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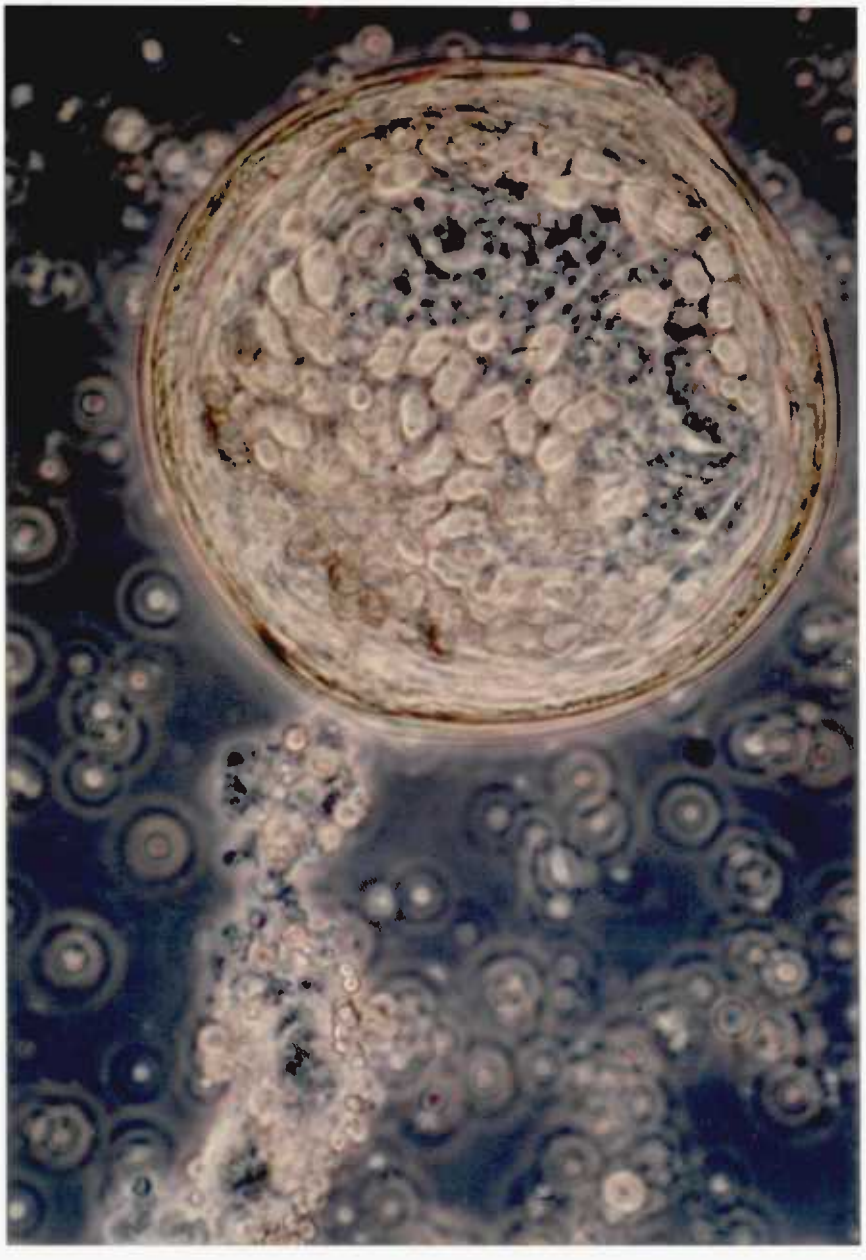

STUDIES ON
MONIEZIA BENEDENI (MONIEZ, 1879)
AND
M. EXPANSA (RUDOLPHI, 1810).

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THESIS SUBMITTED FOR THE DEGREE OF
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*A search was made for an alternative intermediate host which could be conveniently maintained in the laboratory. Three species of insect were investigated: Tribolium confusum, Periplaneta americana, and Locusta migratoria migratoides. No development occurred in any of these species.

Eggs of M. benedeni and M. expansa were hatched in sterile conditions using a variety of mechanical and chemical techniques as a preliminary to in vitro culture.

ABSTRACT

Heather Mary Paterson

Studies on Moniezia benedeni (Moniez, 1879) and M. expansa (Rudolphi, 1810).

Studies were made on the developmental stages of Moniezia benedeni and M. expansa with a view to reproducing the life cycle in the laboratory using in vivo and in vitro techniques.

Several species of oribatid mite, the intermediate hosts, were extracted from turf, infected with the larval stage of the tapeworm, and maintained in culture for several months.

Cysticercoids developed in the mites within 6 - 10 weeks at a temperature of 25°C and relative humidity of 100%.

*See facing page

Attempts were made to culture the oncosphere of M. expansa to the cysticercoid stage in vitro. A wide range of culture media and physical conditions were employed. Although the organisms survived for up to 21 days, no development occurred.

During initial efforts at in vitro culture, large numbers of bacteria were discovered to be always present in the eggs of the tapeworm. Microbiological and biochemical tests were employed to isolate and identify the species. The bacteria were identified as Aeromonas hydrophila, Escherichia coli and Pseudomonas putida.

Cysticercoids from laboratory infected mites were successfully excysted in vitro. The procedure consisted of treatments in an acid-pepsin solution followed by a mixture of pancreatin and sodium taurocholate.

Excysted larvae were maintained in in vitro culture for up to 19 days but no development or strobilisation occurred.

Preliminary experiments investigating the effect of a Moniezia expansa infection in lambs were performed. No pathology or symptoms of disease were recorded.

A comprehensive literature review on many aspects of the life history of M. benedeni and M. expansa is included.

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CHAPTER I

GENERAL INTRODUCTION AND AIMS OF RESEARCH

With the discovery of an intermediate host, and thus the complete life cycle of Moniezia expansa by Stunkard in 1937, many questions relating to the life history of the tapeworm have been subsequently solved. However, over forty years later the pathogenicity of the tapeworm remains a subject of controversy and debate.

Moniezia is cosmopolitan and infects a variety of animals from hippopotamuses to cattle and from reindeer to sheep. Accounts of the disease are similarly as varied and a number of different symptoms have been reported. These include anaemia; digestive disturbances, such as diarrhoea; stunting; emaciation; and loss of wool. In extreme cases, infections have proved fatal, but in others no adverse effects of any kind have been evident.

Owing to the difficulty of producing the life cycle in the laboratory little detailed information is available on the growth, development and physiology of either the larval, or the adult, stages of Moniezia. A project was, therefore, designed to produce a system for examining the nature of the disease using both in vivo and in vitro techniques. All aspects of the life cycle were considered, but emphasis was placed on the production of large numbers of the infective stage, the cysticercoid. This would then allow for controlled experiments in the final host, and hence the possible elucidation of the pathogenicity of the disease.

In Great Britain, Moniezia expansa is the most frequently occurring species and primarily attacks young lambs. The majority of the

research reported here was therefore carried out using this species and its ovine host. When material was available, however, comparative studies were also performed on the related species, M. benedeni.

Classification

Moniezia expansa and Moniezia benedeni are Cyclophyllidean cestodes classified by Spasskij (1951) as outlined below.

ORDER	:	Cyclophyllidea	Beneden in Braun, 1900.
SUBORDER	:	Anoplocephalata	Skrjabin, 1933.
SUPERFAMILY	:	Anoplocephaloidea	Spasskij, 1949.
FAMILY	:	Anoplocephalidae	Cholodowsky, 1902.
SUBFAMILY	:	Monieziinae	Spasskij, 1951, characterised by the possession of a reticular uterus.
GENUS	:	Moniezia	Blanchard, 1891, characterised by the following:- (1) Vagina below seminal vesicle; (2) Reproductive organs duplicated; (3) Excretory organs simple; (4) Bladder-like external and internal seminal vesicles not formed; and (5) Uterus in form of transverse tube with outgrowths.

Moniezia expansa (Rudolphi, 1810) is characterised by the possession of interproglottidal glands which extend the full width of the proglottid. It has a cosmopolitan distribution in Bovoidea, Cervoidea, Camelidae and Suidae.

Moniezia benedeni (Moniez, 1879) is characterised by the possession of interproglottidal glands arranged in a short row in the centre of the proglottid. It has a cosmopolitan distribution in Bovoidea, Cervoidea and Camelidae.

Life Cycle

Moniezia benedeni and M. expansa infect farm animals, particularly goats, sheep and cattle, throughout the world. In Great Britain, M. benedeni is recognised as a parasite of cows and calves, and M. expansa as a parasite of sheep and lambs. Mixed infections do occur, however. The disease is primarily one of young stock as infections are gained soon after weaning when the lambs and calves begin to graze.

The life cycle of Moniezia expansa is shown in figure 1.

Adult tapeworms are found in the small intestine, most frequently in the middle third of the ileum (Frietas & Costa, 1970). Worm burdens vary enormously, ranging from a single worm to over 200 per host. Stoll (1938) has discussed this question, and reported a daily infection rate of between 1 and 2 worms. However, naturally acquired infections usually involve small numbers of worms; Worley, Jacobson & Barrett (1974) have given an average value for the total worm burden of 3.

Experimental infections can be higher; Kates & Goldberg (1951) reported worm burdens of between 9 and 114.

In multiple infections, growth of the worms is not uniform and all

FIGURE 1:a.

THE LIFE CYCLE OF MONIEZIA EXPANSA

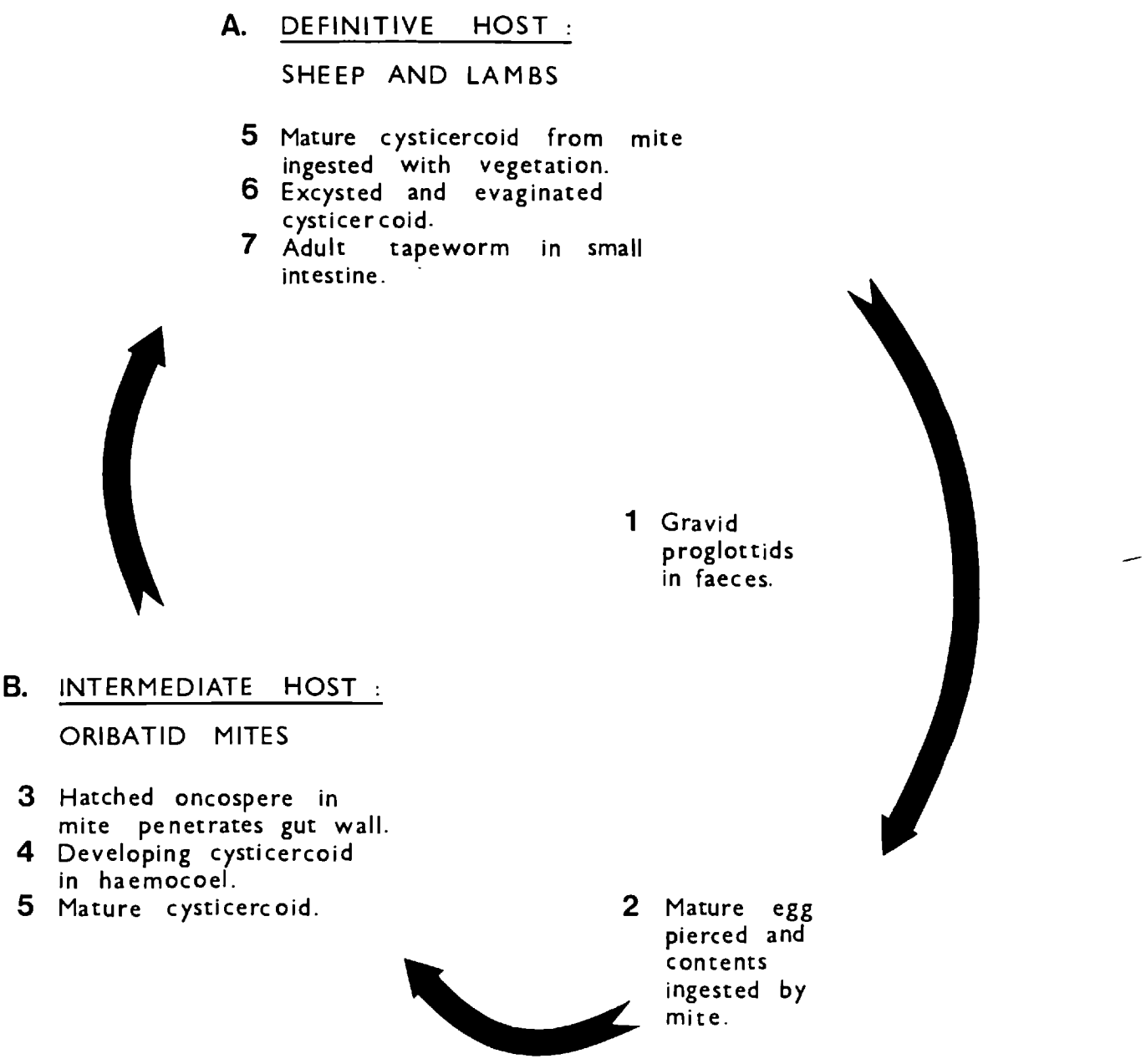
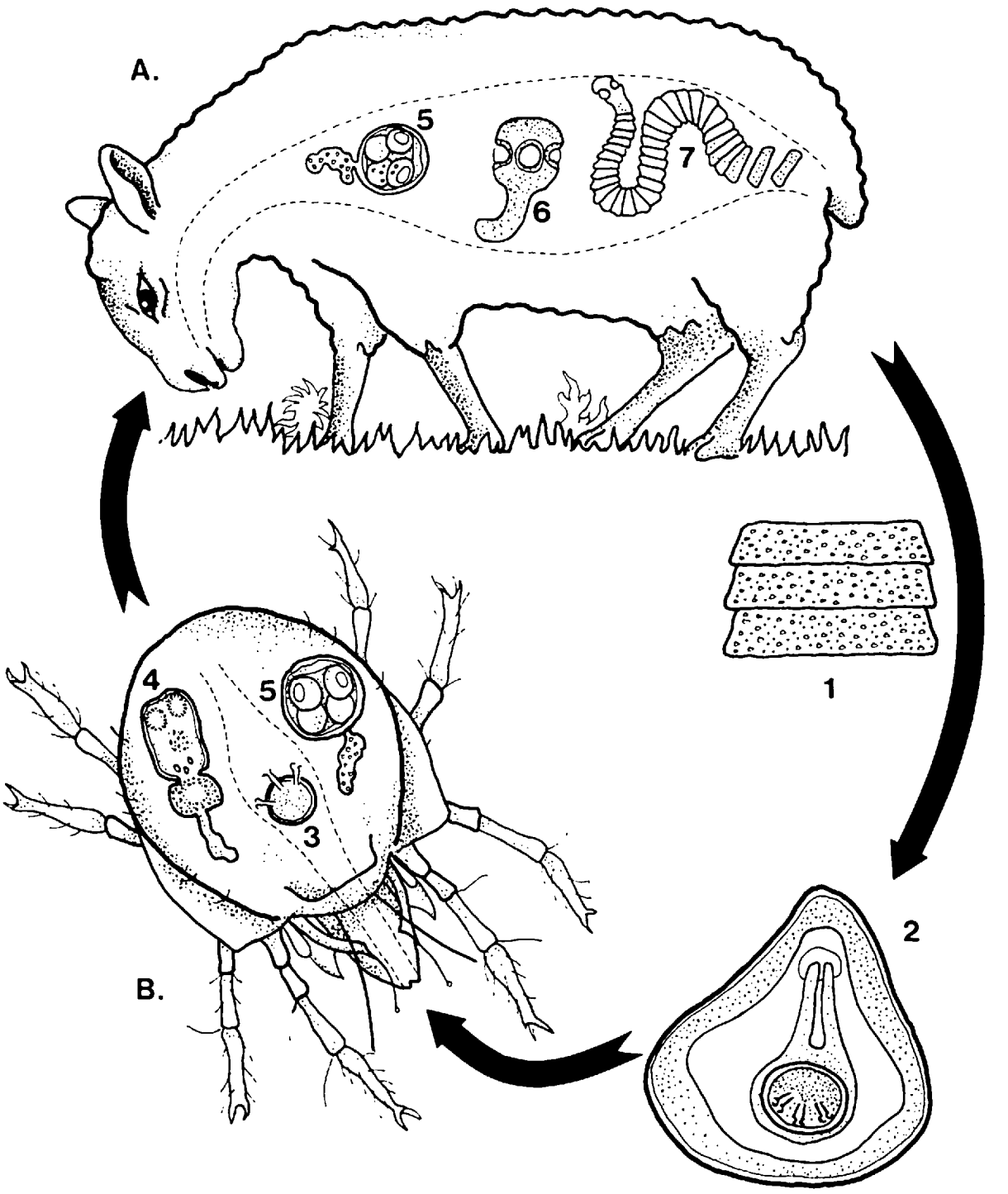


FIGURE I:b.

THE LIFE CYCLE OF MONIEZIA EXPANSA



stages of development can be observed.

Mature worms may measure anything from a few, to several hundred centimetres.

The scolex is unarmed with four prominent suckers and measures 0.36 to 0.80 mm in width. The proglottids are broader than long and measure up to 1.6 cm in breadth in M. expansa, and 2.6 cm in breadth in M. benedeni.

The mature proglottids are covered with straight microtriches without spines (Howells, 1965) and are organised as shown in figure 2. They contain two sets of genital organs. The ovaries and the vitelline glands form a ring on either side, median to the longitudinal excretory canals, while the testes are distributed throughout the central area. At the posterior border each proglottid contains a row of interproglottidal glands, arranged around small pits. Reports by Singh & Singh (1958); Logachev & Dimitrova (1961); and Howells & Erasmus (1969) have described their morphology and histochemistry, but the function of the glands is, as yet, unknown. Their arrangement provides the chief diagnostic feature for distinguishing the two species. M. expansa has a row of glands extending the width of the proglottid, whereas M. benedeni has only a short row confined to the centre of the proglottid. The ultrastructure of the cuticle and subcuticular tissues has been described by Howells (1965).

Little work has been carried out on the growth rate of M. expansa. From histological studies, Lui & Zuidaric (1972) described the cytological development and reported that there is no region of localised

MATURE PROGLOTTID OF MONIEZIA EXPANSA

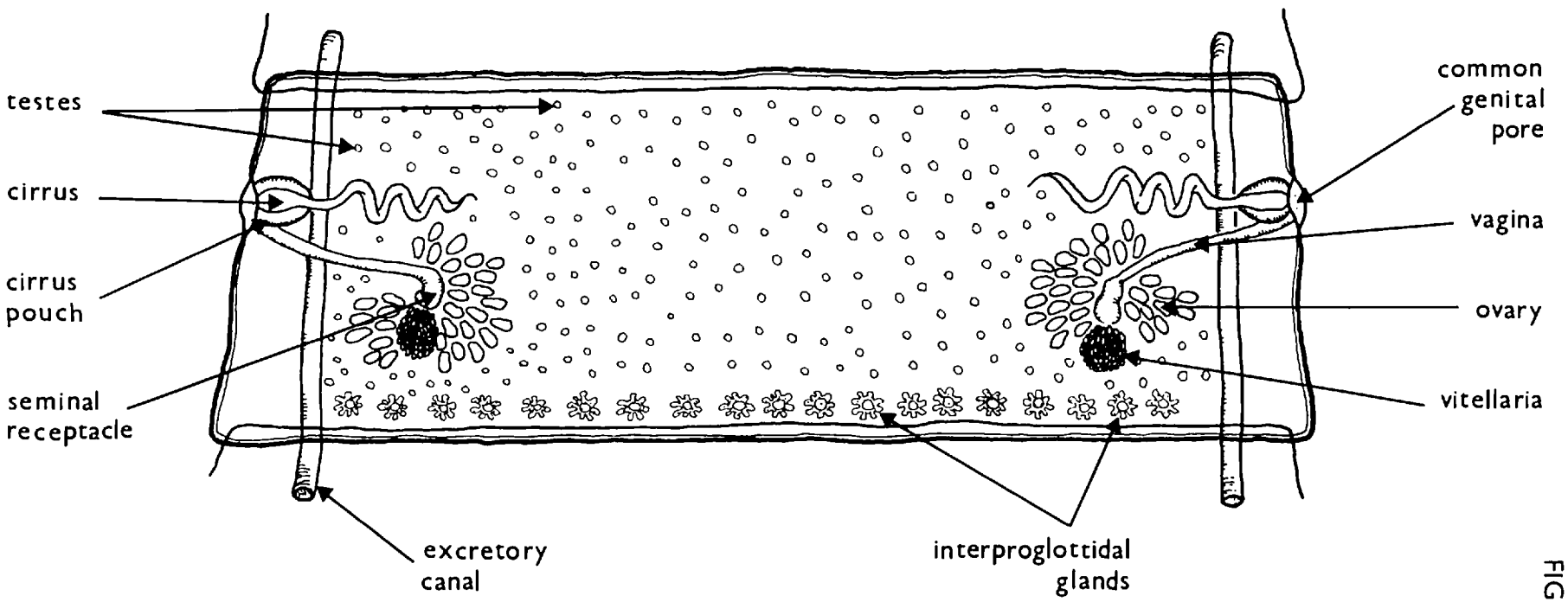


FIGURE .2.

growth, cell division occurring throughout the proglottids so that each one grows continuously until gravid. Curtice (1890, quoted in Haberman & Carlson, 1946) stated that the worms grow approximately one yard per month, and Rayski (1947) reported growth of from one to three inches every 24 hours. Graber & Gruvel (1969) described the growth rates of single worm infections and noted that the rates are considerably slower in multiple infections.

Prepatency can last anything from 25 to 55 days with an average for M. expansa of 37 days. M. benedeni takes longer to develop (Kuznetsov, 1968; Alkov, 1972; and Narsapur, 1976b), whether in lambs or calves. Kuznetsov (1968) also reported a difference which was dependent on the age of the host. Older lambs were more susceptible to M. benedeni and prepatency was shorter than in younger lambs. For M. expansa the situation was exactly reversed.

Patency is similarly as variable, from 11 days (Kassai & Mahunka, 1965) to 265 days (Kuznetsov, 1968) and Rayski (1947) reports as much as 350 days. On average, infections of both species last 2 to 3 months. Hansen, Kelley & Todd (1950) state that there is a correlation between the worm burdens and duration of patency. They found that for small worm burdens (5 worms, or less) the infection (prepatency and patency) lasted for more than 210 days and for large worm burdens (up to 60 worms) the infections lasted only 55 days.

Eggs are voided with the faeces in proglottids shed from the worm. The presence of these in the faeces forms the basis of diagnosing an infection, in conjunction with egg counts using normal flotation methods. Normally eggs remain undispersed in the faeces on the

pastureland, but occasionally they may be disseminated by birds (Kozlov, 1974) which have been observed feeding on the tapeworm proglottids and excreting undigested eggs containing live and infective oncospheres. Eggs can remain viable in wet faeces for up to 40 days (Potemkina, 1951) but do not survive prolonged freezing or desiccation (Obitz, 1934). However, Kuznetsov (1959d) found that eggs did not survive more than 9 days in faeces.

The structure of the mature egg is shown in figure 3; Plate 1. The oncosphere (Plate Ic) is surrounded by three envelopes (Rybicka, 1966). The whole is bounded by a rigid coat - the egg shell - which is sculptured and has a hexahedral form. Within this the outer envelope is in two parts made up of the subshell, oily droplets and the subshell membrane; next, the inner envelope comprising an external, granular layer and the embryophore, which in anoplocephalid tapeworms is modified into a pyriform, or pear-shaped, apparatus (Plate IIa). Finally, the third envelope, or oncospheral membrane, lies within the pyriform apparatus surrounding the oncosphere.

(Plate 11b)
The oncosphere measures 20µm in diameter and bears six hooks, 10µm in length, at the base of which are two penetration glands, described by Sinitsin (1931) and Reid (1948). Ultrastructure and development of the oncosphere have been described by Rybicka (1964; 1966). Moczon (1972) and Caley (1975) have described some of the properties of the various envelopes and membranes, and the latter also discussed the role of the pyriform apparatus in the infection of the intermediate host.

The intermediate hosts are members of the mite order Oribatei, known

FIGURE .3.

DIAGRAM TO SHOW THE STRUCTURE OF THE EGG

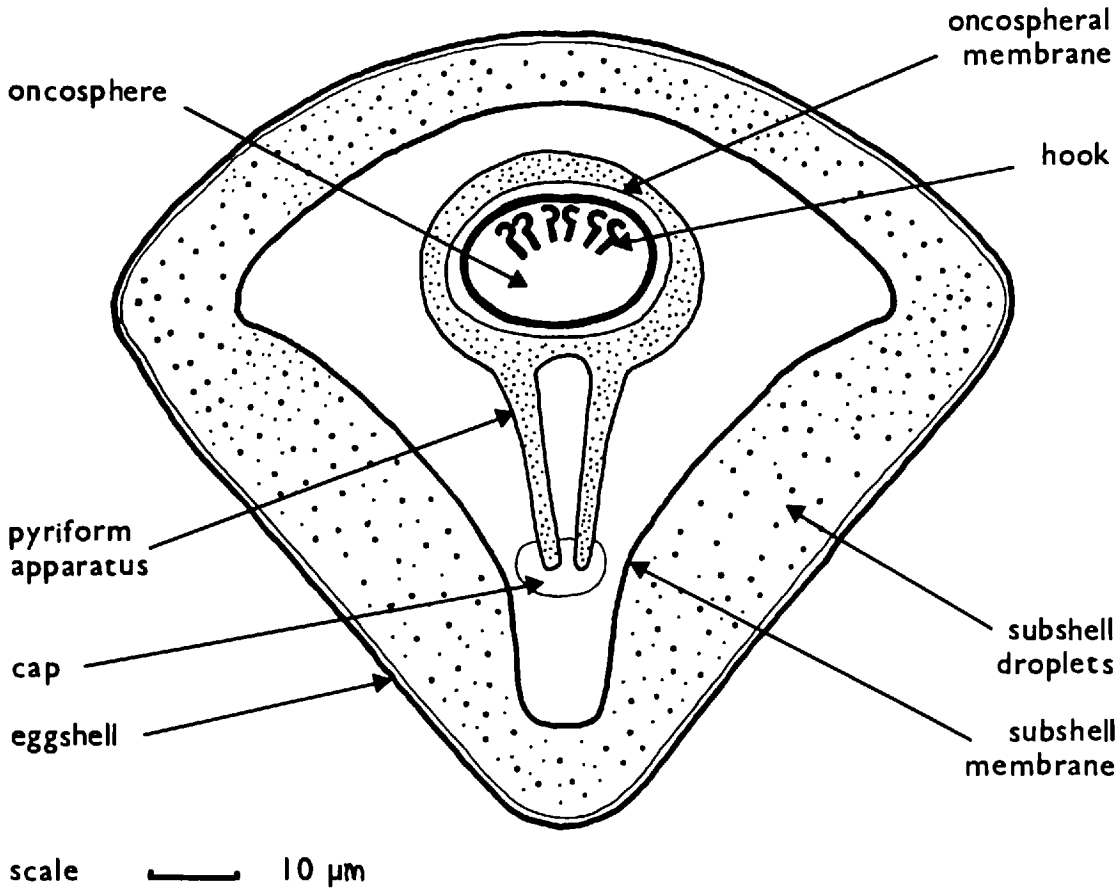
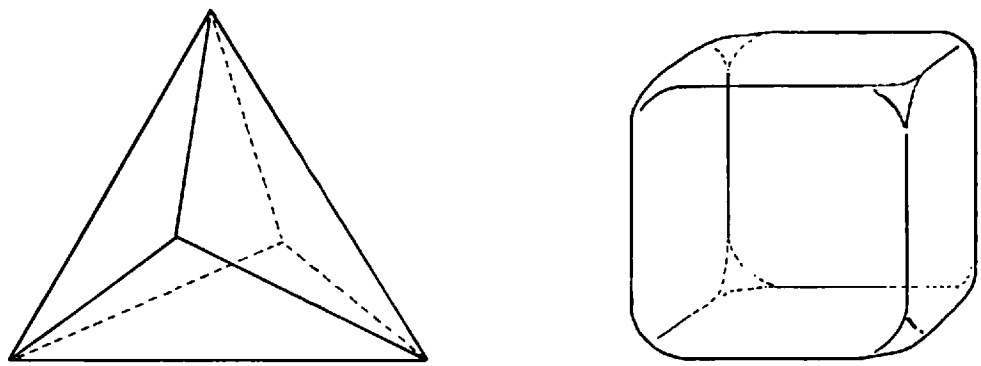


DIAGRAM TO SHOW THE SHAPE OF MONIEZIA SPP. EGGS



M. expansa

M. benedeni

PLATE I

(a) Mature eggs of Moniezia expansa: x 250.

(b) Mature eggs of Moniezia benedeni: x 250.

(c) A crushed egg, Moniezia expansa, showing contents: x 720.

(1) Ruptured egg shell containing lipid droplets.

(2) Sub shell membrane containing bacteria.

(3) Pyriform apparatus containing hexacanth oncosphere.

PLATE I

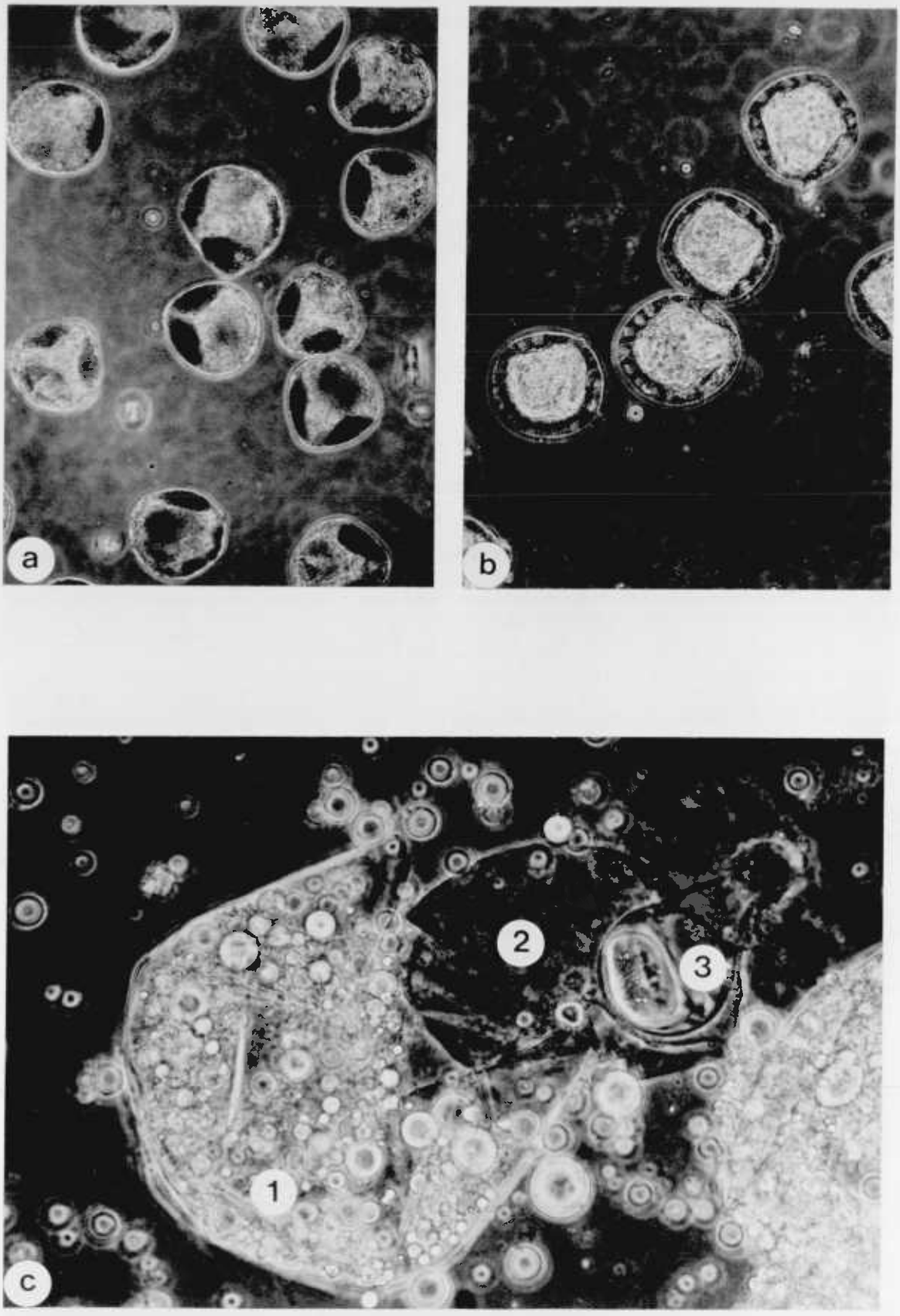


PLATE II

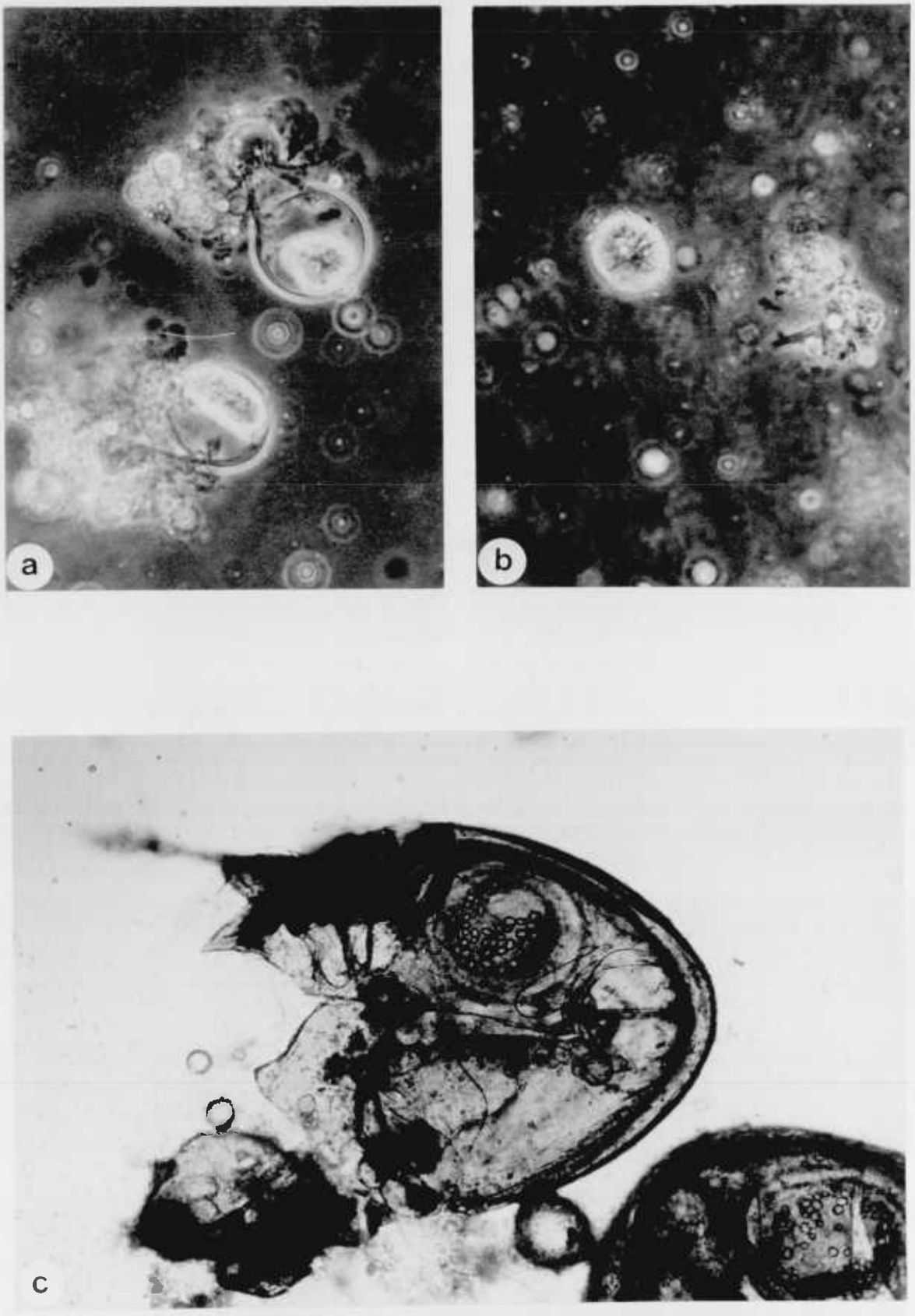
Larval stages of *Moniezia expansa*

(a) oncosphere in pyriform apparatus: x 600.

(b) hatched oncosphere: x 600.

(c) crushed mite, *Scheloribates laevigatus*, containing a cysticercoid: x 180.

PLATE II



as soil or moss mites, and many species, genera and families have now been implicated. Table II-1 lists the species and countries of origin so far described. Details of the mites and their life histories are given in Chapter II.

The mites become infected by ingesting the tapeworm eggs. Most evidence now suggests that, contrary to the work of Kassai and Mahunka (1965), the mites suck out the contents of the egg having pierced it in some way, and do not ingest it whole (Krull, 1939b; Anantamaran, 1951; Allred, 1954; Dubinina, 1958).

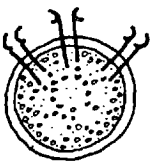
Once within the gut of the mite, the pyriform apparatus is digested by proteolytic enzymes, the oncosphere hatches from its membrane, penetrates the gut wall and enters the mite haemocoel. Here it lodges and development of the cysticercoid begins. Potemkina (1951) divided the development into convenient stages, as follows:- (1) oncosphere; (2) megalosphere; (3) metamere; (4) scolex appearance; (5) invagination; (6) larva cyst; and (7) infective larva cyst. These stages are shown diagrammatically in figure 4; and Plate III shows live stages dissected from laboratory infected mites.

Details of the development of the cysticercoid have been given by Stunkard (1938); Potemkina (1941); Anantamaran (1951); Edney & Kelley (1953); and Narsapur (1976b). Caley (1976) described the structure and ultrastructure of the mature cysticercoid.

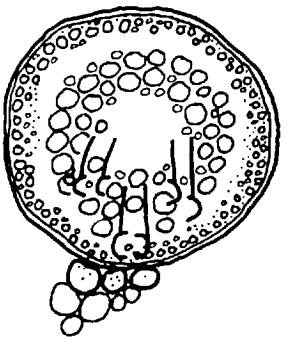
Cysticercoids take anything from 28 days (Narsapur, 1976b) to 180 days (Potemkina, 1948) to reach maturity, i.e. the infective larva cyst stage. The main factor controlling the rate of development

FIGURE .4.

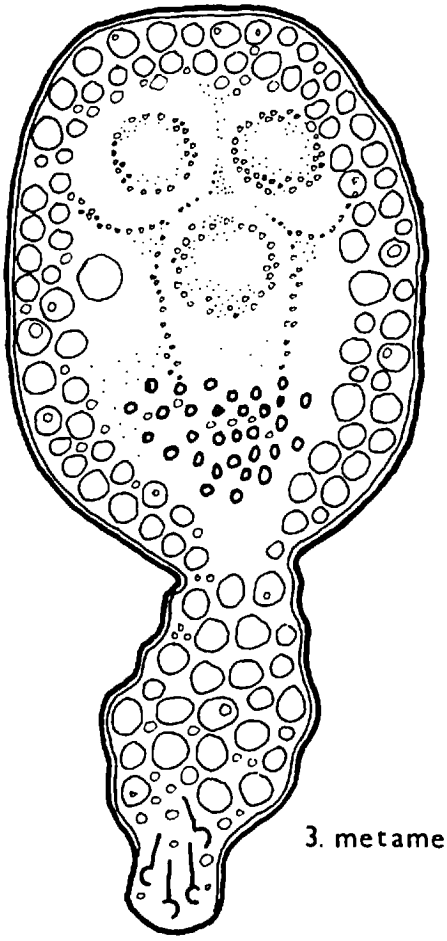
STAGES IN THE DEVELOPMENT OF MONIEZIA EXPANSA CYSTICERCOIDS



1. oncosphere

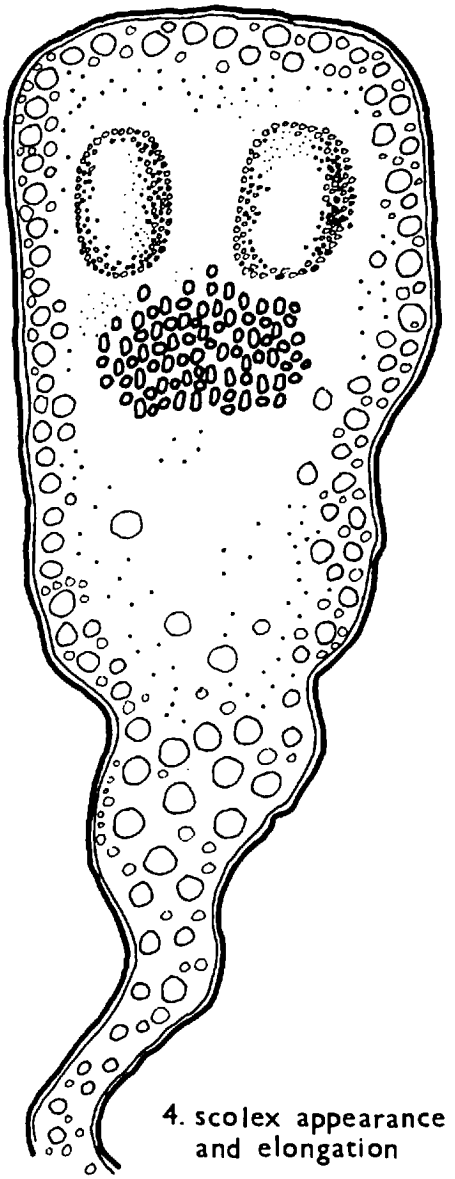


2. megalosphere



3. metamere

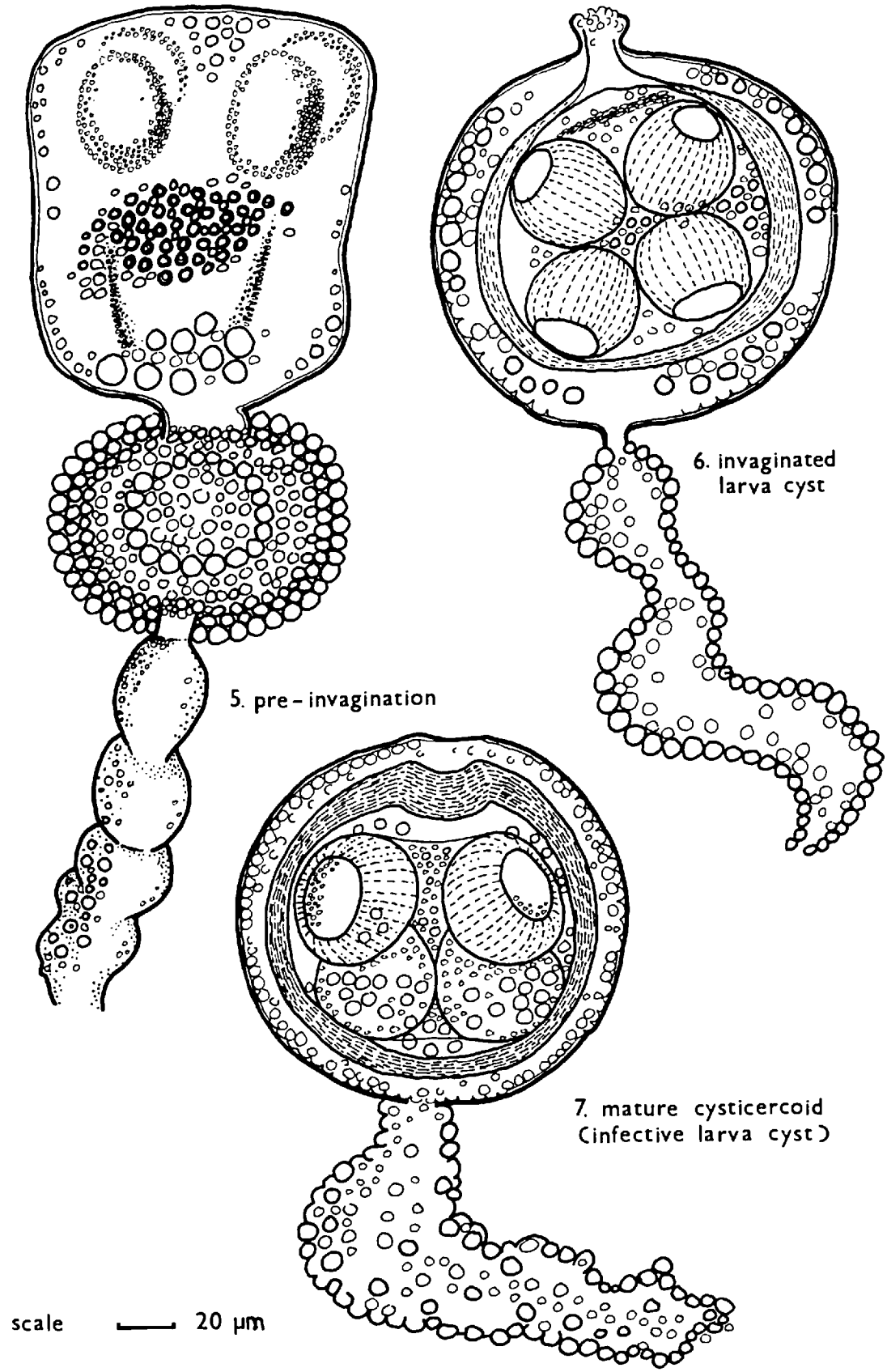
scale —— 10 µm



4. scolex appearance and elongation

(After Potemkina 1948, in Spaskij 1951.)

FIGURE 4.
continued



appears to be temperature. Several authors (Potemkina, 1948; Freeman, 1952; Anantamaran, 1951; Kuznetsov, 1970; Nazarova, 1970; and Narsapur, 1976b) have reported that cysticercoids, either in laboratory cultures or occurring naturally, develop more quickly at higher temperatures.

Cysticercoids measure between 150 μm and 200 μm in diameter, compared with the average size of the mites they infect which is 550 μm. However, mite eggs are also relatively large and thus the internal organs of the female mites are naturally capable of withstanding a great deal of compression when eggs are present. This explains why mites are apparently unaffected by the presence of as many as 20 to 30 cysticercoids in their haemocoel (Nadakal, 1960). Kates & Runkel (1948) reported a single mite containing 13 cysticercoids, and Jurasek (1962) observed a maximum of 16. Normal values are between 2 and 5 cysticercoids per mite. In such multiple infections, cysticercoids develop irregularly and different stages can be observed in the same mite, and the size of the cysticercoid is very often reduced.

It has been stated by Kates & Runkel (1948) that the presence of the cysticercoids causes cessation of egg production in the mites. Similar observations have not been recorded elsewhere, but during the current research only two out of several thousand mites examined were found to contain both eggs and cysticercoids; in both cases the mite contained a single egg and a single cysticercoid. It would appear, therefore, that egg production does, indeed, stop. It is possible that in the competition for food materials, the cysticercoids are successful and this results in the regression or expulsion of any developing eggs. A detailed investigation of the anatomy of the reproductive systems of both sexes of the mites is necessary to reveal whether, in the female, egg production is affected in any way or, in

the male, the phenomenon of parasitic castration occurs.

A full description of the life history of the mites will be given in Chapter II. However, one aspect of their behaviour - namely their daily migrations onto the grass stems whilst the cows and sheep are grazing - makes them ideally suited as intermediate hosts. The infective agents, the cysticercoids, are thus successfully transmitted to the final hosts.

The structure of the cyst wall, described by Caley (1976), protects the larva within the cysticercoid from being crushed. In favourable conditions, the wall is digested, the larva evaginates, and establishes itself in the gut of the definitive host.

CHAPTER II

THE INTERMEDIATE HOST - A REVIEW

Introduction

The complete life cycle of Moniezia expansa was not known until 1937 when Stunkard successfully infected a species of oribatid mite with the eggs of the tapeworm. Thus, an intermediate host was discovered and the theories of direct development, such as those discussed by Sinitsin (1931); Obitz (1934); and Stunkard (1934), were finally disproved. Since then, numerous species of oribatid mite have been found to serve as intermediate hosts for anoplocephalid tapeworms. A list of those species recorded in the literature is given in Table II-1.

The mites belong to a cosmopolitan order of the Acari, known as Oribatei, or Cryptostigmata. They are most frequently found in soil where they are algivorous and fungivorous or feed on decaying wood and leaves of higher plants. They range in size from 200 to 1500 μm, and are recognised as being the most heavily sclerotised of the mite orders.

Life Cycle

The life cycle of cryptostigmatid mites involves four immature stages; the egg hatches into a six-legged larva which then moults successively into the eight-legged proto-, deuto-, and trito-nymphs, and finally into the adult. The facies of the developmental stages are usually markedly different from those of the adults.

The development from egg to adult takes from 2 to 6 months depending on the species of the mite. Stunkard (1944) found that Galumna sp. developed in 65 - 94 days at 25°C; Sengbusch (1954) recorded time intervals of 60.9 days for Galumna longipluma and 87.3 days for G. ithacensis, both at 25°C. He also noted a temperature dependent rate of development - lower temperatures delayed mite growth. G. nervosus developed in 47.1 days at 25°C, but took 63 days at 20°C. Some of the so-called lower oribatid mites, such as Nothrus spp. and Platynothrus spp., exhibit parthenogenicity. Hartenstein (1962b) recorded a development time for P. peltifer of 5 months.

The average life span of the mites is 18 - 20 months. Soldatova (1945) working with Galumna obvius, Scheloribates laevigatus and S. latipes, found that they survived in culture for 19 months 14 days, 18 months 10 days, and 18 months 28 days, respectively. Potemkina (1959b) reported that mites infected with Moniezia expansa survived for 24 months, and those with M. benedeni for 18 - 19 months. No reasons were given for this difference, however.

Eggs are normally laid in the spring by the adults which then die during the following summer and autumn. Juveniles develop in the spring and summer, and overwinter by hibernating in the deeper soil layers.

Habits and Habitats

The normal habitat of the mites is the top layer of the soil, 3 to 4 cm from the surface. The mites are most abundant in rough, old pasture-land where a layer of moss and organic matter covers the soil. Culti-

vated grasslands harbour few mites, but as the vegetation builds up, so does the mite population. This process takes approximately 3 years (Rayski, 1952). Numbers of mites in a given area vary enormously (Krull 1939b; Kates & Runkel, 1948; Edney & Kelley, 1953). Hartenstein (1961), studying a woodland area, and Wallwork & Rodriguez (1961), studying pastureland, found that the mites were distributed in clumps, i.e. they had an aggregated distribution. Preferences of habitat, shown by the presence of large numbers of mites in a particular area, have been reported. Krull (1939b) described a preference for a pasture of soft textured grass shaded by conifers; Rajski (1961) for meadows rather than woods; and Jurasek (1961) reported finding mites more often near sheep folds and on plots where sheep had been kept.

Several authors have noted that mites avoid excess sunlight (Krull, 1939b; Rajski, 1958) and prefer moist habitats (Krull, 1939a; Potemkina, 1959b; Atalla & Hobart, 1964). Such observations led to the discovery of the fact that the mites execute daily migrations in response to the moisture content of the grass and soil. Authors who have discussed these migrations include Rajski (1958); Ulyanov (1961); Wallwork & Rodriguez (1961); and Kassai & Mahunka (1965). Details of the migrations are summarised below. Mites are usually found in the upper 3 - 9 cm of soil during those periods of the day when the pastures are dry and/or under extremes of heat or cold. When the pastures become damp, such as during the early morning or evening dew times or after a shower of rain, the mites migrate upwards onto the vegetation and into the top 0.5 cm of soil.

The stimulus for the migration is not precisely known. Frank (1960)

found that phototaxis was not responsible; Ulyanov (1961) proposed the theory that the increase in soil water content caused a decrease in the air content and thus the ventilation. Hence, the mites moved upwards to compensate for this lack.

This behavioural characteristic ideally suits the mites to their role of intermediate hosts and vectors for a parasite whose final host is a herbivore. These animals gain their infection by ingesting infected mites whilst they graze. The cows and sheep most often feed when the grass is damp, that is, at the same times as the mites move onto the grass stems. The infection is thus effectively transmitted from one host to the other. Krull (1939a) reported that in favourable conditions for mite migrations, sheep can ingest more than 1200 mites per pound of grass.

Mites feed on a number of food materials, including moss, lichen, hyphae and spores of fungi, decayed organic matter, and tapeworm eggs. Mites become infected with the larval stages of Moniezia by ingesting the oncospheres. Eggs of the tapeworm are manipulated by the mites and a hole pierced in the shell; the contents of the egg are then sucked out. This feeding is facilitated by the angular shape of the eggs and by their being well anchored and dry (Krull, 1939b).

Mite populations remain fairly constant with regard to species composition throughout the year, although some seasonal variation in numbers does occur (Macfadyen, 1952). Invariably, there are one or two dominant species of mite which make up as much as 70% of the total population.

It is these species which are most often found to serve as the intermediate hosts for the tapeworms in that particular area (Kates, 1965). However, even with this high incidence of potential host species, natural infection rates of the mites are low, rarely exceeding 12%. Kates & Runkel (1948) have reported typical values of 3.9% of Galumna virginiensis and 2.8% of Scheloribates laevigatus found naturally infected. Both were dominant species in an area in the U.S.A. Rayski (1952) has recorded a natural infection rate of 13.9% for Scutovertex minutus in Scotland.

Extraction and Cultivation in the Laboratory

Studies on the larval stages of Moniezia are difficult owing to this low infection rate of the intermediate hosts. Several hundreds of mites have to be examined before any detailed work can be performed on naturally occurring infections. For this reason, it has proved necessary to culture potential intermediate hosts and infect them in the laboratory. The mites are first extracted from their natural habitat, pieces of turf. Several methods have been used, but all are based on the funnel-desiccation systems of Berlese (1905) and Tullgren (1918). A heat and light gradient is set up by drying out the turf using a light bulb, or equivalent. Mites, being negatively phototropic, and to escape desiccation, move downwards through the soil and are collected in a container below.

Chambers used for culturing and rearing mites are varied in design, but all incorporate the same basic principles. The chief problems are concerned with the choice of a suitable substrate, and the maintenance of correct moisture conditions. Ideally, the system must also prevent excessive growth of contaminants, such as fungus, and must allow for the regular observation of the mites during the course of the study.

Systems used successfully include filter paper cells (Jones, 1950; Cleat, 1952); plaster-charcoal cells (Rhode, 1956; Bhattacharyya, 1962); and a dental plaster cell (Hooper, 1970). Several have used organic bases such as soil or leaf-litter (Potemkina, 1959a; Kassai & Mahunka, 1965; Graber & Gruvel, 1969; Narsapur, 1976a), dung (Rao & Choquette, 1951), or sphagnum moss (Hansen, Kelley & Todd, 1950); Edney & Kelley, 1953).

The correct moisture level, measured by the relative humidity (RH), is maintained either by the regular addition of water onto some absorbent surface such as filter paper (Hansen, Kelley & Todd, 1950; Edney & Kelley, 1953; Sengbusch, 1954; Nadakal, 1960; Littlewood, 1967; Narsapur, 1976a), or by placing the chambers on a wet base such as sand (Kassai & Mahunka, 1965; Hooper, 1970). When it is necessary to know the precise RH of the environment, a series of saturated salt solutions can be used (Atalla & Hobart, 1964). Zinc sulphate, as used by Sengbusch (1954), gives a RH of 82%.

The cultures are normally maintained at ambient room temperatures or in incubators at $20^{o}C$ - $25^{o}C$, and RH values of between 80% and 100%.

Several food preference experiments have been carried out on the mites (Bhattacharyya, 1962; Hartenstein, 1962a; Hooper, 1970). Most food materials are vegetable matter such as potato and carrot, or organic matter from the soil and leaf litter. The alga Pleurococcus, scraped from the bark of trees, is one of the more convenient foodstuffs as it is easy to obtain and is not as prone to fungal growth as the others.

Laboratory Infection

Laboratory infection of mites with Moniezia spp. has been successfully performed on numerous occasions. Eggs from gravid proglottids are smeared onto wet filter paper and offered to the mites in place of food. Infection rates of over 80% have been achieved in this way. (Narsapur, 1976a). Some authors have found more success if the eggs are mixed with sheep faeces first (Rao & Choquette, 1951; Nadakal, 1960).

Description of Species

Individual descriptions of the mite species serving as intermediate hosts for Moniezia spp. are numerous. Useful reviews on several aspects of the biology of these species are given by Rayski (1945) and Rajski (1959).

Development of Moniezia spp.

Factors affecting the development of Moniezia benedeni and M. expansa in the mite have been discussed in Chapter I. Detailed descriptions of the development stages from oncosphere to cysticercoid have been given by Stunkard (1938); Potemkina (1941); Anantamaran (1951); Edney & Kelley (1953); and Narsapur (1976b).

TABLE II-1 : INTERMEDIATE HOSTS OF MONIEZIA BENEDENI AND M. EXPANSA

Country	W/C	Species	B/E	Reference	Date
AFRICA:					
- Fort Lamy and Tchad	W	Africacarus calcaratus	a	Graber & Gruvel	1967
	W	Allogalumna pellucidi	a		and
	W	Galumna baloghi	a		1969
	WC	Scheloribates fimbriatus africanus	E		
	W	Scheloribates parvus conglobatus	a		
	WC	Scheloribates perforatus	E		
	WC	Unguizetes reticulatus	E		
ARGENTINA:	W	Zygoribatula lata	E	Yanarella	1971
AUSTRALIA:					
- Queensland	W	Zygoribatula longiporosa	B	Roberts	1953

TABLE II-1 (continued)

Country	W/C	Species	B/E	Reference	Date
CANADA:					
- East Quebec	C	Scheloribates laevigatus	E	Rao & Choquette	1951
CHINA:	W	Galumna curvum	E	Lin, Ho & Sung	1975
	W	Galumna longipluma	E		
	W	Galumna virginiensis	E		
	W	Oribatella sp.	E		
	W	Scheloribates chauhani	E		
	W	Scheloribates laevigatus	E		
CZECHOSLOVAKIA:	C	Achipteria sp.	E	Jurasek	1962
	C	Galumna sp.	E		
	C	Liacarus sp.	E		
	C	Scheloribates sp.	E		
CZECHOSLOVAKIA:	WC	Achipteria coleoptrata	BE	Prokopic	1967
- Sumava Region	W	Ceratoppia bipilis	X		
	WC	Galumna eliminata	BE		
	WC	Galumna obvius	BE		
	WC	Liacarus coracinus	BE		
	WC	Scheloribates laevigatus	BE		
	W	Scheloribates latipes	X		
	W	Trichoribates trimaculatus	E		

TABLE II-1 (continued)

Country	W/C	Species	B/E	Reference	Date
GREAT BRITAIN:					
- England	C	Euzetes globulus	E	Caley	1976
	C	Platynothrus peltifer	E		
	C	Xenillus tegeocranus	E		
- England	C	Euzetes seminulum	E	Present work	
	C	Liebstadia similis	E		
	C	Notaspis coleoptrata	E		
	C	Platynothrus peltifer	E		
	C	Scheloribates laevigatus	E		
	C	Scheloribates latipes	E		
- Scotland	W	Scutovertex minutus	E	Rayski	1952
HUNGARY:	W	Ceratozetes mediocris	X	Kassai & Mahunka	1965
	W	Pergalumna formicarius	X		
	W	Pergalumna nervosus	X		
	W	Pergalumna sp.	X		
	W	Scheloribates laevigatus	X		
	W	Scheloribates latipes	X		
	W	Scutovertex minutus	X		
	W	Zygoribatula exarata	X		

TABLE II-1 (continued)

Country	W/C	Species	B/E	Reference	Date
INDIA:	C	Galumna sp.	BE	Anantamaran	1951
	C	Scheloribates madrasensis	B		
	W	Protoschelobates sp.	BE	Nadakal	1960
- Bombay	C	Scheloribates fimbriatus	BE	Narsapur	1976a
	C	Scheloribates laevigatus	BE		
JAPAN:	W	Oribatula venusta	E	Fukui	1958
NEW ZEALAND:	W	Multoribates scheloribatoides	E	Ramsay	1966
- Wellington	W	Zygoribatula magna	E		
POLAND:	W	Achipteria coleoptrata	BE	ZyromskaRudzka	1974
- Carpathians	W	Liebstadia similis	BE		
	W	Platynothrus peltifer	BE		
	W	Punctoribates punctum	BE		
	W	Scheloribates laevigatus	BE		
	W	Scutovertex minutus	BE		
	W	Trichoribates incisellus	BE		
RUMANIA:	W	Hermaniella granulata	E	Zarzara	1971
	W	Platynothrus peltifer	E		

TABLE II-1 (continued)

Country	W/C	Species	B/E	Reference	Date
USA:	C	Galumna sp.	E	Stunkard	1937
	C	Galumna virginiensis	E	Hansen, Kelley & Todd	1950
- Kentucky	C	Galumna virginiensis	E	Edney & Kelley	1953
- Maryland and South Dakota	W	Galumna emarginata	E	Kates & Runkel	1948
	W	Galumna virginiensis	E		
	W	Oribatula minuta	E		
	W	Peloribates curtipilus	E		
	W	Protoschelobates seghetti	E		
	W	Scheloribates laevigatus	E		
- Washington	C	Galumna emarginata	X	Krull	1939a
USSR:	C	Galumna obvius	X	Soldatova	1945
	C	Scheloribates laevigatus	X		
	C	Scheloribates latipes	X		

TABLE II-1 (continued)

Country	W/C	Species	B/E	Reference	Date
USSR (continued)	C	Achipteria sp.	BE	Potemkina	1951
	C	Adoristes ovatus	BE		
	C	Galumna obvius	BE		
	C	Galumna nervosus	B		
	C	Phthiracarus sp.	b		
	C	Punctoribates sp.	BE		
	C	Scheloribates laevigatus	BE		
	C	Scheloribates latipes	BE		
	C	Trichoribates sp.	BE		
- Moscow	W	Adoristes ovatus	x	Shumakovich & Sosipatrov	1967
	W	Ceratozetes gracilis	x		
	W	Galumna nervosus	x		
	W	Hermaniella granulata	x		
	W	Platynothrus peltifer	x		
	W	Protoribates badensis	x		
	W	Punctoribates hexagonus	x		
	W	Punctoribates punctum	x		
- Amur Region	W	Scheloribates laevigatus	x	Kramnoi	1973
	W	Scheloribates latipes	x		

TABLE II-1 (continued)

Country	W/C	Species	B/E	Reference	Date
USSR (continued)					
- Armenia	C	Ceratoppia bipilis	BE	Svadzhyan	1962
	WC	Liacarus coracinus	BE		
	C	Liebstadia similis	BE		
	C	Liebstadia sp.	BE		
	WC	Scheloribates laevigatus	BE		
	WC	Scheloribates latipes	BE		
	C	Zygoribatula cognata	BE		
- Azerbaidzhan	W	Scheloribates laevigatus	x	Alieva	1970
	W	Scheloribates latipes	x		
	W	Scutovertex minutus	x		
	W	Trichoribates incisellus	x		
	W	Zygoribatula frisiae	x		
- Gorkov-skoye Region	W	Ceratoppia bipilis	x	Shaldibina	1953
	W	Eremaeus hepaticus	x		
	W	Platynothrus peltifer	x		
- Kazakhstan	W	Zygoribatula frisiae	B	Sokolova & Panin	1960

TABLE II-1 (continued)

Country	W/C	Species	B/E	Reference	Date
USSR (continued)					
- Omsk	W	Galumna obvius	BE	Al'kov	1972
	W	Galumna sp.	BE		
	W	Platynothrus peltifer	BE		
	W	Punctoribates punctum	BE		
	W	Scheloribates laevigatus	BE		
	W	Scheloribates latipes	BE		
	W	Spatiodamaeus subverticillipes	BE		
	W	Trichoribates incisellus	BE		
	W	Trichoribates trimaculatus	BE		
- Stavropol	W	Scheloribates laevigatus	x	Akbaev et al.	1975
	W	Scheloribates latipes	x		
- Tadzhikstan	W	Liebstadia similis	B	Ilyasov	1970
	W	Punctoribates punctum	BE		
	W	Zygoribatula cognata	E		
	W	Zygoribatula skryabini	BE		
- Turkman	W	Galumna flagellata	BE	Orekhov	1960
	W	Galumna type minor	BE		
	W	Scheloribates laevigatus	BE		
	W	Scheloribates latipes	BE		

TABLE II-1 (continued)

Country	W/C	Species	B/E	Reference	Date
USSR (continued)					
- Uzbekhistan	C	Scheloribates fimbriatus	B	Nazarova	1970
	W	Scheloribates laevigatus	BE		
	WC	Scheloribates latipes	BE		
	WC	Scheloribates semidesertus	BE		
	W	Scheloribates sp.	BE		
	W	Xyloribates monodactilis	BE		
	WC	Zygoribatula skryabini	BE		
- Volga	W	Carabodes sp.	B	Kuznetsov	1959b
	W	Ceratozetes sp.	BE		
	W	Galumna sp.	BE		
	W	Scheloribates sp.	BE		
	W	Scutovertex minutus	BE		
	W	Trichoribates incisellus	BE		
	W	Zygoribatula frisiae	BE		
YUGOSLAVIA:	W	Ceratozetes gracilis	E	Frank & Zivkoric	1960
	WC	Galumna nervosus	E		
	C	Galumna sp.	E		
	W	Hermaniella granulata	E		

TABLE II-1 (continued)

Country	W/C	Species	B/E	Reference	Date
YUGOSLAVIA (continued)	W	Liacarus coracinus	E	Frank & Zivkoric	1960
	W	Liebstadia similis	E		
	WC	Notaspis punctatus	E		
	WC	Pelops planicornis	E		
	WC	Protoribates laphotrichus	E		
	WC	Protoribates seghetti	E		
	WC	Punctoribates punctum	E		
	WC	Scheloribates laevigatus	E		
	WC	Scheloribates latipes	E		
	W	Scutovertex minutus	E		
	W	Protoribates laphotrichus	E	Rukavina et al.	1960

NOTES: W = natural infection

C = experimental infection

B = host for M. benedeni

E = host for M. expansa

[a = host for Stilesia globipunctata

b = host for Thysaniezia giardi

mite species quoted with references to hosts for Moniezia.]

x = host species (Moniezia)not specified

CHAPTER III

THE LABORATORY CULTURE AND INFECTION OF THE INTERMEDIATE HOST

Introduction

Very little work has been carried out on the potential intermediate hosts of Moniezia benedeni and M. expansa in England. As previously described (Chapter II), the intermediate hosts are species of oribatid mite, normally found in the soil and moss of grassland areas. Because natural infection rates of the mites with the tapeworm larvae are low, methods for culturing and infecting the mites in the laboratory were investigated. Further studies could then be performed using the developing cysticercoids dissected from the mites.

Materials and Methods

Oribatid mites were obtained from turf collected from an old, well-established pastureland in Berkshire, Southern England. Care was taken to ensure that the land had not been used for grazing animals for a number of years to avoid the chance of mites being already infected with cysticercoids. Turfs were set up in a Kempson bowl extractor, after the method of Kempson, Lloyd & Gelhardi (1963) and left for up to 10 days. Mites were collected live on wet filter paper placed in the bottom of the bowls. Every 2 or 3 days oribatid mites were removed from the extractor and transferred to culture chambers where they were maintained at 25°C and 100% relative humidity. Culture chambers consisted of 9 cm diameter petri dishes filled with absorbent wadding material, such as cotton-wool, and lined with filter

paper. Mites were fed on a suspension of the alga Pleurococcus which was scraped from the bark of trees. The culture chambers were kept moist by the daily addition of a few drops of water, and were kept in the dark in an incubator at 25°C. It was found necessary to replace the filter paper lining of the chambers every 3 or 4 weeks to avoid fungal contamination, and to remove dead mites and faecal waste. The chambers proved satisfactory for maintaining up to 400 mites. They also allowed for the easy observation and identification of the mites when necessary.

Eggs of M. benedeni and M. expansa were obtained from strobilae of the tapeworms removed from lambs slaughtered at Manchester abattoir. Ripe proglottids were broken up using a vortex homogeniser to release the eggs, which were then filtered and washed in water. Mites were infected by allowing them to feed on the eggs of Moniezia. The mites were first starved for 7 to 10 days and then offered a suspension of the tapeworm eggs which had been smeared on the filter paper in the culture chambers. After 3 days mites were transferred to new culture chambers and fed as normal.

The infections were monitored by the periodic dissection of some of the mites. Heavily sclerotised mites could be examined by placing them in a drop of physiological saline on a slide and gently crushing them with a cover slip. Controlled pressure broke open the mites and revealed the contents without damaging them (Plate 11c). Softer bodied mites were first dissected using fine mounted needles. Records were made of host species; age of infection; and number and stage of development of any cysticercoids.

Only mites measuring more than 0.5mm, the average size for species

acting as intermediate hosts, were used for the infection experiments. Quantitative results were recorded for M. expansa only, as eggs of this species were easily available. Experiments involving M. benedeni were recorded by a positive host infection.

Before the mites could be identified, they were first cleared, or macerated, in a 50% aqueous solution of lactic acid. This treatment effectively bleached the mites so that diagnostic features, such as chaetae, could be seen. The mites were transferred to a small amount of the lactic acid on a cavity slide, and covered with a cover slip. The preparation was then warmed gently on a warming plate until the specimen was sufficiently cleared.

Mites were identified to species level using the keys given by Willmann (1931).

Results

The following 13 species of oribatid mite, belonging to 12 genera and 8 families, were identified from the turf samples:-

Hypochthonius rufulus	- Hypochthoniidae
Platynothrus peltifer	- Camisiidae
Nothrus palustris	- Camisiidae
Belba pulverulenta	- Belbidae
Oppia clavipectinata	- Eremaeidae
Liebstadia similis	- Oribatulidae
Scheloribates laevigatus	- Notaspididae
Scheloribates latipes	- Notaspididae
Euzetes seminulum (globula)	- Notaspididae
Punctoribates (Minunthozetes) semirufus	- Notaspididae

Notaspis (Achipteria) coleoptratus	-	Notaspididae
Pelops occultus	-	Pelopsidae
Steganacarus magnus	-	Phthiracaridae

Where synonyms for generic or specific names are in common use, they are given in parentheses.

Table III-1 summarises the results of the experiments involving Moniezia expansa.

Table III-2 gives the frequency distribution of the cysticercoids.

TABLE III-1 INFECTION OF ORIBATID MITES WITH MONIEZIA EXPANSA

	No. of mites collected	% of total population	No. of mites dissected	No. of mites infected	No. of cysts	% of infected mites	Average no. of cysts per mite
H. rufulus	3	0.04	3	0	0	0	0
P. peltifer	218	3.02	108	3	3	2.8	1.0
N. palustris	76	1.05	31	0	0	0	0
L. similis	249	3.45	88	29	49	33.0	1.7
S. laevigatus	3205	44.40	1509	513	767	34.0	1.5
S. latipes	926	12.83	441	79	104	18.0	1.3
E. seminulum	343	4.75	238	1	1	0.4	1.0
N. coleoptratus	1991	27.59	804	8	9	1.0	1.1
P. occultus	200	2.77	76	0	0	0	0
S. magnus	6	0.08	6	0	0	0	0
Overall totals	7217	-	3304	633	933	19.2	1.5

The maximum number of cysticercoids removed from a single mite was 7, dissected from Scheloribates laevigatus.

TABLE III-2 FREQUENCY DISTRIBUTION OF M. EXPANSA CYSTICERCOIDS IN MITES

Species	No. of mites with cysts								Total no. mites dissected	Total no. mites infected	Total no. cysts
	0	1	2	3	4	5	6	7			
P.peltifer	105	3	0	0	0	0	0	0	108	3	3
L.similis	59	16	9	1	3	0	0	0	88	29	49
S.laevigatus	996	346	108	41	13	1	3	1	1509	513	767
S.latipes	362	60	16	1	1	1	0	0	441	79	104
E.seminulum	237	1	0	0	0	0	0	0	238	1	1
N.coleoptratus	796	7	1	0	0	0	0	0	804	8	9

Infection of oribatid mites with Moniezia benedeni

The following species of oribatid mite were successfully infected with M. benedeni:

Scheloribates laevigatus

Scheloribates latipes

Platynothrus peltifer

The development of the cysticercoids in the mite intermediate host was similar to that described by previous workers (Stunkard, 1938; Anantamaran, 1951; Potemkina, 1951; Edney & Kelley, 1953; Narsapur, 1976b). Growth was irregular; individual cysticercoids developed at different rates. Mature cysticercoids were found as early as 28 days, and as late as 84 days post infection. Stages in the development corresponding to those described by Potemkina (1951) are shown on Plate III.

Discussion

The 13 species of oribatid mite listed above are all common inhabitants of moss and soil. It is difficult to compare the list with others recorded for similar areas as the habitats, although all designated as "grassland", differ widely in their characteristics such as soil-type, pH etc. However, it is of interest to note that of the 13 species identified from the rough pastureland investigated, 7 are common to those listed by Rayski (1952) for a piece of "waste-land" adjacent to a field; and 5 are common to those listed by

PLATE III

Developing cysticercoids of Moniezia expansa: stages dissected from mites.

(a) Day 7 x 250.

Increase in size to form megalosphere.

(b) Day 14 x 250.

Increase in size and elongation.

(c) Day 28 x 350.

Elongation and development of tail appendage.

(d) Day 35 x 300.

Continuation of above, with appearance of calcareous corpuscles.

PLATE III.1

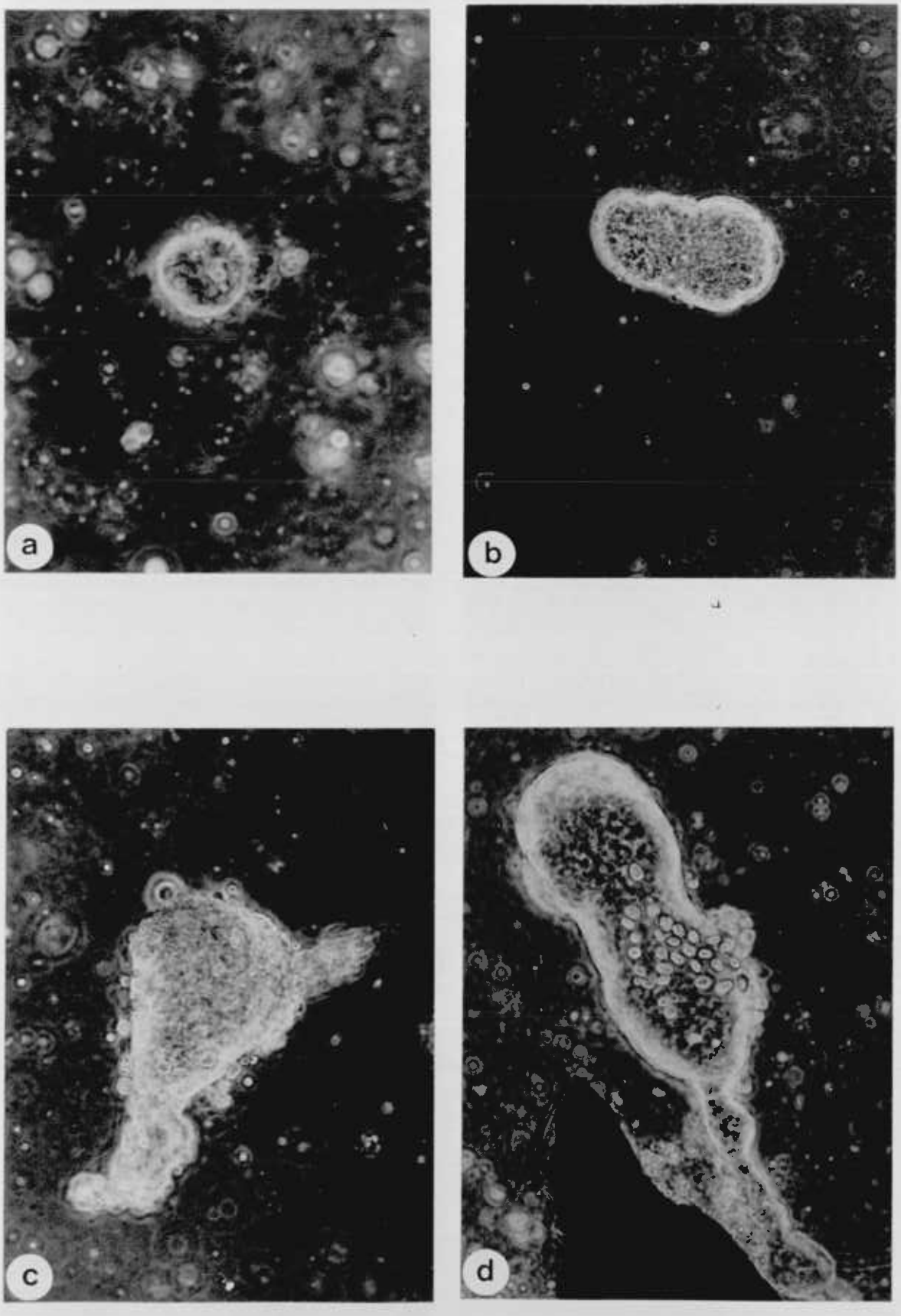


PLATE III - continued

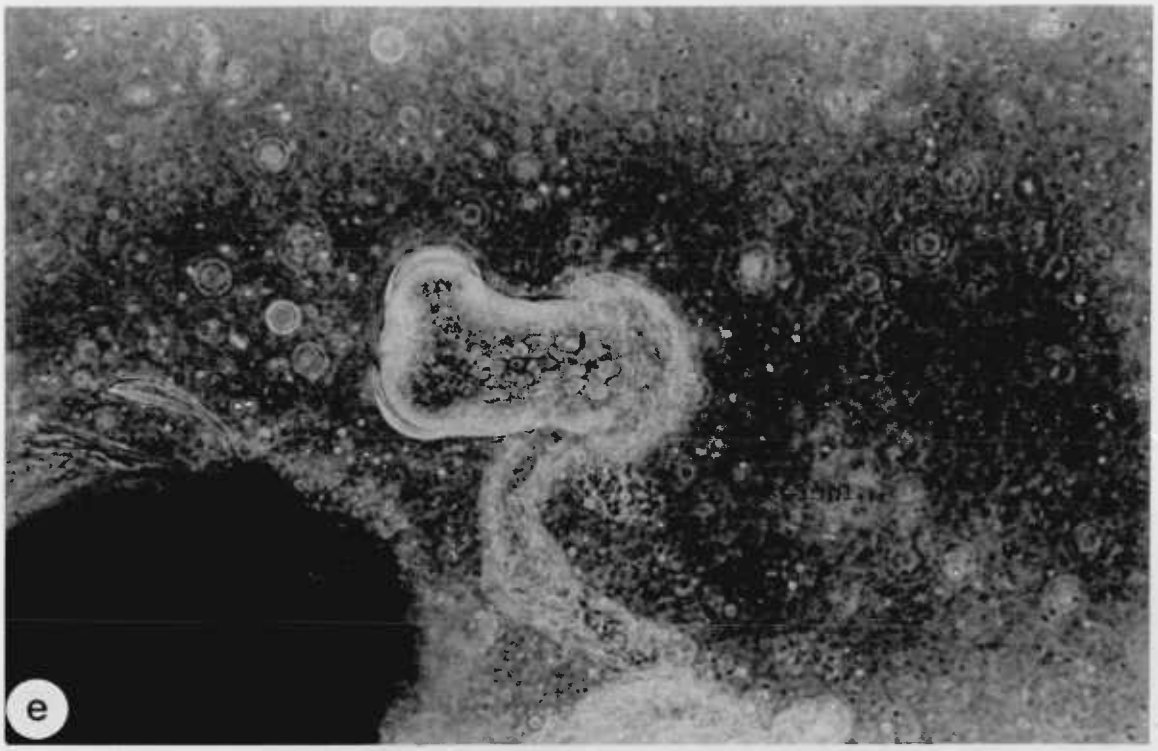
(e) Day 42 x 300.

Development of scolex.

(f) Day 49 x 300.

Increase in size to pre-invagination stage.

PLATE III.2



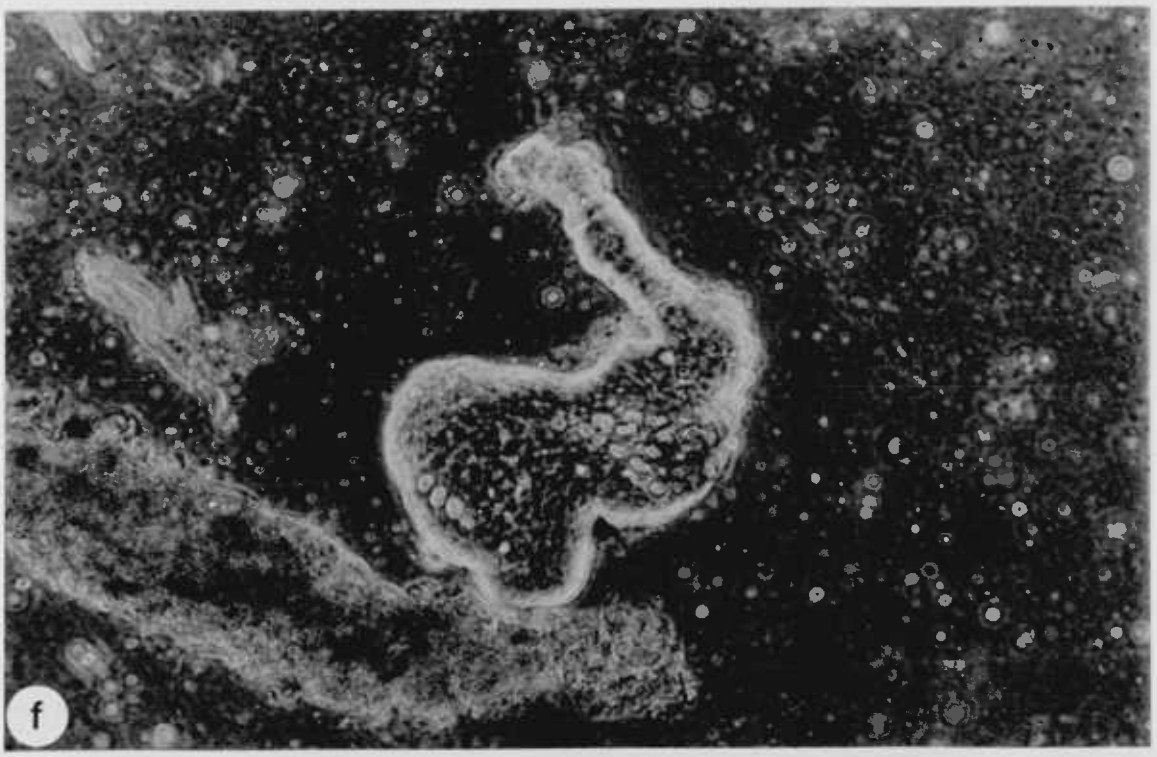


PLATE III - continued

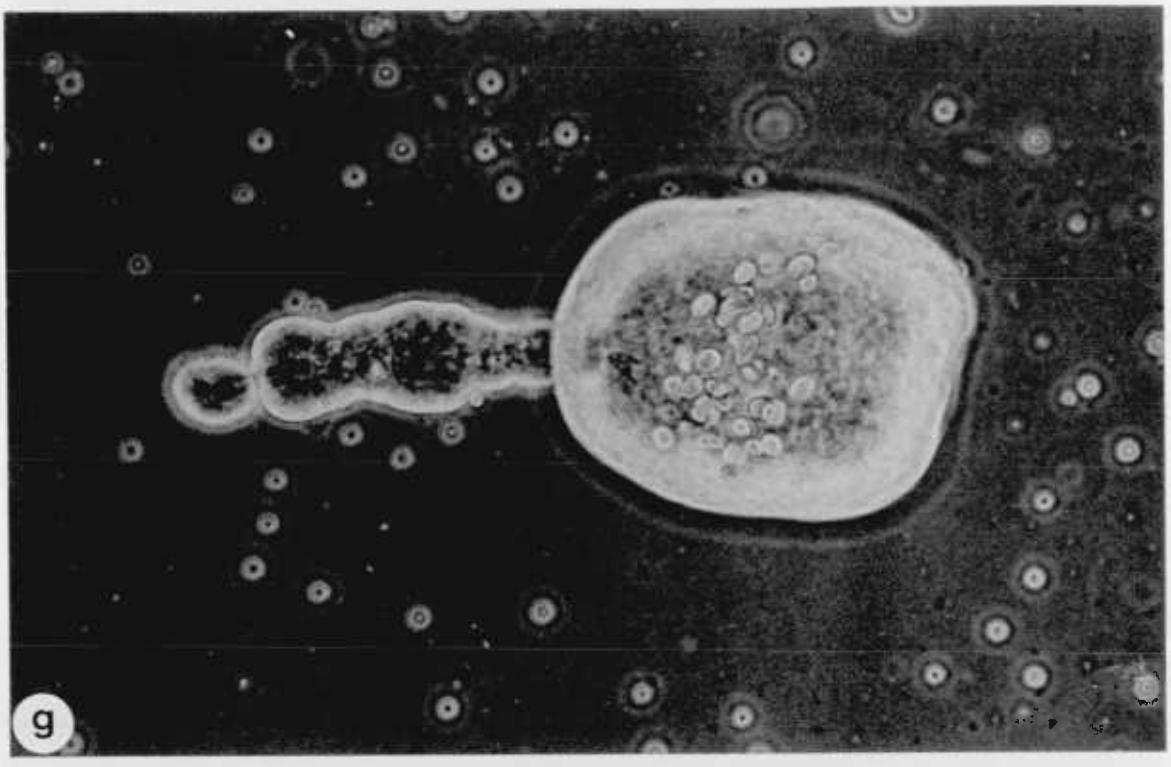
(g) Day 56 x 480.

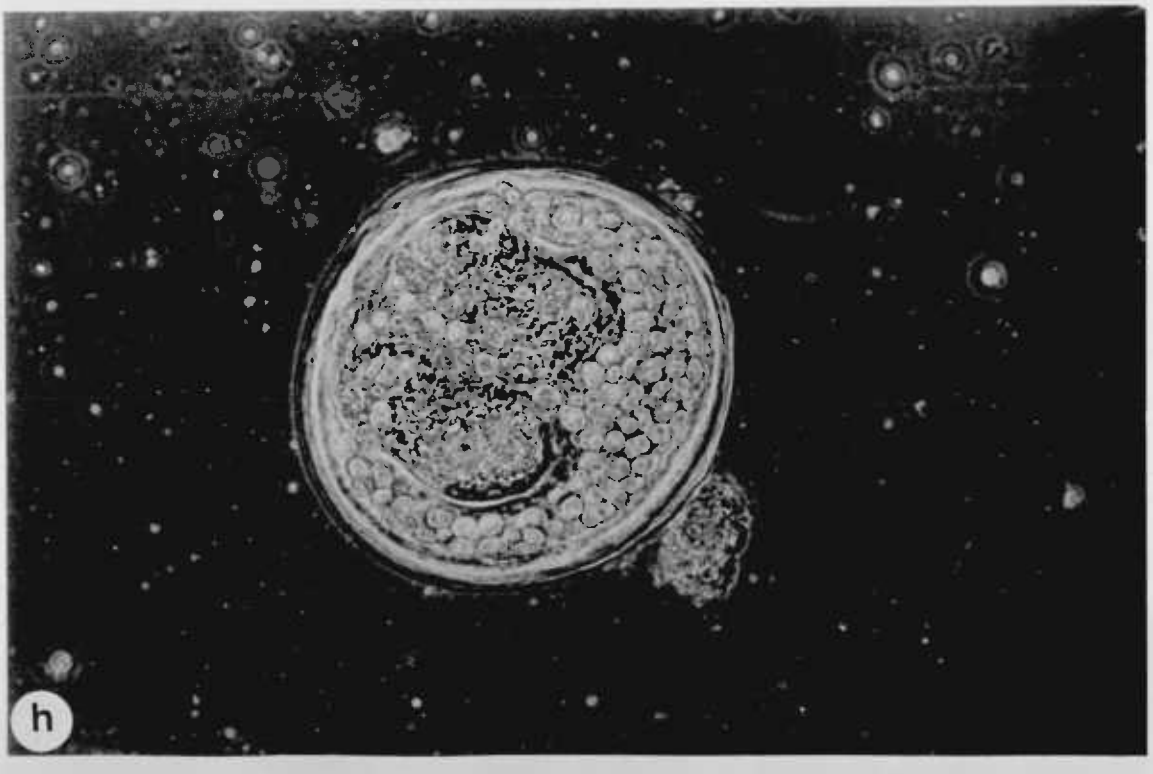
Post-invagination, forming larva-cyst.

(h) Day 63 x 300.

Mature larva-cyst, or cysticercoid, showing outline of suckers and many calcareous corpuscles within cyst wall.

PLATE III.3





Sheals (1957) for "grassland, with a mineral soil".

The population structure of the mites remained fairly constant throughout the duration of the investigation. Apart from two species - Hypochthonius rufulus and Steganacarus magnus, of which less than 10 specimens were collected - all the species were found in the same average proportions. Two dominant species were identified, Scheloribates laevigatus and Notaspis coleoptratus, which together formed over 70% of the total number of mites collected.

Of the 10 species investigated for their potential as intermediate hosts for M. expansa, 6 species were successfully infected. No new hosts are recorded; one species, Pelops occultus, previously known to act as a vector, was not infected during these experiments. The two species of Scheloribates, S. laevigatus and S. latipes, and Liebstadia similis were most frequently infected, having infection rates of 34%, 18% and 33% respectively. Scheloribates is a cosmopolitan genus and several species, of which S. laevigatus occurs most often, have been implicated as intermediate hosts for M. expansa in many countries. (Table II-1)

Three of the species infected with M. expansa were also found to serve as intermediate hosts for M. benedeni. They are Scheloribates laevigatus, S. latipes and Platynothrus peltifer. They do not, however, necessarily represent the complete list of species which can be infected with cysticercoids of M. benedeni. A comprehensive investigation, similar to that performed for M. expansa, was not possible with this other species as supplies of the eggs for the infection experiments were limited.

The potential of a particular mite species to act as intermediate host is not always realised. Many of the experimentally infected mites (designated by "C", Table II-1) have not been subsequently confirmed as such in the wild (designated by "W", Table II-1). Kates (1965), in his review of helminth transmission, also emphasises the point that quite often there is only a single, or primary, mite species acting as vector in a particular habitat. This is perhaps due to some behavioural differences, such as a food preference, which make one mite more likely to ingest tapeworm eggs than another. Such behaviour is probably suppressed by the uniformity imposed by laboratory culture. However, used in conjunction with investigations on naturally infected mites, artificial infections provide a valuable means of studying the development of the larval tapeworms.

Summary

The oncosphere to cysticercoid stage of the life cycles of Moniezia benedeni and M. expansa was reproduced in the laboratory. The intermediate hosts, species of Oribatid mite, were extracted from turf, infected with tapeworm eggs, and maintained in culture for several months. Cysticercoids developed in the mites within 6 to 10 weeks at a temperature of 25°C and relative humidity of 100%.

Details of the oribatid fauna of a pastureland in Southern England are given, together with infection rates for those species which were found to act as intermediate hosts.

CHAPTER IV

A SEARCH FOR AN ALTERNATIVE INTERMEDIATE HOST

Introduction

Several species of oribatid mite, the natural intermediate host of Moniezia, have been successfully infected and cultured in the laboratory, as described in Chapter III. However, the techniques involved were often laborious and did not prove satisfactory for producing large numbers of cysticercoids. Attempts were made, therefore, to find an alternative host which could be conveniently and easily maintained in the laboratory.

Materials and Methods

Eggs of Moniezia expansa, obtained from ripe proglottids, were washed and kept in physiological saline until required. The following insects were maintained in the laboratory: the flour beetle, Tribolium confusum; the cockroach, Periplaneta americana; and the locust, Locusta migratoria migratoides.

Two methods were used to infect the insects: (1) direct feeding of whole eggs; and (2) injection of partially hatched eggs into the haemolymph. Starved T. confusum readily ingest hymenolepid tapeworm eggs, and a similar technique was used for the eggs of M. expansa. Adult T. confusum were starved for 10-14 days and then given M. expansa eggs which had been smeared on filter paper. The eggs were left for 3 or 4 days during which time the beetles were observed feeding on them. After 4, 6 and 8 weeks samples of beetles were dissected and examined for developing cysticercoids.

Before oribatid mites become infected they first pierce the egg, suck out the contents containing the oncosphere, which then is released from the pyriform apparatus by enzymes and finally settles in the haemolymph. The second method involved injecting the eggs straight into the haemocoel of the other insects. The egg shells, however, had first to be removed as they are not affected by enzymes and their presence would have restricted any development of the oncosphere. Eggs were, therefore, crushed in a glass homogeniser or tissue grinder to release the contents and the suspension so formed was injected in 50µl volumes into the insects through the arthroidial membranes. A second group of insects - cockroaches and locusts - were injected with a suspension to which enzymes had been added to digest the pyriform apparatus. This overcame the possibility of the correct enzymes for this purpose not being present in the insects. Samples of haemolymph were extracted from the insects after 4, 6 and 8 weeks to check for the presence of developing cysticercoids. After 10 weeks the insects were sacrificed and the total volume of the haemolymph examined.

Results and Discussion

None of the insects was found to harbour developing cysticercoids at any time throughout the experiment. Other experiments have similarly failed to infect non-acarine hosts with *M. expansa*. Stunkard (1937; 1938) tried unsuccessfully to infect ants and some unspecified insects during his experiments to find the true intermediate host. Success was also limited with various species of non-oribatid mite. Stunkard (1938) using *Rhizoglyphus* managed to recover oncospheres from the body cavity, but no development occurred. Rao & Choquette (1951), however, tried tyroglyphids, *Uropoda sp.*, *Parasiticus sp.* and *Hypoaspis sp.*

without any success. One species of anoplocephalid has, however, been developed in insects: Svadzhian (1963) infected psocids with Thysaniezia giardi.

All the published unsuccessful experiments have, to date, relied on the prospective host feeding on the eggs. As has been suggested here, this in itself is not always sufficient in releasing the oncosphere and development may be prevented. It was hoped that by injecting a suspension of hatched oncospheres such problems would be overcome and a host suitable for laboratory maintainance, which was easier to culture than the oribatids, could be found. However, this experiment would suggest that the haemolymph of insects does not provide a suitable environment for developing cysticercoids.

Summary

Attempts were made to find an alternative intermediate host for Moniezia expansa in the laboratory. Three species of insect were chosen because of the ease and convenience with which they can be maintained in the laboratory. The species were

(1) the flour beetle, Tribolium confusum;

(2) the cockroach, Periplaneta americana; and

(3) the locust, Locusta migratoria migratoides.

No development was observed in any of the species investigated.

CHAPTER V

IN VITRO HATCHING OF THE EGGS OF MONIEZIA BENEDENI AND M. EXPANSA

Introduction

The eggs of Moniezia are composed of a series of membranes and envelopes (figure 3), all of which have to be removed to effect hatching of the oncosphere. Caley (1975) successfully hatched the eggs of M. expansa using a combination of mechanical and chemical treatments. However, oncospheres thus produced were not sterile, a prerequisite for in vitro culture. Therefore, a system was devised which would allow for the production of sterile oncospheres.

Materials and Methods

General techniques

All apparatus and glassware was sterilised by autoclaving before use. Sterile procedures were carried out in a Lamina flow Cabinet which was regularly cleaned and swabbed with ethanol.

Solutions were sterilised by vacuum filtration through a 0.2µm millipore filter and stored at 4°C.

Two types of balanced salt solution (BSS) were used during the experiments, either Hank's or Earle's. The composition of these is given in Appendix I.

Unless otherwise stated, experiments were performed at room temperature.

Eggs

Tapeworms were obtained from the intestines of lambs slaughtered at Manchester abattoir. Gravid proglottids were examined microscopically to ascertain the maturity of the eggs. The criteria chosen for this were:

(1) characteristic shape, i.e. triangular hexahedron for M. expansa and rectangular hexahedron for M. benedeni (immature eggs are spherical); and

(2) localisation of the oily droplets at the edges of the egg (figure 3).

Gravid proglottids were then homogenised using a vortex-type homogeniser at slowest speed to release the eggs. After filtering to remove the tissues, the eggs were washed thoroughly in a sterile balanced salt solution to which antibiotics (streptomycin and penicillin at 100 U/ml) had been added.

When necessary, eggs were stored in Universal containers (Sterilin) at 4°C. for a maximum of 10 days without apparent reduction in viability. For use in experiments, the eggs were allowed to equilibrate to room temperature.

Each process was performed using eggs of both M. benedeni and M. expansa.

Hatching

The hatching process can be divided into two parts: (1) breaking and/or removal of the egg shell and subshell membrane; and (2) removal of the pyriform apparatus and oncospheral membrane.

(1) The egg shell of Moniezia is impermeable and resists attack by enzymes (Caley, 1975). The simplest method of releasing the contents involves crushing the eggs until they break. For in vitro culturing purposes this mechanism must allow for clean and sterile oncospheres. The following techniques were employed.

(a) Grinding and crushing the eggs in glass homogenisers.

1 ml of egg suspension was placed in either a Griffith's tube or tissue grinder and ground with 2 ml BSS for approximately 2 minutes.

(b) Grinding and crushing with glass beads.

Eggs were treated as described by Berntzen & Voge (1965) for removal of the shell.

(c) Sonication.

3 ml of egg suspension in 1 x 2" glass specimen tubes were subjected to sonication at a maximum amplitude of 8 microns for time intervals varying from 15 to 120 seconds. The tubes and their contents were kept bathed in ice to counteract the heating effect of the sonicator, for the duration of the treatment.

(d) Sodium hypochlorite.

1 ml of egg suspension was mixed with a solution of sodium hypochlorite (NaOCl) to give final concentrations of 0.1; 0.5; 1.0; 5.0; and 10.0% NaOCl, for varying time intervals. The suspension was then thoroughly washed in fresh sterile BSS.

(2) Based on the results of Caley (1975), sterile solutions of Chymotrypsin (Sigma[1]) or Pancreatin (Sigma[2]) at concentrations of 0.1% in a BSS pH 7.2, were used for the digestion of the pyriform apparatus. 2-3 ml of the egg suspension from the earlier part of the experiment, i.e. eggs without their shells or subshell membranes, were added to the enzyme solution and left for up to 60 minutes. Observations were made at regular intervals to monitor the digestion of the pyriform apparatus. This treatment caused activation of the oncosphere and the hatching process was completed by the swimming movements of the hooks which released it from the oncospheral membrane.

To minimise contamination of the final culture suspension, it proved necessary to attempt to remove debris in the form of broken fragments of the egg shell, unbroken eggs, and oily droplets. These last were removed by washing well in sterile BSS. To remove the rest, several filtration techniques were tried; the most effective employed a Gooch crucible, pore size 1, through which the suspension was allowed to filter under gravity. Oncospheres, which measure approximately 20µm, were able to pass unharmed through this and were collected in sterile BSS in fresh Universal containers.

Hatched oncospheres were then available for culture.

FOOTNOTES: 1 = Trypsin - Type II: Crude from Hog Pancreas. Activity: 1,000 - 1,500 BAEE units/mg. Chymotrypsin: Activity 500-1,000 ATEE units/mg.

[2] = Pancreatin Grade VI. From Hog Pancreas. Activity Equivalent to 4 x National Formulary (N.F.)

Results

Hatching

(1) Breaking and/or removal of the egg shell and subshell membrane.

(a) Grinding and crushing the eggs in glass homogenisers:-

Both types of homogeniser were found to be equally efficient for breaking open the eggs of both species of Moniezia. Between 80% and 90% of eggs were broken in this way.

(b) Grinding and crushing with glass beads:-

This process proved unsatisfactory for breaking the eggs of either species. Less than 5% were successfully broken even after prolonged treatments.

(c) Sonication:-

Sonication did result in the breaking of eggs of both species. However, time intervals producing sufficient numbers of broken eggs, i.e. >30%, proved too disruptive and the contents of the eggs were also destroyed. As a satisfactory balance could not be achieved, this method was not pursued.

(d) Sodium Hypochlorite:-

Treatment with NaOCl resulted in the removal of the egg shells. This, followed by thorough washing in sterile BSS, apparently left the oncospheres unaffected, judged on the basis of their continued motility, even though NaOCl is known to be highly toxic (Laws, 1967). This process produced different results

for the two species. For M. expansa a concentration of 5% NaOCl for 15 minutes proved successful in removing 90% of the egg shells. For M. benedeni a concentration of 20% was required to remove the same proportion of egg shells in a similar time.

(2) Removal of the pyriform apparatus and oncospheral membrane. Both Pancreatin and Chymotrypsin proved equally satisfactory in digesting the pyriform apparatus and activating the oncosphere. The process was 100% efficient for both M. benedeni and M. expansa after a treatment lasting 15 minutes.

Discussion

The following criteria were decided upon as a means of finding a suitable system for hatching the eggs of M. expansa and M. benedeni as a preliminary to in vitro culture.

(1) Oncospheres must be produced in sufficient numbers for culture.

(2) Oncospheres must be clean and sterile.

(3) Oncospheres must be processed as quickly as possible to reduce effects such as loss of viability, and to comprise a minimum of stages to avoid chances of contamination.

Initially, NaOCl treatment seemed to suit all these criteria. NaOCl acts by attacking the di-sulphide bonds in the shell (Caley, 1975), allowing it to be digested completely. The subsequent washing removes the oily droplets, thus eliminating the need for a further filtration stage. However, over 75% of the eggs treated in this way retained the subshell membrane which encloses the oncosphere and pyriform apparatus.

Although the membrane has been shown to have some permeability (Caley, 1975) it was thought that in culture it could act as a barrier to high molecular weight compounds in the media, and therefore inhibit development of the oncospheres. With this in mind, the NaOCl treatment was not used.

The system found to be most suitable was as follows:-

(1) Eggs suspended in a 0.1% solution of pancreatin in sterile BSS, pH 7.2, were crushed in a glass homogeniser. The contents of the egg thus come into immediate contact with the enzyme. The pyriform apparatus is digested, and the activated oncospheres hatch.

(2) The new suspension, containing the oncospheres, is filtered through a sterile Gooch crucible to remove debris.

(3) The filtered oncospheres are washed, several times, in the culture medium about to be tested.

Thus, the oncospheres could be efficiently processed in as short a time as possible.

In general, both species of Moniezia were found to have the same requirements for successful hatching, and eggs of both species were hatched by the methods described above. However, during the early experiments, slight differences were noted between the two. M. benedeni appeared to have a tougher and more resistant egg than M. expansa. This was shown most clearly by the NaOCl treatment where a higher concentration was required to achieve the same result. Also,

in the mechanical methods of breaking the eggs, M. benedeni appeared to have the stronger egg and consequently smaller proportions of shells were broken each time.

The other main difference between the eggs of the two species is their shape. M. expansa has a hexahedral egg of triangular section, whereas M. benedeni has a hexahedral egg of rectangular section (Plate I a and b). Such angular shapes are unusual and occur much less frequently than the normal spherical cyclophyllidean egg. It is probable that such shapes are more easily manipulated by the mite intermediate host as it pierces the egg and sucks out the contents. To date, there is no record of any rigid host specificity - mites of several species can become infected by either species of Moniezia. The differences in shape, therefore, are not related to this event in any way; they are related to the requirements of the oncosphere rather than the potential cysticercoid.

Once the eggs are on the ground, having been voided with the faeces, they are subject to the full range of external environmental conditions. Whilst still in wet faeces they can survive for up to 40 days (Potemkina, 1951) but are vulnerable, when unprotected, to extremes of heat and cold and, in particular, to desiccation (Potemkina, 1951; and Kuznetsov, 1959d). The two species of Moniezia differ in their seasonality. M. benedeni eggs are found on the pasture much later in the year than those of M. expansa and hence are prone to harsher environmental conditions. M. benedeni, therefore, has a tougher egg with a thicker shell. The rectangular-shaped egg more closely

resembles a sphere than the triangular one and can therefore withstand greater all-round pressures. In this respect, it is a stronger structure, and thus the eggs of M. benedeni are suited to their different life history.

Summary

As a preliminary to the in vitro culture of the oncosphere to the cysticercoid stage, eggs of Moniezia were hatched in sterile conditions based on the techniques described by Caley (1975). Eggs obtained from ripe proglottids were first checked to ascertain their maturity and then subjected to various processes to effect hatching. Differences between the two species, M. benedeni and M. expansa, as characterised by their eggs, are discussed.

CHAPTER VI

INTERSPECIFIC ASSOCIATIONS : HELMINTHS AND MICRO-ORGANISMS

Introduction

During initial attempts to culture the cysticercoid of Moniezia expansa from the oncosphere, in vitro, heavy bacterial contamination of the media occurred. Stringent sterile procedures and the use of normal antibiotics, such as streptomycin and penicillin, failed to eliminate or even reduce this growth. Close examination of the eggs revealed, unexpectedly, the presence of large numbers of bacteria between the subshell membrane and pyriform apparatus (Figure 3; Plate I.c). Using standard microbiological and biochemical methods the bacteria were identified. Experiments were then performed to find an effective antibacterial agent to control the contamination.

Materials and Methods

Eggs obtained from ripe proglottids were washed thoroughly in sterile physiological saline (0.85% NaCl) for 10-15 minutes to remove any bacteria adhering to the outside of the shell. They were then crushed in a sterile glass homogeniser to release the contents. Drops of this suspension were examined microscopically to find the precise location of the bacteria in the egg. Sections of the worm containing immature and developing eggs were also examined for the presence of bacteria.

Isolation and Purification

The general procedure is outlined below. Standard microbiological and biochemical methods, described by Cowan & Steel (1965), were employed throughout. Details of media recipes and formulae are given in Appendix II.

The crushed egg suspension was diluted x 10 with physiological saline and, from this, cultures were set up on blood agar plates or in nutrient broth, either aerobically at 4°C, room temperature, or 37°C, or anaerobically at 37°C. The media used were non-specific and promoted growth of all types of bacteria. Plates were inoculated by the method of agar streaking so that individual colonies could be grown, and the plates were cultured upside down to prevent spreading caused by condensation. Liquid broths were all inoculated using one drop of the diluted suspension.

After 24 hours, all cultures revealed some growth of colonies, most having occurred aerobically at 37°C. Three distinct types of colony were apparent on the blood agar plates, two of which were haemolytic, shown by pale areas around the colony where the blood had been lysed, and one which was not. Using a sterile wire loop, colonies were scraped from the plate and each mixed with 0.5 ml saline. Drops of this were then placed in the following cultures: (1) nutrient broth; (2) nutrient agar; (3) blood agar; and (4) MacConkey's agar. All cultures were set up aerobically, at 37°C.

Examination after 24 hours revealed that all colonies had grown on all media and each was a pure colony of a single species of bacterium. Smears were taken from the blood agar for Gram staining and processed

after Lillie's (1928) method, thus:

(1) Smears passed through flame to kill and fix bacteria.

(2) Placed in ammonium oxalate - crystal violet for 30 seconds.

(3) Washed in water.

(4) Placed in Lugol's iodine solution for 30-60 seconds.

(5) Washed in water.

(6) Decolorised with a few drops of acetone.

(7) Washed in water.

(8) Counterstained with weak carbol-fuchsin for 1-2 minutes.

(9) Washed in water and allowed to dry.

Slides were then observed using oil immersion at x100 to check the Gram reaction and morphology. (Gram positive organisms, i.e. those which have the ability to resist decolorisation by ethanol or acetone, are blue or purple; Gram negative organisms are red.)

Characterisation tests and identification

Colonies were taken from the blood agar plates and a suspension made in physiological saline. The following tests were then performed to characterise the bacteria on the basis of their biochemistry. Unless otherwise stated, cultures were incubated aerobically at 37°C. (See Appendix II for composition of media, etc.)

Primary Tests:

Motility. Young broth cultures, incubated at 37°C, were examined microscopically for signs of motion in a "hanging drop" method.

Catalase activity. A few drops of 3% H_2O_2 were placed on well developed colonies grown on nutrient agar, and the culture examined immediately, and after 5 minutes, for bubbles of gas.

Oxidase activity (Kovács, 1956). 2-3 drops of oxidase reagent were placed on a piece of filter paper and the culture smeared across it with a platinum wire loop. A positive reaction was indicated by the appearance of a dark purple colour.

Acid from carbohydrates. Peptone water containing one of the following carbohydrates: glucose; lactose; maltose; mannitol; salicin; sucrose; and xylose; was inoculated and examined daily for up to 7 days for acid or gas production.

Oxidation and fermentation of glucose (Hugh & Leifson, 1953). Duplicate tubes were inoculated by stabbing with a straight wire. To one tube was added a layer of paraffin to a depth of about 1 cm. Both were incubated for up to 14 days. Results were given by the following colour combinations:

	Open tube	Sealed tube
Oxidation	yellow	green
Fermentation	yellow	yellow
No action	blue or green	green

Secondary Tests:

Arginine hydrolysis. 5 ml arginine broth was inoculated and after incubation for 24 hours, 0.25 ml Nessler's reagent was added. Arginine hydrolysis was indicated by the development of a brown colour.

Citrate utilisation. Citrate media was inoculated with a straight wire and incubated at 37°C. The culture was examined daily for up to 7 days for turbidity, which indicated growth. Positives were confirmed by subculture to Koser's citrate medium.

Decarboxylase reactions (Møller, 1955). Tubes of the four media (arginine, lysine, ornithine and control) were inoculated with a straight wire through the paraffin layer, incubated and examined daily. The media first became yellow due to acid production from the glucose; later if decarboxylation occurred, the media became violet. The control remained yellow.

Gelatin liquefaction. Nutrient gelatin was inoculated with a straight wire and incubated. Examinations were made for up to 30 days for growth and presence of liquefaction.

Gluconate oxidation (Carpenter, 1961). Gluconate broth was inoculated and incubated for 2 days. One Clinitest tablet (Ames and Co.) was placed in the culture and the whole boiled for 10 minutes. The formation of a brown, orange or tan precipitate constituted a positive reaction.

Hydrogen sulphide production. A tube of triple sugar iron agar was inoculated by stabbing the butt and streaking the slope. Observations were made daily for up to 7 days for blackening due to H_2S production.

Indole production. To a 48-hour culture in peptone water was added about 1 ml of ether. The whole was shaken and 0.5 ml Ehrlich's reagent run down the side of the tube. The presence of indole was indicated by a pink or red colour in the solvent.

Nitrate reduction. Nitrate broth was inoculated and incubated for up to 5 days. 1 ml nitrite reagent A followed by 1 ml reagent B were

added. A red colour indicated the presence of nitrite. To tubes not showing a red colour was added powdered zinc (up to 5 mg/ml of culture). A red colour indicated nitrate present in the medium (i.e. not reduced by the organism). Absence of a red colour indicated nitrate absent from the medium (i.e. reduced by the organism to nitrite, which in turn was reduced).

Nitrite reduction. Nitrite broth was inoculated and incubated for 7-14 days. Nitrite reagents were added as for the nitrate test. A red colour indicated nitrite present and absence of a red colour indicated nitrite absent, i.e. reduced by the organism.

Urease activity. A slope of Christensen's urea medium was inoculated heavily and examined after 4 hours and daily for 5 days. A red colour indicated that urea had been hydrolysed.

All results were confirmed by repeating the tests with two different batches of eggs.

Antibacterial agents

The action of a number of antibacterial agents on the three isolated bacteria was tested by the agar diffusion method. Filter paper discs impregnated with the following antibacterial agents were used: Ampicillin (PN); Chloramphenicol (C); Orbenin (OB); Erythromyacin (E); Penicillin (P); Streptomycin (S); Oxytetracycline (OTC); and Sulphamethoxazole and Trimethaprin (SXT). Those to which the bacteria were most sensitive were further investigated to find an effective concentration for controlling the contamination in in vitro culture media. The minimum inhibitory concentration (MIC) was first found from a

series of dilutions of the agent used against the bacteria, and a value well above this was chosen. In each case, the value used was 100 µg of antibacterial agent per ml of medium.

Tests were then carried out on the bacteria using the isolated cultures either singly, or in combination, and the original egg suspension containing all 3 species, both before and after filtering to remove debris such as unbroken eggs and fragments of egg shell. Cultures were set up with nutrient broth, bacterial inoculation, and antibacterial agent, and incubated for up to 4 days. Observations were made every 24 hours and any bacterial growth noted.

Alternative antibacterial agents

Although the antibacterial agents proved successful in controlling growth of the isolated bacteria, they were limited in their control of the relatively high concentrations of bacteria in the original egg suspension. They would, therefore, be of little use in controlling the contamination in the *in vitro* culture.

Alternative agents were sought, with the idea of including them in a washing stage after the eggs had been crushed. As this stage would have to be incorporated into the preliminaries to *in vitro* culture, the processes involved in hatching the eggs, as described in the previous chapter, were repeated here also to check the effect of the agents on the viability of the oncospheres. The agents used were chloros, formaldehyde, alcohol, silver nitrate and Hibitane (chlorhexidine gluconate, I.C.I. Ltd.). Landureau's medium, modified by Voge & Green (1975), proved most convenient and was chosen as the final culture medium for the tests. Washed eggs were thus subjected to the following procedure:

(1) crushed in sterile homogeniser;

(2) washed in one of agents listed above for 1 - 2 hours;

(3) washed in saline to remove agent;

(4) treated with 0.1% pancreatin solution to digest pyriform apparatus;

(5) inoculated into Landureau's medium and incubated at room temperature for up to 3 days.

After 24, 48 and 72 hours the cultures were examined for signs of bacterial growth and to check the appearance of the oncospheres. Any cultures not showing signs of growth were subcultured into nutrient broth and incubated at 37°C.

Further experiments were performed with any successful antibacterial agents to find suitable concentrations and the duration of the washing stage. The crushed egg suspension was washed using various concentrations of the agent for time intervals of 30, 60, 90 and 120 minutes. At the end of each treatment the suspension was rinsed in saline to remove all traces of the agent and then used to inoculate nutrient broth cultures. These were incubated at 37°C and observed after 3 days for bacterial growth.

The washing stage was incorporated into the hatching procedure, thus providing sterile oncospheres for in vitro culture.

Results

Microscopical examination of the eggs revealed that the bacteria were present between the subshell membrane and the pyriform apparatus. (Plate I). Bacteria were also located in clusters around the centre

of eggs which were not fully developed, i.e. before the membranes and egg shell had formed.

The results for the characterisation tests are given below in Table VI-1.

TABLE VI-1 CHARACTERISATION TESTS

Primary	1	2	3
Gram reaction	-	-	-
Morphology	rod	rod	rod
Motility	+	+	+
Catalase	+	+	+
Oxidase	+	-	+
Glucose (acid)	+	+	+
Carbohydrates (f/o)	F	F	0
Anaerobic	+	+	+
Haemolytic	+	+	-
MacConkey's agar	+	+	+
Secondary			
Arginine hydrolysis	N	N	+
Citrate utilisation	N	-	+
Decarboxylase: Control	-	-	-
Lysine	-	+	N
Arginine	+	+	N
Ornithine	-	+	N
Gelatin liquefaction	+	-	-

TABLE VI-1 CHARACTERISATION TESTS - continued

	1	2	3
Gluconate oxidation	+	-	+
H_2S production	+	-	N
Indole production	+	+	-
Nitrate reduction	N	-	+
Nitrite reduction	N	-	N
Urease activity	-	-	+
Lactose (acid)	+	+	-
Maltose "	N	N	-
Mannitol "	+	+	-
Salicin "	N	N	-
Sucrose "	+	+	-
Xylose "	-	N	+

N = test not performed

From identification tables given in Cowan & Steel (1965), the three bacteria were identified as follows:-

(1) Aeromonas hydrophila, a Gram negative, motile bacillus measuring 1-2μm x 0.6μm.

(2) Escherichia coli, a Gram negative, motile bacillus measuring 2-3μm x 0.6μm.

(3) Pseudomonas putida, a Gram negative, motile bacillus measuring 1-2μm x 0.6μm.

The results for the antibacterial agent tests are given below.

TABLE VI-2 RESULTS OF AGAR DIFFUSION SENSITIVITY TESTS

		1	2	3
Ampicillin	PN	R	R	R
Chloramphenicol	C	S	S	R
Orbenin	OB	R	R	R
Erythromyacin	E	ss	ss	ss
Penicillin	P	R	R	R
Streptomycin	S	S	Sø	R
Oxytetracycline	OTC	S	ss	ss
Sulphamethoxazole	SXT	S	r	Sø

R = Resistant

r = Resistant, less than R

S = Sensitive, Sø major zone of inhibition

ss minor zone of inhibition

The three antibacterial agents most effective against the bacteria were Streptomycin (S), Oxytetracycline (OTC) and Sulphamethoxazole (SXT). These were further investigated as shown below.

TABLE VI-3 RESULTS OF SENSITIVITY TESTS ON SELECTED ANTIBACTERIAL AGENTS

	ES	ESF	1	2	3	1+2	2+3	1+3	1+2+3
S	R	R	R	S	R	R	r	R	R
OTC	R	R	S	S	S	S	S	R	S
SXT	R	R	r	r	S	r	S	R	r

ES = egg suspension

ESF = filtered egg suspension

1, 2 & 3 = isolated bacteria species

None of the agents was effective against the bacteria in the egg suspensions.

Alternative antibacterial agents - Observations

Landureau's medium incubated at room temperature for 72 hours:-

Control (medium, oncospheral suspension, no agent)

Dense bacterial growth.

Oncospheres intact and healthy.

Chloros (medium, oncospheral suspension, NaOCl at 15%)

No bacteria.

Oncospheres disintegrated.

Formaldehyde (medium, oncospheral suspension, formaldehyde at 1%)

Bacteria present, less than control.

Oncospheres intact and healthy.

Alcohol (medium, oncospheral suspension, alcohol at 90%)

Bacteria present, less than control.

Oncospheres intact and healthy.

Silver Nitrate (medium, oncospheral suspension, $AgNO_3$ at 1%)

Precipitate (probably AgCl) formed on mixing solutions.

Experiment not continued.

Hibitane (medium, oncospheral suspension, Hibitane at 1%)

No bacteria.

Oncospheres intact and healthy.

Formaldehyde and Hibitane experiments were subcultured into nutrient broth.

Nutrient broth incubated at $37^{o}C$ for 3 days:-

Formaldehyde - heavy bacterial growth.

Hibitane - no bacteria.

After further 21 days, Hibitane experiment still free from contamination; oncospheres disintegrated, no longer visible.

TABLE VI-4 RESULTS OF HIBITANE WASHINGS

Concentrations of 1; 0.5; 0.2; and 0.1 per cent Hibitane were used.

Control = nutrient broth inoculated with unwashed crushed egg suspension.

%	30	60	90	120 minutes wash	
1.0	+++	+++	+	-	
0.5	+++	+++	+	-	
0.2	+++	+++	++	-	
0.1	+++	+++	++	-	
Control	+++	+++	+++	+++	

+++ = heavy bacterial growth

++ = moderate bacterial growth

+ = light bacterial growth

- = no bacterial growth

Thus, a washing stage using 0.1% Hibitane for 120 minutes was incorporated into hatching procedure.

Discussion

The three species of bacteria isolated from the eggs of Moniezia expansa are not individually uncommon, but together, and such a location, are extremely rare. Escherichia coli is frequently found in the intestine of man and many other animals; Aeromonas hydrophila is aquatic and infects reptiles and amphibians to which it is pathogenic; Pseudomonas putida inhabits water and soil and is generally non-pathogenic.

Finding a function for the bacteria is difficult without a detailed study of the zoology and biochemistry of the egg. Explaining the method by which they may arrive there, is more easy. E. coli, as mentioned above, is an enterobacteria and would therefore be found naturally in the gut of sheep; A. hydrophila and P. putida, in soil and water, might contaminate the grass on which the animal feeds. All three species could enter the uterus of the worm via the genital pore and thus "infect" the developing eggs.

The fact that the bacteria do occur in what is normally accepted as a sterile environment, i.e. an egg, would suggest some form of symbiotic relationship. Also, the bacteria have been found in all eggs in both species of Moniezia studied so far, and apparently produce no ill effects in the worms or the oncospheres. The eggs, as previously described (page 17), contain a large amount of lipid material within the shell which could provide a suitable food source for the bacteria. What benefit the bacteria contribute to the relationship can, as yet, be only conjecture. They could, perhaps, be involved in some necessary biochemical reaction resulting in a substance beneficial to the oncosphere, or one that is required in the formation of the egg and its envelopes. This last is supported by the fact that the bacteria are present around the undeveloped eggs before any of the envelopes are complete. However, once the eggs are mature, the bacteria are apparently trapped by the shell and cannot be released until the eggs are broken. This suggests that the bacteria could also be of some importance to the mites. As already described, the mites are reported to "suck out the contents of the egg shell"; thus, they must ingest the bacteria as well as the oncosphere. The gut flora of the mites could, perhaps, be supplemented in this way.

A similar, complex interrelationship between Moniezia, a micro-organism and the mites has been well documented by Dissanaike (1955; 1957a, b; 1958) and Shigina (1972). In this instance, an example of hyperparasitism, Moniezia is parasitised by the microsporidian Nosema helminthorum, which is also a parasite of oribatid mites.

Hyperparasitism is not restricted to micro-organisms, however. Demidova (1957; 1958; 1960) described the relationship between Moniezia and the hookworm, Bunostomum trigonocephalum.

Thus, interrelationships between Moniezia and other organisms are not uncommon. It is not surprising, therefore, to learn of the possibility of another instance, even though this new case involving the bacteria has yet to be fully understood.

Reports of helminths harbouring micro-organisms have been reviewed by Buchner (1965); Woodruff (1968); Lee (1971); and Kozek & Marroquin (1977). The majority of cases described involve nematodes, which, with their migrating larval stages, can spread micro-organisms throughout the body of their hosts. Examples of trematodes acting as vectors include the well known salmon-poisoning disease caused by Neorickettsia helminthoeca which is transmitted by Nanophyetus salmincola. More recently, Bekkouche & Dupouy (1976) observed bacteria in the somatic cells and ovocytes of Polystoma integerrimum, although any effects of these on the frog host were not discussed. Until the present findings of bacteria in the eggs of Moniezia, the only cestode reported as a vector of micro-organisms was Taenia crassicollis which was found in association with the Rift Valley fever virus (Findlay & Howard, 1951).

As is, perhaps, apparent from the examples given above, authors tend to emphasise situations where the helminths serve as vectors for a second disease. Hence, the relationship between micro-organism and final host is discussed, rather than that between micro-organism and helminth. However, the success of such systems depends, finally, on the success of the transmission, so that the micro-organisms cannot afford to harm their vectors. Thus, some form of mutual coexistence, such as symbiosis or commensalism, is to be expected.

One final point arising from the results of this work remains to be discussed: namely, the resistance of the bacteria to the majority of the antibiotics and antibacterial agents employed. This resistance was, in fact, due to just one of the three species found - Pseudomonas putida. Both Aeromonas hydrophila and Escherichia coli are known to be sensitive to several antibiotics, as shown by the agar diffusion sensitivity tests. P. putida, however, is notoriously difficult to eradicate once it has become established as the organisms are resistant to most commonly used antibiotics. Closely related species have even been found in "antiseptic" solutions of benzalkonium chloride, used for rinsing contact lenses (Clancy, 1974). Of the antibacterial agents available, only Hibitane (chlorhexidine gluconate) was found to be effective against this species, both in isolation and in combination in the egg suspension. Thus, sterile oncospheres, hatched from the eggs, could be produced by the incorporation of a washing stage in the original procedure.

Summary

Three species of bacteria were found in the eggs of Moniezia expansa. Using standard microbiological and biochemical methods they were isolated, grown in pure culture and identified. The species were Aeromonas hydrophila, Escherichia coli, and Pseudomonas putida. A suitable antibacterial agent - a chlorhexidine derivative - was found to control the contamination.

The occurrence of interspecific associations between micro-organisms and helminths is discussed.

CHAPTER VII

IN VITRO CULTURE OF THE ONCOSPHERE TO CYSTICERCOID STAGE OF MONIEZIA EXPANSA

Introduction

In vitro culture is now recognised as a valuable tool for studying the biology of endoparasites. Techniques involving the use of complex tissue-culture media have enabled the production of controlled and regulated environments and several species of parasite have been cultivated.

The basic principles, techniques and problems of *in vitro* culture of helminths have been discussed by Smyth (1976).

The major requirement for the culture of any organism is a suitable medium in which growth and development can take place. The life cycle of cyclophyllidean tapeworms generally involves more than one host. In families other than the Taeniidae, there are two - one a vertebrate and the other an invertebrate. To reproduce the complete life cycle *in vitro*, therefore, requires two very different culture systems. Medical influences have directed research towards the production of synthetic mammalian-type media and there are now several of these commercially available. Invertebrate culture has also received much attention and organisms from several phyla have now been studied (Lutz, 1970; Maramorosch, 1976).

This combination of vertebrate and invertebrate hosts occurs in the life cycle of *Moniezia expansa*. Details of the adult, or sheep, system are given in Chapter X; only the larval, or mite, stages will be considered here.

Members of the cyclophyllidean family, Anoplocephalidae, have not so far been used for *in vitro* studies. Certain hymenolepid species, however, which are similar to *Moniezia expansa* in using an invertebrate host, have successfully been cultured *in vitro*. They include *Hymenolepis citelli* (Voge & Green, 1975); *H. diminuta* (Voge, 1975); *H. microstoma* (Seidel, 1975); and *H. nana* (Berntzen, 1970). All these four species have an insect as intermediate host and hence the majority of the *in vitro* work has utilised an insect tissue culture medium. Much success has been gained with a modification of Landureau's cockroach medium (Landureau, 1966; 1968). However, the intermediate host for *Moniezia* is an acarine arachnid and relatively little work has been performed on these animals. General descriptions of arachnid haemolymph are given by Florkin & Scheer (1971) and Maramorosch & Shope (1975). References to various species of tick, scorpion and spider are available from these sources. Work on oribatid mites, however, is restricted to that of Streiff & Taberly (1964) who cultured organs of the mites *Xenillus tegeocranus* and *Platynothrus peltifer* *in vitro*.

Attempts were therefore made to produce a suitable culture system for the oncospheres of *M. expansa*. A variety of different media were used, including those available commercially as well as others based on the works described above.

Materials and Methods

General procedures

All apparatus and equipment was sterilised by autoclaving under pressure before use. Procedures were carried out in a lamina flow cabinet which was regularly cleaned and swabbed with ethanol before use.

Two types of culture vessel were used. Small, glass Leighton tubes (Bellco), measuring 16 x 93 mm and having an approximate capacity of 15 ml, were useful for continuous observation of the culture. Disposable plastic tubes (Sterilin), with a capacity of 10 ml, were also employed. For some procedures requiring larger volumes of liquid, such as washing and rinsing stages, Universal containers (Sterilin), with a capacity of approximately 25 ml, were used.

All media and salines were sterilised by vacuum filtration through 0.2 µm millipore filters (Gelman). Solutions were stored at 4°C until required.

Various additives were mixed with some, or all, of the media. To maintain the sterility, all had antibiotics and fungicides added before storage. These consisted of penicillin and streptomycin at 100 units per ml of culture medium, and amphotericin B or "Fungizone" (Flow) at a concentration of 2.5 µg/ml of culture medium.

Other substances added to the culture media included foetal calf serum (Gibco Bio-Cult) at 10 or 20 per cent, and lobster haemolymph (Gibco Bio-Cult) at 10 or 20 per cent. The majority of experiments used

non-inactivated serum. However, on some occasions the foetal calf serum was inactivated by heating at 56°C for 30 minutes.

Culture vessels containing 2 or 4 ml of the final culture medium were incubated at 25°C. Both aerobic and anaerobic systems were employed. For aerobic conditions, air was used as the gas phase, and for anaerobic 10% carbon dioxide with 90% nitrogen was used. In the latter case, the gas mixture was bubbled through the medium for 1 - 2 minutes at the start of each experiment, and also each time the culture medium was changed.

Culture vessels were either kept stationary, in which case the organisms settled to the bottom of the tubes, or were subjected to gentle agitation to mix the contents. Tubes were either placed in a shaking water bath which shook the vessels at a rate of approximately 80 shakes per minute; or were placed on a rotator apparatus which rotated the vessels vertically at about 25 revolutions per minute.

Cultures which were maintained for more than 7 days were subjected to a medium change every subsequent 4 or 5 days. The change involved the upper volume of medium being removed and replaced with fresh, sterile medium. When necessary this procedure was preceded by gentle centrifugation of the culture (less than 1,000 revolutions per minute) to concentrate the organisms at the bottom of the vessel.

Culture Media

The following media were used during attempts at *in vitro* culture of *M. expansa*.

The composition of each is given in Appendix I.

Commercially obtained media:

(1) Medium 199 (Difco). Concentrations of 25, 50, 75 and 100% medium were used. Foetal calf serum at concentrations of 10 or 20% was sometimes added.

(2) Medium CMRL 1066 (Flow). Used at 100% concentration with 10 or 20% foetal calf serum.

(3) Eagle's Medium, with Earle's Balanced Salt Solution (Difco). Used at 100% concentration with 10 or 20% foetal calf serum.

(4) Medium NCTC 135 (Gibco). Used at 100% concentration with 10 or 20% foetal calf serum.

(5) Medium 858 (Difco). Used at concentrations of 25, 50, 75 and 100%. Foetal calf serum at 10 or 20% was added.

(6) Medium McCoy 5A (Flow). Used at 100% concentration with 10 or 20% foetal calf serum.

(7) Grace's Insect Tissue Culture Medium (Gibco). Used at 100% concentration with 10 or 20% lobster haemolymph.

Prepared Media:

(1) Landureau's Cockroach Medium (Laundureau, 1966; 1968). Used at 100% concentration with 10 or 20% foetal calf serum.

(2) Rehacek's Tick Medium A, used for growth of Rhipicephalus sanguineus cells. (Rehacek & Brzostowski, 1969) Used at 100% concentration with 10 or 20% lobster haemolymph.

(3) Streiff & Taberley's Medium (Streiff & Taberley, 1964). Used at 100% concentration.

In contrast to the highly complex media listed above, cultures were also set up using more simple solutions. For this purpose Hank's and Earle's (Difco) balanced salt solutions with glucose were tested. Foetal calf serum at 10% was added.

Oncospheres

Oncospheres were obtained from mature eggs removed from the terminal proglottids of M. expansa. The eggs were hatched as described in Chapter V.

Sterile oncospheres were rinsed twice in the test medium before inoculation.

Results

No growth or development was observed in any of the culture systems employed. However, the culture media differed in their ability to maintain oncospheres in an apparently healthy condition. The progress of the cultures was divided into two arbitrarily defined stages to allow for some comparison of the systems. Hatched oncospheres set up in a particular culture system gradually ceased any movement, but remained in an apparently healthy or normal state for some days. This was Stage 1. Slow disruption followed, Stage 2, whereby the oncospheres became granular in their appearance and lost any limiting membrane. This disintegration continued until the oncospheres were no longer distinguishable in the medium.

The duration of Stage 1 was taken as the criterion for assessing the different media and culture systems used. Table VII-1 summarises the results of the experiments. Values are given for the duration of Stage 1, i.e. the time the oncospheres remained apparently healthy. Some cultures developed heavy bacterial and fungal contamination, which treatments with antibiotics and fungicides would not reduce. Such cultures were discarded and are not included in the table.

The onset of Stage 2, and thus death, was recognised partly by direct observation and also by the reaction of the organisms to the addition of the vital stain - Trypan blue. Dead oncospheres lost the selective permeability of their membranes and became evenly stained with the dye.

The four "best" media were 199, McCoy's 5A, Grace's, and Landureau's. All these maintained oncospheres for up to 14 and, in some cases, 21 days.

Variation in physical conditions, such as whether the cultures were stationary or agitated, had no observable effect on the oncospheres.

Changing from aerobic to anaerobic gas phases also did not, apparently, affect the oncospheres.

TABLE VII-1: Results of in vitro culture experiments

Media selection

Aerobic cultures in Leighton tubes; stationary at 25°C.				
Experiment No.	Medium	%	Serum, etc. %	Duration of Stage 1 (days)
1, 2	199	25	None	2 - 7
3, 4		50	"	2 - 7
6		100	"	2 - 7
11, 12		100	Foetal calf 10	14 - 21
8, 9, 10		100	" " 20	14 - 21
2, 3, 4, 5	CMRL 1066	100	Foetal calf 10	10
3	Eagle, Earle's	100	Foetal calf 10	3
4, 5		100	" " 20	3
3, 4, 5	NCTC 135	100	Foetal calf 20	7
1	858	50	None	2
2		75	"	2
4, 5		100	"	4
6, 7, 8		100	Foetal calf 20	7

TABLE VII-1 - continued

Experiment No.	Medium	%	Serum, etc. %		Duration of Stage 1 (days)
1, 3, 4, 5, 6	McCoy's 5A	100	Foetal calf	10	21
8, 9, 10		100	" "	20	14 - 21
1, 9, 10	Grace's Insect	100	Lobster haemolymph	10	18 - 21
2, 3, 4, 5		100	" "	20	14 - 21
3, 4, 5, 6	Landureau's	100	Foetal calf	10	14 - 21
7, 8, 10		100	" "	20	10 - 21
1, 2, 3, 4, 5	Rehacek's	100	Lobster haemolymph	10	1 - 7
3, 4, 5	Streiff & Taberley's	100	None		1 - 5
1, 2, 3, 4	Hanks	100	Foetal calf	10	2 - 5
1, 2, 3, 4	Earle's	100	Foetal calf	10	3

TABLE VII-1 - continued

Varying conditions		
Cultures in Medium 199 with 20% foetal calf serum		
Experiment No.		Duration of Stage 1 (days)
11, 12	Anaerobic, Leighton tube, stationary	14 - 21
13	Anaerobic, Leighton tube, shaking	14 - 21
14	Aerobic, plastic tube, shaking	14 - 21
15, 16	Aerobic, plastic tube, rotating	contaminated
Cultures in McCoy's 5A with 20% foetal calf serum		
11, 12	Anaerobic, Leighton tube, stationary	7 - 14
13, 15	Aerobic, plastic tube, shaking	contaminated
16, 17, 18	Aerobic, plastic tube, stationary	7 - 14
Cultures in Grace's with 10% lobster haemolymph		
12, 14	Anaerobic, Leighton tube, stationary	14 - 21
16, 17, 18	Anaerobic, plastic tube, stationary	14 - 21
19, 20	Aerobic, plastic tube, shaking	14 - 21
Cultures in Landureau's with 20% foetal calf serum		
11, 12, 13	Anaerobic, Leighton tube, stationary	10 - 14
14, 16	Aerobic, plastic tube, shaking	14 - 21
18, 19, 20	Aerobic, plastic tube, rotating	7

Discussion

As discussed by Smyth (1976), the problems surrounding *in vitro* culture are numerous and success is not always easy to achieve. Many factors have to be taken into consideration. Ideally, an *in vitro* culture system should support normal growth and development of an organism, culminating in its reproduction. The medium and environment supplied for the organism must, therefore, closely mimic all those "normal" conditions found *in vivo*.

The site of a parasite in its host is complex, being composed of an infinite combination of physical and chemical conditions. Even when these natural characteristics are known, it is extremely difficult to reproduce them exactly *in vitro*. Nutrition and excretion of the parasite in the host animal are continuous processes; in what is effectively a closed system in culture, the obtaining of food and elimination of waste materials may be hampered. Food sources may be incorrect or inadequate and toxic substances may build up to poison the system. Few parasites are found unattached in their host - they have some support or attachment site. This site will have particular characteristics which have to be correctly reproduced. Many parasites have complex life cycles involving various developmental stages and several hosts. Trigger stimuli initiate the necessary changes in growth or behaviour which bring about the continuation of the cycle. These triggers are often based on very slight changes in the parasite's environment, such as an increase in a particular host hormone, and are consequently very difficult to recreate *in vitro*.

Having developed an appropriate system, successful growth of a culture is still not guaranteed. Often, systems support only limited growth of

the organism, the life cycle is not completed, or growth is abnormal. Smyth (1969) and Smyth & Davies (1974) have discussed such limitations on the *in vitro* culture of some cestodes, and also the methods available for overcoming them.

Incomplete or partial development then imposes the further problem of how to assess the efficacy of a particular system. Davies (1976), working with several species of Digenea, emphasised the need for closer examination of cultured organisms and has proposed various criteria for judging normal growth and development. However, during attempts to culture the oncospheres of *Moniezia expansa* *in vitro* no growth or development occurred. Methods of comparing and assessing the different media utilised were, therefore, restricted to subjective observations.

It is important to distinguish between the actual culture of an organism when growth and differentiation occur, and its "maintenance" when it merely survives without development. During these experiments the latter situation occurred; no growth or development of the oncospheres was observed. True culture is, of course, the ideal, but, as stated by Smyth (1976), "....survival is a justifiable aim in preliminary attempts with difficult or new material".

The choice of the commercial media was arbitrary, chiefly based on their availability. The prepared media were chosen on the basis of their previous success in promoting growth of either oncospheres or arachnid tissues, as was described earlier in this chapter. Because little *in vitro* work had been attempted using *M. expansa*, experiments were empirical in their approach. It was hoped that, by using a wide range

of different types of culture media, an appropriate one could be found which would be suitable for the *in vitro* culture of *M. expansa* oncospheres.

The results indicated that four media were more successful at maintaining the oncospheres than the others. Of these, two - Grace's and Landureau's - are designated as insect tissue culture media, and the other two - Medium 199 and McCoy's 5A - are commercially available, general-purpose tissue culture media. From their chemical composition (Appendix I), no particular similarities can be found. Hence, at this stage it is not possible to say what factors are responsible for their greater ability to support the oncospheres in culture.

The fact that conditions such as pH, gas phase and movement had no effect on the cultures can perhaps be explained on the basis that the oncospheres were in a relatively stable state. It is recognised that during periods of growth, organisms can be adversely affected by changes in their environment. As no growth of the oncospheres occurred, such factors would be less important. It is to be expected, however, that conditions of pH, gas phase, etc., would be of more consequence during the successful culture of the oncospheres.

In addition to those associated with *in vitro* techniques, problems also arise due to the nature of the organism itself. Oncospheres of *M. expansa* are small, approximately 20 μm in diameter, thus making the handling and observation of them more difficult. Problems associated with the hatching of the eggs to obtain clean and sterile oncospheres have been discussed in earlier chapters (Chapters V and VI).

The normal development of oncosphere to cysticercoid takes anything from 6 to 10 weeks in the natural intermediate host. Successfully cultured cysticercoids, such as Hymenolepis microstoma (Seidel, 1975) and H. diminuta (Voge, 1975) developed in only 3 weeks. The extended development period of M. expansa is related to the seasonality of its life cycle; mites are infected one year, but not usually infective until the next. However, this feature necessitates a prolonged period of in vitro culture, which means that the complex and finely balanced constituents of the media have to be maintained for a similar length of time.

The successful development of Moniezia expansa in vitro is thus dependent upon the correct interrelationship of innumerable factors and characteristics of the organism, the culture medium, the culture vessel, and all associated conditions. The above attempts by no means represent an exhaustive list of possible experiments, but are preliminary observations and provide a useful framework for further research.

Summary

Attempts were made to culture the oncospheres of Moniezia expansa to the cysticercoid stage in vitro. A wide range of media, including those commercially available and some prepared in the laboratory, were tested. The effects of various physical conditions such as pH, gas phase, and culture vessel, were also investigated.

No growth or differentiation of the oncospheres was observed in any of the culture systems used. However, oncospheres were maintained in an

apparently healthy condition for up to 21 days in 4 of the media tested. These media were Medium 199; Grace's Insect Tissue Culture Medium; Landureau's Cockroach Medium; and McCoy's 5A Medium.

Problems associated with the in vitro culture of helminths are discussed.

CHAPTER VIII

THE ADULT TAPEWORM A REVIEW

Introduction

Tapeworms belonging to the genus Moniezia are widespread in their distribution, being found in a number of different animals throughout the world. The adult worms are primarily recognised as parasites of farm animals, particularly young fatstock. The two most common species, Moniezia benedeni and M. expansa, are generally found in cattle and sheep, respectively. Mixed infections do occur, although one species is usually dominant. Several other animals are also infected, however, especially deer and antelope which, in some areas, serve as important reservoir hosts for the disease. A list of these species as reported in the literature is given in Table VIII-1.

Accounts of the epidemiology of the disease are numerous. Over 35 countries have reported incidences, including most of the U.S.S.R. and U.S.A. Major references to these are given in Table VIII-2.

Life Cycle

The final hosts gain their Moniezia infections whilst grazing infected pastures. Oribatid mites containing cysticercoids are ingested with the vegetation. Once within the gut of the animals, the cysticercoids are released from the mites and, in response to the host's enzymes, excyst and become established in the small intestine. Few data are available on the development of the worms in the gut. Freitas & Costa (1970) reported that specimens of M. benedeni, in cattle, were located

along the entire length of the small intestine, but were most numerous in the middle third.

The scolex of both M. benedeni and M. expansa is small and unarmed, and therefore any attachment mechanism can only be provided by the four suckers. Because of this, it is unlikely that the scolex itself does much harm to the gut of the host. Casarosa (1964) has reported finding the scolex of M. expansa bur ied deeply into the mucosal layer, and has described the histology of this site of attachment.

The worms reach patency in 30 - 40 days making it possible to diagnose an infection at this stage. Gravid proglottids and eggs are shed from the worm and appear in the faeces of the sheep or cow. Faecal egg counts can be made using the standard flotation methods (Kotelnikov & Khrenov, 1976).

Pathology

The infection lasts an average of 3 months. During this time, certain symptoms of the disease may become apparent. For reasons which are not yet fully understood, the effects of an infection are not predictable, and it can establish itself in different ways. It may be either symptomless, or it may be pathogenic. In the former, sheep and cows are not adversely affected in any way; the infection is gained and lost without any indication of its presence. If it is pathogenic, the infection is further designated as either acute or chronic. The chronic form is generally accepted as the mild variety of the disease and the acute form as severe. A complete spectrum of effects does

occur, however, so that it is not always possible to categorise a particular instance of the disease.

Several symptoms are associated with the chronic disease. They are: digestive disturbances such as diarrhoea and scouring; abnormal blood pictures, particularly anaemia, with lowered haemoglobin levels, reduced packed cell volumes and red blood cell counts, and increase in eosinophils and monocytes; and general unthriftiness of the animal indicated by reduced weight gains in fatstock and poor quality fleeces and wool. After an infection is lost, either naturally or after anthelmintic treatment, animals improve and these symptoms disappear.

In contrast, the acute disease is often only recognised by the sudden death of an animal which is subsequently found to harbour tapeworms.

The literature is divided between the three main varieties of the disease and there is thus much debate and controversy as to whether Moniezia is a serious pathogen or not. Kates & Goldberg (1951) have given a useful review of the early accounts of Moniezia infections up to 1950. Reports discussed by them are not included here.

Amongst those authors who do not consider Moniezia to be a serious pathogen are Kates & Goldberg (1951) in the U.S.A.; Edney & Kelley (1953) in the U.S.A.; Brunsdon (1964) in New Zealand; Ferreira & Viana (1971) in Brazil; and Worley, Jacobson & Barrett (1974) in the U.S.A. Their conclusions are based on both natural and experimental infections.

In opposition to the above, somewhat more authors have found Moniezia infections associated with some, or all, of the symptoms already described. They include Gordon (1950) in Australia; Thomas, Downey & Dreadon (1956) in New Zealand; Stampa (1967) in South Africa; Davydov (1969) in the U.S.S.R.; Petrovic (1970) in Yugoslavia; Yanarella (1971) in Argentina; Euzeby (1973) in France; Nilsson (1973) in Sweden; Thakur & Mishra (1973) in India; Efner (1974) and Zyromska-Rudzka (1974) in Poland; and Duwel (1975) in Germany. Cases of sudden deaths of animals with Moniezia include those described by Lafenetre (1948) in France; Hansen, Kelley & Todd (1950) in the U.S.A.; Shleikus (1958: cited in Potemkina, 1965) in the U.S.S.R.; and Lyashenko & Teplov (1974) also in the U.S.S.R.

Reasons for the severity of the disease, and especially the sudden deaths, are not, as yet, very clear. Various suggestions have been made. Of the sudden death reports which have included autopsy findings, several have described the gut of the animals occluded with a mass of worms (Cameron, 1934; Tableman, 1946; Campbell, Todd, Cox & Khrohn, 1956). Such mechanical impaction of the intestine would, undoubtedly, cause serious harm to the host animals. Other workers have described secondary developments associated with tapeworm infections which contribute to the severity of the disease. Thomas, Downey & Dreadon (1956) and Vibe (1976) have given accounts of serious digestive disorders which they called enterotoxaemia and enterotoxicosis, respectively. McBeath, Best & Preston (1977) observed that lambs with M. expansa infections were prone to fly "breech strike" because of contamination of the breech area after scouring. Animals which were cured of tapeworms, and hence were not scouring, were not as susceptible to attacks of this kind. Such disorders have a serious effect on

the general health of the animals concerned and therefore must not be overlooked when the pathogenicity of M. expansa is discussed.

Some groups of workers have also reported pronounced behavioural abnormalities immediately preceding death (Tableman, 1946; Demidov & Sorokin, 1959; Euzeby, 1973). The convulsions and staggering described by these authors suggests that the nervous system was also involved in some way.

It has often been stated that Moniezia is most likely to be pathogenic when animals are undernourished or lacking in a particular nutrient or mineral such as calcium or phosphorus (Cameron, 1934; Gordon, 1950). Little research has actually been carried out to test this, although some experiments have found a correlation between the quality of foodstuffs and the seriousness of an infection (Rukavina, Delic, Cankovic & Markotic, 1960). Lesinsh (1970) also recorded that sheep grazing on copper-enriched pastures gained less serious infections than those on untreated pastures.

Mature Moniezia tapeworms are amongst the largest known. Individuals have been found measuring up to 600 cm in length and 2 cm wide. They thus require a considerable amount of nourishment which can only be gained to the detriment of their host (Cameron, 1934). Similarly, the worms must also produce large amounts of metabolic waste which could prove toxic to their host (Tableman, 1946).

Fernandez & Aranda (1945) tested the effect of an extract of Moniezia expansa worms on portions of gut removed from rabbits and guinea pigs. The extract was found to increase peristalsis and reduce the tonus of

the guts, which would indicate the presence of some toxin. If similar effects occurred in the gut of the host animal, the worms would effectively be causing the gut contents, i.e. the host's food, to pass through more quickly and hence prevent complete digestion. Surprisingly, no further work has been carried out to investigate this.

Control and Treatment

Many anthelmin tics are now available for the successful treatment of Moniezia infections in sheep and cattle. These range from simple chemicals, which have been in use since the first half of this century, such as lead arsenate and copper sulphate, to the more complex substances such as 2-(4-thiazolyl)-5-isopropoxycarbonyl aminobenzimidazole, known commercially as Cambendazole. Table VIII-3 lists those anthelmintics which have proved successful.

Immunity

Chronic infections with Moniezia benedeni and M. expansa are normally present for an average of 3 months. At the end of this period worms are spontaneously expelled. In some instances of very heavy worm burdens this expulsion occurs much earlier (Hansen, Kelley & Todd, 1950). An immunity is acquired so that, in general, infections do not recur. As mentioned previously, tapeworms are found primarily in young stock, i.e. as soon as the animals are allowed to graze on an infected pasture, although infections in older animals (1 - 2 year olds) do occur. This was at first believed to indicate the absence of any immunity (Rayski, 1947). However, some infections persist for a considerably longer period than 3 months, so it is possible that worms

found in older stock were from an early infection which had not been lost.

Seasonality

The seasonality of Moniezia infections is well documented. Details for particular areas can be found in the references given in Table VIII-2. In general, two-peaked infections occur, one in the Spring when young stock first comes onto the pasture, and the second, smaller one, in the Autumn. When both species are present, each has its own pattern, separated from the other by 2 or 3 months. Further details of these cycles, in different locations, can be found from the references given in Table VIII-2.

The question of the pathogenicity of Moniezia benedeni and M. expansa remains unsolved and in dispute. Whatever the answer, the fact remains that in certain circumstances Moniezia infections can cause serious economic losses to farmers. Young fatstock develops poorly and cannot be marketed as quickly and extra expenses are incurred either from the cost of feeding or the cost of dosing the animals to alleviate the symptoms. There remains, therefore, the need for much further research into those areas in which our understanding and knowledge of the disease is incomplete.

TABLE VIII-1 : RESERVOIR HOSTS FOR MONIEZIA SPECIES

Host	Common name	Sp.	Country	Reference	Date
Adenota kob thomasi	Adenota kob	D	Africa	Dinnik	1963
Aepyceros melampus	Impala	B.E	"	Round	1968
Alcelaphos boselaphus	Hartebeest	B	"	Round	1968
Antidorcas marsupialis	Springbok	E	"	Round	1968
Bradypus griseus	Grey 3-toed sloth	B	Costa Rica	Flores-Barroeta et al.	1958
Bubalus bubalis	Buffalo	E	India	Varma	1956
Capra cylindricornis	Caucasian tur	E	USSR	Asadov & Sadikhov	1961
Capreolus capreolus	Roe deer	E	"	Asadov & Yaliev	1971
" "	" "	E	Switzerland	Dollinger	1973
Cephalophus dorsalis	Bay duiker	E	Africa	Round	1968
C. harveyi	Red duiker	X	"	"	"
C. montana	? duiker	E	"	"	"
C. monticola	Blue duiker	E	"	"	"
C. silvicultur	Yellow backed duiker	E	"	"	"
Cervus canadensis	Elk	E	USA	Worley et al.	1969.
C. elephas sibiricus	Altai-wapiti	E	USSR	Pryadko et al.	1964
Connochaetes taurinus	Blue wildebeest	B,E	Africa	Round	1968
Damaliscus korrigum	Topi	E	"	"	"
Gazella thomsoni	Thomson's gazelle	E	"	"	"
Giraffa camelopardalis	Giraffe	E	"	Pester & Laurence	1974
Hippopotamus amphibia	Hippopotamus	A,E,X	"	Round	1968

TABLE VIII-1 (continued)

Host	Common name	Sp.	Country	Reference	Date
Hippotragus equinus	Roan antelope	B,E	Africa	Round	1968
H. niger	Sable antelope	E	"	"	"
Kobus ellipsiprymmus	Water buck	E	"	"	"
K. kob	Buffon's kob	D	"	"	"
K. varondi	Puku	B	"	"	"
Lama glama	Llama	E	Brazil	Rego	1963
Odocoileus hemionus	Mule deer	E	USA	Honess & Winter	1956
O. virginianus	White tailed deer	B,E	USA	Prestwood	1971
Okapia johnstoni	Okapi	E	Africa	Round	1968
Oreotragus oreotragus	Klipspringer	E	"	"	"
Oryx gazella	Gemsbok	E	"	"	"
Ovibos moschatus	Musk ox	X	Canada	Samuel & Gray	1974
" "	" "	X	USA	" "	"
" "	" "	X	Norway	" "	"
Phacocoerus aethiopicus	Wart hog	B,M	Africa	Round	1968
Rangifera tarandus	Reindeer, Caribou	R	USSR	Kolenakov	1938
" "	" "	E	"	Polyanskaya	1961
" "	" "	R	"	Zelinskii et al.	1971
Raphicerus campestris	Steenbok	E	Africa	Round	1968
R. sharpei	Sharpe's gripbok	E	"	"	"
Redunca arundinum	Reedbuck	B,D	"	"	"
" "	"	E,P	"	"	"

TABLE VIII-1 (continued)

Host	Common name	Sp.	Country	Reference	Date
Redunca fulvorufula	Mountain reedbuck	E	Africa	Baker & Boomker	1973
Rupicapra rupicapra	Chamois	E	USSR	Asadov & Sadikhov	1961
Saiga tatarica	Saiga	X	"	Kuznetsov	1959a
" "	"	B,E	"	Petrov	1976
Sylvicapra grimmia	Grey duiker	E	Africa	Round	1968
Taurotragus oryx	Eland	B,E	"	"	"
Tayassu albirostris	Peccary	B	Brazil	Rego	1961
Tragelephas scriptus	Bushbuck	E	Africa	Round	1968
T. strepsiceros	Kudu	E	"	Condy	1972

NOTES

A = Moniezia amphibia
B = M. benedeni
D = M. monardi
E = M. expansa
M = M. mettami
P = M. pallida
R = M. rangiferina

X = Moniezia sp.

TABLE VIII-2 : EPIDEMIOLOGY OF MONIEZIA

Country	Reference	Date
AFRICA		
Congo	Graber	1975
Nigeria	Ayeni	1973
AMERICA - NORTH		
Canada	Frechette & Gibbs	1971
United States	Kates	1965
- Georgia	Ciordia	1975
- Idaho	Worley et al.	1974
- Kansas	Leland et al.	1973
- Montana	Worley et al.	1974
- Wisconsin	Cox & Todd	1962
AMERICA - SOUTH		
Argentina	Yanarella	1971
Brazil	Costa et al.	1974
"	Guimaraes et al.	1976
Venezuela	Vilchez	1954
ASIA		
China	Lin et al.	1975
India	Narsapur	1976b
"	Raina	1973
Iraq	Kadhim	1972
Japan	Fukui	1959
"	Fukui	1960a
Korea	Lee & Lee	1971
Turkey	Guralp et al.	1975

TABLE VIII-2 (continued)

Country	Reference	Date
AUSTRALASIA		
Australia	Gordon	1950
New Zealand	Brunsdon	1976
EUROPE		
Belgium	Pouplard et al.	1970
Bulgaria	Bankov	1975
Czechoslovakia	Prokopic	1967
France	Euzeby	1973
Germany	Burger	1973
Great Britain		
- Scotland	Rayski	1947
- Wales	Hooper	1970
Hungary	Vargo	1973
Iceland	Richter	1974
Norway	Helle	1971
"	Helle	1973
Poland	Lepojev	1970
"	Kozakiewicz	1976
Portugal	Carvalhovarela	1975
Romania	Olteanu et al.	1963
"	Fromunda et al.	1976
Sweden	Nilsson	1973
Switzerland	Eckert	1973
Yugoslavia	Petrovic et al.	1970
USSR (GENERAL)	Alkov	1972
"	Vibe et al.	1973
- Central Zone	Shubaderov	1973
- Buryat	Zherebotsova	1970
- Kazakh	Artemev	1973
- Latvia	Lesinsh	1973

TABLE VIII-2 (continued)

Country	Reference	Date
USSR (continued)		
- Omsk	Kadenatsii & Gapon	1975
- Turkmen	Orekhov	1960
- Ukraine	Panchin et al.	1975
- Volga	Kuznetsov	1959c
WEST INDIES		
Cuba	Dobsinsky	1970
Guadeloupe	Euzeby & Graber	1973

TABLE VIII-3 : ANTHELMINTICS USED AGAINST MONIEZIA BENEDENI AND M. EXPANSA

COMPOUND (Common name)	DOSE	COUNTRY	REFERENCE	DATE
ALBENDAZOLE	10 mg/kg	USA	THEODORIDES et al.	1976
AMINOACRIDINE	0.2 mg/kg	USSR	POTEMKINA	1954
	-	USSR	OREKHOV	1960
ARSENIC:				
Unspecified	-	USSR	AKRAMOVSKI et al.	1957
Calcium arsenate	0.3 and 0.5 gm	INDIA	SAXENA et al.	1972
	-	USSR	SHAKIEV	1973
Lead arsenate	0.5 gm	USA	McCULLOCH et al.	1941
	0.5 gm	USA	RADELEFF	1944
	1.0 gm	USA	HABERMAN & CARLSON	1946
	0.5 and 1.0 gm	USA	WARD & SCALES	1946
	1.0 gm	USA	ALLEN et al.	1948
	-	USA	FOSTER & HABERMAN	1948
	0.5 to 1.5 gm	USA	LINK et al. MORGAN et al.	1950 1950
	0.5 gm	IRAN	MAGHAMI et al.	1959
	-	USSR	OREKHOV	1960
	-	AUSTRALIA	GORDON	1972
Tin arsenate	0.5 gm	USSR	CHUBABRIYA	1955
	0.5 gm	USSR	GARKAVI	1956
	-	USSR	OREKHOV	1960
	-	USSR	LYASHENKO	1973
	-	USSR	SHAKIEV	1973
	0.8 gm	USSR	KADENATSII et al.	1975
	-	USSR	KOPBOSYNOV	1976
BANMINTH	-	POLAND	TARCZYNSKI & ROMANIUK	1975
BITHIONOL	7.0 mg/kg	JAPAN	FUKUI	1960b
	0.15 g/kg	USSR	KADENATSII et al.	1975
	-	USSR	SMYCHKOV	1975

TABLE VIII-3 (continued)

COMPOUND (Common name)	DOSE	COUNTRY	REFERENCE	DATE
CAMBENDAZOLE	30 mg/kg	CANADA	GIBBS & GUPTA	1972
	25 mg/kg	AFRICA - SOUTH	HORAK et al.	1972
	30 mg/kg	USA	KATES et al.	1973
	-	MEXICO	SERRANO et al.	1973
	40 mg/kg	USA	KATES et al.	1974
	-	NEW ZEALAND	SLAUGHTER	1977
COBALT CHLORIDE	-	USSR	DIKOV & GARBUZOV	1968
	-	USSR	ZHILIN	1969
COPPER:				
Cupric aceto arsenite	2.5 mg/kg	NEW ZEALAND	THOMAS	1962
Cupric hydroxy carbonate	-	USSR	DOLNIKOV et al.	1964
Cupric sulphate (solution)	1.125%	USSR	ISTOMIN	1946
	1.0%	USSR	POTEMKINA	1946
	1.0%	YUGOSLAVIA	KATICH	1947
	1.0%	USSR	PASKALSKAYA	1959
	2.0%	USSR	OREKHOV	1960
	2.0%	USSR	OLTEANU et al.	1962
	-	USSR	DOLNIKOV et al.	1964
	2.0%	USSR	RADIONOV	1964
	-	USSR	DIKOV & GARBUZOV	1968
	-	USSR	SHONOV	1969
	1.0%	USSR	ZHILIN	1969
	-	USSR	KATSOVA et al.	1973
	2.0%	USSR	AKBAEV et al.	1975
	-	USSR	SMYCHKOV	1975
DAVAINEX	-	USSR	ZAJICEK et al.	1972
DEVERMIN	0.2 g/kg	USSR	KADENATSII et al.	1975
	-	USSR	SMYCHKOV	1975
DICHLOROPHEN	150 mg/kg	JAPAN	FUKUI	1960b

TABLE VIII-3 (continued)

COMPOUND (Common name)	DOSE	COUNTRY	REFERENCE	DATE
DIPHENTHANE-70	0.5 g/1 lb	USA	ENZIE et al.	1953
	3.0 and 7.5 gm	GREAT BRITAIN	HARRIES	1953
FENBENDAZOLE	10 mg/kg	GERMANY	DUWEL et al.	1975
	5 mg/kg	GREAT BRITAIN	McBEATH et al.	1977
FILIXAN	0.3 gm/kg	USSR	POTEMKINA	1954
	-	USSR	SHAKIEV	1973
GENTAMICIN	-	USA	PANITZ	1975
LEVAMISOLE	8 mg/kg	USA	KATES et al.	1973
LINTEX	50 mg/kg	AFRICA - SOUTH	STAMPA & TERBLANCHE	1961
	-	AFRICA - SOUTH	STAMPA	1967
MANSONIL	-	VENEZUELA	PULGAR	1969
	-	YUGOSLAVIA	PETROVIC et al.	1970
MEBENDAZOLE	20 mg/kg	USA	KATES et al.	1974
MORANTEL TARTRATE	12.5 mg/kg	USA	KATES et al.	1973
NICOTINE SULPHATE	0.8 g/kg	INDIA	KATIYAR & GARG	1966
PHENASAL	-	USSR	KATSOVA et al.	1973
	-	USSR	LYASHENKO	1973
	-	USSR	VIBE	1974
	100 mg/kg	USSR	AKBAEV et al.	1975
	0.2 g/kg	USSR	KADENATSII et al.	1975
	-	USSR	SMYCHKOV	1975
	125 mg/kg	USSR	KOPBOSYNOV	1976
PHENOTHIAZINE	24 g	USA	McCULLOCH et al.	1941
	-	USSR	DIKOV & GARBUZOV	1968
	-	BULGARIA	DONEV	1975
	-	USSR	SMYCHKOV	1975

TABLE VIII-3 (continued)

COMPOUND (Common name)	DOSE	COUNTRY	REFERENCE	DATE
PRAZIQUANTEL	15 mg/kg	BULGARIA	BANKOV	1976
PYRETHUM	0.5 gm	ITALY	SAVI	1948
RESORANTEL	65 mg/kg	GERMANY	DUWEL	1975
SAGIMID	0.07 g/kg	USSR	KADENATSII et al.	1975
SULPHENE	0.085 g/kg	USSR	KADENATSII et al.	1975
TERENOL	65 mg/kg	GERMANY	BEHRENS et al.	1970
	62.5 mg/kg	GERMANY	DUWEL	1970
	65 mg/kg	GERMANY	PFEIFFER	1970
TETRAMISOLE	-	BULGARIA	DONEV	1975
THIABENDAZOLE	-	AUSTRALIA	GORDON	1972
	-	BULGARIA	DONEV	1975
TRICHLOROPHEN	-	BULGARIA	DONEV	1975
	150 mg/kg	USSR	KHALIDOV	1975
YOMESAN	-	GERMANY	ZETTL	1962
	50 mg/kg	INDIA	KATIYAR & GARG	1966
ZANIL	45 mg/kg	USSR	FROYD	1976

CHAPTER IX

PRELIMINARY INVESTIGATIONS OF MONIEZIA EXPANSA INFECTIONS IN LAMBS

Introduction

As described in Chapter VIII, the pathogenicity of Moniezia expansa remains a controversial subject. One of the several symptoms ascribed to the disease is that of anaemia. However, comprehensive investigations into the blood picture of infected lambs are rare. A preliminary experiment was designed, therefore, to monitor a wide range of blood parameters in animals with and without infections of M. expansa. (If any particular trends were observed from these results, further, more detailed, experiments were to be performed.)

Materials and Methods

Six Lonkswale-Cross lambs, aged between 8 and 9 weeks, were used. All had acquired a natural infection of Moniezia expansa, diagnosed by faecal egg counts, whilst grazing on rough hill pastures on a farm in Wincle, Cheshire. The lambs were brought indoors and penned in two groups, A and B, and were fed on a diet of hay and water ad libitum.

Faecal egg counts taken to check the tapeworm infection revealed the presence of gastrointestinal nematodes. In order to limit observations to those produced by M. expansa, all 6 lambs were dosed with Levamisole (I.C.I. Ltd.) at the recommended level of 7.5 mg/kg body weight to eliminate all the roundworms. All animals were subsequently weighed.

For the analysis, 10 ml of blood were taken from the jugular vein of

Haemoglobin was measured on a Coulter Haemoglobinometer - Cyanomethaemoglobin. Red and white blood cell counts were measured on a Coulter Counter - Model 2F. Packed Cell Volume was measured by the Microhaematocrit Method, using a Hawkesley Microhaematocrit Centrifuge.

Platelets were measured using Wrights Stain with an Ames Hema - Tek Slide Stainer.

Other parameters were measured using a Technicon SMA 12/60 Micro Plus Auto Analyser.

each animal and collected in heparinised tubes ready for processing. Two sets of samples were taken in this way.

The following parameters were measured:-

Haemoglobin (HB) g/dl

Red blood cell count (RBC) x 10^{12}/l

Packed cell volume (PCV) % volume

White blood cell count (WBC) x 10^{9}/l

Neutrophils (N) %

Lymphocytes (L) %

Monocytes (M) %

Eosinophils (E) %

Basophils (B) %

Platelets (P) x 10^{9}/l

Sugars (SUGAR) mg/100 ml

Urea (UREA) mg/100 ml

Total Protein (TP) mg/100 ml

Albumin (ALB) mg/100 ml

Total Bilirubin (TBIL) mg/100 ml

Alkaline Phosphatase (ALP) mU/ml

Alanine Amino Transferase (ALT) mU/ml

Aspartate Amino Transferase (AST) mU/ml

Creatine Kinase (CK) mU/ml

Sorbitol Dehydrogenase (SDH) mU/ml

Glutamate Dehydrogenase (GLDH) mU/ml

Sodium (Na) mM

Potassium (K) mM

Calcium (Ca) mM

Inorganic Phosphorus (InP) mM

The analyses were performed by the Haematology Department, I.C.I. Pharmaceuticals Ltd., Cheshire. For methods see facing page.

To ascertain the effects, if any, of the disease on the blood picture, 3 lambs (Group A) were cleared of their infection by dosing with Terenol (Hoechst Ltd.) at 100 mg/kg body weight, so providing 3 "healthy" animals for comparison with the 3 infected ones. After 14 days the second set of blood samples were taken and the same parameters measured.

Regular faecal egg counts and weighings of the animals were carried out.

The complete regime of the experiment was as follows:-

Day	1	6 lambs penned in 2 groups, A and B.
		Weighed, A and B.
		Faecal egg counts taken, A and B.
		Levamisole dose (7.5 mg/kg) A and B.
	3	Weighed, A and B.
	8	Blood sampled, A and B.
	9	Terenol dose (100 mg/kg), A only.
	15	Faecal egg counts taken, A and B.
		Weighed, A and B.
		Levamisole dose (7.5 mg/kg), A and B.
	22	Blood sampled, A and B. Faecal egg counts taken, A and B.
	26	Weighed, A and B.
		Sacrificed.

At the end of the experiment all 6 lambs were sacrificed and any tapeworms present were counted and weighed. Checks were also made for any abnormalities or pathology caused by the tapeworms in the gut or related organs.

Results

TABLE IX-1 RESULTS OF THE BLOOD ANALYSES

(Top figure Analysis 1, bottom figure Analysis 2)

Lamb	HB g/dl	RBC $x10^{12}/l$	PCV Vol.%	WBC $x10^{9}/l$	N %	L %	M %	E %	B %	P $x10^{9}/l$
A1	14.3	10.0	42	13.0	10	89	1	0	0	534
	14.4	9.3	40	11.8	7	91	2	0	0	462
A2	14.4	9.7	45	11.6	40	59	1	0	0	536
	14.8	9.5	44	8.6	24	71	3	2	0	232
A3	14.4	9.9	43	7.4	13	86	0	1	0	477
	14.8	9.5	43	10.8	20	76	3	1	0	349
B1	14.2	9.1	43	8.6	20	71	0	9	0	521
	14.4	9.3	43	12.0	17	78	1	4	0	389
B2	13.6	9.4	41	7.1	12	88	0	0	0	296
	14.8	9.5	42	11.3	13	86	1	0	0	402
B3	15.6	11.2	46	8.8	17	83	0	0	0	487
	14.5	9.5	42	11.9	21	75	2	2	0	396
*Average	12.9	10.8	36	5.9	36	54	8	1.7	0	-

*Average values from Schalm, O.W. (1961) for healthy lambs 60-80 days old.

TABLE IX-1 - continued

Lamb	SUGAR mg/ 100ml	UREA mg/ 100ml	TP mg/ 100ml	ALB mg/ 100ml	TBIL mg/ 100ml	ALP mU/ml	ALT mU/ml	AST mU/ml	CK mU/ml	SDH mU/ml
A1	2.5	5.6	70	34	5	100	12	138	213	8
	4.4	6.7	67	34	3	136	12	137	136	7
A2	2.6	7.6	64	33	5	60	13	119	193	21
	4.1	8.9	58	32	3	108	12	119	157	9
A3	3.8	7.3	63	37	4	86	12	142	334	10
	4.5	8.7	63	37	3	121	11	97	171	6
B1	3.7	3.5	65	36	4	88	10	95	1028	5
	4.9	5.7	65	35	2	125	10	125	133	3
B2	4.2	4.9	63	34	5	103	12	262	862	38
	5.6	7.3	65	34	3	108	9	124	145	5
B3	3.3	5.9	69	40	3	102	15	84	435	10
	4.4	7.5	61	38	2	143	9	78	113	4

TABLE IX-1 - continued

Lamb	GLDH mU/ml	Na mM	K mM	Ca mM	InP mM
A1	3	142	4.9	2.55	2.86
	21	141	5.7	2.61	1.78
A2	84	145	4.2	2.51	2.26
	25	142	5.4	2.37	1.93
A3	19	146	4.3	2.62	2.45
	12	144	5.3	2.60	2.18
B1	3	139	4.7	2.66	2.18
	0	143	5.6	2.67	2.46
B2	3	147	4.7	2.37	3.25
	3	142	5.5	2.67	1.77
B3	17	145	5.1	2.61	3.43
	4	141	5.5	2.70	2.21

TABLE IX-2: FAECAL EGG COUNTS

	Day 1		Day 15		Day 22	
	M	N	M	N	M	N
A1	3,500	✓	0	x	0	x
A2	24,000	✓	0	x	0	x
A3	6,200	✓	0	x	0	x
B1	9,000	✓	5,000	x	0	x
B2	106,000	x	7,000	x	3,000	x
B3	28,000	✓	12,000	x	1,800	x

M = M. expansa egg counts (eggs per gram)

N = Nematodes, present or absent.

TABLE IX-3 WEIGHTS (in kilograms)

	Day 1	Day 15	Day 26
A1	17	17	19
A2	16	17	16
A3	18	19	19
B1	21	24	29
B2	21	21	23
B3	18	18	18

TABLE IX-4: POST MORTEM WORM COUNTS

A1	No worms.
A2	No worms.
A3	No worms.
B1	No worms.
B2	Worms:- 77.32g; 67cc; 11 scoleces; no pathology.
B3	Worms:- 56.29g; 58cc; 7 scoleces; no pathology.

Discussion

The results show no real differences in the blood pictures or weight gains of the healthy or, more accurately, the cured lambs and the infected lambs. The parameters chosen for the blood analysis are far-ranging enough to provide a comprehensive guide to the state of health of the animal concerned. Haemoglobin, packed cell volume and cell count values indicate normal or anaemic blood and show response to disease, such as an increased number of eosinophils and lymphocytes in damaged tissues. Creatine kinase which maintains an ATP reservoir, total protein, sugars and alkaline phosphatase values reveal the overall condition of the animal. Sorbitol dehydrogenase, glutamate dehydrogenase, alanine amino transferase, aspartate amino transferase and the bilirubin levels are normally low and when raised abnormally are excellent indicators of liver damage or disease. Thus, it would appear that, under the conditions of this experiment, these infections of Moniezia expansa in the three lambs were not pathogenic and did not cause any ill effects. There are, however, a number of other considerations to be made before conclusions of any kind can be drawn.

Two unavoidable factors related to experimental technique could well have influenced the results.

Firstly, no true controls were available for comparison. Ideally, three groups of animals should have been used: uninfected animals which had not been in contact with the disease; infected animals; and animals which had been cured of their infection. Without reference to the first of these groups, it is possible that residual effects of the infections present in the cured animals would obscure any differences.

However, it is also important that all groups should have been subjected to similar conditions of food and habitat before the start of the experiment. For this criterion to have been met, all lambs would have been grazing on hill pastures. All would then have been prone to tapeworm infection, and it would not be possible to state with any certainty that uninfected animals had not been in contact with the disease.

Alternatively, individuals of two groups of lambs could have been reared in a controlled, tapeworm-free environment. A known, experimental infection could then have been given to one group and the other, uninfected, group would have served as controls. Unfortunately, owing to a lack of the infective agents of M. expansa, such experimental infections were not possible.

Secondly, the number of experimental animals used for the experiment was very low - 3 only in each group, so that some changes in the blood picture could have been overlooked. Much larger numbers would be required to provide any statistically significant results.

The approximate ages and life histories of the lambs were known, but no facts or figures were available relating to their infections as these had been naturally acquired before the start of the experiment. Although faecal egg counts cannot, with any certainty, be used to indicate the level of an infection, but only reflect the general picture (Bezubik, Stankiewicz & Sinski, 1974), initial examination of the lambs revealed high numbers of eggs and proglottids in the faeces which were noticeably decreased by Day 22 of the experiment, i.e. when the second blood samples were taken. This would suggest that the infection, or worm burden, had been reduced in some way or that patency had ceased, and therefore there was no real difference between the two groups of animals. Kotel'nikov & Khudoshin (1976) report that abrupt dietary changes can result in destrobilisation of the tapeworms. It is possible, therefore, that the moving of the lambs from the hill pastures and a grass diet to the pens and a hay diet resulted in an early loss or reduction of their infections during the course of the experiment. One of the 3 lambs in Group B which was not dosed for tapeworms had lost its infection when examined at post mortem. Kates, Colglazier, Enzie, Lindahl & Samuelson (1973) found that Levamisole given at 8 mg/kg body weight was partially effective in removing tapeworms, although in a later experiment (Kates et al., 1974) they reported no loss of tapeworms. However, the Levamisole dose, necessary for removing the nematodes, could have adversely affected the tapeworms in this experiment, thus causing the observed reduction in the infection.

Reports of other experimental infections attempting to elucidate the pathogenicity of M. expansa in lambs are similarly inconclusive. Shorb (1939), Kates & Goldberg (1951) and Brunsdon (1964) found no clinical evidence of the disease and no significant effect on liveweight gains. No blood parameters were measured. In all cases the number of

experimental animals used was low. However, the type of infections varied in that some were naturally acquired (Brunsdon loc. cit.) and some were artificially produced (Shorb, loc. cit. and Kates & Goldberg, loc. cit.). The worm burdens, too, varied, ranging from 4 to 114. Results of all these investigations still produced similar conclusions.

Stampa (1967) and Hansen, Kelley & Todd (1950) report significant weight gain differences between parasitised and non-parasitised animals. Hansen et al. (loc.cit.) also examined the blood picture of the lambs and found a depression of haemoglobin and packed cell volume levels bordering on anaemia. The only other known experiment examining the blood picture of infected lambs was performed by Ulyanov (1962). He injected the animals with an extract of the tapeworms and recorded a reduction in haemoglobin, erythrocytes and leukocytes, total protein, blood enzyme activity and blood alkaline reserves. No experimental details were available.

Factors such as age of lambs and their diet are important when considering such results. Shorb (loc.cit.) points out that at 5 months his lambs were older than the average lamb acquiring infections. Stampa (loc.cit.) also found that, when cleared of their infections, lambs under 7 months old had comparatively greater weight gains than those above this age.

Stampa (loc.cit.) used lambs from different locations and therefore on different diets for his experiments. He found that lambs raised on poor, arid pastureland and therefore undernourished, had much greater weight gains after dosing for tapeworms than those raised on good pastureland which were well-nourished. He also points out that the

animals used in Brunsdon's experiment (loc.cit.) were well fed.

The pathogenicity of Moniezia expansa has been discussed in some detail in the previous chapter. The experiments described here do not give the final answer to the controversy, but help to shed some light on the problems and indicate areas where more research is needed.

Summary

Preliminary investigations were made into the effect of infections of Moniezia expansa in lambs. A wide variety of blood parameters were measured and results for infected lambs were compared with those for lambs which had been cured of their infections. No apparent differences were recorded.

Experiments involving artificial infections of Moniezia in lambs are discussed.

CHAPTER X

IN VITRO EXCYSTATION AND CULTURE OF THE CYSTICERCOIDS OF MONIEZIA EXPANSA

Introduction

The in vitro culture of several cestode species has now been achieved and the techniques have proved of great value for immunological, biochemical and physiological studies. Reviews of the literature have been given by Taylor & Baker (1968), Silverman & Hansen (1971), and Smyth (1976). However, very little research of this type has been carried out with Moniezia expansa. Attempts were made, therefore, to find a suitable growth-promoting medium for the in vitro culture of the mature worms from the cysticercoids.

Larvae had first to be excysted from the protective cyst wall of the cysticercoid. Based on the work of Rothman (1959), a combination of enzyme and bile salt solutions was tested.

Successfully excysted larvae were then set up in a variety of culture media.

Materials and Methods

General procedures

All apparatus and glassware was sterilised by autoclaving under pressure before use. Salines and media were sterilised by vacuum filtration through a 0.2 µm millipore filter (Gelman). Both were stored at 4°C if not required for immediate use. The composition of all salines and media used is given in Appendix I.

Sterile procedures were performed in a lamina flow cabinet which was cleaned and swabbed with ethanol before use.

Cysticercoids

Oribatid mites which had been infected with eggs of M. expansa and cultured in the laboratory were dissected in Hank's balanced salt solution (HBSS), as described in Chapter III. Cysticercoids were collected and stored for short periods at room temperature in sterile HBSS in groups of 10 prior to each experiment.

Excystation

The following solutions were tested for their efficacy in promoting excystation. All were made up in sterile HBSS and, unless otherwise stated, were at a final pH of 7.2 - 7.4. Before the start of an experiment, all solutions to be used were warmed to 37°C - the temperature at which all experiments were performed.

A. Acid Pepsin Solutions:-

Solutions of pepsin (Sigma[1]) at concentrations of 0.5% (Solution A1) and 1.0% (Solution A2) were adjusted to pH 2 by the addition of a few drops of 1M hydrochloric acid.

B. Enzyme and Bile Salt Solutions:

(1) Trypsin (Sigma[2]) at 0.3% and sodium taurocholate (Sigma) at 0.1%;

FOOTNOTES: 1 = Pepsin (E.C. No. 3.4.4.1) from Hog Stomach Mucosa, 1 : 10,000
Activity: 700 - 1,000 units per mg protein.

[2] = Trypsin - Type II: Crude from Hog Pancreas.
Activity: 1,000 - 1,500 BAEE units/mg.
Chymotrypsin.
Activity: 500 - 1,000 ATEE units/mg.

(2) Pancreatin (Sigma[3]) at 0.3% and sodium taurocholate at 0.1%;

(3) Pancreatin at 0.5% and sodium taurocholate at 0.5%;

(4) Pancreatin at 0.5% and sodium taurocholate at 0.1%;

(5) Pancreatin at 0.1% and sodium taurocholate at 0.1%.

Batches of cysticercoids were treated with one of the solutions from A for 30 minutes, followed by one of the mixtures from B. Care was taken when the solutions were changed to ensure that all traces of the acid solution were removed before the second treatment. The cysticercoids were, therefore, rinsed in sterile HBSS at this stage.

Separate experiments were also performed with the omission of one of the three reagents. Cysticercoids were thus treated with the following combination of solutions:-

C.1 Acid pepsin (0.5%) followed by pancreatin (0.5%);

C.2 Acid pepsin (0.5%) followed by sodium taurocholate (0.5%); or

C.3 A single treatment with a mixture of pancreation (0.5%) and sodium taurocholate (0.5%).

Observations were made during the course of the experiments to monitor any changes occurring. These were made using an inverted microscope (Zeiss) at 37°C.

FOOTNOTE: [3] = Pancreatin - Grade VI from Hog Pancreas.
Activity equivalent to 4 x National formulary (N.f.)

In vitro culture

Three commercially prepared media were utilised for the attempts at in vitro culture. These were Medium 199, Medium NCTC 135 and Medium 858. Their composition is given in Appendix I.

One or more of the following additives were occasionally included in the media:-

(1) Foetal Calf Serum (Gibco Bio-Cult): FCS, used at concentrations of 10% or 20%. It was first inactivated by heating in a water bath at 56°C for 30 minutes.

(2) Bovine Embryo Extract (Flow): BEE 50, used at a concentration of 25%.

(3) Cow Bile: C BILE, at 5%.

(4) Sheep Bile: S BILE, at 0.1%.

A medium consisting of 858 and several additives (Appendix I), designated as S10 (Smyth, in press), was also used.

Monophasic cultures were set up using 4ml of the above media in glass Leighton tubes (Bellco), size 16 x 93 mm. The tubes were placed in a shaking water bath (100 shakes per minute, set to shake for 2 minutes in every 20) at 37°C.

Diphasic cultures were also set up using either coagulated new-born calf serum (Flow) or nutrient agar (Flow) as the base. The serum was coagulated by heating at 75°C for 30 minutes. In each case, 1 ml of the liquid base was placed in the bottom of the Leighton tubes and allowed to solidify, producing a horizontal surface.

Cultures were maintained aerobically, with air as the gas phase, at pH 7.2 - 7.4. After an initial 5 days in culture, and then every subsequent 3 days, 2 ml of the medium were removed and replaced with 2 ml of fresh medium.

Cultures were observed, using an inverted microscope at 37°C, every 24 hours to check for growth.

Results

Excystation

The results for the excystation experiments are given in Table X-1. Recorded observations used in the table are described in detail below.

"No Change" - the treatment had no visible effect on the cysticercoid (Plate IV-1a)

"Swelling" and "Disruption" - of the cyst wall (Plate IV-1, c and d).

"Excystation" - the complete removal, by digestion, of the cyst wall (Plate IV-2,e)

"Activation" - the inverted scolex alternately expanded and contracted; this occurred with or without the cyst wall intact.

"Evagination" - scolex evaginated, leaving sac-like remains of cyst containing calcareous corpuscles (Plate IV-2,f).

In summary, the results show that the process of excystation can be divided into 3 stages: (1) breakdown and removal of cyst wall; (2) activation; and (3) evagination. The system which proved to be the most effective at promoting excystation was a 30-minute acid-pepsin (0.5% or 1.0%) treatment, followed by a 10-minute treatment in a mixture

of pancreatin (0.5%) and sodium taurocholate (0.5%). However, evagination did occur without pre-incubation in acid pepsin.

TABLE X-1: Results of Excystation Experiments

Experiment No.	Treatment	Duration of Treatment (minutes)	Observations
1	A1	30	No change
	B1	120	No change
2	A2	60	No change
	B1	60	Swelling and disruption
		120	No further change
3	A1	30	No change
	B2	5	Swelling and disruption
		10	Activation
		20	Excystation
		30	Evagination
4	A1	30	No change
	B3	2	Swelling and disruption and activation
		5	Excystation
		10	Evagination
5	A2	30	No change
	B3	1	Swelling and disruption and activation
		3	Excystation
		10	Evagination

TABLE X-1 - continued

Experiment No.	Treatment	Duration of Treatment (minutes)	Observations
6	A1	30	No change
	B4	5	Swelling
		10	Disruption and activation
		20	Excystation
		30	Evagination
7	A1	30	No change
	B5	5	Swelling
		10	Disruption
		20	Activation
		30	Excystation
		40	Evagination
8	A1	30	No change
	C1	20	Swelling
		30	Disruption and activation
		40	Excystation
		50	Evagination
9	A1	30	No change
	C2	30	Activation
		120	No further change
10	C3	20	Swelling and disruption
		30	Activation
		40	Excystation and evagination

PLATE IV

Excystation of Moniezia expansa cysticercoids

(a) Normal cysticercoid dissected from mite: x 300.

Post acid pepsin solution:

(b) 30 minutes: x 300.

Post bile salt and pancreatin:

(c) Swelling of cyst wall: x 300.

(d) Disruption of cyst wall: x 300.

PLATE IV.I

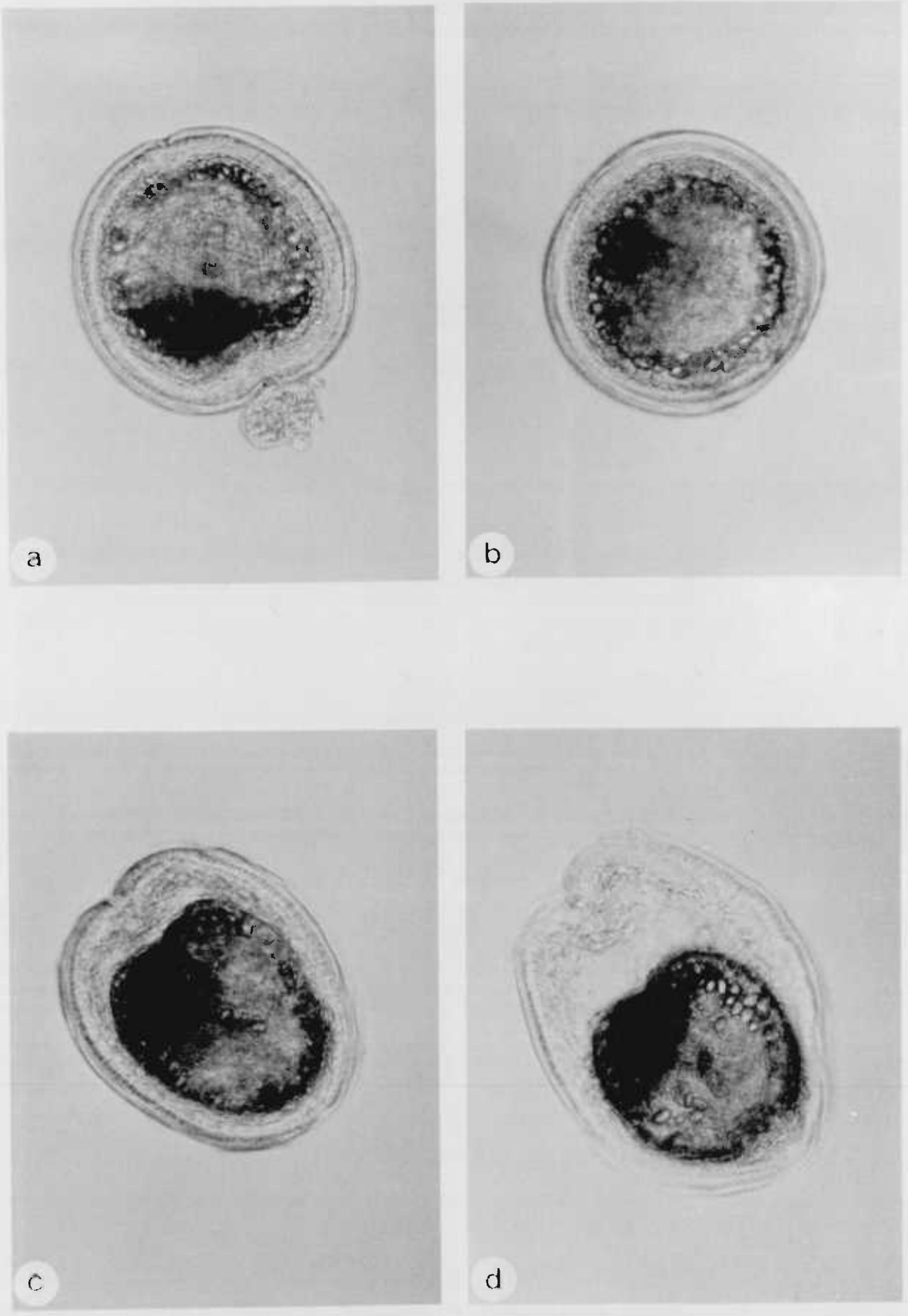
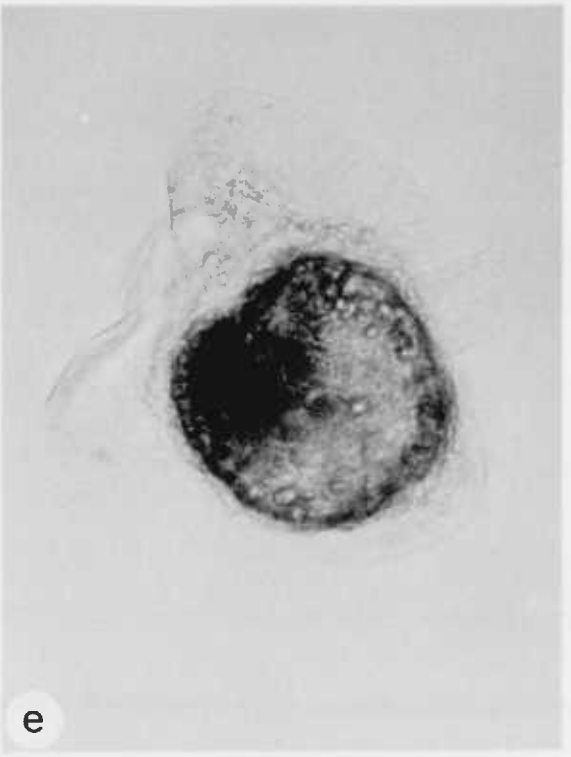


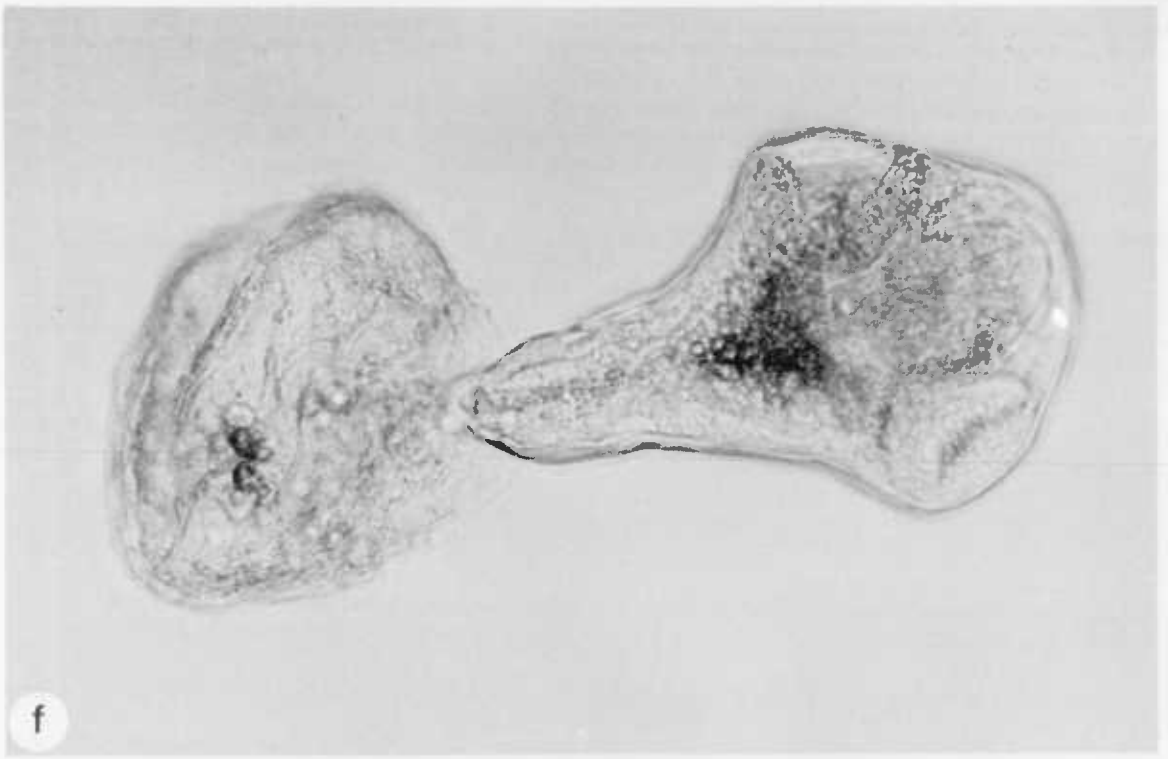
PLATE IV - continued

(e) Digestion of cyst wall: x 300.

(f) Evaginated cysticercoid: x 370.

PLATE IV.2





In vitro culture

The results are summarised in Table X-2. The "duration of culture" records the number of days observations were made on the excysted larvae. The cultures were terminated when the larvae appeared moribund or dead.

Where the phrase "no development" has been used to describe the cultures, it implies no growth or differentiation, rather than no actual change. Towards the end of some cultures, larvae did degenerate due to the apparently detrimental effects of some of the media.

Dimensions of the larvae were difficult to obtain owing to the continuous contracting and expanding of the organisms. Measurements were, therefore, taken when the larvae were at rest. Newly excysted larvae measured approximately 210 µm in length, with the scolex region measuring approximately 130 µm in diameter. 14-day old larvae measured approximately 300 µm in length and 180 µm in diameter, thus showing an overall increase in size. (Plate V)

No development, i.e. differentiation or strobilisation, of the larvae was observed. Some cultures sustained actively contracting larvae for several days and these larvae showed a slight increase in size as described above.

Of the media employed, medium 199 and medium NCTC 135, with the addition of foetal calf serum at 10% and sheep's bile at 0.1%, proved most effective in maintaining healthy cultures.

TABLE X-2: Results of in vitro experiments

Experiment No.	Medium	Additive	Base	Observations	Duration of culture (days)
Monophasic Cultures					
1	858	-	-	No development	3
2	199	-	-	No development	6
3	135	-	-	No development	5
4	S10	As formula	-	No development	3
5	199	10% FCS	-	No development. Active for several days.	10
6	199	20% FCS	-	As above	10
7	199	10% FCS 5% CBile	-	No development	1
Diphasic Cultures					
8	199	10% FCS 25% BEE_{50}	-	No development	7
9	199	10% FCS	Serum	No development. Active for several days.	10
10	135	10% FCS	Serum	No development. Active for several days.	11
11	199	10% FCS 25% BEE_{50}	Serum	No development	7
12	199	10% FCS 0.1% SBile	Serum	No development. Active. Slight size increase.	19
13	135	10% FCS 0.1% SBile	Serum	No development. Active. Slight size increase.	17
14	199	10% FCS 0.1% SBile	Agar	No development. Active.	14
15	135	10% FCS 0.1% SBile	Agar	No development. Active.	12

PLATE V

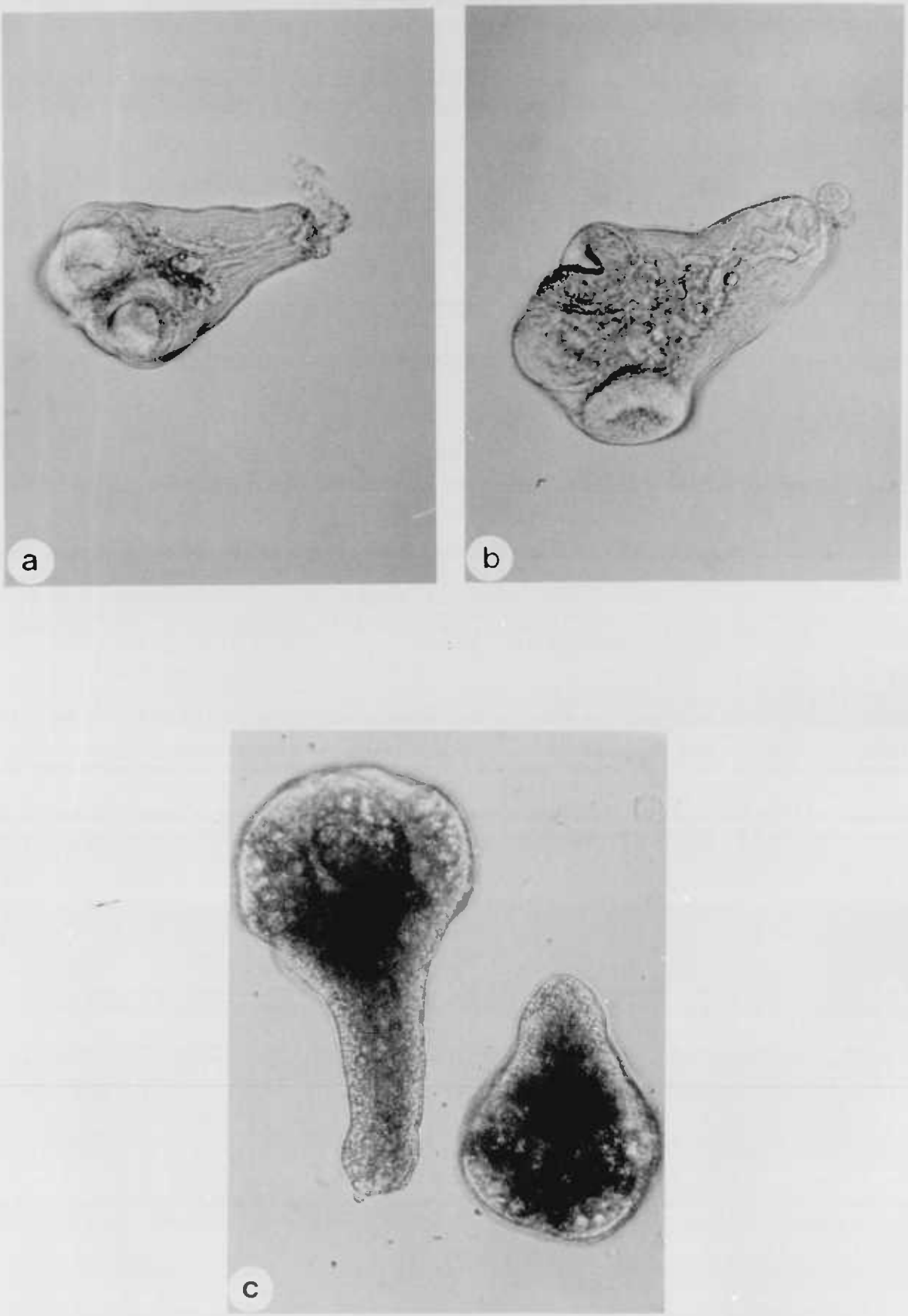
Larval Moniezia expansa in culture

(a) Day 1: x 260.

(b) Day 5: x 260.

(c) Day 15: x 260.

PLATE V



Discussion

The results reveal that the cysticercoids of Moniezia expansa have similar requirements for successful excystation to those reviewed by Rothman (1959). Caley has described the structure and function of the cysts of Hymenolepis microstoma (Caley, 1974) and Moniezia expansa (Caley, 1976). An outer collagenous layer protects the larva from mechanical damage on entry to the host, whilst an inner myelin-like layer protects it from the harsh, acidic environment in the stomach. Excystation is thus effected by the removal of these encapsulating layers.

The acid pepsin solution initiates the break-down of the fibrous outer wall and digestion of the cyst is brought about by the action of the pancreatic enzyme, enhanced by the presence of the bile salt.

However, some important differences between the excystation of M. expansa cysticercoids and those of other species were observed. Trypsin, the pancreatic enzyme most commonly used for the procedure, was ineffective in promoting excystation of M. expansa cysticercoids. Chymotrypsin, present in the crude preparation of trypsin used during these experiments, was similarly ineffective. Pancreatin, however, was successfully utilised in their place. It is interesting to note that during efforts to hatch the eggs of this species the pyriform apparatus was digested by pancreatin and chymotrypsin, but not by trypsin on its own. It is possible, therefore, that the substitution of pancreatin for trypsin is a diagnostic character peculiar to the anoplocephalid worm.

It was also noted that the larvae could be excysted without an acid pepsin treatment. Rothman (1959) reported that this pre-treatment served only to enhance the effect of the following solutions and did not take any active part in digesting the cyst. This is supported by the observation made in the present work that no visible change was apparent in the cyst wall even after an extended period in the acid pepsin solution. Hence, a prolonged treatment in pancreatin and the bile salt solution proved sufficient. It would appear that pancreatin is the major substance promoting excystation, as in its absence no larvae were found to excyst. Sodium taurocholate was necessary for rapid excystation and activation of the larvae.

Thus, these results suggest that the requirements for rapid excystation include all three solutions - acid pepsin, pancreatin and sodium taurocholate. However, each one does not function independently, but interacts to enhance the effect of the others.

The initial attempts at culturing M. expansa from excysted larva to mature adult, which have been described here, proved unsuccessful. The general principles of in vitro culture have already been discussed (Chapter VII) and, therefore, the problems relating to it will not be repeated here.

The culture systems utilised were chosen on the basis of their previous success in promoting the in vitro development of certain other species of cestode. Modifications of Medium 858, including S10, have been used by Smyth (in press) to culture the strobilar stage of Echinococcus granulosus. Seidel (1971) used Medium NCTC 135 with various additives to grow Hymenolepis microstoma to the prepatent adult from the cysticercoid. Several pseudophyllideans have also been cultured using

Medium 199, including Spirometra mansanoides (Mueller, 1966). The addition of substances such as sera to the final culture medium has proved of great value. This subject has been reveiwed by Heath & Elsdon-Dew (1972). Often, some particular physical characteristic of the culture system is important for the normal development of the organism. Smyth & Davies (1974) found that E. granulosus produced abnormal, or so-called "monozoic" forms in monophasic media; the use of coagulated serum as a solid base was necessary for strobilisation.

Similar systems have not, as yet, resulted in more than the maintenance of larval Moniezia expansa tapeworms in vitro. However, much useful information regarding beneficial media and additives has been gained which will provide a basis for further in vitro studies.

Summary

Cysticercoids of Moniezia expansa grown in laboratory infected oribatid mites were excysted prior to in vitro culture. The process of excystation occurred in three stages: (1) digestion of the cyst wall; (2) activation; and (3) evagination. Cysticercoids were subjected to treatments in acid pepsin and a mixture of pancreatin and sodium taurocholate.

Sterile larvae, successfully excysted, were maintained in culture for up to 19 days. Although a slight overall increase in size was observed, no strobilisation occurred.

CHAPTER XI

GENERAL DISCUSSION AND SUMMARY

Since Stunkard discovered the mite intermediate host, thereby completing our knowledge of the life cycles, numerous authors have contributed towards our understanding of the biology of Moniezia benedeni and M. expansa. However, it is clear that there are still areas of research which have been neglected and where work remains to be done. For example, tapeworms of the genus Moniezia have rarely been used for in vitro culture studies, and the pathogenicity of the adult worms in their final host remains a controversial topic. The present research has, it is hoped, made some attempts to fill these gaps.

The principles and techniques of in vitro culture of helminths have been discussed at length elsewhere (Chapters V and VII). Moniezia uses two hosts to complete its life cycle, which conveniently divides any culture work into two sections: (1) the oncosphere to cysticercoid stage; and (2) the cysticercoid to mature worm. However, before treating them individually, it is necessary to emphasise those aspects which apply to both.

As a preliminary to in vitro culture, the organisms involved must be prepared so that they are at the appropriate stage for further development to occur. They must also be prepared so that they are in a clean and sterile condition. Hence, oncospheres must be hatched from the egg shell and surrounding membranes and cysticercoids must be excysted and evaginated from the protective cyst.

The method of hatching M. expansa eggs was first described by Caley (1975). Further techniques had to be developed, however, to obtain oncospheres suitable for culture. A method has now been established which enables large numbers of eggs to be hatched, cleaned and sterilised, so that the above requirements are satisfied. Cleaning and sterilising the eggs proved to be more difficult than was at first expected. Graham & Berntzen (1970) found that Hymenolepis diminuta oncospheres would not develop in the presence of debris such as broken shell fragments and unhatched eggs. The filtration stage was thus an important part of the procedure. Concern for the sterility of the oncospheres led to an unexpected discovery regarding the biology of the egg. Persistent contamination of the cultures was found to be related to the presence of large numbers of bacteria contained in the eggs. This somewhat unusual problem was finally overcome by the identification of the bacteria and the finding of an effective antibacterial agent.

Reports of similar associations between micro-organisms and helminths are becoming more frequent. The phenomenon of these interspecific relationships has been discussed in Chapter VI. The role of the bacteria in the eggs of Moniezia spp. is at present conjectural. Further work is necessary to establish the nature of the association.

For the first time, cysticercoids of M. expansa have been excysted in vitro. The procedures involved fortunately leave the larvae in a sterile condition so that no other special techniques are required as preparation for in vitro culture.

Unfortunately, attempts to culture the oncospheres and the cysticercoids *in vitro* met with little success. However, some cultures were maintained for several days in an apparently healthy condition. These initial experiments gave much useful information regarding the type of media and culture systems suitable for further research.

The nature of the pathogenicity of *M. expansa* and *M. benedeni* infections in sheep and cattle is a subject which has promoted an enormous amount of discussion amongst farmers, veterinary surgeons and scientists (Chapter VIII). The chief factor preventing agreement is the lack of experimental work to investigate the effect of the tapeworms. However, the extensive and comprehensive experiments necessary would involve the use of large numbers of animals and even larger numbers of the infective agent - the cysticercoids. Workers so far have relied on obtaining cysticercoids from infected mites, which tends to be a painstaking and laborious procedure. Nevertheless, it is a necessary one if no alternative source is available.

Following the lack of success in producing cysticercoids *in vitro*, laboratory cultures of the intermediate host were set up. Techniques for the extraction, maintenance and infection of the mites were developed so that an efficient system for producing cysticercoids in the laboratory was established. Initial experiments were also performed in an effort to find an alternative laboratory intermediate host which would be easier to maintain than the mite. Had these proved successful, it was hoped that a more convenient and less time-consuming system could be developed allowing for the simple and rapid production of cysticercoids.

Owing to the unusual difficulties in obtaining the infective stages in sufficient quantities, some experiments were performed using naturally infected animals. Extensive investigations were carried out into the blood pictures of infected animals and those which had been cured of the disease and were therefore apparently healthy. The results indicated little or no difference between the two groups of animals and therefore suggested that in those particular conditions the disease had no effect. These experiments are far from producing conclusive evidence in support of any arguments surrounding the pathogenicity of M. expansa. However, they do serve to emphasise the kind of problems involved in such research, and also provide data which may be useful for further comparative work. There is thus still much to be learnt regarding the effects of this parasite.

An alternative line of research, and one which to date has received little attention, involves the forecasting of the course of a disease from one year to the next. The continuation of the life cycle of Moniezia is made possible only by the successful transmission of the organism from mite to sheep or cow. Thomson, Downey & Dreadon (1956) recorded a particularly high infection of M. expansa in sheep after an abnormally mild winter and early spring. These conditions ideally suit the habits and behaviour of oribatid mites, and it could be assumed, therefore, that transmission was similarly favoured. Conversely, conditions of drought, or extreme heat or cold would not suit the mites and transmission would not be favoured. It thus seems possible that the severity of an infection could be forecast from the climatic observations made during the winter of the preceding year, much as is currently done for outbreaks of Fasciola hepatica (Ollerenshaw, 1974). The value of such a system would be considerable and would eliminate the dilemma of the farmers who have to decide whether or not to dose

their animals.

The results of the work presented here can be briefly summarised under three main headings: (1) the development of techniques; (2) preliminary investigations; and (3) new discoveries. Techniques for handling and infecting oribatid mites in the laboratory and for preparing organisms for *in vitro* culture have been established. Preliminary investigations have been made into suitable systems for *in vitro* culture of oncospheres and tapeworms, into the finding of an alternative and more convenient laboratory intermediate host, and into the effect of the tapeworms on their final host. A system for excysting cysticercoids *in vitro* has been developed. Bacteria have been discovered in the eggs of the tapeworm and have been isolated and identified, enabling a suitable antibacterial agent to be found.

These findings have formed a basis for further work to be performed on the biology and epidemiology of *Moniezia benedeni* and *M. expansa*. Experiments to find a growth-promoting medium for the development of the oncosphere to cysticercoid stage, and also the adult worm, can now be undertaken. If successful, investigations into the pathogenicity of adult *Moniezia* in the final host could then be made using two methods: (1) from controlled experimental infections using cultured cysticercoids; and (2) from biochemical and physiological studies made on the mature worm *in vitro*.

APPENDIX I: SALINES AND MEDIA - IN VITRO CULTURE

SALINES

Earle's — pH 7.2 - 7.4

Ingredients per litre - grams (g)

Bacto Dextrose	1.0
NaCl	6.8
KCl	0.4
CaCl	0.2
$MgSO_4$	0.2
NaH_2PO_4	0.125
$NaHCO_3$	2.2

Gas Phase: 5% CO_2 in air.

Gey's — pH 7.2 - 7.4

Ingredients per litre - grams (g)

Bacto Dextrose	1.0
NaCl	8.0
KCl	0.375
$CaCl_2$	0.275
MgCl	0.21
Na_2HPO_4	0.15
NaH_2PO_4	0.025
$NaHCO_3$	2.2

Gas Phase: Air

Hank's pH 7.2 - 7.4

Ingredients per litre - grams (g)

Bacto Dextrose	1.0
NaCl	8.0
KCl	0.40
$CaCl_2$	0.14
$MgSO_4$	0.10
$MgCl_2$	0.10
KH_2PO_4	0.06
Na_2HPO_4	0.06
$NaHCO_3$	0.35
Phenol Red	0.02

Gas Phase: Air

MEDIA

199, Morgan & Morton Medium 150 pH 7.2 - 7.5

Ingredients per litre

L-Arginine	70 mg	Calcium Pantothenate	0.01 mg
L-Histidine	20 mg	Biotin	0.01 mg
L-Lysine	70 mg	Folic Acid	0.01 mg
L-Tyrosine	40 mg	Choline	0.5 mg
DL-Tryptophane	20 mg	Inositol	0.05 mg
DL-Phenylalanine	50 mg	p-Aminobenzoic Acid	0.05 mg
L-Cystine	20 mg	Vitamin A	0.1 mg
DL-Methionine	30 mg	Calciferol	0.1 mg
DL-Serine	50 mg	Menadione	0.01 mg
DL-Threonine	60 mg	a-Tocopherol Phosphate	0.01 mg
DL-Leucine	120 mg	Ascorbic Acid	0.05 mg
DL-Isoleucine	40 mg	Glutathione	0.05 mg
DL-Valine	50 mg	Cholesterol	0.2 mg
DL-Glutamic Acid	150 mg	L-Glutamine	100 mg
DL-Aspartic Acid	60 mg	Adenosinetriphosphate	1 mg
DL-Alanine	50 mg	Adenylic Acid	0.2 mg
L-Proline	40 mg	Ribose	0.5 mg
L-Hydroxyproline	10 mg	Desoxyribose	0.5 mg
Glycine	50 mg	Bacto-Dextrose	1000 mg
L-Cysteine	0.1 mg	Tween 80	5 mg
Adenine	10 mg	Sodium Acetate	50 mg
Guanine	0.3 mg	Iron (as Ferric Nitrate)	0.1 mg
Xanthine	0.3 mg	Sodium Chloride	8000 mg
Hypoxanthine	0.3 mg	Potassium Chloride	400 mg
Thymine	0.3 mg	Calcium Chloride	140 mg
Uracil	0.3 mg	Magnesium Sulfate	200 mg
Thiamine Hydrochloride	0.01 mg	Disodium Phosphate	60 mg
Riboflavin	0.01 mg	Monopotassium Phosphate	60 mg
Pyridoxine Hydrochloride	0.025 mg	Sodium Bicarbonate	350 mg
Pyridoxal Hydrochloride	0.025 mg	Bacto-Phenol Red	20 mg
Niacin	0.025 mg	Triple distilled water	1000 ml
Niacinamide	0.025 mg	Carbon Dioxide	to pH 7.2

858 Ingredients per litre pH 7.2 - 7.4

L-Arginine	70 mg
L-Histidine	20 mg
L-Lysine	70 mg
L-Tyrosine	40 mg
L-Tryptophane	10 mg
L-Phenylalanine	25 mg
L-Cystine	20 mg
L-Methionine	15 mg
L-Serine	25 mg
L-Threonine	30 mg
L-Leucine	60 mg
L-Isoleucine	20 mg
L-Valine	25 mg
L-Glutamic Acid	75 mg
L-Aspartic Acid	30 mg
L-Alanine	25 mg
L-Proline	60 mg
L-Hydroxyproline	10 mg
L-Cysteine	260 mg
Glycine	50 mg
Pyridoxine Hydrochloride	0.025 mg
Pyridoxal Hydrochloride	0.025 mg
Biotin	0.01 mg
L-Glutamine	100 mg
Folic Acid	0.01 mg
Choline Chloride	0.5 mg
Inositol	0.05 mg
p-Aminobenzoic Acid	0.05 mg
Vitamin A	0.1 mg
Calciferol	0.1 mg
a-Tocopherol Phosphate	0.01 mg
Menadione	0.01 mg
Ascorbic Acid	50 mg
Cholesterol	0.2 mg
Diphosphopyridine Nucleotide	7 mg
Triphosphopyridine Nucleotide	1 mg
Coenzyme A	2.5 mg
Cocarboxylase	1 mg
Flavin Adenine Dinucleotide	1 mg
Uridine Triphosphate	1 mg
Glutathione	10 mg
Adenine Desoxyriboside	10 mg
Guanine Desoxyriboside	10 mg
Cytosine Desoxyriboside	10 mg
5-Methyldesoxycytidine	0.1 mg
Thymidine	10 mg
Bacto-Dextrose	1 g
Ethanol	16 mg
Sodium Acetate	50 mg
Sodium Glucuronate	4.2 mg
Calcium Chloride	0.2 g
Magnesium Sulfate	0.2 g
Monosodium Phosphate	0.14 g
Sodium Bicarbonate	2.2 g
Iron (as Ferric Nitrate)	0.1 mg
Sodium Chloride	6.8 g
Potassium Chloride	0.4 gm
Tween 80	5 mg
Phenol Red	0.02 g
Triple Distilled Water	1000 ml

CMRL 1066	Ingredients per litre	pH 7.2 - 7.4

L-Arginine	70 mg	Biotin	0.01 mg
L-Histidine	20 mg	Folic Acid	0.01 mg
L-Lysine	70 mg	Choline Chloride	0.5 mg
L-Tyrosine	40 mg	Inositol	0.05 mg
L-Tryptophane	10 mg	p-Aminobenzoic Acid	0.05 mg
L-Phenylalanine	25 mg	Ascorbic Acid	50 mg
L-Cystine	20 mg	Socium Acetate	50 mg
L-Methionine	15 mg	Sodium Glucuronate	4.2 mg
L-Serine	25 mg	L-Glutamine	100 mg
L-Threonine	30 mg	Ethanol	16 mg
L-Leucine	60 mg	Bacto-Dextrose	1 g
L-Isoleucine	20 mg	Phenol Red	0.01 g
L-Valine	25 mg	Sodium Chloride	6.8 g
L-Glutamic Acid	75 mg	Potassium Chloride	0.4 g
L-Aspartic Acid	30 mg	Liver Coenzyme Concentrate	0.10 g
L-Alanine	25 mg	Tween 80	5 mg
L-Proline	40 mg	Cholesterol	0.2 mg
L-Hydroxyproline	10 mg	Glutathione	10 mg
L-Cysteine	260 mg	Adenine Desoxyriboside	10 mg
Thiamine Hydrochloride	0.01 mg	Guanine Desoxyriboside	10 mg
Riboflavin	0.01 mg	Cystosine Desoxyriboside	10 mg
Nicotinic Acid	0.025 mg	5-Methyldesoxycytidine	0.1 mg
Nicotinamide	0.025 mg	Thymidine	10 mg
Calcium Pantothenate	0.01 mg	Calcium Chloride	0.2 g
Glycine	50 mg	Magnesium Sulfate	0.2 g
Pryidoxine Hydro-chloride	0.025 mg	Monosodium Phosphate	0.14 g
		Sodium Bicarbonate	2.2 g
Pyridoxal Hydro-chloride	0.025 mg	Triple Distilled Water	1000 ml

EAGLE EARLE'S	Ingredients per litre	pH 7.2 - 7.4	
L-Arginine	17.4 mg	Nicotinamide	1 mg
L-Cystine	12 mg	Calcium Pantothenate	1 mg
L-Tyrosine	18 mg	Pyridoxal Hydrochloride	1 mg
L-Histidine	8 mg	Thiamine Hydrochloride	1 mg
L-Isoleucine	26 mg	Riboflavin	0.1 mg
L-Leucine	26 mg	Inositol	1.8 mg
L-Lysine	26 mg	TC Phenol Red	5 mg
L-Methionine	7.5 mg	Bacto-Dextrose	1000 mg
L-Phenylalanine	16.5 mg	Sodium Chloride	6800 mg
L-Threonine	24 mg	Potassium Chloride	400 mg
L-Tryptophane	4 mg	Monosodium Phosphate	125 mg
L-Valine	23.5 mg	Magnesium Sulfate	100 mg
Biotin	1 mg	Calcium Chloride	200 mg
Folic Acid	1 mg	Sodium Bicarbonate	2200 mg
Choline Chloride	1 mg	Triple distilled water	1000 ml
5% Glutamine	0.6 ml		

GRACE'S	Ingredients per litre	pH 6.9	
Beta-Alanine	200.00 mg	$NaHCO_3$	350.0 mg
L-Alanine	225.00 mg	$NaH_2PO_4.H_2O$	1013.0 mg
L-Arginine HCl	700.0 mg	Alpha-Ketoglutaric acid	370.00 mg
L-Asparagine	350.00 mg		
L-Aspartic acid	350.00 mg	Fructose	400.00 mg
L-Cystine	22.00 mg	Fumaric acid	55.00 mg
L-Glutamic acid	600.00 mg	Glucose	700.00 mg
L-Glutamine	600.00 mg	Malic acid	670.00 mg
Glycine	650.00 mg	D-Succinic acid	60.00 mg
L-Histidine	2500.00 mg	Sucrose	26680.00 mg
L-Isoleucine	50.00 mg	Biotin	0.01 mg
L-Leucine	75.00 mg	D-Ca pantothenate	0.02 mg
L-Lysine HCl	625.00 mg	Choline chloride	0.20 mg
L-Methionine	50.00 mg	Folic acid	0.02 mg
L-Phenylalanine	150.00 mg	i-Inositol	0.02 mg
L-Proline	350.00 mg	Niacin	0.02 mg
DL-Serine	1100.00 mg	Para-aminobenzoic acid	0.02 mg
L-Threonine	175.00 mg	Pyridoxine HCl	0.02 mg
L-Tryptophane	100.00 mg	Riboflavin	0.02 mg
L-Tyrosine	50.00 mg	Thiamine HCl	0.02 mg
L-Valine	100.00 mg		
$CaCl_2$	750.0 mg		
KCl	4.10 mg		
$MgCl_2.6H_2O$	2280.0 mg		
$MgSO_4.7H_2O$	2780.0 mg		

LANDUREAU'S	Ingredients per litre		pH 7.0 - 7.2
L-Arginine HCl	2000 mg	$MgSO_4.7\ H_2O$	670 mg
L-Aspartic acid	200 mg	KCl	894 mg
L-Glutamic acid	1000 mg	$CaCl_2$	484 mg
L-Alanine	120 mg	NaCl	7420 mg
-Alanine	45.5 mg	$NaHCO_3$	424 mg
L-Cysteine	101 mg	$NaH_2PO_4.H_2O$	10 mg
L-Glutamine	559 mg	Glucose	2500 mg
Glycine	200 mg	Trehalose	6900 mg
L-Histidine HCl	404 mg	a-Ketoglutaric acid	365 mg
L-Leucine	249 mg	Citric acid	15.3 mg
L-Lysine	12.4 mg	Fumaric acid	5.8 mg
L-Methionine	492 mg	L-Malic acid	58 mg
L-Proline	748 mg	Succinic acid	5.9 mg
L-Serine	83 mg	Yeast extract	1 g
L-Threonine	20 mg	Lactalbumin hydrolysate	3.5 g
L-Tyrosine	362 mg	Triple distilled water	1000 ml
L-Valine	152 mg		

McCOY'S 5A Ingredients per litre pH 7.2 - 7.4

L-Alanine	13.36 mg	Biotin	0.2 mg
L-Arginine HCl	42.14 mg	Calcium Pantothenate	0.2 mg
L-Asparagine H_2O	45.03 mg	Choline Chloride	5 mg
L-Aspartic Acid	19.97 mg	Folic Acid	10 mg
L-Cysteine	24.24 mg	Inositol	36 mg
L-Glutamic Acid	22.07 mg	Nicotinamide	0.5 mg
L-Glutamine	219.15 mg	Nicotinic Acid	0.5 mg
L-Glycine	7.51 mg	Pyridoxal HCl	0.5 mg
L-Histidine HCl H_2O	20.96 mg	Pyridoxine HCl	0.5 mg
L-Tryptophane	3.06 mg	Riboflavin	0.2 mg
L-Phenylalanine	16.52 mg	Thiamine HCl	0.2 mg
L-Tyrosine	18.12 mg	Vitamine B_{12}	2 mg
L-Lysine HCl	36.54 mg	Glutathione	0.5 mg
L-Methionine	14.92 mg	Bacto-Peptone	600 mg
L-Isoleucine	39.36 mg	Dextrose	3000 mg
L-Leucine	39.36 mg	Calcium Chloride	100 mg
L-Valine	17.57 mg	Potassium Chloride	400 mg
L-Threonine	17.87 mg	Magnesium Sulfate	200 mg
L-Serine	26.28 mg	Sodium Chloride	6460 mg
L-Hydroxyproline	19.67 mg	Monosodium Phosphate	580 mg
L-Proline	17.27 mg	Sodium Bicarbonate	2200 mg
p-Aminobenzoic Acid	1 mg	Phenol Red	10 mg
Ascorbic Acid	0.5 mg	Triple distilled water	1000 ml

NCTC 135 — Ingredients per litre — pH 7.2 - 7.4

L-Alanine	31.48 mg	Choline Chloride	1.25 mg
L-a-Amino-n-Butyric Acid	5.51 mg	Cyanocobalamin (Vitamin B_{12})	10 mg
L-Arginine	31.16 mg	l-Inositol	0.125 mg
L-Asparagine	9.19 mg	p-Aminobenzoic Acid	0.125 mg
L-Aspartic Acid	9.91 mg	Vitamin A	0.25 mg
L-Cystine	10.49 mg	Calciferol	0.25 mg
D-Glucosamine	3.85 mg	Menadione	0.025 mg
L-Glutamic Acid	8.26 mg	a-Tocopherol Phosphate	0.025 mg
L-Glutamine	135.73 mg	Diphosphopyridine Nucleotide	7 mg
Glycine	13.51 mg	Triphosphopyridine Nucleotide	1 mg
L-Histidine	26.65 mg	Coenzyme A	2.5 mg
L-Hydroxyproline	4.09 mg	Cocaboxylase	1 mg
L-Isoleucine	18.04 mg	Flavin Adenine Dinucleotide	1 mg
L-Leucine	20.44 mg	Uridine Triphosphate	1 mg
L-Lysine	38.43 mg	Glutathione	10 mg
L-Methionine	4.44 mg	Ascorbic Acid	50 mg
L-Ornithine	9.41 mg	Deoxyadenosine	10 mg
L-Phenylalanine	16.53 mg	Deoxyguanosine	10 mg
L-Proline	6.31 mg	Deoxycytidine	10 mg
L-Serine	10.75 mg	Thymidine	10 mg
L-Taurine	4.18 mg	5-Methylcytosine	0.1 mg
L-Threonine	18.93 mg	Tween 80	1.25 mg
L-Tryptophane	17.50 mg	D-Glucuronolactone	1.8 mg
L-Tyrosine	16.44 mg	Sodium Glucuronate	1.8 mg
L-Valine	25 mg	Sodium Acetate	50 mg
Thiamine Hydrochloride	0.025 mg	Sodium Chloride	6800 mg
Riboflavin	0.025 mg	Potassium Chloride	400 mg
Pyridoxine Hydrochloride	0.0625 mg	Calcium Chloride	200 mg
Pyridoxal Hydrochloride	0.0625 mg	Magnesium Sulfate	200 mg
Niacin	0.0625 mg	Monosodium Phosphate	140 mg
Niacinamide	0.0625 mg	Sodium Bicarbonate	2200 mg
D-Pantothenate, Calcium	0.025 mg	Bacto-Dextrose	1000 mg
D-Biotin	0.025 mg	Phenol Red	20 mg
Folic Acid	0.025 mg	Ethyl Alcohol	40 mg
		Triple distilled water	1000 ml

REHACEK'S	Ingredients per litre	pH 6.8

L-Alanine	71 mg	Thiamine (HCl)	0.02 mg
L-Arginine (HCl)	8 mg	Riboflavin	0.02 mg
L-Aspartic acid	23 mg	Ca pantothenate	0.02 mg
L-Glutamic acid	63 mg	Pyridoxine (HCl)	0.02 mg
Glycine	42 mg	p-Aminobenzoic acid	0.02 mg
L-Histidine	44 mg	Folic acid	0.02 mg
L-Isoleucine	13 mg	Niacin	0.02 mg
L-Leucine	50 mg	i-Inositol	0.02 mg
L-Lysine	44 mg	Biotin	0.01 mg
L-Phenylalanine	43 mg	Choline chloride	0.20 mg
L-Proline	38 mg	Bovine albumin	10,000 mg
DL-Serine	67 mg		
L-Threonine	44 mg	Penicillin 'G' (Na salt)	30 mg
L-Tyrosine	33 mg	Streptomycin sulphate	100 mg
L-Valine	164 mg	Phenol red (0.7%) (pH 6.8)	7.14 ml
NaCl	6300 mg		
NaH_2PO_4	3910 mg		
$KHCO_3$	1630 mg	Triple distilled water	1000 ml
$CaCl_2$	330 mg		
$MgSO_4$	290 mg		
Glucose	5000 mg		
Inositol	400 mg		

SIO	pH 7.2 - 7.4
Medium 858	130.0 ml
Glucose (30% aqueous soln.)	2.8 ml
Foetal calf serum	50.0 ml
Yeast extract (5% soln. in 858)	18.0 ml
Dog's bile (5% soln. in Hanks BSS)	0.7 ml

STREIFF & TABERLEY'S

	Concentration in parts
1% Agar in Gey's solution	7
6% Filtered Seawater plus 1% Glucose in triple distilled water	6
50% Albumen in Gey's solution	3

APPENDIX II: MEDIA AND REAGENTS - BACTERIOLOGY

STAINING REAGENTS

Ammonium oxalate - crystal violet

Solution A: Crystal violet 10 g

Ethanol (95%) 100 ml

Mix and dissolve.

Solution B: Ammonium oxalate 1% aq. soln.

For use mix 20 ml of solution A and 80 ml of solution B.

Carbol-fuchsin, strong

Solution A: Basic fuchsin 10 g

Ethanol (95%) 100 ml

Mix and dissolve in a stoppered bottle and keep at 37°C overnight.

Solution B: Phenol 5 g

Distilled water 100 ml

Mix and dissolve.

For use, pour 10 ml of solution A into 100 ml of solution B.

Carbol-fuchsin, weak

Dilute one volume of strong carbol-fuchsin with 10-20 volumes of distilled water.

Lugol's iodine

Iodine 5 g

Potassium iodide 10 g

Distilled water to 100 ml

Dissolve the KI and iodine in 10 ml of the water, and adjust to volume with distilled water.

BASIC NUTRIENT MEDIA

Nutrient broth

Beef extract	10 g
Peptone	10 g
NaCl	5 g
Water	1000 ml

Dissolve the ingredients by heating in the water. Adjust to pH 8.0-8.4 with 10 N-NaOH and boil for 10 min. Filter, adjust to pH 7.2-7.4, and sterilize at 115°C for 20 min.

Nutrient agar

Nutrient broth, as above, gelled by the addition of 2% agar.

Peptone water

Peptone	10 g
NaCl	5 g
Water	100 ml

Dissolve the solids by heating in the water. Adjust to pH 8.0-8.4 and boil for 10 min. Filter, adjust to pH 7.2-7.4, and sterilize at 115°C for 20 min.

ENRICHED MEDIA

Blood agar

Defibrinated blood	50 ml
Nutrient agar	950 ml

Melt the nutrient agar, cool to 50°C and add the blood aseptically. Mix and distribute in tubes or plates.

MacConkey agar (modified from MacConkey, 1908; Report, 1956)

Peptone	20 g
NaCl	5 g
Sodium taurocholate	5 g
Water	100 ml

Dissolve the peptone, NaCl and bile salt in the water by heating. Adjust to pH 8.0, boil for 20 min, cool and filter.

Agar	20 g
Lactose	10 g
Neutral red, 1% aq.soln.	10 ml

Add and dissolve the agar by boiling and adjust to pH 7.4. Add the lactose and indicator soln., mix and sterilize at 115°C for 20 min.

CHARACTERISATION TESTS: REAGENTS AND MEDIA

Arginine broth (Niven et al. 1942)

Peptone (tryptone)	5 g
Yeast extract	5 g
K_2HPO_4	2 g
Glucose	0.5 g
Arginine monohydrochloride	3 g
Distilled water	1000 ml

Dissolve by heating, adjust to pH 7.0, boil, filter, and sterilize at 115°C for 20 min.

Citrate, Koser's (modified from Koser, 1923: Report, 1956)

NaCl	5 g
$MgSO_4.7H_2O$	0.2 g
$NH_4H_2PO_4$	1 g
K_2HPO_4	1 g
Distilled water	1000 ml

Dissolve the salts in the water.

Citric acid	2 g

Add to the salts solution and adjust to pH 6.8 with N-NaOH. Filter through a sintered-glass funnel. The medium should be colourless. Sterilize at 115°C for 20 min.

All glassware must be chemically clean and alkali-free.

Decarboxylase medium (Møller, 1955)

Peptone	5 g
Beef extract	5 g
Pyridoxal	5 mg
Glucose	0.5 g
Distilled water	1000 ml
Bromthymol blue, 0.2% soln	5 ml
Cresol red, 0.2% soln.	2.5 ml

Dissolve the solids in the water by heating. Adjust to pH 6.0. Add the indicators, mix, and distribute into four equal volumes. Sterilize at 115°C for 20 min.

For use make the following additions:

1. L - arginine hydrochloride 1%
2. L - lysine hydrochloride 1%
3. L - ornithine hydrochloride 1%
4. no addition

re-adjust to pH 6.0 if necessary.

Distribute the 4 media in 1 - 1.5 ml volumes into small tubes (67 x 10 mm or 3 x 3/8 inch rimless) containing liquid paraffin to a height of about 5 mm and previously sterilized. After distribution sterilize at 115°C for 10 min.

Ehrlich's reagent

p-dimethylaminobenzaldehyde	1 g
Absolute ethanol	95 ml
Conc. HCl	20 ml

Dissolve the aldehyde in the ethanol and add the acid. Protect from light.

Gelatin, nutrient

Beef extract	3 g
Peptone	5 g
Gelatin	120 g
Water	1000 ml

Add the gelatin to the water and allow to stand for 15-30 min. Heat to dissolve the gelatin; add and dissolve the other constituents. Adjust to pH 7.0, and sterilize by heating at 115°C for 20 min.

Gluconate broth (Shaw & Clarke, 1955)

Peptone	1.5 g
Yeast extract	1 g
K_2HPO_4	1 g
Potassium gluconate	40 g
Distilled water	1000 ml

Dissolve in the water by heating. Adjust to pH 7.0. Filter, and sterilize at 115°C for 20 min.

Note: The potassium gluconate may be replaced by 37.25 g of sodium gluconate.

Hugh & Leifson's O-F medium (Hugh & Leifson, 1953)

Peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Agar	3 g
Distilled water	1000 ml
Bromthymol blue, 0.2% aq.soln	15 ml

Dissolve the solids by heating in the water. Adjust to pH 7.1, filter, and add the indicator. Sterilize at 115°C for 20 min.
Add a sterile solution of the appropriate carbohydrate aseptically to give a final concentration of 1%. Mix and distribute aseptically in 10 ml volumes into sterile tubes of not more than 16 mm diameter.

Hydrogen peroxide

H_2O_2, 3% aq.soln. ('10 volume')

Protect from light and store in a cool place. Keep in a bottle closed with a glass stopper, paraffined cork or plastic screw-cap.

Nessler's reagent

Dissolve 5 g potassium iodide in 5 ml freshly distilled water. Add cold saturated mercuric chloride solution until a slight precipitate permanently remains after thorough shaking. Add 40 ml 9 N-NaOH. Dilute to 100 ml with distilled water. Allow to stand for 24 h.

Alternative formula: dissolve 8 g potassium iodide and 11.5 g mercuric iodide in 20 ml water and adjust to 50 ml. Add 50 ml 6N-NaOH. Mix and allow to stand for 24 h.

Notes: The water used in its preparation must be ammonia-free. Allow the reagent to settle before use. Protect from light.

Nitrate broth

KNO_3	1 g
Nutrient broth	1000 ml

Dissolve the KNO_3 in the broth, distribute into tubes containing inverted Durham's tubes, and sterilize at 115°C for 20 min.

Note: Nitrite must not be present in this medium.

Nitrite broth

$NaNO_2$	0.01 g
Nutrient broth	1000 ml

Dissolve the $NaNO_2$ in the broth, distribute into tubes, and sterilize at 115°C for 20 min.

Nitrite test reagents

Solution A: 0.8% sulphanilic acid in 5 N-acetic acid

Dissolve by gentle heating.

Solution B: 0.6% dimethyl-α-naphthylamine in 5 N-acetic acid

or

0.5% α-naphthylamine in 5 N-acetic acid

Dissolve by gentle heating.

Zinc dust or 10% zinc dust suspended in 1% methycullulose solution (Steel & Fisher, 1961).

Oxidase reagent

1% tetramethyl-p-phenylenediamine aq.soln.

Peptone water sugars

To 900 ml peptone water add 10 ml phenol red indicator and sterilize at 115°C for 20 min. Dissolve 10 g of the appropriate sugar in 90 ml water and steam for 30 min. or sterilize by filtration. Add this to

the sterile base, distribute into sterile tubes with inverted Durham's tubes and steam for 30 min.

Notes: The addition of some carbohydrates may cause an acid reaction; in these instances add sufficient 0.1 N-NaOH to restore the original colour.

Triple sugar iron agar (TSI) (Report, 1958)

Beef extract	3 g
Yeast extract	3 g
Peptone	20 g
Glucose	1 g
Lactose	10 g
Sucrose	10 g
$FeSO_4.7H_2O$	0.2 g
NaCl	5 g
$Na_2S_2O_3.5H_2O$	0.3 g
Agar	20 g
Distilled water	1000 ml
Phenol red, 0.2% soln.	12 ml

Heat to dissolve the solids in the water, add the indicator solution, mix and tube. Sterilize at 115°C for 20 min. and cool to form slopes with deep butts.

Urea medium, Christensen's (Christensen, 1946)

Peptone	1 g
NaCl	5 g
KH_2PO_4	2 g
Agar	20 g
Distilled water	1000 ml

Dissolve the ingredients by heating, adjust to pH 6.8, filter, and sterilize at 115°C for 20 min.

Glucose	1 g
Phenol red, 0.2% soln.	6 ml

Add to the molten base, steam for 1 h., and cool to 50-55°C

Urea, 20% aq. soln.	100 ml

Sterilize by filtration and add aseptically to the base. Distribute aseptically into sterile containers and allow to cool as slopes or plates.

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