STUDIES ON THE *IN VITRO* CULTURE OF TAENIIDAE AND ON THE ANTIGENIC PROPERTIES OF THEIR METABOLIC PRODUCTS

by

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PREFACE

I composed this thesis myself and it is the record of my own work that was done in the Department of Tropical Animal Health, Royal (Dick) School of Veterinary Studies, University of Edinburgh, under supervision of Dr. M.M.H. Sewell. None of it has been submitted to any other university.

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"Forsan et haec olim meminisse iuvabit" (Virgil)

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ABSTRACT

The dissertation is divided into five parts, namely studies on the parasite-host models *in vivo*, *in vitro* culture of *Taenia taeniaeformis* oncospheres, *in vitro* growth and maintenance of mature larvae of *Taenia saginata* and *T. taeniaeformis*, the antigens released by the latter *in vitro* and finally the immunogenic properties of these antigens.

In Part I, four strains of *T. taeniaeformis*, from Belgium (B), Iraq (I), Malaysia (M) and Scotland (S), were compared as regards their infectivity for CF1 mice and Sprague-Dawley rats. Two strains, S and M, appeared to be almost exclusively adapted to mice and rats respectively. Mice were generally more susceptible than rats for strains B and I. Thin layer starch gel electrophoresis of extracts of *T. taeniaeformis* of these four strains showed different patterns for the isoenzymes of hexokinase.

Part II is concerned with *in vitro* culture of *T. taeniaeformis* starting with oncospheres. Techniques for hatching and activating oncospheres based on peptic digestion followed by incubation in an artificial intestinal solution, gave unsatisfactory results. Better results were obtained by first disintegrating the embryophores with sodium hypochlorite, followed by stationary incubation in a culture medium containing trypsin, bile and serum. Attempts to cultivate these oncospheres *in vitro* were not as successful as those described by Heath (1973).

Part III describes attempts to monitor the maintenance of *T. taeniaeformis* strobilocerci *in vitro*. The weight of the larvae,

glucose uptake and lactic acid production were measured at regular intervals. None of these parameters proved to be suitable for comparing different media; the response was either too slow or too irregular to be reliable. Different types of culture for T. saginata metacestodes were tried. The best growth was obtained by using a diphasic medium with disrupted coagulated serum as the solid substrate. The most developed forms showed segmentation and early development of the sexual organs.

Studies concerned with the nature of the metabolic antigens are described in Part IV. These were primarily concerned with establishing whether these antigens were materially different from somatic antigen or were merely the result of progressive disintegration of the worms in vitro. Immunoelectrophoresis, ELISA for detecting antibodies and ITAS-ELISA for detecting antigen each allowed the metabolic and somatic antigens to be distinguished. The former appeared to be similar to cyst fluid, in so far as precipitating antigens were concerned, whereas in the ELISA and ITAS-ELISA studies metabolic antigen seemed to give more specific results. Cyst fluid or somatic antigen gave less specificity with sera from mice infected with T. taeniaeformis or Taenia crassiceps. Furthermore, as the level of somatic antigen increased in culture fluids, so did the specificity decrease.

This release of antigens into culture media was studied in relation to time for different culture media and also by comparing this for dead and living larvae maintained under the same in vitro conditions. The results suggested that the release rate for antigen as measured by ITAS-ELISA could provide a useful means of monitoring

such cultures, since the inverse relationship between this and the integrity of the cultured metacestodes was more consistent than any of the parameters studied in Part III or the release rate for metabolic antigen.

Finally, a study of the efficacy of these metabolites as immunogens was undertaken in mice and rats and is described in Part V. The results of these experiments were inconsistent; some batches of culture antigen gave significant protection but no relationship between this and their protein content or the proportion of metabolic and somatic antigen could be demonstrated.

PART I *In vivo* establishment and maintenance of *Taenia taeniaeformis* and *Taenia crassiceps*

Chapter 1 INTRODUCTION

The influence of taeniid parasites on both human and animal pathology and the economic importance of some of the taeniids have been fairly well described but are not always clearly understood.

Numerous studies have been undertaken aimed directly or indirectly at developing means of controlling these parasites. Frequently, model systems have been used in these studies for obvious economical and safety reasons. *Taenia taeniaeformis*, having a cosmopolitan distribution is one of the most frequently chosen models, but *Taenia crassiceps* larvae have recently received more attention. As will be further discussed in Chapter 2, several workers have realised that the infectivity of *T. taeniaeformis* for the commonly accepted intermediate hosts, mice and rats, is rather variable. Indeed, the susceptibility of these intermediate hosts has been the subject of numerous investigations.

Generally, it has been assumed that rats and mice can both be infected with this parasite, but with sometimes quite marked difference in susceptibility between different strains of these hosts. However, the problems encountered in establishing a laboratory culture of *T. taeniaeformis in vivo* proved to be of such an extent that it was partly because of these that attention was turned to other aspects than the original purpose of this work, namely the study of the immunogenicity of antigens obtained during *in vitro* cultures. It is therefore necessary to discuss these problems in some detail, in particular for *T. taeniaeformis*, as this was the main species used in

this work. Similar problems may also occur with T. crassiceps but this parasite was merely used as a source of material for comparative serological studies described in Part IV.

Without discounting the work undertaken by many other taxonomists and mainly for practical reasons, the nomenclature used in this work is based on that of Verster (1969). The references for the scientific names of parasites are given in Wardle and McLeod (1968). Taenia saginata, Goeze,1782, was preferred to Taeniarhynchus saginatus, as the former is the most commonly used name, while Taenia taeniaeformis (Batsch, 1786) was preferred to Hydatigera taeniaeformis, based on Esch and Self (1965).

The larval stages are referred to as larvae, metacestodes or occasionally by the more specific name for T. taeniaeformis, strobilocerci. Admittedly, this usage was rather based on common practice and may be technically incorrect as discussed by Freeman (1970). Similarly, where observations have been made concerning numbers of living and dead T. taeniaeformis larvae encapsulated in liver cysts, for convenience these are referred to as living and dead cysts respectively, as has been done by various authors including: Miller and Gardiner (1932), Greenfield (1942) and Dow and Jarrett (1960). The name Taenia crassiceps (Zeder,1800) Rudolphi,1810, appears to be generally accepted.

Chapter 2 REVIEW OF LITERATURE

2.1 Taenia crassiceps

Baer and Studer (1949) reported the possibility of maintaining T. crassiceps by intraperitoneal passage in mice and hamsters. This important feature of these larvae, namely that asexual reproduction by exogenous budding occurs, was further studied by Freeman (1962). The latter concluded that intraperitoneal inoculation resulted in a greater level of reproduction than if the larvae were implanted s.c. or intrapleurally. However it appeared that his original strain, the strain ORF (Ontario Research Foundation), obtained from a fox (Vulpes fulva) could no longer resume its normal life cycle after several i.p. passages in mice. A second strain, the Toi strain was isolated from a marmot (Marmota monax). Taylor,(1963[a, b]) used the ORF strain for in vitro studies, while Dorais and Esch (1969) compared the growth rate of this abnormal ORF strain with a strain, isolated by themselves from a vole (Microtus pennsylvanicus) and called the KBS strain (Kellogg Biological Station). The former strain showed a faster increase in population size but the frequently observed abnormal morphological features were thought to be the result of genetic mutation. Esch and Smyth (1976) compared the in vitro development towards the adult stage of larvae of the ORF, Toi and KBS strains. Whereas complete development occurred with the Toi and KBS strain, no growth was observed with the ORF strain. Schiller (1973) described morphologic abnormalities in scoleces from KBS strain larvae and observed that such abnormal larvae failed to develop to the strobilar stage when fed or implanted in dogs.

Bilqees (1969) and Bilqees and Freeman (1969) studied the early development of the Toi strain in mice. Chernin (1975[a]) reported that rats were resistant to infection with the native Toi strain but later derived a rat adapted Toi strain by previously injecting the rats i.p. with T. crassiceps homogenate. This strain, named the ERS strain (Ewell Rat Strain), was reported (Chernin 1975[b]) to maintain its ability to reproduce in mice. Chernin (1975[b]) further reported that the sex of the mice did not affect their susceptibility to infection, but both the Toi and ERS strains developed more rapidly in female mice.

2.2 Taenia taeniaeformis

This parasite has been considered to provide the best model for studies on cysticercosis and hydatidosis (Miller 1931[a, b] and Campbell 1938[a]). Miller and Gardiner (1932) studied passive immunity in rats against larval T. taeniaeformis, and pointed out that the lack of adequate criteria for estimating the viability and infectivity of the eggs was a serious handicap in such experiments. Hinz (1962, 1965) aimed at establishing standard procedures for drug screening using T. taeniaeformis and Leid (1977) found this parasite to be very suitable for studying host-parasite relationships.

Several authors have studied the development of T. taeniaeformis to the mature stage in the cat. Miller (1932) found cats not to be resistant to superinfections and also that their susceptibility to this parasite was not influenced by existing infections with Dipylidium spp. or ascarids. Furthermore, the maturity of T. taeniaeformis was determined by the time since infection and not by the number of segments in the strobilocerci when ingested by the cat, since a number

of these were digested off.

Joyeux and Baer (1937) described the early development of *T. taeniaeformis* in cats, as did Hutchison (1959), making a distinction between "true larval strobila and pseudostrobila"; this last feature will be further discussed in Part III. Hutchison (1959) found that the parasites were mainly situated in the middle third of the small intestine. Eggs were formed after 16 days but these did not contain fully developed hexacanth embryos until day 33 after infection. Day 36 was the earliest day by which proglottides were seen to be shed. Singh and Rao (1966) did not observe egg production in the uterus before day 32. This point was discussed by Smyth (1976) who suggested the possibility of parasite strain differences.

Singh and Rao (1966) agreed with Hutchison that the sex of the cats had no significant effect on their susceptibility to *T. taeniaeformis*. Gonzalez and Mishra (1975) found adult cats to be less susceptible than kittens, but differed from previous authors in that they found *T. taeniaeformis* only in the last third of the small intestine, and did not observe eggs in the uteri before the 40th day of infection. Although there seems to be agreement that the minimum age of strobilocerci has to be around 60 days for these to be infective for cats, Singh and Rao (1966) suggested that their infectivity may be higher when they are older. In Britain this parasite seems to be common. Dubey (1966) reported a prevalence of 4.5%, while Cowper (1978) in a survey in Swansea found 15.2% of the cats harboured *T. taeniaeformis*.

Whether or not there is doubt as to the range of definite hosts (Verster 1969), there certainly is regarding the possible intermediate

hosts and the various factors influencing this. Apart from mice and rats, the larval stages of this parasite have also been reported in various other rodents and lagomorphs and even in man. Bacigalupo (1922, cited by Singh and Rao 1977) recorded this parasite in a child, naming it Taenia infantis, while Štěrba, Blažek and Baruš (1976) reported it in the liver of a man. There are several reports in the literature concerning occasional findings of these larvae in wild animals, including: Mahon (1954) who found it in the back muscles of Lepus americanus and also reviewed the previous records in rabbits; Olexik (1976) described it in the grey squirrel (Sciurus carolensis); Roman (1978) during a survey in the region of Lyon (France) found it in voles and wild rats but was surprised not to find it in the house mouse (Mus musculus). Dye, Kemple and Wantland (1949) in Illinois found over eighty per cent of the wild rats to be infected with T. taeniaeformis. Flynn (1973) listed various reports of the prevalence of this parasite in wild rats, ranging from 16 to 45% in the USA and Canada and 58 to 75% in Japan.

On the other hand there are various reports of attempts to experimentally infect wild animals or laboratory animals other than mice or rats. Thus Schiller (1949) in Wisconsin reported unsuccessful attempts to infect rabbits and hamsters.

Wantland (1953) (Illinois) was unable to infect Syrian hamsters with normal or predigested eggs, despite the incidental finding of a strobilocercus in the liver of a hamster subjected to an experimental infection with Trichinella spiralis. Olivier (1962[a]) in Maryland, reported a complete inability to establish infections in Syrian hamsters whereas some guinea-pigs showed small liver lesions, but no living

larvae after incidental infections. Tripathi and Ray (1976) in India, reported incidental infections in two adult golden hamsters, which died presumably from the infection, strobilocerci being found in the liver. Singh and Rao (1971) were unable to infect adult or young guinea-pigs, rabbits or adult hamsters. Orihara (1962), in Japan found early development followed by degeneration of cysts in the liver of experimentally infected voles (Microtus spp.) whereas Mongolian gerbils (Meriones spp.) appeared to be totally refractive.

Incidental infections of laboratory rats and mice is discussed by Jones (1967), Bell (1968) and Flynn (1973) in relation to the hygienic conditions under which these animals were kept.

Various authors have studied the development of T. taeniaeformis in mice and rats. Crusz (1948) reviewed the literature on this subject and described larval development in rats, Hutchison (1958) studied this feature in mice concluding that a minimum period of 60 days is necessary before the strobilocerci reach infectivity for cats, although they continue to grow thereafter and also that growth appeared to be retarded in heavy infections. Singh and Rao (1967) studied the morphological development of this parasite in rats, comparing their data with those of the previous workers.

Banerjee and Singh (1969[a, b, c]) and Banerjee (1972) described the early stages of infection in the rat and the related histopathology in this host.

The variation in susceptibility, especially of rats and mice, towards T. taeniaeformis infection has been intensively studied and various interfering factors have been described. The variability caused by the nature of the eggs, as commented on by Miller and

Gardiner (1932), has been mentioned earlier. In relation to this there are various reports concerning the proglottides from which the eggs to be used are expelled. Hutchison (1958) and Banerjee and Singh (1969[a, b, c]) used the last six gravid proglottides from T. taeniaeformis, recovered from cats intestines. Jones, Segarra and Wyant (1960) made an extensive study of this aspect and concluded that only the last six proglottides contained morphologically mature eggs. Hinz (1965) used eggs only from those proglottides with a length-width index of 4 to 1. Olivier (1962[a]) found eggs from the last two segments to be just as infective as those from free proglottides but those from more anterior segments gave lower infectivity rates.

The dose of eggs administered per animal has varied considerably. Some authors like Heath and Pavloff (1975) have incorporated an estimation of the maturity of the eggs administered to the experimental animals but most authors have presumably given only the total number of eggs administered, since they describe no means for distinguishing between infective and non-infective eggs. Nevertheless criteria for in vitro differentiation of unsuitable eggs and those containing fully developed hexacanth embryos have been described by various workers, as will be discussed in Part II.

The existence of an optimal number of eggs for the development of mature larvae in experimental hosts was suggested by Hinz (1962, 1965), based on his findings that higher numbers of eggs do not necessarily give higher numbers of mature larvae. He recommended giving 200 to 500 eggs per mouse but emphasised that the infectivity should first be tested in vitro, since the number of tapeworms per

cat seemed to have an influence on the infectivity of the eggs, the latter being more infective, when originating from a cat harbouring several tapeworms.

The relation of the strain, age and sex of the experimental intermediate hosts to their susceptibility to T. taeniaeformis infection has been examined by various authors. Some have grouped these hosts according to their susceptibility. Thus Olivier (1962[a]) distinguished three groups: animals which are completely refractory to the infection like hamsters, others like guinea-pigs and some mouse strains, where the oncospheres reach the liver but do not survive long enough to develop into infective larvae and a third group, rats and some mouse strains where development proceeds to the infective stage. Orihara (1962) made a similar classification in which Mongolian gerbils, being totally resistant, were in the first group. Some mouse strains, including CF1, in which only the early phase development occurs were in the second group, while the third group included those animals like rats, some mice strains including strain A in which full development occurs. Both authors reported wide variation in the susceptibility between different strains and age groups of these hosts.

Although the importance of the host strain is now generally acknowledged, it is not always possible to compare the published results, especially in earlier work, when the strains of laboratory rats and mice were less well established and the hosts were often referred to as "albino" mice or rats.

Miller (1931[a]) found Wistar rats to be a suitable strain to work with, whereas for mice strain A was reported to be highly susceptible by Hutchison (1958) and Dow and Jarrett (1960). The latter also found

White Swiss and Porton mice to have a similar susceptibility to each other although it was lower than for strain A. However Olivier (1962[a]) found the White Swiss mouse to be completely refractory and Turner and McKeever (1976) agreed. Singh and Rao (1977) in India found their local albino mouse strain to be more susceptible than two foreign strains. Olivier (1962[b]) observed that resistant mice could be rendered susceptible by administering cortisone twelve days before infection. Zelentsov (1972) tested 13 inbred mouse strains for their susceptibility and selected seven suitable ones. Mitchell, Goding and Rickard (1977) also studied various inbred mouse strains and found that the strains most susceptible to T. taeniaeformis infection were those deficient in complement components C_5 and C_4.

The influence of the age of the host on its susceptibility to Taenia saginata has been described by Froyd (1964). Earlier reports on this phenomenon with T. taeniaeformis in rats were given by Curtis, Dunning and Bullock (1933) and Greenfield (1942); the latter found rats, less than 25 days or over 60 days old to be very resistant. Similarly, Hutchison (1958) found strain A mice to be susceptible only between the ages of 45 and 75 days. Dow and Jarrett (1960) also observed age resistance in mice but not within the same limits as the previous author. Strain A and White Swiss mice were highly susceptible at 14 and 21 days of age and became more resistant thereafter, whereas Porton mice did not show age resistance before 42 days of age. Hinz (1965) recommended the use of mice at 2 to 3 weeks old, while Singh and Rao (1971) found four week old rats to be most susceptible, since only a few larvae developed in older rats. Kwa and Liew (1977) found age resistance in rats to be a limiting factor in

studying the long term effectiveness of immunogens. Turner and McKeever (1976) reported that the proportion of the infective dose of oncospheres penetrating the intestinal wall was higher in younger mice than in older, and that there were also differences in the defence reactions in the livers in different age groups. It was suggested that the impairment of penetration in the older mice may have been the result of disruption of the hatching and activation process.

Although some authors have reported male rats to be more susceptible than females, there has been some controversy on this aspect as well. Campbell and Melcher (1940) reported male rats to be more susceptible than females, with castration enhancing susceptibility in the latter, decreasing it in the former. However, the data given by Greenfield (1942) did not seem to confirm this sex related susceptibility. Fortuyn and Feng (1940/41) found female mice of some strains less resistant to infection than males, whereas Mitchell, Goding and Rickard (1977) stated that male mice were generally more susceptible than females. However this may have been merely strain related because Dow and Jarrett (1960) found only slightly higher susceptibility in male than in female mice in two strains while Hinz (1965) could not find any significant differences between the numbers of infective larvae in livers from male and female mice. In both of the latter reports it was concluded that sex related differences in susceptibility were of only minor importance.

Some workers such as Musoke and Williams (1975[a, b]), experimenting with T. taeniaeformis do not seem to have encountered difficulties in infecting both rats and mice, or at least they made no

comments on this aspect, whereas others including Heath and Elsdon-Dew (1972) reported that they were unable to infect mice with their strain of T. taeniaeformis. Hinz (1962) in attempting to standardise procedures for drug screening, preferred to work with mice rather than with rats, since he observed that more mature larvae formed in the former and also that the percentage of infected hosts was higher for mice (72%) than for rats (48%).

Ferretti, Marconi and Monaco (1970) and Ferretti, Gabriele, Marconi, Monaco and Piccinato (1972) reported that a strain of T. taeniaeformis obtained from cats in Sardinia was infective to some inbred mouse strains, whereas in rats, the larvae did not develop well and were destroyed in the liver after some weeks. In some of the mouse strains, they noticed the development of sterile cysts, i.e. small cysts without a scolex. In particular in strain CF1 the number of these cysts was always very high, though administration of cortisone gave an increase in the number of entire strobilocerci.

2.3 Identification of taeniidae based on isoenzyme differentiation

Le Riche (1977) and Le Riche and Sewell (1977, 1978[a]) described a method for identifying taeniid cestodes from differences in their isoenzymes. This method is based on principles described by Markert and Møller (1959), whose definition of isoenzymes is used in this dissertation. The former workers compared the isoenzymes by electrophoresis in thin-layer starch gels (Wraxall and Culliford 1968 and Kilgour and Godfrey 1973). The zymograms of adenylate kinase, glucose phosphate isomerase (G.P.I.), glutamate dehydrogenase, hexokinase and malate dehydrogenase, from various taeniid species were found to show

different patterns. The position of the most cathodal bands for G.P.I. formed the most useful criterion as a clearer banding was obtained than with the other enzymes. Hexokinase was found to be useful for confirming the results obtained with glucose phosphate isomerase. Le Riche and Sewell (1978[b]) also used the same technique to identify strains of *Echinococcus granulosus*. Similar principles were applied using isoelectric focussing by McManus and Smyth (1979) to differentiate *E. granulosus* strains and all enzymes tested except adenylate kinase, showed different isoenzyme patterns for horse and sheep strains; hexokinase was not tested in this work.

Chapter 3 MATERIALS AND METHODS

3.1 Experimental animals

All mammalian species were SPF animals obtained from the Centre for Laboratory Animals, University of Edinburgh. The mice strains used were the inbred strain A and the random bred strain CF1. Two random bred rat strains were used: Sprague-Dawley and Porton-Wistar. For the work described later, New Zealand White rabbits, and albino Duncan-Hartley guinea-pigs were used. Cats used for maintaining the cycle of _T. taeniaeformis_ were obtained from the breeding colony at the above centre. Following infection they were kept at the "Wellcome Animal Research Unit", University of Edinburgh, since no suitable facilities were available in the animal house where the other species were kept. Although the experimental rabbits, guinea-pigs and cats were not kept in strictly SPF conditions, they were regularly screened for adventitious infections.

3.2 Taenia crassiceps

Toi strain metacestodes kindly supplied by Dr. J. Chernin, were administered intraperitoneally to six week old CF1 mice, using a 2 ml syringe with a sterile (16 x 1" "Monoject 200" Sherwood) disposable needle. After three months these mice were killed by cervical dislocation, the abdominal skin was removed and an incision made in the abdominal wall. The larvae therein were collected into 145 $mmol.l^{-1}$ sodium chloride (OXOID) with 100 i.u. ml^{-1} penicillin (Sodium benzylpenicillin - Glaxo), 100 $\mu g.ml^{-1}$ streptomycin (Streptomycin sulphate BP - Glaxo) and 2 $\mu g.ml^{-1}$ amphotericine B ("Fungizone" Squibb & Sons) (referred to as normal or physiological saline plus antibiotics). The abdominal cavity was rinsed out with this saline. The larvae were

then placed in a 100 mm diameter, 63 μm pore size sieve (Endecotts) and flushed with normal saline with antibiotics. After being washed the structures described by Chernin (1975[a]) as "white bodies" were removed. To maintain the culture, mice were infected with fifteen larvae on the same day, as described above.

3.3 Taenia taeniaeformis

The procedure for maintaining this parasite was generally that described by Jones, Segarra and Wyant (1960) and Leid and Williams (1974[a]). The proglottides expelled by cats, which had been infected for at least two months, were collected, washed and stored at 4°C in normal saline plus antibiotics. Not more than two weeks before being used, these proglottides were placed in a petri dish with normal saline plus antibiotics and eggs obtained by dividing the segments longitudinally with a scalpel blade. The contents of the petri dish were then sieved in 100 mm diameter test sieves (Endecotts). The eggs passed through the top sieve with a pore diameter of 150 μm and were retained by a second sieve with a micropore plate of 20 μm pore size. The remains of the segments on the top sieve were pressed by hand, wearing surgical gloves and rinsed several times with saline plus antibiotics. The lower sieve was then inverted over a 250 ml glass beaker and both sides of the sieve rinsed with normal saline plus antibiotics under moderate pressure from a syringe. This beaker was left at 4°C overnight to allow the eggs to sediment. Next day the supernatant was sucked off and the concentration of eggs estimated by carrying out several counts of the number of viable eggs present in 0.1 ml samples. These were taken while the beaker was on the magnetic stirrer, placed on a slide and examined microscopically.

The viability was estimated by morphological criteria as described by Heath D.D. (personal communication, 1977) i.e. only those eggs were counted which had thick shells and contained oncospheres with the hooks in line rather than splayed out. These counts were always made immediately before using the eggs. Meanwhile the eggs were stored in normal saline plus antibiotics at 4°C.

Groups of 3 to 4 week old CF1 mice or Sprague-Dawley rats were regularly infected in order to have a constant supply of larvae for *in vitro* studies and to maintain the life cycle. To infect these animals they were held supine by tail and neck skin and, using a cannula on a 1 ml syringe, 100 viable eggs were administered to mice or 200 to rats in a volume of normal saline that never exceeded 0.2 or 0.4 ml respectively. The cannula was made by removing the bevel from a 23 G 1 needle for mice and a 20 G 1½ needle for rats ("Yale microlance" Becton-Dickinson), the tip was smoothed and for further protection covered by a 5 mm length of Silicone rubber tubing.

To infect cats, mice or rats, which had been infected for at least three months, were killed by cervical dislocation. Their livers were removed and the viability of the larvae in them checked by removing some of them from the cysts into warm saline and examining them under a stereo microscope. Twenty-five intact cysts, surrounded by liver tissue were then given to each cat by mouth. The cats were previously checked for intestinal parasites and starved for 18 h before infection. Usually they swallowed these cysts without difficulty. The cats were around sixteen weeks old at this stage and both sexes were used.

After the first two months of infection, proglottides were daily

collected. After being infected for about 8 to 12 months the cats were dewormed and replaced by new ones. Cats were never reinfected. On some occasions eggs were obtained after autopsy, when only the last six segments and occasionally those found detached lower in the intestine were used and then only if they looked mature, i.e. uniform in size.

3.4 Origins of the strains of *T. taeniaeformis*

T. taeniaeformis eggs were obtained from four different geographic regions: the Belgian strain (B) was the one routinely used in most of the work and was obtained from "Janssen Pharmaceutica" of Beerse, Belgium. This strain is maintained in these laboratories but is regularly renewed by autopsying local stray cats. The Scottish (S) strain was obtained by autopsies from stray cats. The cadavers were obtained from the Portobello Dog and Cat Home(Edinburgh) and autopsied about one hour after the cats were killed. An attempt was also made to obtain this strain by trapping local brown rats (*Rattus norvegicus*) and house mice (*Mus musculus*). Eggs were also obtained from Iraq (strain I), being obtained by autopsy of a local stray cat in Baghdad. The Malaysian strain (M) was obtained from a laboratory in Kuala Lumpur.

Both mice and rats were infected with all four of these strains and all except the S strain were maintained in cats. Eggs of all strains were used for comparing the susceptibility of male CF1 mice and Sprague-Dawley rats. However, shortage of accommodation for the cats prevented the maintenance of more than two strains at one time. Since the *in vitro* work was based on the B strain, and the M strain was needed for studies in rats, as discussed in Part V, the I strain

was only maintained temporarily, before the M strain was established in cats.

3.5 Enzyme electrophoresis

The thin layer starch gel method of Wraxall and Culliford (1968) and modified by Kilgour and Godfrey (1973) was used as described by Le Riche (1977). Polymorphic forms of three enzymes: glucose phosphate isomerase (EC 5.3.1.9.), hexokinase (EC 2.7.1.1.) and adenylate kinase (EC 2.7.4.3.), were compared, using the same methods and materials as described by Le Riche (1977) and Le Riche and Sewell (1977, 1978[a, b]) except that:

For the zymogram of hexokinase the electrophoresis buffer used was 0.4 $mol.l^{-1}$ Tris HCl pH 8, which avoided overheating the gel as described by Le Riche and Sewell (1978[a]).

In the developing overlay phenazine methosulphate (PMS) was replaced by 50 μ l Meldola Blue (MLB) (8-dimethylamino-2, 3-benzophenoxazin - BCL) at a concentration of 6 $mg.ml^{-1}$ in water (Melrose T.R., personal communication, 1979, and Melrose, Brown and Sharma, 1980).

MLB as an electron transmitter is reputed to be more stable and sensitive than PMS (Turner and Hopkinson 1979 and Burd and Usategui-Gomez 1973).

The adenylate kinase method used was described by the Metropolitan Police Forensic Science Laboratory (1978), the developing overlay being modified by Melrose, T.R.(personal communication 1978) and consisted of 3 ml of 0.5 $mol.l^{-1}$ Tris-HCl buffer (BDH) pH 8 + 27 mg glucose (AnalaR BDH) + 3 ml of 0.1 $mol.l^{-1}$ $MgCl_2$ (AnalaR BDH)

+ 6 mg ADP (sodium salt of adenosine 5' - diphosphate Grade I, Sigma) + 0.9 ml (5 $mg.ml^{-1}$) NADP (monosodium salt of nicotinamide adenine dinucleotide phosphate - Sigma) + 1 ml at 2 $mg.ml^{-1}$ MTT [3- (4,5 - dimethyl thiazolyl-2) -2,5- diphenyl tetrazolium bromide - Sigma] + 50 U hexokinase (Type C - 130 - Sigma) + 24 U glucose-6-phosphate dehydrogenase Type XI Sigma) + 50 μl of 6 $mg.ml^{-1}$ Meldola blue. Several samples were taken from T. taeniaeformis adults and larvae of each of the different strains. These fresh samples were stored whole at $-20^{o}C$, in normal saline in small vials. Storage and preparation of the samples for enzyme electrophoresis was as described by Le Riche and Sewell (1978[a]).

Chapter 4 Infection of mammalian hosts with different strains of *T. taeniaeformis*

4.1 INTRODUCTION

This work was originally undertaken in an attempt to obtain a *Taenia taeniaeformis* strain to which rats were susceptible, so as to facilitate the immunological studies. For logistic reasons associated with keeping the cats, it was not possible to conduct all these trials at the same time using strictly comparable material. The results given are therefore compiled from several independent experiments with male mice and rats.

4.2 Experimental design

4.2.1 Autopsy of the cats: A *post mortem* examination on 81 cats (33 males and 48 females) was done over a period of four months. The intestines were opened over their entire length and any worms found were removed for morphological examination. When *T. taeniaeformis* was found its identity was further confirmed by studying the isoenzymes of the glucose phosphate isomerase.

4.2.2 Experiments with CF1 mice and Sprague-Dawley rats: Six experiments were undertaken. Table I.1 gives the order in which these were done. However, the results are arranged so as to facilitate the comparison between the age groups and the strains with which the mice or rats were infected. This appears to be justified since there were no significant differences between the corresponding groups where the B strain was used in different experiments.

Post mortem examination of these animals was done two months after infection. The livers were carefully dissected and the numbers

Table I.1 Order of experiments for comparing the infectivity of four strains of <u>T. taeniaeformis</u> (B: Belgian, S: Scottish, I: Iraqi, M: Malaysian). The numbers in parentheses for strain B refer to the numbers of the animals as given in Appendix Table I.1.

Age in weeks	MICE (CF1)					RATS (Sprague-Dawley)			
	3 to 4	4 to 5	5 to 6	6 to 7	> 12	3 to 4	4 to 5	5 to 6	6 to 7
Experiment No.									
1	S B (1-7)	S							
2		B (1-10)	B	B	B				
3						S	S B (1-6)		
4	I	I B (11-15)				I B	I	I B	B
5	M B (8-15)	M				M			
6							M B (7-15)	M	M

of living and dead cysts were counted. The data from animals containing living cysts were analysed by Wilcoxon's two sample test (Sokal and Rohlf 1969). The numbers of mice with living cysts were compared using the chi-square test.

For mice infected with B strain eggs the numbers of living cysts in each age group were examined by Kendall's Multiple Rank Test (Kendall 1975).

4.2.3 Additional observations

Ten 4 to 5 week old male Porton-Wistar rats were infected with the B strain at the same time as the animals in Experiment 3 and rats of the same strain but in groups of 3 to 4, 7 to 8 and over 12 weeks of age were used together with those in Experiment 5.

At the same time as Experiment 4, 12 week old strain A male mice were infected with strains B and I of _T. taeniaeformis_, ten in each group.

In an attempt to select a strain to which rats were susceptible, some of the small numbers of the B strain larvae found in rats were passed on to cats. Three such passages were made, but the infectivity for rats, after the third passage was still unsatisfactory. Eggs of this "adapted" strain were used in one experiment (Part V, Experiment 5, Table V.7). The results show the low infectivity although this strain was not used in 3 week old Sprague-Dawley rats.

4.3 RESULTS

4.3.1 Nineteen cats were experimentally infected with _T. taeniaeformis_. Eleven with strain B, two with strain I and six with strain M. Not

many observations were made on the infected cats; all but two became infected (one given strain B and one strain M). When the number of worms were recorded after anthelmintic treatment, there was considerable variability, ranging from one to twenty mature adult worms.

Observations on the intestinal helminth population in local cats in Edinburgh: detailed results are given in Table I.2. Toxocara cati was found in 27.2% of the cats, T. taeniaeformis was found in 8.6%, Toxascaris leonina in 1.2% and Dipylidium caninum was found in 50.6%; 28.4% were not infected with intestinal helminths.

Table I.2 Prevalence of intestinal helminths found at autopsy of of 81 cats originating from the Edinburgh area in 1977

	Male cats	Female cats
Dipylidium caninum	12	19
Taenia taeniaeformis	4	1
Toxocara cati	6	5
D. caninum + T. cati	4	4
D. caninum + T. taeniaeformis + T. cati	0	1
D. caninum + Toxascaris leonina	0	1
T. taeniaeformis + T. cati	1	0
None	6	17

The numbers of T. taeniaeformis encountered per cat were as follows: in the two female cats: one and two, in the male cats: 1, 3, 7, 14 and 22. Samples from all worms were examined against known T. taeniaeformis controls by electrophoresis for the isoenzyme patterns of glucose phosphate isomerase and showed the same pattern as that described by Le Riche and Sewell (1978[a]).

4.3.2 Comparison between different age groups of Sprague-Dawley rats and CF1 mice as to their susceptibility to infection with four strains of T. taeniaeformis. The results of these comparisons are given in Table I.3. Full data are given in Appendix Tables I.1, I.2, I.3 and I.4. The chi-square test on the numbers of animals per group with living cysts versus group total, gave the following results:

- for mice 3 to 4 weeks old: B - S - I were not significantly different from each other whereas M differed significantly from the others (χ^2 (1) = 16***).
- for mice 4 to 5 weeks old: B not significantly different from I and S not significantly different from M. However B plus I differed significantly from M plus S (χ^2 (1) = 27***).
- for rats 3 to 4 weeks old: S strain was significantly different from B plus I plus M (χ^2 (1) = 12***) with no significant differences amongst these three.
- for rats 4 to 5 weeks old: there were no significant differences between B and M or S and I. S plus I strains differed significantly from B plus M (χ^2 (1) = 15***).

The effect of the age of the host on the numbers of living cysts was examined on the groups with the widest ranges i.e. the B strain in mice and the M strain in Sprague-Dawley rats. For the former the correlation between the numbers of living cysts and age of all the mice including those without living cysts was highly significant by Kendall's Multiple Range test (Z = 1.44**).

For the M strain in Sprague-Dawley rats the age resistance was less obvious. Comparisons between all groups with each other by Wilcoxon's two sample test revealed that the only significant difference lay between the 5 to 6 week old rats and those 6 to 7 weeks old (U = 76*).

Table I.3 Mean numbers of living metacestodes of four strains of *Taenia taeniaeformis* per infected host and age group (at time of infection) ± standard deviation, excluding animals without living cysts. Also shows the proportion of animals with living cysts to the total number in each group.

STRAIN	CF1 MICE		SPRAGUE-DAWLEY RATS			
	3 to 4 weeks	4 to 5 weeks	3 to 4 weeks	4 to 5 weeks	5 to 6 weeks	6 to 7 weeks
Belgian (B)	72.6 ± 38.8 14/15	37.1 ± 12.5 14/15	26.9 ± 22.8 14/20	8.0 ± 7.1 12/15	0 0/10	0 0/10
Scottish (S)	31.9 ± 19.1 9/10	112.0 ± 10.6 3/10	1.7 ± 0.6 3/10	1.7 ± 0.6 3/10	-	-
Iraqi (I)	114.2 ± 14.5 9/10	42.9 ± 22.3 9/10	49.9 ± 29.1 18/20	23.7 ± 28.7 3/10	14.2 ± 13.5 4/10	-
Malaysian(M)	1.3 - 0.6 3/10	0 0/10	79.4 ± 55.2 10/10	104.3 ± 66.2 10/10	101.0 ± 33.3 10/10	72.6 ± 13.0 10/10

STRAIN	CF1 MICE		
	5 to 6 weeks	6 to 7 weeks	Over 12 weeks
Belgian (B)	19.4 ± 11.8 8/15	10.4 ± 7.7 10/15	56.5 ± 46 2/15

Between strains, corresponding age groups of rats and mice and different age groups of rats or mice were compared on the basis of the numbers of living cysts, omitting the zero results i.e. where no living cysts were detected. The results of these comparisons are given in Table I.4.

For all four strains, the results obtained between rats and mice were significantly different except for the Iraqi strain where the numbers of living cysts in the 4 to 5 week age group of mice were not significantly different from these in the three infected rats of the corresponding age group.

Age related differences within each host species were detected for all groups of mice with all four strains. For strain M, no living cysts were found in mice of 4 to 5 weeks old. For rats only the B strain showed any age related effect. That such an effect was not detected with the S and I strains is perhaps irrelevant since it is probably due to the small number of rats with living cysts in the two age groups infected with the S strain and in the 4 to 5 week old rats infected with the I strain. The level at which this resistance is acting is not clear. However the numbers of dead larvae, although not always accurate since clear distinction was not always possible, does indicate that this resistance was not always acting at the gut level.

The results obtained with strain B and M in Porton-Wistar rats are shown in Appendix Table I.5. Although the limited extent of this experiment did not allow for detailed comparison it was clear that once again in this host the B strain was not suitable for immunological experiments, whereas the M strain again seemed to be suitable.

Table I.4 A diagrammatic representation of the comparisons of the number of living T. taeniaeformis cysts of four different strains in two age groups of mice and rats.
Abbreviations: B, S, I and M are respectively the Belgian, Scottish, Iraqi and Malaysian strains. The U statistic is given in the parentheses between the groups being compared where significant differences were revealed by Wilcoxon's two sample test: (=) means not significantly different.

Age in weeks	3 to 4	4 to 5	3 to 4	4 to 5	3 to 4	4 to 5	3 to 4	4 to 5
Mice	B ←(150**)→	B	S ←(27**)→	S	I ←(81**)→	I	M	
	↑ (164.5***) ↓	↑ (168***) ↓	↑ (27**) ↓	↑ (9*) ↓	↑ (162***) ↓	↑ (=) ↓	↑ (30**) ↓	
Rats	B ←(131.5**)→	B	S ← (=) →	S	I ← (=) →	I	M ← (=) →	M

No living cysts were found in the mice of strain A, neither infected with strain B or I strain.

Sixteen brown rats of both sexes were captured alive, including six juveniles. None of these showed any signs of infection. Similarly no signs of T. taeniaeformis infections were found in fourteen wild mice.

4.4 Conclusions

A strict comparison between the numbers of living cysts is only possible within each strain, since the infectivity of the eggs originating from different strains might not have been the same. Criteria for estimating this will be further discussed in Parts II and V.

Although the strains B and I still retained considerable infectivity for rats aged 4 to 5 weeks, this was considerably lower than that of strain M and the former at least was no longer infective for slightly older rats. The age effect in rats was obvious by 4 to 5 weeks for strain I and by 5 to 6 weeks for strain B, although strain I was still able to infect some rats aged 5 to 6 weeks. Age related resistance in rats became significant for the Malaysian strain by 6 to 7 weeks, though all the animals were still infected at a useful level for experimental purposes. Although the situation is not absolutely clear cut, the results allow the conclusion , for practical purposes, that B, I and S strains are relatively mouse specific, whereas the M strain is a rat adapted strain. However, this statement is strictly only acceptable for the host strains tested. As the review of literature shows, different strains of host show considerable differences in susceptibility. T. taeniaeformis strain M in

Sprague-Dawley rats seemed to be the best combination for immunological experiments in that the host-age effect was less marked, indeed non-existent, according to Ambu and Kwa (1980) working with the same strains of parasite and rats. The observed incidence of T. taeniae-formis in cats (8.6%) does not differ much with previous findings in Britain: Dubey (1966) 4.5%, Hutchison (1957) 7.7% in Scotland and Cowper (1978)15.2% in Wales. However both the present findings and those of Hutchison (1957) concerning D. caninum (both 50.6%) differ from Cowper (1978) who found 13% of the cats positive for this parasite.

Chapter 5 Enzyme electrophoretic studies with four strains of *T. taeniaeformis*

5.1 INTRODUCTION

Further evidence of the reality of the strain differences of *T. taeniaeformis* in addition to the differences in susceptibility of mice and rats was sought by comparing the zymogram patterns of the four strains for three enzymes.

5.2 Experimental design

For each enzyme several different samples of each parasite strain were compared and also the positions of the samples on the gel were frequently altered. The comparison of the patterns was primarily based on the relative positions of the bands.

5.3 Results

A typical zymogram for glucose phosphate isomerase, adenylate kinase and hexokinase from extracts of adult *T. taeniaeformis* is shown on Plate I.1. Only the pattern for the isoenzymes of hexokinase consistently showed intraspecific differences. Extracts from the Belgian and the Malaysian strains only gave one band, that for the Malaysian strain being in the most anodic position. The Iraqi and Scottish strain gave multiple bands of which the most anodic may have been in the same position as the single band from the M strain. However the heaviest band seen with the Scottish strain was consistently situated more cathodally than any band seen with the other strains.

5.4 Conclusion

The isoenzyme studies appeared to confirm the existence of several strains of *T. taeniaeformis* as already indicated from their differing

infectivity for different intermediate host species or strains.

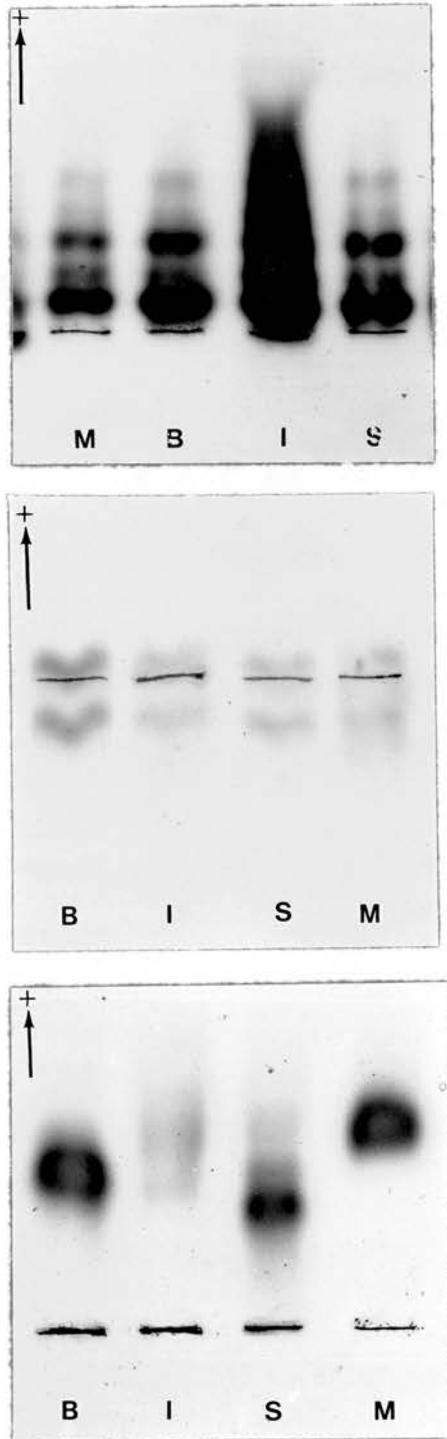

Plate I.1. Zymograms for isoenzymes of glucose phosphate isomerase (Top), adenylate kinase (Middle) and hexokinase (Bottom) in homogenates of adult *Taenia taeniaeformis* of different origins.
(B: Belgian, I: Iraqi, M: Malaysian, and S: Scottish strains).

Chapter 6 DISCUSSION AND CONCLUSIONS

Differences in susceptibility of various host strains of rats and mice to infection with _T. taeniaeformis_ and age related resistance in both hosts are well documented. It now appears that there is an additional factor , in that different strains of this parasite are better suited to either rats or mice. Within the mouse and rat strains used in these experiments, it seems that the Malaysian and the Scottish strains are clearly adapted to infect rats or mice respectively. The results also suggest that there may be differences in both overall infectivity and in their susceptibility to age related resistance in the host within the three mouse adapted strains. Although the Belgian and Iraqi strains are capable of infecting both mice and rats, effective age related resistance appeared earlier in the latter. However this resistance develops very rapidly over the period 3 to 6 weeks of age and some of these apparent strain differences may have resulted from minor variations in the average age of the animals used in different experiments. In addition, although several of these differences were significant, it is not possible to be confident that they are strain related, because they were observed in different trials, which were not always strictly comparable.

Further evidence that the parasite strain differences are consistent for several rat or mice strains is given by Vanparijs, O. (personal communication 1979) who confirmed that several attempts to infect rats with strain B were unsuccessful; equally, Kwa, B.H. (personal communication 1978) and Ambu and Kwa (1980) could not find a mouse strain susceptible to strain M. However, this does not exclude the possible existence of other strains of _T. taeniaeformis_, equally

infective for both these hosts, or indeed for other intermediate hosts.

Ferretti, Gabriele, Marconi, Monaco and Piccinato (1972) reported that their strain of T. taeniaeformis produced considerable numbers of sterile larvae in CF1 mice. Equally Orihara (1962) did not consider CF1 mice to be a good strain. However in the present study CF1 mice seemed to be quite suitable as hosts for the appropriate strains, which may indicate that even so called mouse-strains may differ in their infectivity for different host strains. On the other hand, both strain-related and age-related resistance may be affected by other factors such as overchallenge. If this is so and if animals are naturally infected with much lower doses than those generally used in experiments, this would tend to improve the chances of survival for the parasite species.

T. taeniaeformis in cats from around Edinburgh is not uncommon (8.6%) although it should be noted that in view of the source of the cats, this rate and those for the other parasites may well not be representative of the whole cat population, but of those kept in less hygienic conditions. On the other hand, no infected wild rats or mice were found in the same area and although the number of animals examined was quite low, respectively sixteen and fourteen, this might indicate that the S strain is adapted to another intermediate host species.

Other differences encountered between the four strains relate to the disintegration of the embryophoral blocks in the hatching and activation process of the oncospheres. This will be further discussed in Part II. No detailed morphological comparisons were made, as the range of the various measurements given in the literature (reviewed

by Verster 1969) is fairly wide and may be overlapping. However, for the two strains routinely maintained, strain B and M, the characteristic ratio length to width as 4 to 1, described by Hinz (1965) for mature proglottides containing viable eggs, seemed correct for strain B, whereas most of these proglottides of strain M appeared to be rather short.

There may well be no direct relationship between the different zymogram patterns and the host selectivity but both parameters indicate heterogenicity within this species. The biochemical differences reported between T. taeniaeformis larvae from rats and mice (Von Brand and Bowman 1961) also support such a relation. Furthermore, it is noteworthy that the two strains which differ most in their infectivity namely strains S and M, are also the most disparate as regards their hexokinase zymogram patterns.

If such a strain difference exists within this species it would by no means be a unique situation for helminths. Thus Thorson (1970) discussed the possibility of genetic differences for strains of Trichinella spiralis on the observation of differences in their host (rat) susceptibility.

Verster (1969) summarises the discussions concerning the possibility of Taenia ovis being subdivided into subspecies on the basis of the susceptibility of different intermediate hosts. For T. crassiceps also, as reviewed in Chapter 2, strain differences based on differences in host receptivity may be present, but the non-physiological method of maintaining this species in laboratories may partly account for this.

Williams and Sweatman (1963) opted for the existence of a new subspecies of Echinococcus - Echinococcus granulosus equinus, finding not only differences in host susceptibility between this and other subspecies but also morphological differences. This was further investigated by Hatch and Smyth (1975). Thompson (1976) even concluded that further strain differences exist within the horse strain of E. granulosus from the same intermediate host species but from different geographical origins, based on the unsuccessful attempts to infect experimental animals, previously reported to be susceptible.

These observations for T. taeniaeformis may not allow a clear distinction to be made between the several strains, but possible interference from this factor in immunological work or other experiments may be important, and will be further discussed in Part V. An indication of this may be seen in the work described by Heath and Elsdon-Dew (1972). Their strain of T. taeniaeformis was not infective for mice and also it appeared that mouse serum would not support oncospheral development. In contrast, the reverse situation occurred with rats and rat serum. On the other hand, possible antigenic variation between these strains of T. taeniaeformis might add to the usefulness of this parasite for experimental work.

PART II: *In vitro* culture of oncospheres

Chapter 7 INTRODUCTION AND REVIEW OF LITERATURE

7.1 Hatching and activating of oncospheres

An estimation of the viability of eggs is an important element in various aspects of the study of cysticercosis. Repeatedly checking viability by administering the eggs to the corresponding host is an impractical possibility, hence the need for a reliable rapid method.

An important factor in the epidemiology of cysticercosis is the widespread dispersal and longevity of *Taenia* eggs (Götzsche 1951, Jepsen and Roth 1952) and many experiments have been undertaken to check for the viability of these eggs under different conditions (Penfold, Penfold and Phillips 1937, Silverman 1954[a], 1956, Lucker and Douvres 1960 and Gemmell 1977) as have been experiments in preventing the spreading of cysticercosis by destruction of the eggs (Parnell 1965, Laws 1967 and Gall and Wikerhauser 1969). Age and other external factors may influence the infectivity of the oncospheres (Gemmell 1977) and this also makes it important to estimate the viability of different batches of eggs when these are to be used in experiments related to immunological responses or drug treatments in the intermediate host. Finally, since in recent years, the *in vitro* cultivation of taeniid oncospheres has become an important topic, a necessary first step: hatching and activation of the oncospheres requires more attention.

7.1.1. Definitions

The morphological features of Cyclophyllidean eggs, in particular those of Taeniidae, have been described by various authors: Wardle and McLeod (1968), Park (1971), Morseth (1965) and Nieland (1968) described

the ultrastructure of the oncosphere and its envelopes. Smyth (1963, 1976) commenting on the confusion in nomenclature related to the description of these envelopes, accepts Rybicka's (1966) view: Out of the four primary envelopes some secondary envelopes may be formed. For the Taeniidae the capsule and outer envelopes are either very thin or, according to some authors, shed within the uterine tract (Wardle and McLeod, 1968) others (Gönnert 1970) believed it is always present when the eggs are expelled from the segments, and may be preserved for hours afterwards. The main protective structures of the oncosphere consist of the embryophore which is a secondary envelope, and the oncospheral membrane immediately surrounding the oncosphere.

The ultrastructure of the embryophore is described by Morseth (1965) and Nieland (1968), the main features being the embryophoral blocks, composed of a keratin-type protein, (Morseth 1966), and the granular layer with its basal membrane. According to some workers (Silverman, 1954[a]; Meymarian 1961 and Laws 1968[b]) these blocks are bound together by a cement substance, although Smyth (1969[a]) points out that there is no direct evidence for this, but admits the likelihood of its existence. Nieland (1968) suggests that the closely associated circular bodies described by Morseth (1965) are mitochondria "involved in the synthesis or maintenance of the integrity of the cement substance visible between the blocks".

Freeing the oncosphere from the protective envelopes is a process which involves seven stages according to Gönnert (1970) but in summary it can be described in two steps: Passive disintegration of the embryophore by digestion of the cement binding (Silverman 1954[a]) which holds the blocks together, followed by activation of the hexacanth embryo,

so that it frees itself from the oncospheral membrane either by actively rupturing it with its hooks or, according to Orlov and Kosminkov (1972), by a further passive process, resulting from the combination of the digestive action of the intestinal enzymes and enzymes secreted by the oncosphere itself.

This process is commonly described as hatching and activation of the oncosphere. However, although the definition of an activated or motile oncosphere is generally accepted, the use of the term hatching is not always applied to the same phenomenon. Thus Nieland (1968) stated that most of the hatched oncospheres in his studies retained their oncospheral membranes. Park (1971) distinguished hatched non activated oncospheres, activated oncospheres whose hooks move in order to rupture the membrane and the subsequent hatched oncospheres freed from the enclosing membrane. Silverman (1954[a]), Laws (1967) and Beveridge, Rickard, Gregory and Munday (1975) make a similar distinction. Other authors (Gallie and Sewell 1970 and Gemmell 1977) clearly regarded hatching as including both the disaggregation of the embryophoral blocks and the disruption of the oncospheral membrane.

In this work this latter definition is applied: hatching and activating being two terms used inseparately and indicating the processes resulting in the liberation of the oncosphere from both the embryophore and oncospheral membrane so that a hatched and activated oncosphere is a freely moving organism usually with visible penetrating gland secretions (named by Silverman 1954[b], 1955).

7.1.2. Techniques

Bullock, Dunning and Curtis (1934) found indications that hatching and activation of _Taenia taeniaeformis_ eggs, or in their words,

digestion of the shells showing free and often motile oncospheres, occurs in the small intestine of the rat and concluded that the gastric juice plays no role in this digestion. He could achieve this event <u>in vitro</u> using natural juices obtained by washings from rat's intestines, also using extracts of its pancreas or aqueous solutions of pancreatin, amylopsin, steapsin and trypsin but the presence of fresh or desiccated ox bile with each of these enzymes was found to be essential to obtain complete disintegration. Strong bases such as sodium hydroxyde caused the same effect but killed the oncospheres, but these were resistant to quite strong acids and weak bases. In his opinion the hydrogen-ion concentration of the intestinal juices was a determining factor in the susceptibility or resistance of different host species to this parasite. Silverman (1954[a]) mentioned the work of Isobé in 1922 who was able to obtain disintegration of embryophoral blocks of <u>Taenia saginata</u> eggs after successive treatment with acid pepsin and alkaline pancreatin, but concluded that the critical factor in obtaining hatched and activated oncospheres was the concentration of ions, so he transferred the eggs, free from their embryophoral blocks, to a third solution containing NaCl, Na_2CO_3 and pig bile.

Penfold, Penfold and Phillips (1937) could not successfully hatch <u>T. saginata</u> oncospheres after treatment with artificial gastric solution (AGS) followed by artificial intestinal solution (AIS). Silverman (1954[a]), commenting on these experiments and those by himself and other previous workers, stressed the importance of considering the hatching behaviour from tapeworms belonging to the family of the Taeniidae separately from these of other Cyclophyllideans and points out the importance of quantitative data on these mechanisms. Indeed, whereas for Cyclophyllidea other than Taeniidae hatching of the eggs can occur in

simple solutions (Smyth 1963, 1969[a]), in the latter it is a more complicated phenomenon probably partly because of their thicker embryophore. Experiments with hatching and activating eggs of _Taenia pisiformis_ and _T. saginata_ leads Silverman (1954[a]) to conclude both that the former does not require pretreatment with AGS, whereas the latter does and that the addition of cholesterol to pancreatin and bile salt - which can replace the whole bile - can confer a high hatching and activation rate for _T. pisiformis_ (63%) and _T. saginata_ (74%). The addition of trypsin helped to standardise the technique in so far as it activated the trypsinogen in the crude pancreatin, resulting in a shorter time (one hour to 30 minutes) for the oncospheres to become activated. He laid down his criteria for determining the viability of oncospheres, freed from the embryophores, namely: motility, general appearance and the reaction to vital stains. He applied this technique to the same parasites, to determine the variation in state of maturity of eggs from different segments originating from the same worm (Silverman 1954[b]) and to study the _in vitro_ effect of serum on activated oncospheres (Silverman 1955). Here an interesting observation emerges: Serum had a protective effect on oncospheres in that they remained active in it for 3 h or more, whereas this activity ceased within one hour in intestinal juice. Silverman (1956) reported that it was impossible to hatch eggs after 3 to 4 months of storage due to changes in the binding cement substance between the embryophoral blocks. In the same paper he mentioned the deleterious effects of bacterial contamination on eggs. Numerous workers have used Silverman's technique with variable results. Urquhart (1958) and Gallie and Sewell (1970) reported poor activation rates with this technique on _T. saginata_ eggs. Mango and Mango (1972) gave high percentages (82 to 96%) of viability using

the same technique on T. saginata eggs. Williams and Colli (1970) found that many oncospheres from Echinococcus granulosus and to some extent from Taenia hydatigena could not be activated by Silverman's method, in which they used fresh sheep bile instead of bile salt, but were still infective as judged from the difference in activation rates and infectivity: thus batches of E. granulosus eggs with 5 to 0 % activation rates gave 15 to 17.3% development into cysts. Froyd and Round (1960) and Froyd (1961) used Silverman's technique for T. saginata eggs in experiments determining the immunoresistance of hosts at the intestinal phase and by-passing it by parenteral (s.c. and i.m.) injections with H & A oncospheres. As with Froyd (1964) they indicate good activation rates but no data are given. Gemmell (1962, 1964[a]) used the same technique, when determining an early immune response at the intestinal level and a late immune response at the predilection site, with T. hydatigena, Taenia ovis and T. pisiformis. Wantland (1953), in attempts to infect hamsters with in vitro predigested eggs of T. taeniaeformis found no visible effect of the AGS but an aqueous solution consisting of 1% desiccated ox bile and 0.5% pancreatin at pH 7.2-7.6 gave him fast (in 5 to 20 minutes) disintegration of the embryophoral blocks, releasing active oncospheres. Nieland (1968) used this technique for his electron microscopic observations on eggs of T. taeniaeformis. Although he reported that all eggs hatched within 1½ h, he admitted that most of these hatched oncospheres still retained their oncospheral membranes.

Meyers (1957), when trying to determine the viability of Echinococcus eggs by inducing in vitro the motility of oncospheres, used pepsin to which some minutes later trypsin was added but it is described as being essential that together with the pepsin a complexing

agent, such as sodium phosphate, which binds traces of heavy metals, should be used.

The pretreatment with Meyers' AGS has been frequently used by other workers: Parnell (1965), Morseth (1966) for T. hydatigena and T. ovis. Laws (1967, 1968[a,b]) used it also, but for a much larger incubation time (45 minutes) for T. hydatigena and T. ovis, but found such a pretreatment not necessary for T. pisiformis or E. granulosus. Gemmell (1977) also used it for T. hydatigena and T. ovis. These authors combined this AGS with Silverman's AIS, or slightly modified forms of this (Parmeter and Gemmell 1974).

Jones, Segarra and Wyant (1960) indicated that eggs of T. taeniaformis from the last 6 proglottides were morphological mature and eggs from the same proglottis show relatively uniform hatchability when freshly removed from these proglottides. On the other hand, if they were stored in saline at 10°C, hatching reached the same degree but required longer incubation times: 2 to 3 hours as opposed to 30 minutes when freshly removed. Time of storage between 3 days and one month gave similar hatching results. As the authors pointed out, this is quite different to T. saginata eggs as described by Silverman (1954[b]) and to the eggs of T. hydatigena and T. ovis as later described by Gemmell (1977). The hatching method was that described by Wantland (1953), but the pH was kept at 7.2 using sodium hydrogen carbonate. However, it is not clear whether these authors, when stating that their hatching levels were 80% meant merely disintegration of the embryophoral blocks or complete hatching. The former was accepted as the definition for hatching in a later paper by Huffman and Jones (1962).

Meymarian (1961) tried several hatching and activating solutions

for Echinococcus granulosus and concluded that AGS did not seem to have any effect on these eggs whereas strong alkalines without enzymes could dissolve the cement that holds the embryophoral blocks together. Here he suggested that this cement is not protein. The eggs could be freed from the proglottides and their embryophoral blocks disintegrated, by combining pancreatin, trypsin and $NaHCO_3$ at pH 10 but the proportion of activated oncospheres increased to reach up to 52% on occasion, if the effect of these enzymes was enhanced, by using cholesterol and sheep bile, the latter being more effective than bile salts.

Huffman and Jones (1962) selected a H & A medium for T. taeniaeformis eggs which was similar to Silverman's technique but with an increased amount of pancreatin (1.5%) and 5% ox bile, pH 7.0. This medium was somewhat - but not significantly - more effective than other combinations with lower concentrations of bile or pancreatin or at pH 7.6. With this technique they compared the H & A percentage with the viability of the oncospheres, as assessed from their resistance to various vital stains and their infectivity in rats. They concluded that bile concentration is the only critical factor for DEB, and that its presence is essential for activation, which is independent of the concentration. Their activation levels were low (up to 4%) and more oncospheres were resistant to vital stains, while there was no direct correlation with mobile oncospheres or oncospheres freed from their embryophore. There appeared to be a correlation, although not a high one, between the latter and the infectivity to rats, but it was not close enough to allow in vivo prediction from in vitro results. Motility was not considered to be a good parameter for estimating viability because it was thought to be valid only for a parti-

cular stage in the development of a batch of stored eggs, which are already at varying stages of development when naturally released from proglottides. Parnell (1965), in a study on the effect of taeniid ovicides on the eggs of T. hydatigena and T. ovis assesses the effectivity of chemicals by their influence on activity and hatchability. He used Meyers' (1957) AGS for 45 minutes followed by Silverman's digestion fluid to which Tween 80 is added for 20 minutes. Although DEB occurred in the controls, usually at levels around 80%, the percentages of activated oncospheres were much lower. He concluded that the effect of potential ovicides is more difficult to assess on activity than it is on hatching, presumably using the last term to indicate DEB. He remarked that due to the disappearance of a number of eggs even in the controls, during the H & A procedure, an error was introduced in the assessed H & A proportions. These experiments were further discussed by Laws (1965). The study of ovicides and especially of those chemicals based on sodiumhypochlorite has given interesting results: Penfold, Penfold and Phillips (1937) reported that very dilute solutions of sodiumhypochlorite were ineffective against T. saginata eggs in sewage. Boray (1954) stated that hypochlorite down to a concentration of 1% had a shrinking effect on oncospheres of E. granulosus, but did not dissolve them after one hour's contact. He recommended therefore using a 5% solution to protect people handling this material. A similar observation (Anon., 1961) was made regarding T. hydatigena eggs, in that after treatment with sodiumhypochlorite solution containing 0.1% available chlorine they still retained their infectivity. Meymarian and Schwabe (1962) estimated the effectivity of sodiumhypochlorite (Chlorox) and other chemicals against E. granulosus eggs by hatching and activating them after treatment with

Meymarian's technique (1961). Even when hatching and activation followed 90 minutes treatment with 5% sodiumhypochlorite, a higher percentage of oncospheres showed activity than in the control group. In the former group all the oncospheres lost their embryophore due to the sodiumhypochlorite, but this was not the case in the control group even after the H & A treatment.

Mackie and Parnell (1967), using the techniques of Parnell (1965) tested various chemicals for their ovicidal activity against T. hydatigena and T. ovis eggs. Hypochlorites such as Milton or Chlor-o-gene, needed to be applied at high concentrations to give reduction of the activation levels of 50%.

Furthermore, these authors remarked that the practical use of these solutions is limited by their loss in strength, so that fresh solutions should always be used. It also appeared from this work that various antibiotics had virtually no effect on H & A. Similar work with chemical ovicides has been done by Laws (1967) using eggs of T. hydatigena, T. ovis, T. pisiformis and E. granulosus. The first two species only were pretreated with Meyers' AGS (1957) for 45 minutes. Eggs from all 4 species were submitted to Silverman's AIS (1954[a]) for 10 minutes, in the case of those pretreated with AGS and 20 minutes for the others. Although optimal times for maximal H & A varied for different batches of eggs, the tests with hypochlorite showed interesting results. The action of freshly prepared sodium- and potassium- hypochlorites (Commercial names: Chlorox, Clor-o-gene, Janola), was dependent on the concentration of active chlorine. Between 0.1 and 6.1 % active chlorine there was an expansion of the

embryophoral blocks followed by their disaggregation but both this and the effect on the oncospheres was pH dependent. Thus there was a rapid effect around pH 12, but slow or none at all between pH 7 and 10. Again oncospheres showed considerable tolerance: even after 2 hours exposure to a solution containing 1.5% active chlorine around pH 12 the oncospheres could be activated. With calciumhypochlorite such as the commercial product "H.T.H." however, there was no marked effect on the eggs at any pH level even with concentrations of up to 10% available chlorine. Such treatment prevented the subsequent action of sodiumhypochlorite upon the eggs but this could be reversed by EDTA treatment. The effect of sodiumhypochlorite was explained by this author as a two-stage event: firstly, the peptide linkages of the outer embryophoric membrane are broken by chloramine formation which allows ingress of hydroxyl ions. With this the blocks increase in size and disintegration follows. Thus the physical factor in this event is at least as important as the chemical. Calcium may have a protective effect by being adsorbed onto the egg surface.

In a series of experiments with eggs of T. hydatigena, T. ovis, T. pisiformis and E. granulosus, Laws (1968[b]) studied the phenomenon of the DEB, which he described as hatching. In his opinion the interstitial component or cement substance is converted to a hydrophyllic colloid by the initial enzymatic action of the AIS. These colloidal particles expand due to osmotic pressure and as a result of this, together with the expansion of the embryophoral blocks under the influence of hydroxyl ions, the embryophore disintegrates. According to this author this might explain the inhibition of DEB under certain conditions, such as using hypertonic solutions, when working with immature eggs - i.e. where colloid formation has not yet been completed

or when aged eggs are stored under contaminated conditions, the interstitial colloid being attacked by bacteria. Further, it explains why physical processes applied to the egg such as heating or dehydration would denature the colloid structure, and so prevent DEB. Because of the rapid DEB due to hypochlorite and enzymes such as trypsin both of which are proteolytic, this author does not agree with Meymarian's (1961) suggestion that this cement is a non-proteinaceous substance. Pretreatment with AGS is also regarded as non essential because even eggs from species like T. hydatigena and T. ovis, already described by Parnell (1965) as belonging to the group which required AGS - pretreatment, could release activated oncospheres after mechanical disruption of the embryophore or its disintegration by sodiumhypochlorite followed by AIS treatment. It is noteworthy that in one of his experiments, it seems to be the presence of atmospheric oxygen that is the factor responsible for slower H & A rates occurring in stored T. hydatigena eggs as opposed to freshly obtained eggs.

Gall and Wikerhauser (1969) tested some chemical compounds as potential ovicides against T. saginata. For testing the viability of the treated eggs, they used Silverman's method and the methods used by Wikerhauser (1960) for activating Fasciola hepatica metacercariae. This consisted of adding to the AGS either a combination of trypsin and pancreatin or trypsin and ox bile - the latter gave the best result for F. hepatica metacercariae. It appeared that Wikerhauser's solutions and Silverman's gave similar results but the former produced a higher rate of activation. Wikerhauser, Žuković and Džakula (1971), in attempts to immunise calves against bovine cysticercosis

with H & A oncospheres of T. hydatigena and T. saginata reported that with the solutions of Wikerhauser (1960), with the AIS being trypsin and ox bile, they usually had a higher percentage of hatched and activated oncospheres for T. hydatigena than for T. saginata.

Gönnert, Meister, Strufe and Webbe (1967) concluded that for T. solium and T. saginata, peptic predigestion followed by a tryptic digestion are both required to rapidly break the embryophore down into "keratin crystals".

Smyth (1963, 1969[a]) gave a summary of the work done on that subject so far, the presence of a cementing substance between the embryophoral blocks, and the necessity for pretreatment are discussed. In activation, which he described as the release from the oncospheral membrane, Smyth suggested that the important role of bile might be due to its surface active properties and the subsequent increase of permeability of the oncospheral membrane. Together with the contention that there is a species dependent difference in the cement substance (Smyth, 1963) he suggested that bile may also be a factor in the host differentiation mechanism, a suggestion already made by Berberian (1957). Smyth (1969[a]) quoted a H & A solution modified by Heath in a personal communication in which the use of intermediate host bile together with pancreatin and sodiumhydrogen carbonate in the AIS is reported to give up to 95% activation, allowing for differences due to particular species and particular proglottides.

In attempts to cultivate oncospheres from E. granulosus, T. hydatigena, T. ovis, T. pisiformis and Taenia serialis in vitro (Heath and Smyth, 1970), it was inevitable that H & A required special

attention. For this purpose, eggs were dissected out of the mature segments and stored in saline with antibiotics. After the pre-treatment with pepsin + 1% concentrated HCl in screw capped culture tubes, the eggs were centrifuged and supernatant removed and AIS, consisting of 1% pancreatin, 1% sodiumhydrogen carbonate and 5% bile from one of the intermediate hosts, was added without washing the eggs. The authors took care to retain the released CO_2, and the 30 minute AIS incubation was executed on a roller. After that, the eggs were washed 3 times and activation was estimated by the use of neutral red which stained the activated oncospheres, and trypan blue which stained the dead oncospheres only. With this method the authors claim 100% DEB. Activation for all species varied from 70% to nearly 100%. Better results were obtained when large numbers of eggs, of around one million, were used. Smaller numbers of 10 to 5 000 often gave less than 50% activation. Storage of the eggs gave a progressive decrease in activation rate and so freshly collected eggs were preferred. This method was used by Rickard and Bell (1971[a]) for T. hydatigena and T. ovis but with rather low proportions of motile oncospheres of 5.8% and 3.2% respectively despite using fresh eggs in presumably high quantities. Other experiments in which this method was used were those of Heath and Lawrence (1976) for E. granulosus, Rickard and Adolph (1976), Rickard, Adolph and Arundel (1977) and Heath (1976), who recorded 70% H & A for T. pisiformis and Rickard, White and Boddington (1976) and Rickard, Boddington and McQuade (1977) for T. ovis. Coman and Rickard (1977) studied the ageing process of T. pisiformis eggs outside the host and segments. They concluded that there were at least 4 stages in this process, one of these being

where eggs could be successfully H & A even when they were beyond the infectivity stage in rabbits. They also used the method of Heath and Smyth (1970) but with a variation in treatment times. Activation was assessed by means of 1% trypan blue and they concluded that this was a more reliable estimation of viability and less vulnerable to AIS treatment times than counting motile oncospheres. Although this assessment gave much higher counts and was less erratic it tended to lead to overestimation of viability.

In further experiments with in vitro cultures of T. saginata and T. taeniaeformis (Heath and Elsdon-Dew, 1972), the H & A technique as described by Heath and Smyth (1970) was used for T. saginata but AGS was omitted for T. taeniaeformis and, according to their principle of using the corresponding host bile, a suitable substituent for rat bile was found in a mixture of 40% pig bile, 40% ox and 20% rabbit bile. It appeared that bacterial contamination interfered with H & A of T. saginata. Heath (1973[a]) working with eggs of T. ovis, T. serialis, T. taeniaeformis, T. hydatigena and T. pisiformis, improved and further simplified the technique of Heath and Smyth (1970) by controlling bacterial contaminants using as much as possible material from autopsies and washing the egg solutions with Hibitane prior to treatment with AGS and AIS. Because the object was in vitro culture of these oncospheres, both solutions were passed through a 0.22 µm pore size Millipore filter. No tissue culture roller was used, and most important of all, lamb bile, stored at -14°C, had sufficient activity on all species of oncospheres. Other observations about the use of bile were made by Beveridge, Rickard, Gregory and Munday (1975) comparing H & A results for Anoplotaenia dasyuri and T. pisi-

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formis oncospheres. Basically using the method of Heath and Smyth (1970), they obtained high percentages of activated oncospheres for A. dasyuri with bile from different host species or even without bile. With T. pisiformis they found an effect of bile on the DEB but on H & A its presence or absence in the mixture of pancreatin with sodiumhydrogen carbonate made no difference. Pretreatment with AGS for A. dasyuri allowed DEB of some eggs but when this was accompanied by mild shaking, the majority showed DEB, an observation already made by Silverman (1954[a]) for T. saginata. No such effect was noticed on T. pisiformis eggs but pretreatment with AGS followed by AIS resulted in a higher H & A percentage than with AIS alone.

In the technique of Gallie and Sewell (1970) more attention was paid to the presence of carbondioxide in the AIS. Unlike Heath and Smyth (1970) they washed the AGS out and added hydrochloric acid to the AIS, which contained sodiumhydrogen carbonate, pancreatin, trypsin and 20% v/v ox bile, immediately closing the containers thereafter. After one to 6 h at $39^{\circ}C$ they obtained H & A percentages from 40 to 80% with most of their T. saginata egg batches. They also mentioned the fact that increased storage times required longer incubation times. Later, (Gallie, G. personal communication 1976) this technique was improved by using a higher amount of trypsin ($1\ mg.ml^{-1}$) and, adding a reducing agent; L-cysteine hydrochloride ($0.316\ mg.ml^{-1}$) to the AIS and also performing the incubation with AIS on a blood cell suspension mixer.

Gönnert (1970) commented on several aspects of the release of the various protective layers of an oncosphere. His findings were based on studies with eggs of T. taeniaeformis, T. saginata, T. pisiformis and T. solium. Firstly he stated that the egg capsule is

always present when the eggs are released from the segments. He distinguished 7 successive stages in the H & A process, and insisted that peptic pretreatment is a necessary step because exposure to AIS alone, only results in a partial, slow and atypical DEB. Even a short (5 minutes) incubation in an acid pepsin solution followed by AIS or trypsin solution alone gave typical disintegration into single keratin blocks, the cement being chemically modified by the AGS, without necessarily changing its morphological appearance. It was mainly trypsin that was responsible for breaking up the basal membrane of the granular layer. Bile salts activated the oncosphere which then ruptures the oncospheral membrane with his hooks. These findings were tested and confirmed in vivo with T. taeniaeformis by Thomas (quoted by Gönnert, 1970) who found that half the eggs given by the oral route to a mouse developed into cysticerci in the liver as opposed to only 8% if they were injected into the duodenum and 0.5% injected into the lower part of the intestine. These findings do not seem to be entirely in agreement with Bullock, Dunning and Curtis (1934) and the results obtained for Taenia crassiceps eggs by Freeman (1962) who assumed that eggs of this species did not need a gastric pretreatment.

Park (1971) found that eggs from T. saginata needed pretreatment with AGS whereas hatching and activating those of T. solium or T. pisiformis could be achieved with only AIS. Again trypsin was considered to be the most effective enzyme responsible for hatching. The use of fresh bile and bile salts gave a more effective H & A than sodiumtaurocholate or sodiumdeoxycholate.

Rickard and Bell (1971[b]) used a different AIS for T. ovis and T. taeniaeformis respectively. For the former, 1% pancreatin with sodiumhydrogen carbonate plus 5% fresh sheep bile was used and for the latter the AIS was modified from Silverman (1954[a]) by adding sodiumglycocholate, sodiumtaurocholate and increasing the amount of trypsin. The tubes were gassed with 5% CO_2, 10% O_2 in N_2 and placed on a roller. They obtained for T. ovis 10% for T. taeniaeformis 60% H & A oncospheres.

Another description of the hatching mechanism is given by Orlov and Kosminkov (1972). Without mentioning a particular technique they describe how the oncospheres of T. saginata hatch from the oncospheral membrane after the embryophore is broken up by AGS followed by AIS. Contradicting the suggestion that the rupture of this membrane is partly due to the movement of the embryonic hooks, they state that it is only the interaction between the intestinal enzymes and enzymes secreted by the oncospheres themselves which dissolve this membrane at a well defined spot, located at the anterior end of the oncosphere. The oncosphere emerges through a circular aperture formed at this spot but it is then vulnerable to the lytic action of the intestinal juice and perishes rapidly. Another variant on Silverman's (1954[a]) technique was used by Mitchell G. (personal communication, 1977) for T. saginata oncospheres. Apart from tauroglycocholate he also used taurocholate and glycocholate, each at a concentration of 0.25 $mg.ml^{-1}$ and trypsin was omitted from the AIS. After treatment with AGS and AIS, he considered that a reasonably good result was obtained from a batch of eggs if the DEB is about 100% and motility plus visible penetration glands secretion is anything above 3-4%.

A description of the sequence of events during the hatching of T. hydatigena oncospheres is given by Gemmell (1977). In this work the interrelation between AGS (Meyers, 1957) and AIS (Silverman, 1954[a]) is given. It appears that for both T. hydatigena and T. ovis pretreatment always resulted in an increased proportion of hatched oncospheres as opposed to AIS alone. Furthermore the pretreatment time was an important factor. For T. ovis short (15 min.) pretreatment times appeared to result in more DEB. However, although fewer oncospheres showed DEB, if this time was extended to more than 30 minutes, more became activated later. Gemmell again studied the variability in the H & A characteristics for eggs from different segments and tapeworms and also the influence of AIS during or after the hatching process. These and other experiments lead him to the conclusion that the extent of in vitro H & A depends on the particular developmental phase in which the oncospheres are at the time. He distinguishes six phases which can occur together amongst populations of eggs in a single mature segment. This classification of the life span into different phases is not rigid and even outside the host, given adequate conditions, eggs will continue to proceed to fully competent stage and beyond. Gemmell then suggested that it might be expected that fully competent oncospheres (in his phase 3) will be less affected by adverse conditions such as high temperatures, desiccation, microbial contamination etc. than others.

Smyth (1976) gave a review of factors influencing H & A and pointed out that to date more attention has been paid to the influence of enzymes and bile than to the physical conditions such as pH, redoxpotential and CO_2 content.

7.2 *In vitro* development of oncospheres

Although *in vitro* cultures of cestode oncospheres have been attempted by various workers including Voge (1967[a], 1967[b]), Voge and Seidel (1968), Berntzen (1967, quoted by Heath and Smyth, 1970), Berntzen (1970) and Graham and Berntzen (1970), not much work had been done with Taeniid oncospheres before 1970. Mendelsohn (1935) kept larvae of *T. taeniaeformis* *in vitro* for some weeks but he started with larvae which had been allowed to grow for 15 days in their intermediate host. He reported invagination of the head, without noticeable formation of suckers.

Webster and Cameron (1963) used oncospheres of *Echinococcus multilocularis* but did not achieve much development *in vitro*.

Descriptions of early *in vivo* development of taeniid larvae have been given by several workers, amongst them: McIntosh and Miller (1960) for *T. saginata*, Šlais (1966, 1970, 1973), for *T. solium* and *T. saginata* and Crusz (1948) Hutchison (1958) and Šlais (1973) for *T. taeniaeformis*.

As summarised by Voge (1967[a]), *in vivo* development includes cavity formation and an increased cellular density in the anterior end which is followed by differentiation and development of a scolex orientated inwards. Similar observations were made by Bilqees and Freeman (1969) with *T. crassiceps* larvae and by several other authors including Bilqees (1969) Singh and Rao (1967) and Banerjee and Singh (1969[a]).

Rickard and Bell (1971[b,c]) briefly described the development of previously hatched and activated oncospheres of *T. taeniaeformis* and

T. ovis placed in diffusion chambers and implanted in their respective intermediate hosts, rats and lambs. After 7 days embryos appeared as solid balls of cells, sometimes showing a cavity after 3 weeks. Their size was up to 1.8 mm diameter and they looked like cysts with a cone shaped protuberance, probably the scolex anlage. Similar experiments were briefly reported by Parmeter (1974, 1975[a]), who placed T. hydatigena oncospheres, in diffusion chambers, in sheep.

The first report of in vitro growth of taeniid oncospheres came from Heath and Smyth (1970) working with E. granulosus, T. hydatigena, T. ovis, T. pisiformis and T. serialis, followed by Heath and Elsdon-Dew (1972) for T. taeniaeformis and T. saginata and Heath (1973[a]) with an improved method for T. ovis, T. serialis, T. taeniaeformis, T. hydatigena and T. pisiformis. Not only are these papers important for the study of post oncospheral differentiation but the practical consequence of using the excretory and secretory products of these larvae as vaccines (Rickard 1971-72, Rickard and Bell 1971[a], Heath 1976, Rickard and Adolph 1976, Rickard, White and Boddington 1976, Rickard, Boddington and McQuade 1977, Rickard, Adolph and Arundel 1977, Osborn 1977-78 and Lloyd 1979) gives them considerable importance. Another approach was that adopted by Parmeter and Gemmell (1974) and Parmeter (1974-75[b]) who used chick embryos as experimental hosts for T. hydatigena oncospheres and obtained almost normal development.

Heath and Smyth (1970) used a standard basic culture medium (Medium 858) supplemented with glucose, potassium ions, antibiotics and appropriate host serum at 20% v/v. The cultures were kept at

37°C in roller tubes under a gas phase consisting of N_2 plus 10% O_2 and 5% CO_2. While admitting that the composition of their media was basically empirical, as is frequently in similar experiments in other fields, these authors could come to some conclusions. Thus, fresh serum without prior inactivation from the young, natural intermediate host, supported growth better than that from older animals. No development occurred when sera of other species or sera from infected animals was used. The authors saw this as a possible explanation for the fact that the best results were obtained for T. pisiformis, the only parasite for which they could provide serum exactly corresponding to such standards. They stated that when 5 000 activated oncospheres were placed in 10 ml of medium contained in Leighton tubes, placed on a roller device, they yielded 10 mature larvae. After 10-15 days in vitro the organisms were transferred to larger flasks containing 100 ml medium. The medium was changed twice weekly after packing the developing oncospheres by gentle centrifugation. In this experiment all species showed early cystic development and a considerable increase in size, but the most successful was with T. pisiformis oncospheres, which achieved scolex development, starting with the appearance of scolex anlage on day 5, as compared to day 13 or 14 for T. ovis.

Heath and Elsdon-Dew (1972) studied the role of serum in vitro cultures using oncospheres from T. saginata, T. taeniaeformis and T. pisiformis. The basic medium differed from that of Heath and Smyth (1970) in that they used NCTC-135 with antibiotics added. The gas phase was air and the Leighton tubes were incubated while stationary. To this medium 20% serum from the natural host was added.

On day 3 cavity formation was visible and muscle systems able to change the shape of the young larvae were seen on day 9. It was noted again by the authors that different sera had a variable effect on the stimulation of the growth of the larvae. The absence of stimulatory factors or the presence of inhibitory factors in some sera, even in foetal calf serum may have caused this variable response by the oncospheres. The stimulatory factors were mainly present in the macromolecules (α and β globulins) but the inhibitory factors were not removed by precipitation of the γ globulin fraction. Thus it was either impossible to remove these factors or the inhibition was due to a relative absence of stimulatory factors.

An account of the early in vitro development of T. saginata oncospheres is given by Orlov and Kosminkov (1972), who used Medium 199 + 10% v/v serum. They describe the formation of a slit-like cavity after one day, which progressively increases in size, and eventually exceeds the size of the solid part of the parasite. They named this the procysticercus.

Heath (1973[a]) further improved the in vitro culture methods used with T. ovis, T. serialis, T. hydatigena, T. pisiformis and T. taeniaeformis. The culture medium was again NCTC 135, but glucose, antibiotics, washed rabbit blood cells (1% v/v) and inactivated foetal calf serum were added. The concentration of the serum decreased from 50% in the first week to 20% the next 2 weeks and 10% thereafter. The gas phase was air and the cultures were kept stationary in disposable flasks. Initially 0.1 ml of the medium was used for each oncosphere, but this was progressively doubled twice a week at each change, until the final amount was 10 ml per larvae. Ten per cent

of the eggs gave rise to developing oncospheres. All oncospheres from these species developed into immature cysticerci with invaginated scoleces at a rate comparable to that seen in vivo, although their development rates varied in every culture. The rabbit blood cells seemed to provide the growth stimulation factors present in the specific host sera, which could thus be replaced by the cells plus the foetal calf serum. T. taeniaeformis larvae developed a layer of microvilli on day 4 and blood cells appeared to stick to that layer. These larvae showed rapid expansion after 15 days in vitro with development of muscle systems and scolex anlagen on day 20. At that time they were showing an intense spiral movement. After scolex development these movements changed to contractions on day 26. Scoleces with fully formed suckers, some evaginated, were visible on day 43. This technique was used by Heath (1976) for T. pisiformis.

Heath and Lawrence (1976) developed oncospheres from E. granulosus to immature hydatid cysts of 2 cm diameter after 120 days. They tried several media concluding that the medium described by Heath (1973[a]) using 20% calf, foetal calf or rabbit serum to be suitable. RPMI 1640 and McCoy's 5A gave them similar results to NCTC 135. Cultures without blood cells or with rabbit serum showed a similar growth rate to the others for the first 2 weeks but thereafter the growth rate diminished.

Rickard and Bell (1971[a]) used the method of Heath and Smyth (1970) for T. ovis and T. hydatigena, omitting extra glucose and potassium. They used serum from 3 week old parasite-free lambs, placing 40 000 H & A larvae in 5 ml medium, which was changed every

2 days. After 8 days, 0.5% of the larvae developed to the optimal extent described by Heath and Smyth (1970) while 20% did not develop at all and the rest were in an intermediary stage.

Rickard and Katiyar (1976) cultivated T. pisiformis in a similar way to Heath and Smyth (1970) and Rickard and Bell (1971[a]), using glucose enriched medium 858 and varying concentrations of whole rabbit serum or various fractions of it. Best development occurred with 20% normal rabbit serum.

Parmeter (1974-75[c]) reported the beneficial use of lamb liver extract as a supplement in culture media for larval cestodes.

A further development was the observation by Heath and Lawrence (1977-78) that incorporating a growing cell line in the culture media, allowed them to grow up to 10^5 T. ovis oncospheres at one time.

Lloyd (1979) cultured T. taeniaeformis oncospheres in RPMI 1640 with HEPES buffer and 10% v/v mouse serum for 10 days. No results in relation to in vitro growth were given.

Rickard, White and Boddington (1976), Rickard, Boddington and McQuade (1977) and Rickard and Adolph (1977) used Rickard and Bell's (1971[a]) technique with 20% v/v serum from colostrum deprived lambs for culturing T. ovis oncospheres as did Rickard, Adolph and Arundel (1977) for T. saginata oncospheres and Rickard and Adolph (1976) for T. saginata, T. hydatigena and T. ovis. For T. saginata lamb serum was replaced by inactivated foetal calf serum. In this work development of T. saginata was described as being minimal, although the oncospheres remained active for 2-3 days and showed enlargement. Few details were given of this work, since the in vitro growth aspect

was not the main subject being studied, but rather the immunogenic value of the released metabolites of the developing larvae. A review of these techniques with suggestions for further work is given by Voge (1973, 1978).

Chapter 8 MATERIALS AND METHODS

8.1 Hatching and activating oncospheres

8.1.1. List of materials routinely used

Pepsin 1:10 000 (Sigma) (E.C. 3.4.23.1.)

Pancreatin Grade III 3 x NF (Sigma)

Trypsin Crude II (Sigma) (E.C. 3.4.21.4.)

RPMI 1640 with 25 $mmol.l^{-1}$ HEPES buffer and L-glutamine (Gibco-Europe)

Modified RPMI 1640: as above plus

5% v/v 100 mmol (5 $mmol.l^{-1}$) sodiumpyruvate (Gibco-Europe)

100 i.u. ml^{-1} penicillin (sodiumbenzylpenicillin Glaxo)

100 $\mu g.ml^{-1}$ streptomycin (streptomycin sulphate BP - Glaxo)

Foetal calf serum (Heat inactivated - Gibco - Europe)

8.1.2. Eggs, segments and strains

The methods of preparation and storage of eggs and segments are described in Part I, Chapter 3. The Belgian strain of T. taeniaeformis was used in all sets of experiments. When this was used together with other strains, as in sets No. 6 and 11, the strains are indicated as in the description given in Part I.

8.1.3. Preparation prior to H & A

The experiments were routinely done in screw cap tubes (Pyrex - Corning) of maximum capacity 8.5 ml, 500 to 5 000 eggs in normal saline being added to each tube. These were then centrifuged on a Mistral 4 L Centrifuge (MSE) at 1500 g for 10 minutes, the supernatant

then being drawn off until about 1 ml was left in the tubes. For experiments, mentioned in set 7, and from then onwards, the same type of tube was used, but with a larger capacity of 16.5 ml. In these tubes, the eggs were left in 2.5 ml normal saline plus antibiotics after centrifugation.

8.1.4. Techniques

The following is an outline of the basic techniques tried in this work by methods as close as possible to the authors' description, any modifications being given for each set of experiments.

(a) Gallie and Sewell (1970) modified by Gallie G. (personal communication 1976).

AGS consists of 1% w/v (10 $mg.ml^{-1}$) pepsin (Sigma) and 17.5 $\mu l.ml^{-1}$ (0.2 $mol.l^{-1}$) concentrated hydrochloric acid ("AnalaR" BDH) in distilled water, pH 1.

AIS contained:		
	Pancreatin (Sigma)	10 $mg.ml^{-1}$
	Trypsin (Sigma)	1 $mg.ml^{-1}$
	Ox bile	20% v/v
	L-Cysteine hydrochloride (BDH)	316 $\mu g.ml^{-1}$ (2 $mmol.l^{-1}$)
	$NaHCO_3$ (AnalaR BDH)	10 $mg.ml^{-1}$ (119 $mmol.l^{-1}$)
	in distilled water	

After the eggs were incubated at 38°C in the AGS for 1½ h they were centrifuged at 1000 g for 10 minutes, followed by two washings in physiological saline. AIS was then added till the tubes were almost completely filled, when a further 14 $\mu l.ml^{-1}$ 1.14 mol hydrochloric acid ("AnalaR" BDH) was added. In order to prevent the loss of the carbondioxide so released, the caps were screwed on immediately. The tubes were then incubated at 38°C on a blood cell suspension mixer (Baird & Tatlock), set at 26 rev. min^{-1} at an angle of 30 degrees.

Incubation times varied from 30 min. to 6 hours.

(b) Silverman (1954[a])

The same AGS as for Gallie (1976) was used. AIS was prepared as follows:

as follows:	pancreatin (Sigma)	10 $mg.ml^{-1}$
	trypsin (Sigma)	400 $\mu g.ml^{-1}$
	Cholesterol (BDH)	300 $\mu g.ml^{-1}$
	Sodiumtaurocholate (puriss. Koch-Light)	10 $mg.ml^{-1}$
	Sodium hydrogen carbonate	10 $mg.ml^{-1}$ (119 $mmol.l^{-1}$)
	in distilled water.	

Incubation with AGS followed by two washings was done as described above, then AIS was added and the tubes incubated on the mixer at 38°C for times ranging from 30 minutes to 3 hours.

(c) Heath and Smyth (1970)

The AGS was pepsin (Sigma) 10 $mg.ml^{-1}$ and 10 $\mu l.ml^{-1}$ of 11.4 mol (114.6 $mmol.l^{-1}$) hydrochloric acid ("AnalaR" BDH) in physiological saline.

AIS: pancreatin 10 $mg.ml^{-1}$

Sodium hydrogen carbonate ("AnalaR" BDH) 10 $mg.ml^{-1}$ (119 $mmol.l^{-1}$)

Lamb bile 5% v/v

in distilled water

After 1 hour's incubation at 38°C in AGS followed by centrifugation at 2300 g for 3 minutes but no washings, the AIS was added. The tubes were incubated at 38°C on the mixer for ½-2½ h.

(d) Heath and Elsdon-Dew (1972)

No AGS was used, the tubes with eggs were filled with Heath and Smyth's (1970) AIS in which lamb bile was replaced by a mixture of 2% v/v ox bile, 2% v/v pig bile and 1% v/v rabbit bile. Incubation was from 30 min. to 2 h on the mixer at 38°C.

(e) Wantland (1953)

Incubation in AIS alone, stationary, at 38°C from ½ to 1½ h. This AIS contained pancreatin (Sigma) 5 $mg.ml^{-1}$ and desiccated ox bile (Oxoid) 10 $mg.ml^{-1}$ in physiological saline.

8.1.5. Preparation of the AGS and AIS

These solutions were prepared on a magnetic stirrer, AGS for ½ h at room temperature and AIS at 38°C initially (Set 1) for half an hour but from Set 2 and onwards for about 2 hours. Then they were passed through an Nr 52 filter 11 cm diameter (Whatman) using a Buchner filter. Sodium hydrogen bicarbonate ("AnalaR" BDH) was added as indicated and the pH adjusted to between 7.2 and 7.5. Lamb, pig and ox bile were collected at the local slaughterhouse and rabbit bile in the laboratory. These were passed through an Nr 52 filter (Whatman) and stored in universal bottles at -20°C. The same batch of each bile was used in all experiments. On occasion the bile was sterilised either by autoclaving before storage, or by filtration in AIS, as described under the in vitro procedures.

8.1.6. Pretreatment with AGS replaced by sodiumhypochlorite

In all the experiments where NaOCl was used, this was obtained in the form of "Deosan" (Diversey). It was stored according to the manufacturer's instructions which should provide a stable solution for up to 10 months with a minimum available chlorine content of 10% w/w. A 1/7.5 dilution of this stock solution pH 11.7 was made in deionized distilled water. In a preliminary experiment, it had been observed that the embryophores of *T. taeniaeformis* eggs disintegrated in 4 minutes when exposed to a 1/15 dilution. Hence 2.5 ml Deosan

1/7.5 was added to a 2.5 ml suspension of eggs. After 4 minutes at room temperature, the tubes were filled with deionized distilled water and centrifuged at 1000 *g* for 5 min. This was followed by 2 washes with deionized, distilled water, with centrifugation at 1000 *g* for 10 min. and AIS was then added. The whole procedure was carried out at room temperature, the Deosan dilution being freshly prepared before each experiment.

8.1.7. Modifications

Set 1

Modifications to the described techniques included doubling the concentration of pepsin and comparing pepsin (1:2500 BDH) with the usual type. Ox bile was compared with lamb bile and both autoclaved and non autoclaved bile were used.

Set 2

The oncospheres were agitated by rapidly sucking the AIS up into a Pasteur capillary pipette (Short form 146 mm-Harshaw) and blowing it out, about twenty times. This process, later referred to as "agitating", was carried out each time a technique otherwise gave an unsatisfactory result. If it resulted in an improvement it was then routinely done immediately before each assessment of the H & A rate. In order to avoid such a poorly controlled treatment glass beads ("Ballotini" 3 mm-Jencons) were used in one of the experiments.

Set 3

Gallie's technique (see 8.1.4a) was modified in several experiments, with one or more of the concentrations of pancreatin, trypsin and bile being doubled in the AIS. L-Cysteine hydrochloride was replaced in one experiment by 200μg. ml^{-1} (1.13 $mmol{\cdot}l^{-1}$) L-ascorbic

acid ("AnalaR" BDH) and 100 $\mu g.ml^{-1}$ (0.3 $mmol.l^{-1}$) reduced glutathione (Sigma).

Set 4

The reducing agent and hydrochloric acid as described by Gallie (see 8.1.4a) were sometimes added to the AIS described by Heath and Elsdon-Dew (1972).

Set 5

Segments were stored in the refrigerator in saline with antibiotics from 1 day to 2 weeks after being naturally expelled from cats. Concentrations of both ox and lamb bile were used at 20%, 10%, 5% and 2.5% v/v in distilled water, leaving the concentrations of the other ingredients as described.

Set 6

Eggs from strains B and M were collected from segments naturally passed in the faeces of cats. Segments of strains I and S were obtained by autopsy. A reducing agent and hydrochloric acid were added as described for Gallie (see 8.1.4a) to the AIS of Heath and Smyth (1970) or Heath and Elsdon-Dew (1972). On some occasions pancreatin grade VI (4 x NF Sigma) was compared to the usual grade III. The stains Trypan Blue ("Vital" GURR - BDH) and Neutral Red (GURR - BDH) both at concentrations of 1 $mg.ml^{-1}$ in distilled water were tried as a test for viability. Gallie's AIS was modified by decreasing the concentration of lamb or ox bile to 10% v/v in water.

Set 7

The same modification to Gallie's AIS as described in Set 6 was used. Treatment with sodiumhypochlorite replaced AGS treatment.

Set 8

The AIS was made up in RPMI-1640 (Moore, Gerner and Franklin, 1967) with 25 $mmol l^{-1}$ HEPES buffer (Good, Winget, Winter, Connolly, Izana and Singh, 1966) and L-glutamine (Gibco-Europe). This medium was further modified as described. To this medium 10% v/v sterile foetal calf serum (Gibco-Europe) was added. Where trypsin (1 $mg.ml^{-1}$, 5 $mg.ml^{-1}$ or 10 $mg.ml^{-1}$) pancreatin (1 $mg.ml^{-1}$, 10 $mg.ml^{-1}$) or bile were used, these ingredients were added to modified RPMI-1640 and kept at 39°C on a magnetic stirrer for 2 h. Immediately before use they were passed through MF membrane filters of successively 0.8, 0.45 and 0.22 µm pore sizes (Millipore Ltd.) and the serum then added.

Set 9

Trypsin 10 $mg.ml^{-1}$ and bile 10% v/v were prepared in distilled water and in modified RPMI-1640 with foetal calf serum as previously described.

Set 10

The basic hatching and activating techniques to which other modifications were related was as follows: Pretreatment of the eggs with NaOCL and washes as described above. This was followed by incubation with trypsin 10 $mg.ml^{-1}$ plus ox or lamb bile 10% v/v in modified RPMI-1640 plus 10% v/v foetal calf serum (pH 7.4) prepared as described for Set 8. Immediately after adding this to the onco-spheres they were sedimented by centrifugation at 1000 g for 10 minutes and the supernatant was drawn off, to leave about 2 ml in the tubes, which were then transferred into a water bath at 38°C. Before each assessment of H & A, which was made at regular intervals from 10 min. to 5 h of incubation, they were agitated as described

above. The optimal time of incubation was taken to be the time at which the highest percentage of activated oncospheres was counted. Modifications to this were: AIS consisting of trypsin 10 $mg.ml^{-1}$ and ox bile 10% v/v prepared in distilled water with either L-Cysteine hydrochloride 316 $\mu g.ml^{-1}$ or with $NaHCO_3$ and HCl as described above for the AIS according to Gallie's technique (see 8.1.4.a). Trypsin 10 $mg.ml^{-1}$ and ox bile 10% v/v in either Hanks (HBSS- Gibco-Europe) to which penicillin 100 i.u. ml^{-1}, streptomycin 100 $\mu g.ml^{-1}$ and $NaHCO_3$ 1.75 $mg.ml^{-1}$ was added or in modified RPMI-1640.

Set 11

The basic hatching and activating technique as described for Set 10 was used to compare different batches of eggs, all collected and prepared in the same way but with different storage times at 4°C in physiological saline with antibiotics. One batch was collected from fresh segments, obtained after dosing a cat, without being stored.

8.1.8. Evaluation of hatching and activating oncospheres

Before such an assessment was made, in Sets 1-9, the tubes were taken out of the incubator, centrifuged at 1000 g for 10 minutes and the supernatant drawn off to leave about 2 ml of AIS plus oncospheres, which was kept at 38°C. In Sets 10 and 11 sequential observations were made on aliquots from the same tubes. Agitation, as described for Set 2 was carried out as necessary. Using a pasteur pipette a small sample of the AIS with oncospheres was placed on a microscopic slide and examined without a coverslip under the microscope (Visking) at magnifications of x100 and x400. As previously described a hatched and activated oncosphere was considered to be

an oncosphere which is freed from both embryophore and oncospheral membrane, with visible movement of the hooks unhindered by this membrane and usually showing gland secretions (Plate II.1.g). The loss of an embryophore is referred to as disintegration of embryophoral blocks (DEB) regardless as to whether the oncosphere was set free by complete disintegration of these blocks or due to cracking of the embryophore leaving pieces of the blocks adhering together although the latter was rather unusual. One hundred oncospheres were counted, a distinction being made between visually intact eggs, oncospheres showing DEB only and hatched and activated oncospheres. These results are expressed as percentages, the percentages of hatched and activated oncospheres given being the proportion of the total number of oncospheres counted, not a percentage of those that had lost their embryophore. According to the circumstances several of these counts were made. Any given incubation time means the time before which a tube was taken out of the incubator and processed as described.

8.2 In vitro culture of oncospheres of T. taeniaeformis

8.2.1. Prevention of contamination

All manipulations related to *in vitro* cultures and so requiring sterile conditions were carried out under a laminar air flow cabinet (Microflow). All glassware, filter systems and containers, which were not sterile when purchased or which were washed and reused, were wrapped in aluminiumfoil, sealed with autoclave tape (3 M & Co.) and sterilised by autoclaving at a pressure of 103 kPa for a minimum of 20 minutes, using a portable autoclave (Tuttnauer Co.) and allowed to cool afterwards under the laminar air flow cabinet. Liquid

components of the culture media which could not be sterilised by autoclaving were sterilised by filtration through an MF membrane filter (Millipore) of pore size 0.22 μm, diameter 25 mm fitted with a prefilter (Whatman, GF/A, diameter 21 mm) according to the manufacturer's instructions in a Swinnex filter holder (Millipore). Prior to use this filter system was autoclaved as described above. Care was taken to maintain a constant level of antibiotics throughout the whole procedure. Accordingly the commercially purchased natural and defined components of the culture media were supplemented with 100 i.u. ml^{-1} sodium benzylpenicillin BP (Glaxo Labs. Ltd.) 100 μg. ml^{-1} streptomycinsulphate BP (Glaxo) and initially with 2 μg.ml^{-1} amphotericine B ("Fungizone" Squibb and Sons) though this antifungal agent was omitted in later work.

8.2.2. <u>Culture media and vessels</u>

(a) <u>First Method</u>: initially the procedure described by Heath (1973[a]) was followed. To NCTC-135 medium with L-glutamine (GIBCO-Europe), supplemented with antibiotics was added, an additional 3 mg ml^{-1} (16.6 mmol.l^{-1}) D-Glucose ("AnalaR" BDH), which had been previously dissolved in the same medium and sterilised by filtration. Immediately before use 1% v/v packed rabbit blood cells and 50% v/v foetal calf serum (heat inactivated - GIBCO-Europe) were added. The concentration of foetal calf serum was halved from the second week of culturing onwards. The rabbit red cells were obtained by bleeding a rabbit aseptically from the ear vein into heparin 24 hours before the culture fluid was prepared. The cells were washed 3 times, being centrifuged at 2300 g for 15 minutes in phosphate buffered

saline (PBS Dulbecco 'A' pH 7.3 Oxoid) on each occasion, stored at $4^{o}C$ in PBS overnight and supplemented with 3 $mg{\cdot}ml^{-1}$ D-Glucose ("AnalaR" BDH). For cultures in this medium disposable Nunclon Delta Tissue Culture flasks size 40 ml (Nunc) were used.

(b) Second Method: the culture medium consisted of RPMI-1640 with L-glutamine and 25 $mmol{\cdot}l^{-1}$ HEPES buffer (GIBCO-Europe) purchased in its liquid form, modified as described with antibiotics and sodium pyruvate (GIBCO-Europe). Immediately before use foetal calf serum (heat-inactivated, GIBCO, Europe) was added at the same concentration as described for the first medium. This medium was used in glass Petri dishes (Pyrex-Gallenkamp & Co.) 9 cm diameter. The bottom dish was divided into 2 compartments one small (A) and one larger (B) using a barrier made of either a double layer of nylon bolting cloth of aperture size 20 µm ("Nybolt") or of a single strip of MF filter membrane, pore size 8 µm (Millipore). This barrier, the dimensions of which were 8 x 1 cm, was reinforced by sticking a glass rod along the top long side using Silicone rubber cement (Dow-Corning). The bottom edge and the two sides of this barrier were also stuck to the bottom plate of the petri dish with the Silicone cement. After allowing the cement 24 hours to harden, the barrier was checked for leaks with water, dried and sterilised by autoclaving as described. A layer of Silicone grease ("Silicone Stopcock grease" Dow-Corning) on the rim of the top plate provided a tight seal with the bottom plate. The oncospheres were cultured in the larger compartment. All media were stored at $4^{o}C$, except sodium pyruvate which was held at room temperature and foetal calf serum at $-20^{o}C$. Before use the media were allowed to warm up to $38^{o}C$ in a warm water bath. Defined media were used within a month of being purchased.

8.2.3. Preparation of oncospheres

Approximately 5 000 eggs were used, divided into 4 sterile tubes. The oncospheres were at first hatched and activated by the method of Gallie (see 8.1.4.a) modified as described for Set 2, and later using the basic hatching and activating technique described for Set 10. All solutions were sterilised by filtration. Cultures were only undertaken using batches of eggs for which the proportion of hatched and activated oncospheres reached 10% or above. The oncospheres to be cultured were washed 5 times in culture medium without blood cells, with centrifugation at 1000 g for 10 minutes, and then transferred into the culture vessels at a maximum concentration of 2000 oncospheres (i.e. total number of intact eggs, DEB's and H & A oncospheres) per culture vessel. Medium was added up to 15 ml in the Nunclon flasks or 25 ml in the petri dishes.

8.2.4. In vitro culture procedures

Cultures were kept stationary at 38°C. The gasphase was air, and the media were changed every 48 hours. For the Nunclon flasks this was done by placing them in upright position 3 h before each change, allowing the oncospheres to sediment, drawing off 10 ml of the supernatant and replacing it with fresh medium without rabbit blood cells. Changing the media in the petri dishes was simpler as the aperture size of both the nylon and MF membrane was small enough for surface-tension to prevent the medium passing through the section as long as it remained dry on one side. By the first change, after 48 hours in culture, the diameter of the growing oncosphere should have exceeded the 20 μm and the culture medium could be allowed to flow through the barrier. This was done by gently touching the membrane on the dry side with a sterile hard object or by wetting it

with culture medium. Once the medium flowed through, up to 20 ml could be removed from compartment A with a sterile syringe and replaced by fresh medium introduced into compartment B. If desired this procedure could be repeated several times to rinse the oncospheres with no risk of losing them. By the next change, the fluid being in compartment A was transferred to B, the medium flowing then from B to A was removed. At regular intervals, usually when changing the media, the oncospheres were examined using an inverted microscope (Nikon) to which a camera was attached. Cooling of the cultures could not be avoided during the microscopic examination.

Chapter 9 Hatching and activation of the oncospheres

As described in the review of literature many factors play a role in the hatching and activating process. Accordingly there were many variables in the experiments and these will not be discussed individually. Instead the results have been aggregated into sets of similar procedures and according to their relevance to the main aim of these experiments, namely devising a reliable procedure for obtaining sufficient activated oncospheres in order to proceed with *in vitro* culture studies. The experiments grouped together in numbered sets were not necessarily done at the same time or with the same batches of eggs.

Set 1

Three techniques were compared: Gallie (see 8.1.4.a), Heath and Smyth (1970), Silverman (1954[a]), for incubations from ½ to 4 hours. None of them gave DEB at higher levels than 2% and the rest of the eggs looked microscopically intact. No hatching and activating was achieved. The use of lamb bile instead of ox bile, with these autoclaved or not, did not appear to affect these results. Nor did the use of pepsin from either commercial sources used at concentrations of 10 $mg.ml^{-1}$ or 20 $mg.ml^{-1}$.

Set 2

When the eggs, after treatment by Gallie's procedure with incubation for 5 hours in AIS, were vigorously agitated, about 50% of them showed loss of the embryophore. Hatching either did not occur or did so only at low levels, reaching 11% on only one occasion . Pretreatment with AGS varying from 1 h to 18 h or no pretreatment did not seem to influence these results. Loss of the embryophore either

did not occur or was not above 20%, when glass beads were placed in the tubes during either AGS or AIS incubation on a roller, or when the AGS and AIS solutions were passed over a 0.22 μm pore size membrane filter prior to use. This loss of effect was overcome by leaving the AIS on a magnetic stirrer at 38°C for about 2 h before use. It was also easier then to pass the solution through the filter after which it had the same effect as a conventionally filtered AIS. This prolonged preparation resulted as a whole in a better DEB and occasionally higher, but still unsatisfactory proportions of H & A oncospheres. This was rarely more than 30%, after incubation times varying from 2 to 5 h. In contrast, agitation did not influence the results obtained with Silverman's (1954[a]) technique.

Set 3

In these series of experiments several modifications of the technique described by Gallie (see 8.1.4.a) were tried. If the concentration of pancreatin, trypsin or bile was doubled individually and of all three components together and the rest of the technique being the same as described by Gallie, there was no difference after incubation of up to 5 h. DEB remained about the 50% level and H & A low, i.e. approximately at 10%. However, when L-cysteine hydrochloride was omitted from the intestinal solution, no DEB occurred. The action of the cysteine could however be replaced by ascorbic acid and reduced glutathione. Doubling the L-cysteine hydrochloride or adding it to ascorbic acid with reduced glutathione gave no further improvement. If the sodiumhydrogen carbonate and subsequent addition of hydrochloric acid was omitted, the eggs remained intact even after several hours of incubation.

Set 4

The technique described for T. taeniaeformis eggs by Heath and Elsdon-Dew (1972) had no visible effect on the eggs, no DEB occurred and neither could physical stimulation nor prolonged incubation induce it. However agitation and incubation for up to 2 h together with adding a reducing agent and hydrochloric acid to the AIS gave some improvement in that an increased percentage of oncospheres showed DEB (from 1 to 70%) and H & A (from 0 to 17%).

Set 5

Whole segments, collected from faecal samples were predigested in AGS before being submitted to the AIS, both solutions prepared according to Gallie (see 8.1.4.a). After the pretreatment in AGS, all the segments were digested, but much debris remained. However, after 2 to 3 h incubation in AIS followed by shaking only 10 to 40% of the eggs were still visually intact, while hatching and activation seemed to be generally improved, being usually between 10 to 20% but sometimes even up to 40%. However it was also noticed that activated oncospheres did not seem to be very resistant to the AIS and their number decreased after 1 to 2 h. Several concentrations of bile were used in the AIS and it appeared that it made no difference whether the concentration was 20 or 10% v/v for either lamb or ox bile. Lower concentrations of 5 and 2.5% v/v seemed to affect both DEB and H & A, requiring longer incubation times as the events occurred at a much lower rate.

Set 6

Various strains of T. taeniaeformis were compared using various methods. For strain S: the method as described by Gallie (see 8.1.4.a)

almost always resulted in a DEB of all the eggs after they had been incubated in the AIS for 1 to 3 h without agitation and up to 8% of the oncospheres were hatched and activated. Agitation resulted in an increase of the latter, occasionally up to 18%. The same results were obtained if AGS pretreatment was omitted. When using the techniques described by Wantland (1953), Heath and Smyth (1970) and Heath and Elsdon-Dew (1972), all oncospheres again lost their embryophore but few were activated. The use of pancreatin grade VI (Sigma) or grade III (Sigma) or adding reducing agent made no marked difference. Adding hydrochloric acid to the AIS described by Heath and Elsdon-Dew (1972) gave better but inconsistent results, (from 11 to 23% hatching and activation). Both trypan blue and neutral red were tried as indicators of activation and both stained about the same percentage of oncospheres (e.g. about 10%) of specimens taken from the same tube, with no other visual indication that the stained oncospheres were different in viability from the non-stained. For strains I and M, only the technique of Gallie (see 8.1.4.a) modified by using 10% v/v sheep or ox bile instead of 20% ox bile, was tried. The I strain showed loss of the embryophores of all the eggs, while 3 h incubation in AIS and agitation yielded 25% active oncospheres. Strain M behaved rather like the B-strain when using the same method, in so far as not all the eggs had lost their embryophoral blocks. Agitation again seemed to raise the percentage of activated oncospheres, which occasionally reached 44%, but contrary to the result with strain B agitation had no apparent effect on the DEB of the M-strain eggs.

Set 7

AGS pretreatment was replaced by treatment with sodium hypochlorite followed by 1-2 hours (Gallie, see 8.1.4.a) AIS treatment modified

by using 10% v/v lamb or ox bile and agitation before counting. After 4 minutes NaOCl treatment all the embryophores had disintegrated and later the percentages of activated oncospheres was over 30% and up to 58%, though different batches of eggs often required different incubation times in AIS. Again, once the oncospheres became active they were vulnerable to destruction by the AIS, but more consistent results were obtained. Agitation seemed to improve both hatching and activation rates. The sequence of photographs in Plate II.1.a.b.c.d.e.f.g shows how this happens. The loss of the embryophore occurred, regardless of whether the eggs were freshly expelled or stored for up to 2 months in saline at 4°C, for the two strains tested, namely the B and M strain. Several samples of a batch of T. saginata eggs were tested using the same technique and eggs which had been stored at 4°C for two weeks. The influence of NaOCl on these eggs appeared to be the same as on T. taeniaeformis eggs, but 15 to 25 minutes exposure to NaOCl was necessary to achieve DEB of nearly all the eggs.

Set 8

All embryophores were disintegrated with NaOCl. Then followed incubation for 3 hours followed in modified RPMI 1640 with 10% v/v foetal calf serum alone or with trypsin and sometimes pancreatin. Activated oncospheres were present only when RPMI 1640 + FCS + trypsin 5 $mg.ml^{-1}$ and 10 $mg.ml^{-1}$ was used at levels of 8% and 36% respectively. Combining pancreatin with the trypsin at various concentrations gave no additional activation. When 10% v/v ox bile was included in the RPMI 1640 with 10 $mg.ml^{-1}$ trypsin and 10% v/v foetal calf serum, half of the oncospheres were hatched and activated after 2 hours incubation. Lower percentages of bile were reflected in lower activation rates. When the trypsin was omitted from this latter mixture,

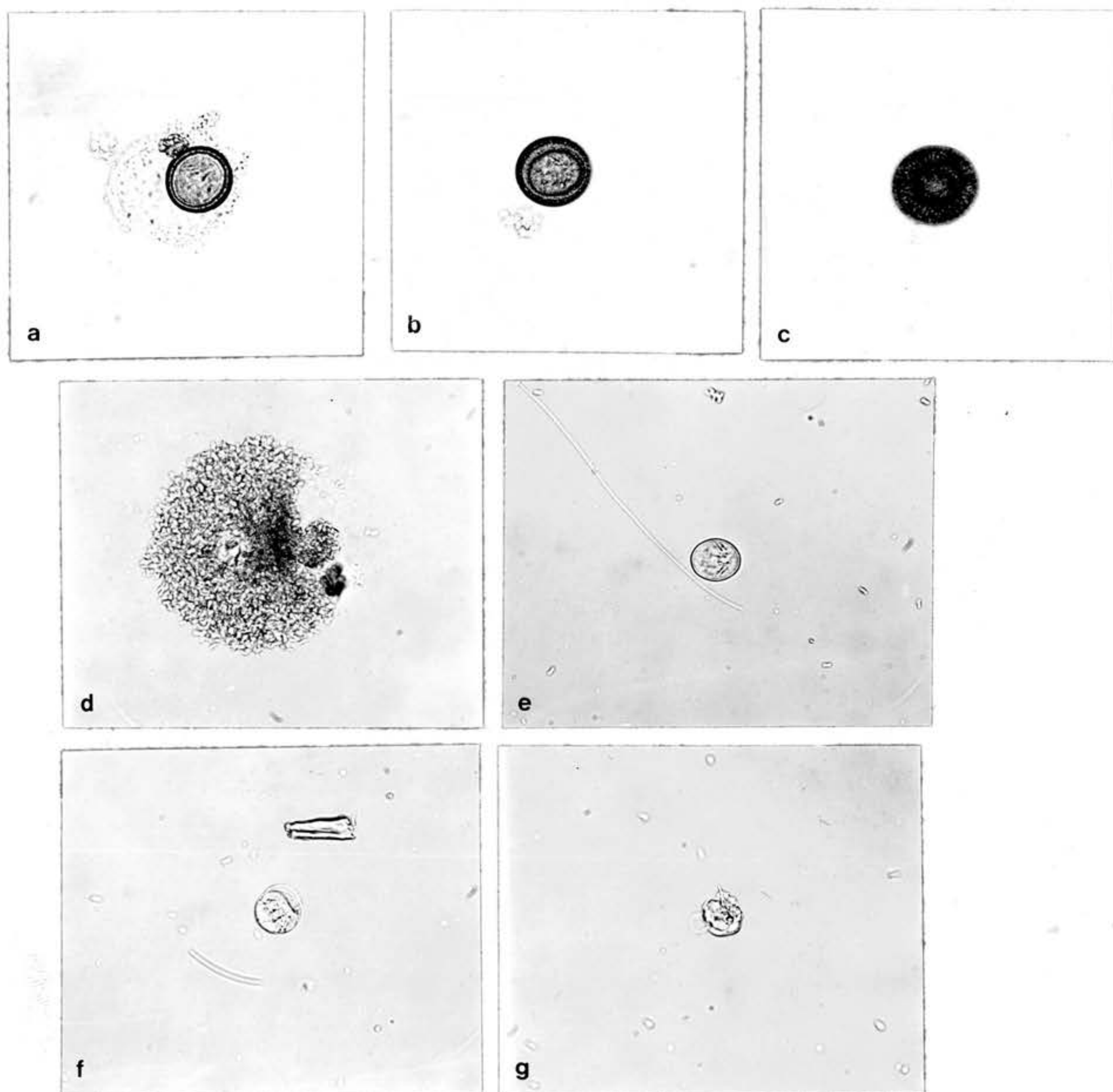


Plate II.1 *Taenia taeniaeformis* (Strain M) (Transmitted light x 340)

a. Egg with remnants of capsule and outer envelopes.

b,c: Under the influence of sodiumhypochlorite, the embryophore darkens and thickens until

d. the embryophoral blocks are dispersed after about 4 minutes

e. leaving an oncosphere within its membrane.

f. Activated oncosphere in the process of hatching under the influence of trypsin and bile.

g. Hatched and activated oncosphere, with moving hooks and visible gland secretions.

several oncospheres showed activity but none of them hatched from the oncospheral membrane.

Set 9

Treatment with AGS (Gallie, see 8.1.4.a) followed by incubation for up to 3 hours with trypsin 10 $mg.ml^{-1}$ and bile 10% v/v in distilled water had no visible effect on the eggs. Sodium hypochlorite treatment followed by the same AIS solution also prepared in distilled water gave the expected loss of all embryophores but none of the oncospheres were activated. In the same experiment when peptic digestion was replaced by NaOCl treatment followed by 2 hours incubation in AIS (Gallie see 8.1.4.a, modified by using 10% v/v ox bile) and agitation before counting, there was a higher proportion (58%) of H & A oncospheres. In both cases within an hour or less the number of activated oncospheres which could be observed was reduced by half when they were left in the AIS. In another experiment pretreatment with NaOCl followed by AIS after a set incubation time (3 hours) gave the following response in terms of activation: trypsin + bile in RPMI + FCS (30%), trypsin + bile in H_2O (0%) AIS (Gallie, see 8.1.4.a) with 10% v/v ox bile prepared in water (12%), prepared in RPMI + FCS (4%).

Set 10

Pretreatment with NaOCl and AIS treatment with trypsin 10 $mg.ml^{-1}$ and ox bile 10% v/v in modified RPMI 1640 and 10% v/v FCS not incubated on a rotator but left stationary at 38°C in a water bath gave activation rates of 50% or above with several batches of eggs. This method allowed regular counts to be made at predetermined intervals on the same tube, making the determination of the optimal incubation

time more accurate and allowed for observation of the effect of AIS on H & A oncospheres. Pretreatment with NaOCl followed by AIS treatment with trypsin 10 $mg.ml^{-1}$ and ox bile 10% v/v prepared in water gave no activation. Adding L-cysteine hydrochloride had no influence but if $NaHCO_3$ + HCl was added the activation level was 20% after an optimal time of 3½ hours. If trypsin and bile were prepared in RPMI or HBSS to which 1.75 $mg.ml^{-1}$ (21 $mmol.l^{-1}$) sodium hydrogen bicarbonate was added, apparent activation rates for RPMI were 22% after 1 hour, but another hour later it was down to 8%. For HBSS the activation rate was 12% after 1 hour but half an hour later only 4% was recorded. Preparation of these in 10% v/v FCS with RPMI gave levels of 56% also after 1 hour incubation, which was maintained for at least 2 hours afterwards.

Set 11

Eggs of different ages of two strains were compared using NaOCl pretreatment with AIS consisting of modified RPMI 1640 with 10% v/v FCS plus trypsin 10 $mg.ml^{-1}$ and ox bile 10% v/v. For some batches a comparison was made with estimations of viability by morphological criteria suggested by Heath, D. (personal communication 1977). Results are given in Table II.1.

Table II.1 Effect of storage of _Taenia taeniaeformis_ eggs on hatching and activating

Period of Storage	Strain	Percentage of eggs showing: DEB	H&A	Optimal incubation time (hours)	Visual assessment of viability (%)
Fresh (autopsy)	B	100	53	2	-
1 week	"	100	54	5	18.2
1 month	"	100	50	2	-
1 month	"	99	43	$2\frac{1}{2}$	-
2 months	"	100	91	4	18.5
1 week	M	100	59	5	22.5
2 weeks	"	100	32	4	17.4
2 months	"	100	20	4	-
2 months	"	99	34	4	7.7

Chapter 10 In vitro cultivation of T. taeniaeformis oncospheres

First Method

This method was tried on 7 different occasions, cultures being maintained for 3 weeks. No signs of development were noticed in most of these cultures, although until the 6th day the oncospheres, which had not escaped from their membranes, were still showing movement. The only exception was in one culture in which an organism, approximately 50 µm in diameter was seen. It appeared as a mass of cells with no recognizable structures. It was not seen again after day 20 of culturing.

Second Method

This method was tried on 5 occasions. No development was seen on two occasions, as the cultures were abandoned when yeast contamination became apparent shortly after starting. In three other instances some development was achieved. On two of these occasions photographs were taken from several cultures which consistently showed a similar type of development. Nevertheless the full development process described by Heath (1973[a]) was never obtained. In these cultures by day 4 a number of organisms started to show post-oncospheral reorganisation, in that they doubled in size, were floating with a slit-like cavity and gentle contractions could be seen. The appearance described by Heath (1973[a]) as "a ball of cells" was seen and by day 8 (Plate II. 2.a.) some larvae clearly showed cavity formation, although not all to the same extent. On day 10 (Plate II.2.b.) some larvae showed an irregular surface, but it was not clear whether this was due to droplets formed by secretion or was part of the layer of microvilli described by Heath (1973[a]), but irregularly formed in this case. The

contractions of these larvae were more noticeable, changing the shape of the larvae frequently. On day 12 more of the cell mass seemed to be aggregated on one side. Apart from the usual contractions some pseudopodia-like movements could be seen (Plate II.2.c.), but it was not clear whether these cytoplasmic protrusions were merely the result of a collapsed bladder. In one petri-dish culture yeast contamination became apparent at this stage, so an extra 2 $\mu g.ml^{-1}$ fungizone was therefore added. The larvae then started to show very intensive movements both contractions and described cytoplasmic protrusions. Two days later these movements ceased. It was therefore decided to omit fungizone from the other cultures and following the introduction of sodium hypochlorite into the hatching and activating process, yeast contamination was not seen again. On day 14 (Plate II.2.d.) it was noticed that some of these protrusions were bigger and although still moving they had a permanent character at that time. However, two days later the appearance of the larvae changed in that some of them had rapidly increased in size, measuring over 200 μm across (Plate II. 2.e.). They were now more or less spherical with a conical protuberance, and a rather dense cellular mass. Others still retained the clear cavity, had an irregular shape and remained smaller in size but all of them were still showing contractions. On day 21 (Plate II.2.f.) the contractions were less strong and had more of a wave-like appearance. From then on little further development could be seen, but it was thought interesting that on day 25, one of these larvae (Plate II.2.g.), in which the distinction between cavity and solid part could be seen, showed an in-and-out movement of the cellular mass, which was thought to be the scolex anlage. Between the 21st day and the 30th day most of these organisms seemed to disappear, probably because they

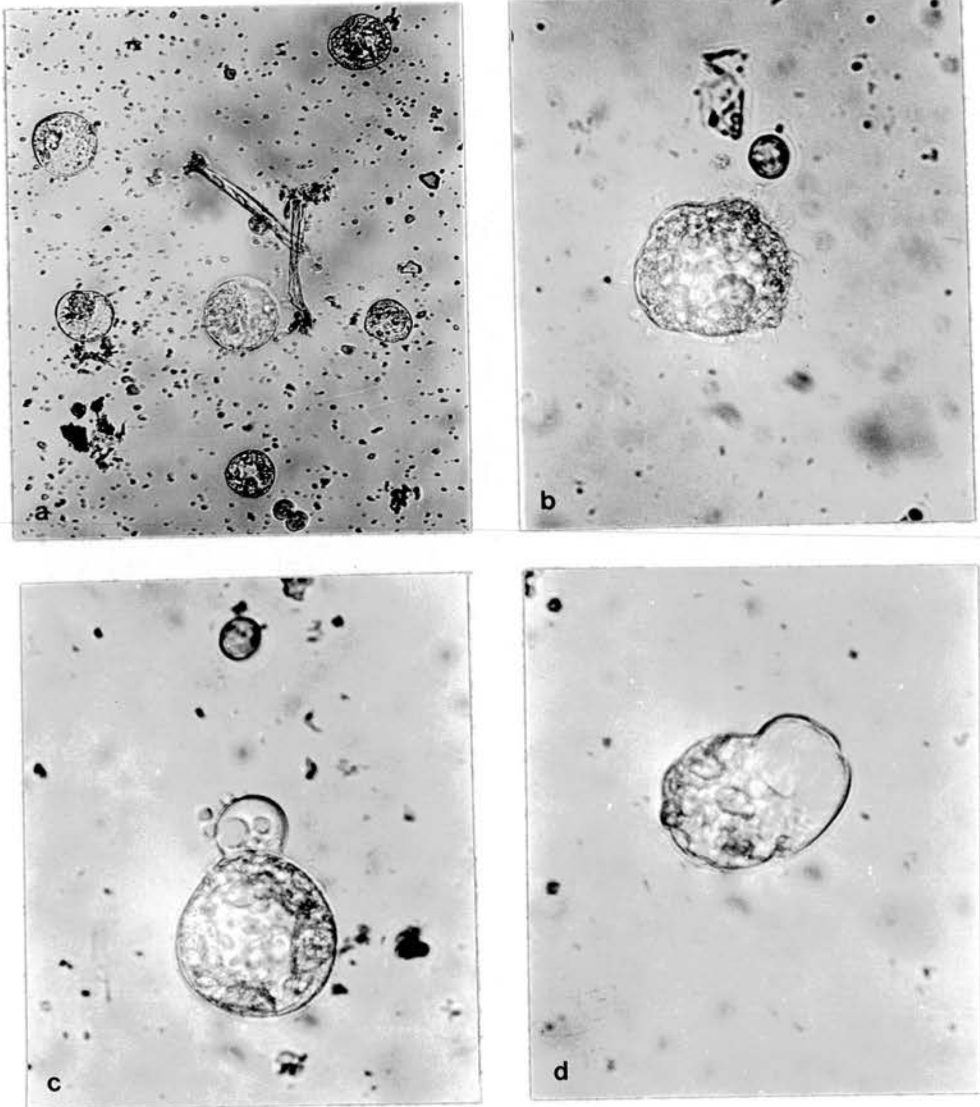


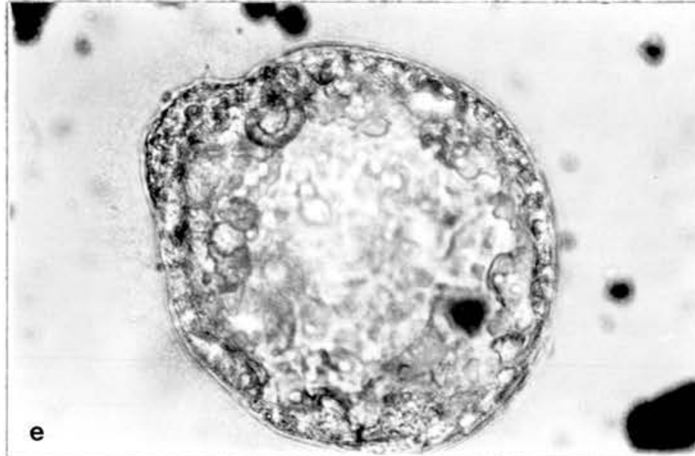
Plate II.2. Development of *Taenia taeniaeformis* oncospheres (strain B) *in vitro.*
(Transmitted light a x 150
b , c, d x 300)

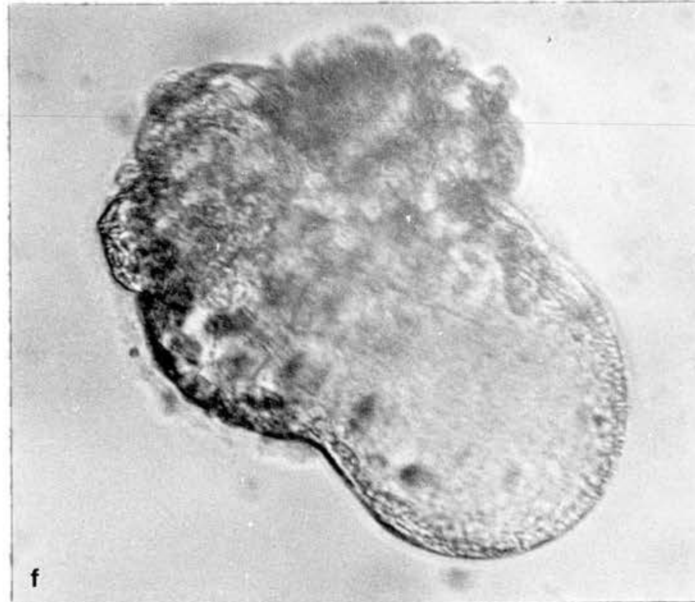
a. Day 8 : Post oncospheral reorganisation with cavity formation. Also shows the marked variation in development seen at this time.

b. Day 10: Irregular surface with possible layer of microvilli.

c. Day 12: The shape of the larvae is constantly changing, showing movements superficially resembling pseudopodia formation.

d. Day 14: Slow contraction which do not affect the shape of the larvae as much as before.





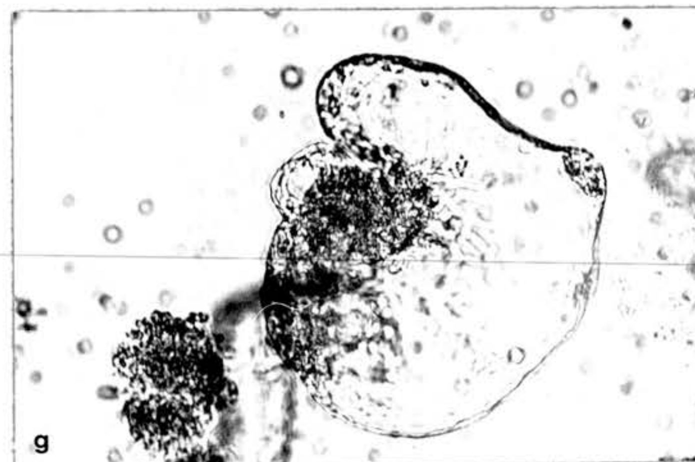


Plate II.2 (Contd)

(Transmitted light x 300)

e. Day 16 : Increase in size. The conical protuberance is characteristic of this stage.

f. Day 21 : The cellular mass becomes denser and wave-like contractions can be seen over the surface.

g. Day 25 : There is a clear separation between the cavity and the dense cellular mass, which showed invaginating and evaginating movements.

died and disintegrated rapidly. From this period on, transparent spheres were often seen floating at the surface of the cultures but it was not clear whether these had anything to do with these larvae or were merely the result of their breakdown. However they were never seen in the absence of larvae - i.e. in compartment A and may be the same structure as were observed by Heath (1973[a]).

The above description and series of photographs are given in the order in which events are thought to have occurred because of similarities between cultures in different petri-dishes at the same times. However in one culture dish several of these developmental forms could coincide, perhaps due either to a delayed start in the development of some oncospheres, or to inhibition of further development of others. On balance, the former explanation is preferred, since disintegration appeared to occur quite rapidly. Exact counts of developing oncospheres were not made but it was estimated that after 2 weeks in culture, between 5 and 10% of the hatched and activated oncospheres had shown further development.

Chapter 11 DISCUSSION AND CONCLUSIONS

11.1 Hatching and activating of oncospheres

It appears from both the literature and the results described above that it is difficult to determine the exact necessitities to obtain high and consistent hatching and activation rates with T. taeniaeformis. As pointed out by Gemmell (1977) and various other authors, that there may be much heterogenicity amongst different batches of eggs and sometimes even in the same batch originating from the same segment, so that they give a different response to apparently similar methods. Even the quantity of eggs is reported to be important (Heath and Smyth,1970). Hence it seems obvious that a single method, which works for all mature eggs, from different species is not yet obtained.

In the experiments, obtaining consistent DEB was the first problem. Related to this, the longer it takes for that disintegration to occur, the longer the delay will be for the next step to occur,as it does not appear as though AIS can have any effect on the embryo and its membrane as long as the embryophore is protecting it. For the Belgian strain of T. taeniaeformis DEB was helped by mechanical agitation, as Silverman (1954[a]) already reported for T. saginata eggs. On the other hand the other strains like I and S gave no difficulties in this respect, with strain M being in an intermediary position in that only partial DEB occurred which was not much enhanced by agitation. This difference in behaviour was not consistently associated with the strain differences described in Part I.

It seems unlikely but cannot be excluded that the differences in manipulation of eggs from these strains may account for the differ-

ences in response to AGS: eggs from strains I and S were collected by autopsies, while eggs from the M and B strains were obtained from segments freshly collected from cats' faeces. If strain differences between T. taeniaeformis are apparent in the H & A behaviour of the eggs, this will be an extra complication in comparing results of different authors, yet on the other hand it may provide a useful tool for studying the hosts selectivity based on the action of their intestinal enzymes and bile, a possibility discussed by Silverman (1954[a]), Berberian (1957), Smyth (1963) and by Beveridge, Rickard, Gregory and Munday (1975). However, despite the differences in the DEB, hatching and activating the oncospheres was a common problem for all these strains. From the experiments, it appears that rapid DEB tended to help the further process, but the question whether AGS is required is not answered.

The improved H & A response after AGS was used to digest the segments tends to confirm the findings of Jones, Segarra and Wyant (1960), that eggs fresh from segments hatch faster than those which have been stored, although in this case, speed was not the factor determined, but percentage of H & A oncospheres. Although the use of NaOCl is not a physiological approach - and therefore subject to criticism (Gönnert 1970) - it does at least allow the H & A problem to be studied separately from DEB, provided that the sterilising and oxidising activity of the hypochlorite does not affect the oncospheres during the short time of contact. Despite the incomplete character of the experiments, due to uncontrollable limitations, some firm observations can be made. Thus, bile appears to be essential as has been reported by most if not all authors, and although it may be

replaced by bile salts, unfortunately as Smyth (1962[b], 1963) points out, these salts are often impure and may influence the H & A. When bile was used without enzymes it stimulated activity but seemed to fail inducing the hatching. This may support Orlov and Kosminkov's (1972) suggestion that the hatching from the oncospheral membrane is not caused by active rupturing due to the hook movements but by a passive digestive process. However it may also be that the presence of the enzymes allows a better penetration of bile followed by a higher and more adequate activation.

The fact that it appears to be trypsin rather than pancreatin which stimulates the H & A may be confirming the findings of Wikerhauser Žuković and Džakula (1971) but it is not possible to draw definite conclusions of this.

Reducing agents seem to improve the H & A, as was found by Gallie (personal communication, 1976) for cysteine with T. saginata eggs. Soulsby (1965) also mentioned their beneficial effect on exsheathment of third stage nematode larvae. Clegg and Smyth (1968) described the stimulating effect of CO_2 and reducing agents on the excystment of Fasciola hepatica metacercariae. Both this and the absence of $NaHCO_3$ may explain the ineffectiveness of trypsin and bile prepared in water as opposed to other media.

The presence of $NaHCO_3$ in the technique of Heath and Elsdon-Dew (1972) had little influence on the eggs of the B-strain until HCl was added. Thus for the B-strain, apparently more than for the others, CO_2 seems to act on the embryophore. Whether the same applies to the oncospheral membrane is not clear, but it may be that it is the release of CO_2 by agitating the solution that has the effect on the

hatching and activation. As stated above "agitation" is a rather subjective action and it would be preferable if it could be replaced by a more controllable tool, perhaps by bubbling CO_2 gas through the AIS solution.

A very decisive factor in the estimation - as opposed to the efficacy of H & A seems to be the survival of oncospheres afterwards. The harmful effect of the AIS on hatched oncospheres, already reported by Silverman (1955), and by Orlov and Kosminkov (1972), may lead to considerable underestimation of the degree of H & A especially in experiments with longer incubation times. To obtain more reliable estimates of viability various workers, including Coman and Rickard (1977) have preferred vital staining, since it shortens the incubation time in AIS. However in the experiments this did not prove to be of much help and the use of such stains was abandoned. Parnell (1965) does not mention whether it was for the same reason that he stopped using them. On the other hand, as Gemmell (1977) pointed out, it may well be that only fully competent oncospheres resist destruction by AIS, as likewise, NaOCl may be eliminating immature or degenerated eggs.

The protective action of serum (Silverman 1955, Orlov and Kosminkov, 1972) may be the only explanation of the higher levels obtained in the last sets of experiments. Although the technique, to which is referred as the basic technique in Set 10, gave the best results, there may seem to be some contradiction in that serum is used together with trypsin. The inactivation of trypsin by serum is well recognized in tissue cultures as described by Wallis, Ver and Melnick (1969) and by Paul (1975). However, trypsinisation in tissue

cultures is usually done at much lower concentrations of this enzyme. So either the excess of trypsin overcame the enzyme inhibiting activity of serum, the inhibition may be prevented by the heat inactivation of the serum, either the breakdown products of trypsin are still active for this purpose, or the protective role of serum lies in the inactivation of noxious enzymes present together with the trypsin or the bile or in the extra buffering effect on the solution. Whatever the mechanism, serum alone or trypsin in water had no effect whereas the combination of both of these was very effective.

11.2. In vitro culture of oncospheres

The growth obtained for T. taeniaeformis in these experiments did not achieve the level of development described by Heath (1973[a]) or the early in vivo development described by Singh and Rao (1967) and Crusz (1948) for the same parasite.

After 11 days of in vitro culture Heath (1973[a]) obtained fragile thin walled cysts, which were soon showing rapid spiralling movement. Although the development of muscle systems, and what was believed to be scolex anlage might have occurred at about the same time in the present series of experiments, the size of the larvae in these cultures was much smaller (4-6 x) than these described by Heath (1973[a]).

Even when the development during the first 10 days is compared with the descriptions given by Heath and Elsdon-Dew (1972), they are still under-sized. Furthermore, the rapid spiralling movement was never seen and the presence of a layer of microvilli was not clear, though this might have required other microscopic procedures to the simple inverted microscope used in this study.

It is possible that these differences are caused by the defined components of the media, but the composition of these media relative to the needs of the organisms remains a problem which is usually only solved in an empirical way (Heath and Smyth 1970). Furthermore, Heath and Lawrence (1976) found no substantial difference, when using RPMI-1640, NCTC 135 or McCoy's 5A in in vitro cultures of Echinococcus granulosus. HEPES is one of the few buffers described by Good, Winget, Connolly, Izana and Singh (1966) as being chemically satisfactory for use in culture media. With a pKa of 7.31 at 37°C, the optimum buffering capacity is just at the desired pH range. Therefore Paul (1975) found that the use of this buffer made it unnecessary to gas the cultures with CO_2. Although its buffering capacities may well be overcome by the high $NaHCO_3$ content in RPMI-1640 (Gibco), gassing the media was not carried out but cultures were maintained in a closed vessel and, under normal conditions, no drastic pH changes were noticed. Nevertheless a reduced $NaHCO_3$ content in RPMI-1640 might have been a more logical approach. HEPES is not supposed to be cytotoxic (Shipman 1969) and it can support higher rates of protein synthesis in bacterial cultures than Tris or phosphate buffer (Good, Winget, Winter, Connolly, Izana and Singh 1966) but as it contains a piperazine derivate, it may have had harmful effect on the helminths though piperazine itself is not particularly cestocidal. Nevertheless the fact that in these experiments oncospheres grew better when cultured by the second method may indicate that neither the use of RPMI-1640 nor HEPES had an adverse effect on the oncospheres, though to prove that, both systems should run simultaneously. Equally, the addition of rabbit blood cells did not seem to stimulate oncospheres to grow in the first method. There was some growth when they

were not used and this tends to support the observation by Heath and Lawrence (1976) that growth of E. granulosus oncospheres in cultures without blood cells was comparable to those where they were present for the first 2 weeks of culture. However the presence of the blood cells improved the growth rate thereafter. Perhaps the beneficial effect of rabbit blood cells combined with foetal calf serum is not so marked in the very early development than it is at a later stage. On the other hand, the probable variability of the undefined compounds of natural origin may also be responsible for inconsistent results.

It seems unlikely that the two different types of vessels used in these experiments are responsible for their different outcome. With the petri dish system it was technically easier to change the media and even to rinse out the eventual metabolites and catabolites, which may have a harmful or beneficial effect, without apparently stressing the organisms or losing some of them than with the Nunclon flasks. On the other hand, it was more difficult to take the petri dishes for microscopic observations. The Nunclon-flasks were handier and quicker for this purpose and hence less vulnerable to cooling.

A possible explanation for the discrepancies between both culture systems might be the number of eggs and hatched and activated oncospheres. Silverman and Hansen (1971) acknowledge the existence of an optimal larval concentration as an influence on in vitro development. However Voge (1978) remarks that the question is still open as to whether a culture vessel should be incubated with thousands of oncospheres, on the supposition that the death of the majority benefits the few survivors. In the literature (Heath and Smyth, 1970

Heath and Elsdon-Dew, 1972 and Heath 1973[a]) the number of cultivated oncospheres at the time of setting up the cultures is often not stated but, considering the fact that in these reports, segments were collected from autopsies or after purging hosts and, given the good H & A reports, the number of activated oncospheres introduced to the cultures may have been much higher than in the above experiments and the proportion of activated oncospheres versus non H & A was higher. In any case, Heath's (1973[a]) optimum development rate of 0.1 ml of medium for each developing oncosphere, counted after 24 hours of in vitro culture is a concentration which could not frequently be achieved so as to allow strictly comparable studies. The number of activated E. granulosus oncospheres, reported by Heath and Lawrence (1976), where 100 activated oncospheres per ml medium were used or the 40 000 hatched and activated T. ovis oncospheres per culture tube used by Rickard and Bell (1971[a]) were numbers which could not be emulated in the study. The fact that the cultures of Heath and Lawrence (1976) and of Rickard and Bell (1971[a]) were successful - although the latter was only maintained for 8 days - may indicate that, at least for these parasites, a high initial density of oncospheres is not harmful. If such a high density is beneficial to the survivors, this may explain the difference in results.

Both the in vitro work and the attempt to improve hatching and activating methods were done simultaneously. Hatching and activation and the survival of the activated oncospheres during the pre-culture period, were better for the latter cultures than they were at first. When sodium hypochlorite was used, not only was the hatching and

activation proportion increased but these cultures proved to be more successful. Furthermore, contamination, in particular from yeasts, ceased to be a problem after the use of NaOCl despite the fact that fungizone was omitted from the last cultures, which suggests that yeasts were introduced in the cultures from an early stage and perhaps only temporarily kept under control by the anti-fungal agent present in the saline in which segments and eggs were kept prior to processing.

PART III *In vitro* culture of metacestodes

Chapter 12 INTRODUCTION AND REVIEW OF LITERATURE

The main aim of the *in vitro* work described in this and the previous Part was the study of the metabolites formed. It can be seen from Part II that this study, starting with oncospheres, had several limitations. Thus the number of growing oncospheres was unsatisfactory due to a limited supply of eggs and also to the difficulties encountered for a long time with hatching and activating. Furthermore the necessary media contained relatively high quantities of serum. Therefore attention was turned to culturing fully developed metacestodes. Originally, and for some purposes continuously, there was a need to use a defined medium. Successful development of helminths in a defined medium is still at an early stage, especially for taeniids, and the *in vitro* work with *Taenia taeniaeformis* metacestodes was largely limited to maintenance. Nevertheless the very interesting subject of *in vitro* growth towards the adult stage was studied as far as time and availability of material allowed.

12.1 General aspects of *in vitro* culture of helminths

Transferring helminths from their natural habitat in an *in vitro* system, has been tried for several decades and has received increased attention as the range of possible applications was realised. Indeed the latter have often been the motivation for the work rather than a consequence of it. Furthermore, whereas the main aim of these studies often lay originally in obtaining a better knowledge into the biology of the parasite, it was soon realised that *in vitro* studies could help to provide an insight into the host - parasite

relationships. Weinstein (1958) stated some of the possibilities of *in vitro* studies, namely that they could offer a better understanding of the pathology of helminthiases, specific nutritional requirements could be revealed and an explanation could be found as to how competition for these nutrients within the host may produce clinical signs.

It was soon clear that, in order to abandon empirical methods with the associated risks of unjustified extrapolation from *in vitro* results to *in vivo* circumstances, a better fundamental knowledge of the biochemistry of both the parasite and the host was imperative. Hence numerous reports, often using short term *in vitro* maintenance of whole parasites or parasitic tissues, have been produced and have sometimes led to direct application in the development of anthelmintics, or at least to a better understanding of their action, with the aim of improving their activity and reducing their toxicity for the host (Saz 1970 and Van den Bossche 1976). In recent years, the study of the excretory and secretory products released by parasites *in vitro* has received more attention, as already mentioned in Part II, such products have somtimes been found to be worthwhile considering as diagnostic and protective antigens (Denham 1967).

Since *in vitro* techniques cover such a wide field with such a variety of applications, it was soon necessary to define the terminology: Baker and Ferguson (1942) used the term axenic culture indicating that no other organism was present than that being cultured. Later, Dougherty (1959) added terminology for when one or more associated organisms are present, and also discussed the equally important terminology describing the components, which made up the

media. Dougherty (1959, 1963) proposed the term holidic for a medium, consisting entirely of chemically defined molecules, meridic for when the medium contains both defined and undefined components, and oligidic, for when the entire medium or most of it consists of crude materials, or uncertain status as nutritional requirements. Smyth (1962[a]) stated the aim of axenic cultures to be to devise an effective holidic culture medium. Silverman (1965) and Silverman and Hansen (1971) stressed this aim and pointed out that because many ingredients for cultivating helminths in vitro are taken over from tissue cultures, supplemented with undefined complex material necessary for successful differentiation, they may have limitations due to difficulties in reproducibility and instability, often compounded by ill-defined specifications.

Clegg and Smyth (1968) and Smyth (1976) made a distinction between in vitro culture and survival; the former indicating growth and development under controlled artificial conditions requiring higher metabolic activity by the parasites to allow for protein synthesis and cellular differentiation, whereas for mere survival metabolism may be at a lower level. These latter definitions are adhered to in the present study and chemically known components are referred to as defined components, undefined components being these of natural origin, usually serum.

Reviews of literature on in vitro culture of helminths or cestodes in particular have been given by many authors including: Hobson (1948), Weinstein (1958), Dougherty (1959), Read and Simmons (1963), Silverman (1965), Berntzen (1966), Hopkins (1967), Taylor and Baker (1968, 1978), Clegg and Smyth (1968), Smyth (1969[a], 1976) and

Silverman and Hansen (1971).

Silverman (1965), Clegg and Smyth (1968), Silverman and Hansen (1971) and Smyth (1976) gave an account of the basic problems encountered with parasites _in vitro_ both in general and for cestodes (Smyth, 1969[a]). The following summarises these accounts, using as illustrations some examples which are thought to be particularly relevant.

(a) _Sterility_: Helminths often live _in vivo_ in non-sterile habitats or they may be obtained without sterile precautions, and care should be taken to avoid introducing micro-organisms into the culture system. Recovery under sterile conditions is not always practical, but although this used to be difficult to overcome, the use of antibiotics in the media and washing procedures mean that this is no longer a major problem. However, although using antibiotics or antifungal agents may control the micro-organisms it does not necessarily mean that a culture is strictly axenic, so the use of the term "_in vitro_ culture" is perhaps preferable. On the other hand antibiotics or antifungal agents may themselves affect the parasites in culture. Berntzen (1962) reported that penicillin G, streptomycin hydrochloride and others, interfered with the growth of _Hymenolepis diminuta_ and _H. nana_, whereas Turton (1974) found no such effect on the former species. Penicillin and Streptomycin are now routinely used in most media at concentrations of 100 i.u. and 100 $\mu g.ml^{-1}$ respectively.

(b) _Physico-chemical and nutritional requirements_: Numerous experiments have been described concerning the biochemistry and physiology of cestodes(reviewed by Read and Simmons 1963, Smyth 1969[a] and 1976

Von Brand 1973, and Bryant 1975 and 1978) and results in this field have been very useful and have often had direct application in chemotherapy (Saz 1970), thus understanding the biochemical relation between host and parasite may reveal pathways of greater physiological importance to the parasite than to the host, so providing a rationale for therapy.

Biochemical studies diverted at critical periods in the life-cycles of helminths (e.g. change of environment, host etc.) (Smyth 1976) may also provide a useful tool for assessing _in vitro_ growth and conversely a deeper insight of the _in vivo_ situation would give more information in the _in vitro_ requirements. _In vitro_ physico-chemical characteristics such as temperature, pH, pO_2, pCO_2, redoxpotential and osmotic pressure are not always easy to control or maintain at a constant level; on the other hand, it is often not known how important they are, and even the exact data on the parameters in the parasites' natural habitat are often lacking (Voge, 1973 and Smyth, 1976). Bryant (1975, 1978) concluded that the environment may itself determine the metabolic pathways used, the critical factor being oxygen tension or concentration of CO_2. These factors in the natural environment may also change under the influence of the parasite as Podesta and Mettrick (1974) showed in the case of _H. diminuta_ in the rat intestine, a finding which led them to the conclusions that determining whether a helminth's metabolism is anaerobic or aerobic cannot merely be deduced from their localisation and that the influence of the parasites on that environment may explain their versatility in CO_2 fixation and aerobic energy metabolism.

Hansen (1978) stressed the necessity of assessing the metabolic role of CO_2 and the effect of pO_2 for each system. Hopkins (1967) discussed the importance of the gasphase and other factors with reference to H. nana and commended the approach of studying the physical parameters of H. nana's natural environment and using it as a guideline for in vitro requirements. Similar deductions could be made for the nutritional requirements.

Clegg and Smyth (1968) suggested that special attention should be paid to the aminoacid proportions in media for those parasites whose in vivo habitats, such as the intestine, are known to have a constant level of aminoacids while also, taking into consideration that materials released by cytolysis may also contain important growth factors. Thus, Cunningham and Slater (1974) attempted to design a more consistent, and ideally defined culture medium for tissue cultures of Glossina morsitans, using aminoacid analysis of the haemolymph. Similarly Pullin (1973) tried to develop a defined medium for larval Fasciola hepatica analysing mucus and haemolymph of Lymnea truncatula.

Alternatively workers have started with a complex medium in which optimal development occurred and then by adding or omitting certain substances could assess their importance. This approach enabled Rose (1973) to detect the lack of influence of liver concentrate formerly used in vitro with Ostertagia circumcincta. Douvres (1979) similarly showed a positive influence by pepsin and reducing agent for defined periods of time on the in vitro growth of Trichostrongylus axei. As for nutritional requirements, Smyth (1976) stated that since most of these are unknown in vivo and the

parasites have access to complex biological materials, it is difficult to compose artificial defined media. Nevertheless defined media supplemented with serum and also with yeast extract, whole blood or blood components etc. have often proved suitable substitutes for the in vitro requirements. On the other hand, the undefined components may interfere with the utilisation of some of the defined substances in the medium, and they may also contain additional amounts of these defined substances. Accordingly, the addition of an undefined substance is only desirable when its omission gives consistently poorer results (Hopkins 1967).

A similar conclusion was reached by Fioravanti and MacInnis (1976[a]) for H. diminuta, who showed that introducing blood or blood components to culture media may alter the primary effect of additives due to interfering substances in the former. Although growth in defined media has been obtained for certain phases of the life cycle of some parasites, as when Stromberg, Khoury and Soulsby (1977) achieved growth of third stage to fourth stage larvae of Ascaris suum, on other occasions the influence of a particular component was very clearly determined. Thus Seidel (1971) found that strobilisation and maturation of H. microstoma could occur in vitro only in the presence of hemin; this was further studied by Khan and De Rijcke (1976) who found that the stimulating effect of haemoglobin and hemin could not be replaced by bilirubin.

A supporting matrix may be an essential requirement for in vitro growth of some parasites, for others it does not seem to have a similar influence. Schiller (1965) found that a diphasic medium, with a blood-agar base allowed H. diminuta to grow to the adult

stage. Davies and Smyth (1978) found various diphasic media, proven to be of value with other helminths, unsuitable for F. hepatica metacercariae in vitro. Similar to the use of mono- or diphasic media and the possible explanations of their influence on the parasite is the use of either static or flow cultures. As Hopkins (1967) pointed out, the latter may be desirable for these parasites which live in vivo in sites where there is a flow such as the intestine, blood vessels or bile ducts. It could provide a better distribution of nutrients and eliminate the accumulation of waste products. However both Hopkins and Smyth (1976) pointed out that for many culture systems a similar effect could be achieved by regularly shaking the culture vessels and renewing the medium every 2 to 4 days, thus overcoming at least one disadvantage of a flow system, which is that it is more difficult to obtain the necessary specimens on which to assess growth directly (Smyth and Davies 1974[a]).

Nevertheless flow culture systems often seemed to have been more advantageous than static cultures. Berntzen (1961, 1962, 1966) constructed a continuous flow apparatus which, together with a complex medium led to a breakthrough in the cultivation of H. diminuta and was subsequently modified for H. nana. Martinetto and Capucinelli (1968) maintained F. hepatica in a flow system which they considered to be superior to a static culture. Sewell (1968) could maintain this parasite in a relatively simple medium in a continuous flow system. Lehner (1977) and Lehner and Sewell (1979) reported that flukes in such an apparatus tended to migrate against the flow of the medium suggesting that they were seeking the freshest

medium, least contaminated with their own excretory products.

The complex adjustments of a parasite's metabolic adaptation to a new host may require an initial stimulus to set off the relevant phase of development which may need to be replicated in vitro (Smyth 1976). In particular for cestodes, evagination of the mature metacestode has often been described e.g. by Riccetti (1975) for Taenia solium, Featherston (1969, 1971) for Taenia hydatigena, Malkani (1933), Edgar (1941) and Rothman (1959) for several species.

Further descriptions of the excystation and evagination with a review of the factors influencing these were given by Read and Simmons (1963) Smyth (1963) and Šlais (1970). Esch and Smyth (1976) discussed the necessity for artificial enzymatic pretreatment of Taenia crassiceps metacestodes prior to in vitro culture, often these metacestodes are already evaginated on removal from the host. Although it had an effect on some aspects, it was not found to be a prerequisite for strobilisation or organisation in vitro.

The fact that trying to simulate as much as possible the in vivo circumstances of a parasite can produce complex problems may be further illustrated by the observations made by Hopkins and Bråten (1969) and Hopkins (1970) on the ability of H. diminuta to migrate in the small intestine. The effect of population density both in vivo and in vitro has been discussed by Read and Simmons (1963) and Silverman and Hansen (1971). Similarly Douvres and Malakatis (1977) were concerned about the optimum number of Ostertagia ostertagi larvae in vitro and Lehner and Sewell (1979) made

some observations on the effects of changing the numbers of flukes in culture.

Davies and Smyth (1978) also noted that the difficulty of reproducing the various physicochemical factors may introduce a source of variation _in vitro_ cultures and so explain phenomena such as unsynchronized somatic and germinal development found for _F. hepatica_ metacercariae. Hopkins (1967) drew attention to physicochemical influences which may be harmful on parasites _in vitro_ such as X-rays or even the influence of light.

(c) _Evaluation of growth or survival in vitro_

Whatever the reason may be for _in vitro_ work, the evaluation criteria for monitoring the culture or even merely to distinguish between survival and growth of the organisms are important (Clegg and Smyth 1968 and Smyth 1969[a]). Criteria for this have often been discussed. Thus Silverman (1965) enumerated seven criteria in which morphological features play an important role.

An important base-line for such criteria may be provided from _in vivo_ observations, but knowledge of _in vivo_ development is not sufficiently adequate for all parasites to allow a clear distinction between normal and abnormal development _in vitro_. On the other hand, rapid evaluation of cultures on these grounds is less effective with parasites which require long development times in their natural hosts. For these, recognition of small changes is important (Smyth and Davies, 1974[a]). Hence, Smyth and Davies (1974[a, b]) divided the early development of _Echinococcus granulosus_, before segmentation, into 5 stages. This approach, adapted for the

particular taeniids in question has been used by several authors: Esch and Smyth (1976), Osuna and Guevara Pozo (1976), Osuna Carrillo, Mascaró Lazcano, Guevara Pozo and Guevara Benitez (1978) and Osuna Carrillo and Guevara Pozo (1978) used as early parameters evagination or neck elongation, appearance of excretory canals, banding and segmentation with further the development of the reproductive organs being taken into account at a later stage. Although Bell and Smyth (1958) described how mitotic activity may be a useful parameter for assessing early development in vitro, this presents some technical difficulties (Smyth 1962[a], 1969[a]). Moreover, Smyth (1967) found it was not a very suitable parameter since the measurement of the nuclear division rate does not indicate whether differentiation is taking place in a cystic or strobilar direction as is the case of Echinococcus spp.

Smyth (1972[a, b]) found cell differentiation to be an important aid in understanding the mechanisms involved at important stages of the parasites' life cycle, such as when changing to another host, which are accompanied by major metabolic changes (Jha and Smyth 1969, 1971).

Flame cell activity was one of the criteria used for assessing the vitality and infectivity of Taenia saginata metacestodes by Ceretto and Arru (1967). Davies (1978) reported ultra structural techniques as a method of assessing development in vitro, especially referring to studies on spermatogenesis.

The activity of the parasites in a culture system was also often referred to as a criterion: Smyth (1967) compared the activity rates of E. granulosus in different media, while he and Esch and

Smyth (1976) paid much attention to the activity of suckers, rostellum and hooks. Smyth (1969[a]) warned that movement may still occur even when the metabolic functions are abnormal. Voge (1978) concluded that both rapid contractions and lack of motility are usually indications of inadequate in vitro conditions.

A simple apparatus using the Wheatstone bridge principle was described by Jewsbury, Homewood and Marshall (1977), which allowed them to monitor the activity of Schistosoma mansoni in vitro and which could even be applied to a flow culture system.

Various workers trying to evaluate in vitro growth and survival used biochemical or radiochemical methods to measure the uptake of nutrients or release of metabolites. Thus NH_3 production was monitored by Sewell (1968) and by Lehner and Sewell (1979) during maintenance of adult F. hepatica in a flow culture system. Von Brand, Churchwell and Eckert (1968) measured glucose and glycogen uptake and the production of succinic, lactic and acetic acid in studying the metabolism of Taenia taeniaeformis. Taylor (1966), Taylor, McCabe and Longmuir (1966) and Taylor and Haynes (1966) studied the metabolism of Taenia crassiceps in vitro, observing the production of appreciable quantities of lactic acid, which may have accounted for the reduction in pH noted by Taylor (1963[a,b]) and could be used as a criterion for normal survival. Estimations of in vitro uptake and release of substances using radioactive labelled precursors were reported by Taylor (1966), Haynes (1973), Morcock (1974) for T. crassiceps and by Nash (1979) for S. mansoni. Fioravanti and MacInnis (1976[a]) evaluated in vitro maintenance of H. diminuta using several metabolic indices including wet weight

and glycogen and protein levels and applied these to determine the beneficial or noxious influences of certain additives (Fioravanti and MacInnis 1976[a, b]). Seed, Boff and Bennett (1978) described phenol oxidase activity as a marker related to egg production for S. mansoni.

12.2 Reports of in vitro cultures with Taenidae

12.2.1 Echinococcus spp.

As well as developing into a strobilated adult worm in the carnivorous final host, protoscoleces of Echinococcus spp. can multiply in the same or a new intermediary host, forming a new hydatid cyst. This feature gave rise to an outstanding series of in vitro experiments by J.D. Smyth during 1962 - 1976, mainly with E. granulosus. Smyth (1962[a]) described the two routes which protoscoleces may take for their development into cysts. As observed from in vitro cultures they either become vescicular and progressively enclosed in a laminated envelope or a posterior bladder may be formed, gradually "absorbing" the scolex region and again forming a cyst, surrounded by an envelope.

These were monophasic cultures containing undefined components such as hydatid fluid, bovine serum etc. In these cysts there was evidence of "protoscoleces anlagen" formation but no sign of development towards the adult stage was noted. Similar findings were made by Gurri (1963), Pauluzzi, Sorice, Castagnari and Serra (1965) and Brudnjak, Cvetnić and Wikerhauser (1970). Smyth and Smyth (1964) discussed the genetic possibilities for the occurrence of variants in Echinococcus, favoured by this feature of their

biology. Smyth, Howkins and Barton (1966) in further attempts to stimulate development of <u>E. granulosus</u> <u>in vitro</u> towards the strobilar stage had no success by changing the usual physico-chemical conditions. They resolved the first problem of accelerating the evagination of the protoscoleces by successive pretreatment with pepsin to digest the dead material and germinal membranes, followed by treatment with pancreatin, trypsin and dog bile, as Smyth (1962[b]) had shown the lytic effect on scoleces from <u>E. granulosus</u> of bile from herbivores.

Based on the findings of Smyth (1964) that the scolex of the adult parasite is deeply buried and attached with its hooks in the crypts of Lieberkühn in the dog's intestine, where the rostellum is extended with formation of secretion droplets, they developed a culture medium with a liquid and solid phase consisting of coagulated bovine serum which allowed the protoscoleces to remain evaginated and became elongated, followed by strobilisation. Since no such effect was seen on agar base it was concluded that the function of the solid phase was not merely support but also nutritive. Ultrastructural studies of the scolex revealed the presence of microtriches restricted to the anterior region of the evaginated scolex, making it almost a tissue parasite rather than an intestinal one. The characteristic "tetanus" of the protoscoleces in liquid media (Smyth, Howkins and Barton 1966) described as a decline in activity of the suckers and a rigid state of the larvae did not occur in this diphasic medium. The absence of development beyond simple segmentation was thought to occur because the physico-chemical milieu was not optimal. Smyth and Howkins (1966) were able to

obtain development to egg producing strobila *in vitro* from *E. granulosus* obtained from dog's intestines before they had developed to the fully mature stage. Both in monophasic (liquid) and diphasic media ova were produced but this happened faster in the latter, which led them to conclude that the later stages of maturation, when microtriches were present over the whole tegument, were less dependent on a contact stimulus. A further description of these experiments about the conditions which determine the direction of development is given by Smyth (1967). Factors inducing vesicularisation were low pH, high pO_2 and also when the intimate contact of scoleces with the coagulated serum base was hindered. The development of protoscoleces reached a 3 - proglottides strobilate stage with presence of shelled eggs but no fertilisation. For an early evaluation of the growth response, the mitotic index (Bell and Smyth, 1958) was not useful because of the two possible types of development, the degree of activity proved being more reliable. An attempt to explain the factors, controlling the direction of development led Smyth, Miller and Howkins (1967) to the following hypothesis: A "strobilization stimulus" may result from the contact of the scolex with a protein substrate either as a result of extra-cellular or membrane (contact) digestion of the latter at the inter-face between host and parasite, or a nervous stimulus received by the microtriches and the rostellar hairs. This stimulus may release a "strobilization organizer" leading towards strobilar growth. They distinguished seven stages in *in vivo* growth towards oncosphere-producing adults, which takes 40 days on average in the dog. The distinction between these stages and the time at which they occurred was taken as a criterion for studying the *in vitro* development and

was applied to compare the effects of several factors in the gaseous, liquid and solid phases of the media. Pure bovine coagulated serum was found to be better for providing the growth stimulus than enriched serum, suggesting that this stimulus is of an "all or none" type. Medium 858 proved to be better than Parker 199. Either anaerobic or an over-high oxygen concentration were unsatisfactory. A relationship between these in vitro findings and host specificity in Echinococcus was discussed by Smyth (1968) and Smyth (1971) commented on other phenomena observed during in vitro cultures. Some organisms grew towards the adult stage with genitalia being formed as normal, yet they remained monozoic, rather than forming the characteristical scolex plus 3 segments form. Both these and intermediate forms were present in the same cultures indicating that their formation is the result of the artificial conditions rather than mutation. The reverse condition also occurred, namely 3 segmented worms with no genitalia. Hence it was concluded that the differentiation of somatic and germinal tissue could take place independently from each other. It was suggested that this may have led to misinterpretations in the classification of species or subspecies and that the evolution to the strobilated form perhaps started as a subdivision of a monozoic form rather than from modified budding. Later findings, (Smyth 1972[b]) defined more clearly the significance of the host/parasite interface. An ingenious culture apparatus described by Smyth (1969[a]) could be modified to provide simultaneously both a disturbed and an undisturbed interface. Only in the latter did segmentation and sexual maturity occur. Studies of the ultra-structural changes occurring during the differentiation from protoscolex to the adult stage (Jha and Smyth 1971) involved

greater activity by the rostellar glands, with the presence of fine mitochondrial droplets at the tip of the rostellum. The combination of these findings suggest that membrane or contact digestion was the correct hypothesis and, if so this may result in an additional benefit to the parasite in that, being coated with host material would make it less recognisable to the host. Smyth and Davies (1974[a]) distinguished 5 stages during *in vitro* development of *E. granulosus* protoscoleces. After evagination, the calcareous capsules started to disappear, excretory canals became visible with bladder formation. Banding then appeared, followed by segmentation with the presence of genital "anlagen". Several factors related to *in vitro* culture were further examined. Generally, the development time *in vitro* was only a few days longer than *in vivo*. It was noticed that the *receptaculum seminis* of worms *in vitro* was still empty of semen, this lack of impregnation being the cause of the infertility. In order to resolve this a study of self insemination in *E. granulosus in vivo* was made by Smyth and Smyth (1969). Furthermore, they noticed a variable *in vitro* response according to the origin of the protoscoleces and to the natural components of the medium, especially the foetal calf serum. This, plus the relatively long time necessary for development, made it difficult to determine the growth producing factors in the serum. An additional complication was the fact that only the sheep strain of *E. granulosus* responded to this culture system. Horse strain protoscoleces did not even segment although they survived. These findings were further expanded (Smyth and Davies 1974[b, c]) and led to the conclusion that the *in vitro* strain difference could even be

used for strain identification, probably because the different strains have different physiological and nutritional requirements.

In vitro cultures with material from Echinococcus multilocularis, using tissue from germinal membranes or protoscoleces, were tried by Raush and Jentoft (1957). Usually cyst formation with incompletely developed protoscoleces was obtained. On one occasion, Webster and Cameron (1963) obtained segmented worms without genitalia in a fully defined medium in paper tube cultures. Smyth and Davies (1975) obtained sexually mature but unsegmented monozoic forms from protoscoleces in monophasic media which indicated that their membrane digestion hypothesis may not apply to E. multilocularis in the same way. Again, the factors responsible for the suppression of strobilisation may have been numerous. These findings thus suggested that not only are the true mechanism and influence of various nutrients and physico-chemical conditions complex but they are probably even specific for each species, or even strain of the worm.

Rickard, Mackinlay, Kane, Matossian and Smyth (1977) studied the lytic effect of normal serum on E. granulosus protoscoleces and confirmed previous findings that it was mediated by complement. Thompson, Dunsmore and Hayton (1979) studied the secretory activity of the rostellum of adult E. granulosus in the dog's intestine. Rostellar gland secretion, associated with the invasion into the crypts of Lieberkühn was thought to have a protective function rather than being a trigger mechanism for strobilisation. Short term in vitro maintenance of adult E. granulosus was also used by Herd, Chappel and Biddell (1975) and Herd (1977) to obtain secretory

antigens which were used as immunogens in dogs.

12.2.2 Taenia crassiceps

Taylor (1963[a, b]) maintained T. crassiceps metacestodes in a fully defined medium. Eagle's medium enriched with glucose $1mg.ml^{-1}$ and sodium taurocholate allowed longer survival - up to 31 days - than other media tested such as 199, NCTC 109 and Waymouth's medium. Some signs of development were observed in the form of growth, development of lateral excretory canals and some larvae even showed internal segmentation. Metacestodes, which had been treated with enzymes, were more active and grew longer than untreated controls. About 200 larvae were placed in roller tubes in 25 ml medium, changed every 2 days; pH 7.4 was found to be optimal. Media were evaluated by survival for which 3 criteria were used: motility of the larvae, flame cell activity and production of acid metabolites. Depletion of vitamins was less harmful to the larvae than depletion of amino-acids.

Taylor (1966) and Taylor, McCabe and Longmuir (1966), found criteria such as radioactive labelling of chemicals with a study of some of the released metabolic products such as lactic acid, useful for monitoring in vitro maintenance. It was concluded that anaerobic glycolysis was a more important source of energy to these parasites than respiration. Glycogen was found to constitute the major reserve of carbohydrates, which remained at a relatively constant level when glucose was present in the medium.

Robinson, Silverman and Pearce (1963) reported bud formation in cysticerci which developed into fully formed scoleces. Strobilisation of mature larvae was also seen. Not many details were given

in this preliminary report apart from the use of medium 199 in roller tubes.

Voge (1963) reported survival of metacestodes, for up to 4 months in a tissue culture medium. Various other media, recommended for development of other cestodes were found to be unsuitable.

Esch and Smyth (1976) obtained growth to the strobilar stage. Medium 858 was enriched with 25% v/v foetal calf serum and yeast extract, glucose, KCl and antibiotics. A solid substrate was provided in the form of heat coagulated bovine serum. An interesting observation was that larvae penetrated the solid base, whether it was previously disrupted or not, and migrated through it immediately they were introduced into the culture and for several days thereafter. Again the authors commented that different batches of serum and metacestodes may have been responsible for inconsistent results. Metacestodes which had undergone enzymatic pretreatment showed better development than others, although this was not an essential prerequisite and it did not digest the bladder which could remain attached throughout the whole development. Alternatively it detached mechanically when the metacestodes migrated through the solid substrate, or it was eventually shedded together with the terminal proglottides. In this work several developmental stages were distinguished and evaluation of different culture systems was made by comparing the time required to proceed to these different stages. Seven stages were noted, beginning with neck elongation, not necessarily due to formation of new tissue and appearance of osmoregulatory canals on day 1 of culture. After 2 days fluting of these canals appeared, banding on day 3, proglottidisation on day 4, formation of

cirrusstreak on day 7 and finally on day 13 the genital pores appeared. Cultures could be maintained as long as 80 days but development did not proceed further than that of stage 7. When no coagulated serum base was used, development did not proceed beyond the proglottidisation stage and the larvae became vesiculated and died. An agar base did not allow any form of development.

Siebert and Good (1979) studied the effect of various sera on T. crassiceps metacestodes in vitro. Controls were maintained in RPMI 1640 in which they remained viable for at least 14 days. After 7 days, normal sera from rabbit, goat, guinea pig and foetal calf serum had no other than a supporting effect. These authors suggested that unheated sera do not have a lytic effect on these metacestodes because of their constant exposure to host body fluids, whereas Echinococcus spp. are protected from these by a cyst wall.

Schiller (cited by Voge 1978) used a diphasic medium, the solid phase consisting of Difco-nutrient agar and defibrinated rabbit blood, and the liquid phase of Hank s'balanced salt solution enriched with glucose and antibiotics. Each cyst introduced in this medium had an average of 157.3 progeny when cultivated for 162 days. The cysts increased 6 to 10 times in diameter after approximately 2 weeks with completely developed scoleces and secondary buds.

12.2.3 Taenia serialis

Smyth (1969[b]) discussed the factors controlling differentiation into the cystic or strobilar directions of these metacestodes in vitro. These appeared to be similar to those discussed for the sheep strain of E. granulosus, following similar culture procedures: Peptic pretreatment followed by a pancreatin plus dog bile mixture

(bile concentration 5% v/v) for 17 h, evaginated all protoscoleces. After that growth in monophasic medium was compared with that in diphasic, the liquid phase being Parker 858 plus coenurus cyst fluid 20% v/v, potassium ion level was raised to 60 $\mu g.ml^{-1}$, the level present in the host's intestine, and glucose was raised to 300 $\mu g.ml^{-1}$. The solid phase consisted of heat coagulated bovine serum in which holes were punctured. As with T. crassiceps the scoleces burrowed themselves in the holes and some of them became separated from the bladder, which is described as a "stalk". After 8 days, the scoleces detached from the serum, segmentation of the neck occurred on day 11 formation of early genital primordia on day 20. When coenurus cyst fluid was replaced by hydatid fluid vesiculation became apparent. The stalk regions, without scolex, remained virtually intact during this period, but after the first 27 days they became vesicular to form a well developed cyst by day 51, without signs of developing protoscoleces. This also happened in monophasic media in a few metacestodes. Thus, for T. serialis also, strobilar growth was induced by directed contact with a solid protein substrate whilst, as with E. granulosus, the potential to develop in a vesicular direction was retained.

12.2.4 Taenia hydatigena

Osuna and Guevara Pozo (1976), Osuna Carrillo and Guevara Pozo (1977) and Osuna Carrillo, Mascaró Lazcano, Guevara Pozo and Guevara Benitez (1978) reported the growth of metacestodes of this species to an infertile adult stage. They used a diphasic medium consisting of coagulated bovine serum, with CMRL 1066 plus FCS, yeast extract, glucose and KCl as liquid phase and a controlled gas phase. A pepsin

treatment was followed by an evagination process consisting of 18 - 20 hours agitation in trypsin, pancreatin, and dog bile prepared in Hanks. After that a process described as "the separation of the mass from the scolex" occurred after 72 hours in a mixture of the evagination fluid and the liquid phase of the culture medium over the solid substrate. The scoleces were then transferred to the described diphasic medium at a concentration of 6 free scoleces per 5 ml liquid and 5 ml solid medium. After these two stages the excretory canals became visible after 2 further days in culture, fluting of these canals and banding was complete for all the worms by day 6. Segmentation of some metacestodes started then and genital primordia were seen at day 18 and 20, when the length varied between 35 and 40 mm. Some larvae survived up to 56 days but no further development was seen after day 20. The separation of "the mass" or forebody (Wardle and McLeod 1968) may be due to the influence of 3 factors, namely the active movement of the scolex, the direct action of the enzymatic treatment or the action of endocellular enzymes stimulated by the intestinal enzymes. Again, the development closely followed the development rate <u>in vivo</u> up to the appearance of genital primordia.

12.2.5 <u>Taeniae pisiformis</u>

Using the same technique and culture media as for <u>T. hydatigena</u> Osuna Carrillo and Guevara Pozo (1978) reported similar results regarding evagination and separation of the forebody for these metacestodes. The excretory canals became visible after day 3, banding after day 8 and segmentation on day 10 - 11.

12.2.6 Taenia solium

Bruckner (1979) reported cultures of pieces of human racemose cysticerci. They were placed in RPMI 1640 supplemented with 10% v/v FCS, 10% v/v pooled human serum or human cerebrospinal fluid. Some growth was obtained in FCS and cerebrospinal fluid being described as reformation of the bladder with subsequent characteristic contractions.

12.2.7 Taenia taeniaeformis

Mendelsohn (1935) reported *in vitro* survival of metacestodes for 35 days. Since then several similar types of *in vitro* cultures allowing survival of these metacestodes have been used for different purposes: Kwa and Liew (1977) and Ayuya and Williams (1979) studied the immunogenic capacities of antigens released in the cultures. Wilmoth (1945) kept metacestodes *in vitro* for 24 days, comparing several media and studying their metabolism. Metacestodes survived best in a simple medium under aerobic conditions. They were able to withstand fairly wide pH and temperature ranges. Cat serum was not beneficial. Numerous experiments have been done with these parasites attempting to reveal aspects of their metabolism, often using whole larvae or part of their tissues. Thus Haskins and Olivier (1958) reported a lower production of nitrogenous material by *T. taeniaeformis* metacestodes than by nematode larvae, one third of their total excretory nitrogen appearing as urea.

Von Brand and Bowman (1961) found that metacestodes originating from rats had a higher metabolic rate than those from mice, differences between larval and adult metabolism were found to be of a quantitative rather than a qualitative nature as judged by their

production of lactic, pyruvic, acetic and succinic acid, glycerol and ethanol under both anaerobic and aerobic conditions. Similarly the relation between size and metabolic rate was studied by Von Brand and Alling (1962). The relation between leakage and absorption of glucose and other sugars for aerobic and anaerobic metabolism (Von Brand, McMahon, Gibbs and Higgins 1964, Von Brand, Churchwell and Higgins 1966) revealed that absorption of glucose by larvae required external glucose levels of 100 - 200 mg % w/v. Glucose leakage could be modified in the presence of other sugars, it was not increased in hypotonic media but both glycerol and glucose could be absorbed from the medium and consumed in large amounts by the larvae.

The utilisation of glucose and glycerol and the resulting metabolic end products was studied by various authors including Waitz (1963). He examined the glycolytic enzymes in extracts of _T. taeniaeformis_, one of his conclusions being that lactic dehydrogenase was present with the favoured reaction in the direction from pyruvate to lactate.

Von Brand, Churchwell and Eckert (1968) concluded that utilising glucose or glycerol gave identical end products: succinic, lactic and acetic acid, the last two being independent of the oxygen availability and it was assumed that they were all originating from dismutation of pyruvic acid.

Further metabolic studies were made by Von Brand, Nylen, Martin and Churchwell (1967), Von Brand and Stites (1970) and Weinbach and Von Brand (1970). Von Brand and Weinbach (1975) studied the role of the calcareous corpuscles and Nieland and Weinbach (1968) studied

the function of the bladder in the nutrition of metacestodes. It appeared that this organ constitutes a large reservoir of glucose and other nutrients while on the other hand it provides a large area for absorption and assimilation of nutrients and may serve as a waste disposal system.

Chapter 13 MATERIALS AND METHODS

13.1 Sterilisation procedures

The same precautions to prevent contamination as described for the *in vitro* experiments in Part II 8.2.1 were used. In addition to these, large quantities of media were sterilised by filtration through a 142 mm diameter, 0.22 μm pore size membrane filter (Millipore). This filter was fitted into a 142 mm disc filter holder (Millipore), the inlet of which was plugged with non absorbent cotton wool, and the outlet connected to silicone tubing, wrapped in aluminiumfoil and autoclave tape. The whole outfit was assembled and sterilised by autoclaving, according to the manufacturer's instructions. It was then allowed to cool in a laminar air flow cabinet (Microflow). Another 142 mm filter holder containing a 124 mm depth filter (Type AP 15 Millipore) as prefilter on top of a 142 mm diameter, 0.22 μm pore size membrane filter was fitted on top of the sterilised filterholder and could, if necessary, be replaced during the procedure. Freshly prepared medium was placed in a 11.4 litre stainless steel pressure vessel (Millipore) which was filled with nitrogen to a pressure of 345 kPa. The medium was then forced through the two filter holders and the silicone tubing into previously sterilised glass bottles. Filter holders and glass bottles were kept under the laminar air flow cabinet from immediately after being autoclaved until the end of the procedure. After filtration, the bottles were closed with air tight stoppers containing a silicone lining and kept at 4°C until use.

13.2 Culture vessels

Disposable sterile polystyrene petri dishes (Sterilin Ltd.)

size 50 x 13 mm and 90 x 15 mm were used for T. saginata and T. taeniaeformis respectively. The rim of the top lid was sealed using silicone stopcock grease (Dow-Corning) which was applied using a sterile syringe of 2 ml capacity. Where diphasic media were used, glass universal bottles (United Glass Ltd.) with a total capacity of 30 ml for T. saginata and glass bottles of 190 ml capacity (Gibco, media bottles) which were used for T. taeniaeformis. Both types had screw caps with a silicone lining, and were washed and sterilised by autoclaving before use.

13.3 Preparation of media

All liquid media were commercially purchased and used within a month. Storage and addition of antibiotics has been described in Part II. Most frequently, the medium used was that described in Part II as modified RPMI (without fungizone). For some experiments with T. taeniaeformis metacestodes RPMI 1640 (Gibco) was purchased in powdered form, and prepared according to the manufacturer's instructions to give the same concentration as the liquid commerical form, except that when additives were used, this was done in such a way that all media contained the same basic concentrations of the RPMI 1640 components. Thus, distilled, deionized water with or without extra additives as desired, was added to the powder, in the same total volume for all media. HEPES buffer (Gibco) to a final concentration of 25 $mmol.l^{-1}$, antibiotics and fungizone and sodium hydrogen carbonate ("AnalaR" BDH) at 2 $mg.ml^{-1}$ were also added. The pH of this medium was adjusted to 7.2, as according to the manufacturer subsequent sterilisation through membrane filters causes a slight pH increase, leaving the final pH at 7.4. As with

the commercially purchased liquid media they were stored after preparation at 4°C and used within a month of preparation.

For the solid substrate in diphasic media, calf serum was allowed to coagulate by leaving it at 80°C for 90 minutes in an oven. Special Bobby Calf serum (Gibco) was used since FCS (Gibco) did not coagulate under these conditions. This serum was coagulated in previously sterilised culture vessels. The volumes of serum to be coagulated was 5 ml for _T. saginata_ and 25 ml for _T. taeniaeformis_ metacestodes. The gasphase for all cultures was air. Antibiotics were used in all liquid phases and initially fungizone was also added, but this was later on discontinued.

13.4 Treatment of metacestodes prior to culture

Only B-strain _T. taeniaeformis_ metacestodes were used for _in vitro_ work. In order to have a regular supply of worm material every month, a group of 3 week old mice were orally infected as described in Part I Chapter 3.3. Mice were killed by cervical dislocation, rather than using chemicals, which may have affected the larvae. Working under the laminar air flow cabinet, the skin from the ventral region was removed, the abdomen incised, after washing the hair off, and the liver dissected out and transferred into a petri dish. Using a pair of scissors a slit was cut in the capsule of each cyst and, while wearing sterile surgical gloves, gentle pressure was exercised with two fingers on the capsule until the metacestode was expelled. This was then removed by hand and transferred into physiological saline with antibiotics at 37°C. _T. saginata_ metacestodes were dissected out in a similar way from a calf's muscles, which had been removed from the carcase and thinly sliced. Whenever metacestodes were encountered they were immediately

removed from the capsules and transferred into physiological saline with antibiotics. Since this procedure was time consuming both meat and saline were usually kept at room temperature. The infected calves came from the control groups in experiments organised by Dr. G.J. Gallie, who was only using half of the carcase himself. Usually these calves were 1 to 2 months old when orally infected with 5 000 to 10 000 T. saginata eggs of Kenyan origin. They were then kept indoors in a parasite free environment for about 2 months and then killed and prepared by standard slaughterhouse procedures. The time lapse between killing the host, dissecting out the parasites and starting the in vitro culture was kept to a minimum, although for T. saginata metacestodes it sometimes took several hours. Since it appeared rather impractical to do this part of the work entirely under sterile conditions, a washing procedure was routinely used, with the additional aim of elminating as far as possible transfer of host material into the cultures. For T. taeniaeformis metacestodes this procedure was as follows: a household nylon strainer of 107 mm diameter, aperture circa 1 mm, was fitted onto a beaker of one litre capacity; this set was sterilised by autoclaving and placed in the laminar air flow cabinet. About 25 metacestodes, freshly liberated from their capsules, were transferred from the saline up to the sieve and rinsed with one litre of warm (37°C) physiological saline, which had been sterilised by autoclaving, cooled and had antibiotics added to it. After that, undamaged larvae were transferred to a petri dish containing the defined parts of the medium. Further washings then followed, by passing them successively through 5 petri dishes containing medium. A sterile needle (Yale sterile disposable hypo-

dermic needles 21G2 Becton Dickinson) was bent and used to transfer the larvae from vessel to vessel without damaging them. T. saginata metacestodes were washed by passing them through 6 petri dishes size 90 x 15 mm, containing warm sterile defined medium. In this case a 5 mm bore sterile glass tube fitted with a rubber teat was used in the same way as a pasteur pipette to transfer the cysts with minimal carry over of fluid. A sterile tube was used for each passage. Finally the cysts were transferred into their complete culture medium to which was added 10% v/v ox bile, sterilised by passage through a membrane filter of 0.22 μm pore size (Millipore). They were left overnight at 37°C to evaginate and the following day, evaginated metacestodes were selected, regardless of whether their cyst bladder was intact or not and further washed 5 times before being placed in the cultures. All the washing procedures and later passages were carried out in the laminar air flow cabinet, using sterile materials and standard aseptic procedures.

13.5 Routine procedures during in vitro culture

Cultures were kept stationary at 37°C in the dark. The gas phase was air but the closed vessels were airtight. The media were first changed routinely after 24 h and then every 48 h. For monophasic cultures in petri dishes this was done by transferring the cysts to a new dish as described for the washing procedures. In cultures where a solid substrate was used or in experiments where diphasic media were compared with monophasic, changing the media involved drawing off and replacing the liquid phase every 48 h. The solid substrate was changed by transferring the metacestodes to a new vessel every 6 days. To do this, some of the liquid phase

was first drawn off and the neck of the vessel flamed with a bunsen. The contents including the worms were then poured into a petri dish. The larvae were picked out and transferred into fresh medium in another vessel. Occasionally, mainly in the event of visible degeneration of the metacestodes, samples from used media were taken and examined for bacteriological contamination. For this purpose, Gram stained smears of debris after centrifuging media were examined. Media and debris were also cultured aerobically and anaerobically on blood agar plates and aerobically in nutrient both at 37°C for 4 days before being examined for bacterial growth.

13.6 Preparation of metacestodes for morphological examination

When the metacestodes were removed from a culture they were processed as described in Cable (1976). First of all they were placed in tap water at 4°C for 6 hours, which killed them and left them flaccid and extended. They were then flattened and placed between glass plates or microscopic slides. Gentle pressure was executed by means of rubber bands or by placing some extra slides on top and AFA Fixative was added. This was a mixture of 10 ml formalin (10% or 40 $mmol.l^{-1}$), 79 ml ethanol (30% or 5.2 $mol.l^{-1}$), 10 ml glycerin (Merck) (concentration 11.62 $mol.l^{-1}$) and 1 ml glacial acetic acid ("AnalaR" BDH) (concentration 17.43 $mol.l^{-1}$). Less acetic acid was used than in the method given by Cable (1976) as otherwise gas bubbles, presumably carbondioxide, were formed and trapped in the tissues. After 24 hours the metacestodes were removed from between the glass plates and stored in this fixative for at least two days in closed vessels. Before staining they were partly dehydrated by successively leaving them in 30% (or 5.2 $mol.l^{-1}$)

and 50% (or 8.65 $mol.l^{-1}$) ethanol for at least 20 minutes. This was followed by 70% (or 12.1 $mol.l^{-1}$) iodine alcohol for one to six hours, then two washings in 70% (or 12.1 $mol.l^{-1}$) alcohol and Semichon's carmine, without counterstain, overnight. The following day the metacestodes were washed twice in 70% (or 12.1 $mol.l^{-1}$) ethanol, destained in acid alcohol [0.5% (or 57 $mmol.l^{-1}$) HCl in 70% (or 12.1 $mol.l^{-1}$) ethanol], washed twice more, and further dehydrated by being left for at least one hour each in 80% (or 13.8 $mol.l^{-1}$) and 95% (or 16.82 $mol.l^{-1}$) ethanol. For clearing the specimens beechwood creosote followed by xylol treatment was used and then they were mounted in D.P.X. ("Depex" - Gurr).

Chapter 14 Maintenance of *Taenia taeniaeformis* metacestodes *in vitro*

Comparison of various parameters for assessing media

14.1 INTRODUCTION

The main aim of the *in vitro* cultures was obtaining metabolic products, and it was assumed that the better the conditions for survival or the slower the degeneration of the worms, the better would be the production of metabolic antigens and the lower the presence of any catabolic antigen. Hence it was desired to devise an optimal maintenance medium and a means of monitoring the well being of the organisms in it. Therefore in two experiments simple defined media were compared with and without the addition of serum.

14.2 Experimental design

The first experiment started with 112-day old strobilocerci divided into three groups. Each group contained five individually marked petri-dishes, four of which contained 5 metacestodes and 25 ml medium each, while one control contained only the 25 ml of medium. All media from the three groups were prepared from RPMI 1640 (Gibco) in powder form. This was the only component for Group 1. For Groups 2 and 3, 10% v/v foetal calf serum (FCS) was added, while Group 3 also contained 5 $mmol.l^{-1}$ sodium pyruvate. The latter was added at the same time as the medium was prepared but the FCS only just before the media was used in a culture. The larvae were weighed at the start of the experiment and when the cultures were changed at 48 h intervals, using an analytical balance (H 20 T Mettler). To do this an empty petri dish was placed on the balance, all 5 worms of one petri dish were weighed in it at

the same time and then quickly transferred to their fresh vessel and medium. The starting weights were used to make homogenous groups.

The used medium from each plate was handled as follows: 15 ml was stored in a closed vial at $4^{o}C$ for determination of free ammonia and glucose concentration, the rest was filtered through a 0.22 µm pore size membrane filter (Millipore) in a 25 mm Swinnex filter holder (Millipore), to remove the tissue particles, occasionally released from the metacestodes. The filtrate was stored in closed vials in the deep-freeze. Free ammonia in the cultures was quantified within 24 h of the samples being obtained. An ammonia probe ("Laboratory Model 8002 - 2" Electronic Instruments Ltd.) was assembled and used according to the manufacturer's instructions. A 9 ml sample was placed in a small beaker on a magnetic stirrer, the probe dipped into it and 1 ml 1 $mol.l^{-1}$ sodiumhydroxide ("AnalaR" BDH) was added. The potential difference across the probe was then determined on the millivolt scale of a pH meter ("Model 291 Mk 2" Pye Unicam). Immediately before assaying the ammonia in the samples the probe was routinely calibrated by plotting the potentials developed by using standard ammonium chloride solutions against the log of the known NH_3 concentration in $mg.l^{-1}$. Thus the probe potentials obtained from the media samples could be converted into NH_3 concentrations by interpolation. The average amount of NH_3 production per larva for each petri-dish over 48 h could then be calculated. Care was taken to assay all the samples and standards at the same temperature. The glucose content in the media was measured by the "GOD/POD Method" (Werner, Rey and Wielinger, 1970,

and Bergmeyer and Bernt 1974) using a kit purchased from The Boehringer Corporation ("Test combination Glucose Colorimetric method GOD/Perid") and the tests were performed corresponding to manufacturer's instructions. Samples were deproteinised with uranylacetate deproteinising solution ("Urac" BCL) or 0.33 N perchloric acid (Koch-Light). The colour reaction was measured with a spectrophotometer (Unicam SP 1800 Pye Unicam) at 436 nm. The glucose concentration, calculated in mg.100ml^{-1}, of the samples were subtracted from their respective group control to give the glucose consumption per one petri dish or, if divided by twenty, the consumption per larvae over 48 h. These measurements were taken within four hours of sampling.

The second experiment differed from the first in that the metacestodes were 106 days old when obtained from the mouse. The media for Group 1 consisted of RPMI 1640 with antibiotics, fungizone and HEPES buffer. For Group 2, 5 mmol.l^{-1} sodium pyruvate was added and for Group 3 the sodium pyruvate and 10% v/v FCS. The same parameters were studied as in the first experiment and in addition the presence of L-Lactate in the used media was determined. This was done at the same time with all the samples that had been stored in the deep-freezer. Samples from each group obtained on the same dates were pooled and two assays were made from each pool, and compared with the control for each group. The method used was described by Gutmann and Wahlefeld (1974). The NADH formed was measured on a spectrophotometer at 340 nm against air. This reading was converted into concentration of L-Lactate per 100 ml according to Bergmeyer (1975). Prior to the reaction hydrazine glycine buffer

(glycine buffer 0.5 $mol.l^{-1}$, hydrazine 0.4 $mol.l^{-1}$ pH 9) was added to the sample, to remove the pyruvate. Next, the sample was deproteinised with 0.6 N perchloric acid. All reagents, including the calibration solutions were obtained from BCL Ltd. in the form of "Testcombination Lactate UV method". The test was performed as prescribed by this manufacturer. From the mean values so obtained for each group, the values of the controls were subtracted and the result divided by twenty, to give an estimate of the mean production of L-Lactate per larvae over 48 hours. The regression of the log transformed data for means of the replicates in each group on time was calculated for each parameter and the significance of the regression coefficient tested by a Student's"t" test. If the regression was significant for any group, analysis of variance was used to show whether the differences between the groups were significant (Sokal and Rohlf 1969).

14.3 RESULTS

First Experiment

For Group 1 it was noted at day 12 that two larvae had degenerated. Degeneration always had the same appearance: swellings appeared in the posterior part above the bladder and progressed anteriorly. Eventually those swellings started round the scolex, giving it an inflated appearance. Approximately 6 days later the posterior part seemed to collapse, releasing debris, and this was followed by the same effect in the anterior part and the scolex, from which hooks became detached. By then there were no signs of movement in these parts, leaving only the middle part of the body intact, and with some motility. An increasing number of worms

underwent this degeneration process, so that by day 38, it was decided to stop the culture for this group, as by this time all but two larvae showed general degeneration. For both Groups 2 and 3 the degeneration started at day 20 but more larvae became more rapidly effected in Group 2 than in Group 3. By day 38 thirteen larvae in Group 2 and seven in Group 3 showed signs of degeneration. When the cultures for both these groups were stopped at day 50, nine larvae in Group 2 were still alive but all had deformed scoleces. In Group 3, fifteen larvae showed motility, of which five had an intact scolex, though the bladder and some posterior segments had been lost.

The weight of the metacestodes in all three groups initially increased and then declined. The decline was partly influenced by the shedding of the bladders, sometimes together with some posterior segments as happened to one worm in Group 1, four in Group 2 and nine in Group 3. The pattern of these changes in weight and the regression formulae are shown in Fig. III.1. The detailed data are given in Appendix Table III.1. Analysis of variance of the combined regression of all these groups indicated that the differences between the regression coefficients were significant ($F = 6.58$ for 2 and 66 degrees of freedom $p < 0.01$). However, the combined regression for Groups 2 and 3 did not reveal a significant difference between their slopes ($F = 0.17$ for 1 and 48 d.f. $p > 0.05$).

Ammonia production which gradually decreased in Group 1, rose for Group 3 and very sharply for Group 2. Fig. III.2 and Appendix Table III.2 show the data and regression formulae. Analysis of variance of the combined regressions revealed significant differences

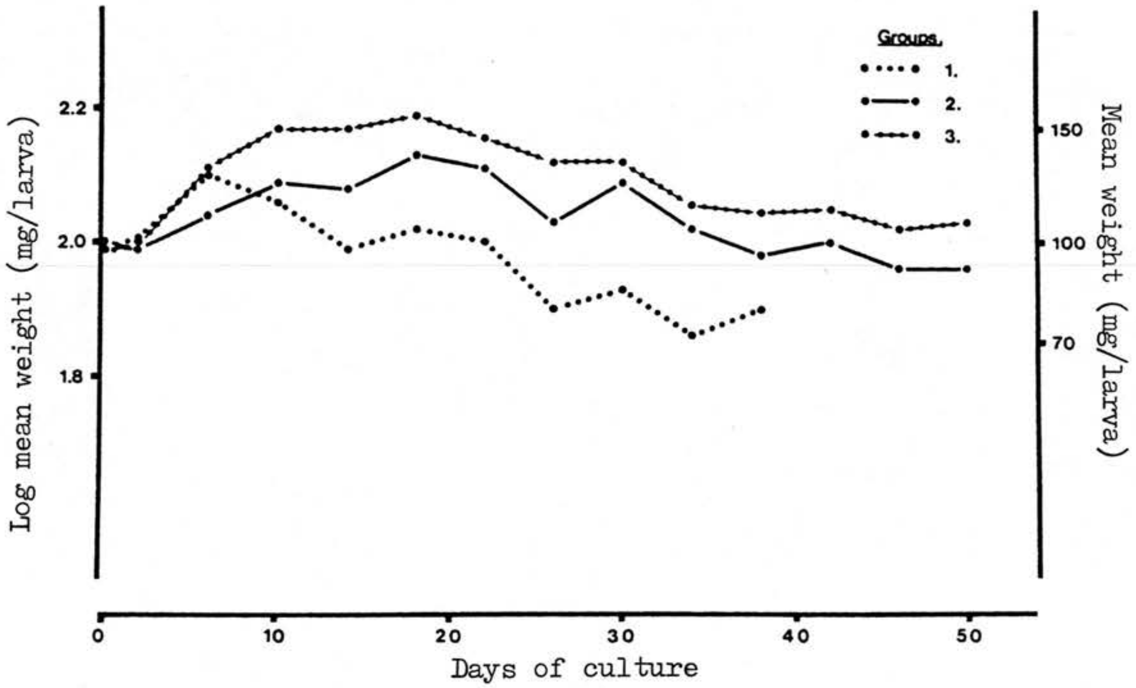


Fig. III.1 Mean individual liveweight of <u>Taenia</u> <u>taeniaeformis</u> larvae maintained <u>in</u> <u>vitro</u> in three different culture media. For Group 1 the medium was RPMI, for Groups 2 and 3 FCS was added and for Group 3 alone, sodium pyruvate was added.

<u>Regression equations</u>

Group 1: $\log Y = 2.091 - 0.006X \quad t_{(18)} = 6.742^{***}$

Group 2: $\log Y = 2.084 - 0.002X \quad t_{(24)} = 2.864^{**}$

Group 3: $\log Y = 2.134 - 0.001X \quad t_{(24)} = 1.854$ NS

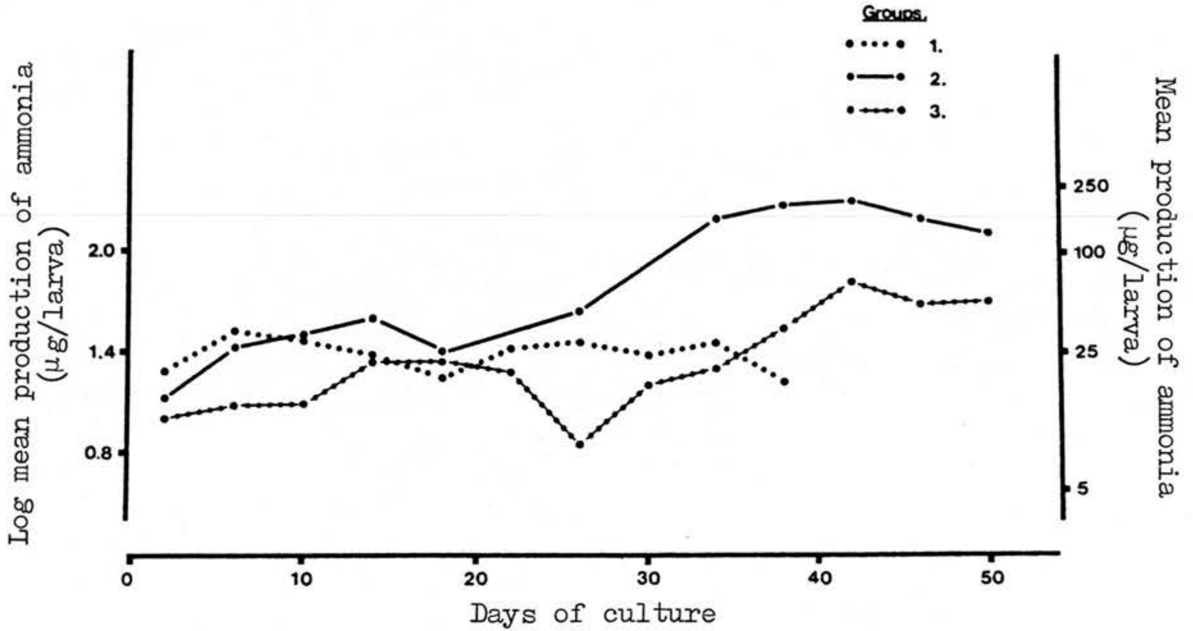


Fig. III.2 Mean production of ammonia per 48 h in μg per <u>Taenia taeniaeformis</u> larva maintained <u>in vitro</u> in three different culture media. For Group 1 the medium was RPMI, for Groups 2 and 3 FCS was added and for Group 3 alone, sodium pyruvate was added.

<u>Regression equations</u>

Group 1: $\log Y = 1.445 - 0.001X \quad t_{(16)} = 0.7$ NS

Group 2: $\log Y = 1.175 + 0.024X \quad t_{(19)} = 10.612^{***}$

Group 3: $\log Y = 0.949 + 0.014X \quad t_{(22)} = 6.427^{***}$

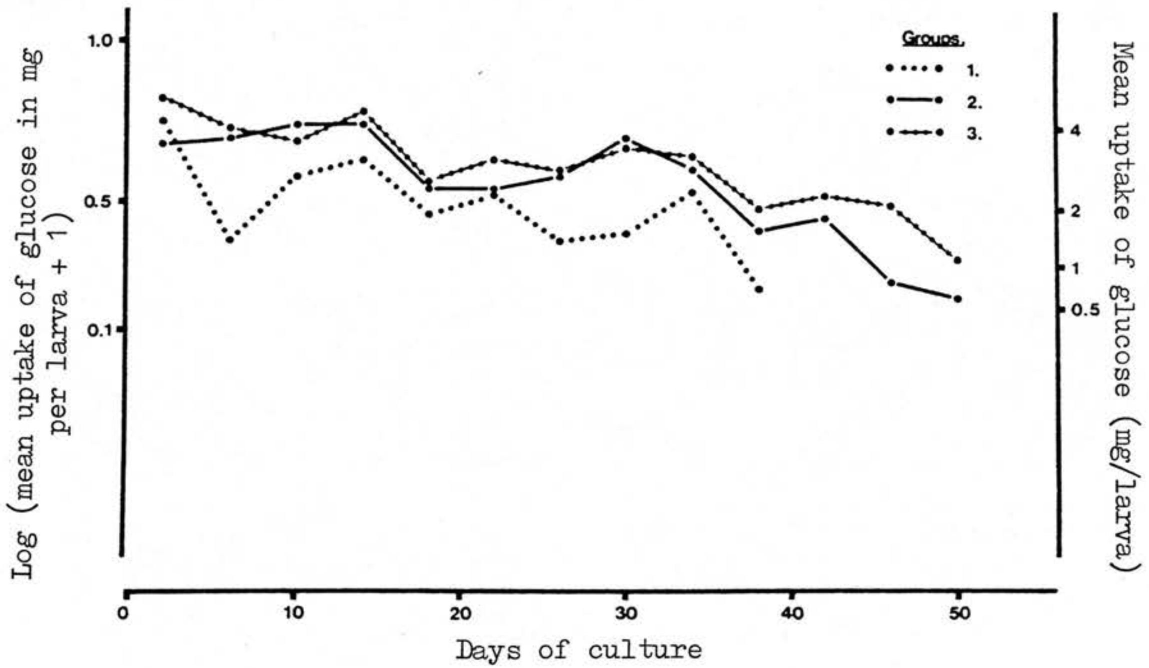


Fig. III.3 Mean uptake of glucose per 48 h in mg per <u>Taenia taeniaeformis</u> larva maintained <u>in vitro</u> in three different culture media. For Group 1 the medium was RPMI, for Groups 2 and 3 FCS was added and for Group 3 alone, sodium pyruvate was added.

<u>Regression equations</u>

Group 1: $\log (Y+1) = 0.637 - 0.009X \quad t_{(17)} = 3.394^{**}$

Group 2: $\log (Y+1) = 0.750 - 0.008X \quad t_{(22)} = 5.913^{***}$

Group 3: $\log (Y+1) = 0.750 - 0.006X \quad t_{(22)} = 5.571^{***}$

between the regression coefficients for all groups ($F = 24.667$ for 2 and 57 d.f. $p < 0.01$) and between Groups 2 and 3 ($F = 9.94$ for 1 and 41 d.f. $p < 0.01$).

The glucose uptake by the larvae in all three types of media decreased gradually, as shown in Fig. III.3 and Appendix Table III.3. Analysis of variance for the three combined regressions indicated no significant differences between the slopes of the three regression lines ($F = 0.81$ for 2 and 61 d.f. $p > 0.05$) whereas the intercepts differed significantly ($F = 17.07$ for 2 and 61 d.f. $p < 0.01$), this clearly resulting from the lower intercept for Group 1.

Second Experiment

As described for the first experiment, degeneration occurred in all three groups, but again it started earlier and became more rapidly generalised in the group which contained the simplest medium, Group 1. By day 8 in Group 1, the posterior swellings had started and by day 14 degeneration had proceeded to most of the scolèces. By day 20 all but four larvae of this group were completely deteriorated, no longer showing any movement and with much debris in the media. It was then decided to stop culturing this group. At this time the larvae in Groups 2 and 3 still had a normal appearance, apart from the fact that 6 in the former and 8 in the latter group had lost their bladder sometimes together with some posterior segments. However from day 24 onwards degeneration started in both these groups and, when the cultures were stopped on day 32, Group 2 had 2 completely degenerate worms and 7 showing scolex degeneration, while the rest (11 larvae) had all lost some of the posterior parts but otherwise appeared normal. In Group 3, six worms were completely

degenerate, five showed scolex degeneration, with only the middle part left intact, while the rest had a similar appearance to the majority in Group 2. The details of the live weight changes are shown in Fig. III.4 and Appendix Table III.4 but none of the regression coefficients were significantly different from zero or from each other. The ammonia production tended to increase with time for all three groups, (Fig. III.5, Appendix Table III.5) but this was only significant for Group 2. Glucose uptake seemed to decrease with time for all groups (Fig. III.6, Appendix Table III.6) but again, this was only significant for Group 2. Variance analysis for the combined regression showed that there were no significant differences between any of the groups as regards slope ($F = 0.422$ for 2 and 32 d.f. $p > 0.05$) or intercept ($F = 2.826$ for 2 and 32 d.f. $p > 0.05$). L-Lactate production did not show a significant trend with time, for any of the groups (Fig. III.7, Appendix Table III.7). Nor was L-Lactate detected in the controls of Group 1 or 2, despite the presence of pyruvate in Group 2. The control for Group 3 had a slight amount of L-Lactate, probably originating from the FCS. As described, this amount was subsequently subtracted from all the L-Lactate values estimated for this group.

At no occasion in either of the two experiments did examination of used media or debris show any sign of bacterial contamination.

14.4 CONCLUSIONS

Although the visual observations in both experiments allowed some discrimination between the media, as to their ability to support the survival of the larvae, in that cultures, consisting of RPMI only had to be terminated earlier than the others because of almost

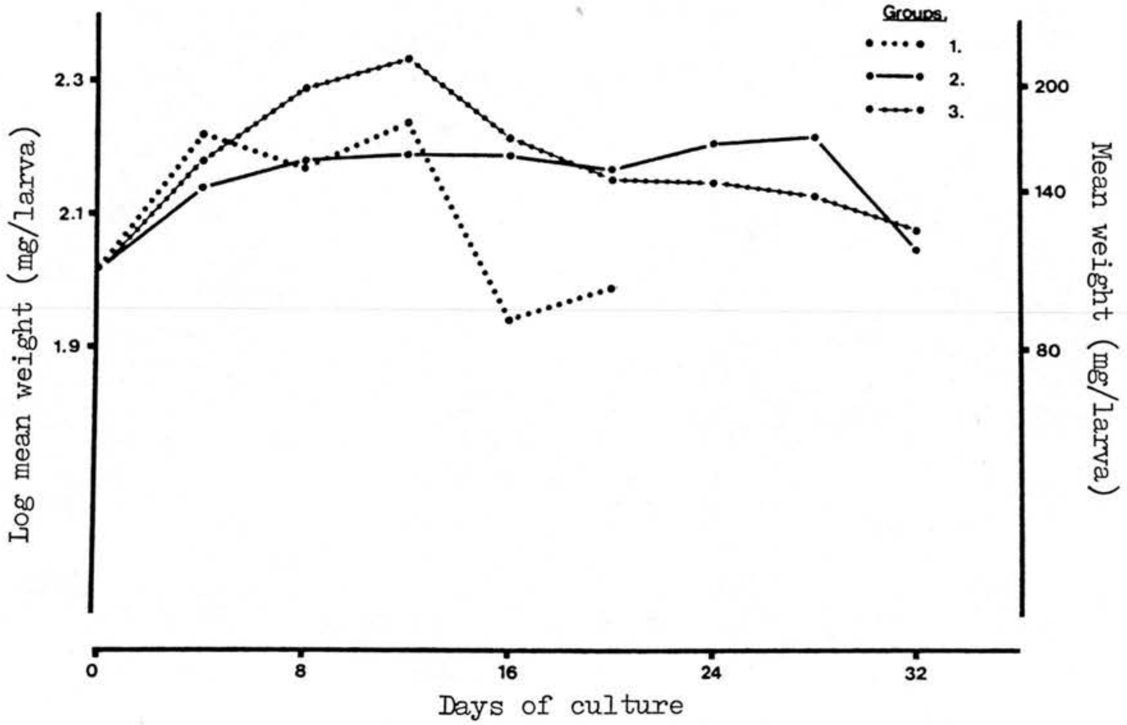


Fig. III. 4 Mean individual liveweight of Taenia taeniaeformis larvae maintained in vitro in three different culture media. For Group 1, the medium was RPMI, for Groups 2 and 3 sodium pyruvate was added and for Group 3 alone, FCS was added.

Regression equations

Group 1: $\log Y = 2.210 - 0.011X$ $t_{(9)} = 2.035$ NS

Group 2: $\log Y = 2.149 + 0.0004X$ $t_{(15)} = 0.231$ NS

Group 3: $\log Y = 2.232 - 0.003X$ $t_{(15)} = 1.148$ NS

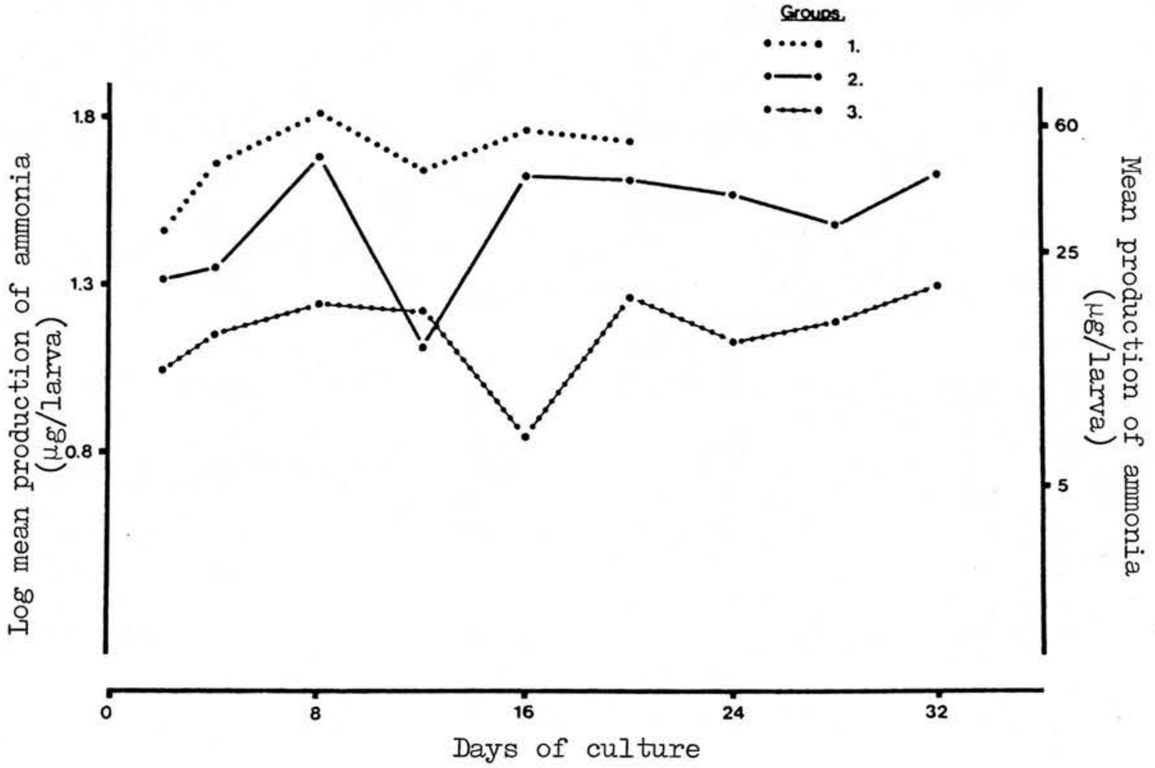


Fig. III.5 Mean production of ammonia per 48 h in µg per Taenia taeniaeformis larva maintained in vitro in three different culture media. For Group 1 the medium was RPMI, for Groups 2 and 3 sodium pyruvate was added and for Group 3 alone, FCS was added.

Regression equations

Group 1: $\log Y = 1.574 + 0.008X$ $t_{(8)} = 1.587$ NS

Group 2: $\log Y = 1.345 + 0.008X$ $t_{(14)} = 2.195$*

Group 3: $\log Y = 1.163 + 0.003X$ $t_{(14)} = 0.773$ NS

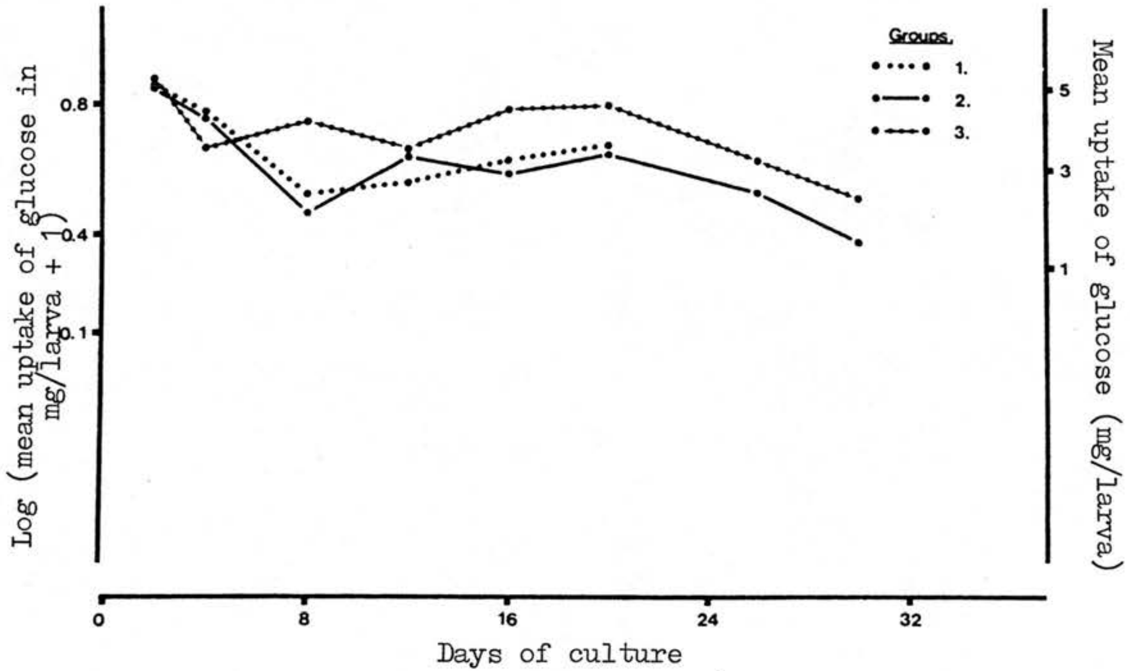


Fig. III.6 Mean uptake of glucose per 48 h in mg per Taenia taeniaeformis larva maintained in vitro in three different culture media. For Group 1 the medium was RPMI, for Groups 2 and 3 sodium pyruvate was added and for Group 3 alone, FCS was added.

Regression equations

Group 1: $\log (Y+1) = 0.733 - 0.012X \quad t_{(8)} = 0.941$ NS

Group 2: $\log (Y+1) = 0.751 - 0.011X \quad t_{(12)} = 3.620^{**}$

Group 3: $\log (Y+1) = 0.780 - 0.006X \quad t_{(12)} = 1.876$ NS

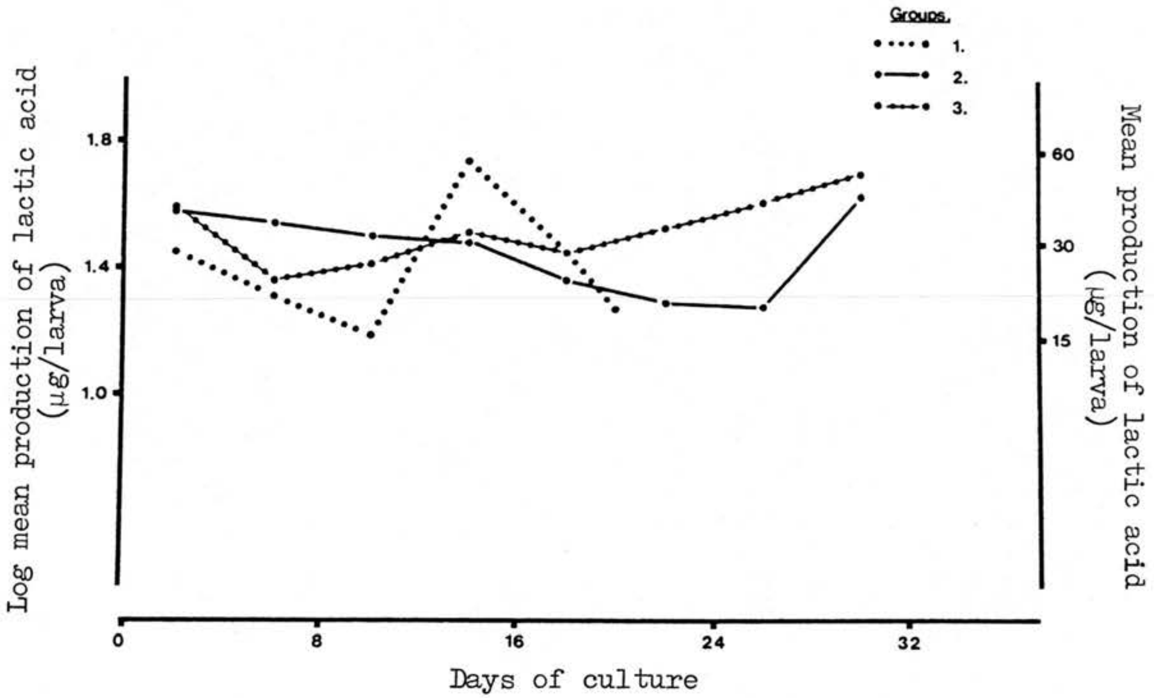


Fig. III.7 Mean production of lactic acid per 48 h in μg per *Taenia taeniaeformis* larva maintained *in vitro* in three different culture media. For Group 1 the medium was RPMI, for Groups 2 and 3 sodium pyruvate was added and for Group 3 alone, FCS was added.

Regression equations

Group 1: $\log Y = 1.381 + 0.001X \quad t_{(4)} = 0.107$ NS

Group 2: $\log Y = 1.540 - 0.005X \quad t_{(6)} = 0.994$ NS

Group 3: $\log Y = 1.405 + 0.007X \quad t_{(6)} = 1.869$ NS

total degeneration of the larvae - none of the other parameters studied reflected this clearly. There was either no clear relationship with time, as in most of the observations in the second experiment, or the changes in the parameter which were time related did not allow a distinction between poor and better media for supporting the larvae. Certainly none of the parameters allowed a conclusion to be drawn as to the nutritive requirements of these metacestodes in vitro more rapidly than could be obtained by a mere visual comparison of their survival in the different media. The weight changes were influenced both by the process of degeneration and also by the occasional loss of bladders which may reflect either a natural part of the change towards the adult stage, or an abnormality if these metacestodes were merely continuing in vitro their normal life within the intermediate host

The rate of ammonia production was clearly not such a valuable parameter in this T. taeniaeformis sytem as it was with F. hepatica (Sewell 1968, Lehner 1977 and Lehner and Sewell 1979). The apparent decline in glucose uptake may be a reflection of the degeneration process occurring in all cultures but it was not significantly related to the degeneration rate. Leakage of glucose from the larvae (Von Brand, Churchwell and Higgins 1966) probably influenced by the progressive degeneration, may well have masked the real uptake. Of the two defined media RPMI 1640, either alone or supplemented with sodium pyruvate, the latter allowed better survival. When FCS was added to both of these, the medium containing pyruvate again seemed to give slightly improved survival. This may indicate that pyruvate has a beneficial effect on these metacestodes.

Chapter 15 Preliminary experiments on survival and growth of *T. saginata* with comparative studies using *T. taeniaeformis* metacestodes *in vitro*

15.1 INTRODUCTION

Using the occasionally available material some simple experiments were set up with *T. saginata* metacestodes trying to stimulate *in vitro* growth with the aim of obtaining a reference for survival or growth against which further experiments could be compared. The lack of previously known parameters for determination of growth or survival, and the rather sporadic way in which these metacestodes became available led to most of these experiments giving inconclusive results, and to their being assessed merely on the basis of the *in vitro* survival of the metacestodes.

15.2 Experimental design

15.2.1 *Taenia saginata* larvae

Experiment 1

Metacestodes were dissected out and then processed either on the same day or after having been left overnight at 4°C. The donor calf was 1 year old and had been infected for 12 weeks. These metacestodes were used to study the effect of the overnight storage on their ability to evaginate.

Experiment 2

Survival of the metacestodes as judged by their movements was compared for two defined media NCTC 135 (Gibco Europe) and RPMI 1640 with 25 $mmol.l^{-1}$ HEPES and L-Glutamine (Gibco) both with and without 10% v/v FCS, thus constituting 4 groups. Eighty evaginated meta-

cestodes were equally divided into 8 petri dishes, two for each group; each vessel contained 10 ml of medium. The host from which the cysts were obtained was a one year old calf which had been infected for 12 weeks. The cultures were changed every 48 h and the absence or presence of movement was observed using a stereo microscope (Vickers).

Experiment 3

Medium RPMI 1640 with HEPES and L-Glutamine (Gibco) with antibiotics was compared with the modified RPMI 1640 described in Part II, Chapter 8.1.1. To both of these 10% v/v FCS was added. Each of the two groups comprised 3 petri dishes, each containing 10 ml of medium and one evaginated metacestode per ml, a total of 60. Change of media and checking for survival after 22 days was as above. The calf from which the cysts were obtained was 5 months old and it had been infected for 13 weeks.

Experiment 4

Sixty metacestodes were divided as in Experiment 3 to form two groups in both of which the same medium was used, namely modified RPMI 1640 + 10% v/v FCS. However the petri dishes in one group were not sealed with silicone grease. The larvae obtained from a 5 month old calf were 12 weeks old at autopsy.

Experiment 5

The modified RPMI + 10% v/v FCS was compared with the same medium containing one of two additives. To one group, lactalbumin hydrolysate (Gibco Europe) was added at a concentration of 6.5 $mg.ml^{-1}$, while hemin (type III Equine, Sigma) was added to another group. The preparation and concentration of the hemin was as described by

Seidel (1971), 100 mg hemin being dissolved in 1.5 ml triethanolamine (Sigma), then further diluted with sterile distilled water and added to the medium to give a concentration of 1 $\mu g.ml^{-1}$. The three groups comprised 2 petri dishes each with 10 ml medium and 10 twelve week old metacestodes in each.

Experiment 6

FCS was compared with human serum (Gibco) and rabbit serum (Gibco), all at 10% v/v in modified RPMI. Each group comprised 4 petri dishes with 10 ml of medium and 10 larvae and a fifth dish of 10 ml of medium with 7 larvae. Age of the larvae was as in the previous experiment.

Experiment 7

The forms of diphasic medium were compared with each other. Two universals were used each containing 10 metacestodes and 10 ml modified RPMI plus 10% v/v FCS. The solid substrates, were both prepared as described above but whereas one was left intact, the other was disrupted using a sterile needle. The age of the donor calf at infection was 1 - 2 months and it was killed 82 days later.

15.2.2 *Taenia taeniaeformis* larvae

Experiment 8

Metacestodes from a 3 month old infection in mice were exposed for 24 h at 37°C to a digestion fluid consisting of pancreatin (Grade III, Sigma) 10 $mg.ml^{-1}$, trypsin (E.C.3.4.21.4) (Crude II, Sigma) 1 $mg.ml^{-1}$, and lamb bile 10% v/v in Hanks BSS (Gibco, Europe). Three groups of 10 metacestodes each were placed in two petri dishes per group with 5 larvae and 25 ml of medium in each and

were treated as follows: one group was left in HBSS one group in the digesting fluid and one group in HBSS to which pieces of inverted fresh small intestine from a cat were added. Additionally 5 worms were placed in a loop of the duodenum from a cat, freshly autopsied; the loop was then placed in a petri dish with HBSS at 37°C.

Experiment 9

Groups of ten 100 day old T. taeniaeformis metacestodes were placed in a diphasic medium, similar to that described for T. saginata. Each bottle contained 25 ml heat coagulated Special Bobby Calf Serum 45 ml modified RPMI and 5 ml FCS. The solid substrate was left intact in two bottles while in two others it was disrupted.

15.3 RESULTS

15.3.1 Taenia saginata

Experiment 1

When metacestodes were treated the same day as the calf was killed, 47 out of 62 (76%) evaginated. In contrast when the meat was left overnight at 4°C before the cysts were dissected out of it, only 28 out of 80 (35%) evaginated, the remaining cysts being at most only partly evaginated (χ^2 = 21.7***). Hence for the following experiments all the in vitro procedures were undertaken on fresh cysts. Furthermore only evaginated larvae were selected for further study.

Experiment 2

The numbers of larvae which survived and were not degenerate in the four media are given in Table III.1.

Table III.1 Numbers of T. saginata metacestodes surviving after several days in vitro

Media	Day of culture 10	12	14	16
RPMI 1640	18	12	10	6
RPMI 1640 + FCS	19	15	11	11
NCTC 135	14	8	6	2
NCTC 135 + FCS	19	15	12	8

On day 16 the difference between the two media was not significant (x^2 = 3.14) while the incorporation of serum appeared to have a significant effect (x^2 = 5.59*)

Experiment 3

When the larvae were kept in RPMI 1640 plus 10% v/v FCS, 18 out of 30 survived and did not show degeneration after 22 days. In the medium containing sodium pyruvate the number surviving was 28 out of 30 (x^2 = 7.5**). However in both media degeneration occurred soon after this time.

Experiment 4

When petri dishes were not sealed, all the larvae were degenerate by 12 days. In the cultures where silicone stopcock grease was applied, the numbers of surviving larvae were 18 out of 30 after 12 days and 5 out of 30 after 24 days.

Experiment 5

The figures for survival when hemin or lactalbumin hydrolysate were added are given in Table III.2.

Table III.2 The numbers of T. saginata larvae surviving *in vitro* in RPMI with different additives.

Group	Treatment	Day of culture 3	8	13	21
1	Modified RPMI + 10% FCS	18	11	9	9
2	Modified RPMI + 10% FCS + hemin	19	10	9	6
3	Modified RPMI + 10% FCS + lactalbumin hydrolysate	16	8	8	3

The difference between Groups 1 and 3 on day 21 is significant ($\chi^2 = 46*$).

Experiment 6

When 10% FCS was added to the modified RPMI, 40 larvae survived out of the initial 47, after 10 days and 33 after 20 days. When human serum was used instead, 30 larvae survived after 10 days, none after 20 days. For rabbit serum, the numbers were respectively 32 and 16 larvae. The difference between the effect of the rabbit serum and the FCS was very significant at 20 days ($\chi^2 = 12.3***$).

Experiment 7

Survival was better in the diphasic medium with the intact solid phase than in any of the monophasic media previously used, but did not exceed 60 days, during which time little development occurred. The best survival was obtained in the medium in which the coagulated serum was broken up, and some degree of development was visible. After 10 days *in vitro* in this culture two larvae had the same appearance as when first evaginated, but in the other eight, the neck was broadened and the osmoregulatory canals were clearly visible. The active body became more distinct from the

passive forebody (Wardle and McLeod 1968) and bladder, which was shed after 18 days by larva and by four others within a further 10 days. After one month in vitro (Plate III.1) 5 larvae, two of them still with the bladders attached, showed clear growth, in that the body had become longer and strobilated at the neck and fluting of the canals was visible. After 2 months all ten larvae were still alive and active. Seven of these larvae were then removed from the culture. Photographs of the most developed of these are given in Brandt and Sewell (1979-80 appended). It measured about 70 mm long and the width of its scolex was 1.80 mm. The width at the middle of the strobila was 0.8 mm. About 150 segments were formed, and after it had been stained with Semichon's carmine (Plate III.2), a cirrus streak formation was visible. Genital pores could not yet be seen, although their probable future location was discernable from the developing cirrus apparatus. Despite the change in shape of the posterior segments, by which they became longer than their width (Plate III.2), genital organogenesis did not seem to be more advanced than in the middle segments: Rather the terminal segments tended to degenerate and become detached. The other six larvae removed from the culture at that time were still alive and active at that time, but did not show so much development, although three of them had grown, being between 20 and 30 mm long, discounting the bladder and forebody. The three remaining larvae were kept in vitro for another two months, remaining very active throughout. Their development was similar as above described, although they were not so long because of the repeated loss of their distal segments. At the end of four months in culture they measured about 60, 50 and 30 mm respectively. At

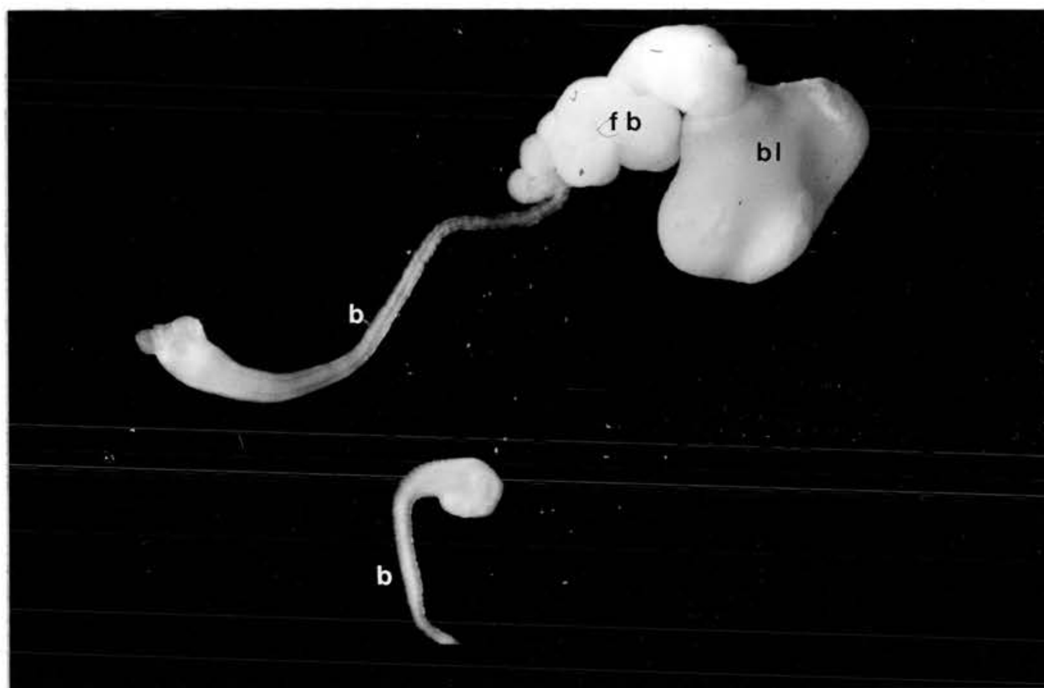


Plate III.1. (incident light x 4.5)

Living *Taenia saginata* after 30 days *in vitro* in a diphasic medium. The shorter specimen has lost its bladder and fore-body. (bl: bladder, fb: fore-body, b: body).

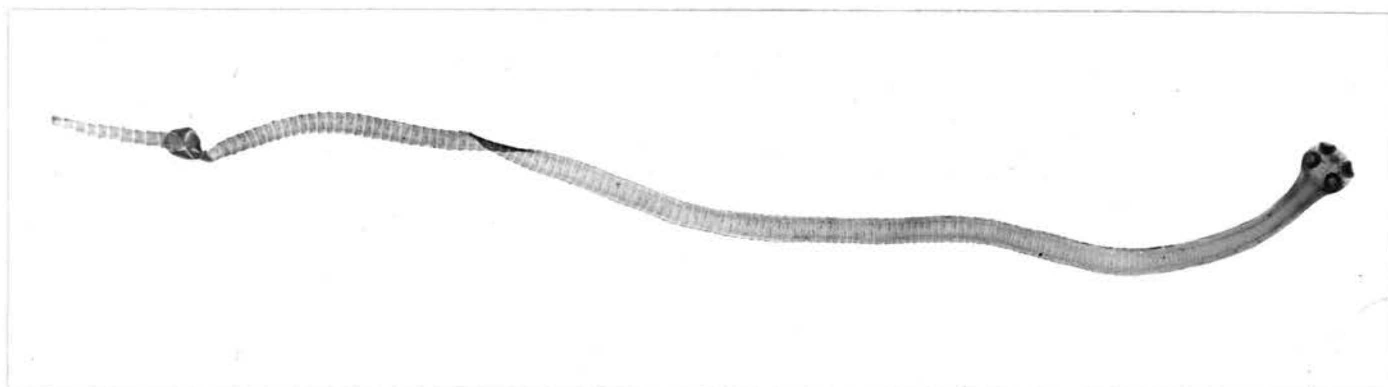


Plate III.2. (Bright field, transmitted light x 3.5)

Taenia saginata developed from a metacestode after 2 months *in vitro* in a diphasic medium, stained with Semichon's carmine. The developing cirrus sacs indicates the incipient location of the genital pores. The terminal 9 mm of the worm broke off during the processing.

no time was penetration or even attachment to the coagulated serum blocks observed.

15.3.2 Taenia taeniaeformis

Experiment 8

None of the digestion techniques applied to these metacestodes showed any result after 24 h and nor were the metacestodes seen to be stuck onto the pieces of intestine.

Experiment 9

The diphasic cultures were stopped after 2 months. No changes could be observed in any of these during and after culture. There was no differences between the cultured larvae and control specimen, taken at the beginning of the *in vitro* culture, even after staining and mounting. As with *T. saginata* none of these metacestodes were ever seen to have penetrated or become fixed to the coagulated serum.

15.4 CONCLUSIONS

The results of the *in vitro* experiments in petri dishes allow of only limited conclusions at best they only indicate that modified RPMI + 10% v/v FCS in sealed petri dishes can sustain for a limited time the survival of the metacestodes and that none of the other procedures would improve on this. The difference between *in vivo* conditions and those provided in these *in vitro* experiments is obviously too great and there is also a lack of knowledge as to the influence of degenerating metacestodes on others. This may explain the fast degeneration in some cultures. Furthermore the degeneration products from the forebody and bladder may affect the remaining parts, normally destined to grow into the adult worm. *In vivo* such an effect

might be eliminated by the intestinal flow and the constant presence of enzymes. Under these circumstances, survival as the only parameter for evaluating *in vitro* conditions cannot be expected to give conclusive results. It only provides a slow measure of the response of the worms to the medium and it is difficult to estimate the efficacy of maintenance or growth, including an abnormal growth. The relatively successful experiment with the diphasic medium may have been a coincidence or it may have been influenced by purely mechanical factors: thus universal bottles are easier to handle than petri dishes and can be more easily kept at the culture temperature while changing the media - e.g. by placing the bottles in a warm water bath.

Chapter 16 A comparison between different types of monophasic and diphasic media for *Taenia saginata* metacestodes in vitro

16.1 INTRODUCTION

The main aim of these experiments was to investigate the reproducibility of the result obtained previously with the medium in which disrupted coagulated serum had been used. A preliminary study was made to ascertain whether the apparent advantage had arisen from the addition of extra nutrients or from the physical support. Morphological parameters were used to compare the different types of culture.

16.2 Experimental design

Experiment 1

Fourteen suitable evaginated metacestodes were available. They were 95 days old at autopsy. Seven of these metacestodes were placed in a culture consisting of a diphasic medium, seven others in a monophasic culture. For the diphasic medium, 5 ml of serum was coagulated in a universal and then broken up before adding 9 ml of modified RPMI plus 1 ml FCS. The same materials were used for the monophasic medium but without coagulating the serum so that this comprised 6 ml of fluid serum and 9 ml of modified RPMI in a universal bottle. Both cultures were maintained and changed as usual. The cultures were terminated after 48 days and the worms fixed and stained. The following measurements were then taken using a microscope with micrometer ocular (Wild) the width of the scolex; the diameter of two of the suckers; the length of the body excluding

the scolex and the bladder plus forebody, present at evagination; the width of the body half-way down its length and the product of these last two parameters.

Experiment 2

Seventy metacestodes, originating from a calf which had been infected at the age of 1 year and autopsied 84 days later, were evaginated and randomly divided into 5 groups with 14 larvae in one universal bottle per group. Groups 1 and 2 were kept in respectively the diphasic and monophasic media as in the previous experiment. Groups 3 and 4 both had the same fluid phase, 9 ml modified RPMI plus 1 ml FCS. However for Group 3 a non nutritive solid phase of Sephadex beads was included. For this purpose 1 g of G 25 Sephadex (Pharmacia Fine Chemicals) was autoclaved in the culture vessel, allowed to cool and 5 ml of modified RPMI was added aseptically. This was left overnight, then the excess of fluid was drawn off before adding the fluid phase for the culture. Group 5 was fixed immediately. All four cultures were stopped after 38 days and the larvae fixed, stained, mounted and measured.

16.3 RESULTS

Experiment 1

After 13 days in the monophasic medium, 2 metacestodes were inactive and showed signs of degeneration, which first started just anterior to the forebody, progressing towards the scolex. This was occurring in five larvae in this group when the cultures were terminated. After staining, only two of these larvae had visible osmoregulatory canals although degeneration was still limited

to the posterior part. One larva in the diphasic medium showed general degeneration and was inactive, while in two others degeneration of the posterior part was starting. All larvae in both groups had retained their bladders and forebodies but in the monophasic group, these parts appeared to be petrified. The measurements for both groups are given in Table III.3.

Table III.3 Mean dimensions (± standard deviation) of _Taenia saginata_ maintained _in vitro_ for 48 days (seven observations of each parameter for each group).

Parameter	Diphasic medium mean	Diphasic medium s.d.	Monophasic medium mean	Monophasic medium s.d.	Student's "t"
Diameter of scolex (mm)	1.06	0.07	0.85	0.13	3.87 **
Diameter of suckers (mm)	0.34	0.03	0.27	0.03	4.31 **
Length of body (mm)	6.12	2.45	2.67	0.91	3.47 **
Width of body (mm)	0.32	0.04	0.26	0.08	1.83 NS
Length x width of body (mm^2)	1.90	0.67	0.72	0.35	4.18 **

Experiment 2

The results at the end of the experiment are summarised in Table III.4. In addition to this it was observed that some larvae, although showing posterior degeneration, had visible osmoregulatory canals. This was so for one larva in Group 1, four in Group 2, the canals were still visible in one completely degenerated larva and four in Group 3. One larva in Group 3 and two in Group 4 were unchanged following evagination.

Table III.4 Numbers of T. saginata showing specified features after in vitro culture for 38 days. Each group comprised 14 individuals.

Group No.	Medium	Degeneration General	Degeneration Posterior only	Canals	Plus strobilisation	Plus bladder and fore-body lost
1	Coagulated serum solid phase	0	4	12	2	1
2	Fortified fluid phase	7	4	8	1	0 (all petrified)
3	Sephadex	8	5	5	0	0
4	Fluid phase only	12	0	0	0	0

The means of the measurements with standard deviation is given in Table III.5. When significant differences were detected by analysis of variance, the data were further analysed by Harter's modification of Duncan's New Multiple Range test (Harter 1960) to reveal significant subsets. In neither of these experiments were larvae observed to penetrate the coagulated serum blocks or hold onto them.

16.4 CONCLUSIONS

The results of these experiments indicated that the diphasic medium with a disrupted coagulated serum solid phase was better than the other media for supporting the cestodes. Both longer survival and more development were obtained with this procedure,

Table III.5 Mean dimensions and significantly differing subsets of groups of T. saginata larvae (14 per group) maintained in vitro for 38 days.

Parameter	Group 1 Coagulated serum solid phase		Group 2 Fortified monophasic		Group 3 Sephadex solid phase		Group 4 Fluid phase only		Group 5 Fixed immediately		Subsets (a) Group Nos.
	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.	
Diameter of scolex (mm)	1.19	0.13	1.07	0.14	1.03	0.19	0.84	0.09	1.19	0.08	4 3 2 1 5
Diameter of suckers (mm)	0.39	0.03	0.35	0.02	0.31	0.03	0.22	0.04	0.41	0.03	4 3 2 1 5
Length of body (mm)	5.24	2.38	3.12	2.11	1.60	0.95	0.90	0.47	0.73	0.30	5 4 3 2 1
Width of body (mm)	0.49	0.15	0.30	0.12	0.29	0.21	0.24	0.08	0.69	0.15	4 3 2 1 5
Length x width of body (mm^2)	2.50	1.49	0.85	0.49	0.40	0.23	0.19	0.09	0.53	0.27	4 3 5 2 1

(a) Subsets within the same tie do not differ significantly from each other ($p<0.01$)

and, furthermore from the measurements in the second experiment the group in this medium was the only one for which the diameters of the scolex and suckers were maintained, growth in length and flattened area of the strobila was maximum and the reduction in the mean width of the body was minimal, the latter parameter being reduced in all cultured groups.

A comparison between the results for the diphasic media in the first and second experiments suggests that the metacestodes in the former were less well developed after 48 days *in vitro* than those after 38 days in the latter. Many of the factors responsible for this difference are unknown, but probably included the difference in the age of the metacestodes at the start and also, perhaps, batch variations in the undefined components. Nevertheless this procedure may have potential value as a reference for further studies on the biochemical and biophysical requirements of these cestodes *in vitro*, with the ultimate aim of devising a chemically defined medium which allows faster and more advanced development.

Chapter 17 DISCUSSION AND CONCLUSIONS

Estimating the value of a medium as regards its ability to support growth or survival of larval cestodes would greatly be facilitated by an early measurable parameter which reliably reflects the viability and biochemical integrity of the cultured forms. Survival and morphology give a reasonable indication of this, but both change too slowly to be convenient. This is especially in work such as this, aimed at devising a suitable undefined reference medium and then trying to improve it in the direction of a defined medium, with the practical aim of studying the antigenic properties of the metabolites. Furthermore, although morphological parameters provided some indication of the value of media for T. saginata, the same parameters could not be used with T. taeniaeformis metacestodes. Singh and Rao (1966) and Gonzalez and Mishra (1975) in studying development in vivo of strobilocerci to adult worms in cats concluded that the morphological changes occurring during this process were limited to genital development and took place exclusively in the strobila. Their observations on the scolex, suckers and hooks indicated no significant changes. Observations of the neck region which may be helpful for T. saginata are of little value for T. taeniaeformis, since even the existence of such a region is considered doubtful by Wardle and McLeod (1968) or at most very short, Esch and Self (1965).

Mere survival of these metacestodes in vitro may also be a parameter of little value and even by 1945 Wilmoth had reported survival for several days in simple media.

Variations in distribution of nutrients such as glycogen within the strobila may explain why the middle part of the T. taeniaeformis larvae was usually the last to degenerate. Such variations were noted by Daugherty and Taylor (1956) in adult Hymenolepis diminuta who found that the glycogen content both decreased anteriorly up to the scolex region and posteriorly from the middle part.

The failure of the biochemical parameters such as glucose uptake, ammonia production or lactic acid production was surprising in view of the successful use of such parameters in other systems (Sewell 1968 and Lehner 1977). Perhaps this arises from the peculiar nature of such long strobilate organisms, where degenerative changes in some of the individual segments may be affecting both contiguous segments and even, through their common organs (osmoregulatory canals, nerves etc.) distant segments. This would be consistent with the observations of Taylor, McCabe and Longmuir (1966), who found that some of these parameters are useful for shorter cestodes such as T. crassiceps.

It may be that the biochemical differences between adult and larval T. taeniaeformis such as those reported by Von Brand and Bowman (1961), Waitz (1963), Von Brand, McMahon, Gibbs and Higgins (1964), Von Brand, Churchwell and Higgins (1966) and Von Brand, Churchwell and Eckert (1968) would afford better parameters for monitoring survival and growth.

Another complication may be that growth as opposed to mere survival of T. taeniaeformis metacestodes in vitro may well take two ways. Either they may continue to grow as larva, producing a pseudostrobila as they do in the intermediate host (Hutchison 1958

and 1959) or they may grow towards the adult stage. If they are leaning towards the adult stage, a large part of the larval strobila is supposed to be shedded (Joyeux and Baer 1937 and Archer and Hopkins 1958), even up to 70% according to Hutchison (1959). Degeneration of this part, with growth or survival in the anterior part, is supposed to distinguish a true strobila from a pseudostrobila (Hutchison 1958 and 1959) and this loss will probably affect both secretions and food uptake.

There is a need for a study of the trigger mechanisms such as digestion or removal by other means of the pseudostrobila which might stimulate development to the adult form. It may be that it would be easier to induce such development in young infective metacestodes (e.g. 60 days old) with smaller pseudostrobila (Hutchison 1959) than in those over 100 days old, with on average more than 10 cm of pseudostrobila, used in the above experiments. Another possibility is that the degeneration products from these posterior segments may have a toxic effect on the rest of the worm, and this may account for the wide variations sometimes seen between cultures. A flow culture system would probably overcome this problem by constantly removing these products. This approach was not used in the present study as it would probably involve working with large volumes in which the metabolic products would be heavily diluted. For this reason and since it was very desirable for the study of the antigenic properties of secretory and excretory products that they should be available within a defined medium, it was decided for the latter purpose to use the procedure in petri dishes with the "modified RPMI" incorporating pyruvate medium. No previous references have

been found in the literature showing that adding pyruvate to culture media for Taeniidae is beneficial but Barrett (1978) described such an effect for Ascaris lumbricoides, and Daugherty (1956) showed that both glucose and pyruvate enriched media would support glycogenesis in Hymenolepis diminuta to about an equal extent. In addition, the fact that pyruvate is included within the metabolic pathways of cestodes has often been mentioned in the literature (Hopkins 1967, Von Brand, Churchwell and Eckert 1968, Smyth 1969[a], 1976, Saz 1970 and Von Brand 1973) but this does not necessarily mean that the addition of it in media is required. Pyruvate could not be found in media in which T. crassiceps larvae were kept (Taylor, McCabe and Longmuir 1966) but their result might have been different if they had previously inhibited the parasite glycolyte enzymes, such as lactate dehydrogenase. If the positive role of some nutrients is not always obvious, a possible negative influence of others is often even more in dispute. An example of this is the value of heat inactivating the sera prior to use in cultures. Herd (1976) reported a noxious effect of complement even in normal sera on E. granulosus in vitro, which could be removed by heat inactivation, while Rickard, MacKinlay, Kane, Matossian and Smyth (1977) found C_3 on the surface of lysing protoscoleces of E. granulosus and suggested that this lysis happened via the alternative pathway. However, Hammerberg, Musoke, Hustead and Williams (1976) working with T. taeniaeformis failed to detect damage during C_3 conversion, and later discussed this in the light of the host-parasite relationships (Hammerberg and Williams 1978). Santoro, Bernal and Capron (1979) reviewed the phenomenon that complement activation by parasites is not always

lethal to these parasites, and stated that the alternative pathway is directly activated by the parasites themselves. However the significance of these works may merely reflect the point raised by Voge (1978) about the need for more precise description of the techniques used. Thus it may be necessary to pay more attention to details of heat inactivation because whereas on the one hand it may be destroying valuable nutrients as well as the labile components of complement (Silverman and Hansen 1971), on the other hand, it may be necessary to heat at a high enough temperature and for long enough to destroy other more heat stable factors such as C_3, and it may even be necessary to repeat the procedure after each time the serum is frozen.

The results with the T. saginata metacestodes when diphasic media were compared with monophasic media may suggest that there are some non-nutritional benefits from coagulated serum, such as those summarised by Smyth (1976), including the physical effects at the parasite-substrate interface. Balis (1963) thought that coagulated serum, which he used in diphasic cultures of Trypanosoma evansi, was superior because it continuously released into the liquid phase a growth factor whereas waste products were diluted by diffusion into it. On the other hand since the solid phase was not changed as often as the liquid phase, a possible conditioning effect is not ruled out. Such conditioning of media has been successfully applied to cell cultures (Cahn, Coon and Cahn 1967) and there may be a similar benefit on some helminths in vitro, especially if there are some products secreted by the parasites themselves, which are necessary for their growth. It is not clear

whether Berntzen (1970) meant something similar when using the term "metabolised NCTC" in the description of his cultures.

PART IV: Studies on the antigenic properties of the products released by *Taenia taeniaeformis* metacestodes when maintained *in vitro*.

Chapter 18 INTRODUCTION AND REVIEW OF LITERATURE

Diagnosis of helminth disease is frequently confirmed by the relatively simple coprological methods or sometimes by detection of larvae or eggs in other than faecal material, but such direct methods are not always sufficient. Thus during the prepatent period or if the production of eggs or larvae is irregular or low, or when the parasite does not complete its full development or is situated in an abnormal location, immunological diagnostic methods may become of value. There is also much interest in the immunology of parasitic infections and of the host parasite relationships from the point of view of prophylaxis. (See Part V). In the development of techniques for such uses, the important role of the antigen soon became apparent. The most obvious readily available antigen, a crude extract of the worms, appeared in many circumstances to result in nonspecific reactions, and this led to attempts to purify or separate out the useful components of the antigen. Various workers have also considered the diagnostic value of the metabolic products released from these parasites. These metabolites have received even more attention as potential protective antigens, and this latter aspect will be dealt in Part V. The aim of this Part is to describe work related to the identification of such metabolites from *Taenia taeniaeformis* i.e. the question as to whether they differ from catabolites or products resulting merely from the decay of the parasite *in vitro*. It was *inter alia* assumed that the latter would be at best, no better than

crude worms or somatic antigen, and probably even less valuable due to breakdown.

Experiments concerned with monitoring the *in vitro* production of these antigens by *T. taeniaeformis* metacestodes and determining some of their characteristics are described. From the different nomenclature used for such metabolic antigens, the term most frequently used in helminthology appears to be ES or "excretory and secretory" antigens, and this has been adopted for this work.

18.1. Detection and function of metabolic antigens

The antigenic nature of secretory or excretory products was first indicated by the observation that, precipitates were formed about helminths exposed *in vitro* to serum from animals infected with the homologous parasite. Sarles (1937, 1938) reported this for *Nippostrongylus muris* (= *N. braziliensis*), distinguishing four types of precipitates, those at the natural orifices of the parasite being the most regularly formed. These observations were related to similar precipitations *in vivo*, and held to be partly responsible for the resistance shown by rats, actively hyperimmunised or protected by passive transfer of immunity (Sarles and Taliaferro 1936 and Taliaferro and Sarles 1939). Thorson (1951) working with the same parasite also concluded that antibodies formed against secretions and excretions had a protective character. Otto (1940) studied similar phenomena for *Ancylostoma caninum* as did Lawler (1940) for *Strongyloides ratti*, both indicating the major role of anti-metabolite antibodies in serum from immune animals. Otto, Shugam and Groover (1942) studied this precipitin reaction for *Necator americanus* with special interest in its specificity. Oliver-González (1946)

studied non-functional and functional antigens - a terminology by which he indicated whether the antigen could produce protective immunity in the host. He suggested a relation between the presence of live worms in the host and functional antigens being formed, based on his experiments on differences in anaphylactic reactions in guinea-pigs against several antigens of Ascaris lumbricoides. Similarly Sprent (1951) investigated such anaphylactic activity for several antigens including metabolic products of A. lumbricoides.

Oral, anal and vulval precipitates formed in vitro by immune serum against Trichinella spiralis were described by Oliver-González (1940) and their possible role in the immune mechanisms was discussed. This was further studied by Jackson and Lewert (1957) who concluded that the precipitates were the result of a reaction of antibodies with antigens on the surface or present in the excretions. Direct evidence of metabolic antigens derived from T. spiralis was reported by Olson, Richards and Ewert (1960) using a gel precipitation test they compared products secreted by living larvae with other antigens; they also discussed the complexity of these antigens. Thorson (1970) gave a review of this type of work with nematodes. Similar work has been reported with cestodes. Thus, Silverman (1955) described precipitates formed around secretions from the penetrating glands of hatched and activated Taenia saginata and Taenia pisiformis onco-spheres, when these were exposed to immune serum and discussed the potential diagnostic value of these phenomena. Smyth (1969[a]) also commented on this and on the secretory granules of taeniid scoleces as a source of antigenic stimulation of the intestine. Various experiments were undertaken whereby attempts were made to remove

this activity of immune serum by absorption with somatic or metabolic antigen. Thus Thorson (1954) found that preabsorbing with ES but not with somatic antigen removed the protective activity of anti-N. braziliensis immune sera. Murell (1971) removed the action of immune serum on the membrane permeability of T. taeniaeformis by absorbing it with either somatic or ES antigen. In contrast, Sewell (1968) working with metabolic products of the trematode Fasciola hepatica found that they were serologically active but would not absorb out these antibodies in sera from infected animals, which formed precipitates around newly excysted living flukes. Jenkins and Wakelin (1977) suggested an association between the protective antigens of Trichuris muris and stichocyte secretions which were also released in vitro.

18.2. Identification of ES antigens

Although, as discussed in the previous Part, various authors have studied the biochemical characteristics of metabolic products of helminths, there has been less interest in combining these studies with immunological aspects. Thorson (1953) found that the respective immune serum could inhibit a lipolytic factor present in the metabolites of N. muris and (Thorson 1956[a]) a proteolytic factor present in secretions of the oesophagal glands of Ancylostoma caninum. He also showed (Thorson 1956[b]) that these secretions were to some extent immunogenic. Soulsby (1963[a]) commented on the probable lability of ES antigens. Apart from the difficulty in collecting large amounts of them, deterioration could occur rapidly presumably because they contain various enzymes. He associated with this Chandler's (1953) contention on the role of enzymes and anti-enzymes in immunity to

helminths and pointed out that it might explain the poor immunogenicity of dead or disintegrated material (Soulsby 1957[a]).

Various workers have expressed their concern about the risk of obtaining abnormal metabolites. Thus, Weinstein (1958) pointed to the need for media in which normal metabolism could occur.

The influence of methods to preserve used media has also often been discussed. Thus, Denham (1967) believed his metabolic T. spiralis antigen to be degraded by freeze-drying. Lyophilisation and, even worse, storage at -20°C was reported to be deleterious by Soulsby (1963[a]) for Ascaris suum metabolic antigen, whereas other workers have not found such an effect (Silverman, Poynter and Podger 1962, Guerrero and Silverman 1969, Simmerman and Leland 1974 and Herd 1975).

Hinck and Ivey (1976) compared ES and hatching fluid from A. suum on the basis of their proteinase activity and found it to be at similar levels. Hammerberg and Williams (1978) found factors which interact with the complement system in serum, to be present in T. taeniaeformis cyst fluid and to be released in vitro. Cyst fluid, especially that originating from T. saginata and Echinococcus spp. has been given attention by several workers as a possible source of antigen (Froyd 1963, Frick and Süsse 1970, Capron, Yarzábal, Vernes and Fruit 1970, Machnika 1974 and Yong, Heath and Parmeter 1978). Kagan and Agosin (1968) stated that it was a complex mixture, containing several substances including end products of carbohydrate and protein metabolism.

Body fluids, especially those from nematodes have received much interest due to discoveries such as the relationship between "self cure" and exsheathing fluid (Soulsby and Stewart 1960), and the

necessity for the presence of the actively metabolising parasite in the immunisation process (Soulsby 1957[a, b]). This led to the contention that metabolic products obtained *in vitro* might contain the same protective antigens as would have been produced by that parasite *in vivo*, provided that these were cultured at the right stage of development (Soulsby 1963[b]). This indicated an awareness of the complexity of these antigens. Apart from these possible variations related to each particular stage of development, Mills and Kent (1965) working with *T. spiralis* were concerned with the possibility that antigen obtained by *in vitro* culture might also be contaminated with products resulting from disintegration and death of the parasites. Based on information obtained with immunoelectrophoresis and gel precipitation, using whole and fractionated antigen these authors accepted the presence of ES antigens in their used media.

A review of this subject is given by Fife (1971), who again stressed the need for more precisely defined media particularly when it is desired to study any ES antigens produced.

Stromberg (1979) determined the molecular weight of immunogens in excretions and secretions of *A. suum* to be around 67 000. Zimmerman and Leland (1974) found only the higher molecular weight fraction of *Cooperia punctata* ES antigen to be serologically active.

Rickard and Outteridge (1974), commenting on their findings that while rabbits could be protected against *T. pisiformis* using an ES antigen they had failed to detect antibodies against this antigen, concluded that there may be no relationship between protecting and serologically active antibodies. They further suggested

that either the protective antibody level was too low to be detected or that the physical form of the antigen prevented such detection. They also considered the possibility that proteolytic enzymes, released in vitro by the developing larvae may have altered and serologically inactivated other secreted enzymes.

18.3 The use of ES products in immunodiagnosis

In discussing immunodiagnosis of helminth infections various authors have concluded that purified antigen might allow for more specific and consistent reactions (e.g. Soulsby 1963[b], Biguet, Capron and Tran Van Ky 1967, Fife 1971, Harrison 1977 - for bovine cysticercosis and Matossian 1977 - for human hydatidosis). Kagan (1974) stated that isolation and purification of parasite antigens had not kept pace with immunodiagnostic methods. Inevitably, following the detection of ES antigens many reports on their diagnostic value were given. Comparing these is not always possible, as not only was there continuous evolution and improvement in the serological and cultural techniques but also the complexity of ES antigen was gradually appreciated.

The serum precipitation reaction in vitro using living larval worms as antigen, for which the "Cercarienhüllenreaktion" described by Vogel and Minning (1949[a,b]) became a classic example, was used by Roth (1946) for diagnosis of human trichinosis. Similarly Silverman (1955) experimented with T. saginata and T. pisiformis hatched and activated oncospheres while Otto, Shugam and Groover (1942) used a similar approach for human hookworm infections, as did Wikerhauser (1961) for bovine fascioliasis.

Sadun and Norman (1957) compared metabolic and somatic T. spiralis antigen by the bentonite floculation reaction and found ES antigen to be more sensitive. This was confirmed by Slanga and Ivey (1962) using the same test but both antigens had equal sensitivity in passive cutaneous anaphylaxis. Oliver - González and Levine (1962) found no difference between larval metabolic and somatic T. spiralis antigen in the agar gel double diffusion technique. Anderson, Sadun, Rosen, Weinstein and Sawyer (1962) had better results with Angiostrongylus cantonensis metabolic antigens, in detecting antibodies in cerebro-spinal fluid by the complement fixation test.

There are several reports concerning metabolic antigens of Schistosoma spp. Kagan and Oliver-González (1958) obtained good results with such antigens from Schistosoma mansoni in haemagglutination tests, as did Minning, Newsome and Robinson (1958) in the complement fixation test. In contrast Sadun, Lin and Walton (1959) using the same tests, found metabolic antigens from Schistosoma japonicum not to be as good as somatic antigens. Madwar, Shoeb and Basmy (1971) used metabolic S. mansoni for an intradermal diagnostic test. Murell, Vannier and Ahmed (1974) found no particular advantages from working with ES antigens from S. mansoni, as many of the antigens present in metabolites were also present in somatic extracts and the latter were easier to obtain.

For F. hepatica metabolites, high specificity was reported by Minning, Newsome and Robinson (1958) in complement fixation tests, in that there was no cross reaction with sera from bilharzia patients. Similar findings were reported by Paris and Capron (1962) in gel diffusion tests using sera from patients with a variety of nematode,

trematode and cestode infections, in that these did not cross react with the reaction between F. hepatica ES antigen and sera from cases of human fascioliasis. Gundlach (1971) had better results with Fasciola metabolites during the prepatent period but at a later stage a somatic antigen was found to be better. Čuperlović and Lalić (1972) and Čuperlović (1972) found that metabolic antigens were also present in crude somatic extracts but only the former were of value for early diagnosis of fascioliasis by the complement fixation test. In contrast, Lehner (1977) found no evidence that metabolic antigen was any better than somatic antigen in the ELISA or immunodiffusion techniques but concluded that as this antigen is probably less complex than a somatic antigen, it might be a more suitable starting product for purification.

Zimmerman and Leland (1974) compared somatic and metabolic antigens from Cooperia punctata in precipitation tests and found that the former also had activity against small intestine mucosal extracts. In the serodiagnosis of visceral larvae migrans caused by Toxocara spp. precipitating antibodies to ES antigen of Toxocara canis and Toxocara cati larvae but not to Toxascaris leonina were found by Hogarth-Scott (1966). Using Toxocara spp ES antigen in the indirect haemagglutination and soluble antigen fluorescent antibody tests, De Savigny and Tizard (1975) concluded this to give highly sensitive and reproducible results for early detection of T. canis antibodies in experimental infection and for diagnosing human larva migrans infections.

Morris, Proctor and Elsdon-Dew (1968) found a fraction of ES antigen to be of great diagnostic value in the serological diagnosis

of porcine cysticercosis by gel precipitation tests but it had no activity in haemagglutination tests whereas an extract of scoleces had such an activity. Similarly Beltran and Gomez Priego (1973) found cyst fluid and metabolites from T. solium metacestodes to be equally effective and better than somatic antigen for detection of experimental or human cysticercosis. Heath and Lawrence (1977-78) reported briefly the use of in vitro products derived from Taenia ovis oncospheres in serodiagnosis.

Finally Araj, Matossian and Frajha (1977) discussed the possibility of the formation of a wide range of antibodies of which only a few are detectable at any one time, when using a complex ES antigen produced during various developmental stages.

18.4 ES and circulating antigens

Although in helminth-immunodiagnosis most of the emphasis has been laid on detecting antibodies, in recent years more attention has been paid to the detection of antigen circulating in the plasma as possibly being more specific (Kagan 1974, 1979). The literature concerning parasite circulating antigens was reviewed by Wilson (1977), who also discussed the possible role of soluble antigen released by the parasites in immune avoidance. Not surprisingly, most work on helminth circulating antigen has been done with schistosomes, as an intravascular parasite. Nash, Prescott and Neva (1974) and Nash, Nasir-Ud-Din and Jeanloz (1977) studied the characteristics of circulating antigen from Schistosoma mansoni and assumed it was either excreted or secreted into the circulation by the worm. They discussed the possibility of using these antigens as a means of quantifying the numbers of schistosomes present. Von Lichtenberg,

Bawden and Shealey (1974) believed that the schistosome gut was the site of production of circulating antigen rather than the tegument. Nash (1974) confirmed previous findings about the polysaccharide nature of the antigen and he also found it only in the epithelial gut cells, whereas Kusel, Mackenzie and McLaren (1975) studied antigen released by S. mansoni in vitro and concluded that culture media contained surface membrane antigens in both soluble and in particulate form. They also considered that these correspond to materials released in vitro, and that they have a possible role in modifying the host immune response. Bawden and Weller (1974) used a complement fixation test to detect S. mansoni circulating antigen, using a rabbit antiserum prepared against a purified somatic antigen, derived from whole worms. Nash (1979) used radioactive labelled nutrient precursors to study the release of excretory and secretory products of S. mansoni. There appeared to be substances of both large and small molecular weight, produced and released from at least two sites, namely the tegument and the gut. Santoro, Vandemeulebroucke and Capron (1979) further studied schistosome circulating antigen and immune complexes using CFT and radioactive labelling with monospecific antisera obtained from antigens fractionated in agarose.

Whereas for schistosomes the circulating antigens have been considered to be derived from ES products, for bovine cysticercosis Van Knapen and Fridas (1977) believed the source to be from degenerating cysticerci; based on the appearance of antigen followed by antibodies in sera and the occasional reappearance of antigen which was believed to follow death of some of the cysticerci. They developed an ELISA test for detecting antigen in which the antisera was prepared

against the somata of Taenia crassiceps larvae.

18.5 ELISA and immunoelectrophoresis (IEP)

IEP has been extensively used in studies on the serological diagnosis of hydatidosis, since the report of Capron, Vernes and Biguet (1967) that the reaction between hydatid fluid and serum from patients showed a specific band "arc 5" even in early infections. This was reported to be the most specific test available for pre-operative diagnosis (Capron, Yarzábal, Vernes and Fruit, 1970 and Varela Diáz, Guisantes, Ricardes, Yarzábal and Coltorti 1975).

Purification of antigens by Bout, Fruit and Capron (1974) gave the test even more practical value. However further studies revealed cross reaction with other taeniids (Varela Diáz, Coltorti and D'Alessandro 1978, Varela Diáz, Coltorti, Rickard and Torres 1977, Yarzábal, Bout, Náquira and Capron 1977). Geerts, Kumar and Aerts (1979) studied the value of IEP for diagnosing bovine cysticercosis and distinguished 2 precipitating lines of diagnostic interest.

Nakane and Pierce described in 1967 a technique for identifying and localising antigen in biological tissues by labelling antibodies with horse radish peroxidase. A technique for the indirect determination of antibodies using antigen adsorbed onto the wall of the polystyrene tubes and enzyme-conjugated anti-immunoglobulin to label the antigen antibody complex was described by Engvall and Perlmann (1971, 1972) and named the enzyme-linked immunosorbent assay (ELISA). Since then this technique had found numerous applications including several related to helminth parasites. This had been facilitated by an increasingly wide availability of commercially produced reagents,

particularly enzyme labelled anti globulin conjugates for many species of animals. The test has been made more practical by further improvements, the most important related to this work being the use of polystyrene microplates instead of tubes (Voller, Bidwell, Huldt and Engvall, 1974). This modification saved reagents, made identification of samples less liable to error and above all simplified and speeded up the washing procedures. Wolters, Kuijpers, Kačaki and Schuurs (1976) and Voller (1978) used ortho-phenylene diamine as substrate, which has the advantages of being more stable and relatively easy to prepare as compared with other substrates which have been described for use with this technique. From an abundance of reports concerned with detecting antibodies for the serodiagnosis of taeniid infections by means of ELISA, sometimes in comparison with other techniques, there seems to be general agreement that ELISA combines the advantages of being both sensitive and practicable (Farag, Bout and Capron 1975, Harrison 1977, Van Knapen and Fridas 1977, Walther and Sanitz 1979, Albert and Hörchner 1979 and Matossian, McLaren, Draper, Bradstreet, Dighero, Kane, Mackinlay and Rickard 1979).

In view of the possibility of obtaining more specific results by detecting circulating antigen (Kagan 1974), several workers have studied the application of ELISA to this procedure. The original procedure for this test was designed for detecting antigen and Kagan (1979) considered that ELISA would be very suitable for this purpose. In parasitology, Voller, Bartlett and Bidwell (1976) used this approach not entirely successfully, for detecting S. mansoni circulating antigen. Van Knapen and Panggabean (1977) obtained encouraging

results detecting Toxoplasma gondii circulating antigen in the acute stage of infection. This, combined with determination of antibody levels, may lead to diagnosis of active toxoplasmosis on a single serum sample. Again, the double antibody sandwich method was used for circulating antigen with the enzyme co-valently linked to anti-toxoplasma immunoglobulins. Harrison (1978) used ELISA to detect antibodies and antigen in sera from cattle with naturally acquired cysticercosis. Antigen levels were reported to be high between ten and twenty weeks post infection.

To avoid the complications and loss of sensitivity associated with enzyme labelling of immune sera, an indirect triple antibody sandwich ELISA for detecting circulating antigen in bovine cysticercosis sera was used by Van Knapen and Fridas (1977). They used T. crassiceps larval somatic hyperimmune serum from two different mammalian species and then a commercial anti-globulin conjugate against the species used in the preceding step. Circulating antigen was again detected but they assumed that this had originated from degenerating cysts.

Chapter 19 MATERIALS AND METHODS

19.1 Preparation of antigens

All T. taeniaeformis antigen originated from the Belgian strain. For preparing saline extracts of adult and larval T. taeniaeformis whole tissue - referred to as larval or adult "somatic antigen" respectively - fresh material was obtained from autopsies or after drug treatment of infected cats. In the latter case, cats were orally dosed with 4 mg arecoline hydrobromide each (Epro Products Ltd.). Only whole (i.e. including scolex) living worms expelled within 30 minutes after treatment were used. Larval material was obtained by dissecting the livers of mice, which had been infected for at least 2 months and up to 6 months. A pool of larvae within this age range was made by selecting normally coloured, living (i.e. motile) larvae. Either material was carefully washed with saline and antibiotics in a household nylon strainer, 12 cm diameter, pore size 1 mm, to remove as much host material as possible. When they appeared clean but still viable they were placed in a universal container with just sufficient saline to cover them and stored at -20°C. For preparing either a larval or adult saline extract as somatic antigen, the material was weighed and cut with a scalpel whilst frozen. A little PBS was added and the whole was homogenised in a top drive homogeniser (MSE) five times for 2 minutes each time, between which it was kept in an ice bath. More PBS was then added to a final concentration of 10% weight/volume and the suspension allowed to extract overnight at 4°C on a magnetic stirrer. It was then centrifuged for 30 minutes at 2000 g and the supernatant stored at 24 h at-20°C to allow a colloidal sediment to form after

being thawed. This suspension was ultracentrifuged at 100000 g (average centrifugal force) for 30 minutes at 4°C on an L_2 65B ultra centrifuge (Beckman) and dialysed overnight in a Visking tubing cellulose membrane (Scientific Suppliers Co. Ltd.) against PBS. This tubing retains molecules in excess of about 20 000 M.W. During all these procedures the temperature was not allowed to rise above 4°C. The protein content of these extracts was routinely estimated by the method of Warburg and Christian (1941) on a U.V. spectrophotometer (SP 1800 Pye Unicam). If necessary the extract was concentrated by dialysis against a solution of polyethylene glycol ("Carbowax 6000" BDH) followed by another estimation. The protein content was eventually adjusted with PBS to 5 $mg.ml^{-1}$. The extracts were then stored in 1 ml aliquots at -20°C or in equivalent amounts lyophilised as a powder in stoppered penicillin flasks. The latter were reconstituted before use by adding 1 ml of distilled deionised water. A slight modification of this process was also sometimes used, in which the initial homogenate from a batch of whole adult worms was prepared using an "LKB X - 25 press cell Disintegrator" (LKB) according to the principle explained by Edebo (1960, 1961[a,b]). For this, the material was transferred to the pre-cooled X-Press cylinder of 25 ml capacity and forced through 4 times at -25°C at 200 MPa using a hydraulic press (P-16 Beckman) the cell being recooled between pressings. The homogenate was removed from the cylinder and further treated and lyophilised as described above. For both methods the yield of soluble protein was around 2 g % of the original wet weight of the whole worms.

Cyst fluid was obtained from metacestodes recovered by autopsy

2 months post-infection, since it was noticed that metacestodes from older infections which had formed more pseudostrobila (as discussed in Part III) had relatively small cyst bladders with less fluid. These metacestodes were washed as described above, but then placed on an inclined petri dish. After perforating the bladder with a sterile needle the cyst fluid was collected and passed through a 0.22 μm pore size membrane filter (Millipore). The protein was estimated and the fluid was stored in small aliquots at $-20^{\circ}C$.

T. crassiceps larval somatic antigen and cyst fluid was kindly supplied by Dr. L.J.S. Harrison, the somatic antigen being prepared much as above except that both cyst fluid and larval somatic antigen were recovered from the same larvae separated by ultracentrifuging them at 100 000 g (average centrifugal force) for 30 minutes at $4^{\circ}C$ (L_2 65 B ultracentrifuge - Beckman).

Medium in which metacestodes had been maintained by the methods described in Part III, is referred to as ES antigen. This material was not fractioned or purified by other means and, as is made clear later in this Part, the use of this name does not imply that it was free from somatic contaminants. Details concerning the culture procedures will be given where relevant but in all experiments with immunoelectrophoresis and for some ELISA studies sufficient cultures were set up to provide enough material to allow for comparisons. The media which is referred to as "Stock ES" was obtained by maintaining a pool of T. taeniaeformis metacestodes, between 4 and 6 months old at autopsy, in defined medium (earlier described as "modified RPMI") in petri dishes at a concentration of one larva per ml. Media were changed daily and kept for a period of about ten

days before the worms were seen to be degenerating. Medium from the first collection was discarded, while the other collections were stored in the fridge until the cultures were terminated, and then pooled, passed through a 0.22 μm pore size membrane filter (Millipore) and stored sterile at -20°C in 100 ml aliquots. These media were concentrated in two ways, freeze-drying or ultrafiltration. For the latter procedure, the medium was pumped through an "On-Line Column Eluate Concentrator" (CEC 1-Amicon) using a peristaltic pump ("Desaga PLG multipurpose peristaltic pump 13 21 00" Camlab). The concentrator cell contained a Diaflo ultrafiltration membrane UM 10 (Amicon), with a molecular weight cut off of 10 000. This was assembled, maintained and used according to the manufacturer's instructions and was tested for leakage using a solution containing Blue dextran (Sigma). The flow rate was adjusted or the concentrated fluid passed over the filter again until the volume of the concentrate was one tenth of the total effluent.

Lyophilisation was performed on a freeze-dryer ("10-140 BA MDC Macro dry ice Mobile" Virtis). For this 50 ml samples were evenly shell-frozen on the wall of a round-bottomed 500 ml flask by gently rotating it in a mixture of isopropylene alochol (BDH) and solid CO_2.

Sublimation was carried out at about 0.5 Pa over about 6 hours. The lyophilised powder was redissolved in distilled, deionised water to a tenth of its original volume and stored at -20°C.

19.2 Preparation of sera

Sera from infected rats and mice were obtained by decapitation of ether-anaesthetised animals. Blood was collected into universal

containers, to a maximum of 10 ml per container and left for one hour at 37°C for the clot to form. This clot was gently loosened, where it adhered to the container and the container was stored at 4°C overnight to allow retraction. Thereafter the clot was removed and the serum centrifuged at 2500 g for 30 minutes at 4°C and stored in capped plastic vials at -20°C. Sera from infected animals was in this Part referred to as pooled sera from rats or mice infected with 200 M or 100 B strain T. taeniaeformis eggs respectively for between two and five months. For T. crassiceps these sera originated from mice which had received fifteen larvae intraperitoneally and then been left for three months as described in Chapter 3. Hyper-immunised sera were raised in rabbits and guinea pigs against somatic antigen from larvae or adult T. taeniaeformis or ES antigen, generally according to the method described by Herbert (1978) and for ES antigen as described by Vaitukaitis, Robbins, Nieschlag and Ross (1971). For convenience the former is referred to as the classic method and the latter as the Vaitukaitis method. Male New Zealand white rabbits 6 months old at the beginning of the experiment and 3 month old albino Duncan Hartley guinea-pigs were used.

19.2.1 Preparation of adjuvant emulsions

All emulsions were of the water-in-oil type and were prepared immediately before use. Either somatic saline extract or 10 x concentrated ES antigen was mixed in equal volumes with Freund's complete or incomplete adjuvant as required. Initially the emulsions were prepared by repeatedly and forcefully injecting the antigen solution in small aliquots into the adjuvant, with vigorous shaking after each injection, until a homogeneous-looking emulsion was formed.

Finally the emulsion was further forced repeatedly through a syringe and needle, to improve the dispersion of the aqueous phase. It was later found to be more practicable to prepare the emulsion with a top drive homogeniser (MSE). Again the antigen was added in small aliquots to the adjuvant and the mixture homogenised for half a minute after each addition, avoiding warming. Emulsions were only used for injections if all but the first droplet retained a spherical form in cold water. No more than one ml of an emulsion was injected in one site.

19.2.2 Hyperimmunisation of rabbits with somatic antigen from adult *T. taeniaeformis* (Ra A.Som)

On day 0 these rabbits each received 2 ml somatic antigen at a concentration of 5 mg soluble protein per ml emulsified in 2 ml Freund's complete adjuvant ("FCA" Difco) i.m. at 4 injection sites. A similar dose in Freund's incomplete adjuvant ("FIA" Difco) was given on day 35 and day 70 and half the dose at two sites on day 366.

19.2.3 Hyperimmunisation of a rabbit with larval *T. taeniaeformis* somatic antigen (Ra L Som)

The rabbit was given 2 ml of larval somatic antigen (5 mg soluble protein ml^{-1}) in 2 ml FCA, injected i.m. in four sites. On day 35 it received a booster s.c. injection of antigen only (10 mg soluble protein total) and was bled on day 42.

19.2.4 Hyperimmunisation of rabbits with ES antigen (Ra ES)

The procedure followed for each of the two rabbits was as follows. On day -10: Serum was collected from each rabbit, inactivated at 56°C for ½ hour and sterilised by filtration. Twenty metacestodes, 6 months old at autopsy, were held for 24 hours in

20 ml modified RPMI. Culture methods were as described before. This medium was then discarded and the larvae transferred to a medium containing 18 ml modified RPMI and 2 ml of rabbit serum. Cultures were changed daily and maintained for eight days. All used media containing sera from a single rabbit, were pooled, filtered through a 0.22 μm pore size membrane filter (Millipore) and 10 x concentrated by ultrafiltration. At day 0, 2 ml of this concentrate was emulsified in 2 ml Freund's complete adjuvant ("FCA" Difco) and injected i.m. into the same rabbit as was the serum donor for the respective *in vitro* culture. The rest was stored at $-20^{\circ}C$. This procedure was adopted as it was considered that serum would probably aid the metabolic processes of the cysts, while on the other hand it would avoid the rabbits forming antibodies against undefined nutrient components of the media. At day 35 the injection was repeated but the antigen was prepared in FIA. This was again repeated at day 63 using half the dose. On day 119 the rabbits were boosted with 5 ml unconcentrated ES antigen given s.c. without adjuvant, originating from a stock ES preparation.

19.2.5 Hyperimmunisation of guinea-pigs with somatic antigen (G.P.a Som)

Each guinea-pig received at day 0, 2 ml of emulsion containing 5 mg soluble protein somatic saline extract in FCA, i.m. at 2 injection sites. On days 49 and 75, they were injected s.c. with somatic antigen only at a concentration of 2.5 mg per animal. Finally they were boosted on day 225 with double this dose. They were bled on days 58, 83 and 232.

19.2.6 Hyperimmunisation of guinea-pigs with ES antigen (G.P. a ES)

For this purpose ES antigen was prepared as described for "Stock ES" during 7 days culture, discarding the media from the first 24 h. The protein concentration of this medium was estimated at 12 $\mu g.ml^{-1}$ and this was concentrated 20 x by freeze-drying.
(a) The classic method G.P. a ES(c): at day 0 each guinea-pig received 1 ml of concentrated ES antigen (≡ 240 μg soluble protein) in 1 ml FCA i.m., divided into two injection sites. They were boosted on days 49 and 75, when they received 0.5 ml concentrated ES without adjuvant s.c. and finally on day 225 they received 10 ml unconcentrated ES antigen s.c. They were bled on days 58, 83 and 232.
(b) The Vaitukaitis method G.P. a ES(v): each guinea-pig received on day 0 0.75 ml concentrated ES antigen (≡ 180 μg soluble protein) in 0.75 ml FCA intradermally, using an all-glass syringe with a sterile disposable needle (25 G 5/8 Becton-Dickinson), divided into 40 injection sites, on the previously shaven and disinfected back. In addition to that they received one s.c. injection of 0.2 ml of Pertussis vaccine (c. 1.6 units Bordetella pertussis The Lister Institute). On day 149 they were boosted with 10 ml unconcentrated ES antigen s.c. They were bled on days 58 and 156.

19.2.7 Collecting serum from rabbits and guinea-pigs

Rabbit blood was collected from the posterior marginal ear vein. For this, the hair was plucked, the area disinfected, a sterile needle (20G 1½ Becton-Dickinson) inserted into the vein and the blood collected through this in a universal container. If

necessary, local hyperaemia was induced by slightly wetting the skin with xylene. Guinea-pig blood was collected by cardiac puncture. The guinea-pigs were starved for 24 hours and the skin around the xiphoid process of the sternum shaved. The animals were anaesthetised with halothane, laid supine and a sterile disposable needle ("21G2" Becton-Dickinson) attached to a 10 ml syringe was inserted, at 30° to the horizontal, bevel uppermost, in the angle formed between the last left rib and the sternum, slowly progressing towards the heart. When the heartbeat was felt the needle was quickly thrust foward into the heart and a maximum of 5 ml of blood was taken. No more blood could be taken at one time without risking the life of the animal. Blood was taken from the rabbits frequently and the times will be given in the results. The sera were then obtained by processing the blood as described above for immune sera. Sera were stored at $-20^{\circ}C$.

19.2.8 Isolation of serum gamma globulins

(a) Precipitation of gamma globulins with ammoniumsulphate

The method described by Campbell, Garvey, Cremer and Sussdorf (1964), with slight modifications including those described by Hebert, Pelham and Pittman (1973) was used. Twenty ml of saturated ammoniumsulphate (760 gram or 5.75 mol $(NH_4)_2SO_4$ to one litre water at $20^{\circ}C$ pH 7) was added to 40 ml serum, dropwise, while stirring, to avoid trapping contaminants. When all the ammoniumsulphate had been added, resulting in a 33% saturation with the salt, the pH was adjusted to 7.8 with 0.5 $mol.l^{-1}$ NaOH and left on the stirrer for 3 h at room temperature. The suspension was then centrifuged at 1500 *g* for 30 minutes and the supernatant discarded. The precipitate was dissolved in 10 ml saline and further purified by twice repeating the

precipitation procedure. The third precipitate was dissolved in 25 ml PBS (Dulbecco 'A' pH 7.3), placed in a dialysis bag (Visking tubing - Scientific Suppliers Co. Ltd.) and left to dialyse against 3 litre PBS at $4^{\circ}C$ to remove the residual ammoniumsulphate. The dialysing fluid was regularly changed and checked after 24 h for the presence of ammoniumsulphate by adding an equal volume of dialysate to saturated bariumchloride. This resulted in a white precipitate if sulphate ions were still present. The protein content was then estimated, adjusted to 5 $mg.ml^{-1}$ and the solution stored in small aliquots at $-20^{\circ}C$.

(b) Isolation of gamma globulin using caprylic acid

The method of Steinbuch and Audran (1969) was used, modified by adjusting the volumes of caprylic acid according to the animal species used (Van Knapen F. personal communication 1978). To 10 ml serum were added 20 ml acetate buffer (60 $mmol.l^{-1}$ pH 4). The pH was adjusted to 4.8 with 0.5 $mol.l^{-1}$ NaOH whilst stirring. Caprylic acid (n-Octanoic acid-BDH) (0.82 ml for 10 ml rabbit serum or 0.66 ml for 10 ml guinea-pig serum) was added dropwise and very slowly using an adjustable micropipette ("Pipetman P" Gilson). After leaving the mixture for 30 minutes on the stirrer at room temperature, it was centrifuged at 2500 *g* for 30 minutes. The precipitate was discarded and the pH of the supernatant raised to 5.7 with 0.5 $mol.l^{-1}$ NaOH. This was then dialysed in Visking tubing against 2 litre acetic acid buffer (15 $mmol.l^{-1}$ pH 5.6) for 30 minutes followed by 24 h dialysis against 2 litre PBS pH 7.3, changing the dialysing fluid three times. After this, the protein was estimated and adjusted to 5 $mg.ml^{-1}$ with PBS and the solution stored at $-20^{\circ}C$ in small aliquots.

Both methods for preparing serum gammaglobulin were done at room temperature up to the dialysis stage, which was performed at 4°C. Both these methods are supposed to yield mainly gammaglobulin but the purity is reported to be higher by the second method (Steinbuch and Audran 1969).

19.3 Estimation of soluble protein

The method of Warburg and Christian (1941) based on the difference in absorbance of a solution at 280 and 260 nm, was found to be useful since it provides a rapid indication of the amount of protein. However, as it is based on the phenylalanine, tryptophane and tyrosine content of the sample it does not strictly measure the total protein concentration. Hence another technique was used for estimating worm-derived protein in defined media and in experiments where such media were compared with other materials such as larval or adult somatic extracts or cyst fluid. The aim was to use a technique which was sensitive enough to detect micrograms of protein per millilitre, while avoiding errors due to other components, particularly the nutrients in the media. It was thought that this last problem could not be entirely overcome by using known standard protein solutions, prepared in unused medium, since the concentration of the nutrients which would potentially interfere with the protein estimation might alter due to the worms' metabolism. Therefore the technique chosen was one based on the specific binding of Coomassie Brilliant Blue G-250 to protein, which results in a change in the colour of the dye, shifting its absorbance at 595 nm. Using a range of known concentrations of a protein standard, a calibration graph was prepared, from which the protein concentration of the sample

could be estimated.

Initially, the microproteinassay described by Bradford (1976) was used. For the preparation of the protein reagent 100 mg Coomassie Brilliant Blue G 250 from two sources (Sigma and Kodak) was allowed to dissolve in 50 ml 95% ethanol by stirring it overnight in a closed vessel. To this 100 ml 85% w/v (or 8.6 $mol.l^{-1}$) phosphoric acid was added, the solution made up to one litre with distilled water, and filtered through a Whatman No. 1 filter paper. One ml of this protein reagent was added to 0.1 ml of the samples or protein standard in a test tube and mixed. The absorbance at 595 nm was measured using glass self-masking cuvettes of 10 mm light path (Hellma) in a SPG-200 spectrophotometer (Pye Unicam). However this protein reagent did not have the required sensitivity and although this could be increased when the protein reagent was prepared with 100 ml 85% v/v (or 13.36 $mol.l^{-1}$) phosphoric acid (the original w/v being assumed to be a misprint), it still did not allow reliable estimations of protein concentrations below 50 $\mu g.ml^{-1}$. Fortunately a more sensitive protein reagent could be obtained when it was prepared according to Sedmak and Grossberg (1977): 60 mg Coomassie Brilliant Blue G 250 (Kodak) was dissolved in 100 ml 1.9% w/v (or 0.19 $mol.l^{-1}$) perchloric acid (Koch-Light) and filtered. To 25 μl samples of the proteinaceous fluid 0.5 ml of this reagent was added. The absorbance read at 620 nm then permitted estimations of between 10 and 75 $\mu g.ml^{-1}$. At higher concentrations linearity was lost. Even better results were obtained with a commerical protein reagent "Bio-Rad Protein Assay" (Bio Rad Laboratories) based on the same principle, the "Microassay procedure" being performed according to

the manufacturers' instructions. Absorbance was measured between 10 and 30 minutes later at 595 nm in the glass cuvette. This was more satisfactory in that it consistently enabled a linear calibration graph to be drawn using bovine gammaglobulin ("Bio Rad Protein Standard") e.g. at concentrations between 10 and 100 $\mu g.ml^{-1}$ over an absorbance range from 0.13 to 1.26.

No interference from the components of the medium was observed, standard solutions prepared in saline or unused medium giving the same readings. Furthermore, this is a rapid procedure which does not require critical timing. All concentrations of immunoglobulins or antigens are expressed in terms of soluble protein, estimated by this technique or by the method of Warburg and Christian (1941).

19.4 Immunodiagnostic methods

19.4.1 The microgel double diffusion immunoprecipitation technique (MGP)

The procedure followed was that described by Gallie and Sewell (1974), being a slight modification from Crowle (1958, 1960 and 1961) who improved Wadsworth's (1957) miniature version of the agar double diffusion technique.

19.4.2 Immunoelectrophoresis (IEP)

The procedure followed was the "microméthode" described by Le Ray (1975), which was a modification of Grabar and Williams (1955)' technique.

19.4.3 Enzyme-linked immunosorbent assay (ELISA)

Generally, the tests were conducted according to the methods and principles described by Harrison (1977). Originally the tests

were performed in polystyrene tubes (Macro-ELISA), but later and for most of the work ELISA was performed in polystyrene plates (Micro-ELISA) ("Mikrotiterplatte 96 K F-Form aus Immulon" Greiner Labor technik).

In between each step there was a washing procedure, consisting of flooding the plates with 0.9% w/v (or 145 $mmol.l^{-1}$) NaCl solution to which 0.05% w/v Tween 20 (Sigma) was added. This solution was left in the wells for 5 minutes and then discarded. The procedure was repeated three times and, after the third washing, the plates were gently tapped onto paper tissue to remove the residual drops of washing fluid from the wells. All buffers were kept at $4^{\circ}C$, while washing solution was kept at room temperature. All dilutions of reagents were prepared immediately before use.

19.4.3.1 ELISA for detecting antibodies

Antigen was diluted in 0.1 $mol.l^{-1}$ sodium carbonate buffer pH 9.6 with 0.02% w/v (or 3 $mmol.l^{-1}$) sodium azide (B.D.H.). Concentrations between 10 and 5 $\mu g.ml^{-1}$ soluble protein were found to be satisfactory. A 300 µl aliquot of this dilution was placed in each well using an adjustable pipette ("Pipetman P" Gilson). The plates were then covered with a lid, incubated for 3 h at $37^{\circ}C$ and left overnight at $4^{\circ}C$ to allow for adsorption of the antigen onto the polystyrene. Serum was diluted in PBS (Dulbecco 'A') pH 7.3 containing 0.05% w/v Tween 20 (Polyoxyethylene sorbitan monolaurate Sigma) with 0.02% w/v (or 3 $mmol.l^{-1}$) NaN_3 and, after the plates had been washed, 300 µl of this solution was placed in each well, and the plates were incubated for 1 h at $37^{\circ}C$ followed by rewashing and incubation with the conjugate dilution. The conjugates were

commercial horse radish peroxidase conjugated IgG against heterologous species IgG diluted in PBS plus Tween 20 as for serum but without NaN_3. Again incubation was for 1 h at 37°C followed by washing and exposure to the substrate. Ortho-phenylene diamine (o.P.D.) (Sigma) was used as substrate as this is easier to prepare and appears to be more stable after the enzyme reaction has been stopped than 5-amino salicylic acid. The substrate was prepared immediately before use by dissolving 35 mg o.P.D. in 100 ml citric acid phosphate buffer (37 $mmol.l^{-1}$ citric acid monohydrate ("AnalaR" BDH) and 126 $mmol.l^{-1}$ disodiumhydrogenphosphate 2 H_2O (BDH) pH 6) and then adding 0.167 ml of 30% w/v (or 14.73 $mmol.l^{-1}$) H_2O_2 ("AnalaR" BDH). 300 µl of this substrate was pipetted into each well and the plate left in the dark for at least ten minutes.

The colour changes were regularly checked thereafter and when considered satisfactory (i.e. the strongest reactions were well developed but still readable) the enzyme reaction was stopped by adding to each well 25 µl of a molar solution of sulphuric acid ("Aristar" BDH). At the same time one ml of this sulphuric acid was added to 12 ml of the remaining substrate which had been left in the dark in a beaker. The relative absorbance of the reaction product was read on a spectrophotometer (SPG - 200 Pye Unicam) at 455 nm or in a filterphotometer ("Vitatron" Fisons MSE) provided with a 450 nm filter, using a glass self-masking cuvette of 1 cm light path (Hellma). In each case zero absorbance was set using the substrate and sulphuric acid mixture. At regular intervals while reading the samples this zero setting was checked and if necessary corrected using the same solution.

19.4.3.2 ELISA for detecting antigen by an indirect triple antibody sandwich technique (ITAS - ELISA)

A slightly modified version of the technique described by Van Knapen and Fridas (1977) was used. The general principles and methods used were similar to those for detecting antibodies, using the same buffers and amounts of fluid per well.

However, as shown in Fig. IV.1 in diagram for the first step the gammaglobulin from antiserum raised against the cestode antigens in rabbits, isolated and diluted in the carbonate buffer to a concentration of 30 $\mu g.ml^{-1}$ soluble protein was allowed to adsorb onto the polystyrene for 3 h at $37^{\circ}C$ and then overnight at $4^{\circ}C$. After 3 washings, the second step was to add the solution in which the presence of any antigen was to be detected - e.g. used culture medium. If dilution of this solution was required, it was done with PBS. Incubation for this and the following steps until the addition of substrate was 1 h at $37^{\circ}C$. For the third step, gammaglobulin from a hyperimmune serum, raised against the same cestode antigen in guinea-pigs, was diluted in PBS plus 0.05% w/v Tween 20 to a concentration of 50 $\mu g.ml^{-1}$ soluble protein and added to each well. For the fourth step a commercial conjugated anti-guinea-pig gammaglobulin was used, such as with ELISA for detecting antibodies. The rest of the procedure was as described for detecting antibodies. The gammaglobulins used in this test were isolated by the caprylic acid method.

19.4.3.3 ELISA for detecting antigen using a double antibody sandwich (DAS - ELISA)

This system differed from ITAS - ELISA in that rabbit gammaglobulins from immune sera raised against cestode antigen and isolated by means of ammonium sulphate as described, were used in both the first

and the third steps. However, the latter step was a combination of the steps 3 and 4 of ITAS - ELISA in that these rabbit anti immunoglobulins were labelled with horse radish peroxidase (E.C. 1.11.1.7.). (Type VI Sigma). Two coupling methods were used for preparing the conjugate. One according to Avrameas and Ternynck (1971) and the other according to Nakane (1975). No fractionation to remove unreacted components was done. Fig. IV. 1 shows a diagram of the consecutive steps in DAS - ELISA.

Fig. IV 1. Diagram of the consecutive steps in ITAS and DAS - ELISA for detecting ES antigen. Washing procedures are applied between steps. Key to abbreviations: RaES Ig, G.P.aESIg Immunoglobulins respectively from rabbit and guinea-pig serum against excretory and secretory antigen from <u>Taenia taeniaeformis</u> larvae.

<u>ITAS - ELISA</u>

1st step	|<	R.aES Ig adsorbed on the polystyrene
2nd step	|< ◇	+ antigen
3rd step	|< ◇ >⊂	+ G.P.a ES Ig
4th step	|< ◇ >⊂o-E	+ R a G.P. Ig enzyme conjugated (Nordic)
5th step	|< ◇ >⊂o-E∋	+ Substrate

<u>DAS - ELISA</u>

1st step	|<	Ra ES adsorbed on the polystyrene
2nd step	|< ◇	+ antigen
3rd step	|< ◇ >+E	+ Ra ES Enzyme conjugated
4th step	|< ◇ >+E∋	+ Substrate

19.4.3.4 Controls and interpretation

Every test was carried out at least in duplicate, with the average result being taken between the readings where there was apparent agreement. Conjugate controls, where the conjugate used at dilutions which will be further described was replaced by an equal volume of normal saline, always yielded a negative result.

Other controls for false positive reactions consisted in replacing sera or immunoglobulins from the infected or hyperimmunised animals respectively with sera or immunoglobulins at the same concentrations from corresponding normal (i.e. not infected or not hyperimmunised) animal species. The antigen control involved using normal saline or unused medium diluted with buffer to the same extent as the antigen. All combinations with either antigen or antibody present or not were included. In order to present the ELISA values in a simple form, the absorbance values for the controls were subtracted from the values for the test in the following order:(+ indicates component present and - indicates component absent or normal sera/gammaglobulin).

For ELISA to detect antibodies:

$$\begin{matrix}\text{Antigen:}\\ \text{Antibody (?):}\end{matrix}\quad \left\{\begin{bmatrix}+\\+\end{bmatrix} - \begin{bmatrix}+\\-\end{bmatrix}\right\} - \left\{\begin{bmatrix}-\\+\end{bmatrix} - \begin{bmatrix}-\\-\end{bmatrix}\right\}$$

For ITAS ELISA & DAS ELISA:

$$\begin{matrix}\text{Rabbit gamma globulin}\\ \text{Antigen (?)}\\ \text{Guinea-pig } \gamma \text{ globulin (ITAS) or Rabbit } \gamma \text{ globulin - enzyme linked (DAS)}\end{matrix}\ \begin{matrix}:\\:\\:\end{matrix}\left\{\begin{bmatrix}+\\+\\+\end{bmatrix} - \begin{bmatrix}+\\+\\-\end{bmatrix}\right\} - \left\{\begin{bmatrix}-\\+\\+\end{bmatrix} - \begin{bmatrix}-\\+\\-\end{bmatrix}\right\}$$

This was checked by incorporating the other control combinations $\begin{bmatrix} - \\ - \\ + \end{bmatrix}$ $\begin{bmatrix} - \\ - \\ - \end{bmatrix}$ $\begin{bmatrix} + \\ - \\ + \end{bmatrix}$ and $\begin{bmatrix} + \\ - \\ - \end{bmatrix}$ but they were consistently insignificant.

In the ELISA for detecting antibodies the $\begin{bmatrix} - \\ - \end{bmatrix}$ combination never gave as strong a reaction as any of the other ones, and similarly for those combinations with normal guinea-pig gamma-globulin in ITAS-ELISA. Hence the above sequence of subtractions. Nevertheless, where it was thought to be useful, because erroneous conclusions might have been drawn from this procedure, the control values are also given.

Chapter 20 Preliminary experiments with ELISA

In order to ensure that the conditions were fairly close to the optima required for the sensitive ELISA technique, a series of preliminary experiments was necessary. A brief account of these is given.

20.1 The commercial conjugates

The correct dilutions of conjugates were usually determined alongside other assays unless specific difficulties were encountered. The dilutions found to be optimal in that they caused little or no background absorbance compared with the conjugate control during the last six months of the work are given in table IV.1.

Table IV.1 Commercial sources of horse radish peroxidase conjugated IgG

Produced in:	against	dilution	Manufacturer
Goat	anti rabbit IgG	1/1250	Nordic
Rabbit	anti goat IgG	1/1000	Nordic
Rabbit	anti guinea-pig IgG	1/1000	Nordic
Rabbit	anti rat IgG	1/500	Nordic
Rabbit	anti rat IgG	1/1500	Miles
Rabbit	anti mouse IgG	1/900	Miles

Since the optimal dilutions vary from batch to batch, the same batches were used as much as possible. A consistently better result was obtained with the anti-rat conjugate from Miles as opposed to that from Nordic. After receipt the conjugates were divided, undiluted, into 0.1 ml aliquots and stored at $-20^{\circ}C$. Each aliquot was only used once.

20.2 Antigen and antibody dilutions

Checkerboard titrations were made to detect the effective antigen/antibody dilution range. Tables IV.2 and 3 give the results of these for rats and mice respectively.

Table IV.2 Checkerboard titrations of serum from rats infected with Taenia taeniaeformis against somatic adult and ES antigen in ELISA. Fourfold dilutions of pooled T. taeniaeformis sera against adult somatic and ES antigen at four soluble protein concentrations. Relative absorbance at 450 nm.

Antigen Concentrations ($\mu g.ml^{-1}$)	Serum dilutions 1/10	1/40	1/160	1/640	1/2560	1/10 240
ES 30 μg	1.52	1.74	1.06	0.36	0.14	0.06
15	1.32	1.64	0.94	0.28	0.14	0.06
10	1.24	1.62	0.84	0.24	0.10	0.02
5	1.38	1.64	0.96	0.34	0.16	0.04
Som 30	1.88	1.60	1.06	0.38	0.16	0.06
15	1.72	1.44	0.94	0.32	0.16	0.06
10	1.90	1.52	1.02	0.40	0.18	0.08
5	1.96	1.68	0.94	0.44	0.26	0.10

Table IV.3 Checkerboard titrations of serum from mice infected with Taenia taeniaeformis against adult somatic and ES antigen in ELISA. Fourfold dilutions of pooled T. taeniaeformis sera against adult somatic and ES antigen at four soluble protein concentrations. Relative absorbance at 450 nm.

Antigen Concentrations (μg.ml-1)	Serum dilutions 1/10	1/40	1/160	1/640	1/2560	1/10 240
ES 30 μg	2.14	1.98	1.84	1.32	0.54	0.24
15	2.20	1.90	1.80	1.10	0.40	0.18
10	2.08	1.92	1.74	1.10	0.44	0.16
5	2.22	1.90	1.72	1.12	0.64	0.14
Som 30	2.10	1.90	1.86	1.48	0.54	0.18
15	2.04	2.08	1.80	1.42	0.50	0.14
10	2.18	2.24	1.86	1.36	0.58	0.14
5	2.24	2.20	1.84	1.50	0.62	0.18

20.3 Hyperimmune sera

The course of the immune response in rabbits is illustrated in Tables IV.4 and 5 for anti adult somatic and anti ES sera respectively.

The specificity of these hyperimmune sera for either somatic or ES antigen was tested by the ELISA for detecting antibodies for both rabbits and guinea-pigs. For the latter a comparison was also made between the classic and the Vaitukaitis methods of hyperimmunisation against ES (Table IV.6).

Table IV.4 Antibody response of rabbits hyperimmunised with adult <u>Taenia</u> <u>taeniaeformis</u> somatic antigen in ELISA. Relative absorbance at 450 nm. Somatic homologous adult antigen at a concentration of 5 µg soluble protein ml^{-1} and serum at a dilution of 1/5000

Day	0	14	28	42	49	63	77	91	105	119	196	364	378
Rabbit No 1	0	0.38	0.41	1.35	1.30	1.26	1.43	1.25	1.31	1.19	1.00	0.64	1.93
2	0	0.35	0.38	1.22	1.25	1.25	1.42	1.23	1.18	1.07	1.03	0.75	1.43
3	0	0.51	0.48	1.30	1.26	1.22	1.37	1.21	1.18	1.13	1.04	0.79	1.47
Injected on day	0		35			70						366	

Table IV.5 Antibody response of rabbits hyperimmunised with <u>Taenia</u> <u>taeniaeformis</u> ES antigen in ELISA. Relative absorbance at 450 nm. ES homologous antigen at a concentration of 5 µg soluble protein ml^{-1} and serum at a dilution of 1/5000

Day	-15	7	14	28	35	42	63	70	119	124
Rabbit No 1	0	0.05	0.14	0.41	0.68	1.36	1.25	1.51	1.16	1.25
2	0	0.09	0.17	0.54	0.68	1.34	0.85	1.17	1.15	1.26
Injected on day	0				35		63		119	

Table IV.6 A comparison of the specificity for somatic and ES T. taeniaeformis antigen of hyperimmune rabbit and guinea-pig sera prepared against these antigens in ELISA. Relative absorbance at 450 nm. Both antigens were used at 5 μg.ml^{-1} soluble protein concentration. Sera were used from the last collection at a dilution of 1/5000 (c) Classic, (v) Vaitukaitis method of hyperimmunisation.

	System	Somatic Antigen	ES Antigen
RABBITS	RaSom No 1	1.53	1.20
	2	1.23	0.86
	3	1.41	0.93
	Ra ES 1	0.22	0.88
	2	0.22	0.90
GUINEA-PIGS	G.P.aSom No 1	1.94	0.91
	2	1.87	0.90
	3	1.55	0.73
	G.P.a ES (c) C_1	0.34	0.99
	C_2	0.34	0.71
	G.P.a ES (v) V_1	0.56	1.39
	V_2	0.89	1.67
	V_3	1.13	1.69

Hyperimmune anti-somatic sera of both species crossreacted with the ES antigen, and a similar crossreaction between the hyperimmune anti-ES sera and the somatic antigen also occurred but this appeared to be proportionately less with rabbit sera. A test run in parallel using 1/10 000 sera gave equivalent results.

The comparison between the two methods of separating immunoglobulins gave the following results: One ml of hyperimmune rabbit anti ES serum yielded 12 mg soluble protein (2.4 ml at 5 $mg.ml^{-1}$) by ammoniumsulphate precipitation, whereas 8 mg was obtained from one ml of the same serum by the caprylic acid method (1.6 ml at 5 $mg.ml^{-1}$). Table IV.7 shows the comparison between sera and immunoglobulin dilutions in ELISA against ES antigen.

Table IV.7 A comparison of the ELISA activity of immunoglobulins separated by the caprylic acid and ammoniumsulphate methods and of the original serum (Ra ES). Relative absorbance at 450 nm. Twofold dilutions of the original serum and the two immunoglobulin solutions were made starting from 1/500 serum or 10 μg soluble protein ml^{-1} for the immunoglobulin solutions. These were compared against ES antigen at 5 $\mu g.ml^{-1}$.

Serum Dilution	1/500	1/1000	1/2000	1/4000	1/8000	1/16 000
Globulin concentration ($\mu g.ml^{-1}$)	10	5	2.5	1.25	0.625	0.3125
Anti ES serum	3.10	2.59	2.07	1.35	0.86	0.48
γ Globulin Capryl.	2.20	1.63	1.09	0.62	0.34	0.19
γ Globulin $(NH_4)_2SO_4$	2.16	1.62	1.02	0.59	0.30	0.18

It appeared that the two separation methods gave similar results in ELISA. However since one ml serum gave $1\frac{1}{2}$ times as much globulin by the ammoniumsulphate precipitation technique compared with the caprylic acid method, the comparison favours the ammoniumsulphate method.

20.4 ELISA for detecting antigen

20.4.1 Detection of ES antigen by adsorption onto polystyrene

Before the sandwich-type ELISA test for detecting antigen was developed, an attempt was made to adsorb the antigen, which was to be detected, onto the polystyrene wall. One of the limitations for detecting antigen in this manner was an apparent limitation of adsorption - e.g. if ES was concentrated a proportionate increase in absorbance was not obtained. This effect could not be entirely accounted for by the fact that the eluate from ES antigen concentrated ten-fold by ultrafiltration gave a positive reading. Thus when the fully-corrected reading for neat ES antigen (12 $\mu g.ml^{-1}$) was 0.97, that for 10 x concentrated ES was 1.25, while the eluate gave a reading of 0.18, when each was used against anti ES rabbit hyper-immunised serum at a dilution of 1/500, after the readings.

Another difficulty was that when FCS was used in the media, its presence in the resultant ES interfered with the ELISA. Confirmation of this is given in Table IV.8. ES antigen (30 $\mu g.ml^{-1}$) was divided into two parts. To one part was added 10% v/v FCS and to the other one 10% v/v unused modified RPMI. Both were then tested against fourfold dilutions of rabbit anti ES serum starting at 1/160. Lower readings were consistently obtained when FCS was present in the assay. The control values were also different tending to be higher when the FCS was present.

Table IV.8 Comparison of ES (30 μg.ml^{-1}) with and without addition of foetal calf serum in ELISA against fourfold dilutions of rabbit anti ES serum or normal rabbit serum. Relative absorbance at 455 nm.

Serum	Antigen	± FCS	1/160	1/640	1/2560	1/10 240
Positive	ES	-	2.59	2.71	1.26	0.72
		+	1.50	1.09	0.89	0.45
Normal	ES	-	0.22	0.07	0.11	0.13
		+	0.16	0.11	0.09	0.12
Positive	Unused medium	-	0.08	0.08	0.03	0.00
		+	0.49	0.21	0.11	0.13
Normal	Unused medium	-	0.02	0.00	0.00	0.00
		+	0.07	0.07	0.06	0.10
Net corrected result		-	2.31	2.56	1.12	0.59
		+	0.92	0.84	0.75	0.30

20.4.2 DAS ELISA

This technique was not satisfactory for detecting antigen in culture media. Neither of the methods for coupling the enzyme to the anti ES immunoglobulins, (Nakane, 1975) and Avrameas and Ternynck (1971), gave sufficiently sensitive reagents and some control combinations gave high readings regardless of the dilution of conjugate (1/100, 1/250 or 1/500).

In Table IV.9 an example of such a test is given, using conjugates prepared by both methods at a dilution of 1/500. A known

active antigen (Stock ES) was used at 30, 15 and 3 μg soluble protein concentration per ml. The immunoglobulins in the first step were used at 25,50 and 100 $\mu g.ml^{-1}$. The other control combinations than those given in the table gave absorbance values below 0.05.

Table IV.9 DAS ELISA with anti-ES rabbit immunoglobulin conjugates (step 3) prepared according to either Nakane (1975) or Avrameas and Ternynck (1971), both at a dilution 1/500. Relative absorbance at 455. +: HISR
-: Normal rabbit serum

Conjugate preparation according to:			Nakane			Avrameas & Ternynck		
Concentration of γ globulin ($\mu g.ml^{-1}$) in first step			25	50	100	25	50	100
Step 1	Step 3	Concentration of Antigen						
+	+	30 $\mu g.ml^{-1}$	0.51	0.46	0.41	0.18	0.16	0.14
		15	0.49	0.41	0.38	0.16	0.15	0.14
		3	0.35	0.28	0.22	0.10	0.09	0.08
+	+	No antigen	0.17	0.15	0.14	0.04	0.04	0.05
-	+	30	0.34	0.30	0.30	0.12	0.10	0.09
		15	0.42	0.40	0.37	0.13	0.14	0.14
		3	0.21	0.18	0.15	0.06	0.05	0.05
-	+	No antigen	0.14	0.12	0.11	0.03	0.03	0.03

20.4.3 <u>ITAS - ELISA</u>

The test was performed as described above. The antigen used in the second step was Stock ES with an original protein concentration

of 30 $\mu g.ml^{-1}$. This was two-fold diluted and to each of these dilutions either 10% v/v FCS or 10% v/v modified unused RPMI was added. In addition, the same antigen was used, undiluted but with 0.05% w/v Tween 20 added to it. The results are given in Table IV.10 with the relevant control combinations and the result of the sequential subtraction to give the net result.

This ELISA system for detecting antigen seemed to be more effective, and ITAS - ELISA was used for all further work. Furthermore, 10% v/v FCS was added to the antigen except in these cases where it was already present in the media. In comparisons between media containing FCS with defined media, 10% v/v FCS was added to the latter and 10% v/v defined media to the former. As appeared from the results some control combinations were lower with, than without FCS.

In an experiment with this ELISA system, the rabbit and guinea-pig anti-ES or normal globulin solutions were used at 25, 50 and 100 $\mu g.ml^{-1}$ and tried in all combinations against ES antigen containing 50 μg soluble protein ml^{-1} and its control; to both of these latter 10% v/v FCS was added. The somatic antigen and its control were similarly treated. Results are given in Table IV.11.

These results seemed to indicate the best combination of immunoglobulins to be 100 $\mu g.ml^{-1}$ for both reagents. However, as there was little difference in the results when 25 and 50 $\mu g.ml^{-1}$ of rabbit and guinea-pig immunoglobulins respectively were used, these latter concentrations were adopted routinely so as to reduce the amount of material needed, especially that from guinea-pig.

Table IV.10 ITAS - ELISA for ES antigen, effect of adding serum to each antigen dilution and 0.05% w/v Tween 20 to the undiluted antigen. Approximate protein concentration of undiluted antigen: 27 $\mu g.ml^{-1}$. Relative absorbance at 455 nm (+ : immunoglobulins from HI serum, - immunoglobulins from normal serum).

Rabbit	G.-pig	Antigen + 10% v/v of	Antigen dilution						
			Neat + Tween	Neat	1/2	1/4	1/8	1/10	1/32
+	+	Unused Medium	1.72	1.82	1.47	1.24	1.02	0.78	0.60
		FCS	1.44	1.29	1.19	1.08	0.86	0.65	0.48
-	+	Unused Medium	0.32	0.49	0.68	0.43	0.28	0.18	0.11
		FCS	0.10	0.11	0.12	0.08	0.07	0.08	0.06
+	-	Unused Medium	0.11	0.11	0.09	0.07	0.07	0.07	0.05
		FCS	0.08	0.09	0.07	0.07	0.06	0.06	0.05
-	-	Unused Medium	0.14	0.10	0.07	0.07	0.06	0.06	0.08
		FCS	0.07	0.09	0.07	0.05	0.05	0.04	0.06
Net corrected result		Unused Medium	1.43	1.32	0.79	0.81	0.73	0.59	0.52
		FCS	1.33	1.18	1.07	0.98	0.78	0.55	0.43

Table IV.11 ITAS - ELISA with various concentrations of gamma globulin ($\mu g.ml^{-1}$) from rabbit (first step) and from guinea-pig (third step) anti ES and anti-somatic sera against the corresponding antigens. Relative absorbance 450 nm.

	ES - Anti ES system			Som - Anti somatic system		
Rabbit γ globulin concentration	25	50	100	25	50	100
Guinea-pig γ globulin concentration 25	1.16	1.09	1.21	1.08	1.26	1.36
50	1.17	1.00	1.31	1.36	1.45	1.56
100	1.34	1.20	1.37	1.33	1.38	1.53

For the same purpose, an attempt was made to use guinea-pig immunoglobulins in the first step and rabbit in the third, at concentrations of 30 and 50 $\mu g.ml^{-1}$ respectively. Otherwise the same materials were used as in the previous test but the antigen concentration was 1 μg soluble protein per ml. The results are given in Table IV.12.

Although this also worked, some of the control combinations gave higher levels of absorbance than was expected. Hence this system was abandoned. For testing the sensitivity of this ITAS - ELISA, both ES and somatic antigen were diluted in modified RPMI with FCS added as described. The soluble protein concentrations of the antigen dilutions, before FCS was added, are given in Table IV.13 together with the corrected results.

Table IV.12 ITAS - ELISA with various concentrations of gammaglobulin from guinea-pig (first step) and from rabbit (third step) anti ES and anti somatic sera against the corresponding antigens. Relative absorbance at 450 nm.
R and G.P.: respectively rabbit and guinea pig immunoglobulins from HIS (+) and normal (-) sera, A: antigen (+) or unused medium (-).

Combination	Rabbit γ globulin concentration (μg.ml-1)	ES - Anti ES system		Som - Anti Somatic system	
	Guinea pig γ globulin concentration (μg.ml-1) →	30	50	30	50
[R +, A +, G.P. +]	30	1.89	2.12	1.92	1.82
	50	2.16	2.12	2.24	2.15
[R -, A +, G.P. +]	30	0.58	0.38	0.39	0.26
	50	0.56	0.49	0.31	0.27
[R +, A +, G.P. -]	30	0.78	0.84	0.56	0.57
	50	0.92	0.51	0.53	0.54
[R +, A -, G.P. +]	30	0.48	0.54	0.45	0.45
	50	0.62	0.57	0.42	0.45

Table IV.13 Sensitivity test with ITAS - ELISA detecting ES and somatic antigen at decreasing concentrations. Relative absorbance at 450 nm.

Concentration of soluble protein in antigen before adding FCS ($ng.ml^{-1}$)	ES - Anti ES system	Som - Anti Somatic system
10 000	1.48	2.38
1 000	1.08	1.97
500	0.92	1.25
250	0.68	0.87
100	0.46	0.49
50	0.29	0.23
25	0.16	0.13
10	0.07	0.05
0	0.00	0.00

20.5 CONCLUSIONS

No unexpected results were found with the ELISA for detecting antibodies when used with the cestode antigens. The antigen concentrations were thereafter kept at 5 μg or 10 μg soluble protein per ml. However, it was to be expected that when this direct procedure was used for detecting antigen, it would only give an indication as to whether this was present or not if defined media were used. When FCS was present there would be competition between antigen and other proteins to be adsorbed onto the polystyrene walls, with a resulting loss of sensitivity and reduced absorbance. However, the ITAS - ELISA seemed to overcome this problem as was confirmed by the further experiments using this test and adding 10% v/v FCS to each dilution of the defined media used as antigen.

When guinea-pig immunoglobulins were used in the first step and rabbit in the third, the control readings were higher than usual. This was not further investigated. However in this study a commercial conjugate prepared from anti-serum raised in goats was used as guinea-pig anti rabbit gammaglobulin conjugate was not available. This use of a serumfraction from an additional species may have accounted for the high controls.

A possible restriction on this technique may arise if the spectrum of antibodies of guinea-pigs and rabbits may not coincide as the technique can only be expected to detect antigen which react with common antibodies in the sera from both species.

There is little doubt that the DAS - ELISA could have been improved. Purifying the conjugates prepared by both methods would very

likely have reduced the background absorbance. But to increase the sensitivity would probably have demanded the use of higher levels of enzyme conjugated immunoglobulins, which would have made the test less practical, unless these antisera were raised in a larger animal such as goats.

Although there may be differences such as the amount of IgM present in the immunoglobulins separated by the ammoniumsulphate method or with caprylic acid, in the ELISA tests both gave similar results as regards the sensitivity of the reagent and satisfactory low background absorbance. However in the one comparison made between these methods, the yield of immunoglobulins expressed as soluble protein concentration was higher for the ammoniumsulphate method, despite the caprylic acid method being simpler. The former method also seems to be safer as far as any possible influence of pH on the immunoglobulins is concerned.

For the guinea-pigs hyperimmunised against ES antigen a better response was obtained by the Vaitukaitis method than by the classic method, although this term for the latter may not be justified since both methods are open to many modifications, which may have altered the result. One such variant may have been the timing; for the guinea-pigs submitted to the classic method three booster injections were given and the serum taken on day 232 from the start. This was compared to sera from those submitted to the Vaitukaitis method boosted once and serum taken on day 156. Similar variations in technique need to be taken into account in considering the experiments related to the specificity of the hyperimmunised sera. Apart from a possible species specific difference in response, which may have

led to the rabbit anti-ES serum being more specific than that from guinea-pigs, boosting the animals several times with the idea of widening the spectrum of antibodies may have led to the production of less specific antibodies (Herbert 1978). It would probably have been better in such experiments to estimate the right moment in the immunisation programme, which would give the optimum compromise between strength and specificity, especially for anti-somatic antibodies. There is also the purity of the antigen to be considered; since, even if ES antigen is largely different from somatic antigen, it seems very probable that, both antigens will be a mixture of each other with the same consequences for the antisera. This question of the purity of the antigens, especially ES antigens has not so far been taken into account, but is partly dealt with in the following experiments.

Chapter 21 A comparison of ES and Somatic Antigen

21.1 INTRODUCTION

Attempts were made to estimate whether ES was really different from somatic antigen, and if so, whether it could be distinguished from the latter in culture media. Furthermore preliminary studies were undertaken relating to the use of these culture antigens for diagnostic purposes.

21.2 Experimental design

Initially, the microgelprecipitation (MGP) technique was used to show the presence of antigen in culture medium. Later these antigens were compared in IEP with larval and adult somatic *T. taeniaeformis* antigen and cyst fluid from *T. taeniaeformis* and *T. crassiceps* metacestodes against sera from hyperimmunised animals and from infected mice.

ES antigen was concentrated 10 x by ultrafiltration and so used in MGP, while for IEP it was further concentrated by freeze-drying and redissolving it to a fifth of its original volume.

Concentrating media such as "modified RPMI" fifty times seemed to be close to the maximum level feasible, as beyond this it becomes too viscous. The protein level of the unconcentrated ES was $30\,\mu g.ml^{-1}$. Cyst fluids, larval and adult somatic antigen had their protein concentration adjusted to $5\ mg.ml^{-1}$. The antigen wells were filled with 12 µl solution and 0.3 ml serum was applied in the troughs. Electrophoresis was performed at $2.5\ V.cm^{-1}$ for $2\frac{1}{2}$ hours.

On one occasion *T. saginata* ES was used. This was obtained from an *in vitro* culture of ten 2 month old metacestodes maintained

at one per ml in modified RPMI for ten days with the medium being changed every 48 h. Used medium was then pooled and concentrated 50 x but no protein estimation was done.

With the availability of the ITAS - ELISA these comparisons were repeated but only with antigens originating from T. taeniaeformis and with whole larval T. crassiceps antigen.

21.3 RESULTS

21.3.1 MGP microgelprecipitation

Medium, concentrated ten times in which 4 month old larvae had been kept for 48 hours per change over 18 days at one larvae per ml, showed precipitating lines in MGP against rabbit anti somatic HIS. However when the successive aliquots of medium were kept separately, it appeared that the antigen was not always released in the same concentration. As shown on Plate IV.1, a strong precipitation line was given with medium from day 9 to 10 and again at day 13 to 14. Similar irregularity was repeatedly observed in other cultures.

21.3.2 IEP immunoelectrophoresis

IEP was used for comparing the different antigens and sera as shown in Plate IV.2 (a) and (b). The patterns shown on slides 1, 2 and 3, where rabbit anti-somatic HIS was used revealed that there were common components present in the somatic antigens used to immunise the rabbits and the culture fluids used as ES antigens.

The somatic antigen mosaic of both larval and adult somatic material seemed to be very similar as shown from their reaction against anti-adult somatic and anti-larval somatic HIS.

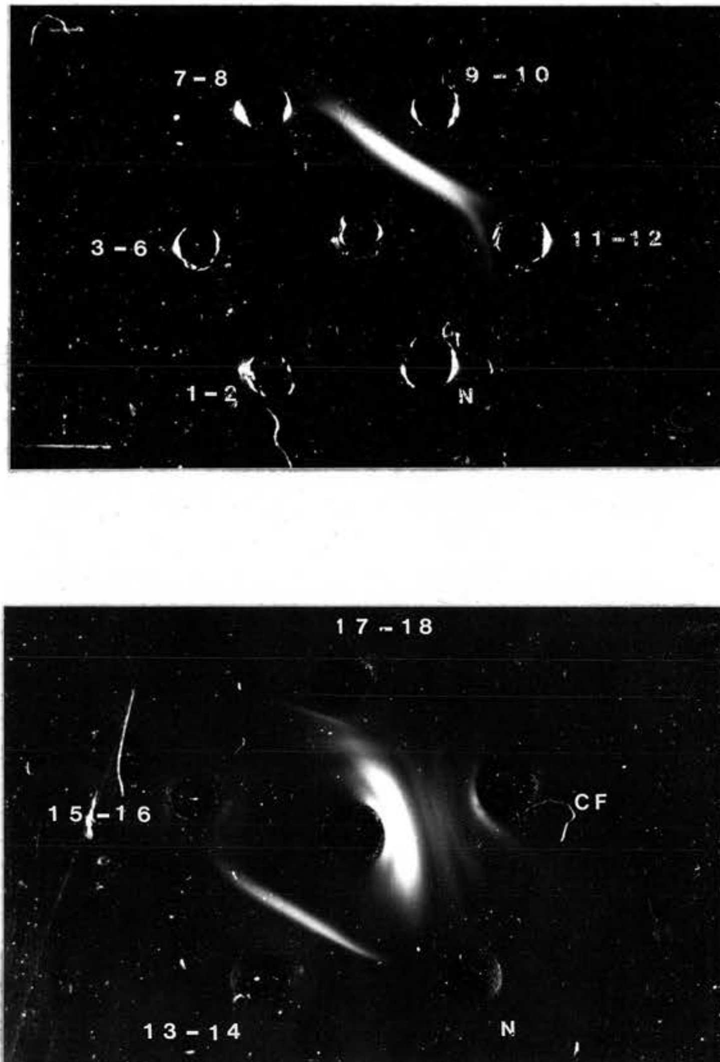
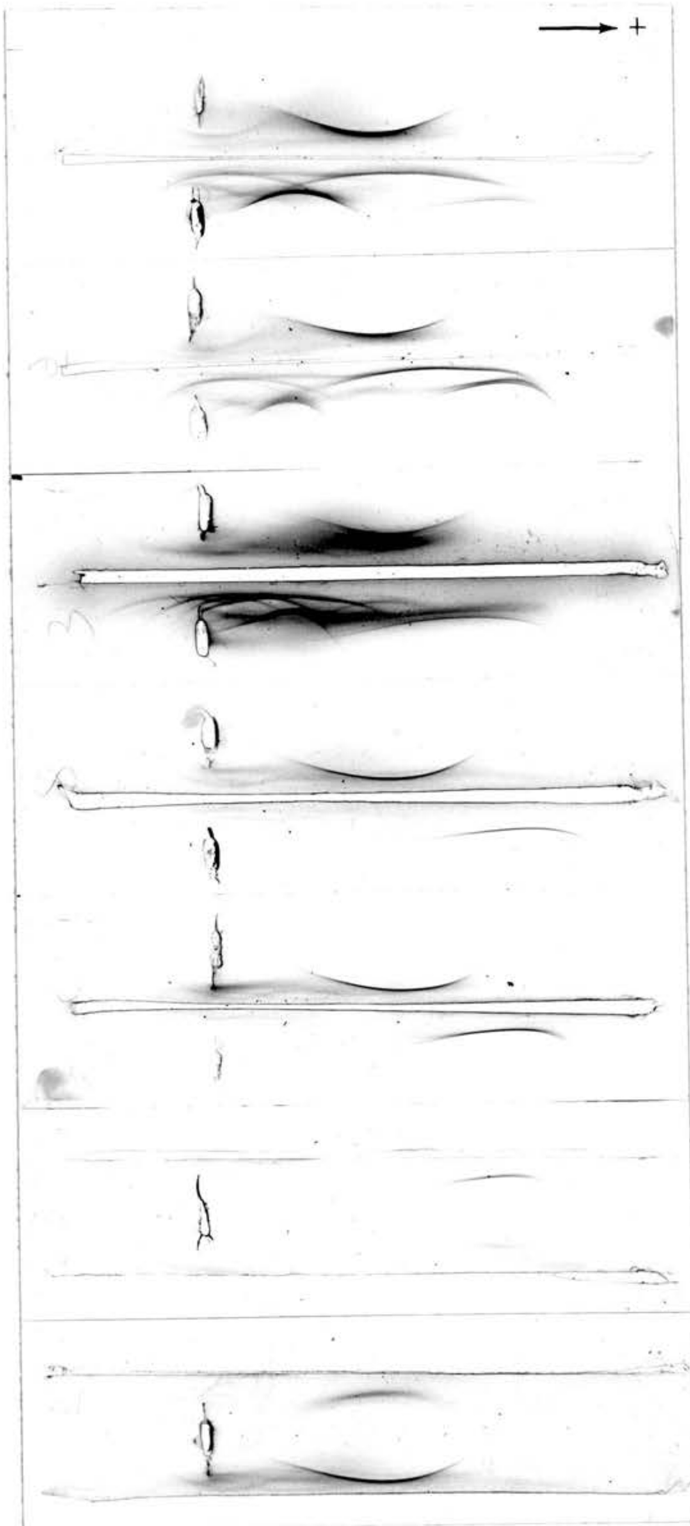


Plate IV. 1. Microimmunogelprecipitation between serum from rabbits, hyperimmunised with *Taenia taeniaeformis* adult somatic antigen in the centre well, and either concentrated homologous ES collected from one culture of metacestodes on the days indicated by the figures in the peripheral wells, unused medium (N) or homologous cyst fluid (CF).

Slide No:



ES *T. taeniaeformis*
HIS - R a A Som *T. taeniaeformis* 1
A Som *T. taeniaeformis*

ES *T. taeniaeformis*
HIS - R a A Som *T. taeniaeformis* 2
L Som *T. taeniaeformis*

ES *T. taeniaeformis*
HIS - R a L Som *T. taeniaeformis* 3
L Som *T. taeniaeformis*

ES *T. taeniaeformis*
HIS - R a ES *T. taeniaeformis* 4
A Som *T. taeniaeformis*

ES *T. taeniaeformis*
HIS - R a ES *T. taeniaeformis* 5
L Som *T. taeniaeformis*

HIS - R a ES *T. taeniaeformis*
A Som *T. taeniaeformis* 6
S - M i.w. *T. taeniaeformis*

HIS - R a ES *T. taeniaeformis*
ES *T. taeniaeformis* 7
S - M i.w. *T. taeniaeformis*

Plate IV. 2(a). Immunoelectrophoresis slides showing the reactions between various sera and antigens as specified. Continuation of this plate: IV. 2(b). Abbreviations - compounded from:
L: Larval, A: Adult, Som: Somatic Antigen, ES: Excretory and secretory antigens obtained from larvae *in vitro*, CF: Cyst fluid, HIS:- : hyperimmunised sera from . . . , R: Rabbit, G.P.: Guinea-pig, a: against, S - M i.w. and S - Rat i.w.: Sera from mice and rats respectively infected with . . . , (c) and (v): Classic and Vaitukaitis method of hyperimmunisation.

This sharing of antigens between somatic and ES antigen in IEP is further revealed on the slides showing their reactions with anti-ES HIS. Again ES shared common components with somatic antigen both from larval and adult origin (Slides 4, 5 and 9). However it can also be seen from Slide 9 that whereas the heaviest line occurring between the anti-somatic HIS and the ES antigen does appear to occur as a minor line between that serum and the somatic antigen, the reverse does not appear to apply to the heaviest line occurring between the latter components.

In view of this extensive sharing of precipitating antigens it was considered that crossabsorption studies using these hyperimmune sera and antigens would not give much information, especially as the hyperimmune sera reflect the same spectrum of activity as the antigens used to raise them. Sera from infected mice and rats were also used in these studies. Once again precipitation lines were formed with all antigens and, as shown on Slides 6 and 7, the general pattern seemed to correspond with that seen when using anti-ES rabbit HIS against both ES and somatic antigens. Furthermore, the pattern seen when cyst fluid from <u>T. taeniaeformis</u> (Slide 8) was used as antigen seemed to correspond more closely with ES antigen rather than with somatic antigen. The main line seen against the ES antigen in the above studies also appeared when sera from rats infected with the M strain was reacted against ES (Slide 10) but here the small cathodic precipitation lines were not seen, although these were present when immune serum from <u>T. crassiceps</u> infected mice (Slide 11) was used. The latter serum also showed a similar pattern to serum from mice infected with <u>T. taeniaeformis</u>, when reacting with somatic larval

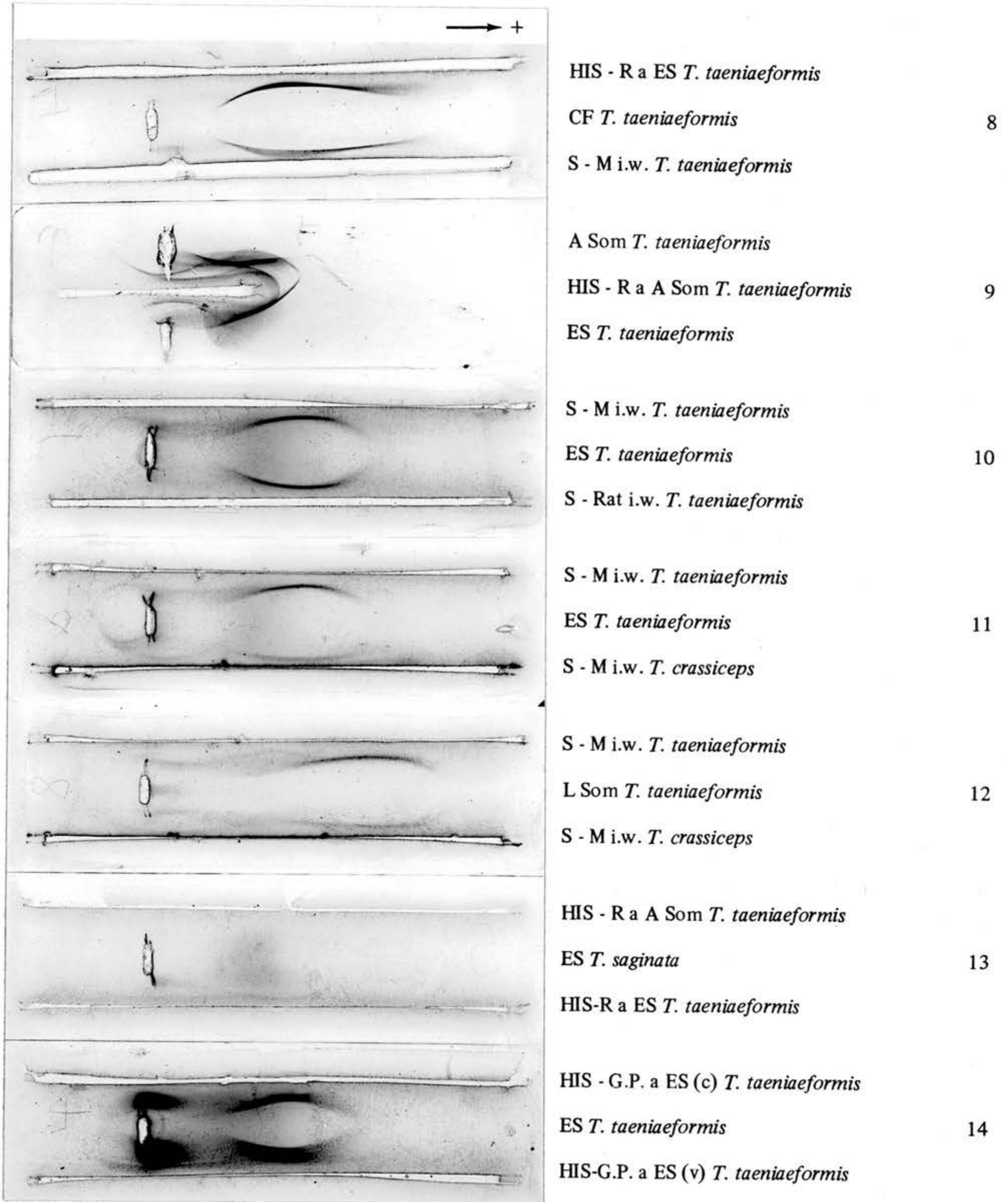


Plate IV. 2(b) (cont.) Immunoelectrophoresis slides showing the reactions between various sera and antigens as specified.

T. taeniaeformis antigen (Slide 12). On the only occasion when T. saginata ES antigen was used, a line appeared against rabbit anti T. taeniaeformis ES HIS in the same region as given with T. taeniaeformis ES antigen but there was no apparent reaction against R a - A som T. taeniaeformis HIS (Slide 13).

Finally guinea-pig HIS against T. taeniaeformis ES, obtained by both immunisation methods, gave a similar pattern (Slide 14) with this antigen. No precipitation lines were seen in the following combinations:

(a) Goat anti-mouse serum (Nordic) versus ES, larval or adult somatic antigen or versus cyst fluid of T. taeniaeformis.

(b) Normal mouse or rabbit sera versus the same antigens.

(c) Unused 50 x concentrated medium versus all the different sera mentioned above.

Consistent patterns were obtained when these experiments were repeated with different batches of antigen and sera.

21.3.3 ELISA and ITAS-ELISA

A similar approach to that for IEP was made with these techniques. Table IV.14 shows the results obtained with the ELISA when T. crassiceps whole larval somatic antigen and T. taeniaeformis adult somatic antigen were used at 25 and 5 μg soluble protein.ml^{-1} and ES antigen at 30, 6, 3 and 1.5 μg.ml^{-1} to detect antibodies in 1/1000 and 1/5000 dilutions of sera from mice infected with either T. taeniaeformis or T. crassiceps.

In another experiment with different batches of sera and antigens larval and adult somatic and ES T. taeniaeformis antigen at concentrations of 25 and 5 μg.ml^{-1} were used to detect antibodies against

Table IV.14 A comparison of three antigens for detecting antibodies by ELISA in serum from mice infected with T. taeniaeformis and T. crassiceps. Relative absorbance at 455 nm.

Serum Dilutions		T. taeniaeformis		T. crassiceps	
Antigen (soluble protein ml^{-1})		1/1000	1/5000	1/1000	1/5000
T. crassiceps	25 μg	0.63	0.23	2.41	1.31
Somatic	5 μg	0.70	0.15	2.04	1.13
T. taeniaeformis	25 μg	0.74	0.24	0.95	0.41
adult somatic	5 μg	0.51	0.12	0.79	0.32
T. taeniaeformis	30 μg	1.39	0.46	0.84	0.34
ES	6 μg	1.16	0.36	0.79	0.30
	3 μg	1.14	0.36	0.71	0.28
	1.5 μg	0.92	0.26	0.49	0.19

Table IV.15 A comparison of three T. taeniaeformis antigens for detecting antibodies by ELISA in serum from mice infected with T. taeniaeformis and T. crassiceps. Relative absorbance at 455 nm.

Serum Dilutions		T. taeniaeformis		T. crassiceps	
Antigen (soluble protein ml^{-1})		1/400	1/1600	1/400	1/1600
Larval somatic	25 μg	1.76	0.80	1.53	0.84
	5 μg	1.66	0.67	1.24	0.59
Adult somatic	25 μg	0.96	0.28	0.99	0.45
	5 μg	0.68	0.18	0.79	0.39
ES	25 μg	1.65	0.69	0.98	0.43
	5 μg	1.43	0.62	0.63	0.29

T. crassiceps and *T. taeniaeformis* in mouse serum at dilutions of 1/400 and 1/1600. The results are shown in Table IV.15.

The experiment was repeated, again with different batches of *T. taeniaeformis* antigens and sera, all antigens being used at concentrations of 5 $\mu g.ml^{-1}$. The antigens were: two batches of ES antigen indicated as ES_1 and ES_2 from two different cultures, from which the media collected after several changes had been pooled and two ES antigens (ES_3 and ES_4), both being a single collection taken after the first 48 h of *in vitro* culture. Furthermore two different batches of adult somatic (A_1 and A_2) and two larval antigens prepared from 5 month old metacestodes, one being a larval somatic (L) antigen and the other pooled cyst fluid (CF). All the antigens were diluted to the same soluble protein concentration.

The sera were pooled from mice infected with either *T. taeniaeformis* or *T. crassiceps* and used at dilutions of 1/400, 1/1600 and 1/6400. The results are shown in Table IV.16.

It was found that ES_3, ES_4 and somatic larval antigen all gave higher control readings than the other antigens for the control which contained antigen and normal mouse serum. This may be due to host (mouse) material present in the larvae at autopsy and subsequently released during this short time in culture.

The same antigens at concentrations of 1, 0.5 and 0.25 $\mu g.ml^{-1}$ were then checked by ITAS - ELISA using both the anti ES and the anti somatic immunoglobulin sandwich.

Results are given in Table IV.17.

Table IV.16 A comparison of eight T. taeniaeformis antigens (all at 5 μg.ml^{-1} soluble protein concentration) for detecting antibodies by ELISA in sera from mice infected with T. taeniaeformis or T. crassiceps Relative absorbance at 450 nm. Key: see Table IV.17.

Serum	T. taeniaeformis			T. crassiceps		
	1/400	1/1600	1/6400	1/400	1/1600	1/6400
Antigen						
A_1	1.10	0.39	0.12	1.01	0.37	0.14
A_2	1.24	0.49	0.14	1.64	0.66	0.19
L	1.72	0.80	0.36	1.20	0.44	0.23
CF	1.67	0.61	0.16	1.42	0.60	0.25
ES_1	2.17	1.13	0.46	0.63	0.23	0.08
ES_2	1.87	0.75	0.29	1.14	0.49	0.18
ES_3	1.47	0.69	0.22	0.27	0.02	0
ES_4	1.35	0.65	0.22	0.30	0.10	0.01

Table IV.17 ITAS-ELISA for detecting the specificity of several antigens. Relative absorbance at 450 nm.
A: adult somatic antigen L: larval somatic antigen
CF: cyst fluid
ES: excretory and secretory antigen from T. taeniaeformis
Subscripts indicate different preparations of similar antigens.

Sandwich		Anti ES			Anti somatic		
Antigen concentration (μg.ml)		1	.5	.25	1	.5	.25
Antigen	A_1	0.18	0.13	0.09	2.16	1.54	1.15
	A_2	0.16	0.09	0.09	2.14	1.39	0.99
	L	0.41	0.24	0.15	1.27	0.81	0.45
	CF	0.20	0.14	0.09	0.46	0.33	0.26
	ES_1	0.65	0.41	0.33	0.39	0.34	0.20
	ES_2	0.77	0.54	0.37	0.85	0.68	0.62
	ES_3	0.97	0.79	0.60	0.29	0.14	0.08
	ES_4	0.96	0.76	0.55	0.28	0.15	0.08

From these ELISA experiments it seemed that adult, larval, cyst fluid and somatic antigen were not very species specific. ES antigens seemed to be more specific and ES_2, the least species specific of the ES antigens clearly contained the highest amount of somatic antigen contaminant.

21.4 CONCLUSIONS

ES antigen and somatic antigen and consequently the antisera raised against these are far from pure. However from the IEP results, ES seemed to have more in common with cyst fluid, while similarly anti-ES HIS seemed to be closer to serum from infected mice than to anti-somatic HIS, although none of these showed any specificity for *T. taeniaeformis* as against *T. crassiceps*. On the other hand in ELISA, ES appeared to be more species specific, even differing in that respect from cyst fluid. From this it seems logical that the more the ES is contaminated with somatic antigen, the less should be its specificity.

These limited preliminary experiments suggested that this is so, although this requires verification. Further experiments were set up to confirm that ES was really being actively produced by the metacestodes *in vitro* rather than just released from degenerating cysts.

Chapter 22 A comparison between the antigens present in the culture fluids about living or dead *T. taeniaeformis* larvae kept under identical *in vitro* conditions

22.1 INTRODUCTION

Two experiments were set up, designed to demonstrate the rate of release of antigens by metacestodes kept *in vitro*, and to study, in respect of this, the relative proportions of catabolic and metabolic antigenic products.

22.2 Experimental design

Groups of living and dead *T. taeniaeformis* metacestodes were submitted to identical *in vitro* cultures: five larvae were kept in 25 ml modified RPMI in each petri dish. The media were changed and sampled every 48 h, being filtered through a 0.22 μm pore size membrane filter (Millipore) and stored at -20°C for later investigation as to their somatic and ES antigen content. *In vitro* procedures were as described in Part III Chapter 13, except that, after washing, half the larvae were randomly selected and killed by transferring them to liquid nitrogen. After this they were placed in the culture vessels and treated in the same way as the living group. This procedure was adopted after it had been found that leaving the larvae in the culture vessel for 3 h at -20°C failed to kill any of them, although the medium was frozen. It was thought that to leave them for a longer time exposed to this temperature might upset the comparison with the living larvae, so the liquid nitrogen procedure was adopted.

In a first experiment using 10 week old metacestodes, the two groups, one with living and one with dead larvae, both included two

replicate plates. In addition there was a third group of two plates in each of which five larvae were placed, their cyst bladders were opened and rinsed and the larvae then discarded. These plates were left together with the other two groups for 48 h. The first two groups were kept till day 6. At the time of this experiment ITAS-ELISA was not available and hence the samples were studied by a direct ELISA, coating the antigen onto the polystyrene and using rabbit anti-ES and anti-somatic HIS.

A second experiment, but with 11 week old living and dead larvae, was carried out under similar circumstances with four plates in both groups. Samples were collected and pooled from the four plates in each group every other day until day 18 of culture. ITAS-ELISA was used to detect somatic and ES antigen, whereas to detect host (mouse) antigen, an indirect ELISA was used as anti-mouse conjugate was not then available. In this system goat anti-mouse ("Ielfo" Nordic) was used in the second step at dilution of 1/500 and anti-goat conjugate in the third step.

22.3 RESULTS

Experiment 1: Rabbit anti-ES, anti-somatic HIS and normal rabbit serum as control were used at 1/100 and 1/500. The results are shown in Table IV.18.

Despite the limitations of this ELISA system for detecting antigen, there seemed to be a clear indication that living larvae continued to produce ES, whereas for the dead larvae there was a sudden release of both somatic and ES antigen, rapidly declining towards the sixth day. As for the media with cyst fluid, the values for ES antigen were not greater than those of the other groups and

the fluid appeared to contain a considerable amount of somatic antigen.

Table IV.18 A comparison by ELISA between living and dead larvae and cyst fluid from T. taeniaeformis held under identical in vitro conditions for the presence of somatic and ES antigen in the medium. The results from two replicate cultures are given for each fluid. Relative absorbance at 455 nm.

Anti somatic		Day 2 1/100	Day 2 1/500	Day 4 1/100	Day 4 1/500	Day 6 1/100	Day 6 1/500
Living	1	0.58	0.46	2.54	2.37	2.05	1.97
	2	1.56	1.00	2.88	2.19	2.62	1.80
Dead	1	2.54	2.51	2.58	1.22	0.36	0.17
	2	2.61	2.09	0.81	0.60	0.22	0.04
Cyst fluid	1	1.67	0.93				
	2	2.66	0.75				
Anti ES							
Living	1	2.12	2.32	2.79	2.38	2.32	2.22
	2	2.89	2.40	2.95	2.61	2.38	2.64
Dead	1	1.62	1.83	1.59	1.16	0.14	0.08
	2	3.00	1.99	0.58	0.64	0.09	0.09
Cyst fluid	1	1.46	1.20				
	2	2.25	1.29				

Experiment 2: The results for ES and somatic antigen for both living and dead larvae are given in Table IV.19.

Table IV.19 A comparison by ITAS-ELISA of the ES and somatic antigen content in cultures of living or dead *T. taeniaeformis* larvae. Relative absorbance at 455 nm.

Day of *in vitro* culture	2	4	6	8	10	12	14	16	18
Somatic antigen									
Living	0.20	0.30	0.25	0.42	0.29	0.18	0.20	0.30	0.30
Dead	0.75	0.47	0.24	0.17	0.16	0.11	0.04	0.02	0.02
ES antigen									
Living	0.73	0.84	0.72	0.88	0.58	0.49	0.70	0.75	0.89
Dead	0.60	1.04	0.29	0.32	0.38	0.28	0.24	0.15	0.01

For living larvae the calculated regressions for both ES $\left[\log (Y + 1) = 0.239 - 0.0002X\right]$ and somatic antigen $\left[\log (Y + 1) = 0.1014 + 0.0002X\right]$ did not reveal a significant change with time: for ES $t_{(7)} = 0.082$, for somatic antigen $t_{(7)} = 0.115$. Furthermore concentrations of ES and somatic antigen were not significantly correlated with each other ($r_{(7)} = 0.661$).

For the dead larvae the release of both ES $\left[\log (Y + 1) = 0.261 - 0.013X\right]$ and somatic antigen $\left[\log (Y + 1) = 0.211 - 0.013X\right]$ was time related $t_{(7)} = 4.009^{**}$ and $t_{(7)} = 5.903^{***}$ respectively and the slopes of the regressions of antigen concentration against time were identical for both antigens, although the intercepts were different which is not surprising considering that in addition to the fact that there is no particular reason for the fluids to contain the same amounts of these antigens, the sera used would probable differ in strength and the two ITAS-ELISA studies for detecting these antigens were not done at the same time.

In this case the apparent concentrations of somatic and ES antigens were significantly correlated $\left[\log (Y + 1) = 0.0135 + 0.727X, r_{(7)} = 0.805^{**}\right]$. The presence of host or host-like antigen is illustrated in Table IV.20.

The estimated concentrations of protein in $\mu g.ml^{-1}$ in the used culture fluids as assayed by the Coomassie brilliant blue micromethod are given in Table IV.21.

Table IV.20 ELISA values indicating the presence of host antigen in cultures with living and dead *T. taeniaeformis* larvae. Relative absorbance at 455 nm.

Day	2	4	6	8	10	12	14	16	18
Living	1.57	0.24	0.05	0.04	0.01	0.07	0.05	0	0
Dead	0.61	0.17	0.08	0.02	0.02	0.10	0.09	0.02	0

Table IV.21 Protein concentrations in $\mu g.ml^{-1}$ for living and dead *T. taeniaeformis* larvae kept under the same *in vitro* conditions.

Day	2	4	6	8	10	12	14	16	18
Living	10	22	14	25	11	14	14	61	38
Dead	800	50	14	21	12	21	22	14	10

22.4 CONCLUSIONS

It is clear from the results that the living larvae are continuing to produce antigen throughout the period of culture. If the alternative that they are only slowly releasing antigen from preformed stores were true then the killed larvae would be expected to release far greater amounts more rapidly. Such production may be either the result of relatively normal metabolic processes or of abnormal catabolism. However, if the latter is true, it is surprising that the rate of production does not fall off with time as the precursors for catabolism are used up. Furthermore the fact that, after the initial loss, the total protein content of the culture fluids from the dead larvae did not fall with time at the same rate as the antigenic activity was falling suggests that at least some of these antigens differ from more degenerative catabolic products.

The fact that the living larvae continued to release the so called somatic antigen at the same rate throughout the time of the culture is probably in part a reflection of the cross reaction between the antiserum and ES antigen but also suggests that the components shared between the somatic and ES antigens are continuing to be formed and released at a similar rate. The relatively small difference in the initial collections from the cultures containing killed larvae in their reactions with anti-ES serum and anti-somatic serum as compared with the same parameters for the living larvae (Fig.IV.2) taken together with the fact that the living larvae appear to release more ES and less "somatic" antigen and the known cross reaction of these sera, suggests that the killed larvae may be releasing true somatic antigen - i.e. that which is not normally present in the ES

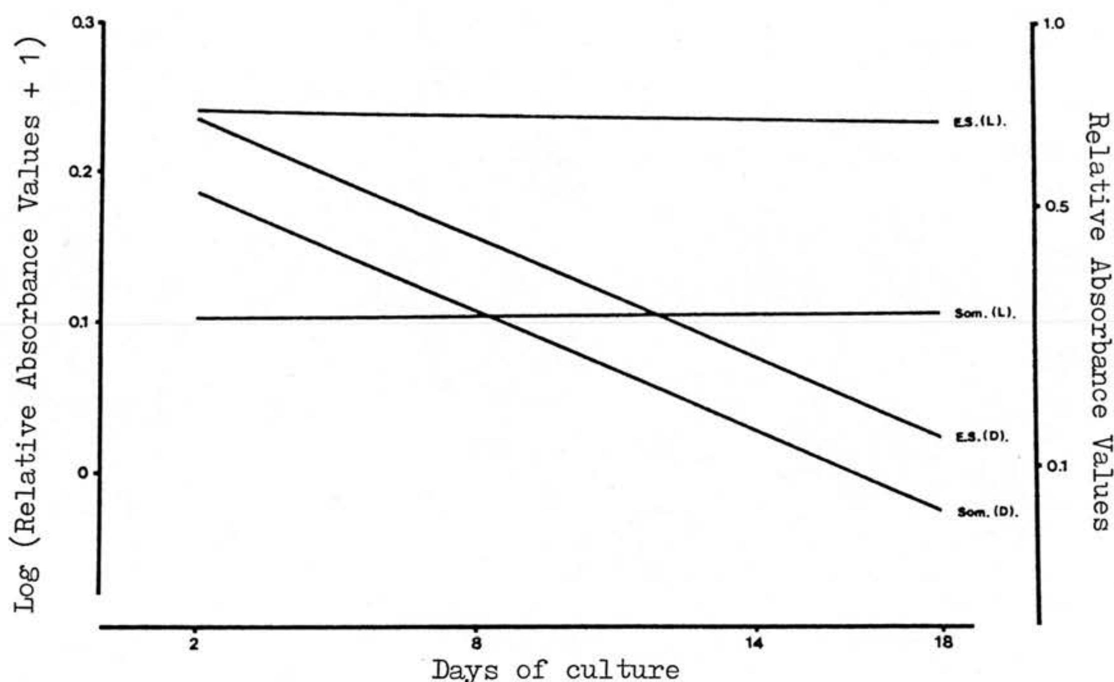


Fig. IV.2 Regressions of the absorbance values by ITAS-ELISA, for both ES and somatic antigen from living (L) and dead (D) Taenia taeniaeformis larvae kept under identical in vitro conditions, on time in days.

Regression equations

Living larvae: ES antigen: $\log (Y + 1) = 0.239 - 0.0002\ X$; $t_{(7)} = 0.082$ NS

Somatic antigen: $\log (Y + 1) = 0.1014 + 0.0002\ X$; $t_{(7)} = 0.115$ NS

Dead larvae : ES antigen: $\log (Y + 1) = 0.261 - 0.013\ X$; $t_{(7)} = 4.009$ **

Somatic antigen: $\log (Y + 1) = 0.211 - 0.013\ X$; $t_{(7)} = 5.903$ ***

products (q.v. I.E.P. results in Chapter 21). Conversely, since these components are not present in ES they presumably are not being released from living larvae in significant amounts.

It again appeared that cyst fluid is not quite the same as ES, in that it seemed to contain a mixture of both these antigens in more equal proportions than the medium obtained on the first days of culture.

Host antigen also seemed to be present and it would be interesting to use ITAS-ELISA to study the origin of this - i.e. whether it was produced or previously absorbed and later released by the larvae *in vitro*.

Chapter 23 The pattern of the release of ES and somatic antigens in different media

23.1 INTRODUCTION

Further comparisons were made relating to the influence of different media on the release of ES and somatic antigens.

23.2 Experimental design

Samples of the medium taken every 48 h in the two experiments described in Part III, Chapter 14, were stored at -20°C.

About one year later, they were examined by the ITAS - ELISA for detecting ES and somatic antigen. They were equilibrated, as described, with either 10% v/v FCS or modified RPMI. The samples were not pooled, and those obtained from each of the four replicate sub-groups and from the control for each group every sixth day from the first experiment and every fourth plus the last day from the second experiment were examined.

Comparisons between ES and somatic antigens were based on the linear regressions of the log transformed relative absorbance values + 1 against time both within and between the groups. Student's "t" tests were performed on the sub-group regressions and F-tests on the group regressions.

23.3 RESULTS

The relative absorbance values at 450 nm are given in Appendix Table IV.1 and 2 respectively for the first and second experiment. Calculated regression lines with the regression formulae for ES and somatic antigen against time in the first experiment were for ES

antigen: Group 1: $\log (Y + 1) = 0.082 + 0.002X$, Group 2: $\log (Y + 1) = 0.082 + 0.002X$, Group 3: $\log (Y + 1) = 0.117 + 0.0005X$, and for somatic antigen: Group 1: $\log (Y + 1) = 0.031 + 0.140X$, Group 2: $\log (Y + 1) = 0.046 + 0.008X$, Group 3: $\log (Y + 1) = 0.014 + 0.009X$. Those for somatic antigen in the second experiment were: Group 1: $\log (Y + 1) = 0.122 + 0.016X$, Group 2: $\log (Y + 1) = 0.234 + 0.005X$, Group 3: $\log (Y + 1) = 0.180 + 0.007X$.

Those for ES antigen will be discussed later in this Chapter. The "t" values for the regression coefficients for the sub-groups are given in Table IV.22 and the F-values for the combined regressions, between the coefficients and the intercepts from the analysis of variance within and between groups are given in Table. IV.23.

From these data it appeared that the concentration of somatic antigen increased with time for all groups in the two experiments and did so faster in the groups for which the medium contained only RPMI (Group 1 in both experiments). When serum or sodiumpyruvate or both were added to this medium the increase was slower. No significant differences in this respect were detected in the first experiment between the regressions for the media containing RPMI + 10% v/v FCS with or without (Group 2) sodiumpyruvate, but in the second experiment all three groups differed significantly.

The release of ES was not so well defined. Although in the first experiment the group regressions varied significantly with time (see F-values for combined regressions in Table IV.23). This relationship was only significant in only five out of twelve sub-groups (see "t" values in Table IV.22). For the second shorter experiment, there was no significant relationship between time and the

Table IV.22 Student's "t" values obtained from the calculated regressions of the log transformed values + 1 against time for somatic and ES antigens released during *in vitro* incubation of larval *T. taeniaeformis* in two experiments, each with four replicate cultures (subgroups) in three different media (groups).

"t" values for the release of		Somatic antigen		ES antigen
First experiment				
Group 1 Subgroup a	$t_{(5)}$	6.436***	$t_{(5)}$	3.974*
b	$t_{(5)}$	6.487***	$t_{(5)}$	4.965**
c	$t_{(5)}$	5.408**	$t_{(5)}$	0.281 NS
d	$t_{(5)}$	7.253***	$t_{(5)}$	1.662 NS
Group 2 Subgroup a	$t_{(7)}$	6.758***	$t_{(7)}$	3.213*
b	$t_{(7)}$	7.816***	$t_{(7)}$	2.273 NS
c	$t_{(7)}$	5.632***	$t_{(7)}$	2.821*
d	$t_{(7)}$	7.128***	$t_{(7)}$	4.089**
Group 3 Subgroup a	$t_{(6)}$	5.678**	$t_{(7)}$	1.370 NS
b	$t_{(7)}$	6.133***	$t_{(7)}$	1.185 NS
c	$t_{(7)}$	8.147***	$t_{(7)}$	1.446 NS
d	$t_{(7)}$	9.298***	$t_{(7)}$	0.845 NS
Second Experiment				
Group 1 Subgroup a	$t_{(4)}$	6.516**	$t_{(4)}$	0.991 NS
b	$t_{(4)}$	20.531***	$t_{(4)}$	1.329 NS
c	$t_{(4)}$	6.033**	$t_{(4)}$	0.904 NS
d	$t_{(4)}$	13.006***	$t_{(4)}$	0.755 NS
Group 2 Subgroup a	$t_{(7)}$	5.150**	$t_{(7)}$	0.166 NS
b	$t_{(7)}$	5.440***	$t_{(7)}$	1.264 NS
c	$t_{(7)}$	4.951**	$t_{(7)}$	0.619 NS
d	$t_{(7)}$	4.909**	$t_{(7)}$	1.592 NS
Group 3 Subgroup a	$t_{(7)}$	5.350**	$t_{(7)}$	0.725 NS
b	$t_{(7)}$	6.934***	$t_{(7)}$	2.018 NS
c	$t_{(6)}$	6.303***	$t_{(7)}$	0.069 NS
d	$t_{(7)}$	7.405***	$t_{(7)}$	0.714 NS

Table IV.23 F-values obtained from the analysis of variance of the log transformed values + 1 for ES and somatic antigen against time compared within and between groups of different media in two experiments.

F-values from:	Combined regression	Between coefficients	Between intercepts
First experiment:			
Somatic antigen			
Group 1	169 ** (1,20)	0 NS (3,20)	0 NS (3,20)
2	183 ** (1,28)	0 NS (3,28)	0 NS (3,28)
3	195 ** (1,27)	0 NS (3,27)	0 NS (3,27)
Anovar: 1 - 2 - 3	644 ** (1,93)	13 ** (2,93)	11 ** (2,93)
1 - 2	95 ** (1,60)	23 ** (1,60)	335 ** (1,60)
1 - 3	102 ** (1,59)	19 ** (1,59)	342 ** (1,59)
2 - 3	461 ** (1,67)	1 NS (1,67)	3.992 NS (1,67)
ES antigen			
Group 1	17 ** (1,20)	0 NS (3,20)	0 NS (3,20)
2	36 ** (1,28)	0 NS (3,28)	1 NS (3,28)
3	5 * (1,28)	0 NS (3,28)	2 NS (3,28)
Anovar: 1 - 2 - 3	66 ** (1,94)	9 ** (2,94)	10 ** (2,94)
1 - 2	69 ** (1,60)	8 ** (1,60)	9 ** (1,60)
1 - 3	37 ** (1,60)	15 ** (1,60)	53 ** (1,60)
2 - 3	41 ** (1,68)	7 ** (1,68)	16 ** (1,68)
Second experiment:			
Somatic antigen			
Group 1	304 ** (1,16)	1 NS (3,16)	1 NS (3,16)
2	103 ** (1,28)	0 NS (3,28)	4.15 * (3,28)
3	162 ** (1,27)	0 NS (3,27)	0 NS (3,27)
Anovar: 1 - 2 - 3	54 ** (1,89)	49 ** (2,89)	20 ** (2,89)
1 - 2	361 ** (1,56)	110 ** (1,56)	51 ** (1,56)
1 - 3	427 ** (1,55)	66 ** (1,55)	38 ** (1,55)
2 - 3	325 ** (1,67)	8 ** (1,67)	19 ** (1,67)
ES antigen			
Group 1	3 NS (1,16)	0 NS (3,16)	0 NS (3,16)
2	2 NS (1,28)	0 NS (3,28)	0 NS (3,28)
3	0 NS (1,28)	1 NS (3,28)	1 NS (3,28)

release of ES antigen.

23.4 CONCLUSIONS

The presence of somatic antigen in the media from all the groups in both experiments seemed to be less erratic and more clearly time related than the parameters used for the same experiments as described in Part III, Chapter 14. This makes it worthwhile considering the value of this antigen as a parameter for assessing the efficacy of different media for maintenance or growth of larvae.

Since for both experiments Group 1 seemed to be visually inferior to both the other groups and also had the fastest rise in somatic antigen level, the relationship between this antigen with catabolism seemed also here to be apparent.

Although good survival of larvae _in vitro_ might be expected to be reflected in higher production of metabolic products or ES antigen, this was not so clearly shown in these studies. In fact it appeared from the second experiment and probably from the first experiment as well, if the results from this were restricted to those obtained over the same period as the former, that the rate of release of ES did not change in a linear manner but had a rather irregular pattern which was closer to a curvilinear relationship with time. This is illustrated in Fig. IV.3, for which Table IV.24 gives the statistical analysis (Snedecor 1956), the regression formulas being calculated on the mean values per group.

This apparent eventual increase in ES antigen concentration may have been due to a faster release because of the progressive degeneration or cuticular damage, which might explain why this rise

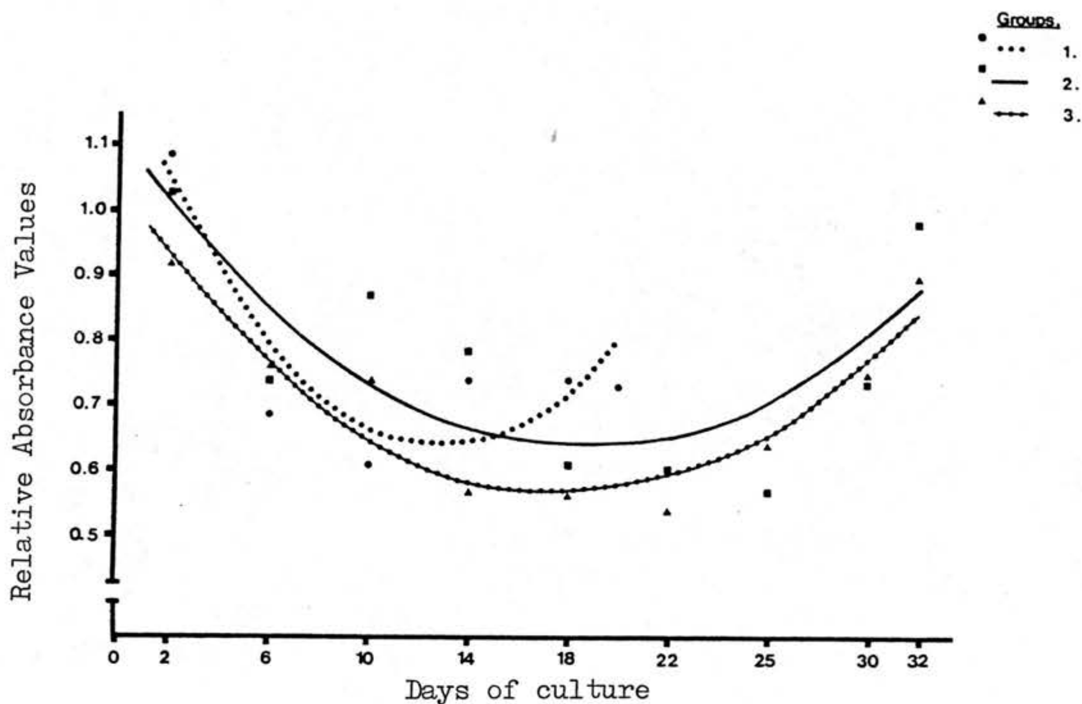


Fig. IV.3 Second Experiment: Curvilinear regressions of the absorbance values by ITAS-ELISA for ES antigen in media recovered from three groups of Taenia taeniaeformis larvae maintained in vitro, on time in days. For Group 1 the medium was RPMI, for Groups 2 and 3 sodium pyruvate was added and for Group 3 alone, FCS was added.

Regression equations:

For Group 1: $Y = 1.1909 - 0.0873\ X + 0.0034\ X^2$

For Group 2: $Y = 1.1185 - 0.0523\ X + 0.0014\ X^2$

For Group 3: $Y = 1.0380 - 0.0538\ X + 0.0015\ X^2$

For statistical analysis see Table IV.24.

was faster in Group 1 than in the other two groups. On the other hand the antisera raised against the ES antigens were not pure and this apparent increase in ES antigen content may have really been a reflection of the increasing concentration of somatic antigens with time in the culture fluid.

Table IV.24 Analysis for linearity and curvilinearity against time for the release of ES antigen by T. taeniaeformis larvae kept in three different media (second experiment).

Parameter	Group 1	Group 2	Group 3
	Variance ratio (F)	Variance ratio (F)	Variance ratio (F)
Linear regression	1.42 (1,4) NS	0.69 (1,7) NS	0.10 (1,7) NS
Curvilinear regression	4.41 (2,3) NS	4.69 (2,6) NS	28.98 (2,6)**
Curvilinearity	5.71 (1,3) NS	8.00 (1,6) *	57.09 (1,6)**

Chapter 24 DISCUSSION AND CONCLUSIONS

It is clear from the results described in this Part that the unrefined antigens released from *T. taeniaeformis* larvae maintained *in vitro* consist of a mixture of antigens with some different characteristics from either the somatic or cyst fluid antigen mixtures. There is undoubtedly some overlapping but each of these is a mixture of antigens, with proportional and probably qualitative differences. In these mixtures the "true" ES antigens, which may for convenience be considered to form one group, distinct from the others such as "somatic" antigens, probably vary with the different *in vitro* methods used to produce them, and also with other factors not studied in this work, such as the age of the metacestodes. The degree of contamination of these ES antigens with somatic antigen clearly varies and at least two factors seemed to be involved: the culture medium and the length of time for which the larvae are exposed to it. There is some indication that the wide range of possibilities for improving *in vitro* cultures may also influence this contamination. However, there are a number of inconsistencies. Thus, the pattern of the release of antigens in the cultures with living larvae described in Chapter 22, Experiment 2, does not correspond closely with the findings described in Chapter 23. The short time scale for the former experiment may be partly responsible for this, but another difference between these experiments was the relatively young age of these larvae in the former (11 weeks) as opposed to that of the larvae in the latter study (16 weeks).

From the results obtained with immunoelectrophoresis, ES antigen seemed to be rather similar to cyst fluid. However complete identity

is very unlikely in view of the results from the ELISA and ITAS - ELISA studies, where ES seemed to be a more species specific antigen than cyst fluid when compared using serum from mice infected with T. taeniaeformis and T. crassiceps. On the other hand, if ES is similar to cyst fluid then an explanation as to why the level recovered in vitro from dead larvae but not for living larvae dropped steadily with time, may be heat lability, in which case there must have been regular renewal or production by the living larvae.

This apparent similarity of ES antigens and cyst fluid antigens in IEP, which was not so apparent in ELISA, may however be due to the insensitivity of the former. The antigen needed to be highly concentrated in order to give a visible reaction in IEP, and this may well not have shown all the reactions occurring. On the other hand, as illustrated by Morris, Proctor and Elsdon-Dew (1968) Harrison (1978) and Harrison and Sewell (1980), not all antigen-antibody reactions detected by one technique are necessarily detected by another. The presence of host antigens may be an illustration of this, they were detected with ELISA but not in IEP. Since it seemed that the concentration of these was particularly high at the beginning of the culture, media from the first 24 h were routinely discarded. The presence of host antigen has been taken into consideration by various authors, as being either antigen produced by the parasites in common with the host (e.g. Capron, Biguet, Vernes and Afchain 1968, Chernin 1977 and Bout, Carlier, Dessaint and Capron 1977) or as contaminant (Varela Díaz, Coltorti, Ricardes, Guisantes and Yarzábal 1976 and Hustead and Williams 1977[a]). The limited study undertaken in the present work would suggest that these host antigens

are contaminative, unless the stimulus to the larvae to produce them was absent in vitro.

Sharing individual antigens amongst the three ES, somatic and cyst fluid antigen complexes has been discussed earlier and contamination of metabolic antigen with catabolic or somatic antigen was also considered by Zimmerman and Leland (1974) for Cooperia punctata, by Rickard and Katiyar (1976) for Taenia pisiformis oncospheres and by Nash (1979) for S. mansoni. Isolating the "true" ES antigens from somatic antigen in the complex mixtures may be difficult or wasteful and another approach might be to improve on the culture methods monitored by ITAS - ELISA for somatic antigen or by monitoring the purity of the ES antigens in the culture fluids by checking them for their specificity for T. taeniaeformis as against T. crassiceps. These methods would probably not permit the acquisition of entirely pure ES antigens but might allow selection of the least contaminated used culture media.

Apart from the immunogenic value of ES, which will be further discussed in Part V, it seems worthwhile to extend these studies in order to check their value as more specific diagnostic antigens. Catabolic contaminants may be one of the explanations for the controversies about the usefulness of ES as diagnostic antigens. On the other hand, in studies for detecting circulating antigen such as those by Bawden and Weller(1974) for S. mansoni and by Van Knapen and Fridas (1977) for bovine cysticercosis, hyperimmunised sera against somatic antigens were used. This might mean, especially in the case of encapsulated parasites, that the circulating antigens mainly detected are those arising from the degeneration of the parasites.

According to the relationship between ES and circulating antigen for S. mansoni (Nash 1979) and the similarity between anti-ES hyperimmune serum and serum from mice infected with T. taeniaeformis indicated by IEP, anti-ES antigen hyperimmune sera might allow the detection of these parasites before encapsulation when their ES products might be expected to be in circulation and could be of value for experimental work. However, if this hypothesis is true, it would require an in vitro culture of the same cestode species against which the diagnosis is aimed and even the stage in the life cycle might be another limiting factor to be considered. Also, it would require the use of fairly specific HIS. In this respect, the method used in this work to hyperimmunise rabbits against T. taeniaeformis ES products, based on the assumption that less somatic antigen would be present in short term cultures with serum enriched media, needs more study to clarify both the influence of serum in general and of serum from different species. The anti-ES hyperimmune rabbit sera appeared to be more specific that those from guinea-pigs, which was not necessarily due to the use of the rabbits' own sera in cultures for preparing the former. Another, rather complex approach might be to make newborn animals tolerant to the undefined components in the medium and to hyperimmunise them later, as is described by Antoine and Neveu (1967).

Further work related to the nature of ES antigen is necessary, one of the aspects which has not been taken into account in this work being the possibility of the particulate nature of some of these ES antigens, such as the surface membrane antigens released by S. mansoni in vitro (Kusel, Mackenzie and McLaren 1975). As well as the question of their existence, possible importance and detectability

by the methods used it would also be necessary to consider the effect on these of the methods by which the media were treated.

The molecular weight of ES antigen has often been discussed as by Nash (1979), Stromberg (1979) and Zimmerman and Leland (1974) and the use of filtermembranes with a molecular weight cut off of 10 000 has been used, before culture, so as to obtain only low molecular weight serum nutrients by Stromberg, Khoury and Soulsby (1977) or, after culture, aiming to separating the excretory material from secretory antigens by Herd, Chappel and Biddell (1975).

On one occasion in this work, antigenic activity was found to be present in the eluate after using such a filter, which led to the abandonment of this method of concentrating and use of freeze-drying instead. This might have been caused by the physical form of the molecules, allowing them to pass through this filter despite higher molecular weights than the limit indicated by the manufacturer, or otherwise, the eluate may have contained fragments broken off of these molecules, still with antigenic or haptenic activity.

Together with the varying opinions of several authors concerning the effects of freeze-drying and deepfreezer storage as reviewed in Chapter 18, another factor has to be taken into account, concerning the possibility that the somatic fractions of the culture antigens may be better preserved than the ES fractions, although no evidence for this was found in the present work.

Another point is, that low molecular weight haptenic substances may have been present along with the ES antigens in the culture fluids which would not have been evaluated in this work, as they would not have stimulated the production of antibodies by the immunised animals.

PART V: Immunogenic properties of the metabolic products of *Taenia taeniaeformis* larvae

Chapter 25 INTRODUCTION AND REVIEW OF LITERATURE

The ability to vaccinate animals against helminth parasites has long been sought and numerous procedures for controlling helminth diseases are based on applied immunology. As will be further reviewed, *in vitro* culture has played an important role in these studies and promising results have formed the basis for further investigations. In the present work, studies on the efficacy of the ES antigens of *Taenia taeniaeformis* as immunogens were initially set back by the age related resistance and strain differences described in Part I. Furthermore, although it was originally intended to investigate ES immunogens obtained from cultures, starting with oncospheres, the obstacles encountered with these cultures, including obtaining sufficiently high numbers of eggs, and hatching and activating these, as considered in Part II, led attention to be turned towards the antigens derived from cultured later larval stages. Obtaining some insight into both the *in vitro* cultural aspects and the release of these antigens, discussed in Parts III and IV, further delayed the work which is to be considered in this Part.

The experiments described here suffered from the shortcomings described in previous Parts and are therefore only of a preliminary nature with rather tentative results.

Despite the continuous development of chemotherapy and better understanding of the biology of parasites, which has facilitated

control and prophylaxis of helminth diseases, there is still a considerable need for preventive measures based on the immunological host-parasite relationships.

The extensive literature on this subject has often been reviewed and discussed by various authors. Campbell (1936) working with <u>T. taeniaeformis</u> in rats distinguished an early and a late immune response, related respectively to the migration and the establishment of the parasite in the tissues. Campbell suggested that the latter was stimulated by the metabolic products of the parasites. Chandler (1953) described various ways in which acquired immunity in the host may be reflected in the worm's development. Soulsby (1957[a], 1958) arrived at some conclusions, based on both his own and previously reported findings. He deduced that the association of protective immunity with the presence of living worms seemed to be related to their metabolic products. It also appeared that the antigens responsible for inducing a protective immunity - called "functional antigens" by Oliver-González (1946) - form only a proportion of the spectrum of antigens present in the helminth and capable of stimulating the production of antibodies.

Further studies led Soulsby (1963[a, b]) to conclude that these "functional antigens" may only be produced at certain stages in the life cycle. In particular, for nematodes, the hatching and moulting fluids from the infective larval stages received much attention. Since then, several valuable reviews relevant to the immunology of cestodes have been published, including these by Gemmell and Soulsby (1968), Smyth (1969[a], 1976), Arundel (1972) and Herd (1975), Clegg and Smith

(1978), Williams (1979), Murray, Robinson, Grierson and Crawford (1979) and Urquhart (1980).

Successful vaccination with somatic antigen against the larval stages of T. taeniaeformis have been reported by Miller (1931[a]), Campbell (1936) and Kwa and Liew (1977), while Gallie and Sewell (1976) vaccinated calves against Taenia saginata metacestodes using a non-living somatic vaccine.

Miller (1931[b]) stated that natural immunity against T. taeniaeformis was more effective than artificially induced immunity and several experiments were based on this observation. Sweatman (1957) reported resistance to superinfection with Taenia hydatigena in sheep, and Urquhart (1958, 1961) investigated this type of resistance against T. saginata in calves. Froyd and Round (1960) and Froyd (1961) studied the possibility of inducing such resistance against T. saginata by injecting hatched and activated oncospheres s.c. or i.m. prior to challenge.

Heath and Chevis (1975) and Heath (1978) used chemotherapy after the parenteral immunisation to overcome the problem of larval development at the injection site in immunised animals.

Wikerhauser, Žuković and Džakula (1971) also experimented on this type of resistance working with T. saginata and T. hydatigena in calves. Experiments described by Gallie and Sewell (1972, 1974[a]) showed that calves could become resistant to superinfections while living cysts from the first infection were still present.

Another approach to produce protective immunity is to use

irradiated eggs. This has been done with T. taeniaeformis (Dow, Jarrett, Jennings, McIntyre and Mulligan 1962) in mice, with T. saginata (Urquhart, McIntyre, Mulligan, Jarrett and Shorp 1963) in calves and with Taenia pisiformis (Beveridge and Rickard 1975) in rabbits.

Gemmell (1964[a, b], 1965) investigated the presence of common antigens between Taenia ovis, T. pisiformis and T. hydatigena and (Gemmell, 1966, 1967) Echinococcus granulosus. These experiments led him to conclude that the important protective immunogens were of metabolic origin rather than purely somatic. Furthermore Gemmell also distinguished two protective reactions, one against the establishment and survival of the parasites, active at the intestinal mucosa level and requiring species specific immunogens, and another at the site of election only acting against growing larvae, associated with cellular responses and not necessarily involving species specific immunogens, as although all species do not have this common functional antigen, some sharing occurs (Gemmell 1969[a]).

Further work (Gemmell, Blundell and MacNamara 1968) revealed the development of absolute resistance to take at least 14 days after immunisation by i.m. injection with activated T. hydatigena oncospheres, followed by homologous challenge. Gemmell (1968, 1969[b]) investigated the possibilities of developing a vaccine against T. hydatigena and T. ovis and concluded that during immunisation with oncospheres, their survival even for a short time could induce a strong resistance against an homologous challenge.

Further studies related to ovine cysticercosis and hydatidosis

were undertaken (Gemmell 1969[c], 1970, 1972[a, b]) and showed acquired resistance to be both age related and not an impermanent state.

Work on the phenomena of resistance to superinfection in lambs and of the cross reactivity of different cestode species in this respect was also done by Heath, Lawrence and Young (1979), while the cross reactivity between Fasciola hepatica and T. hydatigena larvae was studied by Dineen, Kelly and Campbell (1978).

The use of parasite metabolites as immunogens has received attention from various workers. The in vivo use of membrane diffusion chambers, for which a technique was described by Nettesheim and Makinodan (1967) allowed the study of the effect of metabolic products on subsequent challenge-infection, with possibility of controlling the length of exposure to the former. When this technique was successfully adopted by Despommier and Wostmann (1968) for Trichinella spiralis in mice and in similar experiments with T. ovis and T. taeniae-formis by Rickard and Bell (1971[b, c]) it revealed the interesting possibility of parasite metabolites acting as protective immunogens. It appeared that only a few larvae were needed to produce enough diffusible antigen during their early development to stimulate resistance to challenge infections.

So far, the use of ES antigens, obtained from in vitro cultures has been investigated by various workers, mainly using such products obtained from nematode parasites. These include: Silverman, Poynter and Podger (1962), Soulsby (1963[a]), Crandall and Arean (1965), Mills and Kent (1965) and Guerrero and Silverman (1969, 1971).

Rickard and Bell (1971[a]) and Rickard (1971-72) were the first to use ES antigen from cestode parasites. T. ovis and T. hydatigena oncospheres were cultivated in vitro for 8 days and the metabolites so obtained stimulated a high degree of resistance against challenge with T. ovis in sheep. Kowalski and Thorson (1972), working with Mesocestoides corti found ES antigens to be better immunogens than somatic antigens.

Rickard, White and Boddington (1976) further investigated the activity of oncospheral metabolites against T. ovis in sheep and noticed that although ES products could prevent the establishment of the larvae, they had no influence on the few cysticerci which nevertheless became established. Their observation agreed with those of Campbell (1938[a, b, c]) and those of Gemmell (1969[a]), described above concerning the different immunological defence reactions against the early and late phases of parasite development.

Rickard and Adolph (1977) succeeded in vaccinating lambs against T. ovis with homologous ES antigen obtained from oncospheres which had been cultured in vitro for only 24 or 48 h, even when defined media were used.

Rickard and Outteridge (1974) successfully immunised rabbits against T. pisiformis with oncospheral ES, noticing that the functional antibodies were not necessarily the same as those of value for diagnosis. In fact, this discrepancy between "functional" antibodies and detectable antibodies has been frequently made, following the original definition of "functional antibodies" by Oliver-González (1946), among others by Gallie and Sewell (1974[a], 1974[b], 1976), Jacqueline, Vernes, Bout and Biguet (1978) and Kwa and Liew (1978[a])

the latter working with T. taeniaeformis.

Heath (1976) also immunised rabbits against T. pisiformis larvae using homologous oncospheral ES antigen and found the metabolites to be more effective when they were obtained not until after the first 3 days of in vitro culture. Successful vaccination against T. saginata larvae in calves with oncospheral homologous and to a lesser extent with heterologous ES from 2 week cultures were reported by Rickard and Adolph (1976). However, Wikerhauser, Brglez, Džakula, Asaj and Matić-Piantanida (1978) were unable to confirm this using the same methods to produce the ES antigen. In their experiments the vaccinated calves did not show resistance to challenge with 150 000 homologous eggs, which was a much higher dose than that used by the former workers. On the other hand, Lloyd (1979) found ES antigen from homologous and heterologous oncospheres to be protective against T. taeniaeformis in mice when administered either by oral or by i.m. route and against T. saginata in calves when administered i.m.

Kwa and Liew (1977) vaccinated rats against T. taeniaeformis with ES antigen obtained by incubating mature strobilocerci for 24 h and also with somatic strobilocerci antigen. Both antigens were highly effective, provided that the time between vaccination and challenge was not less than 10 days. Similarities between fractions of both these antigen mixtures were studied and resulted in the separation of a purified immunogen, which represented 6.8% w/w and 8.1% w/w of the somatic and ES antigens respectively. The authors did not think that the ES antigen was more effective than somatic antigen and this led them to conclude that it would be better to purify somatic antigen

rather than trying to obtain ES for vaccination purposes. This was in contrast to Leid and Williams (1974[a]) who, finding that it was impossible to absorb out the protective capacity of T. taeniaeformis immune serum using somatic material, concluded that the concentration of certain critical antigens may not have been high enough and suggested that collecting in vitro products might provide a higher concentration of such critical antigen.

Ayuya and Williams (1979) studied the immunogenic activity of ES and somatic antigens, obtained from T. taeniaeformis as described by Kwa and Liew (1977), in rats against the homologous parasite and pointed to the important influence of the route of administration of these vaccines. Oral or intraperitoneal administration was highly effective for both antigens, whereas intramuscular vaccination required the use of adjuvants for successful immunisation.

These last two reports were in accordance with the findings of Musoke and Williams (1976) but differed from those of Heath (1973[b,c], 1974-75) and Heath and Chevis (1975) who found with T. pisiformis in rabbits that the immunity induced by larvae seemed to be limited by their age. Little protective immunity was induced by larvae over 15 days of development and no protection at all by mature larvae. This age-limiting factor was also suggested by the observations of Rickard and Coman (1977).

On the other hand the important role of adjuvants in vaccinations has been the concern of many workers including: Musoke and Williams (1976), Herd (1977), Chedid (1977), Murray, Robinson, Grierson and Crawford (1979) and Williams (1979).

Osborn (1977-78) thought the immunogenic value of T. ovis onco-

spheral ES antigen to be dependent upon the composition of the medium in use and a similar remark was made by Mills and Kent (1965) working with Trichinella spiralis, who thought that the larvae might stop producing the right antigen under inadequate in vitro conditions.

Attempts to use ES antigen from adult E. granulosus to immunise dogs against the adult homologous parasite were undertaken by Herd, Chappel and Biddell (1975) but with rather inconclusive results (Herd 1977). Rickard, Coman and Cannon (1977) investigated this for T. pisiformis in dogs using both oncospheral and adult ES antigens, without eliciting any immunity.

The possibility of controlling cestode infections by combining passive immune transfer via the colostrum, eventually followed this with vaccination of the young animals and the role of ES antigen herein has been discussed by Lloyd (1979), Rickard, Adolph and Arundel (1977) and Rickard, Boddington and McQuade (1977).

Several authors have used passive immune transfer to study the immune mechanisms in cestode infections. Miller and Gardiner (1932) described successful passive immune transfer in rats for T. taeniaeformis. Later Heath and Pavloff (1975) found with T. taeniaeformis that passive immune transfer was effective in preventing development of the oncospheres but not in preventing them from reaching the liver. In contrast, Gemmell, Blundell-Hasell and MacNamara (1969) found no evidence of immune transfer via the colostrum for T. hydatigena in lambs. On the other hand successful transfer of passive immunity against T. saginata in calves was shown by Lloyd and Soulsby (1976).

Several authors have studied the immunoglobulin classes possibly involved in passive immune transfer using T. taeniaeformis (Leid and

Williams 1974[a] and Musoke and Williams 1975[a, b], 1976). These latter authors described the involvement of complement in this immune mechanism as did Mitchell, Goding and Rickard (1977), Musoke, Williams, Leid and Williams (1975) and Hustead and Williams (1977[b]). However Ayuya and Williams (1979) found that serum from donor rats hyper-immunised against somatic antigen appeared to increase the suscepti-bility of receptor rats to challenge infection.

The relationship between the immune mechanism and the possibility of prolonged survival of cestodes in their hosts has been often discussed. Campbell (1938[a, b, c]) concluded that there was existence of two immunological phenomena in rats infected with T. taeniaeformis: early immunity resulting in the absence of liver lesions and late immunity, where the larvae were killed after about 10 days of develop-ment. He suggested that surviving larvae were protected against host antibodies by the cyst wall. Heath and Pavloff (1975) suggested that for T. taeniaeformis the early immunity against reinfection may be induced by the somatic antigen, derived from the high mortality rate of oncospheres during the initial infection, whereas the late immunity may be stimulated by metabolic products from the established larvae. The opposite view as taken by Rickard (1974), Rickard and Outteridge (1974) and Rickard, White and Boddington (1976) who suggested that ES antigen induced resistance against the establishment of larvae whereas the late immunity responsible for killing the established larvae was stimulated by somatic antigen and was possibly more de-pendent on cell mediated immunity.

Musoke and Williams (1975[b]) concluded that older larvae probably evade the host immune reaction by secreting an anticomplementary

factor, rather than solely by protection from the cyst wall. Other workers, such as Siebert, Good and Simmons (1978) have reported immunodepression in the host as possibly being involved in the prolonged survival of T. crassiceps. Varela Diáz, Gemmell and Williams (1972) experimenting with T. ovis and T. hydatigena believed the prolonged survival of the larvae to be associated with fixation of host blocking antibodies on the parasite's surface, which subsequently hinder the action of protective antibodies later produced or prevent a cell mediated immunological action (Rickard, 1974, Kwa and Liew 1978[a], 1978[b]). The role of IgE and IgM antibodies in relation to this has been discussed by Capron and Dessaint (1977) and by Leid and Williams (1974[b]).

Kwa and Liew (1978[b]) experimented with implants in rats of trypsinised larvae of T. taeniaeformis and, observing that these were then more vulnerable to the host defence mechanism, concluded that a coat of blocking antibodies, removed by the trypsin, was involved in the phenomenon of prolonged survival.

The role of cell mediated immunity in cestode infection was studied by Kwa and Liew (1975[a, b]), Rickard and Outteridge (1974), Freeman (1964), Ansari and Williams (1976), Ansari, Williams and Musoke (1976) and various others but as concluded by Williams (1979) the role of cell mediated protective mechanisms in metacestode infections remains uncertain.

Chapter 26 MATERIALS AND METHODS

Before the rat strain of *T. taeniaeformis* became available, two preliminary experiments were undertaken trying to immunise mice with metabolic products obtained during *in vitro* maintenance of larval *T. taeniaeformis* (Experiment 1 and 2). The restrictive age limiting factors in this host, led to an attempt to work with rats using intraperitoneally implanted larvae (Experiment 3 and 4). Finally, obtaining the M-strain allowed one preliminary experiment (Experiment 5) and a final experiment (Experiment 6) to be conducted in rats. In summary the six experiments described in this Part were as follows:

Experiment 1 and 2

Mice were vaccinated with ES and challenged with B-strain eggs.

Experiment 3

Rats were vaccinated with ES and challenged by implanting i.p. B-strain larvae.

Experiment 4

Rats were divided in two groups, one was infected with B-strain eggs, the other not. In each group, subgroups were challenged by implantation of either previously trypsinised or untreated larvae.

Experiment 5

Rats were vaccinated with ES, followed by oral challenge with M-strain eggs.

Experiment 6

Rats were vaccinated with ES collected at different times of *in vitro* culture, and somatic antigen, followed by oral challenge

with M-strain eggs.

26.1 Strains of *T. taeniaeformis* and experimental animals

The non-living antigens used as vaccines were obtained from *T. taeniaeformis* strain B, as were the larvae implanted in Experiments 3 and 4. Eggs of this strain were used as challenge material for Experiments 1 and 2 and in Experiment 4 to infect the rats initially, although in this latter case the eggs originated from an attempt to adapt the B-strain to rats and were from the third rat/cat passage. Rats in Experiments 5 and 6 were challenged with *T. taeniaeformis* M-strain eggs.

CF 1 mice were used in Experiments 1 and 2, males in the former and females in the latter. Male Porton-Wistar rats were used in Experiments 3, 4 and 5. Male Sprague-Dawley rats in Experiment 6. At the start of each experiment, indicated as day 0, all the animals were 3 weeks of age, except in Experiment 3, which started with 10 week old rats.

26.2 Antigens

The *in vitro* procedures were described in Part III. In Experiments 1, 2, 3 and 5, media from the first 24 h were discarded, after that, the media obtained at each change were pooled, filtered through a 0.22 μm pore size membrane filter (Millipore) and stored at -20°C. In Experiments 1 and 3 the fluid was then concentrated by ultrafiltration while in Experiments 2 and 4 this was done by reconstituting it in a smaller volume after freeze-drying, using methods described in Part IV. Further details concerning the *in vitro* work are given in Table V.1.

Table V.1 Specifications for the *in vitro* cultures used to obtain larval ES antigen, in the four preliminary experiments.

Exp.	Age of larvae at day of culture	Medium	Proportion larvae/ml of medium	Time interval in hours for changing the media	Period of culture in days	Concentration factor
1	6 weeks	NCTC + 10% v/v FCS	1/5	48	14	10 x
2	20 weeks	Modified RPMI	1/1	24	14	20 x
3	20 weeks	Modified RPMI + 10% v/v FCS	1/1	24	10	10 x
5	18 weeks	Modified RPMI	1/1	24	10	20 x

In Experiment 6, 16 week old larvae were equally divided in 13 petri dishes, each containing 25 larvae in 25 ml of "modified RPMI". The media were changed every 24 h, but only media from day 0 to 1, 7 to 8, 14 to 15 and 21 to 22 of the culture were retained. On these days the media from the 13 petri dishes were pooled and filtered through a 0.22 μm pore size membrane filter (Millipore). The 325 ml from each of the four pools so obtained was stored at -20°C. In addition, a group of larvae, of the same origin was maintained in 16 petri dishes, each containing five larvae and 20 ml of "modified RPMI". From this group the medium from the first 24 h was discarded, while that from the next 48 h (day 1 to 3 of in vitro culture) was collected and similarly treated as the previous pools. From all five pools, 300 ml was lyophilised, reconstituted in 30 ml and kept at -20°C. The soluble protein content of the five samples was estimated by the Coomassie Brilliant Blue micromethod ("Bio-rad Protein Assay" Bio Rad Laboratories) and the proportion of ES and somatic antigen estimated by ITAS-ELISA as described in Part IV. A soluble saline extract of somatic antigen from fresh, whole, adult T. taeniaeformis was prepared, as described in the previous Parts. The protein content of this extract and the proportion of ES to somatic antigen in it was estimated at the same time as those of the ES samples. The protein concentration of this somatic antigen extract was then adjusted by adding "modified RPMI" to equal that of the pool of concentrated ES antigen with the highest protein content and 30 ml of this adjusted somatic antigen was held with the ES antigens at -20°C. As for the other experiments, a sham vaccine was prepared by freeze-drying 300 ml unused "modified RPMI" and reconstituting it to a volume of 30 ml.

After the first rats in this last experiment, were injected for the first time, which happened to be those from the control group, some animals showed shock symptoms. It was thought that this was due to the hypertonicity of the injected solution. Since it appeared that every medium (including the unused medium from the control group) contained undissolved particles after concentration, they were all centrifuged at 1000 g for 10 minutes, and only the supernatant was injected, after which the rats did not show these symptoms anymore. Nothing abnormal was observed after the injection of the rats in Group 6.6.

26.3 Abdominal implantation of larvae

Metacestodes were removed from the liver capsules of mice which had been infected with T. taeniaeformis for 20 weeks and washed in medium. Those used for Experiment 3 were left overnight at 37°C in "modified RPMI". The larvae used in Experiment 4 were washed in HBSS (Gibco) and then left at 37°C for 1 h 25 minutes, half of them in HBSS, the other half in HBSS + 0.25% w/v trypsin (E.C.3.4.21.4.) (Type II-Sigma) (Kwa and Liew 1978[b]), at the rate of one larva per two ml of solution. After that, they were washed in "modified RPMI" and left overnight in this medium at 37°C. Rats were anaesthetised with ether and the skin on the right ventro-lateral site was shaved, disinfected and incised. An opening was made in the abdominal muscles, and an incision made in the peritoneum through which the worms were transferred into the abdomen. The operation wound was closed with suture clips (12 x 2.5 mm "Michel" Thackray) after which the incision line was sprayed with iodine ("Disphex spray" ICI). The operation, which took about five minutes per rat was done under a laminar air

flow cabinet and asceptic conditions.

26.4 Oral administration of eggs

This was done as described in Part I, the number of eggs used for oral administration referring to the number of viable eggs as assessed immediately before challenge by the morphological criteria, described in Part II. Thus the total number of eggs which each animal received was between 5 and 10 times the indicated dose. The eggs had been obtained from segments not more than 14 days previously. The segments had either been expelled with the faeces, or obtained by autopsy and originated from infections which were at least 10 weeks old. After recovering the eggs they were washed and kept at 4°C in normal saline and antibiotics as described in Part I.

26.5 Blood sampling

These samples were taken by means of heparinised capillary tubes (No. 442 Sarstedt). For mice, about 0.5 mm of the tip of the tail was clipped off with a pair of scissors, while rats were anaesthetised with ether, a small cut with a scalpel blade was made in the tail and the blood taken up into the capillary tubes. The tubes were centrifuged then and the resulting plasma was stored at -20°C in microvials.

These samples were analysed by the ELISA for detecting antibodies described in the previous Part. Adult somatic and a stock ES antigen were used as antigens, both at 10 $\mu g.ml^{-1}$. Plasma was diluted to 1/300 for mice and 1/200 for rats. Relative absorbances were read using a filterphotometer ("Vitatron DCP" MSE) at 450 nm. For Experiments 2, 3, 4 and 5 the plasma from each day for each

group was pooled. In Experiment 6 the plasma from each rat were kept separately but blood was only taken from five animals in each group. The ELISA results, obtained from the first blood samples, before the animals were infected or immunised were used to provide the normal serum control for each animal, as described in Part IV.

26.6 Autopsy and interpretation of results

When experiments were terminated, the mice were killed by cervical dislocation, and the rats by the use of ether. For Experments 1, 2, 4, 5 and 6, the liver was dissected out and the cysts containing living larvae were counted. An attempt was made to count the numbers of dead larvae or liver lesions, but these numbers were not taken into account for describing the results as these lesions could not always be distinguished from the capsules around living larvae, especially in heavy infections.

The Wilcoxon two sample test (Sokal and Rohlf 1969) was used for statistical analysis of the number of living cysts. In addition, the percentage protection was calculated as described by Lloyd (1979) using the following formula:

$$\% \text{ protection} = 100 - \frac{\text{mean number of living cysts per group of immunised animals}}{\text{mean number of living cysts per group of control animals}} \times 100$$

Chapter 27 Immunisation experiments in mice

27.1 INTRODUCTION

At the time of these experiments only two strains of *T. taeniaeformis* were available, B. and S. As shown in Part I, the response in mice over 4 weeks old following infection with these strains was rather variable. Accordingly the challenge infections were given not more than one week after the mice were vaccinated, immediately after weaning at 3 weeks of age.

27.2 Experimental design

For the first experiment five groups, each of five mice, were used as follows: In Group 1.1 each animal received at day 0, 0.5 ml ES antigen i.p. In Group 1.2 each received 0.25 ml ES antigen i.p. The three other groups were control groups: Group 1.3 each animal was injected i.p. with 0.5 ml unused concentrated medium while 0.5 ml of distilled water was given to those in Group 1.4. The animals in Group 1.5 were not injected. All the animals were challenged orally with 100 eggs at day 7 and autopsied on day 35; no blood samples were taken in this experiment.

In the second experiment there were four groups of ten mice each. Each mouse in Group 2.1 and 2.2 received 0.25 ml of ES antigen, corresponding to 60 μg of soluble protein. Mice from Groups 2.3 and 2.4 received concentrated unused medium. The route of administration was per os for Groups 2.1 and 2.3, subcutaneously for Groups 2.2. and 2.4. The mice were challenged with 100 eggs at day 7, blood samples were taken at days 0, 7, 21, 35 and 54 and the autopsies performed on day 54.

27.3 RESULTS

Experiment 1

The mean numbers of cysts containing living larvae, with standard deviations and percentage protection are given in Table V.2. Numbers of living and dead cysts per animal are given in Appendix Table V.1.

Table V.2 Experiment 1: Mean numbers of living cysts and standard deviations and the apparent level of protection in groups of five mice, either vaccinated with ES antigen from *T. taeniaeformis* larvae or controls.

Group No.	Specification	Living cysts mean	s.d.	Apparent % protection
1.1.	ES 0.5 ml	6.8	6.87	76.2
1.2	ES 0.25 ml	19.2	13.72	32.9
1.3	Unused medium	31.6	27.30	
1.4	Water	24.2	27.91	
1.5	Not injected	30.0	38.45	

The only groups that were significantly different from each other were Groups 1.1 and 1.3 (U = 21*). The percentage protection was calculated using the mean number of living cysts of the control groups together, since these were not significantly different from each other.

Experiment 2

The mean numbers of cysts containing living larvae per group with standard deviations and percentage protection are given in

Table V.3. The detailed results for each animal are given in Appendix Table V.2.

Table V.3 Experiment 2: The mean numbers of cysts containing living larvae, with standard deviations, the number of observations per group and the apparent level of protection in mice vaccinated with ES antigen from <u>T. taeniaeformis</u> larvae and two control groups.

Group No.	Specification	Living cysts Mean	s.d.	Number of observations	Apparent % protection
2.1	ES per os	97.5	58.27	10	0
2.2	ES s.c.	39.2	50.35	10	33.6
2.3	Unused medium per os	65.0	61.03	9	
2.4	Unused medium s.c.	53.0	44.76	10	

Statistical analysis of these results revealed no difference between any of the vaccinated groups with any of the control groups: there was a significant difference between Group 2.1 and 2.2 (U = 76*). The percentage protection was calculated using the means of the total number of living cysts from both control groups (Groups 2.3 and 2.4) as these were not significantly different.

The ELISA results are given in Table V.4.

Table V.4 Experiment 2: Relative absorbance at 450 nm obtained with pooled plasma samples from groups of vaccinated and control mice by the ELISA for detecting antibodies using somatic and ES antigen from T. taeniaeformis (challenge on day 7)

Antigen	Group	Plasma from day				
		0	7	21	35	54
SOM.	2.1 ES p.o.	0	0	0	0.26	1.82
	2.2 ES s.c.	0	0	0.03	0.37	1.82
	2.3 sham	0	0	0	0.41	2.08
	2.4 sham	0	0	0	0.28	2.04
ES	2.1 ES p.o.	0	0	0	0.62	2.27
	2.2 ES s.c.	0	0	0.41	1.46	2.53
	2.3 sham	0	0.01	0.06	0.74	2.63
	2.4 sham	0	0	0	0.52	2.29

27.4 CONCLUSIONS

When the results on the protective capacity of ES antigens in mice for the two experiments are considered together, the intraperitoneal route seems to be more successful, although the differences in the methods for obtaining and preparing the antigens for the two experiments rather confound such a comparison. The effect of different amount of vaccine is shown by the difference between Groups 1.1 and 1.2 in Experiment 1 (Table V.2). Furthermore, the period between vaccination and challenge may have been too short for maximum effect, as Kwa and Liew (1977), in similar experiments in rats, only found significant protection when the rats were challenged ten or more days after vaccination.

Chapter 28 Preliminary experiments in rats with implanted *T. taeniaeformis* larvae and larval *T. taeniaeformis* metabolic antigen.

28.1 INTRODUCTION

It was thought that the very variable response of rats to infection with *T. taeniaeformis* eggs of strains B, I and S (Part I) would make experiments with any of these strains in rats rather unreliable. Therefore, abdominal implantations were used, based on the reports by Musoke and Williams (1976) for Experiment 3, and on that by Kwa and Liew (1978[b]) for Experiment 4. For Experiment 5, the M-strain was available but not enough was known about age-related susceptibility of rats to this strain and again only a short period elapsed between immunisation and challenge.

28.2 Experimental design

Experiment 3

Three groups of 10 rats each were used. Each animal in Group 3.1 received at day 0, 0.5 ml concentrated ES antigen, emulsified in 0.5 ml Complete Freunds adjuvant, injected i.m. in four sites and at day 42, 0.5 ml ES antigen only, i.m. Similar schedules were used for the rats in control Groups 3.2 and 3.3 except that unused concentrated medium in FCA was used in Group 3.2 and no adjuvant in Group 3.3.

Experiment 4

Forty 3 week old rats were equally divided into four groups. At day 0 each rat in Group 4.1 and 4.2 was orally infected with 200 eggs, the other groups being left untreated. At day 36, two *T. taeniaeformis* larvae were implanted in the abdomen of each rat. Trypsinised

larvae were used in Groups 4.1 and 4.3 and untreated larvae in those from Groups 4.2. and 4.4. At autopsy numbers of living and dead implanted larvae and the numbers of living and dead cysts in the liver were counted.

Experiment 5

One group (Group 5.1) contained 8 rats and each animal was vaccinated subcutaneously with 1 ml concentrated ES antigen. In the control group (Group 5.2) seven rats were each injected s.c. with 1 ml of concentrated unused medium. Further experimental details of the three experiments are given in Table V.5.

Table V.5 Specifications for three preliminary vaccination experiments against T. taeniaeformis in rats

Exp.	Age of animals at day 0 (days)	Challenge Day	Challenge Age of rat (days)	Dose per rat	Autopsy day	Blood sampling days
3	77	49	126	2 larvae i.p.	77	-4, 7, 21, 49, 63, 77
4	21	36	57	2 larvae i.p.	64	0, 34, 48, 64.
5	21	5	26	200 eggs per os	61	0, 5, 19, 33, 47, 61

28.3 RESULTS

Experiment 3

Most of the larvae were alive in the peritoneal cavity, often in the scrotum and macroscopically unaltered. No signs of peritonitis or inflammation at the operation wounds were observed and all the rats survived to the end of the experiment. In Group 3.1

where ES antigen + FCA was administered only one rat contained one dead larva, the other still being alive. In Group 3.2 (unused medium + FCA) both larvae were dead in one rat, while two other rats had each one dead and one living larva. In Group 3.3. (unused medium only) one rat had two dead larvae and in another one, one larva was dead. The dead larvae were partly resorbed and encapsulated. Table V.6 shows the results of the ELISA for detecting antibodies.

Table V.6 Experiment 3. Relative absorbance at 450 nm obtained by the ELISA for detecting antibodies with pooled plasma samples from groups of vaccinated and control rats challenged on day 49 by i.p. implantation of living <u>T. taeniaeformis</u> larvae.

Antigen	Group	Pooled plasma samples from day -4	7	21	49	63	77
Som.	3.1 ES + FCA	0	0.04	0.02	0.54	1.10	1.67
	3.2 sham (+ FCA)	0	0.03	0.03	0.06	0.43	1.31
	3.3 sham	0	0.02	0.02	0.04	0.66	1.54
ES	3.1 ES + FCA	0	0.12	0.12	1.71	1.35	1.20
	3.2 sham (+ FCA)	0	0.02	0.01	0.05	0.20	0.63
	3.3 sham	0	0	0	0.02	0.17	0.42

<u>Experiment 4</u>

The number of living and dead larvae in the abdominal cavity and cysts in the liver are given in Table V.7. Three rats died before the implantations were done, one from Group 4.2 and two from Group 4.3. At the end of the experiment, liver cysts with living and

dead larvae were clearly distinguishable, the latter being caseous and not larger than 1 mm. There may have been a higher number of larvae present at an earlier stage, if progressive resorption of the dead cysts had prevented all of them being detectable by the time of autopsy.

In some cases parts of the implanted larvae appeared to be dead. As with degeneration in _in vitro_ cultures (described in Part III), the middle part appeared to be the last to be affected.

Table V.7 Experiment 4. Numbers of _T. taeniaeformis_ larvae recovered from the peritoneal cavity and mean numbers ± s.d. of liver cysts in rats. In Groups 4.1 and 4.3 trypsinised larvae were implanted and untreated larvae in Groups 4.2 and 4.4. Prior to implantation rats from Groups 4.1 and 4.2 were orally infected with _T. taeniaeformis_ eggs (strain B from the third rat/cat passage).

Group	No. of rats	Larvae in peritoneal cavity			Liver Cysts			
		Living	Middle part only alive	Dead	Living mean	Living s.d.	Dead mean	Dead s.d.
4.1	4	2			1.2	2.8	28.0	25.0
	1		2		0		20	
	1		1	1	0		2	
	4			2	11.0	14.2	25.0	20.8
4.2	1	2			0		25	
	1	1	1		1		6	
	1		2		47		17	
	6			2	27.2	29.3	25.8	15.2
4.3	1	2						
	1	1	1					
	6			2				
4.4	1	1		1				
	9			2				

In only one instance were a dead, partly resorbed larva and an apparently living and intact larva found in the same rat (Group 4.4), the latter being found in the scrotum and the former in the abdominal cavity. The relative absorbance values at 450 nm, for antibody detected in the ELISA are given in Table V.8.

Table V.8 Experiment 4. Relative absorbance at 450 nm, by ELISA for detecting antibodies, with pooled plasma samples from rats orally infected with T. taeniaeformis eggs (Groups 4.1 and 4.2) or uninfected with abdominal implantation on day 36 of trypsinised (Groups 4.1 and 4.3) or untreated (Groups 4.2 and 4.4) T. taeniaeformis larvae.

Antigen	Group	Plasma samples from day			
		0	34	48	64
SOM.	4.1	0	0.05	1.27	1.38
	4.2	0	0.22	2.08	2.14
	4.3	0	0	0.20	1.78
	4.4	0	0	0.18	1.75
ES	4.1	0	0.10	1.06	1.05
	4.2	0	0.42	1.53	1.45
	4.3	0	0	0.17	0.62
	4.4	0	0	0.16	0.51

Experiment 5

The numbers of living cysts encountered in the liver of each vaccinated (Group 5.1) and control rat (Group 5.2) are given in Table V.9.

Table V.9 Experiment 5. Numbers of living cysts of T. taeniaeformis in rat livers in a group of rats vaccinated with homologous ES antigen and a control group, both challenged with T. taeniaeformis eggs (Strain M) after 5 days.

Group	5.1 (ES vaccinated)	5.2 (sham vaccinated)
Rat No. 1	104	179
2	94	171
3	64	140
4	51	139
5	37	137
6	25	80
7	17	71
8	11	

Statistical analysis between the two groups revealed that they were significantly different (U = 52 **). The percentage protection for the vaccinated group was 61.6%.

The antibody levels in the pooled plasma as detected by ELISA are given in Table V.10.

Table V.10 Experiment 5. The relative absorbance at 450 nm by ELISA for detecting antibodies on pooled plasma samples from rats vaccinated with ES antigen and control rats, challenged with T. taeniaeformis on day 5.

Antigen	Group	Plasma sample from day					
		0	5	19	33	47	61
SOM.	5.1 ES vacc.	0	0.03	0.08	0.13	0.62	0.97
	5.2 sham	0	0	0	0.06	0.79	1.23
ES	5.1 ES vacc.	0	0.11	0.22	0.43	0.92	1.29
	5.2 sham	0	0.04	0.11	0.24	0.79	1.37

28.4 CONCLUSIONS

Although the results obtained in Experiment 3, Group 3.3 are in accordance with Musoke and Williams (1976), in that implanted strobilocerci were able to survive, neither immunisation with homologous ES antigen (Group 3.1) nor FCA (Group 3.2) had any effect on the survival of the implanted larvae, the latter result conflicting with these authors. However, as revealed in Experiment 4 by the poor survival of the implanted strobilocerci in the control group, this method of challenging, the principal feature of these experiments, gives variable results. It is not clear what caused this variability, although some factors, which may influence the survival of strobilocerci under these conditions, such as the age of the implanted metacestodes, have already been observed to bear an influence by Musoke and Williams (1976) and Kwa and Liew (1978[b]).

The times between implanting the larvae and autopsy were virtually the same for all groups within and between the experiments but the age of the rats at the time of implantation differed between the two experiments, being 119 days in Experiment 3 and 76 days in Experiment 4. It seems unlikely, though not to be excluded, that other differences between the control groups of both experiments, such as the short time the larvae were incubated in HBSS, may have influenced the results.

A major difference between these experiments and those of Musoke and Williams (1976) and Kwa and Liew (1978[b]) which could have interfered is that the strain of implanted strobilocerci was a mouse strain, although this can not explain the differences between these experiments. As regards Experiment 5, the same comment concerning time interval

between vaccination and challenge can be made for the experiments described in Chapter 27 and this was taken into account in Experiment 6.

In the ELISA values recorded for these experiments and Experiment 2 (Table V.4) there seemed to be a stronger response against ES antigen in the early stages of infection, which is not surprising for animals immunised with this antigen, but consistently occurred in the control groups as well, whereas towards the end of the experiments there was little difference between both antigens. Implanted strobilocerci on the other hand, seemed to have stimulated production of antibodies which reacted more strongly with somatic than with ES antigen, as seen from the control groups in Experiment 3 (3.2 and 3.3, Table V.6) and the groups in Experiment 4 where the rats were not orally infected (Groups 4.3 and 4.4, Table V.8).

Chapter 29 Immunisation of rats with metabolic antigens from *T. taeniaeformis larvae*.

29.1 INTRODUCTION

The aim of this experiment was to show whether the level of protective immunogen in ES antigen was influenced by the time the larvae were maintained *in vitro*, as was the case for the protein concentration and the proportion of ES and somatic antigen, as demonstrated by the Coomassie Brilliant Blue micro method (Bio Rad) and ITAS-ELISA respectively. Furthermore the knowledge which had been gained on the infectivity of the strains of the parasites, for the different hosts and on the age resistance in the latter allowed a longer period to be left between vaccination and challenge.

29.2 Experimental design

Vaccination of animals;

Seventy rats were equally divided into seven groups and every rat was injected three times intraperitoneally at 48 h intervals (days 0, 2 and 4 of the experiment). Groups 6.1 to 6.4 received three times 1 ml concentrated ES antigen from the cultures containing one larva pro ml and changed every 24 h. For Group 6.1 the medium used was collected on day 1 of culture, for Group 6.2 that from day 8, for Group 6.3 the medium from day 15 and for Group 6.4 that collected on day 22. The rats of Group 6.5 were injected three times with 1 ml ES antigen, from a concentrated pool of the medium which had contained one larva per five ml, the medium being changed after 48 h. The collection made on day 3 of culture was used. Rats from Group 6.6 were injected i.p. three times with 1 ml of somatic antigen on the same days and similarly Group 6.7, the control group, received

one ml of concentrated unused medium on these days.

Challenge of rats and blood sampling:

On day 21 of the experiment all the rats were orally challenged with 250 eggs (M strain) of T. taeniaeformis. Blood samples were taken on three occasions from rats 1 to 5 in each group: before vaccination on day 0, on day 18 and on day 70, the day of autopsy.

29.3 RESULTS

Table V.11 shows the protein content of the five ES antigen samples and the total amount of protein which each rat in the corresponding group received, after three injections of 1 ml each with the concentrated ES antigens and somatic antigen.

Table V.11 Experiment 6. Protein concentration as assayed by the Coomassie Brilliant Blue micro method (Bio Rad Protein Assay") for the 5 samples of ES antigen from larval T. taeniaeformis and somatic antigen, with the corresponding amounts each rat received after 3 x 1 ml i.p. injections and group identification. Rats from Group 6.6 received the same amount of soluble protein as the rats from the group which were vaccinated with the ES antigen that had the highest protein concentration (Group 6.4).

Group	Antigen	Media collected on day	Protein content in $\mu g.ml^{-1}$	Total dose in μg administered per rat
6.1	ES - 1 larva per ml	1	50	1500
6.2	As 6.1	8	20	600
6.3	As 6.1	15	100	3000
6.4	As 6.1	22	140	4200
6.5	ES 1 larva per 5 ml	1-3	17	510
6.6	Somatic antigen	N.A.	140	4200
6.7	Unused medium	N.A.	0	0

N.A. = Not Applicable

The relative proportions of ES and somatic antigen for each sample and three dilutions of somatic antigen was estimated using the ITAS-ELISA technique. Table V.12 shows the results

Table V.12 Experiment 6. The relative absorbance at 450 nm, obtained by ITAS-ELISA for ES and somatic antigens in media in which T. taeniaeformis larvae were maintained and for three dilutions of somatic antigen.

Sample from Group	Values obtained by ITAS-ELISA for detecting: ES antigen	Somatic antigen
6.1	1.01	0.54
6.2	0.68	0.41
6.3	0.86	1.50
6.4	1.11	1.47
6.5	0.80	0.40
Somatic antigen		
140 $\mu g.ml^{-1}$	0.28	1.78
75 $\mu g.ml^{-1}$	0.25	1.51
17 $\mu g.ml^{-1}$	0.21	1.34

The numbers of living cysts recovered from each rat for each group are given in Appendix Table V.3. The mean numbers of living cysts, recovered per group, with standard deviation and the level of protection are given in Table V.13.

Statistical analysis revealed that the only groups which differed significantly from the control group were 6.1 (U = 82 **) and 6.3 (U = 82 **). No significant differences were detected between Groups 6.1 and 6.3. After testing for normality (Snedecor 1956) and applying a "t" test on the square roots of the numbers of living cysts Groups 6.1 and 6.3 again differed significantly from 6.7 ("t" values

respectively 2.81 * and 2.38 *).

Table V.13 The mean number of living cysts plus standard deviation per group of 10 rats, vaccinated with T. taeniaeformis ES antigen after different periods of culture (Groups 6.1 to 6.5), with somatic antigen (Group 6.6) or sham vaccinated (Group 6.7).

Group	Mean number of living cysts	s.d.	% protection
6.1	140.0	90.04	50.0
6.2	219.5	84.23	21.7
6.3	168.0	70.11	40.0
6.4	207.8	147.73	25.8
6.5	245.0	177.21	12.6
6.6	252.4	178.07	9.9
6.7	280.2	142.20	

The raw data obtained with ELISA for detecting antibodies against ES and somatic antigen are given in Appendix Table V.4. Table V.14 shows the means and standard deviations of these results per group, after correction for the corresponding values on day 0.

Table V.14 Experiment 6. Mean values with standard deviations indicating the antibody levels detected by ELISA using ES and somatic <u>T. taeniaeformis</u> antigen against plasma from rats vaccinated on day 0 with ES or somatic <u>T. taeniaeformis</u> antigen and one control group, each challenged on day 21 with homologous eggs. Relative absorbance at 450 nm. The means are derived from 5 observations except for those on day 18, groups 6.3 and 6.5 with somatic antigen which include 4 observations.

Group	Day 18				Day 70			
	Som.antigen		ES antigen		Som.antigen		ES antigen	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
6.1	0.046	0.065	0.058	0.037	0.800	0.244	1.054	0.084
6.2	0.044	0.056	0.066	0.040	1.048	0.256	1.150	0.120
6.3	0.105	0.093	0.296	0.136	0.884	0.061	1.066	0.071
6.4	0.104	0.107	0.190	0.078	1.084	0.393	1.170	0.066
6.5	0.100	0.020	0.088	0.040	1.130	0.168	1.152	0.094
6.6	0.576	0.405	0.028	0.042	1.234	0.271	1.074	0.154
6.7	0.008	0.011	0.000	0.000	0.980	0.157	1.086	0.106

29.4 CONCLUSIONS

It did not appear from these results that there was any direct relationship between protein content or ES/somatic antigen ratio and the level of protective immunogens in the <u>in vitro</u> culture products or for the latter parameter, in somatic antigen. Furthermore, since only antigen collected from the first 24 h and from the 14th day <u>in vitro</u> appeared to be protective, the optimum time of immunogen production or release by the larvae <u>in vitro</u> remains inconclusive.

It may be that these inconclusive results are due to the rats being given too high a challenge dose. Each rat received 250 viable eggs, viability being estimated from their morphological appearance, as 17% of the eggs. Thus each rat received a total of about 1500 eggs. When samples of these eggs were submitted to the basic hatching and activating technique described in Part II Set 10 (see page 69), all the oncospheres were released from the embryophores and 32% of these could be hatched and activated. This is closer to the percentage of viable eggs indicated from the highest number of living cysts encountered in one animal as compared with the total number of eggs administered (41%) and suggests that this procedure may afford a better assessment of viability than the morphological criteria. The poor protection obtained in Group 6.6 vaccinated with somatic antigen is surprising, although somatic extracts from adult worms were used, whereas Kwa and Liew (1977) and Ayuya and Williams (1979) prepared theirs from larval material. Miller (1931[a]) had concluded that the immunogenic properties of larval and adult somatic T. taeniaeformis antigens were the same and no evidence to the contrary was found in the literature.

Although there was a detectable antibody response in ELISA against both somatic and ES antigen, this response was initially stronger against somatic antigen in the rats vaccinated with this antigen (Group 6.6) than against ES and/or somatic antigen in the rats vaccinated with ES. Later on, by day 70, possibly under the influence of the challenge, no marked difference in response against either of the two antigens was noted. This difference in diagnostic and "functional" antibodies has been commented on by various workers as described in Chapter 25.

Chapter 30 DISCUSSION AND CONCLUSIONS

In some experiments protective activity seemed to be present in ES antigen in that significantly fewer cysts with living larvae were found in the livers of the vaccinated mice and rats after challenge, than in control animals, even when the challenge followed vaccination in less than 10 days, the lower limit estimated by Kwa and Liew (1977). However, the overall results were not as good as those reported in similar experiments by Kwa and Liew (1977) and Ayuya and Williams (1979), even when only the results obtained in Group 6.1 (vaccinated with ES antigen from the first 24 h in vitro) are considered. Kwa and Liew (1977) used Freund's Complete Adjuvant but according to Ayuya and Williams (1979) this does not improve the results in this system. The possible explanation that an overdose of eggs was used for challenging, has already been discussed and emphasises the need for better criteria for determining the viability of eggs. Furthermore, the comments made in Part IV Chapter 24 regarding the molecular weights, hapten content and particulate nature of some of these ES antigens may here have to be taken into account as well. In order to avoid the loss of possible immunogenic substances by ultrafiltration or other concentration methods, it was decided to concentrate by freeze-drying. This method presented difficulties in the last experiment, in that the vaccine was painful for the rats. Centrifuging the concentrates greatly reduced this problem, but if particulate immunogens are present in ES antigen, as reported for Schistosoma mansoni (Kusel, Mackenzie and McLaren 1975) and important in relation to protection, these may have been removed by centrifuging. However Rickard and Bell (1971[a]) successfully

vaccinated lambs with oncospheral ES antigen which was centrifuged for 1 h at 16000 *g*. Nevertheless freeze-drying, although useful for preservation of the vaccines, does not seem to be ideal for concentration, especially since the potential loss of material in the eluate in other concentration methods, does not appear to have been important in a number of successful experiments, such as those reported by Ayuya and Williams (1979) and Rickard and Adolph (1976, 1977). Another possibility is that the strain difference in *T. taeniaeformis* may have influenced the results described in Chapters 28 and 29. All vaccine material originated from a mouse strain (strain B) which may not have been able to induce an adequate protection against the rat adapted strains of this parasite used for the challenge. However the reports of cross protectivity between species, such as the ability of *T. saginata* oncospheral ES antigen to induce protection against *T. taeniaeformis* in mice (Lloyd 1979) do not support such hypothesis of interference from the strain difference. It is also possible that any particular strain of *T. taeniaeformis* may respond differently in vaccination experiments carried out under ideal conditions for the parasite as regards age and strain of the host. Thus the resistance of the host to *T. taeniaeformis* eggs may be more effectively enhanced by the immunisation procedure when the susceptibility is already low due to age or strain of the host. Although it was not the original aim of these experiments, it appeared that ELISA for detecting antibodies using the ES antigen allowed relatively early detection of *T. taeniaeformis* infection in rats. A similar report for an ES antigen was made by Gundlach (1971) for *F. hepatica*.

Indirect haemagglutination, reported by Cornish, Le Flore and Smith (1976) to be the most sensitive test for detecting T. taeniaeformis in rats, was also used in this same system by Kwa and Liew (1978[a]). The latter found no detectable levels of antibodies in serum taken during the first four weeks of infection. ES antigen used in ELISA seemed to allow for detection by at least 19 days after infection (cfr. Experiment 5). In the previous Part, ES antigen seemed to be more specific than somatic antigen at least when these were compared against sera from mice infected with either T. taeniaeformis or T. crassiceps, and provided that the ES was not too contaminated with somatic antigen. This, combined with the progress made in recent years in the chemotherapy of cysticercosis, including the development of e.g. Praziquantel (Thomas and Andrews, 1977) may mean that ES antigen is more likely to be of use as a diagnostic antigen than as a vaccine. Such a test would not only be of use in practical diagnostic purposes but also for experimental work on early and late immunity aiding the detection of the early establishment of this parasite in the host.

There are conflicting reports as to the value of ES antigen as a diagnostic antigen (see Part IV, Chapter 18), but equally, ES antigen seems to give variable results as a vaccine even when attempts were made to replicate the same experimental conditions.

Such conflicting reports about ES antigen as a vaccine include those from Lang and Hall (1977) and Rajasekariah, Mitchell, Chapman and Montague (1979) who could induce protection against F. hepatica with ES antigen from immature worms in experimental hosts, whereas

this system was not successful for Lehner and Sewell (1978/1979). More recently, Wikerhauser, Brglez, Džakula, Asaj and Matić-Piantanida (1978) were unable to vaccinate calves against T. saginata using homologous oncospheral ES antigens, although their experimental design was based on that of Rickard and Adolph (1976). The challenge dose given by the latter was 4000 eggs, whereas the former workers were using 150 000 eggs and there may have been other less obvious differences responsible for this.

It is also possible that the techniques for hatching and activating of oncospheres and for in vitro culture of oncospheres and other larval stages have not been described in sufficient detail to allow for them being repeated, so that the ES material is not always the same quality. Such factors may well explain the different results obtained with ES antigens used as either diagnostic or immunising agents.

CHAPTER 31 GENERAL CONCLUSIONS

1. Strain differences in Taenia taeniaeformis were shown by comparing the infectivity of four strains from different origins (two European, one Asian and one from the Middle East) for rats and mice. These four strains showed also different zymogram patterns for the isoenzymes of hexokinase, whereas those for glucose phosphate isomerase and adenylate kinase were identical.

2. The previously reported methods for hatching and activating oncospheres of T. taeniaeformis proved to be unsuccessful with the strains used. Better results were obtained by the successive action of sodium hypochlorite on the embryophores, followed by treatment with trypsin and bile in a culture medium. The serum content of the medium appeared to have a protective role on the fully hatched oncosphere.

3. When mature larvae of Taenia saginata were grown in vitro, a diphasic medium with a solid phase of disrupted coagulated serum, allowed better development than other systems as estimated by morphological criteria.

4. During maintenance of mature T. taeniaeformis larvae in vitro, neither weight, glucose uptake, ammonia nor lactic acid production of the worms proved to be an adequate means of monitoring the culture system.

5. Metabolic products obtained from these in vitro cultures appeared to differ from the somatic antigens of T. taeniaeformis as indicated by immunoelectrophoretic patterns and more clearly

the ELISA technique used to detect antibody or antigen. The use of a metabolic antigen from *T. taeniaeformis* gave a greater specificity in ELISA, than somatic antigen or cyst fluid in tests against sera from mice infected with *T. taeniaeformis* or *Taenia crassiceps*.

6. The level of somatic antigen in media used for *in vitro* maintenance appeared to be inversely related to the well being of the worms.

7. When culture products derived from mature *T. taeniaeformis* larvae were tested as immunogens in mice or rats, the level of protection obtained was usually lower than reported in the literature. Some batches of these antigens showed significant protection but no correlation with this was found with protein levels or with the somatic/metabolic antigen levels.

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Appendix Table I.1. Numbers of living (L) and dead (D) cysts in livers of CF1 mice and Sprague Dawley rats infected with Taenia taeniaeformis strain B

Age at infection in weeks	MICE 3 to 4		4 to 5		5 to 6		6 to 7		Over 12		RATS 3 to 4		4 to 5		5 to 6		6 to 7	
Animal No.	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
1	112	-	57	2	35	5	28	3	89	1	70	20	18	10	-	12	-	49
2	108	-	56	5	28	12	14	8	24	32	59	12	7	21	-	10	-	36
3	65	-	53	5	24	8	13	7	-	32	54	10	4	19	-	5	-	16
4	46	10	42	-	23	10	13	5	-	20	52	10	3	1	-	5	-	15
5	19	2	36	10	23	3	11	1	-	20	30	13	2	-	-	2	-	9
6	5	-	35	2	18	3	9	12	-	11	24	21	-	5	-	2	-	9
7	-	-	29	6	3	8	8	11	-	10	24	15	23	1	-	1	-	9
8	120	-	26	16	1	18	5	6	-	8	19	4	12	3	-	1	-	5
9	115	-	22	1	-	34	2	33	-	7	16	16	12	2	-	1	-	4
10	108	-	14	15	-	32	1	31	-	7	12	11	9	1	-	-	-	2
11	93	-	42	2	-	32	-	21	-	5	10	37	4	3				
12	80	10	39	2	-	29	-	15	-	3	3	6	1	1				
13	75	-	37	12	-	26	-	13	-	2	2	30	1	-				
14	39	20	32	-	-	17	-	6	-	-	2	9	-	10				
15	32	4	-	24	-	6	-	1	-	-	-	51	-	16				
16											-	37						
17											-	26						
18											-	10						
19											-	9						
20											-	6						

Appendix Table I.2. Numbers of living (L) and dead (D) cysts in livers of CF1 mice and Sprague Dawley rats infected with <u>Taenia taeniaeformis</u> strain S

Age at infection	MICE 3 to 4 weeks		MICE 4 to 5 weeks		RATS 3 to 4 weeks		RATS 4 to 5 weeks	
Animal No.	L	D	L	D	L	D	L	D
1	64	2	120	-	2	1	2	-
2	53	-	116	-	2	-	2	2
3	42	-	100	-	1	2	1	-
4	36	4	-	2	-	2	-	5
5	30	-	-	-	-	1	-	-
6	28	3	-	-	-	-	-	-
7	16	4	-	-	-	-	-	-
8	10	1	-	-	-	-	-	-
9	8	-	-	-	-	-	-	-
10	-	10	-	-	-	-	-	-

Appendix Table I.3. Numbers of living (L) and dead (D) cysts in livers of CF1 mice and Sprague Dawley rats, infected with Taenia taeniaeformis strain I

	MICE				RATS					
Age at infection in weeks	3 to 4		4 to 5		3 to 4		4 to 5		5 to 6	
Animal No.	L	D	L	D	L	D	L	D	L	D
1	140	-	76	20	90	6	56	60	33	45
2	130	-	60	27	81	3	14	73	13	30
3	120	2	56	-	80	-	1	17	10	40
4	120	-	53	8	78	-	-	27	1	25
5	110	1	48	2	70	5	-	27	-	16
6	110	-	42	5	68	5	-	18	-	16
7	100	-	29	1	55	3	-	12	-	11
8	100	-	16	-	53	11	-	7	-	9
9	98	2	6	-	43	7	-	7	-	8
10	-	38	-	24	29	25	-	4	-	6
11					25	9				
12					23	38				
13					23	34				
14					18	-				
15					14	2				
16					12	4				
17					8	10				
18					3	50				
19					-	12				
20					-	-				

Appendix Table I.4. Numbers of living (L) and dead (D) cysts in livers of CF1 mice and Sprague Dawley rats, infected with *Taenia taeniaeformis* strain M

	MICE				RATS							
Age at infection in weeks	3 to 4		4 to 5		3 to 4		4 to 5		5 to 6		6 to 7	
Animal No.	L	D	L	D	L	D	L	D	L	D	L	D
1	2	8	-	12	180	1	204	-	153	-	94	-
2	1	5	-	10	141	-	165	-	138	-	84	-
3	1	4	-	6	140	-	160	-	126	-	84	-
4	-	6	-	3	66	-	125	-	122	-	77	-
5	-	4	-	3	61	1	125	-	95	-	74	1
6	-	4	-	3	60	-	105	-	93	-	69	-
7	-	3	-	2	53	-	85	-	90	-	66	-
8	-	2	-	1	46	-	65	-	82	-	65	-
9	-	1	-	-	44	-	8	-	64	-	65	-
10	-	-	-	-	3	-	1	-	47	1	48	-

Appendix Table I.5. Numbers of living (L) and dead (D) cysts in livers of Porton-Wistar rats infected with <u>Taenia taeniaeformis</u> of strains B and M

	Strain B		Strain M					
Age at infection	4 to 5 weeks		3 to 4 weeks		7 to 8 weeks		12 to 13 weeks	
Cysts	L	D	L	D	L	D	L	D
	6	3	180	-	130	-	130	-
	5	2	170	-	40	15	109	-
	2	1	157	1	30	4	95	2
	1	-	147	1	15	3	90	5
	1	1	146	-	13	30	60	5
	1	2	140	-	10	6	45	20
	1	-	121	-	8	13	21	24
	1	-	121	-	-	40	16	20
	1	-	100	-	-	21	7	11
	-	-	71	-	-	20	-	20

Appendix Table III.1 First Experiment

Mean individual liveweights (in mg) of *Taenia taeniaeformis* metacestodes with standard deviations plus number of observations per group taken every 48h

	Group 1			Group 2			Group 3		
Day	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n
0	98.96	10.02	4	99.03	9.57	4	98.79	11.32	4
2	112.58	16.43	4	97.95	15.26	4	102.37	10.94	4
4	128.08	13.90	4	102.37	10.30	4	118.98	7.88	4
6	125.16	13.98	4	110.79	4.58	4	129.67	13.86	4
8	115.24	12.42	4	111.70	17.23	4	140.72	18.75	4
10	116.10	17.12	4	124.19	20.46	4	147.87	12.22	4
12	112.87	21.79	4	129.66	7.38	4	154.69	5.98	4
14	96.74	11.69	4	119.87	12.52	4	149.79	8.15	4
16	106.45	11.53	4	126.27	11.45	4	156.10	5.05	4
18	105.14	8.53	4	135.88	19.96	4	155.59	5.12	4
20	106.28	8.55	4	140.43	20.8	4	146.44	5.03	4
22	100.95	11.58	4	128.61	6.86	4	143.68	16.42	4
24	81.15	10.29	4	112.54	19.06	4	126.85	15.86	4
26	79.28	9.79	4	106.14	14.29	4	132.66	17.71	4
28	83.96	12.42	4	116.59	15.15	4	130.70	11.23	4
30	84.67	17.74	4	124.17	10.09	4	132.80	4.68	4
32	71.76	7.78	4	103.95	8.69	4	119.51	16.21	4
34	72.09	8.09	4	104.96	6.08	4	115.86	15.44	4
36	83.48	9.50	4	103.18	15.61	4	119.06	19.83	4
38	79.30	7.67	4	95.56	11.89	4	109.46	20.37	4
40				101.66	16.68	4	119.32	20.01	4
42				99.66	1.31	4	112.14	19.63	4
44				89.03	1.75	4	109.58	27.60	4
46				91.82	6.70	4	104.21	22.19	4
48				88.83	8.07	4	117.45	23.45	4
50				90.50	9.72	4	107.20	33.98	4

Appendix Table III.2 First Experiment

Mean Ammonia production in µg per *Taenia taeniaeformis* larva with standard deviations plus number of observations per group taken every 48h

	Group 1			Group 2			Group 3		
Day	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n
2	19.1	5.2	4	13.1	3.5	4	9.9	4.4	4
4	34.8	3.4	4	22.6	1.5	4	9.6	1.5	4
6	34.2	4.4	4	26.2	6.0	4	11.9	2.9	4
8	31.4	3.4	4	25.9	3.0	4	15.0	5.0	4
10	28.3	3.5	4	29.3	3.5	4	12.2	2.9	4
12	25.9	1.3	4			0	18.9	7.0	4
14	23.3	1.8	4	39.8	7.5	4	21.8	5.8	4
16	27.6	1.8	4	25.3	1.3	4	17.0	2.7	3
18	16.9	6.0	4	26.0	0.8	3	21.6	1.4	4
20	27.4	1.4	4	25.2	2.6	4	12.6	2.2	3
22	26.5	1.0	4			0	19.0	4.2	3
26	28.2	1.1	4	40.6	4.4	3	6.7	1.7	4
28	28.3	4.4	4	87.2	5.9	4	15.1	5.1	4
30	24.0	1.4	4			0	15.9	5.4	3
32	34.0	1.8	4	126.5	68.0	4	19.3	11.8	4
34	27.9	3.3	4	159.9	60.2	4	19.7	16.8	4
36	27.0	4.9	4	199.3	41.8	4	22.0	22.6	4
38	16.5	6.3	4	191.2	43.2	4	33.9	31.8	4
40				164.2	38.8	4	34.5	37.0	4
42				208.4	53.3	4	64.0	90.6	4
44				147.7	25.8	4	50.4	64.9	4
46				162.7	25.8	4	48.3	59.5	4
48				168.7	62.5	4	56.0	82.3	4
50				132.5	31.0	4	49.7	56.2	4

Appendix Table III.3 First Experiment

Mean glucose uptake in mg per *Taenia taeniaeformis* metacestode with standard deviations plus number of observations per group taken every 48 h

	Group 1			Group 2			Group 3		
Day	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n
2	4.6	0.16	4	3.8	0.80	3	5.6	0.10	3
4	2.5	1.94	4	3.1	0.35	4	4.2	0.36	4
6	1.4	1.27	4	4.0	0.67	4	4.4	0.25	2
8	2.2	0.09	4	3.8	1.13	2	3.1	0.15	4
10	2.8	0.55	4	4.5	0.57	4	3.8	0.31	3
12	3.5	0.36	4	3.7	0.35	2	3.4	0.27	4
14	3.3	0.41	4	4.5	0.71	4	5.0	1.08	4
16	2.9	0.29	4	3.0	0.67	4	2.2	0.99	4
18	1.9	0.56	4	2.5	0.42	4	2.6	0.25	3
20	2.3	0.27	4	2.2	0.47	3	2.6	0.30	3
22	2.3	0.35	3	2.3	0.30	3	3.3	0.47	4
24	0.4	0.14	3	2.0	0.16	4	2.7	0.38	4
26	1.4	0.10	3	2.8	0.19	4	2.9	0.34	3
28	1.4	0.27	3	2.9	0.31	2	3.8	0.24	4
30	1.5	0.34	4	4.0	0.36	2	3.7	0.86	4
32	0.8	0.41	3	3.8	0.70	4	3.5	0.36	4
34	2.3	0.65	4	3.0	0.64	4	3.4	0.38	4
36	1.6	0.40	4	2.4	0.67	4	2.9	0.45	4
38	0.7	0.17	4	1.6	0.24	4	2.0	0.40	4
42				1.8	0.52	4	2.3	0.27	4
44				1.6	0.63	4	2.1	0.78	4
46				0.8	0.36	4	2.1	0.62	4
48				1.4	0.52	4	1.6	0.53	4
50				0.6	0.32	3	1.1	0.36	4

Appendix Table III.4 Second Experiment

Mean individual liveweights (in mg) of Taenia taeniaeformis metacestodes with standard deviations plus number of observations per group taken every 48 h

	Group 1			Group 2			Group 3		
Day	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n
0	105.11	9.70	4	104.93	7.02	4	104.82	3.61	4
2	156.27	17.47	3	135.58	13.96	4	137.37	11.95	4
4	164.63	14.24	4	138.10	13.63	3	151.79	7.26	4
6	169.86	25.31	4	147.70	10.18	4	183.04	8.70	4
8	147.42	13.93	4	151.81	18.23	4	196.72	44.86	4
10	164.20	16.92	4	140.35	20.57	4	203.68	33.34	4
12	172.58	18.37	4	153.53	23.07	4	218.51	38.07	4
14	84.56	4.76	4	150.34	9.95	4	217.20	37.12	4
16	87.35	13.50	4	155.52	11.90	4	162.61	11.17	4
18	92.39	18.65	3	169.70	3.94	4	163.67	19.42	4
20	97.63	17.79	4	148.50	13.93	4	143.95	9.85	4
22				166.44	16.34	4	139.91	6.43	4
24				162.08	11.51	4	142.12	3.88	4
26				153.52	10.91	4	146.43	16.18	4
28				164.60	25.11	4	134.82	19.51	4
30				101.82	19.97	4	124.34	20.02	4
32				113.58	18.89	4	120.52	14.37	4

Appendix Table III.5 Second Experiment

Mean NH_3 production in μg per Taenia taeniaeformis metacestode with standard deviations plus number of observations per group taken every 48 h

	Group 1			Group 2			Group 3		
Day	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n
2	28.61	4.72	4	20.50	4.18	4	11.01	5.64	4
4	46.17	8.30	4	22.39	6.94	4	14.10	3.00	4
6	48.60	5.59	4	28.84	13.35	4	25.01	6.39	4
8	64.01	21.17	4	47.40	11.40	4	17.22	10.08	4
10	35.67	8.50	4	19.65	6.92	4	25.12	2.95	4
12	43.49	8.30	4	12.47	5.12	4	16.54	6.82	4
14	42.65	9.01	4	29.24	2.12	4	19.60	9.18	4
16	57.35	12.55	4	41.71	6.28	4	6.85	3.52	4
18	52.42	8.77	4	30.10	6.01	4	11.40	10.13	4
20	54.22	6.78	4	40.67	4.94	4	18.11	3.72	4
22				38.61	8.39	4	15.44	2.27	4
24				37.45	7.80	4	13.36	4.64	4
26				37.12	4.94	4	23.51	13.27	4
28				30.17	7.80	4	15.47	5.70	4
30				34.59	6.77	4	26.76	8.22	4
32				42.92	6.75	4	20.20	4.04	4

Appendix Table III.6 Second Experiment

Mean glucose uptake in mg per *Taenia taeniaeformis* metacestode with standard deviations plus number of observations per group taken every 48 h

	Group 1			Group 2			Group 3		
Day	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n
2	6.25	0.35	3	6.15	0.75	4	6.35	0.73	4
4	5.02	0.31	4	4.75	0.48	4	3.67	0.39	4
6	3.12	0.15	3	2.72	0.21	4	3.22	0.31	4
8	2.40	0.95	4	1.95	0.34	4	4.65	0.46	4
10	3.80	0.78	4	2.70	0.05	4	3.90	0.10	4
12	2.62	0.43	4	3.37	0.53	4	3.62	0.59	4
14	0.00	0.00	4	3.39	0.52	4	3.11	0.15	4
16	3.25	0.41	4	2.81	0.32	4	5.11	0.48	4
18	3.72	0.25	4	3.15	0.25	4	4.95	1.21	4
20	3.80	0.39	4	3.47	0.27	4	5.33	0.40	3
22				2.49	0.67	4	5.06	0.71	4
26				2.40	0.56	2	3.30	0.30	3
28				1.03	0.08	3	2.11	0.14	4
30				1.40	0.33	4	2.22	0.26	4

Appendix Table III.7 Second Experiment

Mean lactic acid production in μg per *Taenia taeniaeformis* metacestode with standard deviations plus number of observations per group taken every 48 h

Day	Group 1			Group 2			Group 3		
	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n	$\bar{X}$	s.d.	n
2	28.1	0.6	2	38.0	3.7	2	39.3	6.1	2
6	20.3	0.6	2	34.6	2.5	2	22.9	7.3	2
10	15.1	0.6	2	32.0	2.5	2	25.9	1.8	2
14	54.9	11.0	2	31.1	6.1	2	31.6	10.9	2
18	27.7	3.6	2	23.2	1.1	2	27.7	3.1	2
20	18.6	3.0	2			0			0
22				19.4	1.8	2	33.7	0.6	2
26				19.0	2.4	2	41.5	9.2	2
30				42.4	4.9	2	50.6	2.4	2

Appendix Table IV.1 First Experiment

Relative absorbance values at 450 nm for somatic and ES antigen levels by ITAS-ELISA for *Taenia taeniaeformis* larvae maintained in different media

Group	Subgroup	Day 2	8	14	20	26	32	38	44	50
Somatic antigen 1	a	.12	.21	.28	.99	1.75	1.81	1.77		
	b	.07	.11	.17	.62	1.64	1.75	1.77		
	c	.16	.16	.22	.40	1.75	1.80	1.63		
	d	.09	.09	.25	.67	1.61	1.59	1.80		
2	a	.22	.21	.33	.41	1.22	1.43	1.73	1.48	1.67
	b	.24	.22	.30	.44	.97	1.33	1.70	1.55	1.58
	c	.24	.20	.49	.47	1.26	1.66	1.56	1.51	1.46
	d	.14	.18	.28	.47	1.24	1.44	1.45	1.47	1.58
3	a	.19	.13	.16	.56	-	1.50	1.40	0.99	1.86
	b	.17	.12	.16	.45	.94	1.27	1.74	1.04	1.70
	c	.08	.17	.21	.46	.83	1.58	1.55	1.28	1.88
	d	.20	.17	.38	.45	.79	1.44	1.39	1.35	1.76
ES antigen 1	a	.24	.21	.17	.27	.37	.41	.43		
	b	.22	.19	.19	.28	.36	.38	.39		
	c	.42	.19	.29	.44	.41	.44	.35		
	d	.25	.16	.26	.32	.58	.31	.29		
2	a	.31	.23	.24	.35	.40	.45	.43	.35	.47
	b	.35	.29	.23	.38	.31	.43	.45	.38	.42
	c	.28	.38	.36	.33	.32	.53	.49	.45	.45
	d	.32	.29	.35	.30	.30	.50	.44	.50	.51
3	a	.44	.28	.36	.42	.37	.45	.45	.35	.51
	b	.40	.29	.30	.31	.30	.33	.32	.39	.42
	c	.33	.33	.27	.28	.21	.37	.37	.36	.40
	d	.37	.30	.38	.31	.20	.31	.30	.36	.52

Appendix Table IV.2 <u>Second Experiment</u>

Relative absorbance values at 450 nm for somatic and ES antigen levels by ITAS-ELISA for *Taenia taeniaeformis* larvae maintained in different media.

Group	Subgroup	Day 2	6	10	14	18	20	22	26	30	32
Somatic antigen 1	a	.50	.70	1.04	1.46	1.46	1.46				
	b	.41	.55	.89	1.27	1.54	1.73				
	c	.47	.40	1.11	1.20	1.60	1.67				
	d	.43	.58	.78	1.26	1.74	1.76				
2	a	.80	.80	1.10	1.22	1.64	-	1.27	1.50	1.71	1.53
	b	.73	.75	1.17	1.24	1.24	-	1.08	1.42	1.45	1.67
	c	.61	.66	1.14	1.28	1.28	-	1.24	1.22	1.49	1.58
	d	.72	.57	.93	1.04	1.05	-	1.08	.96	1.43	1.49
3	a	.77	.50	.72	.85	1.15	-	1.50	1.52	1.44	1.42
	b	.68	.48	.73	.82	1.17	-	1.48	1.55	1.42	1.62
	c	.67	.46	.70	.91	1.30	-	2.00	1.34	1.40	1.63
	d	.68	.62	.83	.89	.99	-	1.41	1.53	1.28	1.69
ES antigen 1	a	1.28	.64	.70	.84	.66	.85				
	b	0.99	.68	.61	.62	.70	.70				
	c	1.06	.80	.58	.64	.94	.66				
	d	1.02	.63	.55	.84	.65	.72				
2	a	1.14	.79	.94	.93	.51	-	.56	.56	1.06	1.21
	b	1.05	.75	.92	.79	.73	-	.62	.52	.70	.97
	c	.93	.72	.77	.80	.53	-	.67	.67	.65	.93
	d	.98	.70	.83	.64	.66	-	.54	.54	.56	.85
3	a	.95	.76	.62	.58	.53	-	.50	.50	.66	.90
	b	.94	.75	.67	.56	.53	-	.54	.54	.60	.69
	c	.87	.64	.76	.53	.55	-	.55	.74	.79	.79
	d	.92	.78	.89	.61	.62	-	.58	.79	.95	1.20

Appendix Table V.1 Experiment 1

Numbers of living and dead cysts encountered in livers from mice infected with *Taenia taeniaeformis* eggs after vaccination with homologous larval ES antigen or sham vaccination.

(L = living cysts, D = dead cysts)

Group			Mouse No. 1	2	3	4	5	mean	s.d.
1.1	ES 0.5 ml/mouse	L	15	13	5	1	0	6.8	6.87
		D	0	7	61	0	40	21.6	27.55
1.2	ES 0.25 ml/mouse	L	40	23	19	9	5	19.2	13.72
		D	6	26	46	0	0	15.6	20.07
1.3	Unused medium 0.5 ml/mouse	L	74	39	29	10	6	31.6	27.30
		D	27	8	2	3	0	8.0	11.02
1.4	Water 0.5 ml/ mouse	L	58	51	8	4	0	24.2	27.91
		D	5	0	2	1	15	4.6	6.11
1.5	Not injected	L	95	30	22	2	1	30.0	38.45
		D	11	20	0	34	0	13.0	14.42

Appendix Table V.2. <u>Experiment 2</u>

Numbers of living and dead cysts encountered in livers from mice infected with *Taenia taeniaeformis* eggs after vaccination with homologous larval ES antigen or sham vaccination. One animal died in Group 2.3 soon after challenge.

(L = living cysts, D = dead cysts)

Group		Mouse No. 1	2	3	4	5	6	7	8	9	10	mean	s.d.
2.1 ES per os	L	150	150	150	140	120	110	80	75	0	0	97.5	58.27
	D	0	2	2	0	0	5	2	0	1	6	1.8	2.15
2.2 ES s.c.	L	130	120	55	52	34	1	0	0	0	0	39.2	50.35
	D	0	2	2	3	0	50	10	10	30	0	10.7	16.57
2.3 Unused medium per os	L	180	140	90	50	45	40	40	0	0	-	65.0	61.03
	D	3	5	0	5	10	5	10	20	50	-	12.0	15.36
2.4 Unused medium s.c.	L	150	85	75	65	60	35	35	22	3	0	53.0	44.76
	D	0	0	0	0	0	2	5	10	20	10	4.7	6.73

Appendix Table V.3. Experiment 6

Numbers of living cysts encountered in livers from rats infected with *Taenia taeniaeformis* eggs after vaccination with ES antigens or somatic antigen or sham vaccination.

Group	Rat No.										mean	s.d.
	1	2	3	4	5	6	7	8	9	10		
6.1	103	117	210	59	16	75	189	139	333	159	140.0	90.04
6.2	391	192	176	130	290	145	284	130	250	207	219.5	84.23
6.3	139	255	124	146	115	111	99	271	145	275	168.0	70.11
6.4	488	189	288	161	251	348	183	167	3	0	207.8	147.73
6.5	390	445	262	121	460	0	0	107	384	281	245.0	177.21
6.6	375	139	113	149	219	490	180	117	614	128	252.4	178.07
6.7	361	587	129	183	401	287	151	225	314	164	280.2	142.20

Appendix Table V.4. Experiment 6

Results obtained by ELISA (for detecting antibodies) on plasma from rats infected with Taenia taeniaeformis eggs after vaccination with ES antigens or somatic antigen or sham vaccination.

Group	Samples from day	SOM. antigen Rat No. 1	2	3	4	5	ES antigen Rat No. 1	2	3	4	5
6.1	0	0.10	0.12	0.10	0.11	0.09	0.21	0.17	0.18	0.16	0.16
6.2		0.11	0.10	0.08	0.10	0.09	0.16	0.14	0.15	0.14	0.14
6.3		0.10	0.10	0.10	0.12	0.13	0.16	0.14	0.14	0.14	0.12
6.4		0.14	0.14	0.13	0.14	0.14	0.14	0.13	0.11	0.13	0.14
6.5		0.11	0.07	0.10	0.10	0.07	0.17	0.11	0.14	0.14	0.12
6.6		0.11	0.08	0.09	0.07	0.09	0.17	0.14	0.13	0.15	0.20
6.7		0.10	0.10	0.08	0.11	0.12	0.16	0.12	0.13	0.15	0.11
6.1	18	0.26	0.13	0.10	0.15	0.11	0.32	0.20	0.23	0.24	0.18
6.2		0.25	0.09	0.11	0.11	0.13	0.21	0.20	0.16	0.25	0.24
6.3		0.28	0.11	0.14	0.31	-	0.50	0.30	0.36	0.39	0.63
6.4		0.10	0.36	0.17	0.36	0.14	0.32	0.36	0.28	0.42	0.22
6.5		0.20	0.16	0.23	-	0.16	0.32	0.16	0.22	0.24	0.18
6.6		0.16	0.89	0.89	0.31	1.07	0.14	0.15	0.23	0.12	0.23
6.7		0.12	0.10	0.10	0.09	0.11	0.14	0.11	0.11	0.12	0.10
6.1	70	1.21	0.89	0.94	0.96	0.52	1.21	1.30	1.34	1.15	1.11
6.2		1.10	0.86	0.97	1.29	1.50	1.36	1.21	1.17	1.41	1.33
6.3		0.98	0.88	1.02	1.05	1.04	1.25	1.14	1.14	1.31	1.20
6.4		0.60	1.61	1.17	1.50	1.24	1.26	1.35	1.19	1.33	1.37
6.5		1.27	1.13	1.18	1.50	1.02	1.43	1.22	1.25	1.38	1.16
6.6		0.93	1.56	1.36	1.24	1.53	1.04	1.38	1.25	1.11	1.38
6.7		1.16	1.22	0.89	0.92	1.22	1.38	1.21	1.06	1.21	1.24

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Letter

PRELIMINARY OBSERVATIONS ON THE *IN-VITRO* CULTURE OF METACESTODES OF *TAENIA SAGINATA*

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ABSTRACT

Brandt, J.R.A. and Sewell, M.M.H., 1980. Preliminary observations on the *in vitro* culture of metacestodes of *Taenia saginata*. *Vet. Sci. Commun.*, 3: 317—324.

Metacestodes of *Taenia saginata* were cultured in a diphasic medium consisting of a disrupted solid phase of coagulated calf serum and a fluid phase of hepes buffered RPMI-1640 enriched with sodium pyruvate and foetal calf serum. The growing tapeworms formed segments, which showed early development of sexual organs. This culture technique gave better results than methods using monophasic media or an intact solid phase when assessed in terms both of the survival of the cestodes and of five parameters measured at the end of the culture period.

INTRODUCTION

Taylor (1963) first reported the maintenance of a cestode in a fully defined medium using *Taenia crassiceps*. Such a system is of potential value for relatively prolonged biochemical studies and also as a source of sero-diagnostic antigens and non-living vaccines, especially from the metabolic products of the cultured worms as described for *Echinococcus granulosus* by Herd *et al* (1975) and Herd (1977). Similar studies were carried out with *Taenia taeniaeformis* metacestodes by Kwa and Liew (1977) and Ayuya and Williams (1979) although they only maintained the parasites for 24 hours. A diphasic medium, of which the solid component was coagulated serum with an undefined fluid component was successfully used by Esch and Smyth (1976) for *T. crassiceps*. They compared their results with those obtained with *E. granulosus* by Smyth (1967) and Smyth and Davies (1974). The development of both parasites reached a similar level, including the formation of genital pores and sexual organs. Osuna and Guevara Pozo (1976) used a similar culture system and obtained development up to the appearance of genital pores with *Taenia hydatigena*. This paper describes attempts to cultivate *Taenia saginata* metacestodes *in vitro*.

MATERIALS AND METHODS

Fluid phase

RPMI-1640, with L-glutamine and 25mM hepes buffer (Gibco-Europe Ltd., Paisley, Scotland) was used in all cultures as the basic fluid medium. This was supplemented with 100 iu ml^{-1} penicillin, 100μg ml^{-1} streptomycin and 5% v/v 100 mM sodium pyruvate (Gibco). To this basic medium 10% v/v heat inactivated foetal calf serum (Gibco) was added. Ten ml of this mixture was used in each universal container.

Solid phase

Universal bottles, with a silicone lining in the cap, were autoclaved and 5ml special bobby calf serum (Gibco) placed in them and caused to coagulate by heating at 80°C for 90 minutes. This solid phase was then usually broken up with a sterile needle. In one experiment a non-nutritive solid phase was used which consisted of 1 g of G25 Sephadex. This had been autoclaved *in situ* and equilibrated overnight against 5 ml of the basic fluid medium.

Culture procedure

The universals were sealed and kept at 37°C with air as the gaseous phase. The liquid phase was changed every 48 hours and the solid phase every week, both changes being made under sterile conditions.

Metacestodes

The metacestodes were dissected from the muscles of experimentally infected calves. They were washed 6 times by transferring them with 5 mm bore sterile glass tubing, used in the same way as a pasteur pipette, through petri dishes containing sterile basic fluid medium in a laminar flow sterile hood. They were then left overnight at 37°C in the fluid medium with foetal calf serum and 10% v/v ox bile, which had been sterilised by filtration. The following day, the desired number of fully evaginated metacestodes was selected, regardless of whether the cyst bladder was intact or not, and again washed 5 times before being placed in the cultures.

When the metacestodes were removed from the culture they were left in tap water at 4°C for 6 hours to allow relaxation, fixed in acetone-formalin-acetic acid (AFA) and stained with Semichon's carmine.

In two of the experiments the larvae were fixed, stained and compared to each other using five parameters. These were the width of the scolex, the diameter of two of the suckers, the length of the body, excluding the scolex and the bladder plus the forebody (Wardle and Mcleod, 1968) already present at evagination, the width of the strobila half-way down its length and the product of these last two parameters.

Experiment 1

Twenty suitable metacestodes, obtained from a calf 82 days after it had been infected at 1-2 months of age with 5,000 eggs of *T. saginata*, were divided into two cultures. In one of these cultures the solid phase was left intact. Seven of the developing tapeworms were removed from the culture with the disrupted solid culture after 2 months and the remaining 3 left for a further 2 months. The culture with the intact solid phase was terminated after 2 months.

Experiment 2

The metacestodes were obtained from a 1-2 month old calf, which had been infected 95 days previously with 5,000 *T. saginata* eggs. Fourteen metacestodes were divided into two cultures. One was kept in the same diphasic medium as used in Experiment 1. For the second culture the same total components were used, but the bobby calf serum was not coagulated. Thus this comprised a monophasic culture containing 6 ml serum and 9 ml basic medium.

The cultures were stopped after 48 days and the worms fixed, stained and measured.

Experiment 3

The metacestodes originated from a calf infected at the age of 1 year with 12,000 eggs and autopsied 84 days after infection. They were divided randomly into 5 groups of 15 larvae each.

Group 1 was kept in the standard diphasic culture.

Group 2 was kept in the same monophasic medium as used in Experiment 2.

Group 3 was cultured using the non-nutritive solid phase of Sephadex beads.

Group 4 was cultured in 9 ml basic medium and 1 ml foetal calf serum, thus being the fluid phase as used in the diphasic cultures.

Group 5 was fixed immediately.

The four cultures were stopped after 38 days and the developing tapeworms fixed, stained and measured.

RESULTS

Experiment 1

The cestodes in the culture with the intact solid phase never showed much development and were all dead within 2 months. After ten days, eight of the larvae in the culture with the disrupted solid phase showed some development, as the neck had broadened and osmoregulatory canals had become visible. At 18 days one worm lost its bladder and forebody and this occurred in 4 others within the first month. By that time 5 larvae had started to show growth and strobilation at the neck and convolution of the canals. After 2 months all 10 larvae in this culture were still alive and active although 5 of them still had their bladders attached.

At no stage were any of the larvae seen to be penetrating or attaching to the blocks of coagulated serum.

Figures 1 and 2 show the best developed larva of the seven removed from the culture after 2 months. It measured about 70 mm long; the width of its scolex was 1.80 mm and of the middle of the strobila, 0.8 mm. About 150

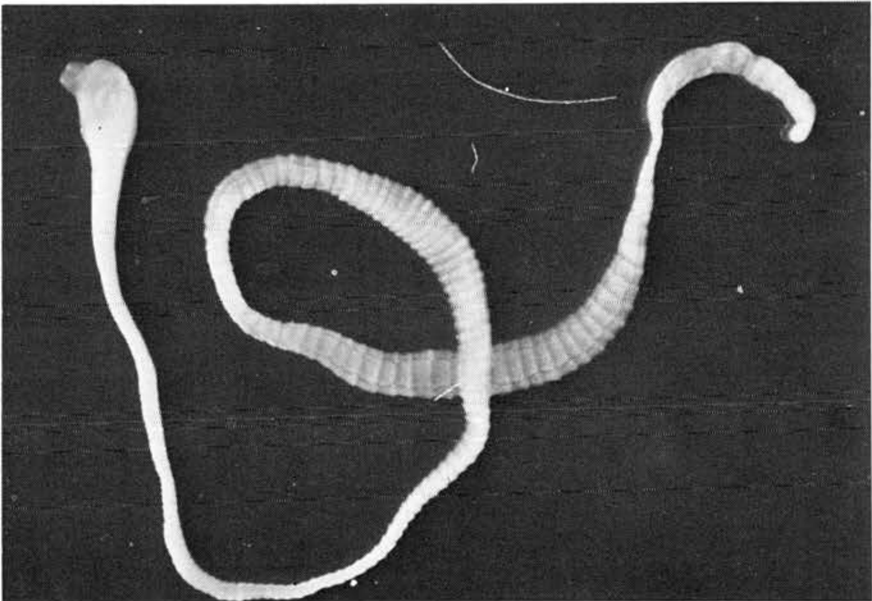

Fig. 1 Living *Taenia saginata* developed from a metacestode 50 days *in vitro* in a diphasic medium. (Incident light x 4.5).

segments were formed and the beginning of what appears to be the cirrus apparatus was present in many of these. Genital pores could not yet be seen, although their probable location was discernible. Although the posterior segments were longer than they were wide, organogenesis did not seem to be more advanced than in the middle ones. Indeed the former seemed rather to be degenerate and liable to become detached.

The remaining larvae, which were kept in culture for another two months, showed a similar development, although they were not as long because of the repeated loss of their distal segment(s). At the end they measured about 60, 50 and 30 mm long respectively.

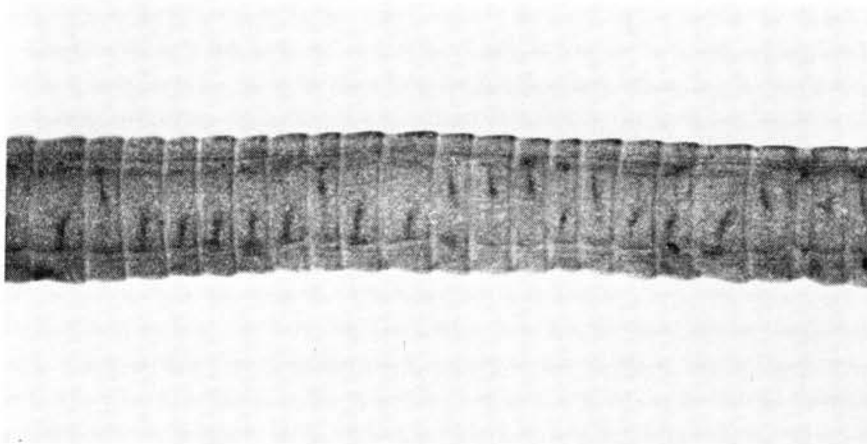

Fig. 2 Part of the strobila of a *Taenia saginata* grown *in vitro* from a metacestode, showing the beginning of the formation of the cirrus apparatus in each segment. (Bright field, transmitted light x22).

Experiment 2

All the larvae in the monophasic medium were inactive and showing signs of degeneration, starting from the posterior part and progressing towards the scolex. In only two of the larvae in this group were osmoregulatory canals visible and degeneration limited to the posterior part.

One larva in the diphasic medium showed general degeneration and was inactive. In two others there was degeneration limited to the posterior part but osmoregulatory canals could be seen in all the larvae.

None of the larvae in either group lost their bladder and forebody but this was petrified in all the larvae in the monophasic system. The measurements for both groups are given in Table 1.

TABLE I

Mean dimensions (± standard deviation) of *Taenia saginata* maintined *in vitro* for 48 days

Parameter	Diphasic Medium	Monophasic Medium	Student's t test
Diameter of scolex (mm)	1.06 ± 0.07	0.85 ± 0.13	3.87**
Diameter of suckers (mm)	0.34 ± 0.03	0.27 ± 0.03	4.31**
Length of body (mm)	6.12 ± 2.45	2.67 ± 0.91	3.47**
Width of body (mm)	0.32 ± 0.04	0.26 ± 0.08	1.83(n.s.
Length x Width of body (mm^2)	1;90 ± 0.67	0.72 ± 0.35	4.18**

Experiment 3

Summarised details of the observation on the cultures are given in Table II and the measurements in Table III. When significant differences were detected by Analysis of Variance, the data were further analysed by Harter's modification of Duncan's New Multiple Range test (Harter, 1960) to reveal significant subsets.

TABLE II

Numbers of *T. saginata* showing specified features after *in vitro* culture for 38 days. Each group comprised 14 individuals.

Group No.	Medium	Degeneration General	Degeneration Posterior only	Canals	Strobilation	Bladder and forebody lost
1	Diphasic	–	4	12	2	1
2	Monophasic	7	4	8	1	– (all petrified
3	Non-nutritive solid phase	8	5	5	–	–
4	Fluid phase only	12	–	–	–	–

TABLE III

Mean dimension and significantly differing subsets of groups of T. saginata maintained in vitro for 38 days

Parameter	Group 1	2	3	4	5	Subsets[a] (no. of groups)
Diameter of scolex (mm)	1.19±0.13	1.07±0.14	1.03±0.19	0.84±0.09	1.19±0.08	4 3 2 1 5
Diameter of suckers (mm)	0.39±0.03	0.35±0.02	0.31±0.03	0.22±0.04	0.41±0.03	4 3 2 1 5
Length of body (mm)	5.24±2.38	3.12±2.11	1.60±0.95	0.90±0.47	0.73±0.30	5 4 3 2 1
Width of body (mm)	0.49±0.15	0.30±0.12	0.29±0.21	0.24±0.08	0.69±0.15	4 3 2 1 5
Length x Width of body (mm^2)	2.50±1.49	0.85±0.49	0.40±0.23	0.19±0.09	0.53±0.27	4 3 5 2 1

[a] Subsets within the same tie do not differ significantly from each other ($p < .01$).

DISCUSSION AND CONCLUSIONS

It is clear that in each experiment the diphasic medium with disrupted coagulated serum allowed both longer survival and more development than the other procedures. Furthermore the measurements from the third experiment show that this is the only group in which the diameters of the scolex and suckers were maintained and that, whereas the mean width of the strobila was reduced in all the cultured groups, this reduction was significantly less in this group.

It is unlikely that every attempt using this procedure will give the same degree of development as that observed in Experiment 1. Indeed a comparison between these cultures suggests that the metacestodes in Experiment 2, after 48 days *in vitro*, were less well developed than those in Experiment 3 after 38 days *in vitro*.

However the procedure would appear to offer considerable possibilities for further studies on the biochemical and biophysical requirements of these cestodes when cultured *in vitro*, as it seems highly unlikely that this simple procedure represents the best attainable conditions.

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List of Abbreviations and Symbols

AGS	Artificial gastric solution
AIS	Artificial intestinal solution
ADP	Adenosine 5' - diphosphate
B	Belgian strain (of *Taenia taeniaeformis*)
DAS-ELISA	Double antibody sandwich enzyme-linked immunosorbent assay
DEB	Disintegration of embryophoral blocks
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ES	Excretory and secretory or metabolic antigens
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
FIA	Freund's incomplete adjuvant
G.P. a ES(c)	Guinea-pig hyperimmune serum against ES antigen raised by the classic method
G.P. a ES(v)	Guinea-pig hyperimmune serum against ES antigen raised by the Vaitukaitis method
H & A	Hatching and activating or hatched and activated
HBSS	Hanks' Balanced Salt Solution (Gibco)
HEPES	4 - (2 - Hydroxylethyl) - 1 - piperazine ethane sulphonic acid
HIS	Hyperimmune serum
I	Iraqi strain (of *Taenia taeniaeformis*)
IEP	Immunoelectrophoresis
Ig	Immunoglobulin
i.m.	intramuscular
i.p.	intraperitoneal
ITAS-ELISA	Indirect triple antibody sandwich - enzyme-linked immunosorbent assay

log	logarithm to the base 10
M	Malaysian strain (of _Taenia taeniaeformis_)
MGP	Microgelprecipitation
MLB	Meldola blue (BCL)
MTT	3 - (4,5 - Dimethyl thiazolyl - 2) -2,5-diphenyltetrazolium bromide
NS	Not significant: in statistics probability more than 0.05
OPD	ortho phenylene diamine (Sigma)
PBS	Phosphate buffered saline (Dulbecco 'A' - Oxoid)
PMS	Phenazine methosulphate
Ra A Som	Rabbit hyperimmune serum against adult somatic antigen
Ra ES	Rabbit hyperimmune serum against excretory and secretory products
Ra L Som	Rabbit hyperimmune serum against strobilocerci somatic antigen
RPMI	Roswell Park Memorial Institute cell culture medium - 1640 (Gibco)
S	Scottish strain (of _Taenia taeniaeformis_)
s.c.	subcutaneous
SPF	Specific Pathogen Free
Tris	Tris - (hydroxymethyl) - aminomethane
-	In tables: Not Done
*	In statistics: probability less than or equal to 0.05
**	In statistics: probability less than or equal to 0.01
***	In statistics: probability less than or equal to 0.001