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ECHINOCOCCUS GRANULOSUS: STUDIES ON THE DEVELOPMENT OF THE METACESTODE TEGUMENT

by

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ABSTRACT

Protoscoleces of the horse strain of *Echinococcus granulosus* were successfully cultured both *in vitro* and in BALB/c mice to produce small hydatid cysts. *In vitro* cysts were formed from vesiculating protoscoleces and also from posterior bladders and portions of brood capsule wall which had detached from the protoscoleces. *In vivo* cysts were produced after intraperitoneal injection of protoscoleces and after their implantation within diffusion chambers. The latter method proved to give a higher yield of cysts although these were generally small in size. Prior to, and during cystic differentiation the ultrastructure of the tegument was monitored using scanning and transmission electron microscopy. Initially the protoscolex tegument showed regional variations in vesicle population and surface projections. Three main types of vesicle were identified, at least two of which were thought to be of Golgi origin. During differentiation considerable change occurred in the tegument and two further vesicle types were observed. The possible function of these and other organelles is discussed with reference to parasite survival and the formation of the carbohydrate-rich laminated layer around the cyst. Production of subsequent protoscoleces within cysts, *in vivo*, involved several developmental stages. Of particular interest were the changes in the surface projections, involving the transitory appearance of microvilli and enlarged microtriches, some of which were involved in rostellar hook formation. Throughout metacestode development and maturation the importance of the Golgi complex was evident. In an attempt to disrupt this system, and hence parasite development, the effects of the ionophore monensin were monitored in cysts and protoscoleces. *In vitro* all parasites were killed by the action of

monensin. Attempts to reproduce these events *in vivo* were much less successful although certain effects were observed. It was concluded that, due to probable difficulties with drug absorption and host toxicity, monensin was an unlikely chemotherapeutic agent for hydatidosis.

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CHAPTER 1

GENERAL INTRODUCTION

Echinococcus Rudolphi 1801 is a small endoparasitic plathyhelminth belonging to the Class Cestoda. It is a true tapeworm (Subclass Eucestoda) lacking a gut, and all metabolic interchange therefore takes place across the syncytial outer covering, the tegument. The parasite belongs to the family Taeniidae, of the order Cyclophyllidea, which as adults characteristically inhabit the intestine of homoiothermic carnivores. Many aspects of the biology of *Echinococcus* have recently been excellently reviewed in the book "The Biology of *Echinococcus* and Hydatid Disease" edited by R.C.A. Thompson (1986). (For a general account of cestode biology, refer to Arme & Pappas, 1983).

The taxonomy of the genus *Echinococcus* is complex and has been reviewed by Kumaratilake & Thompson (1982). A total of 16 species have been described but only four have well defined characteristics and are generally accepted as being taxonomically valid; *E. granulosus* (Batch, 1786), *E. multilocularis* (Leukart, 1863), *E. oligarthrus* (Diesing, 1863) and *E. vogeli* (Rausch & Bernstein, 1972). Of these, *E. granulosus* and *E. multilocularis* are most important in involving humans as intermediate hosts. The present work will, however, only be concerned with *E. granulosus* although reference will be made to *E. multilocularis* where relevant.

Echinococcus has certain unique characteristics which separate it from the major genus of the family, *Taenia*. As an adult, *Echinococcus* is only a few millimetres long and rarely possesses more than four or five segments or proglottides. *Taenia* spp. in contrast, can grow to many centimetres in length and may consist of several

thousands proglottides The basic life cycle of *Echinococcus* (Fig. 1.1) requires two mammalian hosts: the definitive host in which the adult strobilar stage develops; and an intermediate host in which the post-larval or metacestode stage develops. *E. granulosus* exists as an adult in the intestine of dogs and other canids where frequently thousands of worms may be attached to the mucosa of individual hosts by scoleces bearing hooks and suckers. Gravid proglottides and/or eggs are released in the faeces and are ingested by the intermediate host which is often a sheep or horse although other mammals such as camels, pigs, cattle and man can act as suitable hosts depending on the strain of parasite involved (see later). In the intermediate host the larval stage or oncosphere hatches from the egg and invades the bloodstream by penetrating the intestinal mucosa. The oncospheres are then transported in the blood until filtered out by some suitable tissue. The largest numbers of larvae are trapped in the liver and lungs although some may also reach the spleen, muscles, brain or other organs. Once located within these tissues, the oncospheres differentiate into the metacestode stage which is known as a hydatid cyst. This is usually a single fluid filled structure which eventually produces, internally, brood capsules containing protoscoleces which may reach tens of thousands in number. The time taken for development is variable and several months usually elapse before protoscoleces are produced (fertile hydatid). Development within each host species can vary and not all cysts may produce protoscoleces (sterile hydatid).

The protoscoleces are miniature stages composed of a scolex, with hooks and suckers, which is usually invaginated into a posterior non-scolex region. They are uniquely dimorphogenetic and can develop into either adults if devoured by a dog or can differentiate into secondary hydatid cysts if released from the primary cyst within the

intermediate host.

The life cycle of *E. multilocularis* is similar to that of *E. granulosus* except that foxes, other canids and cats can act as definitive hosts and rodents are most frequently intermediate hosts. Additionally the type of hydatid cyst produced is termed 'alveolar' and is more complex than that of *E. granulosus* being a multivesicular or multilocular, infiltrating structure which extends throughout the host tissues. This proliferating nature can result in distant metastatic foci by transport of detached germinal cells in the blood or lymph.

It is the metacestode stage of *Echinococcus* which gives rise to hydatidosis or hydatid disease, a condition which is of considerable medical significance. The fully developed metacestode of *E. granulosus* is typically unilocular (Fig. 1.2). It consists of an inner, nucleated, germinal layer outside which is a tough, non-living laminated layer of varying thickness. A considerable host cellular response, often known as the ectocyst, surrounds the cyst. Asexual proliferation of the germinal layer gives rise to sac-like brood capsules in which the protoscoleces subsequently develop. The germinal layer, brood capsule wall and protoscolex outer covering are tegumentary in nature and consist of a distal cytoplasmic layer attached to underlying, nucleated tegumentary cytons. The nature of these teguments has been the subject of some ultrastructural investigation (Morseth, 1967; Lascano, Coltorti & Varela-Diaz, 1975; Bortoletti & Ferretti, 1973; 1978; Singh & Lee 1979) which has shown that, although the teguments are in continuity, regional variations between and/or within each tissue type exist. Since the germinal layer tegument gives rise to that of the brood capsule which subsequently produces the protoscolex tegument, considerable cytological differentiation must occur during cyst development.

Further information is required, however, before a full appraisal of cyst structure can be gained.

In established cysts, some of the brood capsules and protoscoleces may detach from the germinal layer and lie free in the hydatid fluid at the bottom of the cyst to give rise to what is known as hydatid sand. Daughter cysts, complete with laminated layers may also occur within some primary cysts although no conclusive explanations exist as to whether these originate from free protoscoleces, detached portions of germinal layer or from some other source.

The laminated layer is unique to *Echinococcus* spp. and is so named because of the laminations seen viewed in transmitted light. It is periodic acid - Schiff (PAS) positive and is a polysaccharide/protein complex also containing some lipids (Richards, Ilderton & Yardley, 1987) and with a predominance of galactosamine and glucosamine (Kilejian & Schwabe, 1971). This layer is now widely believed to be of parasite origin although some authors have suggested that host-derived components may also be involved in laminated layer formation (Kilejian & Schwabe, 1971; Bortoletti & Ferretti, 1978). Richards *et al.* (1983a) proposed that the laminated layer is an exaggerated glycocalyx and, in concordance with Coltori & Varela-Diaz (1974) and Bortoletti & Ferretti (1978), suggested that in addition to supporting the cyst this layer may play a significant role in protecting the parasite from immunological attack by acting as a barrier to host cells. Although Golgi-derived vesicles which may be involved in laminated layer formation have been described (Richards, 1984), Thompson (1986) stated that "the laminated layer is of parasite origin and is secreted by the germinal layer, although the precise site of synthesis has still to be convincingly demonstrated". The formation and growth of this structure therefore

requires further investigation in order to extend our knowledge of cyst development.

Cysts of *E. granulosus* can be extremely long lived and ages of up to 16 years and 53 years have been recorded in horses and man respectively (Thompson, 1986). They can also grow to considerable sizes, normally being 50-100mm in diameter but can reach some 500mm in diameter (Smyth, 1981). In man 52-77% of the parasite lesions are in the liver whilst 8.5-44% are in the lungs and the remaining 13-19% in virtually any other bodily location (Schwabe, 1986). The growth of the cyst most commonly results in pressure on other organs and frequently gives rise to upper abdominal pain. Complications include multiple cysts in the same and other organs; rupture of cysts resulting in anaphylactic responses and secondary, bacterial infection (Schwabe, 1986). Death can often result from *E. granulosus* infections particularly in areas of the world where medical facilities are limited. Treatment of human hydatidosis is most frequently accomplished by surgical removal of the cysts. This however, can create problems if protoscoleces are spilled into the peritoneal cavity during the operation as they can subsequently result in multiple secondary hydatid infections. Methods of killing spilled protoscoleces are therefore usually employed such as sterilizing with hypertonic saline (15-20%) or cetrimide (Langer, 1987) although in developing countries, where sterilization of the liver is somewhat crude, formalin is used. Chemotherapy against hydatid disease has only been partially successful, with compounds of the benzimidazole group (Schantz, Van den Bossche & Eckert, 1982; Morris, 1983; Eckert, 1986) and praziquantel (Marshall & Edwards, 1982; Yao Ping, Jun & Xun, 1985) showing some activity against cysts or protoscoleces *in vivo*. There is therefore a need to find alternative compounds or strategies of treatment which are

consistently successful against the disease.

Apart from being a disease of some medical importance, hydatidosis continually results in considerable economic losses in livestock. In areas of Bangladesh some 56% of sheep may be infected with *E. granulosus* whilst in cattle and buffalo of India, infections may be as high as 90% (Schwabe, 1986). Annual losses from condemned offal from cattle, sheep, swine and horses in Great Britain, where hydatidosis is a relatively minor disease, exceeded £70,000 in 1975-77 (Schwabe, 1986) whilst losses from sheep in Australia are over A$1.2 million per year (Kumaratilake & Thompson, 1982).

The geographical distribution of *E. granulosus* is fairly cosmopolitan and may comprise several infection cycles involving definitive hosts such as wild and domesticated dogs, foxes and wolves and intermediate hosts including reindeer, sheep, horses, goats, cattle and pigs. Foci of human infections include the Middle East, Mediterranean countries and parts of Africa. One region where the disease is extremely prevalent is in northern Kenya where one of the several pastoral tribes, the Turkana, has a high frequency of infection. In these people, observed infection rates were around 220 cases for 100,000 but the actual rate is probably much higher (Macpherson, 1983). The incidence of infection is thought to be high because of the close association that the Turkana have with their dogs. Women for example keep dogs as "nurses" to lick infants clean after they defecate or vomit. Additionally human Turkana dead are often laid out in the desert for dogs and wild carnivores to eat (Macpherson, 1983).

In Great Britain and Ireland the incidence of hydatidosis in humans is quite low. In 1981/82, however, some 68 human hydatid operations were performed in the U.K. although some of these are likely to have been immigrants from overseas (Smyth 1984). Small

foci of infection occur in areas such as mid Wales and these are generally in farming areas where there is a close sheep/dog association (Smyth, 1984). The incidence of hydatidosis in British horses, however, may be as high as 60% (Thompson & Smyth, 1975). This figure coupled with the relatively low incidence of human cases suggests that the parasites affecting horses are of a different strain which may not be infective to humans. Failure to infect monkeys with the horse strain (Thompson & Smyth, 1976) also supports this suggestion.

Considerable work has been carried out on parasites occurring in different hosts and it is now widely believed that several strains of *E. granulosus* exist throughout the world. The strains occurring in sheep and horses have received most attention and have been shown to differ considerably in their metabolic requirements (McManus & Smyth, 1978; 1982; McManus & Macpherson 1984; McManus & Bryant, 1986), and in isozyme patterns (Kumaratilake & Thompson, 1979; McManus & Macpherson 1984). In Great Britain at least, human infections appear to be solely due to the sheep strain and the horse strain does not seem to be involved. The possibility that these strains represent separate sub-species (*E. granulosus granulosus* and *E. granulosus equinus*) has been suggested (Williams & Sweatman, 1963) and other authors have even suggested that there is such a great difference in characteristics that the horse strain should possibly be considered as a separate species (*E. equinus*) (Thompson, 1986). For the present study, however, the terms 'horse strain' and 'sheep strain' will be retained.

One important aspect of the strain differences is reflected in the ability of protoscoleces to develop *in vitro*. In the 1960s and 1970s J.D. Smyth carried out extensive studies on the *in vitro* culture of *Echinococcus* to investigate the dimorphogenetic

development of protoscoleces. In his earliest experiments he cultured sheep-derived protoscoleces in a liquid medium to produce miniature hydatid cysts. Development occurred by the protoscoleces swelling or becoming vesicular and secreting a laminated layer, or by producing small posterior bladders which subsequently formed a laminated layer, eventually enveloping the whole parasite. (Smyth, 1962; 1967). Early attempts to culture protoscoleces of sheep origin to adult, strobilate tapeworms were, however, unsuccessful. Between 1966 and 1967, Smyth and his co-workers discovered that a solid, nutritive base (coagulated serum) was necessary in the culture system for strobilate development to occur, thus mimicking the conditions in the dog intestine. Techniques for this 'diphasic' culture system were subsequently improved but attempts to culture the horse strain in either adult or cystic directions were and remained unsuccessful and it was concluded that some conditions necessary for development were missing from the *in vitro* systems. (For a review of the *in vitro* culture of *Echinococcus* see Howell, 1986).

Although a considerable amount of information is available on the morphological cystic development of protoscoleces (sheep strain), Thompson (1986) pointed out that "unfortunately, as with adult *Echinococcus*, there is a pronounced lack of ultrastructural and developmental information". In a similar way, the development of protoscoleces within brood capsules, has only been described at the gross morphological level (Dew, 1922; Pennoit-De Cooman & De Rycke, 1972; Thompson 1976b) and is lacking in ultrastructural details. In view of these gaps in the knowledge of the biology of *Echinococcus* the present study aims to investigate aspects of the cystic development of the horse strain of *E. granulosus* both *in vitro* and *in vivo*, paying particular attention to the ultrastructural changes occurring in the protoscolex tegument during cyst formation and those

occurring in the cyst tegument during protoscolex formation. Since the tegument of parasitic platyhelminths is extremely important in functions such as nutrition and evasion of the host immune response (for reviews of tegument function see Lee, 1966; 1972; Lumsden, 1975 and Threadgold, 1984), a better understanding of the dynamic processes that occur during development may elucidate important systems which could be exploited in combating the parasite.

CHAPTER 2

MATERIALS AND METHODS

2.1 COLLECTION OF PARASITE MATERIAL

The source of all parasitic material used in experimental situations was from hydatid cysts of the horse strain of *Echinococcus granulosus*. In the majority of cases the cysts occurred in the liver (Fig. 2.1) but occasional lung cysts were also used. All of the material was obtained from two local abattoirs; The Red Lion Abattoir, Nantwich, Cheshire and Shelly Bank Abattoir, Huddersfield, Yorkshire. The livers were stored both before and after collection, in a cold storage room for no longer than 4 days prior to use. (Cysts ranged in size from 4-12cm although occassionally very large cysts were also obtained (>15 cm) (Fig. 2.2).

Protoscoleces were removed from the hydatid cysts under aseptic conditions in a laminar air flow cabinet (LAF). The surface of the cysts was first sterilized by painting twice with a 1% solution of alcoholic iodine, allowing the first coat to dry before applying the second. Approximately half of the hydatid fluid was then removed by puncturing the cyst with a 19 guage needle and aspirating the fluid with a 50ml syringe. The top of the cyst was then removed and the brood capsules were gently scraped off the cyst wall, using a sterile plastic pipette (Starsedt), and transferred to sterile, plastic, universal bottles (Sterilin) together with some of the hydatid fluid. The entire contents of each cyst were kept separate by placing them in individual bottles.

Protoscoleces were mechanically released from brood capsules by repeatedly passing the capsules up and down a large bore, blunt

needle with a sterile syringe. The protoscoleces were allowed to sink to the bottom of the universal bottles to enable removal of the hydatid fluid and brood capsule remnants. The protoscoleces were then washed in three 10ml changes of sterile, 0.85% NaCl and finally suspended in 10ml of sterile NCTC 135 culture medium (Flow labs.) (see Appendix 1). All bottles were labelled and stored at $4^{o}C$ for no longer than 48 hours before use. No enzyme or bile pre-treatments, as carried out by Smyth & Davies (1974b), were employed to liberate protoscoleces unless stated otherwise.

Routine samples of primary cyst tissue weretaken by carefully pulling a portion of the laminated layer, with the germinal layer and brood capsules attached, away from the ectocyst (host response layer) and removing it with a pair of scissors. The tissue was placed in 0.85% NaCl and cut into strips of approximately 3 x 10mm, before fixing for electron microscopy.

2.2 INFECTION OF A RODENT, MODEL HOST

2.2.1 Infection by injection of protoscoleces.

BALB/c mice were chosen as experimental laboratory hosts on the basis of availability and reports from other authors (Connor, 1980). Both males and females between 3 and 6 months of age were used at random. The mice were housed in groups of up to 14 and fed and watered *ad libitum* both before and after infection.

Prior to all infections the viability of the batch of protoscoleces to be used was assessed using an eosin exclusion test (Smyth & Barrett, 1980). This was carried out by taking a small sample of protoscoleces (200-300) from the original batch and covering them with a 1% solution of eosin in 0.85% NaCl in a watch glass for 1 min. The eosin was then removed and replaced with 0.85% NaCl. The protoscoleces were examined under a low power binocular microscope and the numbers taking up and excluding the stain counted. Those which took up the stain were judged to be dead and therefore a percentage viability could be calcuated for each batch. Viability in protoscoleces which excluded eosin was confirmed by the microscopic examination of flame cell activity. Only batches of protoscoleces with a viability of greater than 85% were used for experimentation.

Approximately 4000 protoscoleces in 0.1ml of NCTC 135 were injected into each mouse. The number was estimated by first re-suspending the protoscoleces in 10ml of culture medium and taking a 0.1ml sample. The numbers in the sample were estimated by counting and the batch of protoscoleces was diluted with the appropriate amount of culture medium to give approximately 4,000 protoscoleces per 0.1ml when suspended in the medium.

Mice were lightly anaesthetized prior to infection by bubbling

oxygen through a bottle of diethyl ether which was connected to a desiccating jar in which each mouse was placed. The protoscoleces were injected intra-peritoneally and the mice were returned to their labelled cages until required.

The mice were subsequently sacrificed using chloroform and the parasite material removed from the peritoneal cavity. This was either fixed immediately or separated into individual cysts or protoscoleces, depending on the age of infection, and fixed for electron microscopy. Routine eosin exclusion tests were also carried out on the retrieved material, before fixation.

2.2.2 Infection by implantation of diffusion chambers.

Development of secondary hydatid cysts in the absence of a host cellular response was investigated by the implantation of protoscoleces into the peritoneal cavity in diffusion chambers. The chambers were constructed from a kit obtained from Millipore Ltd. which involved cementing 0.3µm cellulose filters to either side of a "Plexiglass" ring (ext. diameter, 14mm; height, 2mm) with an inert cement, to give a chamber with an internal volume of 0.157ml (Fig. 2.3). The chambers were sterilized by exposure to formalin vapour in a sealed tube containing cotton wool soaked in a 10% solution of neutral buffered formalin (Appendix 2). All manipulative techniques were subsequently carried out under aseptic conditions in a LAF.

The chambers were completely filled with approximately 4,000 protoscoleces in NCTC 135 via the filling hole on the side of each chamber (Fig. 2.3) and sealed with a piece of the plastic rod supplied.

Before implantation, the mice were anaesthetized using Sagital (May & Baker) (a sodium pentabarbital preparation given at a dose of

26.4mg/kg body weight), administered by intra-peritoneal injection. This rendered the mice unconscious for approximately 30 min. Only female mice were used for these experiments as in-bred males are more susceptible to this anaesthetic and can frequently die (Wayneforth, 1980).

Operations were carried out on a Formica covered board on to which the previously shaved mouse was attached by means of elastic bands (Fig. 2.4). A longitudinal incision, of approximately 1.5cm, was made in the skin and body wall, slightly to one side of the mid ventral line (Fig. 2.5). The chamber was pushed into the incision (Fig. 2.6) and moved well into the peritoneal cavity. The body wall was sutured with 2 braided silk stitches, tied by the figure of eight method (Wayneforth,1980) (Fig. 2.7) whilst the skin was closed with 3 similar stitches and sealed with Histoacryl Blue (B. Braun) (Fig. 2.8). The mice were then left to recover in a warm room and later returned to normal housing conditions.

2.2.3 Infection by implantation of cultured cysts/protoscoleces.

Secondary hydatid cysts, grown either *in vitro* or *in vivo*, were occasionally implanted directly into mice to assess their ability to continue growing. The same operative techniques as employed in Section 2.2.2 were used to implant the parasites directly (not in diffusion chambers) into the peritoneal cavity after estimations of size and number had been made.

2.3 *IN VITRO* CULTURE OF *ECHINOCOCCUS* PROTOSCOLECES

2.3.1 Standard culture conditions.

In order to investigate the development of secondary hydatid cysts from protoscoleces *in vitro*, a monophasic culture system was adopted. NCTC 135 was chosen as a suitable synthetic culture medium and the standard medium used was NCTC 135 supplemented with 20% v/v heat inactivated, foetal calf serum (FCS) (Gibco).

The NCTC 135 liquid medium was made up from powdered form. To each litre, 2.2g of $NaHCO_3$ was added and the pH adjusted to 7.4. The medium was then sterilized by ultrafiltration through 0.22µm filters, and decanted in 80ml volumes into autoclaved medical flat bottles. To every bottle, 1,000iu penicillin, 1,500iu streptomycin and 500iu gentamycin were added to give a final concentration of 100, 150 and 50iu/ml after addition of the FCS. The medium was stored at $4^{o}C$ until required for use. FCS was purchased in sterilized form and heat-inactivated by incubation at $56^{o}C$ for 30min. The serum was stored at $-20^{o}C$ in 30ml aliquots in sterile plastic universal bottles. All subsequent manipulations were carried out in a LAF.

Cultures were carried out in 10ml round bottomed plastic culture tubes (Sterilin), containing 4ml NCTC 135 plus 1 ml FCS. Each tube was inoculated with approximately 4,000 protoscoleces and a gas phase of air was used in each case. The tubes were incubated at $37^{o}C$ and either rolled in a roller culture unit at 12 revolutions/hour or manually shaken on a daily basis. The medium was renewed every 3-4 days and no adjustments were made between changes unless stated otherwise.

2.3.2 Variations in the standard culture conditions.

A number of variations to the standard culture conditions were carried out and these fall into 7 main categories:

(i) Variation in the inoculation of cultures:

In addition to the use of mechanically freed protoscoleces,parasites were also released by enzymatic digestion and then subjected to a bile salt pretreatment, according to the methods of Smyth (1979). Brood capsules were removed aseptically to sterile universal bottles and treated with 10ml of a 0.5% pepsin solution in Hank's saline (Appendix 2) at pH2.0 for 30 min at 37^{o}C. The protoscoleces were washed several times in Hank's saline and then evaginated using 0.2% sodium taurocholate in NCTC 135 (pH 7.4) for 25 hours. The tissue was then washed in NCTC 135 and transferredto standard culture conditions.

In a number of cases, brood capsules were used directly to inoculate cultures, with approximately 250 capsules being added to each tube.

(ii) Variations in the pH of the culture medium:

The initial pH of the standard medium was 7.4 and this was not re-adjusted between changes of medium. A series of cultures was also set up at different pH values, these being 6.2, 7.4 and 8.5, and 6.8, 7.4 and 8.2. The pH of these cultures was maintained at these values by daily assessment of the colour of the medium indicator (phenol red) and adding an appropriate amount of sterile 0.1M HCl or 0.1M NaOH to return the cultures to the correct pH. All other conditions were the same as for the standard medium.

(iii) Variations in the numbers of protoscoleces per culture tube:

A series of cultures was set up under standard culturing conditions, with approximately 1,000, 1,500, 2,000, 3,000, 5,000 or 10,000 protoscoleces per tube. The pH was not adjusted between changes of culture medium but was measured at the time of change and compared with that of a culture containing no protoscoleces.

(iv) Variations in the serum content of the culture medium:

Concentrations of heat inactivated foetal calf serum used in addition to the 20% in the standard medium, were 0%, 10% and 40% v/v. Cultures containing 20% heat-inactivated horse serum (Gibco) or 20% non-inactivated FCS were also set up.

(v) Variations in the gas phase of the cultures:

Under standard conditions, a gas phase of air was used. Other gas phases employed were 100% O_2 and 95% O_2, 5% CO_2. In each case, culture tubes were gassed by passing the gas mixture through a 0.22μm sterile, "Minisart" filter (Sartorius) connected to a large bore blunt needle, and bubbling it through the medium for 1min. Cultures were re-gassed at each medium change.

(vi) Variations in the basic liquid medium:

In addition to NCTC 135 other synthetic culture media were also employed. These were RPMI 1640, M 199 with Hank's salts and CMRL 1066. (see Appendix 1). The RPMI 1640 and M 199 were obtained in powdered form from Flow Laboratories and made up in a manner similar to NCTC 135. The CMRL 1066 was obtained in sterile liquid form from Gibco. All cultures were carried out, using the additional media above, in a similar manner to section 2.3.1.

(vii) Standard cultures were also set up containing increased N-acetyl glucosamine and N-acetyl galactosamine, (Sigma) added at 3.85 mg/litre.

Where possible the above culture variations were repeated at

least once.

2.3.3 Sampling of *in vitro* cultures.

Sampling of cultures was carried out by first shaking the tubes to re-suspend the parasites and then removing approximately 0.3ml (containing 200-400 protoscoleces) using 1ml sterile plastic transfer pipettes (Sarsedt). The samples were removed to solid watch glasses where counts of various morphological types were made using a binocular microscope. Approximately half of each sample was fixed for electron microscopy whilst the remainder was subjected to an eosin exclusion test to assess the viability of the sample.

In cultures containing approximately 4,000 protoscoleces per tube, no more than three samples were taken from each tube within the first 40 days to prevent a change in protoscolex number from influencing development. For the same reason cultures containing different numbers of protoscoleces (see 2.3.2 iii) were sampled only once per tube.

With all samples, morphological types were photographed using a Reichert Zetopan microscope, with camera attachment, on Kodak Panchromatic X black and white film (32 ASA) or on Kodacolor VR colour film (100 ASA), and printed as desired.

2.4 PREPARATION OF TISSUE FOR ELECTRON MICROSCOPY

2.4.1 Transmission electron microscopy (TEM).

Samples of parasite material to be processed for TEM were fixed in 3% glutaraldehyde (Taab Laboratories) in 0.1M sodium cacodylate buffer at pH 7.2 (Appendix 2) for 8-12 hours at room temperature. The material was washed 3 times in 0.1M sodium cacodylate buffer over a 12 hour period, using a revolving stage. Post fixation was in 1% osmium tetroxide (Taab Labs.), buffered as above for 1.5 hours. After a brief buffer wash, dehydration was performed in a graded acetone series, with 15min in each concentration and 2 changes of 100% acetone. The tissue was infiltrated in 3 changes of Spurr's Premix Resin (Taab Laboratories) over 24-36 hours using a revolving stage and embedded in similar resin in circular moulds (3cm in diameter). Polymerization was carried out at 60^{0}C for 18 hours. Tissue was also embedded in LR White resin (London Resin Company) in a similar manner after dehydration in a graded ethanol series. In addition to the standard fixation procedure, tissue was also fixed in either 3% glutaraldehyde alone or in 1% osmium tetroxide alone.

Sample resin blocks were trimmed using a razor blade and ultra-thin sections (60-90nm thick) were cut on an LKB III Ultratome. Routine 0.5µm sections were also cut and stained with a 1% solution of toluidine blue in borax for examination under the light microscope. The ultra-sections were mounted on uncoated copper grids unless stated otherwise, and stained in 3% aqueous uranyl acetate for 10min and in lead citrate for 5min after a brief wash in distilled water. After a final wash, the sections were viewed in either a Jeol 100 CX II at 80 kV or a Philips EM 200 at 60 kV. Micrographs were taken on either Agfa RA710 film cut in plates (10cm x 8cm) or Kodak Microphen

35mm film. The plates were developed in an Agfa DD3700 automatic processor whilst the 35mm film was developed conventionally using Kodak D19b developer and Kodafix fixative. Micrographs were printed at the desired magnification on Kodabrome II RCF3 or F2 paper, developed in the automatic processor.

2.4.2 Scanning Electron Microscopy (SEM).

Material was fixed for SEM in the same way as for TEM or in a 3:1 mixture of 3% glutaraldehyde and 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 4-8 hours. Dehydration was carried out in a graded acetone series and the specimens were transferred to a Polaron critical point dryer in 100% acetone. When small specimens such as protoscoleces were being processed, the baskets of the dryer were lined with 0.1mm steel mesh to prevent loss of material from the baskets. The tissue was dried using liquid CO_2 for 45min and then mounted on aluminium stubs with either Electrodag high conductive paint or double sided Selotape. The specimens were then coated with a layer of gold (approximately 20-30nm deep) on an EMScope FD 500 sputter coating unit. Stubs were viewed in a Jeol 100T scanning electron microscope and micrographs were taken on Ilford FP4, 120 roll film (125 ASA). Films were developed using Kodak D 76 developer and Kodak 'Kodafix' fixative. Micrographs were printed at the desired magnification on Kodabrome II RC F3 paper.

2.5 LECTIN BINDING ANALYSIS

2.5.1 Analysis using fluorescent lectins.

Protoscoleces from horse liver cysts, 5 month secondary hydatid cysts from mice and 60 day secondary cysts, grown under standard *in vitro* conditions were fixed whole in 3% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, after washing in 0.85% NaCl. Fixation was for 1-2h at room temperature and dehydration and embeddment in LR white resin was carried out as in section 2.4.1.

Sections, 1-3μm thick, were cut on an LKB III ultratome or an LKB pyramatome and flattened on non-fluorescent, "white" glass slides using xylene vapour. Fluorescein isothiocyanate (FITC) labelled lectins were obtained from Sigma and working solutions of 50μg/ml were made up in phosphate buffered saline (PBS) (see Appendix 2). The lectins used are shown in Table 2.1 together with their sugar specificities. Competitive inhibition studies were carried out using 50μg/ml of each lectin made up in either 150 or 300mM solutions of their respective specific sugar in PBS.

One drop of each lectin solution was used to cover sections of each tissue type and slides were then incubated for 1h at room temperature, in a perspex box lined with moist tissue paper, to prevent evaporation. In addition to the sections, whole protoscoleces fixed as above, were also incubated in a few drops of each lectin solution in solid watch glasses. All tissue was then washed in 3x10 min changes of cold PBS ($4^{o}C$). This had to be done carefully in the case of the slides as undue agitation often caused the sections to become dislodged and fall off the slide. Sections were then mounted under a cover slip, in a drop of PBS, and viewed immediately. Whole protoscoleces were viewed on a cavity slide

Table 2.1 **LECTIN SUGAR SPECIFICITIES**

LECTIN	MAJOR SPECIFICITY	MINOR SPECIFICITIES
Wheat Germ Agglutinin (WGA) *(Triticum vulgaris)*	N-Acetyl-D-Glucosamine	None
Soybean Agglutinin (SBA) *(Glycine max)*	N-Acetylgalactosamine	D(+)-Galactose
Peanut Agglutinin (PNA) *(Arachis hypogaea)*	D(+)-Galactose	None
Concanavalin A (Con A) *(Canavalia ensiformis)*	α-Methyl-D-Mannoside	α-D-Glucose, N-Acetyl-D-Glucosamine
Asparagus Pea Agglutinin (APA) *(Tetragonolobus purpureas)*	α-L-Fucose	α-Methyl-D-Mannoside, N-Acetyl-D-Glucosamine, α-D(+)-Glucose

without a cover slip. The tissue was observed on a Reichert "Zetopan" microscope, equipped with a mercury vapour lamp for fluorescence microscopy. Blue light (425nm) was used to illuminate the specimens via a BG 12/3mm exciter filter and a dark ground condenser. The transmitted fluorescence (525nm) was observed using a Sp3 absorption filter and micrographs were taken on Kodak Ektachrome 400 ASA colour slide film using exposures of approximately 2 min. Colour prints were made from transparencies using the "Ektaprint" method.

In addition to controls involving the competitive sugars, sections and whole protoscoleces were also viewed untreated to assess any autofluorescence within the tissue.

Similar lectin binding analysis was also carried out on tissue pre-incubated in neuraminidase (Sigma) at 1 unit/ml in PBS for 1h at 37^{o}C to remove any terminal sialic acid residues.

2.5.2 Analysis using peroxidase conjugated lectins.

In order to try and visualize lectin binding at the ultrastructural level, those lectins which were found to bind using fluorescence microscopy were obtained conjugated to a peroxidase tag from Sigma.

Ultrathin, resin sections of both protoscoleces and secondary cysts were cut and mounted on copper grids. Both osmicated and non-osmicated tissue was used as experimental material. The grids were floated on a drop of each lectin solution (50µg/ml in PBS) placed on a slab of dental wax. Incubation was carried out for 1 hour at room temperature and in a moist environment (see Section 2.5.1). The peroxidase activity was demonstrated by the 3,3'-diaminobenzidine method, modified from Kawari & Nakane (1970) as

follows:

1. The grids were first washed thoroughly in PBS by repeated immersion, and blotted on a piece of filter paper.
2. 5mg of 3,3' diaminobenzidine (DAB) (Sigma) was dissolved in 9ml of 0.05M Tris buffer (see Appendix 2) at pH 7.6, and 1ml of freshly prepared 0.1% hydrogen peroxide (H_2O_2) solution was then added.
3. The grids were attached to glass cover slips using double-sided 'Selotape' and placed in a 10ml syringe. The DAB/H_2O_2 solution could then be repeatedly drawn over the sections in order to prevent non-specific deposits of reaction product from occurring. This was carried out for 15min at room temperature.
4. Sections were subsequently washed in distilled water in the syringe for 2-3 min, removed and allowed to dry before viewing unstained.

Control sections were prepared by omitting a) the H_2O_2 solution, b) the lectin solution and c) by incubating the sections in lectins made up in their respective sugar solution (300mM).

2.6 CYTOCHEMICAL TECHNIQUES.

The following cytochemical techniques were performed on both secondary mouse cysts and protoscoleces at various stages of development:

2.6.1 The thiosemicarbazide-silver proteinate method for carbohydrates. (Thiery, 1967).

The tissue was fixed in 3% glutaraldehyde and processed as normal but without osmication.

1. Ultrathin sections were mounted on gold grids and floated on a 1% aqueous solution of periodic acid for 20-25 min at room temperature.
2. The grids were washed in 2 rapid changes of distilled water and then floated on distilled water for 10 min.
3. The grids were floated on 1% thiosemicarbazide in 10% acetic acid for 30-45 min at room temperature.
4. Two rapid washes in 10% acetic acid were performed followed by 2x15 min washes in 10%, a 5 min wash in 5% and a 5 min wash in 1% acetic acid. Washing was completed with 2x5min changes of double distilled water.
5. A 1% silver proteinate solution was prepared by sprinkling 0.25g of silver proteinate on to the surface of 25ml double distilled water in a crystalizing dish, and leaving untouched for 20-30min. The solution was then thoroughly mixed by stirring.
6. The sections were treated with the 1% silver proteinate solution for 30min, at room temperature, in the dark.
7. Grids were washed several times in distilled water and viewed unstained.

Control grids were prepared by a) omitting the periodic acid, b) omitting the thiosemicarbazide, c) omitting the periodic acid and thiosemicarbazide and d) omitting the silver proteinate.

2.6.2 The demonstration of acid phosphatases (Barka & Anderson, 1962).

1. Whole protoscoleces and 3mm slices of mouse cysts were fixed in 3% glutaraldehyde for 3 hours at 4°C and washed overnight in 0.1M sodium cacodylate buffer at 4°C.
2. The tissue was washed for 10-20min in a buffer wash consisting of 12.5ml of 0.2M Tris/maleate buffer at pH5.2 (Appendix 2) plus 37.5ml of distilled water.
3. A complete incubation medium according to the following formula was prepared:

Stock solution	Volume (ml)	Final conc. (mM)
0.2 M Tris/maleate buffer, pH5.2	10	40
0.1 M sodium β-glycerophosphate	4	8
0.02 M lead nitrate	6	2.4
distilled water	30	

The tissue was incubated in the above medium for 45min at 37°C.

4. The material was then washed for 60min in 2 changes of a buffer wash (see 2).
5. The tissue was then embedded as normal and ultra-thin sections viewed both stained and unstained.

Control tissue was prepared by a) omitting the sodium β-glycerophosphate and b) omitting the lead nitrate.

2.6.3 The demonstration of thiamine pyrophosphatase (Novikoff & Goldfischer, 1961).

1. The tissue was fixed and washed as in 2.6.2.
2. A complete incubation medium was prepared according to the following formula:

Stock solution	Volume (ml)	Final conc. mM
0.02 M lead nitrate	2.0	4.0
0.05 M manganous chloride	1.0	5.0
0.2 M Tris/maleate buffer, pH7.2	4.0	80.0
thiamine pyrosphosphate (cocarboxylase)	11.5mg	2.5
distilled water	3.0	

3. The tissue was incubated in the above medium for 1 hour at 37°C.
4. The material was then washed for 30min in a buffer wash solution (12.5ml of 0.2M Tris/maleate buffer, pH 7.2, plus 37.5ml of distilled water.
5. Normal processing of material was carried out for TEM both with and without osmication and ultra-thin sections were viewed both stained and unstained.

Control tissue was prepared by a) omitting the thiamine pyrophosphate and b) omitting the lead nitrate and manganous chloride.

2.7 EFFECTS OF MONENSIN ON PROTOSCOLECES AND CYSTS

2.7.1 *In vitro* experiments.

The sodium ionophore monensin was obtained as a crystalline, sodium salt from Sigma and as a gift from Lilly Research Laboratories. A 10μM solution of the ionophore was made up by dissolving 6.929 mg in 1ml of absolute ethanol and carefully adding this to 999ml of NCTC 135 culture medium pH 7.4. No other antibiotics were added. The solution was sterilized by ultra-filtration through

a 0.22μm filter and decanted into 100ml bottles for storage at 4°C.

Protoscoleces:

Approximately 10,000 fresh protoscoleces were incubated at 37°C in 10ml of the 10μM monensin solution in sterile plastic universal tubes. Samples of approximately 300 protoscoleces were taken for electron microscopy at the following times:

Expt. A) 0 min, 2 min, 5 min, and 15 min.

Expt. B) 0 min, 10 min, 30 min, and 60 min.

Expt. C) 0 min, 1 hour, 3 hours, 8 hours, 12 hours, 24 hours and 36 hours.

Control cultures were set up in NCTC 135 plus 0.1% ethanol and sampled at the end of each incubation period.

Additional experimental and control cultures were also performed to observe the motility of protoscoleces in warm culture medium and to assess their viability using an eosin exclusion test (see Section 2.2.1). These samples were taken at 0 min, 1 hour, 3 hours, 8 hours, 12 hours, 24 hours, 30 hours and 36 hours, and the experiments replicated a total of three times. Similar viability assessments were also carried out using 5μM and 2.5μM solutions of monensin in NCTC 135, with samples being at 0 hours, 1 hour, 3 hours, 8 hours, 12 hours, 24 hours, 36 hours, 48 hours, 72 hours and 84 hours. Control cultures were sampled at: 0 hours, 24 hours, and 84 hours.

Secondary mouse cysts:

Cysts were removed from mice between 9 months and 1 year post-infection, washed 3 times in sterile 0.85% NaCl at 37°C and incubated in 10ml of 10μM monensin in NCTC 135 at 37°C. The incubations were carried out in sterile, plastic, universal tubes with approximately 100 cysts, of between 2 and 5mm in external diameter, per tube.

Six cysts were sampled for electron microscopy at the following

times: 0 min, 2 min, 5 min, 15 min, 30 min, 1 hour, 3 hours, 8 hours, 12 hours, 24 hours 36 hours and 56 hours.

Control incubations using NCTC 135 plus 0.1% ethanol, were sampled at 15 min, 24 hours, and 56 hours. All incubations and sampling were carried out twice.

Additional incubations, set up for morphological observation, were carried out as above in 10μM, 5μM and 2.5μM solutions of monensin in NCTC 135. Twenty to fifty cysts were placed in each tube and these were monitored, within the culture tubes, under a binocular microscope, at 0 hours, 24 hours, 48 hours, 72 hours, and 96 hours and any morphological effects noted. Cysts from each solution and a control solution were sampled at the end of each incubation period for electron microsocpy. All incubations for morphological observation were carried out a total of three times.

In vitro cultured cysts:

Incubations, set up as above, were carried out using approximately 2,000 *in vitro* cultured cysts (108 day), complete with laminated layers, in 10μM monensin. Approximately 50 cysts were sampled for electron microscopy at 0 min, 2 min, 5 min 15 min, 30 min, 1 hour, 8 hours, 24 hours and 56 hours with control samples being taken at 1 hour and 56 hours. Similar 10μM monensin incubations were also performed using 32 day *in vitro* cultured cysts with samples being taken at 0 hours, 8 hours, 24 hours and 56 hours. Controls were sampled at 56 hours.

2.7.2 *In vivo* experiments.

To assess the effects of monensin on *Echinococcus* within rodent hosts, a series of experiments was carried out using various modes of drug administration.

Expt. 1. Intraperitoneal injection of 10μM monensin.

Five mice, with a 10 month infection, were injected i.p. with 0.2ml of 10μM monensin in NCTC 135 daily; 2 mice, with a similar infection, were injected with 0.2ml of NCTC 135 plus 0.1% ethanol daily and 2 infected mice were left untreated. All injections were carried out under light anaesthesia as in Section 2.2.1. Test mice were killed after 0 days, 7 days, 10 days, 14 days and 21 days and the cysts were removed and processed for electron microscopy. Control mice were killed after 10 days and 21 days and treated in a similar manner.

Expt. 2. Intraperitoneal injection of 20μM monensin.

Five mice, with a 10 month infection, were injected as in Expt. 1, with 0.5ml of 20μM monensin in NCTC 135 plus 0.1% ethanol. Two control mice were injected with 0.5ml of NCTC 135 plus 0.1% ethanol and 2 mice were left untreated. Test mice were killed after 7 days, 21 days 30 days, 50 days and 60 days; injected control mice were killed after 30 days and 60 days; and uninjected control mice were killed after 30 days and 60 days.

Expt. 3. Administration of monensin by gavage.

A 2.5mg/ml suspension of monensin was made up in a 5% solution of acacia (BDH chemicals). Assuming that each mouse weighed approximately 25g, 0.1ml of this suspension would give an equivalent dose of monensin of 10mg/kg body weight. Gavage was carried out by lightly anaesthetizing the mouse with ether (see Section 2.2.1) and placing either a metal gavage needle or a 10cm piece of plastic tubing (2mm in diameter), attached to a 19 guage needle, into the oesophagus and injecting the suspension into the stomach via a 1ml syringe. This procedure had to be done carefully as the mice occasionally died after gavage, presumably due to the needle/tubing entering the trachea rather than the oesophagus. The conditions of

monensin administration are shown below and those mice which died after gavage are denoted (x) whilst those which were sacrificed are denoted (k).

Six mice with a 7 month infection were gavaged 0.1ml of the monensin suspension daily and cysts were removed at 4 days (x), 10 days (k), 15 days (k), 20 days (x), 30 days (k) and 57 days (x).

Four control mice with a similar infection, were gavaged 0.1ml of 5% acacia solution daily and cysts removed after 10 days (k), 15 days (k), 30 days (k) and 47 days (x).

Two infected mice were left untreated and killed after 47 days and 57 days.

Three mice were pre-gavaged with monensin (0.1ml/day) for 10 days prior to i.p. infection with 4000 protoscoleces and subsequently gavaged 0.1ml/day after infection. Parasites were removed 8 days (x), 20 days (x), and 38 days (x) post infection.

Two mice were treated in a similar manner with 0.1ml/day of 5% acacia solution and parasites removed after 28 days (k) and 38 days (k) post infection.

Three mice were infected, i.p., with 4,000 protoscoleces and subsequently gavaged 0.1ml/day of the monensin solution. Parasites were removed after 7 days (k), 40 days (k), and 52 days (k) post infection.

Two mice were treated in a similar manner with 0.1ml/day of 5% acacia solution and parasites removed after 7 days (x) and 40 days (x) post infection.

Two mice were infected, i.p., with 4,000 protoscoleces and left untreated. Parasites were removed after 20 days (k) and 40 days (k) post infection.

All removed parasite material was inspected for morphological changes and processed for electronmicroscopy.

Expt. 4. Incorporation of monensin into feed.

A sample of Romensin, a cattle feed additive containing 10% monensin, was obtained as a gift from Elanco Products. Normal mouse feed, in the form of pellets was ground down in an electric mill and 3g of Romensin was added to 997g of the ground feed to give a final monensin concentration of 300mg/kg. The mixing of the feed was done carefully in small amounts, to ensure an adequate distribution of the drug within the food. The monensin-containing feed was then reconstructed into pellets using a press and individual pellet maker. Six mice, with a 12 month infection, were fed the drug-containing pellets *ad lib.* whilst 3 control mice were maintained on a diet of normal pellets.

2.8 STEREOLOGICAL ANALYSIS.

A limited stereological analysis was carried out on certain tissue using a Kontron Videoplan computerised image analysis system at the Department of Zoology, Queen's University of Belfast. The tissue analysed was: a) protoscoleces subjected to 10μM monensin *in vitro* for 1 hour; b) control protoscoleces, incubated in NCTC 135 plus 0.1% ethanol for 1 hour; c) 9 month-old mouse cysts subjected to 10μM monensin *in vitro* for 1 hour; d) 9 month-old mouse cysts incubated in NCTC 135 plus 0.1% ethanol for 1 hour; e) protoscoleces taken directly from horse cysts. All micrographs were taken at random from at least 3 individual parasites and printed at X 39,100.

In tissues a) and b) the mitochondria and Golgi complexes of the tegumentary cytons were analysed; in tissues c) and d) the tegumentary mitochondria were analysed and in tissue e) the distribution of certain tegumentary vesicles within the scolex and soma regions of the protoscolex was investigated. In each case the area, perimeter and numbers of each organelle within a reference area of cytoplasm were measured, enabling the calculation of the following stereological parameters; the volume fraction (vv), the surface to volume ratio (sv), the mean volume (m.vol), and the mean surface area (m.sur).

Where additional information into the size etc. of various structures is given, the means and standard errors were calculated where (n) > 30, unless stated as different.

CHAPTER 3

MORPHOLOGICAL DEVELOPMENT OF HYDATID CYSTS *IN VITRO*

3.1 INTRODUCTION

The aim of *in vitro* culture of parasites is to provide a medium which contains all of the factors necessary for maintenance and development of the organisms so that their biochemistry, developmental biology and physiology can be studied satisfactorily, isolated from host influences. Ideally every molecule of the medium should be defined and the physio-chemical properties completely controlled (axenic culture). In practice however, many culture systems contain various animal body fluids or tissue extracts, the entire components of which are not completely defined. Replicating the exact physio-chemical conditions of the host also provides difficulties and often the "triggers" necessary for certain aspects of development cannot be reproduced.

Over the past 60 years the *in vitro* development of *Echinococcus* spp. has been the subject of considerable investigation (for reviews see Taylor & Baker, 1978; Smyth, 1985; Howell, 1986). The earliest work was carried out by Dévé (1926) who cultured protoscoleces of *E. granulosus* in 2 ml of hydatid fluid plus 0.5-1.0 ml fresh unheated horse serum. Some protoscoleces were seen to swell or become vesicular, increasing in volume by 2 or 3 times after 14 days. Coutelen (1927a, 1927b) succeeded in maintaining *E. granulosus* protoscoleces for 31 days in hydatid fluid plus human ascitic fluid or in hydatid fluid plus "extrait globulaire". Approximately 1/50 of the protoscoleces became vesicular and increased dramatically in volume although none produced a laminated layer. In 1928 however,

Dévé cultured *E. granulosus* protoscoleces for 43 days in equal parts of hydatid fluid and human ascitic fluid and obtained some cysts with laminated layers.

Little subsequent work was done until 1957 when Rausch & Jentoft cultured portions of germinal layer of *E. multilocularis* cysts in Hank's balanced salt solution supplemented with vole embryo extract and HeLa cells for 134 days. In this medium the tissue proliferated and produced vesicles by the 29th day. After 55 days, some vesicles allegedly contained up to 20 protoscoleces although no indication of the formation of a laminated layer is given. Gurri (1963) also failed to produce cysts with a laminated layer by culturing protoscoleces of *E. granulosus*.

In 1962, Smyth carried out an extensive culture programme to study the cystic development of protoscoleces of the sheep strain of *E. granulosus*. This initially involved removing brood capsules, aseptically, from primary cysts and treating them with a pepsin solution to remove the brood capsule wall and any dead protoscoleces (which were reported to be from 5 to 50% of all protoscoleces present). The freed protoscoleces were then cultured in a variety of media, comprising various combinations of natural (hydatid fluid, bovine serum and bovine amniotic fluid) and synthetic (Parker M 199) media and animal tissue extracts (beef embryo and chick embryo), with a gas phase of air. Medium "L" (2 ml bovine amniotic fluid + 8 ml of M 199) provided the best conditions. In this protoscoleces developed in two ways; they either began to swell or vesiculate during the first few days of culture, eventually secreting a laminated layer after 31-48 days; or produced a swelling at the posterior end, known as a posterior bladder. This bladder was seen to grow during the first few weeks until it was 2-3 times the size of the protoscolex, and became bilobed before a laminated layer was secreted around it

after 31-48 days. This layer was initially confined to the bladder but subsequently enveloped the entire protoscolex. By 55 days the posterior bladders had become reduced in size while the protoscoleces became increasingly vesicular until after 80 days when only a large clear cyst with a laminated layer remained. The cultures were carried on for 112 days when many of the cysts were greyish in appearance, containing lipid deposits whilst others were clearly degenerating. Similar success was also obtained by Pauluzzi, Sorice, Castagnari & Serra (1965) culturing protoscoleces from sheep in Medium 199 and various sera.

Other authors obtained parallel types of development when culturing protoscoleces of *E. multilocularis*. Yamashita, Ohbayashi, Sakamoto & Orihara (1962), culturing in 0.5% lactalbumen in Hank's solution + bovine serum, bile and mouse liver extract, at pH 7.4, obtained protoscoleces which developed in three ways. After 3-4 days some individuals vesiculated, became globular, and a laminated layer and increased numbers of calcarious corpuscles were present after 20 days. Other protoscoleces developed a bladder which appeared at the posterior or anterior end and which grew as the protoscolex degenerated. A laminated layer was eventually secreted around this bladder. The third type of development involved a mixture of the two previous types i.e. the protoscoleces vesiculated and produced a bladder. These too produced a laminated layer, whereas protoscoleces which did not vesiculate degenerated after 80 days. Cultures incorporating a high bile concentration or those which contained no serum had a high protoscolex mortality within the first 20 days.

Webster & Cameron (1963), using Morgan's M 150 or CMRL 1066 plus glycogen, at pH 7.4, also observed different types of development in *E. multilocularis* protoscoleces. These were seen to either vesiculate entirely or to vesiculate at the anterior or posterior end

only. In all cases the suckers were eventually absorbed and the hooks lost. Cysts with a laminated layer and a strand of supporting tissue, running from anterior to posterior, were eventually seen in the cultures. The authors also noted that varying the conditions of culture affected the type of development. A high serum concentration increased the proportion of vesiculating protoscoleces as did a pH of 7.0-7.4. Lowering the pH to 6.8 increased the number of protoscoleces which segmented, showing adult development, whilst a pH of 5.0 or 8.0 caused the protoscoleces to die.

The effects of altering the culture conditions were further investigated by Smyth (1967). Protoscoleces of the sheep strain of *E. granulosus* were cultured in a basic medium of Parker M 199 or M 858 + 20% hydatid fluid, under a gas phase of 5% CO_2, 8% O_2 in N_2, after being released from brood capsules by artificial gastric fluid (pepsin, trypsin and pancreatin). Protoscoleces were again found to either vesiculate and produce a laminated layer after 21-28 days; or produced posterior bladders which, after about 12 days, became sticky and subsequently produced a laminated layer. This second type of development was more common, because generally only about 10% of protoscoleces vesiculated in the first 2 weeks. The proportions of vesiculating protoscoleces were however affected by the gas phase and the pH of the medium. High O_2 tensions (95% O_2, 5% CO_2) or anaerobic conditions were found to increase the proportion of vesiculating protoscoleces (75% and 24% respectively after 48 hours compared with <10% using 10% or 20% O_2). Similarly, an acid pH of 6.5 produced 75% vesicular protoscoleces after 4 days whilst a pH of 7.4 produced <5% vesicular protoscoleces after 8 days. High pH values of 7.8-8.0 were also seen to increase vesiculation and the author concluded that vesiculation was not due to any one factor but probably reflected a response to any abnormal conditions encountered by the parasite. No

conclusions were drawn about the proportions of protoscoleces developing posterior bladders, since altering the enzyme pretreatments had no effect, although a high bile concentration initially suppressed bladder formation for a short time.

Other authors obtained somewhat similar results with various media and in 1976, Heath & Osborn demonstrated the parasite origin of the laminated layer, which had previously been questioned, by culturing protoscoleces of the sheep strain of *E. granulosus* in the synthetic medium NCTC 135 which had no serum or tissue supplements. This medium was also used to culture oncospheres of *E. granulosus* to cysts, when supplemented with 20% FCS (Heath & Lawrence, 1976). In the study by Heath & Osborn (1976) protoscoleces, released from brood capsules by artificial gastric fluid, formed posterior bladders (50%) or vesiculated (40%) and formed laminated layers after 28-56 days. When the parasites were given a shorter enzyme treatment however, vesiculation was almost 100% and the first laminated layers appeared after 17 days. The authors therefore suggested that the formation of posterior bladders was evidence of damage caused by prolonged pepsin treatment. Another interesting phenomenon reported by these authors was the apparent fusion of posterior bladders to form a single vesicle to which protoscoleces were attached on the outside. In general, these structures did not produce laminated layers unless 10-40 protoscoleces were present. Coutelen (1927a, 1927b), Smyth (1962) and Brudnjak, Cvetnić & Wikerhauser (1970) also reported similar structures in the early stages of culture but interpreted these as being brood capsules which had ruptured and everted so that the protoscoleces were attached to the outside of the brood capsule wall. Smyth (1962) reported that these vesicles, when shaken free of protoscoleces, went on to produce a laminated layer similar to that of vesicular protoscoleces. Brudnjak *et al.* (1970), however, found

that these structures, with attached protoscoleces, did not develop laminated layers although vesicular protoscoleces, in the same medium, did. There therefore seems to be no consensus concerning the precise origin or fate of these vesicles or the posterior bladders.

In most of the early cultures of *Echinococcus*, protoscoleces were seen to develop only in the cystic direction. Smyth, Howkins & Barton (1966) and Smyth (1967), however, found that, for development in an adult direction, protoscoleces must be evaginated and cultured in a diphasic medium, comprising a liquid and solid nutritive phase. The authors reported that evagination of protoscoleces was accelerated by exposing them to a bile solution after enzyme pre-treatments, thus replicating the conditions in the dog gut. Various solid nutritive bases were tried and it was found that heat-coagulated bovine serum produced the best development. With this as a solid phase and M 858 as a liquid phase, under 10% O_2, 5% CO_2 in N_2, protoscoleces of the sheep strain of *E. granulosus* segmented, producing 2 or 3 proglottides. It was therefore postulated that contact with a solid nutritive base (possibly replicating conditions in the crypts of Lieberkuhn of the dog intestine) somehow initiated a strobilization stimulus which resulted in the ultimate production of proglottides. Subsequent modifications to this diphasic culture technique are reviewed by Smyth, Miller & Howkins (1967) and Smyth & Davies (1974b).

Although the sheep strain of *E. granulosus* has been cultured successfully in both the adult and cystic direction, the horse strain has proved more difficult. In numerous attempts to culture horse-derived protoscoleces to adults (diphasic medium), the parasites only evaginated and grew slightly with no subsequent development, even after 40 days (and presumably neither did they show any cystic development) (Smyth & Davies 1974a; Smyth, 1979; Smyth & Davies,

1979; Smyth, 1985). There therefore seems to be some physiological differences between these two strains. Only Benex (1968a, 1968b) has reported some success in culturing parasitic material of horse origin. Using M 199, supplemented with chick serum or amniotic fluid, it was reported that pieces of germinal layer, when cultured alone, produced cysts after 3 weeks, which subsequently produced a laminated layer (Benex 1968b). Protoscoleces, however, when cultured, did not form a laminated layer unless some fragments of "germinal layer" remained attached (Benex, 1968a). It is not clear, however, in the latter study, whether the protoscoleces originated entirely from horses or were derived from both horses and cattle.

Subsequent work, in monophasic media, has shown the vesicular and posterior bladder development of horse-derived protoscoleces but has failed to produce cysts with laminated layers (Richards, Bridges, Rogan & Arme, 1984; Casado Escribano & Rodriguez Caabeiro, 1985; Casado Escribano, Rodriguez Caabeiro & Hernandez Rodriguez, 1985). Sheep-derived protoscoleces, cultured in the same media, however produced a well developed laminated layer (Casado Escribano & Rodriguez Caabeiro, 1985; Casado Escribano *et al.*, 1985).

The conditions required for the formation of a laminated layer in the horse strain must therefore be met before development of this structure, and ultrastructural tegumentary changes associated with its development, can be studied. The following results report the development of protoscoleces of horse origin in various monophasic media. The variations in culture conditions were, however, not designed to investigate the specific effects of altered physiological parameters in detail, but rather to find optimum conditions for maximum production of cysts with well developed laminated layers.

3.2 RESULTS

Aspects of the following observations have been published in two papers, Rogan & Richards, 1986a and Richards & Rogan, 1986.

3.2.1 Morphological types found during *in vitro* culture.

Protoscoleces of the horse strain of *E. granulosus* were seen to develop in a broadly similar manner to protoscoleces of the sheep strain of *E. granulosus* and *E. multilocularis* when cultured in a monophasic medium (see Introduction for references). Certain differences in such development were however observed.

Vesiculation of protoscoleces:

Invaginated protoscoleces (approximately 100 μm in length (Fig. 3.1)and evaginated protoscoleces (approximately 175 μm in length (Fig. 3.2) commenced vesiculation from the second day of culture onwards. Invaginated protoscoleces first took on a slightly swollen, angular appearance (Fig. 3.3) before forming a globular structure from approximately 7 days onwards. These vesicular protoscoleces remained invaginated and internal strands of supporting tissue often connected the invaginated scolex to the distal end of the protoscolex. This tissue either took the form of a central column (Fig. 3.4) or a series of narrower, individual strands radiating from the scolex region (Fig. 3.5). At this stage the number of calcareous corpuscles had become reduced and those remaining were associated with the internal supporting tissue (Figs. 3.4, 3.5). These vesicular protoscoleces were approximately 140-180 μm in diameter after 35 days.

Evaginated protoscoleces vesiculated in several ways. Firstly

the entire protoscolex vesiculated uniformly, initially forming a slightly pear-shaped structure (Fig. 3.6) and then a spherical structure with the 4 suckers towards the mid-line and the crown of hooks at the apex (Fig. 3.7) at about 10 days. Secondly the posterior portion (soma) of the protoscolex vesiculated before the anterior scolex region. Swelling started in the posterior half of the soma (Fig. 3.8) and proceeded anteriorly until the whole of the soma had vesiculated while the scolex remained unchanged (Fig. 3.9). Vesiculation of the scolex again proceeded anteriorly, first in the sucker region (Fig. 3.10) and then in the rostellum (Fig. 3.11). The final vesiculated protoscolex was roughly spherical (150-200 μm in diameter at 35 days) but differs from those formed by entire protoscolex vesiculation in the more anterior position of the suckers.

The third type of vesiculation was initiated in the scolex (Fig. 3.12) and proceeded posteriorly, eventually forming a globe-like structure similar to protoscoleces showing entire vesiculation. This third type of evaginated vesicularisation was less common than the previous 2 types which occurred with equal frequency. As with the invaginated type of development, evaginated vesicular protoscoleces possessed strands of supporting tissue running from anterior to posterior, and a reduction in the number of calcareous corpuscles (Figs. 3.10, 3.11).

In successful cultures, the first signs of laminated layer formation involved the vesicular protoscolex becoming sticky and attracting any debris within the culture medium (Fig. 3.13). The earliest that this stage was recorded was 14 days but generally protoscoleces started to produce a laminated layer after 28-49 days. As development continued the layer appeared as a thin colourless zone around the vesicular protoscolex (Fig. 3.14). With time the

thickness of this layer increased (15-35 μm at 40 days) but remained colourless and lacked laminations (Fig. 3.15). At about 56 days the laminated layer of some cysts took on a slightly tanned appearance when viewed under transmitted light. This tanning became more intense after 70 days (Fig. 3.16) when 30-80% of developing cysts had this appearance whilst others had a clear laminated layer. Only in these tanned cysts were laminations in the layer seen although the outermost region frequently remained colourless (Fig. 3.17). Cysts which had been cultured for long periods (70+ days) generally possessed 4-7 laminations in the laminated layer but as many as 10-20 laminations were recorded in some cysts (Fig. 3.18). It is interesting to note that both the laminations and the tanned appearance of the laminated layer disappeared when the cysts were placed in 0.1 M HCl resulting in a completely clear layer within seconds.

In the late stages of culture many developing cysts still retained remnants of protoscolex characteristics, i.e. the presence of rostellar hooks and suckers although the calcareous corpuscles had virtually disappeared (Fig. 3.19). The suckers, although still recognisable, were, however, showing signs of degeneration (see section 6.2.3). Subsequent loss of the hooks seems to have been accomplished in a number of ways. Firstly developing cysts often produced an elongated "neck" region which contained the hooks at the distal end (Fig. 3.20). This appeared to be the first stage in budding off the hooks as occasionally small clusters of hooks were seen within the laminated layer but detached from the rest of the cyst (Fig. 3.21). A second method of hook loss may have occurred when the hooks fall into the centre of the cyst cavity. Occasionally cysts were seen where the cytoplasm surrounding the hooks had pulled away (Fig. 3.22), thus releasing the hooks which may subsequently

fall into the cyst cavity. Although not frequently observed, some developing cysts were seen to possess an accumulation of tissue at one end (Fig. 3.23). This may represent a "healed" region after the loss of the entire scolex from an organism showing posterior vesiculation. In the majority of cases, however, the hooks were lost by the budding method.

In the early stages of cyst development the germinal layer was seen, under high magnification, to be composed of cells which had long processes extending from a central region, therefore giving the tissue a stellate appearance (Fig. 3.24). As the cysts aged and the laminated layer increased in thickness and intensity, the germinal layer had a much darker appearance (Fig. 3.25) and the cellular processes were shorter and compacted (Fig. 3.26). This darkening of the germinal layer seemed to be the first stage in cyst degeneration and was increasingly evident from approximately 84 days onwards. It is important to note that cysts in this condition did not take up eosin. Only when the germinal layer was fragmenting and clearly dead (Fig. 3.27) did the tissue take up the stain. The maximum time which living cysts were maintained in culture was 8 months. The viability within this period was indicated by a negative eosin reaction and by a reduction in the pH of the culture medium (see section 3.2.2). The cysts in late stages of development were 673 ∓ 26.2 μm in external diameter with a laminated layer thickness of 172 ∓ 17.2 μm, and no evidence of brood-capsule of protoscolex formation was observed.

Posterior bladder and vesicle development:

In general, relatively few protoscoleces developed posterior bladders during culture (0%-10%). These occurred on both evaginated and invaginated protoscoleces and occasionally on vesiculating protoscoleces, but were generally only present during the first 14

days of culture. The bladders appeared from the first day of culture and under standard culture conditions were usually small (<30 μm in diameter) (Fig. 3.28), although limited, subsequent growth may have occurred. These bladders, when attached to protoscoleces, did not usually appear to take part in laminated layer formation. Occasional developing cysts were observed to have a posterior swelling (Fig. 3.29) but these were more likely to be attributed to extensive posterior vesiculation.

When cultures were inoculated with brood capsules however, the number and size of posterior bladders was considerably different. Immediately after inoculation of the medium with intact brood capsules, a proportion of them were seen to have become ruptured and everted (Fig. 3.30) with a number of protoscoleces attached to the collapsed brood capsule wall. The cultures were therefore heterogenous, containing intact and ruptured brood capsules and some free released protoscoleces. Presumably the original scraping of the parent cyst wall (and subsequent washings) caused damage to many of the brood capsules wich resulted in their rupture when pipetted into the medium. Many of the free protoscoleces also retained, to varying degrees, a proportion of the attachment stalk and in some cases a portion of brood capsule wall (Fig. 3.31). Within the first few hours of culture the stalk remnants attached to the protoscoleces were seen to swell and form the bladders (Figs. 3.32, 3.33). By day 4, 20%-40% of the free protoscoleces possessed small posterior bladders, the size of which ranged from 24 μm to 106 μm in diameter. From 4-7 days a number of small free vesicles (20-80 μm in diameter), devoid of any protoscolex remnants, were observed (Fig. 3.34). Later (15-20 days) the cultures contained increased numbers of these small free vesicles whereas the proportion of protoscoleces with small posterior bladders decreased (20.95%-38.2% at 4 days compared with

4.14% and 6.72% after 26 and 15 days respectively.) It therefore seemed that these small free vesicles were posterior bladders which had become detached from the protoscoleces. Between 20 and 30 days in culture the small free vesicles secreted a laminated layer and developed into miniature cysts (Fig. 3.35). The cysts formed in this manner differed from those developed from vesiculating protoscoleces in that they were much smaller and no sucker or hook remnants were present. As in the standard cultures, small posterior bladders attached to protoscoleces did not develop a laminated layer although a small number did survive for relatively long periods in culture (up to 70 days).

Early in the same cultures the ruptured brood capsules everted and became swollen (166-490 μm in diameter) and now carried the attached protoscoleces on the outside (Fig. 3.36). The number of protoscoleces attached to the swollen vesicles ranged from 2 (Fig. 3.37) to 37, with many being evaginated (Fig. 3.38) and observed to be active. This number seemed initially to be controlled by the amount of ruptured brood capsule which was involved in the swelling process. Intact brood capsules remained so generally for less than 7 days, after which they formed ruptured, everted brood capsules.

The ruptured everted brood capsules persisted throughout the culture period, often with an apparent decrease in the number of attached protoscoleces as the culture proceeded. However, at a time when other morphological types (i.e. vesicular protoscoleces) in the culture were developing a laminated layer, these forms were devoid of such a layer. Only after 70 or more days did some of them start to produce a laminated layer. At this stage, however, the attached protoscoleces were inactive and appeared to have lost their structural integrity (Figs. 3.39, 3.40).

Also present between 4 and 7 days were large free vesicles

(150-250 μm in diameter) without attached protoscoleces (Fig. 3.41), presumed to be derived from the ruptured brood capsules. By 20-30 days these large vesicles had produced a laminated layer (Fig. 3.42) and their size clearly distinguished them from the miniature cysts produced from the detached posterior bladders. The lack of scolex remnants also distinguished these cysts from those formed from vesicular protoscoleces. Occasionally some of the large vesicles retained 1 protoscolex which was present, although inactive, even after laminated layer formation (Fig. 3.44). The pathways involved in cystic development are shown diagramatically in Fig. 3.45.

Adult - type development:

The above morphological types were present in most monophasic culture systems employed. In addition to these cystic forms, however, certain cultures also produced a number of individuals which had started differentiation in an adult, strobilate direction. These were usually present in small numbers (<5%) and could be recognised by their enlarged, evaginated form (300-550 μm in length), lack of calcareous corpuscles, presence of excretory canals and highly active behaviour (Fig. 3.45). In no case did any of these "worms" produce proglottides. The proportion of organisms showing this adult-type development appeared to decline late in the cultures (beyond 84 days). In one standard culture however, 12.39% of the organisms were of the adult type at 60 days (2.84% cysts with laminated layes; 1.23% vesicular protoscoleces; 83.53% dead). Included in these organisms were 4 individuals which had apparently become fused together (Fig. 3.46). Similar structures had been observed previously but only in very rare cases. Approximately 250 of the adult-type individuals were separated from the others and placed in a new diphasic medium, comprising a solid phase of 2 ml

nutrient agar and a liquid phase of 4 ml NCTC 135 plus 1 ml inactive FCS. These forms continued to grow for some time, some reaching a maximum length of 890 µm whilst others died. After 17 days in diphasic medium (77 days total culture) it appeared that some of the "worms" had started to reverse their development by becoming vesicular (Fig. 3.47) and at 25 days in diphasic medium (85 days total), 20.8% of the parasites had vesiculated whilst 24.4% were still showing adult development and 54.8% were dead. One week later however, all the organisms were dead.

3.2.2 Proportional changes in morphological types present in standard and varied culture conditions.

Due to the relatively crude sampling techniques and long duration of the cultures, repetition of the different variations was limited and the numerical values obtained from these studies did not lend themselves to detailed statistical analysis; a problem also observed by Smyth (1967). With the standard cultures the number of repeated runs was sufficient to calculate means and 95% confidence limits for the major sampling times. For variations of the culture conditions, percentages could only be compared with those of standard cultures set up with the same batches of protoscoleces and culture components.

Standard cultures:

In order to calculate means and 95% confidence limits, the percentages of each type of protoscolex occurring at each sample time had to be transformed using an arcsine transformation (Sokal & Rohlf, 1981), with the calculated mean values being reconverted to percentages. The data from individual cultures are listed in

Appendix 3 while the transformed means and confidence limits are tabulated in Table 3.1 and back transformed means shown graphically in Fig. 3.48. Individuals from each sample were sorted into 7 morphological types: invaginated protoscoleces; protoscoleces with posterior bladders (invaginated, evaginated or vesiculating); protoscoleces showing any type of vesiculation; developing cysts with laminated layers; protoscoleces showing adult development; and dead protoscoleces.

The initial protoscoleces used to inoculate cultures were almost totally in the invaginated state (93.61%). After 1 day however this proportion dropped to about 32% and continued to drop steadily until 21 days when almost all had developed into other morphological types. From 1-10 days, evaginated protoscoleces were the most numerous type found in cultures (42-60%). These started to decline considerably between 7 and 21 days, and after 56 days were present only in small numbers (<10%). During this time, the proportion of vesiculating protoscoleces rose, forming a plateau at approxiamtely 30%-35% from 21-56 days, after which the percentage decreased. Developing cysts, with a laminated layer were increasingly evident after 28 days, so that after 120 days 43.65% of the organisms present possessed a laminated layer. (The highest recorded proportion at this time was 66.6%.) Since the proportion of protoscoleces with posterior bladders was generally less than 10% for the first 14 days, it appeared that these forms were not involved in cyst production. There is, however, a possibility that the bladders may be lost while their associated protoscoleces vesiculate to form cysts. It seems therefore that there is a gradual transformation from invaginated protoscoleces to evaginated ones, then vesiculating ones and finally fully developed vesiculated cysts with laminated layers. This transformation however, does not occur at the same rate for each protoscolex, therefore resulting in the heterogeneous population of

TIME	INVAGINATED	EVAGINATED	POSTERIOR BLADDER	VESICULAR	LAMINATED LAYER	ADULT	DEAD
0 day	75.36 (6.05)	6.92 (5.04)	0	0	0	0	11.66 (6.59)
1 days	34.57 (9.58)	50.46 (7.85)	12.76 (5.12)	0	0	0	6.76 (6.47)
4 days	33.62 (16.09)	42.27 (12.29)	13.68 (4.13)	19.02 (5.26)	0	0	9.15 (6.06)
7 days	25.60 (8.44)	46.65 (5.81)	7.41 (4.9)	27.10 (8.81)	0	1.09 (2.88)	9.42 (4.16)
10 days	32.31 (11.3)	37.15 (13.32)	11.34 (6.68)	25.5 (9.46)	0	0	
14 days	19.51 (12.64)	35.37 (8.53)	5.18 (9.12)	36.5 (10.16)	0.71 (2.12)	1.97 (4.39)	15.34 (11.34)
21 days	4.77 (6.42	47.8 (4.62)	2.07 (3.36)	33.61 (6.38)	3.02 (2.85)	5.67 (10.6)	16.1 (3.35)
28 days	7.97 (8.26)	45.49 (14.21)	0.86 (2.27)	31.35 (9.91)	5.98 (2.76)	3.13 (5.47)	19.66 (15.56)
35 days	3.92 (4.92)	27.7 (16.98)	0	34.83 (15.72)	8.99 (4.2)	4.99 (4.01)	35.85 (15.28)
49 days	0	18.96 (7.49)	0	36.47 (10.57)	19.21 (1.33)	2.63 (5.87)	39.21 (12.02)
56 days	0	14.0 (8.57)	0	37.06 (11.14)	22.7 (1.76)	2.38 (3.27)	40.0 (11.36)
70 days	0	4.45 (4.71)	0	29.02 (12.19)	29.96 (6.65)	0	42.75 (9.38)
77 days	0	2.95 (3.53)	0	23.35 (13.7)	31.24 (10.01)	0	47.03 (11.66)
120 days	0	0	0	4.30 (3.98)	4o.35 (7.43)	0	47.58 (8.17)

Table 3.1 Arcsin transformed mean percentages of morphological types occurring during *in vitro* culture (standard deviations in brackets).

protoscoleces observed in cultures. The numbers of dead organisms was seen to remain relatively low (<10%) in the first 28 days, after which there was a considerable increase, reaching a peak of 54.51% after 120 days.

The presence of protoscoleces showing adult development was generally only detectable after 14 days, since prior to this these individuals appeared similar to normal evaginated forms or early vesiculating forms. Their presence in standard cultures however did not generally last beyond 70 days.

Culture variations: (Data listed in Appendix 3)

Inoculation of cultures with enzyme and bile salt treated protoscoleces:

When protoscoleces, pre-treated with pepsin and sodium taurocholate solutions, were used to inoculate cultures, the proportion of evaginated protoscoleces was initially higher than in cultures with untreated individuals (73.2% and 78.19% compared with 58.2% and 49.3% respectively after 1 day). The number of protoscoleces with posterior bladders also appeared elevated at this time (10.25% and 5.77% compared with 3.97% and 3.14%, at 1 day). Vesiculation in these cultures however, did not seem to be affected although the number of cysts producing a laminated layer was lower (10.16% compared with 29.16%, after 70 days). This lower cyst production may have been due to the higher early mortality observed in the treated organisms (28.24% compared with 8.61%, at 14 days), although in the first of the incubations the mortality of both treated and untreated protoscoleces was very high throughout and the results of this incubation are therefore not conclusive.

Effects of altering the basic medium used:

When protoscoleces were cultured using M199 or RPMI 1640 as a basic medium, supplemented with 20% FCS, the proportions of cysts with laminated layers were considerably lower than similar cultures set up using NCTC 135 (9.16% and 12.66% with M199 after 70 days compared with 42.55% and 29.16% with NCTC 135; RPMI 1640 - 10.13% and 15.14%). Both M199 and RPMI 1640 also did not sustain much parasite survival beyond 70 days suggesting that these media were less suitable for culturing protoscoleces. Interestingly, however, cultures employing RPMI 1640 produced a higher number of vesiculating protoscoleces early on than NCTC 135 (84.51% and 72.21% after 14 days using RPMI 1640 compared with 35.8% and 32.53% with NCTC 135). This suggests that high vesiculation is not always correlated with high cyst production.

Cultures employing CMRL 1066 were much more productive than those using M199 or RPMI 1640 and were more similar to those set up using NCTC 135 (50.26% and 34.3% with laminated layers after 70 days with CMRL 1066 compared with 44.5% and 37.44% using NCTC 135). This suggests that CMRL 1066 is a suitable alternative medium to NCTC 135 for the culture of protoscoleces.

Effects of altered serum contents:

When cultures were set up in the absence of serum (i.e. in NCTC 135 alone), a high proportion of evaginated protoscoleces and a low proportion of vesiculating protoscoleces was evident when compared with standard cultures (NCTC 135 alone: 75.58% and 58.77% evaginated, 11.33% and 20.64% vesiculating; NCTC 135 + 20% inactive FCS: 18.14% and 55.35% evaginated, 48.39% and 25.29% vesiculating, at 14 days). A much higher death rate was also observed in the absence of serum, resulting in the death of all parasites by 49-70 days. It is important to note, however, that even in the absence of serum, some vesicular individuals produced a laminated layer, although the proportions were generally less than 1%.

Increasing the content of inactive FCS to 40% in some instances resulted in a higher initial vesiculation (26.77% compared with 10.44% in standard cultures at 9 days) but in other cases, vesiculation was at a similar rate to standard cultures and eventual production of cysts with laminated layers was also similar to standard cultures in all cases (20.01% and 30.01% compared with 23.7% and 29.34% respectively at 70 days).

Reducing the content of inactive FCS to 10% however, seemed to reduce the eventual percentage of cysts with laminated layers (11.97% compared with 29.34% in standard cultures after 70 days), although the numbers of living organisms at this stage was similar to that of the standard cultures (62.4% compared with 61.3% at 70 days) which may possibly suggest that there is merely a delay in production of a laminated layer by some protoscoleces.

When 20% FCS, which was not heat-inactivated, was employed in cultures there was a considerable decrease in the proportion of cysts with laminated layers produced (8.3% and 6.8% compared with 29.34% and 38.96% in standard cultures at 70 days). These differences may

be due to the high numbers of dead protoscoleces occurring early in these cultures (40.13% and 31.24% compared with 9.26% and 6.93% in standard cultures at 28 days). Another feature evident in these incubations was a relatively high proportion of protoscoleces with posterior bladders early in the culture (6.1% and 11.36% after 4 days).

Cultures containing 20% inactive horse serum did not appear to differ from standard cultures, with laminated layers first appearing around cysts between 14 and 21 days and continuing to form throughout the incubation (27.63% and 32.14% compared with 29.34% and 38.96% in standard culture after 70 days). The number of dead organisms in cultures with horse serum however, was slightly higher than standard cultures in the later stages of the incubations (53.77% and 56.91% compared with 38.7% and 37.6% at 70 days).

Effects of a gas-phase with a high O_2 content:

Results from cultures employing a gas-phase of 100% O_2 or 95% O_2, 5% CO_2 also did not seem to differ from standard cultures with the possible exception of a higher initial evagination rate (62.24% and 45.0% in 100% O_2; 73.3% in 95% O_2, 5% CO_2; 48.0% and 35.3% in air, at 4 days). Eventual numbers of cysts produced were equally high in all cultures, and there appeared to be no increase in the vesiculation rate of protoscoleces.

Effects of supplementing the standard medium with additional hexosamines:

The addition of glucosamine and galactosamine did not have any beneficial effect on the numbers of cysts producing laminated layers nor on the survival of the parasites (34.19% and 42.73% with laminated layers compared with 36.13% and 44.2% in standard cultures

at 120 days).

The influence of pH on cultures:

A low pH of 6.2 appeared to maintain protoscoleces in the invaginated state for longer (70.2% compared with 18.19% at pH 7.4, after 3 days) but also resulted in the early death of all protoscoleces after 14-21 days. Within this period some of the protoscoleces vesiculated but no laminated layers were produced. With a high pH of 8.5, there was little difference in the evagination rate compared with a pH of 7.4 although there may have been a slight reduction or delay in the vesiculation process (52.91% and 23.7% vesiculating at pH 8.5, compared with 57.84% and 50.07% at pH 7.4 after 14 days). The number of cysts with laminated layers does, however, appear to be reduced with 4.13% and 8.24% produced at pH 8.5 after 60 days compared with 13.55% and 17.09% at pH 7.4. The layers formed in cultures at pH 8.5 were however thin and poorly developed. In addition, the proportion of dead organisms at this stage was higher at pH 8.5 (56.10%and 57.2%) than at pH 7.4 (28.19% and 38.7%). There did, however, seem to be a slight increase in the number of protoscoleces showing adult development in some of the cultures at pH 8.5 (5.97% compared with 2.17% at 60 days).

Within the narrower pH range of 6.8-8.2, cystic development was much better, with high numbers of cysts producing laminated layers (after 77 days in the range of 29.77%-52.56%). A pH of 8.2 gave the lowest yield of cysts but the highest proportion of protoscoleces showing adult development (8.97% after 77 days).

Effects of culturing different numbers of protoscoleces:

Results of cultures incorporating different protoscolex numbers showed some interesting effects. When low numbers were employed (below 2,000), no cysts with laminated layers were produced and the death of all protoscoleces occurred by 80 days. Surprisingly, however, these cultures produced high numbers of individuals showing adult development (in the range of 47.3%-60.81% after 40 days). The pH of these cultures had become elevated and ranged from 7.6-8.7 with an average of 8.04.

Above 2,000 protoscoleces, the number of parasites showing adult development dropped dramatically (0-2%). Cysts with laminated layers appeared more often although numbers of 4,000-5,000 produced more cysts earlier than numbers of 2,000-3,000 (6.72%-15.0% compared with 1.95-2.2% after 40 days). The eventual numbers of cysts produced, however, were high in all cases when 3,000 or more protoscoleces were present. The pH of these cultures ranged from 6.9-7.8 with an average of 7.38.

Cultures with approximately 10,000 protoscoleces resulted in a high number of cysts produced (33.5%-42.24% after 120 days) although many of these were showing signs of degeneration by 80-120 days. The pH of these cultures ranged from 6.7-7.27 with an average of 6.9.

Other observations:

When protoscoleces were removed from cysts to culture tubes of hydatid fluid and maintained at 37^{o}C a large proportion evaginated within the first 24 hours. Vesiculation of some individuals occurred after 3 days but cultures could not be maintained for longer than 5 days because of contamination.

3.2.3. Lectin binding analysis of *in vitro* derived cysts.

Lectin binding analysis of cysts cultured *in vitro* for 60 days showed that wheat germ agglutinin (WGA) (Figs. 3.50, 3.51), soybean agglutinin (SBA) (Figs. 3.52, 3.53) and peanut agglutinin (PNA) (Fig. 3.54) all bound to the laminated layer indicating the presence of N-acetylglucosamine, N-acetylgalactose-amine and galactose respectively. Neither Concanavalin A (Con A) (Fig. 3.55) or Asparagus Pea agglutinin (APA) (Fig. 3.56) bound to the cysts showing the absence of mannose and/or glucose and fucose respectively.

3.3 DISCUSSION

In the *in vitro* studies reported in this chapter, the choice of a suitable synthetic medium was based on reports by other authors who had successfully used NCTC 135 to culture either *Echinococcus* spp. (Smyth & Davies, 1974b; Heath & Osborn, 1976; Heath & Lawrence, 1976; Sakamoto, 1978) or other taeniid cestodes (Heath & Elsdon-Dew, 1972; Heath, 1973) and in particular the work of Heath & Osborn (1976) who obtained laminated layers very early on in their incubations, when culturing sheep-derived protoscoleces in NCTC 135 alone.

Similarly , a pH of 7.4 was chosen since this value was the most quoted for *Echinococcus* cultures (Smyth, 1962, 1967; Yamashita *et al.*, 1962; Webster & Cameron, 1963) and because the normal pH of most mammalian body fluids is 7.35-7.45 (Lockwood, 1971). A gas phase of air had also been previously reported in successful *Echinococcus* cultures (Smyth, 1962; Pauluzzi, *et al.*, 1965; Brudnjak *et al.*, 1970; Heath & Osborn, 1976; Casado Escribano & Rodriguez Caabeiro, 1986) and was chosen in preference to a gas phase of 8-10% O_2, 5% CO_2, in N_2, used by Smyth (1967; 1979; 1985) and his co-workers (Smyth *et al.* 1967; Smyth & Davies, 1974b) to culture protoscoleces to the adult stage.

Supplementation with 20% inactive FCS was employed on the basis of this being a common additive in several cestode cultures (Pauluzzi *et al.*, 1965; Heath & Lawrence, 1976; Sakamoto, 1978; Heath & Elsdon-Dew, 1972; Heath, 1973; Casado Escribano & Rodriguez Caabeiro, 1986).

Development within the standard medium chosen was quite adequate to study the differentiation of protoscoleces although clearly it did not support the development of all protoscoleces for indefinite periods of time. Considerable variation was also present between

cultures set up under identical conditions, making direct comparison difficult. These variations are likely to have arisen for several reasons. Firstly, the exact components of the medium may not be exactly the same each time, with batches of FCS being particularly noted for their non-uniformity (Smyth, 1967; 1979; Smyth & Davies, 1974b; 1979). Secondly, although attempts to produce uniformity in the medium were carried out by rotating or mixing, surface related or spatially related factors may play an important role in differentiation and even minor variations in the pH, Eh, pO_2 or pCO_2 may influence the growth and behaviour of protoscoleces. Finally, batches of protoscoleces from different primary cysts are likely to vary considerably in age, viability and developmental potential. Although all samples were subjected to an eosin exclusion test to assess the viability, protoscoleces excluding the dye may not necessarily be capable of development. The failure of this test to show the death of chemically killed protoscoleces (Robinson & Arme, 1985) supports this suggestion and the test should only be used as an indication of protoscoleces which have been dead for some time where the tegumentary membrane is permeable to the dye. Similarly , the death of cysts with laminated layers is also difficult to detect using eosin until the cysts are starting to degenerate rapidly. The test does, however, still have some value in indicating the condition of the organisms within the cultures, over time.

The developmental processes which occur during the *in vitro* culture of protoscoleces of the horse strain of *E. granulosus* seem to be essentially the same as those occurring in the sheep strain and in *E. multilocularis* (see Introduction for reference). Evagination is not essential for cyst formation since some protoscoleces were observed to vesiculate in the invaginated state. Most, however, vesiculated once evaginated. The evagination process appears to

involve an activation of protoscolex motility, followed by a gradual emergence of the suckers and finally the rostellum (also see Marchiondo & Andersen, 1984) which are presumably held within the soma by a system of muscle fibres (Smyth, 1964). The exact nature of the stimuli resulting in evagination are, however, not clear and will be discussed later.

Similarly , the factors influencing vesiculation may be complex and undefined and the process may well represent a response to conditions "abnormal" to the dog intestine as suggested by Smyth (1967). Whatever the factors are, they appear generally to take some time to act since even after 28 days only about 27% may be vesiculating, although some protoscoleces vesiculate within the first few days of culture. The factors influencing the type of vesiculation are also not known but it is possible that certain stimuli, such as surface contact or nutrient absorption across the tegument, may affect different areas of the protoscolex to different degrees. Alternatively, the time spent in the invaginated state may affect development. Since it is known that both the scolex and soma regions have a fundamentally different surface ultrastructure (Smyth *et al.*, 1966; Morseth, 1967; Smyth, 1972; Marchiondo, & Andersen, 1983; McManus & Barrett, 1985), absorption of substances across the tegument of the soma alone may be reduced compared to absorption by the scolex and soma together, in the case of evaginated protoscoleces. If a substance in the medium affects vesiculation then this may explain why some areas vesiculate before others.

During vesiculation there appears to be a reduction in the numbers of calcareous corpuscles present, although Yamashita *et al.*, (1962) reported an increase in these structures during vesiculation of *E. multilocularis* protoscoleces. A similar reduction is also seen during development of *E. granulosus* protoscoleces to strobilate forms

(Smyth & Davies, 1974a; 1974b;1979) which may suggest that some component of the corpuscles is being used up during differentiation. The calcareous corpuscles of cestodes are known to contain both organic and inorganic substances, with calcium, magnesium, phosphates and carbonates being present in relatively large amounts (von Brand, Scott, Nylen & Pugh, 1965; von Brand, Nylen, Martin & Churchwell, 1967; Nieland & von Brand, 1969; Kegley, Brown & Berntzen, 1969; Kegley, Baldwin, Brown & Berntzen 1970; Baldwin, Berntzen & Brown, 1978). Their functions, however, are virtually unknown although it has been suggested that one function may be to act as a phosphate reservoir for the metabolic needs of cestodes, which, because of their dependence on carbohydrate utilization, would require large amounts of phosphate for phosphorylation of hexoses and for other phosphorylating processes (von Brand & Weinbach, 1965; Nieland & von Brand, 1969). If this is the case, then differentiation of protoscoleces would depend on an increased metabolism and may therefore deplete phosphate stores in the corpuscles. Calcareous corpuscles are, however, present in fully formed hydatid cysts and in a variety of adult cestodes and this may indicate that once the parasites are established in their respective locations, then they can re-accumulate the components of the corpuscles.

The whole process of vesiculation is interesting in view of the fact that generally no protoscoleces vesiculate within the hydatid cyst. It may be argued that, since the temperature in the cyst is approximately 37^{o}C, some inhibitory factor must be present in the hydatid fluid which prevents both evagination and vesiculation. This, however does not explain why freed protoscoleces both evaginate and vesiculate when cultured, at 37^{o}C, in hydatid fluid alone. Nor does it explain why cultures incorporating a large hydatid fluid content produce a large number of vesicular or even cystic organisms

(Deve, 1926; 1928; Coutelen, 1927a; 1927b; Smyth, 1962; 1967; Smyth *et al.*, 1967; Casado Escribano & Rodriguez Caabeiro, 1985; Casado Escribano *et al.*), or the presence of daughter cysts within the primary cyst. It seems therefore that hydatid fluid alone does not have an inhibitory effect on freed protoscoleces. This then poses the question of how many free protoscoleces are actually present in live cysts within the intermediate host? It is widely believed that the material found at the bottom of cysts, known as "hydatid sand", is comprised of detached brood capsules and free protoscoleces. One must then ask the question of how long these organisms have been detached from the germinal layer and whether this detachment is a natural occurrence or an accidental one, brought about by handling and storage of carcasses, livers, etc. It may be the case that actually within the intermediate host, very few protoscoleces are released from brood capsules and few brood capsules are detached from the germinal layer. If the brood capsule wall and/or the germinal layer have a direct inhibitory effect on cystic development, of protoscoleces, then actual release of the protoscoleces may therefore be the trigger for both evagination and vesiculation and may provide an explanation for the origin of daughter cysts. These structures appear to be more common in sheep or human cysts and Thompson (1976c) reported that at that time daughter cysts had not been previously recorded in horses. In the present study only two equine hydatids with daughter cysts were found over a three year period. In both cases, the tissue of the primary cyst appeared necrotic and degenerate (see Fig. 3.49), as also reported by other authors (De Rycke & Pennoit-De Cooman, 1978) although the numerous daughter cysts were healthy and possessed viable protoscoleces. The degeneration of the germinal layer may therefore have removed the inhibition on the protoscoleces, thus enabling them to vesiculate and form cysts.

Clearly these hypothetical suggestions require further investigation, but the isolation of some substance from hydatid tissue which inhibits cyst development may be beneficial to any chemotherapy investigations.

Within the cultures, once vesiculation is complete, the formation of a laminated layer begins when the protoscolex takes on a sticky nature. This often causes the developing cysts to clump together, a factor which may affect sampling unless the cultures are adequately shaken before hand. The layer first appears as a clear zone around the parasite and then increases in depth for some time before any tanning or laminations appear. It is not known what the nature of the tanning process is or what significance the laminations have, but similar changes from a clear laminated layer to one with laminations have also been reported by Smyth (1962) and Casado Escribano & Rodriguez Caabeiro (1986). Heath & Osborn (1976) suggested that the laminations may be connected with the number of media changes and recorded a maximum of 8 laminations. Casado Escribano & Rodriguez Caabeiro (1986) recorded a maximum of 12 laminations whilst in the present study 10-16 laminations were not uncommon in well developed laminated layers. It seems likely that these laminations may result from compaction of the laminated layer components and may represent periods of growth.

The tanning process may also represent compaction but it is more likely to represent some sort of polymerization of the laminated layer components as suggested by Kilejian & Schwabe (1971). If laminated layer precursor material is synthesized and secreted from the germinal layer, then extrinsic factors, such as pH or ionic strength, may result in polymerization of this material in a manner similar to that of collagen production (Jackson, 1968). This may therefore explain why the laminated layer loses its tanned

appearance when placed in solutions with a low pH which may act to break any cross-linkages.

Once the laminated layer has become well developed, the last of the protoscolex remnants appear to be lost, i.e. the hooks and suckers. The suckers seem to degenerate and be re-absorbed (Webster & Cameron, 1963) but little reference has been made to the fate of the rostellar hooks. From the present study it seems that the most frequent mode of hook loss is by an extension of the cyst cytoplasm into a "neck" region which contains the hooks at the apex. The hooks and a small portion of cytoplasm are then budded off into the laminated layer and are subsequently lost from the cyst altogether. For this process to occur, there must be some movement or growth of the cytoplasm, within the laminated layer, to form the "neck" region. Release of the "hook bud" into the laminated layer must also require some movement before the hooks are finally jettisoned. It is likely that this is brought about by actual growth of the laminated layer outwards from the germinal layer, causing the hooks, within the layer, to gradually move away from the cyst. The outer edge of the laminated layer must therefore be being continually removed and, in the *in vivo* situation this is likely to occur by the host macrophages phagocytosing the outer edge. (Richards Arme & Bridges, 1983b). *In vitro* it may occur, to some degree, by abrasion against other cysts or the culture tube walls.

The other methods of hook loss occurred less regularly although Yamashita *et al.* (1962) also observed the falling of hooks into the cyst cavity, when culturing *E. multilocularis* protoscoleces. Loss of the entire scolex region seems to be a dramatic measure, if it actually occurs. It is not, however, totally unfeasable as it is known that portions of germinal layer at least, can regenerate into cysts. (Benex, 1968b; Sakamoto, 1978). The soma region could have

similar regenerative properties and "repair" any wound, left by scolex removal, in order to differentiate into a cyst.

As the cysts increase in age they begin to show signs of degeneration with the germinal layer becoming much darker (84 days +) and finally degenerating totally, causing this layer to collapse. Smyth (1962) also observed the darkening of the germinal layer with age and interpreted this as an increase in the amount of lipid present. The author suggested that by the formation of the laminated layer the parasite cuts itself off from nutrients which leads to degeneration. This may be the case, but it does not explain why such changes do not occur *in vivo*. It is, however, unlikely that any *in vitro* culture system can be as good as the *in vivo* situation, and, at best, probably maintains the organisms alive rather than sustaining healthy growth. The nutrients required for sustained growth might therefore be in short supply *in vitro*, and the laminated layer, once formed, may indeed act as a transport barrier, further limiting the uptake of perhaps, an already limited nutrient supply. It is interesting to note, however, that even after being cultured for 80 days *in vitro*, a large number of cysts have the ability to survive and continue to grow once transplanted into mice. This clearly suggests that some factor is missing *in vitro* which prevents the continued growth of cysts, once the laminated layer has become well developed. The ability of *in vitro* cultured cysts to continue growing *in vivo* makes transplantation a useful technique for infecting mice when protoscoleces are in short supply (a frequent event in the present study). *In vitro* culture could therefore be used as a method of "storing" protoscoleces for cystic development as it is known that protoscoleces do not survive well after 30 days storage at $4^{o}C$ (Andersen & Loveless, 1978; Casado, Rodriguez Caabeiro & Hernandez, 1986).

The occurrence of protoscoleces with posterior bladders is of interest in the present study. These structures have been recorded in the majority of *Echinococcus* cultures (see Introduction for references) but there is no concensus concerning the precise origin or fate of such bladders. In *E. granulosus* Smyth (1962; 1967) commented that after 12 days they began to secrete a sticky PAS +ve coat which gradually enveloped the rest of the protoscolex to form the laminated layer. Yamashita *et al.* (1962) also reported that posterior bladders of *E. multilocularis* were involved in the development of cysts with laminated layers, whilst in contrast, in the cultures of Gurri (1963), Benex (1968a) and Brudnjak *et al.* (1970) protoscoleces with posterior bladders did not produce cysts with laminated layers. The present observations show that in standard cultures, relatively few posterior bladders are produced and that these, when attached to protoscoleces generally do not seem to contribute to laminated layer formation. The numbers produced however, can be increased by inoculating cultures with brood capsules which subsequently rupture. These results suggest that the posterior bladders are not osmotic swellings resulting from tegumental damage, as reported by Benex (1968a) and Heath & Osborn (1976), but are portions of the original attachment region of the protoscolex (the evidence for this is presented in Chapters 5 and 6). The initial variation in their size is thought to reflect the degree of attachment stalk and brood capsule wall that is retained on the release of the protoscolex (Fig. 3.57). The proportion of bladders produced therefore seems to be dependent on the mode of protoscolex release and the mechanical methods used in the present studies may remove the brood capsule wall and attachment stalk more efficiently than other methods.

The small free vesicles seen early in brood capsule cultures

were of a similar size range to the posterior bladders and had a similar surface ultrastructure (see Chapter 6). It is therefore proposed that they are formed by detachment of posterior bladders from the parent protoscoleces. Their ability to produce a laminated layer and form a miniature cyst contrasts with those remaining attached to a protoscolex, and represents an additional pathway of cyst development *in vitro*, not previously reported.

The vesiculation of ruptured everted brood capsules has been previously described by Brudnjak *et al.* (1970) who reported the failure of such vesicles with attached protoscoleces to form a laminated layer *in vitro*. In the majority of cases a similar failure was recorded in the present study, with only a minority producing a layer, and then only after 70 or more days in culture. The work of Heath & Osborn (1976) suggested that large "vesicles" with attached protoscoleces could develop a laminated layer early in culture. They regarded such vesicles as not of brood capsule origin, as suggested in the present study, but rather resulting from coalescence of posterior bladders, with a laminated layer only forming if 10-40 protoscoleces were present. Examination of their micrographs, however, shows that a large number of the protoscoleces in contact with the developing cyst are dead or degenerating, a feature commonly found in the present study and attributed to free, dead protoscoleces within the cultures, adhering to the sticky laminated layer.

The present results clearly show that everted, vesiculated brood capsules devoid of protoscoleces, can, *in vitro*, develop a laminated layer and form hydatid cysts in the same way as the small free vesicles, and as reported earlier for the sheep strain in culture (Smyth, 1962). Of interest was the occurrence, within these cultures, of a small number of large vesicles, each with a laminated layer and a single attached protoscolex, early on. From their size,

these vesicles were interpreted as everted brood capsules which had retained a single protoscolex. This raises the question of why those everted brood capsules with numerous attached protoscoleces did not develop laminated layers, with the exception of a few doing so very late in culture. One interpretation might be that viable protoscoleces, once activated, may inhibit laminated layer formation when attached to the brood capsule surface, and that when only one remains the inhibitory influence is insufficient and a laminated layer develops. The retarded development of the laminated layer in a minority of everted brood capsules with attached protoscoleces might suggest that, with age, and the possible degeneration of the protoscoleces, such an inhibition decreases to a point where the production of a laminated layer commenced.

Such a "protoscolex inhibition" hypothesis might provide an alternative explanation for the formation of daughter cysts, as was previously discussed. If brood capsules within hydatid cysts were released from the germinal layer and everted retaining their protoscoleces, then the situation recorded *in vivo* by Dissanaike & Paramanaanthon (1961) and Gill & Rao (1967) would obtain and no laminated layer would develop. However, if the released brood capsules became devoid of protoscoleces then the everted capsules could perhaps develop in a cystic direction in a manner demonstrated in the present study, and produce daughter cysts with a laminated layer.

The occurrence of a number of protoscoleces showing adult development is also of interest in the present study. These individuals were highly active evaginated forms which elongated but never segmented. They possessed well developed excretory canals and lacked calcareous corpuscles (similar to stage 3 in Smyth & Davies, 1974b) and their surface ultrastructure was one of spined

microtriches entirely (see Chapter 6). This development was therefore similar to other cultures of horse-derived protoscoleces in diphasic media (Smyth & Davies, 1974a; Smyth, 1979; Smyth & Davies, C., 1979; Smyth, 1985) but occurred in a monophasic medium. Although these morphological types were generally present in small percentages, their existence indicates that contact with a solid nutritive base, as first suggested by Smyth (1967), is not essential to initiate strobilar development although it may be required to maintain it. Strangely, the fate of these individuals in culture was either death or vesiculation. It is likely, however, that this vesiculation does not represent a de-differentiation into a cystic form as protoscoleces showing adult development did not survive or produce cysts when transplanted into mice by i.p. injection or in diffusion chambers (see Chapter 4). The vesiculation therefore probably represents an osmotic swelling prior to death. The existence of these forms may, however, be confused with true vesicular forms, thus influencing sampling in the late stages of cultures.

In a few cultures, 2-4 of the adult-type worms were seen to be fused together. These organisms were probably originally protoscoleces which were attached to a common piece of brood capsule wall which was subsequently absorbed into the rapidly growing strobilar forms. A similar theory may be put forward to explain the reported presence of "an additional scolex" on certain strobilar forms of *E. multilocularis* cultured *in vitro* (Smyth, 1979; Smyth & Davies, C. 1979).

The results from altering the culture conditions showed that none of the alterations increased the number of cysts produced and some variations produced a lower yield.

Of the other synthetic media tried in place of NCTC 135, only

CMRL 1066 (a modified M 858) proved to be equally as good. Both M 199 and RPMI 1640 produced a much lower yield of cysts which were poorly developed. These results are surprising, particularly in the case of M 199 which had previously been used successfully by other authors in culturing protoscoleces of sheep origin (Smyth, 1962; 1967; Pauluzzi *et al.*, 1965; Brudnjak *et al.*, 1970; Casado Escribano & Rodriguez Caabeiro, 1986), although Smyth and his co-workers subsequently changed from M 199 to M 858 in order to culture protoscoleces to adults (Smyth *et al.*, 1967; Smyth & Davies, 1974b; Smyth & Davies, C, 1979; 1985). Casado Escribano *et al.*, (1985), however, failed to produce cysts with laminated layers when culturing protoscoleces of horse origin in a medium based on M 199, whilst sheep-derived protoscoleces produced cysts in the same medium. This difference in development between the two strains may therefore be due to differences in the specific components of each of the synthetic media (see Appendix 1 for full details). Apart from one or two amino acids, NCTC 135 and CMRL 1066 differ from M 199 and RPMI 1640 mainly in the nucleotides and coenzymes. In NCTC 135 and CMRL 1066 coenzyme A, trilithium salt, $2H_2O$; deoxyadenosine H_2O; deoxycytidine HCL; deoxyguanosine H_2O; FAD, disodium salt; NAD; NADP, sodium salt, H_2O; and UTP, trisodium salt, $2H_2O$ are all present, but are absent from M 199 and RPMI 1640, although M 199 does contain adenine sulphate; 5'-AMP; ATP disodium salt; guanine HCl; hypoxanthine; uracil and xanthine. These differences in nucleotides and coenzymes may therefore affect certain processes more such as protein synthesis, nucleic acid synthesis, lipid synthesis, carbohydrate metabolism and the functioning of certain enzymes, specifically in the horse strain than in the sheep strain of *E. granulosus*.

Another factor which NCTC 135 has, and which the others lack, is

glucosamine. Since the laminated layer of hydatid cysts has been reported to have a high content of galactose, galactosamine and glucosamine (Kilejian *et al.*, 1962; Korc *et al.*, 1967; Kilejian & Schwabe, 1971), the presence of glucosamine in the medium may facilitate laminated layer formation. The laminated layers formed in the cultures of Smyth (1962; 1967) Pauluzzi *et al.* (1965), Brudnjak *et al.* (1970) and Casado Escribano & Rodriguez Caabeiro (1986), which employed M 199, may have been dependent on the extensive supplementation of this medium with other nutrients. The results of the present lectin binding studies on cysts cultured in the standard medium (NCTC 135 + 20% v/v inactive FCS) show that galactose galactosamine and glucosamine are present in the laminated layer. This suggests that the parasites are either absorbing these components directly from the medium or are manufacturing them from existing nutrient stores and/or absorbed nutrients. It would be interesting to find out whether the same lectin binding properties are present in the few cysts formed in unsupplemented NCTC 135. This would indicate whether or not the parasite could manufacture galactose and galactosamine from a medium where the only carbohydrates present are glucose and glucosamine, or whether the FCS is required for these molecules.

Surprisingly the supplementation of the standard medium with additional hexosamines did not appear to increase the proportion of cysts producing laminated layers although there is no evidence to suggest that the nature of the laminated layer had not been altered in these cultures.

The influence of the serum content seems to be an important factor in cultures since when serum is absent, very few cysts are produced and there is a high protoscolex mortality. Serum concentrations of 10-40% v/v increased the proportions of

protoscoleces which vesiculated, a feature also observed by Webster & Cameron (1963), and also the numbers of laminated layers produced. Concentrations of 20% and 40%, however, produced more cysts than 10% indicating that the amount of serum is important. It should be noted that even after many years of vertebrate tissue culture, the factors present in serum which are responsible for promoting growth in cells and tissues are almost entirely unknown (Clarke & Stoker, 1971).

The inclusion of 20% v/v inactive horse serum did not significantly increase cyst production, which suggests that the factors necessary for growth are present in both horse and foetal calf sera and that there appears to be no species-specific component which is beneficial to horse-derived protoscoleces.

When 20% FCS which was not inactivated was employed, there was a much lower cyst production and a higher protoscolex mortality in the early stages of the incubations. This suggests that some heat-labile factor/s in the serum, probably complement, were resulting in the early death of protoscoleces. Similar results have also been reported by other authors incubating both *Echinococcus* protoscoleces (Herd, 1976; Kassis & Tanner, 1976; Rickard, MacKinlay, Kane, Matossian & Smyth, 1977) and newly excysted cysticercoids of *Hymenolepis* spp. (Bøgh, Christensen & Andreassen, 1986), in a variety of fresh normal sera from different animals. There is considerable evidence to suggest that this lytic reaction is a response to complement activated, in the absence of specific antibodies, by the alternative activation pathway. In the previous studies mentioned large proportions of protoscoleces were lysed within a few hours whilst, in the present study, parasites appeared to survive for longer periods, with some eventually forming cysts. This extended survival may be due to the fact that when serum is stored at -20^{o}C, complement activity may not be totally preserved as temperatures of

-40 or -80^{0} C are normally used to store such sera (Herd, 1976).

The inclusion of a gas phase with a high O_2 content also did not increase the proportions of cysts produced. Neither did it cause an increase in the rate of vesiculation as reported by Smyth (1962). This variation may again be due to strain differences since it is known that sheep and horse-derived protoscoleces have differences in their metabolism (McManus & Smyth, 1978; 1982; McManus & Bryant, 1986). The sheep strain has been reported to consume greater amounts of O_2 *in vitro* than the horse strain (McManus & Smyth, 1978) and has a higher dependence on the tricarboxylic acid (TCA) sequence of carbohydrate metabolism. Since sufficient oxygen is a pre-requisite for the classical oxidative functioning of the TCA cycle, the sheep strain may therefore be metabolically adapted to a higher oxygen tension than the horse strain (McManus & Smyth, 1982). Since vesiculation and reorganisation of the protoscoleces are likely to be energy dependent processes, a higher oxygen tension may be required for the sheep strain than for the horse strain.

The enzyme and bile pre-treatments used to release protoscoleces from brood capsules and to stimulate their evagination did not appear to have any beneficial effects on cyst production. Although the pepsin treatment appears to release protoscoleces, the majority of dead protoscoleces were not digested by the treatment. Smyth (1962) also reported that not all of the dead protoscoleces were removed by the pepsin but the remainder were removed by subsequent washing. In the present study, washing alone was found to remove the vast majority of dead protoscoleces, once released from the brood capsules. Pepsin treatment also appeared to result in a higher proportion of protoscoleces with posterior bladders. These bladders may have originated from portions of brood capsule wall, as suggested earlier, but are more likely to represent areas of tegument which had

been damaged by prolonged enzyme treatment as postulated by Heath & Osborn (1976), and may explain the high mortality observed. If this second reason holds then there appears to be two types of bladders; ones which originate normally from the attachment stalk/brood capsule wall, and ones which originate from tegumental damage. Although similar in appearance, it is important to distinguish between these two types since those which have "natural" bladders are likely to be viable and capable of cyst development whilst those showing damage are unlikely to develop.

The bile treatment did cause a slightly higher initial evagination but, in standard cultures, a high evagination rate occurred in the first few days, without any bile treatment. It is not known what the exact triggers for evagination are and, although bile appears to play some part, other factors must certainly come into play. Maintaining protoscoleces at $4^{o}C$ will keep them invaginated for longer, whilst incubation at $37^{o}C$ (even in hydatid fluid) will result in evagination of a large proportion of protoscoleces within the first few days. Temperature therefore appears to be important but it does not explain why protoscoleces do not evaginate within the primary cyst. Detachment from the brood capsule or germinal layer may therefore be important as was discussed earlier.

The influence of pH appeared to affect development to some degree. Low pH values of 6.2 caused protoscoleces to remain invaginated for longer but also resulted in the early death of all organisms: a similar observation to that of Webster & Cameron (1963) who observed death at pH 5.0. The high numbers of invaginated individuals might therefore be slowly dying and unable to evaginate. There was no evidence of a higher vesiculation rate at high pH values (8.5) as reported by Smyth (1967). Although cysts with laminated

layers were produced at pH values of 8.2-8.5, there were considerably fewer than at pH 6.8-7.4 which seemed to provide optimum cyst production. Again there may be strain differences in the optimum pH for cyst development, in the same way that there appears to be a species-specific difference in the pH required for adult development between *E. multilocularis* with a pH of 6.8 (Webster & Cameron, 1963) and the sheep strain of *E. granulosus*, with a pH of 7.4 (Smyth, 1967 1979; 1985). This may also be reflected in the fact that any adult-type development observed in the present study appeared to increase slightly at higher pH values (8.2) whilst no such development occurred at pH 6.8. It is important to note that in cultures where the pH was kept at one value, daily variations did occur. This was due to the combination of an increase in pH due to the loss of CO_2 from the liquid medium to the atmosphere above the medium, and the decrease in pH due to the acid waste products of the parasites themselves (Agosin, 1957; 1968; McManus & Smyth, 1978; 1982). These daily fluctuations may be enough to influence development even though the average pH was maintained at a constant value.

These pH changes were also important in the cultures employing different numbers of protoscoleces. When low numbers were used the amount of acidic waste products was not enough to counteract the rise in pH due to loss of CO_2 and these cultures therefore had a high pH of about 8.0. Similarly when approximately 10,000 protoscoleces were used the drop in pH caused by the waste products was greater than the rise caused by loss of CO_2 and these cultures had a low pH of about 6.9. Only when numbers of 3,000-5,000 were used, were the two effects balanced out to give an average pH of around 7.4. There were however, still fluctuations in the pH of these cultures over time, probably caused by increased or decreased activity of the

organisms themselves.

The most striking feature to note about these cultures was that when low numbers were employed (<2,000), a low yield of cysts was produced but a high proportion of protoscoleces showed adult development. Although these cultures had a high average pH (8.0), cultures with 4,000 protoscoleces, maintained at pH 8.2, did not show such exaggerated adult development. This therefore suggests that actual numbers of protoscoleces influence development. This influence could be due to a number of factors such as nutrient availability (although when some cultures were occasionally set up with double the volume of medium, there was no increase in adult development) or possibly some sort of crowding effect which promotes cystic differentiation.

The present results show that *in vitro* development of equine *E. granulosus* protoscoleces is affected by several parameters. The standard culture medium employed appeared to be the simplest to use and produced high yields of cysts. In subsequent chapters the ultrastructural development of the tegument will be investigated for protoscoleces cultured in this standard medium.

CHAPTER 4

MORPHOLOGICAL DEVELOPMENT OF SECONDARY HYDATID CYSTS *IN VIVO*

4.1 INTRODUCTION

Investigations on the *in vivo* development of secondary cysts from experimentally infected protoscoleces of *E. granulosus* have been carried out in a variety of hosts. Several rodents have proved to be successful laboratory models including white mice (*Mus musculus*) (Webster & Cameron, 1961; Gurri, 1967); NMRI mice (Pennoit-De Cooman, De Rycke & Outryve, 1974; De Rycke & Pennoit-De Cooman, 1978a); Quackenbush mice (Heath, 1970); BALB/c mice (Pauluzzi & De Rosa, 1969; Connor, 1980; Richards, Arme & Bridges 1983b) and gerbils (*Meriones unguiculatus*) (Thompson, 1976a; 1976c), with a proportion of cysts produced in each host species, being fertile. On the other hand Cotton rats (*Sigmodon hispidus*), DBA/1J mice, C57 BL/6J mice (Webster & Cameron, 1961), sheep (*Ovis domestic*), Guinea-Pigs (Cavia porcellus) and white rats (*Rattus norvegicus* var. Wistar albino) (Heath, 1970), were shown to be unsuitable for cyst production.

In successful laboratory models infection rates showed considerable variation and ranged from 30% to 95%. The number of cysts produced also varied considerably both between and within the same host species and appeared to be influenced by the numbers of protoscoleces injected. Gurri (1963) found that only 2.5% of protoscoleces injected intraperitoneally (i.p.) into "white mice" formed cysts and suggested that not all protoscoleces were capable of cyst formation. Pauluzzi & De Rosa (1969) injected 2350-4890 protoscoleces into BALB/c mice and obtained 241-597 cysts respectively, indicating that 10.25-12.22% of protoscoleces formed

cysts. When 10,627 protoscoleces were used, however, this proportion dropped to 4.8%. Pennoit-De Cooman *et al.* (1974) also stated that not all protoscoleces were in a physiological state to form hydatids and observed that the more protoscoleces injected, the more cysts were produced. This increased production was not linear, however, and the authors proposed that a crowding effect may have been occurring at high protoscolex densities. The optimum number of protoscoleces for maximum cyst production was found to be 2000-5000. Several authors also noted that the proportions of cysts produced varied considerably even when the same numbers of protoscoleces were injected. This variation was, however, reduced when all the protoscoleces were from the same primary cyst; suggesting that each batch may have a different cyst-producing potential (Pauluzzi & De Rosa, 1969).

The early development of secondary hydatids has only received limited attention and the initial stages of differentiation were reported to be similar to that which occurs *in vitro* (see Chapter 3) although most of the protoscoleces were seen to vesiculate in the invaginated condition (Heath, 1970; Thompson 1976c). Laminated layers were observed to develop at times ranging from 15-20 days (Gurri, 1963), 35 days (Heath, 1970) to 6-8 weeks (Cameron & Webster, 1969).

Subsequent growth and development resulted in cysts of widely differing sizes within individual hosts 6-12 months post-infection (Pauluzzi & De Rosa, 1969; Pennoit-De Cooman *et al.*, 1974; Heath, Christie & Chevis, 1975; Thompson, 1976a; De Rycke & Pennoit-De Cooman, 1978a; Connor, 1980; Richards *et al.*, 1983b). These occurred within the peritoneal cavity as either free cysts or as cyst masses which were encapsulated in a substantial host cellular response. In general the cysts in the centre of cyst masses were the smallest

(0.5-1mm in diameter 1 year p.i.) whilst those on the outside of the masses, and the free cysts were progressively larger (up to 40mm in diameter 1 year p.i.) (De Rycke & Pennoit-De Cooman, 1978; Heath *et al.*, 1975; Richards *et al.*, 1983b). Pennoit-De Cooman & De Rycke (1978) & Richards *et al.* (1983b) postulated that in the small cysts within the cyst masses, growth was inhibited by pressure from other cysts. Pennoit-De Cooman & De Rycke (1978) referred to these small cysts as being in a "latent phase" and showed that, if transplanted into parasite-free mice, they could continue growth at a similar rate to free cysts in the original infection.

In general, the above studies have concentrated on aspects of the fully formed cysts and little emphasis has been put on the transition of protoscoleces to cysts. An understanding of gross morphological changes occurring during secondary cyst formation is essential for investigations on the ultrastructural development. The present chapter therefore describes the morphological events associated with cystic differentiation and growth.

4.2 RESULTS

4.2.1 Development after intra-peritoneal injection of protoscoleces

Within the first three weeks post infection (p.i.) very little morphological development was observed in the injected protoscoleces, which remained mostly in the invaginated condition. All parasites recovered were, however, found in clumps of up to 700 individuals, surrounded by host leucocytes (Fig. 4.1). Individual mice possessed up to 12 of these protoscolex masses which varied in length from 1.5-7mm and in width from 1.0-5mm. The total number of protoscoleces retrieved from individual mice, in the first month p.i., ranged from 1,250 to 3,506 of which the viability was 51% ∓ 4.8 at 5 days .p.i. (N=8) and 7.2% ∓ 11.9 at 18 days p.i. (N=8).

After 14 days some of the protoscoleces had become misshapen and irregular in outline (Figs. 4.2, 4.3). Others had begun to show some signs of vesiculation (Fig. 4.4) by becoming swollen in the posterior soma region. At this stage, however, no spherical vesicular forms, similar to those occurring *in vitro*, were found.

The first laminated layers were observed after 28 days p.i. and appeared as a clear zone, approximately 1.2-16μm thick, surrounding the developing cyst. The shape of these cysts varied considerably. Some were evaginated and fully vesicular, with the suckers and rostellar hooks being clearly visible at the anterior end (Fig. 4.5). Others were invaginated showing little, if any, vesiculation (Fig. 4.6). Intermediate vesiculating forms were also found in addition to cysts which had a very irregular, angular outline although still possessing a relatively thick laminated layer (Fig. 4.7).

Between 30 and 54 days p.i., mice generally possessed less than

10 cysts with laminated layers. These were mostly of irregular shapes (Fig. 4.8) although occasionally, the cysts were fully vesicular and showed signs of sucker degeneration (Fig. 4.9). The calcareous corpuscles were generally much reduced in number although some parasites still possessed substantial proportions of these structures (Fig. 4.10). All cysts still retained the rostellar hooks which in many cases were condensed into a bud at the apex of the cyst (Fig. 4.11), projecting into the laminated layer. In the majority of cysts this laminated layer was still clear in appearance but in others it was up to 31.4μm thick, and starting to darken or take on a "tanned" appearance when viewed under transmitted light,and produce laminations. Subsequent development of the cyst involved the formation of a "neck" region, at the distal end of which the "hook bud" appeared to be becoming detached (Fig. 4.12).

By 90 days p.i. between 20 and 47 cysts with laminated layers were found in individual mice. These occurred either free or as cyst masses encapsulated in a host cellular response. The diameter of the hydatids ranged from 390-600μm with a maximum laminated layer thickness of 165μm. The cysts now had a more rounded appearance although some still had an irregular shape, even after 132 days (Fig. 4.13). The laminated layers had become progressively darker and possessed more laminations (Fig. 4.13) although these were not evident at the periphery of the layer.

Between 6 months and 12 months p.i.; the size of the hydatids showed a much greater variation (Fig. 4.14) and ranged from 0.5-6.5mm at 6 months p.i. to 0.6-31mm at 12 months p.i. where the laminated layer had a maximum thickness of 215μm and possessed up to 25 laminations. In general the free cysts tended to be larger than those within the cyst masses. The thickness of the laminated layer was also not constant between cysts from the same infection and in

many cases small cysts had a comparatively thicker laminated layer than large ones (Fig. 4.15). The germinal layer of these cysts was now devoid of any hook or sucker remnants and possessed newly formed calcareous corpuscles.

The number of cysts obtained from each mouse, 6-12 months p.i., ranged from 16 to 706 with a mean of 247.6 ∓ 48.32 thus indicating that only 6.2% of the injected protoscoleces, formed cysts. Only on rare occasions, however, were no cysts found in mice autopsied 6-15 months p.i. In the majority of cases all cysts produced were sterile and only on 5 separate occasions was a fertile cyst found, between 12 and 15 months p.i. These however were not always the largest cysts in the infection.

The increase in size of cysts during the first year of infection was difficult to investigate since there was such great variation in the diameter of cysts taken from each mouse. During sampling, however, the largest and smallest cysts in each mouse were measured as well as a random sample of 10-15 of the remaining cysts in order to obtain an average diameter. Fig. 4.16 shows a graph of this mean cyst diameter (taken from at least 3 mice on each occasion) against time. From this graph it can be seen that the growth rate is not constant but apparently accelerates between 210 days (7 months) and 270 days (9 months) after which the rate slows down, giving a mean cyst diameter of approximately 3.125mm. Again it must be stressed that because of the variability in cyst dimensions and the fact that the whole population was not measured, many individuals were either larger or smaller than these mean values. This variability was most evident later in the infection as shown by the standard error bars in Fig. 4.16.

A graph of mean laminated layer thickness against time (Fig. 4.17) was also plotted for the same population of cysts. This shows

that there was a constant increase in the thickness of the laminated layer up to 132 days after which there was an apparent reduction in mean thickness between 132 days and 180 days. After this period there was a second increase, levelling off around 270 days at a mean diameter of around 140μm. This apparent reduction in the depth of the laminated layer may have been artifactual but may also reflect a true developmental process as will be discussed in Section 4.3.

4.2.2 Development of cysts within diffusion chambers

The cystic development of protoscoleces within diffusion chambers showed some similaraties to both *in vitro* development and *in vivo* development after i.p. injection. After 4 days 69-81% of the protoscoleces were in the invaginated state whilst 8-10% were starting to vesiculate (still invaginated). Occasional parasites possessed small posterior bladders but these amounted to less than 1% of the total. By 12 days, although most of the protoscoleces were still invaginated, 19%-25% were undergoing vesiculation. This figure increased to 68%-73% at 25 days, of which 6%-11% possessed a thin laminated layer. Morphologically these developing cysts were of the evaginated vesicular type and still possessed rostellar hooks and sucker remnants. Throughout the first 35 days the proportion of dead protoscoleces ranged from 8%-16.5%.

One interesting feature in one of the 35 day samples was the presence of large evaginated protoscoleces (Fig. 4.18) similar to those showing adult-like development during the *in vitro* culture. These were approximately 300μm in length and amounted to 9.4% of the protoscolex population. Their adult-like characteristics were verified by examinations under TEM (see Chapter 6).

The 90 day samples revealed 2000-2350 individuals within each

chamber of which 18%-32% were dead protoscoleces and 68%-82% were developing hydatids with laminated layers. These were small and fairly uniform in size, with the largest being 1000μm in diameter and the mean being 287∓4.9μm and this was generally lucent in appearance and lacking laminations (Fig. 4.19). Occasional cysts, however, had a much darker laminated layer with a limited number laminations (Fig. 4.20). Some of the hydatids showed little evidence of the original scolex region of the protoscolex but many others possessed a "neck region" with an associated "hook bud" in the process of detachment (Figs. 4.19, 4.21).

Chambers removed after 7 months (210 days) contained 1800-2100 developing cysts with laminated layers. These again were small and relatively uniform in size (1135 ∓ 135μm in diameter) and had a laminated layer 127.8 ∓ 15.12μm in depth (Fig. 4.22) which possessed several laminations.

The single chamber retrieved after 8 months (240 days) had ruptured and 3 large cysts 18-28mm in diameter were present free within the peritoneal cavity. Within the ruptured chamber 84 cysts were present and ranged in diameter from 1.8-14mm in diameter.

Two final chambers removed after 9 months (270 days) possessed 2100-2300 cysts which had a mean diameter of 1,525μm∓174 and a mean laminated layer thickness of 132 ∓ 21μm. The mean number of cysts produced in all diffusion chambers 3-9 months p.i. was therefore 1661 ∓ 279 (N=7) and reflected a 41.5% cyst-production assuming an initial infection of 4000 protoscoleces.

When cysts grown in chambers for 9 months were transplanted into the peritoneal cavity of parasite-free mice, 18 out of 30 (3 mice,10 cysts each) of the organisms were retrieved after 14 months. These cysts had a mean diamter of 6.68mm ∓ 0.89 thus reflecting a 4.38% increase in cyst diameter.

4.3 DISCUSSION

The present studies show that BALB/c mice are acceptable hosts for the growth of secondary hydatids of the horse strain of *E. granulosus*. Results are in accordance with previous reports employing this strain of mice (Pauluzzi & De Rosa, 1969; Connor, 1980; Richards, Arme & Bridges, 1983b) but suggest that this host may not necessarily give the best results. Although there was a considerable percentage infectivity, the number of fertile cysts (i.e. possessing protoscoleces) produced was extremely low; a feature also observed by Connor (1980). Other authors, however, have achieved 30-65% fertile cysts by growing the horse strain of *E. granulosus* in gerbils (Thompson, 1976a). If fertility is a criterion of better growth and development then perhaps the gerbil would make a more suitable laboratory host.

The present studies showed that, from approximately 4000 protoscoleces, 0.4%-17.65% (mean=6.2%) formed cysts. These data and the sizes of the cysts are comparable to the results of other authors using a variety of hosts (Gurri, 1963; Pauluzzi & De Rosa, 1969; Frayha, Lawlor & Danjani, 1971; Pennoit-De Cooman *et al.* 1974) and suggest that the vast majority of protoscoleces do not form cysts and presumably die.

The viability of protoscoleces in the present studies dropped rapidly in the first 18 days suggesting that they are most vulnerable in the early stages of development *in vivo* i.e. before the formation of the laminated layer. It must be stressed that in these early stages only 1,250-3,500 organsism were retrieved, suggesting that either all of the protoscoleces were not located, or some had disintegrated or that the numbers injected were not always close to 4,000. It is likely that the latter reason holds true due to the

crude estimations of protoscolex numbers used. This problem is probably common to most of the previous studies and only Pennoit-De Cooman *et al.*, (1974) have devised a complex electronic counter, modified from a Coulter-type counter, to estimate protoscolex numbers more accurately.

The variability in the number of cysts produced in each mouse also seems to be a common feature in previous reports and various hypotheses have been put forward to explain this. The physiological state of each protoscolex batch may vary so that some parasite infections may be more viable than others (Gurri, 1963; Pennoit-De Cooman *et al.* 1974). It is likely that this variation in physiological conditions is a function of the age of each "donor" cyst. Since it is known that all protoscoleces are not formed in cysts at the same time (Dew, 1922; Pennoit-De Cooman & De Rycke, 1972; Thompson, 1976c; Rogan & Richards, 1987) then the population of protoscoleces with any one cyst will show a heterogeneous age profile. Each batch will therefore contain a proportion of very old protoscoleces and a proportion of very young individuals, both of which may be incapable of forming cysts.

Another possible factor involved in number of cysts formed may be the sex of each host animal. There seems to be no consensus of opinion on this idea since Frayha *et al.* (1971) stated that male mice made better hosts whilst Pennoit-De Cooman *et al.* (1974) reported that female mice produced better cyst infections. Thompson (1976a), however, observed no difference in cyst production between male and female gerbils. Although no specific choice, of host sex, was made in the present study records showed that male mice were used in approximately 66% of cases thus reducing any host-sex variability that may exist but not eliminating it.

In addition to batch variation it seems most likely that the

factor most influencing cyst numbers is the intensity of the host immune response which may vary from mouse to mouse. Clearly the percentage of cysts produced after injection (approximately 6.2%) is considerably elevated if the parasites are placed in diffusion chambers (approximately 41.5% cyst production). This finding indicates that the host cellular attack may play a major role in the killing of protoscoleces and that when this element of the host response is eliminated or substantially reduced*, by using diffusion chambers, more cysts are produced. The death of the remaining protoscoleces in the diffusion chambers is likely to be due to the humoral immune response (which would not be eliminated from the chambers) since it is known that protoscoleces, *in vitro*, are susceptible to host serum components (Herd, 1976; Kassis, Goh & Tanner, 1976; Rickard *et al.* 1977). The ability of the small cysts, produced in the chambers, to continue growth when transplanted to unconfined conditions in naive mice suggests that they are similar to the "latent cysts" of Pennoit-De Cooman & De Rycke (1978). It also makes this a useful technique for producing and maintaining large numbers of cysts for a variety of experimental procedures. The continued growth of these cysts also emphasises the point that the parasites no longer seem susceptible to the host immune response after a certain stage of development - a phenomenon which may well depend on the presence of an established laminated layer.

In normal development *in vivo* the processes leading up to the formation of the laminated layer appear to be different from that *in vitro*. In the initial few weeks following infection there seems to be little morphological development and protoscoleces were mainly in the invaginated condition. This contrasts with the relatively rapid evagination and vesiculation observed *in vitro*. Once morphological changes started to occur they involved the protoscoleces often taking

* see Section 6.2.2

on irregular shapes, and sometimes forming a laminated layer before any vesiculation. These findings therefore contrast with the suggestions of Heath (1970) and Thompson (1976c) that early cystic differentiation *in vivo* was similar to that *in vitro*. In the present study cysts generally only became spherical and fully vesicular after the laminated layer had become well established. This suggests that events in the *in vitro* system may be abnormal.

It seems likely that the sequence of events involving vesiculation and laminated layer formation has become somewhat reversed in the *in vitro* system. The stimulus initiating vesiculation is therefore perceived before that initiating laminated layer formation. However, subsequent differentiation and the end products of the two culture systems are similar, thus confirming the value of the *in vitro* system.

Early development in diffusion chambers appeared to show features more akin to *in vitro* development. The early vesiculation of a proportion of the protoscoleces, the presence of posterior bladders and the occurrence of adult-type parasites are all features associated with *in vitro* differentiation and which also occur to some extent within the diffusion chambers. It seems possible that the protoscoleces, in the chambers were influenced by the NCTC 135 medium in which they were initial suspended. The conditions inside the chambers may therefore, not approximate to the environment within the peritoneal cavity for some time and may represent a "pseudo *in vitro*" system.

It seems, however, that events in all the cultivation systems are similar with respect to laminated layer development. The layer starts off as a clear amorphous zone around the developing cyst and subsequently takes on a tanned appearance, possessing laminations, under transmitted light. This change in appearance may be a result

of some polymerization activity present after initial secretion of the laminated layer precursors or may additionally result from compaction and shrinkage of the layer itself. This second reason may be supported by the data on laminated layer thickness over time. These data appear to indicate that the laminated layer increases in depth to a point after which it is reduced and subsequently increases again. The reduction in depth occurs at the time when the cyst is undergoing maximum increase in volume. Internal hydrostatic pressure and external pressure from adjacent cysts or organs may therefore compress the laminated layer maxtrix and compact it to form the colouration and laminations. It must be stressed however, that due to the great variability in the size of cysts from each mouse, true data on growth of cysts may be hard to obtain; a problem also commented on by Thompson (1976a).

Throughout the *in vivo* development of secondary hydatids the most striking event to occur is the formation of the laminated layer. As this structure appears early on in differentiation it must be of considerable benefit to the parasite. It would therefore be of great value to examine how this layer aids the cyst, particularly in the evasion of the immune response.

CHAPTER 5

STRUCTURE OF THE GERMINAL LAYER, BROOD CAPSULE WALL AND PROTOSCOLEX TEGUMENT

5.1 INTRODUCTION

The body surface of metacestodes, like that of adult tapeworms, is covered with a continuous cytoplasmic layer, supported by a band of connective tissue and joined, by cytoplasmic processes, to underlying nucleated cell bodies. The whole structure is now collectively known as a tegument (Threadgold, 1962) and the ultrastructure of both metacestode and adult cestodes has been reviewed by Lee (1966; 1972), Smyth (1972), Lumsden (1975), Lumsden, Voge & Sogandares-Bernal (1982), Lumsden & Hildreth (1983), Ubelaker (1983) and Threadgold (1984). The terminology involved in describing this ultrastructure, however, is not consistent and is often confusing (Arme, 1984). Fig. 5.1 is a composite, generalised diagram of the cestode tegument showing both adult and metacestode features, and indicates the terminology which will be adopted in the present study together with commonly used alternative terms.

The three main regions of the tegument are the distal cytoplasm, internuncial processes and tegumentary cytons and these are present in all of the surface coverings of the hydatid cyst of *E. granulosus*. The terms "germinal layer" and "brood capsule wall" are therefore used to describe structures which are tegumentary in nature but which also have other tissues, such as muscle, excretory tubules and calcareous corpuscles associated with them.

The Germinal Layer

The germinal layer tegument of *E. granulosus* hydatids has been described by Morseth (1967), Bortoletti & Ferretti (1973), Lascano, Coltorti & Varela Diaz (1975), Lopez-Campos, Linares, Alonso, Aneiros & Diaz Flores (1978), Singh & Lee (1979), Conder, Marchiondo, Williams & Andersen, 1983, Richards, Arme & Bridges (1983a) Threadgold (1984) and Casado Escribano & Rodriguez Caabeiro (1986) and has been shown to consist of a distal cytoplasm connected to underlying, nucleated tegumentary cytons by internuncial processes. The distal cytoplasm possessed truncated microtriches with little or no electron-dense spine and was in direct contact with the laminated layer which was composed of a microfibrillate matrix containing aggregates of electron-dense granules. The laminations in this layer have been attributed to different degrees of compaction of these components (Morseth, 1967; Lopez-Campos *et al.*, 1978; Singh & Lee, 1979; Richards *et al.*, 1983a; Casado Escribano & Rodriguez Caabeiro, 1986).

The laminated layer has been shown to be strongly PAS positive and to be a protein-carbohydrate complex, with glucosamine, galactosamine and galactose being the major carbohydrate components (Kilejian, Sauer & Schwabe, 1962; Korc, Hierro, Lasalvia, Tolco & Calcagno, 1967; Kilejian & Schwabe, 1971). Kilejian *et al.*, (1962) also suggested that this structure had infra-red spectral characteristics similar to chitin. Certain lipids have also been detected (Richards *et al.* 1987 but whether these are of host or parasite origin is not clear. Richards (1984) demonstrated that the carbohydrate component possessed 1,2-glycols, that the protein complex was predominantly basic with identifiable S-S groups and that acid mucopolysaccharide material was present in the laminated layer of secondary cysts from mice. Glycogen, however, was not detected

(Kilejian *et al.*, 1962). There seems to be some doubt as to whether calcium was present in the laminated layer since Morseth (1967) reported a negative reaction for calcium using the Alizarin Red S test whilst Cameron & Fitzpatrick (1925) obtained a positive reaction using the Macallum method. Richards (1984) also detected divalent cations, which may have been calcium, using the Von Kossa test and the chloranilic acid reaction.

Within the distal cytoplasm numerous electron-lucent, membrane-bound vesicles, often containing an electron-dense granule, have been observed (Morseth, 1967; Bortoletti & Ferretti, 1973; 1978; Lascano *et al.*, 1975; Richards *et al.*, 1983a; Threadgold, 1984). These have been suggested as being involved in either pinocytosis (Lascano *et al.*, 1975) or exocytosis since Richards *et al.*, (1983a) reported that the electron-dense granules were the same as those occurring in the laminated layer and were formed in the Golgi complexes of the cytons for eventual release from the distal cytoplasm. Richards (1984) also indicated that the microfibrillate matrix of the laminated layer was formed by release of carbohydrate containing material from Golgi-derived vesicles. Other authors have reported that there are very few other organelles such as mitochondria or electron-dense secretory bodies within the distal cytoplasm compared with that of other metacestodes (Bortoletti & Ferretti, 1973; Lascano *et al.*, 1975; Singh & Lee, 1979; Conder *et al.*, 1983).

The perinuclear cytoplasm of the tegumentary cytons have been shown to possess varying numbers of mitochondria, electron-lucent vesicles and Golgi complexes whereas ribosomes were rare and always occurred free (Bortoletti & Ferretti, 1978). Occasional dense lysosomal bodies have also been observed (Bortoletti & Ferretti, 1973; Lascano *et al.*, 1975; Singh & Lee, 1979; Threadgold 1984),

although Richards *et al.* (1984) reported that single cisternae of smooth endoplasmic reticulum, at the periphery of the cytons, may be involved in the formation of two types of autophagosomes/autolysosomes.

Other cell types present in the germinal layer were muscle cells, glycogen storing cells and flame cells with their associated collecting ducts (Morseth, 1967; Bortoletti & Ferretti, 1973; Lascano *et al.*, 1975). The presence of calcareous corpuscles has also been noted (Lascano *et al.*, 1975).

Considerable variation in the ultrastructure of cysts of *E. granulosus* from different host species (Bortoletti & Ferretti, 1978; Richards *et al.*, 1984) and in cysts from the same host species (Richards *et al.*, 1984) has been reported and these lie mainly in the thickness of the laminated layer, numbers of microtriches and vesicles, amount of stored glycogen and the extent of autophagic activity.

The Brood Capsule Wall

The ultrastructure of the brood capsule wall has been described by Morseth (1967), Bortoletti & Ferretti (1973), Lopez-Campos *et al.*, (1978) and Threadgold (1984). The polarity of the tegument is reversed so that the distal cytoplasm is directed in to the lumen of the capsule while the cytons lie around the outside, bathed in the hydatid fluid. The distal cytoplasm is thinner than that of the germinal layer and also possesses truncated microtriches. Internally, numerous electron lucent vesicles are present which extend into the tegumentary cytons where Golgi complexes and mitochondria have also been observed. The thickness of the distal cytoplasm and the whole of the brood capsule wall were found to vary with the age of the capsule and the degree of protoscolex formation

(Bortoletti & Ferretti, 1973), being thicker in younger capsules and in areas where protoscoleces develop. Muscle tissue and flame cells were also reported to be present in the brood capsule wall.

The Protoscolex Tegument

The ultrastructure of the tegument of protoscoleces of *E. granulosus* and *E. multilocularis* is basically similar and has been described by Morseth (1967), Sakamoto & Sagimure (1970), Bortoletti & Ferretti (1973), Kassis, Goh & Tanner (1976), Lopez-Campos *et al.*, (1978), Singh & Lee (1979), Kaminja, Tukamoto & Oku (1982) Marchiondo & Andersen (1983), Threadgold (1984), and McManus & Barrett (1985). It was found to show regional variations in ultrastructure, particularly between the scolex and the posterior region which has been termed the "soma" in the present study. Fully formed, spined microtriches were present over the whole of the scolex, including the rostellum where they obscured the rostellar hooks. These projections were polymorphic, with long, filamentous types being present over the rostellum, and broader, blade-like forms being present over the suckers. In the soma region microtriches were absent whilst small knob-like elevations, with an electron-dense cap, were abundant. These have been considered to be undeveloped microtriches by some authors (Morseth, 1967; Kassis *et al.*, 1976). The soma region also possesses a thick PAS positive glycocalyx which is not present over the scolex.

Within the distal cytoplasm numerous vesicles of various types were present in both the scolex and soma although the functions of these were not known. Mitochondria were rarely seen in the distal cytoplasm but were present in the nucleated tegumentary cytons which possessed other organelles similar to the cytons of the germinal layer tegument.

The previous work on the ultrastructure of the teguments of the hydatid cyst and protoscolex has only described the basic details. If a full investigation into the development of these structures, (e.g. the protoscolex tegument into the germinal layer or the germinal layer into the protoscolex tegument via the brood capsule) is to be performed then more detailed observations are required.

5.2 RESULTS

5.2.1 Ultrastructure of the germinal layer tegument

In hydatid cysts of over 9 months in age, grown in mice, the distal cytoplasm of the germinal layer was 2.199 ∓ 0.09μm in depth (Fig. 5.2). The outer edge was bounded by a plasma membrane which was continuous over the tegumentary projections. These projections, which will be referred to as truncated microtriches, were cylindrical structures, 0.438 ∓ 0.015μm in height and 0.119 ∓ 0.002μm in diameter. They tapered slightly at the distal end where occasionally, an accumulation of electron-dense material was present (Fig.s 5.3, 5.4a). The shaft of these microtriches had an inner, electron-dense shaft support which, in longitudinal section, appeared as two peripheral bands, 9.25 ∓ 0.375nm thick, lying parallel to the microthrix membrane and approximately 10nm from it.

The core of the shaft, in favourable sections, possessed several micro-filamentous structures, approximately 3.65nm in diameter and 8.0nm apart. These filaments ran the length of the shaft and terminated in the region of a base plate which appeared circular (22.9nm in diameter) in all section planes indicating its spherical nature (Figs. 5.3, 5.4a).

The truncated microtriches projected into the laminated layer which in 9 month old cysts was 14.4 ∓ 19.35μm in depth before fixation. In TEM this layer appeared to have a microfibrillate matrix and possessed clusters of electron dense granules which individually had a diameter of 30.95 ∓ 0.96nm (Fig. 5.2, 5.5). These granules except for a region ranging from 0.6μm to 1.8μm thick immediately adjacent to the apical plasma membrane (Fig. 5.6). Their distribution, however, was not random and accumulation of the

clusters into bands was frequently observed (Fig. 5.7) and presumably corresponded to the laminations seen with the light microscope.

Within the distal cytoplasm numerous electron lucent, membrane bound vesicles were present (Fig. 5.3). These were generally oval or elongated in shape, 226.69 ∓ 8.63nm long and 95.53 ∓ 5.57nm wide. A large number of these vesicles contained a single electron-dense granule which was 32.77 ∓ 1.08nm in diameter. In unstained sections these granules (Fig. 5.8), and those of the laminated layer (Fig. 5.9), appeared to have an internal alveolar structure suggesting that both types of granules had the same origin. In general the vesicles within the distal cytoplasm had a random orientation but in the region immediately adjacent to the apical plasma membrane, they frequently were orientated with their long axis perpendicular to the membrane (Fig. 5.10). Although these occurred very close to the apical membrane, fusion with the membrane was never observed. Electron-dense discoid bodies were usually not present in the tegument.

At the base of the distal cytoplasm, invaginations of the basal plasma membrane extended between 0.435μm and 1.095μm into the distal cytoplasm near occasional moderately dense mitochondria with few cristae (Fig. 5.11). Also present in the internuncial processes and the tegumentary cytons were mitochondria which were tubular in nature, of varying lengths and approximately 0.208 ∓ 0.007μm in diameter. The nuclei were 2.423 ∓ 0.011μm in diameter and had a large prominent nucleolus (Fig. 5.12). The perinuclear cytoplasm contained numerous vesicles with electron-dense granules (35.38 ∓ 1.38nm in diameter) similar to those of the distal cytoplasm (Fig. 5.12). In addition to these, other vesicles with varying numbers of smaller granules (17.47 ∓ 0.71nm in diameter) were also observed (Fig. 5.13) particularly in the region of the Golgi

complexes which appeared to be composed of loosely stacked cisternae. The presence of these very small granules suggests that the larger ones may be formed by fusion of the smaller ones. In addition, small vesicles (approximately 55.8nm in diameter), devoid of dense contents, were also osberved in the perinuclear cytoplasm (Fig. 5.14). These may have been transition vesicles between the Golgi complexes and the endoplasmic reticulum which occurred only as a series of single cisternae around the periphery of the cytons (Fig. 5.15). Occasional vesicles (approximately 132nm in diameter) with flocculent peripheries were present within the perinuclear cytoplasm (Fig. 5.15) but rarely within the distal cytoplasm. Ribosome-like bodies approximately 17nm in diameter, were present on the ER cisternae in some cases (Fig. 5.16) but not in all. Similarly such structures sometimes occurred associated with the outer nuclear membrane and free within the cytoplasm (Fig. 5.13) or were totally lacking. It is possible that some of these bodies could be β-glycogen particles, which are 15-40nm in diameter (Threadgold 1976), but most of the glycogen seemed to occur in the α-rosette formation within separate cytoplasmic extensions of the tegumentary cytons (Fig. 5.14). These extensions were simple sac-like structures which had a very pale cytoplasm and varying amounts of glycogen granules ranging from none (Fig. 5.15) to numerous (Fig. 5.17). No evidence of glycogen-storing "cells" (Bortoletti & Ferretti, 1973) was observed. Large lipid droplets which may or may not have been osmiophilic were also present in the extensions.

Other organelles occurring in the perinuclear cytoplasm were very occasional microtubules, approximately 20nm in diameter, (Fig. 5.18) and two types of structures which may represent autophagosomes (Fig. 5.19). The first of these were large vacuoles, between 0.195μm and 1.85μm in diameter, which had a moderately dense periphery and

flocculent contents. The size of these large vacuoles may be affected by fusion of smaller, similar vesicles which were often seen in close proximity to each other (Fig. 5.20). The second type of vacuole was 0.686μm to 1.087μm in diameter and also had flocculent contents. It differed from the first type, however, in possessing an asymmetrical periphery of dense lamellar material with a periodicity of approximately 6.2nm between the laminations, possibly suggesting a that these structures are residual bodies of membraneous origin (Figs. 5.18, 5.21). The absence of conspicuous lysosomal bodies may support the suggestion that both types of vacuole may be involved not only in autophagocytosis but in autolysis also. Richards *et al.* (1984) suggested that the lamellar type may be involved in autophagy and degradation of glycogen granules and membraneous organelles. The non-lamellar type, on the other hand, may be involved in sequestration of Golgi-derived products and may represent a type of storage lysosome.

Other cell types present in the germinal layer were flame cells and their associated collecting ducts, calcareous corpuscle producing cells and muscle cells or myocytons. These myocytons, in addition to the muscle fibrils also possessed glycogen-containing cytoplasmic extensions but could be distinguished from tegumentary cytons by their pale cytoplasm and narrower mitochondria (0.173 ∓ 0.014μm) (Fig. 5.22). The tegumentary distal cytoplasm was separated from these other tissues by an inconspicuous basement lamina (Fig. 5.6) which was less well developed than in the protoscolex (see Section 5.2.3).

From the many cysts examined, considerable variation was seen in the tegumentary tissue. This variation was mainly confined to the quantity of components such as ribosomes, Golgi complexes, endoplasmic reticulum, glycogen and lipid. The detailed nature of

the mouse cysts could not, however, be predicted from their external appearance and there was no significant difference in the general structure of the germinal layer of murine cysts compared with that of the horse cysts examined in the present study.

5.2.1.2 Histochemistry

(a) Lectin binding analysis:

Lectin binding analysis at the light microscopic level indicated that the laminated and germinal layers of hydatid cysts grown in mice bound wheat germ agglutinin (WGA) (Fig. 5.23), soybean agglutinin (SBA) (Fig. 5.24) and peanut agglutinin (PNA) (Fig. 5.25) indicating the presence of N-acetylglucosamine, N-acetylgalactosamine and galactose respectively. Asparagus pea agglutinin (APA) did not bind to either layer (Fig. 5.26) and sections appeared similar to unstained sections which showed a slight autofluorescence of the germinal layer (Fig. 5.27). This suggests that fucose is absent from the cyst. Concanavalin A (Con A) did not bind to the laminated layer but did bind strongly to the germinal layer indicating the presence of mannose and/or glucose in that region only (Fig. 5.28). With the exception of WGA, binding of all other lectins could be completely inhibited by the addition of the respective sugar, thereby confirming their specificity. With WGA the fluorescence was reduced although some still occurred indicating that at least a proportion of WGA binds non-specifically. Pretreatment with neuraminidase did not affect lectin-binding indicating that no additional binding sites were exposed on removal of any terminal sialic acid residues which may have existed.

When lectin binding studies were carried out at the electron microscope level, using peroxidase labelled lectins, definition was not good. Although there was a clear positive reaction in the

laminated layer (Fig. 5.29) for WGA, SBA and PNA, non-specific reaction deposits made it impossible to locate any specific binding within the germinal layer (Fig. 5.30), even when a 10 fold dilution of the DAB components was made.

(b) Ultrastructural cytochemistry

Ultrastructural carbohydrate localization was much more precise when the thiosemicarbazide-silver proteinate method was used. Here sections incubated in the complete medium showed a strong reaction in the laminated layer and the glycogen-containing processes of the cytons (Fig. 5.31). Within the distal cytoplasm of the germinal layer tegument, however, very little reaction product was present, especially in the vesicles (Fig. 5.32). A slight deposit was, however, present around the dense granules of these vesicles. This could have been non-specific since similar deposits were seen around the granules and any fissures in the laminated layer of control sections incubated in a silver proteinate solution alone (Fig. 5.33) and around the distal cytoplasm granules when incubated in a medium lacking the periodic acid (Fig. 5.34).

The reaction product in the laminated layer was absent between the truncated microtriches (Fig. 5.32). This was also the region where the microfibrillate matrix in normal stained sections was absent and may reflect an artifactual shrinkage and pulling away of the laminated layer or a region of unpolymerized material.

Within the tegumentary cytons the reaction product was scarce (Fig. 5.35) and mainly limited to both the lamellar (Fig. 5.36) and non-lamellar (Fig. 5.37) vacuoles/autophagosomes. Localization within the perinuclear vesicles or Golgi complexes was not observed.

5.2.1.3 X-ray microanalysis

In order to further analyse the nature of the electron-dense granules in the germinal layer and laminated layer, a limited amount of energy dispersive X-ray microanalysis was carried out by the Zoology Department of The Queen's University of Belfast. Sections, 0.3µm thick, of 9 month old mouse cysts were mounted on aluminium grids and analysed in a Jeol 100CX transmission electron microscope fitted with a Link systems 860 microanalyser.

The analysis of the granule clusters in the laminated layer showed that the most significant elements present were calcium and phosphorous (Fig. 5.38) possibly indicating that calcium phosphate was a constituent of the granules (N.B. the tissue was processed using a non-phosphate containing buffer). The other peaks which occurred in the spectra were constantly present throughout the analysis and can be identified from known sources i.e. aluminium from the grid; chlorine from the resin; and copper from the grid holder arm.

The spectra for the granules within the germinal layer distal cytoplasm (Fig. 5.39) showed the same calcium and phosphorous peaks but to a lesser degree, possibly due to single granules being analysed rather than clusters (in the laminated layer). Again the same background elements were present. When the two spectra were compared together (Fig. 5.40) it was clear that both the granules in the germinal and laminated layers had the same components but in different amounts.

5.2.2 Ultrastructure of the brood capsule wall tegument

The distal cytoplasm of the mature brood capsule tegument was considerably thinner than that of the germinal layer and protoscolex

being approximately 0.406 ∓ 0.037μm in depth. The surface of the distal cytoplasm possessed truncated microtriches similar to those seen on the germinal layer tegument (Figs. 5.41, 5.42). The density of these projections was, however, much less than the germinal layer with their being totally absent in some areas. A flocculent glycocalyx was observed on the internal surface of the brood capsule particularly near where protoscoleces were present (Fig. 5.42). This glycocalyx gave a positive reaction for carbohydrates using the thiosemicambazide-silver proteinate method (Fig. 5.43). The distal cytoplasm possessed numerous membrane-bound vesicles which were elongate in shape (approximately 265nm in length and 84nm in breadth) and were almost totally electron lucent (Fig. 5.41). Occasionally vesicles did, however, have a moderately dense granule within them (Fig. 5.41). Mitochondria were observed in the distal cytoplasm (Fig. 5.41) and in the perinuclear cytoplasm of the cytons (Fig. 5.44) but were generally few in number. The cytons were connected to the distal cytoplasm by very short internuncial processes (Fig. 5.44) and in many cases the nuclei actually appeared to lie in swellings of the distal cytoplasm (Fig. 5.42). The perinuclear cytoplasm was typically dense but was poorly supplied with organelles. Golgi complexes were not detected although infrequent electron-lucent vesicles were present (Fig. 5.44). Similarly endoplasmic reticulum was rare although ribosome-like bodies were frequently seen (Fig. 5.44). Occasional electron-dense bodies which may have been lysosomes or residual bodies were also present (Fig. 5.42). The cytons possessed cytoplasmic extensions containing glycogen granules (Fig. 5.42) but these were frequently poorly supplied with glycogen (Fig. 5.44).

The cytons were randomly distributed around the brood capsule except for the regions of the protoscolex attachment stalks where

more cytons were present. Each stalk (Fig. 5.45) was 1.8-3.9μm in length and possessed a significant distribution of truncated microtriches (Fig. 5.46, 5.47). Vesicles within the distal cytoplasm of the stalk often contained electron-dense structures (Fig. 5.46) similar to those of the protoscolex soma (see Section 5.2.3). The remainder of the internal structure of the attachment stalk was composed of muscle fibrils, cytons (Fig. 5.46) and flame cell collecting ducts which appeared to run the length of the stalk towards the protoscolex (Fig. 5.45).

Muscle fibrils and collecting ducts were also observed around the whole of the brood capsule (Fig. 5.48) and a basement lamina was not observed. In SEM the ducts could be seen to cross the brood capsule in several directions (Figs. 5.49, 5.50).

5.2.3 Ultrastructure of the protoscolex tegument

The structure of the protoscolex is such that it is divided into two main regions when in the evaginated state (Fig. 5.51). These have been termed the scolex and the soma. When the protoscolex is in the invaginated condition the scolex is inverted inside the soma (Fig. 5.52). Ultrastructurally the tegument was found to show variations between these regions.

(a) The soma tegument:

The distal cytoplasm of the soma tegument varied in depth from 2.963 ∓ 0.147μm at the posterior end to 1.866 ∓ 0.119μm at the mid-soma region. The apical surface possessed a thick glycocalyx (0.535 ∓ 0.02μm in depth) which had a microfibrillate appearance when viewed in TEM (Fig. 5.53). Below the glycocalyx, characteristic cestode microtriches were absent but numerous small, blunt elevations

(Fig. 5.4b 5.53) were evident. These were dome shaped, approximately 0.127 + 0.005μm in height and 0.146 ∓ 0.02μm in width. An inner electron-dense "cap" was present in these structures which was approximately 14.75nm thick and which occurred some 12nm from the apical membrane. This electron dense material was not continuous between adjacent blunt elevations.

Within the distal cytoplasm, numerous membrane bound vesicles were present and these appeared to be of 3 main types. The first type (T_1) were vesicles which were oval or elongate in shape, 230.4 ∓ 23.2nm long and 148.5 ∓ 8.73nm wide (Fig 5.54). These were electron lucent or slightly flocculent but possessed an electron dense core which was "comet" shaped in longitudinal section or circular (54.2 ∓ 0.99nm in diameter) in transverse section. The second type of vesicle (T_2) was disc-shaped and of a similar size to the T_1 vesicles but possessed dense flocculent contents which were frequently, particularly evident at the periphery of the vesicles (Fig. 5.55). The third type of vesicle (T_3) was a simple electron lucent structure of similar size and shape to the other two types (Fig. 5.55). In the soma tegument the T_1 and T_3 vesicles were the most numerous whilst the T_2 vesicles were very rare but were seen in greatest numbers in the anterior soma region.

Occasionally mitochondria of moderate electron density and possessing few cristae were observed in the basal region of the distal cytoplasm (Fig. 5.54). Below these a basal plasma membrane was present which often infolded into the distal cytoplasm to form shallow "basal infolds" 0.185μm-0.315μm in length (Fig. 5.55). Below the distal cytoplasm a thick basement lamina was evident, approximately 0.455 ∓ 0.07μm thick and having a fibrous appearance under TEM. This band of connective tissue separated the distal cytoplasm from underlying circular and longitudinal muscle fibrils

(Fig. 5.54).

Internuncial processes connected the distal cytoplasm to the tegumentary cytons which lay below the muscle (Fig. 5.56). These cytons typically had a dense cytoplasm and a central nucleus, 1.68 ∓ 0.135μm in diameter which possessed a prominent nucleolus (Fig. 5.57). Within the perinuclear cytoplasm mitochondria, with few cristae, were evident (Fig. 5.57) and were elongate or tubular with a diameter of approximately 0.250 ∓ 0.01μm. The density of the perinuclear cytoplasm was partially due to the presence of numerous small granules, approximately 18nm in diameter (Fig. 5.57). These may have been β-glycogen particles but the negative thiosemicarbazide-silver proteinate reaction in the cytons (see Section 5.2.3.2) does not support this suggestion. The association of some of these particles with single cisternae of endoplasmic reticulum, which occurred only around the periphery of the cytons (Fig. 5.58), suggests that they are more likely to be ribosomes. Glycogen was however present, in the α formation, within cytoplasmic extensions of the tegumentary cytons (Fig. 5.57) which often contains lipid droplets of moderate electron-density.

Golgi complexes were only occasionally observed in protoscoleces fixed directly after removal from primary cysts. Such complexes generally appeared inactive, lacking associated vesicles (Fig. 5.57). On other occasions however the Golgi complexes were more prominent, consisting of stacked cisternae which often contained electron-dense contents in the cisternae of the maturing face (Fig. 5.59) and transition vesicles in the forming face. The vesicles associated with these Golgi fields appeared to be originally of uniform electron density (Fig. 5.59) but subsequently appeared as electron-lucent vesicles with a rounded dense core (Fig. 5.59). These were present in the perinuclear cytoplasm and in the internuncial processes and

probably were equivalent to the T_1 bodies of the distal cytoplasm since occasionally they had the "comet" shaped core (Fig. 5.60). Since no distinct lysosomal-like bodies were identified in the perinuclear cytoplasm, some of the vesicles occurring near the Golgi complexes may have been hetero/autolysosomes.

(b) The scolex tegument

The scolex tegument varied from that of the soma in a number of ways. The thick glycocalyx was absent, and fully formed, spined microtriches occurred in place of the blunt elevations (Fig. 5.61). The microtriches were polymorphic with three main types being present. Over the suckers and base of the scolex, microtriches with short, broad bases or shafts and long electron-dense spines predominated (Figs 5.62 5.4c). The shaft of these projections (0.239 ∓ 0.013μm in height) was 0.466 ∓ 0.019μm wide at the base, narrowing to 0.294 ∓ 0.011μm where a curved base plate, approximately 19.88nm in depth, was present. This structure appeared linear in cell section planes indicating that it was not tubular in nature. The shaft possessed an inner peripheral electron-dense shaft support, approximately 11.28nm from the single apical plasma membrane. The thickness of the support was asymmetrical and was more pronounced in the anterior aspect of the microthrix. In the core of the shaft numerous longitudinal microfilaments were observed in favourable sections (Fig. 5.62). These were approximately 3.65nm thick and had a periodicity of approximately 8.25nm. They ran from the base of the shaft to a region approximately 10nm from the baseplate. The spine was 1.199 ∓ 0.052μm in length and was seen to possess an inner very dense medulla separated from the membrane by a less dense cortical layer approximately 11.28nm thick (Fig. 5.62). The medulla region commenced approximately 11.34nm above the base plate and possessed

laminations. In transverse section the spine had a rhomboid shape with the laminations of the medulla appearing as tubules approximately 7.8nm in diameter (Fig. 5.63). In this section plane the electron-density of the medulla appeared to be more intense around the edges.

A second type of microthrix was also present in the sucker region of the scolex (Figs. 5.61, 5.4d). This type had a longer narrower shaft (approximately 0.364μm long and 0.138μm wide) which was cylindrical in shape and possessed a shorter narrower spine (approximately 0.876μm x 0.138μm). The shaft had an peripheral, symmetrical, electron-dense shaft support and the core possessed similar microfilaments to those of the first type of microthrix. The baseplate was also similar but occasionally had a circular profile suggesting that it may be more tubular in nature. The spine, like that of the first type, possessed a cortex and a laminated medulla.

The third type of microthrix was confined to the rostellar region (Figs. 5.64, 5.4e). These microtriches had a cylindrical shaft, approximately 0.245μm in length 0.072μm in diameter, which possessed a thickened asymmetrical, electron dense shaft support. The baseplate in many cases appeared circular (approximately 20.5nm in diameter) but in others (Fig. 5.65) appeared elongate, possibly suggesting its tubular nature. The spine was the most conspicuous part of these microtriches being extremely long (>2μm) and filamentous and best seen in SEM where they obscured the rostellar hooks (Fig. 5.66). The spines were approximately 0.129μm in diameter and had a laminated dense medulla approximately 20nm in width which gradually tapered to a point.

The distal cytoplasm of the scolex tegument was considerably thinner that of the soma (0.716 ∓ 0.034μm) except in the region of the rostellar hooks where the depth often exceeded 8.3μm (Fig. 5.67).

The types and proportions of the vesicles in the distal cytoplasm were, however, quite different from the soma. T_1 bodies were absent or very rare in the sucker and rostellar teguments whilst the T_2 vesicles with their flocculent contents, were much more numerous. Simple T_3, electron lucent vesicles were also present (Fig. 5.62). Stereological analysis (see Appendix 5) (Steer 1981) showed that the mean volume fraction occupied by the T_2 vesicles in the scolex tegument was $0.939 \times 10^{-1} \mu m^3/\mu m^3$ whilst in areas of the soma tegument where T_2 vesicles were present it was $0.879 \times 10^{-2} \mu m^3/\mu m^3$ the difference being significant at the 0.05 level (Students *t* test). Infolds of the basal plasma membrane of the distal cytoplasm were also present in the scolex. In the rostellar region however, these were much longer (up to 3μm) than in any other region. These often occurred by the sides of the hooks (when see in transverse section) (Fig. 5.68) which were bounded by a single plasma membrane. Hooks were never observed projecting out from the tegument and uncovered by it. In SEM, hooks which were extended appeared to be still covered by a layer of microthrix-bearing tegument, suggesting that they are entirely intra-tegumentary structures (Fig. 5.69). The basal infolds may therefore play a part in protrusion of the hooks.

Other structures associated with the scolex tegument were occasional sensory bulbs (Fig. 5.70). These consisted of a bulb-like expansion of a dendrite within the tegument, from which a cilium process extends to the exterior. The basal body at the base of the cilium was a simple cylinder. The bulb was held in place by a circular septate desmosome and had an internal electron-dense ring or collar. Also within the bulb were electron-lucent vesicles and mitochondria. These sensory bulbs were not observed in the soma tegument.

The tegumentary cytons of the scolex were similar to those of

the soma except for the Golgi complexes. Here more of the Golgi cisternae had electron dense contents (Fig. 5.71) and vesicles with dense flocculent contents, similar to the T_2 vesicles, were present in the perinuclear cytoplasm (Fig. 5.72). It seems therefore that the cytons of the scolex are responsible for the manufacture of the T_2 vesicles whilst those of the soma manufacture the T_1 vesicles.

5.2.3.2 Histochemistry

(a) Lectin binding analysis

Sections of protoscoleces were seen to bind WGA (Fig. 5.73) SBA (Fig. 5.74) and PNA (Fig. 5.75) to their external soma surface indicating the presence of N-acetylglucosamine, N-acetylgalactosamine and galactose respectively in the glycocalyx. APA was not observed to bind (Fig. 5.76), indicating the absence of fucose and sections appeared similar to unstained sections in showing a slight autofluorescence of the hooks. Con A did not bind to the external surface of protoscoleces (Fig. 5.77) but bound strongly to areas within the body of the protoscolex. This suggested that mannose and/or glucose were not present in the glycocalyx. Binding of all lectins except for WGA could be inhibited by the addition of their respective sugar (Fig. 5.78) indicating their binding specificity. With WGA not all the binding could be inhibited, indicating a possible non-specific binding component. When whole protoscoleces were examined the soma surface of invaginated protoscoleces fluoresced brightly (Fig. 5.79) compared with untreated protoscoleces which showed autofluorescence in the hooks and calcareous corpuscles (Fig. 5.80). With evaginated protoscoleces, however, only the soma fluoresced brightly (Fig. 5.81) while the scolex showed little lectin binding indicating that it was the thick glycocalyx on the soma which was responsible for most of the binding.

As with the hydatid cyst, lectin binding at the ultrastructural level was not sufficiently specific to allow precise carbohydrate localization in the tegument.

(b) Ultrastructural carbohydrates localization

Protoscoleces exposed to the thiosemicarbazide - silver proteinate reaction showed a dense reaction product in the area of soma glycocalyx (Fig. 5.82). Within the distal cytoplasm and tegumentary cytons very little reaction was obtained in most of the soma tegument (Fig. 5.82). In the anterior soma, however, some of the tegumentary vesicles showed a strong carbohydrate localization (Fig. 5.83). The distal cytoplasm of the scolex tegument possessed a large number of vesicles with a dense reaction product (Fig. 5.84) which was not present in control material (Fig. 5.85, 5.86). In addition to the vesicles, a slight reaction was observed on the spine material of the microtriches (Fig. 5.84). These deposits may have been non-specific although they did not occur to the same degree in control sections incubated without the periodic acid (Fig. 5.85). A positive result was also obtained for the glycogen-containing extensions of the tegumentary cytons (Fig. 5.87) and also for individual vesicles within these cytons (Fig. 5.88). It seems therefore that the carbohydrate-containing vesicles were confined to the scolex and anterior soma tegument and presumably corresponded to the T_2 vesicles observed in routine sections.

5.3 DISCUSSION

The results presented in the present chapter are in general agreement with previous reports on the ultrastructure of *Echinococcus* spp. metacestodes, but also provide additional information necessary for a more complete understanding of the subsequent developmental processes that occur.

The Hydatid Cyst

The appearance of the laminated layer of the hydatid cyst indicated that it was composed of two main components, a microfibrillate matrix seemed to be responsible for the positive thiosemicarbazide-silver proteinate reaction for carbohydrates. The carbohydrates present, as evidenced by the lectin binding analysis, were N-acetyl-glucosamine, N-acetylgalactosamine and galactose, and results were therefore in agreement with analysis carried out by other methods by different authors (Kilejian *et al.*, 1962; Korc *et al.*, 1967; Kilejian & Schwabe, 1971). The use of lectins to detect specific carbohydrate residues has been established as a powerful tool for studying properties of cell surfaces (for a review see Sharon & Lis, 1972) and they enable an accurate localization of the components to be made. In the present study, the sugars mentioned above were present in both the laminated and germinal layers although quantification of the amount of binding could not easily be made. Binding of Con A was limited only to the germinal layer and this was probably due to the large amount of glycogen present in the tegumentary and muscle cyton processes. The inability of N-acetyl-glucosamine to inhibit all the binding of WGA to the parasite surface has also been reported by other authors (Stein & Lumsden, 1973; Simpson & Smithers, 1980; Rogan & Threadgold, 1984)

and may be due to a non-specific electrostatic binding component.

The electron-dense granules within the laminated layer, appeared to have a calcium component, possibly calcium phosphate, as indicated by the X-ray microanalysis. This is in accordance with Cameron & Fitzpatrick (1925), Cameron & Webster (1969) and also with Richards (1984) who detected divalent cations which may have been calcium. Results, however, are contrary to the findings of Morseth (1967) and this discrepancy may be due to the accuracy of the methods used or in the preparative techniques involved. The granules within the germinal layer vesicles also gave a positive calcium result with X-ray microanalysis and are presumably of the same origin as those of the laminated layer as suggested by Richards *et al.* (1983a). These appeared to arise within the Golgi complexes of the tegumentary cytons and be transported to the distal cytoplasm for ultimate exocytosis. The presence of a region of the laminated layer immediately adjacent to the distal cytoplasm which was free of granules may suggest that extrusion of these granules could be a sporadic process. This could also explain the laminations seen in the laminated layer and the observations made by Richards *et al.* (1983a) where incubation of *in vivo* derived cysts in an *in vitro* culture medium caused an increase in the extrusion of granules from the germinal layer. This transplantation to an *in vitro* situation may therefore have provided the stimulus for release of the granules.

Formation of the carbohydrate matrix component of the laminated layer is not well understood although Richards (1984) reported the presence of carbohydrate-containing vesicles within the distal cytoplasm which presumably contributed to the laminated layer. In the present study, no such vesicles were observed using the same methods as Richards (1984) although occasional flocculent vesicles were observed in the cytons. It is again possible that these vesicles

may be present in the distal cytoplasm in sporadic bursts with long, quiescent periods inbetween resulting in their absence from the tegument on most occassions and producing a type of "punctuated equilibrium".

The functions of the laminated layer are not fully known but it is likely to give support to the cyst and to act as a barrier to the cellular immune response of the host. The possible presence of calcium in this layer may therefore aid in the support function and explain why the region immediately adjacent to the germinal layer, which lacks the granules, often presents a zone of weakness during tissue processing causing the laminated layer to pull away from the germinal layer. If calcium is present, these granules may also affect the host's complement system by acting as complement consuming factors in the same way as suggested for the calcareous corpuscles of *E. multilocularis* (Kassis & Tanner, 1976; Richards & Williams, 1982) which also occur in the germinal layer and may act as a calcium store. These granules may therefore act as an additional immunoprotective mechanism.

The truncated microtriches of the germinal layer tegument have a highly reduced spine or do not possess one at all and are similar to projections occurring on certain post-oncospheral stages of some other cestodes (Lumsden, Oaks & Mueller, 1974; Lumsden *et al.* 1982; MacKinnon & Burt, 1984; MacKinnon, Jarecka & Burt, 1985) where they have been referred to as either microvilli or developing microtriches. If, in the normal spined microthrix, the spine is involved in abrasion or anchorage within the host, as has been previously suggested (for reviews see Lee 1966; 1972; Lumsden 1975; Lumsden *et al.*, 1982; Lumsden & Hildreth, 1983; Threadgold, 1984), then there is no need for such a function in the hydatid cysts where the laminated layer separates the living parasite tissue from the

host tisssue. The truncated microtriches of the germinal layer never develop long spines and cannot therefore be considered as developing microtriches which are formed complete with spines. The function of the truncated microtriches is more likely to be concerned with surface area amplification for nutrient absorption (Bortoletti & Diaz, 1978), as indeed are the spined microtriches of adult cestodes (Threadgold, 1984; Threadgold & Robinson, 1984). Within the truncated microtriches the electron-dense shaft support may give rigidity to the whole structure and maintain its upright shape. Material from this region may also contribute to the small amount of spine material that may be present. The baseplate of these projections is unusual in its spherical nature which has also been observed by Bortoletti & Ferretti (1973), Lascano *et al.* (1975) and Marchiondo, Conder & Andersen (1985), but the function of this structure is unknown.

At the base of the distal cytoplasm the basal plasma membrane was seen to form shallow infolds. These basal infolds have been reported in the tegument of other cestodes (Baron, 1971; Hopkins, Law & Threadgold, 1978; Lumsden *et al.*, 1982; Ramirez-Bon, Merchant, Gonzalez-Del Pliego & Canedo, 1982; Lumsden & Hildreth, 1983; Threadgold & Dunn, 1983; Richards & Arme, 1984) and in various stages of the life cycle of *Echinococcus* spp. (Jha & Smyth, 1971; Lascano *et al.*, 1975; Singh & Lee, 1979). Their functions are, however, largely unknown although similar structures have been reported to be involved in osmoregulation in certain trematodes (Threadgold & Brennan, 1978). It is also possible that this basal membrane may be important in regulating the selective transport of nutrients into the hydatid fluid.

Below the basal plasma membrane a very sparse basement lamina was evident in some sections. The lack of development of this

connective tissue layer is probably due to the fact that the support function of a basement lamina has been taken over by the laminated layer on the outside and the hydatid fluid on the inside. These are not present in the protoscolex where a well developed basement lamina occurs.

The tegumentary cytons of the germinal layer often had large numbers of free ribosomes. Although their size was similar, (18nm) these structures could be distinguished from β-glycogen particles by their lack of a positive carbohydrate reaction using the thiosemicarbazide-silver proteinate method. Their identification could have been further investigated, however, by using a cytochemical staining procedure for RNA.

The presence of these ribosomes associated with endoplasmic reticulum (ER) was comparatively rare and the amount of ER was also considerably less than in other animal tissues (Threadgold 1976). The ER was limited to a series of single cisternae around the periphery of the cytons - an unusual location but one which has been observed in the myocytons of other cestodes (Baron, 1968). The proportions of ribosomes and ER suggest that the cytons are involved in synthesis of protein, mainly for their own growth, with little being directed towards secretion. The ER and/or the Golgi complexes may, however, be involved in the production of autolysosomes as suggested by Richards *et al.* (1984) although no direct evidence of this was observed. The numbers and size of these structures may reflect the metabolic state of the cysts, and the variation in the amount of autophagic activity may be connected with the location of the cyst within the host. Alternatively Richards *et al.* (1984) suggested that smaller cysts at the centre of cyst masses had a greater amount of autophagic activity due to their growth being physically inhibited by other cysts. It was suggested that these

small cysts may have been producing greater amounts of organelles and secretory products than was necessary - the excess being autophagocytosed.

The Brood Capsule Wall

Observations on the structure of the mature brood capsule wall are in agreement with those of other authors (see Introduction for references). The function of the brood capsule tegument appears to vary with the degree of protoscolex formation, and this will be discussed further in Chapter 7. In mature capsules from horse cysts, where protoscolces are fully formed, the activity of the brood capsule tegument may be reduced, possibly explaining the poor supply of organelles found in the tegumentary cytons. The function of the brood capsule at this stage may simply be to act as a retaining sac for the already formed protoscoleces. The presence of truncated microtriches on the internal surface, however, indicates that it may also act as an absorptive surface, and it is not inconceivable that the electron-lucent vesicles (similar to T_3 vesicles of the protoscolex) could be pinocytotic in nature. Since it is known that mature brood capsules may contain up to 50% dead protoscoleces (Smyth, 1962), the brood capsule may act to resorb the breakdown products from the interior of the capsule for recycling.

The ultrastructure of the protoscolex attachment stalk has not been previously described and the present study indicates that it possesses characteristics of both the brood capsule and protoscolex teguments and therefore represents a transition zone. The presence of flame cell collecting ducts in the stalk suggests that these could be in continuity with those of the protoscoleces. This would mean that excretory products would be removed from the protoscoleces to the hydatid fluid outside the capsule and not accumulate within it.

The Protoscolex

The most striking feature of the protoscolex tegument ultrastructure is the regional variation which exists between the scolex and the soma. This variation would suggest that each region had a separate function.

The scolex tegument is similar to that of adult cestodes in possessing spined microtriches and is therefore more committed to an adult type of development than the soma. The structure of the blade like microtriches, occurring over the suckers and lower portion of the scolex, is basically similar to the microtriches of other cestode species (Baron, 1968; Featherston, 1972; Polyakova, Kristova & Kirilova, 1978; Hulínská, 1980; Richards & Arme, 1981; Lumsden & Hildreth, 1983; MacKinnon & Burt, 1983; Threadgold, 1984; Holy & Oaks, 1986) and adult *Echinococcus* (Morseth 1966; Jha & Smyth, 1969). The functions of the internal components of these projections are, however, not clear. Within the shaft the inner electron-dense shaft-support may give rigidity to the structures (Richards & Arme, 1981; MacKinnon & Burt, 1983) and maintain their posteriorly directed orientation. This would explain why this structure is asymmetrical, being thicker on the anterior side of the microthrix. This orientation is likely to be important if the microtriches act as anchoring structures to maintain the position of the scolex within the dog intestine. In addition to support, the shaft support, which is in continuity with the spine, may represent material which is being transported to be incorporated into the spine. The core microfilaments within the shaft have also been reported in other cestode microtriches (Grammeltvedt, 1973; Lumsden, 1975; Lumsden *et al.*, 1982; Lumsden & Hildreth, 1983; Holy & Oaks, 1986) and have been suggested to be composed from actin (5-7nm in diameter, Threadgold, 1976). These are also similar to the core filaments in the

microvilli of the mammalian intestine (Threadgold, 1976) and may similarily result in the microtriches being mobile, bringing about a movement in the external environment and reducing any unstirred water layers which may exist. It is not clear how these filaments could bring about contraction as no terminal web, as occurs in the intestine, was observed. The base plate may act as an anchoring structure although the microfilaments appeared to terminate before this. It is possible, however, that there may be some connection between the filaments and the baseplate which is not preserved by normal fixation.

Within the electron-dense spine of the microtriches the canals have previously been referred to as microtubules (Jha & Smyth, 1969; MacKinnon & Burt, 1983) although Lumsden (1975) stated that they had a smaller diameter than true cytoskeletal microtubules which are 20-30nm in diameter (Threadgold, 1976). In the present study the canals in the spine were approximately 7nm thick which is considerably less than that of true microtubules. The function of these canals is unknown although that may be involved in the transport of material up or down the spine. Each microthrix was covered by a single unit membrane with a sparse glycocalyx and no evidence of a double membrane structure, as reported by Jha & Smyth (1969) for adult *E. granulosus*, was observed.

The microtriches on the rostellar region of the protoscoleces were morphologically very different from those on the suckers although they had the same basic internal components. These filamentous rostellar microtriches have been reported by other authors in *Echinococcus* protoscoleces (Sakamoto & Sagimura, 1970; Marchiondo & Andersen 1983; 1984) and adults (Jha & Smyth, 1971; Thompson, Houghton & Zaman, 1982) and also on the rostellum or other cestodes (Featherston, 1972). The rostellar microtriches may be

involved in nutrient absorption, but additionally might provide a loose sheath for the hooks, thus preventing them from becoming ensnared or enmeshed during the process of evagination (Marchiondo & Andersen, 1983; 1984).

The distal cytoplasm of the scolex tegument possesses carbohydrate-containing T_2 vesicles which are considerably more numerous than in the soma. The function of these vesicles is, however, unknown. Their involvement in glycocalyx formation is unlikely unless there is a rapid turnover of the apical membrane of the scolex tegument compared with that of the soma which has a much more extensive glycocalyx and fewer T_2 vesicles. Vesicles with a similar appearance (see Lumsden *et al.*, 1982; and Lumsden & Hildreth, 1983 for reviews), some with carbohydrate contents (Trimble & Lumsden 1975), have been reported in many other cestode species and a diverse number of functions have been attributed to these inclusions; for example, secretion, formation of the microtriches, formation of the glycocalyx, elaboration of the cytosol component of the distal cytoplasm, formation of the basal lamina and endocytosis. Since the vesicles in the present study are of Golgi origin it is most likely that they are involved in secretion or the maintenance of the microtriches. It is interesting to note that, using the thiosemicarbazide-silver proteinate stain, a small amount of deposit was present over the spine and electron-dense shaft support, possibly indicating that these had a carbohydrate component. The T_2 vesicles may therefore be involved in maintaining these projections and this would explain their abundance in the scolex tegument. The Golgi complexes producing the T_2 vesicles were only found in the tegumentary cytons of the scolex although some of the vesicles were present in the distal cytoplasm of the soma, mainly in the anterior region. This suggests that there may be some posterior movement of

the T_2 vesicles from the distal cytoplasm of the scolex to that of the soma. Microtubules, which may be associated with such movement were not, however, observed in the distal cytoplasm, although they have been observed in other cestodes (Richards & Arme, 1985).

The tegument of the soma differed considerably from that of the scolex, particularly in the glycocalyx, projections and inclusion bodies. The glycocalyx had the same lectin-binding properties as the laminated layer which unfortunately meant that the lectins could not be used as markers of laminated layer formation during the cystic transformation of protoscoleces. The soma glycocalyx is much thicker than that reported for other cestodes and it is possible that it has some protective function for the parasite. When the invaginated protoscolex is ingested by a dog the scolex is contained within the glycocalyx-bearing soma and may therefore be protected from host enzymes by it. Similarly , when liberated within the peritoneal cavity of the intermediate host, the soma tegument with its glycocalyx may initially protect the protoscolex from the host immune response and allow time for the laminated layer and any other immuno-protective mechanisms to become established. The blunt elevations may act to reduce the surface area exposed to either enzymes or immunological agents. Similar structures have also been reported in other metacestodes (Mackinnon & Burt, 1982; 1984) where the authors suggested that they reduced the surface area exposed to host enzymes. The role of these blunt elevations in the subsequent development of protoscoleces will be discussed in later chapters.

Within the distal cytoplasm of the soma, the T_1 vesicles were considerably more numerous than in the scolex. The function of these vesicles is unknown although they may be involved in the release of secretory products. Their absence in the scolex would, however, seem to indicate that they could be concerned with maintenance of the

thick soma glycocalyx although they did not appear to be carbohydrate in nature.

The simple T_3 vesicles, which occurred in both the scolex and soma distal cytoplasm, may have been pinocytotic in nature although there was no direct evidence for this. Endocytotic vesicles have, however, been demonstrated in other metacestodes using ruthenium red as a marker (Hopkins, Law & Threadgold, 1978; Threadgold & Hopkins, 1981; Threagold & Dunn, 1983; 1984). Such marker studies and enzyme-cytochemical staining, if carried out on *Echinococcus* protoscoleces, may also reveal further functions of the cytoplasmic vesicles.

Although numerous vesicles of various types were observed in the protoscolex distal cytoplasm, little evidence of their synthesis was apparent in the cytons. The relative inactivity of the Golgi/ER system is probably due to the protoscolex being a quiescent stage which is not active until exposed to the correct triggers (Smyth, 1967; 1979; 1985; Smyth & Davies, 1974b). The activity of the cytons is therefore likely to be increased once adult or cystic development commences.

In conclusion it seems that the tegumentary ultrastructure of the protoscolex and hydatid cyst are considerably different. If differentiation from one form to another is to proceed then significant changes must occur concerning the tegumentary organelles. This differentiation will be investigated in subsequent chapters and the possible functions of certain organelles further elucidated.

CHAPTER 6

ULTRASTRUCTURAL CHANGES OCCURRING IN THE PROTOSCOLEX TEGUMENT DURING CYSTIC DEVELOPMENT *IN VIVO* AND *IN VITRO*.

6.1 INTRODUCTION

Although there have been many studies on the tegumentary ultrastructure of various cestode species, little work has been done on the differentiation from one life cycle stage to another. Those reports which exist have concentrated on differentiation from the oncosphere to the metacestode (Charles & Orr, 1967; 1968a; 1968b; Braten, 1968; Smyth, 1972; Grammeltvedt, 1973; Hulínská, 1980; Lumsden *et al.*, 1982; Engelkirk & Williams, 1982; 1983; Ubelaker, 1983; MacKinnon & Burt, 1984; Richards & Arme, 1984). Virtually all of the above studies have reported changes in the vesicle population of the tegument and modifications of the surface microarchitecture. Each species, however, was found to have its own modifications although certain features were held in common.

Electron microscopical investigations concerning *Echinococcus* metacestodes are limited to brief descriptions of oncosphere - cyst development (Sakamoto & Sugimura, 1970); cyst-protoscolex development (Sakamoto & Sugimura, 1970) and protoscolex-adult development (Jha & Smyth 1971) and, to date, no workers have described the protoscolex-secondary cyst differentiation. Sakamoto & Sugimura (1970) described the initial formation of the cyst germinal layer of *E. multilocularis*, from undifferentiated cells of the oncosphere. The authors were, however, unable to come to any conclusions about the formation of the laminated layer, and whether or not vesicles of the distal cytoplasm were involved. They did, however, observe

laminated layer material surrounding some of the host cells and concluded that this layer may not be formed purely from a "metabolic substance" of parasite origin.

The structure of the germinal layer tegument and the presence of the laminated layer are obviously important in cyst survival. Clearly the parasite must have some method or methods of evading the host immune response and investigations into cyst wall maturation may elucidate systems which could be exploited to reduce parasite survival. Since the handling of *Echinococcus* oncospheres, in the laboratory, is potentially hazardous, the protoscolex offers a relatively safer alternative for such studies.

6.2. RESULTS

6.2.1 Ultrastructural aspects of cystic development *in vivo* after intraperitoneal injection

Investigations on the ultrastructural changes occurring in the protoscolex tegument during cystic development in mice were often difficult to carry out due to the high mortality of parasites in the first few weeks. It was therefore necessary to try and separate events which were a result of immunological attack and subsequent degeneration from those involved in cyst differentiation.

In the first few days post-infection (p.i.), groups of protoscoleces were encapsulated by host leucocytes. In these early stages the host cells were primarily a mixture of eosinophils and macrophages (Fig. 6.1); the macrophages being in an activated state and possessing many residual bodies and extensive pseudopodia (Fig. 6.2). Early changes in the tegument were confined to the soma region whilst the scolex, although occasionally evaginated, remained relatively unchanged. From 2-4 days p.i. there was almost total loss of the soma glycocalyx accompanied by a flattening of the blunt elevations (Figs. 6.3, 6.4). Within the distal cytoplasm the T_1 vesicles were apparent in some regions (Fig. 6.4) but were greatly reduced in others (Fig. 6.5). The flocculent T_2 vesicles, however, were more numerous in the soma distal cytoplasm and were present together with the simple T_3 vesicles which persisted throughout development (Figs. 6.3, 6.5). The T_2 vesicles, which occasionally varied in the intensity of the flocculent contents, arose from Golgi complexes in the soma tegumentary cytons. These appeared more active due to the large numbers of ribosomes and peripheral rough ER (Fig. 6.6). Mitochondria within the cytons were also more numerous and

from day 4 to day 30 were 263.3 nm ∓ 8.5 in diameter. This was significantly larger than those present at day 0 ($p<0.01$, Students t test). The extensions associated with the tegumentary cytons were generally rich in glycogen deposits (Fig. 6.3), only rarely showing signs of depletion (Fig. 6.6).

In many cases the protoscolex tegument had a vacuolated appearance when viewed at low magnifications. At higher powers, however, it could be seen that this appearance was caused by a detachment of the distal cytoplasm from the basement lamina (Fig. 6.7). It was not clear, however, whether this detachment was artifactual, a feature of cystic development or a response to the host immunological attack. In severe cases it is likely that the latter was true since residual body-like structures were also observed in the distal cytoplasm (Fig. 6.7) indicating autophagocytosis. Detachment was also common in protoscoleces associated with eosinophils that were degenerating (Fig. 6.8) but occurred, to a lesser extent, in parasites at all stages of development, including 0 day protoscoleces. This may suggest that a limited amount of distal cytoplasm detachment may be artifactual but that in severe cases it may precede stripping of the distal cytoplasm and its subsequent engulfment by macrophages (Fig. 6.9).

From 7-12 days p.i. the most striking developmental feature was the dramatic increase in T_2 vesicles both in the distal cytoplasm (Fig. 6.10) and cytons (Fig. 6.11) of the soma tegument. These vesicles originated from the Golgi complexes in the cytons (Fig. 6.11 inset) which were present together with large numbers of ribosomes suggesting that the tissue was highly active in synthesis and secretion. The carbohydrate nature of the T_2 vesicles was verified by the periodic acid-thiosemicarbazide reaction (Fig. 6.12). The T_1 vesicles at this stage had almost totally disappeared.

Increased numbers of elongate mitochondria (often up to 1.5μm in length) occurred in the distal cytoplasm (Fig. 6.13) and in the cytons where, occasionally, they occupied virtually all of the perinuclear cytoplasm (Fig. 6.14).

Many areas of the soma tegument had started to produce the truncated microtriches characteristic of the germinal layer (Figs. 6.10, 6.13), by 7-12 days p.i. These were formed initially below the apical membrane and parallel to the tegumentary surface (Fig. 6.15). The electron-dense material of the now flattened blunt elevations appeared to contribute to the shaft support of the early truncated microtriches although subsequent projections formed *de novo*. The origin of the components of these truncated microtriches including the spherical baseplate, was not evident although participation of the T_2 vesicles cannot be ruled out as they were often observed close to the developing projections (Fig. 6.13, 6.16). Once formed the microtriches were subsequently uplifted to their elevated position (Fig. 6.16).

During initial formation and uplifting of the truncated microtriches no laminated layer was present but it was interesting to note that from about 10 days onwards, the host cells immediately surrounding the parasites were degenerate giving rise to a considerable amount of cellular debris, (Fig. 6.17) possibly indicating some cytotoxic action. Even at this stage of development (just prior to laminated layer formation) the developing parasites were still susceptible to immunological attack and several possessed a tegument which was highly disrupted (Fig. 6.18).

From 12-30 days p.i. electron-lucent vesicles with electron-dense granules which will be referred to as 'G' vesicles, had begun to appear in the distal cytoplasm and cytons of both the scolex and soma tegument. In some cases, particularly in the scolex,

the dense granules were also present in some of the T_2 vesicles (Fig. 6.19). Within the cytons of these parasites both T_2 vesicles and 'G' vesicles were present in the same perinuclear cytoplasm (Fig. 6.20). This suggests that the cytons were differentiating and producing granule-containing 'G' vesicles instead of T_2 vesicles.

The appearance of these 'G' vesicles in the tegument was followed by the formation of the laminated layer (20-40 days p.i.). In general this first appeared, beyond the developing truncated microtriches and around the entire immature cyst, as a few strands of amorphous material containing clumps of electron-dense granules, identical to those of the distal cytoplasm 'G' vesicles (Fig. 6.21). In some cases, however, the early laminated layer also possessed many membraneous vesicles (Fig. 6.22). It was not clear, however, whether these vesicles were of parasite origin or from degenerating host cells.

During the initial stages of laminated layer formation the numbers of T_2 vesicles within the distal cytoplasm remained high (Fig. 6.23). As development progressed, however, this population of vesicles began to decrease (Fig. 6.21) until they were totally absent from approximately 48 days p.i. onwards (Fig. 6.25). Similarly, in the tegumentary cytons the T_2 vesicles became totally absent and replaced by 'G' vesicles (Fig. 6.26). The individual granules within these vesicles appeared to arise from a fusion of much smaller granules present in the vicinity of Golgi fields (Fig. 6.26) and were therefore presumably derived from the Golgi complexes. Although these granules appeared to be present in the cytons, distal cytoplasm and laminated layer, no evidence of exocytosis of these structures or any other vesicle type was observed. Many vesicles were, however, seen in close proximity to the apical plasma membrane and presumably fused with it.

Also present in the perinuclear cytoplasm at the time of laminated layer formation, were other irregularly shaped vesicles, ranging in diameter from 348nm to 640nm and possessing a dense periphery and strands of moderately dense material within their lumen (Fig. 6.26). The larger forms were identical to the carbohydrate-containing, storage lysosomes of the mature germinal layer. The smaller forms (117nm - 230nm in diamter) designated as T_4 vesicles were observed in association with the Golgi complexes which also occasionally possessed flocculent contents in the cisternae of the maturing face (Fig. 6.27). These T_4 vesicles frequently appeared to fuse together (Fig. 6.26) to form intermediate stages in the formation of the early storage lysosomes which were increasingly present in the cytons as development progressed. The T_4 vesicles were also occasionally present in the distal cytoplasm of the maturing cysts (Fig. 6.28) and, since no other carbohydrate-containing body was present, presumably contributed to the carbohydrate matrix of the laminated layer.

Within the remainder of the developing cyst the basement lamina and longitudinal and circular muscle fibrils were still evident (Fig. 6.23) although the suckers showed signs of degeneration (Fig. 6.29).

As development of the cysts during the second and third months p.i. continued, growth of the laminated layer was rapid (Fig. 6.30). The outer edge was, however, continually being phagocytosed by a new generation of macrophages (Fig. 6.31). In this peripheral zone the accumulations of electron-dense granules often appeared larger than within the interior of the laminated layer (Fig. 6.32).

Within the tegument, the soma region had taken on the appearance of the mature germinal layer tegument. The scolex region, however, still possessed rostellar hooks and spined microtriches beneath the laminated layer. Only after 54-70 days p.i. were the spines of the

microtriches shed into the laminated layer, and these projections replaced by a population of truncated microtriches (Fig. 6.33). Similarly, at this stage, the hooks had become dislodged from the tegument and formed into a bud surrounded by a layer of cytoplasm and a portion of laminated layer (Fig. 6.34). From approximately 90 days p.i. onwards this hook bud was released into the laminated ,layer (Fig. 6.35). This loss of the hooks, together with degeneration of the suckers and basement lamina connective tissue, therefore resulted in the parasite becoming truly cystic. As the volume of the cysts increased the tegumentary cytons became more numerous presumably by division from existing nuclei and the tegument took on its mature appearance. It did, however, often possess limited glycogen reserves and numerous lipid droplets (Fig. 6.36) during subsequent growth. Only after 4-6 months growth were abundant glycogen deposits re-established (Fig. 6.37).

6.2.2 Ultrastructural aspects of cystic development in diffusion chambers

In general, cystic development of the tegument in diffusion chambers was similar to that of protoscoleces free in the peritoneal cavity. There were, however, some important differences.

During the first 12 days p.i. little differentiation of the tegument occurred. T_2 vesicles had only slightly increased in the soma distal cytoplasm and the glycocalyx was not reduced in thickness (Fig. 6.38). In many cases this glycocalyx had a dense deposit on its surface which was not present on free protoscoleces. This deposit may have been a non-specific residue of the cement used in chamber construction.

During the first two weeks in chambers death of several

protoscoleces was evident although not as obvious as in free i.p. infections. This appeared to be caused by lysis and stripping of the tegumentary distal cytoplasm (Fig. 6.39) and suggests that the parasites were subjected to immunological attack which, due to the pore size of the chambers, was presumably humoral in nature.

From 12 days p.i. onwards the truncated microtriches began to appear on the soma tegument (Fig. 6.40) and were formed, by uplifting, prior to and during laminated layer formation (Fig. 6.41). Whilst these projections were first developing remnants of the soma glycocalyx were still evident although somewhat reduced (Fig. 6.40).

Formation of the laminated layer was concurrent with the appearance of the 'G' vesicles, T_4 vesicles and carbohydrate-containing storage lysosomes. All forms were observed in the perinuclear cytoplasm, particularly in the vicinity of the Golgi complexes (Figs. 6.42, 6.43). The laminated layer, when first formed, was comprised solely of a microfibrillar matrix and no electron-dense granules were observed (Fig. 6.41). As development proceeded occasional host cells were entrapped in the laminated layer (Fig. 6.44) suggesting that some chambers were not totally impervious to host leucocytes, although the pore size was sufficiently small. The laminated layer of these cysts often possessed membraneous vesicles (Fig. 6.45) but generally no electron-dense granules were observed. In the region adjacent to invading host cells, however, patches of granules were recorded (Fig. 6.44). The 'G' vesicles within the tegument were always present together with numerous carbohydrate-containing storage lysosomes (Fig. 6.45).

In mature cysts of over 7 months in age, the laminated layer seemed to vary in appearance. Most areas did not possess dense granules but had dense fibrillae with a similar distribution (Fig. 6.46). In other areas, however, patches of dense granules were

present (Figs. 6.46, 6.47). The remainder of the cyst was identical to those developed freely in the peritoneal cavity.

6.2.3 Ultrastructural aspects of development *in vitro*

6.2.3.1 Cystic development

Development of the tegument of vesicular protoscoleces *in vitro* was generally similar to that occurring *in vivo* although more akin to development within diffusion chambers.

During the first week of culture little ultrastructural changes occurred. At the parasite surface the soma glycocalyx was still evident and the blunt elevations were relatively unaltered (Fig. 6.48). Within the soma distal cytoplasm the T_1 bodies were much in evidence and only occasional T_2 bodies were present (Fig. 6.48). Similarly the soma tegumentary cytons were also relatively inactive.

From 7-14 days, however, the soma tegument became more dynamic. Golgi complexes, producing T_2 vesicles, and mitochondria were more evident within the cytons (Fig. 6.49) and glycogen within the cytoplasmic extensions appeared depleted (Fig. 6.49). In the scolex region little differentiation had occurred and T_2 vesicles continued to be manufactured by the Golgi complexes and transported to the distal cytoplasm (Fig. 6.50).

From 14 days onwards many vesiculating protoscoleces showed a reduction in the amount of soma glycocalyx material although it was still clearly visible (Fig. 6.51). The blunt elevations also had decreased in height whilst there was an increased presence of T_2 vesicles accompanied by a decrease in T_1 vesicles (Fig. 6.51). In many cases the protoscolex vesiculation process and loss of connective tissue somewhat "stretched" the tegument resulting in a decrease in the depth of the soma distal cytoplasm and producing

large areas of "bare" distal cytoplasm with very few tegumentary cytons (Fig. 6.52). It is quite likely that parasites in this condition may cease development due to the lack of cytons since it is known that not all vesicular protoscoleces form cysts (see Chapter 3). In other protoscoleces adequate cytons were present and possessed many mitochondria which occasionally were quite swollen (Fig. 6.53). Truncated microtriches were present on some developing cysts from approximately 14 days onwards (Fig. 6.53) and appeared to be formed by uplifting from the tegumentary surface as in the *in vivo* situation (Fig. 6.54). In most cases a small amount of glycocalyx material was still present during initial formation of these structures (Fig. 6.53).

The vesicle population in the distal cytoplasm at this stage was mainly one of electron lucent T_3 vesicles although several T_2 bodies were also present. As further development occurred, prior to laminated layer formation, more T_2 vesicles were present together with 'G' vesicles (Fig. 6.55). As formation of the laminated layer began, from approximately 20 days onwards, flocculent T_4 vesicles were evident in the cytons (Fig. 6.56) and in the distal cytoplasm (Fig. 6.57). Some of these had condensed into larger storage lysosomes although those within the distal cytoplasm usually remained small (Fig. 6.57). The asymmetrical, laminated residual bodies were not, however, present at this stage.

The appearance of the laminated layer was quite different from that occurring *in vivo*. In the initial stages of its formation *in vitro* it appeared only as a diffuse network of microfibrils (Fig. 6.58) without aggregates of electron-dense granules. As this layer became progressively thicker, from approximately 84 days onwards, it appeared to possess accumulations of denser fibrillate components (Fig. 6.59). These denser fibrillae never took on the appearance of

the electron-dense granules occurring in the distal cytoplasm (Fig. 6.59) and in the laminated layer of cysts grown *in vivo* (see Fig. 6.59 inset) although they had a similar distribution to the latter.

The scolex tegument at this late stage, whilst covered by a relatively thick laminated layer, still possessed the hook bud and some spined microtriches. These were however, in the process of being replaced by truncated microtriches (Fig. 6.60). Both the distal cytoplasm and cytons possessed many T_4 vesicles some of which also contained electron-dense granules of various sizes (Fig. 6.60).

In the later stages of development residual bodies, with lamellated peripheries began to appear in the perinuclear cytoplasm (Fig. 6.69) and occasionally in the distal cytoplasm (Fig. 6.62). The origin of these structures was unclear but it is likely that their lamellae are of membraneous origin and may result from breakdown of membraneous structures such as mitochondria.

Late in development, from 110 days onwards, several cysts appeared to have a large number of lipid droplets associated with the tegument (Fig. 6.63). This possibly indicates a degeneration of the cyst tissue and in several cases was accompanied by a gross swelling of some of the mitochondria (Fig. 6.63). Since it is known that cysts begin to take on a darkened colouration and die in prolonged *in vitro* culture, these ultrastructural features may represent the first stages of tissue degeneration.

6.2.3.2 Ultrastructure of posterior bladders and free vesicles formed during *in vitro* culture

The posterior bladders and free vesicles produced in the first weeks of culture had an identical ultrastructure. The distal cytoplasm was thin (0.3μm) and possessed mainly electron lucent T_3

vesicles (Fig. 6.64). The cytons were relatively inactive and possessed few organelles (Fig. 6.64). Large areas of distal cytoplasm were free from projections (Fig. 6.65) but other regions possessed significant populations of truncated microtriches (Fig. 6.64) even in the initial days of culture (Fig. 6.66). Flame cell collecting ducts were also associated with the walls of the posterior bladders/vesicles (Fig. 6.65). The ultrastructure of these bladders and vesicles was therefore identical to that of the brood capsule wall (see Chapter 5) and confirms the suggestion that the bladders/vesicles originated from portions of brood capsule wall and attachment stalk which were retained by protoscoleces after release. Free vesicles, detached from protoscoleces, developed laminated layers in a similar manner to vesiculating protoscoleces.

6.2.3.3 Development of forms showing adult-like characteristics *in vitro*

Protoscoleces showing adult-like development could be identified from approximately 28 days onwards. The most striking feature about these individuals was the development of spined microtriches on the soma tegument (Fig. 6.67). These structures formed parallel to the tegumentary surface, in the same way as the truncated microtriches, and were subsequently uplifted to an upright position (Figs. 6.68, 6.69). Microthrix development was accompanied by a greatly increased population of T_2 vesicles in the distal cytoplasm. These were generally densely flocculent (Fig. 6.70) but occasionally had a less dense appearance (Fig. 6.69). The T_2 vesicles were produced in vast quantities in the scolex and soma tegumentary cytons (Fig. 6.71) and could clearly be seen to be formed by the Golgi complexes (Fig. 6.72). These complexes were much more numerous and active than those

of the original protoscolex and their association with the peripheral rough ER could be best observed in specimens fixed in osmium tetroxide alone (Fig. 6.73).

The cytons also possessed an extremely dense cytoplasm, numerous ribosomes and increased numbers of mitochondria (Fig. 6.71) which were enlarged (346 ± 14·04 µm in breadth). The tegumentary tissue was clearly very active in synthesis and secretion although glycogen reserves were generally well maintained (Figs. 6.71, 6.67). The 'G' vesicles and carbohydrate-containing storage lysosomes, characteristic of cystic development, were however, absent from the tegument. Protoscoleces showing adult-like development in diffusion chambers possessed identical features to those present *in vitro*.

6.3 DISCUSSION

Observations on tegumentary differentiation in protoscoleces maintained in the peritoneal cavity; in diffusion chambers or *in vitro* was essentially similar in all three cases. Discussion of this differentiation process will therefore be primarily concerned with the *in vivo*, free protoscolex situation. Variations that existed in the other environments will also be considered.

During the first month post-infection, protoscoleces were encapsulated in a considerable host cellular response, similar to that reported by other authors (Smyth & Heath 1970; Ali-Kahn & Siboo 1980; Richards *et al.* 1983b). The involvement of eosinophils in causing damage to schistosomula of *Schistosoma mansoni* has been well documented (for review see Butterworth, Vadis & David, 1980), although a direct role for these cells in the killing of cestodes, has not been convincingly demonstrated. They are, however, commonly found closely associated with developing metacestodes (for review see Rickard & Williams, 1982) and are presumed to have some deleterious effects. Similarly , activated macrophages have been shown to have a protoscolicidal effect on *E. multilocularis in vitro* (Baron & Tanner, 1977) and it is therefore highly likely that the host cellular response is responsible for high protoscolex mortality in the first weeks p.i. This idea is backed up by the fact that survival of protoscoleces in diffusion chambers was much greater (see Chapter 4). The present ultrastructural observations, show, however, that the parasites are still susceptible to immunological attack within the chambers, presumably from a humoral response. There is no evidence that antibody has a restrictive role in the growth of fully formed cysts of *Echinococcus* (Ali Kahn, 1974; Rickard & Williams, 1982), but during development protoscoleces may be more susceptible. Supporting

evidence for this comes from *in vitro* studies where protoscoleces were lysed by the action of complement activated by the classical and alternative pathways. (Kassis & Tanner, 1976; Herd 1976; Rickard *et al.*, 1977).

Clearly the host mounts an intense immunological attack on the parasites often causing severe mortality. The host immune system, however, is not "swamped" by the high parasite burden since when small numbers of protoscoleces are employed correspondingly lower numbers of cysts are produced (Pauluzzi & De Rosa, 1969). Protoscoleces which survive must be able to evade this host response and develop into cysts. Since various changes occur in the parasite during development this evasion may involve several different processes.

Initially the protoscoleces are in the invaginated condition and are therefore enclosed by a tegument bearing a very thick glycocalyx. This glycocalyx is considerably more extensive than in other cestodes and may initially form a barrier against immunological attack. This may operate as a physical barrier preventing close adherence of leucocytes to the apical tegumentary membrane. In addition it may also bind host immunoglobulins which act as blocking antibodies and interfere with cell mediated effector mechanisms, as suggested for *Taenia pisiformis* (Williams, Picone & Engelkirk, 1980).

Since the glycocalyx is lost *in vivo* in the first two days (presumably by phagocytosis, since it persists for longer in diffusion chambers), other mechanisms must aid in survival. The soma tegument initially showed an abundance of T_1 vesicles which gradually become depleted. The function of these inclusion bodies is unknown but their limitation to the exposed soma tegument may suggest that they have some immunoprotective activity. It is possible that biochemical changes in the nature of membranes can alter

susceptibility to lysis even when surface antigens are displayed and complement is fixed (Williams *et al.* 1980). Presumably the conformation or nature of molecules associated with or inserted in the membrane alter the susceptibility to complement once it becomes fixed. Substances from the T_1 vesicles may therefore be incorporated into the apical membrane and elicit some resistance to complement lysis. Alternatively the T_1 and any other excretory/secretory products may act as complement consuming factors, being released at a rate compatible with the consumption of a complement distant from the parasite surface (Williams *et al.*, 1980). The susceptibility of protoscoleces to complement lysis *in vitro* may be explained by the fact that *in vivo* complement accumulation around the sequestered organisms is limited by the rate of diffusion of complement proteins towards the parasite. *In vitro*, where no such rate-limiting barriers exist, complement is in excess and protoscoleces are susceptible to lysis (Kassis & Tanner, 1976; Herd, 1976; Rickard *et al.*, 1977).

Other excretory/secretory products, (possibly involving T_1 vesicles) released from or on the surface of protoscoleces may have an immunosuppressive action on the cell mediated host responses since it has been shown that lymphocyte transformation (Dixon, Jenkins & Allan, 1982) and macrophage activation (Robinson & Arme, 1985) are affected by the presence of protoscoleces. Whatever mechanisms operate, the ability and effectiveness of each protoscolex to mount an evasive strategy will determine whether or not it will survive. The high mortality in the early stages of infection therefore indicates that there is a fine balance involved in this evasion. Since subsequent protoscolex mortality is not so great it appears that parasites surviving the initial attack can maintain or increase evasion throughout development.

The present ultrastructural studies indicate that there is

increased activity within the cytons, involving accelerated synthesis and secretion. Mitochondria also appeared more numerous and enlarged indicating increased metabolic activity (Threadgold, 1976). Similar cellular activity has also been reported for other developing metacestodes (Smyth, 1972; McCaigue, Halton & Hopkins, 1986) and for developing adults of *E. granulosus* (Jha & Smyth, 1971). No evidence of the formation of mitochondria from infolds of the basal tegumentary membrane, as reported in the latter study, however, was observed in the present study. The increased activity and depletion of glycogen reserves was presumably required for the differentiation processes of the parasites. Secretory activity appeared to mainly involve the T_2 vesicles, between 7 and 30 days p.i. Similar flocculent, carbohydrate-containing bodies have been reported for many other cestodes (see Chapter 5) and have been suggested to be involved in a variety of functions such as contributing to the glycocalyx, microtriches, basal lamella or cytosol of the tegument or containing secretory substances processed for exocytosis. In the present studies, the T_2 vesicles were initially concentrated in the scolex region but become numerous in the soma tegument at a time when the truncated microtriches were forming. Additionally high numbers of T_2 vesicles in the microthrix-bearing adult-type tegument supports the suggestion that the vesicles may play a part in formation or maintenance of both spined and truncated microtriches or their associated membranes.

The formation of truncated microtriches has not been previously described but seemed to involve initial formation of the projections parallel to and below the parasite surface, with susbequent uplifting. This is similar to the formation of spined microtriches as reported for certain other cestode species (Timofeev & Kuperman, 1972; Lumsden, Oaks & Muller, 1974; Hess 1980; Richards & Arme,

1984). It is therefore possible that the T_2 vesicles could contribute to the electron-dense material of the shaft support in the truncated microtriches and the shaft support and spine of the spined microtriches. The origins of the other microthrix components, however, remains unknown.

In cysts formed *in vitro*, the increased presence of T_2 vesicles was not as dramatic as *in vivo*. This may reflect a slower, more gradual formation over time. Presumably after the initial formation of the projections *in vivo*, others are produced as the cyst grows. Although T_2 vesicles were not commonly seen in mature cysts, formation of additional truncated microtriches may involve a more localized production of these bodies.

At the time when the surface projections were changing, the parasites were still occasionally susceptible to immunological attack. Again many protoscoleces were able to withstand or evade this attack and additional evasive mechanisms may be operating at this stage. From about 10 days p.i. a large number of host cells in the immediate vicinity of the parasites were degenerated, possibly indicating that the protoscoleces had some cytotoxic effect on host cells. It is possible that this degeneration could be due to natural death but Annen, Köhler, Eckert & Speck (1980) and Heath (1986) reported that substances present or released from hydatid fluid or cysts, had a cytotoxic effect on host leucocytes. It is therefore not inconceivable that such a substance could be released from protoscoleces during cystic transformation and may aid in parasite survival prior to and after the appearance of the laminated layer.

The initial formation of the laminated layer was concurrent with the appearance of the conspicuous 'G' vesicles in the tegumentary cytoplasm. These vesicles presumably contributed to the aggregations of dense granules in the laminated layer, as suggested

by Richards *et al.* (1983b). From the X-ray microanalysis carried out (see Chapter 5) on these granules, a significant calcium peak was detected. Since it has been reported that the laminated layer of *Echinococcus* cysts exhibits specific antibody binding and complement fixation (Ali Kahn & Siboo 1980; Heath, 1986). These granules could act as anticomplementary factors. Operation may be as suggested for the calcareous corpuscles of other cestodes, by binding complement non-specifically and rendering it unable to attach to the parasite surface (for reviews see Williams *et al.*, 1980; Rickard & Williams, 1982; Rickard, 1983).

With cysts grown *in vitro*, however, the dense granules were not present in the laminated layer although they were present in the germinal layer vesicles. Denser fibrillae were, however, present in the laminated layer with a similar distribution as the granules. It is possible that when the granules are released from the germinal layer they become soluble and only when the substance comes into contact with a host factor does it reprecipitate. This may explain why there is frequently a granule free zone immediately adjacent to the tegument. The precipitating factor is presumably missing *in vitro* and could originate from host leucocytes since the granule accumulations were often larger and denser at the external periphery of the laminated layer of peritoneal cysts. Similarly cysts developed in diffusion chambers often showed only a patchy distribution of granules which may reflect incomplete diffusion of cellular products into the chambers.

Formation of the carbohydrate matrix of the laminated layer was unclear but seemed to involve flocculent T_4 vesicles and their associated storage lysosomes since these were the only carbohydrate-containing organelles present in the later stages of laminated layer formation and in mature cysts (see Chapter 5). It

appeared that the T_4 vesicles were synthesized by the Golgi complexes as suggested by Richards (1984) and often coalesced to form the larger storage lysosomes. Since the T_4 vesicles were observed comparatively rarely in the distal cytoplasm of developing cysts, and very rarely in mature cysts, it is possible that their release occurs in sporadic bursts. Production, however, appears to be fairly continuous during development and the storage lysosomes may act to autophagocytose excess Golgi products as suggested by Richards *et al.* 1984. Alternatively these organelles may store the Golgi products for eventual release to the exterior, in full or as re-budded T_4 vesicles although no direct evidence of this was observed. Release of the matrix material may depend on the rate at which the peripheral laminated layer is phagocytosed by host cells. Since these macrophages never reach the tegumentary membrane, the laminated layer serves as highly efficient barrier and an additional immunoprotective mechanism.

Completion of cystic differentiation occurs in the original scolex region when the spined microtriches and rostellar hooks are jettisoned. The budding off of the hooks in a cytoplasmic vesicle has not been previously described and illustrates the reparative, dynamic nature of the tegumentary tissue - a factor which may be important in repair of immunological damage. Clearly, therefore, the fully formed cyst is able to survive in its host, probably as a result of a range of immunoprotective mechanisms. The most likely of these might be the possession of anticomplementary factors, cytotoxic substances and the laminated layer but additionally, cysts may mask some antigens by the incorporation of host immunoglobulins, since host IgG has been identified in the hydatid fluid of *E. granulosus* cysts (Coltorti & Varela-Diaz, 1972; 1975).

CHAPTER 7

ULTRASTRUCTURAL DEVELOPMENT OF THE TEGUMENT ASSOCIATED WITH THE MATURATION OF PROTOSCOLECES IN BROOD CAPSULES

7.1 INTRODUCTION

The morphological development of protoscoleces of *Echinococcus* spp. within brood capsules has been described in a number of studies (Dew, 1922; Sakamoto & Sugimura, 1970; Pennoit-De Cooman & De Rycke, 1972; Bortoletti & Ferretti, 1973; Šlais, 1973; Thompson, 1976a; Ubelaker, 1983) and shown to involve the formation of buds, the tegument of which is derived from an up-pushing of the brood capsule wall. Each bud then develops two constructions which delimit the rostellar and the sucker regions and only later is the non-scolex (soma) region formed. Differentiation is complete after rostellar hook formation and the scolex then invaginates into the soma region.

The majority of studies have involved light microscope observations, and detailed information on the ultrastructural development, particularly of the protoscolex surface is limited. Studies on the fully formed protoscolex show the scolex surface to possess spined microtriches and the soma region to be characterised by small, blunt elevations (see Chapter 5). it is also known that the internal surface of the brood capsule, from which the protoscolex will arise, possesses truncated microtriches (without spines) (see Chapter 5). These projections have been previously referred to, by other workers, as both microtriches (which, in cestodes, typically possess spines) and microvilli (which characteristically lack a base plate and shaft-support material) thereby causing confusion in the literature. As the terms "microtriches" and "microvilli" will be

referred to in the present chapter, their use will be as defined above.

Since the brood capsule wall gives rise to the protoscolex tegument, then some transformation or replacement of the projections must occur during development. In addition the components of the tegument, especially the vesicle population, must also mature before differentiation of the fully formed protoscolex is complete. The present chapter therefore describes the maturation of the tegument associated with protoscolex development.

7.2 RESULTS

Material for the examination of developing protoscoleces was obtained from occasionally fertile cysts produced in mice or from equally rare fertile daughter cysts from horse livers. In addition two fertile cysts from a gerbil infection were obtained courtesy of Mr. D.L. Morris of the Department of Surgery, University Hospital, Nottingham.

When all of the above cysts (totalling 9 in number) were dissected and examined, the internal surface of the germinal layer possessed several papillae (Figs. 7.1, 7.2). In SEM the larger papillae possessed a bulbous tip at the end of a stalk originating from the germinal layer (Fig. 7.3). The entire surface of these papillae was covered with cells with many cytoplasmic processes (Fig. 7.3). When examined in LM these papillae could be identified as immature brood capsules possessing protoscoleces at various stages of development. These capsules contained between 0 and 19 protoscoleces and a developmental sequence similar to that reported by earlier authors (see Introduction) was observed.

The earliest stage recorded was that of a small brood capsule attached to the germinal layer but devoid of protoscoleces (Fig. 7.4). Protoscolex formation began with a multiplication of cells forming a small swelling at the distal end of the brood capsule which subsequently grew to form a small bud (Fig. 7.5.). As development proceeded, constrictions delimiting the hooked rostellum from the rest of the scolex were evident (Fig. 7.6). Only after full development did invagination occur (Fig. 7.7) and subsequent protoscoleces form (Fig. 7.8).

Many of the larger, more mature brood capsules had ruptured and everted so that the developing protoscoleces were projecting

outwards. This arrangement therefore allowed observation of the various stages using SEM (Fig. 7.9). These are diagrammatically summarised in Fig. 7.60.

Prior to protoscolex formation the brood capsule, seen in TEM, consisted of a cavity surrounded by a uniform layer of cells (Fig. 7.10). The distal cytoplasm lining the cavity possessed mainly electron lucent T_3 vesicles and a scant population of truncated microtriches (Fig. 7.11). The cells at the periphery of the capsule were undifferentiated and possessed large nuclei and abundant small mitochondria (Fig. 7.11).

The earliest protoscolex stage (Stage 1) observed in EM consisted of a simple bud arising from the brood capsule wall (Figs. 7.12, 7.13). This bud was composed of a distal portion projecting into the brood capsule cavity (Fig. 7.13) and a proximal portion extending slightly to the outside of the capsule (Fig. 7.14). Both regions were packed with numerous undifferentiated cells. Because of the collapse of the brood capsule wall in SEM preparations, both regions of the early developing buds projected from the capsule wall (Fig. 7.12).

The tegument of the proximal and distal regions of these stage 1 protoscoleces differed in ultrastructure. In the distal region, which was to become the scolex, the tegumentary syncytium was thin (approximately 0.82µm in depth) and possessed only a small number of lucent T_3 vesicles and occasional small mitochondria (Fig. 7.15). The surface projections consisted mainly of occasionally truncated microtriches (Fig. 7.15) but towards the posterior of the distal region, microvilli, lacking shaft support material and extending up to 0.865µm in length (approx. 71nm in width) were also present (Fig. 7.16).

In the proximal region of these Stage 1 protoscoleces, which was

to become the soma, the tegument was more convoluted but also possessed truncated microtriches and a greater population of long microvilli (Figs. 7.17, 7.18), up to 2.125µm in length. The vesicles of this portion of the tegument were more numerous but again were mainly comprised of T_3 vesicles. Occasional vesicles containing rod like bodies were, however, also observed (Fig. 7.17). The origin of these vesicles was not elucidated but it is possible that they may represent the precursors of the T_1 vesicles.

Stage 2 of development involved the formation of two constrictions in the bud; one in the distal or scolex region, delimiting the rostellar and sucker portions and the other separating the scolex from the area which will become the soma (proximal region) (Fig. 7.19). The surface ultrastructure of the scolex was primarily one of truncated microtriches although short microvilli were also becoming more evident (Figs. 7.20, 7.21). The presumptive soma still possessed truncated microtriches and numerous long microvilli on the surface of the distal cytoplasm which was also more abundant in vesicles. The vesicle population, however, was now primarily composed of T_3 vesicles together with vesicles with electron dense cores which were presumably of T_1 origin (Fig. 7.22). In addition to these, a third type of inclusion body was present. These T_0 vesicles (Fig. 7.22) were round, densely flocculent and stained for carbohydrate using the periodic acid - thiosemicarbazide method (Fig. 7.23). The T_0 vesicles differed from T_2 vesicles in size and shape, being larger and more rounded (192nm in diameter compared to T_2 maximum diameter - 142nm). The T_0 bodies were limited to the presumptive soma and absent from the scolex where the T_2 vesicles would eventually predominate. These T_0 vesicles were occasionally observed in close association with the apical membrane (Fig. 7.24) and presumably contributed to the formation of the now apparent

glycocalyx (Fig. 7.23). Both the T_0 and T_1 vesicles were observed in the perinuclear cytoplasm of the tegumentary cytons (Fig. 7.25) thus suggesting that they were of Golgi origin, although Golgi complexes were rarely seen, presumably due to the limited amount of tissue available for examination.

Stage 3 of development involved organisation of the internal tissues and further differentiation of the rostellum into a cone-shaped region and also a broadening of the sucker area (Fig. 7.26). On the rostellum, increasing numbers of microvilli were evident and a band of large, triangular shaped projections were present near the rostellar base (Fig. 7.27). In TEM these projections appeared to be very broad microtriches (approximately 0.41μm in diameter, with spines approximately 0.54μm in length) arising from the tegumentary surface (Fig. 7.28). The shaft of these type 1 large microtriches (M_1) had conspicuous, electron-dense shaft support material which was particularly evident along the longer, anterior side of the shaft (Fig. 7.28). This material was in contact with the spine and presumably contributed to it. This material, however, did not appear to be dependent on T_2 vesicles which were only occasionally present in the scolex at this stage. Presumably the shaft support material arose from precursors free in the distal cytoplasm but possibly concentrated in a region, just below the apical membrane, which was darker in appearance (Fig. 7.28).

The M_1 large microtriches were considerably broader than the typical spined microtriches (approximately 0.27μm in diameter) which, by this time, were becoming evident on the sucker region together with microvilli (Fig. 7.29). The earliest formed sucker microtriches appeared to arise from existing truncated microtriches by accumulation of electron-dense material from the shaft support to form the distal spine (Fig. 7.29). Microtriches were, however, also

observed forming *de novo* and parallel to the tegumentary surface, and to become subsequently up-lifted into their normal position (Fig. 7.30). The soma region, at this stage, still possessed truncated microtriches and long microvilli.

At Stage 4, differentiation of the rostellum continued with the formation of a rostellar ridge (Fig. 7.31). The M_1 large microtriches were concentrated in this area and had increased dramatically in size (spine length approximately 1.2μm; diameter approximately 0.9μm) (Figs. 7.32, 7.33) whilst microvilli had also increased in size and number (Fig. 7.32). Although more numerous, the M_1 microtriches were considered to be the precursors of the rostellar hooks but at this stage they still retained their microthrix appearance in TEM (Fig. 7.34).

As development progressed (Stage 5) the rostellar hooks became more pronounced (Fig. 7.35) and projected from the base of the rostellum. At high power in SEM (Fig. 7.36) the hooks were ridged and uncovered by the tegument. In TEM (Fig. 7.37) the hooks could still be seen to have a microthrix configuration although the spine was now very long and curved. Neither the 'handle' or 'guard' components of mature hooks were evident at this stage.

Numerous long microvilli were present over the entire surface of the protoscolex including the suckers which now also possessed abundant spined microtriches (Fig. 7.38) and numerous T_2 vesicles in both the distal and perinuclear, tegumentary cytoplasm (Fig. 7.39).

Below the double row of hooks and above the suckers, a second band of enlarged microtriches was present (Figs. 7.40, 7.41). These M_2 large microtriches occasionally appeared as two individual projections in close proximity and may have been formed by a fusion of smaller individual microtriches (Fig. 7.42) in contrast to the enlargement process of the M_1 large microtriches. The individual

projections appeared to fuse to form a graded size range of M_2 microtriches, increasing from posterior to anterior(Fig. 7.41). Abundant T_2 vesicles were also present in the region of the M_2 microtriches (Fig. 7.43). Rostellar microtriches however, were not yet present and microvilli still predominated.

With increased differentiation (Stage 6), the microvilli on the suckers were gradually lost (Fig. 7.45). The M_2 large microtriches were still present (Figs. 7.46, 7.47) and in TEM appeared like small hooklets. They were, however, not involved in hook formation as the hooks were already formed (Fig. 7.47).

At this stage the soma was becoming elevated from the brood capsule surface (Fig. 7.44) and still possessed microvilli and truncated microtriches. These projections, however, were gradually being outnumbered by small blunt elevations (Fig. 7.48). These structures were occasionally present underneath existing microvilli (Fig. 7.48) but the vast majority were not associated with the microvilli. The blunt elevations therefore seemed to form independently of the microvilli. The T_0 vesicles of the soma tegument were now greatly reduced in number and a thick glycocalyx was present. Numerous T_1 vesicles were present in the distal cytoplasm (Fig. 7.48) and the cytons which appeared to be actively manufacturing these bodies (Fig. 7.49).

Further developmental stages of protoscoleces involved continued elevation of the soma surface from the brood capsule wall (Fig. 7.50) and subsequent loss of the soma microvilli and truncated microtriches. The microvilli were also completely lost from the suckers leaving the spined microtriches as the only tegumentary projections in this region (Fig. 7.51). The large M_2 microtriches in the region immediately below the rostellum also disappeared, presumably by shedding since enlarged microthrix spines were

occasionally observed free in the brood capsule cavity (Fig. 7.52).

Protoscolex differentiation was finally concluded in the rostellar region. In Stages 5 and 6 the rostellum was covered by long microvilli and the rostellar hooks projected outwards covered only by a membrane (Fig. 7.53). Subsequent development involved the loss of the microvilli and a covering of the hooks by the rostellar tegument (Fig. 7.54). It was unclear, however, whether this covering was a result of growth of the tegument over the hooks or by retraction of the hooks into the tegument. The entire surface of the rostellum then became covered by long, filamentous microtriches (Fig. 7.55) which were formed initially parallel to the tegumentary surface prior to uplifting (Fig. 7.56). In TEM the rostellar hooks now consisted of the characteristic blade, handle and guard portions (Fig. 7.57) and could no longer be recognised as being of microthrix origin. A tegumentary layer bearing microtriches, always covered these hooks even when extended in fully formed active protoscoleces (Fig. 7.58). Only when differentiation was complete did invagination of the protoscoleces take place (Fig. 7.59).

In the vast majority of cases, the protoscoleces formed inside the brood capsule via the sequence of events mentioned above. Occasionally, however, protoscoleces were seen developing on the outside of the brood capsules. This occurred both in very early capsules (Fig. 7.61) and in more mature ones (Fig. 7.62). In section these protoscoleces appeared to be well developed in possessing rostellar hooks and large M_2 microtriches (Fig. 7.63) on the interior of the protruding protoscolex. The external surface (ie. that outside the brood capsule) was cellular and not tegumentary in nature (Fig. 7.63). These protoscoleces therefore appeared to have an "inside out" configuration and presumably could become incorporated into their orthodox position inside the capsule by the sequence of events

shown diagrammatically in Fig. 7.64.

7.3 DISCUSSION

The present results indicate that protoscolex formation is initiated in the brood capsule shortly after the formation of the capsule cavity. As no earlier stages of brood capsule formation were observed the origin of the cavity and its surrounding tegument cannot be commented on. A similar difficulty was encountered by Bortoletti & Ferretti (1973) and Thompson (1976b) who indicated that it was impossible to state whether the brood capsules arose from a cell cluster, inside which the cavity is formed by lysis or from an introflexion of the distal cytoplasm of the germinal layer. Undoubtedly further information on brood capsule formation could be gained from examination of less developed fertile cysts.

Initial formation of protoscoleces in immature brood capsules appears to take place one at a time. As the volume of the capsules increases, protoscoleces at various stages of development can be observed in the same brood capsule, at the same time, indicating that development is asynchronous. Initial stages involve a multiplication of undifferentiated cells that lead to an up-pushing of the brood capsule tegument to form a small bud. This raised portion of the tegument therefore forms the initial covering of the presumptive scolex region and is initially fairly simple in structure, possessing few vesicles and a sparse population of truncated microtriches.

The presumptive soma region however, although not yet elevated, has a greater vesicle population and possesses long microvilli in addition to truncated microtriches. These microvilli subsequently become evident over the entire surface of the immature protoscoleces. The presence of these projections is of interest in that they have not previously been reported and do not occur on either the fully formed protoscolex or the hydatid cyst. The microvilli are therefore

transitory structures and occur in *Echinococcus* only during the early stages of protoscolex formation. In other cestode species however, they occur both on the metacestode and the developing presumptive adult (Mount, 1970; Timofeev & Kuperman, 1972; Lumsden, 1975; Hulínská, 1980; Lumsden *et al.*, 1982; Engelkirk & Williams, 1982; 1983; Richards & Arme, 1983; MacKinnon & Burt, 1984; Threadgold 1984). The fully developed protoscolex possesses microtriches on the scolex region and the possibility of microvilli being the precursors of these structures has to be considered, since Hulinska (1980) (who refers to spineless microtriches) and MacKinnon & Burt (1984) report the transformation of microvilli into microtriches in *Taenia hydatigena* and *Paricterotaenia paradoxa* respectively. The present study provided no evidence to support such a transformation in *Echinococcus*: the microvilli appeared to be shed in a manner similar to the shedding of microvilli from the developing scolex region of *Hymenolepis diminuta* (Richards & Arme 1984) and from the surface of the developing metacestode of *T. taeniaeformis* (Engelkirk & Wiliams, 1983). The function of these projections, in addition to surface amplification, therefore remains unclear as they do not appear to play a part in the formation of subsequent structures. It is possible, however, that the loss of microvilli and their replacement by microtriches on the scolex, may be associated with the display of different antigenic determinants at the apical membrane, as suggested for *T. taeniaeformis* by Engelkirk & Williams (1983). Although the environment within the cyst cavity is free from host cells, immunoglobulins have been detected (Coltorti & Varela-Diaz 1972; 1975). Whether these antibodies are capable of fixing complement remains to be seen, but the microvilli/microthrix change may produce an antigenic shift, preventing binding of the immunoglobulin to the maturing parasite. Clearly immunocytochemical analysis would be

required to investigate the possibility of stage specific antigens in juvenile and fully formed protoscoleces. Alternatively the presence of microvilli may be a feature common to all taeniid development but being more relevant in species where the microvillar surface is exposed to the full immunological response.

Whilst the microvilli are still present, spined microtriches form on the scolex of the protoscoleces. These projections appear to develop in one of two ways in *Echinococcus*. The first formed of these arose by accumulation of electron-dense material at the tip of existing truncated microtriches, therefore indicating a transformation from truncated to spined microtriches as also reported by MacKinnon & Burt (1984) and MacKinnon, Jarecka & Burt (1985) in *Paricterotaenia paradoxa* and *Haplobothrium globuliforme* respectively. The origin of the electron-dense material was unclear but seemed to occur in a band adjacent to each microthrix and just below the apical membrane. The presence of T_2 vesicles in the tegument at this stage was rare so their participation in contributing to spine material seems unlikely. This material therefore presumably arose from precursor material occurring in the cytosol of the tegument and not in membrane-bound vesicles.

T_2 vesicles were, however, present in greater numbers when subsequent microtriches developed, *de novo*, beneath and parallel to the apical membrane. These microtriches were subsequently uplifted in the same way as reported for certain other cestode species (Timofeev & Kuperman, 1972; Lumsden, Oaks & Mueller, 1974; Hess, 1980). The precise origin and assembly of the component parts of the microtriches was not elucidated, although participation of the T_2 bodies cannot be ruled out as other authors have postulated the involvement of membrane-bound vesicles in microthrix formation (Lumsden *et al.*, 1974; Hulínská, 1980; Richards & Arme, 1984;

Threadgold, 1984).

Of particular interest in the present study was the presence of very large microtriches, two types of which were apparent. The first type (M_1) arose early in scolex development and was initially formed by uplifting from the tegumentary surface. During maturation the spines of these microtriches increased dramatically in size and formed the precursors of the rostellar hooks, in a manner similar to that of the homologous "hooklets" reported for *T. crassiceps* (Bilqees & Freeman, 1969; Mount, 1970). Growth of the spine to form the blade of the hook is probably brought about by the transfer of electron-dense material up the sides of the M_1 microthrix shafts as reported for *T. crassiceps* by Mount (1970). Subsequent development of the hooks occurred after they had been enveloped by the rostellar tegument. Similar development occurs in *T. crassiceps* where the hooks are covered by hypertrophied tegument (Mount 1970) (Referred to as the "hook organ" by Bilqees & Freeman, 1969).

In these cases the initial microthrix-like "hooklet" serves as a framework upon which secondary thickening, to form the guard and handle of the hook, occurs. Mount (1970) suggested that dense, membrane-bound vesicles may contain the "hook protein" involved in secondary thickening. Similarly, in the present study, T_2 vesicles in the rostellar tegument may be involved in hook formation although whether they contribute to the actual substance of the hook remains unresolved and could be further investigated by cytochemical methods.

The second type of large microtriches (M_2), which may be homologous to the "superfluous hooklets" reported by Bilqees & Freeman (1969) for *T. crassiceps*, are also interesting. They are formed at the same time as the hooks and slightly below them, but they do not appear to contribute to hook formation and eventually disappear. It is possible that they may represent M_1 large

microtriches which do not form hooks but split to form individual normal microtriches. It is more likely, however, that they arise from a fusion of the existing microtriches and are subsequently shed from the tegument. One possible explanation for their transitory presence may lie in their close proximity to the hook bearing region and the evidence that the hooks develop from microtriches (in this case the M_1 microtriches). This process is presumably under the influence of a hook-forming stimulus and it is not inconceivable that this is not sufficiently precise as to prevent enlargement and subsequent fusion of microtriches in adjacent areas. The presence of M_2 microtriches only below the developing hooks might be explained by the fact that anterior to the hooks the surface still possesses microvilli which presumably remain unaffected by the stimulus. Certainly a lack of precision in the hook-forming mechanism occurs in *Echinococcus*, resulting in variation in hook number (Smyth, 1964).

One of the last events involved in protoscolex formation was the elevation of the soma region. The early soma tegument, however, seemed to be developing prior to elevation and was active in the production of T_1 vesicles. Again no suggestions as to the function of these vesicles were apparent but they did not seem to be involved in glycocalyx formation. This function was attributed to carbohydrate containing T_0 vesicles which were present only as the soma tegument was maturing and not when it was fully formed. This suggests that the thick soma glycocalyx is formed prior to full differentiation and does not appear to be renewed. Autoradiographical studies, however, may confirm whether or not there is a turnover of the glycocalyx material in the fully formed protoscolex prior to and after release from the cyst.

The formation of the blunt elevations on the soma surface did not involve the microvilli although they were occasionally present at

the time of formation. The involvement of the truncated microtriches in blunt elevation formation however, could not be determined. Their apparent loss during development may have been due to shedding, in the same way as the microvilli, or they may have been resorbed into the tegument for subsequent incorporation into the blunt elevations. Similar elevations occur on the surface of the cysticercoid of *Ophryocotyle insignis* (MacKinnon & Burt 1983) where it was suggested that they might serve to reduce the surface area exposed to host enzymes. A similar function may operate in *Echinococcus*, protecting protoscoleces from host enzymes in the dog or from immunological attack in the intermediate host. It may be argued, however, that the soma surface of *Echinococcus* must, because of the dimorphic potential of the protoscolex, remain structurally uncommitted. However, the loss of the truncated microtriches from the developing soma surface remains unexplained since they appear to be structurally 'suited' to both modes of development (characterising the hydatid stage and being capable of transformation into spined microtriches).

It is, however, unlikely that a full explanation of the surface changes during protoscolex formation can be gained purely from structural considerations, since these do not take into account any changes in surface antigenicity that might be occurring.

In the vast majority of cases the protoscoleces were observed developing on the interior of the brood capsule. In a few cases, however, occasional protoscoleces were seen on the outside of the capsules; a feature also reported by Dew (1922), Pennoit-De Cooman & De Rycke (1972) and Thompson (1976c). Dew (1922) and Pennoit-De Cooman & De Rycke (1972) intepreted the position of these protoscoleces as being due to elastic forces resulting from muscle activity and causing the protoscoleces to be pushed outwards. These authors also suggested that contractile forces might be capable of

forcing the protoscoleces back into the brood capsules where completion of maturation could occur. Thompson (1976c), however, suggested that the existence of these 'external' protoscoleces was a result of overcrowding within the brood capsule and that they ultimately degenerated and detached from the capsule wall.

The present observations show that the external protoscoleces are often fairly well developed, possessing hooks, suckers and M_2 microtriches. Their presence on young brood capsules containing no other protoscoleces suggests that there existence is not due to overcrowding and it therefore seems quite possible that they could be forced back into the capsule to complete their development. Pennoit-De Cooman & De Rycke (1972) proposed that this change of protoscolex position from outside to inside may occur quickly at a point where the "muscular contraction force" of the developing protoscolex is in equilibrium with the "cuticle elastic reversing force" (i.e. the contractile nature of the brood capsule wall as shown by its ability to turn inside out on rupture).

The results in the present chapter have clearly shown that gross differentiation of protoscoleces is accompanied by significant changes in the tegument and in particular the surface microarchitecture and have therefore added to our knowledge and understanding of the developmental biology of *Echinococcus*.

CHAPTER 8

INVESTIGATIONS ON ATTEMPTED DISRUPTION OF CYSTIC DEVELOPMENT USING MONENSIN

8.1. INTRODUCTION

Although effective chemotherapeutic agents against adults of *Echinococcus* exist (for example praziquantel, Thomas & Gonnert (1978)) those used against the hydatid stage are not consistently successful (Benzimida-zoles - Heath, Christie & Chevis (1975); Schantz, Van den Bossche & Eckert (1982); Morris (1983); Eckert (1986): praziquantel - Marshall & Edwards (1982); Yao, Ping, Jun & Xun (1985)). There is therefore a need to improve the efficacy of these existing drugs or exploit new compounds with anti-hydatid activity.

The importance of the Golgi system in maturation of cysts and protoscoleces of *E. granulosus* has been elucidated in the previous chapters. Events such as laminated layer and glycocalyx formation, maintenance of membrane integrity, secretion and formation of microtriches all appear to involve Golgi-derived vesicles which therefore contribute to the immunoprotective and nutritive properties of the organism. Since these are obviously important functions, any disruption of the Golgi system may reduce parasite survival.

The carboxylic ionophore monensin, which binds Na^+ and K^+, altering transport of the cations across membranes, has been shown to affect the Golgi complexes of various mammalian tissues and cell lines. Swelling of the Golgi cisternae and inhibition of secretory product transport have been reported often *in vitro* with treatments ranging from 10 min to 4h (Griffiths, Quinn & Warren, 1983;

Tartakoff, 1983; Ellinger & Povelaka, 1984; Strous, van Kerkhof, Willemsen, Slot & Geuze, 1985; Ono, Manners, Shimada, Kuwano & Mifune, 1985). In addition, monensin has been used routinely in the treatment of coccidiosis in poultry and lambs (Shumard, Callender & Reid, 1970; Leck, Fayer & McLoughlin, 1976; McDougald & Dunn, 1978; Calhour, Carrol & Shelton, 1979; Chapman, 1984) where it causes an increased sodium influx and gross swelling in *Eimeria* sporozoites (Smith, Galloway & White, 1981; Smith & Galloway, 1983).

There are, however, no reports on the treatment of helminth parasites with monensin although Fairweather & Skuce (1985) and Skuce & Fairweather (1985) stated that the ionophore affects the Golgi cisternae in the tegumentary and vitelline cells of *Fasciola hepatica*. In view of this, the present chapter describes attempts to disrupt cystic development and maturation of *E. granulosus* by the action of monensin *in vitro*. Furthermore a preliminary *in vivo* study was carried out to investigate the potential of monensin as an anti-*Echinococcus* agent.

8.2 RESULTS

Aspects of the following observations have been published in a research paper (Rogan & Richards, 1986b).

8.2.1 Effects of monensin on the protoscolex *in vitro*

Prior to exposure to monensin, protoscoleces had a typical tegumentary ultrastructure as described in Chapter 5. The cytons were relatively inactive, possessing few mitochondria and occasional Golgi complexes with narrow cisternae (Fig. 8.1).

The first effects of 10μM monensin were apparent, in both the scolex and soma tegument, after 15min. These mainly consisted of a swelling of the Golgi cisternae, particularly of the maturing face (trans cisternae) (Fig. 8.2).

After 1 hour the swelling of the Golgi stacks was more severe, with cisternae associated with the forming face (cis cisternae) also being affected (Fig. 8.3). Increased numbers of swollen, electron-lucent vesicles were also apparent in the vicinity of the Golgi fields (Fig. 8.3). Occasional mitochondria also appeared swollen whilst others and the peripheral ER remained unchanged (Fig. 8.3). At this stage several tegumentary cytons possessed two types of residual body (Fig. 8.4). The first type was characterized by the presence of multilaminate stacks of electron-dense material, the laminations of which had a periodicity of approximately 7nm, and vesicles of moderate electron density (Fig. 8.4 inset). The second type lacked the multilaminate stacks and contained general debris material (Fig. 8.4). The distal cytoplasm appeared relatively unaltered although occasional small residual bodies, lacking multilaminate stacks, were present at the base (Fig. 8.4). The

numbers of mitochondrial profiles appeared elevated in the cytons and internuncial processes. Stereological analysis (see Appendix 4) confirmed that both the mean volume ($0.044\mu m^3$) and mean volume fraction of mitochondria ($0.148\mu m^3/\mu m^3$) were significantly greater than the same parameters in control protoscoleces ($0.0135\ \mu m^3$ and $0.069\mu m^3/\mu m^3$, respectively) ($P<0.01$ in both cases Students *t* test) analysis of the Golgi membranes in monensin-treated organisms was not, however, significantly different from control parasites (mean surface/volume ratio = $3.65\ \mu m^2/\mu m^3$ and $3.36\mu m^2/\mu m^3$ respectively) ($P>0.1$ Students *t* test). This suggests that, initially, swelling of the Golgi complexes was confined to existing membranes and did not involve production of additional cisternae.

Cytochemical attempts to detect acid phosphatase and thiamine pyrophosphatase activity in the Golgi complexes were inconclusive. In most of the control protoscoleces neither enzyme could be detected although occasional cytons possessed a slight deposit in the Golgi region for both acid phosphatase (Fig. 8.5) and thiamine pyrophosphatase (Fig. 8.6). In monensin treated tissue no reaction products were observed in any protoscoleces for either enzyme (Figs. 8.7, 8.8).

From 3-8 hours, the swelling of the Golgi cisternae and associated vesicles was more pronounced and numerous large electron-lucent vacuoles characterized the cytons (Fig. 8.9). In several cases the distal cytoplasm appeared normal but in others large vacuoles, together with both types of residual body, were present (Fig. 8.10). Some of the protoscoleces examined also had severe disruption of the tegument near the base of the scolex (Fig. 8.11), involving intensive vacuolation and shedding off of the microtriches.

By 12 hours many protoscoleces had tegumentary cytons which

contained numerous vacuoles and enlarged mitochondria with dilated cristae (Fig. 8.12). Other cytons were occasionally multinucleate (Fig. 8.13) whilst the distal cytoplasm often possessed large vacuoles (Fig. 8.14). In areas of the distal cytoplasm which were not disrupted, the vesicles appeared turgid and less flattened than normal (Fig. 8.12).

After 24 hours in 10μM monensin many of the protoscoleces had, tegumentary tissue which in TEM lacked contrast (Fig. 8.15), appearing dead. The cytons contained large vacuoles, mitochondrial remnants and general debris (Fig. 8.16) whilst the distal cytoplasm was often vacuolated (Fig. 8.17), highly folded (Fig. 8.18) and possessed large cytoplasmic blebs (Fig. 8.19). When examined by SEM, the soma surface of many protoscoleces was contorted into numerous ridges whilst the suckers of the scolex were highly profiled (Fig. 8.20a,b). These features and the fact that protoscoleces had ceased moving after 1 hour suggests that they may have gone into a spastic paralysis. Many others showed more severe effects, being collapsed and possessing numerous blebs (Fig. 8.21a,b). In all cases the treated protoscoleces appeared quite different from controls (Fig. 8.22) which in TEM had normal tegumentary ultrastructure with the cytons possessing un-swollen Golgi cisternae (Fig. 8.23).

Throughout the 10μM monensin incubations there was some variability in the degree of ultrastructural damage and even at 24 hours some protoscoleces were only in the initial stages of Golgi swelling (Fig. 8.24). This variability was also reflected in the viability figures as assessed by eosin exclusion (Table 8.1).

	Mean % viability			
Time(hrs)	10μM monensin	5μM monensin	2.5μM monensin	Control
0	97.3	98.6	98.2	97.3
1	97.8	95.2	98.3	-
3	97.6	97.3	96.9	-
8	96.8	95.4	95.0	-
12	81.2	93.2	96.3	-
24	46.5	86.1	87.6	96.8
30	5.3	-	-	-
36	0.0	49.4	61.5	-
48	0.0	21.0	39.3	-
72	0.0	0.0	9.8	-
84	0.0	0.0	0.0	94.9

TABLE 8.1 Table showing % viability of protoscoleces incubated in various concentrations of monensin.

These data show that, in the 10μM solution, only after 12 hours was there a significant drop in viability. Even at 24 hours, when many protoscoleces were showing severe ultrastructural damage, 37% still retained the ability to exclude eosin. Only after 36 hours were all protoscoleces dead.

In the 5μM and 2.5μM solutions a similar trend was observed, albeit slower. In 5μM monensin all protoscoleces were dead after 72 hours whilst in 2.5μM total mortality occurred after 84 hours. This suggests that the effects of monensin are fatal to protoscoleces and are both dose and time dependant.

8.2.2 Effects of monensin on cysts *in vitro*.

When murine hydatid cysts were incubated in 10μM monensin, the first effects were again seen after 15min. These involved the cytons possessing many more electron-lucent vesicles (Fig. 8.25) and very large, carbohydrate containing storage lysosomes. Within the distal cytoplasm and the cytons several of the G' vesicles has a dense periphery and extremely large electron-dense granules (up to 8nm in diameter) (Figs. 8.26, 8.27).

After 1 hour the vesicles and storage lysosomes of the cytons appeared more swollen and increased mitochondrial profiles were apparent (Fig. 8.28). Many of the mitochondria were enlarged (Fig. 8.29) and stereological analysis indicated that the mean volume of the mitochondria ($0.066\mu m^3$) was significantly greater compared to control incubated cysts ($0.028\mu m^3$) ($P<0.02$, Students *t* test).

In many cases, the membranes bounding the tegumentary vesicles were irregular and appeared to have lost their integrity (Fig. 8.30). These and some of the mitochondria were often encapsulated into what appeared to be autophagic vacuoles (Fig. 8.30). The nuclei, however, remained intact except for occasional myocytons where the nuclear membrane had 'blown out' (Fig. 8.31).

By 3 hours the increased numbers of mitochondria were still evident and clusters of them were often observed just below the base of the distal cytoplasm (Fig. 8.32). In several cases the mitochondrial cristae of both the tegumentary and myocytons were dilated (Fig. 8.33) indicating breakdown of the organelles. Additionally the tegumentary mitochondria were often found in close

proximity to large membraneous whorls which were increasingly present (Fig. 8.34).

After 8 hours the tegumentary tissue appeared pale (Fig. 8.35) and the cytons were characterized by large vacuoles, mitochondria and membraneous whorls (Fig. 8.35). The mitochondria possessed dilated cristae, which often appeared circular, and were clearly degenerating (Fig. 8.36). They were often surrounded by a series of membranes (Fig. 8.37) and presumably gave rise to the membraneous whorls after their degeneration (Fig. 8.38). In most cases the distal cytoplasm, although pale, appeared relatively unaltered (Fig. 8.38).

Between 24 and 48 hours the tegumentary cytons were a mass of large membraneous vesicles (Fig. 8.39). The same was true of the distal cytoplasm (Fig. 8.40) which was only loosely attached to the laminated layer in a few places. Incubated control tissue after 56 hours still showed a normal ultrastructure with cytons possessing un-swollen Golgi complexes, vesicles and mitochondria (Fig. 8.41). The distal cytoplasm, however, occasionally had high numbers of mitochondria close to the basal membrane (Fig. 8.42).

When viewed by LM the control cysts appeared normal with the germinal layer being closely associated with the laminated layer (Fig. 8.43). When cysts incubated in 10μM monensin were examined, however, it was evident that, from 8 hours onwards, some cysts showed a "pitting" of the germinal layer (Fig. 8.44). This subsequently resulted in the germinal layer detaching from the laminated layer (Fig. 8.45) and eventually collapsing into the centre of the cyst (8.46). From the ultrastructural studies, cysts with detached germinal layers were judged to be dead. This detachment could therefore be used as a LM criterion of death, and the proportion of cysts showing detachment (or being dead) throughout incubation in

various concentrations of monensin is shown in Table 8.2.

	average % cysts with detached germinal layers			
time(hrs)	10μM monensin	5μM monensin	2.5μM monensin	Control
0	0	0	0	0
24	33.3	8.7	0	0
48	98.7	59.3	12.1	0
72	100	100	43.7	0
96	100	100	100	0

TABLE 8.2 % cysts with detached germinal layers during *in vitro* monensin incubations.

Clearly the effects of monensin on cysts is also time and dose dependant with all cysts being dead in the 10μM solution often approximately 48 hours. The same was true in the 5μM solution after 72 hours and in the 2.5μM solution after 96 hours.

The ultrastructural effects of monensin were also apparent in 32d *in vitro* cultured cysts (Figs. 8.47, 8.48) and in 108d cultured cysts (Fig. 8.49) after exposure to a 10μM solution for 0.5-24hrs.

This suggests that the ionophore affects both mature and immature cysts as well as protoscoleces.

8.2.3 *In vivo* effects of monensin

A summary of the monensin dosage administered in each condition is given in Table 8.3.

Treatment	Stock	Amount given daily	mg/kg body weight
In vitro	10μM=6.929mg/l =6.9ppm	-	-
In vivo injected	10μM=6.929mg/l =6.9ppm	0.2ml=1.38μg i.p.	0.055
In vivo injected	20μM=13.858mg/l =13.8ppm	0.5ml=6.9μg i.p.	0.276
In vivo gavage	2.5mg/ml=2,500ppm	0.01ml=0.25mg oral	10.00
In vivo Feed	300mg/kg=300ppm	5-10g=1.5-3.0mg oral	60-120

TABLE 8.3 Summary of the monensin dosages employed in investigations

Administration by injection

Since the 10μM solution of monensin had an effect on parasites *in vitro*, it was decided to inject this solution (0.2ml) into the peritoneal cavity of infected mice (10months) daily over a 21 day period. Results of the autopsies are shown in Table 8.4 where cysts were examined by LM for evidence of germinal layer pitting and detachment. Representative samples were also fixed for TEM.

On LM morphological observations there appeared to be no difference between treated and control cysts allowing for the natural variation which exists in infections. The same was true for the TEM observations except that some treated cysts had a distal cytoplasm

10μM MONENSIN INJECTED				CONTROL INJECTED			
time (days)	Nos. cysts	extern. diameter	comments	time (days)	Nos. cysts	extern. diameter	comments
0	74	2.89 ± 0.77	all normal				
7	116	3.1 ± 1.20	all normal				
10	359	7.39 ± 0.93	all normal	10	227	1.88 ± 1.01	all normal
14	143	2.42 ± 0.92	all normal				
21	121	5.37 ± 0.66	all normal	21	133	6.79 ± 0.37	all normal

TABLE 8.4 Results of autopsies of mice injected with 0.2ml of 10μM monensin daily.

possessing enlarged electron-dense granules (Fig. 8.50) in some regions. There was no other evidence of any monensin-induced effects.

When the concentration of monensin was raised to 20μM and 0.5ml was injected daily, the following autopsy results were obtained. (Table 8.5).

Most treated cysts examined by TEM had a normal tegumentary ultrastructure. Those showing slight pitting after 21 and 30 days did not show undue damage although there was some evidence of an increase in mitochondrial profiles and autophagic activity (Figs. 8.51, 8.52). In the one cyst which had a detaching germinal layer after 60 days, the tegumentary tissue was considerably affected. The distal cytoplasm was severely vacuolated (Fig. 8.53) and the cytons were characterized by numerous large vacuoles and membraneous whorls indicative of mitochondrial degeneration (Fig. 8.54). Other cysts examined from the same mouse, however, had a comparatively normal ultrastructure.

Administration by gavage

Results from autopsies of mice with a 7 month infection gavaged with 0.25mg monensin daily are shown in Table 8.6 and those with a new infection are shown in Table 8.7.

The autopsies carried out on mice gavaged with monensin (Table 8.6) showed that a small number of cysts were apparently affected in that they showed pitting and detachment of the germinal layer. When the cysts showing pitting after 10 days were examined by TEM, the vesicles of the distal cytoplasm were irregular in shape and many enlarged mitochondria were evident near the basal membrane (Fig. 8.55). These were also evident in the cytons where a considerable amount of tegumentary tissue was degenerate (Fig. 8.56). Other cysts examined at 10, 15 and 20 days appeared normal in ultrastructure.

20μM MONENSIN INJECTED				CONTROL INJECTED				UNTREATED CONTROL			
time (days)	nos. cysts	ext. diam(mm)	comments	time (days)	nos. cysts	ext. diam(mm)	comments	time (days)	nos. cysts	ext. diam(mm)	comments
7	49	2.01±0.9	all normal								
21	293	5.17±0.83	1 cyst pitted								
30	268	8.25±1.17	7 cysts pitted	30	114	2.43±1.43	all normal	30	216	6.75±0.8	all normal
50	176	4.33±1.37	all normal								
60	210	6.05±0.87	1 cyst detaching	60	77	2.97±1.62	all normal	60	119	4.22±0.4	all normal

Table 8.5 Results of autopsies of mice injected with 0.5ml of 20μM Monensin.

MONENSIN TREATED				CONTROL GAVAGE				UNTREATED CONTROL			
time (days)	nos. cysts	ext. diam(mm)	comments	time (days)	nos. cysts	ext. diam(mm)	comments	time days	nos. cysts.	ext. diam(mm)	comments
4	156	8.29±0.95	all normal								
10	291	5.75±0.32	8 pitted	10	64	10.2±0.94	all normal				
15	119	4.23±0.75	all normal	15	133	4.23±0.7	all normal				
20	207	3.10±0.83	all normal								
30	53	8.72±1.73	5 detaching	30	124	6.79±1.1	all normal	47	28	3.27±0.6	all normal
57	97	10.2±0.9	all normal					57	219	2.19±1.1	all normal

Table 8.6 . Autopsy results from mice, with a 7 month infection, administered with 0.25mg monensin daily by gavage.

Pre-gavage/gavage Monensin			Gavage Monensin			Gavage control			untreated control		
time (days p.i.)	nos. PSC* retrieved	details	time (days p.i.)	nos. PSC retrieved	details	time (days p.i.)	nos. PSC retrieved	details	time (days p.i.)	nos. PSC retrieved	details
8	1500	48.7% dead	7	2000	61% dead	7	2500	50% dead			
20	978	72.0% dead	40	875	13 cysts**				20	1570	4 cysts**
38	650	80 cysts**	52	1020	27 cysts**	40	450	40 cysts**	40	967	97 cysts**

Table 8.7 Autopsy results from mice gavaged with 0.25mg monensin daily, from 10 days prior to infection (pre gavage/gavage) and from the time of infection (gavage).

* PSC = protoscoleces; ** cysts here are defined as those parasites with a visible laminated layer.

The treatment of mice for 10 days prior to and at the time of infection did not appear to influence the eventual number of cysts produced, compared with control mice, over a 40 day period (Table 8.7). Parasites examined throughout the treatment by TEM showed no significant differences in ultrastructure compared with that of control parasites at the same stage of development.

Incorporation of monensin in feed

Autopsies on mice given monensin-containing feed (300mg/kg) revealed the following results.

The results in Table 8.8 suggest that incorporating monensin in feed has slightly more significant effect on established cysts than the other methods of administration. In all cases, however, the majority of hydatids were relatively unaffected by the ionophore.

The pitted cysts examined at 25 days generally had a normal tegumentary ultrastructure although some had a distal cytoplasm which was vacuolated in places (Fig. 8.57). The affected cysts examined at 42 days and 49 days, however, were clearly necrotic. These possessed a detached germinal layer and a tegument which was highly vacuolated and contained numerous residual and membraneous bodies (Fig. 8.58). Although administration of monensin in the feed appeared to have more effect on cysts it must be stressed that from 35 days onwards the treated mice had diarrhoea, moved slowly and with difficulty and occasionally died. This suggested that the ionophore was having a toxic effect on the mice and the experiment was terminated after 49 days.

MONENSIN TREATED				CONTROL			
Time (days)	Nos. cysts	extern. diameter	details	Time (days)	Nos. cysts	extern. diameter	details
25	116	6.89 ± 0.86	6 pitted	25	143	5.99 ± 0.8	all normal
35	420	2.31 ± 1.11	all normal				
42	136	10.11 ± 2.03	1 detached 1 collapsed	42	197	11.27 ± 1.9	all normal
49	74	5.07 ± 0.75	2 detached				
49	179	4.23 ± 0.6	all normal	49	223	4.25 ± 0.71	all normal

TABLE 8.8 Autopsy results from mice administered monensin in feed (1.5-3.0mg monensin daily).

8.3 DISCUSSION

The present results show that, *in vitro* at least, monensin has a rapid, lethal effect on protoscoleces and cysts. Although slight differences in effects were observed between the two stages, basically similar events were common to both (i.e. swelling of the Golgi membranes; swelling and multiplication of mitochondria; increased autophagy and degeneration of tegumentary tissue).

The initial swelling of the Golgi cisternae and associated vesicles, and particularly those associated with the maturing face is in accordance with other reports (Griffiths *et al.* 1983; Tartakoff, 1983; Strous *et al*, 1985) although Ellinger & Povelka (1984) reported that Golgi complexes of rat gut absorptive cell showed an overall swelling that was not associated with any Golgi subcompartment.

The mode of action of monensin in causing such swelling is not well understood. The ionophore is a metabolite of *Streptomyces cinamonensis* and its capacity to bind Na^+, K^+ and protons results in a 1:1 cation exchange when inserted into biological membranes (Tartakoff, 1983). The precise effect of the Na^+ and K^+ concentrations on the Golgi is not known but it has been suggested that the swelling may be due to the presence of an impermeant weak acid within the cisternal space, the protons of which exchange with Na^+ resulting in a passive water influx (Tartakoff, 1983). Alternatively, the normal compressed form of the cisternae may be due to ion pumps that ultimately result in a water efflux. The increase in cation permeability caused by the monensin may therefore relax this condition and result in swelling (Tartakoff, 1983). This explanation is supported by the fact that ouabane, an inhibitor of Na^+/K^+ ATPase pumps, also produces swelling of the Golgi cisternae in rodent sensory neurones (Threadgold, 1976). Additionally monensin

has been shown to increase Na^+/K^+ ATPase activity in sporozoites of *Eimeria tennella* (Smith & Galloway, 1983) and in cardiac tissue of animals showing monensin toxicosis (Langston, Galey, Lovell & Buck, 1985) presumably in an attempt to correct membrane ionic imbalances.

The significance of Golgi swelling is unclear but it is presumably detrimental. Attempts to study any effects on the enzyme activity, associated with the Golgi complexes, were inconclusive as cytochemical techniques for localization of the Golgi markers, thiamine pyrophosphatase and acid phosphatase were unsuccessful. It is possible, however, that a pH shift brought about by the association of monensin and protons, may influence enzyme activity. Inhibition of intracellular transport, reported for other cells in response to monensin (Griffiths *et al.*, 1983; Cowen, Sakamoto & Sakamoto, 1985), may also be occurring in *Echinococcus*. Previous reports have suggested that synthesis of material is unaffected by monensin, resulting in accumulation in the Golgi cisternae. In the present experiments, enlarged electron-dense granules were observed in the tegumentary tissue of cysts exposed to monensin and may reflect an accumulation of a Golgi-derived product.

Similar accumulations of secretory vesicles have also been reported for *E. granulosus* cysts treated with mebendazole (Verheyen, 1982) where the drug is thought to effect internal transport via the microtubules. In this case vacuolation and disruption of the distal cytoplasm also occurred, possibly suggesting a coalescence of the secretory vesicles within the distal cytoplasm.

The altered cation concentrations caused by the ionophore would also presumably affect the muscle system and may cause the spastic paralysis observed in protoscoleces. Swelling of the mitochondria, however, may be a result of several factors. Langston *et al.* (1985) stated that monensin indirectly increases the intracellular calcium

concentrations by increased calcium influx, release of bound calcium from the mitochondria and decreased efflux of calcium. This in combination with sodium influx caused a swelling of the mitochondria. Alternatively any increased activity of the Na^+/K^+ ATPase pumps would require an increased demand for ATP and hence elevated respiratory activity as reported for *Eimeria* (Smith & Galloway, 1983). Such activity may cause a swelling and proliferation in the mitochondria and this may be a symptom of general stress since similar effects occur in *Echinococcus* protoscoleces during cystic development (see Chapter 6); after exposure to very different agents such as complement (Kassis *et al.* 1976) and in other cestodes under immunological attack (McCaigue *et al.*, 1986). It is likely that, since mitochondria ultimately degenerate, both increased activity and ionic imbalance may be occurring to cause swelling. Interesting observations could however, be made on what effects monensin has on the respiratory rates of the parasites.

The presence of residual bodies in protoscoleces and membraneous whorls in cysts suggests that increased autophagy was occurring. This is presumably an attempt to remove degenerate organelles such as mitochondria and has also been reported for cysts of *Echinococcus* spp. exposed to mitomycin C (Marchiondo & Andersen, 1985) and mebendazole (Verheyen, 1982) and for other parasitic platyhelminths exposed to various drugs (Clarkson & Erasmus, 1984). Clearly, however, ultimate death of the parasites is likely to be a result of a variety of factors, including impaired secretory and mitochondrial activity, and vacuolation and rupture of the tegument. This latter effect is common to several drug treatments such as mebendazole (Verheyen, 1982) mitomycin C (Marchiondo & Andersen, 1985) and praziquantel (Morris, Richards & Chinnery, 1986; Morris, Taylor, Daniels & Richards, 1987) and presumably finally results in the

parasites becoming eosin positive in the eosin exclusion test.

The variation in susceptibility of protoscoleces to the effects of monensin is similar to that reported for protoscoleces of *E. multilocularis* exposed to complement *in vitro* (Kassis *et al.* 1976) and may reflect age differences within each sample. Since it is known that protoscoleces and brood capsules are not all formed at the same time (see Chapter 7), the population of protoscoleces within each hydatid will contain recently formed individuals as well as older ones which may be more susceptible to drug treatment. However, the fact that both protoscoleces and cysts were rapidly killed in a 10μM solution suggests that monensin is an effective agent against *Echinococcus in vitro*. The *in vitro* killing of protoscoleces was considerably more rapid than that recorded for albendazole (Chinnery & Morris, 1986) and praziquantel (Morris *et al.* 1986, 1987). Attempts to replicate the results *in vivo*, however, were largely unsuccessful. Only a small number of cysts showed any effect of the monensin treatment after oral or intraperitoneal administration of relatively high doses. The fact that some cysts, although small in number, showed both slight and severe effects is encouraging and suggests that if the amount of monensin reaching the parasites could be increased, higher mortalities may be obtained.

With the intraperitoneal injection, only 0.5ml of a 20μM solution, daily, had any significant effect. Clearly, since this strength of monensin would, by extrapolation, have a dramatic effect *in vitro*, some of the solution was not reaching the cysts in the peritoneal cavity. This was probably due to lack of circulation in the peritoneal fluid resulting in a very localized area of administration. For this to be increased, a considerably greater volume would have to be injected. This is, however, unfeasible as damage to the host may occur through excess peritoneal fluid and

exposure of the internal organs to high doses of monensin.

Administration of the ionophore orally appeared to be a more suitable mode of delivery and resulted in some cysts having degenerated tissue, with others possessing enlarged granules in the secretory vesicles, and proliferating mitochondria. Again if the amount of drug reaching the parasites could be increased better results may have been obtained.

Pharmokinetic studies (Donoho, Manthey, Occolowitz & Zornes, 1978; Herberg, Manthey, Richardson, Cooley & Donoho, 1978; Donoho Harberg Zornes & Van Ruyn, 1982; Davison, 1984; Donoho, 1984) on the fate of monensin administered orally to cattle, chickens and rats have shown that the ionophore is rapidly metabolised into a series of metabolites with 40-95% of the dose being excreted in the faeces. Bilary excretion appeared to be the major route of elimination and only 0.03-0.4% of the monensin or its active metabolites remained in the liver tissue. Other tissues such as fat, kidney or muscle retained negligible amounts of monensin and it is therefore likely that the concentration within the peritoneal cavity, in the present feeding studies, would similarly be low. The experimental situation involved in these studies is, however, somewhat false when considering primary cysts from natural infections, where the majority of cysts are in the liver or the lungs. Since significantly greater quantities of monensin may be retained in these tissues, natural infections may be more susceptible to drug action.

In considering using monensin in the treatment of hydatid disease the problem of toxicity must be overcome. In the present study, mice were showing some of the clinical signs of monensin toxicity after receiving 60-120mg/kg of the ionophore daily for 35-49 days. The LD_{50} for mice has been reported to be 125mg/kg (Todd, Novilla & Howard, 1984; Langston *et al*. 1985) so clearly the

administered dose was approaching this value. Cattle, sheep, dogs and monkeys have been reported to withstand moderate doses (LD_{50} 12-160mg/kg; Todd *et al.* 1984; Langston *et al.* 1985) although horses are highly susceptible to monensin toxicosis (LD_{50} < 2mg/kg). Any further work would therefore have to monitor host organs for signs of toxicity and maintain the levels of monensin administered, below 12mg/kg.

In conclusion it seems that the potential use of monensin in the treatment of animal hydatid disease is limited unless a higher dose, which is not toxic to the host, can be directed to the parasites, primarily in the liver. The use of drug carriers and delivery systems may provide greater efficacy in this situation. It would also be of interest to know whether incorporating monensin in the feed had any prophylactic activity against oncospheral infections since several animal feedstuffs contain monensin (e.g. Romensin) as a growth promoter (Begstrom & Maki, 1976; Boling, Bradley & Campbell, 1977; Calhoun, Carroll, Livingston & Shelton, 1979; Leitgeb, Mader, Lettner & Yshirch, 1985).

CONCLUDING COMMENTS

The present results clearly show that protoscoleces of the horse strain of *E. granulosus* can be successfully cultured *in vitro* and in BALB/c mice to produce miniature hydatid cysts. Success *in vitro* may be due to a number of factors which proved to be important in influencing development. Firstly, the choice of base medium is crucial, with NCTC 135 and CMRL 1066 being better than M 199 or RPMI 1640. Secondly, the numbers of protoscoleces per tube can affect development, with low numbers favouring adult-like development and optimum cystic development obtained with approximately 4,000 protoscoleces per tube. Thirdly controlling pH variations to maintain the pH between 6.8 and 7.4 is beneficial for cystic development. Finally, the relatively gentle agitation employed in the present system may mean that the micro-conditions surrounding the parasite could show considerable un-defined variations, some of which may be suited to cystic development. Indeed, within a single culture tube, the existence of many morphological types, suggests that conditions within the medium may not be totally homogeneous.

During culture, morphological, cystic development was similar to that described for the sheep strain of *E. granulosus* and involved both vesiculation and posterior bladder formation. The origin of the bladders was shown to be from the protoscolex attachment stalk, sometimes with portions of brood capsule wall. The fact that these bladders and large portions of brood capsule wall could, when detached from protoscoleces, form cysts is interesting. It seems possible that there may exist a balance of inhibitory influences within the hydatid. In the intact cyst, protoscoleces nor brood capsules normally produce cysts. Similarly, during *in vitro* culture, the same is true when many protoscoleces are attached to brood

capsules. When protoscoleces are freed from the brood capsule (and from other protoscoleces), however, both tissue types can form cysts. It is therefore plausible that protoscoleces could inhibit cystic development in both brood capsules and other protoscoleces for some time at least. In this light, the formation of daughter cysts is also interesting. These structures appear to be associated with degenerating primary cysts and, because of their numbers being in hundreds rather than thousands, their formation may be more dependent on ruptured everted brood capsules (without protoscoleces attached) rather than vesiculating protoscoleces.

Normal development of protoscoleces in the peritoneal cavity of mice differed from the *in vitro* situation primarily in terms of a slower rate of protoscolex vesiculation (i.e. it often occurred after laminated layer formation). Subsequent differentiation was, however, similar in both cases.

Growth of cysts in diffusion chambers proved to be an effective way of protecting the parasites from the host cellular response and increasing the number of cysts produced. This is therefore a useful method of producing a controlled number of cysts and may be of considerable benefit in chemotherapeutic studies where the effects of drugs in killing the parasites may be masked by an efficient host cellular attack. This is particularly important at the time of infection with protoscoleces (thus mimicking spillage at surgery) where, in the normal case, many protoscoleces are naturally killed by the host immune response. Implanting the parasites in diffusion chambers would therefore mean that the protoscolicidal effect of a drug would be easier to assess.

During both *in vitro* and *in vivo* cystic development considerable changes in the tegument occurred and, although the functional significance of these events remain speculative, some may be

concerned with immunoprotection. Amongst the major events are changes in the tegumentary vesicles and include a loss of T_1 vesicles in the soma region, an initial increase in T_2 vesicles followed by a decline, and the appearance and maintenance of the granule-containing 'G' vesicles. The T_2 vesicles appear to be involved with microthrix-bearing portions of the tegument and may contribute to the formation of spined and possibly truncated microtriches. The exact construction of these projections still remains unclear but involves assembly of the various components below and parallel to the apical membrane, prior to subsequent uplifting.

The presence of the laminated layer is important in protecting the cysts from immunological attack since most parasite deaths, *in vivo*, occurred prior to its formation. It seemed therefore that there was a period of approximately 30 days when the parasites were most susceptible to attack. Additionally, cysts with an established laminated layer (derived from *in vitro* cultures or diffusion chambers) grew well when injected into naive mice. The formation of this layer appeared to be dependent on two types of Golgi-derived vesicle, the 'G' vesicles and the T_4 vesicles. The 'G' vesicles were always present in the tegument of developing and mature cysts and possessed calcium-containing granules. Similar aggregations of these granules were also present in the laminated layer of peritoneal cysts although there was generally a granule free region immediately adjacent to the apical membrane. This region may have been artifactual but may also suggest that either extrusion of the granules was on a sporadic basis or that the nature of the granules was somehow changed on release from the tegument. Evidence from the *in vitro* cultures (where the granules were not present) and the diffusion chambers (where the granule distribution showed regional variation) suggests that the granules may be released from the

tegument in a more soluble form to be re-precipitated by some host factor, possibly originating from associated leucocytes. The presence of calcium within these granules is significant in that they may act as complement consuming factors, thus providing an immuno-protective mechanism. It would be interesting to verify this function by detecting components of the complement cascade within the laminated layer, possibly by the use of monoclonal antibodies.

The formation of the carbohydrate matrix of the laminated layer seemed to be dependent on the T_4 vesicles. Their presence in the distal cystoplasm of the germinal layer, however, was relatively rare and in many sections examined they were absent. Within the cytons, T_4 vesicles appeared to be incorporated into carbohydrate-containing storage lysosomes and their release from the distal cytoplasm may have been on a sporadic basis, being absent most of the time. The function of the storage lysosomes is, however, unclear. They may represent a site for the sequestration of excess laminated layer material produced by the Golgi complexes. Alternatively they may represent an intermediate stage between formation of material in the Golgi complexes and release at the surface.

Observations from the early *in vivo* and *in vitro* cultures showed that there is a change in the appearance of the laminated layer with time, progressing from a colourless layer to a laminated structure, appearing 'tanned' in transmitted light. It is likely that this change is due to the compaction and/or polymerization of the laminated layer components. The early layer may therefore be less viscous and capable of 'flowing' over the parasite surface, perhaps accounting for earlier reports by Smyth (1962, 1967) which stated that *in vitro* the posterior bladders produced a laminated layer which subsequently enveloped the whole protoscolex.

Subsequent development after laminated layer formation involved

loss of all protoscolex remnants such as the suckers and hooks. It is important to say, however, that the final stages of cystic development took up to three months to occur. This suggests that in the initial period of infection, the parasites were in a state of considerable re-organization and may therefore show biochemical or metabolic characteristics different from either protoscolex or cyst. In this respect, further physiological work on the cystic stage should be limited to those parasites obtained more than 3 months post-infection. On the other hand the periods of time involved in the various phases of cystic development have now been more accurately defined thus permitting further evaluation of the physiological changes which occur in this period of differentiation. Similarly the efficacy of chemotherapeutic agents can now be assessed for developing stages as well as cystic and protoscolex stages in an attempt to find the most vunerable stage in the parasite life cycle.

Although formation of fertile cysts containing protoscoleces was not obtained *in vitro*, those formed *in vivo* showed interesting developmental features, particularly in respect to the changes in surface microarchitecture. The presence of true microvilli has not been previously reported for developmental stages of *Echinococcus* other than the oncosphere. The significance of these projections is unclear as they do not appear to be involved in the formation of subsequent structures. Their function may, however, be reflected in changes in the characteristics of the apical membrane which may also be associated with antigenicity. The formation of the rostellar hooks appears to be similar to that reported for other taeniid species and shows clearly that these structures are derived from enlarged microtriches which subsequently become enveloped by the rostellar tegument.

As yet the significance of the blunt elevations of the soma

region is unclear though it appears that they offer a reduced surface area compared to a region of microthrix-bearing tegument. This, and the fact that released protoscoleces are generally invaginated into the soma suggests that the soma tegument may have a more protective function than that of the scolex. The thick glycocalyx formed by a population of vesicles present only at the time of protoscolex formation (T_0), also emphasises the protective nature of the soma. It is not clear, however, whether such protection is mainly required for development within the intermediate host, the definitive host or both. Similarly the T_1 vesicles of the soma tegument may provide some sort of initial protection for the parasite in the first few days after release from the hydatid cyst.

The ultrastructural observations presented here show that throughout differentiation of *E. granulosus* cysts and protoscoleces, Golgi-derived vesicles play an important role. This secretory system is therefore a good target for potential chemotherapeutic agents. In an attempt to exploit this system monensin proved to have rapid protoscolicidal and cysticidal effects *in vitro* which involved affecting the Golgi complexes in the first instance. Ultimate death of the parasites, however, is likely to have been caused by a variety of factors resulting from the ionic imbalance brought about by the ionophore. The use of monensin in veterinary studies seems unlikely due to the rapid metabolism and excretion of the compound and the potential toxicity to the host. The toxicity problem, however, is more aplicable to horses which are much more sensitive to monensin than sheep. The potential Golgi-disrupting action of this type of compound is, however, important. It may open up new chemotherapeutic regimes which could be effective in preventing the formation of the laminated layer and hence prevent protoscolex/cyst differentiation.

In conclusion the work presented in this thesis illustrates the

complex changes which occur in *E. granulosus* protoscoleces during cystic differentiation. In particular the dynamic nature of the tegument is emphasised and possible immunoprotective mechanisms raised. In view of these observations further research should be directed towards investigating the chemical nature of the secretory vesicle contents, and the various factors involved in laminated layer formation. Additionally the possible existence of stage specific antigens could be examined, using such methods as surface radio-labelling, in an attempt to gain a further understanding of how *E. granulosus* survives and proliferates within its intermediate host.

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APPENDIX 1

COMPONENTS OF THE VARIOUS CULTURE MEDIA EMPLOYED DURING *IN VITRO* CULTURE.

NCTC 135 MEDIUM

Ingredient	mg/litre
L-Alanine	31.48
L-α Amino-n-butyric acid	5.51
L-Arginine HCl	31.16
L-Asparagine H_2O	9.19
L-Aspartic acid	9.91
L-Cystine, disodium salt	12.41
L-Glutamic acid	8.26
L-Glutamine	135.7
Glutathione	9.33
Glycine	13.51
L-Histidine HCl H_2O	26.65
L-Hydroxyproline	4.09
L-Isoleucine	18.04
L-Leucine	20.44
L-Lysine HCl	38.43
L-Methionine	4.44
L-Ornithin HCl	9.41
L-Phenylalanine	16.53
L-Proline	6.13
L-Serine	10.75
Taurine	4.18
L-Threonine	18.93
L-Tryptophan	17.50
L-Tyrosine disodium salt	20.43
L-Valine	25.00
L-Ascorbic acid	50.00
Biotin	0.025
Choline chloride	1.25
Calciferol	0.25
D-Calcium pantothenate	0.025
Folic acid	0.025
Phositol	0.125
Menaphithone sodium bisulphite $3H_2O$	0.048
Nicotinic acid	0.0625
Nicotinamide	0.0625

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NCTC 135 MEDIUM cont...

Ingredient	mg/litre
p-Aminobenzoic acid	0.125
Pyridoxal HCl	0.0625
Pyridoxine HCl	0.0625
Riboflavin	0.025
Thiamin HCl	0.025
DL-α Tocopherol phosphate, disodium salt	0.025
Vitamin A acetate	0.29
Vitamin B12	10.00
$CaCl_2 2H_2O$	264.9
KCl	400.0
$MgSO_4 7H_2O$	204.8
NaCl	6800
$NaHCO_3$	2200
$NaH_2PO_4 2H_2O$	158.3
Cocarboxylase	1.00
Coenzyme A, trilithium salt, $2H_2O$	2.50
Deoxyadenosine H_2O	10.00
Deoxycytidine HCl	10.00
Deoxyguanosine H_2O	10.00
FAD, disodium salt	1.00
D-Glucosamine HCl	3.85
D-Glucose	1000
D-Glucuronolactone	1.80
5-Methylcytosine	0.10
NAD	7.00
NADP, sodium salt H_2O	0.98
Sodium acetate	30.14
Sodium glucuronate H_2O	1.80
Phenol red sodium salt	20.00
Thymidine	10.00
Tween 80	12.50
UTP, trisodium salt $2H_2O$	1.00

CMRL 1066 MEDIUM

Ingredient	mg/litre
L-Alanine	25.00
L-Arginine HCl	70.00
L-Aspartic acid	30.00
L-Cysteine HCl	233.3
L-Cystine disodium salt	23.66
L-Glutamic acid	75.00
L-Glutamine	100.0
Glutathione	10.00
Glycine	50.00
L-Histidine HCl H_2O	20.00
L-Hydroxyproline	10.00
L-Isoleucine	20.00
L-Leucine	60.00
L-Lysine HCl	70.00
L.Methionine	15.00
L-Phenylalanine	25.00
L-Proline	40.00
L-Serine	25.00
L-Threonine	30.00
L-Tryptophan	10.00
L-Tyrosine disodium salt	49.72
L-Valine	25.00
L-Ascorbic acid	50.00
Biotin	0.01
D-Calcium pantothenate	0.01
Choline chloride	0.50
Folic acid	0.01
i-Inositol	0.05
Nicotinic acid	0.025
Nicotinamide	0.025
p-Aminobenzoic acid	0.05
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.01
Thiamin HCl	0.01
$CaCl_2$ $2H_2O$	264.9

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CMRL 1066 MEDIUM cont...

Ingredient	mg/litre
KCl	400.0
$MgSO_4$ $7H_2O$	200.0
NaCl	6800
NaH_2PO_4 $2H_2O$	158.3
$NaHCO_3$	2200
Cholesterol	0.20
Cocarboxylase	1.00
Coenzyme A, trilithium salt $4H_2O$	2.61
Deoxyadenosine H_2O	10.00
Deoxycytidine HCl	10.00
Deoxyguanosine H_2O	10.00
FAD, disodium salt	1.00
D-Glucose	1000
5-Methyl deoxycytidine	0.10
NAD	7.00
NADP, sodium salt $2H_2O$	1.00
Phenol red sodium salt	10.00
Sodium acetate	50.00
Sodium glucoronate H_2O	4.20
Thymidine	10.00
Tween 80	5.00
UTP, trisodium salt $2H_2O$	1.00

M 199 MEDIUM (WITH HANK'S SALTS)

Ingredient	mg/litre
L-Alanine	25.00
L-Arginine HCl	70.00
L-Aspartic acid	30.00
L-Cysteine HCl	0.0987
L-Cystine disodium salt	23.66
L-Glutamic acid	66.82
L-Glutamine	100.0
Glutathione	0.05
Glycine	50.00
L-Histidine HCl H_2O	21.88
L-Hydroxyproline	10.00
L-Isoleucine	20.00
L-Leucine	60.00
L-Lysine HCl	70.00
L-Methionine	15.00
L-Phenylalanine	25.00
L-Proline	40.00
L-Serine	25.00
L-Threonine	30.00
L-Tryptophan	10.00
L-Tyrosine disodium salt	49.72
L-Valine	25.00
L-Ascorbic acid	0.05
Biotin	0.01
Claciferol	0.10
D-Calcium pantothenate	0.01
Choline chloride	0.50
Folic acid	0.01
i-Inositol	0.05
Menophthone sodium bisulphite $3H_2O$	0.019
Nicotinic acid	0.025
Nicotinamide	0.025

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M 199 MEDIUM (WITH HANK'S SALTS) cont...

Ingredient	mg/litre
p-Aminobenzoic acid	0.05
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.01
Thiamin HCl	0.01
DL-α Tocopherol phosphate disodium salt	0.01
Vitamin A acetate	0.1147
$CaCl_2 2H_2O$	185.5
$Fe(NO_3)_3 9H_2O$	0.10
KCl	400.0
KH_2PO_4	60.00
$MgSO_4 7H_2O$	200.0
NaCl	8000
$NaHCO_3$	350.0
$NaH_2PO_4 2H_2O$	
Na_2HPO_4	47.50
Adenine sulphate	10.00
5'-AMP	0.20
ATP, disodium salt	10.00
Cholesterol	0.20
2-Deoxyribose	0.50
D-Glucose	1000
Guanine HCl	0.30
Hypoxanthine	0.30
D-Ribose	0.50
Sodium acetate	36.71
Phenol red sodium salt	17.00
Thymine	0.30
Tween 80	5.00
Uracil	0.30
Xanthine	0.30

RPMI 1640 MEDIUM

Ingredient	mg/litre
L-Arginine	200.0
L-Asparagine H_2O	56.82
L-Aspartic acdi	20.00
L-Cystine, disodium salt	59.16
L-Glutamic acid	20.00
L-Glutamine	300.0
Glutathione	1.00
Glycine	10.00
L-Histidine	15.00
L-Hydroxyproline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lysine HCl	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Tyrosine disodium salt	24.86
L-Valine	20.00
Biotin	0.20
D-Calcium paniothenate	0.25
Choline chloride	3.00
Folic acid	1.00
i-Inositol	35.00
Nicotinamide	1.00
p-Aminobenzoic acid	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamin HCl	1.00
Vitamin B12	0.005
$Ca(NO_3)_2$	69.49
KCl	400.0
$MgSO_4 7H_2O$	100.0
NaCl	6000
$NaHCO_3$	2000

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RPMI 1640 MEDIUM cont...

Ingredient	mg/litre
$NaH_2PO_42H_2O$	
Na_2HPO_4	800.7
D-Glucose	2000.0
EPES	
Phenol red sodium salt	5.00

APPENDIX 2

BUFFERS AND FIXATIVES

1) Sodium cacodylate buffer 0.1M pH 7.2

Solution A: Sodium cacodylate (0.2M)
Sodium cacodylate 42.8g
($Na(CH_3)_2AsO_2.3H_2O$)
Distilled water 1000ml

Solution B: Hydrochloric acid (0.2M)
(HCl)

For a 0.1M solution (pH 7.2) add 4.2ml of solution B to 50ml of solution A and make up to 100ml with distilled water.

2) 3% Glutaraldehyde in sodium cacodylate buffer (pH 7.2)

To 10ml of 25% aqueous glutaraldehyde ($HCO(CH_2)_3(HO)$, as supplied, add 73.3ml of sodium cacodylate buffer (0.1M, pH 7.2).

3) 1% Osmium tetroxide in sodium cacoxylate buffer (pH 7.2).

To 5ml of 4% aqueous osmium tetroxide (OsO_4), as supplied, add 15ml of sodium cacodylate buffer (0.1M, pH 7.2).

4) 10% Neutral buffered formalin (pH 7.0).

37% formaldehyde (HCHO) solution 100ml
Distilled water 900ml
Acid sodium phosphate, monohydrate 4g
($NaH_2PO_4.H_2O$)
Anhydrous disodium phosphate 6.5g
(Na_2HPO_4)

Buffers and fixatives cont....

5) Tris/Maleate buffer (0.2M pH 5.2 and pH 7.2

Solution A: Tris(hydroxymethyl)aminomethane-maleate (Tris/Maleate) (0.2M)

Tris ($(CH_2OH)_3C-NH_2$) 30.3g
Maleic acid ($CHCO_2H$) 29.0g
distilled water 500ml

Solution B: 4% Sodium hydroxide

Sodium hydroxide (NaOH) 4g
distilled water 96ml

For desired pH add the following volume of solution B to 40ml of solution A and dilute to 100ml with distilled water.

Solution B	pH
20ml	7.2
6ml	5.2

6) Tris buffer (Tris/HCl buffer) (0.05M) pH 7.6

Solution A: 0.2M Tris

Solution B: 0.1N HCl

To 10ml of solution A add 15ml of solution B and dilute to 40ml with distilled water.

7) Phosphate buffered saline (PBS)

Solution A: Sodium chloride (NaCl) 8.09g
Potassium chloride (KCl)).29g .
Disodium phosphate (Na_2HPO_4) 1.159g
Potassium phosphate (KH_2PO_4) 0.29g
distilled water dilute to 800ml

Solution B: 0.19g calcium chloride ($CaCl_2$) in 100ml distilled water

Solution C: 0.19g magnesium chloride hexahydrate ($MgCl_26H_2O$) in 100 ml distilled water. Filter

Mix solutions A, B and C in order.

Buffers and fixatives cont....

8) Hank's balance salt solution (pH 7.2-7.4)

Clacium chloride ($CaCl.2H_2O$)	185.5 mg/l
Potassium chloride (KCl)	400.0 mg/l
Potassium phosphate (KH_2PO_4)	60.0 mg/l
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	200.0 mg/l
Sodium bicarbonate ($NaHCO_3$)	350.0 mg/l
Disodium phosphate (Na_2HPO_4)	47.5 mg/l
Sodium chloride ($NaCl_2$)	8000.0 mg/l
Glucose ($C_6H_{12}O_6$)	1000.0 mg/l

APPENDIX 3

NUMERICAL DATA ON PROPORTIONS OF MORPHOLOGICAL TYPES EXISTING DURING *IN VITRO* CULTURE VARIATIONS.

Abbreviations used:

Iv. = Invaginated protoscoleces.

Ev. = Evaginated protoscoleces.

P.B. = Protoscoleces with posterior bladders.

Ves. = Vesiculating protoscoleces

L.L. = Protoscoleces/cysts with a laminated layer

Ad. = Adult

Dd. = Dead protoscoleces

psc = Protoscoleces

FCS = Foetal calf serum (inactivated unless stated as different)

The data for culture variations is presented together with reference data from concurrent standard cultures.

I Alterations in the basic medium used.

% individuals in M199 + 20%v/v FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
14d	0	8.15	0	10.03	0.15	0	81.66
35d	0	0	0	7.67	2.10	0	90.22
60d	0	0	0	0	8.57	0	91.43
70d	0	0	0	0	9.16	0	90.84
120d	0	0	0	0	0	0	100.0

% individuals in NCTC 135 + 20%v/v FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
14d	0	16.5	0	35.8	1.23	0	46.41
35d	0	6.06	0	22.38	3.14	2.09	66.31
60d	0	1.02	0	3.52	39.47	0	56.19
70d	0	0	0	5.13	42.55	0	52.32
120d	0	0	0	0	36.19	0	63.81

% individuals in M199 + 20%v/v FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
7d	18.5	59.1	3.16	8.12	0	0	10.12
14d	0	63.57	0	15.14	0	0	21.29
35d	0	0	0	54.43	1.97	0	43.6
60d	0	0	0	39.33	10.96	0	49.71
70d	0	0	0	6.18	12.66	0	81.16
120d	0	0	0	0	3.21	0	96.79

% individuals in NCTC 135 + 20%v/v FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
7d	18.67	63.55	2.13	13.02	0	0	2.63
14d	0	43.21	0	32.53	0	0.71	8.61
35d	0	11.2	0	70.24	1.25	2.12	15.19
60d	0	0	0	50.77	27.03	0	22.2
70d	0	0	0	37.44	29.16	0	33.4
120d	0	0	0	1.13	44.2	0	54.67

% individuals in RPMI 1640 + 20%v/v FCS

Time	I.V.	Ev..	P.B.	Ves.	L.L.	Ad.	Dd.
14d	0	10.62	0	84.51	0.45	0	4.42
35d	0	24.64	0	12.5	2.85	0	60.0
60d	0	0	0	0	11.08	0	88.92
70d	0	0	0	0	10.13	0	89.87
120d	0	0	0	0	0	0	100.0

% individuals in NCTC 135 + 20%v/v FCS

Time	I.V.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
14d	0	16.5	0	35.8	1.23	0	46.41
35d	0	6.06	0	22.38	3.14	2.09	66.31
60d	0	1.02	0	3.32	39.47	0	56.19
70d	0	0	0	5.13	42.55	0	52.32
120d	0	0	0	0	36.19	0	63.81

% individuals in RPMI 1640 + 20% v/v FCS

Time	I.V.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
7d	25.17	42.19	0	25.44	0	0	7.2
14d	0	22.17	0	72.21	0	0	5.62
35d	0	1.63	0	51.34	1.4	0	45.48
60d	0	0	0	5.23	8.63	0	86.14
70d	0	0	0	6.91	15.14	0	77.95
120d	0	0	0	0	0	0	100.0

% individuals in NCTL 135 + 20%v/v FCS

Time	I.V.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
7d	18.67	63.55	2.13	13.02	0	0	2.63
14d	0	43.21	0	32.53	0	0.71	8.61
35d	0	11.2	0	70.24	1.25	2.12	15.19
60d	0	0	0	50.77	27.03	0	22.2
70d	0	0	0	37.44	29.16	0	33.4
120d	0	0	0	1.13	44.2	0	54.67

% individuals in CMRL 1066 + 20%v/v FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
49d	0	1.07	0.53	39.6	27.24	0	31.54
70d	0	0	0	18.47	50.26	0	30.26
120d	0	0	0	0	55.3	0	44.7

% individuals in NCTC 135 + 20%v/v FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
49d	0	1.53	0.2	46.75	32.44	0	19.08
70d	0	0	0	21.96	44.50	0	33.52
120d	0	0	0	0.97	48.33	0	50.7

% individuals in CMRL 1066 + 20%v/v FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
7d	2.19	58.54	5.19	31.09	0	0	5.18
14d	0	27.58	2.69	63.47	0.15	0	6.08
35d	0	2.37	0	76.2	10.12	0	28.2
60d	0	0	0	32.7	25.19	0	42.11
70d	0	0	0	18.12	34.3	0	46.58
120d	0	0	0	0	49.7	0	50.3

% individuals in NCTC 135 + 20%v/v FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
7d	18.67	63.55	2.13	13.02	0	0	
14d	0	43.21	0	32.53	0	0.71	
35d	0	11.2	0	70.24	1.25	2.12	
60d	0	0	0	50.77	27.03	0	
70d	0	0	0	37.44	29.16	0	
120d	0	0	0	1.13	44.2	0	

II Alterations in the serum content of the media

% indiviausl in NCTC 135 (without serum)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
3d	60.4	25.83	0	2.01	0	0	11.74
9d	14.16	68.67	0	3.43	0	0	13.73
14d	10.83	72.2	0	2.53	0	0	14.44
18d	3.19	75.58	0	11.33	0	0	9.9
28d	4.56	62.36	0	4.94	0	0	28.13
35d	0	7.06	0	4.70	0	0	88.23
42d	0	5.12	0	3.87	0.37	0	90.66
49d	0	0	0	0	0	0	100.0

% individuals in NCTC 135 + 20% FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
3d	64.42	14.42	0	20.55	0	0	0.61
9d	26.86	58.20	0	10.44	0	0	4.47
14d	32.25	18.14	0	48.39	0	0	1.21
18d	4.96	70.24	0	21.06	0.83	0	2.89
28d	8.58	78.78	0	10.09	0.5	0	2.02
35d	2.31	57.91	0	8.47	0.77	0	30.50
42d	1.77	34.16	0	22.66	6.2	0	35.21
49d	0	13.67	0	37.2	11.97	0	37.16
70d	0	4.21	0	28.27	23.7	0	43.82

% individuals in NCTC 135 + 40% FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
3d	55.19	18.71	8.95	15.2	0	0	1.95
9d	17.61	49.13	3.1	26.77	0	0	3.39
14d	12.9	24.81	0.52	59.12	0	0	2.65
28d	6.12	19.72	0	69.79	1.2	0	3.17
35d	0	18.97	0	57.3	2.01	0	21.72
42d	0	5.22	0	61.18	5.3	0	28.3
49d	0	1.72	0	42.19	14.21	0	30.12
70d	0	0	0	39.04	20.01	0	40.95

% individuals in NCTC 135 (without serum)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
0d	89.82	0.7	0	0	0	0	9.48
4d	37.93	39.08	3.45	10.72	0	0	8.62
7d	45.1	39.12	0.54	10.87	0	0	4.35
14d	10.4	58.77	0	20.64	0.2	0	10.19
21d	0	24.19	0	21.03	1.01	0	53.77
56d	0	5.24	0	10.96	0	0	83.8
70d	0	0	0	0	0	0	100.0

% individuals in NCTC 135 + 10% FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
0d	89.82	0.7	0	0	0	0	9.48
7d	5.17	69.21	3.6	13.85	0	0	8.17
14d	0.12	70.21	1.39	15.15	0	0	13.13
21d	0	47.14	0	34.7	0	2.02	16.14
56d	0	11.71	0	47.63	7.76	0	32.9
70d	0	3.21	0	47.22	11.97	0	37.6

% individuals in NCTC 135 + 20% FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
0d	89.82	0.7	0	0	0	0	9.48
4d	2.58	74.74	2.05	9.77	0	0	10.82
7d	13.02	63.91	0	17.74	0	1.77	3.55
14d	4.83	55.35	1.33	25.29	0	4.97	8.23
21d	0	53.24	1.85	14.3	1.39	19.91	9.26
56d	0	10.95	0	40.79	12.12	1.16	34.9
70d	0	1.23	0	30.73	21.34	0	38.7

% indidivuals in NCTC 135 + 40% FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Od.
0d	89.82	0.7	0	0	0	0	9.48
7d	10.87	57.02	2.1	15.24	0	0	14.71
14d	2.13	47.19	0	28.2	0	0.13	22.35
21d	0	42.2	0	19.2	0.73	0	37.87
56d	0	7.23	0	38.21	8.95	0	45.61
70d	0	0	0	27.1	30.01	0	42.8

% individuals in NCTC 135 + 20% horse serum (inactive)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
0d	89.82	0.7	0	0	0	0	9.48
4d	19.91	57.14	1.73	17.31	0	0	3.89
7d	21.67	39.86	2.79	30.06	0	0.7	4.89
14d	9.32	31.69	0.57	52.3	0.3	0	6.12
21d	0	22.84	0	56.11	2.1	0	18.95
56d	0	2.67	0	32.82	12.59	0.4	41.52
70d	0	0.19	0	18.41	27.63	0	53.77

% individuals in NCTC 135 + 20% FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
0d	89.82	0.70	0	0	0	0	9.48
4d	2.58	74.74	2.05	9.77	0	0	10.82
7d	13.02	63.91	0	17.74	0	1.77	3.55
14d	4.83	55.35	1.33	25.29	0	4.97	8.23
21d	0	53.24	1.85	14.3	1.39	19.91	9.26
56d	0	10.95	0	40.79	12.12	1.16	34.9
70d	0	1.23	0	30.73	29.34	0	38.7

% individuals in NCTC 135 + 20% FCS (not inactive)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
0d	89.82	0.7	0	0	0	0	9.48
4d	11.07	52.57	6.1	2.21	0	0	3.69
7d	28.3	15.39	3.4	47.24	0	0	5.66
14d	15.19	19.24	0	38.12	0	0	27.45
21d	14.13	10.0	0.49	33.15	2.1	0	40.13
56d	5.21	12.34	0	27.61	5.6	0	49.24
70d	0	5.23	1.97	27.3	8.3	0	57.2

% individuals in NCTC 135 + 20% horse serum (inactive)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
4d	27.61	58.23	1.33	10.13	0	0	2.7
14d	7.34	54.37	0.17	29.93	0	0	8.19
28d	0	14.3	0	63.31	6.11	0	16.28
56d	0	2.81	0	52.87	15.02	0	29.3
70d	0	0.12	0	10.83	32.14	0	56.91

% individuals in NCTC 135 + 20% FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
4d	20.1	48.0	7.2	22.4	0	0	2.3
14d	15.3	40.3	6.28	35.0	0	0	3.12
28d	1.2	62.1	0	26.97	2.8	0	6.93
56d	0	5.21	0	48.34	18.24	0	28.21
70d	0	0	0	23.44	38.96	0	37.6

% individuals in NCTC 135 + 20% FCS (not inactive)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
4d	22.4	47.4	4.95	18.54	0	0	6.71
14d	12.19	35.08	2.13	31.26	0	0	19.34
28d	2.0	20.95	0	45.70	0.11	0	31.24
56d	0	4.24	0	19.37	2.09	0	74.3
70d	0	0.76	0	21.21	6.8	0	71.23

III Alterations in the hexosamine content.

% individuals in NCTC 135 + 20% (+ additional glucosamine and galactosamine)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
0d	87.95	1.25	0	0	0	0	10.8
10d	59.32	15.68	2.12	6.35	0	0	16.52
18d	8.14	57.35	1.36	15.21	0.84	0	17.10
28d	0	29.02	2.19	48.09	1.07	0	19.63
35d	0	27.14	0	38.43	9.62	1.09	23.72
70d	0	2.17	0	62.31	21.79	1.23	37.12
120d	0	0	0	8.57	34.19	0	52.24

% individuals in NCTC 135 + 20% FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
0d	84.95	6.25	0	0	0	0	8.8
10d	21.86	42.47	3.99	28.8	0	0	2.51
18d	6.17	24.45	2.57	33.3	1.07	0	8.42
28d	0	14.62	0	57.21	3.14	5.01	10.19
35d	0	0.67	0	56.71	8.19	2.36	18.12
70d	0	0	0	54.88	15.95	0	28.4
77d	0	0	0	46.79	22.14	0	31.07
120d	0	0	0	3.7	36.13	0	60.17

% individuals in NCTC 135 + 20% FCS (+ additional glucosamine and galactosamine)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
7d	44.78	39.7	2.97	8.18	0	0	4.37
35d	11.12	21.4	0	45.76	7.62	0	14.1
70d	0	1.22	0	50.97	25.41	0	22.4
120d	0	0	0	6.75	42.73	0	50.52

% individuals in NCTC 135 + 20% FCS

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
7d	37.6	8.44	5.14	11.57	0	0	2.39
35d	14.21	29.03	0	35.76	4.93	0	16.07
70d	0	6.79	0	36.87	31.7	0	24.64
120d	0	0	0	1.97	44.2	0	58.83

IV Effect of enzyme and bile pretreatment

% individuals in culture with pepsin and bile pretreatment

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
1d	12.57	78.19	5.77	0	0	0	3.47
7d	3.13	62.14	9.13	14.69	0	0	10.91
14d	0	41.77	1.2	28.79	0	0	28.24
35d	0	20.91	0	32.17	0.17	2.59	44.16
70d	0	2.0	0	6.8	10.16	1.73	79.31

% individuals in culture without pretreatments

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
1d	45.59	49.3	3.14	0	0	0	1.97
7d	18.67	63.55	2.13	13.02	0	0	2.63
14d	0	43.21	0	32.53	0	0.71	8.61
35d	0	11.2	0	70.24	1.25	2.12	15.19
70d	0	0	0	37.44	29.16	0	33.4

V Variations in the gas phase

% individuals in NCTC 135 + 20% FCS (+ 100% O_2)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
4d	18.2	62.24	7.9	6.24	0	0	5.42
10d	3.63	30.28	8.95	13.33	0	0	4.37
14d	0	72.71	5.25	15.77	0	0	6.27
28d	0	57.82	0	28.24	3.6	0	10.34
56c	0	26.03	0	39.97	15.10	0	18.19
70d	0	0	0	32.76	27.14	0	40.10
120d	0	0	0	0.8	31.2	0	68.0

% individuals in NCTC 135 + 20% FCS (+ air)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
4d	20.1	48.0	7.2	22.4	0	0	2.3
10d	12.4	57.44	5.1	21.3	0	0	3.76
14d	15.3	40.33	6.28	35.0	0	0	3.12
28d	1.2	62.1	0	26.97	2.8	0	6.93
56d	0	5.21	0	48.34	18.24	0	28.21
70d	0	0	0	23.44	38.96	0	37.6
120d	0	0	0	0	40.17	0	59.83

% individuals in NCTC 135 + 20% FCS (+ 95% O_x 5% CO_2)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
4d	7.19	77.3	8.95	4.37	0	0	2.19
10d	0	72.3	4.77	19.17	0	0	3.76
14d	0	68.01	3.19	20.63	0	0	8.17
28d	0	30.24	0	60.08	0.95	0	8.73
56d	0	7.14	0.24	58.73	13.77	0	20.12
70d	0	0	0	0	39.2	0	35.19

% individuals in NCTC 135 + 20% FCS (+ 100% O_2)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
1d	65.2	29.7	5.1	0	0	0	0
4d	47.3	45.0	4.6	3.1	0	0	0
10d	43.6	48.2	5.1	4.1	0	0	0
14d	10.2	58.1	6.2	22.9	0	0	2.6
28d	0	64.2	0.7	30.1	0.2	0	4.8
35d	0	38.75	0	41.7	6.95	0	12.6
56d	0	21.87	0	40.08	16.15	0	21.9

% individuals in NCTC 135 + 20% FCS (+ air)

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
1d	48.1	47.2	4.7	0	0	0	0
4d	50.2	35.3	9.3	5.2	0	0	0
10d	49.7	34.9	8.5	6.7	0	0	0.2
14d	27.5	35.2	7.2	28.6	0	0	1.5
28d	9.71	55.14	1.1	28.2	0.65	0	5.2
35d	2.1	22.4	0	52.85	4.16	0.2	18.29
56d	0	18.7	0	48.1	14.2	1.0	29.0

VI Variations in the pH of the culture medium

% individuals in NCTC 135 + 20% FCS at pH 6.2

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
3d	70.2	6.1	4.76	19.47	0	0	3.34
5d	52.18	11.3	5.34	24.21	0	0	6.97
8d	50.01	18.17	0	20.33	0	0	11.49
14d	0	0	0	0	0	0	100

% individuals in NCTC 135 + 20% FCS at pH 7.4

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
3d	18.19	43.99	7.01	29.2	0	0	1.61
5d	20.20	31.22	6.35	41.3	0	0	0.93
8d	9.95	34.42	1.02	51.24	0	0	3.37
14d	11.25	20.63	0	57.84	0	0	10.28
30d	0	15.99	0	71.24	0.5	0	12.27
60d	0	2.97	0	55.29	13.55	0	28.19

% individuals in NCTC 135 + 20% FCS at pH 8.5

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
3d	19.43	44.98	5.12	29.71	0	0	0.76
5d	10.71	50.64	0	37.22	0	0	1.43
8d	8.19	33.10	0	50.98	0	0	7.73
14d	8.0	29.4	0	52.91	0	0	9.69
30d	0	19.41	0	48.78	0	2.47	29.34
60d	0	12.27	0	26.33	4.13	1.17	56.10

% individuals in NCTC 135 + 20% FCS at pH 6.2

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
5d	54.95	19.02	1.93	20.12	0	0	3.97
8d	52.14	18.58	5.69	18.3	0	0	5.29
14d	16.13	34.41	12.14	22.9	0	0	14.42
21d	0	0	0	0	0	0	0

% individuals in NCTC 135 + 20% FCS at pH 7.4

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
5d	14.04	30.12	2.97	51.38	0	0	1.49
8d	10.42	31.72	1.3	52.55	0	0	4.01
14d	3.19	38.53	0	50.07	0	0	8.21
21d	0	37.99	0	44.35	0.05	0	17.6
35d	0	21.9	0	41.8	4.1	1.4	35.4
60d	0	4.32	0	37.72	17.09	2.17	38.7

% individuals in NCTC 135 + 20% FCS at pH 8.5

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
5d	20.14	52.46	1.73	22.4	0	0	3.27
8d	18.33	55.41	1.95	20.17	0	0	4.14
14d	1.47	56.47	0	23.7	0	0	18.36
21d	0	47.88	0	31.81	0	0.97	19.34
35d	0	12.79	0	42.0	2.3	3.21	39.7
60d	0	0	0	28.59	8.24	5.97	57.2

% individuals in NCTC 135 + 20% FCS at pH 6.8

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
5d	41.39	18.97	7.91	29.76	0	0	2.97
15d	13.74	45.55	2.16	31.43	0	0	7.12
40d	0	5.36	0.35	42.5	7.14	0	44.64
77d	0	1.24	0	10.08	48.66	0	40.02
96d	0	0	0	0.75	50.12	0	49.13

% individuals in NCTC 135 + 20% FCS at pH 7.4

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
5d	22.4	59.08	5.29	10.11	0	0	3.12
15d	10.01	39.23	4.19	40.6	0	0	5.97
40d	0	3.0	0.02	39.74	8.51	1.42	47.31
77d	0	1.92	0.64	9.23	52.56	0	35.64
96d	0	0	0	1.39	39.95	0	58.66

% individuals in NCTC 135 + 20% FCS at pH 8.2

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
5d	8.17	61.31	5.34	14.21	0	0	0.97
15d	15.24	43.77	7.19	28.44	0	1.13	4.23
40d	0	5.78	1.41	42.19	4.22	2.65	43.75
77d	0	1.22	0	14.79	29.77	8.98	45.25
96d	0	0	0	4.38	34.32	1.03	60.27

VII Alterations in the numbers of protoscoleces per tube.

% individuals in cultures with 1,000 psc/tube

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
40d	0	20.27	0	3.38	0	60.81	15.54
80d	0	0	0	0	0	0	100
120d	0	0	0	0	0	0	100

% individuals in cultures with 1,500 psc/tube

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
40d	0	20.34	0	4.65	0	58.14	16.86
80d	0	0	0	0	0	0	100
120d	0	0	0	0	0	0	100

% individuals in cultures with 2,000 psc/tube

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
40d	0	37.36	0	20.66	2.2	2.0	37.8
80d	0	17.33	0	9.27	18.55	0.8	54.03
120d	0	0	0	8.16	31.0	0	60.8

% individuals in cultures with 3,000 psc/tube

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
40d	0	41.38	0	17.93	2.07	0.69	37.93
80d	0	22.65	0	9.37	14.06	0	53.9
120d	0	0	0	12.0	42.66	0	45.33

% individuals in cultures with 4,000 psc/tube

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
40d	0	9.36	0	67.25	6.72	0	38.01
80d	0	0	0	14.2	39.6	0	46.2
120d	0	0	0	1.8	50.45	0	47.74

% individuals in cultures with 5,000 psc/tube

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
40d	0	4.28	0	49.25	15.0	0	44.97
80d	0	0	0	8.68	48.35	0	42.96
120d	0	0	0	1.8	61.46	0	36.7

% individuals in cultures with 10,000 psc/tube

Time	Iv.	Ev.	P.B.	Ves.	L.L.	Ad.	Dd.
40d	0	0	0	51.95	8.85	0	39.2
80d	0	0	0	21.13	32.97	0	45.9
120d	0	0	0	6.03	42.24	0	51.73

pH of culture media during incubations

initial pH = 7.4

	0-4d	4-8d	8-12d	12-16d	16-20d	36-40d	78-80d	Mean
0	8.6	8.35	8.0	8.0	8.8	9.0	8.7	8.49
1,000	8.2	8.1	7.7	7.7	7.6	9.1	8.7	8.01
1,500	8.36	7.79	7.9	7.7	7.8	8.4	8.6	8.06
2,000	7.84	7.84	7.28	7.16	7.6	7.8	7.6	7.59
3,000	7.8	7.75	7.55	7.3	7.45	7.3	7.5	7.52
4,000	7.86	7.62	7.31	7.18	7.29	7.1	7.0	7.34
5,000	7.82	7.55	7.5	7.21	7.1	6.95	6.9	7.29
10,000	7.16	7.27	6.96	6.88	6.8	6.78	6.7	6.93

APPENDIX 4

DATA FROM STEREOLOGICAL ANALYSIS OF TEGUMENTARY TISSUE

Abbreviations used:

VV = Volume fraction of an organelle.

SV = Surface to volume ratio of an organelle.

M.VOL. = Mean volume of an organelle.

M.SUR. = Mean surface area of an organelle.

REF AREA = Reference area of cytoplasm measured.

I Analysis of T_2 vesicles in 0 day scolex tegument

	VV $\mu m^3/\mu m^3$	SV $\mu m^2/\mu m^3$	M.VOL. μm^3	M.SUR μm^2	REF AREA μm^2
	0.117	6.544	0.105×10^{-2}	0.589×10^{-1}	7.382
	0.674×10^{-1}	3.779	0.100×10^{-2}	0.563×10^{-1}	4.312
	0.932×10^{-1}	5.822	0.7311×10^{-3}	0.457×10^{-1}	2.369
	0.743×10^{-1}	4.602	0.752×10^{-3}	0.466×10^{-1}	2.825
	0.977×10^{-1}	5.082	0.123×10^{-2}	0.638×10^{-1}	8.818
	0.731×10^{-1}	4.001	0.123×10^{-2}	0.672×10^{-1}	3.829
	0.134	7.632	0.968×10^{-3}	0.550×10^{-1}	2.727
Mean	0.9387×10^{-1}	5.3517	0.995×10^{-3}	0.562×10^{-1}	4.608
Std. error	0.939×10^{-2}	0.5295	0.756×10^{-4}	0.305×10^{-2}	0.949

II Analysis of T_2 vesicles in 0 day soma tegument (where present)

	VV $\mu m^3/um^3$	SV $\mu m^2/um^3$	M.VOL. μm^3	M.SUR. μm^2	REF.AREA μm^3
	0.388×10^{-2}	0.192	0.153×10^{-2}	0.758×10^{-1}	13.02
	0.127×10^{-1}	0.632	0.141×10^{-2}	0.703×10^{-1}	6.460
	0.709×10^{-2}	0.267	0.289×10^{-2}	0.109	10.24
	0.115×10^{-1}	0.564	0.130×10^{-2}	0.638×10^{-1}	10.18
Mean	0.879×10^{-2}	0.414	0.178×10^{-2}	0.797×10^{-1}	9.97
Std. error	0.203×10^{-2}	0.108	0.373×10^{-3}	0.100×10^{-1}	1.34

III Analysis of Golgi cisternae of monensin treated protoscoleces (*in vitro*, 10μM monensin, 1hr)

	VV $\mu m^3/\mu m^3$	SV $\mu m^2/\mu m^3$	M.VOL. μm^3	M.SUR. μm^2	REF AREA. μm^2
	0.158	6.075	0.259×10^{-2}	0.997×10^{-1}	5.045
	0.926×10^{-1}	3.725	0.274×10^{-2}	0.110	3.567
	0.883×10^{-1}	3.001	0.363×10^{-2}	0.123	3.023
	0.117	4.216	0.342×10^{-2}	0.123	9.976
	0.129	2.801	0.143×10^{-1}	0.311	14.910
	0.136	2.926	0.115×10^{-1}	0.246	12.910
	0.163	3.588	0.128×10^{-1}	0.282	14.360
	0.986×10^{-1}	2.899	0.563×10^{-2}	0.165	7.823
Mean	0.123	3.654	0.707×10^{-2}	0.182	8.95
Std. error	0.102×10^{-1}	0.388	0.175×10^{-2}	0.298×10^{-1}	1.701

IV Analysis of Golgi cisternae of monensin control protoscoleces (*in vitro*, 1hr)

	VV $\mu m^3/\mu m^3$	SV $\mu m^2/\mu m^3$	M.VOL. μm^3	M.SUR. μm^2	REF AREA. μm^2
	66×10^{-1}	1.933	0.784×10^{-3}	0.570×10^{-1}	9.646
	0.331×10^{-1}	2.390	0.786×10^{-3}	0.567×10^{-1}	13.680
	0.664×10^{-1}	4.712	0.640×10^{-3}	0.454×10^{-1}	2.281
	0.521×10^{-1}	6.626	0.210×10^{-3}	0.267×10^{-1}	3.420
	0.587×10^{-1}	5.213	0.475×10^{-3}	0.415×10^{-1}	5.794
	0.156×10^{-1}	0.600	0.231×10^{-2}	0.885×10^{-1}	1.884
	0.245×10^{-1}	2.108	0.486×10^{-3}	0.418×10^{-1}	8.813
Mean	0.397×10^{-1}	3.369	0.813×10^{-3}	0.511×10^{-1}	6.503
Std. error	0.738×10^{-1}	0.818	0.260×10^{-3}	0.735×10^{-2}	1.661

V Analysis of the tegumentary mitochondria of monensin treated protoscoleces (*in vitro* 10μM monensin 1hr)

	VV $\mu m^3/\mu m^3$	SV $\mu m^2/um^3$	M.VOL. μm^3	M.SUR. μm^2	REF AREA μm^2
	0.1913	3.844	0.180×10^{-1}	0.362	5.045
	0.261	3.656	0.497×10^{-1}	0.698	3.567
	0.688×10^{-1}	1.621	0.132×10^{-1}	0.312	3.023
	0.192	3.162	0.319×10^{-1}	0.523	9.976
	0.172	2.264	0.683×10^{-1}	0.902	14.910
	0.158	1.757	0.843×10^{-1}	0.939	12.910
	0.396×10^{-1}	0.536	0.623×10^{-1}	0.852	14.360
	0.103	1.923	0.237×10^{-1}	0.441	7.823
Mean	0.148	2.345	0.440×10^{-1}	0.629	8.950
Std. error	0.258×10^{-1}	0.399	0.924×10^{-2}	0.890×10^{-1}	1.701

VI Analysis of tegumentary mitochondria of monensin control protoscoleces (*in vitro*, 1hr)

	VV $\mu m^3/\mu m^3$	SV $\mu m^2/um^3$	M.VOL. μm^3	M.SUR. μm^2	REF AREA. μm^2
	0.344×10^{-1}	0.861	0.114×10^{-1}	0.285	9.646
	0.100	2.143	0.202×10^{-1}	0.432	13.680
	0.823×10^{-1}	0.314	0.296×10^{-2}	0.113	2.281
	0.119	2.726	0.134×10^{-1}	0.306	3.420
	0.512×10^{-1}	1.042	0.175×10^{-1}	0.356	5.794
	0.116	3.186	0.683×10^{-2}	0.187	1.884
	0.556×10^{-1}	1.194	0.228×10^{-1}	0.490	8.813
Mean	0.693×10^{-1}	1.638	0.136×10^{-1}	0.310	6.503
Std. error	0.162×10^{-1}	0.400	0.270×10^{-2}	0.497×10^{-1}	1.661

VII Analysis of tegumentary mitochondria of monensin treated murine cysts (*in vitro*, 10μM monensin, 1hr)

	VV $\mu m^3/\mu m^3$	SV $\mu m^2/\mu m^3$	M.VOL. μm^3	M.SUR. μm^2	REF AREA. μm^2
	0.310×10^{-1}	0.478	0.480×10^{-1}	0.739	83.41
	0.997×10^{-1}	1.347	0.685×10^{-1}	0.925	24.74
	0.164	2.148	0.869×10^{-1}	1.138	15.03
	0.825×10^{-1}	1.062	0.717×10^{-1}	0.924	14.94
	0.728×10^{-1}	1.089	0.525×10^{-1}	0.785	8.981
Mean	0.900×10^{-1}	1.225	0.655×10^{-1}	0.902	29.42
Std. error	0.217×10^{-1}	0.271	0.700×10^{-2}	0.696×10^{-1}	13.73

VIII Analysis of tegumentary mitochondria of monensin control murine cysts (*in vitro*, 1hr)

	VV $\mu m^3/\mu m^3$	SV $\mu m^2/\mu m^3$	M.VOL. μm^3	M.SUR. μm^2	REF AREA. μm^2
	0.764×10^{-1}	1.193	0.626×10^{-1}	0.978	6.403
	0.397×10^{-1}	0.746	0.252×10^{-1}	0.474	9.939
	0.491×10^{-1}	1.117	0.128×10^{-1}	0.292	3.959
	0.947×10^{-1}	2.045	0.167×10^{-1}	0.361	10.170
	0.659×10^{-1}	1.240	0.247×10^{-1}	0.465	34.09
Mean	0.652×10^{-1}	1.268	0.284×10^{-1}	0.514	12.91
Std. error	0.975×10^{-2}	0.213	0.887×10^{-2}	0.121	5.42

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