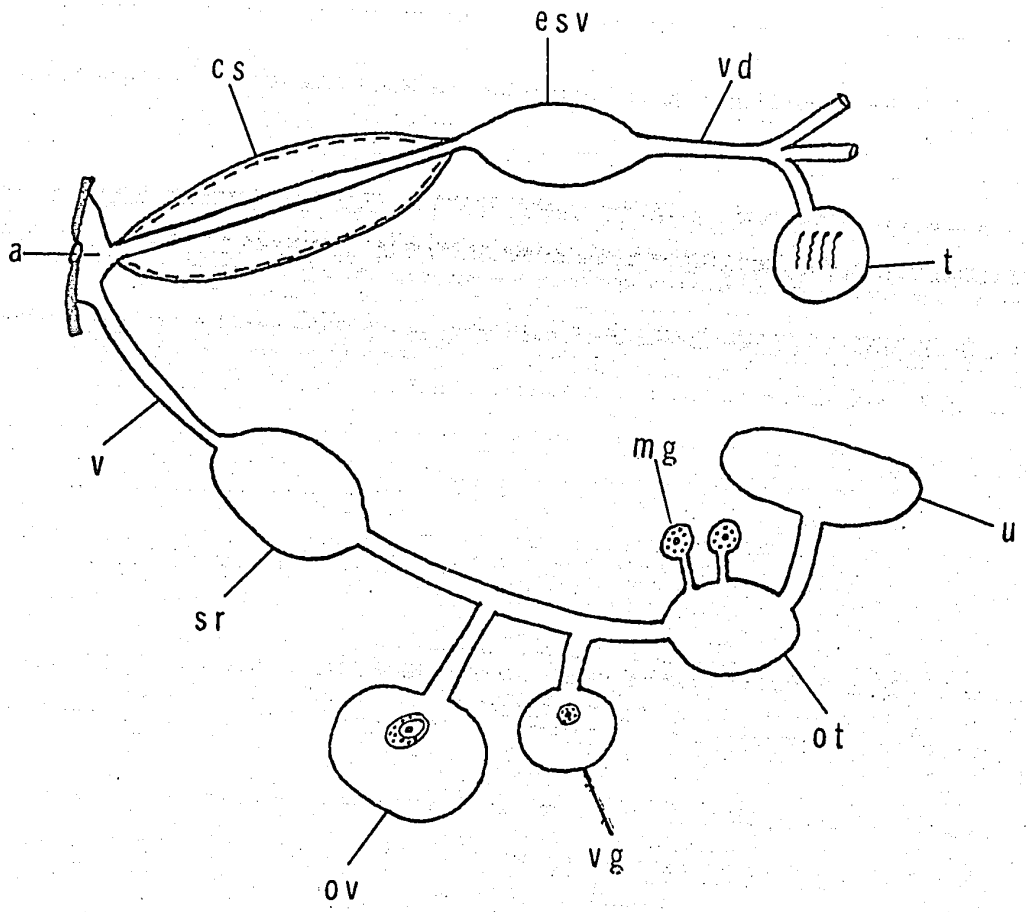
1. Excystation -- completed in the small intestine
2. Attachment of the worm to intestinal wall
3. Cell division
4. Segmentation (formation of proglottids or strobilisation)
5. Organogeny
6. Gametogenesis
7. Sperm transfer and fertilisation
8. Formation of the egg (embryogenesis proper)

Excystation in H. diminuta appears to be a rapidly accomplished process. The cysticercoid is apparently "primed" in the stomach and the process completed in the small intestine. The young worm escapes from its larval envelopes into the lumen of the gut through a slender canal. For successful excystation a combination of pepsin, trypsin, bile salts and a temperature corresponding to the host's body appear to be essential (Rothman, 1959). Rothman (1959), Goodchild and Harrison (1961) observed that younger cysticercoids excysted much faster than older cysticercoids. Details regarding the physiology of excystation are available in the reports of Read (cited by Read and Simmons, 1963), Rothman (1959), and Smyth (1963a). The present investigation is not concerned with excystation, but rather with events following excystation. There is no published information regarding the structural organisation of the young worm except for the brief reports of Goodchild and Harrison (1961) and Roberts (1961).

The proglottids, once produced, rapidly differentiate to become egg producing units. This differentiation has been very adequately summarised by Bell and Smyth (1958) who wrote that "the processes taking place in the maturation of a pseudophyllidean[1] plerocercoid to an adult cestode or a metacercaria to an adult trematode

are essentially concerned with the development of genitalia for the elaboration of the egg." This statement would hold true for a cyclophyllidean cestode like H. diminuta as well. The process of reproduction in the adult tapeworm culminates in the formation of the so-called egg. Mature tapeworm proglottids are thus efficient egg-producing units, and a study of development in this tapeworm would be very much concerned with the process of reproduction.

It would be appropriate at this point to give a brief and generalised description of the reproductive system of H. diminuta (see Fig. 1). Each proglottid contains both the male and female reproductive structures. The spermatozoa are produced in 3 testes and they are transferred through a vas deferens and seminal vesicle to eventually arrive at a copulatory organ--the cirrus complex, which is situated laterally in the proglottid. The cirrus complex opens into a small enclosed space called the genital atrium, which communicates laterally with the exterior through an opening called the atriopore. A slender vagina leads inwards from the atrium and opens into a large, dilated, tubular structure called the receptaculum seminis, in which sperms received in copulation are stored prior to fertilisation. The egg is initially assembled in a centrally located chamber



t - testis
vd - vas deferens
esv - external seminal vesicle
cs - cirrus sac
a - genital atrium
v - vagina
sr - seminal receptacle
ov - ovary
vg - vitelline gland
ot - ootype
u - uterus
mg - cell of Mehlis' gland

Fig. 1.--Diagrammatic representation of the reproductive system of H. diminuta.

called the ootype, which is surrounded by a collection of gland cells which discharge their secretion into it. These gland cells constitute Mehlis' gland. A large ovary, producing oocytes, and a smaller compact vitelline gland, which produces the vitelline cells, are situated close to the ootype. The oocyte, spermatazoan and a vitelline cell arrive at the ootype through specific ducts, and, together with the secretion of Mehlis' gland, are all assembled in this chamber to form the egg. Fertilisation apparently takes place in the ootype. The egg is then ejected into the uterus where it completes its development. Every mature proglottid is built on the same plan and has the identical function of producing eggs.

Adult tapeworms possess a highly successful reproductive process, which is evident from the great numbers of potential offspring produced for propagation. Reproductive physiology is thus a subject of considerable importance. There has been no over-all study of the reproductive process in tapeworms, rather it has been studied in piecemeal fashion. Consequently, information pertaining to the reproductive physiology of these animals comes from several different sources.

Detailed morphological and anatomical descriptions

of the reproductive system of tapeworm species are many. References to such accounts are available in the texts by Hyman (1951), Wardle and McLeod (1952), and Joyeaux and Baer (1961). Zschokke's description of the reproductive system of H. diminuta is the only detailed account available to date with regard to this species. His study, published in 1889, does not provide sufficient information and is therefore considered to be inadequate. A knowledge of functional morphology is essential for a meaningful study of physiology. Good recent examples of such accounts are those of Rees (1961), and Rees and Williams (1965). Material prepared in taxonomic investigations has been used for morphological studies of the reproductive system, and specimens used in such investigations are generally prepared as stained whole mounts. This is a rather inadequate technique since structural details could easily be concealed, with only the gross morphology of the genitalia being visible.

Gametogenesis has been studied extensively in trematodes, but to a very much lesser extent in cestodes. Amongst the published accounts of gametogenesis in cestodes are those of Douglas (1963) and Rybicka (1964a). Rybicka (1966b) gives a brief account of gametogenesis in H. diminuta. Rosario (1964) studied spermatogenesis in H. diminuta and H. nana using the electron microscope.

The fine structure of the cestode spermatazoan has been investigated by Gresson (1962), Rosario (1964), and Lumsden (1965). The physiology of the male reproductive system in cestodes has been largely ignored except for studies on spermatogenesis and sperm ultra-structure.

The formation of the gametes is an early step in the reproductive process. The ovum and spermatazoan unite in fertilisation, and the fertilised ovum combines with the products of the vitelline gland and Mehlis' gland, eventually forming the egg. The so-called egg is actually a larva enclosed by one or more protective envelopes. The importance of the egg in the life cycle of a parasitic platyhelminth cannot be overemphasised, and consequently the formation of the egg and especially the protective coverings have received considerable attention. The elaboration of these protective coverings in the egg is generally referred to as egg-shell formation (Smyth and Clegg, 1959), and it has been the subject of a review by these two authors.

It was originally believed that the shell or capsule of the trematode egg was secreted by Mehlis' gland, which was thus also referred to as the "shell" gland. Subsequently Leuckart in 1886 (cited by Smyth and Clegg, 1959), through careful observations, concluded

that the shell was actually formed from material present in the vitelline cells. This conclusion was substantiated later by many other workers--Dawes (1940), Markell (1943), Rees (1939), to mention a few. The chemical nature of the vitelline cell material and egg-shell remained obscure until precise histochemical techniques for detecting chemical components in tissues were developed. The investigations of Stephenson (1947), Smyth (1951a, 1951b, 1954b) and others showed that the egg-shell in trematodes was a quinone-tanned protein, or sclerotin, which originated from shell precursors present in the vitelline gland cells. A similar study was extended to a pseudophyllidean cestode Schistocephalus solidus by Smyth (1956), where it was similarly shown that the egg-shell was a sclerotin secreted by vitelline cells which contained the sclerotin precursors. The chemistry of egg-shell formation in Diphylobothrium latum, another pseudophyllidean tapeworm, was studied by Bogomol'va and Pavlova (1964) (cited by Rybicka, 1966a), who confirmed the observations of Smyth (1956).

The formation of the embryonic envelopes in cyclophyllidean cestodes follows a different pattern. the vitelline gland is small and compact, and generally one vitelline cell (unlike in trematodes and pseudophyllidean cestodes) is associated with the fertilised

oocyte in the formation of the egg. The materials in the vitelline cell form a thin capsule which surrounds the fertilised ovum. The ovum undergoes cleavage and some of the macromeres formed in cleavage form the embryonic envelopes which invest the developing larva. Rybicka (1966b) made a histological study of embryogenesis in H. diminuta which includes a description of the formation of the embryonic envelopes. No detailed histochemical observations have been made pertaining to the formation of the embryonic envelopes in cyclophyllidean cestodes (equivalent to egg-shell formation in trematodes and pseudophyllidean cestodes). Johri (1957) studied the formation of the egg in a cyclophyllidean cestode Multiceps smythii, but his study was rather inconclusive. He noted that egg-shell formation in this species was greatly contrasted with that in trematodes and pseudophyllidean cestodes. "Phenol protein" granules (i.e., sclerotin precursors) were reported to be absent in the vitelline cells, though acidophil proteins were present. Acidophil proteins and small amounts of basic protein were present in the ova. The embryophore was found to show positive results with the performic acid-Schiff reaction, indicating the presence of a keratin-like protein. Johri was unable to trace the origin of the embryophore. This study, being

the only such study of "egg-shell" formation in a cyclophyllidean cestode, is unfortunately incomplete. Fragmentary information is available regarding the chemical nature of the embryonic envelopes (see Rybicka, 1966a). Morseth (1965) studied the development of the taeniid embryophores with the electron microscope. Morseth (1966) also investigated the chemical composition of the taeniid embryophore using several techniques. He concluded that there was "strong evidence for classifying the embryophoric blocks of these species as keratin-type proteins" (Morseth, 1966).

Histochemical observations on the vitelline cell and ovum correlated with the formation of the embryonic envelopes would be informative in a study of reproductive physiology. Such a study has not been done with reference to a cyclophyllidean cestode.

Histochemical information pertaining to the reproductive physiology of cyclophyllidean cestodes is fragmentary. Most of the information to be furnished below concerns Hymenolepis diminuta (some, if not most of these observations were made in studies which were not directly concerned with reproductive physiology).

(a) Spermatazoa.--The presence of RNA in the spermatazoa of H. diminuta was observed by Cheng and

Jacknick (1964). Glycogen was detected in the sperms of H. diminuta by Hedrick and Daugherty (1957), Cheng and Dyckman (1964), and Rybicka (1967).

(b) Ova (oocytes).--The ova of H. diminuta lack glycogen (Hedrick and Daugherty, 1957; Cheng and Dyckman, 1964; Rybicka, 1967). The granular nature of the oocytes of cyclophyllidean tapeworms has been observed by several workers. Löser (1965a) described granules occurring in the cytoplasm of oocytes and according to him they represent stored materials ("Reservestoffe") for the embryo. Rybicka (1966b) observed vitelline material in the form of granules in the oocytes of H. diminuta. The histochemical nature of these granules is obscure. Johri (1957) reported that the oocytes of Multiceps smythii contained "acidophil" protein. The nature of the material in the oocyte is undoubtedly important since the fertilised ovum, in its subsequent development, produces the embryonic envelopes.

(c) Vitelline cells.--The vitelline cells contain material which forms a thin capsule surrounding the developing fertilised oocyte. The precise chemical nature of the vitelline material is unknown. Rybicka (1964b) noted that the vitelline cells in Moniezia

expansa showed metachromasia with toluidine blue which probably indicated the presence of acid mucopolysaccharides. Johri (1957) observed "acidophil" proteins in the vitelline cells of Multiceps smythii. The absence of sclerotin precursors in the vitelline cells was noted by Johri (1957) in M. smythii, and in H. diminuta by Llewellyn (1965). Some glycogen is present in the form of small globules, in the vitelline cells of H. diminuta (Hedrick and Daugherty, 1957; Cheng and Jacknick, 1964; Rybicka, 1967; Lumsden, 1965b). The presence of glycogen in the vitelline cells has led to the interpretation that these cells may have a nutritive role as well (Rybicka, 1966a). Löser (1965a) described the granules in the vitelline cells of H. diminuta as being stained a bright red with azan.

(d) Mehlis' gland.--The role of Mehlis' gland in the reproductive process of parasitic platyhelminths has long been a subject of controversy (see reports by Smyth and Clegg, 1959; Löser, 1965b; Ebrahimzadeh, 1966). Histochemical research shows that, in the species of trematodes and cestodes studied, the secretion of this gland is PAS positive and fast to digestion with saliva or diastase. Pearse (1960) recognizes 4 main groups of substances which show a positive PAS reaction, which is unaffected by treatment with diastase. They are:

neutral mucopolysaccharides; muco- and glycoproteins; glycolipids; unsaturated lipids and phospolipids.

The secretion of Mehlis' gland in H. diminuta too is PAS positive and diastase fast (Hedrick and Daugherty, 1957; Rybicka, 1967; Löser, 1965b). Löser (1965b) observed that the secretion of Mehlis' gland in cestodes stained a deep blue with azan. Hanumantha-Rao (1960) suggested that the secretion was a phospholipid-like substance. As to its function, the most plausible one to date appears to be that suggested by Dawes (1940), according to whom the secretion "forms a thin film which serves as a basis upon which the secretion of the 'yolk cells' is deposited to form the thin rudimentary capsule . . ." (Dawes, 1940). Burton (1963) observed that isolated Mehlis' gland kept in physiological solutions was capable of synthesising membranes. He suggested that the function of the secretion was to form "basic capsular membranes" on the inside of which the shell proper would be formed. Löser (1965b) comes to a similar conclusion regarding the role of Mehlis' gland in cestodes. He describes two kinds of cells occurring in Mehlis' gland of cestodes: large "mukosen" gland cells and smaller, less numerous, "serosen" gland cells. Both cell types open into the lumen of the ootype through slender ducts. The secretion of the "mukosen"

gland cells, which stains a deep blue with azan, is discharged into the ootype and forms a thin membrane against which the vitelline granules are deposited. These vitelline granules spread out and coalesce to form a shell. Löser did not carry out any special detailed histochemical studies on Mehlis' gland, except examining it for periodic acid-Schiff-reactive substances. Clegg (1965) recently reported his findings on Mehlis' gland and egg-shell formation in Fasciola hepatica. He observed that the cells of Mehlis' gland contained granules of secretion composed largely of lipo-protein. The lipo-protein secretion formed very thin membranes on both inner and outer surfaces of the egg-shells. This study appears to substantiate the observations of some earlier workers mentioned above. Clegg's study is the only such detailed investigation of Mehlis' gland in a parasitic platyhelminth. Thus it would be both interesting and informative to see, using histochemical methods, whether Mehlis' gland in a cyclophyllidean cestode, like H. diminuta, possesses similar characteristics.

A study of egg formation in several cestode species was made by Löser (1965a, 1965b), in which neither histochemical techniques were used, nor was the formation of the egg-shell or embryonic envelopes

studied in detail. Löser was more concerned with the structural organisation of the female reproductive apparatus in relation to egg formation, as well as the physiology of Mehlis' gland correlated with egg formation. His first report has the title "Der Feinbau des Oogenotop bei Cestoden." The term "Oogenotop" was coined by Gönnert (1962) to describe a complex of structures which was concerned with the elaboration of the egg in Fasciola hepatica. This complex consists of:

(a) Afferent ducts (oviduct, vitelline duct; uterus) which conducted the component parts of the future egg (i.e., oocyte or ovum, vitelline cells and sperm) to

(b) the ootype--a centrally located chamber into which opens

(c) Mehlis' gland cells.

Within the ootype, the ovum, sperm, vitelline cells and secretion of Mehlis' gland are moulded to form the egg, which is then ejected into

(d) the uterine duct and uterus which also function as different ducts, conducting the eggs away from the ootype.

Gönnert believes that the functions of the different parts of this complex are coordinated by strategically located nerve cells. The central theme of

his account is that the different parts of the female reproductive apparatus, in the region of Mehlis' gland, form a complex organ--the Oogenotop--and that these parts function in a smoothly coordinated manner as to form an efficient, egg-producing organ. Löser adopted Gönnert's idea of the Oogenotop in his study of egg formation in cestodes. He first discussed the concept of the Oogenotop; this is followed by a detailed account of its anatomy and histology, a major portion of which is devoted to Mehlis' gland. Brief descriptions of the Oogenotop of several cestode species are provided, and the description of this complex with reference to H. diminuta is very brief with no accompanying diagram. Unlike Gönnert, Löser does not describe nerve cells associated with different regions of the Oogenotop, although he does subscribe to the idea that nerve cells must coordinate the functioning of the different parts. He does describe certain cells associated with the Oogenotop, which for the lack of sufficient information, he prefers to call "X-cells."

Löser's second report (1965b) is titled "Die Eibildung bei Cestoden." He recognizes 4 main types of eggs, which are correlated with the 4 different types or varieties of Oogenotop complexes possessed by the different cestode species (Löser, 1965b, page 565).

A given species of tapeworm has a particular form of Oogenotop, corresponding to which a characteristic egg is produced. An appreciable portion of this study, too, is devoted to the physiology of Mehlis' gland.

It is apparent, then, that useful though it is, the concept of the Oogenotop is limited in a sense when one is considering the reproductive physiology of a tapeworm. The Oogenotop, as visualised by these authors, is composed of

(a) afferent ducts leading into

(b) a central chamber--the ootype--where the egg is initially assembled,

(c) Mehlis' gland surrounding the ootype, and

(d) efferent ducts which move the eggs out of the ootype.

The male part of the reproductive system is thus completely excluded from the complex. Further, very brief attention, if any, is paid to the ovary and vitelline gland. Nevertheless one must appreciate the approach of these authors, since it focusses attention on the egg-producing property of the trematode and cestode reproductive system; and also induces one to view the different ducts and glands as being part of a well-coordinated and efficient organ complex which is responsible for the elaboration of the egg.

The scolex is a very characteristic anatomical feature of the cestode body. One of its functions is to facilitate the attachment of the worm to the host tissue, for which it is provided with organs of attachment. In the Cyclophyllidea these organs generally include 4 prominent suckers and several hooks. The scolex of H. diminuta lacks hooks, though 4 well-developed suckers are present. The functional morphology of the scolex in different species, including its mode of attachment to the host intestine, has been investigated in recent years by Rees (1956, 1958, 1961), Smyth (1964a), and Rees and Williams (1965). Such a detailed study does not appear to have been made for H. diminuta, although it is a common laboratory-maintained parasite. Smyth (1963) reported secretory activity of the scolex of Echinococcus granulosus, which appears to be the first such report in the literature. Observations of living hydatid worms under high power revealed "small viscid droplets" being secreted by the rostellar region into the medium surrounding the worm. This phenomenon was not observed in worms "earlier than 32 days development in the dog" (Smyth, 1963b). These observations led to a more detailed study of the scolex of E. granulosus, the results of which were reported by Smyth (1964a). The secretion droplets, observed previously, were

believed to be produced by a group of spindle-shaped secretory cells which formed a rosteller gland. The droplets were extremely labile and histochemical tests on these cells led Smyth to suggest that the secretion was probably a lipoprotein or lipid-protein coacervate. He speculated that the secretion may function as a hormone involved in growth regulation, or it may have histolytic properties in lysing host tissue. That it may have antigenic properties was suggested as a third possibility. This study of Smyth was concerned with the rostellar gland and the mode of attachment of the scolex to the host intestine, thus there was no information regarding the disposition and organisation of the nervous system within the scolex.

In the cestodes, the elements of the nervous system appear to be concentrated in the scolex forming a "brain" (Hyman, 1951; Wardle and McLeod, 1952; Bullock and Horridge, 1965). The anatomy of the nervous system of cestodes is very difficult to study by light microscopy, and it is therefore not surprising that adequate descriptions of this system are very scarce. Wilson (1965) investigated the neuroanatomy of H. diminuta and H. nana by studying the distribution of acetylcholinesterase within the scolex. The localisation of this enzyme enabled her to furnish a description of the gross

morphology of the nervous system. She observed, in the scolex of H. diminuta, a central nerve ring with 2 lateral ganglia from which nerves arose running anteriorly and posteriorly. The rostellum was described as being innervated "by a series of approximately 15 rosteller rings." She also described a structure showing intense acetylcholinesterase activity which occurred at the tip of the rostellum. This was referred to as the apical organ. As to the function of the rostellum, Wilson speculated that it probably served as a chemosensory organ involved in growth regulation. Wilson's description of the nervous system of H. diminuta appears to be the first on record. It clearly shows the gross organisation of the nervous system in the scolex, and the presence of a definite sac-like rostellum. However, due to the particular methods of investigation used in the study, it shows a lack of structural detail, at the cytological level, regarding the nervous system, rostellum, and the relationship between the two.

In vitro cultivation of parasitic forms is considered to be a very useful method of study for obtaining information about the parasite's environment, and its role in the growth and differentiation of the parasite (Berntzen, 1966). Smyth, Hawkins and Barton (1966), and Smyth (1967), using in vitro methods, investigated some

of the factors involved in the control of differentiation of E. granulosus. They found that the protoscolex could differentiate in two directions, leading to either a cystic or a strobilate form. In order to obtain the strobilate (corresponding to the adult) form from protoscolices, 2 conditions had to be satisfied. Firstly, the scolex had to evaginate, and once evaginated, it had to come into intimate contact with a substrate which was not only nutritive but also suitably firm in texture. They speculated that contact between parasite and host tissue may be "a part of the stimulus inducing growth in the strobilar direction. . ." They also observed the presence of a number of very fine hairs at the tip of the rostellum which would come into contact with the host tissue. According to these workers the hairs "undoubtedly represent sensory receptors, and their exposure by evagination of the scolex and subsequent contact with a suitable substrate may result in the production of a stimulus necessary for strobilisation. This stimulus could possibly operate through a neurosecretory mechanism."

Information pertaining to the physiology of the cestode scolex, which has been reviewed above, indicates that a very useful field of investigation awaits exploration. A study of the functional morphology of

the scolex of H. diminuta might provide information relevant not only to this tapeworm but also to tapeworms in general. The structure of the scolex, and its development from the cysticercoid, is here examined in some detail in the hope of eliciting information relevant to the role of the scolex, or "head," in the control of the development of the worm.

The review of the literature in this introductory chapter indicates some of the deficiencies in our knowledge regarding the developmental biology of adult cyclophyllidean tapeworms in general, and H. diminuta in particular. The present study, reported in the following chapters, is an attempt to correct some of these deficiencies.

A study of physiological processes necessarily involves the factor of time. Tapeworms thus form excellent material for studying the physiology of development and reproduction, since in a single worm of sufficient maturity all of the developmental stages are available for analysis. However, tapeworms are of relatively simple construction, and this fact coupled with their parasitic habit, renders them difficult to manipulate experimentally. One cannot, for example, perform simple operations on these animals and remove

organs as one could on an insect. Furthermore, compared with the organs of an insect, the important structural entities within a tapeworm proglottid are relatively small and inaccessible. Apart from *in vitro* experiments very little room is left to manoeuvre in. On the other hand, descriptive techniques like histology, cytology and histochemistry judiciously applied to worms of known age, will yield considerable information concerning the developmental physiology of these parasites. It is hoped that the results obtained will encourage more critical work in this field and especially discourage the perpetuation of uncritical work and dogmatic statements.

CHAPTER II

EXPERIMENTAL PROCEDURES

1. The Intermediate Host

The flour beetle Tribolium confusum was used as the intermediate host in the investigation. A stock colony of these beetles was kept in a large jar, containing whole wheat flour, maintained at room temperature. A small amount of vitamin B was added to the whole wheat flour. Beetles used for infection with the tapeworm eggs were obtained from this colony. Prior to bring infected, the beetles were starved for a period of 4-5 days. Gravid tapeworm proglottids were recovered from rats carrying an infection of 3-week-old tapeworms, which ensured an appreciable recovery of these gravid proglottids. The rats were killed with an overdose of ether, and the tapeworms removed from the intestines were kept in a dish of distilled water placed in a refrigerator for 18-24 hours. After this period in the cold, which served to relax the worms, the gravid proglottids were cut into short pieces and placed on strips of moist filter paper contained in Petri-dishes. The starved beetles were introduced into the dishes and

allowed to feed on the gravid proglottids overnight. At the end of this period they were transferred to Petri-dishes containing whole wheat flour. These dishes were maintained at room temperature.

To obtain the cysticercoids, the infected beetles were dissected in a few drops of 0.85% saline on a glass slide under a binocular dissecting microscope. The liberated cysticercoids were sucked up by a pipette and transferred to test tubes containing about 2 ml of 0.85% saline. The cysticercoids were obtained in this manner from beetles about 2-3 weeks after they had been infected with the tapeworm eggs.

2. The Definitive Host

Young female Wistar strain rats weighing approximately 150 gm were used as the definitive hosts. The following technique was employed to infect the rats. A small bore rubber catheter, attached at one end to a glass syringe, was introduced into the stomach of a lightly etherised rat. A small volume of saline (1-2 ml) containing the cysticercoids was introduced into the syringe, and then gently expelled into the stomach of the rat by pressure exerted with the plunger. All infected rats were individually caged and maintained on

a diet of Purina Rat Chow and water.

The observations of Roberts (1961) and Goodchild and Harrison (1961) were very useful in determining the number of cysticercoids to be administered to the rats. Goodchild and Harrison (1961) observed that administering a hundred or more cysticercoids per rat had no effect on the growth and size of the tapeworms during the first 3 days of growth. The following observations of Roberts (1961) were particularly relevant.

(a) The period of most rapid growth was the first 7 days. Population density had no effect on the growth of the worms in this period.

(b) Population density had no effect on the rate of maturation and length of the pre-patent period. The pre-patent period was 16-17 days in infections of one cysticercoid per rat or a hundred cysticercoids per rat.

Personal experience and the experience of others (Goodchild and Harrison, 1961; Roberts, 1961) indicated that, for the recovery of an appreciable number of worms in the first 2 days of growth in the definitive host, a fairly high infective dose of cysticercoids would be necessary.

To obtain young worms on the first 3 days of

growth the minimum dosage level was 150-200 cysticercoids per rat. For the recovery of worms 4 days to 18 days or more after infection, the level was 20-25 cysticercoids per rat. This latter quantity of cysticercoids was found to be a little excessive when recovering older worms, especially at 13 days after infection and periods beyond that, since the worms were quite large by then and folded back and forth in the intestinal lumen. This necessitated much care in the opening of the intestine and in the subsequent removal of the worms in order to avoid damaging the worms in the process.

At the outset it would be expedient to define or clarify what is meant by the expression "a 15-day-old" or "20-day-old" worm. In this report, when reference is made, for example, to a 15-day-old worm, it means that the worm in question was recovered after a period of 15 days from the day of infecting the rat host with the cysticercoids. In other words, the expression "15 days old" here refers to the period of time between the inoculation of the rat host with cysticercoids and the sacrifice of the same host for the recovery of the tapeworms. This does not necessarily mean that all the worms recovered from a particular rat after a certain period of time would all be in the same state of development. Some cysticercoids could excyst faster

than others on being administered to a rat, and the worms developing from these cysticercoids would be "older" than the worms that developed from the cysticercoids which were slower to excyst. Thus a few worms from each population would have to be studied to ascertain the level of development attained on a particular day. When administering the cysticercoids to a rat, care was taken to note the time of infection so that the rat could be sacrificed as close to the scheduled hour as possible.

All the rats were killed with a heavy dose of ether. The entire small intestine was then quickly removed through a small incision in the abdomen, and immediately dissected in warm (37°C) Tyrode's physiological solution. The tapeworms were removed from the intestine and placed immediately in the appropriate fixative. Care was taken to ensure that as many "complete worms" (i.e., worms with both scolex and terminal proglottid intact) as possible were obtained. When in doubt about this, the worms, prior to fixing, were examined in a dish of warm Tyrode's solution under a binocular dissecting microscope to make sure that this morphological criterion of "completeness" was satisfied. This problem seldom arose when recovering worms up to 5 and 6 days after infection, since their comparatively

small size greatly facilitated both cutting the intestine open as well as the gentle removal of the worms with forceps. In infections of 15 or 18-day-old worms, for example, the large, writhing mass of worms in the intestinal lumen necessitated much caution in the removal of worms to avoid damaging them. It is very easy to sever a single worm in several places because of this to and fro folding of the strobila.

In experiments to study the parasite "in situ," a somewhat different procedure from the above was adopted. The rats were killed by a sharp blow to the head, and the intestines quickly removed into a dissecting dish. The intestines were then carefully cut open, pinned onto the dish and exposed to appropriate fixatives in the following manner. A short length of intestine (3-4 inches long) was cut open at a time and the sides pinned down, after which a sufficient amount of fixative was pipetted onto the exposed region. This procedure of obtaining the parasites was adopted to minimise any possible alterations of the normal in situ picture. Worms 3 days and older were easily detected in situ. Two-day-old worms were located with great difficulty, even when using a binocular dissecting microscope. All attempts to locate day-old-worms in this manner were unsuccessful.

3. The Treatment of the Parasites after Removal from the Definitive Host

The treatment of the parasites soon after removal from the environment of the host intestine is very important when histological, and especially histochemical techniques are to be employed in studying their biology. Since most of the studies were carried out on material sectioned for microscopy, the process of tissue fixation is very important.

The purpose of fixation is to try to preserve the tissues, cells and cell constituents as close to the life-like state as possible, maintaining the structural integrity of the various cells and tissues, as well as the reactivity of their chemical constituents. This is an ideal condition and difficult to duplicate in reality, but could be approximated with certain appropriate fixatives. To obtain satisfactory fixation not only is the choice of a suitable fixative important, but prompt fixation is also imperative. Thus when dealing with parasitic organisms it is especially important that fixation should be as prompt as possible, since, with the removal of the parasite from its usual environment, tissue and metabolic deterioration would set in. Great delay in fixation should thus be avoided. A survey of the literature shows that this routine is not strictly

adhered to. Many workers introduce a preliminary treatment of the worms before placing them in the selected fixatives. Thus it is common practice to relax the tapeworms in ice-cold water, tap water (Rybicka, 1964a) or in an ice bath (Rybicka, 1966b) before fixing them. Goodchild and Harrison (1961) placed the specimens in cold normal saline to relax them, after which they were transferred to the various fixatives. Douglas (1961) relaxed specimens of Baerietta diana in chloretone before transferring them to the fixing solutions. In the present investigation such preliminary pre-fixation procedures were avoided. Whenever a delay in fixation was brought about by some unavoidable circumstances, the freshly removed tapeworms were kept in Tyrode's solution at 37°C until transferred to the fixative, so that a certain amount of metabolic activity could be maintained by the worms--a procedure recommended by Johri and Smyth (1956).

Worms were relaxed in cold water only for two purposes. The first was for preparing stained whole mounts of tapeworms, for which the standard procedure is to compress adequately the specimens before fixing, so that the internal structures are rendered visible on staining. However, tapeworms like Hymenolepis diminuta are too big for this procedure, so relaxing them in water

was resorted to. The specimens were placed in a dish of distilled water kept in the refrigerator for 18-24 hours, then transferred to dilute 8% formalin and preserved in this solution until required. The relaxation procedure was occasioned a second time when obtaining gravid proglottids for infecting beetles. Tapeworms were again placed in a dish of distilled water in the refrigerator for 18-24 hours and then the gravid proglottids fed to the previously starved beetles, which is a procedure used by Schiller (1959). Connected with this, it is interesting to mention an observation of Collings and Hutchins (1965) made in a study of motility and hatching of the onchospheres of Hymenolepis microstoma. They observed that eggs placed in water kept in a refrigerator overnight, and then transferred to certain test solutions like saline, beetle extract and sera, showed a greater degree of onchosphere motility and hatching than those eggs which were placed in similar media directly after removal from the host. They believed that a temperature sensitive mechanism operated in the onchosphere which appeared to initiate onchosphere motility, which is a prerequisite for hatching.

The details pertaining to the histological and histochemical techniques used in this investigation are provided in an appendix at the end of the dissertation.

The histochemical techniques used in this study are of importance and they are discussed in the appendix, so that the details and other relevant information concerning these techniques will not interfere with the main narrative.

THE PROGLOTTID

CHAPTER III

THE DEVELOPMENTAL PATTERN OF *H. DIMINUTA* IN THE WHITE RAT

The pattern of development was studied by infecting rats with known standard doses of cysticercoids, and removing the worms at daily intervals from 1 day up to 18 days after infection. The degree of development and differentiation attained on each day was noted by studying appropriately stained sections of the last few proglottids of the strobila (i.e., the proglottids at the posterior terminal region of the worm). This region of the worm would represent the "oldest" part of the strobila, and thus would, for any given day, show the maximum level of development attained by the worm on that particular day. There are, in fact, 2 regions of the worm that could be studied in this manner: the posterior terminal region of the strobila consisting of the "oldest" proglottids, and the anterior-most region which is the scolex. The study of the development of the scolex will be discussed in a later chapter.

1. The Sequence of Development of Hymenolepis diminuta in the White Rat as Observed under the Author's Experimental Conditions

Day 1.--Great difficulty was encountered in recovering the young worms on this day. (Goodchild and Harrison (1961) devised a method for recovering the young worms, which commenced with scraping the intestinal mucosa and further dissecting the scrapings in saline. The dishes of saline containing the scrapings were kept exposed to a 45-watt bulb in a reflector, and after 2-3 hr, young worms crawling on the bottom of the dish were easily recoverable. This method was considered to be unsatisfactory by the author, and so was not tried out.) Presumably, on the first day, the cysticercoids would have excysted and the young worms secured lodgement among the intestinal villi.

Day 2.--Transverse sections of the posterior terminal region of the strobila show a dense group of cells, constituting an anlage of cells, in the central region of the body parenchyma (see Fig. 2). These cells are somewhat spindle-shaped. The excretory vessels are also visible and their course lies within this anlage. Beneath the tegument is a layer of conspicuous cells, each with a prominent nucleus.

Note: The male and female reproductive tracts lie with their longitudinal axes parallel to the long axis of the proglottid. Thus a transverse section of the proglottid would be a longitudinal section of the cirrus sac and seminal receptacle, for e.g.; and a longitudinal section of the proglottid would cut both cirrus sac and seminal receptacle (for e.g.) transversely.

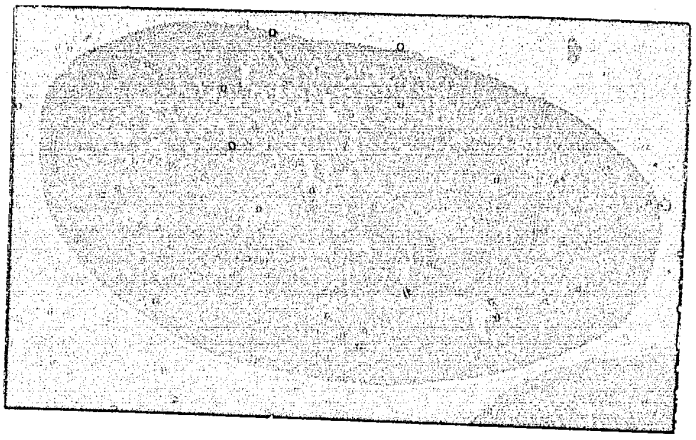


Fig. 2.--Transverse section of the posterior terminal region of a two-day old worm. Note central anlage of cells.
(Bouin's, paraldehyde fuchsin with Halmi's, x 615)

Fig. 3.--Transverse section showing primary anlage of cells. Note the muscles separating the cortical parenchyma (c) and the medullary parenchyma (m).
(Bouin's, paraldehyde fuchsin with Halmi's, x 330)

Day 3.--The primary anlage is much clearer now and appears somewhat oval-shaped in transverse section. The excretory vessels are visible as two distinct groups, and the two groups, each consisting of a dorsal and ventral vessel, lie close to each other. Immediately beneath the tegument is a distinct and well-defined zone of tissue, which was not apparent on the previous day.

Day 4.--The primary anlage is still visible, and consists of a central knot of cells and a thin peripheral band of cells. The two groups of excretory vessels lie more laterally now, and are thus at an appreciable distance from each other. A prominent band of longitudinal muscles is visible in the parenchyma now and it sharply separates the parenchyma into an outer cortical zone and an inner medullary zone (see Fig. 3).

Day 5.--On the fifth day the state of development shown by the posterior end of the strobila is a distinct advance on that of the first 4 days. Then only an anlage of cells was visible; now all the structures of the reproductive apparatus, in different stages of differentiation, are present. The formation of proglottids has commenced, and the transverse excretory duct, which lies at the posterior limit of each proglottid, is visible. The ovary, vitelline gland and

Mehlis' gland are easily recognisable. The seminal receptacle is formed and resembles a thin slender tube. The terminal region of the male reproductive tract is still in the process of differentiation; the external seminal vesicle may be formed, while the cirrus sac and its ejaculatory duct and internal seminal vesicle are still being defined (see Figs. 4, 5, and 6). The testes show signs of activity as evidenced by the presence of the early stages of spermatogenesis within them. However, no mature spermatazoa were observed within the testes.

Day 6.--The reproductive apparatus in the terminal proglottids appears to be completely formed now, with the constituent glands and ducts being completely differentiated. All stages of spermatogenesis were observed in the testes, including mature spermatozoa. No spermatozoa were observed in the terminal parts of the male reproductive tract (i.e., in the seminal vesicles and ejaculatory duct) (see Fig. 7). In some instances the vitelline gland showed signs of incipient productivity with a few vitelline cells exhibiting distinct vitelline granules in the cell cytoplasm.

Day 7.--The oocytes in the large ovary are clearly recognised, each with a prominent nucleus and nucleolus, and distinct granules in the cytoplasm.

Fig. 4.--Transverse section showing the immature ovary (o), Mehlis' gland (M) and vitelline gland (v). (Bouin's, paraldehyde fuchsin with Halmi's, x 510)

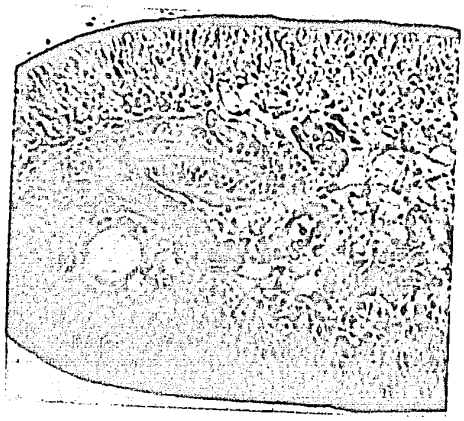


Fig. 5.--Transverse section showing seminal receptacle (s), differentiating cirrus sac (c) and external seminal vesicle (e).
(Bouin's, paraldehyde fuchsin with Halmi's, x 400)

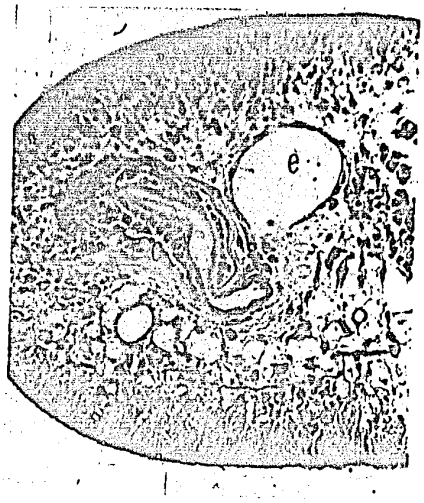


Fig. 6.--Transverse section showing further differentiation of the cirrus sac (c) and external seminal vesicle (e).
(Bouin's, paraldehyde fuchsin with Halmi's, x 385)

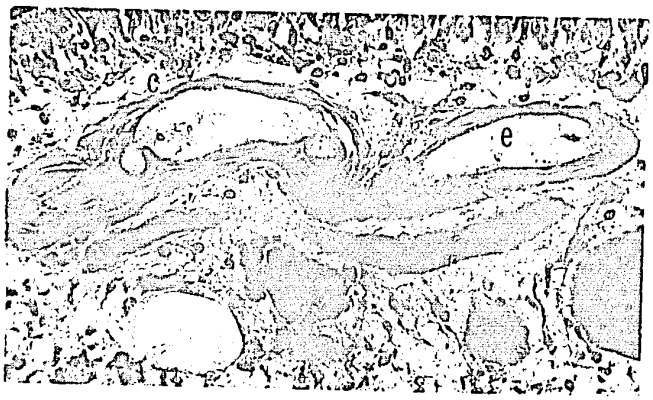


Fig. 7.--Transverse section showing completely formed cirrus sac (c) and external seminal vesicle (e). Note the absence of spermatozoa in these two structures.
(Susa, Azan, x 315)

Similarly the vitelline gland is a compact mass of vitelline cells, each with its large nucleus and cytoplasm studded with secretion granules. Mehlis' gland is visible as a compact intensely staining structure. In most of the proglottids examined, spermatozoa were observed in both external and internal seminal vesicles, while no spermatozoa were visible in the seminal receptacle. In a few instances, spermatozoa were observed in the seminal receptacle as well, indicating that the transfer of spermatozoa from the male tract to the female tract had commenced. In still some other cases, a further advance in physiological state was observed: in addition to spermatozoa being present in both male and female reproductive tracts, a few "eggs" were also observed in the slender uterus. Thus, three phases of the reproductive process were noted to occur on this day. They are:

1. The appearance of spermatozoa in the male genital tract.
2. The transfer of spermatozoa from the male to the female genital tract.
3. The release of spermatozoa from the seminal receptacle into the ootype, with the concurrent release of oocytes from the ovary, vitelline cells from vitteline gland and the secretory granules from Mehlis' gland,

into the ootype. The oocyte, vitelline cell, spermatozoan and secretory granules of Mehlis' gland are the component parts of the egg, and, within the ootype, they are assembled together to form the "immature" egg, which is then ejected into the uterus to complete its development there.

Day 8.--A greater number of eggs were observed in the uterus, which is still a rather slender tube confined to the dorsal region of the proglottid. In some of the proglottids examined, the eggs in the uterus were undergoing cleavage. The seminal receptacle is larger and has a swollen, distended appearance, which could be correlated with the need to accommodate the increasing number of spermatozoa entering into its lumen.

Day 9.--The uterus is somewhat more extensive now and occupies a wider area within the proglottid. The eggs within the uterus are undergoing cleavage. The testes, ovary, vitelline gland and Mehlis' gland are all visible, as well defined structures, in a functional and active state. The seminal receptacle is increasing in size and volume (see Figs. 8 and 9).

Day 10.--Most of the eggs within the extensively branched uterus are still undergoing cleavage. The testes are visible and they contain spermatozoa. The

Fig. 8.--Transverse section showing the mature female reproductive system. o, ovary; M, Mehlis' gland. Note the vitelline duct (at arrow) leading from the vitelline gland, and containing vitelline cells; also note the uterus (u) containing developing eggs. (Susa, Azan, x 550)

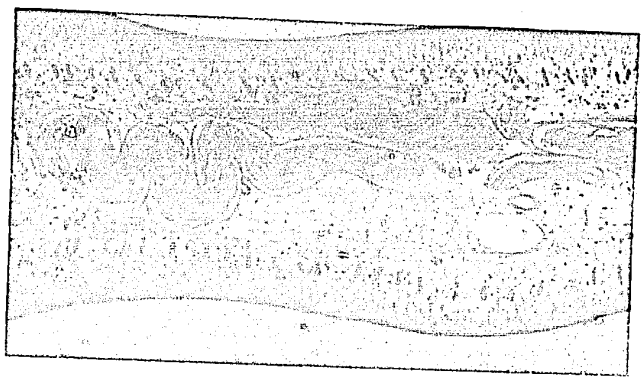


Fig. 9.--Transverse section showing spermatozoa in both external seminal vesicle (e) and seminal receptacle (s). Note the prominent swollen condition of the seminal receptacle.
(Bouin's, paraldehyde fuchsin with Halmi's, x 140)

ovary is very much reduced in size and resembles a small sphere, consisting of a few oocytes. The vitelline gland, too, is greatly reduced in size and contains a few vitelline cells. The seminal receptacle is very prominent, resembling a swollen tube which is, in some instances, thrown into small dorso-ventrally directed loops (see Figs. 10 and 11).

Day 11.--The uterus, with its numerous tubular branches, occupies a very large area of the proglottid, and it is the most prominent structure to be seen. The testes, ovary, vitelline gland and Mehlis' gland are not visible and are presumably spent and atrophied. The only remnant of the male reproductive tract is the muscular cirrus sac enclosing the ejaculatory duct and internal seminal vesicle. The large and voluminous seminal receptacle, containing abundant spermatozoa, is still present. The embryos within the uterus, formed from the cleaving eggs, show a more advanced state of differentiation: a distinct shell is being formed at the outer margin of the differentiating embryo.

Days 12-15 inclusive (see Fig. 12).--The uterus gradually enlarges, and its numerous tubular branches ramify and extend throughout the proglottid. Very little parenchymatous tissue is present, and this is

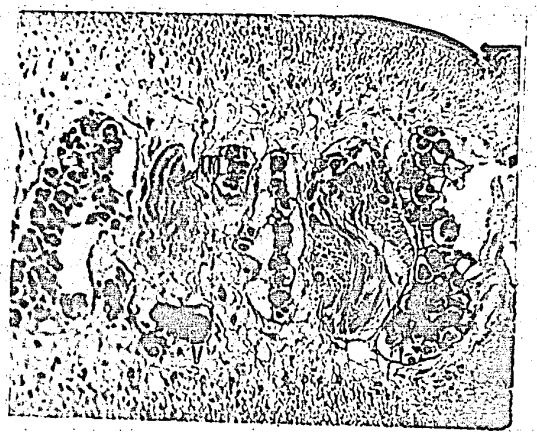


Fig. 10.--Transverse section of a proglottid showing the shrunken vitelline gland (v) and Mehlis' gland (m). Note the testis with spermatozoa and branches of the uterus containing embryos. (Bouin's, aldehyde fuchsin, x 190)

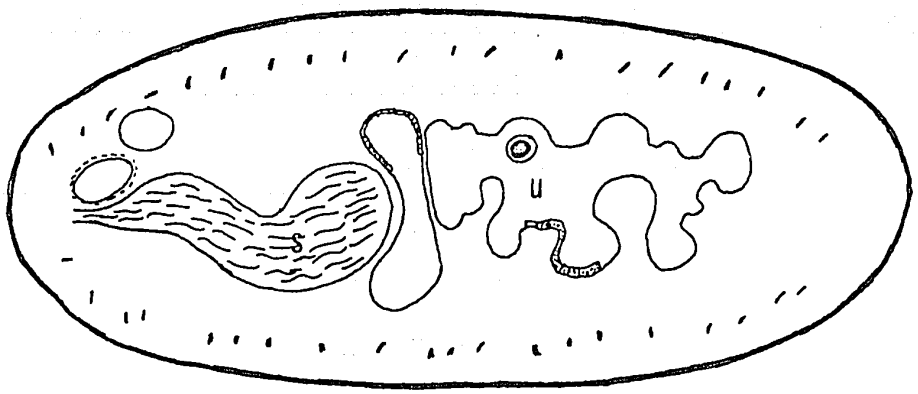


Fig. 11.--Diagrammatic transverse section of a proglottid. Note seminal receptacle (s) and the expanding uterus (u). The epithelium of the uterus is indicated at two points. (x 100)

confined to a thin zone beneath the tegument, and to patches between the branches of the uterus. A part of the cirrus sac is still present, and similarly, a shrunken remnant of the once capacious seminal receptacle is present, and it still contains spermatozoa. All traces of the other reproductive structures have completely disappeared. The embryos continue with their progressive development, and by the 13th day are readily recognisable as developing onchosphere larvae with hooks and penetration glands. The formation of the embryonic envelopes surrounding the larva is also well under way. The uterine epithelium is thick and has prominent nuclei.

Day 16.--The embryos in the uterus have completed their development and are identified as typical six-hooked larvae surrounded by 3 distinct membranes, or embryonic envelopes. A fragment of the muscular cirrus and part of the seminal receptacle may still be observed. The uterine epithelium is very thin and is not easily recognised now. The entire proglottid is filled with eggs.

Days 17-18.--The condition of the proglottids on these days is similar to that of the 16th day. The epithelium of the uterus is very thin and has collapsed at many points so that one can no longer recognise the

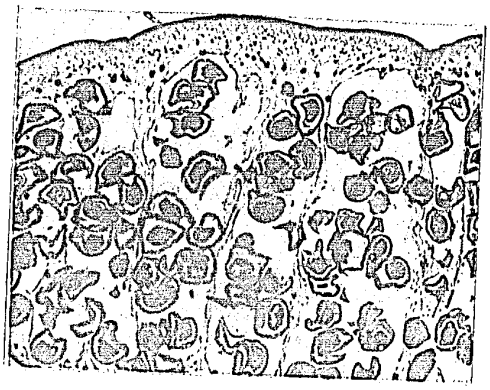


Fig. 12.--Transverse section showing the uterus, containing eggs, filling the entire proglottid. Note uterine epithelium at arrow.
(Bouin's, paraldehyde fuchsin with Halmi's, x 105)

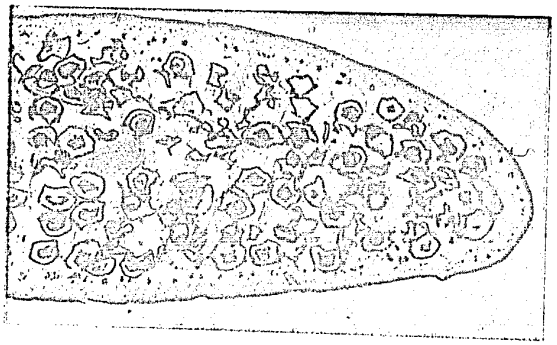


Fig. 13.--Transverse section of a gravid proglottid.
(Bouin's, paraldehyde fuchsin, x 80)

uterus as a branched tubular structure (see Fig. 13). Eggs are more abundant in the faeces of the rat host, indicating that more proglottids are being shed now.

The pattern of the development of H. diminuta in the rat, as described in the last few pages, can be broadly summarised as follows:

Days 1-5.--The excystation of the cysticercoids and subsequent attachment of the young worm to the intestinal wall occurs. Strobilisation (proglottid formation) commences on the 5th day, and the histogenesis of the constituent parts of the reproductive apparatus begins.

Days 6-7.--The formation of the reproductive apparatus is completed. The activities pertaining to sexual reproduction commence.

Days 8-9.--The first phase in egg-formation is at its height, leading to the appearance of the "immature," assembled eggs in the uterus.

Days 10-11.--The decline in the functional importance of the ovary, vitelline gland and Mehlis' gland sets in. The emphasis now shifts over to the expanding uterus, which rapidly gains prominence as the single important structure in the proglottid.

Days 12-15.--The accent is now on the uterus and the progressive development of the eggs within its numerous ramifying branches.

Days 16-18.--The uterus is no longer easily recognisable as a tubular branched structure. The entire proglottid is occupied by completely developed "eggs" (i.e., 6-hooked larvae surrounded by the 3 embryonic envelopes). The shedding of these gravid proglottids (apolysis) begins.

2. The Practical Application of the Timetable of the Developmental Events

Information pertaining to the sequence of events in the developmental physiology of the parasite, especially with regard to the precise timing of these events, was an essential prerequisite for the subsequent study of the reproductive physiology of the animal.

A tapeworm of sufficient ontogenic maturity (for example, an 18-day-old worm obtained under the author's experimental conditions) would show all the developmental stages, from the histogenesis of the reproductive structures to proglottids with completely developed eggs. Thus, using just one such worm, one could study the different phases in sexual reproduction. The

difficulty in adopting this method becomes apparent when it is realised that a worm of this age would consist of several hundreds of proglottids, and would be as long as 10-14 inches. It is almost impossible to point out to a particular region of the fixed strobila and accurately state what the developmental level of the proglottids in that particular region would be like. Exceptions to this would be the posterior terminal proglottids which would contain the mature eggs, and the young proglottids, lying immediately posterior to the neck region, which would be at a developmental level of early histogenesis. It is a very tedious task, then, to pick out, in the fixed strobila of an 18-day-old worm, the proglottids where, for example, the transfer of spermatozoa from male to female genital tracts had commenced, or those in which early embryogenesis was occurring. Only by the trial and error sectioning and staining of the different regions of the strobilua could one arrive at the required proglottids.

But when a broad timetable of the events in development, as the author has obtained, is available, then the difficulty in the investigation emphasised above is greatly reduced. Thus, to obtain some information regarding the physiology of the ovary, vitelline gland or Mehlis' gland, one would recover worms 8-9 days

after infection of the rat host and study the posterior terminal proglottids of these worms. As the timetable indicates, it is at this age when the first phase of egg-formation is at its height and the ovary, vitelline gland and Mehlis' gland are very prominent in the penultimate proglottids of the worm. There is the possibility that some worms in a population would not be of the exact developmental state as indicated in the timetable of events. If such a worm is in a more advanced state of development than anticipated, it would then be necessary to examine a number of proglottids, anterior to the penultimate few, in order to obtain the proglottids in the appropriate developmental state. The histological examination of strobilas of different ages showed that there was no difference (except in size) between, for example, the posterior terminal proglottids of an 8-day-old worm and the proglottids, with the corresponding state of development, from the strobila of an 18-day-old worm.

The author would like to emphasise one point here. It should always be borne in mind that the timetable of developmental events constructed by the author refers to the observations of results obtained from rigidly controlled experimental conditions as outlined in Table 1. These conditions were always adhered to, so

TABLE 1.—The experimental conditions pertaining to the recovery of tapeworms on each day of growth

Day(s) after infection	Number of cysticercoids per rat	The relevant details pertaining to the rat host
1 2 3	150-200	Female Wistar strain white rats, weighing about 150 gm. Individually caged. Given food and water ad lib.
4-18 inclusive	20-25	As above

that, with a reasonable degree of confidence, one could infect a rat with a specific number of cysticercoids and obtain worms showing the anticipated state of development.

TABLE 2.--The development of *Hymenolepis nana* and *H. microstoma*

(see notes at foot of table)

Days after Infection	The visible features indicating developmental state	
	H. nana	*H. microstoma*
1		No segmentation, no grossly visible anlagen
2		Same as Day 1
3	Primordia visible	Some internal segmentation, appearance of anlagen
4	Appearance of testes, ovary, vitelline gland, seminal vesicle and seminal receptacle.	External segmentation, male and female anlagen visible
5	Uterus, preonchosphere visible	Same as Day 4
6	Meta-onchosphere	Testes in few proglottids
7	Onchospheres and onchosphere coat	Testes defined
8	Vitelline membrane present in eggs	Early mature to mature segments
9		Mature segments
10	Egg-shell visible	Same as Day 9
11	Gravid proglottid with completely developed eggs.	Disappearance of female glands. Few pre-onchospheres visible
12		Pre-onchospheres no hooks
13		Semi-gravid proglottids with onchospheres
14		Near gravid proglottids
15		Gravid proglottids

Notes

1. The development of H. nana is from the report of Berntzen (1962). The tapeworms were raised in mice. The material for study was fixed in formalin and prepared as whole mounts stained in picrocarmine. Pre-onchosphere = primary ball of cleaving embryonic cells; meta-onchosphere = differentiated embryo lacking hooks; onchosphere = embryo with hooks.

2. The development of H. microstoma is from the report of de Rycke (1966). The tapeworms were raised in Swiss albino mice (Mus musculus). The worms were fixed in Carnoy and stained in Feulgen; and also fixed in 10% formalin and Bouin and stained in Best's carmine and Mayer's haematoxylin. All specimens were prepared as whole mounts.

3. The development of Echinoccoccus granulosus is based on the report of Hutchison and Bryan (1960). The worms were raised in dogs. Fixatives used were 10% neutral formalin, Gilson's fluid, Alcohol-formalin-acetic acid and hot 70% alcohol. The specimens were prepared as whole mounts stained in carmine and haematoxylin.

4. The development of H. diminuta was studied by the author under the conditions shown in Table 1. The worms were prepared for general histological study according to the methods outlined in the first part of the Appendix, found at the end of this dissertation.

3. A Comparison of the Developmental Pattern of some Different Tapeworms

It is of some interest to compare the developmental patterns of different cyclophyllidean tapeworms. Table 2 is a brief comparative summary of the developmental pattern of two other hymenolepidid species. The hydatid organism, Echinococcus granulosus, is exceptional in two respects when compared with the other tapeworms considered above. Firstly, it is very much smaller than the other tapeworms, with a mere 3 or 4 proglottids comprising the strobila; and secondly, its development in the definitive host is a very much slower process. A brief description of the development of this tapeworm, based on a report by Hutchison and Bryan (1960), is given below.

Growth pattern of Echinococcus granulosus in the dog (see notes on page 62)

Week 1.--No sign of proglottid formation. The young worm resembles the protoscolex, but it is longer.

End of week 2.--Further elongation of the worm is observed. One proglottid has formed, but there is no sign of organogeny in this proglottid.

Week 4.--Two proglottids are now visible. The terminal-most proglottid shows a well-developed reproductive apparatus with testes, ovary, vitelline gland, cirrus

complex and genital atrium. The sub-terminal proglottid shows the first traces of organogeny with the appearance of anlagen.

Week 5.--Three proglottids now comprise the strobila. The terminal proglottid has a slender uterus containing developing embryos. All the other reproductive structures in this proglottid are beginning to disappear.

The reproductive apparatus in the sub-terminal proglottid is still differentiating. A few testes are visible in it. The 3rd proglottid is very much smaller than the other two and shows no signs of organogeny.

Week 8.--The terminal proglottid: the uterus is now much larger and contains the developing embryos. The cirrus sac and a part of the seminal receptacle are still visible.

2nd proglottid: the differentiation of the reproductive apparatus continues, and more testes are visible. The cirrus sac, vagina and seminal receptacle are clearer and more defined. The vitelline gland and ovary are also visible.

3rd proglottid: it is still very small and shows no signs of organogeny.

Week 9.--The terminal proglottid: the uterus is

somewhat larger. Remnants of the cirrus sac and seminal receptacle are still visible.

2nd proglottid: the reproductive apparatus has been completely formed in this proglottid.

3rd proglottid: a trace of organ formation is evident with the appearance of the early anlagen.

Week 17.--The terminal proglottid: the uterus has enlarged and it shows lateral sacculations. The remnants of the cirrus sac and the seminal receptacle are no longer visible.

2nd proglottid: a fully-developed reproductive apparatus is present.

3rd proglottid: the state of development is the same as that in the 9th week.

The prepatent period in Echinococcus granulosus appears to be quite long when compared with the prepatent period in the Hymenolepidid species considered. According to Smyth (1964b) it takes about 7 weeks for the eggs to be detected in the faeces of the host. Sweatman and Williams (1963) observed the prepatent period to be of about 8 weeks duration in the dog. Similarly, Yamashita et al (cited by Smyth, 1964b) state that eggs were observed in the faeces of dogs 7 to 9 weeks after infection. In E. granulosus it takes as

much as 4 weeks for the reproductive structures of the terminal proglottid to be formed, and a further 4-5 weeks before the mature eggs in this proglottid are passed in the faeces. This is very slow compared with the Hymenolepidid species considered earlier.

Another feature of interest in E. granulosus is the great discrepancy in size, state of development as well as rate of development shown by the constituent proglottids of a single strobila. For example, at a period of 9 weeks after infection, the terminal proglottid is large and is entirely occupied by the uterus which contains eggs; the second proglottid is smaller and shows a completely formed reproductive apparatus, but lacks a uterus and eggs; the third proglottid is very much smaller than the other two and within it only a trace of the genital primordia is visible. The rate of development, too, is markedly different in the 3 proglottids. In the terminal proglottid, by the end of the fourth week, the reproductive apparatus was fully developed and at the end of 9 weeks (i.e., about 5 weeks later) eggs were clearly visible in the expanding uterus. In the second proglottid the genital anlagen appeared in the fourth week and by the ninth week (i.e., 5 weeks later) the reproductive apparatus was

completely formed. However, even after 17 weeks (i.e., 9 weeks after the reproductive structures were completely developed) there were no signs of egg production in this proglottid. The 3rd proglottid has the slowest rate of development; a trace of the genital primordia was visible at 9 weeks, and at 17 weeks (i.e., 8 weeks later) the state of development in this proglottid was still the same.

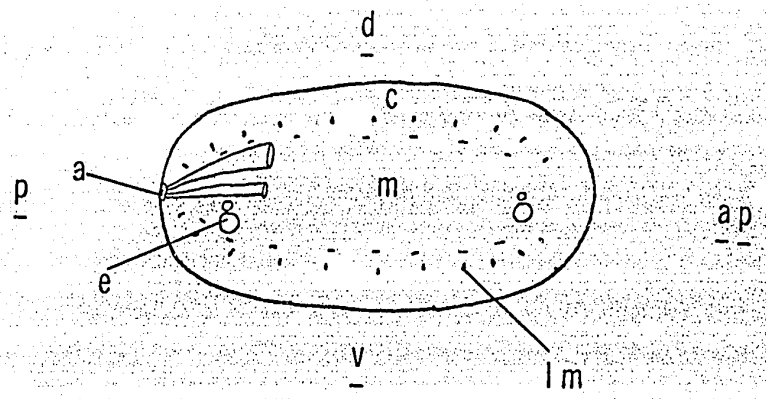
A close examination of the developmental pattern of the 4 species of tapeworms considered, shows that, despite differences in the timing of events and rates of development, the sequence of developmental events is the same in all. First there is segmentation or proglottid formation, followed by organogeny leading to the formation of the functional reproductive apparatus. Then ensues sperm transfer from the male tract to the seminal receptacle of the female tract, fertilisation and appearance of eggs in the uterus. Finally the uterus expands with the gradual disappearance of all the other reproductive structures in the proglottid, and the development of the embryos within this uterus progresses, culminating in the formation of hooked onchosphere larvae surrounded by the embryonic envelopes.

CHAPTER IV

THE MALE REPRODUCTIVE SYSTEM--PART I

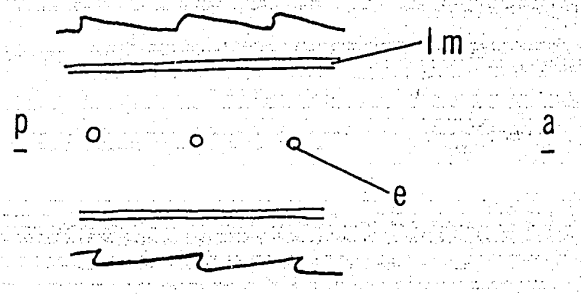
The reproductive system of each proglottid in the strobila contains both male and female systems. In other words, this tapeworm is a monoecious animal whose body consists of several monoecious "units," arranged one behind the other, and showing progressive differentiation from the anterior end of the strobila to the posterior end.

Two conspicuous layers of longitudinal muscles divide the parenchyma of the proglottid into an outer cortical zone and an inner medullary zone. The entire reproductive system lies in the inner medullary parenchyma (see Fig. 14). The two groups of excretory ducts, situated at the two lateral ends of the proglottid, mark the limit of the lateral extension of the reproductive system, except on the poral side (i.e., the lateral border of the proglottid where the genital atrium is located) where the vagina and a part of the cirrus sac extend beyond the excretory ducts (see Fig. 14). The posterior extension of the reproductive apparatus is limited by the transverse excretory duct which unites the two ventral excretory ducts (see Fig. 15).



d - dorsal
v - ventral
p - poral
ap - aporal
a - genital atrium
e - excretory duct
lm - longitudinal muscle
c - cortical parenchyma
m - medullary parenchyma

Fig. 14.--Diagrammatic transverse section of a proglottid.



p - posterior
a - anterior
e - posterior excretory duct
lm - longitudinal muscle

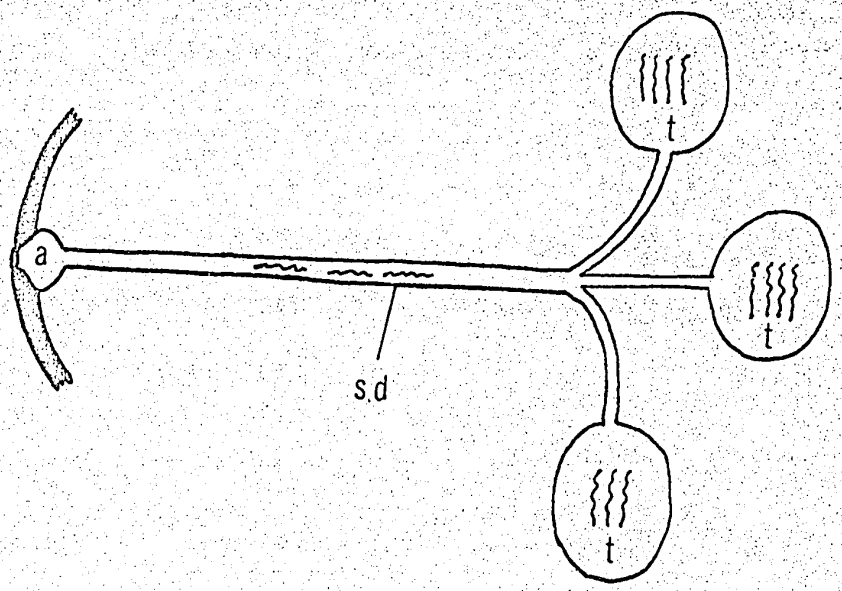
Fig. 15.--Diagrammatic longitudinal section of a part of the strobila.

Within these boundaries lies the major portion of the reproductive system, and its constituent structures occupy all of this enclosed area, with parenchymatous tissue occurring in the spaces between these structures.

The male reproductive system basically consists of three testes and a sperm duct which serves to conduct the spermatozoa, produced by the testes, to the female tract (see Fig. 16). The sperm duct is fundamentally a single duct which shows physiological specialization along its length. The sperms must traverse all of the different physiologically specialized regions of the duct before they enter the vagina of the female system. The major portion of the sperm duct lies dorsally and in the anterior part of the proglottid.

Each proglottid usually contains three testes, each of which is enclosed by a thin capsular membrane. Supernumerary testes may be encountered within individual proglottids of a single worm. The testes are oval in cross section, and more circular when viewed in longitudinal sections. Two testes are situated aporally between the ovary and the two aporal lateral excretory ducts (see Fig. 17). The third testis lies porally, between the ovary and the poral lateral excretory ducts.

A very slender, thin-walled duct called the vas



t - testis
sd - sperm duct
a - genital atrium

Fig. 16.-Diagrammatic representation of the male system composed of 3 testes and a sperm duct.

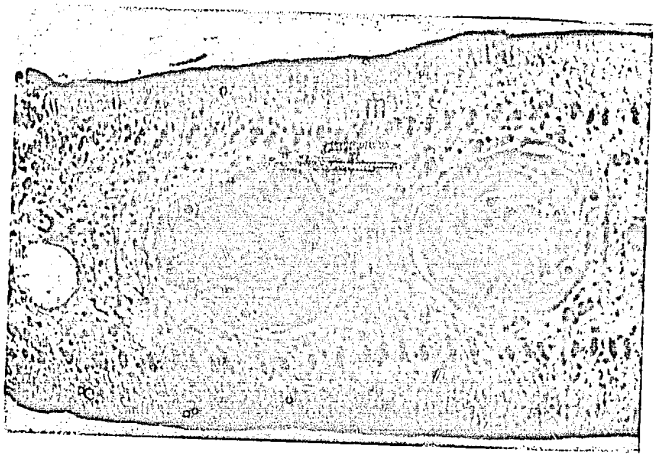


Fig. 17.--Transverse section showing 2 testes. Also note the excretory ducts (arrow) and the longitudinal muscles (m).
(Bouin's, paraldehyde fuchsin, with Halmi's, x 155)

efferens leads out from the inner dorso-lateral aspect of each testis. The three vasa efferentia run inwards and anteriorly to meet in the mid-dorsal region of the proglottid, and form a common, larger channel called the vas deferens. This is the first region of the sperm duct (see Fig. 18). The lumen of the vas deferens is wider than that of the vas efferens, and it is lined by a thin cellular epithelium. The vas deferens lies anteriorly in the proglottid and dorsally, just beneath the longitudinal muscles of the proglottid. It is easily observed in mature proglottids because of the numerous spermatozoa within its lumen (see Fig. 19).

The vas deferens is a long straight tube and at its poral end it opens into the next region of the sperm duct which is called the external seminal vesicle. This sac-like structure may be considered to be a dilated extension of the vas deferens, and its epithelium is identical with that of the vas deferens and continuous with it. The external seminal vesicle (see Fig. 20) possesses two characteristic features; the first is that it increases in size to form a capacious sac, and secondly, its outer surface is studded with numerous unicellular gland cells, which are often erroneously referred to as "prostate glands." The lumen of the external seminal vesicle becomes considerably reduced in

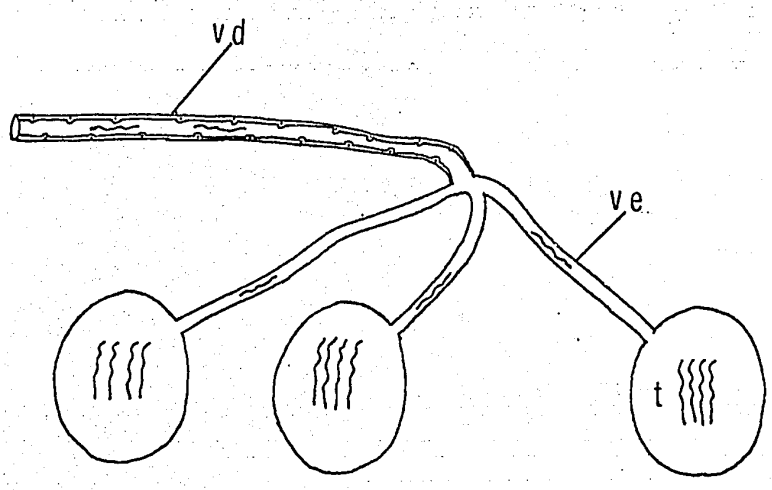


Fig. 18.--Diagrammatic representation of testes (t), vasa efferentia (ve), and the vas deferens (vd).

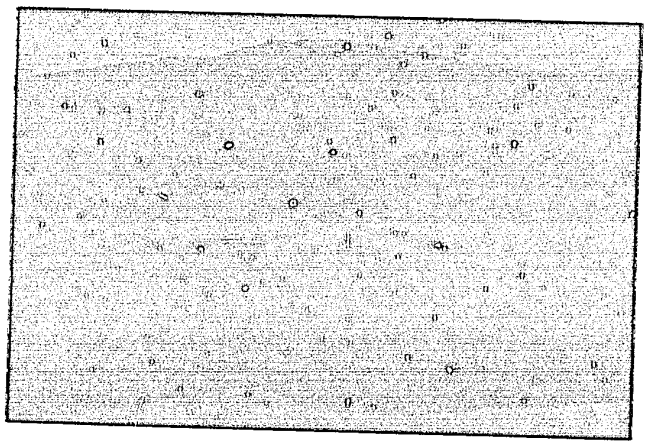
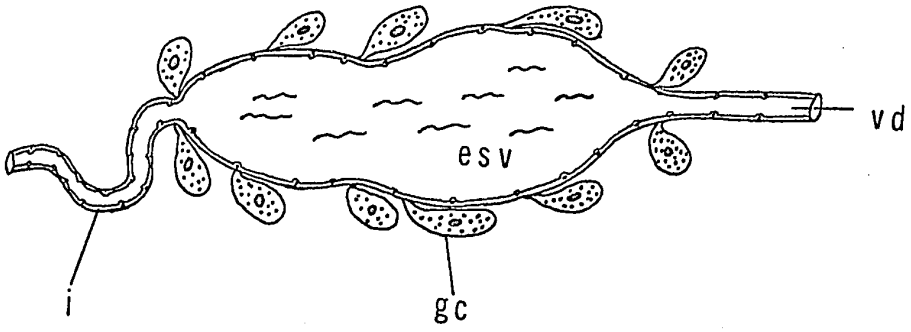


Fig. 19.--Transverse section showing the vas deferens (at arrow) with spermatozoa in its lumen. (Bouin's, paraldehyde fuchsin, with Halmi's, x 190)



vd - vas deferens
esv - external seminal vesicle
gc - gland cell of the external seminal vesicle
i - isthmus

Fig. 20.--Diagrammatic longitudinal section of the external seminal vesicle.

diameter as it leads into the cirrus sac. This narrow part of the seminal vesicle is here termed the isthmus of the external seminal vesicle.

The cirrus sac extends to the genital atrium, and it does not lie dorsally throughout its course, but slopes gradually downwards (i.e., ventrally) and at the same time curves posteriorly to open into the genital atrium (see Figs. 21 and 22). The cirrus sac is a muscular structure traversed by a duct. The sac is elongate, cylindrical and of almost uniform diameter, and tapers sharply at its two ends (see Fig. 23).

The wall of the cirrus sac (see Figs. 24 and 25) is composed of 2 layers of muscles. On the outside is a layer of powerful longitudinal muscles, inner to which is a layer of circular muscles. These two layers of muscle extend along the entire length of the cirrus sac. Internal to the circular muscle layer, and closely attached to it, is a thin cellular layer with conspicuous nuclei. This layer is a mere one cell thick. There are, then, three main components of the wall of the cirrus sac: an outer longitudinal muscle layer, an inner circular muscle layer which rests on a thin cellular membrane.

The duct traversing the cirrus sac is divisible

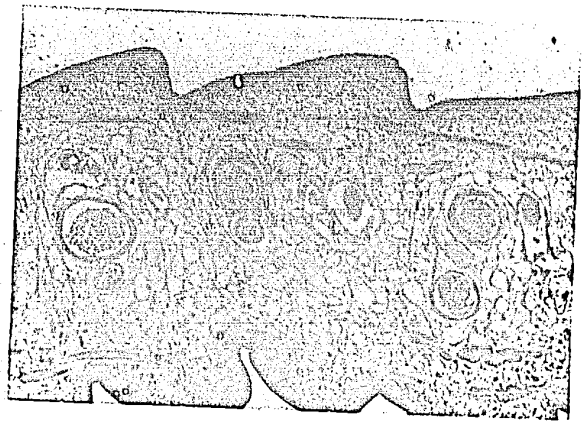


Fig. 21.--Longitudinal section of a part of the strobila. Note dorsal and anterior position of the cirrus sac (at arrow).
(Bouin's, paraldehyde fuchsin with Halmi's, x 190)

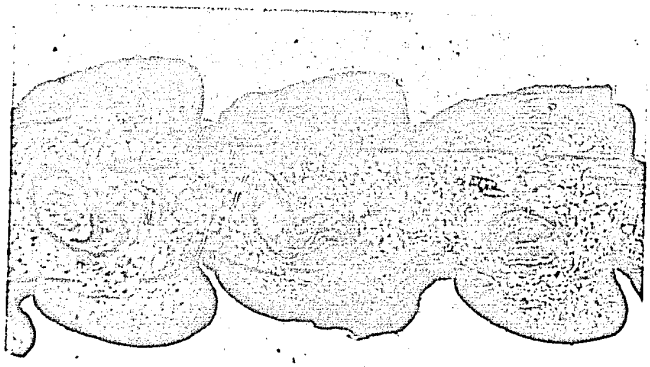
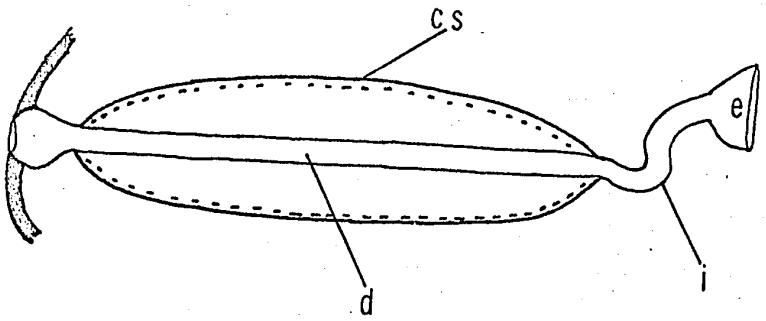


Fig. 22.--Longitudinal section of a part of the strobila. Note the ventral and more posterior position of the cirrus sac (at arrow).
(Bouin's, paraldehyde fuchsin with Halmi's, x 155)



cs - cirrus sac
d - duct
e - external seminal vesicle
i - isthmus

Fig. 23.--Diagrammatic longitudinal section of the cirrus sac (cs) enclosing a duct (d).

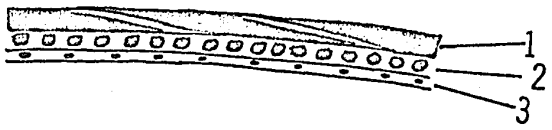
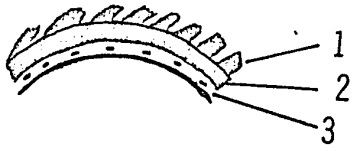


Fig. 24.--Diagrammatic longitudinal section of the wall of the cirrus sac.



1 - outer longitudinal muscles
2 - inner circular muscles
3 - cellular membrane

Fig. 25.--Diagrammatic transverse section of the wall of the cirrus sac.

into two parts--a proximal part and a distal part (see Fig. 26). The distal part, as we shall see shortly, is further divisible into 2 smaller regions. The duct in the cirrus sac is then divisible into 3 distinct regions which, as we shall see later, are physiologically specialized zones.

The proximal duct is greatly distended to form a capacious sac called the internal seminal vesicle (see Fig. 27). The internal seminal vesicle is joined to the external seminal vesicle by the slightly coiled isthmus of the external seminal vesicle, and it has a thin cellular epithelium which lies very close to the inner cellular membrane of the wall of the cirrus sac.

The sperm duct in the distal half has a very much narrower lumen than that of the internal seminal vesicle (see Figs. 26 and 27). The cellular epithelium lining the internal seminal vesicle gradually becomes taller in the zone marking the transition from internal seminal vesicle to the distal duct (see Fig. 27; also Fig. 44 in Chapter V). The epithelium is distinctly taller in the distal duct proper, and it continues to line this duct for about half its length. On the external surface of this part of the distal duct (i.e., the part with the tall epithelium) are found well-developed prominent longitudinal muscles. These muscles will be called the

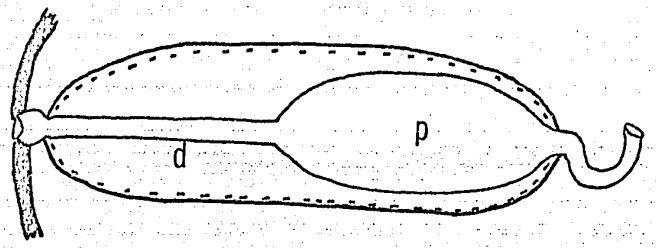
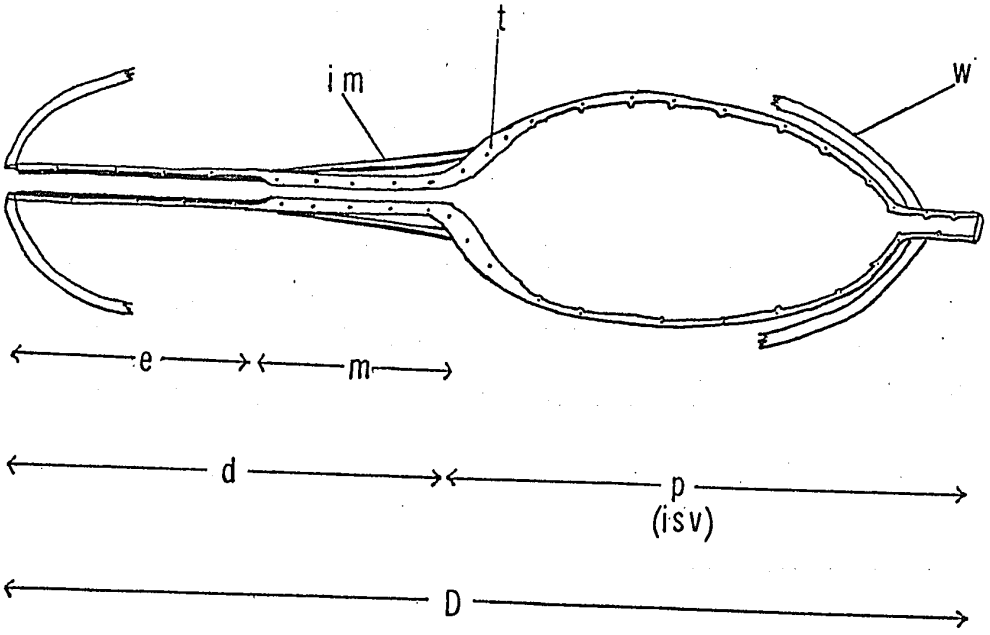


Fig. 26.--Diagrammatic longitudinal section of cirrus sac showing the proximal zone (p) and the distal zone (d) of the duct within it.



p - proximal half of the duct
d - distal half of the duct
isv - internal seminal vesicle
e - ejaculatory duct
w - wall of cirrus sac
m - muscular duct
im - inner longitudinal muscles
t - transition zone

Fig. 27.--Diagrammatic longitudinal section of the duct (D) traversing the cirrus sac.

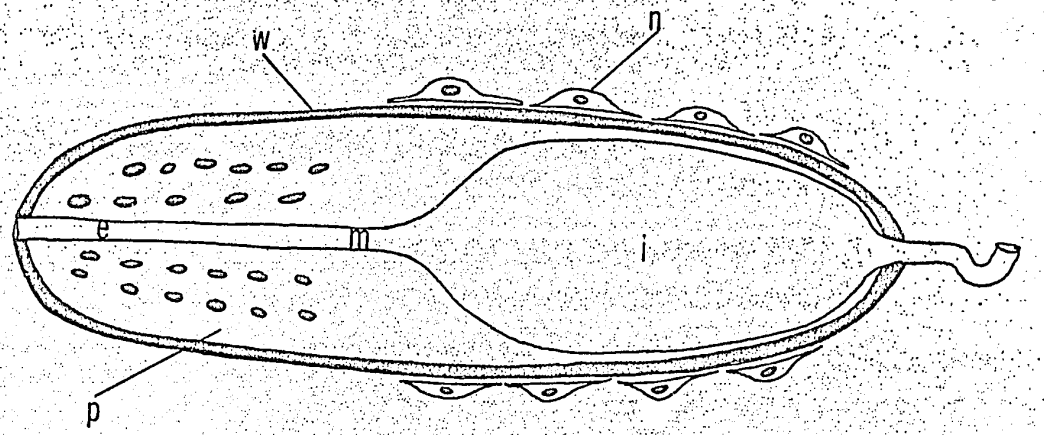
inner longitudinal muscles to distinguish them from the outer longitudinal muscles of the cirrus sac. These inner longitudinal muscles are closely attached to the wall of the duct, extend throughout its length and terminate proximally in the region marking the transition from internal seminal vesicle to the distal duct (see Fig. 27). Summarising then, the first half of the distal duct, lying adjacent to the internal seminal vesicle, is characterised by a tall epithelium and longitudinal muscles on its external surface. This is the muscular duct (see Fig. 27).

The second half of the distal duct is the third specialized part of the sperm duct lying within the cirrus sac. Here, the lumen of the duct is wider than that of the muscular duct, and its epithelium is thin, unlike that of the muscular duct. It is important to realise that the epithelium lining this duct is identical with that lining the lumen of the internal seminal vesicle, external seminal vesicle and vas deferens. A characteristic feature of the duct here is that inner to the thin nucleated epithelium and resting on it, is a prominent intensely staining intima, which is found only in this region of the sperm duct. The part of the distal duct with this intensely staining intima is the ejaculatory duct (see Fig. 27). This ejaculatory duct

communicates directly with the genital atrium. The wall of the cirrus sac terminates at the tip of the ejaculatory duct.

The lumen of the internal seminal vesicle is capacious, and its wall lies very close to the wall of the cirrus sac. On the other hand the lumens of the muscular duct and ejaculatory duct are very much narrower, with the result that there is an appreciable space between the walls of these two ducts and the wall of the surrounding cirrus sac. This space is occupied by a solid mass of parenchymatous tissue in which are found many densely staining cells (see Fig. 28).

Finally, associated with the wall of the cirrus sac are two groups of very prominent cells, one of which lies in the parenchyma immediately dorsal to the wall, and the other lies immediately ventral to the wall (see Fig. 28). Each group consists of 5 to 6 cells which extend in a line over part of the cirrus sac (see Fig. 28). Each cell is approximately triangular, in cross sections of the proglottid, with a tall central region tapering to two narrow ends. There is a prominent nucleus in the wide central region of the cell. The base of the cell lies very close to the outer longitudinal muscle layer of the cirrus sac, and one end of it is



w - wall of the cirrus sac

n - nerve cell

p - parenchyma with dense cells

Fig. 28.--Diagrammatic longitudinal section of cirrus sac.

Note: (1) the close apposition of the wall and the epithelium of the internal seminal vesicle.

(2) the distribution of the nerve cells.

(3) the wide lumen of the internal seminal vesicle (i) and the narrow lumens of the muscular duct (m) and ejaculatory duct (e)

Also see Figure 31.

drawn out into a thin long process. In cross sections of the cirrus sac, the process from each cell can be discerned to make contact with its outer longitudinal muscles (see Figs. 29 and 30).

Recapitulating then, the sperm duct consists of several distinct regions. They are the vas deferens, external seminal vesicle and the duct traversing the cirrus sac. The cirrus sac (see Fig. 31) has a complex structure with a muscular wall, with which are associated a few "nerve" cells. The sac encloses a duct which is continuous with the external seminal vesicle and vas deferens on its inner aspect, and which opens into the genital atrium on the poral side. This duct is divisible into three distinct regions: a large expanded internal seminal vesicle, a narrower muscular duct with a tall epithelium and muscles attached to its walls, and, finally, the ejaculatory duct which has a characteristic deeply staining intima.

This chapter shows that the spermatozoa, in their journey to the vagina of the female system, traverse a single long sperm duct organised into several physiologically distinct regions. It is now necessary to examine the functional significance of these regions.

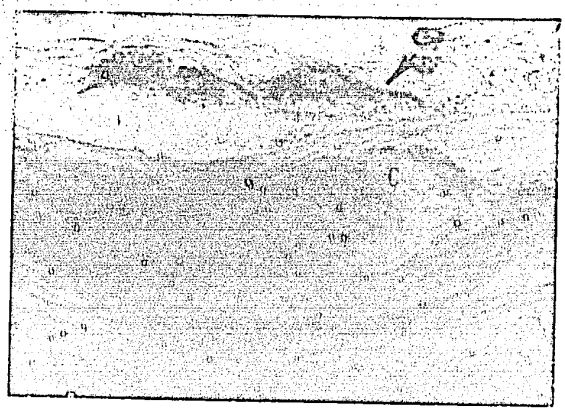


Fig. 29.--Transverse section passing through the cirrus sac (c). Note nerve cell (arrow).
(Carnoy's, Pyronin G, x 975)

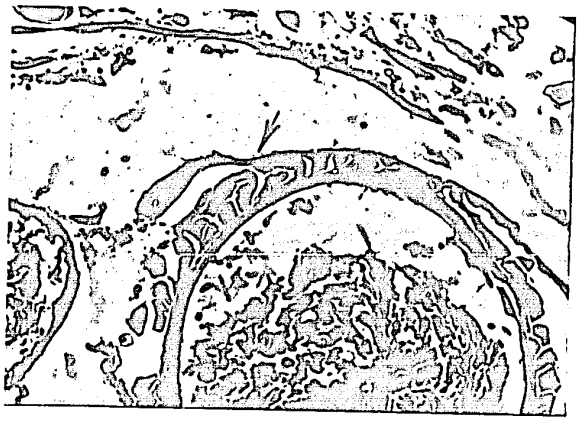


Fig. 30.--Longitudinal section passing through the cirrus sac. Note nerve cell process making contact with the outer longitudinal muscles of the cirrus sac (at arrow).
(Osmium-ethyl gallate, phase contrast, x 1000)

Fig. 31.--Transverse section passing through the cirrus sac; i, internal seminal vesicle; m, muscles of the muscular duct; e, ejaculatory duct; a, genital atrium; w, wall of cirrus sac; n, nerve cell. See also Fig. 28.
(Bouin's, paraldehyde fuchsin with Halmi's, x 575)

CHAPTER V

THE MALE REPRODUCTIVE SYSTEM--PART II

The functional significance of the different parts of the male system will be examined in this chapter. The testes are the first to mature and release their products into the male tract. The spermatozoa leave the testes, travel through the vasa efferentia and vas deferens, and enter the external seminal vesicle.

1. The External Seminal Vesicle

It is, at first, very small and almost oval-shaped in cross sections of the proglottid (see Fig. 32). With the arrival of spermatozoa in its lumen, this vesicle gradually increases in size to become a large capacious sac packed with spermatozoa (see Fig. 33). The spermatozoa soon move into the internal seminal vesicle. The prominent large size of the external seminal vesicle with its large volume of spermatozoa implies that it functions as a place where the spermatozoa are temporarily stored. Presumably large quantities of spermatozoa are produced by the testes, which then have to be removed from the testes regularly, and as

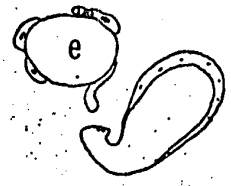


Fig. 32.--The external seminal vesicle (e) in diagrammatic sectional view when devoid of spermatozoa.

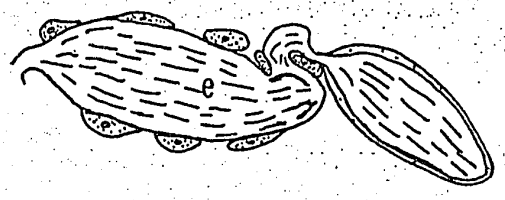


Fig. 33.--The external seminal vesicle (e) in diagrammatic sectional view when packed with spermatozoa.

often as possible, so that there would be space within the testes for succeeding generations of spermatozoa. Until such time as the spermatozoa are transferred to the female reproductive tract, they would have to remain within some storage point along the male tract: the seminal vesicles appear to have this function.

We saw earlier that the second characteristic feature of the external seminal vesicle was its possession of numerous gland cells. These cells, which are found throughout the length of the external seminal vesicle, are almost oval in section and are closely applied to seminal vesicle. The cells undergo a cycle of development which is correlated with the entry of spermatozoa into the seminal vesicle. Before the spermatozoa arrive, the cytoplasm of the gland cell is diffuse; but as spermatozoa appear, proteinaceous granules are elaborated. These increase in number and staining intensity as the vesicle fills with spermatozoa (see Figs 34, 35 and 36).

What is the nature and function of this secretion? A survey of the literature shows that there are cells associated with the cirrus sac or seminal vesicle of many cestodes and trematodes, which are somewhat loosely referred to as prostate gland cells or prostate glands (see Hyman, 1951; Joyeaux and Baer, 1961). Davies and

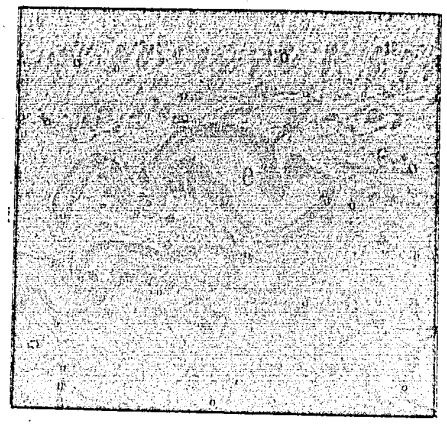


Fig. 34.--Transverse section passing through the external seminal vesicle (e). Note the absence of spermatozoa; the gland cells (at arrow), each with a prominent nucleus, but diffuse cytoplasm.
(Bouin's, mercury bromphenol blue, x 400)

Fig. 35.--Transverse section passing through the external seminal vesicle. Note the gland cells (at arrow) are larger and have granules in the cytoplasm; also note the swollen condition of the receptacle packed with spermatozoa.
(Bouin's, mercury bromphenol blue, x 400)

Rees (1947), in their study of the bird tapeworm Andrepigynotaenia haematopodis, suggested the possibility that these cells produced a secretion which aided in the penetrating action of the male copulatory organ. Kouri and Nauss (1938) noted the strong morphological similarity between Mehlis' gland and the prostate gland in Fasciola hepatica. According to these workers, the similarity in appearance and structure of these two glands, as well as their respective locations in proximity to converging ducts of the reproductive apparatus, suggests that they have similar functions. This function, they believed, was in supplying "a lubricant to facilitate the ready passage of genital products such as vitellogenous cells, spermatozoa and ova through narrow ducts." (Kouri and Nauss, 1938). Such ideas are only speculative and have yet to be substantiated. In order to make any plausible suggestions regarding the possible functions of the secretion of these gland cells, some knowledge regarding its chemical nature is essential. Simple histochemical tests were carried out with a view to obtaining this information.

The histochemistry of the secretion produced by these gland cells

The results of the histochemical tests are shown in Table 3 (see also the Appendix for details of the

TABLE 3.--Histochemical observations on the secretion of the gland cells of the external seminal vesicle

Histochemical tests	Results
PAS	+
PAS after diastase	+
PAS after lipid extraction with hot methanol:chloroform	+
Best's carmine	-
Alcian blue, pH 2.5	-
Toluidine blue	Blue colour, no metachromasia
Mercury bromphenol blue	+
Millon's reagent	faint +
Ninhydrin-Schiff	-
DDD	-
Performic acid-Alcian blue (PFAB)	+

histochemical procedures, and the rationale of the tests). The granules are PAS positive and fast to digestion with diastase. The PAS reaction is unchanged by extraction with methanol:chloroform, thereby excluding lipids. There is clearly a carbohydrate moiety, other than glycogen, in the granules (see Fig. 37). Protein is also present in the granules: they stain intensely with mercury bromphenol blue and faintly with Millon's reagent. The granules stained intensely with PFAB, but gave no reaction with DDD. This indicates the presence of abundant cystine in the protein. Thus the granules are probably a carbohydrate-protein complex. The granules failed to stain with Alcian blue at pH 2.5, and further do not exhibit metachromasia with Toluidine blue, which reactions exclude acid mucopolysaccharides. The granules then fall into the category of glyco- or mucoproteins of Pearse's classification (Pearse, 1960). The granules also stained intensely with paraldehyde fuchsin after oxidation (see Fig. 36), and failed to stain in sections not exposed to the oxidant. This indicates the presence of amino acids rich in sulphur (see Appendix under the use of paraldehyde fuchsin).

Fig. 36.--Transverse section showing the gland cells of the external seminal vesicle. Note the dense staining of the cells.
(Bouin's, paraldehyde fuchsin after permanganate oxidation, x 400)

Fig. 37.--Transverse section showing the PAS-positive nature of the secretory granules of the gland cells of the external seminal vesicle.
(Lison's, PAS, x 420)

The fate and function of the secretion

The secretory granules are not observed within the lumen of the external seminal vesicle, although a few granules may be observed in the epithelium lining the lumen of the vesicle. The failure to observe the secretory granules within the lumen of the vesicle could be due to either the very small size of the granules, and thus the likelihood of their being obscured by the mass of spermatozoa within the vesicle; or the granules may contain short-lived and exceptionally labile material which is rapidly metabolised by the spermatozoa and are thus not observed within the vesicle. A combination of both of these possibilities cannot be excluded.

We cannot say anything further about the nature and possible function of this secretion. That it is of some importance is evident from the observation that the secretion is not present in the cells of the seminal vesicle prior to the arrival of spermatozoa, and that its appearance is correlated with the presence of sperms within the vesicle. The cells become swollen and packed with the secretion. The secretion granules may perhaps be some essential metabolite required by the spermatozoa, which substance they obtain here on their way to the

female tract. Or the secretion may contain some factors essential for the maturation of the spermatozoa. It is of some interest to note that in the rabbit, the spermatozoa mature while passing through the epididymis. The ability to fertilise is suddenly acquired when the spermatozoa pass through the distal region of the corpus epididymis (see Orgebin-Crist, 1967). On the basis of her experiments on sperm maturation, Orgebin-Crist stated that "it seems. . . the spermatozoa need the special environment created by the epithelium of the corpus epididymis." (Orgebin-Crist, 1967). Is it possible that a similar situation obtains here?

One can only speculate with the information available. The relative inaccessibility of this secretion, the paucity of its quantity and the inevitable difficulty to be encountered in experimenting with spermatozoa outside the proglottid, do not enable us at the present time to experiment further to learn more about this secretion. We then still await any definite statements regarding the function of the gland cells of the external seminal vesicle. In view of this, the continued use of the term prostate gland, which has functional overtones, is to be discouraged.

The cirrus sac

The spermatozoa leave the external seminal vesicle, travel through the isthmus and enter the internal seminal vesicle within the cirrus sac.

2. The Internal Seminal Vesicle

The spermatozoa are stored temporarily in the internal seminal vesicle before being ejaculated into the genital atrium. The spermatozoa are presumably forced out of the internal seminal vesicle by the pumping action of the cirrus sac brought about by contractions of its muscular wall. The close apposition of muscular wall and epithelium (see Figs 28 and 31 in Chapter IV), probably contributes towards a more effective translation of the force of muscular contraction on the wall of the internal seminal vesicle. The paired cells, lying adjacent to the cirrus sac in the region of the internal seminal vesicle (see Figs. 30 and 31 in Chapter IV), which are tentatively called "nerve" cells, may initiate and control this muscular activity.

3. The Muscular Duct

Contractions of the muscular cirrus sac wall force spermatozoa from the capacious internal seminal vesicle into the remaining part of the sperm duct which has a much narrower lumen. This part of the sperm duct is made up of the muscular duct and the ejaculatory duct, which are separated from the wall of the cirrus sac by the solid mass of parenchymatous tissue (see Figs. 28 and 31 in Chapter IV). The narrowing of the sperm duct at this point will increase the pressure on the spermatozoa, thus propelling the semen out of the male tract with considerable force. The solid parenchymatous mass would also prevent any dilation of these ducts and a consequent diminution in the force of ejaculation (see Figs. 38, 39, 40 and 41). The pumping action of the cirrus sac is probably further enhanced by the longitudinal muscles of the muscular duct; contractions of these muscles will shorten the duct, thereby forcing the spermatozoa towards the atrium (see Figs. 40, 42 and 43). If the circular muscles in the wall of the cirrus sac contract simultaneously, the ejaculation of spermatozoa could be expected to be forceful and rapid. Spermatozoa are never observed in the muscular duct, which suggests that not only is the process of ejaculation very rapid, but also that the muscular duct acts as an effective

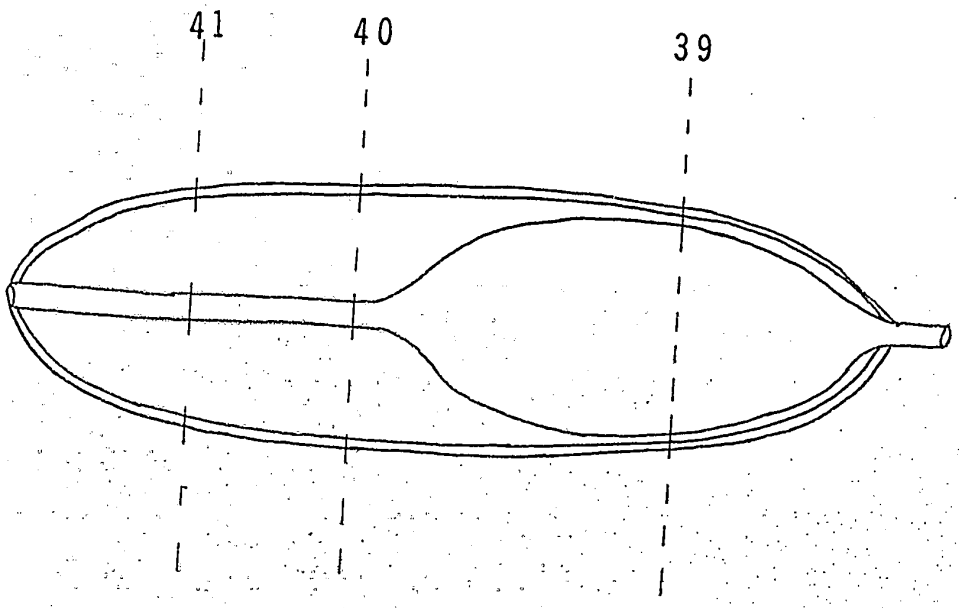


Fig. 38.--Diagram of the cirrus sac indicating the regions depicted in Figures 39, 40 and 41. Also see Figure 28.

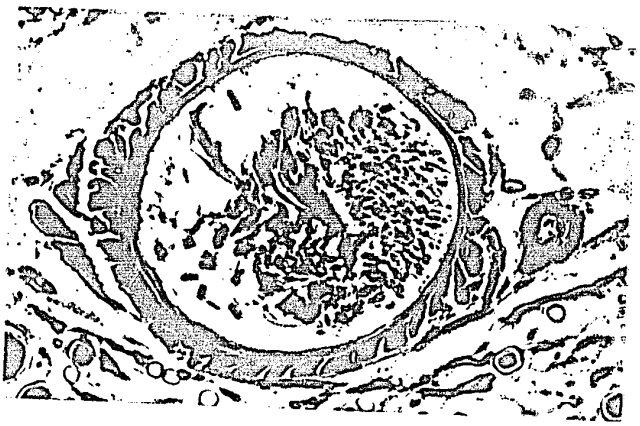


Fig. 39.--Longitudinal section passing through the internal seminal vesicle. Note the wide lumen of the vesicle, and the close proximity of its epithelium to the muscular wall of the cirrus sac.
(Osmium-ethyl gallate, phase contrast x 1,170)

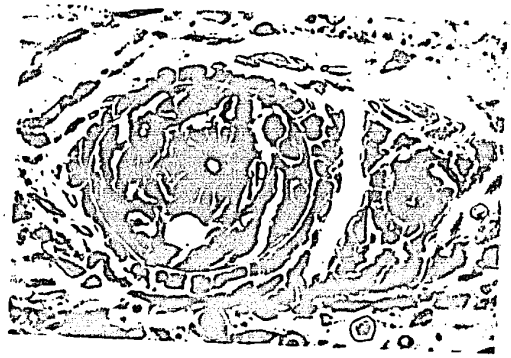


Fig. 40.--Longitudinal section passing through the muscular duct. Note the narrow lumen and muscles of the duct (at arrow); p, parenchyma.
(Osmium-ethyl gallate, phase contrast, x 750)

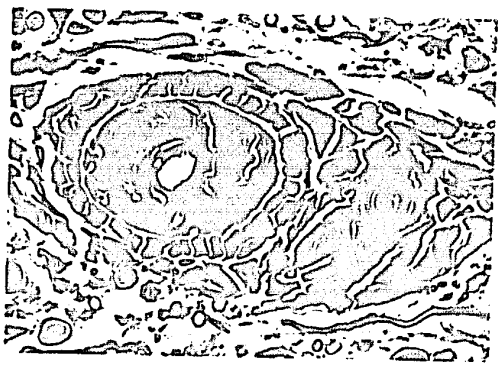


Fig. 41.--Longitudinal section passing through the ejaculatory duct. Note the epithelium and the intima of the duct (at arrow).
(Osmium-ethyl gallate, phase contrast, x 750)

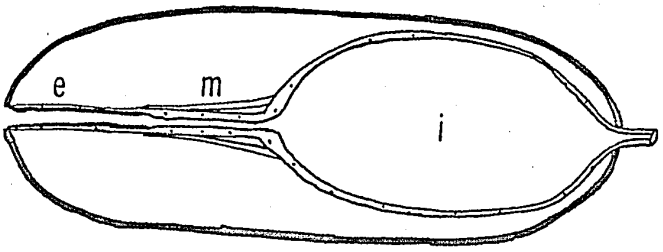


Fig. 42

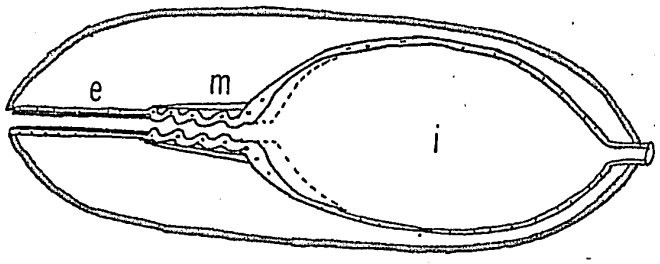


Fig. 43

Figs. 42 and 43.--Diagrammatic longitudinal sections of the cirrus sac showing "resting" state (Fig. 42) and contracting state (Fig. 43) of the muscular duct (m).

i - internal seminal vesicle

e - ejaculatory duct

valve to prevent leakage of spermatozoa. This is probably a consequence of the small lumen of the duct, which is in turn partly due to the tall epithelium which lines it (see Figs. 40 and 44).

4. The Ejaculatory Duct

This duct is easily recognized by its deeply staining intima, which is proteinaceous as indicated by its staining with mercury bromphenol blue. The intima also stains with the PFAB procedure, which shows that it is rich in protein disulphide groups and thus possibly represents a "keratinised" material with some structural stability (see Fig. 45). Such a rigid structure may serve two functions: it may furnish a more effective lining for the ejaculatory duct than an epithelium of less rigid material, and it may also act as a rigid support against which the muscular duct could contract.

The testes commence the production of spermatozoa at an early stage in development, and the spermatozoa soon appear in the male reproductive tract, which is essentially a single long sperm duct showing physiological specialization along its length. This duct is responsible for conducting the spermatozoa to the female tract. The

Fig. 44.--Transverse section passing through the cirrus sac. Note epithelium of the muscular duct (at arrow); w, wall of cirrus sac; i, internal seminal vesicle.
(Bouin's, paraldehyde fuchsin with Halmi's, x 900)

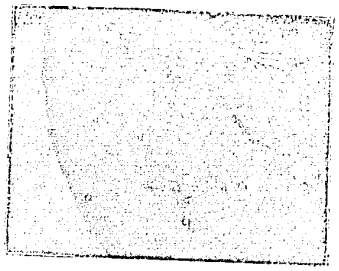


Fig. 45.--The ejaculatory duct in section. Note the positive staining of the intima (arrow).
(Performic acid-Alcian blue, x 425)

spermatozoa come into contact with a secretion in the internal seminal vesicle, and are stored in the seminal vesicles until such time as they are forcefully expelled into the genital atrium and vagina during copulation. The process of sperm transfer or copulation will be dealt with in Chapter VII.

CHAPTER VI

THE FEMALE SYSTEM

> It is the task of the female reproductive system to receive and store the products of the male system, to produce eggs, to ensure that the eggs and spermatozoa meet and to deposit the eggs.
>
> Davey in Reproduction in Insects (1965a)

The female system in the proglottids of this tapeworm consists of two main parts or regions: the first, consisting of the vagina and the seminal receptacle, is responsible for receiving and storing the spermatozoa; the second part is responsible for the elaboration of the egg, and to it belongs the ovary, vitelline gland, Mehlis' gland, ootype and uterus.

1. The First Part of the Female System

a. The vagina

The vagina leads inwards from the genital atrium (see Fig. 46). The vestibulum of the vagina is wide and drawn out in the antero-posterior direction. The vagina proper is narrow and flattened at first, and its epithelium, like that of the vestibulum and the genital atrium, is "cuticularised." The flattened vagina makes a ventral dip (see Fig. 46) and is now a cylindrical

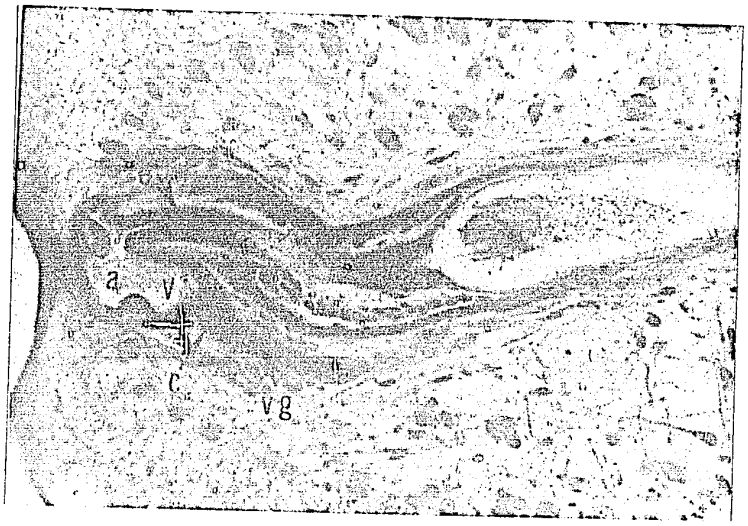


Fig. 46.--Transverse section passing through the poral end of both male and female reproductive tracts. a, genital atrium; e, ejaculatory duct; v, vestibulum of the vagina; vg, vagina; c, circular muscles of the vagina.
(Bouin's, paraldehyde fuchsin with Halmi's, x 485)

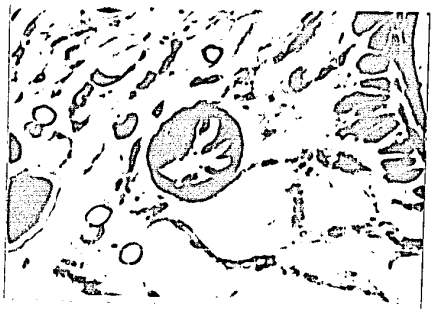


Fig. 47.--Longitudinal section passing through the vagina. Note the ciliated epithelium. A part of the cirrus sac is visible in the right hand corner.
(Osmium-ethyl gallate, phase contrast, x 1,560)

tube, circular in cross section, with a ciliated epithelium (see Fig. 47). Circular muscles invest the vagina throughout its length. The vagina is situated ventrally in the proglottid, between the dorsally situated cirrus sac and the more ventrally located excretory ducts (see Fig. 48).

b. The seminal receptacle

The vagina is continuous with the seminal receptacle which could be distinguished from the former by its wider lumen, and epithelium which lacks cilia (see Fig. 48). In the younger proglottids it is a thin slender tube, but in the more mature proglottids, with the gradual accumulation of spermatozoa in its lumen, it becomes a large, voluminous, sac-like structure, thrown into one or two dorso-ventrally directed loops. It is now packed with spermatozoa. The wall of the receptacle has circular muscles, and it becomes thicker at its inner end, at which point is found a muscular sphincter. The sphincter guards the passage leading to the sperm duct, which connects the seminal receptacle with the egg-elaborating region of the female system.

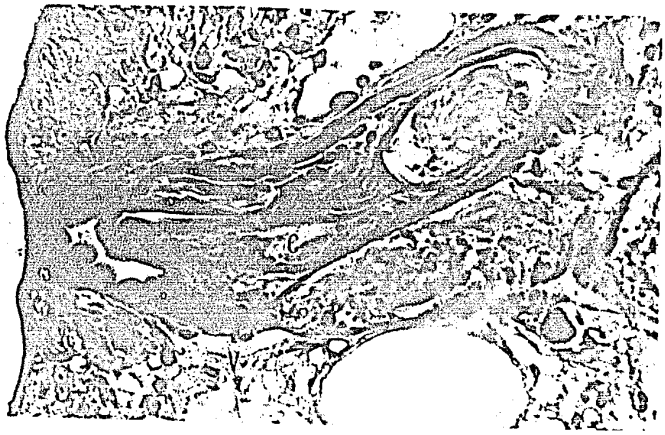


Fig. 48.--Transverse section passing through the poral end of male and female reproductive tracts. c, cirrus sac; v, vagina; sr, seminal receptacle; the arrow indicates the two excretory ducts. (Bouin's, Azan, x 550)

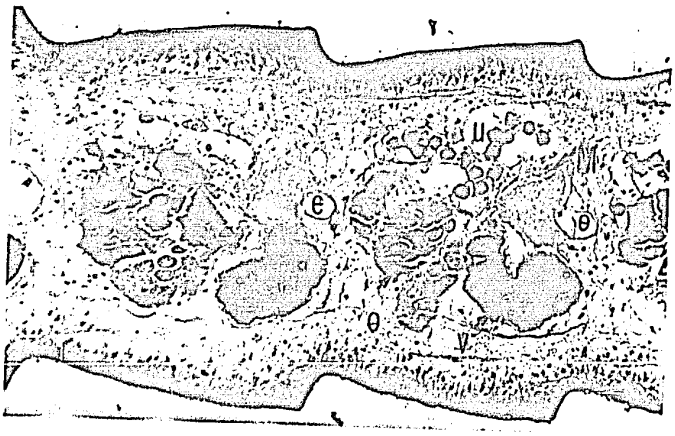


Fig. 49.--Longitudinal section of a part of the strobila showing the female reproductive system; o, ovary; m, Mehlis' gland; v, vitelline gland; u, uterus; e, posterior transverse excretory duct. (Bouin's, paraldehyde fuchsin with Halmi's, x 130)

2. The Egg-elaborating Region of the Female System

a. The ovary

The major part of the ovary lies in the anterior half of the proglottid, and at its widest part it is of an appreciable height (see Fig. 49). The ovary has two lobes, each of which has several small blunt projections; thus the outline of the ovary is irregular. The lobes describe a very gentle curve as they extend posteriorly and the posterior border of the ovary is slightly concave, with the vitelline gland lodged within this cavity. The two lobes of the ovary are connected by a short narrow isthmus from the dorsal surface of which arises the oviduct. A very delicate membrane forms the outer wall of the ovary; electron microscopic studies show that the ovary of *F. hepatica* is enclosed within a thick (80-130 mμ) basement membrane and an outer capsule containing muscular tissue (Bjorkman and Thorsell, 1964). The primary oocytes have a characteristic appearance, each containing a large nucleus with a prominent nucleolus, and many dense granules in the cytoplasm.

b. The vitelline gland

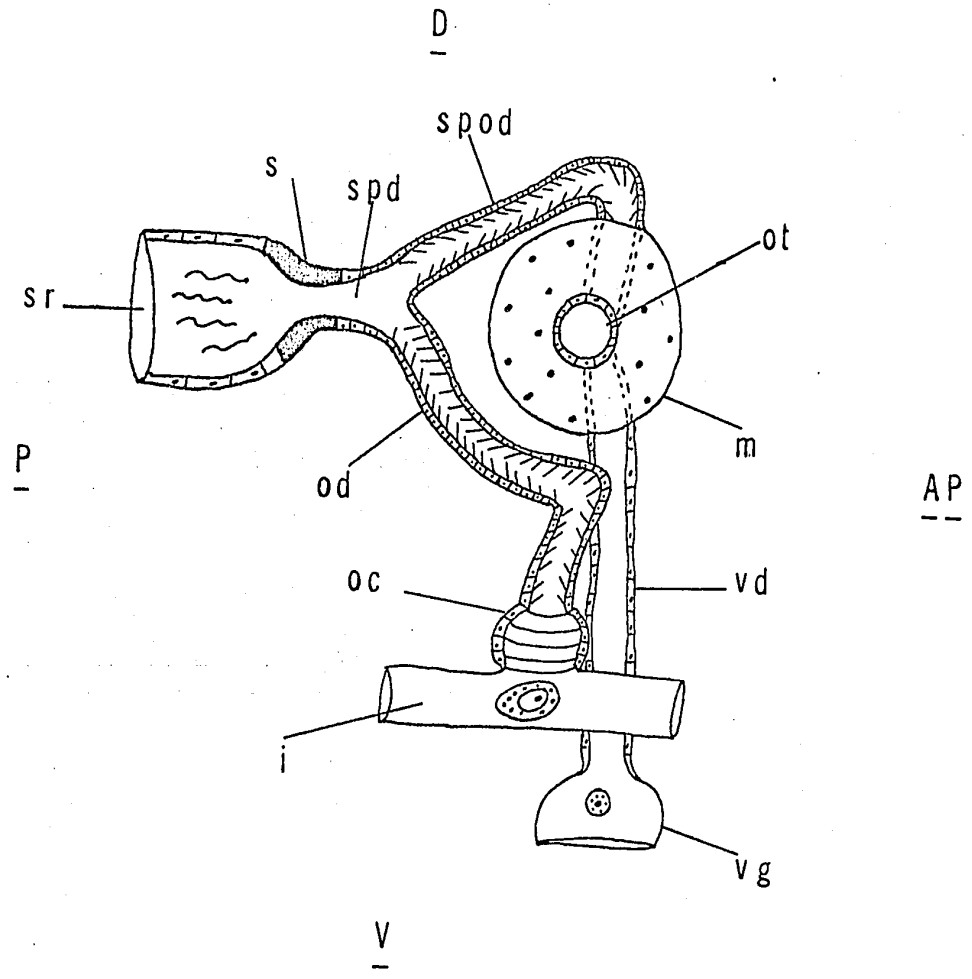
The vitelline gland is situated in the posterior part of the proglottid, ventral to the ovary and Mehlis'

gland (see Fig. 49). The immature vitelline gland is dorso-ventrally flat, but later develops three lobes and is more rounded. It is a compact gland in H. diminuta as opposed to its diffused condition in trematodes and pseudophyllidean cestodes. The immature vitelline cells are spindle-shaped with prominent nuclei and very little cytoplasm; but the mature cell is spherical and its cytoplasm is packed with granules. The cell boundaries of these cells become obscure so that the mature gland resembles a mass of spherical granules among which are scattered the larger nuclei. The short vitelline duct leads out from the anterior-most, central lobe of the gland.

c. The arrangement of the different ducts and glands of the egg-producing region

The ducts belonging to this region are the oviduct, spermduct, sperm-oviduct, vitelline duct, ovo-vitelline duct and uterine duct. Associated with these ducts are two chambers--the small ootype and the larger uterus (for a diagrammatic representation of the relationships of the ducts and glands, and their disposition in the proglottid, see Figs. 50, 51 and 52).

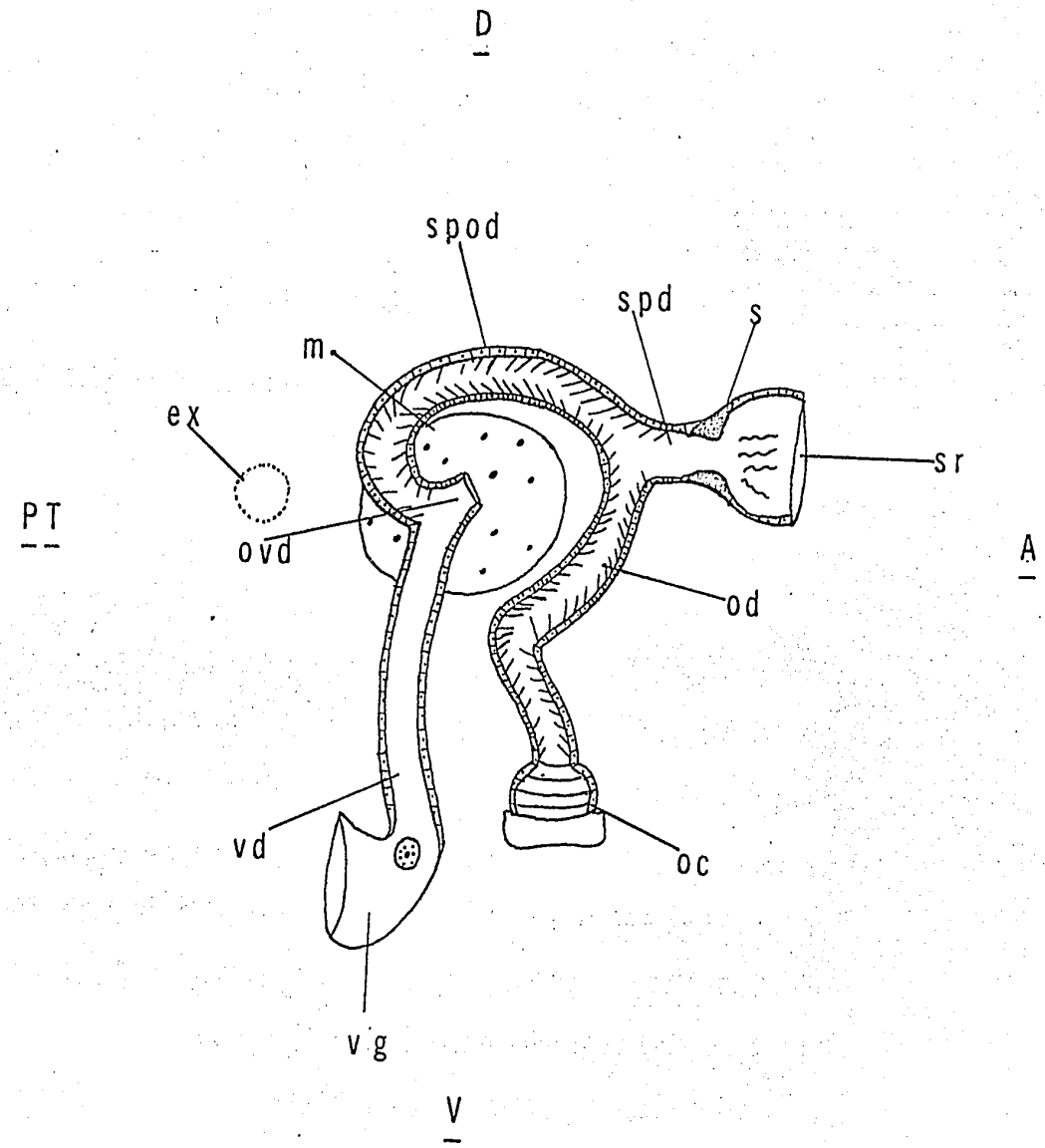
The oviduct, arising from the dorsal surface of the isthmus, has two recognizable regions: a proximal



D - dorsal
V - ventral
P - poral
AP - aporal
sr - seminal receptacle
s - sphincter
spd - sperm duct

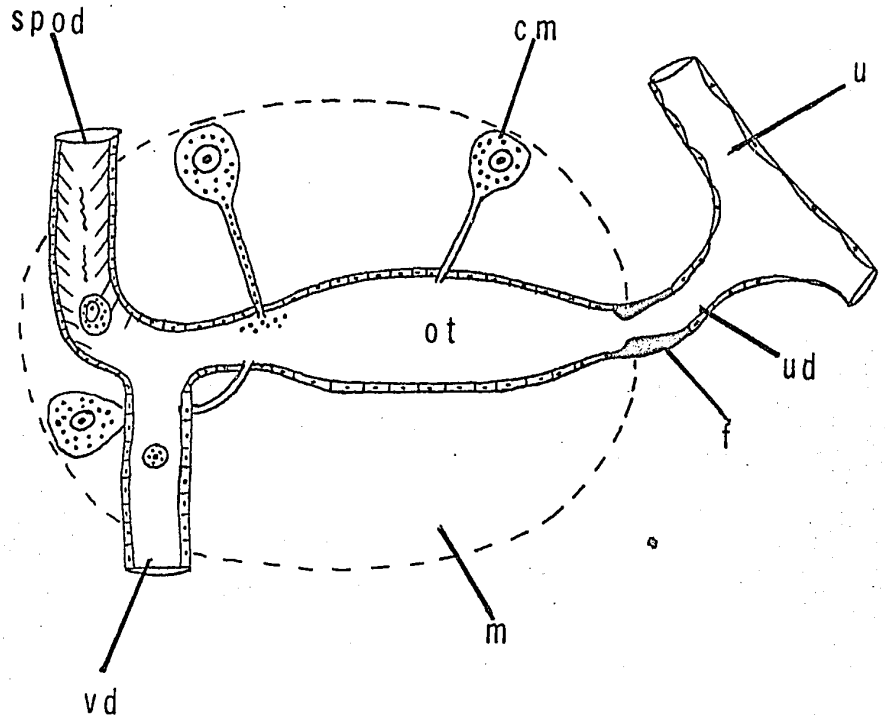
spod - sperm-oviduct
i - isthmus of ovary
oc - oocapt
od - oviduct
vg - vitelline gland
vg̃ - vitelline duct
m - Mehlis' gland
ot - ootype

Fig. 50.--Diagrammatic sectional view of the egg elaborating region of the female system (viewed transversely from the anterior).



A	- anterior	spod	- sperm-oviduct
PT	- posterior	m	- Mehlis' gland
D	- dorsal	ex	- posterior transverse excretory duct
V	- ventral	ovd	- ovo-vitelline duct
sr	- seminal receptacle	vd	- vitelline duct
s	- sphincter	vg	- vitelline gland
spd	- sperm duct	oc	- oocapt[illegible]
od	- oviduct		

Fig. 51.--Diagrammatic sectional view of the egg elaborating region of the female system (viewed longitudinally).



f - flap-like valve of
ud - uterine duct
u - uterus
cm - gland cell of Mehlis' gland
spod - sperm-oviduct
vd - vitelline duct
m - Mehlis' gland
ot - ootype

Fig. 52.--Diagrammatic sectional view of the ootype, Mehlis' gland, uterus and associated ducts.

oocapt, with prominent circular muscles, lying adjacent to the isthmus, and a distal cylindrical tube with a ciliated epithelium and an outer coat of circular muscles. The oviduct runs dorsally towards Mehlis' gland, turns porally and continues along the margin of Mehlis' gland and joins the unciliated muscular sperm duct to form the sperm-oviduct (the sperm-receiving region and the egg-producing region are now connected). The sperm-oviduct has a muscular coat investing a ciliated epithelium. It runs a short distance aporally along the dorsal margin of Mehlis' gland, turns posteriorly and continues along the posterior margin of Mehlis' gland, and, at about the level of the posterior excretory duct, it turns inwards, within the gland, to meet the vitelline duct. The vitelline duct, a part of which lies among a few cells of Mehlis' gland, is muscular and lacks a ciliated epithelium. It ascends to join the sperm-oviduct to form the short, unciliated ovo-vitelline duct which opens into the ootype.

d. The ootype and uterus

The ootype or egg chamber is a cylindrical tube with a slightly expanded central region and tapering ends. It lies obliquely in the proglottid, and thus is not readily seen in cross sections of the proglottid.

A jacket of circular muscles invests the thin unciliated epithelium of the ootype, which is one cell thick and encloses the ootype lumen. Densely staining material lines the ootype lumen. The ootype communicates with the uterus through a short uterine duct which has a thicker epithelium, and two flap-like "valves" at its entrance (see Fig. 53). The uterus, situated dorsally, has a thin epithelium of flattened elongate cells. In immature proglottids it resembles a thin, long, cylindrical tube, but as development progresses, it makes a spectacular increase in size.

e. Mehlis' gland

This is made up of several unicellular glands arranged around the ootype. Each gland cell has a swollen basal region, which contains a nucleus and several small cytoplasmic granules, and a thin tapering proximal region which is the duct. The ducts are visible in thin sections (1-2 μ thick), and they are observed to pierce the epithelium of the ootype to open into its lumen (see Fig. 53). All of the gland cells do not occur at the same level, but they are distributed in 2 or 3 compact rows around the ootype, giving the gland its characteristic dense, compact appearance in H. diminuta (see Fig. 54).

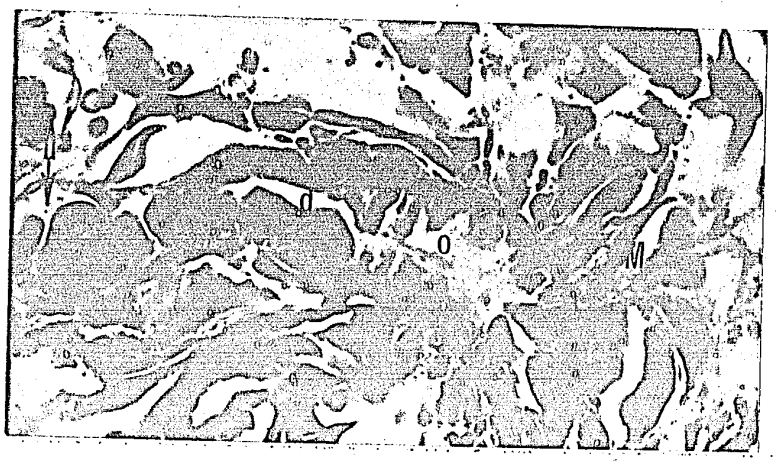


Fig. 53.--Section passing through the ootype. m, gland cell of Mehlis' gland, the arrow shows the duct of the gland cell entering the ootype; o, the ootype; d, uterine duct; u, uterus. Note the epithelium of the uterine duct.
(Osmium-ethyl gallate, phase contrast, x 1,530)

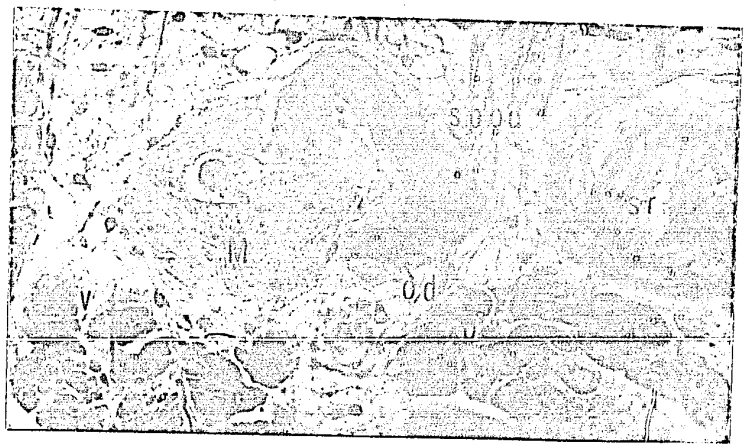


Fig. 54.--Transverse section passing through the female reproductive system; sr, seminal receptacle; spod, sperm-oviduct; m, Mehlis' gland; v, vitelline gland; od, oviduct; o, ovary. Note spermatozoa in seminal receptacle; the oocytes lack the prominent cytoplasmic granules.
(Bouin's, paraldehyde fuchsin with Halmi's, x 480)

Only the structure of the female reproductive system was described in this chapter. It is more convenient to consider the details pertaining to the physiology of the female system in subsequent chapters. In the next chapter we will consider the communion between the male and female reproductive tracts in terms of the transfer of spermatozoa.

CHAPTER VII

THE TRANSFER OF SPERMATOZOA

In tapeworms, fertilisation is internal and copulation is therefore necessary. There does not appear to be much precise knowledge regarding copulatory processes in tapeworms. It is of interest to remind ourselves that the tapeworm body or strobila is made up of several proglottids serially arranged, each of which possesses both male and female reproductive systems.

1. Copulation and Sperm Transfer in Tapeworms in General

Wardle and McLeod (1952), in their monumental work on tapeworms, state that

> In spite of the general belief that an extruded cirrus can be inserted readily into the vagina of a proglottid in another part of the strobila, few observations exist to support it. Some authors have thrown doubt upon the supposition. There is a strong suspicion that in the majority of tapeworms there is a simple emission of semen into the fluid environment and an active migration of sperm cells from the environment into the vagina.

Hyman (1951), on the contrary, writes that

> Self fertilisation by eversion of the cirrus into the vagina of the same proglottid is probably the most common method of impregnation in cestodes. Copulation between different proglottids of the

> same strobila, or when opportunity affords, of different strobila, is probably frequent. The insemination in such cases may be mutual or the younger anterior male segments may inseminate the more posterior segments that have reached the female phase.

Joyeaux and Baer (1961) believe that autocopulation is the general rule prevailing amongst most cestode species. They thus write

> Il semblerait que chez la majorité des cestodes, l'auto-copulation entre éléments mâle et famelle du même anneau soit la règle, surtout quand celui-ci est beaucoup plus large que long. Il est en effet très frequent d'observer le cirre introduit dans le vagin du même anneau.

Sandars (1957) provides a description of the anatomical arrangement of the terminal regions (i.e., the structures at the atrial end) of the male and female systems in the tapeworm Anoplotaenia dasyuri. The organisation of the ducts in this region, as interpreted by her, show that this tapeworm possesses an efficient means of ensuring autocopulation in each proglottid (see Sandars, 1957, page 321). The literature on tapeworms contains many descriptions of evaginated or everted copulatory organs of the male system (see Hyman, 1951, for example). These reports are many and not documented here; the report of Rees and Williams (1965) is just one such example, where a prominent evaginated cirrus, armed with backwardly directed spines, is described. Do these everted cirri, which presumably are

copulatory organs, imply the occurrence of cross copulation between different proglottids of the same or different strobila? An everted cirrus has been observed by the author in the terminal proglottid of several specimens of Echinococcus granulosus (unpublished observations). The presence of an everted cirrus in this tapeworm is an interesting feature when one considers the great disparity in the developmental states shown by the different proglottids of the same strobila. It appears to be very unlikely that there is sperm transfer between the sub-terminal and terminal proglottids of the same strobila because the reproductive system is still in the process of being formed in the sub-terminal proglottid, when the terminal proglottid is already observed to have abundant spermatozoa in the seminal receptacle and a uterus containing developing embryos. Does this imply the occurrence of copulation between adjacent worms?

Some interesting observations on sperm transfer in the pseudophyllidean tapeworm, Schistocephalus solidus, were made by Smyth (1946, 1950, 1954a) in his in vitro experiments using the progenetic larva of this tapeworm. The eversion of the cirrus was observed in larvae cultured in peptone broth at 40°C. The cirrus in each proglottid was observed to evert and invaginate

periodically; at its fully extended state, this considerably long cirrus was observed to wave about for a moment and then eject a mass of spermatozoa. The temperature of the culture medium was critical and had to be near 40°C or else cirrus activity would cease immediately. Although the worms in the culture medium were in close apposition, the cirri were observed to evert directly outwards, and copulation between worms did not occur. Histological examination of these worms revealed that the lumens of the receptacula were all devoid of spermatozoa, which contrasted with the condition in worms matured in birds, where masses of spermatozoa were always observed in the receptacula. Subsequent experiments, where worms were cultured in a special thin cellulose tubing, resulted in the successful insemination of the receptacula. Worms cultured at the bottom of the tube (outside the thin cellulose tubing) showed empty receptacula. These results suggested that some form of compression, such as would be present in the gut of the bird, and simulated by a thin cellulose tubing in <u>in vitro</u> culture methods, was essential for successful insemination. According to Smyth, the compression, brought about by the close apposition of the strobila and the wall of the gut, could either force the everted cirrus to be bent back and be inserted into the

vagina, or it could create a confined space between the worm and gut within which a concentration of ejaculated spermatozoa would accumulate. The spermatozoa would then either be forced into the vagina, probably aided by chemotaxis, or else sucked in by the vagina. Writing about copulation (insemination) in tapeworms, Smyth (1962) says that "whether self fertilisation within the same proglottid or cross fertilisation between different proglottids occur, copulatory processes are only made possible in cestodes, by the compression of the strobila against the intestinal wall. Thus fertilised eggs are only produced in vitro if the strobila is compressed during maturation: this is normally achieved by an artificial gut of cellulose tubing." This statement may certainly be valid for Schistocephalus, but whether such a broad generalisation, to include tapeworms in general, is justified, is questionable.

The assumption that spermatozoa are ejected into the environment (i.e., the gut of the host) before they enter the vagina (see Wardle and McLeod; Smyth, above) must also assume that the viability of the spermatozoa is not affected by the contents of the gut. This has never been tested.

There are two families of cyclophyllidean tapeworms whose members show interesting morphological

features regarding the reproductive system (see Hyman, 1951). Tapeworms of the family Acoleidae, which are parasitic in birds, are hermaphroditic, but the female system either lacks a vagina or the vagina does not open to the exterior. According to Hyman (1951) the spermatozoa are transferred through hypodermic impregnation, and the cirrus is accordingly heavily armed with large hooks. A similar process is suggested to occur in the protogynous bird tapeworm Andrepigynotaenia haematopodis which is believed to lack a vagina (Davies and Rees, 1947). The members of the family Dioecocestidae are said to be dioecious with separate sexual forms. In Dioecocestus, the male, which is reported to be more slender than the female, possesses a stout, spinose cirrus; the female has a vagina lacking an external opening. The male of Infula has a large muscular cirrus while the female possesses a slender vagina. In such forms then, cross copulation between different strobilas presumably takes place.

The tapeworm Shipleya inermis has caused much controversy as to whether it is hermaphroditic or truly dioecious. It was first described by Fuhrmann in 1908 from a charadriform bird Gallinago gigantea from Brazil. (reference in Schell, 1959). Fuhrmann thought it was protandrous. Baer (1940) described several specimens of

this parasite from Wilson's snipe, Capella delicata, and he too considered it to be hermaphroditic and protandrous. On the contrary, Voge and Rausch (1956) studied specimens obtained from dowitchers--Limnodromus species--and came to the opposite conclusion that the tapeworms were definitely dioecious with distinct male and female specimens. More recently Schell (1959), after a careful study of several specimens of this tapeworm obtained from the bird Limnodromus griseus, concluded that this cestode was hermaphroditic and protandrous. According to Schell, the male reproductive system develops first, followed by a rapid transition to the female system. This transition was so rapid that very few strobilas could be found in a transitional state, showing both male and female reproductive organs; thus, most of the strobilas showed either male or female reproductive structures. If the worms are protandrously hermaphroditic how does copulation take place? If they are truly dioecious, then cross copulation between male and female strobilas must occur.

There are, then, three possible ways in which insemination may be effected: sperm transfer may occur between proglottids of different strobilas, or between proglottids of the same strobila, or thirdly, between the male and female tracts of the same proglottid. It

is unlikely that spermatozoa are ejaculated into the intestinal environment and then sucked in, probably along with some contents of the gut, into the vagina. The transfer of spermatozoa between proglottids of the same strobila is no different, genetically speaking, from that of insemination within the same proglottid.

2. Copulation and Sperm Transfer in H. diminuta

Jones et al (1963) have this to say about copulation in relation to the genetics of H. diminuta. "The strobila may be compared only loosely to a Mendelian population, for mating is not in any sense random, being restricted to the insemination of mature proglottids by the male systems of protandrously precocious younger individuals of the same chain." This seems to imply that the copulatory organ of the anteriorly located proglottid is inserted into the vagina of a more posteriorly situated proglottid; or else the worm folds on itself so that such proglottids could be in apposition for insemination.

According to Wilson (1965) interstrobilar copulation (meaning copulation between proglottids of different strobilas) has been observed. She thus writes,

> Observations of Hymenolepis diminuta (Douglas and Schiller, unpublished data) indicated that intrastrobilar copulation occurs in vitro. When two mature worms are placed together, there is considerable activity with regard to voluntary strobilar alignment of the sexually mature proglottids, in each of the two worms, until these portions of the strobila have moved into juxtaposition for copulation. During copulation, the cirrus is protruded from the cirrus pouch of one individual and inserted into the genital atrium of a corresponding proglottid in the other worm.

There are, then, two mechanical possibilities: either the intromittent organ can be thrust out through the atriopore, or the products of the male system are poured out of the copulatory organ into the atrium of the same proglottid. If insemination between strobilas occurs, or indeed, if one protandrous proglottid is to fertilise another proglottid further down the same strobila, then the first possibility must obtain. The burden of the evidence presented in this section of the chapter renders the second possibility more likely.

a. Anatomical considerations

We have already examined the arrangement of the ducts which are important in copulation (see Chapters IV, VI, and Fig. 46). The products of the male system arrive in the ducts of the cirrus sac which we can equate with the copulatory organ. The cirrus sac opens into the genital atrium as does the vagina of the female

system (see Fig. 46). A brief description of the genital atrium follows.

The genital atrium is a small muscular chamber which communicates with the exterior through a small atriopore situated ventro-laterally in the posterior part of the proglottid. Several longitudinal muscle fibres radiate outwards from the atrium, in the region of the atriopore, and run parallel to the proglottid margin, but do not extend very far inwards (see Figs. 55 and 56). The activity of these muscles presumably determines the opening and closing of the atriopore. Circular muscles, forming a muscular jacket, invest the whole atrium which has an inner cuticularised epithelium. There is a cluster of cells, associated with the atrium, which extends inwards as far as the muscular duct. Each of these cells, here termed the atrial gland cells, has a swollen basal region containing a nucleus and a distal duct-like process which extends towards the atrium (see Figs. 56 and 57). It was not determined whether these cells elaborated a specific secretion. The genital atrium becomes narrow at its inner end, into which end open the vagina and ejaculatory duct. One could also consider, for descriptive purposes, the atrium to be a chamber which constricts at its inner end to form two separate ducts--the dorsal ejaculatory duct and the

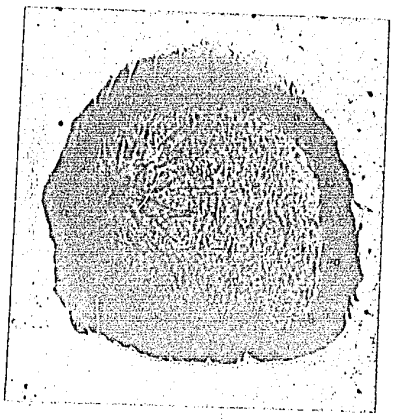


Fig. 55.--Longitudinal section of a proglottid passing through the atriopore; m, longitudinal muscle; a, atriopore.
(Bouin's, paraldehyde fuchsin x 330)

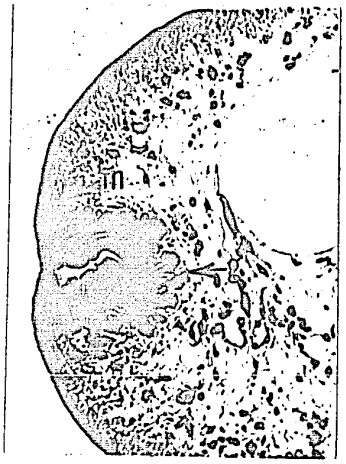


Fig. 56.--Transverse section passing through the atrium. Note muscles (m) and atrial gland cells (g). (Bouin's, paraldehyde fuchsin, x 300)

Fig. 57.--Same as Fig. 56, seen under higher magnification x 1000.

ventral vagina (see sequence of Figs. 58, 59 and 60). It is important to realise that the genital atrium, while it also communicates with the exterior of the worm, forms a chamber into which open the copulatory organ of the male and the vagina of the female.

The examination of the histological arrangements, described above and in previous chapters, indicates that an eversible intromittent organ, responsible for sperm transfer, is unlikely to be formed in the proglottids of this tapeworm. It could be argued that the ejaculatory duct may be evaginated to form an intromittent organ. A situation of this nature obtains in the tapeworm Acanthobothrium coronatum, where a large evaginated cirrus is observed. The invaginated cirrus, lying within the cirrus sac, consists of a wide terminal region and an elaborately coiled, thin, long inner region which is continuous with the coiled vas deferens, also lying within the cirrus sac (see Rees and Williams, 1965, page 640, Fig. 28). The invaginated cirrus encloses a cavity lined with spines. The long tubular cirrus evaginates to form the proboscis-like copulatory organ on whose outer surface now lie the spines, and the vas deferens within the cirrus sac is almost a straight tube now (see Rees and Williams, 1965, page 640, Fig. 27). It is clear that the formation of this everted copulatory organ is

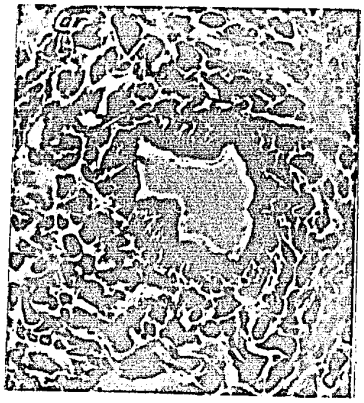


Fig. 58

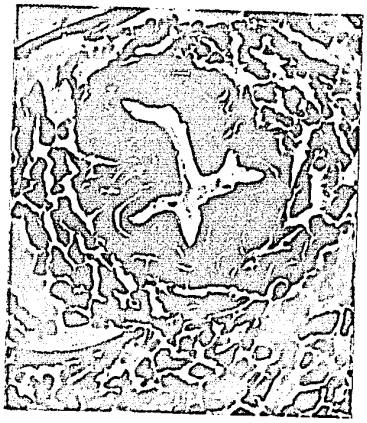


Fig. 59

Fig. 60

Fig. 58.--Longitudinal section passing through the atrium. Note glandular zone around atrium.

Fig. 59.--Longtidunal section passing through inner end of atrium. Note the constriction forming two regions.

Fig. 60.--Longitudinal section passing further inwards showing the dorsal ejaculatory duct (e) and ventral vagina (v).

(All 3 figures: Osmium-ethyl gallate, phase contrast, x 1000)

made possible by the presence, within the cirrus sac, of the long coiled vas deferens as well as the very long and specialized terminal part of the male reproductive tract. Such a situation does not obtain in H. diminuta (see Fig. 46).

Copulation between different strobilas may be effected in the following manner. If the lateral margin of the proglottid was pulled in and the genital atrium simultaneously flattened, the tip of the ejaculatory duct may come to lie near the atriopore. The atriopore of this proglottid would then have to be closely apposed to that of the recipient proglottid so that spermatozoa could be ejaculated from the male tract of the "donor proglottid" into the atrium of the other. Such a method does not seem to be very effective, and it does not fit into a scheme which envisages the formation of an eversible intromittent organ. Fertilisation between proglottids appears to be a difficult process requiring considerable morphological specialization.

b. Observations on singly-established worms (i.e., worms individually raised in rats)

If crossing is essential, then it clearly follows that strobilas individually raised in rats will be sterile. This could be tested by raising a single worm in a "clean" rat (i.e., a rat not infected with any

other worms). Six female rats, each weighing about 150 g, were caged individually and kept under observation for a period of 3 weeks, during which daily examinations of their faeces were made to detect the presence of any helminth eggs. The faecal examinations indicated that all 6 rats were uninfected. At the end of the 3 weeks, each rat was infected with a single cysticercoid of H. diminuta. All 6 rats were sacrificed 3 weeks after infection, and on autopsy, 4 of the 6 rats were found to be infected, each with a single large specimen of H. diminuta.

Microscopic examination of the eggs obtained from the gravid proglottids of these worms, showed that they were no different from the eggs obtained from worms of a larger population; motile hexacanth larva, enclosed by the characteristic embryonic membranes, were visible. Sections of mature proglottids of all 4 worms showed spermatozoa in testes, seminal vesicles, and the seminal receptacle of the female tract. Histological examinations further showed that there was no difference in the structure and anatomical arrangement of the reproductive system in the proglottids of worms raised either alone or as members of a larger infective population; the only difference is one of size, the tapeworm raised alone being much larger than one from a larger population.

The gravid region of each of the singly-established worms was fed to one of 4 separate batches of flour beetles, and each batch of beetles was maintained in a separate Petri-dish. After 2 weeks the infected beetles were dissected for cysticercoids and 4 groups of cysticercoids were thus obtained. These cysticercoids were then fed to 8 rats, 2 rats for each batch of cysticercoids. All 8 rats were sacrificed 3 weeks after infection, and autopsy revealed that all 8 rats were infected with tapeworms; gravid proglottids of these tapeworms were in turn used to infect further populations of flour beetles.

Thus, insemination and subsequent egg development occurred although intrastrobilar copulation and fertilisation were eliminated. Intrastrobilar copulation may occur, but it is not essential. There remain, therefore, only two possibilities: either insemination occurs within the same proglottid or spermatozoa from an anterior proglottid are transferred to a more posterior proglottid on the same strobila. The suggestions of various authors (Jones *et al*, Douglas and Schiller--see above) that there is an eversible intromittent organ in *H. diminuta*, which is thrust through the atriopore of one proglottid into the atrium of another proglottid, is rendered unlikely by an examination of the histological

arrangements described previously. Consider again the suggestion that sperm transfer takes place between different proglottids of the same strobila: it is difficult to envisage what selection forces acted in evolution to bring about such a mode of fertilisation. There is, after all, no genetic difference between the case where one proglottid fertilises another on the same strobila, and the case where insemination occurs on the same proglottid: both are instances of self-fertilisation.

It appears then, that the most likely mode of insemination in H. diminuta is by autocopulation, i.e., the transfer of spermatozoa from the male tract to the female tract of the same proglottid.

c. Other histological observations

Further support for the contention that insemination within the same proglottid is probably the rule, comes from an examination of the seminal receptacle during the reproductive process. At first the receptacle is a slender long tube with a lumen devoid of spermatozoa. It gradually enlarges with the arrival of spermatozoa, until it becomes a voluminous sac-like organ folded on itself (see Figs. 61, 62 and 63). This pattern of development is the same in tapeworms raised singly or as members of a larger population. Thus, the transfer

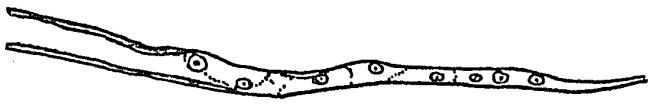


Fig. 61

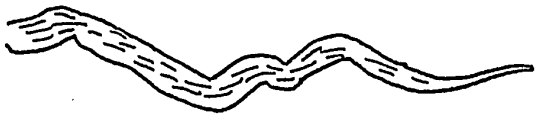


Fig. 62

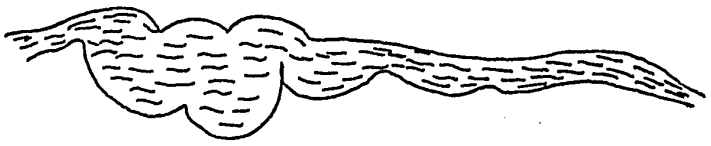


Fig. 63

Figs. 61, 62 and 63.--Diagrammatic view of the seminal receptacle in different stages of development. Note the lumen in Figs. 62 and 63 containing spermatozoa.

of spermatozoa into the female tract is clearly a gradual process rather than an abrupt one. It therefore follows that if insemination from one proglottid to another is the rule, then penetration of the atriopore of one proglottid by the intromittent organ of another must be a prolonged or regularly occurring process. Such a phenomenon has not been observed during the course of this investigation, and it is further incompatible with the histological observations of the anatomy of the reproductive system reported in earlier pages of this dissertation.

In worms, raised either singly or as members of a larger population, the mature proglottids are arranged one behind the other, each proglottid showing a swollen seminal receptacle packed with spermatozoa. This is a very characteristic feature of the strobilas of H. diminuta, and this serial, unbroken sequence of sperm-packed seminal receptacles is most likely to be the result of autocopulation, rather than that of an inefficient or haphazard mode of sperm transfer, such as the strobila folding on itself for the apposition of the atriopores of two proglottids to effect sperm transfer.

d. A hypothetical scheme for sperm transfer in H. diminuta

This process is envisaged to be as follows: spermatozoa are forcefully expelled from the internal seminal vesicle into the atrium of the same proglottid by co-ordinated muscular contractions of the cirrus sac and muscular duct. The force exerted by the muscular contractions of the atrium and vagina, combined with the ciliary activity of the vaginal epithelium, would expel the spermatozoa from the atrium, through the vagina into the seminal receptacle. Although spermatozoa are readily observed in the internal seminal vesicle and seminal receptacle, they are rarely, if ever, seen in the intervening ejaculatory duct, genital atrium and vagina, which indicates that the process of sperm transfer may be very rapid. One can only speculate about the role of the atrial gland cells. It is possible that they elaborate a secretion which initiates contractions of the atrium and proximal vagina, or else the cells may produce enzymes or some metabolites essential for the spermatozoa. The presence of the atriopore is difficult to account for in this scheme of events. If it were always to remain open, then there may be some loss of spermatozoa during copulation. On the other hand, it may remain closed during the passage of the spermatozoa from the male to the female tract through the atrium,

and could be opened by contraction of the longitudinal muscles, to release excess amounts of spermatozoa which may interfere with the pumping action of the atrium. This function of the atriopore is admittedly a purely speculative idea with no evidence at present to support it.

3. Some Consequences of Copulation in Tapeworms

If tapeworms were all capable of self-fertilisation, as has been shown in H. diminuta, they would then be at an advantage, in that the need, for 2 strobilas to be present in the same host to ensure copulation and eventual egg-production, can be eliminated. One tapeworm larva could infect the definitive host, mature and produce numerous offspring. The reproductive potential is further enhanced by strobilisation, so that there are many reproductive units, each capable of producing a large number of eggs.

The reproductive units or proglottids in a strobila must be genetically uniform or identical, since they are produced by a process akin to fission or vegetative budding. Thus sperm transfer between two proglottids on the same strobila would still be a form of selfing. Even if cross-fertilisation between two different strobilas

occurs, there is the possibility that the "mating" strobilas would be genetically very similar, if not identical. Such a condition is almost certain to exist in a population of adult hydatid tapeworms (E. granulosus). Consider a population of these worms within the intestine of the dog: it is very likely that these worms developed from the protoscolices of the same hydatid cyst, which in turn developed from a single egg. Thus, most, if not all, the individuals of the population would be genetically alike, excluding of course, the rare possibility of mutants within this population. It appears, then, that whether copulation between different strobilas or auto-copulation occurs, genetically speaking, we would be witnessing a form of selfing in this population of tapeworms.

Tapeworms like E. granulosus and H. diminuta could then be considered to be self-fertilising hermaphrodites, and if mutations were to occur, they would occur in both eggs and sperms, and homozygous offspring would eventually be produced carrying the recessive mutant genes. Factors like the physical impossibility for gene flow between strobilas in different hosts, polyembryony as shown by the larval hydatid organism, and the habit of selfing in the adult worms will enable the mutations to become homozygous and thus be expressed. Such a form of sexual

reproduction would then promote the rapid formation of new well-adapted races or strains (see Smyth and Smyth, 1964).

Selfing then would confer on tapeworms the ability to produce numerous offspring even when a single adult individual is present in the host animal; and it further enables the organism, already endowed with a high reproductive potential, to exploit favourable niches by promoting the formation of new, stable, highly-adapted races or strains.

CHAPTER VIII

THE FORMATION OF THE EGG--PART I

The previous chapter showed how the spermatozoa arrive in the seminal receptacle of the female tract. They remain here until they move into the ootype where fertilisation occurs, and where the constituent parts of the egg, that is, the oocyte, vitelline cell, and secretion of Mehlis' gland, are assembled. The formation of the egg in H. diminuta proceeds in two stages: the first stage, occurring in the ootype, is comparatively brief and constitutes the assembly of the component parts of the egg; the second stage, occurring in the uterus, is of longer duration, during which the development of the egg is completed.

The structure of that part of the female system which is responsible for the elaboration of the egg, was described in Chapter VI. A histochemical study of the ovary, vitelline gland and Mehlis' gland was informative in studying the physiology of egg formation. These observations are tabulated and discussed in the first part of the chapter.

1. Histochemical Observations

a. The ovary

The immature oocytes have a large nucleus and a homogeneous cytoplasm. The mature oocyte (i.e., the oocyte when ready to be ejected into the oviduct) is oval in shape with a nucleus, containing a prominent nucleolus, and conspicuous cytoplasmic granules (see Fig. 68). The histochemical observations on the oocyte are summarised in Table 4.

These observations show that the oocytes, while lacking both lipid and carbohydrate, contain appreciable amounts of protein and ribonucleic acids. Some of the protein contains sulphur as sulphydryl groups which is indicated by the positive result with the DDD reagent (see Fig. 64, and Appendix under tests for proteins). This is significant when considering the subsequent development of the fertilised ovum. Ribonucleic acid (RNA) is present in the nucleolus and in numerous small cytoplasmic granules. This is a characteristic feature of the oocytes of many species (Davenport and Davenport, 1965a, 1965b; Davenport, 1967). Some of the protein may be associated with RNA in the form of ribonucleoprotein complexes, which is reported to occur in the oocytes of many species (see same authors above). The nucleolus is

TABLE 4.—The histochemical observations on the oocytes

Test	Observation
PAS	-
Best's carmine	-
Alcian blue, pH 2.5	-
Toluidine blue	Blue colour; no metachromasia
Osmium-ethylgallate	No osmiophilia
Mercury bromphenol blue	Granules coloured red
Ninhydrin-Schiff	+
Millon's reagent	+
DDD	+
DDD after N-Ethyl maleimide	-
Performic acid-Alcian blue	-
Pyronin-Methyl green	Granules and nucleolus stained red
Pyronin-Methyl green after Ribonuclease	Staining of granules and nucleolus abolished

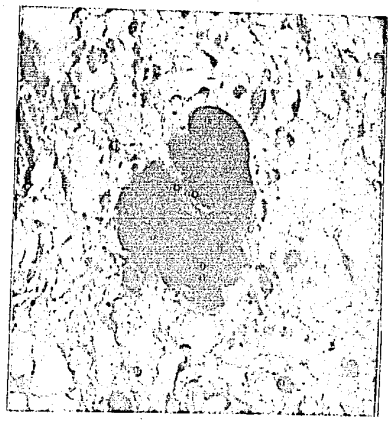


Fig. 64.--Section of a part of the ovary. Note intense positive staining of the oocytes.
(Formol-calcium, DDD, x 385)

Fig. 65.--Transverse section passing through the ovary and vitelline gland. Note positively staining granules of the vitelline cells (arrow), and the failure of the oocytes (o) to be stained.
(Bouin's, Performic acid-Alcian blue x 370)

known to contain RNA and protein in the form of ribonucleoprotein complexes, and the principal function of the nucleolus is to synthesise ribosomal RNA (Birnstiel, 1967). Most of the RNA in the cytoplasm is contained in ribonucleoprotein particles called ribosomes (Harris, 1965). Electron microscope studies of the ovary of F. hepatica (Bjorkman and Thorsell, 1964) show that the oocytes are characterised by their high content of free ribosomes in the cytoplasm; and some of these ribosomes are aggregated in clusters. It is likely that a similar situation obtains in the oocyte of H. diminuta.

b. The vitelline gland

The mature vitelline cells contain small spherical granules in the cytoplasm. The histochemical observations on the gland (see Table 5) show that, in addition to these prominent vitelline granules, there are small deposits of glycogen present in the cytoplasm. The vitelline granules contain lipids, proteins and acid mucopolysaccharides, but lack glycogen and other PAS positive materials. The granules stain intensely with the PFAB procedure (see Fig. 65) which indicates the presence of high concentrations of cystine and protein-bound disulphide groups (see Appendix under tests for proteins). The acid mucopolysaccharides present are not

TABLE 5.--Histochemical observations on the vitelline gland*

Test	Observation
PAS	-
Best's carmine	-
Alcian blue, pH 2.5	+
Alcian blue, pH 1.0	-
Toluidine blue	γ metachromasia
Millon's reagent	+
Mercury bromphenol blue	+
Ninhydrin-Schiff	-
DDD	-
Performic acid-Alcian blue (PFAB)	+
PFAB without oxidation	-
Osmium-ethyl gallate	granules strongly osmiophilic

* The tabulated results refer only to the vitelline granules. With the PAS procedure small granules of glycogen were detected in the cytoplasm. These granules contain only this substance, and are distinct from the vitelline granules, which are PAS negative.

sulphated, as indicated by the failure of the granules to be stained with Alcian blue at pH 1.0. The granules are strongly osmiophilic indicating their lipid content (see Fig. 66), and some of this lipid may be associated with protein to form a lipoprotein, which is suggested by the positive results obtained on staining with acidified protoporphyrin (see Appendix, under tests for lipids). These granules, containing these substances, would be excellent material for the formation of protective envelopes and similar structures.

It is of some interest to compare the histochemical observations on the vitelline cells of H. diminuta, which is a cyclophyllidean tapeworm, with the histochemical characteristics of the vitelline cells of a pseudophyllidean cestode and a trematode. Such a comparison is furnished in Table 6. In Fasciola and Schistocephalus, the egg which is passed to the exterior has a characteristic egg shell which is made of a resistant protein. The bulk of the material forming this shell comes from the vitelline cells which contain the shell-precursors in the form of basic proteins, phenols and phenol oxidases. The phenol is oxidised to form a quinone, which then combines with the protein to form a cross-linked, stable protein called sclerotin. The sclerotin forms the resistant egg shell enclosing

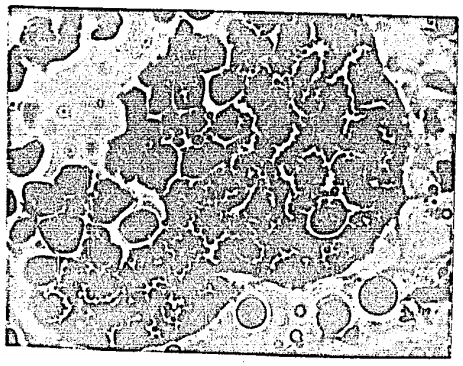


Fig. 66. Transverse section passing through the vitelline gland. Note the strongly osmiophilic vitelline granules.
(Osmium-ethyl gallate, phase contrast, x 760)

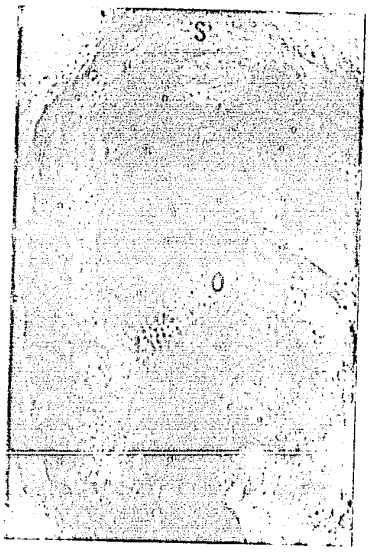


Fig. 67.--Transverse section passing through Mehlis' gland. Note the deeply staining gland cells (arrow); s, sperm-oviduct; o, ootype.
(Formol-calcium, AB-PAS, x 800)

TABLE 6.--Comparative histochemical observations on the vitelline gland of 3 selected platyhelminth species

Class	Name of organism	Components of vitelline cell	Reference
Trematoda (Digenea)	Fasciola hepatica	basic proteins phenols polyphenol oxidase	Smyth, 1954b
Cestoda (Pseudophyllidea)	Schistocephalus solidus	basic proteins phenols polyphenol oxidase	Smyth, 1956
Cestoda (Cyclophyllidea)	Hymenolepis diminuta	a. acid mucopolysaccharide lipid basic protein; some protein with protein-bound disulphide groups, and some protein as lipoprotein	observations made in the present investigation
		b. Phenols and polyphenol oxidase absent	Llewellyn, 1965

the ovum (Smyth and Clegg, 1959). The egg shell is resistant to pancreatic digestion (Llewellyn, 1965).

The chemistry of the vitelline cells of H. diminuta indicates that an entirely different situation obtains in the cyclophyllidean vitelline gland. The larva is not enclosed by a single sclerotised shell, but by several embryonic envelopes which are derived from the cleaving ovum itself. The vitelline cells contribute very little, if any material towards the formation of these envelopes. Another contrasting feature is that these envelopes are not resistant to pancreatic digestion (Llewellyn, 1965). The loss of the tanning agents in the vitelline cells, and the inability of the embryonic envelopes to resist pancreatic digestion, is believed to be correlated with the phase in the life cycle where the larva is released in the gut of the intermediate host; the digestive enzymes of the host are partly responsible for the hatching of the egg by disrupting the enclosing envelopes (Llewellyn, 1965).

c. Mehlis' gland

The histochemical observations on the cells of this gland are shown in Table 7 (also refer the Appendix under histochemical techniques for explanations of the tests). The results indicate that the granules elaborated

TABLE 7.--Histochemical observations on Mehlis' gland

Test	Observation
PAS	+
PAS after diastase	+
PAS after hot methanol:chloroform	+
Best's carmine	-
Alcian blue, pH 2.5	+
Alcian blue, pH 1.0	-
Toluidine blue	purple β metachromasia
Millon's reagent	faint positive
Mercury bromphenol blue	+
Ninhydrin-Schiff	+
DDD	-
Performic acid-Alcian blue	-
Osmium-ethyl gallate	granules strongly osmiophilic

by this gland contain carbohydrate, protein, lipid and some acidic substance, probably acid mucopolysaccharide. The positive PAS reaction, which is unaltered by diastase digestion and lipid extraction, coupled with the positive reactions for proteins, indicates that there is a carbohydrate-protein complex in the secretory granules. A strong osmiophilic reaction as well as a positive reaction with acidified protoporphyrin, indicate that a lipoprotein is also present. The secretion is then a carbohydrate-lipid-protein complex.

These results are in agreement with the observations reported by Clegg (1965) who showed that Mehlis' gland in F. hepatica elaborated secretory granules which contained carbohydrate, lipid, protein and some acidic group. Extraction of this secretion and further analysis showed that it was a lipoprotein with a high (60%) protein content, some phospholipid and a small amount of carbohydrate (Clegg, 1965). According to Clegg the acidic group, responsible for the positive reaction with Alcian blue, is probably the phosphate radical. The secretion in H. diminuta stains with Alcian blue only at pH 2.5, showing that the reaction is not due to the acidic sulphate group. The combined PAS-Alcian blue procedure of Mowry (1963) stained the granules pink-purple (see Fig. 67).

2. The Assembly of the Egg (see Figs. 50, 51, 52)

The spermatozoa are prevented from leaving the seminal receptacle by a muscular sphincter located at its inner end. There appears to be some mechanism which determines the timing of sphincter-relaxation, permitting the exit of spermatozoa. It was observed on many occasions that while the seminal receptacle was large and distended with abundant spermatozoa in its lumen, both the ovary and vitelline gland, of the same proglottid, were immature: most of the oocytes lacked the prominent cytoplasmic granules, and only a few vitelline cells contained vitelline granules (see Fig. 54 in Chapter 6). The sphincter prevents the exit of spermatozoa at this stage. But whenever the sphincter was observed to have relaxed, allowing the exit of spermatozoa into the sperm duct (see Fig. 68), both ovary and vitelline gland were in a functionally mature state. Spermatozoa, then, appear to be released only when the ovary and vitelline gland are ready to discharge their products into the ootype.

Spermatozoa are easily observed in the sperm duct but never in the sperm-oviduct or ootype; oocytes are occasionally observed in the oocapt, never in the oviduct and sperm-oviduct, and only rarely in the ootype;

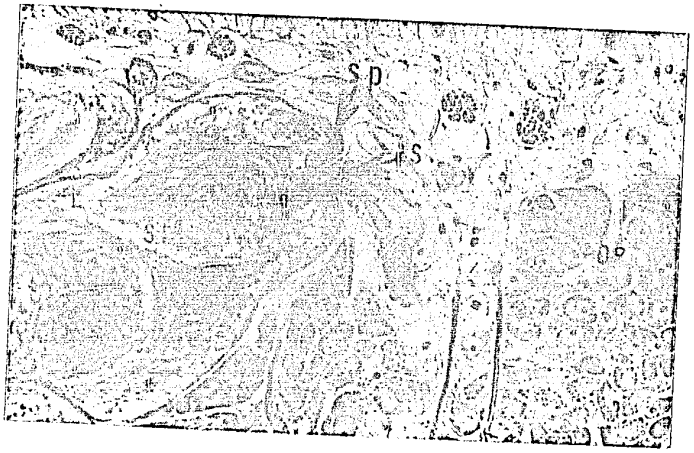


Fig. 68.—Transverse section passing through a part of the female system. Note (1) the swollen seminal receptacle (sr) containing spermatozoa; (2) the muscular sphincter (sp); (3) spermatozoa in the sperm duct (s); (4) the ovary (o) with the oocytes showing the characteristically prominent nucleus and cytoplasmic granules. (Bouin's, paraldehyde fuchsin with Halmi's, x 400)

on the other hand, as many as 3 or 4 vitelline cells can be observed within the vitelline duct. These observations indicate that the oocytes and spermatozoa are moved rapidly within the ducts whereas the vitelline cells are transported more slowly. The contractions of the muscular walls of the oviduct and sperm-oviduct, together with the sweeping motions of their ciliated epithelia, would move the ova and spermatozoa rapidly to the ootype. The vitelline duct is not ciliated and depends only on contractions of its muscular walls to transport the vitelline cells. To compensate for this, however, is the comparatively short length of the vitelline duct, so that the vitelline cells do not have to be moved very far to enter the ootype.

The events leading to the assembly of the egg in the ootype are envisaged to be as follows (see Figs. 50, 51 and 52). The sphincter of the seminal receptacle relaxes, releasing a stream of spermatozoa into the sperm duct; an oocyte, ejected from the ovary, is moved up the oviduct, and is then joined by the spermatozoa and together they are rapidly transported along the sperm-oviduct towards the ootype. At the entrance to the ovo-vitelline duct, the spermatozoa and oocyte are joined by a vitelline cell, and they all then enter the ootype. The flap-like "valves" of the uterine duct are

presumably apposed to prevent the premature exit of the egg-precursors into the uterus. It is possible that the ovo-vitelline duct closes temporarily so that the ootype is effectively isolated for a brief space of time. Meanwhile, the cells of Mehlis' gland discharge their secretion into the lumen of the ootype. The oocyte, which has by now been fertilised, and vitelline cell are surrounded by the secretion granules of Mehlis' gland. The valves of the uterine duct open, and the oocyte and vitelline cell, enclosed by the secretion of Mehlis' gland, are ejected, through the uterine duct, into the uterus, by muscular contractions of the wall of the ootype. The valves close again and the entrance to the ootype is opened to allow the next group of egg-precursors to enter.

The ootype is thus visualised as an efficient mould or assembly point where the component parts of the egg are assembled before the egg is exported to the uterus, where subsequent development takes place. It is probable that only one egg is assembled at a time, and the mechanism can be visualised as a continuous process, where the component parts of the egg are delivered, one batch at a time, to the ootype, wherein they are assembled and passed on to the uterus. This process of assembly must be very rapid since oocytes are rarely

observed in the ootype. Bjorkman and Thorsell (1963) have estimated that the rate of egg formation *in vitro* in *F. hepatica* is about 2 eggs per minute.

The serial replication of these assembly points, brought about by strobilisation, confers on the tapeworm a high egg-producing capacity, within a limited space of time. If only one such assembly unit (ootype) were present, then a much lesser number of eggs would be assembled within a given space of time. Increasing the quantity of oocytes and vitelline cells, by increasing the size or numbers of the ovary and vitelline gland, will not result in an increased egg production, within a given period of time (e.g., the time spent by the parasite in the definitive host). However, if there were an increase in the number of ootypes as well, then an increase in the number of egg-assembling units would result, and there would be a greater number of eggs elaborated within this period of time. This point has been emphasised by Llewellyn (1965) in his discussion of the survival value of strobilisation in the evolution of cestodes.

The formation of the egg is a complex process in which the functions of the different parts of the reproductive apparatus, participating in this process,

must be well synchronised; and this may well be mediated by nerve cells, as suggested to occur in F. hepatica by Gönnert (1962).

CHAPTER IX

THE FORMATION OF THE EGG--PART II

In this chapter we shall examine some features of interest in the development of the egg in the uterus.

1. The Oocyte and the Egg

The fertilised oocyte in the uterus stains intensely and does not show the characteristic nucleus and cytoplasmic granules observed earlier. It undergoes cleavage, forming an embryo composed of many cells, some of which separate to form a granular syncitial envelope enclosing the rest of the cells. These cells multiply and differentiate to form the larva with hooks and glands. The granular envelope gives rise to the 3 embryonic envelopes enclosing the larva, and these are the outer shell, a middle envelope and an inner embryophore adjacent to the larva. The embryonic envelopes and the larva together constitute the mature egg (see Fig. 69).

Histochemical tests for proteins (Mercury-bromphenol blue, Ninhydrin-Schiff) showed that protein

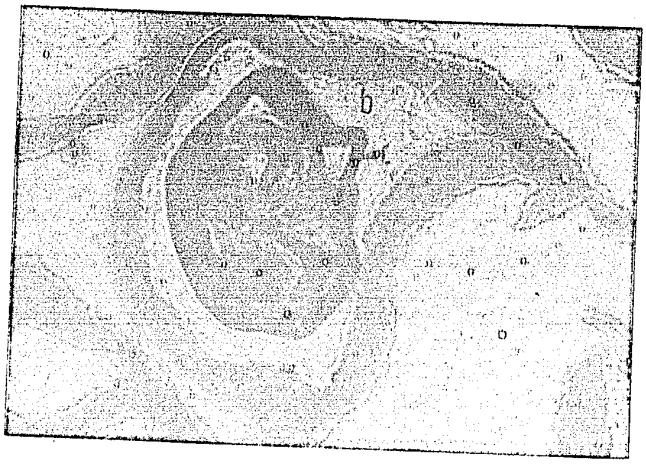


Fig. 69.--Section of a mature onchosphere (the egg). a, outer shell; b, middle envelope; c, embryophore; d, hexacanth larva. (Bouin's, paraldehyde fuchsin with Halmi's, x 1000)

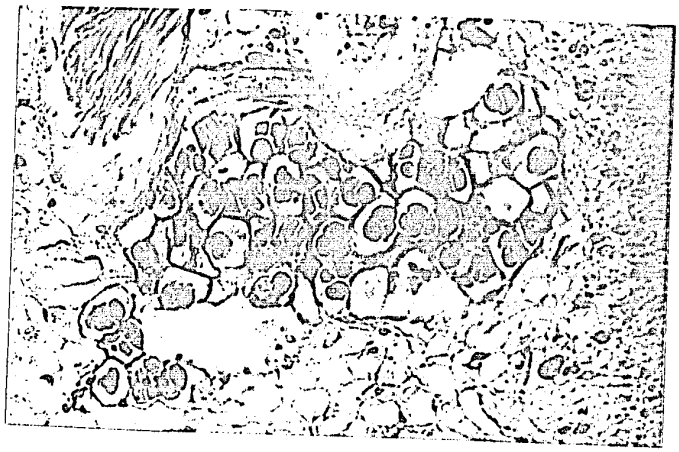


Fig. 70.--Transverse section showing a branch of the uterus containing developing embryos. Note the vitelline capsules (arrow) enclosing the embryos. (Bouin's, paraldehyde fuchsin, with Halmi's, x 480)

was present in envelopes and larva. Of greater interest were the tests for proteins containing sulphydryl and disulphide groups (DDD and PFAB--see Appendix). The envelopes and larva did not stain with the DDD reagent, indicating the absence of sulphydryl groups. On the other hand, the embryophore and hooks stained intensely with the Performic acid-Alcian blue test, which indicates the presence of cystine and protein disulphide groups. The oocyte, on the contrary, lacks protein-bound disulphide groups, although it has appreciable amounts of protein sulphydryl groups (see Chapter VIII). It is evident then, that during embryogenesis, some of the protein of the oocyte, containing sulphydryl groups, is involved in the formation of the embryophore and hooks, during which the sulphydryl groups are oxidised to form stable disulphide bonds. The embryophore and hooks are rigid structures and the presence of protein-bound disulphide groups in these structures is not unusual.

2. The Vitelline Cells

The vitelline granules are freed from the vitelline cells and coalesce to form a capsule which encloses the developing oocyte. The formation of this capsule probably begins in the ootype and it is completed in the uterus. Both capsules and vitelline

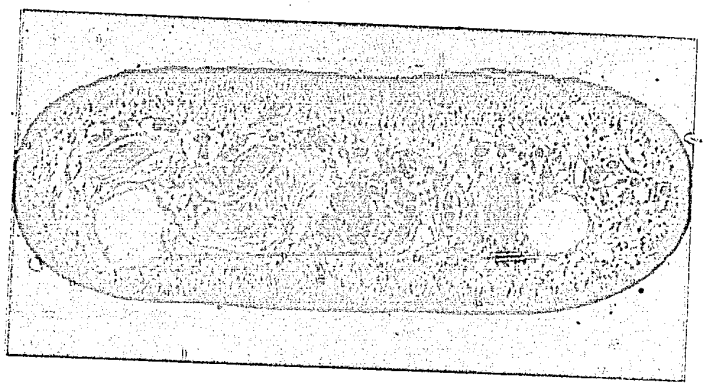


Fig. 71.--Transverse section of a proglottid. Note slender uterus, located dorsally (arrow), containing eggs.
(Bouin's, paraldehyde fuchsin with Halmi's, x 120)

granules show identical staining reactions with PFAB, Millon's reagent, Alcian blue and Mercury bromphenol blue. They are also both strongly osmiophilic.

The capsule is easily observed during the cleavage of the ovum and the early stages of embryogenesis (see Fig. 70). It remains intact after the differentiating embryo has formed the outer syncitial granular envelope, but it is no longer detectable after the true outer shell of the egg has been formed. Its function during early embryogenesis is clearly protective; the presence of protein-disulphide groups in its chemical constitution indicates its structural stability.

The fate of the capsule in the later stages of embryogenesis is uncertain. The capsule may disintegrate; but fragments, which would be produced by disintegrating capsules, were never observed. It is possible that the capsule does remain in the mature egg, as a very thin membrane, closely adhering to the outer shell. Such a membrane would not be easily visible in the kind of histological preparations used in this study. Another possibility is that the capsule is incorporated into the composition of the outer shell.

3. Mehlis' Gland and Egg Formation

The secretory granules elaborated by this gland are selectively stained with the AB-PAS procedure of Mowry (1963) (see Appendix), and the granules are observed in the lumen of the ootype. Using the AB-PAS procedure, Clegg (1965) showed that the granules from Mehlis' gland formed 2 membranes, one of which acted as a template on which the egg shell, derived from the vitelline cells, was formed. The membranes showed up very clearly against the egg shell, with the AB-PAS procedure. In H. diminuta, however, the AB-PAS sequence failed to demonstrate such a membrane, which, if it exists, would presumably form a template against which the vitelline capsule would form. With the AB-PAS sequence the vitelline capsule stained green because of its affinity for Alcian blue.

Thin sections of material, prepared according to the osmium-ethyl gallate procedure, failed to demonstrate such membranes. The failure to observe membranes using this procedure may be explained by the fact that both the vitelline granules, forming the capsule, and the secretory granules of Mehlis' gland, presumed to form membranes, are strongly osmiophilic, and it was not possible, therefore, to differentiate separate membrane and capsule.

It is likely that the membrane, formed from these granules, is very thin and only more sensitive methods, like electron microscopy, would detect it. One should also consider the possibility that the membrane, if it is formed, becomes incorporated into the vitelline capsule, thereby making its detection very difficult.

Several other theories have been advanced to explain the significance of the secretion of Mehlis' gland (see Smyth and Clegg, 1959; Löser, 1965b; Ebrahimzadeh, 1966, for details and references). Thus it is suggested that the secretion acts as a lubricant, as a gamone which activates the spermatozoa, as a substance which liberates the vitelline material from the vitelline cells, and as a secretion in which eggs are suspended. These suggestions are not proven as yet. The present investigation shows that the secretion of Mehlis' gland in H. diminuta is a complex of several substances, and is histochemically similar to the secretion of Mehlis' gland in F. hepatica; the secretion is discharged into the ootype where it associates with the oocyte and vitelline cell. Further investigations are necessary to clarify the role of this secretion.

4. The Uterus

The development of the uterus has been described in Chapter III, where it was seen that it expands rapidly, during egg formation from a slender tube, lying in the dorsal part of the proglottid, to a large branched, tubular structure which fills the entire proglottid. The uterine epithelium which forms the wall of the uterus is inconspicuous at first, but later becomes prominent; the nuclei of the epithelial cells are larger and the cytoplasm has many proteinaceous granules in it, as demonstrated with mercury bromphenol blue. By the time the uterus reaches its maximum extent in the gravid proglottid, the epithelium is thinner and less conspicuous, and in some areas it appears to have disintegrated. At this stage it fills the proglottid (see Fig. 71; also Figs. 10, 11, 12, 13 in Chapter III). The uterine epithelium may contribute some materials for the formation of the eggs; such a process has been suggested to take place in some trematodes (see Ebrahimzadeh, 1966).

The process of egg formation is a neat, two-stage process which allows the participating structures to function at their maximum capacities. During the first stage, called egg assembly (see Chapter VIII) which is

confined mainly to days 8, 9 and 10 (see Chapter III), the ovary, Mehlis' gland, vitelline gland and seminal receptacle "export" their products to the ootype, where the eggs are assembled and then ejected into the uterus, within which they undergo cleavage. By the end of this period the ovary, vitelline gland and Mehlis' gland are spent. In the second stage, occurring in days 11-16 (see Chapter III) the uterus expands to fill the proglottid and the embryos within it complete their development to form the characteristic eggs.

THE SCOLEX

CHAPTER X

THE STRUCTURE OF THE SCOLEX

The remainder of this dissertation will be devoted to the study of the scolex and its significance in the biology of the parasite. In this chapter we shall examine the structure of the scolex, of both the cysticercoid and the worm in the definitive host.

1. The Cysticercoid

The cysticercoid has a scolex lying within a cavity enclosed by several membranes (see Fig. 72). The scolex has 4 suckers and a rostellum within which are many cells with prominent nuclei but hardly any cytoplasm. Scattered nuclei are observed outside the rostellum, and no other structures were observed in the present investigation. The worm in the definitive host, on the other hand, has a scolex with a more complex organisation. The cysticercoid excysts within the gut of the definitive host during which process the scolex emerges from the central cavity, and soon fastens itself to the wall of the intestine (see Fig. 73). The scolex increases in size and its internal organisation is much clearer now.

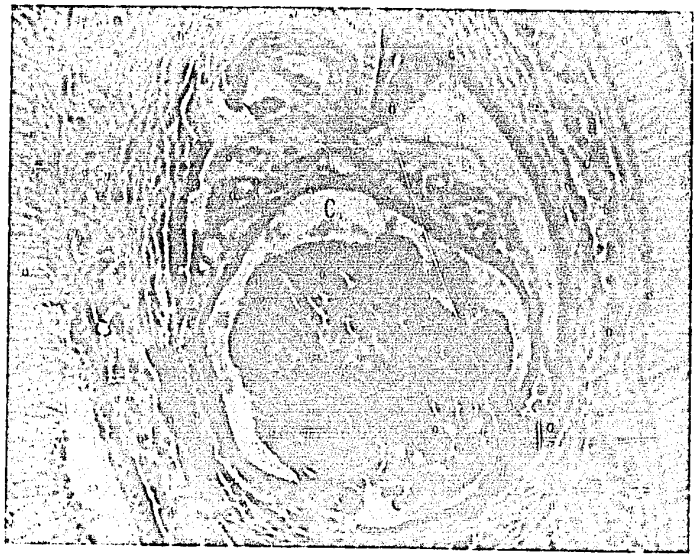


Fig. 72.--Horizontal section of a cysticercoid. Note the envelopes enclosing the central cavity (c); the arrow indicates the rostellum of the larval scolex. (Bouin's, paraldehyde fuchsin with Halmi's, x 560)

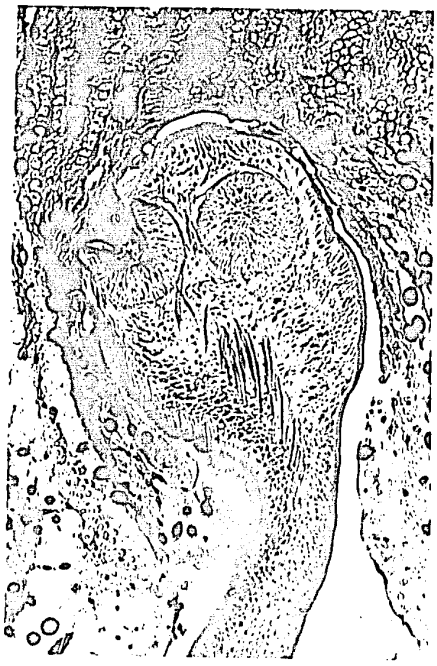


Fig. 73.--Horizontal section of the scolex of the adult worm <u>in situ</u> in the intestine of the rat. Note the sucker gripping the epithelial lining of the villus. (Bouin's, Azan, x 200)

2. The Worm in the Definitive Host

The scolex has 4 prominent suckers, and a well-developed rostellum, in addition to which are observed muscle fibres, excretory ducts, nerve tracts and a brain. We shall now examine all of these elements in greater detail.

a. The suckers and the attachment to the host's intestine

The scolex, lying at the anterior end of the strobila, is almost rectangular in cross section and can be visualised as being made up of a right and a left half, with each half having a dorsal and ventral sucker (see Fig. 74). Each muscular sucker encloses a deep cavity, and at their widest point, the inner margins of the suckers, in each half of the scolex, lie very close to each other with very little parenchymatous tissues in between (see Fig. 74).

The worms are located in the small intestine, and sections of worms, fixed *in situ*, show that the scolices of the worms are lodged among the villi. Most of the scolices are located deep within the space between two villi, and are attached to the lining of the villi, with the suckers enclosing "plugs" of the mucosal tissue containing goblet cells (see Fig. 73). While inflammatory

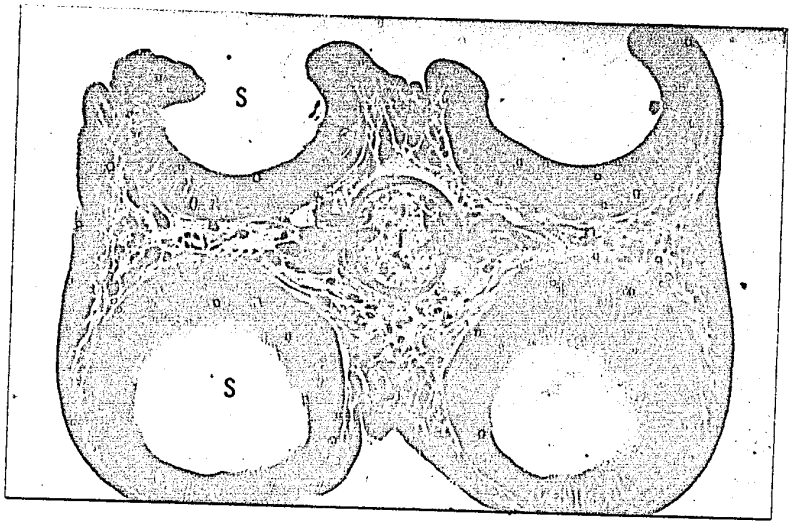


Fig. 74.--Transverse section of the scolex of the adult worm. s, sucker; r, rostellum; t, excretory tubule within the rostellum; n, nerves 4 and 5 of the 2nd pair.
(Susa, Trioxyhaematin, x 450)

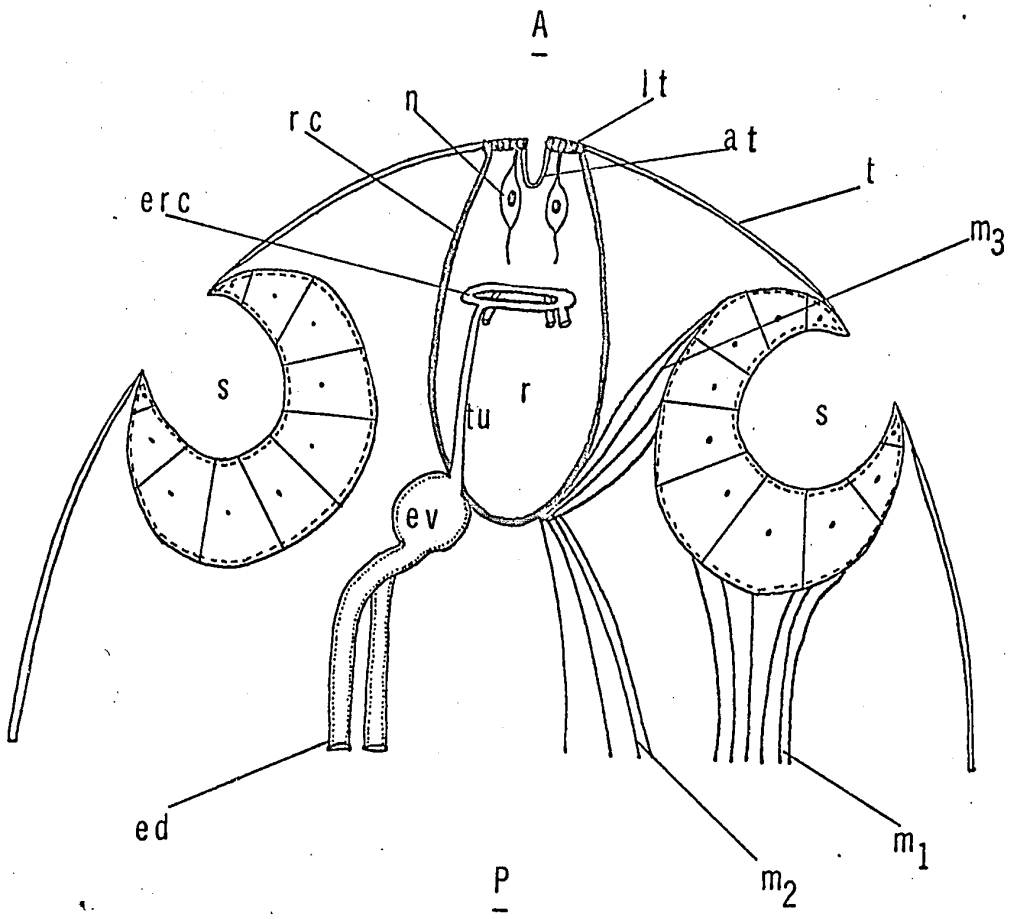
tissue reactions are not obvious, the destruction of goblet cells probably occurs. Thus a few goblet cells adjacent to the anterior end of the scolex appear to be ruptured so that their contents become spread over the surface of the scolex; the mucus is clearly visible as a very thin but distinct layer on the tegument.

b. The rostellum

The rostellum lies between the suckers in the anterior part of the scolex, and extends beyond the anterior margin of the suckers. It is ovoid, with a flattened anterior end, and circular in cross section (see Figs. 74 and 75). The rostellum is enclosed by a prominent rostellar capsule on all sides except at the anterior end, where the capsule is lacking, and the rostellar area is in direct contact with the tegument of the scolex (see Fig. 75). There is a pocket-like invagination of the tegument into this region of the rostellum. Within the rostellum are found large conspicuous cells, excretory ducts, nerve tracts and smaller cells and vacuoles.

c. Musculature

While the detailed musculature of the scolex was not investigated, the disposition of the more prominent muscles was noted. Thick strands of longitudinal muscle



A	- anterior	ed	- excretory duct
P	- posterior	ev	- excretory vesicle
lt	- lateral tegument	tu	- excretory tubule within the rostellum
at	- apical tegument invaginated into	erc	- excretory ring canal
r	- rostellum	m_1	- longitudinal muscles
t	- tegument of the rest of the scolex	m_2	- posterior diagonal muscles
n	- nerve cell	m_3	- anterior diagonal muscles
rc	- rostellar capsule	s	- sucker

Fig. 75.--Diagrammatic sectional view (horizontal) of scolex.

fibres run posteriorly towards the neck region from the base of each sucker. Close to the anterior end of the scolex, and anterior to the suckers, are 4 diagonally arranged bands of muscle fibres, which extend from the two lateral faces of the rostellar capsule towards the corners of the scolex. Four bands of diagonal muscle fibres are attached to the base of the rostellum, of which 2 are attached to the dorso-lateral sides of the rostellum and the other 2 to the ventro-lateral sides. These fibres run anteriorly towards the suckers, and are here called the anterior diagonal muscles of the rostellum (see Fig. 75). Similarly a second set of 4 bands of diagonal muscles extends posteriorly towards the neck region, and here they are called the posterior diagonal muscles of the rostellum. The activity of these muscles presumably gives mobility to the scolex, suckers and rostellum.

d. The excretory ducts

Two groups of longitudinal excretory ducts, or vessels, extend the length of the strobila from the posterior-most proglottid to the scolex. Each group is situated laterally in the medullary parenchyma, and consists of a small dorsal vessel and a larger ventral vessel. These vessels are convoluted in the neck region

before entering the scolex. The two groups of excretory ducts run towards the base of the rostellum and the vessels of each group then unite to form two large vesicles, one on each side of the rostellum, with their inner walls touching the posterio-lateral surfaces of the rostellar capsule (see Fig. 75). Two slender tubules leave each vesicle and enter the rostellum through openings in the rostellar capsule. Four such tubules thus enter the rostellum, and appear as small circular structures in cross section; two of these tubules are situated in the dorsal part of the rostellum and the other two in the ventral part (see Fig. 74). They run anteriorly and then all four unite to form a circular or ring canal which lies horizontally in the rostellum (See Fig. 75).

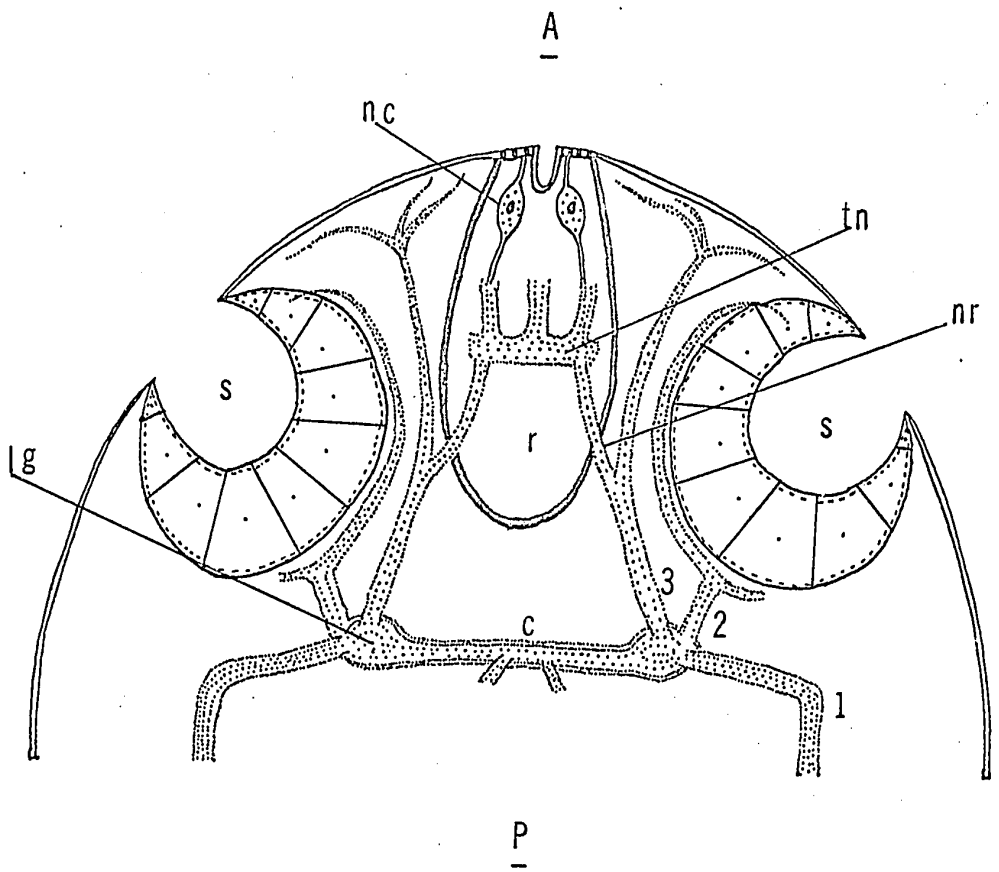
e. The nervous system

The description of the nervous system is based on the examination of serial sections; the nerve tracts and nerve cells were most readily observed in material fixed in Susa and stained with Hansen's trioxyhaematin. Only the major nerve tracts are considered here: the fine details of the nervous system are inconspicuous.

The nervous system in the scolex consists of a brain composed of two lateral ganglia joined by a transverse commissure, several nerves given off by the brain,

and conspicuous nerve tracts within the rostellum. The lateral ganglia of the brain lie posterior to the suckers with each ganglion lying between the dorsal and ventral sucker of its half of the proglottid. A few nerve cell bodies are observed in the ganglia. The ganglia are joined by a thick transverse commissure which lies immediately posterior to the rostellum (see Fig. 76). The commissure contains prominent nerve cell bodies (see Fig. 77). Four nerves are given off from the commissure; two arise from the dorsal aspect of the commissure and extend dorsally between the 2 suckers, and the other 2 nerves arise on the ventral surface and extend ventrad between the 2 ventral suckers. After a short distance, these nerves turn anteriorly and continue forwards between the suckers.

The 2 lateral ganglia <u>each</u> give off 5 nerves; one unpaired nerve and 2 paired nerves. The unpaired nerve arises laterally from the ganglion, runs towards the lateral margin of the scolex, turns posteriorly and continues in the neck and the remainder of the strobila as the main, laterally situated, longitudinal nerve cord (see Fig. 76). The 2 nerves of the first pair (nerves 2 and 3) arise on the dorsal and ventral aspects of the lateral ganglion respectively. Each nerve runs towards the dorsal and ventral sucker respectively, turns inwards,



A - anterior
P - posterior
lg - lateral ganglion of the brain
c - transverse commissure of the brain
nr - nerve to the rostellum
r - rostellum
1 - main lateral nerve
2 - a nerve of the first pair
3 - a nerve of the second pair
tn - transverse median nerve within the rostellum
nc - neurosecretory cell
s - sucker

Fig. 76.--Diagrammatic sectional view (horizontal) of the scolex.

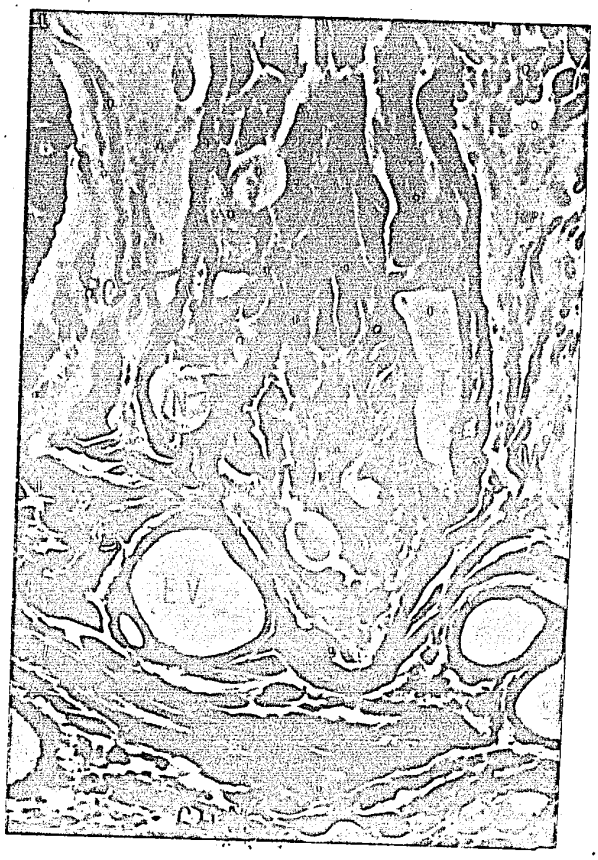


Fig. 77.--Horizontal section of the scolex of the adult worm. RC, rostellar capsule; EV, excretory vesicle; T, excretory tubule within the rostellum; N, nerve tract within the rostellum; the arrow above the excretory vesicle indicates the nerve tract entering the rostellum; M, anterior diagonal muscles; COM, the transverse commissure of the brain; note the nerve cell body.
(Osmium-ethyl gallate, phase contrast, x 1,240)

and continues along the inside base of the sucker for a short distance, and then turning sharply, pursues an anterior course (see Fig. 76, nerve 2). The nerves eventually break up into smaller branches at the anterior end. The nerves of the second pair (nerves 4 and 5) arise from the inner dorsal aspect of the lateral ganglion, and pass inwards and anteriorly towards the rostellum (see Fig. 76, nerve 4; also Fig. 74). The 2 nerves lie lateral to the large excretory reservoir and run anteriorly to come close to the rostellar capsule. A short lateral branch arises from each nerve and passes into the rostellum at a point just anterior to the entrance of the slender excretory tubules into the rostellum (see Figs. 76 and 77). Having given off these branches, the nerves continue their anterior course between the suckers and rostellum, with one nerve lying adjacent to the dorsal sucker and the other adjacent to the ventral sucker. The nerves eventually divide into many fine branches at the anterior end. (This pattern of the distribution of the nerves was described for one half of the scolex, and is identical to that which obtains in the other half.)

Nerve fibres are observed <u>within the rostellum</u>. There are many strands of nerve fibres extending posteriorly towards a prominent transverse nerve tract,

which is median and lies posterior to the circular excretory canal observed within the rostellum. From each end of the transverse nerve tract a thick nerve runs posteriorly and makes its exit from the rostellum by joining with the two short nerve branches which enter the capsule laterally (see Figs. 76 and 77).

f. The large cells in the rostellum

Large conspicuous nerve cells were always observed in the anterior part of the rostellum in sections of the scolex. It was not possible to make an accurate count of the total number of these cells, but an approximate estimate is that there are at least 12 to 14 cells. The cells are typically bipolar, each with a distal filament-like process terminating at the tegument, and a proximal process which extends towards, and makes eventual contact with, the median transverse nerve tract in the rostellum (see Fig. 76). The middle swollen part of the cell contains a prominent nucleus. All of the cells do not occur at the same level, but are evenly distributed throughout the anterior of the rostellum, and packed close to each other. In horizontal sections of the scolex, they give the appearance of being arranged in 2 clusters.

The morphology of the cells, and their disposition in the rostellum in relation to the tegument and nerves,

therefore suggests that they are sensory or neurosensory cells.

g. The tegument at the rostellar surface and the sensory cells

The tegument at the rostellar surface has 2 regions of which one, the apical tegument, is invaginated and lies within the rostellum, while the other, the lateral tegument, covers the rostellum anteriorly. The neurosensory cells come into contact with the latter part (see Fig. 76). The lateral tegument is thick and has a characteristic striated appearance which is best observed in longitudinal sections of the scolex; the striations are due to alternate light and dark staining regions. In thin sections of osmium-fixed material vesicular patches are observed in this otherwise homogeneous, densely osmiophilic tegument. This feature was not observed in the tegument of adjacent regions (see Fig. 78). It is likely that this lateral tegument is a specialized zone containing sensory endings which are associated with the neurosensory cells. Sensory structures associated with nerves are known to occur in the tegument of trematodes and cestodes (Dixon and Mercer, 1965; Morseth, 1967; Morris and Threadgold, 1967; Erasmus, 1967). The electron microscopic observations of these workers show that the sensory structures are bulbous or vesicular in appearance with an outwardly projecting

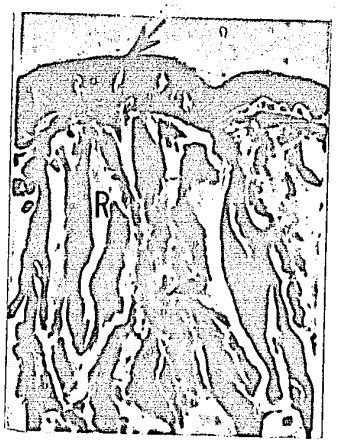


Fig. 78.--Horizontal section of the scolex of the adult worm. R, rostellum; note the vesicular patches (arrow) in the tegument.
(Osmium-ethyl gallate, phase contrast, x 1,240)

cilium. It is also of interest to note that Smyth, Howkins and Barton (1966) reported the occurrence of numerous, delicate hairs at the tip of the rostellum in E. granulosus, which they believe to be sensory receptors.

h. The neurosensory cells

The neurosensory cells, described in a previous section of this chapter, contain material which stains heavily with paraldehyde fuchsin. Various modifications of Gomori's paraldehyde fuchsin stain have been used to demonstrate neurosecretory cells in invertebrates, of which one is the simplified procedure of Cameron and Steele (1959). Using this technique the neurosensory cells in the rostellum were shown to be fuchsinophilic. This characteristic staining property of these cells, combined with their morphological appearance and anatomical disposition in the rostellum, suggest that they are neurosecretory cells: they will be described in greater detail in the next chapter.

CHAPTER XI

THE SCOLEX AND NEUROSECRETION

It was suggested in the previous chapter that the cells in the rostellum of H. diminuta were neurosecretory. A histochemical study of these cells was made and it is reported in the first section of this chapter.

1. The Histochemistry of the Cells in the Rostellum

The histochemical observations on these cells are shown in Table 8. The secretion failed to stain with the PAS procedure and Best's carmine; glycogen, other carbohydrate and carbohydrate-protein complexes are absent. The secretion did not stain with Alcian blue and also failed to show any metachromasia with Toluidine blue, indicating that acid mucopolysaccharides are also absent. The secretion was osmiophilic, indicating the presence of lipids. The secretion is obviously proteinaceous since it stained with mercury bromphenol blue. With performic acid-Alcian blue, granules of secretion, stained blue, were observed in cell body and axon. These granules failed to stain when the oxidant was omitted in the staining procedure. The positive results with PFAB indicate the

TABLE 8.--Histochemical observations on the nerve cells in the rostellum

Test	Observation
PAS	-
Best's carmine	-
Alcian blue, pH 2.5	-
Toluidine blue	blue, no metachromasia
Mercury bromphenol blue	+
Performic acid-Alcian blue	+
PFAB without oxidation	-
Osmium-ethyl gallate	secretion osmiophilic

presence of appreciable amounts of cystine. According to Pearse, the sensitivity of this test is low but it has a high specificity, and therefore, positive results with this procedure are indicative of appreciable quantities of cystine. He further writes that despite its low sensitivity, this procedure is of importance in applied histochemistry in the demonstration of the neurosecretory substance in various species, and "it must be considered the histochemical reaction of choise." (Pearse, 1960). The neurosecretory material of vertebrates and invertebrates shows a high concentration of cystine (Sloper, 1957;

Schreiner, 1966; and other references in Sumner, 1965).

Two widely used procedures in the demonstration of neurosecretory material are Bargmann's chrome haematoxylin-phloxine method and various modifications of Gomori's paraldehyde fuchsin procedure. The method used in this study was the modified paraldehyde fuchsin procedure of Cameron and Steele (1959). With this method the secretion in the cells of the rostellum stained an intense purple colour, and failed to stain when the oxidation step, using acidified permanganate, was omitted in the procedure. According to Sumner (1965; also see Appendix under Aldehyde fuchsin) the staining produced after oxidation is due to strong acids, produced by the oxidation of cystine, which then combine with aldehyde fuchsin by ionic links. The histochemical evidence then favours the view that the cells in the rostellum of H. diminuta are neurosecretory.

Smyth (1964a) described spindle-shaped glandular cells in the rostellum of E. granulosus, which were believed to secrete globules of material anteriorly. The secretory material, which was difficult to fix, showed sudanophilia and also gave a weak performic acid-Schiff reaction; these observations led Smyth to conclude that the secretion was probably a lipoprotein or lipid-protein coacervate.

2. The Cycle of Secretion Shown by the Cells in the Rostellum

The neurosecretory cells undergo a cycle of activity. In the cysticercoid, the cells are recognizable, but fail to stain with paraldehyde fuchsin; the cells are relatively small with a nucleus and very little cytoplasm (see Fig. 72).

By 2 days after infection of the definitive host, before strobilisation has begun, the cells have increased in size and the cytoplasm is faintly fuchsinophilic. By 4 or 5 days after infection the cells have increased further in size, their bipolar nature is more obvious, and, most important, the cytoplasm is intensely fuchsinophilic. Some of the cells at this time exhibit little fuchsinophilia, but by 6 or 7 days all the cells are intensely fuchsinophilic and have a swollen appearance (see Fig. 79). At 16 to 18 days after infection, the cells begin to lose their turgid appearance and there is a decrease in the intensity of fuchsinophilia. Granules of secretion can now be observed in the axons of the cells leading towards the nerves of the rostellum (see Fig. 80). The amount of detectable fuchsinophilic material in the cells decreases until, at 20 days after infection, many of the cells lack the fuchsinophilic material (see Fig. 81). At later stages none of the cells contain the secretion.

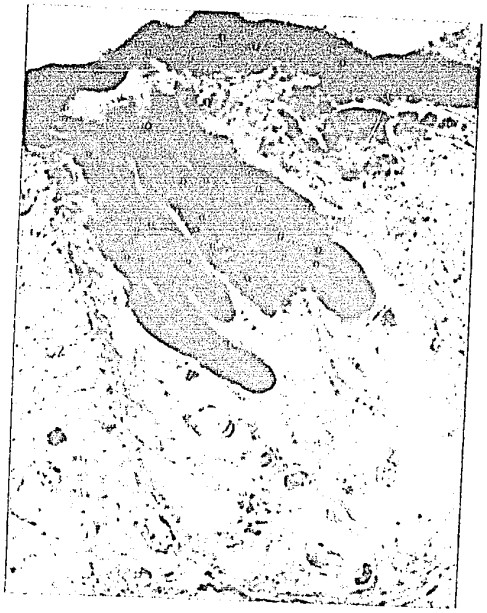


Fig. 79.--Horizontal section of the scolex of the adult worm 7 days after infection. Note the neurosecretory cells, in the rostellum, containing abundant fuchsinophilic material. (Compare with Fig. 72, where only the nuclei of the cells are visible, and they are stained heavily by the counterstain.)
(Bouin's, paraldehyde fuchsin with Halmi's, x 1,300)

Fig. 80.--Horizontal section of the scolex of the adult worm 18 days after infection. Note the granules of secretion (arrow) in the axon; 1, nerve tract; 2, transverse median nerve within the rostellum (see Fig. 76).
(Bouin's, paraldehyde fuchsin with Halmi's, x 1,000)

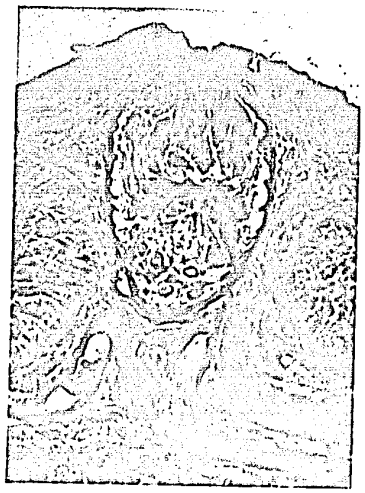


Fig. 81.--Horizontal section of the scolex of the adult worm 20 days after infection. Note the absence of fuchsinophilia in most of the neurosecretory cells.
(Bouin's, paraldehyde fuchsin with Halmi's, x 475)

The cyclic nature of the secretion contributes another piece of evidence for the notion that these cells in the rostellum are neurosecretory. Thus there exists in the rostellum a group of nerve cells which elaborate in a cyclic fashion a material which stains intensely with paraldehyde fuchsin, one of the classical neurosecretory stains. Furthermore, at a particular stage in the life cycle the secretion is observed to be released into the axons. These characteristics contribute sufficient evidence for the first description of neurosecretory cells in a cestode (Davey and Breckenridge, 1967). Neurosecretory cells have been described for other platyhelminths using various techniques (see Table 9).

It is difficult to assign a precise function to these cells on the basis of this investigation. Strobilisation, or the formation of proglottids, begins on the 5th day, and apolysis, or the shedding of gravid proglottids, commences 16 to 17 days after infection. The granules of secretion are released into the axon 16 to 18 days after infection; thus the release of the neurosecretory material is correlated with the shedding of the first proglottid.

TABLE 9.--Reports of neurosecretion in the phylum Platyhelminthes

Class	Name of organism	Mode of observation	Author(s)
Turbellaria	Leptoplana acticola	light microscopy	Turner (1946)
	Polycelis nigra	light microscopy	Lender and Klein (1961)
	Dugesia gonocephala	electron microscopy	Oosaki and Ishii (1965)
	Dugesia dorotocephala	electron microscopy	Morita and Best (1965)
	Procotyla fluviatilis	electron microscopy	Lentz (1967)
Trematoda	Dicrocoelium lanceatum	light microscopy	Ude (1962)
	Fasciola hepatica (cercaria)	electron microscopy	Dixon and Mercer (1965)
Cestoda	Hymenolepis diminuta	light microscopy	Davey and Breckenridge (1967)

3. The Significance of Neurosecretion in the Rostellum

Neurosecretory cells were described and defined on a cytological basis. Whether these cells have an endocrine function has to be demonstrated physiologically, and such a study was not attempted in this investigation. The identification and description of cells as neurosecretory, on a cytological or histochemical basis carries the implication that an endocrine function might eventually be assigned to these cells. The importance of neurosecretory cells lies in their role as mediators in the integration of many activities of the animal body.

The significance of neurosecretory phenomena has been appreciated by biologists for some decades now. Neurosecretion and neuroendocrine functions are well documented amongst the vertebrates and invertebrates; and it is among the invertebrates, especially in the phylum Arthropoda, that this phenomenon has been investigated in greatest detail. However, the significance of neurosecretion, and its importance in developmental events, has been appreciated in the field of parasitology only in very recent years; thus the possibility of "internal secretions" exercising an influence on the developmental events in the life of a parasite has been suggested as well as investigated (Rogers, 1961; Rogers and Sommerville, 1963; Meerovitch,

1965; Davey, 1966; Davey and Kan, 1967).

One of the main difficulties in studying tapeworm development with this approach is due to the parasitic nature of these animals, which makes regular observations of them during development a tedious task, involving the periodic sacrifice of the host animals with its attendant difficulties. There have been reports within recent years, of *in vitro* experiments where tapeworms have been grown from the larval stage to the adult egg-producing stage (Berntzen, 1961, 1962; Schiller, 1965; Sinha and Hopkins, 1967; Hopkins, 1967). This achievement in experimental techniques provides a method, with greater experimental flexibility, for studying development in tapeworms. Such studies have already been attempted (Smyth, Howkins and Barton, 1966; Smyth, 1967).

A combination of *in vitro* experiments and sound histological, histochemical and cytological observations would be very useful in future studies of tapeworm development.

SUMMARY

1. The physiology of development of a tapeworm was investigated by studying two regions of the tapeworm body --the strobila and the scolex.

2. By studying the terminal proglottids of worms taken from rats at successive intervals after infection, a timetable of developmental events was constructed.

3. The important events in the development of the adult worm are:

a. the formation of proglottids which commences on the 5th day after infection;

b. the differentiation of the reproductive system in the proglottid;

c. the transfer of spermatozoa;

d. the elaboration of the egg, which process proceeds in two stages and is of the longest duration in reproduction;

e. apolysis, or the shedding of the proglottids when they are gravid; this commences 16 to 17 days after infection.

4. The male and female reproductive systems have been described in detail. The male system consists of

3 testes and a long sperm duct, composed of physiologically specialised regions, which is responsible for conducting the spermatozoa to the female tract. These regions are the vas deferens, external seminal vesicle, internal seminal vesicle, muscular duct and ejaculatory duct, of which the last three are enclosed within the muscular cirrus sac. The physiological significance of these regions is examined. The gland cells of the external seminal vesicle elaborate a secretion which is a carbohydrate-protein complex.

5. The female system consists of 2 parts: one which receives spermatozoa during copulation and stores them, and the other which is responsible for the elaboration of the egg.

6. Cross-fertilisation between different worms is not essential for the production of fertile eggs. This fact, coupled with a detailed examination of the anatomical relationships of the male and female systems, has led to the conclusion that the transfer of spermatozoa from the male to the female tract occurs within the same proglottid.

7. Histochemical studies were carried out on the ovary, vitelline gland and Mehlis' gland. The oocytes contain protein and RNA, and some of this protein contains appreciable amounts of sulphydryl (SH) groups. The

prominent vitelline granules, in the vitelline cells, contain lipid, protein and acid mucopolysaccharide. The protein moiety contains protein-bound disulphide groups. The cells of Mehlis' gland secrete granules which contain carbohydrate, lipid, protein and some acidic substance.

8. An oocyte, spermatozoon, vitelline cell and the secretion of Mehlis' gland are assembled in the ootype to form the "immature" egg. This assembly process, which is the first stage in egg formation, is examined in some detail. An important point is that this process is a rapid one: all the eggs of the proglottid are assembled in less than 3 days.

9. The assembled egg is ejected into the uterus where it completes its development, to form the hexacanth larva surrounded by embryonic envelopes. The uterus becomes very large during this period and it fills the entire proglottid. The entire process of egg formation requires about 8 to 9 days for completion in each proglottid.

10. The cysticercoid larva, in the haemocoel of the arthropod host, has a scolex with 4 suckers and a rostellum, within which are many small undifferentiated cells. The excysted larva attaches itself to the intestine of the rat and enters into a period of development. The structure of

the scolex, as well as its mode of attachment to the rat intestine, have been described.

11. Particular attention has been focussed on the rostellum, which contains a group of prominent neurosecretory cells which send their axons into a nerve tract leading to the brain. The relationships of these cells suggest that they are also sensory structures.

12. The neurosecretory cells undergo a cycle of elaboration and release of secretion which is correlated with the onset of apolysis.

APPENDIX

APPENDIX

1. General Histological Techniques

a. Fixatives

1. Bouin's fluid

A modified form of this fixative was used. The formula is given in Pantin (1960) page 9; instead of acetic acid, 1% trichloracetic acid was used in making up the fixative (see Halmi, 1952; Ewen, 1962).

2. Heidenhain's Susa

This proved to be an excellent fixative for studies of the scolex. It was made up and used as recommended by Pantin (1960), pages 8-9.

3. Osmium tetroxide

See under tests for lipids.

b. Sections

The fixed tissues were dehydrated, cleared in benzene, infiltrated with wax (melting point, 58-60°C) in a vacuum oven. Sections were cut at a thickness of 5 μ on a Cambridge rocking microtome. The sections were mounted

on clean glass slides smeared with albumen adhesive. After staining, the sections were dehydrated in an ascending series of alcohols, cleared in two changes of xylene and mounted in permount.

Sections of cysticercoids were cut at a thickness of 2-3 μ on the rocking microtome. The cysticercoids were infiltrated with, and embedded in "Ester Wax 1960" (British Drug Houses Ltd.).

c. General Histological Stains

1. Heidenhain's Azan

A simplified modification of this stain (Hubschman, 1962) was used.

2. Hansen's iron trioxyhaematin

Sections of scolices, fixed in Susa, were stained according to the procedure given by Pantin (1960), pages 40-41.

3. Paraldehyde fuchsin with Halmi's Counterstain

Paraldehyde fuchsin was introduced by Gomori (reference cited by Sumner) as a suitable stain for elastic tissue. A number of modifications have been proposed for this staining procedure for its use in

staining neurosecretory cells (Clark, 1955; Cameron and Steele, 1959; Ewen, 1962). In the present investigation the procedure of Cameron and Steele was used, along with Halmi's counter stain--the formula of which was obtained from Humason (1962, page 289). Although this staining procedure is used almost specifically for studying neurosecretory systems, it is also very good for microanatomical studies (see Clark, 1955; Ewen, 1962), and was excellent for studying the histology and cytology of the proglottids of H. diminuta.

The chemistry of paraldehyde fuchsin staining was studied recently by Sumner (1965; also refer this report for references to earlier studies). A critical step in staining with paraldehyde fuchsin involves oxidation with acidified permanganate, after which the sections are exposed to the staining solution. Sumner suggests that the staining produced by paraldehyde fuchsin (a dark purple colour) after oxidation, in all the tissues studied by her, including the preoptic and neurohypophysial neurosecretory material of the Bullhead (Cottus gobio), is due to the strong acids (sulphinic or sulphonic) produced by the oxidation of cystine. The aldehyde fuchsin attaches itself to the acids by ionic links. Spicer et al (1967) similarly state that proteins rich in sulphur amino acids stain darkly in sections which have been previously

oxidised with an oxidant like acidified permanganate. According to these workers, glycogen stains in sections which have been oxidised for 30 minutes at 37°C in a 1% solution of periodic acid. Strongly acidic mucins (e.g., the sulphated mucins in the intestinal goblet cells of the rat) stain in unoxidised sections.

2. Histochemical Techniques

The fixatives used are indicated along with the particulars of the different histochemical procedures. The sections were prepared as before unless otherwise indicated.

a. Carbohydrates

1. The periodic acid-Schiff reaction (PAS)

The reaction is based on two reactions:

i. the oxidation of vic-glycol and glycol like groups into dialdehydes by periodic acid.

ii. the combination of the dialdehyde with Schiff's reagent to form a substituted dye which is red-purple or magenta in colour.

It is important to show that the positive PAS reaction is due to the specific oxidation of glycol and glycol-like

groupings, for which 3 control tests are essential. They are: i. subjecting the sections to Schiff's reagent without periodic acid oxidation; the sections fail to stain; ii. Blocking the reactive groups by acetylation with acetic anhydride in dry pyridine; sections treated thus, and then subjected to the PAS procedure should fail to stain; iii. Deacetylation; where the sections, acetylated as above, are treated with potassium hydroxide which restores the reactive groups, thus making a positive PAS reaction possible. In the present study all PAS-positive substances were identified on the above basis.

According to Pearse (1960) the substances which are PAS-positive are polysaccharides (glycogen), neutral mucopolysaccharides, muco- and glycoproteins, glycolipids, unsaturated lipids and phospholipids. All these substances are unaffected by treatment with diastase, except glycogen which is removed by incubation with this enzyme. Although, from a theoretical point of view, glycolipids and other lipid substances could give a PAS-positive reaction, it is believed that, in paraffin sections, they are either no longer present, or else present in such small quantities that they do not react. Thus, once glycogen has been eliminated, only one class of materials gives a PAS-positive reaction, and they are the carbohydrate-protein

complexes. Acid mucopolysaccharides do not give a positive PAS reaction. To ensure that a PAS-positive reaction is due to carbohydrate and not lipid, sections of material are first treated with pyridine or hot methanol: chloroform, in order to extract any lipid present, and then subjected to the PAS routine.

The fixative used was Lison's "Gendre fluid" at -73°, as given by Pearse (1960), page 788. The Schiff reagent used was that of Barger and De Lamater, prepared according to the method given by Pearse (1960), page 822. The procedure followed was that of McManus outlined by Pearse (1960), page 832, without counter staining. Digestion with malt diastase together with the PAS technique was used as a test for glycogen. The sections were incubated in a 1% solution of malt diastase (Fisher Scientific Co., Fairlawn, N.J.) in distilled water at 37°C for half an hour. Lipid extractions were carried out using pyridine (Pearse, 1960, page 846) or methanol: chloroform.

2. Best's carmine stain for glycogen

This is an empirical method, but remarkably selective. The fixative used was Lison's "gendre fluid." The procedure followed was that outlined by Casselman (1959), pages 100-101. Control sections were treated with malt diastase as in the PAS procedure.

3. Acid mucopolysaccharides

a. Alcian blue

Alcian blue appears to combine as a cation preferentially with acidic groups like carboxyls and sulphates in mucosubstances. Alcian blue, unlike all other basic dyes, shows no affinity for RNA and does not cause non-specific cytoplasmic staining when used at an appropriate pH.

The material used for staining with this procedure was fixed in Bouin's fluid, and Baker's formol-calcium (Pearse, 1960, page 787).

The procedure followed is that outlined by Mowry (1963), pages 410-411.

In order to distinguish carboxyl from sulphate groups, sections were stained in a solution of Alcian blue at pH 1.0, as recommended by Lev and Spicer (1964). At this low pH only, sulphated mucosubstances are stained by the dye.

The dye used was Alcian blue 8 GX (Allied Chemical).

b. Metachromasia with Toluidine blue

This dye is used to reveal acidic groups. Metachromasia here refers to the change of colour from blue through violet to red when the tissue is stained with a

solution of the dye. The monomeric alpha form of the dye is blue; on combination with suitable substrates, 2 main varieties of metachromasia result: γ metachromasia (red) due to the predominance of the γ form of the dye, and β metachromasia (violet) due to the simultaneous presence of α and γ forms of the dye. It is believed that cationic dye molecules are attracted to, and bound by the negatively charged acidic groups in the tissues, to give metachromasia.

The fixatives used were Bouin's fluid and Baker's formol-calcium.

Procedure.--The sections were brought to water and stained for 30 seconds to 1 minute in a 0.1% solution of Toluidine blue O (Fisher Scientific Co., Fairlawn, N.J.) in 30% alcohol. The slides were rinsed in distilled water and observed immediately when still wet. No permanent preparations were made.

4. The combined Alcian blue-PAS procedure (AB-PAS)

This elegant method of Mowry (1963) enables the detection of both acidic and vic-glycol groups in the same tissue sections. With this procedure, complex carbohydrates rich in acidic groups, especially carboxyls, are coloured turquoise blue; neutral carbohydrates containing vic-glycol

groups (e.g., glycogen) are coloured magenta. Carbohydrates having both acidic and oxidisable glycol groups are coloured by both AB and PAS giving deeper blue to purple shades.

The procedure followed is that given by Mowry (1963), page 415. The Schiff's reagent used was the same as that used in the standard PAS reaction, outlined earlier.

Material was fixed in Bouin's fluid and Baker's formol-calcium.

b. Proteins

Most of the tests for proteins in histochemistry are quite specific for particular amino acids, which constitute only a part of the protein molecule. However, positive results with such tests can be interpreted as indicative of the presence of protein, since free amino acids are very unlikely to be present in tissue sections.

1. The ninhydrin-Schiff test

This is used to detect amino groups in alpha amino acids, and is based on 2 reactions:

i. Ninhydrin reacts with the alpha amino acid to yield an aldehyde which is appreciably stable and does not diffuse.

ii. This aldehyde is then demonstrated with Schiff's reagent.

The fixative used was Bouin's fluid.

The procedure followed is that given by Casselman (1959), page 130.

2. Millon test

Among the amino acids it is specific for tyrosine. Millon's reagent produces a complex which is coloured an intense red.

The fixative used was Baker's formol-calcium.

The sections were brought to water; the slides carrying the sections were rested on match-sticks in a Petri dish, and the sections were gently flooded with Millon's reagent (Fisher Scientific Co., Fairlawn, N.J.). The sections were stained for half an hour at room temperature, mounted in glycerine and examined immediately.

3. The dihydroxy-dinaphthyl-disulphide test (DDD)

This is used to detect protein sulphydryl (SH) groups. The reaction proceeds in two steps:

i. The disulphide group in the DDD reagent specifically oxidises the protein sulphydryl to form a protein naphthyl disulphide and another free naphthyl complex.

ii. The protein naphthyl disulphide is coupled with a diazonium salt to form an azo dye. A blue staining

indicates a high concentration of SH groups and a red stain indicates lower concentrations.

The material was fixed in Baker's formol-calcium.

The procedure followed is that given by Casselman (1959), pages 142-143. The DDD reagent was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The diazo compound used was Fast Blue B salt (Edward Gurr Ltd., London).

Control sections were incubated in a 0.1 molar solution of N-ethyl maleimide (Nutritional Biochemicals Corporation, Cleveland, Ohio) in phosphate buffer, pH 7.4, in order to block the SH groups and render them unreactive towards the DDD reagent.

4. The performic acid-Alcian blue test (PFAB)

It is a very sensitive test for the sulphur amino acid cystine containing protein disulphide groups. The essence of the test is the oxidation of cystine by a strong oxidant, performic acid, to form cysteic acid (alanine β sulphonic acid) followed by the demonstration of this sulphonic acid by combination with Alcian blue at a low pH. Material with a high content (4% or more) of cystine appears dark blue, while lower amounts of cystine appear a paler blue (Pearse, 1960).

Material was fixed in Bouin's fluid and Baker's formol-calcium.

The procedure followed is that given by Pearse (1960), page 806.

Controls for the PFAB procedure consisted of sections which were treated in the same manner as the test sections, except that they were not exposed to the oxidising agent.

5. Mercury bromphenol blue

This is not primarily histochemical, although widely used. It was introduced by Durrum for the demonstration of protein in filter paper spots and later adopted as a general stain for protein by Mazia, Alfert and Brewer. This latter procedure was further modified by Bonhag (references in Pearse, 1960).

Fixatives used were Bouin's fluid, and Baker's formol-calcium.

The procedure followed is that given by Pearse (1960), page 792, in which 1% mercuric chloride and 0.05% bromphenol blue in 2% aqueous acetic acid were used.

c. Lipids

The osmium-ethyl gallate procedure of Wigglesworth (1957) was used to detect lipid in tissue sections. Differing opinions are held regarding the validity of using osmium as a histochemical reagent for lipids. The most recent discussion of this subject is that of Adams et al (1967), where new and old evidence concerning the possible reactions of osmium with proteins, lipids, and polysaccharides in tissue sections, is evaluated. These workers conclude "that osmium tetroxide reacts with and is reduced by cis-unsaturated lipids," while on the other hand, no evidence was obtained to suggest that osmium tetroxide is reduced or bound by proteins and polysaccharides in tissue sections.

The procedure followed is that outlined by Davey (1965b), pages 998-999. Sections were cut at a thickness of 1-2 μ on a Cambridge rocking microtome, and mounted sections were examined under both ordinary and phase contrast light microscopes.

Sections of material prepared in this manner were also very useful in the histological and cytological study of both proglottid and scolex.

Lipids and proteins may be associated to form lipoprotein complexes which may be detected with acidified

solutions of protoporphyrin. Sulya and Smith (cited by Searcy and Bergquist, 1960) observed that protoporphyrin was very sensitive in detecting small quantities of phospholipids, triglycerides, cholesterol, etc.; the lipids applied to filter paper, treated with acidified protoporphyrin, and viewed under ultraviolet light were observed to show a bright red fluorescence. Searcy and Bergquist (1960), appreciating this observation, used acidified solutions of protoporphyrin to detect serum lipoproteins; they suggest that protoporphyrin is capable of binding with the lipid moieties of serum lipoproteins.

A "histochemical test" was devised based on these observations: sections of material fixed in Baker's formol-calcium were brought to water, through a graded series of alcohols, stained in an acidified solution of protoporphyrin for half an hour, rinsed in distilled water, and examined, when still wet, under dark field illumination with exciter filters BG 12 and BG 3 on a Zeiss photomicroscope. The presence of red fluorescence was taken to indicate the presence of lipids; and the presence of proteins in the same location thus suggested the presence of some lipoportein. This could be further clarified with separate tests for lipids and proteins (see above).

Protoporphyrin-IX Dimethyl Ester (Sigma Chemical Co., St. Louis, Mo.) was hydrolised according to the procedure of Grinstein (1947).

d. Ribonucleic Acid (RNA)

Pyronin G is used to detect RNA because of the affinity shown by RNA for basic dyes. The material was fixed in Carnoy's fluid--formula in Pantin (1960), page 11. The procedure used was the Pyronin-Methyl green technique as given by Casselman (1959), pages 119-120. RNA is not the only basophilic constituent of tissues, and to obtain valid results, control tests using the enzyme ribonuclease are essential. Control sections were incubated for 1 hour at 37°C in a solution of ribonuclease (Nutritional Biochemicals Corporation, Cleveland, Ohio) in distilled water at a concentration of 0.5 mg RNAse per ml of water. The test solutions were incubated in distilled water at 37°C for the same length of time. The enzyme affects the Pyronin staining of RNA only, and any other staining due to acid mucopolysaccharides and other basophilic substances is not affected. Methyl green stains the nucleus green or blue green.

3. Miscellaneous

Tyrode's physiological solution. The formula for this solution was obtained from the Microtomists Vade-Mecum (ed., Gatenby and Painter), page 732.

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