

DEVELOPMENTAL BIOLOGY OF
ECHINOCOCCUS MULTILOULARIS
IN VIVO AND IN VITRO

by

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Abstract

The in vitro culture of Echinococcus multilocularis from protoscolex to the adult stage, is described. By modifying and improving previous techniques (Smyth 1979a), E. multilocularis has been maintained for longer periods in vitro, resulting in the consistent development of sexually mature adults, with up to three sets of genitalia. Comparative studies on E. granulosus, (sheep origin) emphasized differences in the developmental pattern between the two species in vitro. Intraspecific variation was also highlighted by in vitro culture, using E. granulosus protoscoleces from various intermediate hosts (sheep, horse, cattle, man).

Sexual development of E. multilocularis in vitro, was compared with that in vivo, using material from the same source. Dogs were infected with larvae, routinely maintained in laboratory rodents, and autopsied at 15, 20 and 21 days p.i. Although maturation time in vitro was somewhat slower than that in vivo, the developmental pattern was similar, in that maturation of the genitalia within the first proglottid, was usually completed prior to further strobilar development. Subsequent observations of fixed material from another source, grown in vivo, (16, 18 and 20 days p.i.), revealed a similar mode of sexual maturation.

No shelled eggs were found in vitro, but, by transferring worms that had been partially matured in vivo (21 days p.i.) to culture, developing oncospheres were observed on day 5 in vitro (26 days total).

The gross morphology, structure and ultrastructure of adult E. multilocularis is described. Comparative morphological studies on the maturation of the genitalia revealed some differences between in vivo and in vitro material. The absence of fertilized eggs in vitro, is considered to be due to a variety of factors, rather than just a failure of self-insemination in cultur^{ed} worms, reflecting possible deficiencies in the culture system.

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GENERAL INTRODUCTION

Echinococcus multilocularis (Leuckart, 1863, syn. E. alveolaris Klemm, 1883 and Alveococcus multilocularis Abuladze, 1960) Rudolphi, 1801, is a ^{cl}cyathophyllidean cestode of the family Taeniidae, and the causative organism of alveolar hydatidosis (alveococcosis; alveolaris; alveolar or multilocular echinococcosis) in man.

Life Cycle

The life cycle, which has been extensively studied, involves two mammalian hosts and is shown in Fig.1. The adult stage occurs in the small intestine of carnivores such as foxes, especially of the genera Vulpes and Alopex. Gravid proglottids passed in the faeces, may be picked up by foraging rodents (commonly of the family Cricetidae), which serve as intermediate hosts. Following the usual taeniid pattern, on ingestion, the intermediate hosts' digestive secretions stimulate hatching of the eggs, and hexacanth embryos (oncospheres) are released. These burrow through the intestinal wall and may penetrate the blood system, from where they are transported to any part of the body. The liver and lungs act as primary filters, although infections can occur in many other sites, including the peritoneal cavity. Each larva develops into a multivesicular structure, a semi-solid matrix basically composed of brood capsules containing protoscoleces. Invasion of the body tissues by this cystic mass is continuous, due to asexual multiplication of the larva. Continuation of the life cycle is effected when the intermediate host is eaten by the carnivorous definitive host. This cestode is therefore adapted to hosts having an essentially obligatory predator-prey relationship.

Although E. multilocularis is mainly perpetuated within a sylvatic cycle involving foxes and cricetine rodents (see Table 1 for a full list of recorded natural hosts), other mammals may be affected. Man is susceptible to larvae of E. multilocularis, and domestic dogs and cats commonly act as

Fig.1

Life cycle of Echinococcus multilocularis

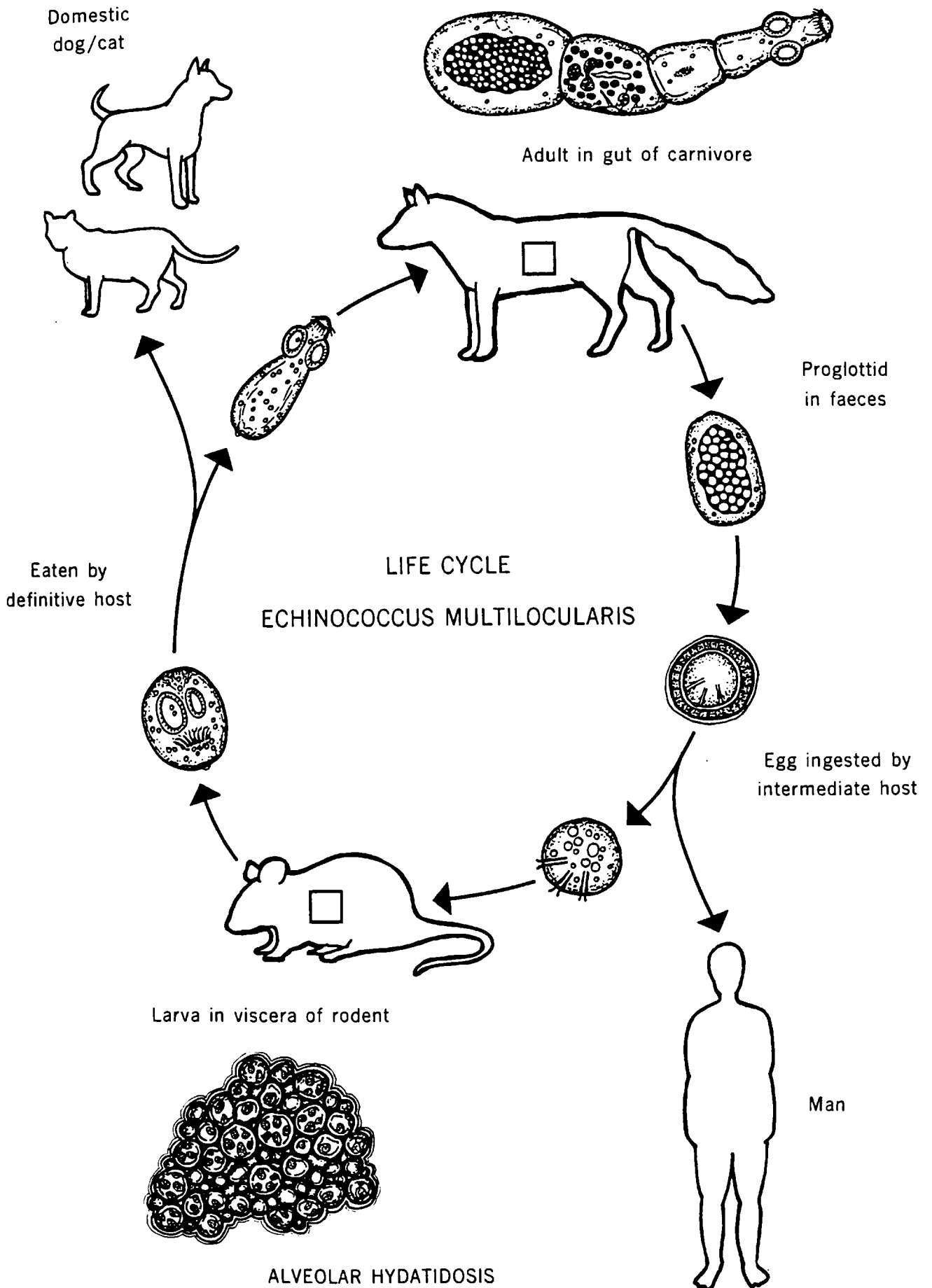


Table 1

Recorded natural definitive and intermediate hosts of
Echinococcus multilocularis

1. Definitive Hosts

(a) Canidae: - Red Fox (Vulpes vulpes), Artic Fox (Alopex lagopus), Domestic dog (Canis lupus f. familiaris), Wolf (Canis lupus), Dog fox, Corsac fox (Vulpes corsac), Raccoon fox (Nyctereutes procyonides), Coyote (Canis latrans)

(b) Felidae: - Domestic cat (Felis domesticus), Spotted cat (Felis hibica sarda)

2. Intermediate Hosts

(a) Cricetidae: - Voles (Arvicola spp., Microtus spp., Clethrionomys spp.), Muskrat (Ondatra zibethicus), Deer mouse (Peromyscus maniculatus), Lemming (Lemmus spp.), Gerbil (Cricetulus barabensis), Northern mole-vole (Ellobius talpinus), Shrew (Sorex spp.)

(b) Muridae: - House mouse (Mus musculus), Striped field mouse (Apodemus agrarius), Norway rat (Rattus norvegicus), Short-tailed mole-rat (Nesokia indica)

(c) Sciuridae: - Long-tailed suslik, Squirrel (Citellus spp.), Red squirrel (Sciurus vulgaris)

(d) Dipodidae: - Jerboa (Allactager elater)

(e) Ochotonidae: - Daurian pica (Ochotona daurica)

(References: - Rausch, 1967a; Eckert et al, 1974; FAO/UNEP/WHO, 1981; Kairov, 1981; Worley et al, 1982; Zeyhle, 1982a)

definitive hosts. In fact, domestic life cycles of E. multilocularis have recently been described and their public health implications discussed, by Leiby & Kritsky (1972) in central North America, and by Eckert, Muller & Partridge (1974) in the Southern Federal Republic of Germany. The potential rapid spread of such a pathogenic disease to man from domestic animals, is of obvious epidemiological importance (see later).

Geographical Distribution

The ecology and geographical distribution of E. multilocularis has been well documented in the past (Rausch, 1967a; Matossian, Rickard & Smyth, 1977). A more recent but brief review appeared in the FAO/UNEP/WHO Guidelines for Surveillance, Prevention and Control of Echinococcosis/Hydatidosis (1981).

The distribution of E. multilocularis, unlike the closely related species E. granulosus, is limited to the Northern Hemisphere, apparently restricted by the host specificity of the larval stage (Rausch, 1967a). To date, the occurrence of naturally infected final and/or intermediate hosts has been reported in: (references, as above; more recent ones indicated, where appropriate) southern and eastern France (Petavy, Rey, Deblock & Cambon, 1981, Houin, Deniau, Liance & Puel, 1982; Houin, Liance, Dumas & Puel, 1982; Pichot, Duriez-Vaucellf, Maisonneuve, Deblock & Petavy, 1982), Switzerland, Austria, central southern Germany (Eckert, 1981) and south west Germany (Zeyhle 1982a,b), Greece, Yugoslavia, Turkey (Yuhse, Yaycioglu, Varinli & Orsal, 1976), Czechoslovakia, Bulgaria (Genov, Svilenov & Polyakova-Krusteva, 1980; Genov, Svilenov, Polyakova-Krusteva & Hlebarov, 1981), USSR (Isakov, 1982; Isakov & Olesova, 1982), northern Iran, Japanese islands and North America, especially Alaska (Wilson & Rausch, 1980), south central Canada (Lee, 1969), and north central U.S.A. (Worley, Feigley, Sterner, Seese & Eastman, 1982; Seese, Sterner & Worley, 1983).

Despite the fact that few data exist on the world wide prevalence and/or incidence of human alveolar hydatidosis, individual cases have been described from many of the countries listed above, known to contain a sylvatic cycle of E. multilocularis. Most recent reports of human infections have come from France (Petavy et al, 1981; Seigneuric, Viard, Thierry, Michiels, Knopk, Camerlynck, Couailler, Charton & Cortet, 1982), Germany (Eckert, 1981), Turkey (Icoz, Ulus & Ozek, 1978; Eldegez, Terzioglu, Mercan, Erzurumlu & Gungel, 1980; Soysop, Solak, Serbest, Karatosun, Yildiz & Kulaklii, 1980), China (Cipeng, 1981) and USSR (Kairov, 1981; Tumol'skaya, Ozeretskovskaya, Sivakov & Panteleeva, 1981). From records of the occurrence of the human disease in Europe, E. multilocularis is now known to be endemic in the region of central and southern Germany, western Austria, Switzerland and eastern France. In addition, cases of alveolar hydatidosis in man are frequently seen throughout the greater part of the territory of the Soviet Union, northern Japan and western Alaska. Outside these known endemic regions, single cases of alveolar hydatidosis have been reported from several other countries; a small number of human infections have been recorded in Italy, and individual cases have been diagnosed in Sardinia, Greece, Yugoslavia, Poland, Hungary, Romania, Tunisia (Robbana, Ben Rachid, Zitouna, Heldt & Hafsia 1981), India, Australia, New Zealand, and even Great Britain (Rausch, 1967a; Matossian et al, 1977; FAO/UNEP/WHO, 1981). It has been suggested that some of these single human cases may have been due to the importation or immigration of infected animals, or, alternatively, infection may have occurred when travelling through endemic areas. However, it must be remembered that the diagnosis of alveolar hydatidosis is often very difficult; many cases of the disease in man may have been overlooked, and the identity of single cases cannot easily be determined. Some suspected alveolar cysts may have been caused by larvae of E. granulosus, comparable perhaps, to the multivesicular form sometimes seen in domestic animals. On the other hand, the larvae of other Echinococcus spp. may manifest themselves differently, i.e. in an alveolar-like form, within man. For example, previous authors (Rausch, 1967a; FAO/UNEP/WHO, 1981)

have suggested that the few suspected cases of human alveolar hydatid disease found in Argentina, Uruguay and Venezuela may have been atypical larval forms of another Echinococcus sp., since there is no other known record of E. multilocularis in the Southern Hemisphere.

Epidemiological and medical aspects

The world wide importance of this zoonotic parasite (and other species of the genus Echinococcus) has recently been emphasised by the production of FAO/UNEP/WHO Guidelines for Surveillance, Prevention and Control of Echinococcosis/Hydatidosis (1981). In another recent publication, Eckert (1981) stresses that due to "the high lethality rate of E. multilocularis infections in man, and (the appearance of) a considerable number of human cases of alveolar and cystic echinococcosis in various areas this zoonosis deserves more attention of veterinary and health authorities". From the public health point of view, the potential rapid spread of alveolar hydatidosis to man has become more apparent over the past few years. The endemic area of central Europe is now known to cover a much wider area than that described by Rausch in 1967a. Recent publications from France (where human deaths from alveococcosis are estimated as 20 per annum), show that several cases of human alveolar hydatidosis have been identified in central and north-eastern areas alongside a sylvatic cycle of red foxes (Vulpes vulpes) and rodents (Arvicola terrestris) (Houin, Liance, Dumas & Puel, 1982; Pichot et al, 1982).

In the endemic areas of Russia, circulation of E. multilocularis occurs between Polar foxes, Red foxes, wolves or dogs (and possibly cats as well) and cricetine or sciurine rodents (Lukashenko, 1968; Lukashenko, Ivchenko & Wolfson, 1970; Kairov, 1981). In the USSR, the occurrence of human infection is most commonly observed in hunters or fur-farm workers of any age. Suggested modes of infection are various and include: direct contact with infested dogs, or skins of infected foxes and dogs, water-borne (e.g. the drinking of

water from non-running reservoirs, snow or ice contaminated by infected definitive hosts), and dust-borne. There is also a possibility of human infection contracted through eating raw berries collected in habitats of infected foxes and other carnivores (Kurashvilli, 1964; Lukashenko, 1971). This mode of infection has also been suggested as a mechanism for transmission in France (Seigneuric et al, 1982).

A similar epidemiological picture emerges in North America (especially Alaska), where the distribution of E. multilocularis is discontinuous. Several sylvatic cycles have been identified, principal final hosts being Arctic or Red foxes, while numerous cricetid rodents act as intermediate hosts. Some autochthonous human infections are thought to have been acquired from domestic dogs and/or cats (Leiby & Kritsky, 1972; Crellin, Marchiondo & Andersen 1981), (also observed in Germany by Eckert et al, 1974). These infections of domestic animals may arise from eating wild rodents, or those associated with human dwellings, such as house mice. This situation must be considered important from a prophylactic point of view, although control measures specifically against E. multilocularis have yet to be introduced.

The case fatalityⁱⁿ rate man, as quoted by Rausch (1975 - cited by Crellin et al, 1981), ranges from 50-70%. The slow growth of the larvae in humans often means that, by the time the disease is clinically manifested, the multivesicular cyst is inoperable (cf. E. granulosus). Schantz, Van den Bossche & Eckert (1982) state that the percentage of surgically resectable cases varies in different case series from 26-58%, with approximately 93% of patients with non-resectable E. multilocularis, dying within ten years.

Chemotherapeutic studies on the disease in man have been severely limited, due to the relatively small number of identified human cases. However, a recent review of Schantz et al (1982) states that, in patients treated for alveolar hydatidosis with mebendazole, the progressive course of the disease appears to be arrested, but the parasites are not

killed. Similar inhibition has been seen in experimentally infected gerbils. Although mebendazole, or other benzimidazole derivatives may not appear to be most suitable chemotherapeutic agents against larval E. multilocularis in man, this therapy cannot be truly assessed without further controlled clinical trials (Muller, Akovbiantz, Amman, Bircher, Wissler, Witassek & Wuthrich, 1982).

Laboratory studies

Numerous studies have been undertaken on various aspects of the biology of the genus Echinococcus. However, most work has been concerned with E. granulosus, possibly due to its' cosmopolitan distribution, its' ability to utilize a wide range of hosts, and the high prevalence of human hydatidosis in many parts of the world. In comparison, alveolar hydatid disease, specific to a smaller number of hosts, is not only apparently restricted to the Northern Hemisphere, but the incidence in man is, at present, relatively low.

Nevertheless, the larval stage of E. multilocularis has proved to be an ideal laboratory model for a variety of studies, due to its' ease of maintenance within rodents by intraperitoneal passage, and its' rapid rate of development, compared with E. granulosus. Many workers have reported the susceptibility of various species and strains of laboratory animals to larvae of E. multilocularis (Lubinsky & Desser, 1963; Lubinsky, 1964, 1967; Kamiya, 1973; reviewed by Smyth & Heath, 1970). In addition, numerous studies have been undertaken on the development, histogenesis and pathology of the larval stage in various experimentally infected hosts of different ages and sex (Cameron, 1960; Sakamoto & Sugimura, 1969, 1970; Ohbayashi, Rausch & Fay, 1971; Hinz, 1972a; Kamiya, 1972; Baron, Rau & Tanner, 1974; Vogel, 1978). The development of larval tissue, especially the germinal cells, of E. multilocularis has been observed both in vitro and in vivo by Yamashita, Ohbayashi, Sakamoto & Orihara (1962), Sakamoto & Kotani (1967), Sakamoto, Yamashita & Ohbayashi, (1967) and Sakamoto (1978). Ali-Khan (1974a,b) studied the host-parasite

relationship of the larvae, referring to the apparent ineffectiveness of host-immune mechanisms against E. multilocularis cysts. More recently, larval E. multilocularis has been used successfully for various chemotherapeutic studies (Campbell & Blair, 1974; Campbell, McCracken & Blair, 1975; Schantz et al, 1982) and also in immunodiagnostic tests (Hoghooghi, Kagan, Schiller & Aminzadeh, 1977).

In contrast, the strobilate stage of E. multilocularis has received little attention. Due to its high pathogenicity, very few workers have been able to study the establishment, development and other aspects of the adult stage in experimentally infected hosts - namely, dogs. However, in vitro culture makes it possible to investigate various aspects of the parasite's physiology, biochemistry, and developmental biology, which cannot be easily and safely determined in vivo.

Previous observations on the in vivo development and maturation of E. multilocularis protos^{col}idesces have been limited to a few authors (Webster & Cameron, 1963; Smyth & Davies, 1975; Smyth, 1978a, 1979a,b; Smyth & Barrett, 1979, 1980a). In addition, in vitro development to egg-containing mature adult worms, has recently been achieved, following partial maturation of the prot^oscoteles in experimentally infected definitive hosts, (Thompson & Eckert, 1982). However the complete development of the strobilate stage of E. multilocularis, to full sexual maturity, has not been previously described in detail, nor directly compared with in vivo worms obtained from experimentally infected animals.

Scope of this study

Although this thesis is primarily concerned with the developmental biology of the strobilate stage of E. multilocularis, Section I, which deals with the in vitro culture of this parasite, also includes some work on E. granulosus from various intermediate hosts (sheep, horse, cattle and man). Due to continual passage of E. multilocularis larvae through

laboratory rodents, a supply of material (protoscoleces) is always available for in vitro culture work.

However, opportunities to culture various 'strains' of E. granulosus were not overlooked, in order to study the intra-specific, as well as inter-specific variations of development in vitro. This section details the various culture techniques employed in order to obtain the best possible development of E. multilocularis in vitro. As an assessment of the efficacy of the culture system used, the maturation of the protoscoleces to the adult stage in vitro is directly compared with in vivo material from experimentally infected dogs. Further comparisons were made by culturing worms which had been partially matured in vivo, and their subsequent development followed.

Section II includes observations on the gross morphology and fine structure of ^{the}strobilate stage of E. multilocularis. Differences noted between in vivo and in vitro material are emphasized and discussed. Continual assessment of cultured worms using both fresh and fixed material, and light and electron microscopy, has enabled the development of the adult stage of the parasite to be studied in depth, with particular emphasis on the reproductive system. Possible reasons for the incomplete development of worms in vitro, i.e. the lack of fertilised eggs in culture, are discussed.

There are few detailed ultrastructural studies on gametogenesis in ^{cyl}cyzophyllideans, and therefore observations of the processes involved in E. multilocularis, as described in Section II, are compared with other electron microscopy studies on the development of the genitalia in a variety of cestodes and trematodes.

SECTION I

In vitro culture of Echinococcus multilocularis; comparative studies with E. granulosus

A. INTRODUCTION

1. Introduction to the in vitro culture of cestodes in general

In vitro culture of cestodes (as well as other parasitic helminths) has long been accepted as an important parasitological tool, a point which has most recently been emphasized by Smyth & Silverman (1981). The advantages of in vitro culture have been previously emphasized by several authors (Weinstein, 1966; Silverman, 1965, 1981; Smyth, 1969c, 1976, 1981; Chew, 1981; Eckert, Gemmell & Wikerhauser, 1981), and therefore will not be elaborated upon here. However, the problems and difficulties of establishing and growing intestinal helminths in culture (Smyth, 1969c, 1976, 1981; Smyth & Davies, 1974b; Smyth & Silverman, 1981) have probably resulted in "some loss of interest in the field" over the past decade (Smyth, 1981).

Much progress was made during the nineteen-sixties and early seventies on the in vitro culture of cyclophyllidean cestodes. Most notable achievements were those of Benⁿtzen (1961-) with Hymenolepis spp. and Smyth (1962-) working on Echinococcus spp. Since then, having established basic techniques for the cultivation of tapeworms (reviewed by Silverman, 1965; Silverman & Hansen, 1971; Smyth, 1969c, 1976; Taylor & Baker, 1978), workers have been able to embark on a greater variety of studies pertinent to a further understanding of the biology of cestodes, parasitic in man and domestic animals, and consequently, potential control measures.

For example, strain differences between E. granulosus of sheep and horse origin were highlighted by in vitro culture (Smyth & Davies, 1974a; Smyth, 1979b, 1982a) - a result

supported later by biochemical evidence (McManus & Smyth, 1978, 1979). This led to an increased interest in the epidemiology of hydatid disease, especially with regard to the potential infectivity of various strains to man.

Smyth (1979b) also tentatively proposed a scheme for the identification of taeniid eggs, based on in vitro hatching requirements and post-oncospherical development. The differentiation of taeniid eggs (being morphologically similar) in faecal, soil and water ^{samples} ~~MPL~~, is of obvious epidemiological importance. To date, no other method of precise identification has been worked out, although Craig (1983) has recently reported some success, with immunofluorescence techniques, differentiating between oncospheres of T. hydatigena and T. pisiformis.

In vitro culture techniques have also been utilized for many chemotherapeutic studies; for example, assessment of the action of anthelmintics on E. multilocularis (Sakamoto, 1973) and the screening of protoscolicidal drugs (Sakamoto & Gemmell, 1975 - cited by Eckert et al, 1981). Such systems are not dependant on the maintenance of cestode pathogens in vivo, and therefore the use of a large number of laboratory animals for preliminary screening of drugs. In addition, Smyth & Barrett (1980b) have utilized in vitro culture as one means of assessing the viability of human hydatid material following surgical removal, especially after chemotherapy.

More recently, Silverman (1981) discussed the possible use of in vitro culture of parasites for vaccine development, although he did not refer specifically to any particular cestode species. However, Eckert et al (1981) reviewed recent immunization work by Rickard and his colleagues (1971-), based on the collection of ES antigens from in vitro cultures of various taeniid species.

Reviewing the above recent applications, it is clear that the need for new, improved and simplified techniques for the in vitro culture of cestodes, represents an urgent problem for the

benefit of these, and other studies. In addition, the immense value of being able to reproduce the life cycles of cestodes, of both medical and veterinary importance (such as Echinococcus spp.), in vitro, i.e. without resource to host animals, cannot be over-emphasized.

The remainder of this introduction to Section I is devoted to two literature reviews. The first concerns the in vitro culture of various stages in the life cycle of E. multilocularis, including some studies on cell culture. The second review, covers the in vitro culture of other cestodes of Canidae. This has been restricted to attempts, and ultimate achievements in obtaining segmentation and sexual differentiation in vitro, since this thesis is only concerned with the maturation of larvae to the adult stage.

2. Review of the literature on the in vitro culture of Echinococcus multilocularis

The first recorded attempts on the propagation of E. multilocularis in vitro was by Rausch & Jentoft (1957) in Alaska, using chopped larval tissue from experimentally infected rodents. Cultures were maintained in a basic medium of 40% human ascitic fluid in Hank's Basic Salt Solution (HBSS), with antibiotics, and supplemented with Hela cells, extract of vole embryo or human plasma. In some experiments the larval tissue proliferated and produced protoscoleces in vitro "about as rapidly as in suitable intermediate hosts such as Microtus and Clethrionomys". Although no attempt was made to infect the definitive host with the protoscoleces thus produced, the viability of the material was confirmed following intraperitoneal injection into natural intermediate hosts, where it continued to grow. Proliferation in the cultures appeared to be limited solely by the size of the culture vessel used.

Cameron (1960) reported the early work of Dr. G.A. Webster on the in vitro culture of larval material (both chopped and intact) from cotton rats and gerbils. Intact cysts readily produced vesicles in culture, eventually forming a multilocular structure. Protoscoleces within the cysts remained viable for "extended periods of time" until degeneration occurred. However, free protoscoleces showed a tendency to differentiate in one of two ways - either they vesiculated, pre- or post-evagination, or, following evagination, they gradually lost their calcareous corpuscles, developed a well defined excretory system, became more elongate, and in some cases, segmented. Occasionally, anomalous forms were observed in vitro, such as second "heads" growing from the tail portion, or two bodies attached to a single scolex. The full results of this work were subsequently published by Webster & Cameron (1963), who, after testing 46 different media, concluded that the developmental course of E. multilocularis (in this case - E. m. sibericensis, the Alaskan strain) in vitro, could be pre-determined by using a particular medium. Best vesicular

development was achieved in Morgan's synthetic medium, M150, with the addition of glucose. Segmentation of the protoscoleces occurred in a modified M150 medium, or Parker's CMRL 1066, with the additions of meat digests and/or dog gut extract. There was no evidence, however, of development of genitalia. A few experiments were carried out on the in vitro hatching and growth of oncospheres, according to the procedures described by Meymarian (1961) for E. granulosus, but contamination proved to be a problem, and no conclusive results were obtained.

During a series of studies on echinococcosis in Japan, Yamashita et al (1962), described in detail the vesicular development of E. multilocularis protoscoleces (Alaskan strain) in vitro, following trypsinization of the larval tissue. Their media essentially consisted of 0.5% lactalbumin in HBSS, with the addition of bovine serum, which was a necessary element for protoscolex survival. Although bovine bile and liver extract initially accelerated vesiculation, it was concluded that these tended to cause degeneration and rupture of the protoscolex. In the best cultures, collections of germinal cells, similar to the early stages in brood capsule formation, were found, but this was the maximum development achieved. With the addition of Hela cells, the results were similar to those recorded by Rausch & Jentoft (1957) in that the protoscoleces became embedded in the cells, prior to enlargement and multilocular vesiculation. The Hela cells apparently provided a substrate on which the larval E. multilocularis could proliferate, although their presence somewhat restricted the rate of cyst growth.

This early in vitro culture work was briefly reviewed by Smyth (1964a), and in the same year, Lukashenko (1964) reported his observations on the in vitro development of E. multilocularis protoscoleces, vesicles and germinative tissue from a cotton rat. Optimal results were obtained using minced germinal membrane in Parker's 199 Medium, supplemented with cotton rat embryo extract, bovine serum and lactalbumin. In successful cultures, he observed the development of vesicles,

ultimately containing protoscoleces with hooks. Their viability was confirmed following infection of puppies, or by intraperitoneal inoculation of cotton rats. In vitro culture of protoscoleces in some media led to the occasional development of strobilate worms with two or three segments, but with no genitalia; a similar result to that reported by Webster & Cameron (1963).

The first report of the in vitro culture of germinal cells of E. multilocularis was by Lukashenko, Brzevsky & Smirnova, (1965 - cited by Sakamoto et al, 1967), using daughter cysts from infected cotton rats. The cells were obtained by trypsinization. The highest cell division recorded was in Medium 199 containing 10% cotton rat embryo extract, 5% lactalbumin and 15% calf serum. A more comprehensive report on the in vitro culture of germinal cells followed by Sakamoto et al (1967). Their observations on the morphology of germinal cells grown in vitro, compared favourably with those of Sakamoto, Yamashita, Ohbayashi & Orihara (1965) on material in situ. The cells rapidly established in a culture medium of HBSS containing 0.5% lactalbumin, 0.1% yeast extract and Medium 199, with 20% calf serum. Following proliferation, ^bBothryoid colonies of round cells formed a monolayer in culture vessels. In subcultures, the cells assumed a spindle, and then an asteroid shape, with a varying number of cytoplasmic processes. However, in contrast to Lukashenko's observations (1964) on minced germinal tissue, no vesicles formed in vitro. In addition, Lukashenko et al (1965) had included cotton rat embryo extract in their culture medium with success, whereas Sakamoto et al (1967) described the "action of cotton rat embryo extract (to be) unstable" and noted "a considerable difference in results" between the two groups.

Subsequently, in 1975, Smyth & Davies described their own observations on the development of E. multilocularis in vitro, following techniques established for the cultivation from protoscolex to strobilate stage of the closely related species, E. granulosus (Smyth, 1967; Smyth & Davies, 1974b). They used material from cotton rats or gerbils, the original larval

tissue being of German origin. Following pepsin treatment, the free protoscoleces were washed in HBSS before being transferred to evaginating solution - basically composed of HBSS, supplemented with medium 'S' (Parker's 858 Medium, glucose, KCL, foetal calf serum and yeast extract) and 0.05% dog bile. Using both mono- and diphasic culture media (variations of medium 'S', and the addition of a coagulated bovine serum base, respectively), Smyth & Davies (1975) were the first to achieve in vitro development of E. multilocularis protoscoleces to sexually mature adults. However, the majority of worms assumed bizarre "monozoic" forms, i.e. with only one set of genitalia, although some "pseudosegmented" adults were obtained in a few cultures. It is also interesting to note that in "poor" cultures, the protoscoleces became vesicular, or abnormal, rather than differentiating into adult worms, as had been observed by earlier workers. The maturation in vitro was compared to that in vivo by infecting cats with protoscoleces (Smyth & Davies, 1975). No "monozoic" forms were observed in these hosts, although due to shortage of material, the authors' description of the worms' development in vivo was somewhat brief.

In 1978a, Smyth compared the unusual phenomena of "monozoic" development in vitro with his observations on the sheep strain of E. granulosus. The latter readily segmented in culture, producing adults with three or four proglottids. He suggested that in E. multilocularis, although full sexual differentiation occurred in vitro, somatic growth was severely inhibited, in contrast to E. granulosus (sheep strain). As in all Echinococcus spp. cultures, no fertilized eggs, or oncospheres were obtained, although ova and/or vitelline cells were sometimes observed in the uterus.

Tissue cultures of E. multilocularis were resumed by Sakamoto (1978a), who compared the development of larval tissue (of Alaskan origin) in vitro and in vivo (in cotton rats). Cells derived from the germinal layer of cysts from cotton rats following trypsin treatment, were cultured in NCTC 135 containing 0.5% lactalbumin, 0.1% yeast extract and one of a

range of inactivated sera. Best results were obtained with 20-30% foetal calf serum. The germinal cells proliferated, assuming a spindle, then asteroid shape, as previously observed by Sakamoto et al (1967). With the addition of cotton rat or chick embryo extracts the processes of the cells spread over the base of the culture flask, and, in addition, syncytia with many nuclei and vacuoles were clearly apparent from day five.

These too, had occasionally been observed by Sakamoto et al (1967), and Sakamoto (1978a), who, in describing them as large, vesicular, syncytical cells, suggested that they were precursors of brood capsules or hydatid cysts. However, the authors' in vivo studies showed that the germinal cells were also capable of developing into cysts.

Further work on the in vitro cultivation of protoscolex to the adult worm was reported in several papers in the late 1970's. Smyth (1979a) gave comprehensive details of the culture techniques found to be most suitable for the establishment of sterile protoscoleces in culture. The medium used - S10 (adapted from that described by Smyth & Davies, 1974b, 1975), contained an increased level of 25% inactivated foetal calf serum, and the author also suggested the use of CMRL 1066 or NCTC 135 as substitutes for Parker's 858. Smyth (1979a) and Smyth & Davies (1979) described the unusual "monozoic" development of the worms in vitro, as mentioned previously, adding that "pseudo-segmented", or "pseudopolyzoic" forms were not unusual in older cultures. However, as before, no fertilized eggs were obtained in vitro. In some cultures (more than 60 days old), aberrant development was occasionally observed, such as the appearance of a second, morphologically normal scolex - a phenomenon which has^d already been described by Cameron (1960).

Smyth & Barrett (1979) gave a detailed account of the maturation of cultured E. multilocularis protoscoleces, using the established in vitro techniques. They described the development of the genitalia from both fresh and stained material, and discussed variations observed in the differentiation of the adult worms, i.e. from the "monozoic" to

the "pseudo-segmented" forms. However, as reported by Smyth & Davies (1975), Smyth (1979a) and Smyth & Barrett (1979), no shelled eggs were ever found in cultured worms. Despite the abundance of apparently normal sperm, insemination had never been observed in vitro, and sperm had never been seen in the receptaculum seminis. It was therefore assumed that fertilization could not proceed in cultured E. multilocularis due to lack of sperm transfer, although vitelline cells and/or ova (presumably unfertilized) were sometimes found within the uterus.

Following observations on the development of adult E. multilocularis and other platyhelminths in vitro, Smyth (1982b) outlined the insemination-fertilization problems encountered in cultured cestodes. He stated that the situation in E. multilocularis was very similar to that observed in E. granulosus, although attempts at growing either species within "compression" systems (as found necessary for Schistocephalus solidus to produce fertile eggs, in vitro), have, to date, been unsuccessful. Although occasionally on subjecting E. multilocularis to some pressure, the cirrus tip was seen at the vaginal entrance, or a very few sperm were observed in the genital atrium or receptaculum seminis; however, no fertile, shelled eggs were obtained, nor haploid embryos formed in vitro, in contrast to the results obtained with the pseudophyllidean cestode Schistocephalus solidus (Smyth, 1954)

Recently, however, adult E. multilocularis have been induced to produce eggs in vitro, following partial development for 20-21 days in definitive hosts, and maturation for a further eight days in culture (Thompson & Eckert, 1982). From earlier observations, these authors anticipated that fertilization would have taken place by 21 days post oral infection, in vivo. The techniques employed were originally described by Smyth & Howkins (1966), who cultured partly developed strobila of E. granulosus from a 28-38 days infection in dogs, and obtained shelled, active oncospheres. Kumaratilake & Thompson (1981) successfully repeated these experiments and subsequently proved the infectivity of the

oncospheres produced in vitro, by oral and intra-peritoneal injections of mice. Thompson & Eckert (1982), using a monophasic medium (Medium 199, supplemented with foetal calf serum, KCL, glucose, cystine and phenylalanine - as used by Kumaratilake & Thompson, 1981), were therefore able to produce in vitro, apparently morphologically normal, thick-shelled eggs of E. multilocularis. The authors were, however, unable to confirm viability of the oncospheres following primary infection of gerbils.

3. Review of the literature on the in vitro culture of other cestodes ^{of} Canidae

3.1 Spirometra mansonoides

In vitro studies on Spirometra mansonoides were first reported by Mueller in 1958, who successfully "cultivated" the second larval stage in vitro. In early experiments, plerocercoids removed from mice, could be maintained in vitro for up to 11 months, in Medium 199 with 10% calf serum, although growth was negligible. However, with the addition of 20% fresh chick-embryo extract, the parasites grew noticeably in length. At 60 days, they were fed to kittens, but the results were inconclusive; only one "sickly" animal produced aberrant eggs. In a subsequent experiment, Mueller (1958) infected the body cavity of mice with proceroids, and removed the immature plerocercoids five days post infection (p.i.). They were then grown in vitro as before, being fed to cats at 60 days. On this occasion, normal adult worms were obtained.

The following year, Mueller (1959a,c) was able to culture plerocercoids from proceroids in vitro, without partial maturation as described above in mice. Proceroids were extracted from copepods, placed directly into culture medium (as before) and then maintained for 60 days in vitro. Infectivity was proved by feeding the cultured parasites to cats from which adult worms with eggs, were consequently harvested. The cycle was completed by the collection and hatching of the eggs, which produced a normal infection in copepods, and finally mice.

A stage further in the in vitro culture of S. mansonoides was reached by Berntzen & Mueller (1963, 1964). Employing the methods of Berntzen (1961, 1962), they successfully grew the parasite to the young adult stage, using either culture-reared plerocercoids (Mueller, 1959c), or those from infected mice. Optimum results were obtained with a very complex medium (designated M 115), in a continuous flow culture system, and a gas phase of 5-10% CO₂ in N₂. Pretreatment with cat bile,

hamster bile or sodium taurocholate, together with trypsin, was necessary for scolex evagination and removal of the 'old' larval tissue (i.e. the posterior part of the plerocercoid) respectively. Furthermore, without the use of this so-called evaginating medium (in Earle's Salt Solution - ESS), differentiation and growth did not occur. In the best cultures, segmentation was observed from day eight, with the maximum development achieved, being the appearance of germinal primordia.

In 1982, Berntzen & Mueller reported their eventual success in being able to culture S. mansonoides from the plerocercoid to the gravid adult in vitro, using culture apparatus described by Berntzen (1966). Spargana raised in laboratory mice were pretreated in evaginating solution and cultured individually, in M 115 (as previously) with the addition of cat bile, and the exclusion of Stock Solution C (Reducing Solution) after day five. Although the maximum length of the segmented worms was little more than that previously observed by Berntzen & Mueller (1964), development to sexual maturity was achieved. Times of segmentation and maturation compared well with known development rates of worms in vitro, with gravid proglottids being seen from day 16. Eggs collected from cultures were incubated to hatching (Mueller, 1959b), and the resultant coraxacidia, on being fed to copepods, developed into procerroids. Berntzen & Mueller (1972) quoted S. mansonoides as being "certainly the largest cestode reared in vitro", and concluded that "if a laboratory substitute could be found for the copepod host, the entire life cycle will have been reproduced by artificial means".

3.2 Mesocestoides corti

The in vitro culture of Mesocestoides corti from the second larval stage, or tetrathyridium, to the sexually mature adult, has only recently been achieved (Barrett, Smyth & Ong, 1982; Thompson, Jue Sue & Buckley, 1982). Earlier in vitro studies with larvae of this species were initiated by Specht and Voge (1965), who were able to maintain tetrathyridia from mice for

up to 4.5 months in a previously used tissue culture medium, modified by the addition of glucose. Asexual multiplication occurred in a few cultures, but not at a rate comparable to that observed in vivo (i.e. in the peritoneal cavity of mice); no sexual differentiation occurred. Subsequently, Voge & Coulombe (1966) tried numerous mono- and diphasic media with different gas phases and pH ranges for the cultivation of M. corti tetrathyridia. The optimum rate of asexual multiplication was observed in diphasic media (NCTC 109, or modified M 115 (Berntzen & Mueller, 1964), inactivated horse serum and lactalbumin, with whole human blood), and was comparable to that in the mouse host. Whole haemolyzed blood with M 115 served as a good maintenance medium and, in some experiments, apparently "induced the formation of normal young adults in 10 to 14 days of culture". However, the authors did not elaborate further; it is assumed that no segmentation occurred.

As mentioned earlier, sexual differentiation of M. corti tetrathyridia in vitro, has only recently been achieved by two independent groups. Similar culture systems were used, modified from those described by Smyth & Davies (1974b), Smyth (1979a) and Smyth & Barrett (1979). Thompson, Jue Sue & Buckley (1982) reported consistent strobilisation of worms using a diphasic culture medium (CMRL 1066 supplemented with glucose, yeast extract, dog bile and foetal calf serum, over a coagulated bovine serum base), with development in vitro being comparable to that in vivo. However, although self copulation was frequently observed, fully developed oncospheres were not produced in vitro. In contrast, Barrett, Smyth & Ong (1982) initially observed only sporadic segmentation, using mono- or diphasic media (as above but with different antibiotics), but a few shelled eggs with hooked oncospheres were obtained from cultured worms. It was therefore considered that the developmental differences in vitro observed by the two groups, indicated the possible occurrence of two strains of M. corti (Barrett, Ong & Smyth, 1982). However, from recent experiments in this laboratory, including the in vitro culture of tetrathyridia from both sources, and isoenzyme studies, it

appears that earlier dissimilar results were probably due to different culture methods employed in the two laboratories. In fact, tetrathyridia can now be consistently grown in vitro, with the majority segmenting and attaining sexual maturity, (as seen by Thompson, Jue Sue & Buckley, 1982), with the production of a few, mature oncospheres (S.J. Ong & N.J. Barrett, personal communication/observation). Since Voge (1967) and Voge & Seidel (1968) have successfully cultured M. corti in vitro from oncosphere to tetrathyridia, it is feasible therefore, that, once a large number of shelled ^geggs can be obtained from cultured worms, the entire life cycle of this parasite could be maintained in the laboratory.

3.3. Taenia crassiceps

Taenia crassiceps larvae have long been recognised as particularly suitable laboratory models for a variety of studies, on account of their ease of maintenance and rapid proliferation in laboratory mice. However, the in vitro culture of the asexually budding cysticerci to the adult worm has received relatively little attention.

In 1963, three papers were published, coincidentally all concerned with the maintenance of T. crassiceps larvae in vitro. Firstly, Voge (1963) was unable to sustain cysticerci from mice, in various media which had previously proved successful for the cultivation of other cestodes (e.g. Mueller, 1959a,c; Berntzen, 1961). However, using the tissue culture medium of Fogh & Lund (1957 - see Specht & Voge, 1965), she effectively maintained the larvae for up to four months in roller tubes, with a gas phase of air, or 10% O₂, 5% CO₂ in N₂. In some cultures the cysticerci increased slightly in length and formed a new bud, but no other development was observed.

In contrast, Robinson, Silverman & Pearse (1963) maintained T. crassiceps larvae in Medium 199, and observed asexual budding in vitro, with the development of new scoleces armed with hooks and suckers. These workers also briefly mentioned strobilization of mature cysticerci, but did not elaborate

further.

In the third publication, Taylor (1963) attempted to culture larvae of the ORF strain of T. crassiceps in a variety of media, after preliminary experiments on the survival times of cysticerci in Balanced Salt Solutions (BSS). Pretreatment with pepsin, trypsin and sodium taurocholate appeared unnecessary for the establishment of the larvae in vitro, although those subjected to enzymic digestion were initially more active and longer than untreated worms. However, the authors' observations of further development in vitro, are somewhat contradictory. In initial experiments approximately 50% of worms developed into strobilicerci during a 31 day culture period in Eagle's Medium; no strobilization was observed. In later cultures, using the same medium, larvae remained active in vitro for 25 days, by which time the majority "showed well developed lateral excretory canals, and, in some cases, signs of internal segmentation". Following unsuccessful attempts with Medium 199, NCTC 109 and Weymouth's medium, Taylor (1963) reverted to using Eagle's Medium, this time supplemented with sodium taurocholate and additional glucose. The author finally concluded that this was "the most successful medium". However, although the larvae grew 5 mm longer than those in Eagle's Medium alone, they did not survive for a greater period of time, and no mention was made of any strobilar development.

The first successful attempt at growing T. crassiceps larvae (KBS and Toi strains) to the strobilate stage in vitro, was reported by Esch & Smyth (1976). The culture protocol was adapted from that described by Smyth (1967) for E. granulosus. A diphasic medium of Parker's 858, supplemented with inactivated calf serum, yeast extract, glucose, KC₂^L and antibiotics, with a coagulated bovine serum base, was found to be suitable for segmentation, the serum base being essential for the early development of the genitalia. Pre-treatment with enzymes and/or bile salts was found to be unnecessary for growth in vitro, as previously observed by Taylor (1963). Esch & Smyth (1976) described the development of the cysticerci in

detail, from neck elongation and the appearance of ~~om~~^smoregulatory canals, to proglottid formation and the appearance of genitalia. The male components of the reproductive system were clearly defined by day 13 (although no sperm were reported), whereas, only the anlagen of vitellaria and ovaries were identified. Despite maintaining cultures for periods of up to 80 days, no further development was recorded.

Subsequently, Rickard & Smyth (unpublished observations - see Chew, 1981) simplified the procedures of Esch & Smyth (1976) and reported some comparable results. However, they were able to achieve strobilate development of T. crassiceps larvae to early sexual development in a mono- rather than diphasic medium (cf. Esch & Smyth, 1976). In both cases, the liquid medium was based on Parker's 858.

Chew (1981) carried out a large number of experiments on the in vitro culture of cysticerci; the best results were obtained with the KBS strain. The authors' results of development in vitro were compared with in vivo material from dogs, and supported by comprehensive ultrastructural studies. Optimum development was achieved using a diphasic medium of a liquid phase similar to that described^b by Esch & Smyth (1976), (replacing KCl with dog bile), and a coagulated newborn calf serum macerate. Pretreatment with pepsin and a bile-complex solution proved advantageous, in contrast to the previous authors' observations. However, the maximum growth observed was less than that described by Esch & Smyth (1976); only the anlagen of the cirrus, ovaries, vitellaria and testes, along with a genital pore, were seen. Nevertheless, Chew (1981) subsequently obtained sexually mature, gravid adults by partial maturation of T. crassiceps in dogs for 17 days, followed by cultivation in vitro for a further 14 days. Ultrastructural studies revealed, however, that only a few normal eggs were produced.

A culture system for the consistent growth of T. crassiceps cysticerci in vitro, to sexually mature, egg-laying adults has yet to be achieved.

3.4 Taenia hydatigena

In vitro studies with Taenia hydatigena have been carried out by only one group of workers from Spain (Osuna-Carrillo and colleagues, 1977-). Preliminary research by Osuna-Carrillo, Mascaro-Lazcano, Guevara-Pozo and Guevara-Benitez (1977), was concerned with the influence of different pH values and dissolved oxygen tensions on the evagination of cysticerci in vitro. Using a solution of pancreatin, trypsin and dog bile in HBSS, they deduced that optimum results (i.e. 100% evagination in 0.5h) were achieved at pH 7.2 with an O₂ tension of 6.1 ppm. Further studies by the same group (Osuna-Carrillo et al, 1978b) demonstrated that 100% evagination occurred most rapidly with trypsin, pancreatin and dog bile used together. Only 75% evagination was achieved when the components were used separately, and, even though 100% evagination occurred with any two of the three supplements to HBSS, the process needed at least 16h rather than 0.5h.

Subsequently, Osuna-Carrillo et al (1978a) were able to describe the in vitro culture of T. hydatigena cysticerci from cattle, to the "non-fertile, adult phase". Following pepsinisation, and evagination (as above), the larvae were cultured in a diphasic medium (CMRL 1066 supplemented with foetal bovine serum, yeast extract, glucose and KCL, gassed with 10% CO₂, 5% O₂ in N₂, over a coagulated bovine serum base). The early stages of the development in vitro of this species were (not surprisingly) very similar to the detailed observations of Esch & Smyth (1976) and Chew (1981) for T. crassiceps, but with segmentation occurring from day seven rather than day four. In spite of much individual variation, the maximum rate of maturation achieved was very similar to that obtained in vivo (as described by Featherston, 1969). However, the segmented worms did not reach full, sexual maturity, although anlagen of the genitalia were apparent from day 17 in vitro. It is conceivable that, as with T. crassiceps, further in vitro experiments with T. hydatigena cysticerci, should result in their development to the mature

adult stage.

3.5 Taenia pisiformis

Osuna-Carrillo & Mascaro-Lazcano (1982) have recently been able to record successful development of Taenia pisiformis to the sexually mature adult, in vitro. Larvae removed from the intestinal mesenterium of infected rabbits were initially subjected to washes in antibiotics and then HBSS. Following manual removal of the pericystic membrane and further rinses in HBSS, the larvae were pepsinised and then incubated in an evaginating solution of trypsin, pancreatin and canine bile in HBSS (as for T. hydatigena). The evaginated cysticerci were cultured in CMRL 1066, with 25% foetal calf serum and additional glucose and KCl, over a base of coagulated bovine serum. The media was gassed ^{with} the 10% O₂ and 5% CO₂ in N₂. Segmentation was observed from day eight and developing female genitalia and genital pores were seen after 16 days in vitro. By 29 days the testes were well developed. However, insemination was never observed, and no eggs were obtained in culture, although some proglottids were shed from day 32. Osuna-Carrillo & Mascaro-Lazcano (1982) compared the development of T. pisiformis in vitro with that of other Taeniidae, and concluded that the tapeworms of canids appeared to have similar requirements; for example, a diphasic medium was necessary for strobilization in vitro. They also suggested that, for insemination to occur, some type of mechanical/physical compression is necessary; (first demonstrated by Smyth (1954) as essential for Schistocephalus solidus in vitro and later discussed by Smyth & Davies (1974b), regarding the lack of shelled eggs in cultured E. granulosus). No other reports on the cultivation of T. pisiformis in vitro have been found.

3.6 Taenia serialis

Taenia serialis was cited by Smyth (1969b) as being a suitable parasite model for cytodifferentiation. By studying the development of protoscoleces (which had been removed from a

coenurus) in vitro, he was able to observe the ability of this species to differentiate in two directions (i.e. strobilar or vesicular), as in Echinococcus spp. The culture methods used were a slight modification of those employed for E. granulosus (Smyth, 1967). Following pre-treatment with pepsin, pancreatin, trypsin and dog bile, evaginated protoscoleces were cultured in Parker's 858 (containing extra potassium and glucose), plus 20% coenurus cyst fluid, with a solid base of coagulated beef serum. Segmentation was observed in all diphasic cultures, but maturation did not proceed past the appearance of genital anlagen. In monophasic media, very few worms segmented, and somatic growth was limited. However, in all cultures some strobila showed abnormal development and eventually became vesicular. In addition, the stalk regions, detached from some scoleces within the first 48 h of culture, also became cystic, but no germinal buds or other signs of developing protoscoleces formed. No further attempts to culture this relatively rare parasite to the sexually mature adult stage in vitro have been reported.

3.7 Echinococcus granulosus

The ability of E. granulosus protoscoleces to develop in one of two directions (as with E. multilocularis) has fascinated workers for many years, especially as early in vitro cultures of this species resulted in cystic, rather than strobilar, differentiation (Smyth, 1962, 1964a). However, in 1966, Smyth, Howkins & Barton were the first to achieve segmentation of E. granulosus protoscoleces in vitro, following the establishment of a basic technique for axenic cultivation (Smyth, 1962). Continuing with a variety of experiments (explained in greater detail by Smyth, 1967, whose in vitro culture techniques provided a basis for the successful culture of many of the canid cestodes included in this review), the authors demonstrated that the conditions required for protoscoleces to differentiate in a strobilar direction in vitro were (a) evagination of the scolex, and (b) a nutritive substrate with which intimate contact could be established. In addition, the usual physiological requirements such as pH, Eh,

gas phase, etc., had to be met within reasonable limits. Failure to provide such conditions (by using a monophasic medium, for example) resulted in the alternative cystic development. Prior to cultivation, scolex evagination ~~evagination~~ was accelerated by pepsinization, followed by treatment with pancreatin, trypsin and dog bile (or equivalent bile salts). In culture, a diphasic medium of Parker's 199 Medium plus 20% hydatid fluid, over a coagulated bovine serum base, and a gas phase of 8% O₂ and 5% CO₂ in N₂, stimulated segmentation from about 30 days in vitro with three proglottids being formed by 60 days. However, the development of the genitalia was severely limited at first (Smyth et al, 1966), although improved techniques resulted in the appearance of genital anlagen and well-defined genital pores, with spermatogenesis being observed in the best cultures (Smyth, 1967). In addition, destrobilization was occasionally noted.

In the same year, Smyth, Miller & Howkins (1967) observed the pattern of strobilar development in vivo, following oral infection of dogs with E. granulosus from sheep, and then autopsying at various intervals; thus, they established criteria for assessment of the development of this species in vitro. The authors continued their culture experiments with a variety of diphasic media, and concluded that segmentation occurred earliest (22 days) in Medium 858 with 20% hydatid fluid, gassed with 5% CO₂, 10% O₂ in N₂, over a bovine serum base. Sexual maturity was attained in this medium, following addition of 5% beef embryo extract at the onset of strobilization, although maturation of the protoscoleces took more than twice as long as that observed in vivo, and very few worms in each culture reached the three-proglottid stage. Furthermore, despite the presence of abundant spermatozoa in the testes and vas deferens, and free cells in the uterus (presumed to be ova), no fertilized eggs were obtained in culture. Following comparisons with in vivo material, Smyth et al (1967) concluded that insemination did not occur in vitro, since the receptaculum seminis was always devoid of sperm and therefore the ova could not be fertilised.

In 1971, Smyth reported the unusual occurrence of "monozfloc" forms of E. granulosus in vitro, as later recorded by Smyth & Davies (1975) during the cultivation of E. multilocularis. This was observed in only one of the "several hundreds of cultures" however, and it was speculated that this development represented "mutant" protoscoleces, or was induced by unusual experimental conditions (although the majority of worms in the same culture vessel, segmented). The "monozoic" phenomena in Echinococcus spp. was later reviewed by Smyth (1978a).

The same author, working on E. granulosus of sheep origin in the U.K., rather than in Australia, subsequently modified the culture media to contain 5% yeast extract, with additional glucose and K⁺, which resulted in the maturation times in vitro approaching those in vivo, in the most successful cultures (Smyth, 1971).

In 1974, a detailed account of the results and problems encountered during the in vitro culture of E. granulosus protoscoleces from sheep, was published by Smyth & Davies (1974b). Although Parker's 858 was the preferred basic medium, the authors stated that CMRL 1066 and NCTC 135 were satisfactory substitutes. An additional alteration to the previously described culture medium was the use of foetal calf serum instead of hydatid fluid, the quality of which was subject to much inter-cyst variation. In an attempt to induce insemination and hence fertilization in vitro, worms were subjected to a variety of "compression" systems (also reviewed by Smyth, 1982b), but these proved unsuccessful, as did initial experiments using a circulating culture system.

Turning their attentions to material from other intermediate hosts, Smyth & Davies (1974a) described the lack of development in vitro of E. granulosus protoscoleces of horse origin. Using their established culture system, in which protoscoleces from sheep readily segmented and developed into sexually mature adults, they observed that larvae of horse origin did not grow past "stage 3" (see later, and Smyth

& Davies, 1974b). Numerous attempts to induce segmentation by varying many of the parameters involved in culture work, proved unsuccessful. Subsequently, these authors concluded that the physiological differences shown by in vitro culture work reflected the existence of two strains of E. granulosus. This was elaborated by Smyth & Davies (1979), who had further modified the culture medium by supplementing it with dog bile, increasing the foetal calf serum level to 25%, and removing the extra K^+ . The evagination fluid had also been simplified to 5% bile, or 0.2% sodium t α urocholate, in HBSS, following pepsinisation of the protoscoleces (see also Smyth & Davies, 1974b). However, although a high percentage of the horse protoscoleces readily evaginated (in contrast to the sheep material), no segmentation could be induced in vitro, despite the further addition of growth factors, vitamins, blood, etc., to the medium.

Smyth (1979b) then reported preliminary experiments on the in vitro culture of E. granulosus larvae of cattle origin. In a small number of cultures, the author observed "virtually no growth", although some worms survived for 33 days in vitro. Thus, he tentatively concluded that "protoscoleces of cattle origin fall into the same developmental pattern as that of the horse strain".

In Spain, Osuna-Carrillo (quoted by Smyth, 1979b), using only a single culture, found that E. granulosus protoscoleces from pigs, also failed to segment in vitro.

Additional studies on the in vitro culture of protoscoleces from a variety of natural intermediate hosts (human, cattle, camel, goat, sheep), have been undertaken in Kenya by Macpherson (1981). Unfortunately, many of the authors' cultures were lost due to fungal contamination, with only a single goat, and one cattle culture being maintained for longer than 16 days. Using the procedures described by Smyth & Davies (1979), and their culture medium S10 (where the basic medium was NCTC 135 or RPMI 1640), Macpherson (1981) observed development to the "presegmentation stage 3" (i.e. "stage 3" of

Smyth & Davies, 1974b) in all viable cultures, by day nine (the camel material survived for only six days). No further growth was seen in the human and sheep cultures (which were lost on day 16), or in the cattle material, one culture of which was maintained for 37 days in vitro. However, the single "successful" goat culture showed signs of segmentation ("banding") by day 21, with two proglottids eventually being formed by 59 days. No reference was made to the appearance, or development of the genitalia. More recent culture experiments by Macpherson (personal communication) have resulted in the survival of E. granulosus from camels for up to 61 days in vitro, and the development of two-segment, adult worms with genital anlagen. Also, further work on cattle material has produced interesting results, in that the protoscoleces segmented from day 24, with three proglottids being observed by day 39 (cf. Smyth, 1979b; Macpherson, 1981). Subsequent to this, Macpherson & Smyth (1984) have described the culture of material from a variety of intermediate hosts in Kenya. Protoscoleces of human, camel, cattle, sheep and goat origin (from Kenya) as well as buffalo material (from India), all developed and segmented in vitro. Genital anlagen were apparent in all cases, but further maturation was only seen in the cattle and camel material, in which testes were observed. Macpherson & Smyth (1984) concluded that, following their observations on the similar pattern^{of} in vitro development of E. granulosus protoscoleces from a variety of hosts in Kenya, and supported by biochemical evidence (McManus, 1981; Macpherson & McManus, 1982), the strains observed may all be potentially infective to man.

The use of in vitro culture as a tool in the epidemiology of hydatid disease, namely by identifying the various strains, which are infective to man has also been emphasised by Smyth, McManus, Barrett, Bryceson & Cowx (1980), who for the first time, cultured E. granulosus protoscoleces of human origin. The material from a Saudi Arabian patient, differentiated to the segmented stage, with the appearance of genital anlagen. The developmental pattern in vitro thus corresponded to that of the sheep strain (UK), (along with the strains studied by

Macpherson & Smyth, 1984) and fitted with circumstantial, epidemiological evidence from Saudi Arabia, in that the sheep/dog strain may have been the source of human infection.

Smyth (1979a) gave a comprehensive account of the in vitro techniques currently employed for the successful culture of E. granulosus (and E. multilocularis) protoscoleces of UK sheep origin, to sexually mature, adult worms (cf. Macpherson & Smyth, 1984). However, fertile eggs have yet to be obtained in any material cultured from the larval stage. Nevertheless, shelled, viable oncospheres have been obtained in vitro by the method of transferring partially developed strobila of E. granulosus, from dogs to culture vessels (Smyth & Howkins, 1966, in the UK; Kumaratilake & Thompson, 1981, in Australia). This same protocol (described earlier) has also proved successful with E. multilocularis (Thompson & Eckert, 1982).

B. MATERIALS AND METHODS

1. Source of material

1.1 Echinococcus multilocularis

Larval E. multilocularis was maintained in laboratory rodents - namely Cotton rats (Sigmodon hispidus, obtained from the National Institute of Medical Research, Mill Hill, London) and Gerbils (Meriones unguiculatus, bred in the Department of Pure and Applied Biology, Imperial College, London) - by intraperitoneal (i.p.) injection. The susceptibilities of these animals to infection has previously been reported by Sadun², Norman, Allain & King (1957) and Norman & Kagan (1961). The primary inoculum was obtained from the London School of Tropical Medicine and Hygiene in 1971, and was originally extracted from a naturally infected intermediate host in Germany.

Infected animals were killed with chloroform and cervical dislocation, and their abdominal cavity opened. Following removal of the larval tissue, the inoculum was prepared in one of two ways: (1) the material was macerated in an equal amount of Hank's Basic Salt Solution (HBSS - Flow Laboratories or Gibco Europe Ltd.) in a Waring Blendor (Waring Products Corporation) for 10-20s at slow-speed, or, (2) the tissue was coarsely chopped with scissors, in a beaker, and an equal amount of HBSS was then added. Although Hinz (1972b) reported that the highest rate of parasitic growth in experimentally infected rodents, was attained with manual, rather than mechanical trituration of the larval tissue, great care was necessary when employing this method. The bursting of brood capsules and consequent, sudden release of hydatid fluid and protoscoleces, in an open, rather than closed, container was obviously, potentially more dangerous to the operator. However, the dissection of the animals, and subsequent procedures were always carried out in a Laminar-flow Cabinet (Slee), and protective glasses was always worn by the operator

and observers.

Whichever method was used, the material was then sieved through a fine-mesh strainer, and any remaining debris was rinsed with a little HBSS. Cotton rats were infected i.p. with 0.5 ml of the resultant inoculum, following anaesthesia with CO₂ gas, whereas gerbils received 0.2 ml, without any anaesthetic. A large bore hypodermic needle was used in both instances - 19G x 1 1/2, 40 mm 11/10 (Gillette Surgical).

1.2 Echinococcus granulosus

Livers and lungs from sheep, and horse livers, infected with the larvae of E. granulosus, were obtained from abattoirs in the UK, most material coming from Birmingham and Bristol respectively. Hydatid fluid containing protoscoleces from cysts in cattle lungs, was sent from University College, Dublin, Eire. Human hydatid material, in the form of complete unilocular cysts, or aspirated hydatid fluid, was collected from the Hospital for Tropical Diseases, London, and the Royal Hallamshire Hospital, Sheffield.

2. General Sterile Procedures

Aseptic techniques for the preparation and maintenance of in vitro cultures, were used throughout. All glassware, instruments and apparatus were sterilised, and the culture procedures described below were carried out in a Laminar-flow Cabinet.

All pipettes were thoroughly soaked in Sodium hypochlori^tde solution (BDH) and the rest of the glassware and other apparatus was washed in Pyroneg (Diversey Ltd.), prior to repeated rinses in distilled water. The ends of pipettes (once cleaned and dried) were plugged with non-absorbent cotton wool, as were other small unstopped openings on apparatus, the larger ones being covered with aluminium foil and autoclave tape. All glassware etc, was then placed inside autoclave bags and

sterilised in a Pulsmatic Autoclave (Getinge) at 2 Kg cm⁻² pressure (135°C for 7 min for pipettes, and 121°C for 20 min for other glassware and apparatus). Dissecting instruments were boiled in a steriliser (Surqual), immediately prior to use.

All culture media and salt solutions were prepared in triple-distilled water (Gibco) and subsequently sterilised, either by vacuum or pressure filtration, through a 0.2 μ m pore size, cellulose acetate filter, and thick glass fibre prefilter (Sartorius; Gelman Hawksley Ltd.; Millipore Corporation). All solutions were then incubated at 37°C for 48h, to test for sterility, prior to the addition of antibiotics.

3. Physical Conditions of Cultures

3.1 Culture Vessels

The following culture vessels were used: -

(a) Leighton tissue culture tubes (Bellco)

These ~~were~~ short style, borosilicate glass tubes were the most frequently used vessels, due to their small, convenient size and excellent optical properties. Three different tubes were tried, the middle ones being the preferred size: -

16 x 93 mm (to contain 5 or 10 ml media)
19 x 105 mm (10 ml)
28 x 120 mm (20 ml or more)

(b) Milk Dilution (M.D.) bottles (Kimax)

For culture work, 20 ml or more, media was used in these 250 ml capacity glass bottles.

(c) Tissue culture flasks (Costar)

These plastic, 50 ml capacity culture vessels were bought, presterilised (by radiation). Either 10 or 20 ml media were used in Costar flasks.

(d) Tissue culture flasks (Sterilin)

These larger, plastic culture containers (200 ml) were also irradiated prior to purchase. At least 50 ml media were used in Sterilin flasks.

(e) Circulatory culture system, or 'stationary' lift (Smyth, 1969c; Smyth & Davies, 1974b)

This custom-made glass apparatus, (it's design based partly on Berntzen's (1962) recirculatory unit, successfully used for the cultivation of Hymenolepis nana), allowed a continual flow of gassed media to pass through the culture system. In the lower flask, through which media was circulated in cellulose tubing, "undisturbed" protoscoleces of E. granulosus have already been grown to maturity in diphasic media (Smyth & Davies, 1974b).

3.2 Agitation

In some experiments, the cultures were not subjected to any movement at all, being kept upright in a water-bath or incubator, but usually one of two different types of agitation were used: -

(a) Shaking water-bath (Gallenkamp)

The culture vessels were gently agitated in a simple harmonic, horizontal motion, which was either continuous (60-70 revolutions per minute) or intermittent (3-4 min shaking, 3 times per hour).

(b) Roller apparatus (LEEC)

The culture vessels, specifically Leighton tubes, were rotated at an angle of approximately 20° to the horizontal, at a rate of 12 revolutions per hour. The apparatus was kept in an incubator.

3.3 Gas phase

The following gas phases were used: -

10% CO₂, 5% O₂ in N₂
5% CO₂, 10% O₂ in N₂
10% CO₂, in N₂
20% CO₂, in N₂
Air

Sterile gas (except to the air phase) was bubbled through the liquid medium in the culture vessels, whenever it was renewed. For the air phase, culture vessel tops were loosened slightly in the incubator.

3.4 pH and temperature

In the majority of experiments, the pH was maintained at about 7.2 to 7.4. Any intentional deviation from this will be detailed in the results. Phenol red in the basic culture media and Balanced Salt Solutions (BSS) acted as a colour indicator. When reconstituting powdered media and BSS, sodium bicarbonate was added in the quantity indicated by the manufacturers. In later experiments, hepes (Flow; Research Organics Inc.) was found to be an excellent additional buffer, and was used at a concentration of 20 or 25 mM. In these cases, the amount of NaHCO₃ added did not exceed 10 mM (i.e. 0.85 g/l), as recommended by one of the manufacturers (Flow).

The routine temperature used throughout, for all cultures, was 37-38°C.

4. Culture Media

Both mono- and diphasic culture media were tried.

4.1 Liquid phase

The culture media was usually made up of commercially available basic tissue culture media, and supplemented with serum, yeast extract, glucose, dog bile and antibiotics. The liquid phase most routinely used, designated S.10.E, was similar to that described as S.10 by Smyth (1979a), Smyth & Barrett (1979) and Smyth & Davies (1979), except that Parker's 858 was replaced by CMRL 1066, and the foetal calf (or bovine) serum (FCS) was not inactivated (see Table 2).

Other liquid media used were named as follows: -

S.10.A : equivalent to S.10 (Smyth, 1979a),
with Parker's (TC) 858

S.10.B : S.10, with NCTC 135

S.10.C : S.10, with RPMI 1640

S.10.D : S.10, with CMRL 1066

[S.10.E : as S.10.D, but FCS not inactivated (see Table 2)]

S.10.F : as S.10.D, but with inactivated newborn calf serum (NBCS) instead of FCS

(all the basic tissue culture media were obtained from Flow, Gibco or Difco Laboratories; FCS and NBCS, from Flow or Gibco).

The components of medium S.10.E (and other defined media described above), were prepared as follows: -

Table 2Liquid culture medium: -S.10.E

CMRL 1066 (Flow;Gibco)	260 ml
Foetal Calf (Bovine) Serum (Flow; Gibco)	100 ml
Yeast Extract (Gibco) (5% in CMRL 1066)	36 ml
Glucose (BDH) (30% in distilled water)	5.6 ml
Dog bile (5% in HBSS (Flow; Gibco))	1.4 ml

including 20 mM hepes (Research Organics; Flow), 10 mM
 NaHCO₃ (BDH) and 100 μ g/ml ~~#~~ Gentamicin (Flow; Gibco).

(a) CMRL 1066

This powdered medium was reconstituted according to the manufacturers instructions, with the addition of NaHCO_3 and hepes in appropriate amounts, so that their ultimate concentration in S.10.E was 10 mM and 20 mM, respectively. One litre batches were usually prepared at a time, the media then being sterilised as described in General Sterile Procedures. CMRL 1066 was stored at 4°C in glass, medical pattern bottles (Gallenkamp).

(b) Serum

In S.10.E, the FCS (which was stored at -20°C and kept thawed for short periods at 4°C) was not inactivated. In other culture media, 100 ml bottles of presterilised sera were heated in a water bath at 56°C for 30 min to inactivate it.

(c) Yeast extract

A stock solution of 5% yeast extract was made up in CMRL 1066 (or any of the other tissue culture media, depending on the basic culture medium used). Prior to sterilisation (as described in General Sterile Procedures), the solution was prefiltered through filters of 5.0 μm , 1.2 μm , 0.8 μm , 0.45 μm and 0.2 μm pore sizes, in sequence. The sterile stock-solution was stored in 100 ml bottles at 4°C.

(d) Glucose

A stock of 30% glucose solution was prepared in distilled water, and then sterilised and stored at 4°C, as above.

(e) Dog bile

Bile was collected freshly from the gall bladders of dogs (slaughtered at a dog pound), and stored frozen. A 5% solution was made up in HBSS, as necessary, sterilised, and stored at 4°C, as previously described. Sometimes, prefiltration was

necessary, as with the yeast extract solution.

(e) Antibiotics

Penicillin and streptomycin, available as a mixed solution of concentration 5000 I.U./ml and 5000 $\mu\text{g}/\text{ml}$ respectively, was kept frozen (-20°C) until required, and then stored at 4°C . In early culture work, these antibiotics were routinely added to the sterilised basic tissue culture medium (e.g. CMRL 1066) which had been incubated for 48h at 37°C , to test for sterility. A level of 2 ml/100ml to gave a final concentration (in CMRL 1066) of 100 I.U./ml (penicillin) and 100 $\mu\text{g}/\text{ml}$ (streptomycin). In later cultures, gentamicin solution (50 $\mu\text{g}/\text{ml}$) was found to be a more suitable antibiotic. It was stored, presterilised, at 4°C , and added to the culture medium (e.g. S.10.E) at a rate of $\frac{2\text{ml}}{100\text{ml}}$ to give a final concentration (in S.10.E) of 100 $\mu\text{g}/\text{ml}$. Balanced salt solutions, such as HBSS, always contained 100 I.U./ml and 100 $\mu\text{g}/\text{ml}$, of penicillin and streptomycin, respectively (unless otherwise indicated).

The liquid culture medium S.10.E (and others as well), was compiled from the above sterile constituents, as necessary, in batches of 201.5 ml or 403 ml (see Table 2). Following incubation for 48h at 37°C , it was kept at the same temperature in medical pattern bottles whilst in use, but for no longer than 4 weeks. Liquid media in cultures were renewed every 2 to 4 days.

Variations of the liquid culture medium S.10.E (apart from those already described) included the addition of extra dog bile, sheep red blood cells (RBC - Flow; Gibco), dog gut extract (see Webster & Cameron, 1963) and beef embryo extract (BEE - Flow; Gibco). H.40, a medium described by Webster & Cameron (1963), who achieved some success in the cultivation of E. multilocularis in vitro, was also tried in one experiment. Precise details of the different media employed, will be given in the Results Section where appropriate.

4.2 Solid phase

Coagulated bovine serum was the most commonly used base in diphasic media. Aliquots of presterilised NBCS were heated in culture vessels at 76°C until the serum became opaque and solidified. The length of time (usually between 1/2h and 1 1/2h) depended on the amount of sera, and culture vessel, used.

Coagulated NBCS was also used to make a macerate, using a Waring Blendor, or tubular homogeniser (or even by chopping the solidified serum with a sterile pipette), and then adding HBSS, or a basic tissue culture medium. Macerate was stored at 4°C until required.

The solid phase was sometimes renewed or replenished during an experiment, but not as often as the liquid media.

As with the liquid phase, extra constituents were sometimes added to the NBCS prior to coagulation (such as sheep RBC, dog blood (collected fresh and stored at 4°C in HBSS), and BEE). Macerate was also made from these mixtures. Alternatively, the serum base was replaced by solidified 2 or 3% agarose (Bio-Rad Laboratories) in HBSS, and was sometimes supplemented with rice starch or BEE.

Other variations in the solid bases included making small holes in the surface with a sterile dissecting needle, or Pasteur pipette, or providing a rough surface by bubbling the serum prior to coagulation.

Details of the different bases used will be given, where appropriate, in the Results section.

5. Culture Techniques

5.1 Echinococcus multilocularis

(a) In vitro

The techniques outlined below, have been adapted from those described by Smyth (1979a). The protocol found to be most satisfactory for the preparation of E. multilocularis cultures is summarised diagrammatically in Fig.2, and detailed below.

(i) Infected rodents (usually 6-12 months p.i.) were killed by chloroform and cervical dislocation. On a dissecting board, the ventral surface of the animal was thoroughly painted with 1% iodine in 90% or 96% alcohol, and allowed to dry. This process was repeated. The abdominal cavity was then carefully opened to expose the multilocular infection. For culture work, cystic masses within the peritoneal cavity (either free, or loosely attached to the mesenteries or alimentary canal) were preferred. It was often impossible to cleanly extract material which had infiltrated the liver, and other viscera.

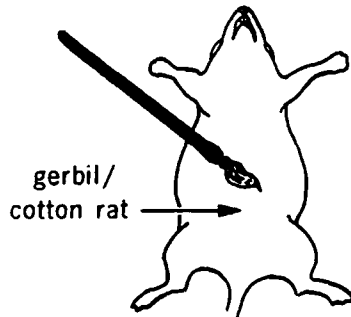
(ii) Approximately 10-20g larval tissue was used for each experiment, in which 10 large (e.g. in M.D. bottles) or 20 small (e.g. in Leighton tubes) cultures were usually set up at a time. Normally the material was processed immediately, but occasionally it was necessary to store the cysts in a covered sterile beaker^k, at 4°C, for up to 3 days (maximum).

(iii) The cysts were transferred to a Waring Blendor, and macerated for 10-20s at slow speed, in 100 ml sterile, 0.5% pepsin (Sigma) in HBSS (at pH 2.0), warmed to 37°C. The homogenate was then transferred to a covered sterile beaker^k (250 ml), and the Blendor rinsed with a further 20 ml pepsin. The mixture was stirred using a magnetic flea, in an incubator at 37°C, for approximately 45 min. This digestion process resulted in 'free' protoscoleces, and its progress was assessed by microscope examination of small samples, taken at intervals. Meanwhile, the pH was maintained at 2.0 (the Phenol red in the

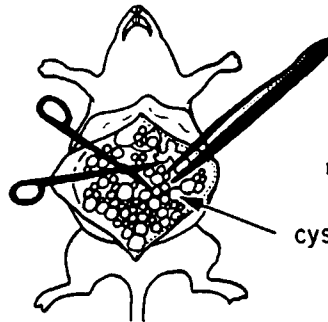
Fig.2

Protocol for the in vitro culture of
Echinococcus multilocularis protocolectes

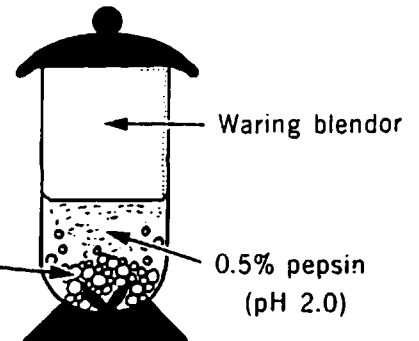
1. PAINT SURFACE TWICE
WITH IODINE



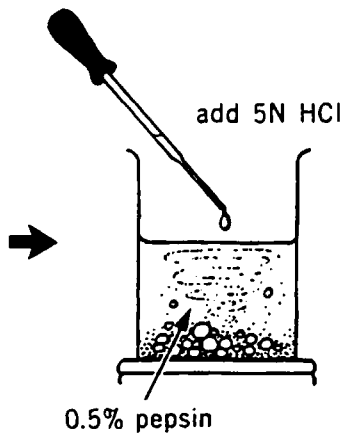
2. REMOVE CYSTS
FROM BODY CAVITY
ASEPTICALLY



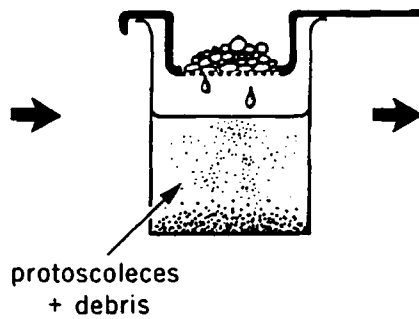
3. HOMOGENISE
IN PEPSIN



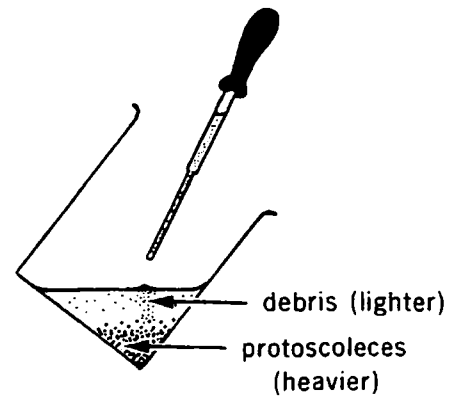
4. DIGEST IN PEPSIN
(MAINTAIN pH AT 2.0)



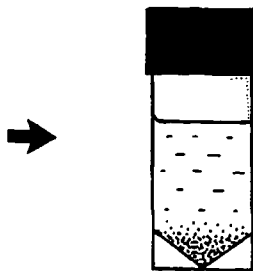
5. SIEVE UNDIGESTED
MATERIAL



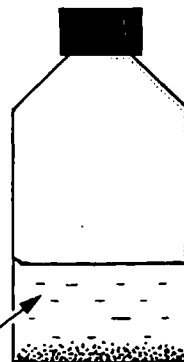
6. REMOVE DEBRIS



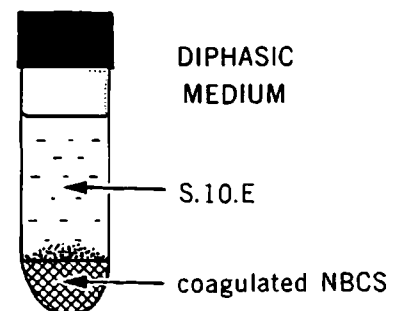
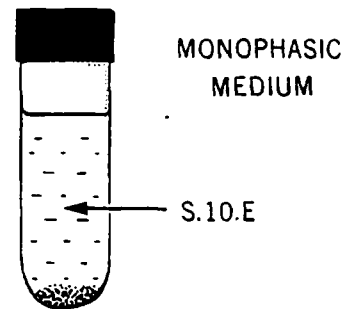
7. WASH IN HBSS $\times 4$



8. EVAGINATE IN
BILE MEDIUM



9. IN VITRO CULTURE



HBSS being a good colour indicator) by the addition, when necessary, of 5N HCl. Generally, the older the infection, the more calcified it was, and therefore, the more HCl was needed to keep the pH at 2.0.

(iv) Following pepsinisation, the mixture was sieved through a fine/medium mesh strainer into another, sterile beaker. The undigested material in the sieve was rinsed several times with HBSS (containing 20 mM hepes), to ensure that the majority of the protoscoleces were collected. The resultant 'free' larvae were then washed (in the beaker) in several changes of HBSS, using 50 or 100 ml at a time. The viable 'heavy' protoscoleces settled to the bottom quickly, whilst the 'lighter' dead larvae and membranous debris, were removed in the supernatant. The protoscoleces were then carefully transferred to 20 ml HBSS in a 30 ml Universal container (Sterilin Ltd); (the larvae readily adhered to glassware at this stage, and therefore, only very small aliquots were transferred at a time, using no more than the bottom 2 cm of the pipette).

(v) The material was subsequently subjected to further washes, this time in a Universal, on a rotator (Eschmann Bros.), in an incubator. Usually four rinses, each of 20-30 min duration, in fresh HBSS, was sufficient.

(vi) Finally, the protoscoleces were carefully transferred (as above) to an M.D. bottle containing gassed 'evaginating solution' (see Table 3), in which they remained for 18-24h, in a continuous shaking waterbath.

Following overnight evagination, the viability of the protoscoleces was assessed by microscopic examination, prior to setting up cultures. The majority of the evaginating solution was removed by pipette, and approximately a 0.5 ml suspension of protoscoleces was transferred to each culture vessel, containing gassed media (these had usually been prepared 24h beforehand, and incubated at 37°C, to test again, for sterility).

For experiments in which a variety of factors were being tested, at least three replicates of each culture type were used, and experiments repeated where possible. Approximately 1 ml settled protoscoleces (pre-evagination) was found to be necessary for these experiments, to be divided between 10 to 20 culture vessels.

(b) In vivo

The efficacy of the culture system described above, was assessed by comparing the development of worms in vitro with those matured in vivo. The protocol for experimentally infecting and collecting material from dogs was modified from that recently described by Thompson & Eckert (1982).

Three greyhounds of unknown age (but assumed to be over two years) were maintained in individual, isolated cages in the Animal Unit, St. Thomas' Hospital Medical School, London, for the duration of the experiment. They were fed daily on dehydrated food, and supplied with water ad libitum. One week prior to oral infection, the dogs were closed with Coopane^R (piperazine adipate) (Wellcome) for roundworms, and Yomesan^R (niclosamide) (Bayer) for tapeworms, according to the manufacturers' instructions.

Approximately 60g larval tissue was removed from a gerbil, 12 months p.i. Following a microscopic evaluation of percentage viability, the material was equally divided into three, and each multilocular mass, roughly chopped with scissors. The dogs were infected at their usual feeding time (i.e. they had last been fed, 24h previously), the larval tissue being mixed with a little canned pet food.

The three greyhounds were autopsied at 15, 20 and 21 days p.i., following euthanasia with Vetanacol^R (sodium pentobarbitol) (Veterinary Drug Co. Ltd.); they had last been fed, 20h previously. The prepatent period of E. multilocularis is known to be 28 days (Thompson & Eckert, 1982) and thus the

last dog was killed, well in advance of mature eggs being produced.

On each occasion, the abdominal cavity was rapidly opened, and the small intestine ligatured at either end, before removal. The gut was temporarily stored (1-1 1/2h) for transport to this laboratory in warmed, non-sterile HBSS, containing antibiotic antimycotic solution (Gibco) (1 ml/100 ml). For removal of the worms, the intestine was equally divided into six parts, each being slit open in a separate glass dish, to which was added 500 ml HBSS, containing 20 mM hepes, penicillin and streptomycin (100 I.U./ml and 100 μ g/ml) and antibiotic antimycotic solution (1 ml/100 ml). Following incubation at 37°C, for between 10 and 40 min, the relaxed worms were collected with Pasteur pipettes from the bottom of the containers, and temporarily stored in Universals containing HBSS for later microscopic examination. Some worms were collected for culture, and these were washed up to nine times in HBSS (containing 20 mM hepes, 100 I.U./ml and 100 μ g/ml penicillin and streptomycin, 2.5 μ l/ml fungizone (Squibb Amphotericin B - Gibco) and 100 μ g/ml gentamicin), over a period of about 3h. Initially the Universals were gently agitated by hand, but were later transferred to a rotator.

In the first experiment (i.e. 15 days p.i.), the worms for culturing, were subsequently washed twice in CMRL 1066, containing 25 mM hepes and antibiotic antimycotic solution (1 ml/100 ml), for 40 min before being put into culture vessels. In the next two experiments however, (i.e. 20 and 21 days p.i.) the worms for transferring to culture, were washed in two changes of S.10.E with 20 mM hepes, for 30 min, instead.

The culture vessels used were medium-sized Leighton tubes on a roller apparatus, and the medium was S.10.E (with hepes).

5.2 Echinococcus granulosus in vitro

The protocol described below for the preparation of E. granulosus cultures, is very similar to that already outlined

for E. multilocularis. Again, it is a slight modification of the techniques detailed by Smyth (1979a), and represented diagrammatically in Fig.3.

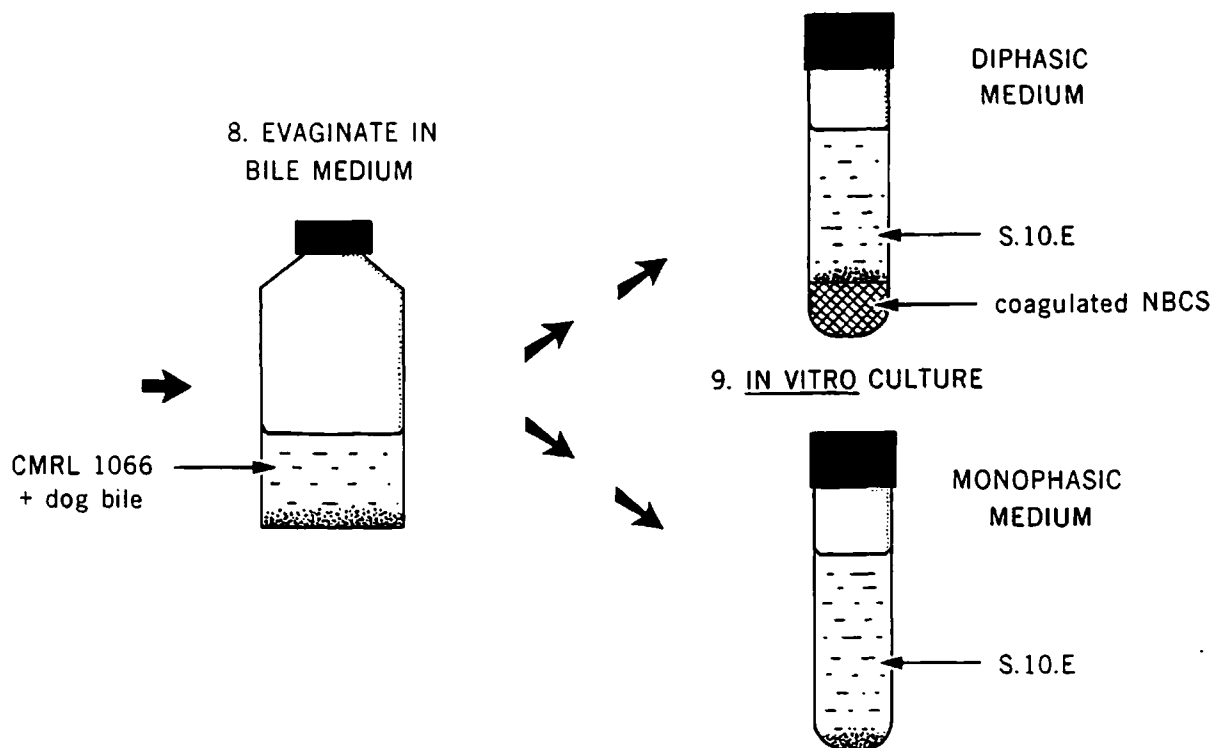
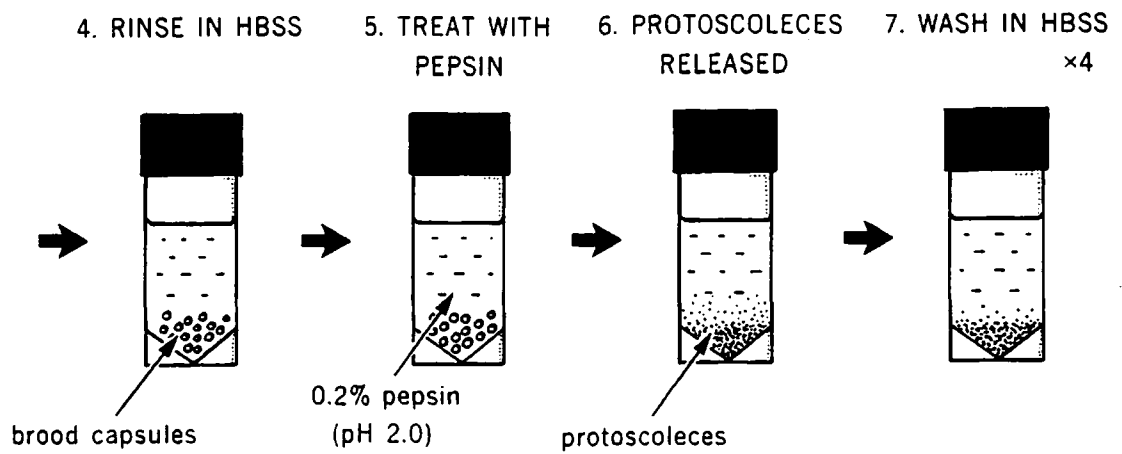
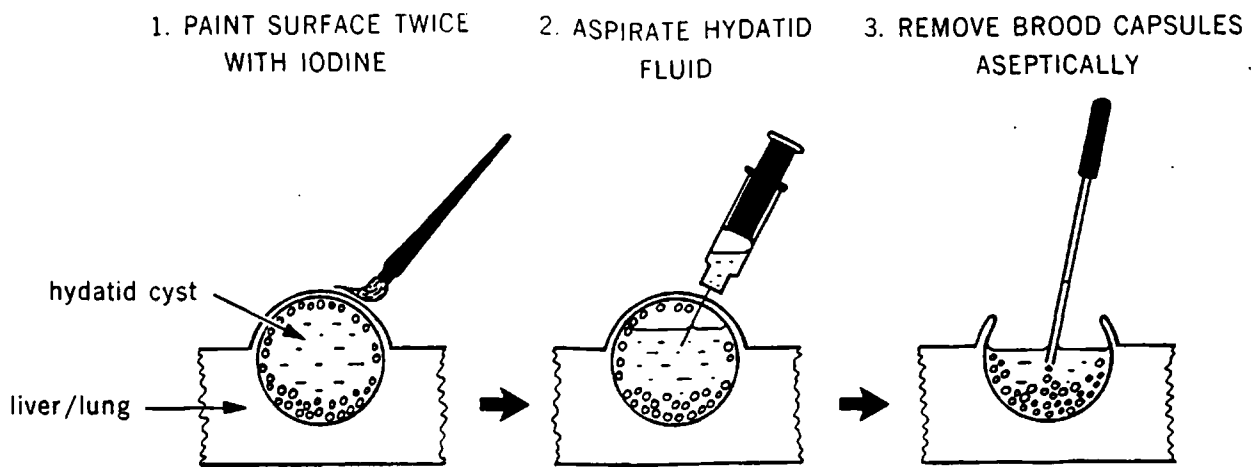
The surface of unilocular cysts was painted with alcoholic iodine, which was allowed to dry, and the process repeated. Using a large bore hypodermic needle (19G x 1 1/2) and syringe of appropriate size, the hydatid fluid was drawn off and transferred to a Universal. The top of the cyst was then opened with scissors. In most cases, the brood capsules could easily be removed with a 1 ml serological pipette and stored in the Universal containing the aspirated hydatid fluid. (this was possible with the sheep material, but several Universals were usually needed for each of the larger cysts found in horse livers). Brood capsules were easily detached from the germinal membrane with the end of the pipette, although they were often found deposited as 'hydatid sand' at the bottom of the cysts. Sometimes the laminated membrane tore easily, and in these cases, in order to extract all the brood capsules, it was easier to transfer the membranes to the Universal as well. No daughter cysts were found in any sheep or horse material, but they were not uncommon in large cysts of human origin.

For each experiment, using material of sheep, horse or human origin, the contents of each cyst (if more than one was available, or used) were placed in separate containers. Sterile cysts (i.e. those without larvae) were abandoned, as were those obviously infected, or degenerate and full of pus.

Prior to further treatment, the sterility and viability of each cyst was microscopically checked; the best ones were then pooled. E. granulosus protoscoleces/brood capsules received in hydatid fluid, rather than in situ (such as the cattle material), were treated from this stage in the procedures. Sometimes storage of the material was necessary prior to culture preparation. Protoscoleces were found to survive in situ for several days at 4°C, or within hydatid fluid in sterile containers without becoming abnormal. However, where possible, fresh material was used.

Fig. 3

Protocol for the in vitro culture of
Echinococcus granulosus protoscoleces
(after Smyth, 1979a)



The next step was to remove as much hydatid fluid as possible, leaving brood capsules and protoscoleces at the bottom of the Universal. The fluid was replaced by 20 ml warmed (37°C) 0.2% pepsin in HBSS (pH 2.0). The digestion process took about 20 to 45 min, on a rotator, in a 37°C incubator, its progress being assessed with the aid of an inverted microscope. The free protoscoleces were then washed in HBSS with 20 mM hepes, at least four times, as for E. multilocularis, and then transferred to the gassed evaginating solution (see Table 3). The techniques for setting up cultures on the following day, was the same as already described for E. multilocularis.

Fig. 4 outlines the procedures in more detail, relevant to the in vitro culture of human hydatid organisms.

Fig. 4

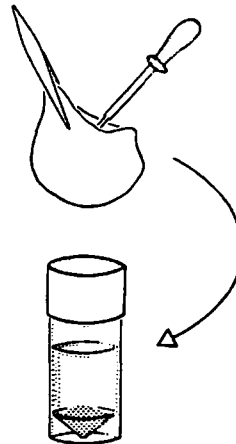
Procedures for the in vitro culture of human hydatid organisms: protoscolex to adult worm.

(drawn for the FAO/UNEP/WHO Guidelines on Surveillance, Prevention and Control of Echinococcosis/Hydatidosis, 1981; see also, Smyth & Barrett, 1980a)

PROCEDURES FOR *IN VITRO* CULTURE OF HUMAN HYDATID ORGANISMS:

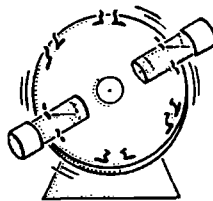
PROTOSCOLEX TO ADULT WORM

1. Collect brood capsules and free protoscoleces aseptically. Store in hydatid fluid or saline.



- check (i) % viability.
(ii) presence of bacteria.

2. Treat with 0.2% pepsin, pH 2.0, on rotator.¹

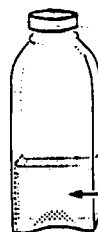


- check release of protoscoleces from brood capsules.

3. Wash protoscoleces in saline on rotator (x4).¹



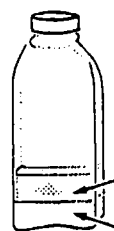
4. Evaginate protoscoleces in liquid medium.



CMRL 1066 + 1% dog bile

- incubate overnight in shaking water bath.^{1,2}

5. Set up cultures with diphasic media.



S.10.E

coagulated newborn calf serum

- incubate as above and change liquid media every 3 days.^{1,2}

(¹ temperature : 37°C; ² gas phase : 10% CO₂, 5% O₂ in N₂)

Table 3Evaginating Solution: -

CMRL 1066	50 ml
-----------	-------

5% dog bile in HBSS	1 ml
---------------------	------

including 25 mM hepes,
Penicillin (100 I.U./ml) and
Streptomycin (100 μ g/ml)

6. Assessment of Development

During in vitro culture of both E. multilocularis and E. granulosus protoscoleces, their development was continually assessed, using either fresh or fixed material.

Cultures were checked, at least every other day, with the aid of an inverted microscope (Zeiss), which was housed in a warm (37-38°C), perspex chamber mounted on a trolley. This allowed prolonged observation when necessary, without the risk of affecting worms by a temperature change. In addition, small samples were removed from some cultures at intervals, and examined microscopically at higher magnifications. Early observations, such as those to test the viability of protoscoleces, prior to culture, were carried out on unstained material, whereas the use of vital stains, such as 0.1% Neutral red (G.T. Gurr Ltd.) in HBSS, were found to be advantageous during the study of mature specimens.

The fixation of material for both light and electron microscopy will be dealt with in Section II. This Section (I) is concerned only with the gross morphological development of protoscoleces in vitro (and in vivo, for E. multilocularis).

Regularly written records of observed development in vitro, were supplemented with both diagrams (drawn with the aid of a Leitz Camera Lucida) and micrographs (taken on a Nikon inverted microscope within a warm chamber, for fresh material, or a Lietz Vario Orthomat system, attached to an Ortholux II, for both fresh and fixed specimens).

C. RESULTS

1. Echinococcus multilocularis

1.1 In vitro

Successful in vitro culture of E. multilocularis largely depended on the quality of the parasite material available. Both cotton rats and gerbils proved to be suitable laboratory hosts for the maintenance of larvae by intraperitoneal passage, but the quality and quantity obtained from each host was subject to much variation. Protoscolex viability (as determined by activity of worms and flame cell movement) post-evagination of not less than about 60%, was found to be necessary for the establishment of 'good' cultures. However, in old infections (> 12 months p.i.), the protoscoleces often contained many calcareous corpuscles and hence appeared very dark; even with a high percentage viability these were found not to be suitable for culture work.

In addition, it was often difficult to achieve consistent results, both during a single culture experiment, and from one experiment to another, for a variety of reasons. Samples of FCS, for example, had to be tested prior to purchase, different batches often producing widely differing results. The quality of the dog bile was also subject to variation, from one host to another. Furthermore, it was found that the number of worms in each culture affected the rate of development of E. multilocularis in vitro. Although cultures were set up on day 1, with each vessel containing approximately the same number of worms, it was difficult to maintain this number of viable worms for the duration of an experiment, due to (1) unavoidable removal of the 'lighter' dead, lysed or vesicular forms, when changing the media, or (2) removal of samples for microscopic examination and assessment of worm development. It was generally concluded that overcrowded cultures (i.e. ones in which the media became quite acidic within 24h, as observed by the yellow colour of the Phenol red indicator in the media),

were as detrimental to the parasites' development, as those in which only a small number of worms (i.e. approximately <500/10 ml media) were found. For reasons outlined above, no statistical analysis of the worm numbers per ml medium for optimum development in vitro, was carried out.

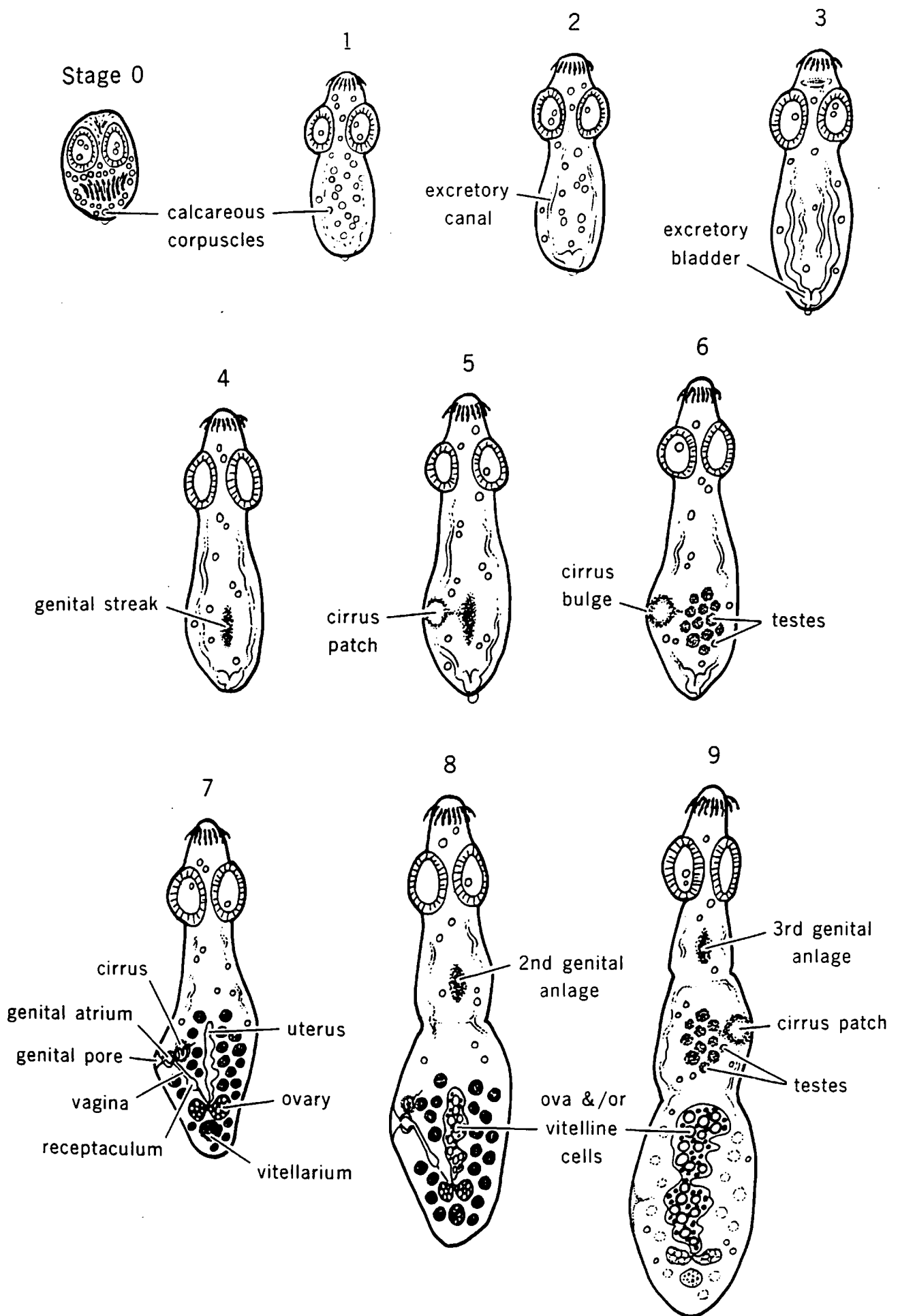
Early stages in the development of E. multilocularis protoscoleces in vitro (observed within the culture vessels), has previously been recorded by Smyth & Davies (1974b), Smyth (1979a,b) and Smyth & Davies (1979); a more recent and detailed account having been given by Smyth & Barrett (1979). The results of this more comprehensive study on the development of this species in vitro, from protoscolex to the sexually mature adult, has resulted in a modification of the previously described stages, redefined (see Fig.5 and Plates 1-11) as follows: -

Stage 0: This stage, as seen within the brood capsules, was defined as an invaginated protoscolex, which contained numerous calcareous corpuscles. Occasionally, a small piece of tissue was observed at the posterior end after pepsinisation, denoting the original point of attachment to the brood capsule.

Stage 1: Evagination of protoscoleces was sometimes stimulated by pepsin treatment, or subsequent washings in HBSS, although, for the majority of larvae, dog bile was the main evagination influence. In all experiments, a certain number of active protoscoleces did not fully evaginate; they tended to become vesicular or degenerate, and were eventually removed from culture, following several changes of media. Others became vesicular, post-evagination. A ring of four suckers and two rows of hooks on scoleces were clearly visible at this stage in culture, although further microscopic examination revealed that, in vitro, the hooks were rarely extruded. In addition, a bilobed excretory bladder and four longitudinal osmoregulatory canals (the larger two of which, were the main collecting ducts) were present at this stage, but difficult to observe under the low-power magnification of the inverted microscope.

Fig. 5

Developmental stages of Echinococcus multilocularis in vitro, S.0 to S.9



Stage 2: Elongation of protoscoleces (the first indication of growth) was associated with a reduction in number of calcareous corpuscles.

Stage 3: By this stage, very few corpuscles were observed, although at no time did all the calcareous corpuscles disappear. In culture vessels, the excretory canals were seen clearly, with microscopic examination revealing a ring-shaped duct within the scolex, to which the canals were connected.

Stages 4 & 5: Genital development of the elongated protoscolex was usually preceded by a period of inactivity, during which time the worms, now clearly dorso-ventrally flattened, sometimes assumed a curled stance, with the scolex bending laterally towards the developing genital opening. Stages 4 & 5, being the onset of sexual development, have been considered together here (although recorded separately - see Table 4). This was because it was often difficult to distinguish which of the two stages appeared first (i.e. the genital 'streak' or the cirrus 'patch') or, whether they arose simultaneously. In cultures, the cirrus anlage appeared as a clear circular patch to one side of the worm, indicating the eventual site of the cirrus pouch. The genital streak, (i.e. primordium), aptly described as a dark cluster of cells (supposedly germinal and germinal-somatic), seen along the midline of the worm lying antero-posteriorly, and next to the cirrus patch. This rapid division of cells at the onset of sexual differentiation often caused a slight posterior swelling. In fixed and stained whole mounts (e.g. Aceto-orcein - see Section II for details), both the cirrus patch and genital streak appeared as a group of darkly staining cells.

Stage 6: Cultured worms became asymmetrical by this stage, the developing cirrus pouch and associated reproductive structures causing the so-called 'cirrus bulge'. The periphery of the genital streak rapidly reorganised into testes anlagen, and the cirrus patch developed into a muscular cirrus pouch. As before, stained squashes of complete specimens, showed the developing testes as clusters of darkly staining cells. The

somatic growth exhibited by this stage was somewhat variable; some worms were regarded as stunted, whilst others grew in length considerably. In the latter, germinal development was usually somewhat retarded.

Stage 7: This next stage has previously been described as the "sexually mature monozoic form" (Smyth & Barrett, 1979). In culture vessels, it was very difficult to distinguish any particular developmental stages between the definite appearance of testes primordia (stage 6) and the mature adult worm (stage 7). More detailed observations, especially of stained material, were necessary in order to clearly identify the appearance and subsequent maturation of the ovaries and other reproductive organs, not yet considered (a comprehensive investigation of the developmental biology of the reproductive system of adult worms is dealt with in Section II). Therefore, for in vitro culture studies, early development of the female genitalia was not recorded, stage 7 was characterized by the appearance of a uterus, being the last, and most obvious, of the reproductive structures to form. This uterus was either observed empty, (sometimes containing only a few crystal-like bodies), or full of cells, specifically ova and/or vitelline cells. At no time were any shelled eggs seen in utero.

Stage 8: This next stage, previously designated "pseudosegmented" development (along with stage 9) by previous authors (see Smyth & Barrett, 1979), was characterized by the appearance of a second genital anlage (i.e. cirrus patch and/or genital streak), anterior to the first.

Stage 9: Subsequently, the appearance of another genital primordium anterior to the first two, with the potential to develop into a third set of reproductive structures, was denoted stage 9. This was irrespective of the maturation stage of the second genitalia. The maximum growth of E. multilocularis protoscoleces achieved in vitro, was the development of up to three sets of genitalia, with the third attaining sexual maturity, although this occurred in only a few cases. The maturation of the second and third genital anlagen

were normally associated with the gradual degeneration of the first and second reproductive systems respectively.

Having characterized the above stages of development in E. multilocularis in vitro, following observations during numerous experiments, it was consequently possible to assess each culture by noting the time at which each stage first appeared (or, more precisely, was first observed). However, due to the inter- and intra-culture variation in development times, the small size of E. multilocularis, and the large number of worms in each culture (estimated to be initially (i.e. on day 1), between 5000 and 10,000, depending on the culture vessel used) it was considered impractical to carry out any statistical studies. Consequently, only the best development achieved in each culture/experiment was recorded, as shown in Table 4, although more detailed observations for each experiment are described below: - (it should be noted that not all experiments attempted are detailed below, due to their inconclusive results, or those prematurely terminated due to bacterial or fungal infections. Also, straightforward culture experiments, for example, when testing FCS batches have been omitted here, but included in Table 4.)

Experiment E.51

Serum-free medium (NCTC 135)

In this experiment the development of E. multilocularis protoscoleces in a serum-free liquid medium was observed. Costar flasks containing 20 ml NCTC 135, over a coagulated bovine serum base (with and without sheep RBC), were kept in a continuous-shaking waterbath. The liquid medium was changed every four days, and new bases, approximately every week. The gas phase was 5% CO₂, 10% O₂ in N₂.

By day 6, many protoscoleces had developed to stage 3. No further growth was observed although the majority remained active up to 25 days in vitro. Increasing numbers of protoscoleces then became swollen and vesicular, eventually disintegrating. By day 33 over 60% in each culture were

Table 4Rate of development of Echinococcus multilocularis in vitro (in days)

(refer to Fig.5 for a description of the developmental stages, S.3 to S.9)

<u>Exp.</u>	<u>Culture</u>	<u>Developmental stages</u>						
	<u>Medium</u>	<u>S.3</u>	<u>S.4</u>	<u>S.5</u>	<u>S.6</u>	<u>S.7</u>	<u>S.8</u>	<u>S.9</u>
E.51	NCTC 135+	6						
	NBCS base							
E.52	S.10.C	5	24	21	24	34	41	
E.52(LIFT)	S.10.B		15	23	23	30	30	
E.53		4	15	22				
E.54	S.10.D	3	18	21	25	44	50	
E.55	S.10.D	4	25	28	41	53		
	(inc. hepes)							
E.55	S.10.D	4	25	28	35	59	68	
	(inc. heppso)							
E.55	S.10.D+	4	22	23	28	38	53	77
	sheep RBC							
E.58	S.10.E*	5	20	23	26	37	44	75
	+/- bases							
E.59	S.10.E	3	18	23	24	37	42	64
E.60	S.10.E	5	22	22	25	32	35	
E.61	S.10.D/E	3	15	18	21	27	29	52
E.62	S.10.E	3	19	19	24	29	40	
E.64	S.10.E	5	23	21	25	31	32	
E.65	S.10.E	4	18	18	19	32	32	
E.67	S.10.E	7	25	25	25	29	35	63
E.68	S.10.E	5	22	18	22	32	39	
E.69	S.10.E	4	18	21	25	32	49	
E.70	S.10.E	7	16	20	21	28	32	50
	+/- sheep RBC							
E.74	S.10.E.	3	17	20	22	29	31	

* = hepes was incorporated in all media from exp.E.58 onwards

vesicular, the rest being inactive. However, a few worms in one culture remained quite active until the cultures were terminated on day 38, but did not show any signs of growth.

Serum-free liquid medium thus apparently stimulated vesicular, rather than strobilar development, even with the addition of a serum base.

E.52

Medium S.10.A v. S.10.B v. S.10.C

This experiment was set up to compare three different liquid media: - S.10.A, S.10.B and S.10.C (see Materials and Methods). Unfortunately during the course of this trial, TC 858 became unobtainable commercially, and therefore cultures growing in S.10.A, were replenished with S.10.B from day 23. It was, however, most encouraging to note comparable results between media S.10.A and S.10.C during the early stages of the experiment, as TC 858 had previously been successfully used as a basic culture medium for several years by workers in our research team at Imperial College (Smyth & Davies, 1975; Smyth, 1979a). This suggested RPML 1640 as being a possible, suitable replacement for Parker's 858.

In all cultures, some protoscoleces had developed to stage 3 by day 5. Little further development was observed in S.10.B, however, with many worms becoming vesicular from day 28, followed by a gradual degeneration of the material. In contrast, in S.10.A and S.10.C, cirrus patches were first noted on day 21, with many testes appearing by 26 days in vitro. Several 'monozoic' adults were apparent by day 34 in S.10.C. The quality of the cultures originally grown in S.10.A gradually declined on using S.10.B as a replacement, but some 'monozoic' development occurred. Many of the cultures in the experiment eventually succumbed to a fungal infection, but one excellent culture (in S.10.C) was maintained for 56 days, during which time two sets of genitalia were observed in a few worms i.e. stage 8. In all sexually mature worms the uterus was always empty.

E.52-Lifts

Circulating culture system (S.10.B)

Two circulating culture systems (lifts) were set up at the same time, with the same larval tissue as E.52, as described above. The medium used was S.10.B, which was changed every week. One lift was gassed with 20% CO₂ in N₂ and the other with 10% CO₂ in N₂.

Protoscoleces in the bottom flask developed well with cirrus patches and testes being observed by day 23. Several 'monozoic', and a few 'pseudosegmented' worms were seen on day 30 (i.e. stages 7 and 8). Lifts were kept running for 58 days, and little difference in development was noted between the two gas phases. The appearance of some sexually mature worms in S.10.B was surprising, considering the lack of development in the same media during the concurrent experiment E.52. However, the gas phase was different; no oxygen was bubbled through the media in this experiment.

Critical observations of development times in lifts were difficult, however, as samples of worms had to be removed for examination, and contamination proved to be a problem, if this particular culture system was disturbed too frequently.

E.53

Medium S.10.C v. S.20 v. S.21

Due to the success achieved with medium S.10.C in E.52, this experiment was set up with S.10.C as the control culture medium, and was compared with two others : (i) S.20, in which all the yeast extract in S.10.C was replaced by BEE, and (ii) S.21, in which only half the amount of yeast extract was substituted by BEE. Monophasic cultures were set up in M.D. bottles in a waterbath, and gassed with 10% CO₂, 5 O₂ in N₂. The media was changed every 3 or 4 days.

In all cultures, many worms became vesicular, although less did so in S.10.C. In this medium, genital anlagen were

observed on day 15, and stage 5 was first seen on day 22, in vitro. No further development was achieved in S.10.C, although many cultures suffered from fungal and/or bacterial infections, the experiment being prematurely terminated on day 28. No sexual development at all, was recorded in S.20 and S.21, although in the latter, somatic growth of some worms was quite marked.

E.54

Medium S.10.C v. S.10.D

In this experiment, medium S.10.C, in which E. multilocularis had previously successfully been cultivated to the mature adult stage (i.e. E.52), was compared with another monophasic medium - S.10.D (i.e. using CMRL 1066 instead of RPMI 1640). Cultures were set up in M.D. bottles in waterbaths, with some being subjected to continuous shaking, and others, intermittent agitation. The media was changed every 3 days, and gassed with 10% CO₂, 5% O₂ in N₂.

The surprising result of this experiment was the poor development observed in S.10.C, whereas the worms in S.10.D (whether agitated or not) grew well, and matured at times comparable to those in previous successful experiments (i.e. E.52 and E.52 lifts). In S.10.C there was no development beyond stage 3, although the worms were generally active and some grew quite long. The cultures with S.10.C were terminated at 42 days, as the material had become increasingly vesicular, whilst those in S.10.D were maintained until day 56.

E.55

Various pH values - 6.0 to 7.5 (S.10.D)

This experiment was established with spare material from E.54, in order to find the optimum pH for maximum growth of the parasites in vitro. Worms which had been cultured for 4 days in S.10.D, as for E.54, were then equally divided between 15

Leighton tubes on a roller apparatus, containing medium S.10.D. The basic tissue culture medium was made up without any NaHCO_3 , but with one of five different buffers (Research Organics Inc.) at a concentration of 25 mM. In S.10.D (the buffer concentration being 20 mM), the recorded pH values were as follows: - mes, pH 6.0; mopso pH 6.75; hepes, pH 6.95; heppso, pH 7.2; bicine, pH 7.5.

Best development was achieved with hepes and heppso buffers, although the rate of maturation was somewhat slower when compared with earlier experiments. The first cirrus patch was seen on day 28, with testes being observed on day 35 (in heppso) and day 41 (in hepes). Sexually mature adults were seen on day 53 in hepes, but not until day 59 with heppso. The hepes cultures were terminated on day 57, whereas those in heppso were continued up to 100 days, with stage 8 appearing on day 68. In the other cultures, the parasites growing in bicine were very stunted and inactive, and rapidly became vesicular. These were discarded on day 18. In mes and mopso, the worms remained quite active for up to 20 days, although little growth occurred. The quality of these cultures subsequently declined, and were terminated on day 35. Consequently it was concluded that best development (in this experiment) was achieved with a pH of around 7.0 (the pH was found to drop by 0.2 to 0.4 between each change of media).

One extra culture was set up during this experiment with medium S.10.D (containing only NaHCO_3 , and no other buffer) plus 1 ml sheep RBC. The worms in this tube grew rapidly with a cirrus patch appearing by day 23, and testes by day 28. Stages 7 and 8 were observed on days 38 and 53, respectively. The first recorded appearance of stage 9 in E. multilocularis in vitro, (i.e. the ultimate appearance of a third genital anlage), was in this culture on day 77. In mature worms however, the majority of uteri were empty. The culture was finally terminated on day 100.

E.58Monophasic (S.10.E) v. various diphasic media

In this experiment, S.10.E (i.e. with non-inactivated FCS) with 20 mM hepes and 10 mM NaHCO₃, was used in all cultures, and tested with a variety of bases. S.10.E only, was used as the control, with bases in diphasic cultures being as follows:

- (i) 3 ml coagulated NBCS, (ii) 2 ml NBCS coagulated with 1 ml BEE, (iii) 2 ml 2% agarose in BSS with 1 ml BEE, and (iv) 3 ml 2% agarose in HBSS. Cultures were maintained in Leighton tubes on a roller apparatus, and the liquid medium, changed and gassed (10% CO₂, 5% O₂ in N₂) every 3 days.

In the monophasic media, genital streaks were seen from day 20, cirrus patches from day 23, and testes from day 26. Mature adults were first seen on day 37, with ova and vitelline cells being seen within the uterus by 41 days in vitro. Stages 8 and 9 were observed on days 44 and 75 respectively. The monophasic cultures were maintained for a total of 106 days, by which time many worms had degenerated, or become vesicular. Similar results were obtained in diphasic cultures, although initial development was somewhat retarded, especially when worms became trapped in the bases with holes. Observations were restricted in these cases, and therefore, in some vessels, the base was macerated with a pipette during the course of the experiment, in order to release the worms.

Consequently, these results indicated that: S.10.E was as suitable as S.10.D for the cultivation of E. multilocularis (and thus discarding the need for the inactivation of FCS); monophasic cultures were preferable to those with solid bases; and hepes, along with NaHCO₃, acted as a good biological buffer.

E.59Medium S.10.E

Following the earlier success with S.10.E and hepes, experiment E.59 was set up using this monophasic medium only,

to further evaluate the maturation rate of E. multilocularis in vitro. The cultures were kept in Leighton tubes on a roller apparatus, the medium being gassed with 10% CO₂, 5% O₂ in N₂, and changed every 2 to 4 days.

Genital streaks were first noted by day 18, and cirrus patches appeared on day 23, with testes first being seen the following day. Cells were first observed in the uterus of mature adults on day 37, with second and third genital anlage being apparent on days 42 and 64, respectively. Cultures were maintained for 101 days, although the condition of the worms gradually deteriorated, especially over the last three weeks. This was not unusual in cultures kept for such long periods.

S.10.E (inc. hepes and NaHCO₃) was consequently considered to be a suitable medium for the culture of E. multilocularis in vitro, due to their long survival and good development, and thus, in subsequent experiments, was used as the control medium.

E.60

Reduced FCS concentration (S.10.E)

In this experiment the level of FCS in S.10.E was reduced in some cultures in order to assess whether the worms could be grown as successfully in liquid media with less than 25% serum. The media were as follows: -

- (i) S.10.E i.e. 65% CMRL 1066, 25% FCS
- (ii) S.10.E II 70% " " , 20% " ,
- (iii) S.10.E III 65% " " , 20% " 5% HBSS
- (iv) S.10.E IV 80% " " , 10% " ,
- (v) S.10.E V 70% " " , 10% " 10% HBSS

Cultures were maintained in Leighton tubes on a roller apparatus. The monophasic media were gassed with 10% CO₂, 5% O₂ in N₂, and changed every 3 days.

In all cultures, stages 4 and 5 appeared between 22 and 25 days, and testes between 25 and 28 days. However, best development was achieved in the control medium S.10.E, in which mature adults were seen on day 32, with vitelline cells in the uterus. In other cultures, development was much slower. A small number of 'monozoic' adults were seen on day 35 in S.10.E II and III (i.e. 20% FCS), with even fewer mature worms in S.10.E IV and V (i.e. 10% FCS). In S.10.E, stage 8 was reached by 35 days, whereas an additional week in vitro was necessary for the 20% FCS cultures to achieve this result. From day 43, the 10% FCS cultures degenerated rapidly, with the worms becoming contracted or vesicular. The cultures were maintained for 64 days, with the majority of the worms in S.10.E having attained sexual maturity (i.e. stages 7 to 9), many with ova and/or vitelline cells in utero. For those in S.10.E II and III, (i.e. with only 5% less FCS), the range of developmental stages observed at any time was much greater, with numerous worms remaining sexually immature by the end of the experiment. In other words, germinal development (but not necessarily, somatic growth) was somewhat inhibited.

A 25% serum level in medium S.10.E was thus regarded as the minimum concentration necessary to support rapid, and relatively consistent sexual maturation.

E.60(a)

Medium H.40

This experiment was run in conjunction with E.60, with the aim of trying a new culture medium, H.40, which had been devised and described by Webster & Cameron (1963). These authors observed some clear segmentation of E. multilocularis in vitro in H.40 (as opposed to the 'pseudosegmentation' seen in S.10.E), although they did not describe any genital development. The liquid medium basically consisted of CMRL 1066 (as in S.10.E) with various supplements, including meat digests and dog gut extract. However, despite carefully following the authors' protocol not only did the preparation of

meat digests and dog gut extract prove to be difficult, but none of the cultures survived for longer than 7 days in H.40. Due to the problems encountered with the medium, this experiment was unfortunately not repeated.

E.61

Increased FCS levels (S.10.E); S.10.E v. S.10.D; plus or minus hepes

In this experiment, three variables in the culture media were considered: -

(i) with or without hepes, (ii) inactivated or non-inactivated FCS, and (iii) 25% or 50% FCS.

The two alternatives in both (i) and (ii) had previously been tested separately, in experiments E.54, E.55, E.58 and E.59, no direct comparison having been made during any one experiment. The third variable factor, (iii), was considered subsequent to the results obtained in E.60. Cultures were kept in Leighton tubes on a roller apparatus, with the media being changed and gassed (10% CO₂, 5% O₂ in N₂) every 2 to 4 days.

The results of this experiment were most encouraging in that, in the majority of cultures, first signs of sexual development (stage 4), were observed as early as 15 days in vitro, with mature 'monozoic' adults being found on day 27; ova and vitelline cells were present in many of the uteri. This was the shortest recorded maturation time to date. Complete development to stages 8 and 9 was achieved on days 29 and 52, respectively. Some cultures were maintained for a maximum of 109 days in vitro, although the majority of worms began to degenerate from day 70.

In all cultures, very similar results were obtained, and development times were certainly comparable. However, best growth was observed in S.10.D and S.10.E (both with hepes), and the usual level of 25% FCS. Although equally good development occurred with 50% FCS, the worms appeared somewhat darker, and thus it was considered unnecessary to raise the serum level in

future experiments.

E.62

Medium S.10.E v. S.10.F

Continuing with S.10.E as the control medium, the use of NBCS as a replacement for FCS, was tested in this experiment (i.e. medium S.10.F). Cultures were set up in Leighton tubes on a roller apparatus, and the liquid medium gassed with 10% CO₂, 5% O₂ in N₂. Media were replaced every 2 or 3 days.

The onset of sexual development (both stages 4 and 5) was observed on day 19 in S.10.E, with testes appearing by day 24, and mature adults (with ova in the uterus) from 29 days in vitro. Later, many mature worms had both ova and vitelline cells in utero, although some uteri remained empty. In S.10.F, sexual development began on day 22, with only a few worms maturing to a maximum developmental stage 7, from day 29. The cultures with NBCS rapidly declined from day 43, whereas those in S.10.E survived better, reaching stage 8 by 40 days, and eventually being discarded on day 68.

One interesting point to note was that during this experiment, the gas phase had to be altered unavoidably to 5% CO₂, 10% O₂ in N₂, and this resulted in a large increase in the number of swollen or vesicular worms in each culture. (In all in vitro cultures, a certain number of worms per vessel, commonly degenerated, or became vesicular, and did not develop normally). Therefore, this new gas phase was not considered to be suitable for the strobilar development of E. multilocularis, especially in the control medium, S.10.E.

E.67

Increased levels of dog bile (S.10.E)

In experiment E.67, an attempt was made to ascertain whether higher levels of dog bile in S.10.E would be advantageous to the growth of E. multilocularis protoscoleces in vitro. Previous observations in this laboratory

demonstrated that increased bile concentrations resulted in greater worm activity, although it is not known whether this is representative of the in vivo situation.

The worms were grown in S.10.E for 9 days in vitro before the addition of extra bile to some cultures; extra bile was replenished at every change of media. The culture vessels were Leighton tubes on a roller apparatus, the gas phase was 10% CO₂, 5% O₂ in N₂, and the media was replaced every 2 to 3 days. Additional bile was added in the following amounts: - (i) 0.05 ml, 5% dog bile in HBSS per 10 ml S.10.E (i.e. per Leighton tube), (ii) 0.1 ml dog bile and (iii) 0.2 ml dog bile (both added as (i)).

Unfortunately, this experiment suffered from bacterial infections on day 17, although it was noted that only a few of the tubes containing extra dog bile were affected, and in these, the infections were relatively low. By washing infected cultures in several changes of HBSS containing twice the usual level of antibiotics, and then placing the parasites in new vessels with fresh media (adding 0.2 ml dog bile to the cultures in S.10.E only), the bacterial infection was eliminated (or at least reduced) in most cases. These results therefore suggested that the bile had some antibiotic effect, in preventing, reducing or eliminating the bacterial invasion. However, the increased bile levels in the media, appeared to inhibit parasite growth, both somatic and germinal. Consequently, as from day 23, the media in all cultures was replaced by S.10.E only. On day 25, stages 4, 5 and 6 were observed in all vessels, and 'monozoic' adults were seen on day 29. In general, despite earlier problems with infected cultures, the worms developed well with many maturing to stage 7, several to stage 8, and a few to stage 9. The uterus of some adults was swollen, but empty, although many clearly contained ova. Cultures were eventually terminated on day 77.

Supplementing S.10.E with extra dog bile was therefore found to be a possible growth inhibitor, although the ^Lytic effect of higher concentrations may have been responsible for

preserving several aseptic cultures, when other concurrent vessels, especially those without extra bile, became infected. Clearly more work would be needed before these speculations can be substantiated. Unfortunately, difficulty in obtaining bile severely limits such experiments.

E.68

Various gas phases; with, or without agitation (S.10.E)

A variety of gas phases were tried in this experiment. All cultures were grown in S.10.E, in Leighton tubes. The controls were gassed with 10% CO₂, 5% O₂ in N₂ (as before), some cultures being kept on the roller apparatus, and other, upright in a beaker (i.e. with no agitation). Air as the gas phase was tested by leaving the tops loose on some 'still' tubes, in the incubator used above, others were placed in a CO₂ incubator (Borolabs) set for 10% CO₂ in air. Media was changed (and gassed where appropriate) every 2 to 4 days.

In the control cultures, development was generally good, with the first cirrus patch appearing on day 18, and testes by day 22. 'Monozoic' adults were seen from day 32, although cells were rarely observed in the uterus. However, in all cultures that were kept 'still' (even with the control gas phase), maturation took somewhat longer and somatic growth was inhibited, especially those in the CO₂ incubator. In addition, the number of worms per culture developing past stage 3, was much less in the non-agitated vessels. The experiment was continued for 60 days, during which time it was observed that, not only were rotated cultures better than 'still' ones, but that a gas phase of 10% CO₂, 5% O₂ in N₂ appeared to be more suitable than air, which was more compatible than 10% CO₂ in air. In air, fewer worms attained sexual maturity than in the control gas phases, and, in 10% CO₂ in air, the worms were stunted and many looked abnormal. From these results, it was difficult to critically assess the optimum levels of CO₂ and O₂, although an oxygen level of greater than 5% appeared to be detrimental to the development of E. multilocularis in vitro (see also experiment E.62).

E.69Various gas phases; with, or without agitation (S.10.E)

This experiment was a repetition of part of the previous one (E.68), in which two variables were tested: - (i) gas phase, i.e. 10% CO₂, 5% O₂ in N₂ versus air, and (ii) rotation of the Leighton tubes on roller apparatus, versus tubes kept upright in beaker. For the first gas phase, sterile gas was bubbled through the medium (S.10.E) as usual, whilst tube tops were left loose for the air phase (as in E.68). The media was changed every 2 or 3 days.

The results of this experiment confirmed those achieved in E.68, in that a gas phase of 10% CO₂, 5% O₂ in N₂ initiated better sexual development and somatic growth, than air. On testing the pH of discarded media, it was proved that in non-gassed cultures (i.e. air phase) the pH was normally between 7.5 and 7.8, a level which had previously been found to be unsuitable for growth in vitro (see E.55). In addition, rotated cultures were more uniform than those kept 'still' (in other words the intra- and inter-culture variation in development was much less in agitated vessels).

The development rate from stage 3 to stage 8 in this experiment was very similar to that recorded in E.68 (see Table 4).

E.70Medium S.10.E plus sheep RBC

This experiment was set up subsequent to the success achieved in one culture in E.55, with the addition of sheep RBC to the medium, S.10.E. In E.55 1 ml RBC was added at the beginning of the experiment, and the worms survived well in the clotted blood at the base of the vessel. No extra blood was added when the medium was changed. In this particular experiment, blood was not added to the cultures until day 18, by which time, sexual development had already begun in some

cultures (genital streaks on day 16). Either one or two drops of sheep RBC were added to the Leighton tubes containing 10 ml S.10.E, and this was repeated every other time the medium was changed at 2 to 3 day intervals. The gas phase was 10% CO₂, 5% O₂ in N₂.

In all cultures, cirrus patches and testes were observed on days 20 and 21 in vitro, respectively, with 'monozoic' adults appearing by day 28. Development continued up to the appearance of a second and third genital anlage (on days 32 and 50), the cultures finally being terminated on day 53. In several worms, a swollen, empty uterus was apparent, but both ova and vitelline cells could clearly be seen in numerous adults in all cultures. It was therefore concluded that the addition of sheep RBC did not stimulate a more consistent or improved development in vitro (compared with the controls in S.10.E only, and earlier experiments). However, the blood was not added until after the onset of sexual differentiation; whether the addition of sheep RBC at the beginning of the experiment would have initiated earlier, or improved germinal development, could only be ascertained by a subsequent experiment.

The results of all the above experiments (and others not included here), shown in Table 4, have been summarised in Fig.6, and some stages shown in Plates 1-11. It must be emphasized here, that due to inter- and intra-culture variation, only the best results (i.e. stage of development) achieved in each experiment have been recorded, and, as mentioned before, it was not considered viable to carry out any statistical analysis on the data.

A line of best-fit is shown on Fig.6, taking into account the points representing the results of 2 or more experiments, and has tentatively been denoted the 'average' (or, mean) development of adult E. multilocularis in vitro. It can be seen that this line, is very close to the best results achieved.

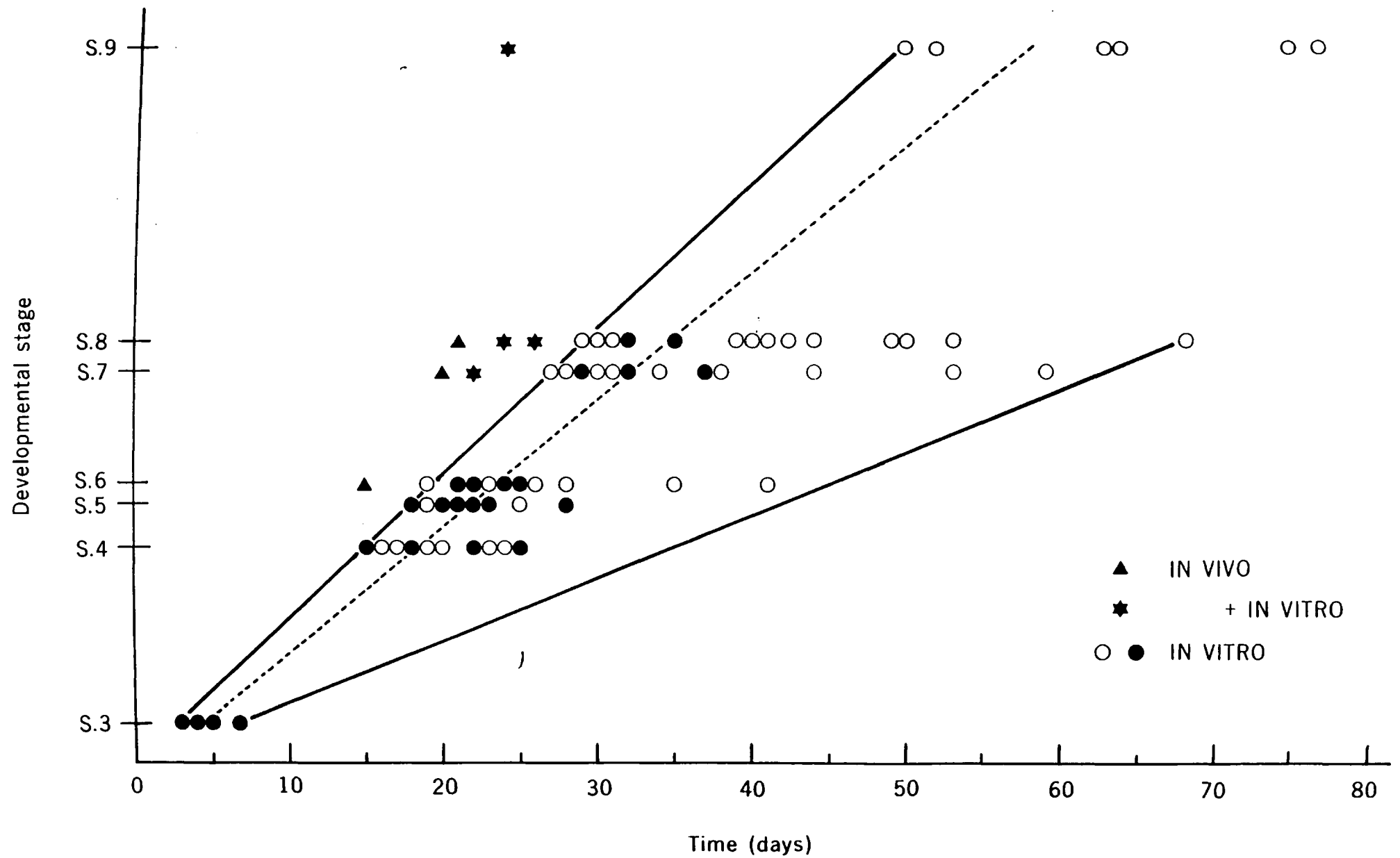
Fig.6

Rate of development of Echinococcus multilocularis in vivo
and in vitro

(○: represents the results of one experiment;

●: represents the results of two or more experiments)

(the bold lines enclose the range of development observed
in vitro; the discontinuous line shows the average, or
mean, rate of growth in vitro)



From the more comprehensive details noted for each experiment (described above), it was apparent that the inter- and intra-culture variation in development was much greater during the earliest experiments. A gradual improvement in the in vitro techniques employed, and the establishment of S.10.E (inc. hepes, NaHCO_3 and gentamicin) as a suitable monophasic culture medium, resulted in the relatively uniform rate of development amongst cultures, which was consistently achieved during latter experiments (i.e. uniformity of maturation, both inter- and intra-culture).

In summary, best results were obtained in 10 ml medium S.10.E (including 20 mM hepes, 10 mM NaHCO_3 and 100 μg gentamicin/ml), gassed with 10% CO_2 , 5% O_2 in N_2 , in Leighton tubes (19x 105 mm) on a roller apparatus. The media was changed and gassed every 2 or 3 days. In this culture system, the majority of the worms developed to stage 7 at least, usually to stage 8, and sometimes to stage 9 (see Fig.5). The latter two stages, which had previously been termed 'pseudosegmentation', were recently considered to be no more than an apparent inability of the cestode to constrict between the genitalia (Smyth, Barrett, Eckert & Thompson, 1982), rather than lack of an interproglottid membrane, the existence of which has been disproved by Mehlhorn, Becker, Andrews & Thomas (1981). Nevertheless, in spite of (i) an improvement in the development rate in vitro, (ii) relatively consistent and uniform maturation of E. multilocularis protoscoleces in vitro, and (iii) increased longevity of cultured worms, during which time, development past the 'monozoic' adult stage was not uncommon, no shelled eggs were ever found in vitro. During culture experiments, numerous attempts were made to induce insemination, and consequently fertilisation, using a variety of "pressure" systems, following the successful in vitro development of Schistocephalus solidus to the egg-containing adult by Smyth (1954). The results obtained with these techniques will be considered in detail in Section II. However, it proved difficult to confine or 'squash' adult E. multilocularis due to its' minute size (<0.5 mm), and worms subjected to such treatment, often did not survive.

It should also be noted that abnormal development of protoscoleces sometimes occurred in vitro, such as the development of a second scolex. These, and other aberrant forms have recently been described by Smyth & Barrett (1979).

1.2 In vivo

The three dogs orally infected with E. multilocularis larvae (with a viability of 80%), all harboured developing adult worms on autopsy at 15, 20 and 21 days p.i. The first and third dogs were also infected with several specimens of another, larger cestode, tentatively identified as Dipylidium sp.

At 15 days, the majority of worms were recovered from the anterior half of the small intestine, with a gradual reduction in numbers in the 4th and 5th sections. None were found in the most posterior part. The maximum development achieved at this time was stage 6; developing testes and cirrus anlagen were clearly seen, with microscopic examination revealing no other genital structures. It was interesting to note that the variation of developmental stages observed was similar to that in cultured worms, in that many were still immature, and worms at stages 4 and 5 were also common.

At 20 days p.i., worms were found throughout the length of the small intestine, with the greatest numbers being recovered from sections 2, 3 and 4. This time, the majority of the parasites were sexually 'monozoic' adults (similar to those observed in vitro, i.e. stage 7), with cells seen in the uterus. There was much less variation in development by 20 days p.i. (although a few immature stages were observed), in contrast to the 15 day old worms.

At 21 days p.i., parasites were again recovered from all sections of the small intestine, with the majority being found in the 3rd and 4th segments. As with the 20 day old infection, some worms were still immature, but many had developed into sexually mature adults, in which the uterus was commonly packed with ova and vitelline cells. A few uteri were empty, (again, as seen in vitro), and some even contained crystals similar to those observed in cultured worms. The majority of the worms had, however, matured to stage 8, with the appearance of the second genital anlage; in some, immature testes were apparent.

In contrast to in vitro worms, however, the demarcation between the two genitalia was more obvious in the in vivo specimens. A number of degenerate or dead worms were also recovered from the dog at 21 days p.i.

Some parasites from each of the infected dogs were washed and transferred to culture, and the results of these subsequent in vitro studies are shown in Table 5, with details as follows:

-

15 days p.i. (D.1)

During the first few days in culture, these worms were quite active, and several grew in length. By day 4, signs of further genital development were noted, accompanied by a period of inactivity. Sexually mature adults were first seen on day 7 in vitro (i.e. $15+7 = 22$ days total), with cells in the uterus being clearly discernable from day 8, although, in some, the uterus remained empty. By day 11, ($15+11 = 26$ days) the rudiments of second genitalia were apparent in some worms, although up until termination of the experiment at 13 days ($15+13 = 28$ days), the majority of worms remained as 'monozoic' adults, usually with only vitelline cells in utero. Immature stages were seen throughout the culture period, as well.

20 days p.i. (D.2)

In culture, these worms appeared to be less active than the younger parasites from the first dog (D.1). Also, the variation in development was less marked, in that very few immature worms were observed, although several did not mature past stage 6 during the culture period. After 4 days in vitro ($20+4 = 24$ days), a second genital anlage was seen in some, in the form of a genital streak. The uterus in the first genitalia was usually packed with cells - both vitelline and ova. No further development was observed by termination of the experiment on day 8 ($20+8 = 28$ days).

Table 5

Rate of development of Echinococcus multilocularis in vitro,
following partial maturation in vivo (refer to Fig.5 for a
description of the developmental stages, S.6 to S.9)

<u>Dog</u>	<u>Worms</u> <u>collected</u>	<u>S.6</u>	<u>S.7</u>	<u>S.8</u>	<u>S.9</u>
D.1	15 days p.i.	15	22(7)	26(11)	
D.2	20 days p.i.		20	24(4)	
D.3	21 days p.i.			21	24(3)

The numbers in the above table, refer to the total age of the worms (days p.i.); those in brackets, denote the number of days in vitro.

The results shown here have also been included in Fig.6, in order to compare the rate of development in vivo, with that in vitro.

21 days p.i. (D.3)

By day 3 (21+3 = 24 days) in culture, the second genital anlage in stage 8 worms, had developed into mature testes; in some, a third genital primordium (streak) was obvious. On day 5 in vitro (21+5 = 26 days), in the best cultures, many worms had grown considerably in length, and, in a few, the penultimate segment had developed to sexual maturity. Several free mature proglottids were also observed in one culture vessel. Detailed microscopic examination of samples removed from cultures, further revealed the presence of large fertilised ova, and in three specimens developing oncospheres were clearly seen (Plates 12-14). The cultures were consequently terminated the same day (5 days in vitro, 26 days in total), the material being fixed as described in Section II. As with the worms from the second dog, there was little variation in development, the majority of worms containing two sets of reproductive structures. Only a few, relatively immature worms (pre-stage 7) were seen. An interesting point to note was that in one vessel, a morphologically abnormal worm was seen on day 2 in vitro, as sometimes observed in worms grown in culture from protoscoleces. This parasite had a well developed second scolex, an abnormality that is occasionally seen evaginated in protoscoleces but also develops de novo in vitro. Either of these possibilities could explain the in vivo situation.

In all the above cultures, using worms from dogs, degeneration of the specimens in vitro was quite marked, considering that the cultures were maintained for a maximum of only 13 days, and development was generally poor.

Detailed observations of the reproductive system of in vivo material, and subsequent development in vitro, is dealt with in Section II, the results being compared with earlier observations on adults grown solely in vitro.

2. Echinococcus granulosus in vitro

As with E. multilocularis, the successful establishment and maintenance of cultures largely depended on the quality of the material available. Once again, viability was important, usually a protoscolex viability of no less than 60% was considered suitable for culture work. In addition, only material from cysts which contained relatively clear hydatid fluid was tested for viability; hydatid cysts with turbid fluid were normally discounted. Other problems encountered in achieving consistent results during culture work, have already been outlined for E. multilocularis.

As shown in Fig.7 the development of E. granulosus protoscoleces in vitro was somewhat different from that of E. multilocularis, one obvious dissimilarity being that segmentation in E. granulosus occurred prior to, rather than after, the appearance of genital anlagen. In addition, the maturation time of this species was much longer than that of E. multilocularis.

The early developmental stages observed in vitro have previously been described by Smyth et al (1967), Smyth & Davies (1974b) and Smyth (1979a), although the stages were numbered differently in each case. In order to assess the results of this particular study, each stage of development has been redefined, and subsequently numbered, following observations of several experiments. The details of the developmental pattern observed in E. granulosus in vitro, was, as follows: - (see Fig.7 and plates 15-20).

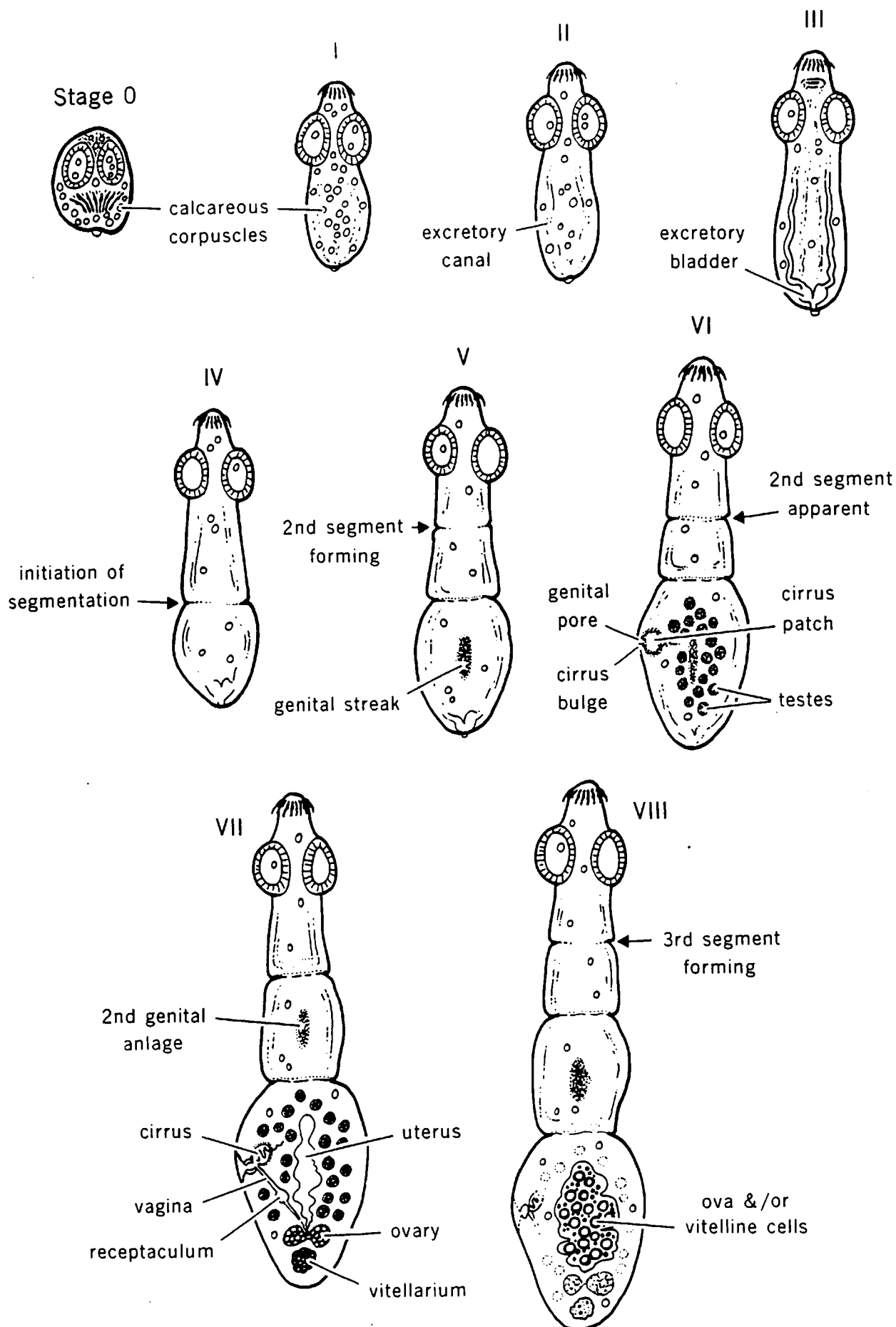
Stage 0: This was the invaginated protoscolex, as seen in the brood capsules (as E. multilocularis).

Stage I: The evaginated protoscolex was defined Stage I (as Stage 1 in E. multilocularis).

Stage II: Somatic growth of the larva, associated with a reduction in the number of calcareous corpuscles, followed

Fig.7

Developmental stages of Echinococcus granulosus in vitro,
S.0 to S.VIII.



evagination (as Stage 2, E. multilocularis).

Stage III: The osmoregulatory canals were now very clearly defined in culture, along with a posterior excretory bladder (as Stage 3 for E. multilocularis).

Stage IV: The onset of segmentation (previously described as "banding"; see Smyth 1979a) was defined by the appearance of a clear 'band'; an apparent thinning of the cytoplasm, which was the demarcation of the first segment.

Stage V: The appearance of a genital streak (see Stage 4, E. multilocularis) within the now more clearly defined posterior segment, denoted the onset of Stage V. The simultaneous formation of another proglottid may be apparent, with the appearance of a second, clear band, anterior to the first.

Stage VI: This stage was characterized by the presence of testes within the posterior segment. At the same time, the cirrus bulge became apparent, related to the development of the cirrus patch and associated structures, such as the genital pore. Again, as with Stage V, the formation of a second proglottid, may or may not be visible at this stage.

Stage VII: The maturation of the posterior segment, to the equivalent Stage 7 of E. multilocularis, i.e. the development of all male and female reproductive organs, with the ultimate appearance of a uterus, was defined as Stage VII. This was also associated with the appearance of a second genital anlage (streak) in the penultimate proglottid.

Stage VIII: This last stage, characterized by the dilation of the uterus in the posterior segment, associated with degeneration of other reproductive structures, was the maximum development achieved in vitro. Normally the uterus was packed with cells, and occasionally the formation of a third proglottid, anterior to the first two, was observed.

As with the E. multilocularis cultures, having

characterized the above developmental stages of E. granulosus in vitro, the results of each experiment were assessed accordingly. However, relatively few experiments were conducted with E. granulosus (on account of difficulties in obtaining suitable material), the results of which are shown in Table 6. Some developmental stages are shown in Plates 15-20. Fig. 8 shows the development of E. granulosus of sheep origin, and also a couple of successful cultures, using material from other intermediate hosts (viz. horse and human). Further details of the in vitro experiments are given below:-

2.1 Sheep material

HS.38

Diphasic medium (S.10.C plus NBCS base)

In this experiment, the worms were cultured in diphasic media (25 ml S.10.C, over 20 ml coagulated NBCS) in M.D. bottles. The vessels were continuously agitated in a waterbath, and the liquid medium was changed and gassed (10% CO₂, 5% O₂ in N₂) every 2 or 3 days.

Protoscoleces matured to stage III by day 5, and remained active, gradually growing in length, until 'banding' (stage IV) was first observed on day 17 in vitro. As in E. multilocularis, the parasites normally became quiescent prior to segmentation and germinal development. Both a genital anlage and the formation of a second segment were obvious by day 28, with testes, genital pore and cirrus bulge clearly seen on day 40. The cultures were then discarded due to a bacterial infection.

HS.40

Medium S.10.C v. S.10.D (both with NBCS bases)

In this experiment, a diphasic medium was used as above, with the previous liquid phase S.10.C, being compared with another, S.10.D. In addition to the M.D. bottles, originally routinely used for E. granulosus cultures, other vessels were

Fig.8

Rate of development of Echinococcus granulosus in vitro

Sheep material: and

Continuous lines enclose the range of development observed in vitro; experiments HS.38 to HS.47.

Discontinuous line between the two, represents the estimated average growth rate (of both sheep and human material).

△: represents the result of a single experiment

▲: represents the result of two, or more, experiments

Human material: (see above; experiment Hu.3)

Horse material

Lower discontinuous line indicates growth rate, taking into account S.VI, at 118 days; experiment H.43.

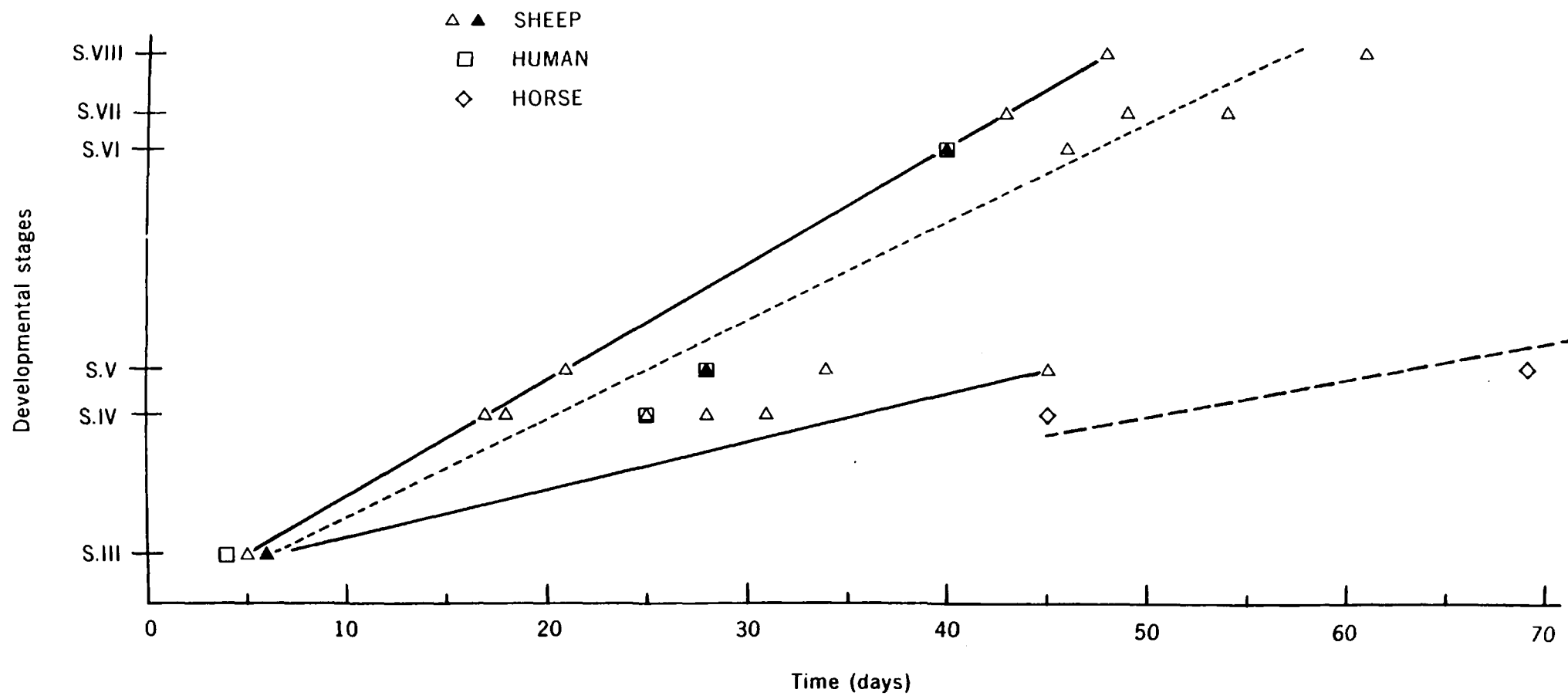


Table 6

Rate of development of Echinococcus granulosus in vitro
(in days)

(refer to Fig.7 for a description of the developmental stages, S.III to S. VIII)

<u>Exp</u>	<u>Medium</u>	<u>S.III</u>	<u>S.IV</u>	<u>S.V</u>	<u>S.VI</u>	<u>S.VII</u>	<u>S.VIII</u>
	HS.38 S.10.C+	5	17	28	40		
	NBCS base						
	HS.40 S.10.D+base	6	28	28	40	49	61
<u>Sheep</u>	HS.43 S.10.E+base	6	31	45			
	HS.45 S.10.E+/-base	6	18	21	40	43	58
	HS.47 S.10.E+base	6	25	34	46	54	
	HS.41 S.10.E+base						
	HS.42 S.10.E+/-base						
<u>Horse</u>	HS.43 S.10.E+/-dog	*	44	69	118		
	blood (no base)						
	HS.44 S.10.E+/-base		45				
<u>Cattle</u>	EC.7 S.10.E+/-base	4					
<u>Human</u>	Hu.3 S.10.D+base	4	28	28	40		

*Stage III was difficult to estimate for horse material
- see Results section.

tried in this experiment: -

- (1) M.D. bottles containing 20 ml S.10.C/D + 20 ml NBCS
- (2) Sterilin flasks containing 20 ml S.10.C/D + 20 ml NBCS
- (3) Costar flasks containing 20 ml S.10.C/D + 10 ml NBCS

All vessels were agitated, and the media replaced and gassed, as in HS.38

The results of this experiment were unfortunately inconclusive in that many cultures succumbed to bacterial infections from 9 days, or fungal contamination from 21 days, in vitro. In the remaining vessels, a larger number of worms than usual became vesicular during the course of HS.40, whilst others were relatively inactive, dark and stunted. However, the onset of sexual development (namely, both stages IV and V) was first observed in two surviving cultures (S.10.D plus NBCS base, in M.D. bottles) on day 28. By day 40, testes and cirrus patches were obvious, with genital pores, clearly seen on day 42. One culture was maintained for 76 days in total, with a second genital anlage appearing in some worms on day 49, and a large swollen uterus full of cells in the first segment, on day 61. A few free proglottids were also seen in the vessel. As observed in E. multilocularis cultures, the E. granulosus protoscoleces which became trapped in the serum bases, generally failed to develop, and, in many cases, it was necessary to macerate the NBCS bases during the experiment, in order to release the worms for observation. This macerate appeared to be a more suitable medium than solid bases with holes.

HS.43

Monophasic (S.10.E) v. diphasic media; M.D. bottles v. Leighton tubes

In this experiment, the development of E. granulosus protoscoleces in M.D. bottles (in a discontinuous-shaking waterbath) was compared with that in Leighton tubes (on a roller apparatus). Both mono- and di-phasic media were tried,

the bases being NBCS, and the liquid phase, S.10.E, including NaHCO_3 and hepes (which had proved successful for the growth of E. multilocularis in vitro). The liquid media were changed and gassed (10% CO_2 , 5% O_2 in N_2) every 2 to 4 days.

The development of protoscoleces in monophasic cultures (both in M.D.'s and Leightons'), was generally poor, (although some worms grew quite long) and had to be terminated before the initiation of segmentation. Banding was first seen in an M.D. bottle on day 31, and by day 37 in Leighton tubes. The appearance of a second band was seen in an M.D. bottle on day 45. There were no signs of any germinal development in any of the cultures, which were terminated on day 52, following a gradual deterioration in their quality.

HS.45

Monophasic (S.10.E) v. diphasic media; M.D. bottles v. Leighton tubes

The culture conditions in the experiment were similar to those in ~~HS~~HS.43; the media, gas phase and culture media being used in M.D.'s and both mono- and di-phasic media in Leighton tubes.

Banding was first observed in diphasic medium in Leighton tubes on day 18, with genital anlagen appearing on day 21. By this time, banding had begun in M.D. bottles, and in monophasic media. The worms in the M.D.'s however, subsequently degenerated, and these were discarded on day 34, by which time, numerous worms in the Leightons (both mono- and di-phasic media), contained 2 clear segments and developing genitalia. Testes were seen by day 40 in diphasic media, 3 days before the same stage was obvious in monophasic media. Due to bacterial infection, only the monophasic cultures were maintained until the end of the experiment on day 63, by which time, stage VIII had been reached (day 48), and some free proglottids seen at the bottom of the tubes. Much intra-culture variation was obvious during this experiment; and the worms showed clear signs of degeneration over the latter three weeks. Since

segmented worms had not previously been obtained in monophasic media, this was an anomalous result and difficult to account for.

HS.47

Monophasic (S.10.E) followed by diphasic media

In this experiment, only a few cultures were set up in Leighton tubes on a roller apparatus, initially in monophasic medium S.10.E, but worms were later transferred to tubes containing a NBCS base.

Banding was observed on day 25, the first genital anlage on day 34, with testes appearing by day 46. A uterus was first seen on day 54, but remained empty, and no further development was observed by termination of the cultures on day 60. As with previous experiments, much variation in development was observed, and the worms gradually deteriorated over the latter part of the experiment.

2.2 Horse material

Several experiments were set up using protoscoleces from cysts in horse livers, but development past stage III was rarely observed.

H.41

Diphasic medium (S.10.E with NBCS base)

In this experiment, diphasic cultures (20 ml NBCS with 20 ml S.10.E) were maintained in M.D. bottles in a discontinuous-shaking waterbath. Extra bile was added to some vessels - namely 1 ml 5% dog bile in HBSS.

Little growth was observed in this experiment, the cultures being terminated on day 41.

H.42

Monophasic (S.10.E) v. diphasic media; S.10.E or NBCS base, with dog blood; M.D. bottles v. Leighton tubes

A variety of factors were compared in this experiment: Leighton tubes versus M.D. bottles; monophasic, versus diphasic media; the addition of dog blood to the liquid phase or NBCS base; NBCS macerate versus a uniform, solid base. In all cultures, S.10.E was the liquid medium, which was changed and gassed (10% CO₂, 5% O₂ in N₂) every 3 to 4 days.

The cultures were all terminated on day 31, by which time only a few in each vessel had grown in length, the majority remaining stunted and inactive, or gradually becoming vesicular.

H.43

Medium CMRL 1066 followed by S.10.E; various extra components

In this experiment, the protoscoleces were grown in basic culture medium only, i.e. CMRL 1066, for the first two weeks, and then transferred to S.10.E (on this occasion, containing no hepes). In some cultures, extra dog bile (0.5 ml, 5% bile in HBSS/10 ml S.10.E), dog blood (0.5 ml 20% blood in HBSS/10 ml S.10.E) or dog gut extract (1 ml/10ml S.10.E - see Webster & Cameron, 1963), were added. The vessels used were Leightons, on roller apparatus, the media being changed and gassed (10% CO₂, 5% O₂ in N₂) every 2 to 3 days.

First signs of segmentation (the first observed in any recorded culture of E. granulosus protoscoleces of horse origin), were noted on day 44, with the appearance of a constriction around the body, forming a clear, posterior bulge ('segment'). This was seen in two vessels, one of which contained dog blood. By day 52, the parasites had grown tremendously in length, with the onset of segmentation now appearing in another vessel - that with 1 ml dog gut extract. By 69 days, two proglottids were apparent in the first two vessels, but no genital anlage. However, by day 118, a stage

VI worm was seen in the only surviving culture, in monophasic medium, S.10.E only. Detailed microscopic examination clearly revealed the presence of testes, and anlagen of other genital structures. In all, only 3 maturing adults were seen by termination of the experiment on day 122 (although several showed indications of segmentation), with a second genital anlagen obvious in one of the worms. The strobila of these worms was surrounded by a thick amorphous coat, the origin of which could not be determined. (Plate\$ 20).

H.44

Monophasic (S.10.E) v. diphasic media; CMRL 1066 v. S.10.E;
NBCS bases plus sheep RBC

This experiment was set up to compare various culture conditions, following the success achieved in the previous experiment. A basic liquid phase of S.10.E (with hepes and NaHCO_3) in Leighton tubes, on roller apparatus, was tested against:- (1) diphasic cultures with a NBCS base, with or without sheep RBC, (2) CMRL 1066 with a NBCS base, again, plus or minus sheep RBC. Cultures were gassed with 10% CO_2 , 5% O_2 in N_2 , the liquid media being changed every 2 to 4 days. After one week, some of the CMRL 1066-only cultures, were changed to S.10.E, the few cultures kept in the basic tissue culture media being discarded by day 20, due to degeneration or vesiculation of the worms. In the other vessels, a large number of worms also became vesicular, but some remained active, and showed signs of growth. By day 34, genital anlagen were seen in the S.10.E-only cultures, but the worms showed no signs of segmentation (much like the early germinal development in E. multilocularis). However, by day 45, elongate worms in this vessel, and some diphasic cultures (with and without blood in the base) had begun to segment (as in H.43). All cultures were terminated by day 59, no further development having been observed.

2.3 Cattle material

Only a few cultures were set up with E. granulosus protoscoleces from cattle, and the small amount of material available for each experiment restricted the number of vessels used each time, to between one and six. Many cultures suffered early on from bacterial infections, probably due to the fact that the material was not received in situ, but had been aspirated from hydatid cysts, at source.

In the longest-running experiment, (EC.7), both mono- and di-phasic cultures (S.10.E, plus or minus NBCS bases, in Leighton tubes) were maintained for only 17 days. Stage III was observed from day 4. However, the worms generally remained inactive, despite growing in length somewhat, and then gradually degenerated from 11 days in vitro (Plate 19).

2.4 Human material

Several human hydatid cysts (or hydatid fluid containing protoscoleces from infected patients) were examined in this laboratory, but only one batch of material (Hu.3), was successfully cultured in vitro. The host was a female from Saudi Arabia, who had four (relatively small) cysts removed from the peritoneal cavity. Only enough material for two cultures was obtained from the cysts. The protoscoleces were placed in M.D. bottles, containing 20 ml S.10.E over a 20 ml NBCS base. The liquid medium was gassed with 10% CO₂, 5% O₂ in N₂, and changed every 3 days.

Stage II was apparent by 4 days in both vessels, although over the first two weeks in vitro, many worms became vesicular, the remainder being generally inactive. However, by day 18, the worms began to elongate, and, both banding and genital streaks were observed in the two cultures on day 28. By day 40, a second segment had begun to develop in a few worms, with the appearance of cirrus patches and testes in some. The cultures were maintained for 85 days, by which time the worms

had degenerated somewhat. No further development was observed. (Plate 18).

The results of all the above E. granulosus cultures have been summarized in Table 6., following the developmental pattern in Fig.7, with the successful experiments being shown in Fig.8.

Most of the culture work on this species was carried out using sheep material, although, compared with E. multilocularis, relatively few experiments were attempted. Many of the early experiments suffered from bacterial infections, and cultures could not be maintained for the length of time required for maturation of E. granulosus, which was much longer than that needed for E. multilocularis. Consequently, for the above reasons, and also, the great intra- and inter-culture variation in development observed in vitro, no conclusive results were obtained, regarding the most suitable culture medium, vessel, gas phase, etc., required for the growth of this species in vitro. Nevertheless, a diphasic culture medium generally induced better development than monophasic (although the most successful horse culture, was in S.10.E only), and a NBCS macerate or solid serum base, was preferable to a 'pitted' serum base, in which the parasites became trapped, resulting in apparent inhibition of their maturation.

Although the immature, evaginated protoscoleces (i.e. stage I), generally appeared more uniform at the onset of the cultures, if compared with E. multilocularis, they contained many more calcareous corpuscles. Consequently, it was more difficult to clearly assess the time of appearance of developmental stage III, as fewer corpuscles were 'lost' in E. granulosus in vivo. This was especially so with the horse material - see Table 6. In addition, details of the morphology of the genitalia were also very difficult to see in cultures, often because the worms were quite dark, and in some (especially horse and human), a thick tegumental coat sometimes

completely enveloped the posterior part of the worm.

As with E. multilocularis, no shelled eggs were ever observed in cultured worms; neither was self insemination seen.

D. DISCUSSION

1. In vitro culture protocol

In order to study the development of E. multilocularis and E. granulosus protoscoleces in vitro, it was primarily necessary to find a suitable culture medium, (ideally) capable of sustaining growth and maturation to the adult stage, at a rate comparable to that observed in vivo. In addition, it was desirable to establish a culture protocol that was relatively straightforward, and readily reproducible. By modifying procedures described by previous authors (cited in the Introduction) for the cultivation of cestodes in vitro, including Echinococcus spp. such a protocol has now been developed, and is described in detail in the Materials and Methods Section.

The culture medium (S.10.E) found to be most suitable, was little different from that recently described by Smyth (1979a) and Smyth & Davies (1979) - i.e. S.10. However, in this study it was found ^{un}necessary to inactivate the FCS (at least, in the batches tested in this laboratory), which thus simplified the procedures involved in the preparation of the medium. S.10 was also modified in other ways; firstly, CMRL 1066 was routinely used, since Parker's 858 medium had become commercially unobtainable, and comparable results were not obtained with other basic tissue culture media (e.g. RPMI 1640, NCTC 135, Medium 199). In addition, 20 mM hepes buffer was routinely included in the media, along with 10 mM sodium bicarbonate. This proved most effective in maintaining the pH of S.10.E, especially in the stock bottles, and it is thought that the addition of hepes was partly responsible for the uniformity of cultures, and relatively consistent results achieved. One other modification of the media, was the introduction of an alternative antibiotic, viz. gentamycin (50 mg/ml). This replaced the more routinely used and relatively inexpensive penicillin and streptomycin (100 i.u./ml and 100 µg/ml respectively). Both Rudin, Healey, Phillips, Gump & Forsyth (1970) and Schafer, Pascale, Shimonaski & Came (1972)

recommended the use of gentamycin in tissue culture, in preference to combined penicillin and streptomycin. Its stability at various pH and temperature levels, its ability to remain active in the presence of serum, and to withstand autoclaving, along with the fact that it "is free from apparent cytotoxicity at bactericidal and antimycoplasmal levels", consequently suggested gentamycin to be a "superior (antibiotic) in tissue culture" (Rudin et al, 1970; Schafer et al, 1972). In this, current investigation, it was certainly found to be a most suitable inclusion in the culture media, and thought to be partly responsible for a reduction in the number of bacterial infections encountered during in vitro experiments. It was considered unnecessary to add an antifungal agent such as fungizone, since fungal contamination was relatively rare, and it was thought preferable to keep antibiotic and antimycotic agents at low levels. Higher than usual concentrations of antibiotics (and fungizone) were used however for obvious reasons, when washing the adult parasites removed from dogs, prior to culture.

One of the major problems in the preparation of the culture medium, was the inevitable variation in the constituents - (such as the water, serum, yeast extract, dog bile, etc), from one batch to another. This inconsistency has already been described by numerous authors with regard to in vitro culture work, and it is generally accepted that variations in the quality of media components, account for some of the developmental differences observed between culture experiments. In an attempt to eliminate this problem, several precautions were observed during the preparation of the media used in this particular study.

The importance of the purity of the water used for reconstituting powdered media and BSS is self-evident. In this laboratory, sterile, triple distilled water was used throughout, in order to maintain uniformity, the water then being resterilized during filtration of the media. Powdered media and BSS were purchased from reliable companies, upon whose quality control we had to depend. This also applied to

other purchased powder constituents such as yeast extract and glucose. However, no such control over the quality of the serum or dog bile could be maintained from one batch to another. For this reason, it was necessary to primarily 'screen' each batch in the culture system, prior to experimental work, by observing worm survival and rate of somatic and germinal development over a period of several weeks.

Due to the relatively long development time of E. multilocularis in vitro, it was impractical to assess the importance (as well as the most appropriate levels) of each and every constituent in medium S.10.E, especially as the optimum development of the worms achieved in vitro, was very similar to that observed in vivo. Suffice to say here, that the importance of a high glucose level, the addition of yeast extract and bile have previously been demonstrated to be necessary for the general establishment, growth and maturation of cestodes in vitro (for example: - De Rycke & Berntzen, 1967; Khan & De Rycke, 1975, 1976 - Hymenolepis microstoma. Smyth & co-workers, 1959 onwards - E. granulosus and E. multilocularis).

Apart from the basic tissue culture medium CMRL 1066, a substantial percentage of the media is composed of serum. In this study, a level of not less than 25% FCS was found to be necessary for the optimum development of E. multilocularis protoscoleces in vitro; a similar level to that recently used by Smyth & co-workers (1974-). Nevertheless, due to the variability of serum batches, it has long been accepted that inconsistent results are often obtained from one culture experiment to another (Khan & De Rycke, 1975 - H. microstoma; Smyth & Davies, 1975 - E. granulosus). A recent publication by Price & Gregory (1982), described measurements of various chemical and physical properties of sera, in an attempt to correlate results with growth promotion properties. They concluded that there might be a relationship between growth hormones, endotoxin and bilirubin levels, and growth promotion, although it was difficult to draw any direct conclusions. They

also mentioned that inactivation significantly lowers several growth enhancing components of serum. This could account for the improved growth of E. multilocularis in vitro observed in S.10.E, if compared with earlier experimental work when the FCS was inactivated (Smyth & Davies, 1975; Smyth, 1979a). With regard to the use of serum in culture media, and the widely different growth promotion properties of different batches, it is most encouraging to note that several cell cultures may now be maintained in serum-free media, using instead, a defined combination of growth factors, hormones and other essential nutrients. (Collaborative Research Inc., 1980, 1981). The advantages of such systems are obvious, especially concerning the uniformity of culture conditions. Preliminary experiments in this laboratory using ITS Premix (Collaborative Research Inc.) containing bovine insulin ($5 \mu\text{g/ml}$ medium), human transferrin ($5 \mu\text{g/ml}$) and selenium (5ng/ml) as a serum replacement (either totally or partially), have proved inconclusive, but further work along these lines may prove enlightening.

The fact that E. multilocularis may be grown from protoscoleces to the strobilate stage in vitro in monophasic media, confirms earlier observations (Smyth & Davies, 1975; Smyth, 1979a; Smyth & Barrett, 1979; Smyth & Davies, 1979). In addition, Thompson & Eckert (1982) demonstrated no differences in development of strobilate worms in either mono- or di-phasic media in vitro, following partial maturation for 20/21 days in vivo. In comparison, it has long been recognised that E. granulosus protoscoleces of sheep origin will only develop to the adult stage in vitro in diphasic media, a monophasic liquid medium initiating cystic, rather than strobilar development (Smyth, 1962, 1967; Smyth et al, 1966, 1967), indicating possible physiological differences between the two species. Nevertheless, both Smyth & Howkins (1966) and Kumaratilake & Thompson (1981) have shown that partially developed E. granulosus protoscoleces from dogs, between 28 and 38 days p.i., will continue to mature in vitro to the gravid stage in both mono- and di-phasic media (as with E. multilocularis). These authors' observations suggest that E. granulosus

protoscoleces require contact with a solid, nutritive base in the early stage of strobilar development only (Smyth & Howkins, 1966). With the aim of simplifying the culture protocol, the use of mono- rather than di-phasic culture media for E. multilocularis during this particular study, was clearly an advantage.

2. Echinococcus multilocularis

In vitro culture procedures offer an important tool for developmental and morphological studies of parasitic helminths, but, as emphasized by Weinstein (1966), results must be interpreted with caution, and substantiated by observations of "material derived from in vivo development in a normal host".

In this current investigation, in vitro culture has enabled a comprehensive study on the development of the strobilate stage of E. multilocularis, following observations of the consistent maturation of protoscoleces to stage 9 (i.e. with 3 proglottids) in culture. These observations were subsequently compared with those of parasites in vivo, using material from the same source (i.e. the larval stage maintained by i.p. passage in laboratory rodents).

Although the in vivo studies were limited to worms collected from only 3 dogs, from the results obtained, it appears that the pattern of development of worms in vitro, closely resembles that observed in vivo. In other words, maturation of the first proglottid to stage 7 (i.e. the sexually mature, so-called 'monozoic' adult) occurs prior to segmentation and the subsequent appearance of genital anlage in the second proglottid. Smyth & Davies (1975), Smyth (1978a, 1979a), Smyth & Barrett (1979) and Smyth & Davies (1979) have previously described such 'monozoic' development of E. multilocularis in vitro, as being abnormal, and suggested that it was due to inadequate nutritive and/or physicochemical factors in the culture system.

Although the mode of development in vivo and in vitro in this study are similar, the growth rate in vitro is somewhat slower, (see Fig.6) suggesting that the in vivo situation is not fully replicated in our culture system.

Many early observations of E. multilocularis adults from both natural and experimentally infected hosts, were solely concerned with the gross morphology of the segmented worms rather than the actual pattern or rate of development (Rausch, 1956; Rausch & Schiller, 1954; Vogel, 1957). These, and other authors, recognised the taxonomic value of certain obvious morphological characteristics in the genus Echinococcus (strobilate stage), although Smyth & Smyth (1964) and Rausch (1968), and others, subsequently emphasized that species should not be differentiated on these criteria alone, since a variety of intrinsic and extrinsic factors could account for certain morphological differences seen in same species.

Very few authors have considered in detail, the development of the adult stage in vivo, with many observations being carried out post-maturation (Yamashita, Ohbayashi & Kitamura, 1958a,b; Smyth & Davies, 1975; Crellin et al, 1981; Thompson & Eckert, 1982, 1983). The findings of these authors, along with those of this current investigation, are summarized and compared in Table 7 and Fig.9. It is clearly apparent that the reported rates and pattern of development in vivo vary considerably. This may be due to the different isolates of E. multilocularis used in the experimental infections, or possibly, host induced. In fact, with regard to the latter, Smyth & Davies (1975) reported poor establishment and growth of E. multilocularis in cats, although, in this particular investigation, the same isolate of E. multilocularis resulted in a large number of well-developed adults in experimentally infected dogs. This observation is consistent with the results of earlier research by Vogel (1957), Rausch & Richards (1971), Crellin et al (1981) and Thompson & Eckert (1983), who have all shown cats to be less suitable than dogs as definitive hosts of E. multilocularis (of N. American or German origin).

On comparing the development of this parasite in experimentally infected dogs, Yamashita et al (1958a,b) obtained two-segmented worms by 15 days p.i. (using material of Alaskan origin); a much faster in vivo growth rate than observed with material (originally isolated in Germany) from

Fig.9

Comparative development of the strobilar stage of
Echinococcus multilocularis, in vivo (dogs) and in vitro

In vitro:

Discontinuous line shows the best results
obtained in this study

In vivo (dogs):

- ▲ after Yamashita et al (1958a,b)
- ◆ after Thompson & Eckert (1982, 1983)
- (◆) personal observations of material sent from
Zurich by Thompson & Eckert (see Section II)
- current study

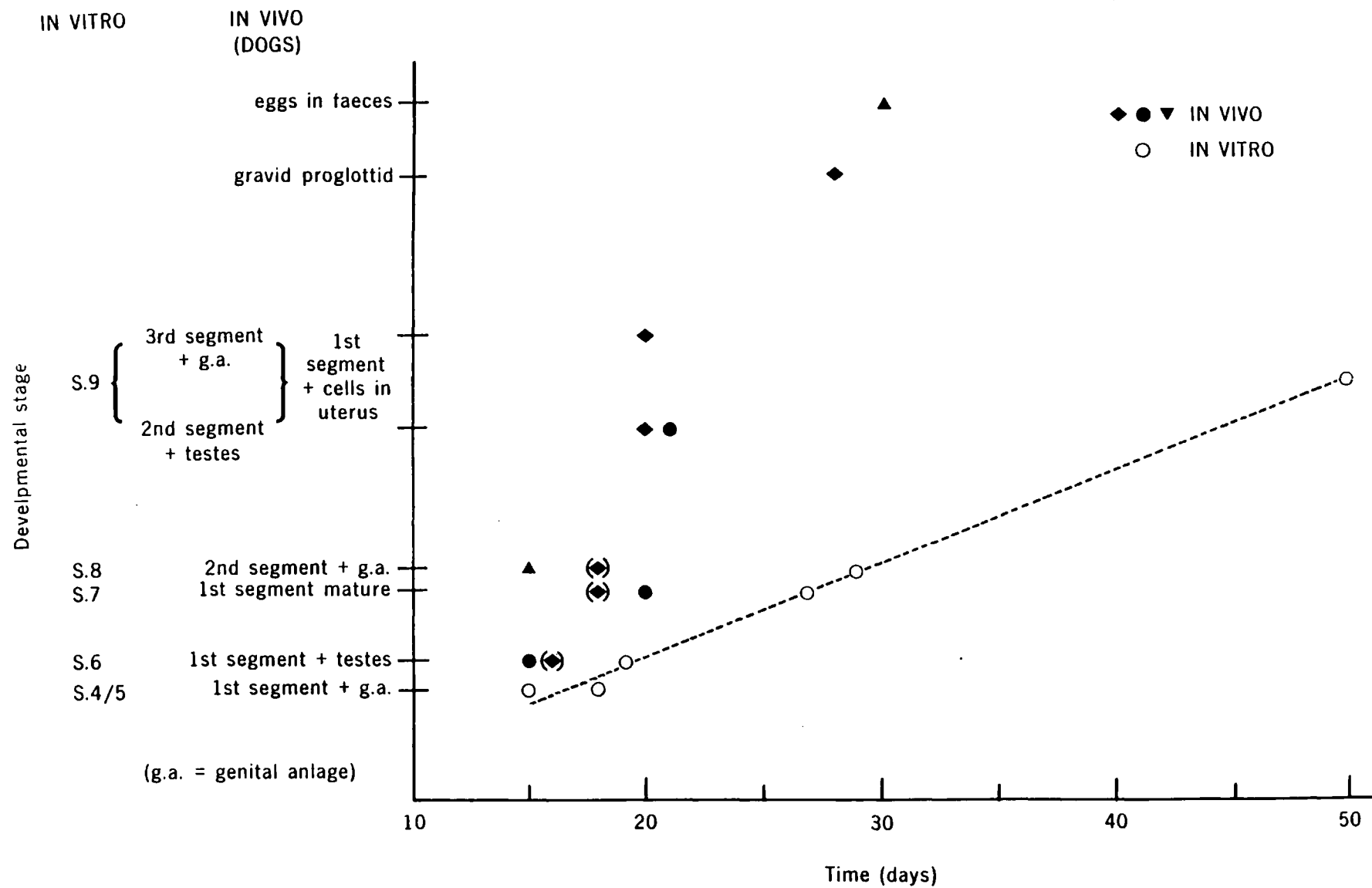


Table 7

Development of the strobilate stage of
Echinococcus multilocularis in vivo; a comparison of studies

<u>Study</u>	<u>Host</u>	<u>Development (days)</u> (<i>c.p.</i> = <i>cirrus patch</i> ; <i>g.a.</i> = <i>genital anlage</i>)
Yamashita <u>et al</u> (1958a,b)	Dog (species not defined)	15d:2 proglottids (1st mature; 2nd with g.a.) 117d:4 " (1st gravid; 2nd mature; 3rd testes; 4th g.a.) 290d:4 " (1st gravid; 2nd mature to gravid; 3rd testes; 4th g.a.)
Smyth & Davies (1975)	Cat (undefined)	11d:1 proglottid 18d:1 " (testes) 25d: ? ("tubular genitalia")
Crellin <u>et al</u> (1981)	(a) Dog (various) (b) Cat (various)	21d: "immature" 21d: "immature" (but smaller than (a))
Thompson & Eckert (1982)	Dog (Appenzeller)	20d:3 proglottids (1st uterus containing unembryonated eggs & vitelline cells; 2nd & 3rd undefined) 21d:3 proglottids (as 20d)
Thompson & Eckert (1983)	(a) Dog (Appenzeller) (b) Cat (Fullinsdorf, Hoffmann La Roche)	20d:3 proglottids (1st uterus containing unembryonated eggs & vitelline cells; 2nd testes & c.p.; 3rd g.a.) 21d:3 proglottids (as 20d) 21d:2 " (1st testes & c.p.; 2nd g.a.) (smaller than (a) - see also Crellin <u>et al</u> , 1981)
Barrett (1984) (current study)	Dog (Greyhound)	15d:1 proglottid (testes & c.p.) 20d:1 proglottid (ova & vitelline cells in uterus) 21d:2 proglottids (1st as 20d; 2nd testes)

this laboratory. Experimental work by Crellin et al (1981), using material collected in Minnesota, resulted in "immature" worms from dogs 21 days p.i.; no reference was made to segmentation. More recently, Thompson & Eckert (1982, 1983) reported development of worms to the three-segment stage by 20 days p.i., with the uterus in the terminal proglottid containing unembryonated eggs and vitelline cells; little further development was seen by 21 days p.i. In contrast, worms collected from a dog 20 days p.i. in this study, possessed only one mature segment, with ova and vitelline cells in the uterus. By 21 days p.i., a second segment containing testes, was observed in the majority of parasites.

Since there are no further published reports on the development of the adult parasite in dogs prior to segmentation, it is not known whether the so-called initial 'monozoic' development of E. multilocularis demonstrated in this study, both in vivo and in vitro (see also, Smyth & Barrett, 1979), is specific to our particular isolate of the species. Nevertheless, we have observed some fixed material from dogs, 16, 18 and 20 days p.i., sent from Zurich by Prof. J. Eckert and Dr. R.C.A. Thompson (see Section II). The 20 day old worms were as already described by Thompson & Eckert (1982, 1983). The 18 day old worms comprised two, or sometime three, segments, with the first proglottid being sexually mature. Of the 16 day old worms, many were immature, but in some, the male genitalia were fully developed in the first segment, with sperm obvious in the testes, genital atrium, and occasionally, in the receptaculum. However, further observations of their material is necessary before it can be ascertained whether full sexual maturity occurs prior to the formation of, and genital development within, a second proglottid. Occasionally, stained (Aceto-orcein), whole mounts of material cultured from the larval stage in this laboratory, revealed the presence of a second genital anlage, prior to full maturation of the genitalia in the terminal segment (Smyth & Barrett, 1979).

Both our material, and that used by Thompson & Eckert (1982, 1983) for in vivo studies, were originally isolated in

Germany. However, the growth rate of their material, was clearly much faster than that used in the study (but comparable to the observations of Yamashita et al, 1958a,b). Since the larval stage in this laboratory has been passaged through rodents for many years, it is conceivable that a new variant of the species has arisen (see Smyth & Smyth, 1964).

One interesting point to note is the so-called 'pseudosegmented' development of E. multilocularis in vitro following several weeks culture, observed and described by Smyth & Davies (1975), Smyth (1978a, 1979a), Smyth & Barrett (1979) and Smyth & Davies (1979). In this study, the demarcation between proglottids (both in vivo and in vitro) was not as obvious as would be expected, or as previously observed in other cestodes. Melhorn et al (1981) have recently demonstrated that, in E. multilocularis (and some other cestodes), no interproglottid membrane exists, and that 'segmentation' is solely defined by a circular constriction of the strobila between each set of genitalia, and, in some cases, an overlapping by anterior proglottids. This investigation confirms these observations, and it is suggested that, in E. multilocularis, the constriction between proglottids, is not that well defined. It has also been noted during this study that, whilst fresh or fixed, unstained material may appear 'pseudosegmented', stained whole mounts often show a clear segmentation pattern. In fact, photographs published by other workers (Thompson & Eckert, 1982, 1983) of adults from dogs (stained and unstained whole mounts), clearly substantiate this point. It is thus proposed that the terms 'segmentation' and 'banding' in describing the development of E. multilocularis in vivo (referred to by Smyth & Davies, 1975 and Thompson & Eckert, 1982, 1983) be interpreted with caution, and should not be considered as specific stages in the maturation of this species, the apparent onset of segmentation being dependent on the subjective observations of the authors.

Although some cultures have been maintained for over three months in vitro, there is commonly a gradual deterioration in the parasites after about six weeks. This was far more obvious

in worms grown in vitro following partial maturation in vivo (also observed by Thompson & Eckert, 1982). However, there are no known recorded accounts of the longevity of the adults within definitive hosts, although Yamashita et al (1958a,b) maintained an experimental infection in a dog for 290 days. In this study, both dead and degenerate worms were recovered from dogs as early as 21 days p.i., well before the formation of gravid proglottids. Thompson & Eckert (1982) did not report on the condition of adults recovered from dogs (20 and 21 days p.i.), but did mention that no destrobilization occurred in their subsequent in vitro studies. The authors thus suggested that destrobilization was not merely a consequence of the terminal proglottid becoming gravid but may be due to physical and/or chemical activities occurring at the host-parasite interface in vivo (Thompson, Dunsmore & Hayton, 1979). In this study however, a few cases of destrobilization were observed in vitro, both in cultures established from the larval stage, as well as those with worms recovered from dogs. Limited observations of this phenomenon, prevent speculation on the destrobilization stimulus in E. multilocularis

In spite of the apparent normal development of the genitalia, with the production of an abundance of sperm, ova and vitelline cells, to date, no fertilized eggs have been found in vitro. It is possible that the culture conditions are lacking in some physico-chemical factors, necessary during the latter stages of worm development. It is of interest of note that at 15 day p.i., the majority of parasites collected from the dog intestine were in the anterior half, whereas at 20 and 21 days p.i., most were found in the 3rd and 4th (of 6) sections. This leads to a tentative suggestion that the requirements of the developing adult worms may alter during the course of maturation. In fact, Kumaratilake & Thompson (1981) stated that mature E. granulosus worms (28 days p.i. from dogs) will continue to develop normally in vitro in monophasic media, whereas early development in vitro is dependant on the addition of a nutritive base in the culture vessels. Therefore, it may be necessary to alter the culture environment during the course of in vitro experiments. Nevertheless, by following methods

described by previous authors (Smyth & Howkins, 1966; Kumaratilake & Thompson, 1981; Thompson & Eckert, 1982) for the in vitro culture of partly matured strobila of Echinococcus spp. from experimentally infected dogs, immature oncospheres of E. multilocularis were obtained. Unfortunately though, due to the pathogenicity of this species in man, further observations on this particular stage of development could not be pursued. However, it demonstrated one important point, namely that following numerous years of i.p. passage of the larval stage through laboratory rodents, E. multilocularis was still capable of developing to the sexually mature, egg-containing adult, albeit the fact that the normality and/or viability of the resultant oncospheres could not be proved. The absence of fertilized eggs in cultured material has previously been attributed to lack of self-insemination, and hence fertilisation in vitro (Smyth, 1982b). However, it is possible that morphological or developmental abnormalities, not discernable at the light microscope level, could account for the limited development seen in vitro, and so, for this reason, detailed morphological studies on the developmental biology of the strobilate stage of E. multilocularis were undertaken (see Section II).

The occasional appearance of abnormal forms in vitro, such as the development of a second scolex, has previously been recorded (Smyth & Barrett, 1979). Although these may reflect on a deficiency in the culture medium, the occurrence of anomalies in protoscoleces of E. multilocularis (and E. granulosus) has previously been observed, both in this laboratory, and by Sakamoto (1978b,c). It therefore seems likely that they represent mutants.

3. Echinococcus granulosus

Due to limited studies on the in vitro culture of E. granulosus protoscoleces in this study, no firm conclusions can be drawn, as to the best culture protocol for optimum development of this particular species. It is, however, of interest to note some developmental differences between the maturation of E. multilocularis and E. granulosus (of sheep origin), in vitro. The rate of development of the latter is certainly much slower, both in vitro and in vivo, as described by previous authors (Smyth & Howkins, 1966; Smyth et al, 1967; Smyth, 1967; 1979a,b; Smyth & Davies, 1975; Thompson, 1977; Kumaratilake & Thompson, 1981; Kumaratilake, Thompson & Dunsmore, 1983), and confirmed in this in vitro study. Another major difference is in the gross morphological changes observed during maturation. In E. multilocularis, the genital anlage is obvious prior to proglottid formation, whereas in E. granulosus, the development of the reproductive structures usually follows the onset of segmentation. The phenomenon of banding as a precursor to true segmentation, has already been described for E. granulosus and briefly discussed earlier for E. multilocularis. In the present study, it was observed that the constriction between proglottids was certainly more marked in E. granulosus, banding clearly indicating the onset of segmentation in vitro. However, ^{one}~~of~~ again, no interproglottid membrane was observed, confirming the results of Melhorn et al (1981).

As mentioned earlier, previous work with E. granulosus has emphasized the need for a diphasic culture medium, for protoscoleces to develop to the strobilar stage in vitro, and this investigation generally confirms that better development is achieved in such conditions. However, in one experiment, segmentation and genital development was observed in liquid medium S.10.E only, but it is difficult to account for this anomalous result. In the same experiment, destrobilization was observed, as also described by other workers (Smyth, 1967; Smyth & Davies, 1974b), although Thompson (unpublished data - see Thompson & Eckert, 1982), stated that no free proglottids

were observed in his own cultures of E. granulosus.

The best observed in vitro growth rate of E. granulosus protoscoleces from sheep, is somewhat slower than the maturation time in vivo, described by previous authors (see Fig.10), although the in vivo development time of sheep material has recently been shown to be quite variable due to intraspecific, or strain differences (Kumaratilake et al, 1983). However, the in vitro results compare favourably with those described from experiments of Smyth & Davies (1974b) and Smyth (1979a,b).

With E. granulosus of horse origin, little growth was observed in vitro, as also noted by Smyth & Davies (1974b) and Smyth (1979a,b), indicating possible physiological differences between the horse and sheep strains. However, in one experiment, a few worms segmented and some genital development was seen, in medium S.10.E. In another, the initiation of segmentation was apparent. This must again be regarded as an anomalous result, since no such development occurred in previous or subsequent experiments, and the time of maturation was far greater than the reported growth rate in vivo (Thompson, 1977). Nevertheless, it is the only occasion where such growth has been observed for E. granulosus protoscoleces of horse origin.

Only a few cultures were established with material from cattle, and in these very little growth was observed in vitro, generally following a developmental pattern similar to the horse protoscoleces (also observed by Smyth, 1979b). However, since the cultures were maintained for no longer than 17 days, these results cannot be accepted as ^{un}equivocal.

One successful culture of human material was maintained for several weeks, during which time, development to the segmented, adult stage was achieved, although full maturity of the genitalia was not obtained. The early developmental pattern of these protoscoleces, thus closely resembled that of the sheep material in vitro (Smyth et al, 1980).

Fig.10

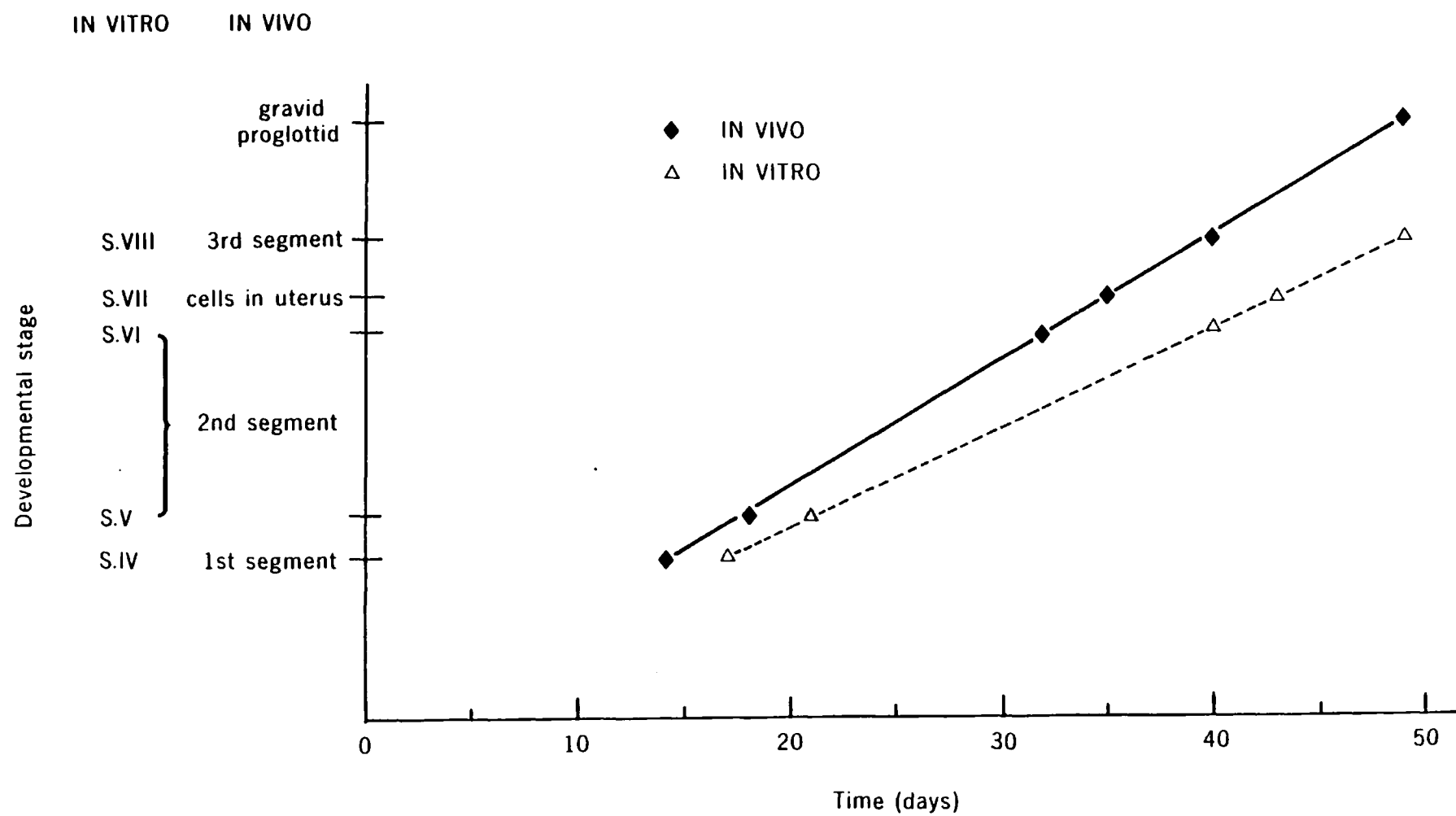
Comparative growth rate of the strobilar stage of Echinococcus granulosus (sheep origin), in vivo and in vitro

In vitro:

Discontinuous line shows the best results obtained in this study.

In vivo:

after Smyth (1967, 1979a,b), Smyth et al (1967) and Smyth & Davies (1974a,b).



Due to the limited number of experiments concerning the in vitro development of E. granulosus protoscoleces from various intermediate hosts in this study, it is difficult to speculate on strain differences, or host influence on parasite development, although these subjects have previously been discussed in detail by numerous authors (Rausch, 1967b; Smyth, 1967, 1968, 1978b, 1979a,b; Smyth & Davies, 1974a; Smyth & Davies, 1979; Macpherson, 1981; Kumaratilake & Thompson, 1982; Kumaratilake et al, 1983). It is however, of interest to note that Macpherson & Smyth (1984) have recently described the growth and segmentation of protoscoleces from a variety of intermediate hosts (human, camel, cattle, sheep, goat and buffalo) in Kenya, using a culture system similar to that described in this study. The pattern of development described by the authors was similar in all strains cultured in vitro; segmentation and the appearance of genital anlagen were noted in all cases, but with testes appearing in camel and cattle cultures only. No sexually mature worms were observed, as was the case with the human material cultured in this investigation. The initial development of the strobilar stage of E. granulosus in vitro, using material collected from a variety of intermediate hosts, thus, in general, shows a similar pattern. This may indicate that they represent closely related strains, although biochemical (McManus, 1981) and isoenzymatic (Macpherson & McManus, 1982) studies have shown some marked differences in their characteristics. However, since the development of adult E. granulosus from various hosts in vitro, has been shown to vary considerably, both in previous studies and in this investigation (for example, the segmentation and sexual development seen in occasional cultures of horse material), it is difficult to draw any specific conclusions at this point, on strain differentiation in E. granulosus, as determined by in vitro culture. Further experimental work is required using material of different origins, in a series of concurrent cultures, maintained under identical conditions. Current research by Macpherson (personal communication) in Kenya, on cross infectivity studies with parasites from various intermediate and definitive hosts,

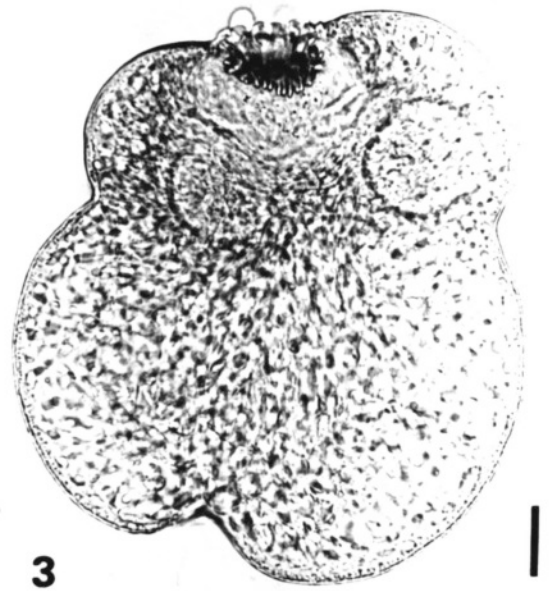
should throw further light on this question of intraspecific variation in E. granulosus.

PlatesSection IAbbreviations

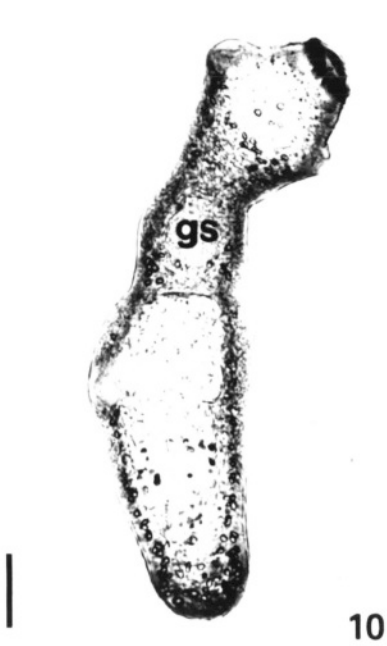
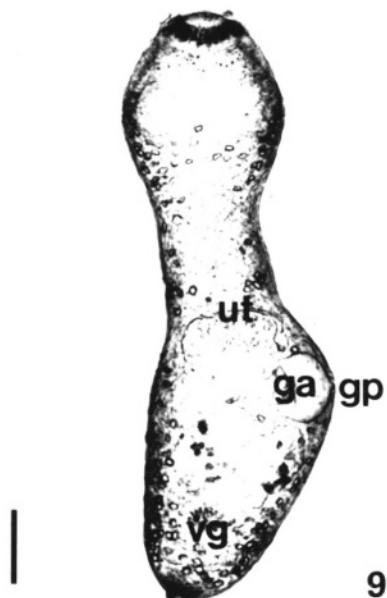
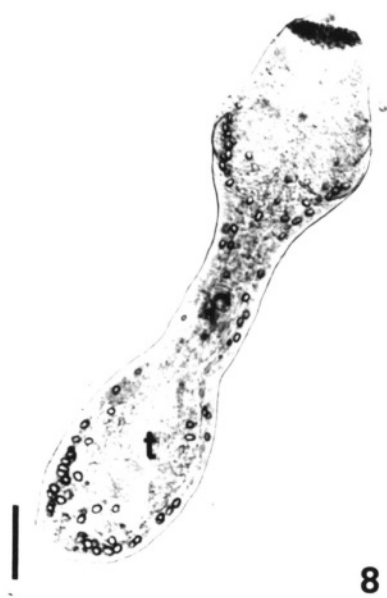
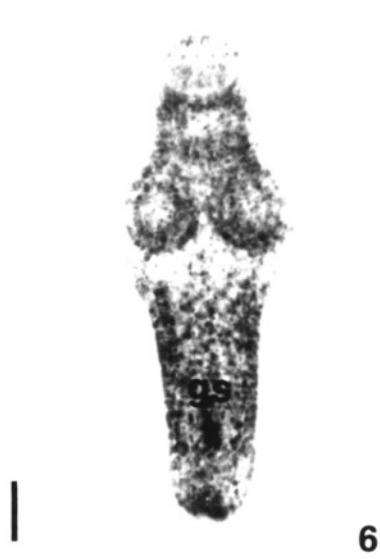
cc : calcareous corpuscles
cp : cirrus patch
eb : excretory bladder
ed : excretory duct
ga : genital atrium
gp : genital pore
gs : genital streak
t : testes
ut : uterus

Plates 1-5E. multilocularis in vitro

- Plate 1 Evaginated protoscolex (Stage 1)
Note presence of calcareous corpuscles.
Scale bar reps. 50 μm
- Plate 2 Vesicular protoscolex (5 days in vitro)
Scale bar reps. 50 μm
- Plate 3 Vesicular protoscolex (8 days in vitro)
Note characteristic darkening of cells
Scale bar reps. 50 μm
- Plate 4 Vesicular protoscolex (8 days in vitro)
Scale bar reps. 50 μm
- Plate 5 Stage 3 (8 days in vitro)
Note excretory ducts, posterior excretory bladder, and reduction in number of calcareous corpuscles.
Scale bar reps. 50 μm



Plates 6-11E. multilocularis in vitro
(refer to Fig.5)Plate 6Stage 4. Scale bar reps. 50 μm Plate 7Stage 5. Scale bar reps. 50 μm Plate 8Stage 6. Scale bar reps. 100 μm Plate 9Stage 7. Scale bar reps. 100 μm Plate 10Stage 8. Scale bar reps. 100 μm Plate 11Stage 9. Scale bar reps. 250 μm



Plates 12-14E. multilocularis in vivoPlate 12

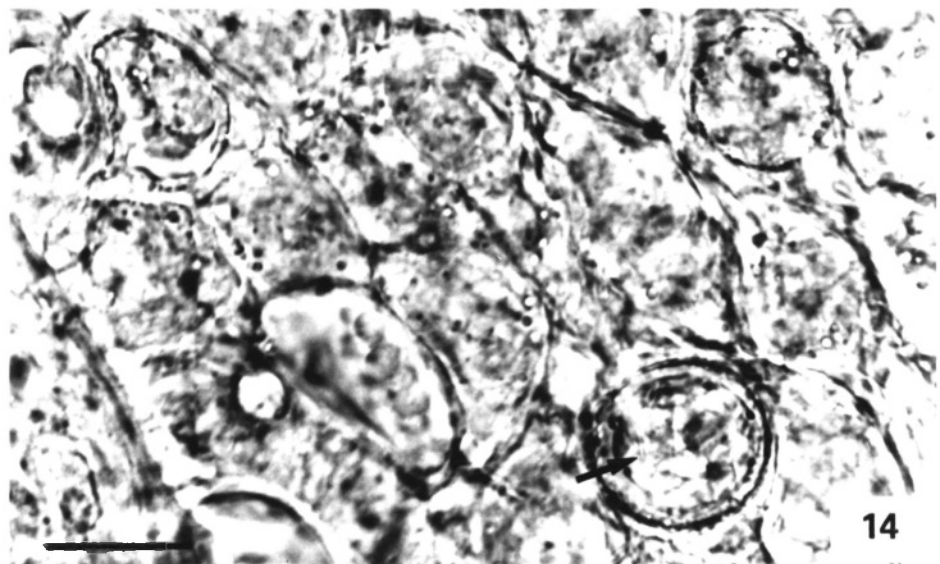
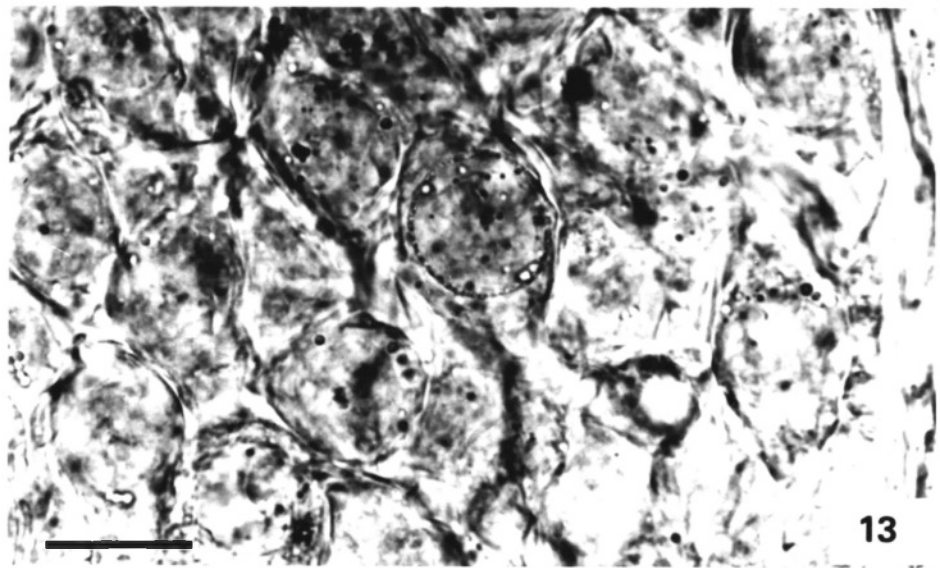
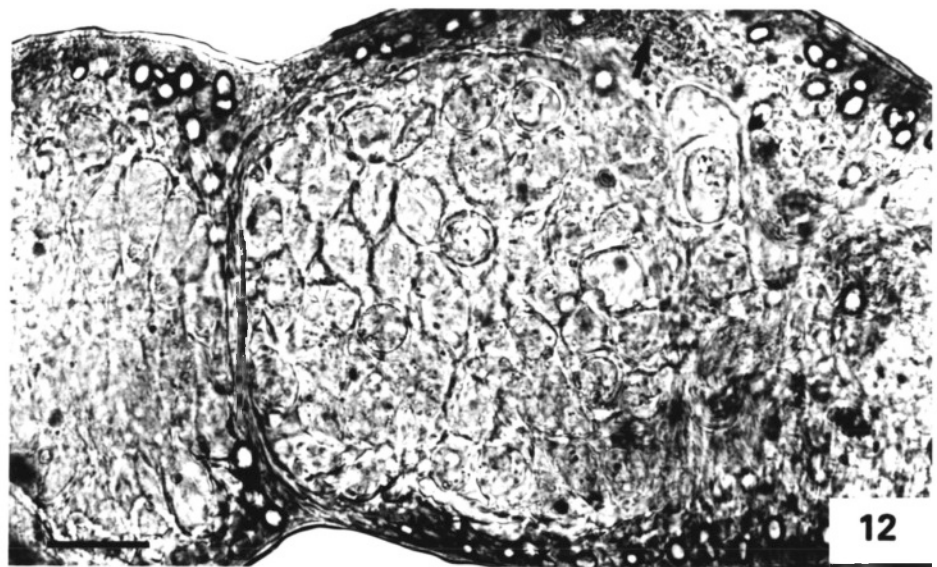
Uterus of terminal proglottid (21 days in vivo
+ 5 days in vitro)
Note the presence of sperm in genital atrium
(arrow) (Neutral red).
Scale bar reps. 50 μ m.

Plate 13

Detail of developing embryos.
Scale bar reps. 20 μ m.

Plate 14

Detail of immature oncosphere.
Note appearance of characteristic taeniid egg
shell and oncospherical hooks (arrow).
Scale bar reps. 20 μ m.



Plates 15-20E. granulosus in vitro
(refer to Fig.7)

- Plate 15 Stages IV/V (sheep material).
Scale bar reps. 100 μm
- Plate 16 Stages VI/VII (sheep material).
Scale bar reps. 100 μm
- Plate 17 Stage VIII (sheep material).
Scale bar reps. 200 μm
- Plate 18 Human material.
Scale bar reps. 100 μm
- Plate 19 Horse material, showing usual stage of
maturation achieved (stage III).
Scale bar reps. 50 μm
- Plate 20 Horse material, showing segmentation.
Note the thick, amorphous coat on the
strobila (arrows).
Scale bar reps. 500 μm

15



16



17



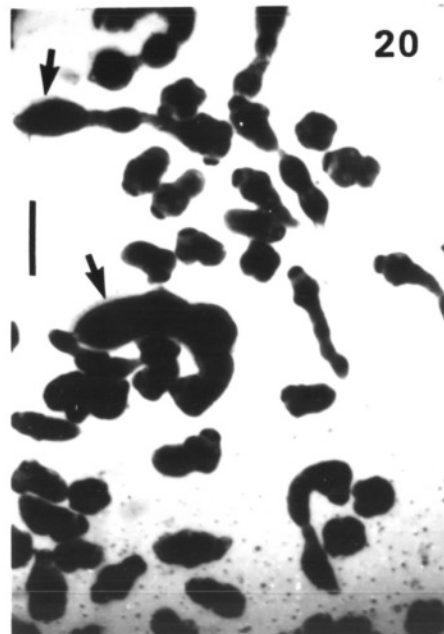
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19



20



SECTION II

Structure and ultrastructure of adult Echinococcus multilocularis in vivo and in vitro, with particular emphasis on the reproductive system

A INTRODUCTION

1. Introduction to the reproductive system of cestodes

Although sexual reproduction is a major feature in the life-cycle of cestodes, the tapeworm reproductive system as a whole, has not been extensively studied, especially amongst the cyclophyllideans (Lumsden & Specian, 1980). Much of the literature on cestode genitalia has been derived from light microscopy, but, over the past decade, several morphological and cytological studies at the electron microscope level have increased our knowledge on the more obvious elements (see later). Nevertheless, comprehensive accounts of the entire reproductive system are scarce. Published reports include those of Sulgotowska (1972, 1974), on the histogenesis of Hymenolepis diminuta; Rybicka (1964a, 1966), on gametogenesis and embryonic development in Dipylidium caninum, and who reviewed early work on other cestode species, and Galkin (1979), on morphogenesis of the genital system of Passerilepis crenata. A more recent account by Lumsden & Specian (1980), described the morphology, histology and fine structure of H. diminuta, at both the light and electron microscope level.

As mentioned by Silverman (1981), the establishment of parasitic helminths in in vitro culture, has made many phases of their life cycles available, ~~many phases of the life-cycle~~ for ultrastructural study. In this current investigation, the in vitro culture of E. multilocularis protoscoleces to the strobilate stage, has enabled detailed observations on the gross morphology and fine structure of the reproductive system to be carried out. These have been supported, to some degree, by observations of material grown in vivo (see Weinstein, 1966).

2. Review of the literature on the structure and ultrastructure of adult Echinococcus multilocularis

Most early publications on the gross structure of the strobilar stage of E. multilocularis outlined certain morphological characteristics, many of which proved to be of some taxonomic value, especially when differentiating between species of Echinococcus (Rausch & Schiller, 1954; Rausch, 1956, 1968; Vogel, 1957; Rausch & Nelson, 1963; Kurashivilli, 1964; Smyth & Smyth, 1964; Rausch & Richards, 1971). Nevertheless, although the gross morphology of E. multilocularis was very similar in all cases, slight variations in some morphological characteristics (such as number and size of proglottids, number and distribution of testes, position of the genital pore), were noted by many of these authors. It was consequently suggested that these differences were due to both intrinsic and extrinsic factors; namely, variations between geographically isolated species, or between worms recovered from different definitive hosts.

The results of Rausch & Schiller (1954), Rausch (1956) and Vogel (1957), were supplemented with diagrams of the mature (and also, gravid) proglottids of E. multilocularis, outlining the organisation of the reproductive structures. More recent publications by Eckert et al (1974), Eckert (1981) and Zeyhle (1982b), briefly described the gross morphology of the adults, as a means of identification, but with little reference being made to the arrangement of the genitalia, or other structures.

An electron microscope study of this species was undertaken by Melhorn et al (1981), but these authors were more concerned with the nature of segmentation in cestodes, rather than the structures encountered in each proglottid (see Section I). Nevertheless, they observed that (1) the uterus in the terminal, gravid proglottid (28 days p.i.) extended into the parenchyma of the penultimate one, (2) the longitudinal muscles were continuous throughout the worm's length, and (3) the parenchymal cells of each segment were not separated by an interproglottid membrane.

Thompson & Eckert (1982, 1983) carried out observations at the light and electron microscope levels, on E. multilocularis, both from experimentally infected dogs, and subsequent in vitro studies. Although most of their results referred to the state of segmentation of the adults, they did, nevertheless, mention the presence of a rostellar gland in mature worms (as observed in E. granulosus by Smyth, Morseth & Smyth, 1969, and Thompson et al, 1979), and briefly described observations of gametogenesis and embryonic development, with the ultimate appearance of shelled eggs in utero.

In 1975, Smyth & Davies reported on the morphology of 'monozoic' adults of E. multilocularis obtained in culture. A more detailed account on the maturation of protoscoleces, and the cytology of the adult genitalia in vitro, was given by Smyth & Barrett (1979). Brief accounts on the comparative morphology of adults in vivo and in vitro, along with preliminary observations on sperm development in adult E. multilocularis, have recently been presented by Smyth et al (1982) and Barrett & Smyth (1983), respectively.

3. Review of the literature on the structure and ultrastructure of genitalia in parasitic platyhelminths

This review considers the observations of previous authors on the maturation of individual elements within the platyhelminth reproductive system. Precise details on the development, morphology and fine structure described in these publications has, however, been omitted here, as they will be considered in the Discussion, where pertinent to observations of E. multilocularis.

3.1 Organ histogenesis

Although histogenesis of the genitalia has not been considered in detail in this study, it is of interest to note the results of other workers on the early development of the reproductive system in cyclophyllideans (Douglas, 1961; Sulgostowska, 1972, 1974; Lumsden & Specian, 1980). It is now generally accepted that the early, primary anlage (primordium), separates into a peripheral primordium (located in the outer medullary region of the proglottid), and a central primordium. Each is comprised of germinative, germinative-somatic and (possibly) somatic cells. A third, somatic, primordium, is also present. The protandric development of cyclophyllideans is initiated by the differentiation of the peripheral primordium into the male organs, and this is followed by the development of the reproductive ducts from the somatic primordium. Subsequently, the central primordium gives rise to the female components.

3.2 Testes (spermatogenesis, spermiogenesis and sperm structure)

A relatively recent publication by Roosen-Runge (1977) on the process of spermatogenesis in animals, reviewed the early knowledge of sperm development in various vertebrate and invertebrate phyla. The author cited several early papers on cestodes and trematodes, stating that most only contained "fragmentary data" on testes and spermatogenesis.

Nevertheless, he concluded that the process of spermatogenesis was apparently similar in platyhelminths (and also "very much alike in all animals"). He also mentioned that the testes of platyhelminths should be more correctly regarded as spermatocysts, since "each contains a single clone of germ cells, and are far removed from the complexity of a metazoan 'organ'". (In this study, the term testis(es) will nevertheless be used, to correspond with the description of other authors).

Most observations on spermatogenesis, spermiogenesis and/or sperm structure in parasitic helminths, have been carried out on digeneans and cestodes, with little attention being paid to monogeneans or acanthocephalans. However, with regard to the latter two groups, Halton & Hardcastle (1976) described spermatogenesis in Diclidophora merlangi. More recently, Justine & Mattei (1982a) briefly described mature sperm of three monogenean families (Heterocotyle sp., Diplectanum sp. and Cleitharticus sp.), and reviewed the observations of previous authors on several other Monopisthocotylea. Spermiogenesis has also been described in three species (Megalocotyle grandiloba, Heterocotyle sp. and Amphibdelloides spp.) by the same authors (Justine & Mattei, 1983a,b,c).

Fewer studies have been undertaken on acanthocephalans, although spermiogenesis and sperm structure have been observed by Whitfield (1971), Marchand & Mattei (1976a,b, 1978) and Marchand (1981).

By far the most comprehensive reports on various aspects of the development and maturation of testes in trematodes and cestodes, are those concerning several species of Digenea. The most recent observations include Burton (1960, 1972) on Haematoloechus medioplexus; Sato, Oh & Sakoda (1967) on Paragonimus miyazakii; Grant, Harkema & Muse (1976) on Pharyngostomoides procyonis; Fujino, Ishii & Mori (1977) on Paragonimus ohirai and Eurytrema pancreaticum; Rees (1979) on Cryptocotyle lingua; Justine & Mattei (1982b) on Gonapodasmius tomex; Robinson & Halton (1982) on Corrigia vitta; and, Erwin

& Halton (1983) on Bucephaloides gracilescens. Each of these authors described spermatogenesis in great detail, and it is now widely accepted that the process involved, are similar in all digenean (and also monogenean) species studied. In other words, a single spermatogonium gives rise to eight primary spermatocytes, by mitosis; subsequent meiotic division, results in 16 secondary spermatocytes and consequently 32 spermatids.

The fine structure of mature digenean sperm has certainly received much attention, and it was, in fact, observations on H. medioplexus by Shapir^fo, Hershenov & Tulloch (1961), which resulted in one of the first descriptions of an unusual flagellar morphology. Subsequently, Silveira & Porter (1964) and Tulloch & Hershenov (1967), reviewed and reported on the flagellar structure of several platyhelminths, both free-living and parasitic. Other studies specific to the fine structure of digenean sperm include those of Morseth (1969), on Dicrocoelium dendricum, and Kitajima, Paraense & Correa (1976) and Justine & Mattei (1981), working on Schistosoma mansoni and S. bovis, respectively. Justine (1982) has also described spermiogenesis in S. bovis.

With regard to cestodes, numerous accounts have been published on the processes involved in spermatogenesis over the past 30 years, although detailed ultrastructural studies are somewhat limited. Amongst the first detailed reports were those of Rybicka (1962) on the pseudophyllidean, Triaenophorus lucii, with the cyclophyllidean species receiving more attention from Douglas (1963 - Baerietta diana and Distoichometra kozloffii) and Rybicka (1962, 1964b - D. caninum). Further observations by Gresson (1962) on Proteocephalus pollanicola, and Rosario (1964) on H. diminuta and H. nana, were enhanced with the publication of electron micrographs. Nevertheless, although a review by Rybicka (1966) on embryogenesis in cestodes questioned the interpretations of many previous authors, regarding the processes observed during spermatogenesis, it was concluded that a similar situation occurred in all pseudophyllidean and cyclophyllidean species. Observations on spermiogenesis and sperm structure were too few

for generalization (Rybicka, 1966).

During the 1960's there followed an abundance of papers concerned with certain aspects of spermatogenesis and sperm ultrastructure amongst cyclophyllidean cestodes; these included, Morseth (1969) on Echinococcus granulosus; Swiderski (1970) on Catenotaenia pusilla, Inermicapsifer madagascariensis and H. diminuta; Featherston (1971) on Taenia hydatigena; Kelsoe & Allison (1972), Kelsoe, Martin, Ubelaker & Allison (1973), Kelsoe, Ubelake & Allison (1977), Robinson & Bogitsch (1978) and Lumsden & Specian (1980), all on H. diminuta, and Taneya (1973) on Ophryocotyloides corvorum. However, it was only recently that the entire process of spermiogenesis was outlined for a cyclophyllidean, namely E. multilocularis (Barrett & Smyth, 1983).

The sperm structure of a ~~pseudophyllidean~~^{another} species has been described by Swiderski (1968a) for Moniezia expansa. Also, recent S.E.M. studies by Yamane, Nakagawa, Makino & Hirai (1982b), using fractured specimens of D. latum, revealed certain stages of spermatogenesis and sperm structure.

Kazacos & Mackiewicz (1972) described spermatogenesis in a caryophyllidean cestode, Hunterella nodulosa, at the light microscope level, and reported it to be similar to that encountered in pseudophyllidean and cyclophyllidean species. Sperm fine structure of another caryophyllidean, Glaridacris catostomi, was described by Swiderski & Mackiewicz (1976). With regard to other cestode orders, Swiderski and his co-workers (1974 -) observed various stages in the maturation of the testes of several Tetraphyllidea, Protoscephalidea and Trypanorhynca. Most studies have been carried out on the former group; representatives of the Tetraphyllidea include - Acanthobothrium fillicolle, Echeneibothrium beauchampi, Oncobothrium uncinatum and Phyllobothrium gracile (Swiderski, 1974; Mokhtar-Maamouri & Swiderski, 1975, 1976a; Mokhtar-Maamouri, 1979, 1982).

Although the process of spermatogenesis within the cestode

orders studied, appears to be analagous, (although different from the digenean situation in that an extra mitotic division of the spermatogonia, ultimately results in 64, rather than 32 spermatids), some differences in the ultrastructure of mature sperm have been noted by many authors (cited above). It has recently been suggested by Euzet, Swiderski & Mokhtar-Maamouri (1981), that obvious differences may be used as criteria in the analysis of cestode phylogenesis, by dividing the class into two groups, according to the number of axonemes (flagella) present, in the sperm (i.e. one, or two).

3.3. Ovaries (oogenesis)

Ovarian development has received relatively little attention, in contrast to studies on gametogenesis in the male system. However, the pattern of oogenesis appears to be similar in all platyhelminths studied, with maturation of the oocytes being completed outside the ovary.

Among the Monogenea, ultrastructural information on ovarian development has come largely from studies on Calicotyle kroyeri, Diplozoon paradoxum and Didiclophora merlangi, by Halton, Stranock & Hardcastle (1976).

Burton (1960) gave a comprehensive account of oogenesis in a digenean, following observations on H. medioplexus. Subsequent studies on the digenean ovary, oogonium and oocyte, with the aid of electron microscopy, have been carried out by Gresson (1962) with Fasciola hepatica; Kaulish (1965) with Gorgoderina vitelliloba; Erasmus (1973) with S. mansoni; and Grant et al (1977) with Pharyngostomoides procyonis.

The majority of observations on oogenesis in cestodes, has been undertaken on cyclophyllidean, rather than pseudophyllidean genera. In 1966, Rybicka reviewed early publications on the cytology of oogenesis, including those of Douglas (1963) on Baerietta diana and Distoichometra kozloffi, and Rybicka (1964b) on Dipylidium caninum. More recent observations are those of Coil (1972), on oogenesis in

Gyrocoelia pagollae, and the ultrastructural study of Lumsden & Specian (1980) on H. diminuta.

3.4 Vitellaria (vitellogenesis)

The morphology, histochemistry and development of vitelline cells in platyhelminths, have been studied in great detail by numerous authors, with particular emphasis being given to the role of the vitelline globules during egg-shell formation.

Studies on the development of monogenean vitelline cells have been carried out by Davies (1940) on Hexacotyle extensicauda, and Halton et al (1974) on Diplozoon paradoxum, Diclodophora merlangi, Dicolodophora denticulata and Calicotyle kroyeri.

Observations of vitellogenesis in several species has resulted in numerous detailed publications of the processes involved. Vitelline cells of H. medioplexus have been studied by Tulloch & Shapiro (1957), and Burton (1960, 1963) subsequently described the association of fertilized oocytes and vitelline cells, in the same species. F. hepatica vitellaria and their role in egg shell formation, have been the object of detailed investigations by Bjorkman & Thorsell (1963) and Irwin & Threadgold (1970, 1972). Other studies include those of Grant et al (1977), who described the maturation of vitelline cells in Pharyngostomoides, and Irwin & Maguire (1979) who observed the vitelline follicles of G. vitelliloba. However, by far the most comprehensive reports on digenean vitellogenesis are those on Schistosoma sp. by Erasmus and his co-workers (Erasmus, 1973, 1975; Erasmus & Davies, 1979; Erasmus & Popiel, 1980; Erasmus, Popiel & Shaw, 1982). Other literature on vitellogenesis in trematodes, has recently been cited by Smyth & Halton (1983).

Amongst the cestodes, vitellogenesis has been studied in several species, representing several different orders. Much of the early cytological and histochemical studies on the vitelline glands of various cyclophyllidean and

pseudophyllidean cestodes, has been reviewed by Rybicka (1966). More recent investigations on the Cyclophyllidea, include the structure, ultrastructure and subsequent role of vitelline cells in capsule formation during embryogenesis (Swiderski, Huggel & Schonenberger (1970b) for Catenotaenia pusilla and Inermicapsifer madagascarensis, and Swiderski et al (1970a,b) and Lumsden & Specian (1980), for H. diminuta). Brief accounts on the structure and function of vitelline cells in other Cyclophyllidea, have been given by Swiderski (1968b) and Coil (1972).

Amongst the other orders, vitellogenesis has been described by Swiderski & Mokhtar (1974) for the pseudophyllidean, Bothriocephalus clarivothrium; Mackiewicz (1968) and Swiderski & Mackiewicz (1976) for three caryophyllidean species (Caryophyllaeus laticeps, Caryophyllaeides fennica and Glaridacris catostomi); and Mokhtar-Maamouri & Swiderski (1976b) for some tetraphyllidean cestodes.

3.5 Mehlis' gland

As with the vitellarium, there is extensive literature on the structure and function of the Mehlis' gland in numerous species of trematodes and cestodes, especially with regard to its role in the process of egg-shell formation.

Amongst the Monogenea, several workers have described the fine structure, function and histochemistry of its secretions: Dawes (1940), for Hexacotyle extensicauda; Ramalingham (1970), for Pricea multae and Promycotyle sp.; and Stranock (1974) and Stranock & Halton (1975) for Diplozoon paradoxum and Calicotyle kroyeri.

In common with the Monogenea, two types of secretory cells have been located in the Mehlis' gland of Digenea. The following authors have reported, to varying degrees, on the morphology, histochemistry and function of the gland in several species: Hanumantha-Rao (1959), Clegg (1965), Thorsell & Bjorkman (1965), Threadgold & Irwin (1970) and Irwin

& Threadgold (1972) on F. hepatica; Burton (1960, 1963, 1967a) and Bogitsch (1970) on H. medioplexus; Coil (1965, 1966, 1972) on Hydrophitrema gigantica, Ogmocotyle indica and Lintonium vipex; Erasmus (1973) on S. mansoni; and Grant et al (1977) on Pharynogostomoides procyonis.

Further references, and description of the Mehlis' gland in monogenean and digenean species have been given by Erasmus (1972) and Smyth & Halton (1983). Smyth & Clegg (1959) reported on the Mehlis' gland in both trematodes and cestodes, in relation to it's role in egg-shell formation.

Much of the early work on histochemical studies and suggested functions of the Mehlis' gland in pseudophyllidean and cyclophyllidean cestodes, has been reviewed by Rybicka (1966). More recent studies include those of Coil (1972) on Gyrocotyle pagollae, and Lumsden & Specian (1980), who detailed ultrastructural investigations on the Mehlis' gland of H. diminuta.

3.6 Reproductive ducts and accessory structures

The overall arrangement of the reproductive ducts in relation to the male and female components, and accessory structures in platyhelminths, has been well documented in the past (see Smyth, 1976; Whitfield, 1982). Details of the various reproductive tracts, such as the vasa efferentia, vas deferens, cirrus duct, genital atrium, vagina, receptaculum seminis, ootype, oviduct, oocapt and vitelline duct (as well as the uterus, which may be regarded as a modified duct), have rarely been the sole subject of a publication. However, many authors (cited previously), especially those undertaking ultrastructural studies, have reported their observations on some of the above mentioned ducts, pertinent to the particular element of the reproductive system under study. For example, morphological descriptions of the Mehlis' gland, invariably include some detail on the structure of the ootype (e.g. Stranock, 1974). Nevertheless, particular attention has been paid by some, to the method of insemination, and consequently,

fertilisation, in cestodes. Williams & McVicar (1968) described aspects of the functional morphology of sperm transfer in some tetraphyllidean species, with reference to both self- and cross-fertilisation. More recently, Smyth (1982b) has reviewed the insemination-fertilisation problems in several pseudophyllidean and cyclophyllidean cestodes cultured in vitro. Smyth et al (1967) gave details of the female reproductive tracts in E. granulosus in vivo, with reference to the receptaculum seminis, pre- and post-impregnation. In subsequent publications (Smyth & Smyth, 1969; Smyth & Davies, 1974b), the process of self-insemination in the same species was described, along with the morphology of the associated structures, including the presence of a vaginal sphincter (see also, Bacigalupo & Rivero, 1949).

Other studies pertinent to the reproductive system, include those of Douglas (1961) and Sulgostowska (1972, 1974) on histogenesis of the reproductive ducts and accessory structures in some cyclophyllideans. More recently, Lumsden & Specian (1980) described the ultrastructure of many of the reproductive tracts mentioned above, in H. diminuta.

B. MATERIALS AND METHODS

1. Source of material

1.1 In vivo

Adult E. multilocularis developed in vivo were obtained from two independent sources: -

(a) Dogs were infected in the UK with larval tissue maintained in laboratory rodents here (as described in Section I). Dogs were autopsied at 15, 20 and 21 days p.i. (as previously reported), thus providing adult worms at various stages of development, pre-patency.

(b) Other dogs were infected in Zurich with material from the Institut fur Parasitologie der Universitat Zurich, and then autopsied at 16, 18 and 20 days p.i. The material collected from these hosts was fixed immediately (for both light and electron microscopy), and then sent to this laboratory for subsequent procedures (see later).

1.2 In vitro

All the adult worms used for observation of development in vitro, were obtained during the culture experiments carried out in this laboratory (see Section I). The majority of the fixed material referred to later, was recovered during the most recent experiments, by which time relatively consistent development had been achieved in vitro (see Section I). Observations were also carried out on fresh and fixed specimens of cultured worms which had been partially matured in vivo (see Section I). All specimens were washed in HBSS prior to fixation.

2. Light microscopy

2.1 Fresh material

Fresh specimens, either from dogs or culture vessels, were sometimes observed unstained, although the addition of a few drops of 0.1% Neutral red in either HBSS or distilled water, proved to be a most convenient stain for observing parts of the genital system. Other vital stains were found to be less satisfactory.

2.2. Fixed whole mounts

Some worms were fixed and stained in Aceto-orcein prepared according to Conn (1961). The specimens were incubated in aceto orcein at 37°C for 10-40 min. Samples were then observed microscopically, carefully flattened between a slide and coverslip. For permanent mounts, the worms were then post-fixed in 90% alcohol, dehydrated, cleared in xylene, and mounted in Canada balsam or DPX.

Other whole mounts were prepared by fixing 'squashed' specimens in 70% ETOH mercuric-acetic or 10% formalin, prior to staining with Gower's Carmine. Permanent mounts were made following dehydration, clearing and mounting, as described above.

2.3 Fixed for sectioning

(a) Wax blocks

A variety of fixatives were used prior to embedding specimens in wax; these were: -

70% ETOH	
10% Formalin	
Baker's fixative	
Bouin's	"
Carnoy's	"
Susa	" (Heidenhain's)
Zenker's	"

Following the required period of fixation, usually overnight, and subsequent treatment (if necessary), the material was dehydrated, cleared in xylene, and embedded in wax by standard procedures. Sections were cut on a Spencer A20 microtome at a thickness of about 7 μm .

A variety of histological and histochemical staining techniques were employed during this study, although the histochemistry of the adult worms does not form part of this investigation. The most routinely used stain was Haematoxylin and Eosin (or Orange G). Other stains employed were: - Modified Casson's Trichrome (Chew, 1981), Sudan Black B, Methyl Green Pyronin Y, Periodic Acid-Schiff, Heidenhain's Iron Haematoxylin, Modified Maximow's technique (Chew, 1981).

Unless otherwise stated, the methods outlined above for the fixation, embedding and staining of specimens, were those described in detail by Pearse (1960), Bancroft (1967) and Drury & Wallington (1967). All stains were purchased from R.A. Lamb.

(b) Resin blocks

Due to the small size of the parasites, it was advantageous to cut thinner (i.e. < 5 μm) sections for light microscopy. This was achieved by preparing the material as for transmission electron microscopy (see later), embedding in a resin such as Agar 100 or Araldite (Agar Aids; TAAB) and then cutting sections with glass knives, on a Reichert-Jung OM4 Ultracut ultramicrotome, at a thickness of 0.5 to 1 μm . Sections were stained with toluidine blue, and viewed under oil. This method was also used for locating particular areas of the worms to be sectioned for transmission electron microscopy.

3. Electron microscopy

For both transmission and scanning electron microscopy (TEM and SEM), the specimens were fixed at 4°C in 4% glutaraldehyde (GTA - TAAB), in 0.1 M sodium cacodylate buffer (pH 7.2) containing 3% sucrose and 0.015% CaCl_2 , for several hours to

overnight. The material was then rinsed in three changes of buffer (containing sucrose and CaCl_2 , as above).

3.1 Transmission electron microscopy

Specimens were post-fixed for one hour at 4°C in 1% Osmium tetroxide (OsO_4 - TAAB) in buffer, with sucrose and CaCl_2 , as above. Following two rinses in distilled water, the material was dehydrated and cleared in a graded series of acetone. Specimens were left in a 1:1 mixture of absolute acetone and resin (Agar 100 or Araldite) on a rotator, overnight. They were then embedded in fresh resin, and subsequently polymerised for 48 h at 60°C . Blocks were trimmed, and thin sections cut, on an ultramicrotome, using both glass and diamond knives. Sections were collected on formvar-coated, copper grids and double stained in uranyl acetate and Reynold's lead citrate for 5-10 min in each. Stained sections were viewed on a Philip's 300 or 301 Transmission Electron Microscope at 60 or 80 kV.

3.2 Scanning electron microscopy

Following fixation and several washes in buffer, the specimens were dehydrated in a graded series of acetone. The material was then subjected to critical point drying for 1 hour, using Polaron E5000 Apparatus with liquid CO_2 , mounted on stubs with double-sided tape, and then gold-coated for 5-10 min in a Polaron E5000 Diode Sputter-Coater. Some specimens were crudely fractured with a hair, prior to sputter-coating. Specimens were viewed on a Phillip's 500 Scanning Electron Microscope at 25kV and a spot size of 320.

C. RESULTS

1. In vivo and in vitro material compared

Following observations of material from in vitro culture, and adult worms recovered from experimentally infected dogs, it appeared that the morphology of the strobilar stage of E. multilocularis was very similar in both cases. Therefore, the description of the structure and ultrastructure which follows is applicable to both in vivo and in vitro material, although any observed differences will be outlined where appropriate.

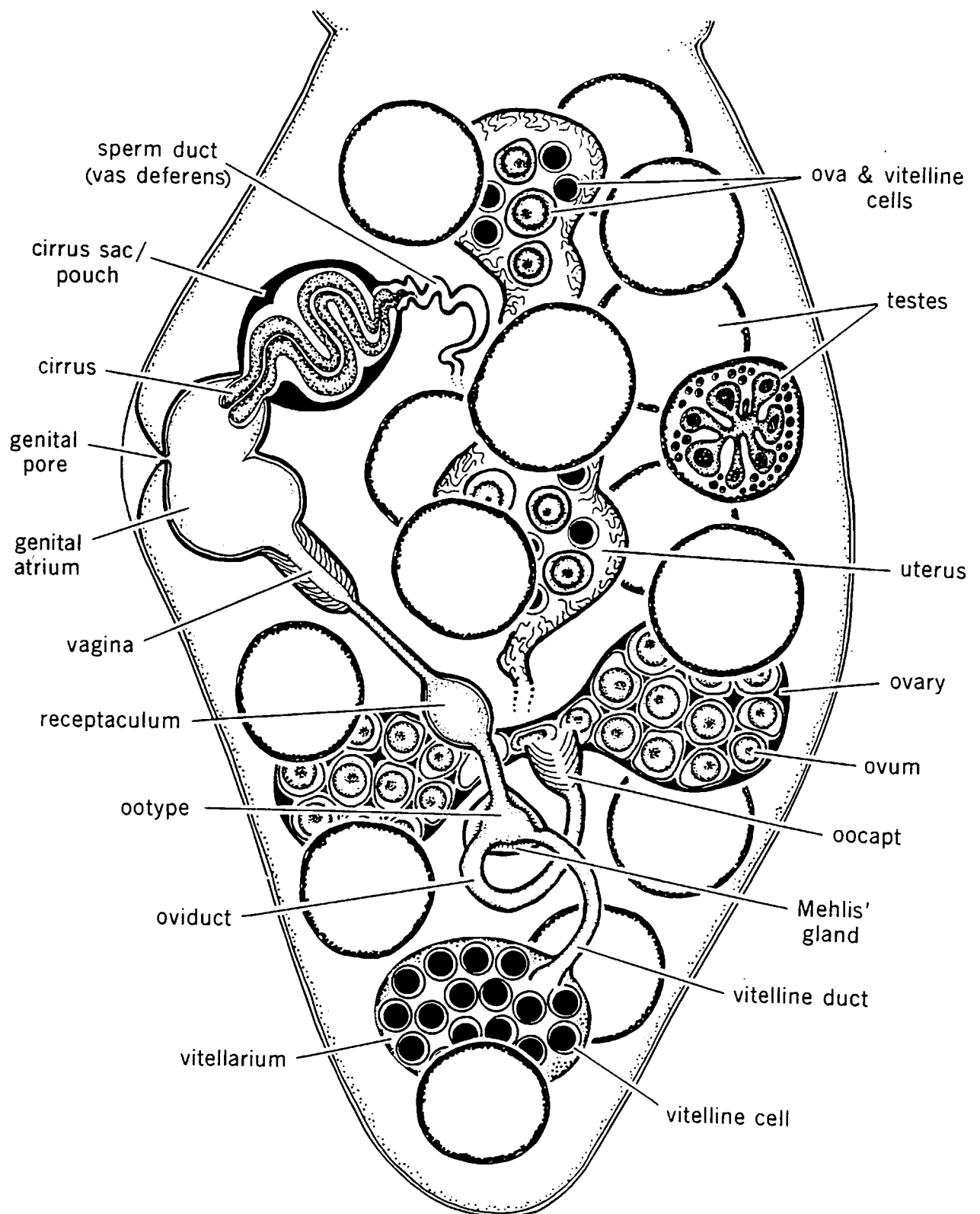
2. Gross morphology of the mature proglottid

The gross developmental pattern of the maturation of the genitalia has already been described in Section I; a more detailed description of the development of the male and female reproductive organs will be given later in the Results section.

The gross morphology of a mature proglottid is shown in Fig.11. The testes are scattered throughout the segment, with the majority situated posterior to the cirrus sac. The number of testes per proglottid ranges from 10 to 20 (in vitro; $\bar{x} = 15$) or from 15 to 25 (in vivo; $\bar{x} = 18$). The vasa efferentia have not been detailed in Fig.11, although in mature worms, when packed with sperm, they are clearly visible. The large sperm duct (vas deferens) leading into the cirrus duct, is also obvious, and again, packed with sperm in mature segments. No seminal vesicle was apparent. The cirrus itself lies inverted and folded within the thick-walled, muscular cirrus sac, situated laterally, in the anterior third of the worm. When everted, the cirrus protrudes into the genital atrium. The genital pore is a small lateral opening, lying in a slight depression just anterior to the middle of the proglottid. It opens into the large genital atrium, which in some mature worms, may contain sperm, and some lipid-like droplets (see later). The vagina is composed of two distinct parts; the proximal, wide neck has a thick and possibly muscular wall, whereas the distal part, is thin-walled, and much narrower. No

Fig.11

Mature proglottid of Echinococcus multilocularis
(semi-diagrammatic); only one testis shown in detail



vaginal sphincter was observed. The vagina runs posteriorly from the genital atrium, toward the mid-line of the segment, where it terminates at the opening of the ovoid receptaculum. In vitro, a few sperm and lipid-like droplets have been occasionally observed in mature segments, whereas in vivo (20 and 21 days p.i. for the UK material; 16 days p.i. onwards, for material sent from Zurich), the receptaculum is commonly packed with sperm. In some fresh specimens, light microscopy revealed the presence of ova and vitelline cells in the receptaculum, although this unexpected observation was probably due to the abnormal flattening with a coverslip, the cells having been forced anteriorly from the ootype.

A short, wide duct leads posteriorly from the receptaculum to the ootype (characteristically triangular, in shape), and associated Mehlis' gland. Two other ducts enter the lower half of the ootype - the oviduct and the vitelline duct. The large vitellarium is situated^a at the posterior end of the segment, although one or more testes usually lie below it. The bilobed ovary is sited anterior to the vitellarium, normally at the level of the receptaculum and ootype. From the isthmus of the ovary, the oocapt leads into the oviduct. Although a connection between the uterus and the ootype has not been observed, it is assumed that the ova and vitelline cells pass directly into the posterior of the uterus from the ootype. The sacciform uterus extends anteriorly to the limit of the mature segment. As maturation continues, the uterus swells, initially in the anterior half of the segment, but eventually filling a large part of the proglottid. This phenomenon has been observed in all cases, irrespective of whether the uterus is packed with cells, or empty. If ova and vitelline cells are present in the uterus, they can be seen moving to and fro in fresh specimens, due to muscular contractions of the worm. As mentioned in Section I, crystal-like bodies have been observed in the uterus, although the nature of these has not been determined.

As soon as ova and vitelline cells begin to fill the uterus, a fairly^a rapid degeneration of most of the reproductive

structures within the proglottid occurs. In vitro, the disintegration and resorption of the genitalia has been observed, beginning with the testes and associated ducts. This is followed by the ovary, and finally the vitellarium. The cirrus, cirrus sac, genital atrium and proximal part of the vagina can usually still be seen in these segments, although in a somewhat degenerate state. This is also true of the gravid proglottids of in vivo material.

3. Structure and ultrastructure of the genitalia

3.1 Male reproductive system

(a) Testes (spermatogenesis, spermiogenesis and sperm ultrastructure)

As briefly described in Section I, the testes were seen to develop from the periphery of the genital streak, the peripheral genital primordium. Although some stages in the maturation of the testes have been observed with the light microscope, electron microscopy provides a more detailed description of the processes involved.

Spermatogenesis within a single proglottid appears to proceed, more or less, synchronously, although within each testis, several different stages of development may be seen (Plate 21). In such sections, primary spermatogonia are generally located around the periphery; later developmental stages are found centrally within the testis lumen.

The testis epithelium is a thin syncytium, containing large nuclei, several mitochondria, scattered ribosomes, some electron lucent vesicles, sparse endoplasmic reticulum (ER) and numerous, characteristic networks of tubules, presumed to be agranular endoplasmic reticulum (AER). The latter were sometimes quite pronounced, and often seen in association with β -glycogen particles (Plates 22 & 23). During initial stages of development, the epithelium appears somewhat convoluted, with several vacuoles, and cytoplasmic extensions separating

the testis into compartments, or follicles (Plate 25). Some sections of in vitro material show that these extensions may persist throughout spermatogenesis and spermiogenesis, each follicle containing one, or more, stages (Plate 24). Although this phenomenon is by no means universal for in vitro material, no similar situation was observed in worms matured in vivo.

In spermatogonia, the onset of mitosis could be determined by the appearance of centrioles (Plate 26). In some sections, nuclear pores were apparent (Plate 27). Following two divisions, four tertiary spermatogonia are formed (Plate 28), and a subsequent division results in a rosette of eight quaternary spermatogonia (Plate 28). The fine structural morphology of all spermatogonial stages is similar; they typically comprise a large, rounded nucleolus, with prominent nucleolus and abundant heterochromatin. The cytoplasm is usually dense, with numerous ribosomes, many mitochondria (small, with a dense matrix and few cristae), a few Golgi complexes and sparse ER. It appears that cytoplasmic continuity is maintained throughout the spermatogonial divisions due to incomplete cytokinesis.

A fourth division results in a plasmodial rosette of 16 primary spermatocytes (Plate 30). The nuclei within these, contain a reduced amount of heterochromatin. The cytoplasm, now more electron lucent, includes many mitochondria, free ribosomes, a few Golgi complexes, some ER, and occasional, whorled lamellae. In addition, paired centrioles are sometimes visible at this stage (Plate 30 inset).

The next cell division gives rise to 32 secondary spermatocytes, similar in morphology to the primary spermatocytes, with the cells still maintaining cytoplasmic continuity (Plate 31). During the spermatocyte stages, a central cytoplasmic body, the cytophore, becomes apparent. Each of the surrounding spermatocytes is attached to the cytophore, by cytoplasmic connections, or strands (Plate 32). The cytophore contains organelles common to the spermatocyte cytoplasm.

The final division of spermatogenesis results in 64 spermatids; plasmodial in nature, with the cytophore still apparent (Plate 34). As subsequent differentiation of the spermatid proceeds (spermiogenesis), the cytophore and residual cytoplasm of the spermatids forms a so-called, residual body. This was observed at both light and electron microscope levels as a densely staining body. TEM sections show the residual cytoplasm to contain numerous mitochondria, occasional Golgi complexes, scattered ribosomes, lamellae whorls and abundant (possibly phagocytitic) vacuoles (Plate 33).

The process of spermiogenesis has been observed at the ultrastructural level, and the sequence of events is outlined in Fig.12. It should be pointed out however, that, although the diagrams show spermiogenesis occurring in a single plane (for ease of description), in reality, electron microscopy has revealed that this is not the case. Therefore, in each of the micrographs accompanying the following sequence of events, not all structures associated with a particular stage can always be identified.

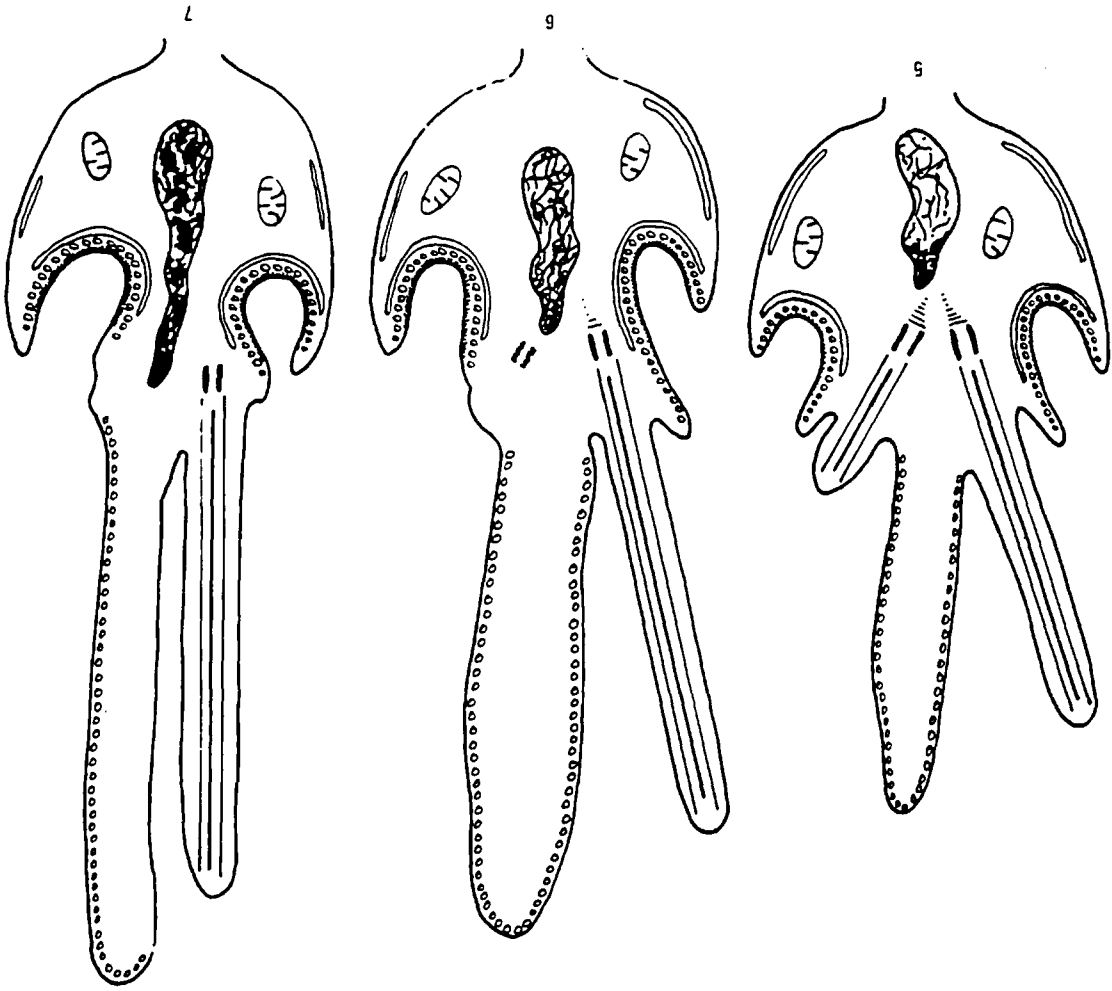
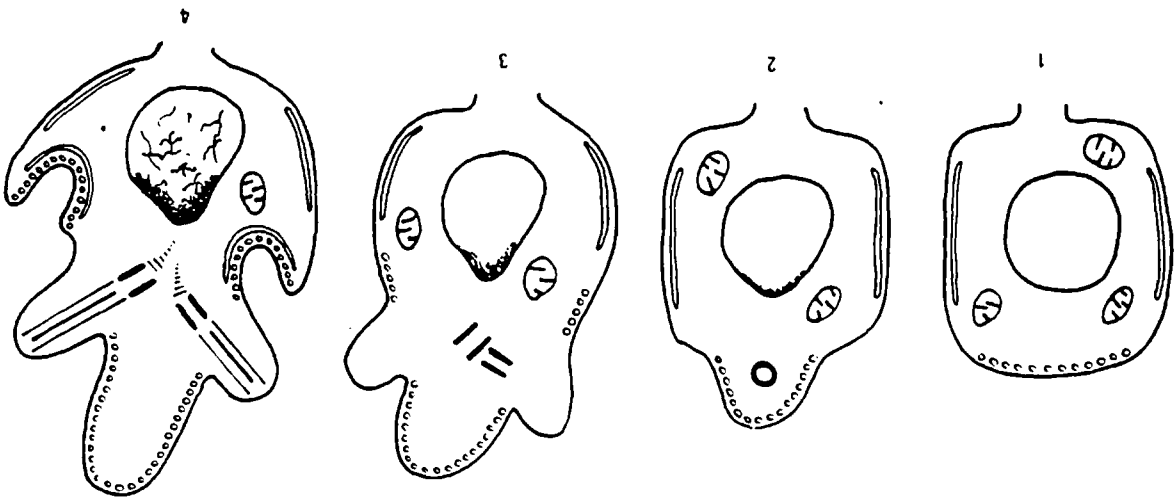
Early spermatids are characterised by the presence of a nucleus, appreciably smaller than that of the spermatocytes, and containing scattered heterochromatin and a compact nucleolus. Within the cytoplasm, numerous scattered mitochondria are apparent (irregularly shaped, with a dense matrix and few cristae), as well as free ribosomes, Golgi complexes, occasional vacuoles, and sparse ER. Initial differentiation is characterised by the presence of a row of microtubules associated with the inner margin of the apical plasma membrane (Fig.12(1); Plate 35). Simultaneously, profiles of ER become aligned along the remaining cell margins. Sometimes, densely staining areas within the cytoplasm are seen, similar in morphology to the nucleolus. Although these are occasionally seen during spermatogenesis, they are more obvious in spermatids at the onset of spermiogenesis.

The next stage is defined by the appearance of a centriole,

Fig.12

Diagrammatic representation of spermiogenesis in
Echinococcus multilocularis (see Plates 38 to 48)

- ( : represents mitochondrion;
-  : represents ER;
- o : represents microtubules;
- // : represents basal bodies;
- /// : represents axonemes;
-  : represents rootlets)



accompanied by the formation of a cytoplasmic knob, or extension of the cell (Plate 36). This centriole apparently divides, resulting in two sited perpendicular to each other at the cell apex (Fig.12(2)). These centrioles become the basal bodies of the developing axonemes, the latter being observed in slightly more advanced stages, along with associated rootlet fibres (Fig.12(3); Plate 37)). This characteristic 'zone of differentiation' becomes more obvious as an involution of the plasma membrane at the base of the cytoplasmic extension, forms a collar around the developing sperm (Fig.12(4); Plate 38). Profiles of ER are seen under the thickening cell membrane in this region (the so-called 'arching membranes'), as the microtubules elongate to form a cytoskeletal ring around the periphery of a median cytoplasmic process. The elongation of the latter, and associated development of two lateral axonemes from the basal bodies has been observed (Fig.12(4); Plate 39). However, later stages are characterised by the continual elongation of only ne axoneme (Fig.12(5); Plate 40). The ultimate appearance of a single-axoneme type sperm (see later), suggests that the second axoneme is aborted during an early stage of its development, although its associated basal body may still be observed in some sections (Plate 41).

Concomitant with this stage of differentiation is the characteristic morphological changes of the spermatid nucleus. Initially the apical part begins to elongate, with chromatin becoming more condensed beneath the nuclear membrane (Fig.12(5); Plates 36 onwards). Eventually the nucleoplasm comprises fine strands of chromatin, resulting in a fibrillar-like appearance (Plate 42). This becomes more obvious as the nucleus continues elongation, and migrates into the median cytoplasmic process (Fig.12²(6); Plate 43). Simultaneously, the remaining axoneme rotates, until the latter is parallel with the median extension, with which it fuses, forming a unipartite structure (Fig.12(7)). A longitudinal groove running the length of the cytoplasmic process, may serve as a guide for the axoneme.

Further constriction of the collar ('arching membranes') in

the 'zone of differentiation', results in separation of the sperm from the residual spermatid cytoplasm. No flagellar roots are seen in the differentiated sperm; it appears that they remain in the resultant residual body (described earlier), which is subsequently resorbed on degeneration of the testis. It is possible that the basal body, on the other hand, may be incorporated into the sperm (see later).

Details of the processes outlined above have also been traced from electron micrographs of the differentiating sperm seen in cross section. These are shown in Plate 44. Occasional areas of dark floccular material may be seen in the cytoplasm; these are similar to the nucleolus-like bodies, described earlier.

The sperm apparently continues to elongate after spermiogenesis, resulting in a reduced amount of cytoplasm surrounding the axoneme. In addition, the nuclear material becomes more condensed, and spirals around the axoneme as it migrates along the sperm (Plate 45). In some sections, sperm near the testis epithelium contain pockets of β -glycogen within the peripheral cytoplasm (Plate 45).

Following spermiogenesis, and subsequent elongation of the sperm, it is apparent that some further maturation of the latter occurs, since the morphology of the sperm in the reproductive ducts, is somewhat different from that of the 'immature' forms in the testes. This maturation process occurs, or is initiated, around the periphery of the testes, often with the sperm in intimate contact with the epithelium (Plate 46). Both light and electron micrographs show the mature testes to contain sperm coiled around the periphery, and the resultant residual body, lying centrally (Plates 47 & 48).

It was difficult to determine the ultrastructure of a complete, mature sperm, due to their coiled nature within the reproductive ducts. Nevertheless, Plate 49 outlines certain distinctive morphological characteristics at various points along its' length. At one end, no peripheral microtubules are

present, only the outer ring of microtubules of the axoneme (detailed later) are seen. (Plate 49,1). A little behind this, a central fibril appears, as well as radial spokes, attached to the axoneme microtubules. Later sections reveal the complete axoneme structure, as seen in cross section.

Peripheral microtubules then become apparent in the surrounding cytoplasm, closely associated with the external membrane. Further along the sperm, the axoneme is contained in a central core of relatively electron-translucent cytoplasm. This is surrounded by an electron-lucent tube, which, itself, is enclosed in a dense cytoplasmic cylinder containing the peripheral microtubules. Behind this, the outer tube becomes more distinct by the appearance of an inner ring of fibril-like units, separating it from the electron lucent area. Later, the outer tube apparently separates into two, each distinguished by the density of the cytoplasm. This becomes more obvious with the appearance of 'radial septa', radiating outwards from the now thickened, ring of fibril-like units, through the inner, less dense cytoplasm; the outer, denser cytoplasm still comprises a ring of peripheral microtubules. These radial septa continue for some way, and then disappear, although the cytoplasm of the outer tube still appears as two cylinders of varying densities. Behind this, the nucleus becomes apparent, lying within the electron lucent area, in close contact with the ring of fibril-like units. Following sections show that the nucleus both curls, and spirals around the axoneme, which is still within the central cytoplasmic core. Eventually the outer cytoplasmic tube returns to its original state of uniform density; the nucleus is still apparent.

The nucleus is seen in sectioned material for about 40-60 μ m; the full length of a mature sperm has yet to be determined, although its average diameter is about 0.4 μ m.

The other end of the sperm is characterised by a gradual reduction in diameter, with the outer cytoplasmic tube becoming thinner; the ring of fibril-like subunits disappears, but peripheral microtubules are still present. The axoneme then becomes almost star-like in appearance, (somewhat resembling

the basal body is ⁿ structure) but with the peripheral microtubules and central fibril present.

It was difficult to ascertain the polarity of mature sperm, and hence the terms 'anterior' and 'posterior' have not been used in the above description. This was due, in part, to the apparent mobility of the nucleus within the electron-lucent area surrounding the axoneme, and bordered by the outer cytoplasmic ring.

The axoneme itself (defined here as 9+'1'), measures 0.15-0.2 μm in diameter, and consists of nine doublet microtubules arranged cylindrically around a central complex (0.05-0.09 μm diameter) and connected by radial fibrils. The two tubules of each doublet are morphologically distinct; one (tubule A), contains an electron dense region, partly occluding the lumen, and bears two lateral dynein arms; the other tubule (B), closely associated with the first, is simple. The central complex comprises a single core, or fibril, surrounded by a electron lucent cylinder, which, turn, is endorsed by a dense, cortical sheath. In longitudinal section, the numerous sub-units of this sheath appear to be arranged in a helical pattern (see Plate 40).

Degeneration of the testes often occurred prior to the release of all sperm into the vas deferens, and was seen from 21 days in vivo and 32 days in vitro (Plate 50). Some sperm showed features which were regarded as abnormal, in that the outer cytoplasmic cylinder was fractured. It was difficult to determine whether this was a consequence of the degenerating genitalia, or whether anomalous forms occurred spontaneously. Alternatively, it could be a fixation artifact.

Although the processes involved in spermatogenesis and spermiogenesis both in vivo and in vitro were similar, premature degeneration of the testes was more common in cultured material.

(b) Vasa efferentia and vas deferens

During spermatogenesis, the vasa efferentia are formed, and often seen lying within the fibrous, basal lamina beneath the epithelium of the testes. The ducts eventually become patent with the testicular lumen.

The initial development of the ducts is associated with the appearance of several microvilli projecting into the lumen from the somewhat convoluted syncytial epithelium (Plate 51). As the vas efferens become more swollen, accommodating an increased number of microvilli, the epithelial lining consequently becomes thinner, and is seen to contain a few mitochondria and several profiles of ER (Plate 52). In some mature worms, cross sections of sperm are seen in the duct lumen. All vasa efferentia eventually connect to the larger vas deferens, which leads to the cirrus duct. Morphological differences between the two, includes a reduction in the number of microvilli in the vas deferens, and the appearance of surrounding circular muscles near the junction with the cirrus duct. Light microscopy commonly revealed the presence of coiled sperm within the ducts of mature in vitro and in vivo worms (Plate 53). Occasionally, in fresh material, some movement of the sperm was observed, although it was not very vigorous. However, the overall muscular contractions of the worm were seen to stimulate some activity, and force the sperm anteriorly through the ducts; there appeared to be no muscles specifically associated with the vasa efferentia.

The cirrus duct runs throughout the cirrus which is housed, inverted and folded, within the cirrus sac. The latter comprises a compact mass of musculature (Plate 54), which, on contracting, causes the cirrus to evert. The epithelium of the proximal part of the cirrus is morphologically very similar to that of the vas deferens (Plate 55). The remainder of the duct epithelium contains an increasing number of electron lucent vesicles and cytoplasmic extensions, resembling modified microtriches (see later)(Plate 56). The nuclei are withdrawn into extensions of the syncytical cytoplasm beneath the surrounding musculature. Electron micrographs reveal the occasional presence of sperm which are certainly more prevalent in in vivo material.

The number and frequency of the modified microtriches increase along the cirrus duct. Near the cirrus tip, they are morphologically similar to those of the tegument (described later), although more filamentous in nature (Plate 57).

Sporadic muscular contractions seen in fresh material, sometimes cause the cirrus to partially evaginate into the genital atrium; occasionally the cirrus has been seen to protrude through the genital pore both in vivo and in vitro (Plate 58).

In an attempt to increase the chances of self-insemination (and consequently, fertilisation) in vitro, mature, cultured worms were subjected to a variety of 'pressure' systems (for example: some were embedded in a semi-solid matrix of NBCS, agar, agarose etc; others were sandwiched between a variety of natural and/or synthetic materials, such as scintered glass coated with serum; some were trapped in a mesh of nylon fibres, or fine, glass capillary tubes; some were treated with stimulants, such as bile, to increase muscular activity; whilst others were subjected to rapid passage to and fro, through fine rubber tubing attached to syringes). However, although cirrus activity was usually stimulated by such treatments, worms subsequently and rapidly degenerated when returned to normal culture conditions. Partial insemination was often observed

microscopically, when squashing fresh specimens beneath a coverslip; this also commonly forced sperm through the cirrus duct into the genital atrium (Plate 59). In some cases, sperm have been seen extruding through the genital pore.

(c) Genital pore and genital atrium

SEM shows the genital pore situated centrally on a lateral protrusion of the tegument, presumably due to the swollen nature of the genital atrium (see later) (Plate 60); sometimes this swelling lies within a depression (Plate 61). These differences are presumably dependent of the state of contraction of the cirrus pouch and/or genital atrium, at the time of fixation. Externally, the genital pore is surrounded by microtriches, forming a relatively uniform pattern over the tegument (see later).

The genital atrium is easily recognisable as a large, swollen part of the reproductive ducts into which the cirrus evaginates, the genital pore opens, and from which the vagina extends posteriorly. Ultrastructurally, the epithelium is of relatively uniform thickness, with the cytoplasm containing extensive ER and some vesicles. Occasional nuclei are seen in cytons beneath the surrounding muscular layer. Infrequent, and relatively short epithelial extensions into the genital atrium, are supported by electron dense material, either elongate or disc-shaped (Plate 64). They somewhat resemble the modified microtriches in the proximal part of the cirrus duct, although, due to the swollen nature of the atrium, they are far more dispersed. The elongate, electron dense bodies appear similar in morphology, and are probably analagous, to the shaft of the tegument microtriches (see later); the characteristic cytoplasmic bases are absent in the genital atrium and proximal part of the cirrus duct, but obvious throughout the remainder of the cirrus duct. A fairly prominent muscle layer beneath the epithelium of the genital atrium probably accounts for the intermittent contractions of the latter, seen in fresh material.

As already mentioned, sperm are commonly observed in the atrium in vivo material from 18 days p.i. (Plate 63). Electron micrographs show the presence of small, membranous vesicles, some of which may form conglomerates of varying sizes, enveloping individual sperm (Plate 62). In fresh material, lipid-like droplets have been observed in the atrium. Also, occasional movement of the sperm has been noted, both in cultured worms, and those from dogs; the 'anterior' forms a characteristic loop, or coil, which then rotates about an (arbitrary?) axis some way behind (Plate 65). The result is a swinging, almost corkscrew-like, movement.

3.2 Female reproductive system

(a) Vagina and receptaculum

Light microscopy reveals that the vagina is composed of two distinct parts; the anterior section, leading from the genital atrium, is thick-walled and wide-necked, almost funnel-shaped; distally it forms a duct leading to the receptaculum. Ultrastructurally, however, there are few morphological differences between the two sections. The epithelium of the vaginal neck somewhat resembles that of the tegument, in that the nuclei of the syncytial cytoplasm are withdrawn beneath a layer of (circular) muscles (Plate 66). The cytoplasm contains numerous electron-lucent vesicles, ribosomes, occasional mitochondria and extensive ER. Some of the microvilli-like epithelial extensions into the lumen, are somewhat similar in morphology to the tegument microtriches (described later). They are composed of both a cytoplasmic base and a short, pointed shaft, surrounded by a plasma membrane, and, in some cases, are branched.

Progressing down the vagina, the nuclei of the epithelium gradually become accommodated within the distal, syncytial cytoplasm (Plate 67). The modified microtriches are gradually replaced by an increased number of filamentous microvilli. Both circular and longitudinal muscles (although somewhat dispersed), are apparent surrounding the lower part of the

vagina (Plate 68). In sections observed with the light microscope the lumen often stains deeply (Plate 69), possibly indicating the presence of a secretion. (In the same sections, the cirrus is also deeply stained).

The receptaculum is generally swollen in mature worms, with fewer microvilli present and an increased folding of the plasma membrane at the luminal interface (Plate 70). The nuclei are contained within the distal cytoplasm (as in the lower part of the vagina), with a reduced amount of muscle beneath the basal lamina. No sperm have been seen in TEM sections, although light microscopy commonly revealed the presence of sperm bundles in vivo, whilst in vitro, sperm were only rarely observed. In fresh specimens, lipid-like droplets of varying size have been seen in the receptaculum. Electron micrographs show membrane-bound vesicles within the receptaculum (and the vagina), but whether these are analagous to the droplets, or even to the densely staining vaginal lumen described above, remains to be demonstrated.

The duct leading from the receptaculum (Plate 71) to the ootype, is relatively short, but ultrastructurally similar to that of the oviduct (see later). The syncytial epithelium posterior to the receptaculum becomes more convoluted at the luminal surface, with cytoplasmic extensions ramifying through the lumen. Microvilli are still present in the duct, as are the membranous vesicles described above. Cytoplasmic organelles in the epithelium are similar to those found in the vagina and receptaculum.

(b) Ovary and oviduct

Oogenesis has not been observed in any great detail, due, in part, to the fact that the processes involved appear to be relatively rapid, following maturation of the male gametes.

The bilobed ovary is seen to develop from the central primordium. Differentiation of the oogonia appears to be more or less, synchronous, with the ultimate appearance of primary

oocytes. These rounded cells (9-12 μ m diameter), with a central nucleus and prominent nucleolus, can be seen at the light microscope level (Plate 72); granular material surrounding the nucleus, is also apparent. In stained sections, one, or more, dense bodies, similar to the nucleolus, have been observed in the cytoplasm (Plate 73).

Ultrastructural observations reveal the cytoplasm to contain many irregularly shaped mitochondria (with few cristae), as well as numerous ribosomes, occasional Golgi complexes, and scattered ER (Plates 74 & 75). The cytoplasm is generally homogeneous in nature, and slightly more electron dense than the nucleoplasm. Occasional dense, nucleolus-like bodies are also apparent. The occurrence of paired centrioles within the cytoplasm is not uncommon, indicating the onset of division.

Smaller cells observed in some TEM sections may represent oogonia (Plate 74). They contain the same cytoplasmic organelles, including occasional paired centrioles, but no nucleolus-like bodies.

The syncytial epithelium of the ovary ramifies throughout the organ, with cytoplasmic extensions apparently delimiting each maturing cell. Extensions of the surrounding, glycogen-rich parenchymal cells may also be seen in sections of the ovary. The oocytes are in fairly intimate contact with the epithelium at selected sites (similar to the spermatogonia within the testes). The epithelial cytoplasm contains numerous irregularly shaped, nuclei, each with prominent nucleolus.

There is also an abundance of small mitochondria, ribosomes and ER. Some intracellular glycogen particles may also be present. Similar particles have been noted in the lumen of the ovary.

Observations on in vitro material reveal that, following oogenesis, rapid degeneration of the oocytes may occur in the ovary. This has been determined in fresh material with the aid of Neutral red; oogonia and oocytes do not normally take up the stain, whereas degenerate cells become granular, and stain red.

Oocytes normally pass into the oviduct via the oocapt,

eventually reaching the ootype, from where they pass into the uterus. The oocapt is quite distinctive in fresh specimens observed microscopically, as funnel-shaped, with characteristic, almost concentric striations. Ultrastructurally, these appear as bands of cells (or possibly a highly nucleated syncytium) from which cytoplasmic extensions infiltrate the lumen (Plate 76). Surrounding the oocapt is a thin band of circular muscles.

The epithelium of the oviduct is syncytial and somewhat complex in nature. It is composed of a large number of cytoplasmic extensions and microvilli, which extend throughout the lumen of the canal (Plates 78 & 79). Some intraluminal vesicles are also present. The cytoplasm is densely packed with mitochondria, ER, electron-lucent vesicles and nuclei (containing nucleoli). Occasional circular muscle fibres surrounding the oviduct are apparent. Light microscopy of sectioned material often shows an affinity for stains, such as Heidenhain's iron haematoxylin (Plate 77), again possibly indicating the presence of a secretion.

As the oviduct nears the ootype, its ultrastructure changes somewhat. The cytoplasmic epithelial extensions become less frequent, although microvilli are still present (Plate 80). At the entrance to the ootype, the lumen contains fewer epithelial extensions and there is an increase in the number of nuclei present (Plate 81).

(c) Vitellarium and vitelline duct

The vitelline gland is formed from the central primordium and its development is concurrent with that of the ovary. However, in contrast to oogenesis, maturation of the vitellarium can be observed in fresh material with the aid of Neutral red, since the vitelline granules readily absorb the stain (Plate 82). Vitellogenesis seems to be a fairly rapid process, the development and subsequent amalgamation of the vitelline droplets resulting in a large, single globule within each cell. In these mature cells, the remaining cell contents are aligned

in a crescent-like shape around the globule (Plate 83).

With the aid of electron microcopy, the processes involved have been followed. Although differentiation of the vitelline cells occurs more or less simultaneously, various stages of maturation may be observed in a single section (Plate 84). Immature cells are irregular in outline. Each contains a nucleus, with nucleolus and scattered chromatin. The cytoplasm contains several scattered mitochondria (of irregular shape, few cristae, and relatively dense matrix), numerous free ribosomes, some Golgi complexes and sparse ER.

The vitelline gland itself, is almost spherical in outline. The thin syncytial epithelium (containing infrequent nuclei, abundant mitochondria, AER and ribosomes) ramifies throughout the vitellarium, in close contact with, and apparently separating, each cell.

As maturation proceeds, granular endoplasmic reticulum (GER) becomes apparent, and several small, electron dense vitelline granules are formed within the cytoplasm (Plate 85). Occasionally, granular, electron dense areas are in association with the vitelline material. Subsequently, small pockets of mainly α -type glycogen become apparent (Plate 86). The vitelline granules increase in size, presumably by coalescence (Plate 87), eventually each mature cell contains a single globule (Plate 88). During these stages, the cytoplasm is packed with GER. In mature cells, the nucleus (now without nucleolus) and cytoplasm are displaced to one side, along with a large amount of glycogen (now accumulated in a single mass). Golgi complexes and rounded mitochondria are still obvious, but GER is rarely seen. The vitelline globule (about $6\ \mu\text{m}$ diameter) almost completely fills the cell (approximately $7\ \mu\text{m}$ diameter). It is not always in direct contact with the cytoplasm, but contained within a membrane bound vacuole of floccular material.

Mature cells pass from the vitelline gland to the ootype via the vitelline duct. The epithelium of the latter is

syncytial in nature bearing occasional filamentous lamellae on its lumⁱenal surface (Plate 89). The ultrastructure of the vitelline duct was much simpler than that of the other female reproductive tracts, but its staining properties with Heidenhain's iron haematoxylin were similar to those of the oviduct (see Plate 77). No subepithelial musculature was observed.

Some differences were noted between in vivo and in vitro material. Firstly, it appeared that a greater amount of glycogen was present in the mature vitelline cell of worms grown in vivo. In vitro, the cell membranes were often indistinct, and intra-lumⁱenal glycogen particles were observed. This apparent premature degeneration of some cells with resultant loss of glycogen would account for the lesser amount of food reserves seen in mature cells.

(d) Ootype and Mehlis' gland

Light microscopy of both fresh and fixed material show the ootype to be triangular in shape, surrounded by a nucleated epithelium. As with the oviduct and vitelline duct, the ootype stains intensely with Heidenhain's iron haematoxylin (see Plate 77). The epithelium was initially considered to be cellular, but, due to abundant ER, and extensive cytoplasmic invaginations and extensions, its nature, at present, remains unclear.

Within the ootype lumen, numerous cilia are present, and interspersed with microvilli (Plate 90). The cilia are typically anchored by basal bodies and rootlets within the epithelium, which also contain intracellular organelles in common with the oviduct, including numerous electron lucent vesicles.

The Mehlis' gland associated with the ootype, is situated beneath the muscular layer surrounding the ootype epithelium (Plate 91). Although not discernable by light microscopy in this study, electron micrographs reveal that the ootype is not

completely surrounded by gland cells, but their exact location has not been determined. Only a single type of cell has been identified. These are somewhat irregular in outline and contain numerous mitochondria, ER, ribosomes and occasional pockets of α - and β - glycogen particles. Numerous electron dense bodies (secretions) are produced in the gland cells, and are transported to the ootype by cytoplasmic extensions ('ducts'), which project through the muscle layer and ootype epithelium (Plates 91 & 92). During transportation, a number of bodies are packaged within membrane bound vesicles of somewhat floccular material, prior to release (Plate 92).

(e) Uterus

The uterus is a blind ending sac, primarily situated anterior to the oviduct. It ultimately occupies almost the entire proglottid following an initial swelling in the anterior part of each segment. The uterine wall is syncytial in nature, with nuclei scattered throughout the cytoplasm (Plate 93). These nuclei are fairly prominent, with an irregular shape, tending towards an oval, or elongate, outline. They contain clumped heter^ochromatin and a distinct nucleolus. The epithelial cytoplasm is dense, containing numerous free ribosomes, membranous lamellae, occasional Golgi complexes and some electron lucent vesicles. The basal plasma membrane is separated from surrounding parenchymal cells by inters^titial material; there is little underlying musculature. The internal surface of the uterine wall, bears numerous, filamentous microvilli, extending into the lumen. Electron micrographs show the lumⁱnal material to be floccular, with scattered membranous vesicles, which are electron lucent.

As already described, light microscopy revealed the presence of both ova and vitelline cells in utero, along with elongate crystals of unknown origin both in vivo and in vitro (Plates 94 & 95). In vitro, the ova (up to 12 μ m diameter - presumably unfertilised) rapidly degenerate, becoming characteristically granular, and staining deeply with Neutral red (see earlier) (Plate 96). This is associated with a

reduction in cell size. Vitelline cells have been seen to degranulate in utero, with the vitelline globule separating into smaller droplets.

In the terminal proglottids of some worms recovered from dogs, and subsequently matured in vitro, a gradual enlargement of the fertilised ova was observed with the ultimate appearance of immature oncospheres, following embryonic cleavage. These maturing eggs were easily distinguished by their large size (22-25 μ m diameter) and, in a few worms, developing oncospherical hooks and characteristic keratin blocks of the egg shell were observed (see Section I).

Few ultrastructural studies have been carried out on the uterine contents of mature worms, both in vivo and in vitro. Nevertheless, ova, vitelline cells and Mehlis' gland secretions have been observed in material from dogs. Since embryonic development and associated egg shell formation does not form part of this thesis, the fate of the fertilised ova, and the role of the vitelline cells and Mehlis' gland secretions, has not been ascertained.

4. Other structural and ultrastructural observations

During this study, the general structure and ultrastructure of the adult worms (other than the genitalia) have been observed. The morphology of the tegument, scolex, sensory organs and excretory system in E. multilocularis, appears to be similar to that described for other cestodes, but for interest, and as a reference to points raised in the discussion, will be briefly outlined here.

4.1 Tegument

The tegument of adult worms in general, resembles that of E. granulosus, as described by Morseth (1966), Jha & Smyth (1969) and Thompson, Houghton & Zaman (1982).

The external epithelial covering of the adults is

characteristically syncytial in nature, with the nuclei found individually within tegumental cytons. These are withdrawn beneath the muscle (circular and longitudinal) layers, and continuous with the distal cytoplasm by cytoplasmic bridges, or connections. The external surface bears numerous microtriches usually directed toward the posterior of the worm. (Plate 97). These microtriches are generally identical in morphology, although their shape, size and frequency may vary from one area to the worm to another. The external plasma membrane covering each microthrix, is often seen surrounded by floccular material, presumed to be either glycocalyx, or exogenous in nature. Each microthrix is composed of two distinct parts, their comparative size being variable: (i) The base, is an extension of the tegument cytoplasm, although it bears no common organelles. Parallel fibrillar-like structures can be seen within the base, lying along the same axis as the microthrix itself. An electron dense plate, or thickening, is obvious beneath the plasma membrane, on the anterior side, with respect to the worm. Also, just beneath the shaft lies a base plate; a tubular structure of electron dense walls containing a less dense central area. (ii) The shaft itself, is more electron dense than the base, although it is composed of fibrils similar to those found in the latter. The shaft tapers to a point, and is usually angled more posteriorly than the base. In cross section, the microtriches appear hexagonal (Plate 98). Scanning electron microscopy, shows that the microtriches completely cover the tegument of the adult worm (Plate 100).

The dense tegumental cytoplasm itself, is fairly uniform and of typical cestode morphology, although its thickness may vary. It is packed with ovoid, electron lucent vesicles, some containing material of a floccular nature, interspersed with membrane bound, electron dense bodies. Numerous mitochondria are seen in close association with the basal membrane, beneath which lies a fibrous basal lamina. The tegumental cytons contain, along with the nucleus and prominent nucleolus, numerous mitochondria, several Golgi complexes, abundant ribosomes, and electron lucent vesicles in common with those

found in the distal cytoplasm. No membrane bound, dense bodies were observed in the cytons.

No differences in tegument ultrastructure have been observed between in vivo and in vitro material.

4.2 Scolex

The scolex comprises the typical taeniid type with four large suckers and a rostellum armed with two rows of hooks (Plate 101).

Ultrastructurally, the suckers are clearly delimited from the remainder of the scolex, by a plasma membrane. Although the morphology of the distal cytoplasm and tegumental cytons is, as already described, the suckers are readily distinguished by a compact mass of glycogen-rich parenchymal cells and interspersed with extensive radial and circular muscles. Little interstitial fibrous material is present, in contrast to the cellular organisation of rest of the scolex. Subtegumental osmophilic globules are common in cytoplasmic extensions ('ducts') throughout the scolex (also described by Specian, Allison & Ubelaker, 1974, for H. diminuta), indicating possible secretory activity. Their origin has yet to be determined. A greater degree of secretory activity was exhibited by in vivo worms. The microtriches on the scolex are more filamentous than those on the strobila, and appear more dispersed in sectioned material.

The two rows of rostellar hooks are clearly distinguishable by their morphology. Each comprises a characteristic handle, with a curved, pointed blade, directed posteriorly. The upper row of large hooks, with prominent blades, is interspersed by a lower ring of hooks, with short blades and relatively long handles (Plate 104). In vivo, the hooks are often seen protruded, a situation which is less common in cultured worms. TEM sections show the hooks embedded within the rostellar tegument (Plate 105), the handles anchored, and possibly formed in specialised cells beneath the sub-tegumental muscle layers

(Plate 106). A "hook-organ" has also been observed by Bilgees & Freeman (1969) in T. crassiceps.

The characteristic, muscular rostellar pad lies within the rostellum at the level of the hooks (Plates 107 & 108) and it is presumed to be responsible for the contraction and extension of the rostellar tip, and possibly hook movement as well. Light microscopy reveals a morphology similar to that described by Thompson & Eckert (1983) in E. multilocularis, and Smyth (1964b), Smyth et al (1969), Smyth, Gemmell & Smyth (1970) and Thompson et al (1979), in E. granulosus.

In the apical rostellum, a cluster of cells lying anterior to the rostellar pad can clearly be seen (Plate 107). These are characteristic of the rostellar gland cells already described by light microscopy of E. multilocularis and E. granulosus adults (authors cited above). Electron micrographs suggest that these cells may be specialised tegumental cytons, containing organelles as previously described (Plate 109). There are, however, few cytoplasmic vesicles, although the number and extent of the Golgi complexes are quite pronounced. Occasional electron dense nuclear inclusions were noted, corresponding to the ultrastructural observations on E. granulosus by Smyth et al (1969) and Thompson et al (1979). The origin of this 'secretion' remains unclear. Cytoplasmic extensions of the cytons form an extensive interconnecting network within the rostellar 'gland'. Parenchymal cells are infrequent, but interstitial material within the intercellular spaces, evident.

The rostellar tip is generally dome-shaped, although its "telescopic mobility" in vivo has previously been described in Echinococcus spp. by Smyth et al (1969) and Thompson et al (1979). An external covering of a meshwork of filamentous microtriches, some of which are branched, is obvious (Plate 102). In TEM sections of both in vivo and in vitro material, the distal cytoplasm of the tegument is somewhat convoluted, and contains large, vacuolar areas (Plate 110); thus confirming earlier observations of in vivo specimens (authors cited

above). These may cause distension of the rostellar tip, and sometimes, in vitro, an abnormally large protuberance results (Plate 103).

4.3 Sensory Receptors

Two different types of sensory organs have been observed within the tegument. The first is a typical unciliated type with a relatively short cilium which protrudes through a gap in the distal cytoplasm (Plate 111). It is anchored by a characteristic basal plate and short rootlet, which are embedded in a specialised cell of relatively electron lucent cytoplasm, containing numerous electron lucent, membrane bound vesicles. Numerous large mitochondria are present, at the base of the cell. The receptor is supported by two rings of electron dense material around the basal plate. Some sections show these rings to be tubular in nature, with a less dense central area (Plate 112). Close to the uppermost ring, desmosomes provide intimate contact with the distal tegumentary cytoplasm.

The second type of (presumed) sensory receptor, is non-ciliated, and is embedded within the distal cytoplasm, although it does not protrude as far as the external plasma membrane (Plate 113). The flattened distal end is attached to the surrounding tegument by desmosomes, in close association with a single, dense collar. Some sections show this collar to be fibrillar in nature, similar in morphology to the shaft of the microtriches (Plate 114). Central to the inner collar lies an indistinct structure (basal body?) (Plate 115), from which extends an expanded striated rootlet (Plate 116). This extends through the floccular cytoplasm of the receptor cell, at the base of which, mitochondria are found (Plate 117). An elongation of each type of cell presumably connects with the nerve trunks.

The presence of ciliated and non-ciliated types of sensory receptors is in agreement with recent observations by Richards & Arme (1982) for Caryophyllaeus laticeps, (see also Whitfield,

1979), although the structures described above, have previously been considered as part of a single sensory ending, in E. granulosus (Morseth, 1967).

4.4 Excretory system

The structure of the excretory system in protoscolecids, as observed by light microscopy, has already been outlined. In adult worms, this general pattern appears to be similar, but with the development of a transverse duct connecting the main longitudinal, collecting ducts at the posterior of each proglottid (excluding the terminal one). Ultrastructural studies reveal the epithelium of these ducts to be a syncytium, in close association with surrounding parenchymal cells, containing occasional nuclei, numerous vesicles (both electron lucent and electron dense), and bearing knob-like extensions on the luminal surface (Plate 118). Infrequent muscle fibres may also be present surrounding the ducts.

Flame cells, associated with the excretory ducts, were seen dispersed throughout the worm during development, although their pattern of distribution was not mapped. Each has the typical taeniid structure of a single cell containing a large nucleus, with prominent nucleolus and scattered chromatin, several mitochondria, ribosomes, ER, some vacuoles, a few Golgi saccules and occasional dense bodies (Plate 119). A closely packed group of flagella (of the typical '9+2' pattern - Plate 120), arising from the cell, are anchored by basal bodies and associated rootlets. The flagellar tips extend into excretory ductules, the epithelium of which is simpler in morphology than the main ducts, into which they run, with the cytoplasm being devoid of vesicles.

Calcareous corpuscles are visible throughout all stages of the worms' development, from protoscolex to adult. However, as previously mentioned, light microscopy has revealed their rapid reduction in numbers during the first few days in vitro; this has yet to be demonstrated in vivo. Although not always properly fixed by the EM techniques used in this study, they

are seen to be bordered by a thin cytoplasmic layer, in close association with surrounding cells, and are composed of one or more concentric whorls of material (Plate 121).

Ultrastructure of the excretory system in E. multilocularis is apparently similar to that in other cestodes; the functional morphology of such systems has recently been discussed by Yamane et al (1982a) following observations of D. latum.

D. DISCUSSION

One of the main aims of this study was to compare the morphology of worms matured in vivo and in vitro.

1. Gross morphology

In general, the gross morphology of the adult worms grown in vivo and in vitro was found to be very similar, with the development of up to three proglottids. The gross anatomy of the mature segment was also found to resemble that detailed by both Rausch & Schiller (1954) and Vogel (1957), but in this study it was concluded that the oviduct led directly into the ootype, rather than into the receptaculum, as described by Vogel (1957). This interpretation was based on the study of fresh worms, and reinforced by examination of serial sections, of in vivo and in vitro material.

The number of testes found in vitro material was certainly less than that encountered in in vivo worms (both UK and Zurich material), a result which may have been due to a lack of nutritive, or other, factors within the culture medium. However, it should be pointed out that the number of testes per segment has been found to vary considerably, and, on average, the material from dogs used in this study contained fewer testes than observed in E. multilocularis by previous authors (Rausch & Schiller, 1954 - 17 to 26; Vogel, 1957 - 14 to 31; Yamashita et al, 1958a,b - 15 to 30; Kurashivilli, 1964 - 18 to 30). These variations could be due to observations of different isolates (strains?) of the species in question, although, unlike E. granulosus, strain differences in E. multilocularis have not been examined in any great detail.

2. Reproductive system

Histogenesis of the genitalia appears to follow a pattern similar to that in other cestodes, with the development of the reproductive system being protandric (Douglas, 1961; Sulgostowska, 1972, 1974).

2.1 Male genitalia

The process of spermatogenesis in E. multilocularis also resembles that in other cestodes, in that a single, primary spermatogonium gives rise to 64 spermatids following four mitotic and two meiotic divisions. Synaptonemal complexes, characteristic of meiosis, were however, not observed in this study. Whether the characteristic plasmodial rosette of the spermatocyte stages, is due to incomplete cytokinesis during spermatogonial divisions, or arises from fusion of the 4^o spermatogonia, has long been under discussion (Featherston, 1971; Kelsoe et al, 1977; Robinson & Bogitsch, 1978). Some workers favoured the fusion theory, although their ultrastructural studies included few observations on the early stages of spermatogenesis (Rosario, 1964; Robinson & Bogitsch, 1978). It seems more likely that cytoplasmic continuity of the cells is maintained throughout (Swiderski, 1970; Featherston, 1971; Taneya, 1973, Mokhtar-Maamouri & Swiderski, 1975; Lumsden & Specian, 1980), the "syncytial connections allowing synchronised activity" (Kelsoe et al, 1977). Ultrastructure of the various stages is similar to that previously described for cestodes (Featherston, 1971; Mokhtar-Maamouri & Swiderski, 1975; Kelsoe et al, 1977; Lumsden & Specian, 1980). However, the occurrence of nucleolus-like bodies (NLB) in the cytoplasm of spermatids, and occasionally in earlier stages, has not been reported in other cestodes, although Robinson & Halton (1982) recently described a similar body in a digenean, for the first time. These inclusions are a common feature of monogenean and digenean oocytes; apparently being aggregates of ribonucleoprotein derived by transport of nuclear material through nuclear pores (clearly observed in this study), they clearly play an important part during germ cell differentiation (to be discussed later). Robinson & Halton (1982) suggested that, since the NLBs disappear during spermiogenesis, they probably provide functional RNAs for protein synthesis during sperm differentiation, rather than for materials needed during early embryogenesis.

The fine structure of cestode testicular epithelium, does not appear to have been described in detail before. Featherston (1971) observed a number of 1^o spermatogonia within a "common envelope" in T. hydatigena, and Kelsoe et al (1973) noted a "narrow testis wall" in H. diminuta. The presence of a non-germinal epithelium has, however, been identified in numerous digenean species, and is more commonly regarded as a "sustentacular layer" (Grant et al, 1976; Robinson & Halton, 1982; Erwin & Halton, 1983). Sato et al (1967) observed "supporting cells" surrounding spermatogonia of Paragonimus miyazakii; Grant et al (1976) suggested that similar cells in Pharyngostomoides procyonis were nutritive. More recently, Robinson & Halton (1982) and Erwin & Halton (1983) proposed that, in addition to supportive and nutritive roles, the sustentacular tissue of Corrigia vitta and Bucephaloides grasilescens may also be involved in the sequestration and intracellular degradation of testicular materials, and the recycling of elements essential to spermatogenesis. Although it seems likely that the sustentacular cells of digenean species are analagous to the syncytial, testicular epithelium in E. multilocularis, the ultrastructure of the latter is certainly less complex, suggesting that it does not play such an important part during spermatogenesis. Nevertheless, the spermatogonia are commonly found in close association with the epithelium, the spermatogonial plasma membrane intermittently making contact with that of the epithelium suggesting a possible nutritive role of the latter. Following spermiogenesis, sperm have also been seen in contact with the testicular lining, indicating that the latter may be involved to some extent in maturation of the male gametes (see later). As the testes mature, the epithelium becomes much reduced, and therefore, although it may be supportive at the onset of spermatogenesis, such a role is not apparent in later stages. However, in the testes of in vitro material, cytoplasmic extensions of the epithelium sometimes separate the gonads into follicles, which may persist until degeneration of the testes. Since no similar situation has been found in worms matured in vivo (in which the epithelium is confined to the periphery of each testis), this has been regarded as abnormal, or

incomplete, development of the epithelium, possibly reflecting some deficiency in the culture medium.

The characteristic networks of AER found in the epithelium, have not previously been described in testes (or other organs) of parasitic platyhelminths. Threadgold (1976) reported the abundance of AER in animal cells concerned with lipid production, glycogen metabolism and impulse conduction. Although the function of the AER in E. multilocularis testes cannot be determined solely from the present ultrastructural studies, it is suggested that it may be involved in glycogen metabolism. The β -glycogen particles seen in association with the AER would support this. Such particles have also been observed in maturing sperm, whereas intracellular glycogen has yet to be demonstrated during any stage of spermatogenesis or spermiogenesis. The intimate contact between the sperm and testicular epithelium following spermiogenesis, lead to the speculation that, not only is the AER concerned with glycolysis, but may also be involved in the transportation of carbohydrate to the maturing sperm (see later).

Early observations on spermiogenesis in cestodes relying on light microscopy, resulted in some confusion of the processes involved, and the subsequent inclusions of the sperm (Douglas, 1963; Kazacos & Mackiewicz, 1972; Taneya, 1973). In fact ultrastructural studies by other workers (Gresson, 1962; Rosario, 1964; Featherston, 1971; Robinson & Bogitsch, 1978), provided little additional information, especially on the mode of formation of a single-axoneme type sperm (as seen in Cyclophyllidea). However, spermiogenesis has been recently, briefly described in E. multilocularis (Barrett & Smyth, 1983), with full details being presented here. Mokhtar-Maamouri & Swiderski (1975) and Mokhtar-Maamouri (1979, 1982) described spermiogenesis in Acanthobothrium fillicolle benedenii, A. fillicolle fillicolle, Onchobothrium uncinatum and Phyllobothrium gracile. In general, the processes involved are similar to those of this study, although in the Acanthobothrium spp. and O. uncinatum, two axonemes are completely formed and incorporated into the cytoplasmic process. The sperm of P.

gracile, on the other hand, contain a single axoneme, and Mokhtar-Maamouri (1979) demonstrated that this was due to abortion of one of the two basal bodies during spermiogenesis. In contrast, each basal body in the spermatids of E. multilocularis initiate formation of an axoneme; one being subsequently aborted during elongation of the other.

Far more comprehensive reports on spermiogenesis come from observations of several digenean species (Sato et al, 1967; Burton, 1972; Grant et al, 1976; Rees, 1979; Robinson & Halton, 1982; Erwin & Halton, 1983). The processes described were similar in all cases and corresponded to the observations of Mokhtar-Maamouri & Swiderski (1975) and Mokhtar-Maamouri (1982) on three Tetraphyllidea (described above), in that complete development of two axonemes, followed their rotation and subsequent fusion with the median, cytoplasmic process, results in the formation of a two-axoneme type sperm. A similar situation is apparent in some monogenean species (Halton & Hardcastle, 1976; Justine & Mattei, 1983a,b), although in one species (Heterocotyle sp.) one of the axonemes is prematurely aborted (Justine & Mattei, 1983b). The latter observation somewhat resembles the process encountered in E. multilocularis.

The presence of a single axoneme within other helminth sperm (specifically Monogenea (Amphibdelloides spp.), Digenea (Schistosoma spp.) and Acanthocephala (Polymorphus minutus and others)), has been attributed to the occurrence of only one centriole within the spermatid 'zone of differentiation' (Whitfield, 1971; Marchand, 1981; Justine, 1982; Justine & Mattei, 1983c).

The presence of a centriole-like body (centriole-type body, centre body or microtubule organising centre) sited between the two basal bodies (centrioles) during spermiogenesis, appears to be universal amongst Digenea (excluding Schistosoma spp.) and tetraphyllidean species studied. In contrast, their absence has been noted in (1) monogenean species (in which two basal bodies occur), and (2) E. multilocularis. (Ultrastructural studies on other Cyclophyllidea, although somewhat limited in detail, describe the occurrence of two centrioles, but do not mention a third, centriole-like body). Although its origin and function remain unclear, it has been suggested that (1) it is involved in the assembly of the peripheral microtubules of the axoneme, (2) it acts as a template for the central complex of the axoneme, or (3) may participate in the construction of the cortical microtubules extending into the median, cytoplasmic process (see Robinson & Halton, 1982). Since all the processes occur in the apparent absence of a centriole-like body, it seems that it may play another role during spermiogenesis, not pertinent to the differentiation of cyclophyllidean, and some monogenean, sperm.

On reviewing the literature concerning spermiogenesis, it is apparent that, although the overall process is somewhat similar in all parasitic platyhelminths studied, the pattern of differentiation and elongation of the sperm is ultrastructurally quite distinct for each species.

The structure of the axoneme in E. multilocularis is similar to that in other free-living and parasitic platyhelminth sperm (Shapiro et al, 1961; Silveira~~a~~ & Porter, 1964; Tulloch & Hershenov, 1967; Silveira, 1969). It differs from a~~a~~ typical flagellum (seen in the flame cells of E. multilocularis), in that the pair of central microtubules are replaced by a single, core complex, and hence the designation, 9+'1'. (The Schistosomatidae are unusual in this respect in that the central core is either absent, or of floccular electron dense material - see Justine & Mattei, 1981; Justine, 1982). The microtubules are most likely formed from the basal bodies; the origin of the central complex in E. multilocularis

remains uncertain. The axoneme is presumably an important structural component of the sperm, and may aid in their movement, although in fresh material the activity of the sperm is not very great. The axoneme also acts as a support, around which the nucleus spirals; the coiled nature of the peripheral microtubules in the cytoplasmic process would suggest that they play a part in guiding the nucleus into a spiral shape around the axoneme. Whether the nucleus migrates to the distal end of the sperm during the latter stages of spermiogenesis, remains uncertain. It is also possible that the nucleus remains mobile, and may migrate towards either end of the mature, or maturing, sperm (Rosario, 1964). If so, this may account for the unusual corkscrew-like movement of the male gametes, seen in the genital atrium (Plate 65), although nuclear movement is unlikely to be rapid.

Since the structure of a complete, mature sperm has not been fully described, it is difficult to correlate the results of this study with observations of other workers. Nevertheless, the presence of a single axoneme, around part of which is a spiralled nucleus, is universal amongst the cyclophyllidean, caryophyllidean and diphyllidean cestodes studied. Two-axoneme type sperm, common to digenean species, are found in pseudophyllidean, proteocephallidean and tetrahyarchidean cestodes. The ~~T~~etraphyllidea are unusual, in that both types are found within this order. The division of cestodes into two groups according to the number of axonemes, has recently been suggested, and possible phylogenetic relationships discussed (Euzet et al, 1981).

Most workers have reported the absence of the basal body and associated rootlets in mature sperm, although Mokhtar-Maamouri (1982) recently observed them in A. fillicolle fillicolle. It is possible that the basal body is also enclosed in the sperm of E. multilocularis, following observations of an unusual structure at one end of the mature gametes.

The lack of mitochondria appears to be characteristic for

all cestode sperm, in contrast to the Digenea. Featherston (1971) described a spiral septate structure in one region of T. hydatigena sperm, speculating that it could be a highly modified form of mitochondrion. Similar structures, apparently derived from mitochondria, have been observed in insect sperm (Phillips, 1974). It is possible that the septate structures described by Featherston (1971) and the morphologically similar radial septa observed in this study, correspond to mitochondrial cristae. However, fusion of the spermatid mitochondria into a single mass (as in Digenea) or nebenkern (as in insects) has not been observed in E. multilocularis; numerous mitochondria are seen in the residual body following spermiogenesis. Further observations are necessary before the origin of the unusual radial, septate structure can be determined.

The inclusion of β -glycogen particles in the cytoplasm of mature sperm appears to be universal for all cestodes. Robinson & Bogitsch (1978) stated that a period of rapid glycogen synthesis followed spermiogenesis in H. diminuta, and proposed that, in the absence of mitochondria, cestode sperm probably depend on glycolysis for their energy requirements. In E. multilocularis, a scheme for the incorporation of glycogen in maturing sperm has been proposed; the method of inclusion of glycogen into sperm of other cestode species has not previously been discussed. However, digenean species also contain glycogen, and Erwin & Halton (1983) have tentatively suggested that the walls of the vas deferens and seminal vesicle of Bucephaloides grascilescens may be secretory in nature, providing substrates for glycogen synthesis. It is not surprising to note that the glycogen found within platyhelminth sperm is of the β -configuration, since this is a readily accessible energy source (Lumsden, 1965).

One other point to note regarding sperm structure is the apparent lack of an acrosome. "Crested bodies" have been observed in some tetraphyllidean sperm (Mokhtar-Maamouri & Swiderski, 1975; Mokhtar-Maamouri, 1979, 1982), but whether these are homologous to the acrosome of vertebrate sperm,

remains to be demonstrated. Burton (1967b) suggested that in H. medioplexus , "considering the stiffness of the sperm , its length, and the limited space for penetration of the ovum, an acrosome probably would provide little advantage".

Few observations have been recorded on the ultrastructure of the sperm ducts. However, the morphology of the vasa efferentia in H. diminuta (Lumsden & Specian, 1980), is similar to that encountered in E. multilocularis ; the well developed muscular layer of the seminal vesicle, corresponding to that observed in this study, around the vas deferens near the junction with the cirrus duct.

The eversible cirrus, and surrounding musculature of the cirrus sac is characteristic amongst cestodes. No other reports on the ultrastructure of this organ have been found, although SEM studies have been carried out on several species, showing the everted cirrus to be covered with spines (Lumsden & Specian, 1980), or microtriches (Yamane et al., 1982b). The process of insemination is considered in detail later.

The ultrastructure of the genital atrium is described, apparently for the first time, in this study. The nature of the lipid-like droplets seen in fresh material, still remains uncertain. They may be comparable to the small vesicles observed in TEM sections, which sometimes conglomerate, forming larger masses, in which sperm may be trapped. It is possible that this material is exogenous, or, alternatively , may be secreted from the genital atrium epithelium. The possible functions of such secretions within the reproductive ducts is considered in detail later.

2.2 Female genitalia

The general structure of the vagina and receptaculum in E. multilocularis closely resembles that outlined by Smyth *et al* (1967), for E. granulosus. In fact, the author's diagrams (ascertained by light microscopy) show, (1) the presence of numerous luminal extensions in the vagina, and (2) the thick-walled, cellular nature of the receptaculum. Although ultrastructural studies have confirmed the first observation, in E. multilocularis, the receptaculum epithelium is syncytial, rather than cellular. It does, however, contain a large number of distal nuclei. Lumsden & Specian (1980) described a similar ultrastructural morphology in H. diminuta, although the presence of 'modified microtriches' in the anterior part of the vagina was not reported. It is likely that the luminal extensions may act as a non-return valve mechanism (Lumsden & Specian, 1980).

No vaginal sphincter, as seen in E. granulosus (Bacigalupo & Rivero, 1949; Smyth & Smyth, 1969) and in H. diminuta (Lumsden & Specian, 1980) was found in E. multilocularis, suggesting that the method of sperm transfer in this species, may be somewhat different (see later).

The occurrence of lipid-like droplets in the vagina and receptaculum of fresh specimens, the densely staining lumen of the vagina (and also the cirrus) in wax sections, and the appearance of luminal vesicles in TEM sections, all point towards a possible secretory role of these ducts. Moreover, the epithelium comprises numerous electron-lucent vesicles, as also observed in the distal cytoplasm of the tegument, again, suggesting a secretory function. Whether intracellular (or even extracellular) digestion also occurs, as observed in the oviduct of S. mansoni (part of which also acts as a receptaculum), by Erasmus (1973), remains to be proved. It is possible that some secretion is necessary to aid transport of the sperm. It may also play a part in (1) a feedback system by regulating the release of ova or vitelline cells from their respective organs, (2) providing necessary substances for final

maturation of the sperm, or (3) providing a chemical gradient, along which the sperm migrate towards the ootype (Erasmus, 1973).

Although oogenesis has not been studied in detail in this investigation, it seems likely that the processes involved are similar to those described for other parasitic helminths. In other words, following mitotic divisions of the oogonia, primary oocytes are formed. Subsequent meiosis may be initiated in the ovary, with completion of the maturation division occurring in the oviduct, ootype, or even the uterus.

Light microscopy of fresh material reveals the structure of primary oocytes to be similar to that described by Johri (1957), Douglas (1963) and Rybicka (1964a, b, 1966) for various cestodes, and Burton (1960) for H. medioplexus. As to the granular material seen surrounding the oocyte nucleus both in the ovary and the uterus, its nature appears uncertain. In a review on embryogenesis in cestodes, Rybicka (1966) stated that (1) this substance was commonly regarded as "yolk" material, accumulated within the oocyte, and (2) the nutritive role of the vitelline cells in Cyclophyllidea is greatly reduced, with the nutritive function probably being carried out by the oocyte. Rybicka (1966) also noted that cyclophyllidean and pseudophyllidean oocytes could possibly be distinguished by their cytoplasmic yolk reserves (namely vitelline particles, or fine granular material). Histochemical studies by Johri (1957) showed the oocytes of M. smythi to contain prominently areas of acidophil proteins. Although Coil (1972) was in agreement with the occurrence of two types of cytoplasmic inclusions in cestode oocytes, he suggested that the small, diffuse granules which were released in the ootype, contributed to the first membranous "shell", whereas the larger granules, remained until cleavage, to be "used later". Burton (1960) on the other hand, considered that the cytoplasmic inclusions of digenean oocytes were neither nutritive, nor shell precursors. He identified the granules around, and in close contact with the nuclear membrane as mitochondria, and stated that the presence of a few distinct bodies (having the same staining characteristics as

the nucleolus), were due to nuclear extrusion, occurring during reorganisation of chromosomal material. Such nucleolus-like, cytoplasmic bodies (NLCBs) have also been observed in stained wax sections in this study, and it seems likely that they are analogous to the RNA bodies described by Burton (1960).

Ultrastructural studies by Koulish (1965), Burton (1967b), Halton *et al* (1976) and Grant *et al* (1977) have all revealed NLCBs, in digenean and monogenean species, similar to the electron dense areas demonstrated in E. multilocularis oocytes. It appears that nucleolar extrusion and associated passage of RNA to the cytoplasm is an important part of oocyte differentiation within digenean and monogenean parasites, and a similar situation could be expected in cestodes. (The NLCB are similar to the NLB described in Corrigian vitta spermatids by Robinson & Halton, 1982 - discussed earlier).

Electron microscopy of E. multilocularis oocytes certainly reveals few cytoplasmic inclusions, with the exception of numerous perinuclear mitochondria. It seems likely that these may account for some, or all, of the granular material seen in fresh preparations. These, and other organelles (ribosomes, ER and Golgi complexes) appear to be universal for oocytes of all parasitic platyhelminths studied. However, the characteristic electron dense cortical granules described by Erasmus (1973), Halton *et al* (1976), Grant *et al* (1977) and Irie, Basch & Beach (1983), may be specific for monogenean and digenean species. No such granules (presumed to be involved in the initial phase of fertilization - Halton *et al*, 1976) have been found during this ultrastructural investigation, or in that of Lumsden & Specian (1980) on H. diminuta.

The appearance of floccular material (morphologically similar to that of the α -glycogen particles in surrounding parenchymal cells) in intercellular spaces between E. multilocularis oocytes and the ovarian epithelial network, has been mentioned. Erasmus (1973) also observed luminal material, and considered the dense matter to be discharged cortical granules and debris from disintegrating ova. Although no

cortical granules were seen in E. multilocularis, it is possible that some degeneration of the cells could account for the floccular, luminal material.

The intimate association of the oogonia and oocytes with the ovary epithelium, is suggestive of a possible supportive and/or nutritive role of the latter. This has also been observed in the testes, and similar, possible functions of the testis' epithelium, have been discussed earlier.

Adjacent to the ovarian isthmus, lies the oocapt, which, being funnel shaped, probably guides the oocytes into the oviduct. Although no sphincter has been identified, the oocapt is surrounded by a thin band of circular muscles. Since this muscular layer is continuous along the oviduct, it seems likely that oocytes are forced into the ootype by peristaltic waves. This movement is probably fairly rapid, as no oocytes have been found in the oviduct of either fresh or fixed material. The oviduct epithelium itself is very complex in nature with numerous luminal cytoplasmic extensions and microvilli generally directed towards the ootype, and it is speculated that, as in the vagina and receptaculum, these may act as a valve mechanism. In contrast, the oviduct of H. diminuta is morphologically very simple (Lumsden & Specian, 1980). The syncytial epithelium bears, on its luminal surface, only small, infrequent microvilli. Within digenean species studied, the epithelium is cellular, but the luminal surface does bear extensive cytoplasmic extensions or lamellae (Erasmus, 1973; Grant et al, 1977). Cilia have also been observed by these authors in the region of the oviduct, nearest to the ootype; a situation which is also apparent in E. multilocularis. However there are no cytoplasmic inclusions in the oviduct of this species to suggest the occurrence of intracellular digestion, as demonstrated in S. mansoni (Erasmus, 1973). Nevertheless, the presence of both cytoplasmic and luminal vesicles indicate a possibly secretory function; also demonstrated by the densely staining oviduct wall seen in light microscope preparations. The potential role of such secretions has already been discussed earlier.

The presence of a single vitelline gland posterior to the ovary is typical amongst the Taenidae. Light microscopy of the mature vitellarium, reveals cells similar in structure to those of other cyclophyllidean cestodes. (Johri, 1957; Rybicka, 1964b, 1966), with a large vesicle (vitelline globule) displacing the cytoplasm and nucleus peripherally. In contrast, Coil (1972), reported the presence of a small number of granules in a vacuole in mature cells of another cyclophyllidean, the dioecious species, Gyrocoelia pagollae.

The maturation of vitelline cells in E. multilocularis has been followed to some extent by light microscopy. Maturing cells are characterized by the presence of several small vitelline granules, which apparently coalesce, eventually forming the large spherical globule, typical of mature cells. Although various stages of maturation had been described in early studies of cyclophyllideans, Rybicka (1966) suggested that the observed differences in vitelline cell morphology was due to different methods of fixation employed by different authors.

Further studies on vitellogenesis have been enhanced by electron microscopy, although little attention has been paid to cestodes. Nevertheless, one ultrastructural study has confirmed earlier observations on the mature cell, in that, amongst the cyclophyllidea, the type of accumulation of vitelline material differs somewhat (Swiderski et al, 1970a, b). Cells of Inermicapsifer madagascarensis were found to contain a large, single vesicle (as in E. multilocularis); in contrast, two or three vesicles were present in Catenotaenia pusilla; whilst in H. diminuta, numerous perinuclear granules were observed. No reference, however, was made to the glycogen content of the vitellaria, although Lumsden & Specian (1980) reported the presence of a large amount in H. diminuta (also demonstrated in this study in E. multilocularis).

Ultrastructural studies on other cestode orders (namely, Pseudophyllidea, Tetraphyllidea and Caryophyllidea), have also

revealed the presence of an enormous glycogen inclusion (Swiderski & Mokhtar, 1974; Mokhtar-Maamouri & Swiderski, 1976b; Swiderski & Mackiewicz, 1976). In fact, Swiderski & Mackiewicz (1976) described the formation and storage of intranuclear glycogen in Glaridacris catostomi, both within the nucleoplasm, and the "membrane-free nuclear vacuole", confirming their earlier observations at the light microscope level (Mackiewicz, 1968). It is generally assumed that glycogen is synthesised and stored within vitelline cells, primarily as nutritive reserves for the developing embryo. It is therefore not surprising, that the α -type particle predominates in E. multilocularis vitellaria, since this configuration constitutes glycogen reserves in cestode tissues (Lumsden, 1965; Swiderski & Mackiewicz, 1976). (This is in contrast to the β -glycogen found in the sperm, where a readily available energy source is required). The possible role played by various cytoplasmic org^anelles, such as mitochondria, GER and ribosomes, during glycogen metabolism in vitelline cells of G. catostomi, has been discussed by Swiderski & Mackiewicz (1976).

Ultrastructural observations of the processes involved in vitellogenesis in E. multilocularis, are generally in agreement with previous studies on cestodes (cited above), Digenea (Bjorkman & Thorsell, 1963; Irwin & Threadgold, 1970; Erasmus, 1973, 1975; Grant et al, 1977; Erasmus & Davies, 1979; Irwin & Maguire, 1979; Erasmus & Popiel, 1980; Erasmus et al, 1982). and Monogenea (Halton et al, 1974). Maturing cells are characterized by the presence of abundant GER, with vitelline granules (presumably proteinaceous in nature), being secreted by the extensive Golgi complexes. Both Swiderski & Mokhtar (1974) and Mokhtar-Maamouri (1976b) have also demonstrated the appearance of lipid droplets during vitellogenesis in Bothriocephalus clavibothrium and Echeneibothrium beauchampi, respectively, although their role appeared uncertain. No lipid was observed in this study, or in other cestode species (Cylophyllidea - Swiderski et al, 1970a,b; Lumsden & Specian, 1980; and Caryophyllidea - Swiderski & Mackiewicz, 1976). In contrast, the presence of lipid was noted in maturing cells of several digenean and monogenean species (authors cited above).

The nature of floccular material seen in association with the vitelline material in E. multilocularis has not been determined; further (histochemical) investigations may prove enlightening.

Irwin & Maguire (1979), noted the absence of glycogen and yolk bodies in mature cells of G. vitelliloba, having stated that, in Digenea, "the composition of food reserve produced and stored in vitelline cells varies between species, but always comprises glycogen, yolk bodies and lipid in varying proportions". Having reviewed the recent literature on vitellogenesis in platyhelminths, it appears that the occurrence of such reserves is certainly not universal; it is consequently speculated that the type and amount of nutritive reserves reflects (1), the number of vitelline cells associating with each oocyte, (2) the nature of egg shell formation, (3) the rate of embryonic development, and (possibly) (4) the life expectancy of the larva within the egg. The yolk bodies observed by Irwin & Threadgold (1970), Halton et al (1974) and Irwin & Maguire (1979), probably correspond to ^{the} ribosomal complexes (whorls) described by Erasmus (1975), Erasmus & Popiel (1980) and Erasmus et al (1982); their absence has been noted in vitelline cells of E. multilocularis and other cestodes studied. The presence of calcareous corpuscles in vitellaria of Schistosoma spp. has also been recorded, (Erasmus & Davies, 1979; Erasmus & Popiel, 1980; Erasmus et al, 1982), although again, not observed in this study.

It is widely accepted that 'nurse cells' are present in the vitellarium of parasitic platyhelminths. In this study, the syncytial epithelium of the vitelline gland has been seen to ramify throughout the organ, with cytoplasmic extensions separating each cell. A similar situation has been observed in the ovary. Although intermittent, intimate contact between the syncytium and enclosed vitelline cells (or oocytes, in the case of the ovary; and spermatogonia, in the testes), is apparent, it was difficult to assess what role the epithelium plays during the process of vitellogenesis, and therefore the term

'nurse cell' has not been adopted here. In fact, for these same reasons, and the fact that the 'cells' were syncytial in nature in G. catostomi, Swiderski & Mackiewicz (1976) used the term, "interstitial cells".

Very few observations on the ultrastructure of the vitelline duct in platyhelminths have been recorded, and published accounts have only been found for digenean and monogenean species. The epithelium of the duct in E. multilocularis differs from that of the above accounts, in that it is syncytial. However, lamellae have been found in F. hepatica (Irwin & Threadgold, 1970), S. mansoni (Erasmus, 1973) and in three monogenean species (Halton et al, 1974). Cilia were not observed in E. multilocularis, but have been reported, either throughout, or in part of, the vitelline duct, by Erasmus (1973) and Halton et al (1974), as well as Grant et al (1977) - for Pharyngostomoides procyonis.

The luminal extensions in the duct of E. multilocularis are certainly far less than those observed in the other reproductive ducts and often seen flattened against the epithelium. However, it is conceivable that they may act as a valve mechanism, as previously suggested for the oviduct and vagina.

Although no other reports on the ultrastructure of the ootype in cestodes has been found, its morphology in E. multilocularis is similar to that in some digenean & monogenean species. Luminal, epithelial extensions, lamellae or microvilli have been observed in F. hepatica (Irwin & Threadgold, 1972), S. mansoni (Erasmus, 1973; Irie et al, 1983), Calicotyle kroyeri and Diplozoon paradoxum (Stranock, 1974; Stranock & Halton, 1975) and Pharyngostomoides procyonis (Grant et al, 1974); the ootype of D. paradoxum is also ciliated.

The problems encountered in locating the Mehlis' gland in cylophyllidean cestodes by light microscopy, has been emphasised by Johri (1957). The "elongate glandular cells" and

the "central aggregation of cell-like structures" described by the author, probably correspond to the gland cells and ootype epithelium, respectively, as seen in TEM sections of E. multilocularis. Coil (1972) on the other hand, stated that in Gyrocoelia pagollae, the Mehlis' gland is highly developed and comprises three types of cells - mucosa, serosa and basal. The latter are probably analogous to the ootype epithelium. Light microscopy studies of the Mehlis' gland by other workers, were concerned mainly with histochemical observations. Rybicka (1966) reviewed early publications on cestodes and trematodes, but did not distinguish between different types of cells. However, Coil (1965, 1972) and Bogitsch (1970) were able to differentiate between serous, or DB (dense body) cells and mucous, or MB (membranous body) cells, in the Mehlis' gland of some Digenea.

Ultrastructural studies by Lumsden & Specian (1980) also revealed the presence of two types of unicellular glands in a cyclophyllidean, H. diminuta. The membrane bound, electron dense granules in the serous cells, morphologically resemble those described for E. multilocularis. It is therefore surprisingly that only a single cell type was observed in this study. Lumsden & Specian (1980) however, did not describe the location of the unicellular glands. It is possible that (1) they occur in different sites and therefore would not necessarily be viewed together, in a single section, and (2) the mucous cells may be less frequent than the other type, and hence overlooked in this study. Other ultrastructural observations have been carried out on numerous monogenean and digenean species, and the occurrence of two cell types has been recorded by Thorsell & Bjorkman (1965), Bogitsch (1970), Threadgold & Irwin (1970), Burton (1967a), Irwin & Threadgold (1972), Stranock (1974), Stranock & Halton (1975) and Grant et al (1977). However, it is of interest to note that Erasmus (1973) and Irie et al (1983) observed only one type of Mehlis' gland cell in S. mansoni, which corresponded to the serous, DB, Type II and S₂ cells described by other workers (cited above), and those observed in this study. The absence of other (mucous, MB, Type I or S₁) unicellular glands in S. mansoni and

E. multilocularis may be due to reasons already mentioned, or possibly because only one cell type exists in these species. Alternatively, it is possible that the ootype epithelium is secretory. This has already been a suggested function of other ducts in E. multilocularis. In fact, Stranock & Halton (1975) have reported secretory activity of the ootype in D. paradoxum, in addition to the secretions produced by two types of Mehlis' gland cells. They also noted that ootype secretion was not uncommon in some digenean species, in which only one cell type had been identified.

It appears that the secretory activity of the Mehlis' gland and ootype in platyhelminths, presents a complex situation, the different types of gland cells and secretory bodies encountered, probably reflecting the nature of egg shell formation within different species or groups. It has recently been emphasised that the secretory products of either type of cell (as classified above) are often morphologically quite distinct from one species to another, possibly reflecting differences in their chemical composition (Stranock & Halton, 1975; Grant et al, 1977). In this study, Mehlis' gland secretions have been observed in the uterus, whereas the corresponding dense bodies in H. medioplexus, F. hepatica and Pharyngostomoides procyonis dissociate immediately on entering the ootype-uterine lumen; it is the secretion from the other cell type which persists in three species. It is consequently apparent that correlations between secretory material of different species, needs to be supported with histochemical evidence.

3. In vivo and in vitro material compared

Although the general structure and ultrastructure of E. multilocularis adults matured in vivo and in vitro is similar, a number of differences have been observed within the reproductive system.

With regard to the male genitalia, it is apparent that, not only are fewer testes found in cultured worms, but premature degeneration of the same would result in fewer sperm being available for insemination, and consequently fertilization. Nevertheless, a large number of sperm have been found within the vasa efferentia and vas deferens of adults in vitro. Consequently, a major problem seems to lie within the process of sperm transfer. Unlike E. granulosus (Bacigalupo & Rivero, 1949; Smyth & Smyth, 1969), E. multilocularis does not possess a vaginal sphincter, which may aid in securing the cirrus during insemination, thus ensuring passage of sperm into the female ducts. However, following observations of innumerable adults, in vivo and in vitro, the cirrus of E. multilocularis has never been seen fully inserted. In fresh material, contractions of the cirrus pouch cause the cirrus to evaginate into the genital atrium; only on rare occasions, has the cirrus tip been observed in the anterior part of the vagina. In worms matured in vivo sperm bundles have regularly been noted, both within the genital atrium and receptaculum. This would suggest that one of two methods of sperm transfer are possible. Firstly, insemination does occur, but is a very rapid process and therefore has not been observed in this study. Sporadic contractions of the cirrus pouch would account for the presence of sperm within the genital atrium. Secondly, sperm are ejected only as far as the latter, from where they migrate to the receptaculum. It is speculated that this second method, not uncommon amongst cestodes (Wardle & MacLeod, 1952), is most likely for E. multilocularis. The obvious lack of sperm transfer in cultured worms may be due to various intrinsic or extrinsic factors. Some chemical stimulant may be required to enhance movement of sperm through the cirrus duct; or physicochemical factors are lacking in our in vitro system

which would increase cirrus activity. In vivo, it has already been suggested that the worms are subjected to some external pressure from surrounding microvilli, and peristaltic motion of the intestine (Smyth, 1982b). Nevertheless, sperm have been observed occasionally (but rarely) in the genital atrium and/or receptaculum of cultured worms, and it therefore seems surprising that fertilization did not occur in at least one instance. However, as already mentioned, rapid degeneration of the ova, both within the ovary and the uterus, is not uncommon in vitro. A similar situation has also been reported in cultured S. mansoni by Irie et al (1983). Although early stages of oogenesis appeared to progress normally, a "disfunction of unknown cause", prevented further maturation to the primary oocyte. Ultrastructural studies of vitellogenesis in E. multilocularis in vitro, have also demonstrated the possible breakdown of vitelline cells during maturation, with the resultant loss of glycogen. Although this observation may not account for the fertilization problem, it demonstrates that the in vitro situation is not fully replicated by the culture system.

Other problems of fertilization in cultured E. multilocularis may be related to the timing of maturity and release of the male and female gametes. In vivo, it appears that sperm may be present in the receptaculum, prior to maturation and release of the oocytes. Migration of the sperm into the oocyte may depend on some stimulus, either from the released oocytes themselves, or the associated secretions of the Mehlis' gland and/or the oocyte, or some other unknown factor. Since oocytes and vitelline cells have been observed in the uterus of adults grown in vitro, independent of whether insemination has occurred, or not, it seems that their release is not necessarily triggered by the presence of sperm in the receptaculum. However, in some worms, the oocytes remain in the ovary, resulting in a swollen, empty uterus. It is difficult to speculate on the exact stimulus required for gamete release, but it may be partly dependant on their maturity; in other words, if the culture media is unable to sustain development of the oocytes to a stage at which they may

be fertilized, then their release from the ovary will not be effected.

A further point to consider, is the apparent rapidity with which ova and vitelline cells pass through their respective ducts into the ootype, and from there, into the uterus. Female gametes have not been observed in either the oviduct or ootype, suggesting that precise timing (of maturaton and release) may be necessary if the sperm and oocytes are to meet, in order to ensure fertilisation.

It is therefore considered that the failure of cultured material to produce viable, shelled eggs, is a combination of a variety of factors, rather than the sole problem of inducing self-insemination in vitro. Further studies to improve the culture system are clearly necessary, before the final goal of producing 'normal' adult worms with viable eggs is likely to be achieved.

Plates

Section II

Abbreviations

AER	:	agranular endoplasmic reticulum
ax	:	axoneme
bb	:	basal body
bd	:	blade
bl	:	basal lamina
c	:	centriole
cc	:	calcareous corpuscle
ce	:	cytoplasmic extension
ci	:	cirrus
cl	:	cilia
co	:	collar
cr	:	crystalline body
cs	:	cirrus sac
cy	:	cytophore
d	:	desmosome
du	:	duct
ed	:	excretory duct
er	:	endoplasmic reticulum
fc	:	flame cell
g	:	glycogen
ga	:	genital atrium
gc	:	Golgi complex
GER	:	granular endoplasmic reticulum
gp	:	genital pore
h	:	hook
ha	:	handle
l	:	lumen
la	:	lamellae
m	:	microtriches
me	:	median cytoplasmic extension
mg	:	Mehlis' gland
mi	:	mitochondrion
mm	:	modified microtriches
ms	:	Mehlis' gland secretion
mt	:	microtubules
mu	:	muscle
mv	:	microvilli
n	:	nucleus

nu : nucleolus
o : oocyte
oc : oocapt
od : oviduct
oe : oviduct or ovary epithelium
og : oogonium
ot : ootype
ov : ovary
pc : parenchymal cell
r : rootlet
rb : residual body
re : receptaculum
rg : rostellar gland
rp : rostellar pad
s : secretions
sb : secretory body
sc : spermatocyte
sd/t : spermatid
sg : spermatogonium
sh : shaft
sp : sperm
t : testes
tc : tegumental cyton
te : testis epithelium
tg : tegument
ue : uterus epithelium
ut : uterus
v : vagina
va : vacuole
vc : vitelline cell
vd : vitelline duct
ve : vas efferens
vg : vitelline gland
vs : vesicles

Plate 21

TEM testis, showing various stages of spermatogenesis.

Scale bar reps. $2.5 \mu m$ (in vivo)

Plate 22

TEM agranular endoplasmic reticulum in testicular epithelium, with associated β -glycogen particles (arrows).

Scale bar reps. $0.5 \mu m$ (in vivo)

Plate 23

TEM pronounced AER.

Scale bar reps. $0.5 \mu m$ (in vivo)

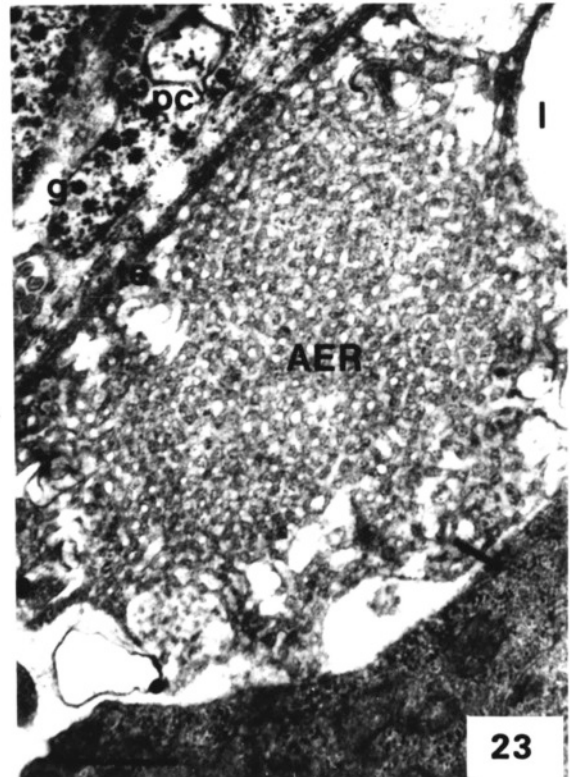
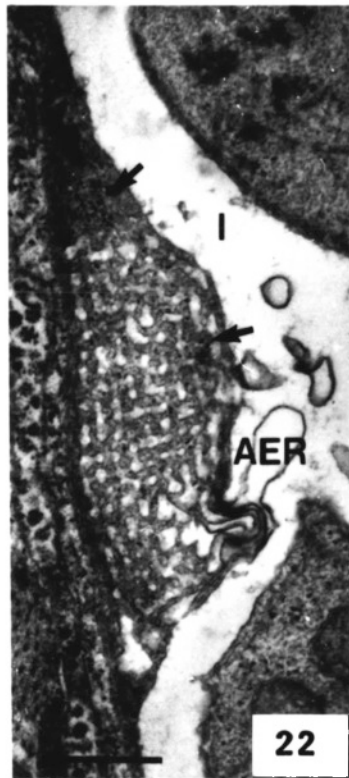
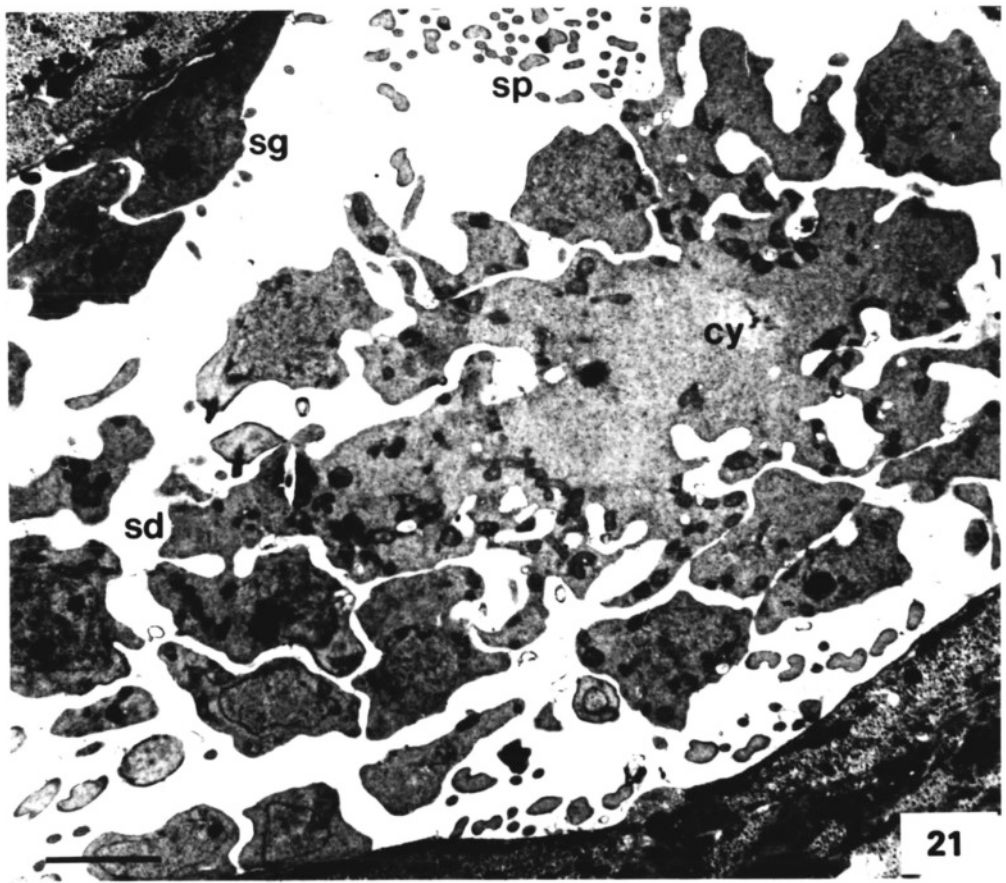


Plate 24

TEM testis showing division of testis into follicles.

Scale bar reps. $2\mu m$ (in vitro)

Plate 25

TEM immature testis, with spermatogonium.

Scale bar reps. $2\mu m$ (in vitro)

Plate 26

TEM spermatogonium. Note presence of centriole (arrow).

Scale bar reps. $1\mu m$ (in vitro)

Plate 27

TEM spermatogonium. Note presence of nuclear pores (arrows)

Scale bar reps. $1\mu m$ (in vitro)

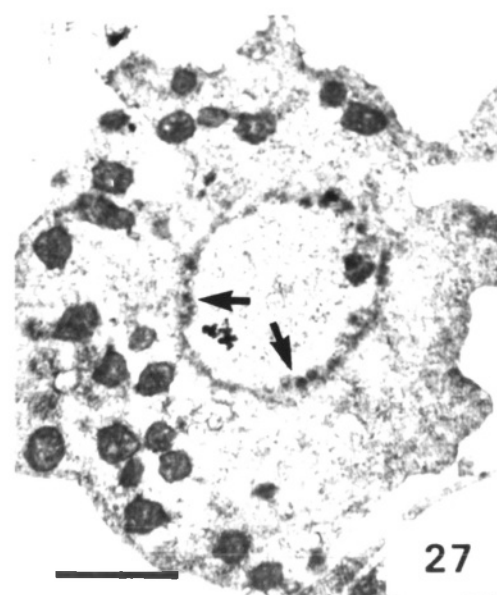
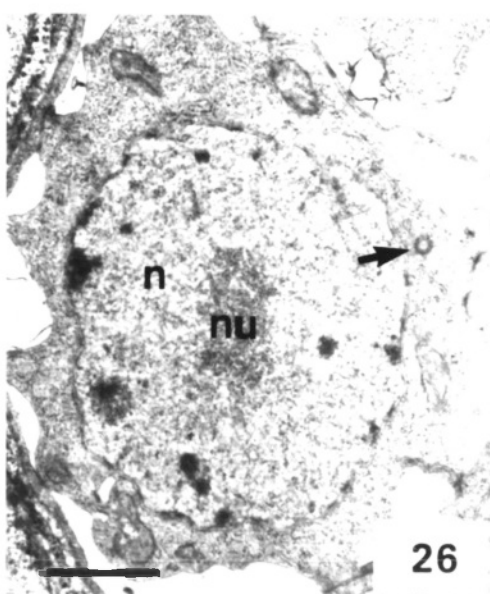
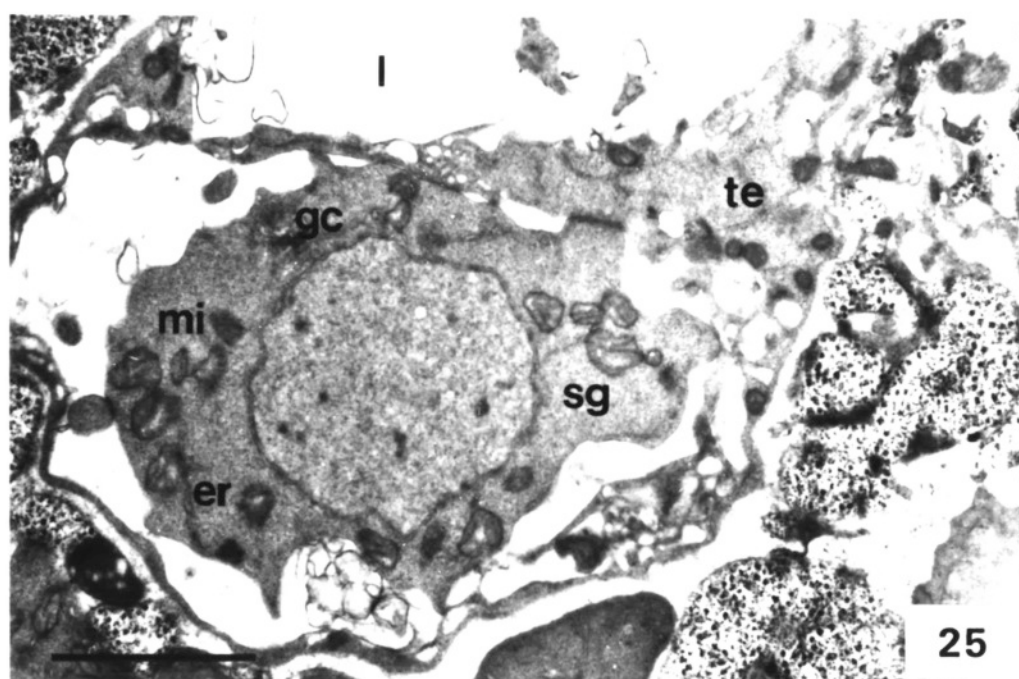
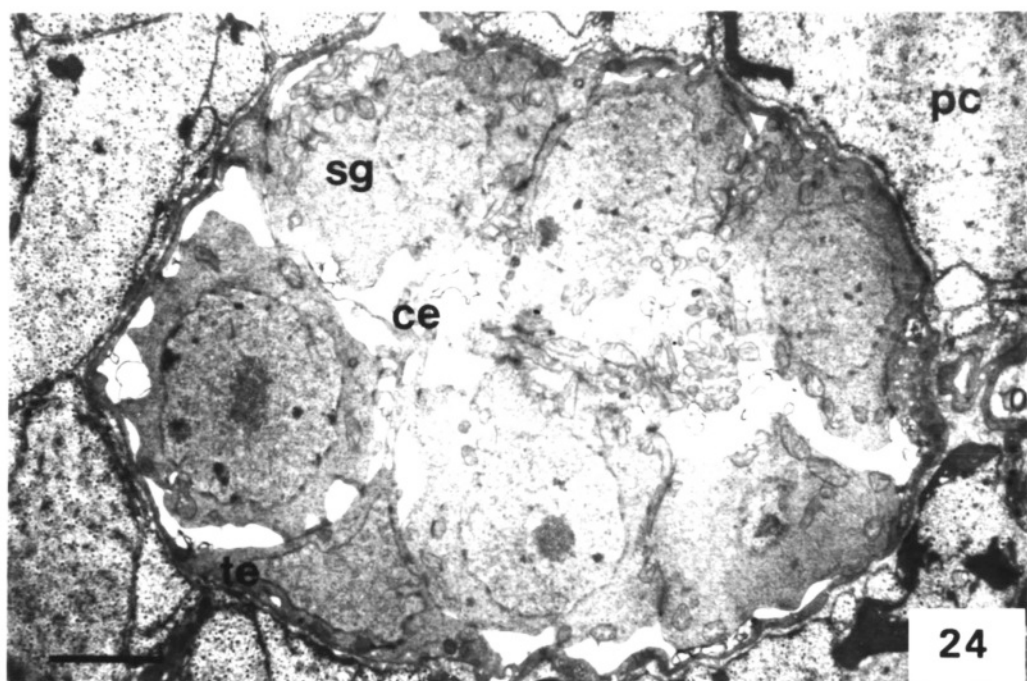


Plate 28

TEM tertiary spermatogonia.

Scale bar reps. 2 μ m (in vitro)Plate 29

TEM quarternary spermatogonia.

Scale bar reps. 2 μ m (in vitro)

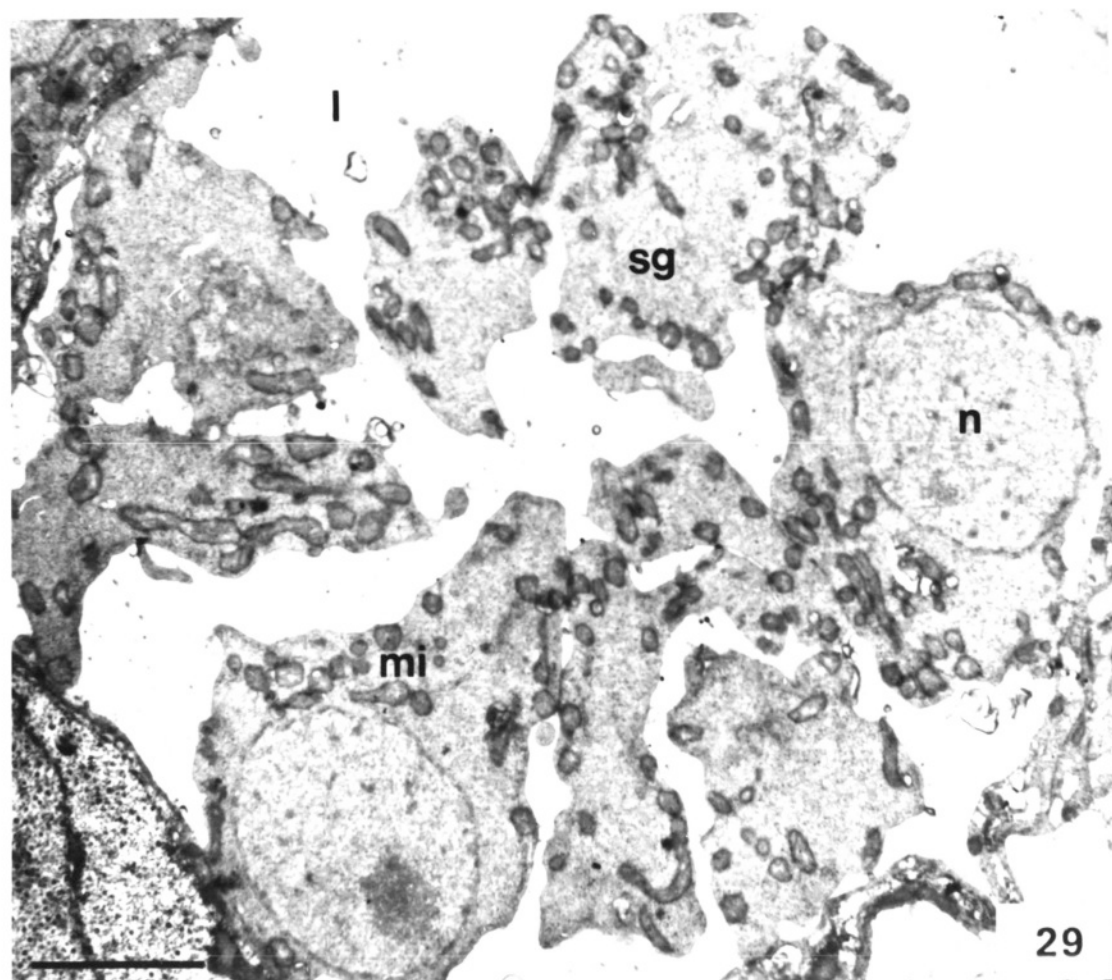
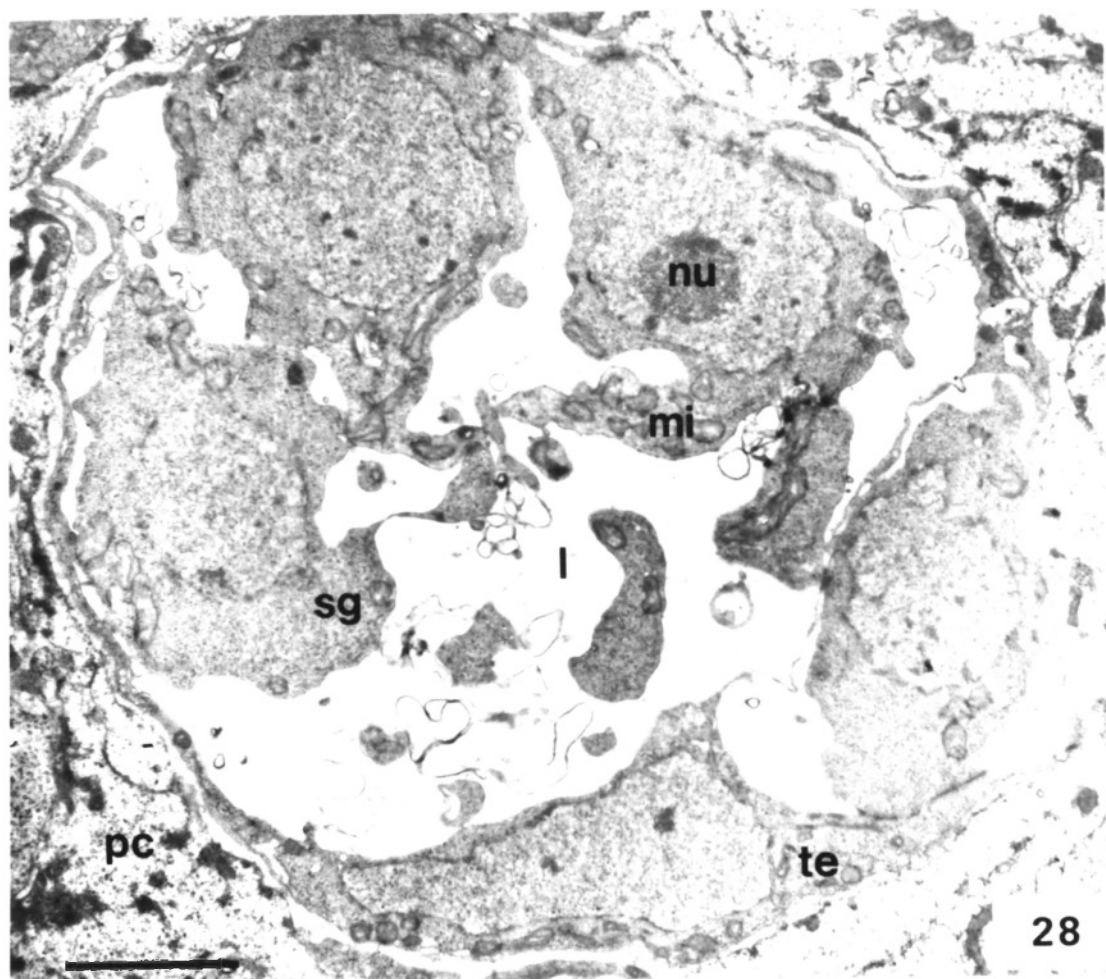


Plate 30

TEM primary spermatocytes.

Scale bar reps. 1 μm (Inset shows the paired centrioles in detail)
(in vitro)Plate 31

TEM secondary spermatocytes.

Scale bar reps. 2.5 μm (in vitro)

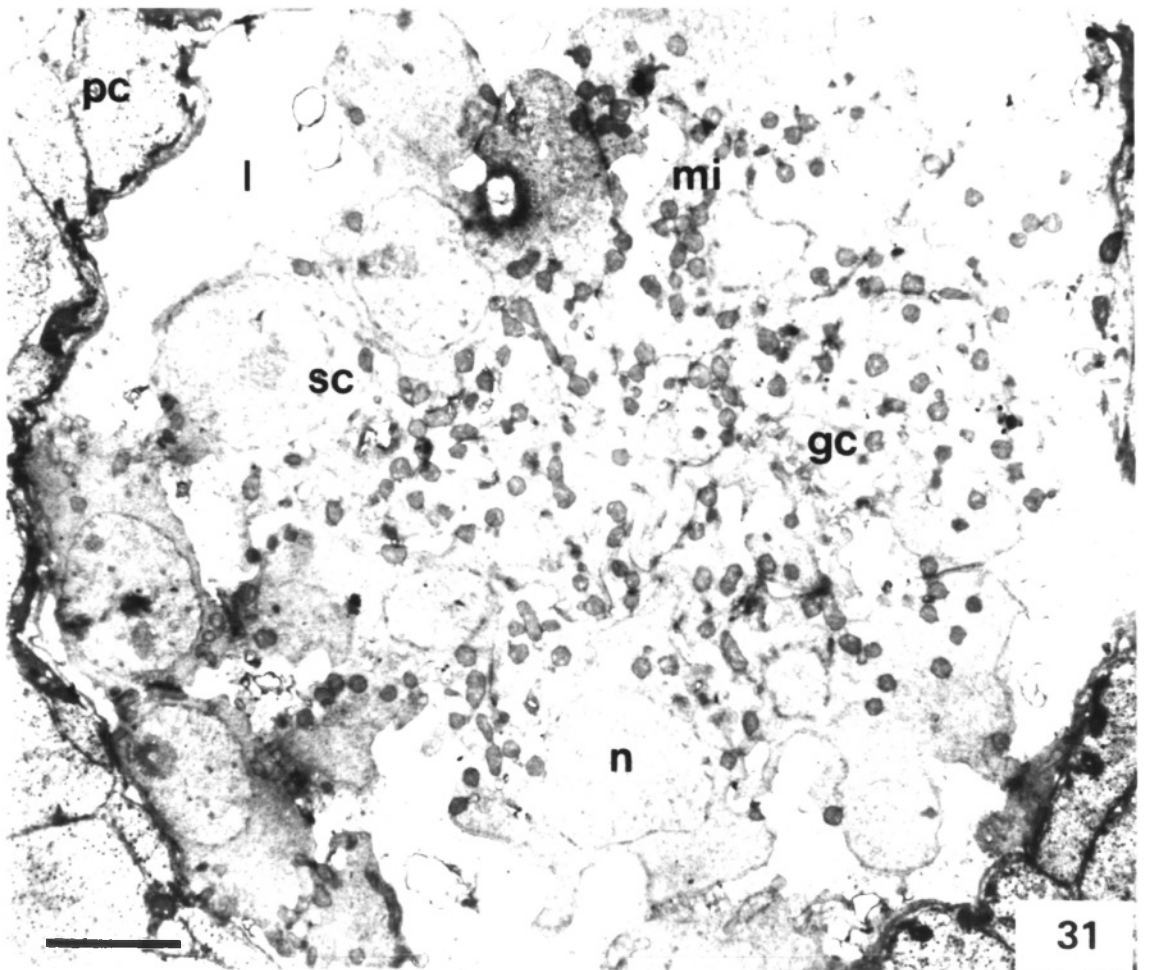
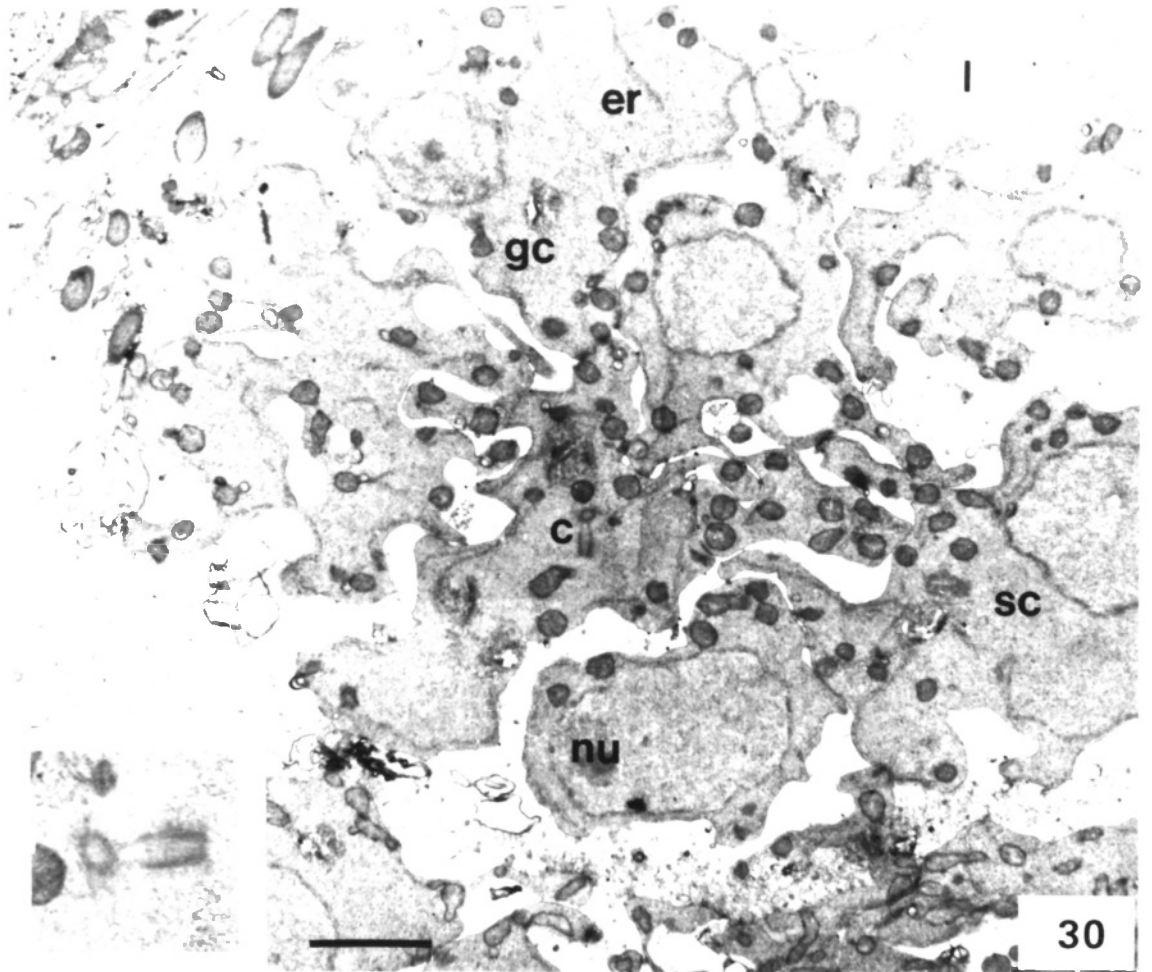


Plate 32

TEM spermatocytes, showing the central cytophore and cytoplasmic extensions.

Scale bar reps. $2\ \mu m$ (in vitro)

Plate 33

TEM testis, showing the residual body.

Scale bar reps. $2\ \mu m$ (in vitro)

Plate 34

TEM testis showing spermatids.

Scale bar reps. $5\ \mu m$ (in vitro)

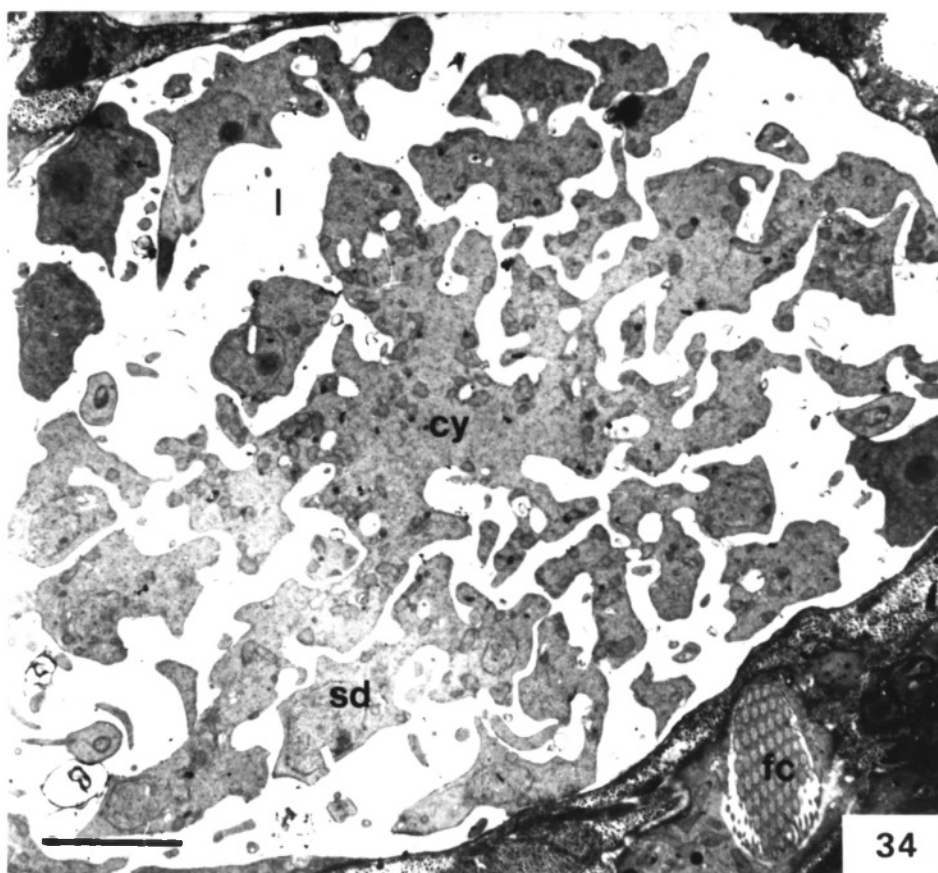
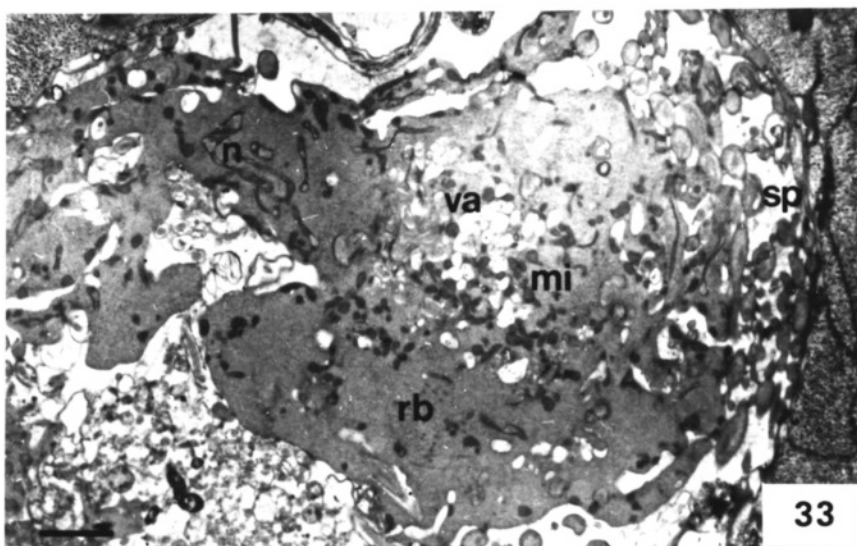
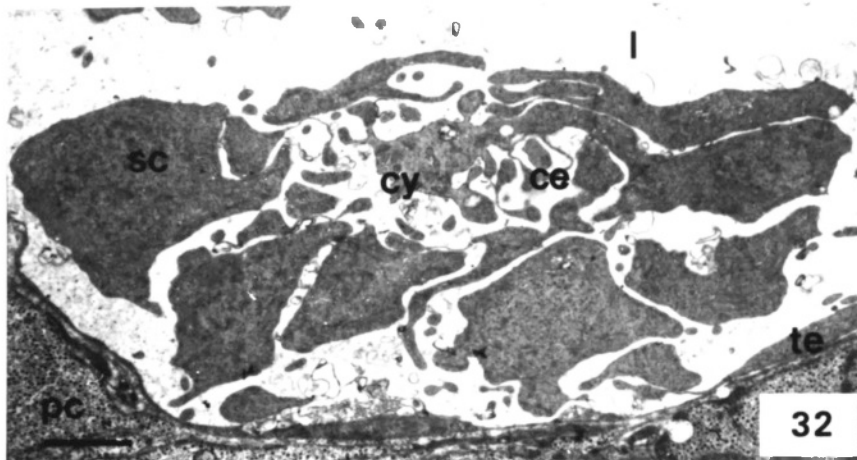


Plate 35

TEM spermatids. Note apical microtubules and nucleolus-like bodies in the cytoplasm (arrows).

Scale bar reps. $1\mu m$ (in vivo)

Plate 36

TEM spermatid, showing presence of a single centriole within a cytoplasmic knob.

Scale bar reps. $0.25\mu m$ (in vitro)

Plate 37

TEM of paired centrioles and associated rootlets during spermiogenesis.

Scale bar reps. $0.2\mu m$ (in vitro)

Plate 38

TEM "arching membranes" (arrows), showing the microtubules and endoplasmic reticulum.

Scale bar reps. $0.25\mu m$ (in vitro)

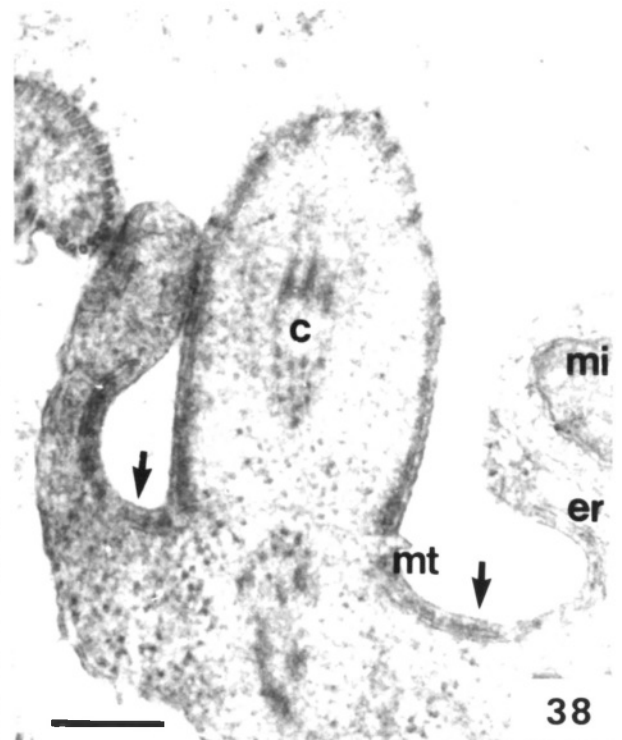
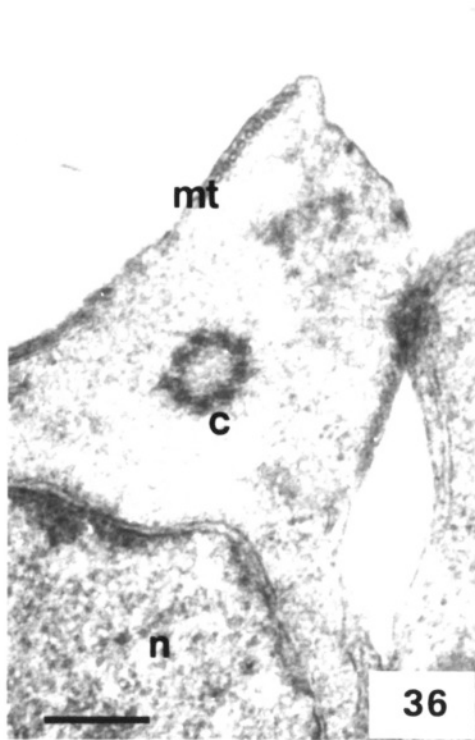
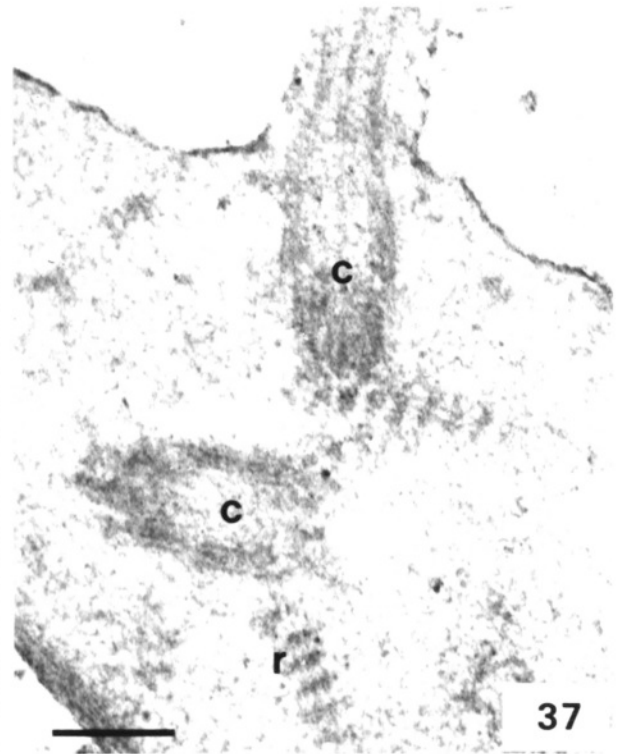
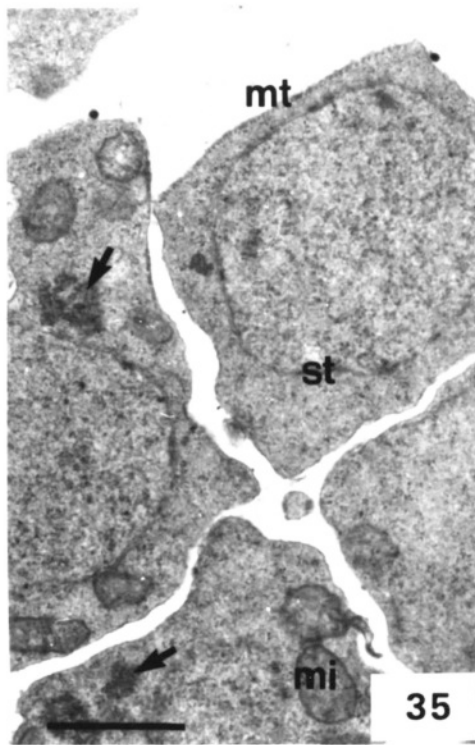


Plate 39

TEM spermiogenesis, showing the development of two axonemes from the paired basal bodies. Note the median cytoplasmic extension with peripheral microtubules.

Scale bar reps. $0.25\ \mu\text{m}$ (in vivo)

Plate 40

TEM elongating axoneme.

Scale bar reps. $0.5\ \mu\text{m}$ (in vivo)

Plate 41

TEM elongating axoneme; basal body of second axoneme apparent.

Scale bar reps. $0.5\ \mu\text{m}$ (in vivo)

Plate 42

TEM condensing spermatid nucleus.

Scale bar reps. $0.2\ \mu\text{m}$ (in vitro)

Plate 43

TEM late spermiogenesis, showing migration of the nucleus into the median cytoplasmic process (arrow).

Scale bar reps. $1\ \mu\text{m}$ (in vitro)

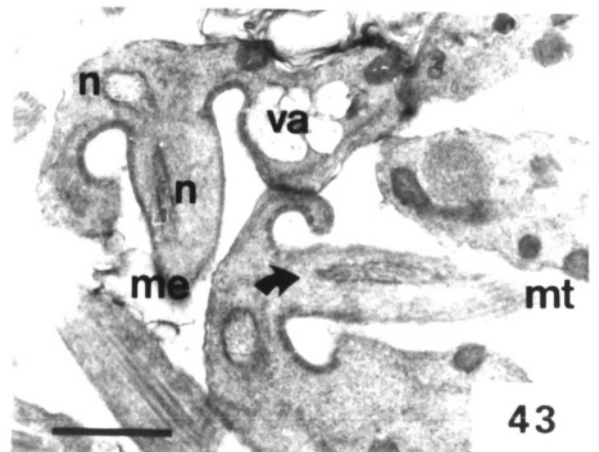
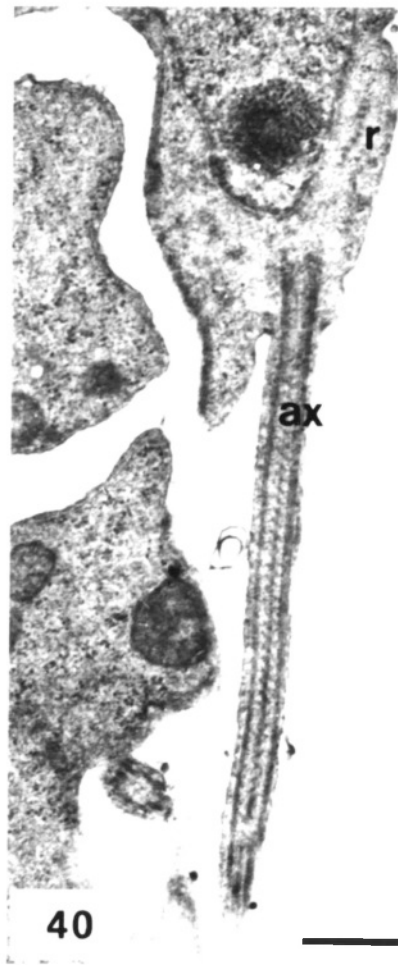
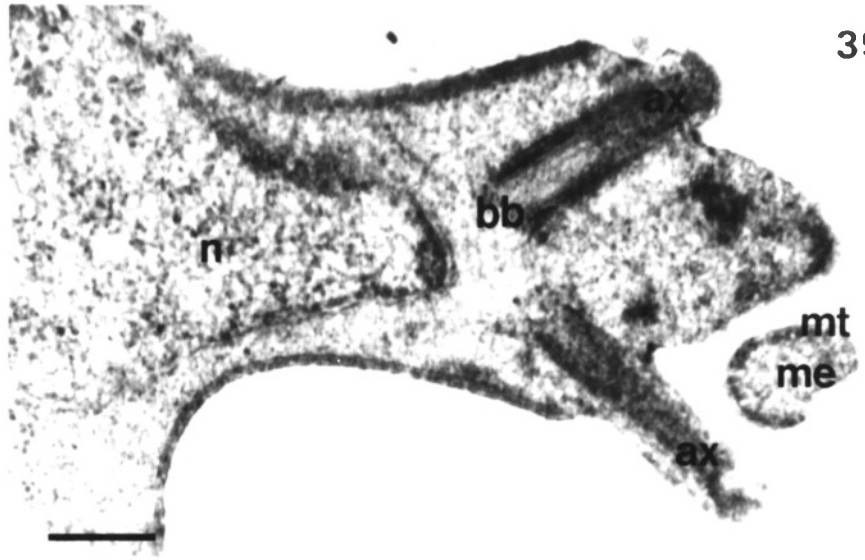


Plate 44

The process of spermiogenesis, as determined by cross sections of TEM (diagrammatic representation; the microtubules being designated by $\circ$, the axonemes by $///$, and the basal bodies by $//$).

H: shows the single axoneme (C) fused with the median cytoplasmic process (A).

I: shows the longitudinal growth of the cytoplasmic process, into which the axoneme is apparently guided.

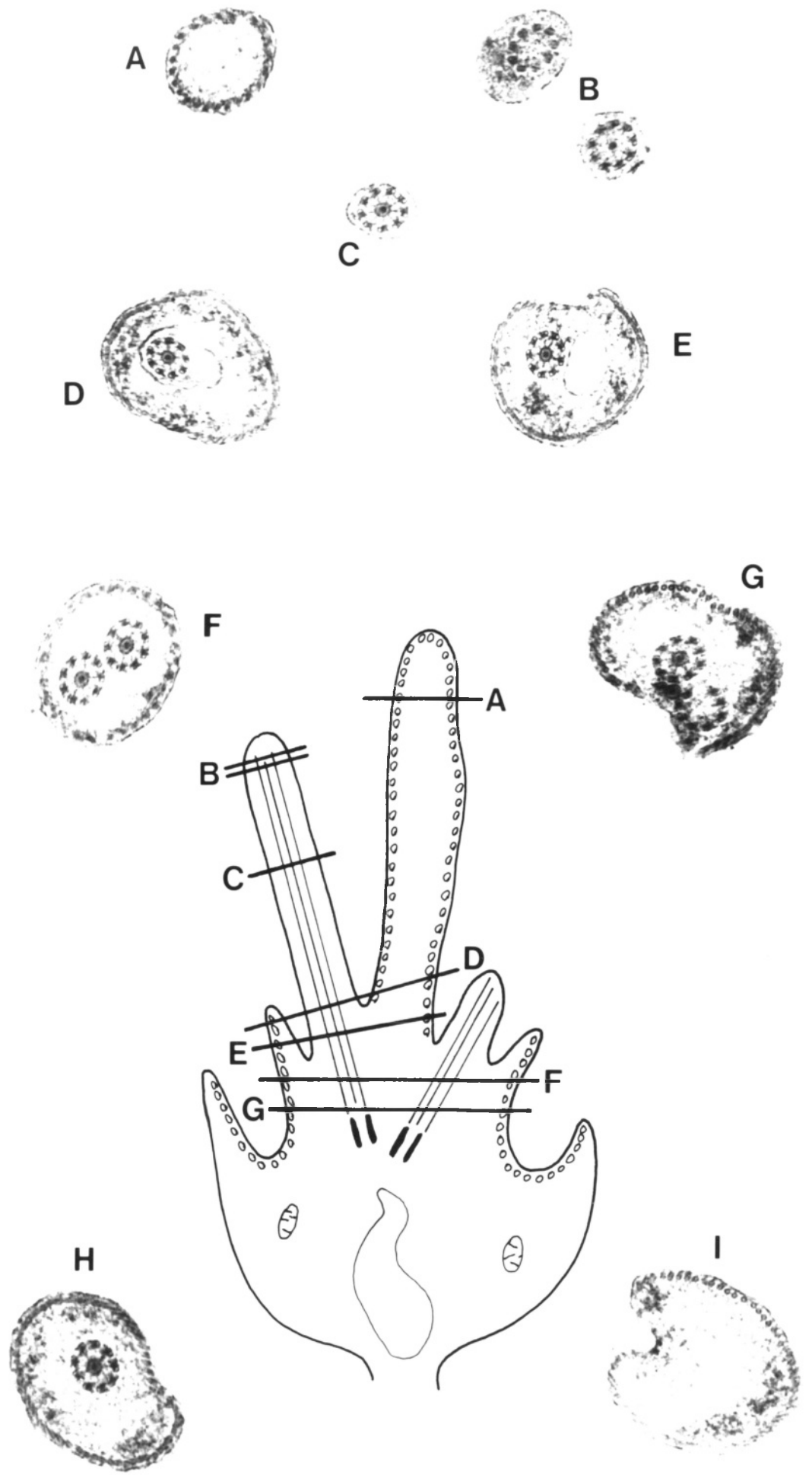


Plate 45

TEM sperm in testis showing the spiralled nucleus and intracellular β -glycogen particles.

Scale bar reps. $0.5 \mu m$ (in vitro)

Plate 46

TEM maturing sperm in close association with testis epithelium.

Scale bar reps. $0.5 \mu m$ (in vivo)

Plate 47

Mature testis showing sperm and residual body (fresh material; Neutral red).

Scale bar reps. $2 \mu m$ (in vitro)

Plate 48

SEM mature testis showing sperm.

Scale bar reps. $5 \mu m$ (in vivo)

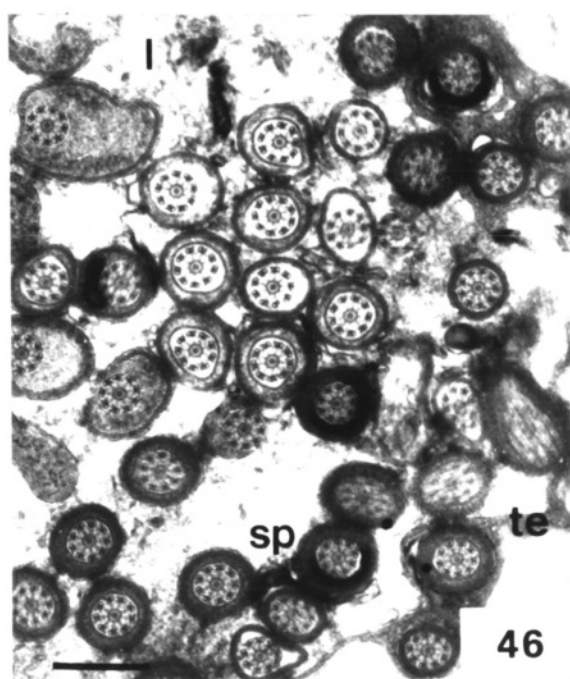
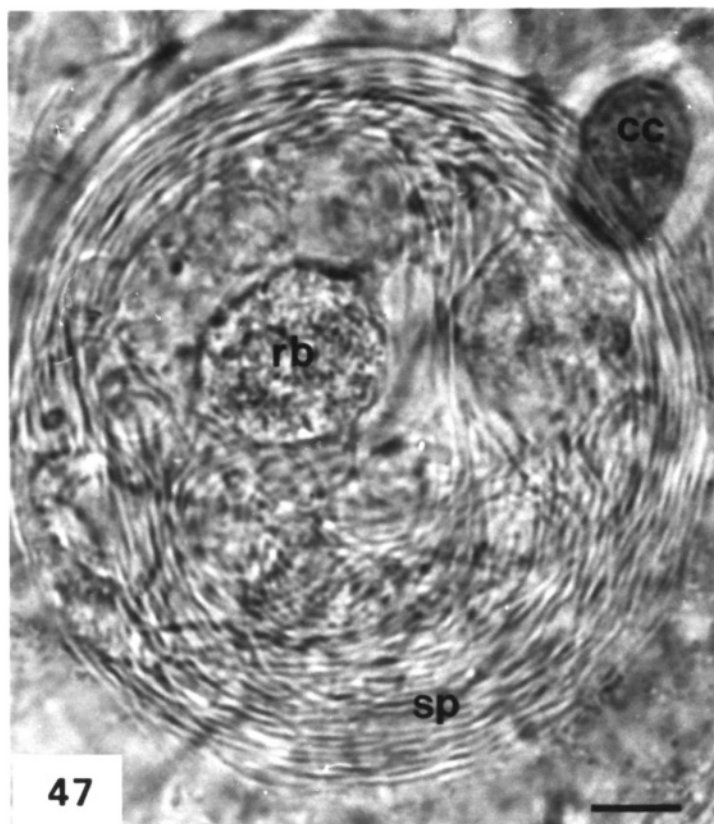
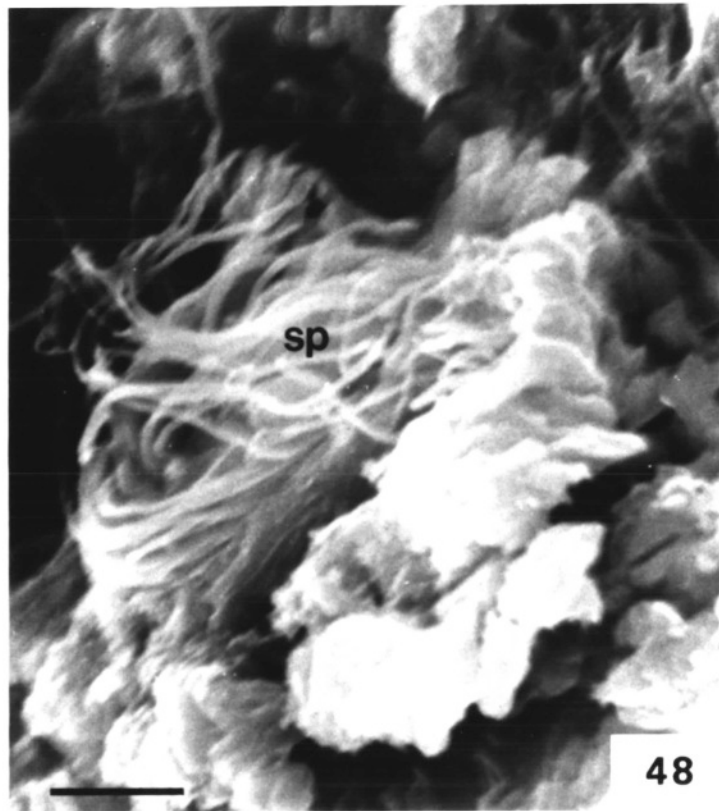
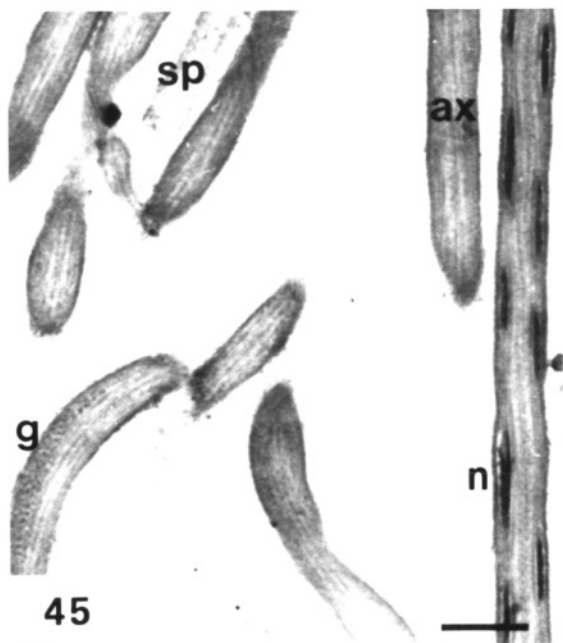


Plate 49Sperm ultrastructure
(refer to text)

The numbers relate to the order in which each of the structures are located; the unusual radial septa are shown in both cross, and longitudinal section.

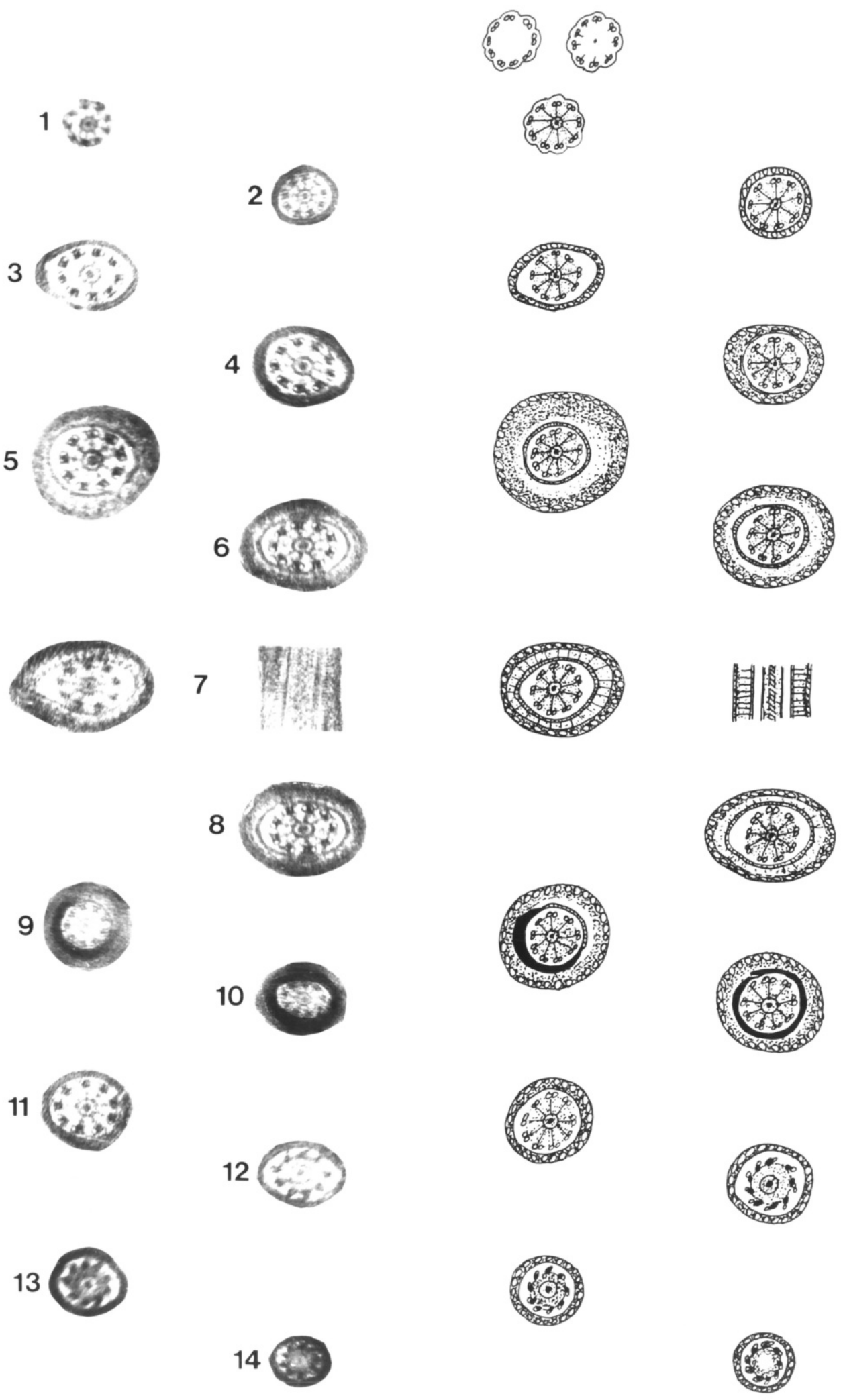


Plate 50

TEM degenerating testis.

Scale bar reps. 1 μm (in vivo)Plate 51

TEM vas efferens.

Scale bar reps. 1 μm (in vivo)Plate 52

TEM vas efferens, containing sperm.

Scale bar reps. 1 μm (in vitro)Plate 53Vas efferens containing coiled sperm
(fresh material; Neutral red).Scale bar reps. 2 μm (in vitro)

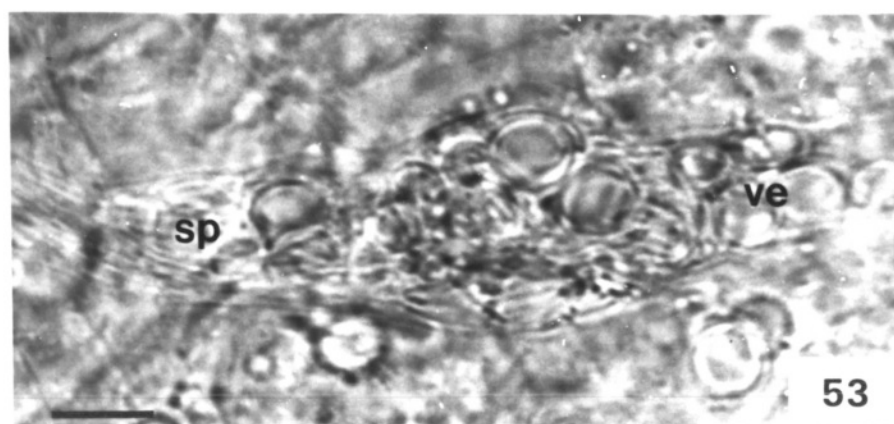
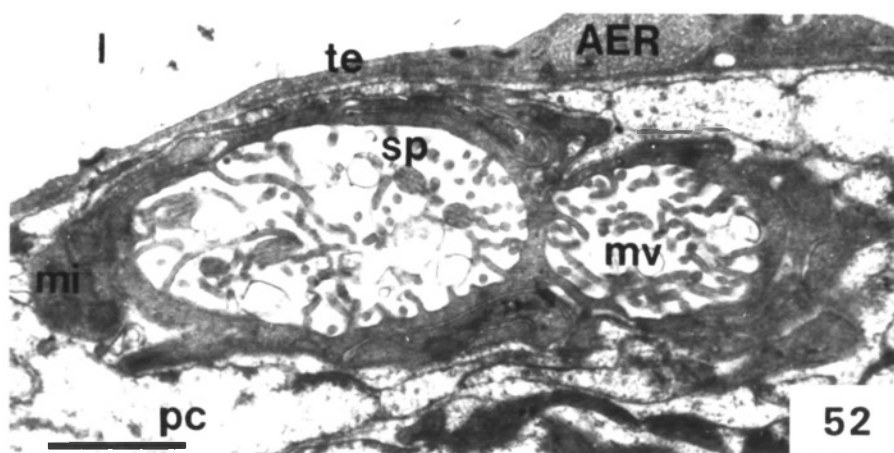
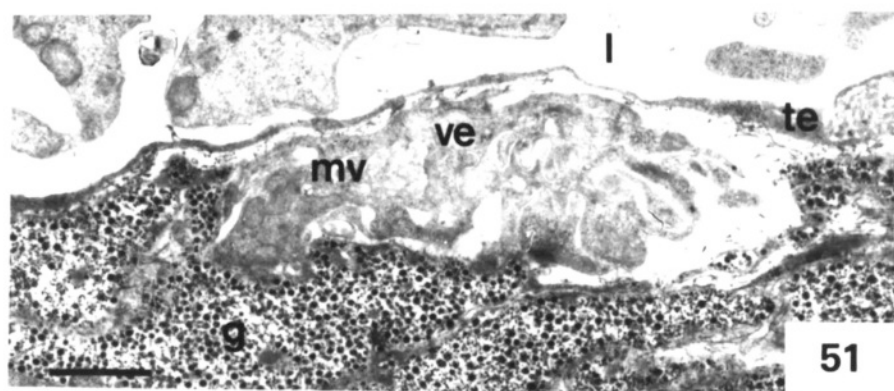
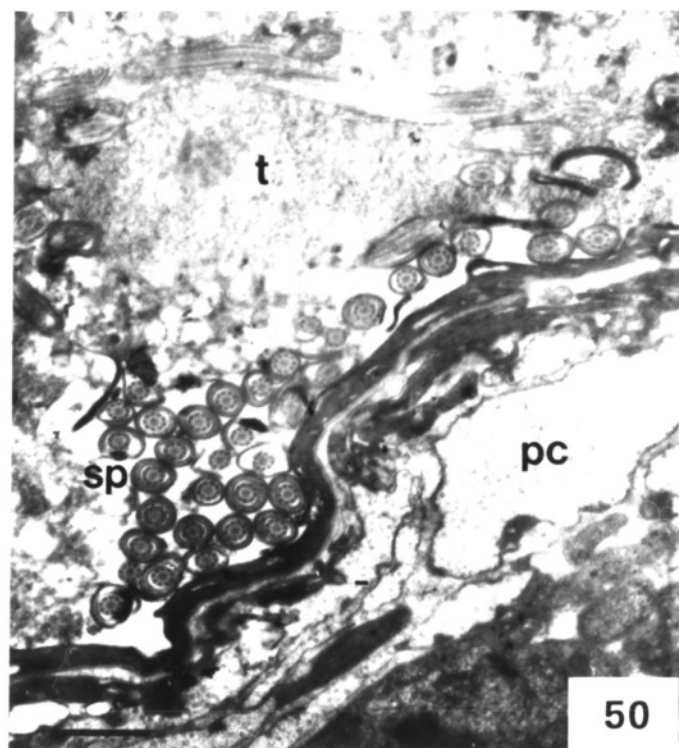


Plate 54

TEM cirrus sac showing the dense layer of circular muscles.

Scale bar reps. 5 μm (in vivo)

Plate 55

TEM initial part of cirrus duct, similar to the vas deferens, containing sperm.

Scale bar reps. 1 μm (in vivo)

Plate 56

TEM cirrus duct showing luminal, modified microtriches.

Scale bar reps. 1 μm (in vivo)

Plate 57

TEM cirrus showing the microtriches in the duct nearest to the genital pore.

Scale bar reps. 2 μm (in vivo)

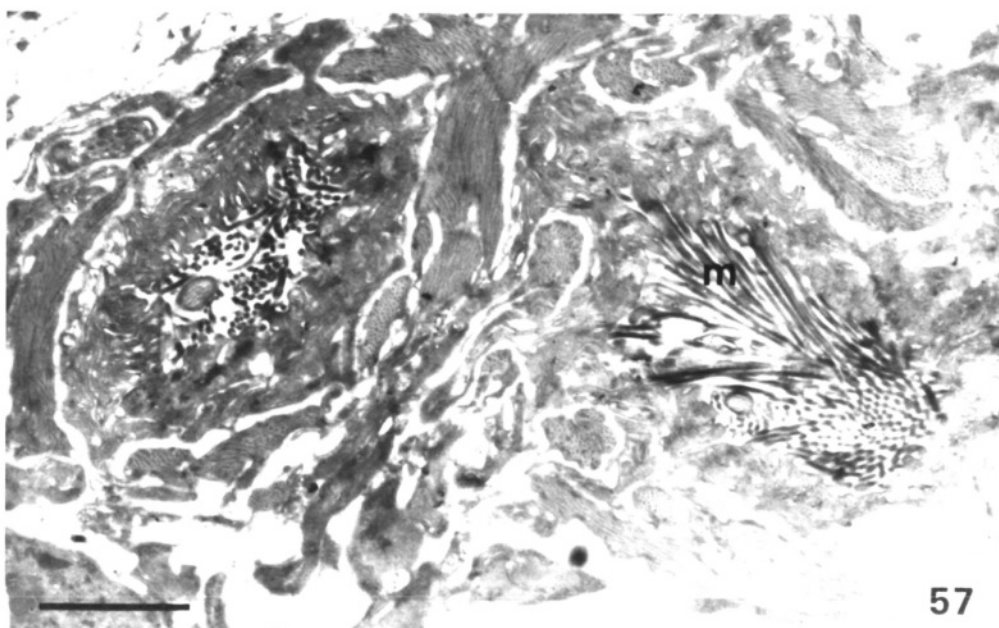
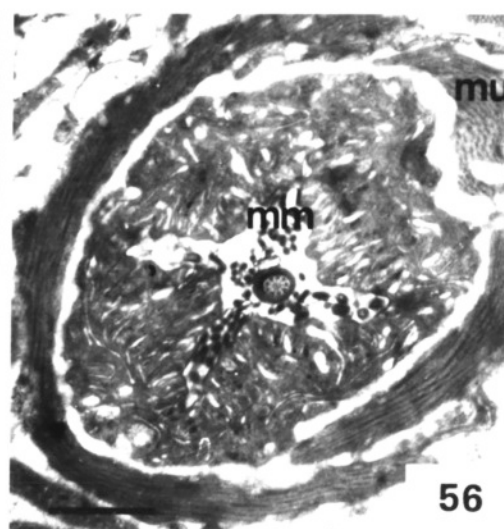
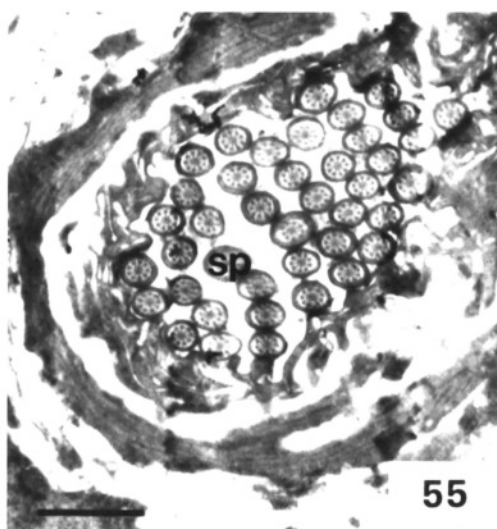
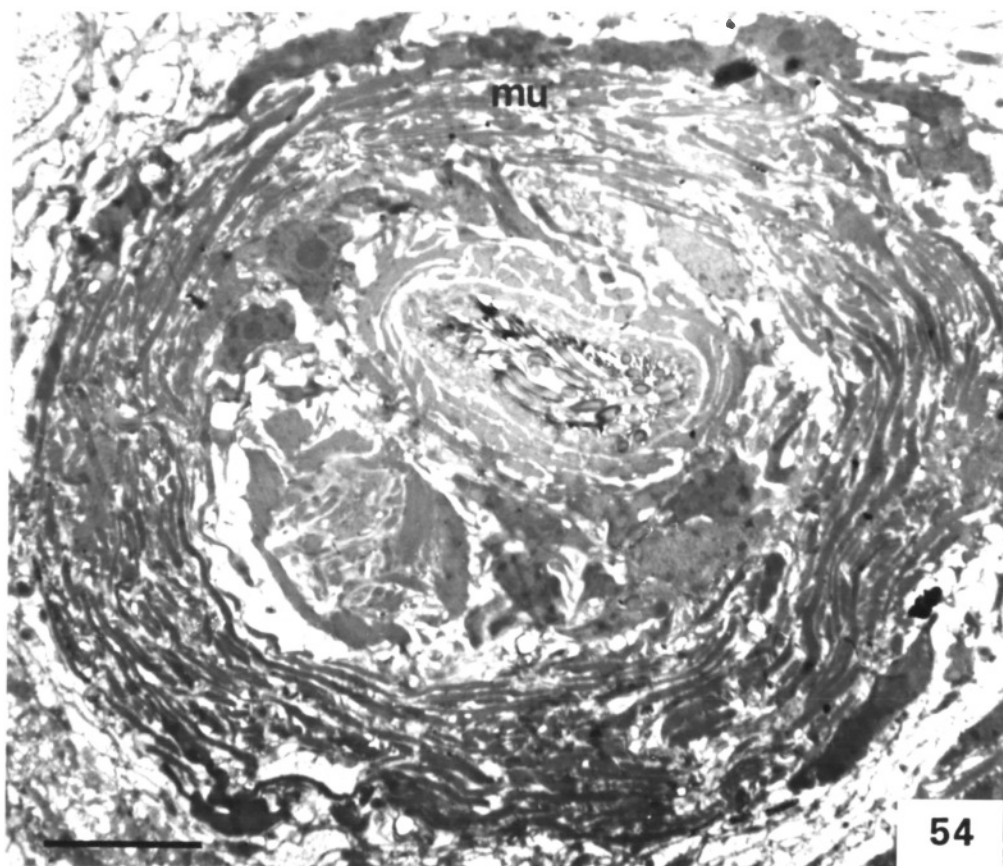


Plate 58

Cirrus protruding through genital pore
(fresh material; Neutral red).

Scale bar reps. 20 μm (in vitro)

Plate 59

Cultured worm flattened beneath a coverslip
showing the cirrus in the genital atrium, and
a bundle of sperm.

Scale bar reps. 5 μm (in vitro)

Plate 60

area around
SEM/genital pore.

Scale bar reps. 10 μm (in vivo)

Plate 61

SEM ~~area around~~ genital pore.

Scale bar reps. 10 μm (in vivo)

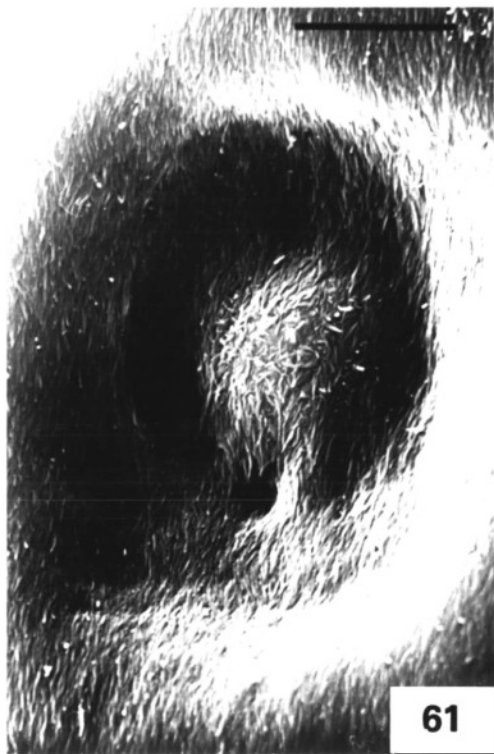
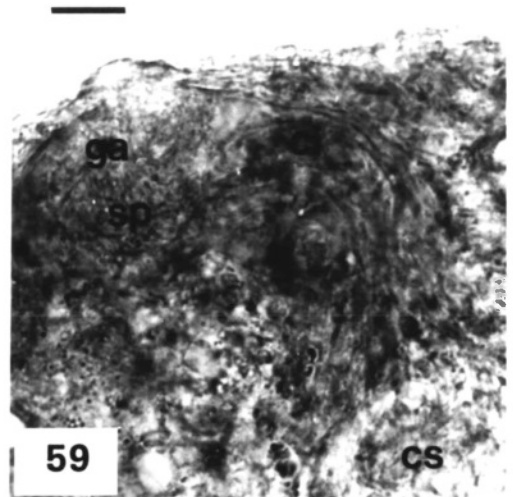
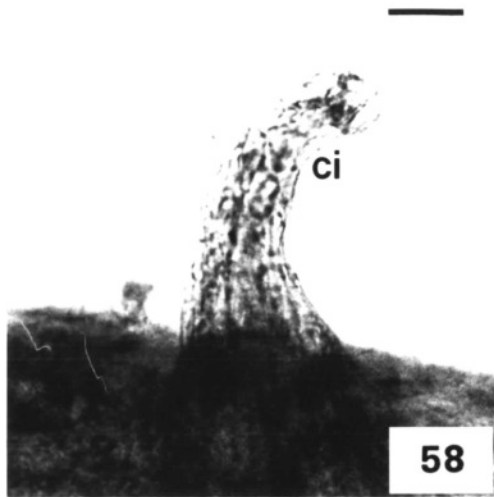


Plate 62

TEM genital atrium, showing the sperm surrounded by conglomerates of vesicles (arrows).

Scale bar reps. $0.5\ \mu m$ (in vivo)

Plate 63

Stained section showing the cirrus tip in the genital atrium, containing a mass of sperm (Haematoxylin and eosin).

Scale bar reps. $20\ \mu m$ (in vivo)

Plate 64

TEM genital atrium epithelium.

Scale bar reps. $2\ \mu m$ (in vivo)

Plate 65

Sperm movement in the genital atrium. (Fresh material; Neutral red).

Scale bar reps. $5\ \mu m$ (in vitro)

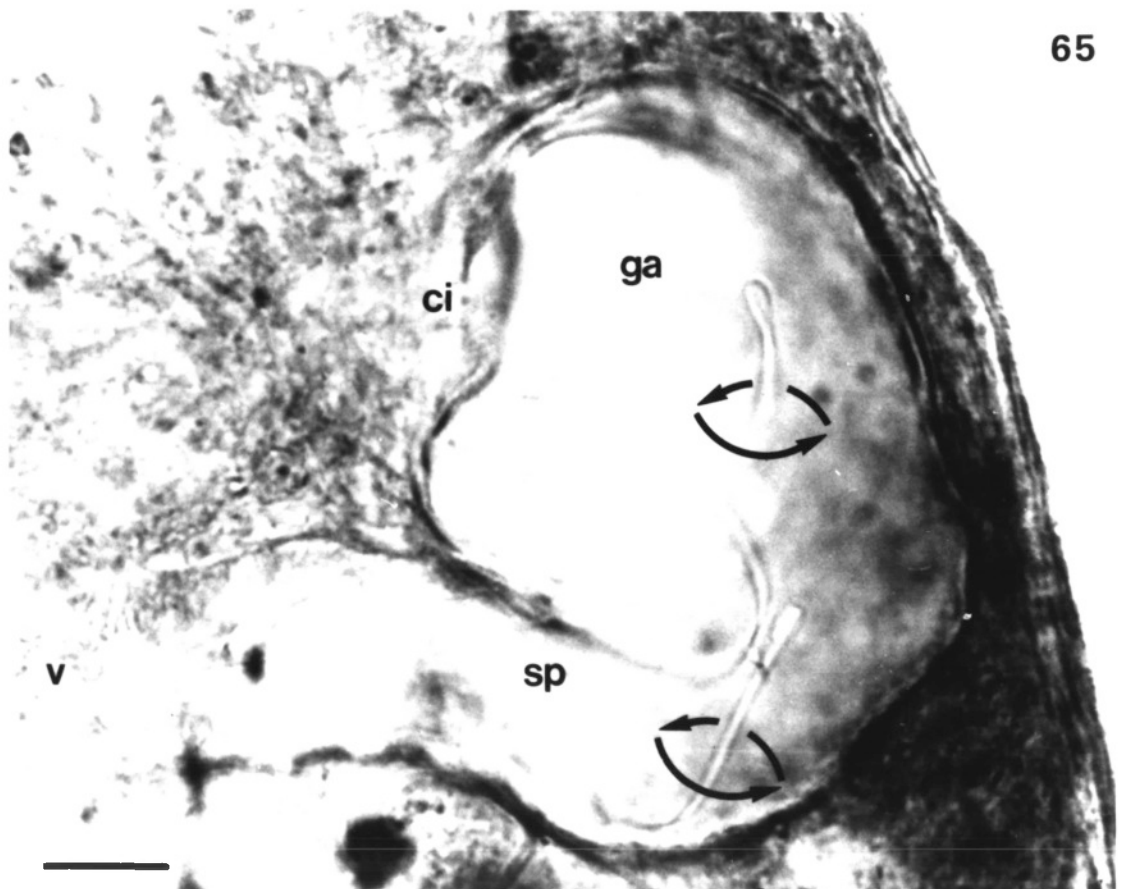
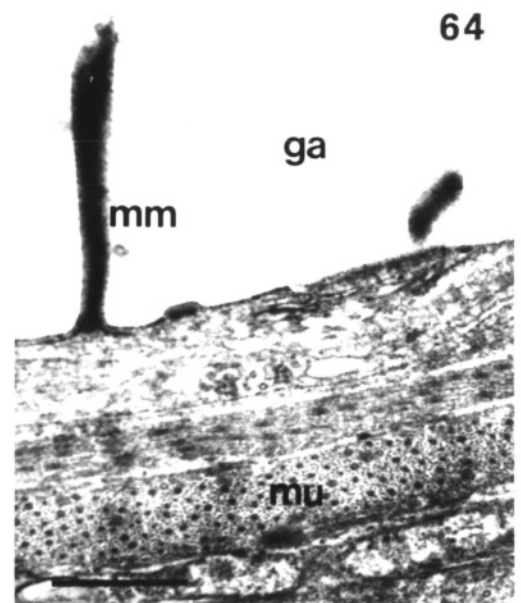
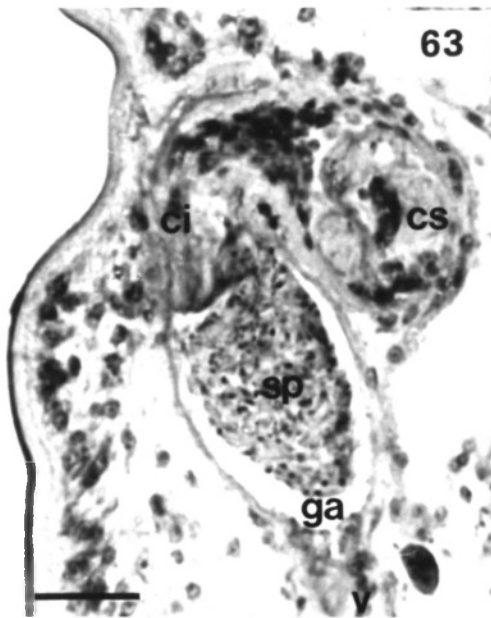
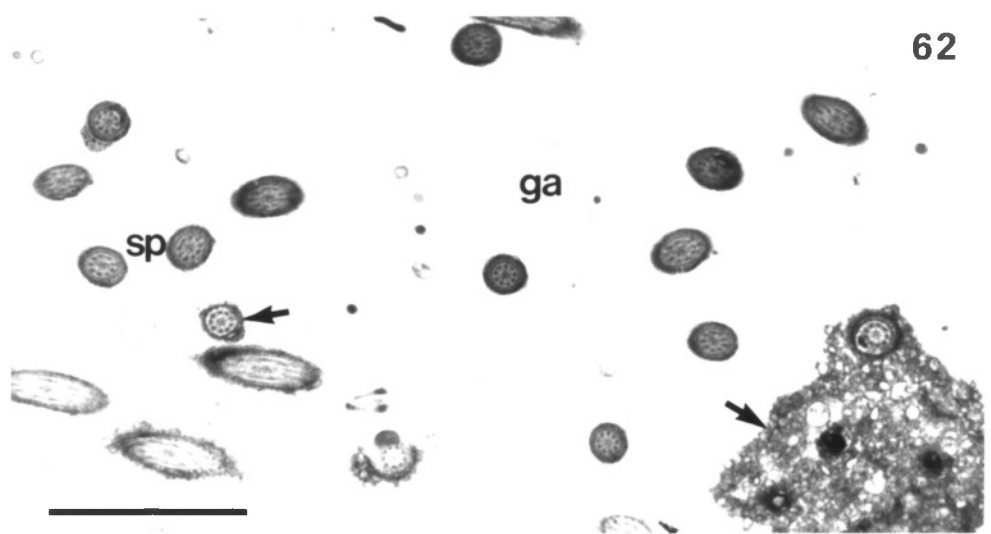


Plate 66

TEM vagina, anterior part.

Scale bar reps. 10 μm (in vitro)Plate 67

TEM vagina, showing the gradual inclusion of the nuclei within the distal cytoplasm.

Scale bar reps. 20 μm (in vitro)Plate 68

TEM vagina, lower part.

Scale bar reps. 2 μm (in vitro)

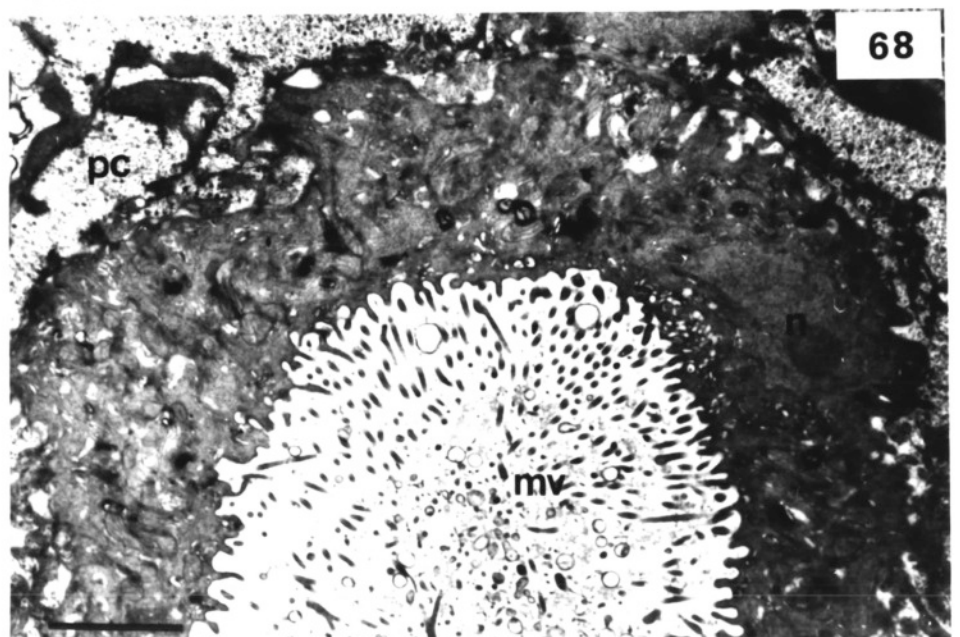
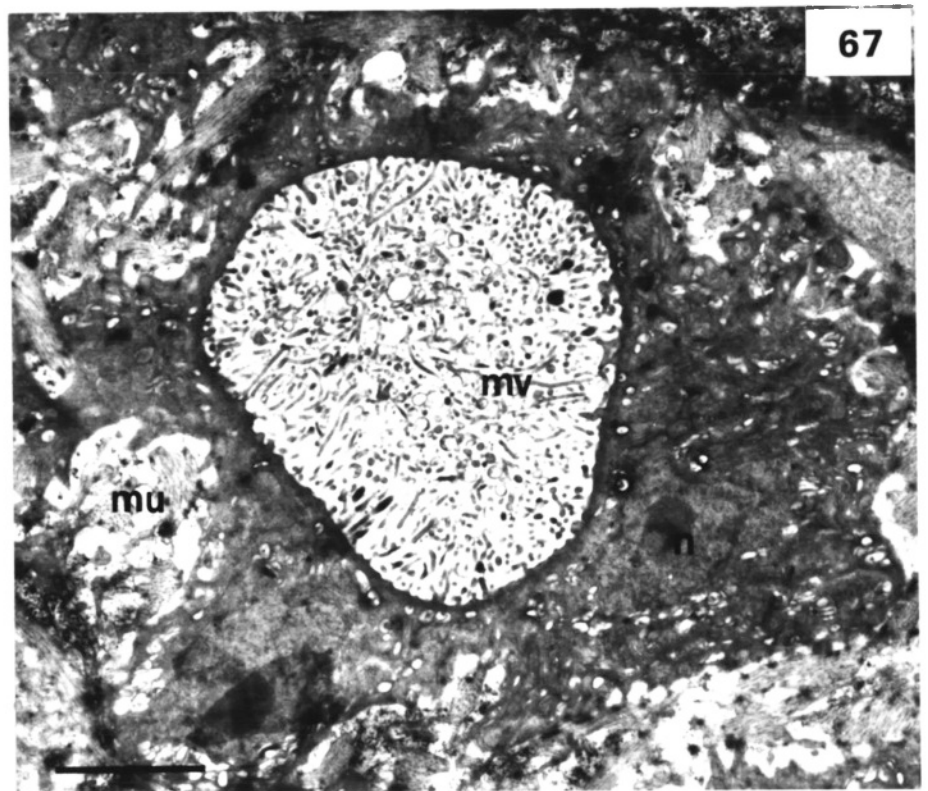
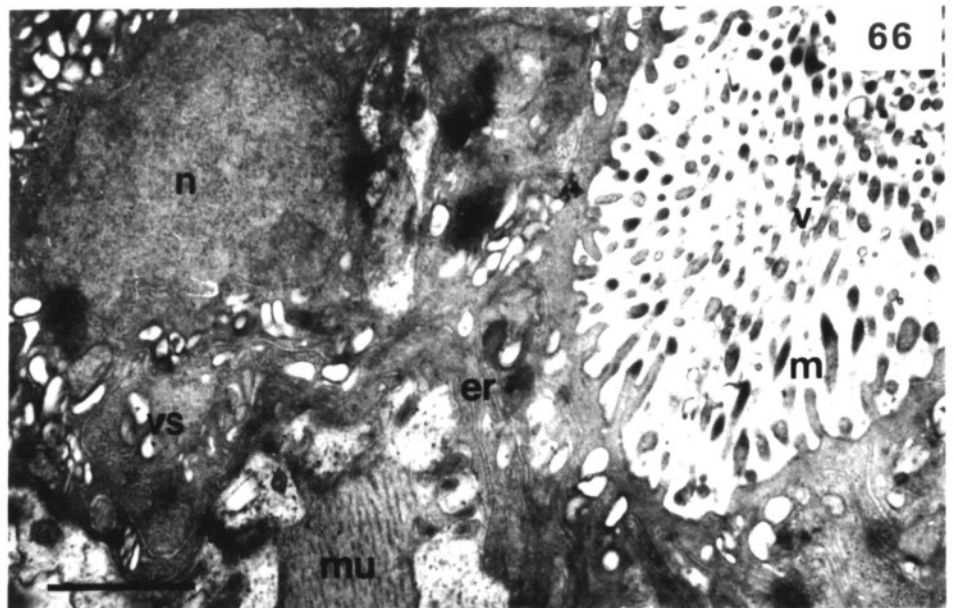


Plate 69

Section of a mature segment showing intensely stained vaginal lumen and cirrus (Heidenhain's iron-haematoxylin).

Scale bar reps. $20\ \mu m$ (in vivo)

Plate 70

TEM receptaculum. Note epithelial extensions, microvilli and lum~~e~~nal vesicles.

Scale bar reps. $2\ \mu m$ (in vitro)

Plate 71

TEM posterior of receptaculum; the lower duct leads to the ootype.

Scale bar reps. $2\ \mu m$ (in vitro)

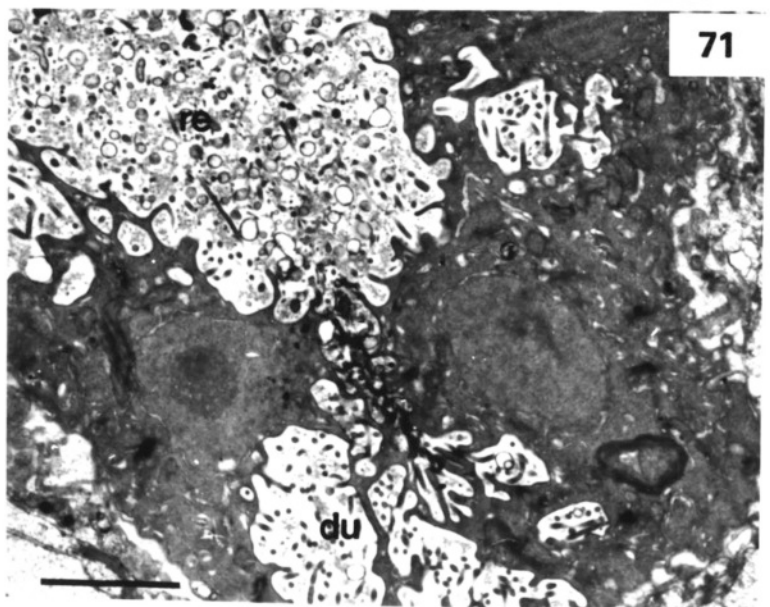
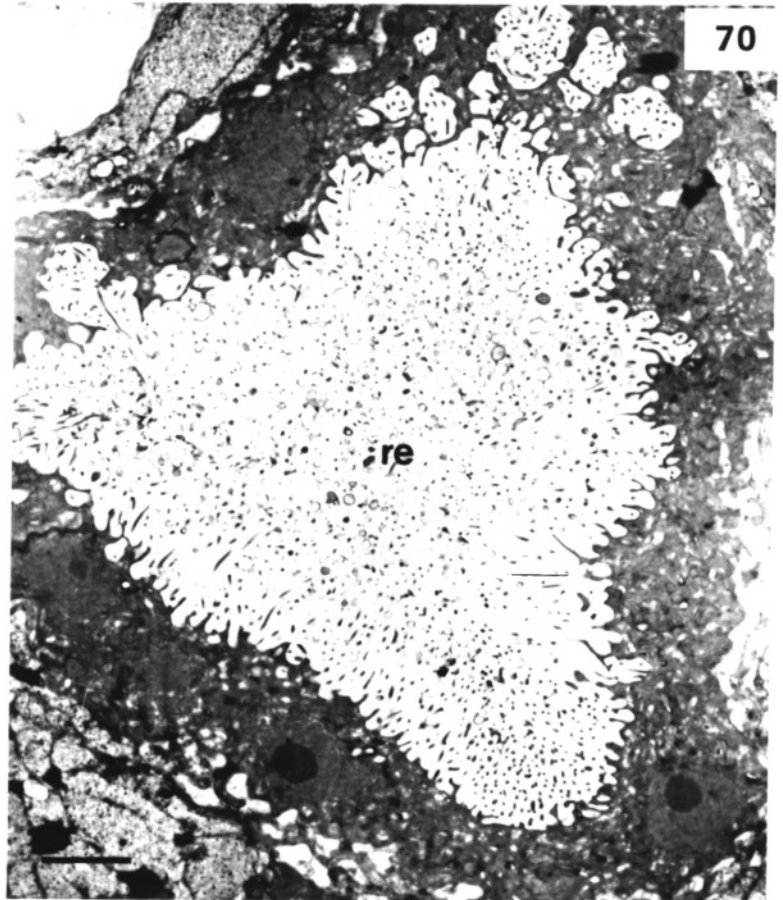
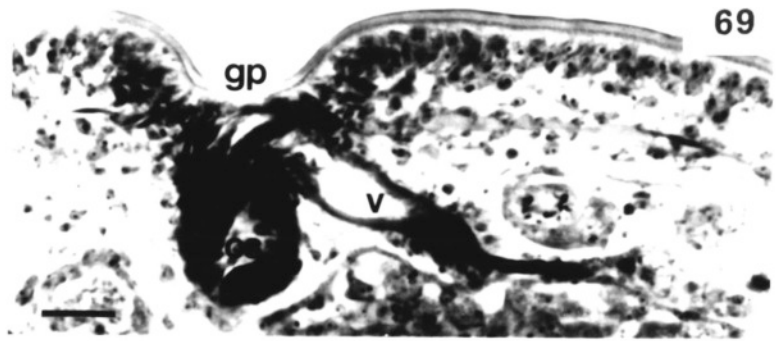


Plate 72

Ovary of fresh, unstained mature worm.
Note the rounded oocytes with large nucleus
and prominent nucleolus.

Scale bar reps. 5 μm (in vitro)

Plate 73

Ovary in stained section showing the
nucleolus-like bodies (arrows) with the same
staining characteristics as the nucleoli
(Heidenhain's iron-haematoxylin)

Scale bar reps. 10 μm (in vitro)

Plate 74

TEM ovary. Note the large oocytes, and
smaller oogonia.

Scale bar reps. 2 μm (in vitro)

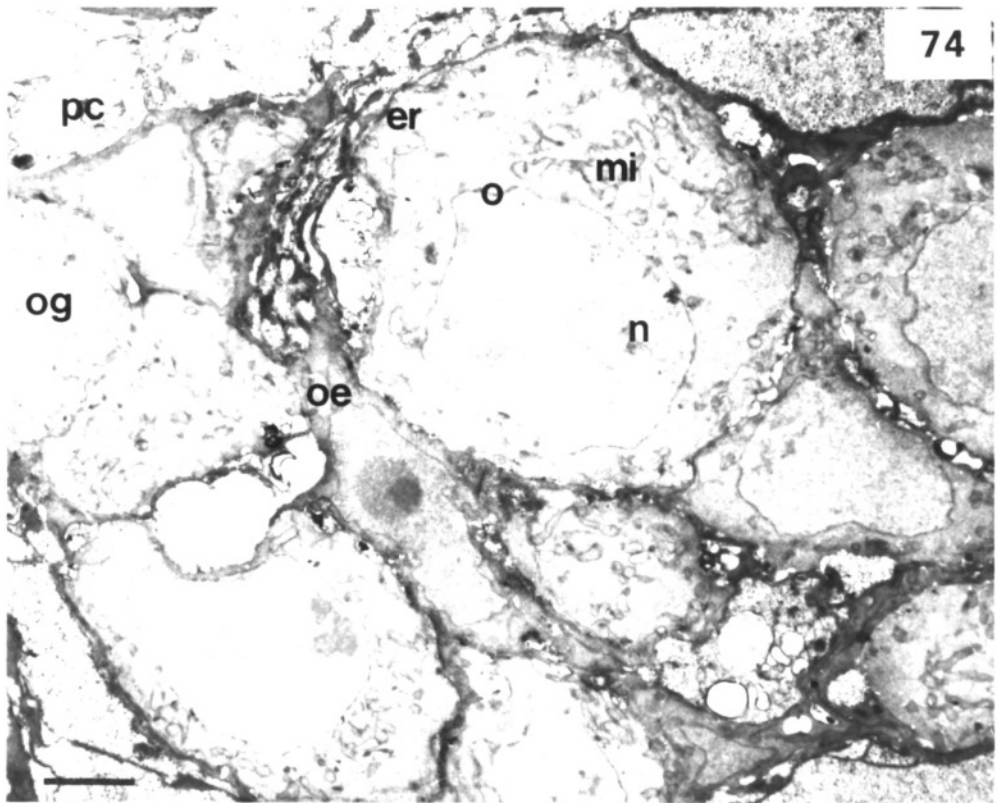
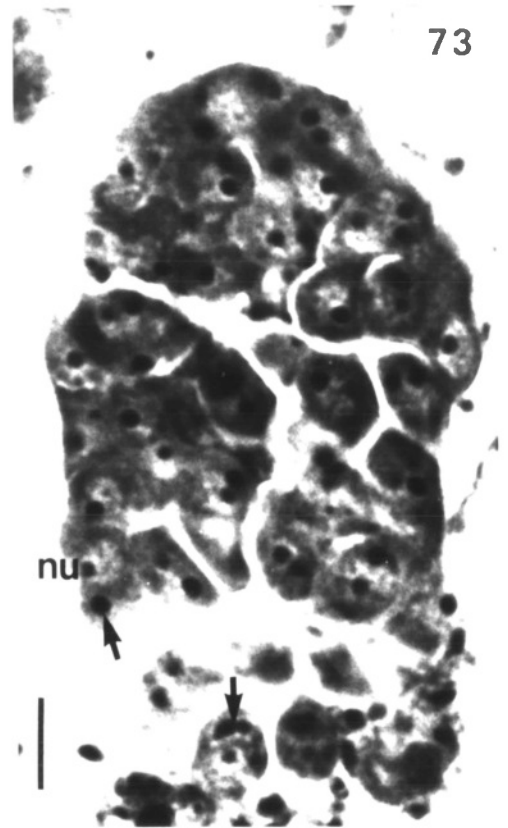
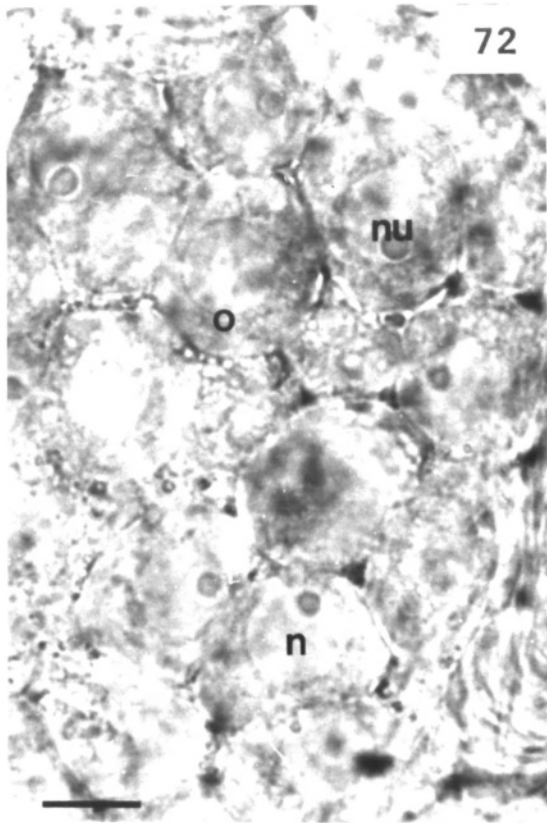


Plate 75

TEM oocyte, showing the nucleolus-like body (arrows).

Scale bar reps. 2 μm (in vitro)

Plate 76

TEM oocapt, with the ovary on the LHS and the oviduct on the RHS.

Scale bar reps. 5 μm (in vitro)

Plate 77

Section of a mature worm showing the intensely stained oviduct, vitelline duct, ootype, anterior duct and receptaculum epithelium (Heidenhain's iron-haematoxylin); the cirrus also shows affinity for the stain.

Scale bar reps. 20 μm (in vivo)

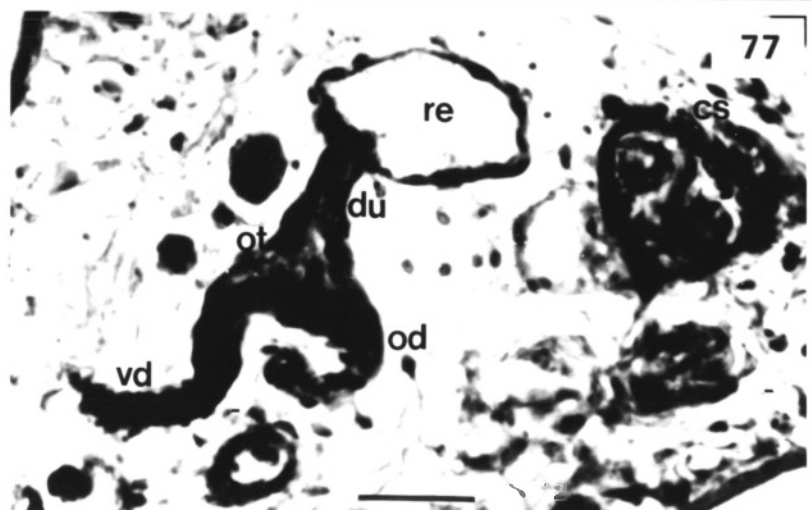
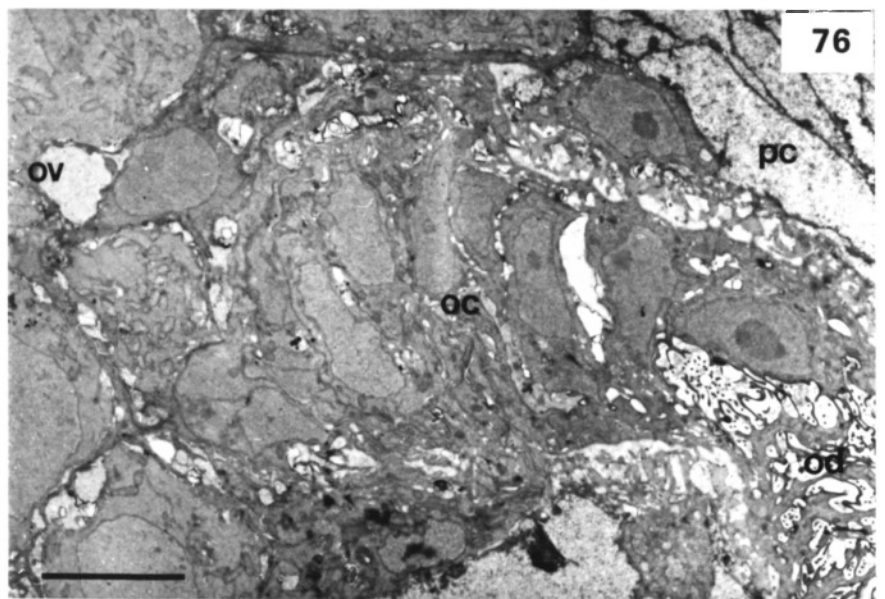
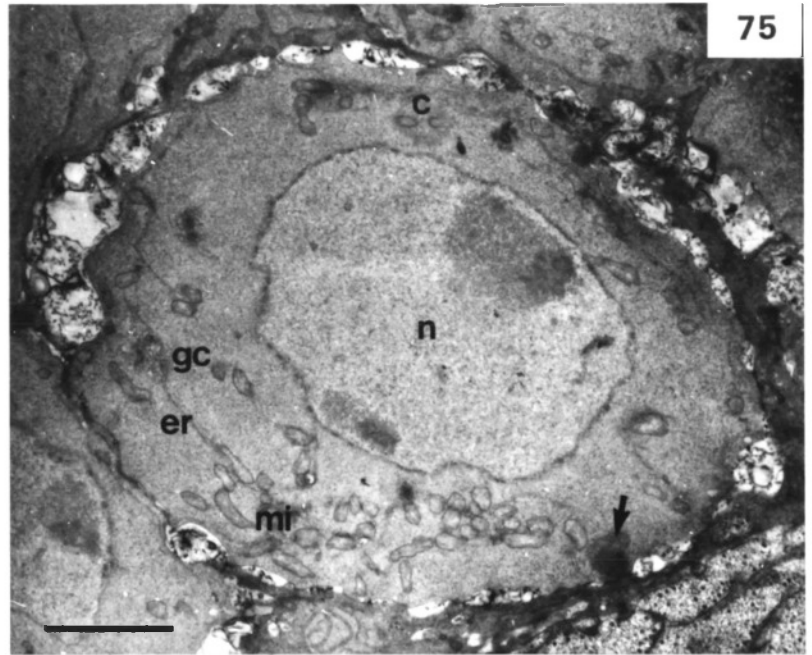


Plate 78

TEM oviduct (cross section), showing the extensive lum^enal cytoplasmic extensions and microvilli.

Scale bar reps. 2 μ m (in vitro)

Plate 79

TEM oviduct (longitudinal section).

Scale bar reps. 2 μ m (in vitro)

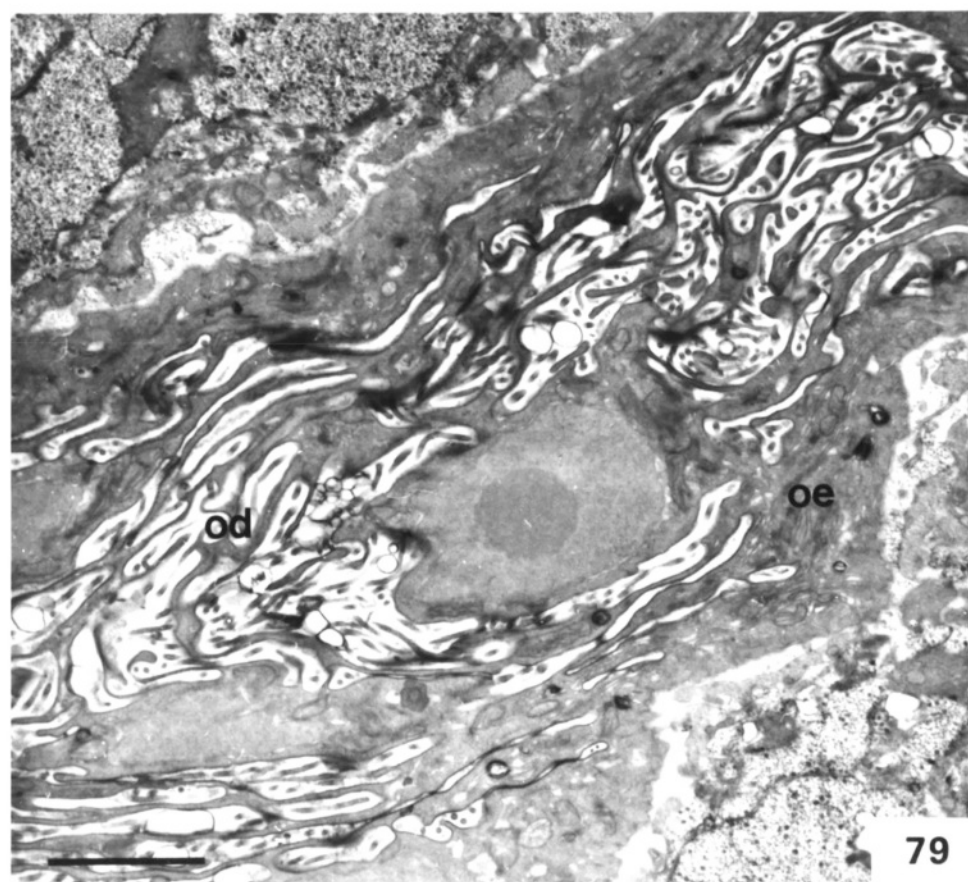
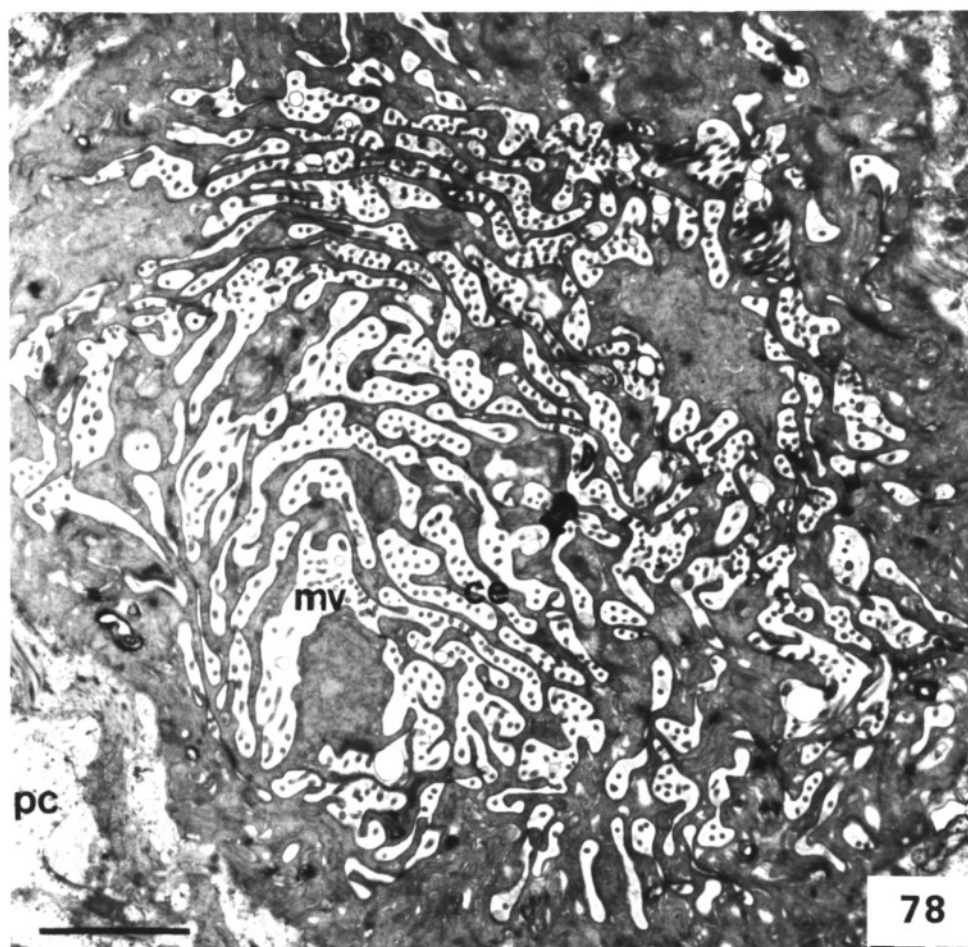


Plate 80

TEM latter part of the oviduct, showing a reduced number of luminal inclusions.

Scale bar reps. $2\ \mu m$ (in vitro)

Plate 81

TEM oviduct, next to the ootype. Note the presence of cilia, on the RHS, as found in the ootype.

Scale bar reps. $1\ \mu m$ (in vitro)

Plate 82

Vitelline gland; fresh material stained with Neutral red. Most cells are mature, with one large globule, although small vitelline granules can be seen (arrow).

Scale bar reps. $10\ \mu m$ (in vitro)

Plate 83

Vitelline gland in unstained fresh material. Mature cells contain a single globule, displacing other cell contents peripherally (arrow).

Scale bar reps. $10\ \mu m$ (in vitro)

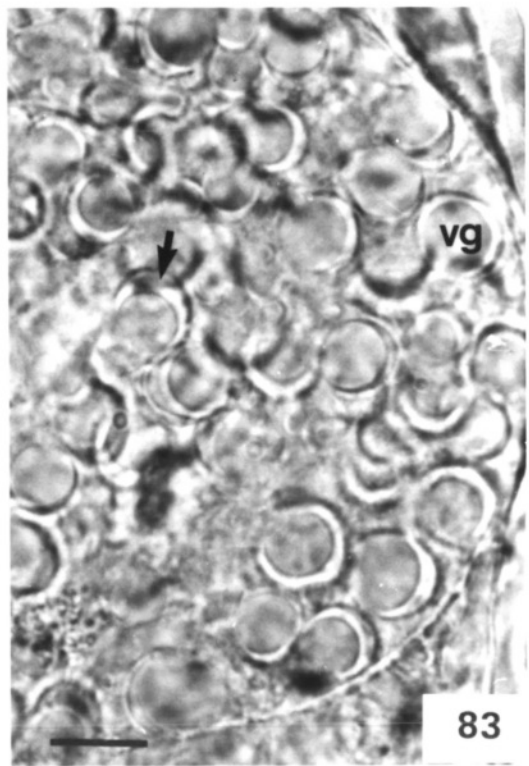
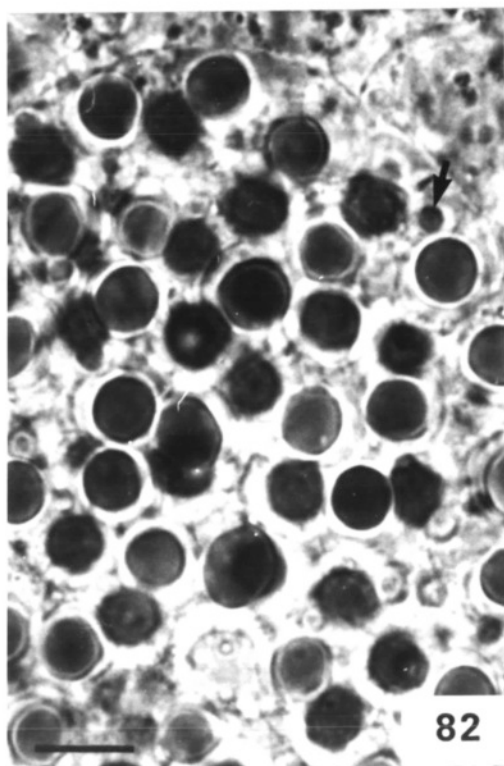
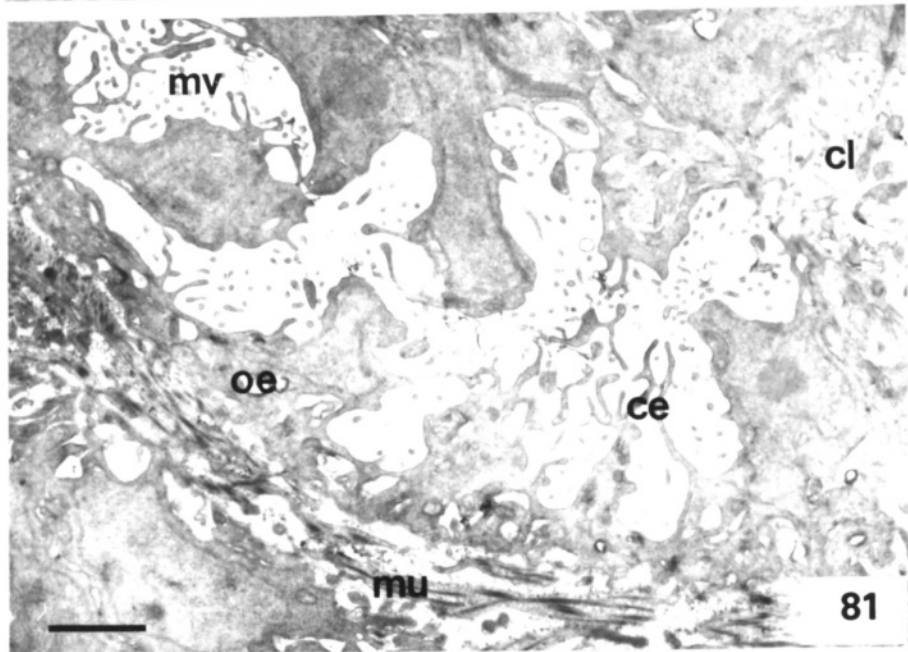
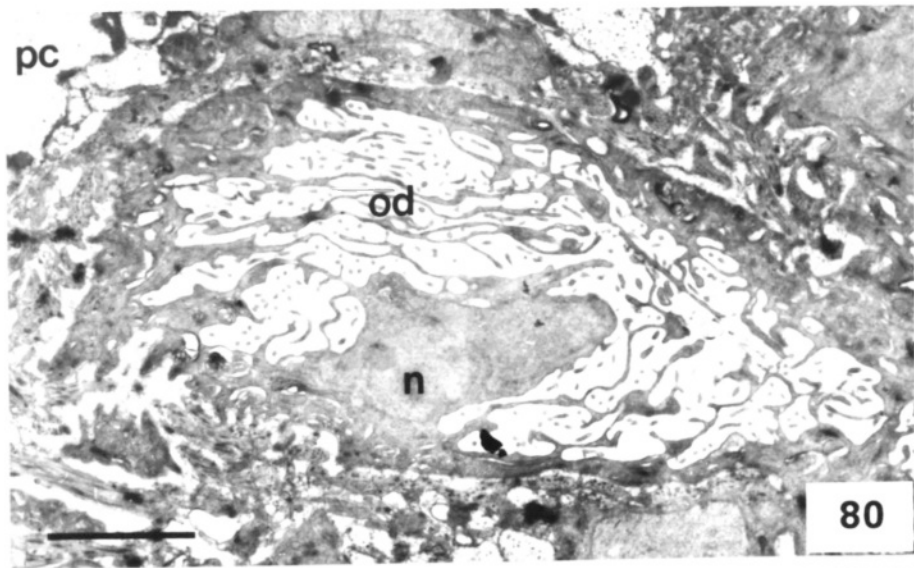


Plate 84

TEM vitelline gland showing cells in varying stages of maturation.

Scale bar reps. 5 μm (in vitro)

Plate 85

TEM maturing vitelline cell; small amount of vitelline material present.

Scale bar reps. 1 μm (in vitro)

Plate 86

TEM maturing vitelline cell with an increased number of vitelline granules, and the appearance of glycogen.

Scale bar reps. 1 μm (in vitro)

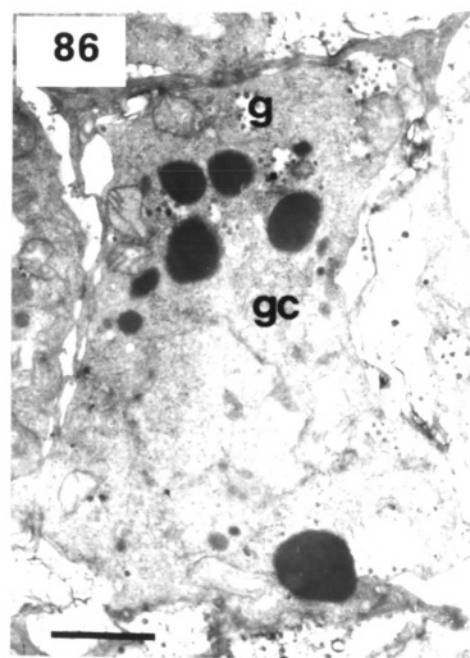
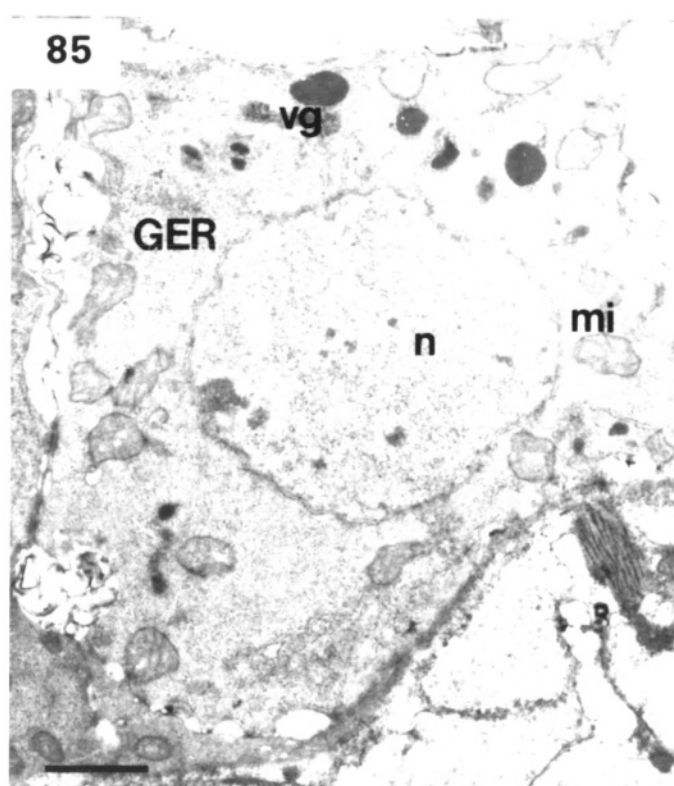
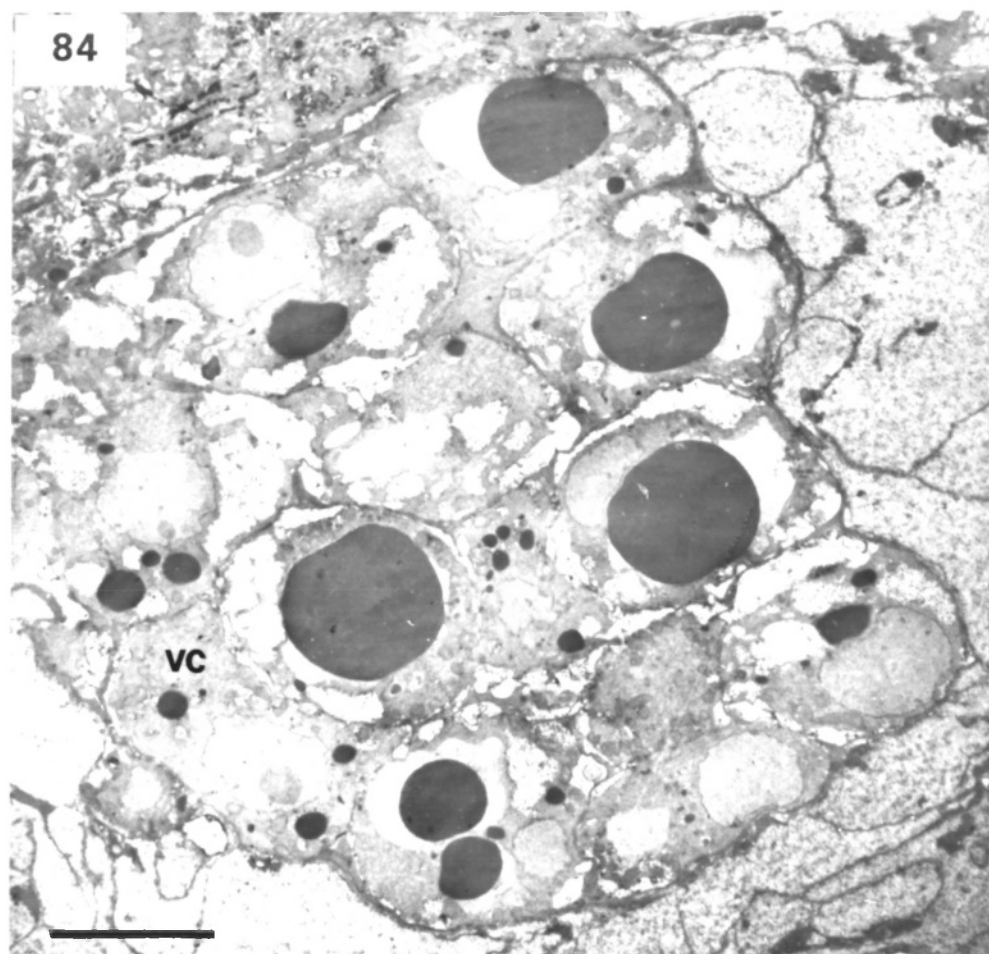


Plate 87

TEM maturing vitelline cell, containing fewer but larger, vitelline granules.

Scale bar reps. 1 μm (in vitro)

Plate 88

TEM mature vitelline cell, with a single globule and abundant glycogen.

Scale bar reps. 1 μm (in vitro)

Plate 89

TEM vitelline duct containing mature vitelline cells.

Scale bar reps. 2 μm (in vivo)

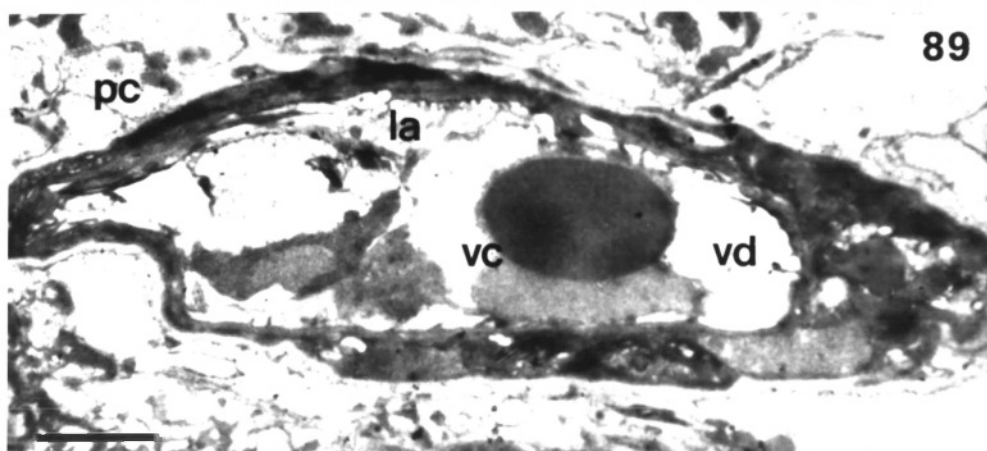
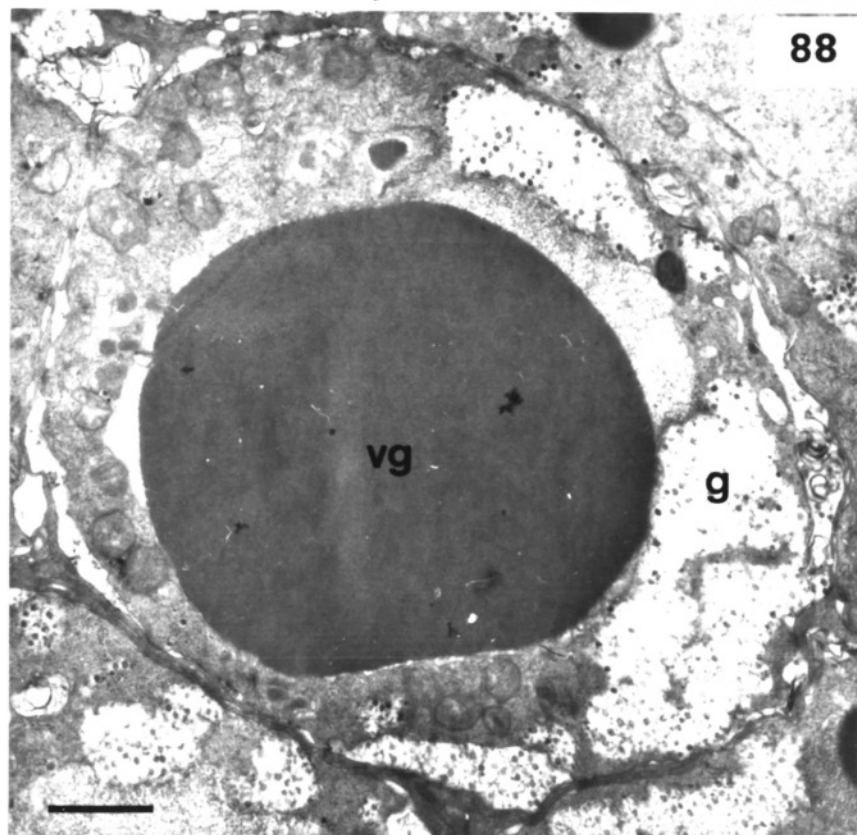
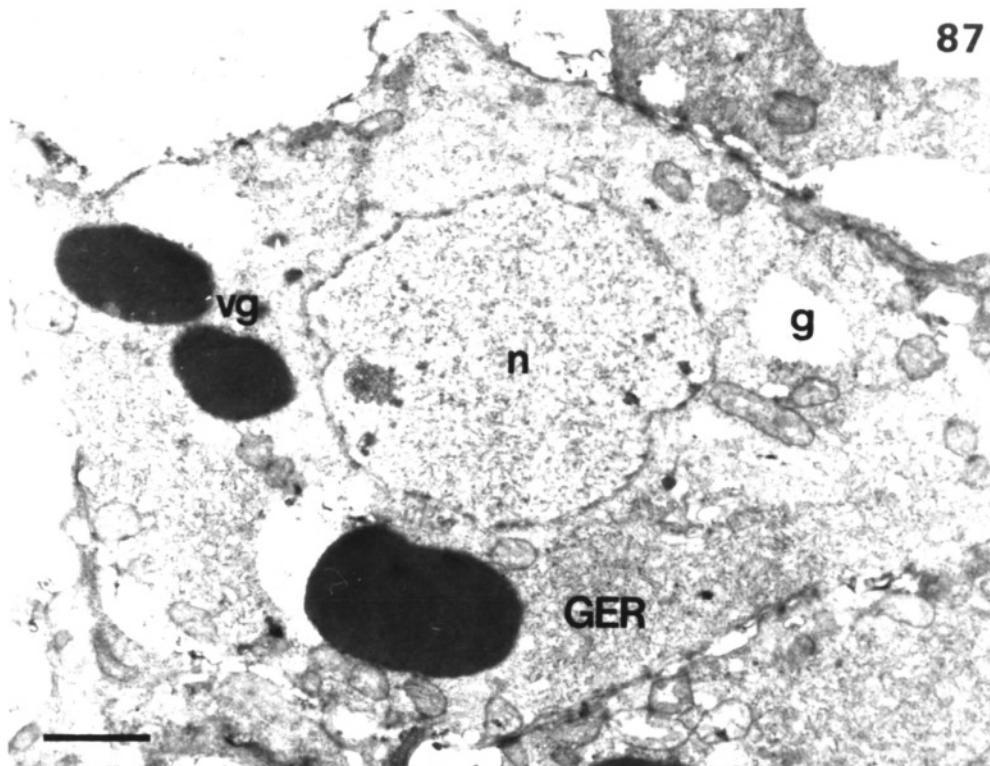


Plate 90

TEM ootype, showing luminal microvilli and cilia.

Scale bar reps. $2\mu m$ (in vitro)

Plate 91

TEM ootype and Mehlis' gland, showing the electron dense secretions.

Scale bar reps. $2\mu m$ (in vitro)

Plate 92

TEM ootype, showing the release of the Mehlis' gland secretions into the lumen (arrow)

Scale bar reps. $1\mu m$ (in vitro)

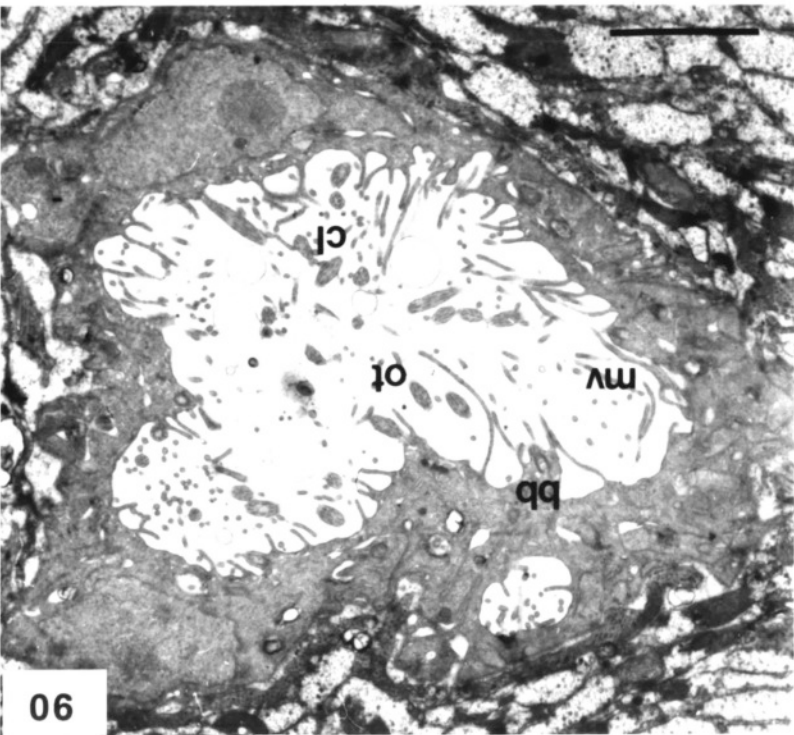
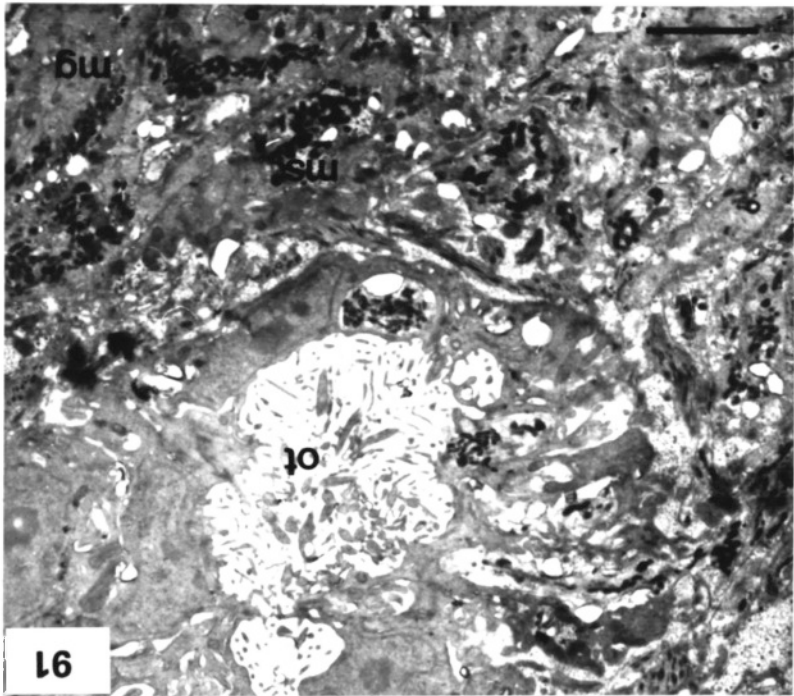
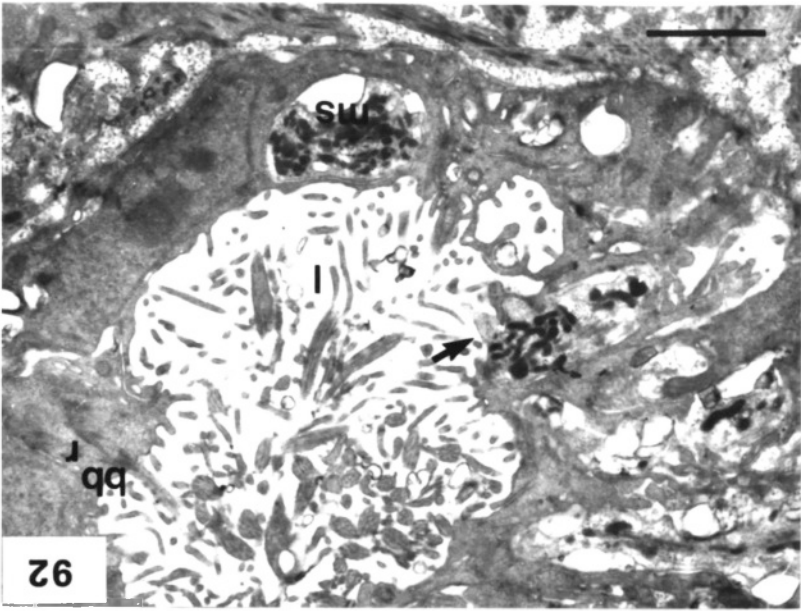


Plate 93

TEM uterus epithelium. Note the Mehlis' gland secretion and crystalline body in the lumen.

Scale bar reps. $0.5\ \mu m$ (in vivo)

Plate 94

Uterus of unstained, fresh material. Note the large oocytes and elongate crystals.

Scale bar reps. $5\ \mu m$ (in vitro)

Plate 95

Uterus of adult worm, containing oocytes and vitelline cells (Heidenhain's iron-haematoxylin).

Scale bar reps. $50\ \mu m$ (in vivo)

Plate 96

Degenerate cells in uterus of unstained, fresh material.

Scale bar reps. $10\ \mu m$ (in vitro)

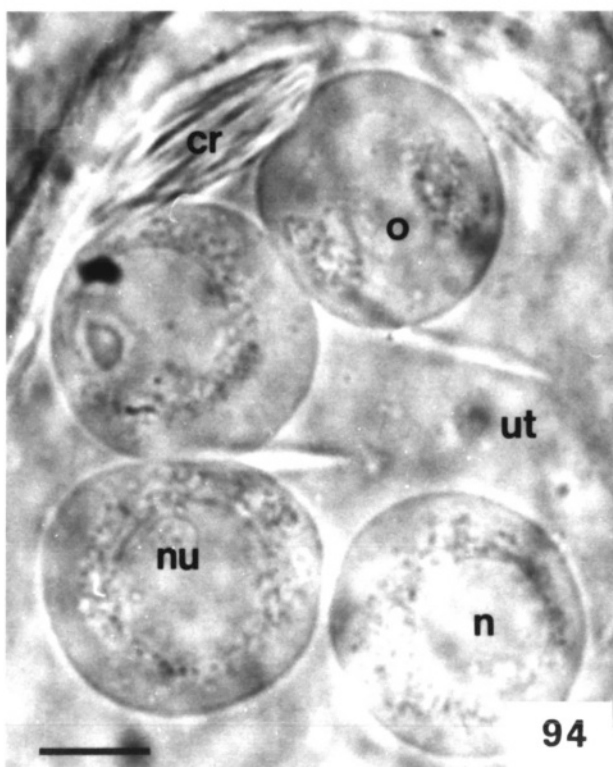
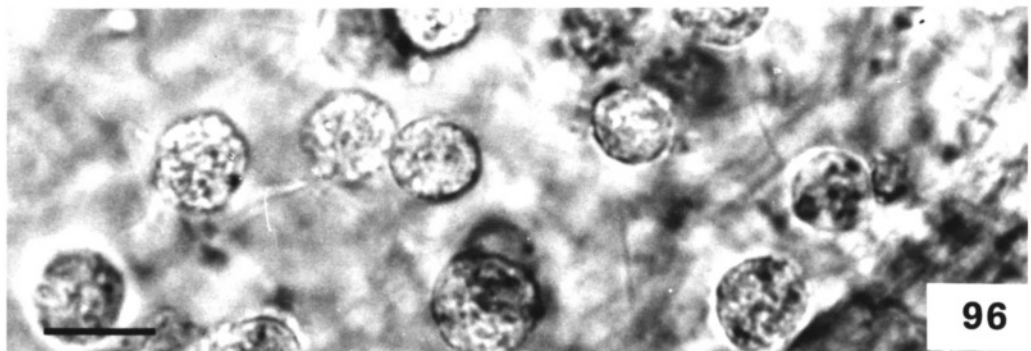
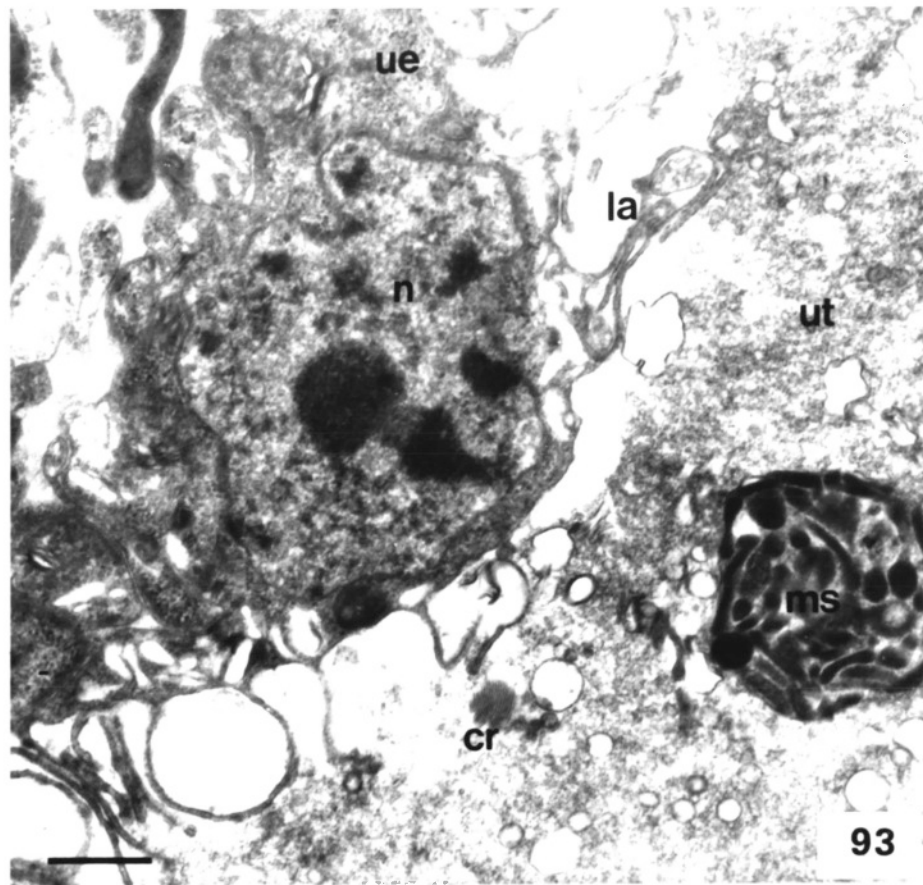


Plate 97

TEM tegument (tegumental cytons not shown).

Scale bar reps. $0.5\ \mu m$ (in vitro)

Plate 98

TEM microtriches. Note electron dense thickening on side of base (arrow) (see also Plate 97).

Scale bar reps. $0.5\ \mu m$ (in vivo)

Plate 99

TEM sucker and scolex: - Scolex^x tegument (RHS), with large tegument~~al~~ cytons; sucker (LHS), with extensive radial muscles.

Note secretions in scolex and sucker.

Scale bar reps. $2\ \mu m$ (in vitro)

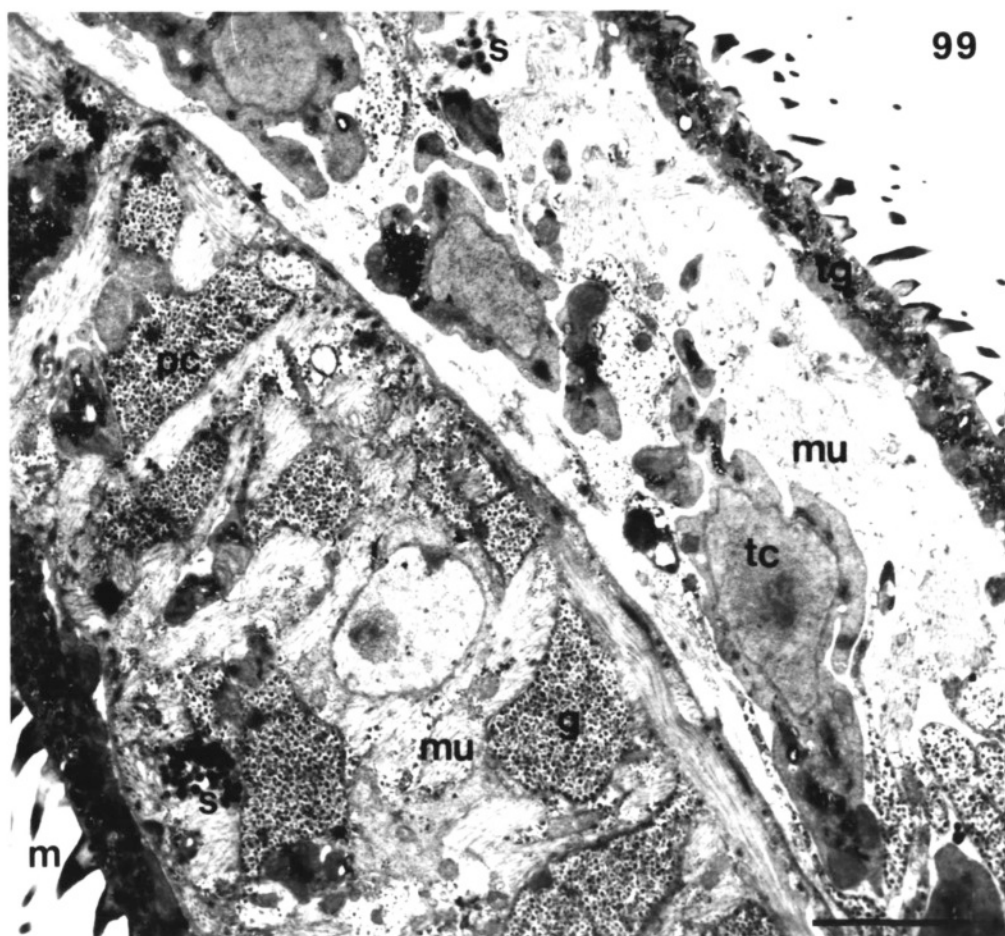
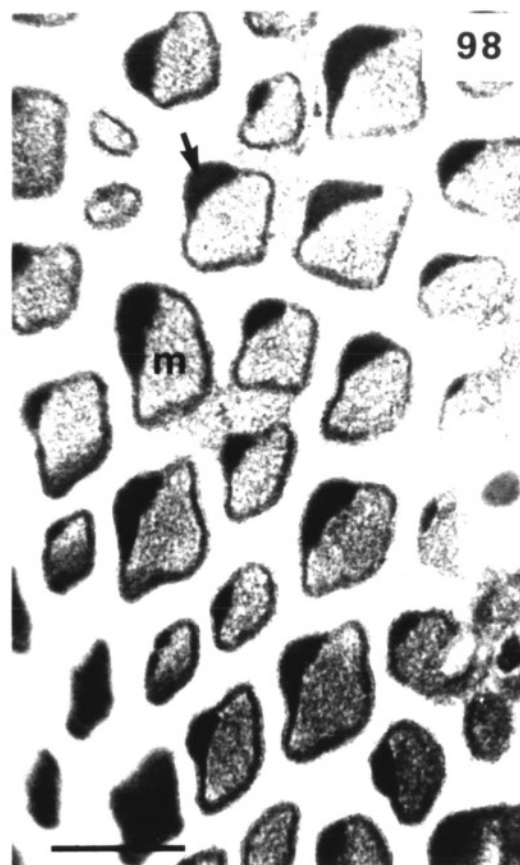
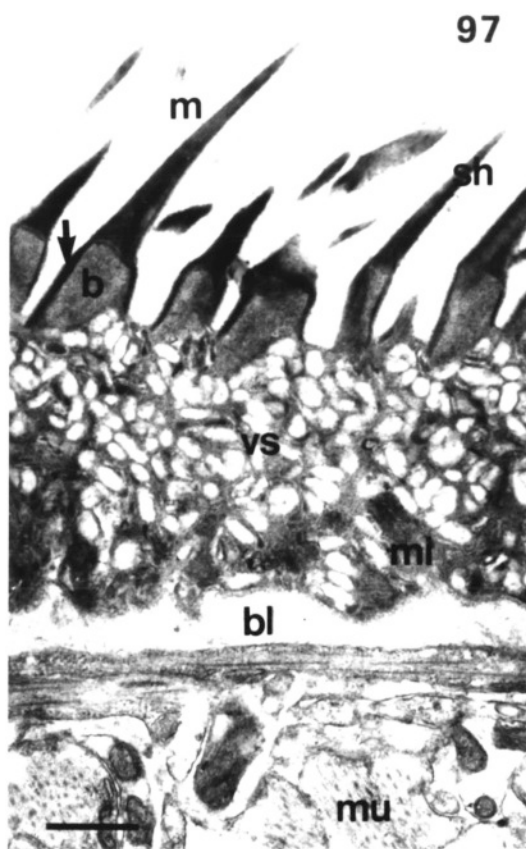


Plate 100

SEM adult worm.

Scale bar reps. 1 *mm* (in vivo)Plate 101

SEM scolex showing the suckers, and two rings of protruding hooks.

Scale bar reps. 25 μm (in vivo)Plate 102

SEM rostellar tip showing the meshwork of microtriches.

Scale bar reps. 20 μm (in vivo)Plate 103

SEM rostellar tip, showing abnormal extension in a cultured worm. Note absence of hooks.

Scale bar reps. 20 μm (in vitro)

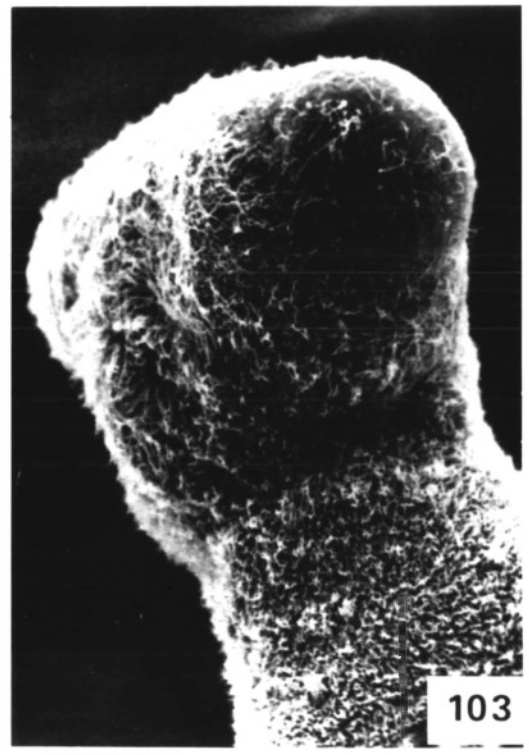


Plate 104

Rostellum of adult worm showing the large (L) and small (S) hooks (Aceto-orcein).

Scale bar reps. 20 μm (in vitro)

Plate 105

TEM rostellum showing the protruding blade part of the hook.

Scale bar reps. 1 μm (in vivo)

Plate 106

TEM rostellum with hook. Note the blade in the distal cytoplasm, and the handle embedded in a subtegumental cell, beneath the muscle layers.

Scale bar reps. 1 μm (in vitro)

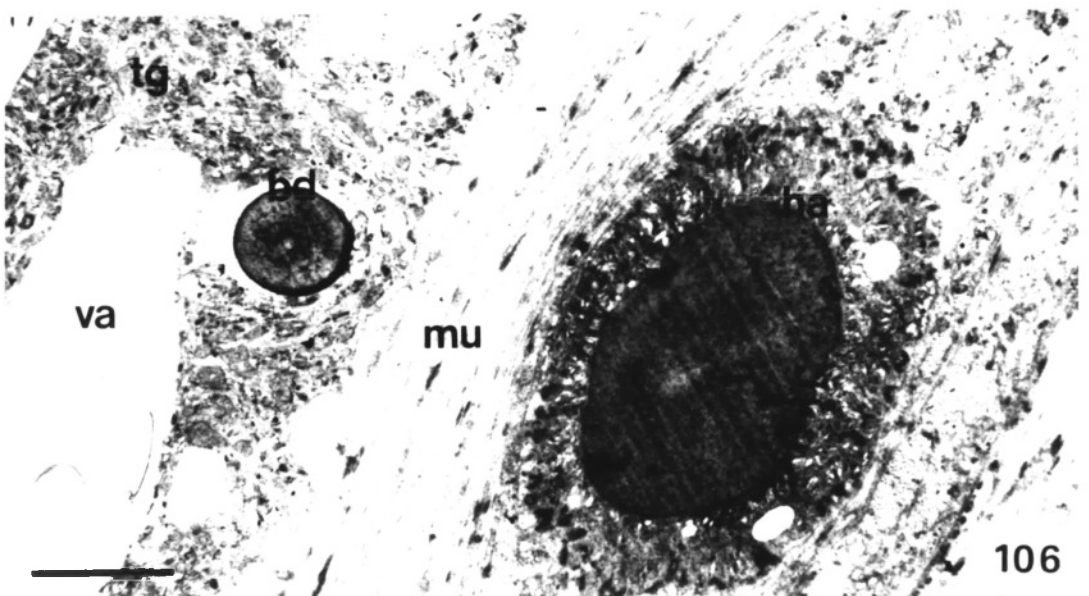
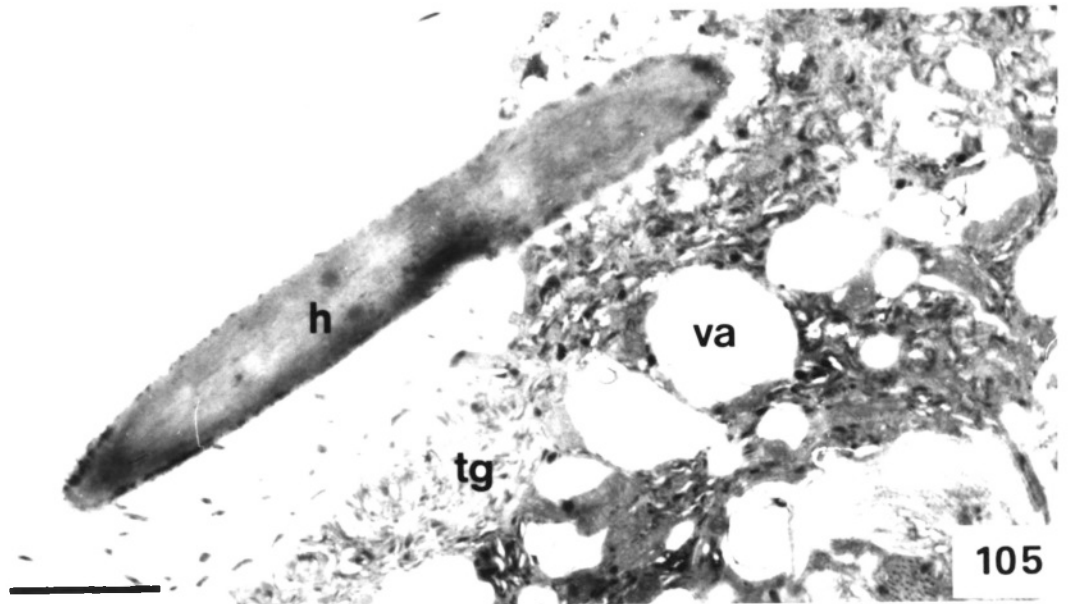
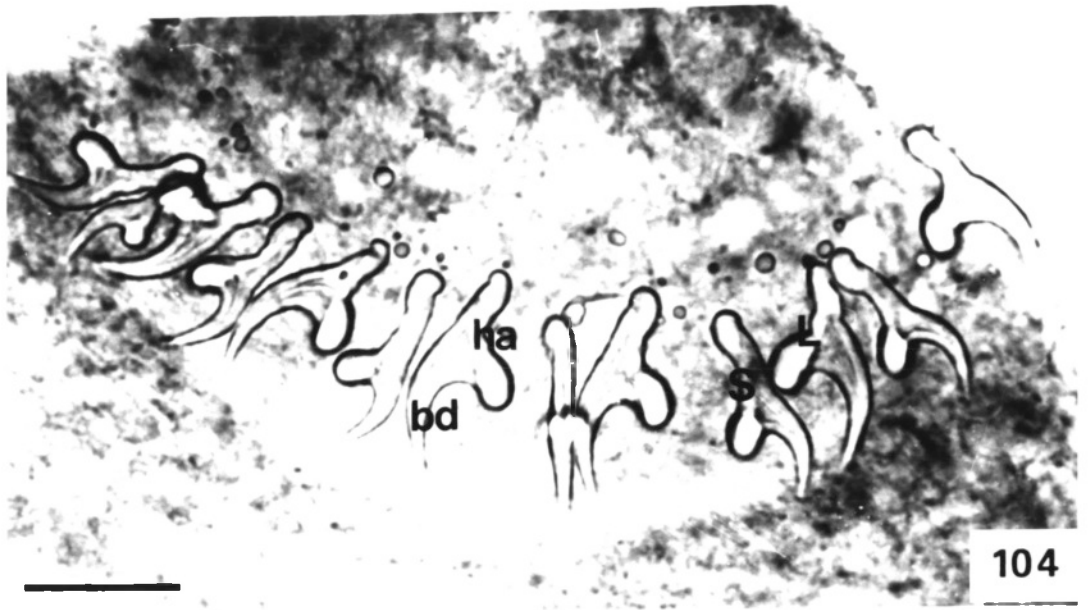


Plate 107

Section of scolex showing the rostellar gland and pad (Haematoxylin and eosin).

Scale bar reps. 50 μm (in vitro)

Plate 108

TEM rostellar pad. Note extensive muscles.

Scale bar reps. 1 μm (in vivo)

Plate 109

TEM rostellar gland, showing the secretory bodies.

Scale bar reps. 1 μm (in vitro)

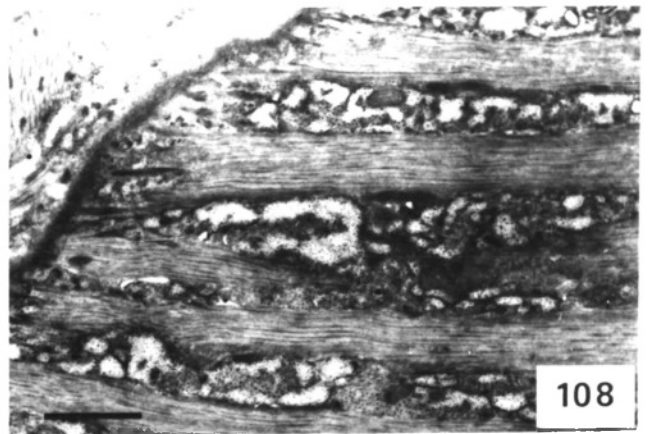
Plate 110

TEM scolex tegument showing the large vacuoles.

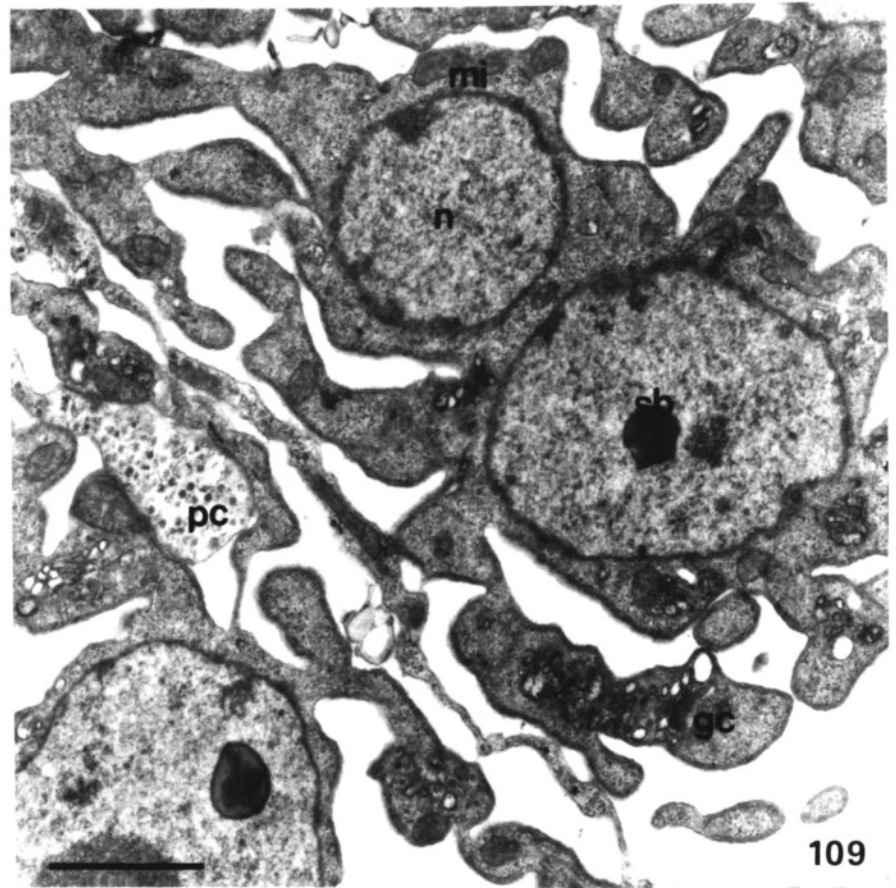
Scale bar reps. 1 μm (in vivo)



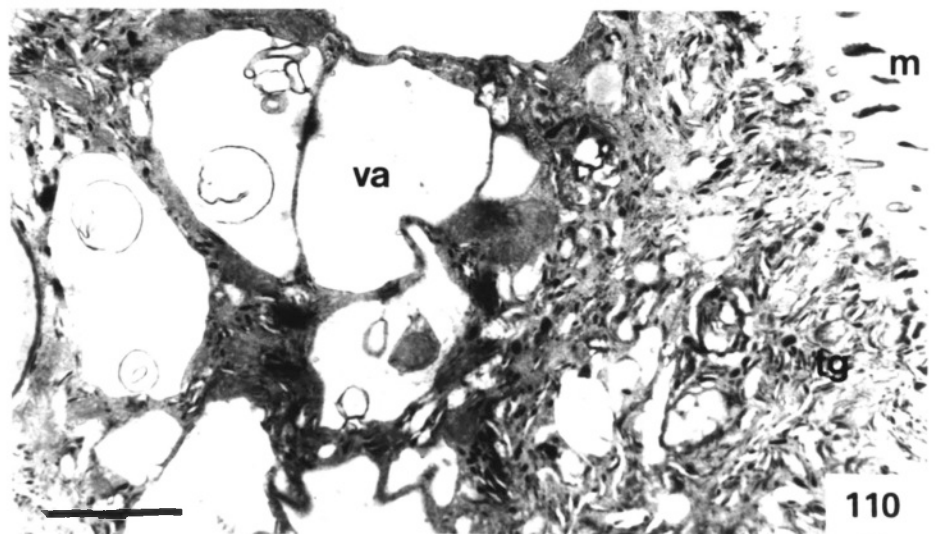
107



108



109



110

Plate 111

TEM ciliated sense organ.

Scale bar reps. $0.5\ \mu\text{m}$ (in vitro)

Plate 112

TEM ciliated sense organ, showing the two collars and central basal body. Desmosomes are also apparent.

Scale bar reps. $0.5\ \mu\text{m}$ (in vivo)

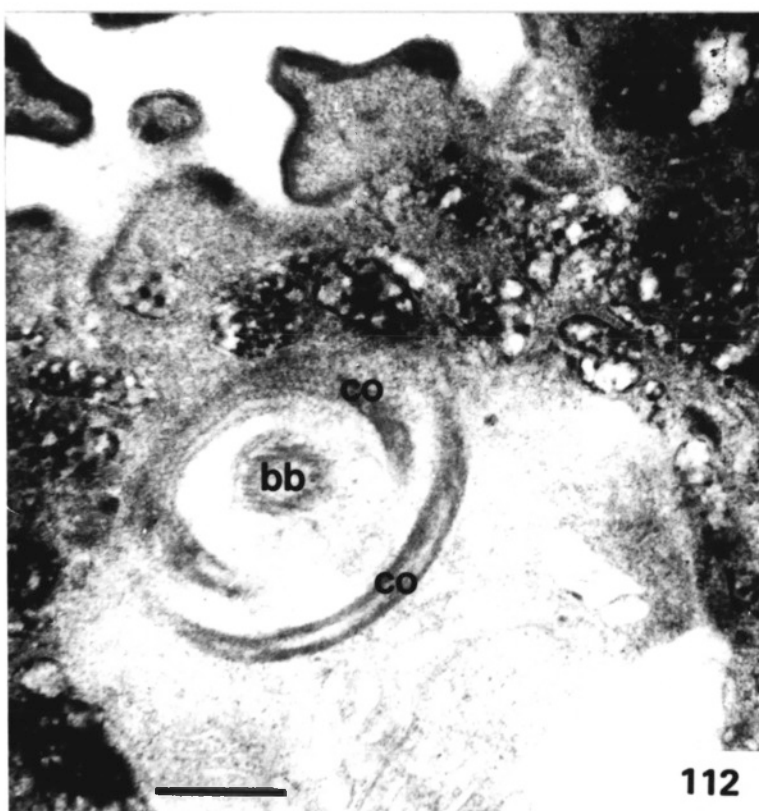
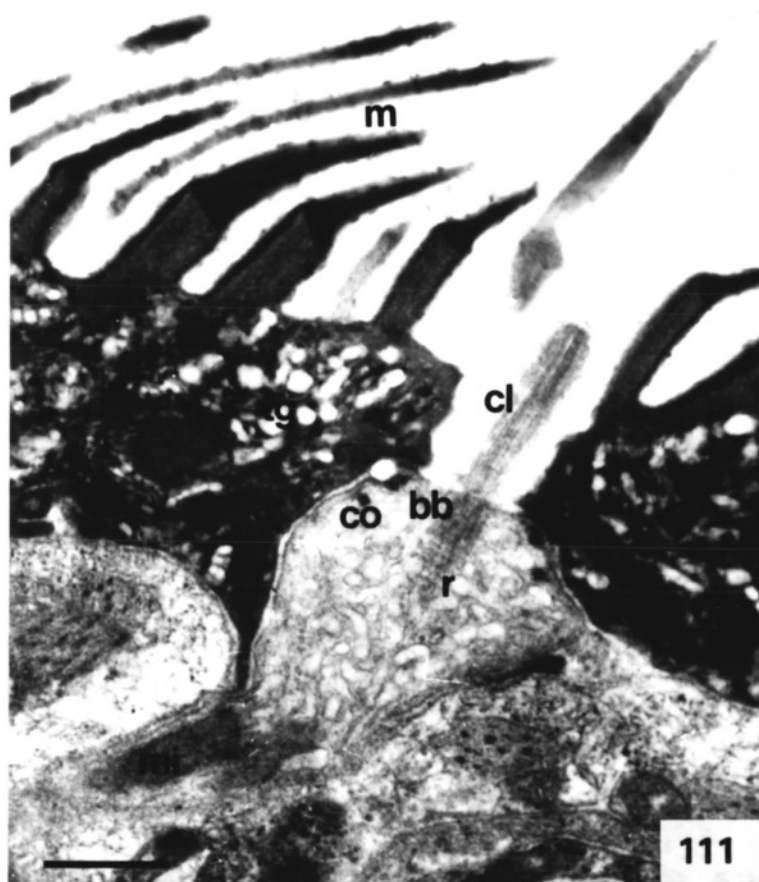


Plate 113

TEM non-ciliated sense organ.

Scale bar reps. $1\ \mu m$ (in vitro)Plate 114TEM non-ciliated sense organ,
showing the electron dense, single collar.Scale bar reps. $0.25\ \mu m$ (in vivo)Plate 115TEM non-ciliated sense organ, showing the
basal body.Scale bar reps. $0.25\ \mu m$ (in vivo)Plate 116TEM non-ciliated sense organ, showing the
expanded rootlet.Scale bar reps. $0.5\ \mu m$ (in vitro)Plate 117TEM base of non-ciliated sense organ,
showing the rootlet fibres and mitochondria.Scale bar reps. $0.25\ \mu m$ (in vivo)

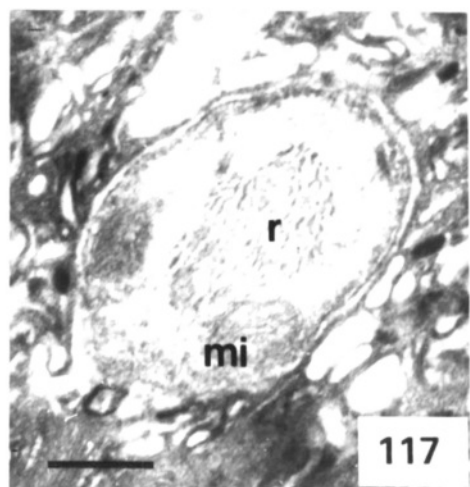
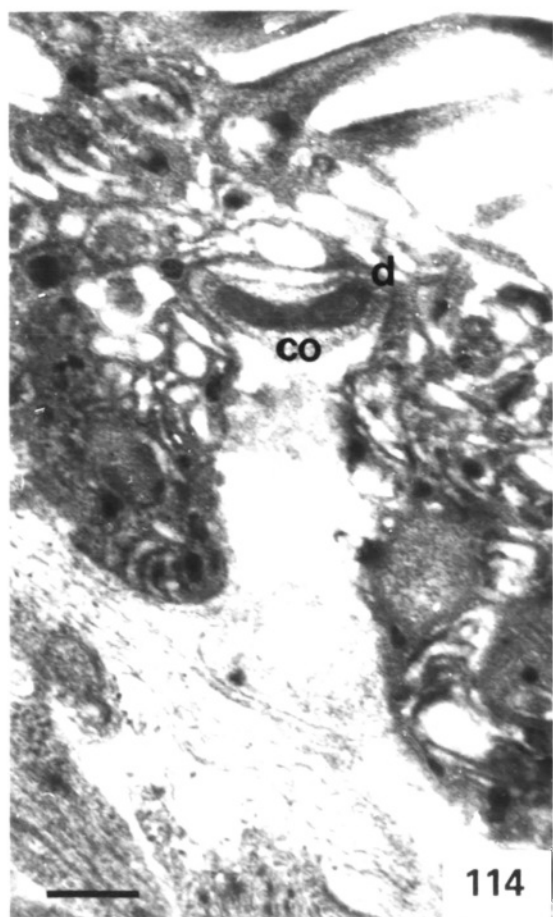
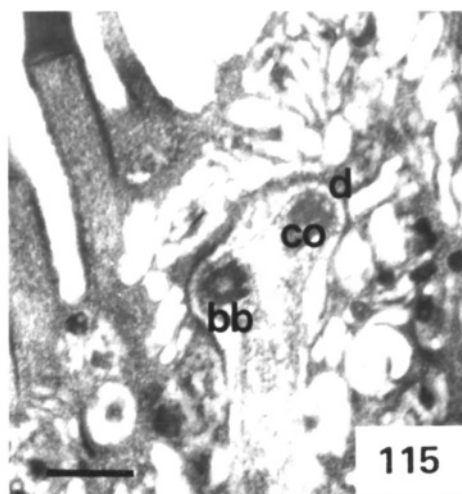
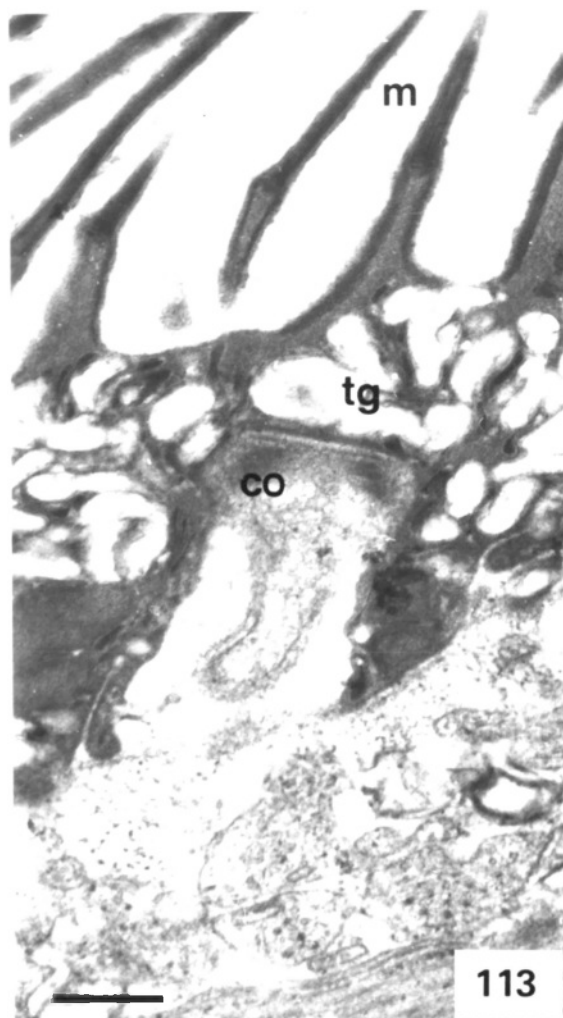


Plate 118

TEM excretory duct showing the knob-like, luminal extensions.

Scale bar reps. $0.25\ \mu m$ (in vitro)

Plate 119

TEM flame cell (longitudinal section).

Scale bar reps. $2\ \mu m$ (in vivo)

Plate 120

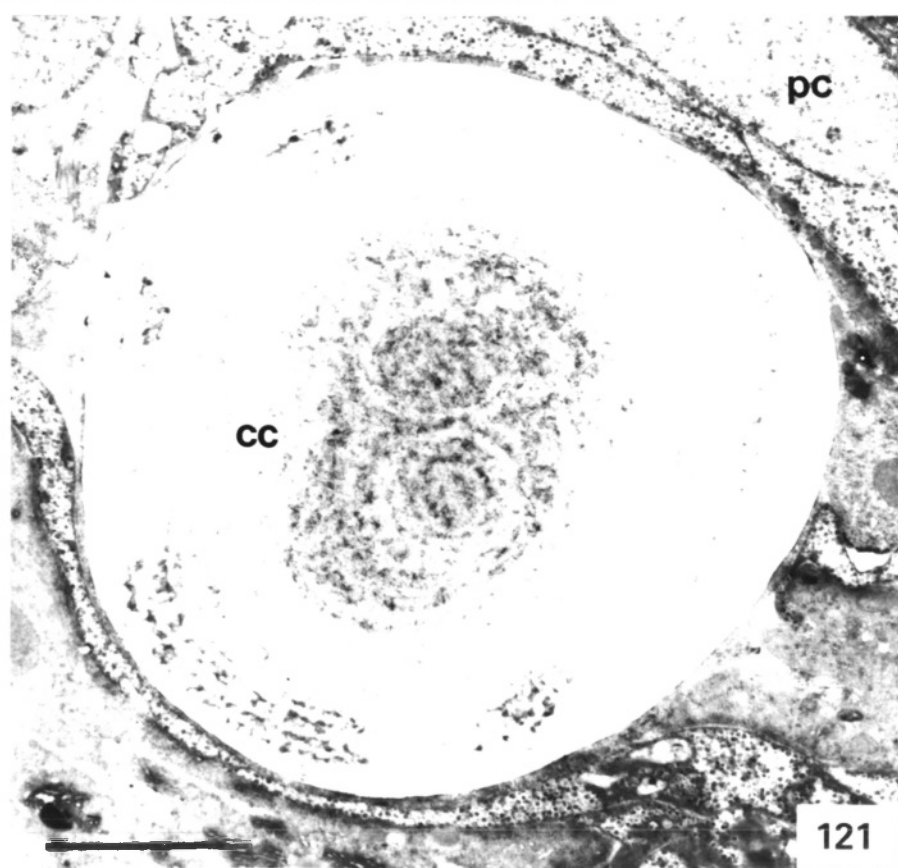
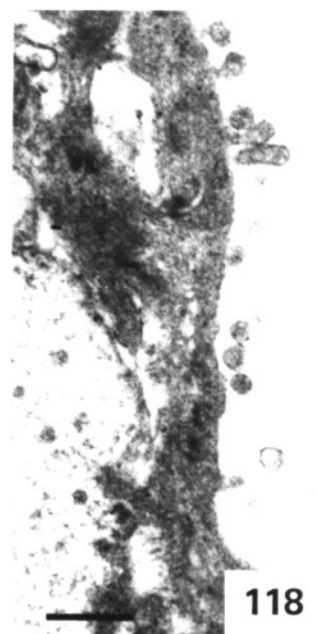
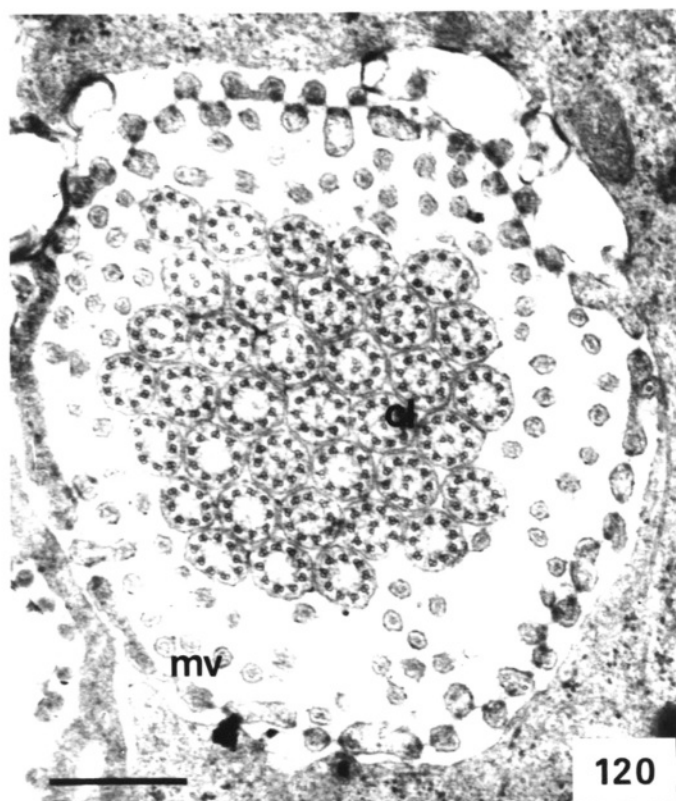
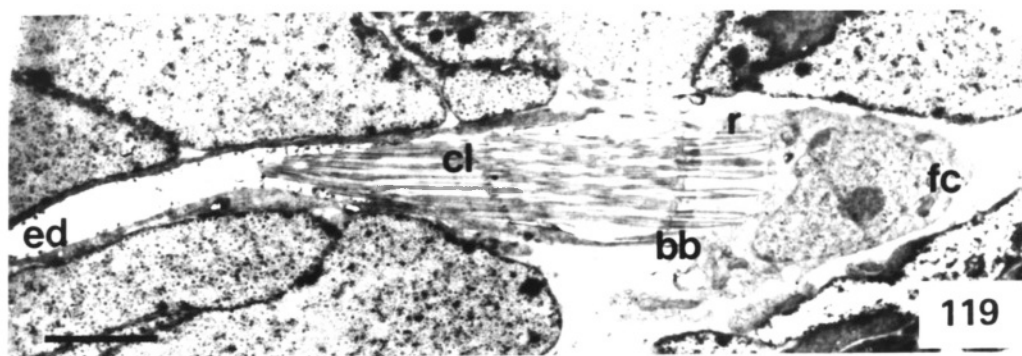
TEM flame cell (cross section), showing the microtubular arrangement of the cilia.

Scale bar reps. $1\ \mu m$ (in vivo)

Plate 121

TEM calcareous corpuscle.

Scale bar reps. $2\ \mu m$ (in vivo)



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SPONTANEOUS SEXUAL DIFFERENTIATION OF *MESOCESTOIDES CORTI* TETRATHYRIDIA *IN VITRO*

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Abstract—BARRETT N. J., SMYTH J. D. and ONG S. J. 1982. Spontaneous sexual differentiation of *Mesocestoides corti* tetrathyridia *in vitro*. *International Journal for Parasitology* 12: 315–322. Tetrathyridia of *Mesocestoides corti*, from the body cavity of mice, maintained in the laboratory by intraperitoneal infection, were used for *in vitro* culture. In an initial experiment, after 50 days asexual multiplication *in vitro* one tetrathyridium spontaneously segmented and developed into a sexually mature adult. Further experiments were carried out in an attempt to determine the conditions favouring segmentation and sexual differentiation. A combination of 5 or 10 ml liquid medium SIOE.H (basically composed of CMRL 1066 and foetal calf serum with supplements) changed every 3 days, in a Leighton tube (19 x 105 mm), rotated at 38°C and gassed with 10 or 20% CO₂, containing between 100 and 200 tetrathyridia, has proved to be most suitable so far. Numerous adult worms with normal male and female genitalia have been obtained in this system. However, segmentation is sporadic, rather than consistent and only a few shelled eggs with hooked oncospheres have so far been obtained, suggesting that impregnation and fertilization *in vitro* is not fully comparable with that *in vivo*.

INDEX KEY WORDS: *Mesocestoides corti*; tetrathyridia; *in vitro* culture; segmentation; sexual differentiation; genitalia; paruterine organ; oncospheres.

INTRODUCTION

MESOCESTOIDES spp. normally mature in the intestine of carnivores (Thompson, 1976; Loos-Frank, 1980b), some species being infective to man (Chandler, 1942). The larvae (or tetrathyridia) are found in the body cavity and liver of various reptiles, birds and mammals (Witenberg, 1934; Voge, 1953; Loos-Frank, 1980a). It is generally believed that an earlier larval stage occurs in arthropods but this has only been demonstrated for *M. lineatus* in oribatid mites by Soldatova (1944). However, the development of *M. corti* from oncosphere to tetrathyridium has been successfully completed *in vitro* (Voge, 1967; Voge & Seidel, 1968). The tetrathyridia, which have a rapid rate of asexual multiplication, are readily maintained by intraperitoneal (i.p.) passage through laboratory mice (Specht & Voge, 1965) and have also been maintained *in vitro* (Voge & Coulombe, 1966).

Recently, the *in vitro* culture of *M. corti* from tetrathyridium to the adult, strobilate worm has been achieved (Thompson, R. C. A. & Jue Sue, L. P., unpublished abstracts, Australian Society for Parasitology Conference, Perth, 1981; Thompson, Jue Sue & Buckley, 1982), using a modified technique developed for *Echinococcus granulosus* (Smyth, 1967; Smyth & Davies, 1974), but fertile eggs were not obtained. Independently (but at a somewhat later date) we have carried out similar experiments with the same species which have also resulted in the

development of adult worms. In this case segmentation was more sporadic but a few shelled eggs were obtained. This paper describes these results, from the first spontaneous development of a sexually mature worm, after 50 days culture, to further experiments in an attempt to determine the conditions favouring sexual differentiation to the adult parasite. The results are compared with the data reported by Thompson & Jue Sue (unpublished abstracts cited above, 1981) and Thompson *et al.* (1982).

MATERIALS AND METHODS

Maintenance of parasites. Tetrathyridia of *M. corti* (originally obtained from Dr. M. Voge, U.C.L.A.) were maintained in the laboratory by i.p. passage through laboratory mice (TFI strain), using 25 to 40 larvae in Hanks balanced salt solution (Flow Laboratories Ltd., U.K.) containing 100 i.u. penicillin/ml and 100 µg streptomycin/ml and 20 mM Hepes (Hopkin & Williams, U.K.) for each mouse.

Collection of tetrathyridia. Sterile larvae were obtained by opening the peritoneal cavity of a mouse (3–8 months post infection (p.i.)), using standard aseptic techniques. The cavity was repeatedly rinsed with sterile Hanks saline (containing antibiotics and Hepes), removing the parasites via a pipette to a 30 ml Universal container (Sterilin Ltd., U.K.)—a technique similar to that described by Specht & Voge (1965). In most cases the thoracic cavity, alimentary canal and all internal organs of infected mice were also thoroughly checked for the presence of *M. corti* larvae.

In vitro culture. Only the tetrathyridia recovered from the peritoneal cavity were used for culture. They were washed at least twice with 20 ml warmed Hanks saline for about 20 min each time in a 38°C incubator, rotating the vial during rinsing. The larvae were then transferred to culture vessels containing prepared culture media. Various types of glass and plastic containers were used [200 ml tissue culture (t.c.) flasks (Sterilin), 50 ml t.c. flasks (Costar, U.S.A.), Milk Dilution (M.D.) bottles (A. H. Thomas Co. U.S.A.) and Leighton t.c. tubes—16 x 93 mm and 19 x 105 mm—(Bellco Glass Inc., U.S.A.)], as were numerous media combinations tried, both mono- and diphasic (see Table 1). Several other factors in each experiment were varied, i.e. the number of tetrathyridia per culture, amount of media, gas phase, use of pepsin and/or trypsin as a pretreatment before washing in Hanks saline, movement of the cultures and time between changing the media (see Table 2). Where possible at least four replicates of each system were set up in any one experiment, with similar experiments being repeated at a later date. Cultures were kept at 38°C either in a water-bath or incubator.

TABLE 1—COMPOSITION OF MEDIA USED FOR *in vitro* CULTURE OF *Mesocostoides corti* TETRATHYRIDIA

Liquid Medium S10E.H (including 20mM Hepes)	
Foetal calf serum (Gibco)	100.0 ml
CMRL 1066 (Flow) including 26,000 I.U. penicillin and 26,000 µg streptomycin (Flow)	260.0 ml
5% Yeast extract (Gibco) in Hanks saline	36.0 ml
30% Glucose (Hopkin & Williams) in distilled water	5.6 ml
5% Dog bile in Hanks saline (or 0.2% Sodium taurocholate (Sigma) in distilled water)	1.4 ml
<i>Variations:—</i>	
Substituting CMRL 1066 with —	
RPMI 1640 (Flow)	
McCoy's 5A (Flow)	
TC 199 (Gibco)	
Substituting Foetal calf serum with —	
Horse serum (Flow)	
<i>Solid bases</i>	
Newborn calf serum (Flow) coagulated at 76°C until it becomes opaque and 'sets'.	

Morphology and histology. The gross morphology of the tetrathyridia and adults of *M. corti* was studied, using both fresh and fixed material, as was their development *in vitro*. Cultures were observed regularly with the aid of an inverted Zeiss microscope, worms being removed as necessary for more detailed study. Live specimens were observed microscopically unstained, in 0.1% neutral red in Hanks saline or in aceto-orcein (Paul, 1970). Some worms were 'squashed' and fixed in mercuric-acetic or the alcohol-formalin fixative used by Wikgren, Knuts & Gustafsson (1970) and later stained in Gower's carmine. Others were fixed in Carnoy's fluid or 5% formal saline and embedded in wax; 7 µm sections were cut and stained with Erlich's haematoxylin and eosin or orange G.

TABLE 2—VARIOUS PARAMETERS USED FOR *in vitro* CULTURE OF *Mesocostoides corti* TETRATHYRIDIA

Number of tetrathyridia/culture:—	20–400	
Amount of media/culture:—	(a) Liquid	(b) Solid
Sterilin t.c. flasks	20 ml	—
Costar t.c. flasks	10 ml	10 ml
M.D. bottles	20 ml	20 ml
Leighton t.c. tubes	5 or 10 ml	2 or 3 ml
Gas phase:—	(a) air (b) 10% CO ₂ , 5% O ₂ , 85% N ₂ (c) 20% CO ₂ , 80% N ₂	
Pretreatment:—	(a) 0.2% pepsin in Hanks saline (pH 2), rotated for 20 min at 38°C. (b) 0.5% trypsin in distilled water, treated as (a)	
Movement of cultures:—	(a) 'Still'—in incubator (b) 'Activated'—either rotated at 1 rev./5 min (Leighton t.c. tubes) or shaken in a waterbath, continuously or intermittently (all t.c. flasks and M.D. bottles)	
Intervals between changing media:—	2–5 days.	

RESULTS

Occurrence in animals

Tetrathyridia were most commonly found in the peritoneal cavity of i.p. infected mice, usually free, but sometimes attached to the mesenteries; many also occurred in the liver. Several months p.i., both the peritoneal cavity and the liver were normally packed with larvae. Occasionally tetrathyridia were found in the intestinal wall or mucosa, usually as small, rounded, calcified forms. No other sites of infection were observed.

In vitro culture

Washing the tetrathyridia in Hanks saline stimulated the evagination of most scoleces; the remainder usually evaginated in culture. In all cultures the larvae were seen to multiply asexually from day 1, either by fission, anteroposteriorly, after replication of the suckers and excretory bladder, or by budding, as described by Specht & Voge (1965). The behaviour of the tetrathyridia *in vitro* was similar to that described by Voge & Coulombe (1966) in that the larvae readily attached to the culture vessels with their suckers and also to the coagulated serum bases in diphasic media. In the latter, it was common to see them wedged and flattened between the coagulated serum and the culture vessel, or completely buried in the base. These worms eventually became immobile and remained 'trapped'; they did not apparently return to the liquid media. The burrowing phenomenon occurred more often in 'still' rather than in 'activated' cultures (see Table 2).

Unless the cultures were very small (<50 worms), the tetrathyridia tended to become closely entwined, especially those which elongated and eventually segmented, attaching firmly to each other with their suckers. Hence in any culture, one or more clumps of worms were common, along with the free parasites and those attached singly to the container or serum base.

There was much variation in the behaviour and development of the parasites between different cultures. Segmentation and sexual differentiation in all experiments was sporadic. In some cultures, no further development other than asexual multiplication (the rate of which eventually slowed down and in some cases ceased) occurred; in others, even under similar conditions, over 50% segmented. There was no evidence of strobilization or asexual multiplication in the larvae trapped in the serum bases. Maturation occurred most often with the following:- 5 or 10 ml SIOE.H using CMRL 1066 (see Table 1) in Leighton t.c. tubes, slowly rotated in an incubator (38°C) with 100-200 worms, changing the media every 2 or 3 days. Segmentation occurred more frequently in the cultures gassed with 20% CO₂ in N₂ than in the other gas phases, with least occurring in air, but less abnormalities were apparent in the gas phase of 10% CO₂, 5% O₂ in N₂. Horse serum and foetal calf serum produced similar results but the latter was used routinely, our sample of horse serum being prone to precipitation of lipid. Segmentation occurred in as little as 15 days culture in the monophasic medium. In one particular experiment, similar results were obtained using diphasic media, but in contrast, strobila development in SIOE.H only, was not apparent until 24 days later. It proved beneficial to change the media less often or to reduce the amount of media used in cultures with few worms (usually <50), whereas using >200 larvae per culture was often detrimental to their development unless the medium was changed every day. In these large cultures the media rapidly became acidic, as shown by the phenol red indicator in the basic t.c. media.

Sexual differentiation was also achieved in Costar and Sterilin t.c. flasks but not in M.D. bottles; substituting CMRL 1066 with other basic t.c. media (see Table 1) was unsuccessful. No segmentation occurred using <100 tetrathyridia per culture and the percentage segmenting in cultures of >200 was reduced. The use of pepsin and/or trypsin as pretreatments did not appear to be necessary for the establishment and development of the parasites *in vitro*.

As is common with *in vitro* culture work, it was difficult to assess the efficacy of the various systems statistically, the main limiting factor being the number of replicates possible. In addition, the high rate of asexual multiplication of the tetrathyridia *in vitro* and the apparent diversity in development and morphology of the adults made standard statistical analysis impossible to apply. There was often much variation in results, both inter- and intraculture and

hence experiments were repeated where possible. The efficiency of the various culture systems was therefore assessed on the best results achieved in each one.

Morphology & histology

The undifferentiated tetrathyridia were dorso-ventrally flattened and asymmetrical in shape, usually convoluted laterally and on average 2 mm long. They had no rostellum, their flat scolex being armed with 4 suckers only; this was normally seen inverted *in vivo* and occasionally in 'unfavourable' *in vitro* conditions. The larvae contained numerous calcareous corpuscles and also possessed an excretory bladder and 4 excretory canals running anteroposteriorly, 2 on each side of the body. However, these were usually not visible until several days in culture when the larvae elongated and began 'clearing' (i.e. the number of calcareous corpuscles was reduced). Stained sections showed a network of muscles at the anterior end below and between the suckers and a thin band of muscles below and close to the tegument surrounding the whole worm.

Many tetrathyridia readily multiplied asexually *in vitro* as previously described and generally remained active. Those which did not survive *in vitro* culture tended to round up, becoming very dark and inactive. In all cultures from day 1 onwards some worms elongated, assuming a symmetrical vermiform shape and became lighter in colour; it was some of these forms which later segmented, from day 15 onwards.

Associated with the beginning of segmentation was the appearance of a round, thin-walled cirrus pouch, eventually containing a coiled muscular cirrus, on the median line at the anterior end of each segment. The testes, situated longitudinally and laterally, usually between the excretory canals and the centrally placed genitalia, as noted by Witenberg (1934), developed at the same time but were not apparent in unstained fresh specimens. Aceto-orcin and Gower's carmine showed the developing testes clearly (Fig. 3). Several segments usually appeared spontaneously and within 5 days the posterior ones were sexually mature, i.e. the male and female genitalia had fully developed (Fig. 1). Meanwhile further segmentation continued, the immature segments usually being broader than long, the converse being true for posterior ones. Sometimes in old cultures the end proglottids became almost 'barrel shaped' and in some cases very dark and abnormal; these were often lost in culture. The actual lengths of worms differed widely, the number of segments ranging from just a few to 30 or more.

In a sexually mature segment the following were apparent: a cirrus coiled in the cirrus pouch, besides which was a thick-walled and much looped vas deferens and a common genital opening lying dorsally to the genital atrium; a bilobed ovary at the posterior end with two smaller vitelline glands, one posterior and ventral to each lobe; testes situated as explained previously, varying in number from 17 to

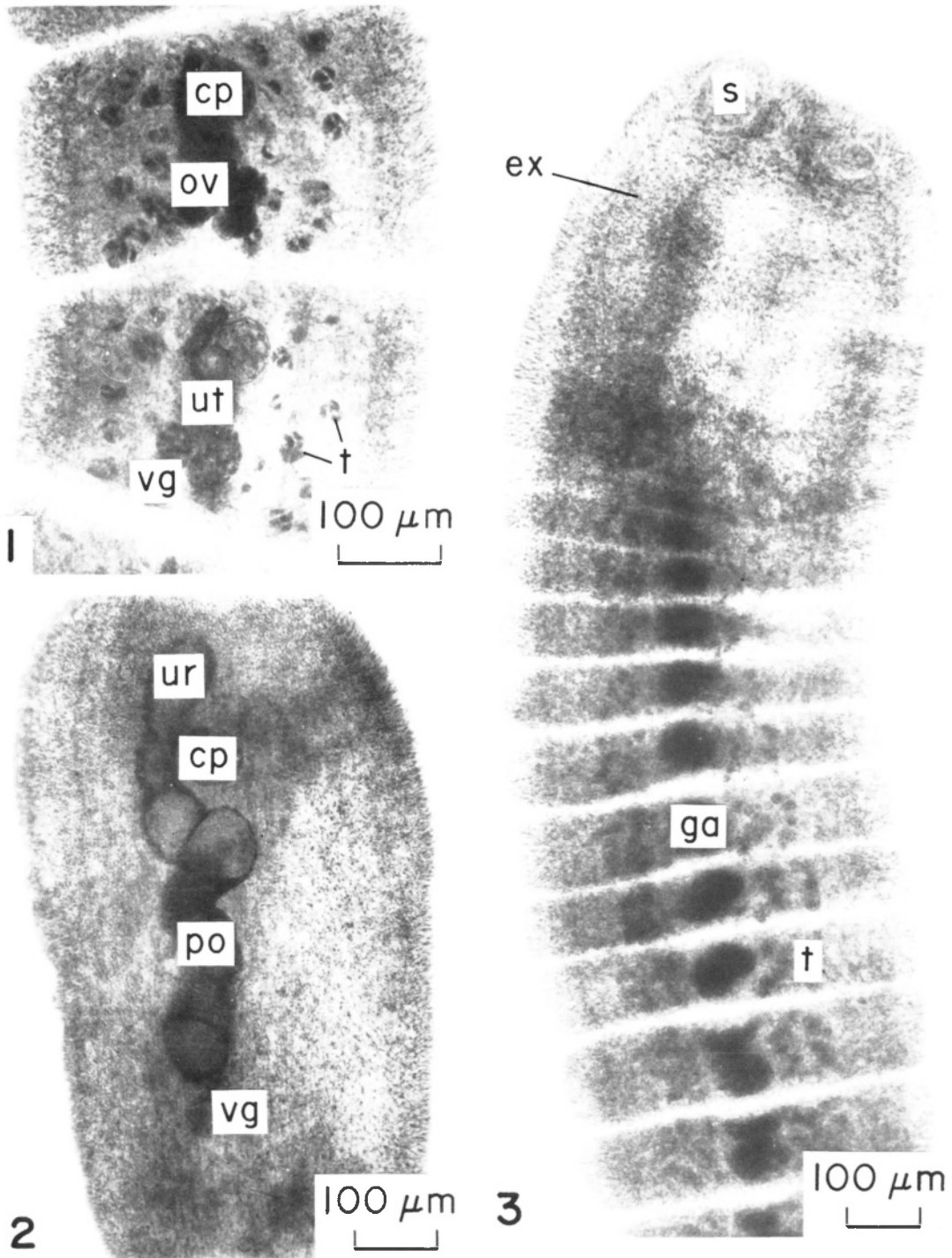


FIG. 1. Mature segments of an adult *M. corti* cultivated *in vitro* (34 days), showing the male and female genitalia.

FIG. 2. Posterior segment of *M. corti* (same worm as Fig. 1), showing an empty paruterine organ (po). Note the cirrus pouch (cp) and the degenerating vitellaria.

FIG. 3. Anterior portion of *M. corti* (same worm as Fig. 1), showing the initial stages of segmentation and sexual differentiation (Gower's carmine).

Abbreviations: cp, cirrus pouch; es, egg shell; ex, excretory canal; ga, genital anlagen; h, hooks; on, oncosphere; ov, ovary; po, paruterine organ; s, sucker; t, testes; ur, uterus remnant; ut, uterus; vm, vitelline material; vg, vitelline gland.

45, on average 29, with approximately half on each side (this is 25% less than that recorded for *M. corti* by Hoeppli, 1925, and 50% less than that mentioned by Witenberg, 1934); a uterus placed along the median line, curved around the cirrus pouch and reaching down to the ovary; two longitudinal excretory canals on each side, one connected posteriorly by a wide transverse commissure. The ova and vitelline cells appeared morphologically normal as did the spermatozoa seen both in mature testes and the vas deferens. The sexual differentiation of *in vitro* cultured worms is summarised diagrammatically in Fig. 4, stages 5 and 6 being sexually mature proglottids.

Paruterine organs were obvious in the posterior segments (Fig. 4, stages 7–9), from 7 days after segmentation began and their appearance was associated

with the degeneration of the ovaries and vitelline glands, in that order. In these segments only one excretory canal was visible on either side. The paruterine organ developed from a dark mass of cells seen at the posterior end of the uterus in mature segments. These cells eventually constitute the thick fibrous wall of the organ, its lumen presumably being derived directly from the lumen of the uterus. The actual development of the paruterine organ showed much variation even within different segments of the same worm. Differences were also noted depending on whether or not ova and/or vitelline cells were present in the uterus, prior to the formation of the organ. If the uterus remained empty the paruterine organ generally took the shape of a partially coiled tube, with a uterus remnant apparent (Fig. 2). However, if it contained any cells, the organ was

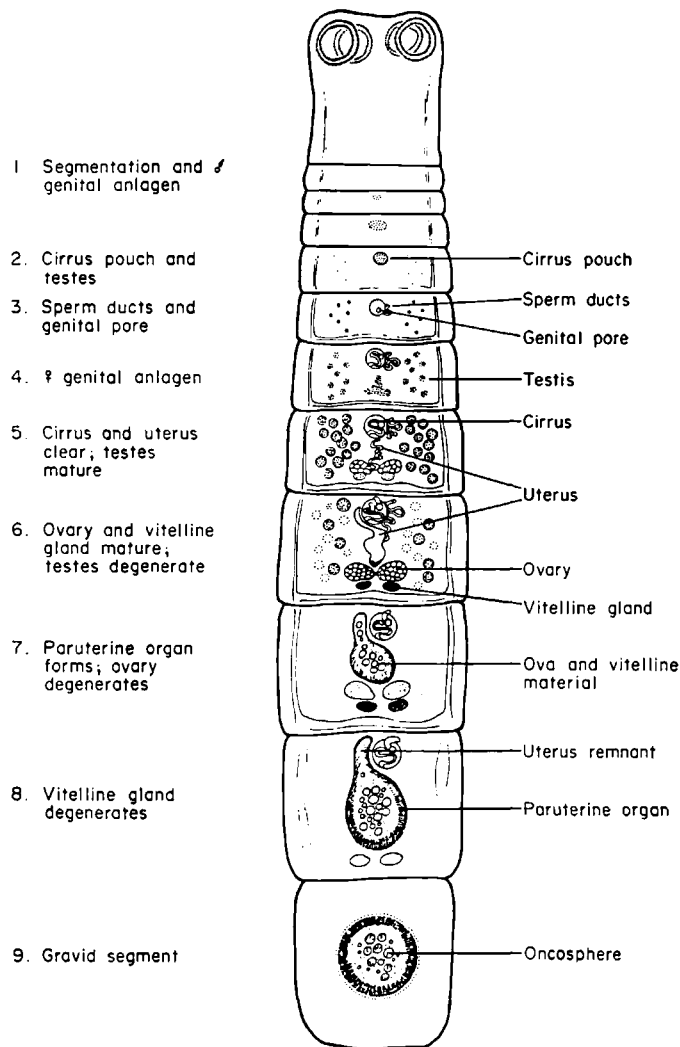


FIG. 4. Diagrammatic representation of the stages of sexual differentiation encountered in *M. corti* cultured *in vitro* from the tetrathyridium.

usually swollen to an ovoid or spherical shape, any uterine remnants eventually being lost (Fig. 4, stage 9). By this stage no excretory canals were evident in the proglottid.

Often in the older segments the long eversible cirrus was seen protruding from the genital opening (also seen by Byrd & Ward (1943) in *in vivo* adults) before the degeneration, or resorption, of the male genitalia, but copulation was never observed. In fresh material only ova and vitelline material were observed in the paruterine organ unless it was empty. However, oncospheres were seen in stained (haematoxylin and orange G) sections of a worm cultured *in vitro* for 112 days (approximately 100 worms in 10 ml SIOE.H in a Sterilin t.c. flask, kept horizontal and still) (Figs. 5 & 6). They were identified by the presence of oncospherical hooks and very thin egg shells and measured, on average, 22 μ m diameter. This compares well with the description of oncospheres from *in vivo* worms, collected by Voge (1967). No oncospheres were recognised in whole mounts of worms from the same culture, stained with Gower's carmine, although this technique was found to be less suitable for morphological studies unless the material had been well flattened. The posterior segments containing the paruterine organ eventually degenerated *in vitro* on becoming dark and vacuolated and were often lost to the medium (usually several proglottids attached together), from where they could be recovered. This material, however, was rarely suitable for microscopy.

The morphology of the adult *M. corti* grown *in vitro* was comparable to that described by Hoeppli (1925), Witenberg (1934) and Byrd & Ward (1943). However, some worms showed abnormal sexual development *in vitro*; for example, one tetrathyridium which had begun to divide asexually by longitudinal fission, then segmented; each proglottid consequently contained two sets of genitalia (Fig. 7).

In some cases, segmentation was incomplete and adults appeared to contain proglottids with two single sets of genitalia, both along the median line.

DISCUSSION

The ability of tetrathyridia of *Mesocostoides* sp. to multiply asexually both *in vivo* (especially in laboratory mice) and *in vitro* (Voge & Coulombe, 1966; Specht & Voge, 1965), makes this species an especially useful laboratory model and enables various culture systems to be tested.

The ability of some larvae to spontaneously segment and develop into sexually mature worms amidst those which persist in asexual multiplication *in vitro*, must be regarded as an unusual phenomenon and one of especial interest in relation to the switch from asexual to sexual differentiation. Unfortunately, segmentation in our *M. corti* material was sporadic rather than consistent. This result is in contrast to that reported by Thompson & Jue Sue

(unpublished abstracts cited above, 1981) and Thompson *et al.* (1982), using *M. corti* of different origin, but in comparable culture systems. In their experiments, most larvae readily segmented and differentiated sexually. In addition to this, there are several other differences evident in the development of *M. corti* in the two culture systems such as the presence of scolex cleft prior to segmentation (Thompson & Jue Sue, unpublished abstracts cited above, 1981). These may be due to slight variations in the culture techniques, or may possibly indicate the occurrence of two 'strains'. This hypothesis is now being further investigated by co-operative experiments between both research groups.

The anatomical descriptions given by Hoeppli (1925), Witenberg (1934) and Byrd & Ward (1943) supposedly for the same species, *M. corti* (Syn. *M. variabilis* and *M. lineatus* forma *litterata*, see Witenberg, 1934), differ slightly in some aspects; this could possibly be due to either the different hosts from which the parasites were obtained, the processing methods employed by the authors, or may again indicate the occurrence of two or more 'strains'. Speciation in the genus *Mesocostoides* may prove to be more complex than previous studies have indicated (Loos-Frank, 1980b).

Copulation has been reported by Thompson & Jue Sue (unpublished abstracts cited above, 1981) in their cultured worms, but has not yet been observed by us. However, ova and vitelline cells have often been found in the uterus of our *M. corti*, eventually being enclosed by the paruterine organ and appear to be morphologically normal, as do the spermatozoa seen in mature testes and sperm ducts. The number of testes found was small compared with *in vivo* material (Hoeppli, 1925; Witenberg, 1934) and this may indicate that the culture system is lacking in certain nutritional factors.

Further studies are needed, using both light and transmission electron microscopy, in order to determine the detailed structure of the reproductive systems. The development of the paruterine organ especially, requires investigation. It has been described by various workers as a thickwalled, egg or uterine capsule, an egg ball, or a paruterine/parauterine organ or capsule (Hoeppli, 1925; Witenberg, 1934; Chandler, 1942, 1946; Byrd & Ward, 1943; Voge, 1967; Thompson, 1976; Thompson & Jue Sue, unpublished abstracts cited above, 1981). However, its development has rarely been studied in detail, with the exception of Chandler (1946), for *M. latus*, and Byrd & Ward (1943), for *M. corti*. *In vitro*, the paruterine organ is stimulated to develop even when the uterus is empty. However, whether or not its development is 'normal' can only be determined by comparison with *in vivo* adults. In stained sections, the paruterine organ of *in vitro* worms containing oncospheres (Fig. 6) certainly resembles that shown by Byrd & Ward (1943) in worms recovered from an opossum.

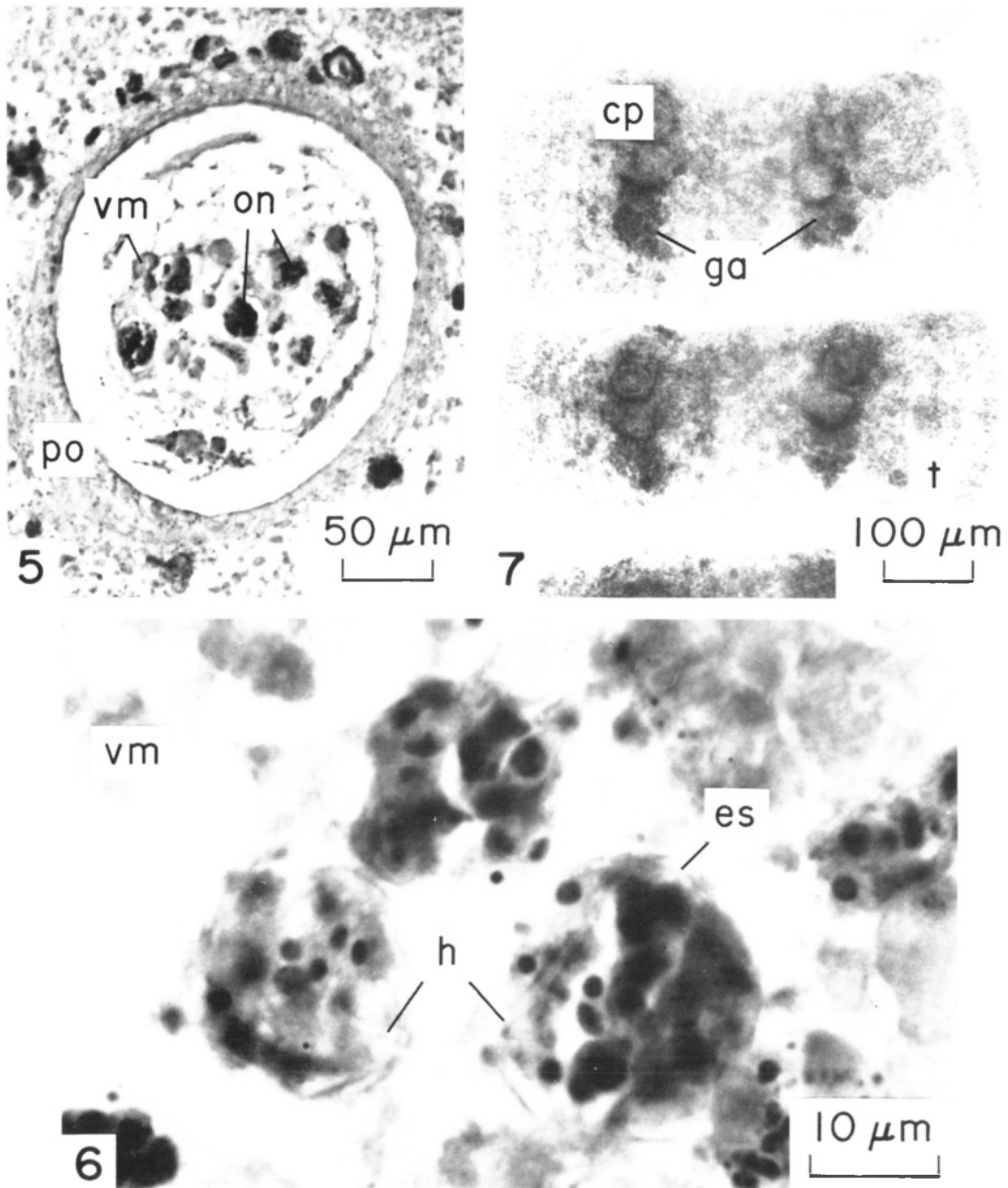


FIG. 5. Section through a gravid segment of *M. corti* cultured *in vitro* (112 days), showing the paruterine organ (po) containing oncospheres (on) (Haematoxylin and orange G).

FIG. 6. Section through a gravid segment of *M. corti* (as in Fig. 5), showing the oncospheres (on) in detail.

FIG. 7. Segments of an adult *M. corti* cultured *in vitro* (112 days), showing the abnormal occurrence of two sets of genitalia per proglottid (Gower's carmine).

Abbreviations: cp, cirrus pouch; es, egg shell; ex, excretory canal; ga, genital anlagen; h, hooks; on, oncosphere, ov, ovary; po, paruterine organ; s, sucker; t, testes; ur, uterus remnant; ut, uterus; vm, vitelline material; vg, vitelline gland.

It is possible that, as for *E. granulosus* and *E. multilocularis* (Smyth, 1979), insemination may prove to be a special problem associated with the *in vitro* culture of cestodes. However, the appearance of oncospheres in one segmented worm, seen in serial sections, indicates that our *in vitro* system is not only capable of supporting the normal sexual differentiation of *M. corti*, but shows that insemination apparently occurred at least in one instance. Alternatively, these oncospheres could represent 'haploid' (i.e. unfertilized) eggs which have been stimulated to develop; a situation which occurs in the pseudophyllidean cestode *Schistocephalus solidus* (Smyth, 1950) *in vitro*.

Due to the lack of a plentiful supply of mature worms, our morphological studies can only be described as preliminary. However, once *M. corti* can be cultured to the adult stage consistently and fresh shelled eggs can be obtained in quantity from them, it should be possible to maintain the whole life cycle of *M. corti* in the laboratory.

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**ECHINOCOCCUS MULTILOCULARIS: FURTHER
OBSERVATIONS ON STROBILAR DIFFERENTIATION
IN VITRO**

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ABSTRACT

Differentiation of *Echinococcus multilocularis* "in vitro" is described in detail. The early phase of development may be divided into seven stages, stage 1-7. The latter stage represents a "monozoic" form in which sexually mature male and female genitalia are present but in which somatic growth has been suppressed. Stage 7 can differentiate further, and several morphogenic patterns can result. Some organisms elongate and develop two or more sets of genitalia, forming "pseudo-segmented" strobila. In others, morphogenesis is inhibited; occasionally supplementary scolices may develop, up to four being found in one case. These results are discussed in terms of cytodifferentiation of cestodes in general.

RESUMEN

Se describe en detalle la diferenciación de *Echinococcus multilocularis* in vitro. La primera fase del desarrollo puede dividirse en siete estadios. El último estadio representa una forma "monozoica", en la cual la madurez sexual del macho y la genitalia de la hembra están presentes pero el grupo somático ha sido suprimido. Los siete estadios pueden diferenciarse algo, y resultar varias formas morfogenéticas. Algunos organismos se alargan y desarrollan dos o más porciones de genitalia, formando es-

trobilos "pseudo-segmentados". En otros, la morfogénesis se inhibe; ocasionalmente pueden desarrollarse escolices suplementarios, encontrándose hasta cuatro en un caso. Estos resultados se discuten en términos de citodiferenciación de cestodos en general.

INTRODUCTION

Techniques for the *in vitro* culture of *Echinococcus granulosus* and *E. multilocularis* from the protoscolex to the sexually mature strobilate stage are now reasonably well established (SMYTH & DAVIES, 1974; SMYTH & DAVIES, 1975; SMYTH, 1979a, 1979b). The pattern of morphogenesis of the strobilar phase of *E. granulosus in vitro* approaches that in the dog gut, although the rate of maturation *in vitro* is slower and fertile eggs have not yet been obtained. In the case of *E. multilocularis*, however, *in vitro* differentiation is strikingly different from that of *E. granulosus* in that although sexual differentiation takes place, somatic segmentation is somewhat suppressed with the result that bizarre, sexually mature, "monozoic" worms are formed (SMYTH & DAVIES, 1975; SMYTH, 1979b). After prolonged culture, a further two or three sets of genitalia may develop but, apparently, no "true" proglottids (i. e. with interproglottid membranes) develop. Although a general account of this monozoic pattern of development has been given (SMYTH & DAVIES, 1974) no detailed description of the various stages occurring *in vitro* has yet been given, nor has subsequent development beyond the monozoic stages been described. Such an account forms the basis of this paper.

MATERIALS & METHODS

1. Separation of culture material

E. multilocularis was obtained from cotton rats (*Sigmodon hispidus*) intraperitoneally infected, the parasite species having been maintained in this laboratory for 8 years. Precise technical details for obtaining sterile protoscoleces free from host material have been given in considerable details elsewhere and will not be repeated here (SMYTH & DAVIES, 1975; SMYTH, 1979b). Essentially the method consists of aseptic removal of cystic

material from the rat, digestion with pepsin, and repeated washings. In previous accounts, it has been especially recommended that a tubular homogenizer be used to homogenize the dense cystic tissue containing *E. multilocularis*. It has been found, however, that a normal homogenizer (of the Waring blender type) may be used, provided that (a) large quantities of 0.5% pepsin (about 200 ml) are used, (b) homogenizing times are < 20 secs. and (c) N.HCl is repeatedly added to remove the calcification in the tissues (which can be very severe in some cases) so that a pH of 2.0 (optimum for pepsin) is maintained over the 1 hr period of digestion at 38°C. Details of this decalcification procedure, which is a key factor in the preparation of sterile protoscoleces, have been given earlier (SMYTH, 1979b).

2. *Culture Media*

Medium S. 10, which used Parker 858 as its basic constituents, was used in previous experiments (SMYTH & DAVIES, 1974) and in some experiments described here. However, Parker 858 is no longer commercially available and other basic media—RPMI 1640 and CMRL 1066—have been used as reasonably successful substitutes but whether they are as entirely adequate as Parker 858 has yet to be critically examined. Results with NCTC 135 have not been very satisfactory but, due to inter-culture variation, precise critical evaluation of the growth properties of media is difficult.

Culture Vessels. Cultivation was generally carried out at 38°C in glass Milk Dilution bottles (20 mls medium), in a discontinuous shaking water bath (3.5 shakes/20 mins.) but some success has also been achieved using Leighton tubes (10-15 mls medium) on a roller tube (1 rotation/5 mins.).

3. *Staining*

Developing worms were removed at intervals and examined both in fresh and stained condition. Neutral Red served as a useful vital stain for general anatomy, being especially valuable for demonstrating the development of the vitellarium but not the testes. Maturation of the testes was best followed by staining in aceto-orcein for 30-60 mins. Before staining it is

essential to wash worms several times in Hank's saline, in order to remove the serum, which otherwise causes precipitates on contact with aceto-orcein. After staining worms may also be mounted in aceto-orcein for general morphology, further cellular detail being obtained by 'squashing' (as for chromosomes) and oil immersion examination. Permanent, stained, whole mount preparations were made by fixing in mercuric-acetic fixative and staining in Gower's carmine.

IN VITRO DEVELOPMENT

1. 'Monozoic' Development (Stages 0-7. Fig. 1)

It is convenient, for the purposes of description, to divide the *in vitro* development into a series of numbered stages, although differentiation is, of course, continuous. Evaluation of success (or otherwise) of a particular culture system can thus be made by identifying the appearance of these stages and assessing the maximum degree of development achieved at any time.

Stage 0. (Fig. 1). An unevaginated protoscolex, fresh from its brood capsule. The hooks and calcareous corpuscles are prominent.

Stage 1. (Fig. 1). A fresh protoscolex evaginated after treatment with bile or bile salt. The numerous calcareous corpuscles are readily seen.

Stage 2. (Fig. 1). The first indication of growth and differentiation taking place *in vitro* is the gradual disappearance of the calcareous corpuscles; these first become translucent and eventually disappear altogether. This process is accompanied by the appearance of excretory canals, the middle regions of which appear first as elongated 'streaks' just visible through the calcareous corpuscles.

Stage 3. (Fig. 1). A characteristic and readily recognisable stage with criteria sharply defined i.e. the calcareous corpuscles have almost entirely disappeared but a few may persist. A noticeable feature is the appearance of the bilobed posterior excretory bladder; the latter connects to the lateral excretory canals which can now be seen throughout their entire length and may sometimes be seen to extend into the scolex region.

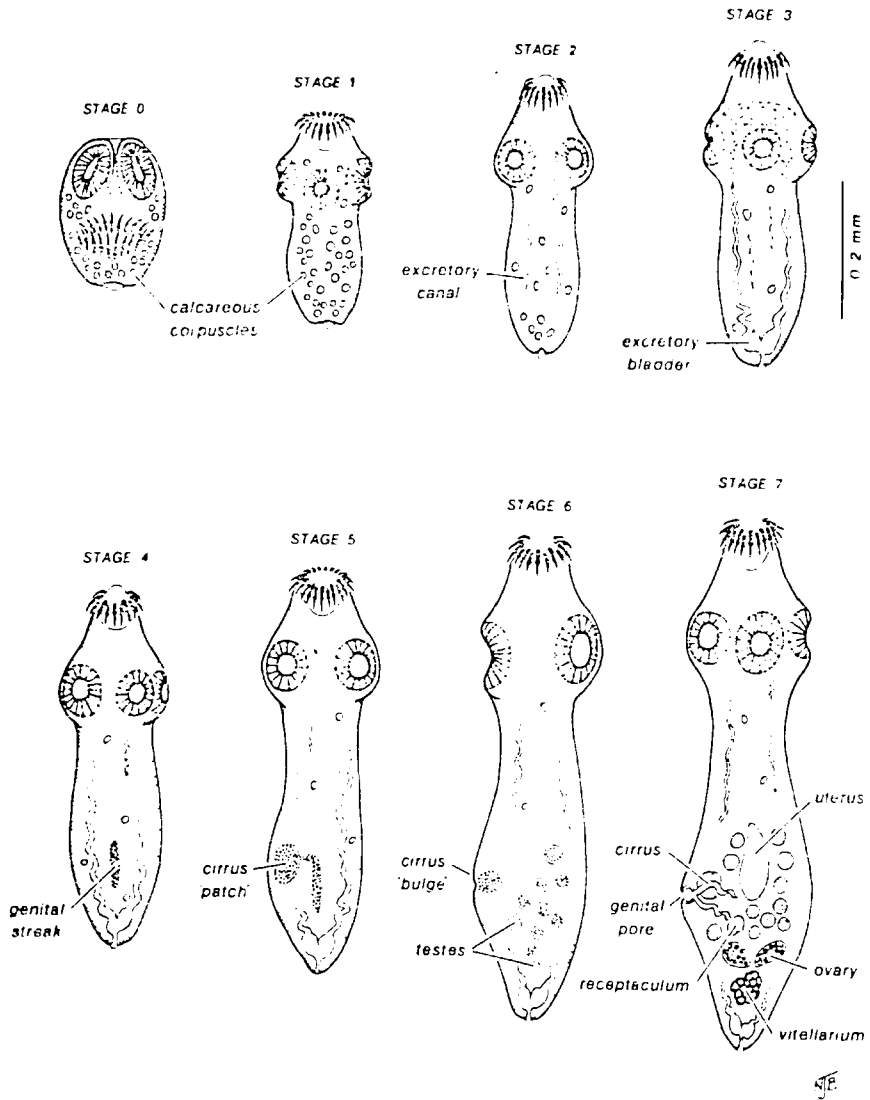


Fig. 1

NOTE: The above stages are virtually identical with the early stages of the development of *E. granulosus* *in vitro* (SMYTH & DAVIES, 1974). Thereafter, however, the *in vitro* development of the two species diverges markedly. In *E. granulosus*, the next phase of development consists of segmentation, followed by the development of proglottids. In *E. multilocularis*, further development *in vitro* generally results in the formation of genitalia, without segmentation, and the development of 'monozoic' forms, as indicated below (Stage 4-7). Some forms, however, undergo a type of pseudosegmentation (Stages IV-X, Fig. 2); these stages are dealt with later.

Stage 4. (Fig. 1). This stage is characterized by the appearance of the 'genital streak'—the anlagen of the genitalia—in the posterior strobilar region. In fresh worms, this may be seen as a somewhat faint, translucent streak which appears in contrast to the darker region of the proglottid. This streak is not always easy to see in fresh preparations, but stands out readily as a dark area in aceto-orcein preparations.

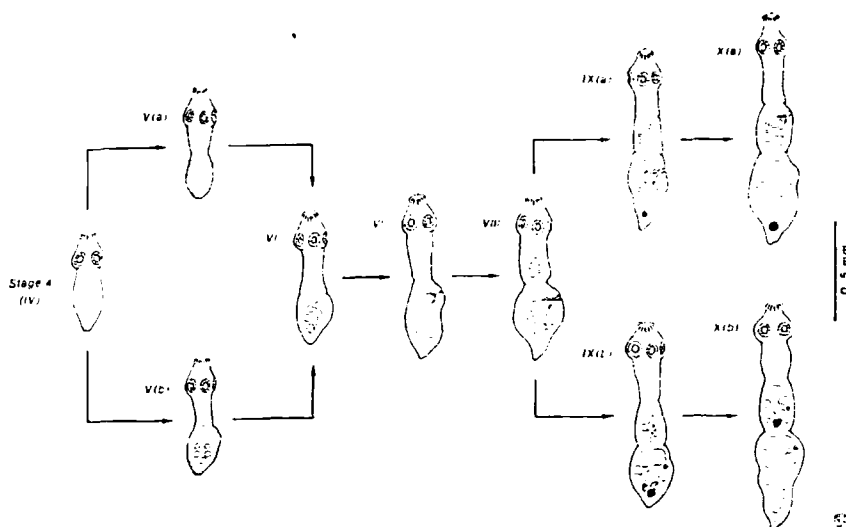
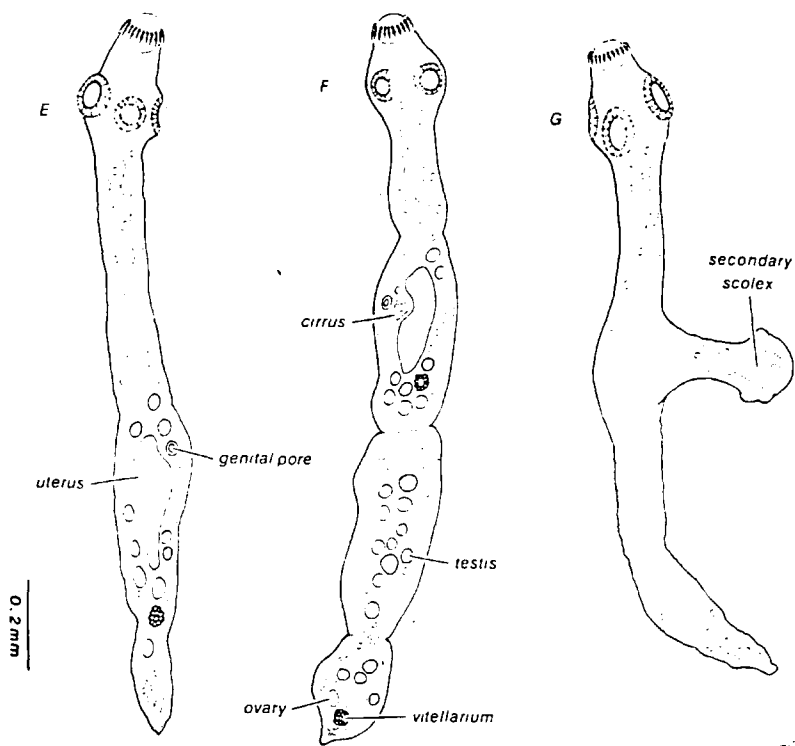
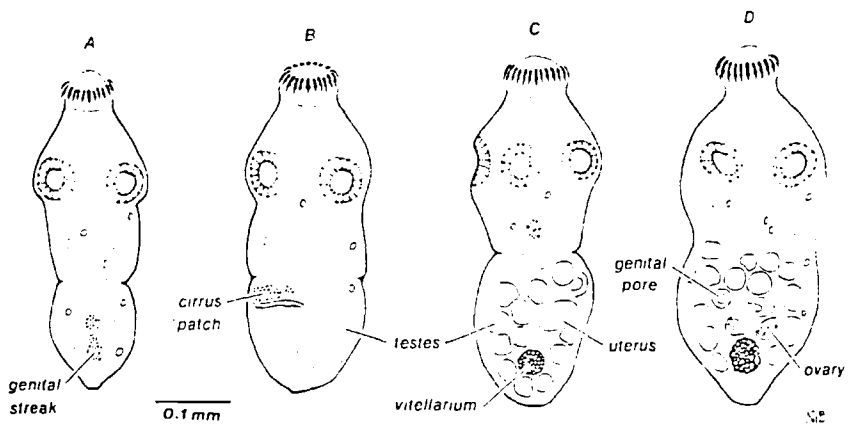


Fig. 2



Figs. 3-4

Stage 5. (Figs. 1, 6). This stage is characterized by the appearance in fresh material of a clear 'patch' which represents the cirrus anlage. It is almost oval in outline and very characteristic, once observed. In aceto-orcein preparations the cirrus anlage stains darkly and appears as a lateral extension of the genital streak.

Stage 6. (Figs. 1, 5). The side of the worm, containing the cirrus patch becomes swollen so that (what we have termed) the cirrus 'bulge' appears and the worm commences to take on an asymmetrical appearance. Testes appear as clear circular patches in fresh preparations, and various stages of spermatogenesis may be seen.

Stage 7. (Figs. 1, 11). This is essentially a sexually mature 'monozoic' form. The asymmetrical appearance, noted in the previous stage, becomes more prominent due to the further development of the cirrus region, and the genital pore is fully developed and may be seen opening and closing spasmodically. In the best developed worms the testes, ovaries and vitellarium are fully developed.

The testes stain especially well in aceto-orcein preparations and mature spermatozoa and developing spermatogonia and spermatocytes may be readily identified. Fresh sperms may also be identified in fresh preparations, but the use of neutral red is of little value in showing up the cytological details of the testes. On the other hand, as mentioned, neutral red is exceptionally useful for showing up the state of the vitellarium in fresh preparations, the vitelline cells taking up the stain readily and standing out as round, heavily stained cells. In contrast, the ovaries do not stain selectively in either aceto-orcein or fresh (neutral red) preparations and their development *in vitro* is difficult to assess.

2. 'Pseudo-segmented' development. (Stages IV-X. Fig. 2).

Although many organisms follow the pattern of 'monozoic' development outlined above, other patterns of development occur which are either alternative to the monozoic pattern or which subsequently develop from it. In most cultures, the population appears to be made up of worms showing many patterns of development, although the monozoic form predom-

minates. To distinguish these variations of development from the monozoic pattern already described, the stages have been numbered in Roman numerals; e.g. Stage V, VI, etc.

Stage IV. (Figs. 1, 2) (= Stage 4. Monozoic). This may be taken as the starting point for the alternative patterns of development.

Stage V. (Fig. 2). Two pathways of differentiation may be followed: V (a) in which the genital anlage of the 2nd proglottid (anterior) appears before the 1st set (posterior) has differentiated, or V(b) in which the 1st set differentiates as far as the cirrus patch and testes stage before the anlage of the 2nd proglottid appears. In both these stages, the developing strobila clearly becomes constricted into anterior and posterior regions, and, although (for convenience) we have used the word 'proglottid' to describe the posterior region, a 'true' interproglottid membrane does not appear to be formed between them. The process could perhaps better be referred to as 'pseudo-segmentation' as it may not be identical with the 'true' segmentation which takes place *in vivo*.

Stage VI. (Figs. 2, 6, 7). The posterior proglottid has testes and cirrus patch and the anlage of the anterior proglottid is present. This stage can clearly arise from either V(a) or V(b).

Stages VII & VIII. (Figs. 2, 8, 9). Sexual differentiation of anterior and posterior proglottid continues. This stage approaches that of an *in vivo* worm, grown in the cat for 25 days (Fig. 12).

Stages IX & X. (Fig. 2). Two pathways may be followed depending largely on the degree of somatic growth which takes place. In IX(a) further pseudo-segmentation into 3 proglottids takes place with only the anlage of the 3rd proglottid (anterior) appearing (Stage X(a)). In IX(b) only 2 sets of genitalia are formed, both of which reach maturity (Stage X (b)).

Later 'normal' development. If cultivation is continued for very long periods, say > 100 days, 3 or 4 proglottids may be formed and rather aberrant-looking elongated pseudo-segmented strobila are formed.

3. 'Multi-scolex' Development

In these long-maintained cultures too, a small proportion of worms may develop one or more supplementary scolices. This

is a remarkable phenomenon and does not appear to follow any regular pattern, nor to occur, apparently, in response to any particular stimulus. A supplementary scolex can apparently arise from any part of the strobila (a) beside the existing scolex (Fig. 13), or (b) some distance down the strobila (Fig. 12). In one case, a grotesque form with 4 scoleces and virtually no strobilar region developed (Fig. 15) but this very aberrant form has not been seen again in cultures.

The development of a supplementary scolex is signalled by the appearance of a small swelling which gradually elongates and differentiates into a sucker. As far as we can judge from morphological criteria, supplementary suckers are normal in morphology and behaviour.

4. *Cytology of genitalia*

The structure of the genitalia during early development and when sexually mature, was studied in fresh, neutral red preparations, aceto-orcein squashes and in routine sections prepared for light microscopy.

As is well recognized in *in vitro* culture systems, intracultural and inter-cultural variations were common and some worms were less well-developed than others or became stunted or showed other abnormalities. In the best-developed worms, the testes apparently developed normally, as evidenced by the appearance of morphologically normal spermatozoa; similarly, in the ovary and vitellarium morphologically normal cells were present. In sterile worms, spermatogenesis was often inhibited and mature spermatozoa were not formed in many of these worms.

Because of their relatively undifferentiated condition, it is more difficult to detect abnormalities in the ovaries and vitellarium. In stunted worms, the ovaries and vitellarium were usually difficult to locate. This could mean either that they had not differentiated or that their presence could not be detected in aceto-orcein squashes or fresh neutral red preparations.

The detailed cytology of *in vitro* worms will not be considered here but will be the subject of further studies.

5. Failure of insemination to occur *in vitro*

As with *E. granulosus* (SMYTH & DAVIES, 1974) insemination did not occur *in vitro* with the result that fertilized eggs were not obtained.

In the best-developed worms, 'cells' could be seen swirling round in the uterine cavity; these were presumed to be ova and vitelline cells. Monitoring of these cells showed that no further development had apparently occurred, since the striated, keratin embryophore—characteristic of fertilized eggs—was not formed.

DISCUSSION

Although the basic technique for *in vitro* development of the strobilar phase of *E. multilocularis* is now well established, it is clear that the development achieved *in vitro* does not yet parallel that *in vivo*. In this respect, *in vitro* differentiation is much more varied than in the case of *E. granulosus* (sheep strain) which readily segments *in vitro* and shows few of the abnormalities reported here for *E. multilocularis*.

We have discussed the 'monozoic' phenomenon elsewhere (SMYTH & DAVIES, 1975) and have emphasised that the widely held view that polyzoic cestodes arose from monozoic forms (LLEWELLYN, 1965; FREEMAN, 1973) makes this a problem of special interest from the evolutionary point of view.

There is little agreement on the origins of somatic and germ cells in cestodes. In the pseudophyllidean cestodes, the Finnish group of Wikgren and his co-workers (WIKGREN & KNUTS, 1970; WIKGREN & GUSTAFSSON, 1971; VON BONSDORFF *et al.*, 1971; GUSTAFSSON, 1977) could only identify *one* basic cell type, the *germinative cell* from which they concluded all types of cells, including germ cells, were derived. In the cyclophyllidean cestodes, on the other hand, DOUGLAS (1961) has reported the presence of *two* primitive cell types, *somatic generative* and *gonal* in *Baerietta diana*. HOWEBER, SULGOSTOWSKA (1972, 1974), working with *Hymenolepis diminuta*, named *three* types of undifferentiated cells, (a) *somatic*, along the excretory canals and nerve cords, (b) *germinative*, in the neck and genital anlagen, and (c) *germinative-somatic* in the neck and immature proglottids.

Preliminary studies by GUSTAFSSON (1976) on cytodifferentiation in *E. multilocularis* and *E. granulosus* suggest that only one primitive cell type exists. In the latter, she has described *germinative* cells with the same characteristics as those described by the Finnish group in the pseudophyllidean cestodes. These cells, which are highly basophilic and have large nuclei and prominent nucleoli, make up about 24% of the cell population.

Similarly, in *E. multilocularis*, only one primitive cell type (the 'dark-stained' undifferentiated cell) has been identified (SAKAMOTO & SUGIMURA, 1970). This again can presumably be considered the equivalent of the germinative cell of the Finnish workers.

It is clear from the results obtained in the present study that the differentiation of the germinative cell in *E. multilocularis* is very sensitive to environmental and/or nutritive conditions. In some cases, it will produce mainly genital cells and few somatic cells so that monozoic forms are produced. In other cases, somatic differentiation proceeds more or less in parallel with that of the genital cells and 'pseudosegmented' forms with 'proglottids' develop. We have termed these 'pseudo-segmented' rather than 'segmented' because it is not clear from light microscope observations whether or not a true inter-proglottid membrane is formed; further e.m. studies are underway to clarify this point. Even in these 2-3 pseudo-segmented forms, the genitalia 'bulge' in the strobila, which suggests that the rate of somatic cell division is out of phase with that of the genitalia, so that the number of somatic cells falls behind that normally formed *in vivo*. Presumably, some environmental or nutritive factor or factors is lacking in the culture medium or conditions.

The appearance of 'supplementary' scoleces on some developing strobila must again be interpreted as the response of a 'germinative' cell to *in vitro* environmental conditions different from those normally encountered *in vivo*. Usually only one supplementary scolex arises (Figs. 4G, 12), and then only after 2 to 3 months *in vitro* culture, but two are not uncommon and in one case (Fig. 15) a bizarre multiscolex form developed.

It is clear that differentiation in this species is especially 'plastic' and provides unusually interesting material for studies

on cytodifferentiation and morphogenesis. The culture medium and the environmental condition provided is at present only reasonably approximate to that produced in the dog gut and further experimentation with the nutritive constituents and the physico-chemical characteristics of this is clearly necessary. Further detailed studies at an ultrastructural level may also help to throw light on the basic problems of cytodifferentiation in this species.

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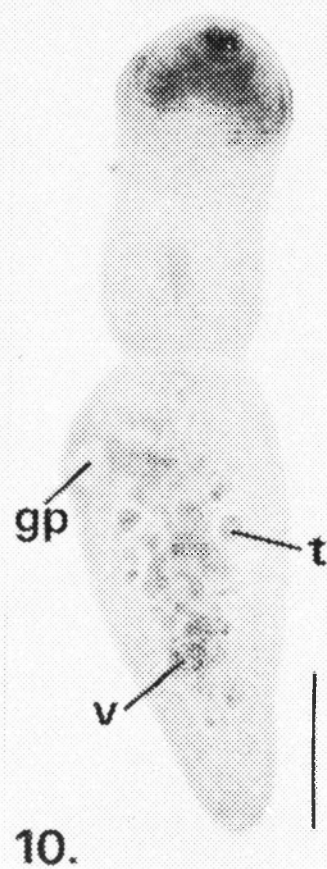
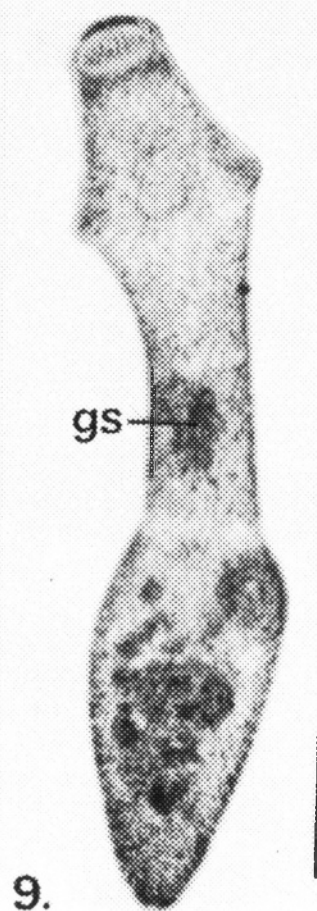
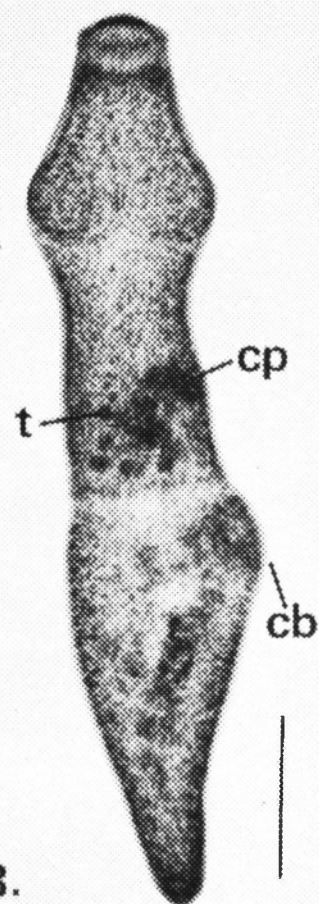
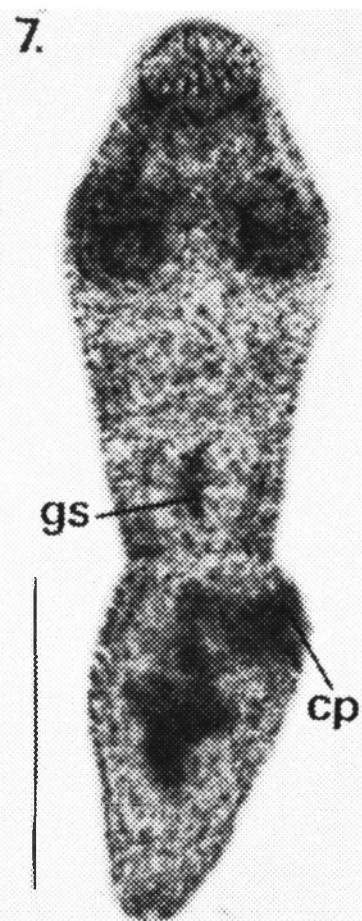
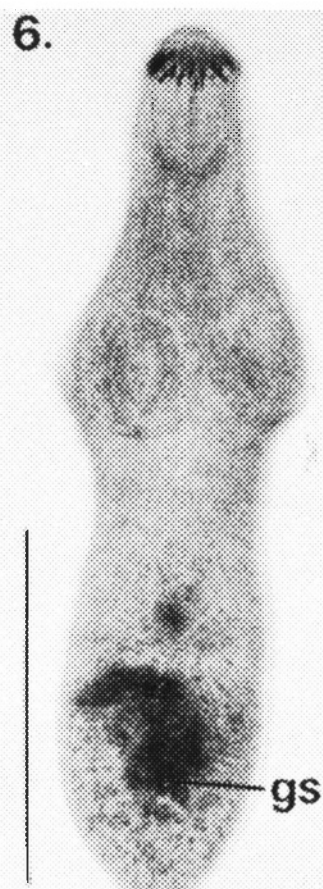
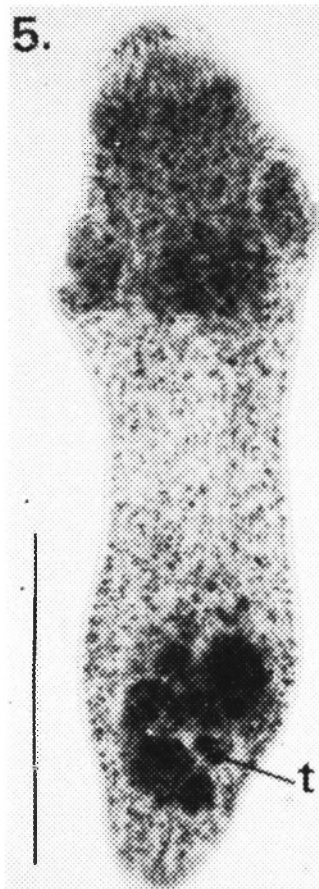
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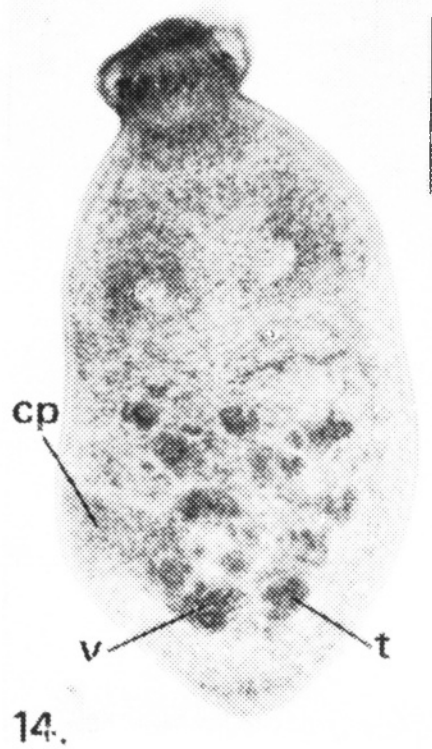
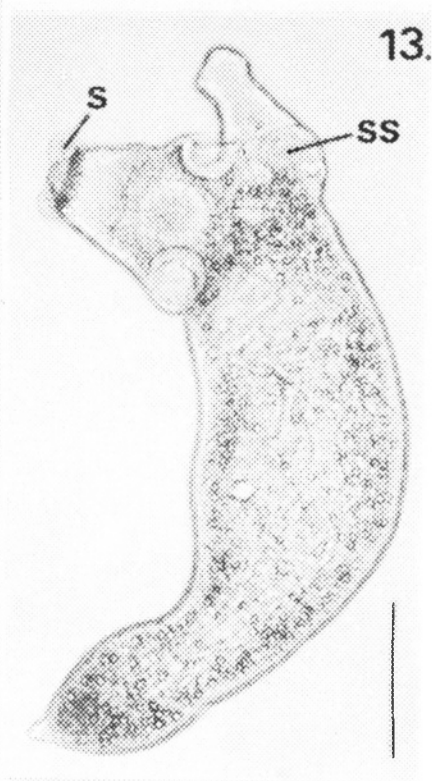
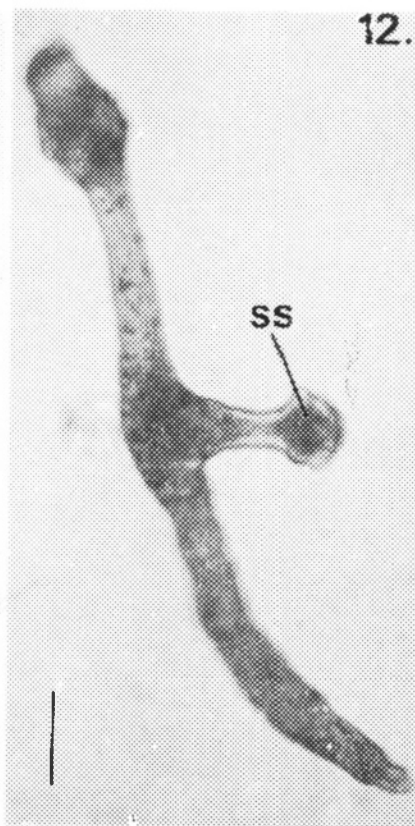
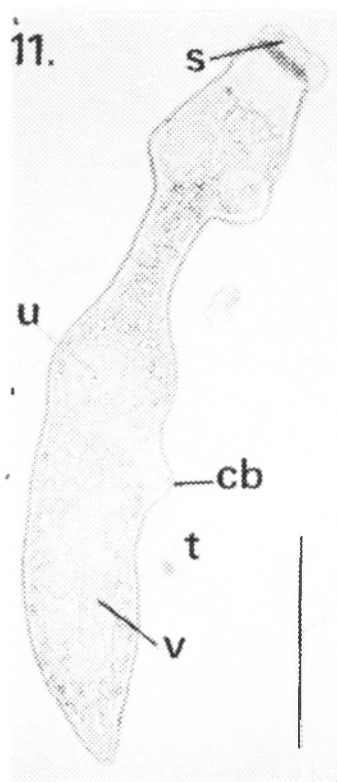
(The scale bar on the micrographs represents 200 μ m)

- Fig. 1.—Stages of development of *E. multilocularis* *in vitro* from protoscolex (Stage 0) to monozoic adult (Stage 7).
- Fig. 2.—Some common variations of strobilar differentiation *in vitro* from Stage 4 (Fig. 1) onwards, based on aceto-orcein and living preparations.
- Fig. 3.—Various stages, seen in Fig. 2, after fixation and staining with Gower's carmine.
- Fig. 4.—E. Elongating monozoic form with well developed uterus.
F. Pseudo-segmented form showing 3 sets of genitalia; interproglottid membrane poorly defined.
G. Secondary scolex developing after prolonged culture.
- Fig. 5.—Stage 6; 55 days culture. Gower's carmine.
- Fig. 6.—Stage V(a)/VI; 33 days culture.
- Fig. 7.—Stage VI; 44 days culture.
- Fig. 8.—Stage VIII; 45 days culture.
- Fig. 9.—Stage VII/VIII; 45 days culture.
- Fig. 10.—25 days in cat (resembles Stage VI/VII).
- Fig. 11.—Fully mature monozoic form; Stage 7.
- Fig. 12.—Form with lateral supplementary scolex; 101 days culture.
- Fig. 13.—Form with anterior supplementary scolex; 43 days culture.
- Fig. 14.—Monozoic form, contracted after fixation; Gower's carmine; 43 days culture.
- Fig. 15.—Multiscoler form; 64 days culture.

ABBREVIATIONS

cb. = cirrus bulge
cp. = cirrus patch
gp. = genital pore
gs. = genital streak
t. = testis
v. = vitellarium





Procedures for testing the viability of human hydatid cysts following surgical removal, especially after chemotherapy

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Summary

The basic problems involved in assessing the viability of hydatid material following surgical removal from human cases are outlined and the use of enzyme digestion techniques is shown to provide a rapid and relatively simple diagnostic procedure. The method is described in detail and examples of its practicability are cited. Infection in laboratory animals provides a back-up diagnostic procedure but cyst development in them is too slow to be of value for most clinical work.

Introduction

The increased use of chemotherapy in the treatment of human hydatidosis has focussed attention on the need for reliable procedures for assessing the drugs in question. If any hydatid material survives drug treatment, there may be a chance of secondary cysts developing. Therefore, following surgical removal of cysts—whether drug treated or not—it is important that the viability of the hydatid material be tested, both quickly and efficiently.

1. Basic Problems

Cysts which are obviously living are readily identified by the presence of protoscoleces which show flame cell activity on microscopic examination, and this method has been widely used by workers to judge cyst viability. However, the situation is much more difficult when the contents of a cyst are in a state of partial degeneration (which is often the case with human material) and brood capsules and/or protoscoleces are not immediately evident. In these cases, the pathologist is faced with a mass of amorphous tissue which has to be treated in order to extract any viable hydatid material.

In such cases, we have recently used enzyme digestion techniques, derived from *in vitro* culture (SMYTH & DAVIES, 1974; SMYTH, 1979; SMYTH *et al.*, 1980) and have found that these provide a rapid, and relatively simple, diagnostic procedure. When these methods are used in conjunction with those already available, the whole provides a set of reliable procedures for assessing cyst viability. The recommended protocol is summarized in Fig. 1, and given in detail below.

2. Treatment of cyst material

This differs initially depending on whether or not the cyst(s) has been removed intact.

a. *If cyst intact*—using a hypodermic syringe, withdraw some hydatid fluid (to reduce pressure) and transfer it to a 30 ml vial and allow it to stand for a few moments. If the cyst is highly fertile, some

brood capsules are often withdrawn with the fluid; these sink to the bottom of the storage vial and can be removed with a Pasteur pipette and examined immediately (see 3a and 3b below). The cyst may then be opened with sterile instruments and examined. In fresh cysts some brood capsules adhere to the germinal membrane, or they occur free as hydatid "sand" deposited at the bottom of the cyst. They may also be contained within daughter cysts. At this stage a portion of cyst wall and germinal membrane may be fixed for histological examination, if desired.

If brood capsules and/or protoscoleces are not immediately apparent, the contents of the cyst may be cut up finely with scissors and the whole passed through a tea-strainer type sieve. If, on microscopical examination, brood capsules or protoscoleces are still not evident in the liquid filtrate, the cyst may be sterile, or dead, or any protoscoleces present concealed in a mass of tissue retained in the sieve. This tissue may be further treated by the enzyme digestion technique, utilizing pepsin or trypsin, as detailed below (3c). Finally, a small sample of chopped cystic tissue (which may contain live germinal membranes but not necessarily protoscoleces) should be injected into appropriate laboratory hosts (e.g. gerbils, mice) to test for subsequent cystic development.

b. *If cyst is not intact*—(as in biopsies) the sample may contain the laminated membrane of the cyst wall with some ill-defined tissues and blood. Direct identification of protoscoleces is difficult in such material and the tissue is best mixed with about five times its own volume of saline (Hanks or other BSS), shaken vigorously and the whole passed through a sieve before examining the liquid and residual tissue, as above.

3. Experimental Procedures

a. *Flame cell activity*: If the protoscoleces, extracted by any of the methods outlined above, are plentiful, a sample can be placed on a microscope slide and examined for flame cell activity. This may sometimes be seen with $\times 40$ objective but more readily at oil immersion levels ($\times 90$ – 100 objective). The activity produces a faint flickering movement readily recognizable, once seen.

b. *Vital staining*: Dead protoscoleces stain readily with eosin, whereas living protoscoleces do not take up this dye. Add a few drops of 0.1% aqueous eosin to the specimens on a slide. If the protoscoleces do not take up the stain, they are viable.

c. *Enzyme digestion*: As indicated above, if the sample provided contains degenerating cystic tissue

PROCEDURES FOR THE DETECTION OF LIVING HYDATID ORGANISMS

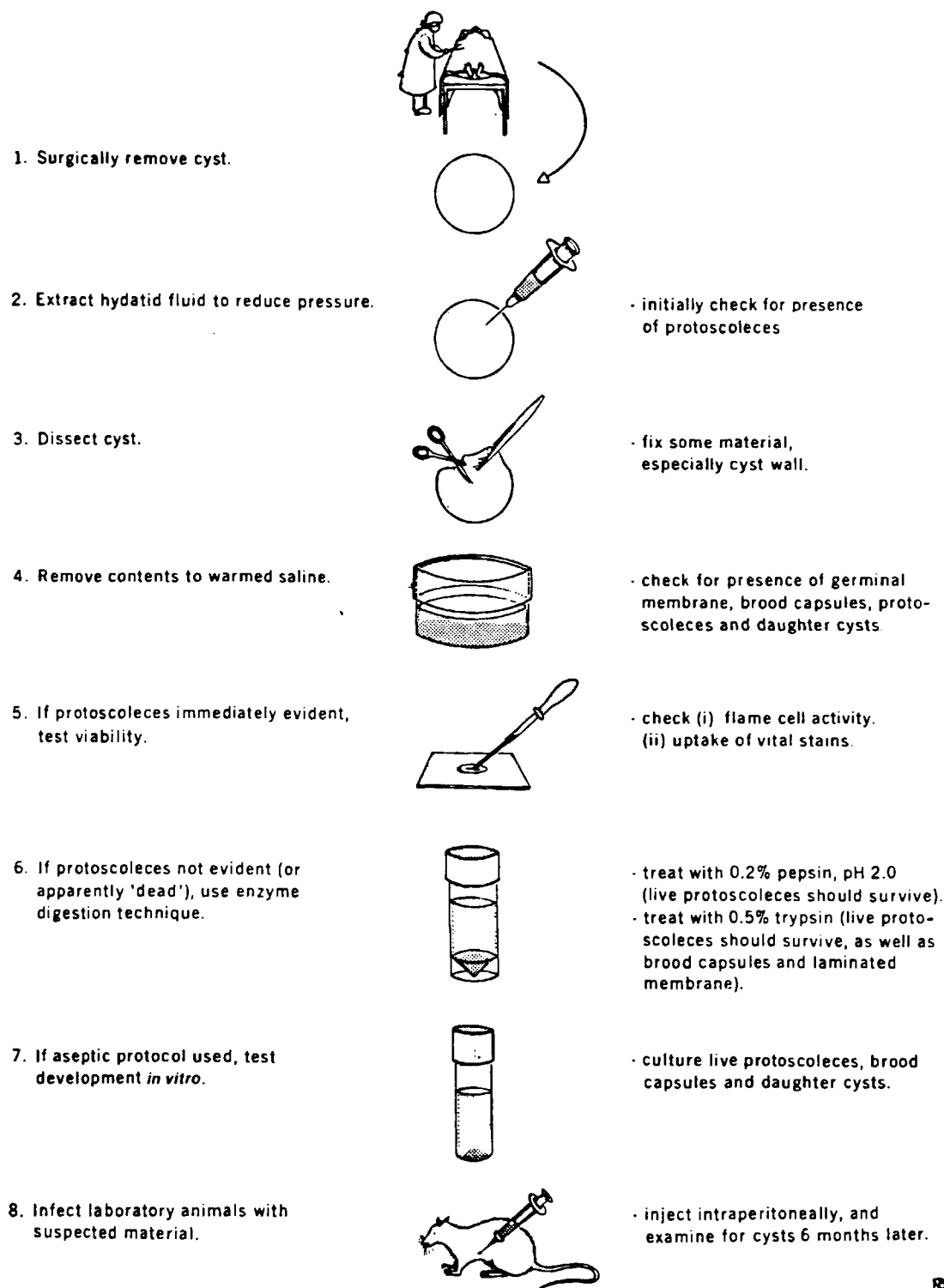


Fig. 1. Outline of procedures for the detection of living organisms in human hydatid cyst material.

and no obvious brood capsules or free protoscoleces, treatment with digestive enzymes is appropriate. Either pepsin or trypsin may be used.

(i) *Pepsin digestion*: (SMYTH & DAVIES, 1974). Treatment with this enzyme digests most of the dead material or at least "clears" it, with the result that the living protoscoleces (which readily survive digestion) become apparent. The method is particularly appropriate for small samples of about 10 to 20 ml. With larger samples, the pH of 2.0 is difficult to maintain (especially if calcified) and the trypsin digestion procedure—see (ii) below—which is less dependent on pH, is more appropriate.

Allow the material to settle in a vial and remove as much clear supernatant fluid as possible. Add (say) 20 ml of 0.2% pepsin (Sigma 1:10,000; activity 1,200 to 2,000 units/mg protein) in Hanks or BSS (adjusted to pH 2.0), and incubate at 38°C for 15 min. If a rotator or shaking water bath is available, this aids digestion but is not essential. Much dead material should normally be digested and live protoscoleces, if present, become apparent. However, longer treatment—up to 75 min—may be necessary in some cases, especially if much fibrous tissue is present in the sample.

Ideally, the digestion process should be followed under an inverted microscope but, if this is not available, samples for examination can be removed at intervals with a Pasteur pipette.

(ii) *Trypsin Digestion*: Digestion in 0.5% trypsin (Sigma Type II; activity 1,000 to 1,500 BAEE units/mg protein) in Hanks (or BSS) at pH 7.4 to 7.8 may be used instead of the pepsin treatment. Two to three hours digestion may be required and, as with pepsin, living protoscoleces survive this treatment and may readily be recognized. If a substantial amount of tissue remains undigested, the whole digest may be poured through a strainer and the liquid examined for live protoscoleces, which readily sink to the bottom.

The viability of the protoscoleces may be tested as before (see 3a and 3b), but treatment with digestive enzymes should cause any live protoscoleces to become active. However, it may be necessary to add a few drops of 0.2% sodium taurocholate or 5% dog bile in order to stimulate body contractions within the protoscoleces (often resulting in scolex evagination), thus confirming their viability.

d. *Infection of laboratory hosts*: Even if all the above tests prove negative for the presence of protoscoleces, living germinal membrane may still be present. As this is capable of regeneration and causing secondary hydatidosis, it is important to test for its viability by injection into laboratory animals. Sheep and horse hydatids will grow in gerbils and mice (HEATH, 1970; THOMPSON, 1976; PENNOIT-DE COOMAN & DE RYCKE, 1970; SCHWABE *et al.*, 1970), and these animals can serve as test hosts. Approximately 0.2 to 0.5 ml of chopped hydatid material (in Hanks or BSS) is injected i.p. into these hosts. Development is very slow, and the lower limits for cyst formation are, as yet, unknown. We have obtained cysts in gerbils six months after inoculation of human (drug-treated) material suspected of being viable.

(e) *In vitro culture to strobilar stage*: *In vitro* culture

of hydatid material is only likely to be successful if (i) the material has been removed and stored under sterile conditions, and (ii) at least 0.3 ml of settled protoscoleces are available. If these conditions are satisfied, the material may be cultured *in vitro*, following the technique of SMYTH & DAVIES (1974). SMYTH *et al.* (1980) have grown segmented worms approaching sexual maturity from human material. This technique, however, is not necessary for testing viability, but could provide valuable additional information regarding the growth rate and developmental pattern of hydatid material from different countries each of which may represent different strains (SMYTH, 1979).

Discussion and Conclusions

We believe the importance of establishing suitable criteria for assessing the viability of hydatid tissues, especially after drug treatment, cannot be over-emphasized. There is now a comprehensive literature on the use of drugs (especially mebendazole) against clinical hydatidosis in man but, in general, the criteria used by different workers have not been consistent and are often ill-defined. The above is an attempt to bring together briefly the various methods used. In particular, we would urge the use of the enzyme digestion technique in doubtful cases for, by its use, it is possible to detect the presence of even a small number of living protoscoleces in an apparently dead cyst. In one human case, following surgical removal of a cyst, standard microscopical examination showed no living protoscoleces and yet after tryptic digestion of the mass of degenerating cystic tissue (which had been treated with mebendazole) we were able to show that living protoscoleces were present, so that a chance of secondary infection was possible. In another instance, being presented with a small (and bloody) mass of cystic material (again, after mebendazole treatment) we were able to demonstrate, within approximately one hour, by use of the pepsin digestion technique, that no protoscoleces were present.

Although failure to demonstrate the presence of viable protoscoleces may be considered a fairly reliable indication of absence of live material, the ultimate criterion must be based on failure of the suspected tissue to grow in laboratory animals. Unfortunately, growth in these is extremely slow and the earliest we have so far been able to detect positively developing cysts (in gerbils) is six months—generally too slow for most clinical work, so that this procedure must be only considered to be a "back-up" diagnostic technique. If the earlier methods failed to indicate a positive result and the tissue was later shown to be viable in laboratory hosts, it does not necessarily follow that secondary hydatidosis would develop in the patient in question. However, this would be a possibility, so that further monitoring of such a patient would clearly be advisable.

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