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HEPATIC MICROSPORIDIOSIS OF JUVENILE GREY MULLET, *CHELON LABROSUS*, WITH PARTICULAR REFERENCE TO PARASITE DEVELOPMENT AND TRANSMISSION

by

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DECLARATION

This work has not been accepted in substance for any other degree, and is not concurrently being submitted for any other degree.

Signed J. R. Ralphs

Candidate

Date 15/11/84

This is to certify that the work submitted here was carried out by the candidate himself. Due acknowledgement has been given to any assistance received.

Signed J. R. Ralphs

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ABSTRACT

James R. Ralphs. Hepatic microsporidiosis of juvenile grey mullet, *Chelon labrosus*, with particular reference to parasite development and transmission.

Microgemma hepaticus gen.n.sp.n. is described from the liver of juvenile mullet, *Chelon labrosus*. Development occurs within xenomas, of diameter 500 µm. These have microvillar surfaces, encircling bands of mitochondria and a reticulate nucleus with many nucleoli. Meronts are plasmodial, divide by plasmotomy and are enclosed within host membranes. Sporonts are free in the cytoplasm and divide by multiple exogenous budding. In sporogenesis the polar sac is formed from golgi vesicles, the manubrium form a dense golgi body and the coiled polar filament form a spiral cisterna linked with the perinuclear membrane system and golgi reticulum. This was also implicated in the formation of the endospore. The polaroplast developed from expanded sacs derived from the manubrium. Uninucleate spores (4.2 x 2.4 µm) possessed 7-9 filament coils and lamellar polaroplast. Foci of infection were associated with liver connective tissue, and histopathological effects limited to adjacent cell necrosis due to pressure atrophy in restricted sites. Host responses result in granuloma formation, with gradual resolution of the lesion. *In vitro* investigations into spore hatching and viability showed exporulation was stimulated by oxidising agents and following pretreatment with media of low pH or high osmolarity. Inoculation of spores into RTG cell cultures resulted in observations of a freshly injected sporoplasm, and a plasmodial stage after 72 hours. The ultrastructure of extruded filaments, spore cases and cell penetration is described. Attempts at transmission of the parasite to mullet by stomach tube, feeding of crustacean spore carriers or intraperitoneal injection were unsuccessful and suggested that larval fishes may be the targets of infection. The possible transmission of the parasite in the marine environment is discussed. On the basis of structural and developmental features the genus *Microgemma* is placed in the family Unikaryonidae. The affiliation of the genus with other fish microsporidia is noted, and wider implications of life cycles and nuclear behaviour to taxonomy and evolution are discussed.

INTRODUCTION

Microsporidia, it has been suggested, form the largest group of parasitic animals, and possibly therefore the largest group of animals (Sprague and Vavra, 1976). Certainly the 725 named and unnamed species listed by Sprague (1977d) form a large and widespread group, with hosts ranging from other parasitic protozoa through the animal kingdom up to and including man. Many new species have been described subsequent to this listing, and every potential host group seems to have its associated microsporidian parasites; it seems probable that the majority of species are yet to be discovered. However, despite the widespread occurrence of members of the group, and the long history of study of the microsporidia (the first named species being Nosema bombycis Nageli, 1857) few detailed descriptions exist which include information concerning developmental stages. This probably results from the fact that they have not been considered as pathogens of major commercial, verterinary or medical importance until recently, particularly with the advent of fish farming and the possible use of microsporidia as biological control agents. New interest in the group has undoubtedly been stimulated by the development of electron microscope techniques and the application of new techniques from immunology and molecular biology in the study of the parasites themselves and their host/parasite interaction. It is only recently, however, that sufficient studies of structural features have been performed to allow a reasonably comprehensive assessment of the interrelationships of species, as included in taxonomic systems proposed by Sprague (1977b) and Weiser (1977). These systems themselves have already been overtaken in some respects by the increasing knowledge of the ultrastructure of type species (e.g. Canning and Nicholas, 1980; Canning et al., 1982) and a

greater understanding of life cycles, notably amongst dimorphic genera and those with meiotic divisions at the onset of sporogony (Canning and Vavra, 1977; Hazard et al., 1979; Loubes et al., 1976; Loubes, 1979). As already suggested by Canning and Vavra (1977), a priority area of research must be the investigation of the developmental biology of the microsporidia and the elucidation of as many life cycles as is possible.

Protozoan diseases of fishes have been shown to be of major significance in both fish farms and fisheries. The potential danger of these pathogens in the wild has long been recognised (Fischthal, 1944; Sandholzer et al., 1945; Haley, 1954; Sinderman, 1970; Hauck, 1984), with the decline of entire fisheries being attributed to microsporidiosis; reports of losses in fish farms have been made by Summerfelt and Warner (1970), McVicar (1975) and McKenzie et al. (1976). Disease is likely to be of considerable importance in fish farming owing to high density of stock; however there is the possibility of control and treatment in these manageable waters provided fundamental data is available on the parasite concerned. There is, therefore, an even greater need today for studies on all aspects of fish microsporidiosis. The establishment of microsporidia under controlled laboratory conditions both in vivo and in vitro offers scope for research into life cycles, development, pathology and host cell control in microsporidian infections.

The purpose of the study undertaken here was to investigate aspects of microsporidian disease in fishes; to this end the host/parasite system chosen was a hepatic microsporidian, Microgemma hepaticus gen.n.sp.n. in juvenile grey mullet, Chelon labrosus. In the investigation of the biology of this parasite research has proceeded into four main areas. In vivo studies involved

observations on the wild population and attempts to establish laboratory infection systems; *in vitro* investigations were performed on factors involved in the triggering of the infection process, partially to learn more about the wild situation but also with the aim of introducing the parasite into tissue cultures; the investigation of the development, structure and histopathological effects of xenomas of *M. hepaticus* on the liver of its host, the progress of the host response and the assessment of the potential pathogenicity of the disease; and the ultrastructural characterisation of the parasite *Microgemma hepaticus*, the study of its development within liver xenomas and the taxonomic implications of its developmental cycle.

REVIEW

The literature review presented below is divided into three main sections, dealing with historical records of fish microsporidiosis and the taxonomy of the group, transmission of microsporidia of fishes experimentally and *in vitro*, and ultrastructure and development of microsporidia, including host/parasite interactions. General reviews of the microsporidia have been presented by Kudo (1924), Vavra (1976a,b) and Canning (1977). Microsporidia of vertebrate hosts were reviewed by Canning (1976).

Historical records of fish microsporidia; taxonomy of the group

The effects of microsporidiosis of fish were first described by Gluge (1836), who observed spherical tumours on the stickleback *Gasterosteus aculeatus*. The causative organism was recognised as a microsporidian and named *Nosema anomala* by Moniez (1887), after the only named genus at the time, *Nosema* Nageli 1857. Thelohan (1892) placed this species in a new genus, *Glugea*, renamed as *Glugea microspora*. In addition, he described two more species from fish hosts, *Glugea destruens*, and an unnamed parasite from the cottid which Gurley (1893) subsequently named *Pleistophora typicalis*. Gurley also emended *G. microspora* (Moniez, 1887) to *G. anomala* (Moniez, 1887). Thelohan (1895) described six new species from fish hosts, including three marine species. These were *Glugea gigantea* from the connective tissue of *Crenilabrus melops*, *G. depressa* from the liver of *Julius vulgaris* and another liver parasite, *G. ovoidea*, from the livers of *Motella tricirrata* and *Cepola rubescens*. Doflein (1898) reported a new species, *G. lophii*, from the nervous system of *Lophius piscatorius* and Hagenmuller (1899) described *Glugea stephani* from the intestine of *Pleuronectes platessa*. These latter two

species have subsequently attracted considerable attention. Thus by the year 1900 twelve species had been described from fish hosts, eleven assigned to the genus Glugea, and one to Pleistophora. In addition, Linton (1901) reported a microsporidian parasitic in the liver of the rockpool fish, Rhombus triacanthus which Woodcock (1904) assigned to the genus Pleistophora. It should be pointed out here that Labbé (1899) had followed Thelohan (1892) in regarding Glugea and Nosema as synonymous, but restored the older name Nosema Nageli 1857. As indicated by Sprague (1977a) the confusion between Glugea and Nosema persisted for a long time, and as recently as 1969 Lom and Weiser reviewed the literature and synonymised the two genera. Not until Cali (1971) demonstrated the diplokaryotic nature of Nosema were the two genera conclusively separated. Between the year 1900 and the publication of Kudo's (1924) major review, the level of research activity on the microsporidia increased and enabled Kudo (1924) to list 32 species from fish hosts. These were contained in four genera: Glugea Thelohan 1892; Pleistophora Gurley; Nosema Nägeli 1857 and Thelohania Henneguy 1892. New fish species of particular interest described during this period were Nosema branchiale Nemeczek (1911) from the gills of Gadus aeglefinus, Glugea hertwigi Weissenberg (1911) from the intestine of Osmerus mordax and Pleistophora destruens Delphy (1916) from the musculature of the grey mullet Mugil auratus. Sprague (1977c) included the latter, along with G. gigantea Thelohan 1895 as a synonym of Ichthyosporidium giganteum (Caullery and Mesnil, 1905) Scwarzewsky, 1914. It is of interest that these authors regarded I. giganteum as a haplosporidian, and its true nature as a microsporidian was not demonstrated until Sprague (1966) and Sprague and Vernick (1968a) examined the type material at ultrastructural level. Glugea lophii

Doflein 1898 was also the subject of several studies; Pace (1908) emended the species to Nosema lophii, this becoming the first Nosema species from a fish host, a move supported by Weissenberg (1911). Subsequently, major differences between this parasite and other fish microsporidia have been recognised, and Weissenberg erected for it the new genus Spraguea Weissenberg 1976, which was later redefined at ultrastructural level by Loubes et al. (1979). Kudo's (1924) monograph remained the only review of the early species, and indeed was the only comprehensive listing of species and generic descriptions, until Sprague (1977c) published his annotated list of species, this despite the great increase in interest in the microsporidia during this period. Many new species were described in the time between these two major listings, and notable species from fish hosts, particularly liver parasites, are included here. Jirovec (1932) described Glugea acerinae from the gut wall of Acercia cernua; Pflugfelder (1952) reported Glugea pseudotumefasciens from many sites, including the liver, in Brachydanio rerio; Schaperclaus (1941) described Pleistophora hyphessobryconis from the musculature of Hyphessobrycon innesi and other hosts, and Schubert (1969) erected a new genus and species, Heterosporis finki, for a parasite of Pterophyllum scalare. Liver microsporidia reported from marine hosts were Glugea machari Jirovec 1934 from Dentex vulgaris, Nosema ovoideum Raabe 1936 from the red mullet Mullus barbatus, and Glugea caulleryi Van den Berghe 1940 from Amnodytes lanceolatus. Raabe (1936) believed N. ovoideum to be identical to G. ovoidea Thelohan 1892, but emended the earlier name. Sprague (1977c) decided that the diagnosis of this species was uncertain and transferred it to the collective genus Microsporidium Sprague 1977c. Specialised reviews of the literature were produced by Putz, Hoffman and Dunbar (1965)

and Putz and McLaughlin (1970), listing 25 and 31 species respectively as parasitic in freshwater and euryhaline fishes. Putz, Hoffman and Dunbar (1965) also described a new species, Pleistophora salmonae, from Salvelinus fontinalis. Sprague and Vernick (1966) made a brief report of a new species of Glugea from Apeltes quadraticus, which in 1968 they described at ultrastructural level as Glugea weissenbergi Sprague and Vernick 1968b. Sprague (1977c) in his annotated list of species, included 76 species of microsporidia parasitic in fishes, contained within eight genera. The largest genera numerically were Glugea Thelohan 1892 and Pleistophora Gurley 1893. The remaining genera, having one or two representatives each, were Thelohania Henneguy 1892; Nosema Naegeli 1857; Ichthyosporidium Caullery and Mesnil 1905; Heterosporis Schubert 1969; Mrazekia Leger and Hesse 1916 and Spraguea Weissenberg 1976. Subsequently, several reports of significance to fish microsporidiosis have been made. A single species has been reported from the liver of grey mullet, Pleistophora sp. from Mugil cephalus (Paperna et al., 1978). New species of Glugea described at ultrastructural level are Glugea plecoglossi Takahashi and Egusa (1977), Glugea atherina Berrebi and Bouix (1978) and Glugea truttae Loubes and Maurand 1981. In addition, Canning et al. (1982) have made an ultrastructural redescription of the type species Glugea anomala (Moniez, 1887). Pleistophora species have also been investigated more fully at ultrastructural level, Canning et al. (1979) describing a species from Blennius pholis which was subsequently named Pleistophora littoralis Canning and Nicholas (1980). This 1980 report also included an ultrastructural description of the type species, P. typicalis Gurley 1893. Two new genera have been described from fish hosts; these are Tetramicra Matthews and

Matthews 1980 from the turbot, Scopthalmus maximus, and Loma Morrison and Sprague (1981a) from the gills of a variety of species. This latter genus includes two previously described species, Nosema branchiale Nemeczek 1911 and Pleistophora salmonae Putz, Hoffman and Dunbar 1965, emended to the new genus (Morrison and Sprague 1981a, 1983). Several microsporidia have been reported as hyperparasites of fish infecting trematodes. These were included in a review by Canning (1975) and further species have been described by Canning and Madhavi (1977) and Canning et al. (1983).

The major contributions to microsporidian taxonomy were reviewed by Sprague (1977a), who then proposed a new revision of the classification (Sprague, 1977b). Weiser (1977) simultaneously proposed an alternative modification. Further reports detailing the precise characters used in the separation of taxa are Canning and Vavra (1977) and Vavra et al. (1981). Although Sprague's (1977a) review was a thorough description of the taxonomic history of the group, it is instructive to follow the groupings of the two major genera parasitic in fish hosts, Glugea Thelohan 1895 and Pleistophora Gurley 1893 through the various taxonomic systems. Thelohan (1892) suggested the first classification, creating the single family Glugeidae, containing three genera, Glugea Thelohan 1892, Pleistophora Gurley 1893 and Thelohania Henneguy 1892. Thelohan placed his family Glugeidae in the order Myxosporidia. The grouping together of Myxosporidia and Microsporidia continued in taxonomy, the groups being regarded as equivalent but linked together within the larger group Cnidosporidia Doflein 1901. This arrangement persisted through many revisions at various ranks, e.g. Kudo (1924), Poisson (1953) and Weiser (1961). Lom and Vavra (1962), Lom and Corliss (1967) and Vavra (1966) showed, however that the two groups were

unrelated, and Sprague (1970) separated them, although in this report he then linked the microsporidia with the haplosporidia. Thelohan's (1892) system was followed by Gurley (1893), Thelohan (1895) and Labbé (1899) with various modifications. Doflein (1898) proposed an alternative system, with the microsporidia divided into two groups, based on the number of spores contained in a pansporoblast. It is of note that Pleistophora and Glugea were grouped together in this system, Glugea being regarded as pansporoblastic. In contrast, Stempell (1909) returned to the ideas of the early workers, and proposed that the microsporidia should be returned to the original rank of order (Balbiani, 1882). He included three families in this order, Nosematidae Labbé 1899, Pleistophoridae Stempell 1909 and Glugeidae Thelohan 1892. This represented the separation of Glugea and Pleistophora species at the highest level yet. The next major revision of the group was made by Leger and Hesse (1922), who proposed an entirely different taxonomic system. The order Microsporidia Balbiani 1882 was retained, but was divided into two suborders, the Monocnidea, for "spores with one polar capsule" and the Dicnidea for "spores with two polar capsules". As the basic divisions of this system were on spore morphology Glugea and Pleistophora were grouped together with all other species of classical form in the family Glugeidae Thelohan 1892 in the order Monocnidea Léger and Hesse 1922. It was later shown (Weiser, 1961; Vavra, 1968) that the major taxonomic division of this system was based on erroneous observations; however the system persisted for a long time and was used in various modifications by many authors, notably Kudo (1924), Poisson (1953), Weiser (1961) and Kudo (1966). Tuzet et al. (1971) were the first to completely revise microsporidian taxonomy after Leger and Hesse (1922), and proposed a

new system. The order Microsporidea, contained within the class Microsporidea Corliss and Levine, 1964, was divided into two suborders, the Apansporoblastina for species where sporogony was without a pansporoblast, and the Pansporoblastina for those with a pansporoblast. Glugea was tentatively regarded as possessing a pansporoblast, and was placed with Pleistophora in the new family Polysporidae, in the suborder Pansporoblastina. Sprague (1977b) developed this system further. The microsporidia were elevated to phylum status (eliminating the link with haplosporidia, Sprague, 1970), and the phylum microsporida divided into two classes, the Rudimicrosporea for those having unusual spore structure and the class Microsporidea for those of classical form. The order Microsporida within this class was divided into two suborders, the Pansporoblastina Tuzet et al., 1971 and the Apansporoblastina Tuzet et al., 1971. In contrast to Tuzet et al., 1971, however, Glugea and Pleistophora were separated. Pleistophora was contained in the family Pleistophoridae of the Pansporoblastina and Glugea, regarded as apansporoblastic, in the family Glugeidae of the Apansporoblastina along with Encephalitozoon and Spraguea. The Microsporidia were also elevated to the rank of phylum in Weiser's (1977) system, published in the same year, and similarly species of unusual spore morphology were separated from the classical forms as the class Metchnikovellidea Weiser 1977. However, the classical forms, class Microsporididea Corliss and Levine, 1964 were divided differently. Two orders were established, the Pleistophoridida where sporogony and spores are uninuclear and the Nosematidida where sporogony and spores are diplokaryotic. The genera Glugea and Pleistophora were placed in the family Pleistophoridae Stempell (1909) in the order Pleistophoridida Stempell 1909. Of these two most recent revisions

of the taxonomy, Sprague's (1977b) system has entered a more widespread use, being adopted by Levine et al. (1980) in their revised classification of the protozoa. However, recent studies show the need for at least partial revision of this system. Canning and Hazard (1982) showed that the genus Pleistophora Gurley 1893 was a composite genus, and indicated that two of the genera separated from it, the classical Pleistophora from fishes and Vavraia Weiser 1977 from mosquitos were greatly different from Polydispyrena Canning and Hazard (1982) which was probably related to dimorphic genera (reviews Canning and Vavra 1977; Maddox and Sprenkel, 1978). Canning et al. (1982) demonstrated that the type species of the genus Glugea Thelohan 1892, G. anomala (Moniez, 1887) was in fact pansporoblastic and was thus more closely related to Pleistophora and Vavraia than to Encephatitozoon and Spraguea, with which Sprague had grouped the genus Glugea.

Transmission of microsporidiosis, in fish hosts and in vitro

There are few reports concerning the successful transmission of fish microsporidiosis under experimental conditions. The successful experiments are included here, along with unsuccessful efforts of interest. In addition, features of the transmission of insect microsporidia considered to be of relevance to general microsporidian transmission are included. Although ecological studies were not a feature of the project undertaken here, certain studies of fish microsporidiosis which may be of relevance to the transmission of Microgemma hepaticus in south western England are included. In vitro studies have been performed with insect microsporidia in various cell lines and papers dealing with exsporulation and infection of cell cultures are reviewed.

The first experimental infections of fishes were performed by Weissenberg (1922) and later, in 1968, described in more detail. These infections were achieved by feeding larval sticklebacks with spore-carrying crustacea and contamination of tank water with spores of Glugea anomala. Dykova and Lom (1978) subsequently achieved infections with this parasite using similar techniques. Varying degrees of success has been achieved with other species of Glugea. Stunkard and Lux (1965) failed to transmit Glugea stephani to winter flounders, and Deslile (1969) failed to infect smelt with Glugea hertwigi. Successful infections of G. stephani were reported by McVicar (1975) and Olson (1976) in pleuronectid flatfishes and of G. hertwigi by Scarborough and Weidner (1979) in smelt. McVicar's (1975) infections were achieved by intraperitoneal injection, contamination of food with spores and force-feeding of spore suspensions; Olson (1976) by feeding with spore-carrying crustacea and amphipods. Scarborough and Weidner (1979) used similar methods. Matthews and Matthews (1980) infected turbot with Tetramicra brevifilum by intramuscular injection of spore suspension; infections via the oral route were, however, unsuccessful. Lom (1969) had previously infected goldfish with the muscle parasite Pleistophora hyphessobryconis by the implantation of infected material into the musculature. Weidner (1973) reported the infection of invertebrates with the fish parasite Glugea stephani; this remains the only report of the infection of an invertebrate with a vertebrate-infecting microsporidian.

Field studies of fish microsporidiosis have been confined mainly to the common Glugea species G. hertwigi and G. stephani. Heaviest infections were described in juvenile fishes by Haley (1954), Stunkard and Lux (1965), Legault and Deslile (1967), Deslile

(1969) and Chen and Power (1972). Stable, low levels of infection in older fishes were reported by McVicar (1975) and Takvorian and Cali (1981). Summerfelt and Warner (1970) in an investigation of the ovarian parasite Pleistophora ovariae showed a different pattern of infection, with higher intensities in young fishes but greater incidence of infection in older hosts. Studies of insect-infecting microsporidia having bearing on general microsporidian transmission are those of Kramer (1970), Maddox (1973), Oshima (1973) and Undeen (1978, 1983) on the longevity of spores and Cerkasova and Vavra (1972) who presented physiological data of relevance to spore viability. Transmission of insect microsporidia has been reported by many authors, reports of interest here being those dealing with horizontal and vertical transmission of dimorphic species (Keller et al., 1966; Hazard and Weiser, 1968; Pilley, 1976; Andreadis, 1983).

Transmission of microsporidiosis to and within cell cultures has been reported by several authors, and is usually dependent on the stimulation of filament extrusion in the cell culture medium. The ability of strong physical or chemical stimuli, notably oxidising agents, to stimulate exsporulation has long been known and reported by many workers, including Thelohan (1895); Kudo (1918); Oshima (1927, 1937) and Lom and Vavra (1963). A more physiological approach has been taken by some authors in studies of the exsporulation of invertebrate species, with spores responding to conditions analagous to those in the insect gut. These include the pretreatment of spores in alkaline media (Ishihara and Sohi, 1966; Ishihara, 1967, 1969; Kurtti and Brooks, 1971; Undeen, 1975; Street et al., 1980) or immersion in media containing alkali metal ions at high pH (Ishihara, 1967; Undeen, 1978, 1983). In occasional cases, spores will

exsporulate directly on immersion in cell culture medium (Higby *et al.*, 1979; Smith *et al.*, 1982). The exsporulation of the fish parasite *Glugea stephani* was investigated by Weidner and Byrd (1982), who showed that exsporulation was dependent on the displacement of internal calcium from the polaroplast. These authors also demonstrated that, in contrast to the insect species, external calcium ions were inhibitory to exsporulation. One other report, Hashimoto *et al.*, 1976, gives physiological data on the exsporulation of fish microsporidia; spores of *Pleistophora anguillarum* were shown to be sensitive to conditions of high and low pH. As *M. hepaticus* is likely to be sensitive to conditions in the host intestine, studies of fish intestinal physiology are relevant here. Intestinal enzymes of the mullet *Mugil cephalus* were reported by Ishida (1935) and Hamid *et al.* (1979). Details of other species were presented by Sera and Okutani (1968), Williams *et al.* (1970), Western (1971) and Clarke (1981). In addition, nutritive information on the mullet *M. cephalus* was given by Shehadeh *et al.* (1972) and Kuo and Shehadeh (1972). For comparative purposes, triggering of excystation of metacercariae of helminth parasites has been described many times, for example, McDaniel (1966) and Dixon (1966), and was reviewed by Smyth and Halton (1983). Triggering of the infection process in cestodes was reviewed by Smyth (1969), and in nematodes by Rogers (1966).

Reports of the development of microsporidia in cell cultures do not include fish parasites, successful *in vitro* infections have been performed only with insect parasites and the mammalian parasite *Encephalitozoon cuniculi*. The earliest report of microsporidian development in cell culture was made by Trager (1937) who obtained some development of *Nosema bombycis*; his experiments were, however, never successfully repeated. Subsequent cell culture work falls into

two main categories; firstly where infected cells and tissues are transferred from infected hosts, and secondly where cell cultures are inoculated with spore isolates. Insect-infecting species of the first group have been reported by Gupta (1964), Kurtti and Brooks (1971), Sohi and Wilson (1976) and Kurtti et al. (1983). The mammalian parasite Encephalitozoon cuniculi is the most studied of this group, with infected cells easily available following immunosuppression of laboratory animals. The life-cycle has been described several times in vitro, notably by Bismanis (1970), Vavra et al. (1972) and at ultrastructural level by Pakes et al. (1973). Shadduck (1976) reviewed the literature on the culture of this species. Examples of the second group are parasites of invertebrates, the first report being that of Ishihara and Sohi (1966). Ultrastructural details of the infection process were shown by Ishihara (1968a) and Weidner (1972) immediately after the inoculation of sporoplasms into cells. Development in vitro has been described by Ishihara, 1967, 1968, 1969; Undeen, 1975; Higby et al., 1979 and Smith et al., 1982, and at electron microscope level by Street et al. (1980). Several reports indicate a low specificity of cell type for microsporidian infections in vitro, with infections occurring in various mammalian and invertebrate cell lines (Ishihara, 1968b; Undeen, 1975; Shadduck, 1976; Smith et al., 1982). Similar observations have been made with other intracellular protozoa in vitro (Strout et al., 1965; Doran and Vetterling, 1967). A single report exists on the survival of microsporidian stages extracellularly, where Weidner and Trager (1973) investigated the effects of ATP on extruded sporoplasms. Reports of methods used in the in vitro culture of fish cells for use in the present study were reviewed by Sigel and Beasley (1973a,b) and Wolf and Quimby (1976).

Ultrastructure, development and host/parasite interactions

The earliest applications of the electron microscope to the study of microsporidia were those of Steinhause (1951) and Krieg (1955), who examined whole mounts of spores. Weiser (1959) was the first to apply ultrathin sectioning techniques, to spores of *Nosema laphygmae*, and identified the spore wall and polar filament. Huger (1960) observed layering in the spore wall and polar filament of *Nosema locustae* spores, and recognised a laminar membranous structure which he named the polaroplast. Subsequent to this early period several authors described features of spore ultrastructure, including Puytorac (1961, 1962), Puytorac and Tournet (1963), Codreanu *et al.* (1965), Vivier (1965, 1966), Vavra *et al.* (1966) and Petri and Schiodt (1966). In 1967 Lom and Corliss reviewed the literature and summarised spore structure as follows ".... their essential components are known : a shell composed of several layers; a coiled polar filament whose basal part is joined with the polar cap at the anterior end of the spore; an anteriorly located swelling organelle, the polaroplast; a fluid-filled vacuole at the posterior end of the spore; and finally the sporoplasm itself". Further details of spore structure have been added by many authors, and were comprehensively reviewed by Vavra (1976a). Cytoplasmic features were described by Lom and Corliss (1967), Schubert (1969), Vinckier *et al.* (1971), Weidner (1972), Liu and Davies (1973), Canning and Sinden (1973), Desportes (1976) and Larrson (1980). Structure and arrangement of polaroplast lamellae were described by Lom and Corliss (1967), Stanier *et al.* (1968), Schubert (1969) and Weidner (1970); the association of the polaroplast with the anchoring disc by Ishihara (1968), Vernick *et al.* (1969) and Weidner (1972). Polar filament structure has been described by Schubert (1969), Kudo and Daniels

(1963), Stanier et al. (1968), Canning et al. (1979) and Matthews and Matthews (1980). Details of filament structure, with reference to the extrusion process, were reported by Lom and Corliss (1967), Sprague and Vernick (1969), Schubert (1969), Vernick et al. (1969), Weidner (1972) and Lom (1972). The appearance of the spore wall has been described many times and Erickson and Blanquet (1969) reported its chitinous nature. Recent reports on its structure are those of Canning and Nicholas (1974), Larrson (1981) and Percy et al. (1982). Puytorac (1962) made the first ultrastructural study of developmental stages, describing meronts and sporoblasts of Mrazekia lumbriculi. This was followed by an investigation of six species by Vavra (1965) recognising sporogony and sporogenesis and describing diplokaryotic nuclei at electron microscope level. Short reports were made by Vivier (1966) and Manier and Maurand (1966) on details of sporogony of Metchnikovella hovassei, Thelohania bracteata and Pleistophora simulii. Many subsequent studies have been made, and microsporidian development and ultrastructure reviewed comprehensively by Vavra (1976a, 1976b). Reports of importance to the study here are detailed below in the broad groupings of merogony, sporogony and sporogenesis. Merogonic development has been described ultrastructurally from many species and shows differences between groups. Development is largely plasmodial in some species, e.g. Glugea (early reports, Weissenberg, 1911, 1913, 1922; Debaisieux, 1920; ultrastructural studies, Sprague and Vernick, 1968a; Berrebi, 1979; Loubes et al., 1981; Canning et al., 1982) and Pleistophora (Canning et al., 1979; Canning and Nicholas, 1980; although Lom and Corliss (1967) indicated the presence of a uninucleate population in P. hyphessoboyconis), and Tetramicra brevifilum Matthews and Matthews 1980. In contrast, Nosema and Encephalitozoon multiply

mainly by binary fission (Ishihara, 1969; Weidner, 1970; Sprague and Vernick, 1971; Canning, 1981; Canning et al., 1983; Spelling and Young, 1983). Nuclear division occurs by acentriolar pleuromitosis (Hollande, 1972), details being given by Sprague and Vernick (1968a), Youssef and Hammond (1971), Canning and Sinden (1973), Morrison and Sprague (1981c) and Batson (1982). Interest has been shown in host/parasite relationships of merogonic stages, with the association of meronts with host cell organellae being a common observation (Szollosi, 1970; Weidner, 1970; Youssef and Hammond, 1971; Canning, 1981; Canning and Hazard, 1982). In addition, Matthews and Matthews (1980) reported the development of meronts within host produced vacuoles. Criteria for the recognition of the change in development from merogonic to sporogonic development were indicated by Vavra (1976b). However, the conditions for the triggering of this change have not been universally established. A nutritional trigger has been suggested for some species (Ishihara, 1969; Sprague and Vernick, 1971; Vavra et al., 1972; Maurand and Vey, 1973; Vavra, 1976b review), and a meiotic division begins the process in others (Loubes et al., 1976a,b, 1979; Hazard et al., 1979; Vivares and Sprague, 1979; Canning and Hazard, 1982). In certain species an intermediate population of cells occurs between merogony and sporogony (Weissenberg, 1913; Debaisieux, 1920; Berrebi, 1979; Loubes et al., 1981; Canning et al., 1982; suggested by Matthews and Matthews, 1980). In others development occurs directly from the meronts, e.g. Pleistophora (Canning et al., 1979; Canning and Nicholas, 1980; Canning and Hazard, 1982) and Nosema and Encephalitozoon (Canning and Sinden, 1973; Canning and Nicholas, 1974; Canning, 1981; Canning et al., 1983). The final sporogonic division into sporoblasts occurs by several methods

resulting in considerable variations at this stage (Vinckier et al., 1970, 1971; Sprague and Vernick, 1971; Loubes et al., 1977; Vivares and Sprague, 1979; Canning et al., 1979; Matthews and Matthews, 1980; Canning and Nicholas, 1980). These are of considerable significance in taxonomy (Sprague, 1977a,b; Canning and Vavra, 1977; Vavra et al., 1981). In certain species sporogonic development results in two spore types, varying in size (Canning et al., 1979; Canning and Nicholas, 1980) or in morphology, as in dimorphic species (e.g. Kellen et al., 1966; Hazard and Weiser, 1968; Loubes et al., 1979; Andreadis, 1983). The final stage of the developmental cycle, spore morphogenesis, has received much attention. The earliest stages of development of the anchoring disc and polar filament were first described by Vavra (1965) and subsequently confirmed by Vinckier et al. (1971), Jensen and Wellings (1972), Vinckier (1975), Loubes et al. (1979) and Loubes et al. (1981). They were described as of golgi origin by Vavra (1965), and Sprague and Vernick (1969) stated that most, if not all of the spore organellae were of golgi origin. Liu and Davies (1972a) indicated that polar filament development was associated with both endoplasmic reticulum and golgi. Sprague and Vernick (1974) suggested the golgi origin of the coiled part of the polar filament, a view supported by Tazikawa et al. (1975) and subsequently by many authors (e.g. Vernick et al., 1979; Morrison and Sprague, 1981c, 1983; Canning et al., 1982; Batson, 1983). In many cases membranous remains of the filament forming system are left in the posterior vacuole of mature spores (Schubert, 1969; Pakes et al., 1973; Sprague and Vernick, 1974; Morrison and Sprague, 1981a,c, 1983). Several authors have reported the occurrence of clumps of filament cores in golgi vacuoles (Jensen and Wellings, 1972; Sprague and Vernick, 1969; Weidner,

1972; Sprague and Vernick, 1974; Berrebi, 1979; Takvorian and Cali, 1983), and Berrebi (1979) suggested a method of filament deposition from these structures. Golgi structure and function in other cells has been described many times, including Beams and Kessel (1969) and Anderson et al. (1970). The origin of the polaroplast from endoplasmic reticulum was reported by Lom and Corliss (1967), Sprague et al. (1968) and Weidner (1970). More recent reports indicate the origin of this structure as golgi vesicles (Jensen and Wellings, 1972; Liu and Davies, 1972a,b; Vinckier, 1975; Canning and Sinden, 1973 and Loubes et al., 1979). Little information is available on the origin of the complete spore wall. Many authors have shown the formation of the exospore from extramembraneous thickenings of sporonts (review Vavra, 1976b); Liu and Davies (1972a) suggested the origin of the endospore from expanded sacs in the sporoblast cytoplasm.

Hypertrophy of infected host cells is a common response to infections with protozoa (e.g. Coccodia : Scholtyseck, 1973; Frenkel, 1977; Melhorn and Frenkel, 1980; Pasternak and Fernando, 1984) and in microsporidia in particular hypertrophy and reorganisation of the host cell can result in characteristic lesions. Reviews of microsporidian interaction with host cells have been produced by Weissenberg (1976) on parasites of fish and annelids and Weiser (1976a,b) on parasites of insects. The first reports dealing in detail with the characteristic lesion of certain fish microsporidia were made by Weissenberg (1911, 1913), who recognised that the cyst of Glugea anomala was a complex of the host cell and parasite stages. Leger and Hesse (1916) described a hypertrophic cyst of Mrazekia lumbriculi, from annelids. Chatton (1920) and Chatton and Courier (1923) recognised the intimate structural and

physiological relation of host and parasite and named the association "complexe xenoparasitaire". Weissenberg (1922, 1949) independently coined the term "Xenoma" for such lesions. Xenomas have frequently been studied since. The classical xenoma of G. anomala was studied in experimental infections by Weissenberg (1922, 1968) who detailed the stages in its development. The first ultrastructural study of a Glugea xenoma was made by Sprague and Vernick (1968a) on G. weissenbergi. Later studies were made on the periphery of G. anomala xenomas (Weidner, 1976; Lom et al., 1979). Berrebi (1979) made the first complete ultrastructural description of the Glugea cyst, following an earlier report (Berrebi and Bouix, 1978) on G. atheriae. These details have recently been confirmed in the type species, G. anomala by Canning et al. (1982). Xenomas of other species having different structure have been reported, Matthews and Matthews (1980) describing Tetramicra brevifilum and Morrison and Sprague (1981a,b,c, 1983) describing Loma species. A common feature of these fish xenomas is the reticulate, hypertrophied host cell nuclei. Studies of nuclear function have not been reported in fish xenomas; however chromosomal changes have been reported in infected insect cells (Pavan, 1969). A development of the xenoma form of infection was described in Ichthyosporidium giganteum, where the parasite makes use of the host response to form an immense multicystic lesion (Sprague, 1969; Sprague and Vernick, 1974; Sprague and Hussey, 1980). The host response to infections of microsporidia in fishes was described by Dykova and Lom (1978) and Matthews and Matthews (1980), and reviewed by Dykova and Lom (1980). Parasites of invertebrates having lesions which show certain similarities to those of fish microsporidia have been described by Puytorac (1962) and Janiszewska et al. (1979) on Mrazekia species

from annelids, and by Spelling and Young (1983) on Nosema herpobdellae from the leech Erpobdella octoculata. Hyperparasitic microsporidia are also of interest here, and have been reported in metacercariae from fishes by Canning and Madhavi (1977), Paperna et al. (1978) and in reviews by Canning (1975) and Canning et al. (1983).

MATERIALS AND METHODS

Chelon labrosus

Collection. O-group Chelon labrosus were collected from St. Johns lake, a creek of the river Tamar estuary, the fish being present from mid-September until the last week in May. Shoals of mullet became trapped in a pool at low tide, and collections were made by passing a 0.5 cm mesh seine net around the pool. The fish were transferred rapidly to polyethylene bins containing creek water, aerated by oxygen cylinder, for transport to the laboratory. On arrival they were transferred to sea water at 18°-20° and treated with a broad spectrum antimicrobial agent as a precaution against bacterial and fungal infections of net-damaged fish. Agents used were methylene blue or malachite green, at a dose rate of 2 mg/litre of sea water. At the termination of treatment the healthiest fish were transferred to the aquarium as stock or maintained in isolation for experimental studies on transmission.

Maintenance. Stock fish were maintained in sea water at a constant temperature of 10° and contained within 50 l polyethylene tanks incorporated in the main aquarium water circulation system. Water quality was maintained by passage through a biological filter bed; the total aquarium capacity was 15,000 litres with a recirculation time of 45 minutes. Fish required for experimental studies were transferred to small perspex tanks (18" x 10" x 10") containing sea water which had been carefully decanted after standing for at least 7 days. This method was employed to reduce the risk of introduction of microsporidian spores. Water quality within the experimental tanks was maintained by undergravel filtration and recirculation by a simple airlift pump. Temperature control was achieved by immersion

of the tanks in a water bath maintained at 12° and individually heating them to the required temperature using elements controlled by a contact thermometer (Gallenkamp). This system enabled temperature control from 12°-30° to an accuracy of ± 0.5°. Experimental fish were allowed to equilibrate for 48 hours after transfer to these tanks before experimental procedures were carried out.

Husbandry. Husbandry included monthly partial water changes in experimental tanks, daily feeding with No. 2 trout pellets and removal of dead fish and uneaten food. Strict hygiene was enforced in handling fish, and nets assigned to each tank were sterilised by immersion in Milton.

Anaesthetics. Fish were anaesthetised before experimental procedures by immersion in a 0.01% solution of MS222 (Ethyl-m-aminobenzoate; Sigma) in sea water. The fish were left in the anaesthetic until swimming activity ceased. Following experimental procedures the fish were transferred to highly aerated sea water until fully recovered and returned to their experimental tanks. Fish were killed by overdose of MS222.

Introduction of spore suspensions into fish. Spore suspensions were introduced into mullet by stomach tube or intraperitoneal injection. The stomach tube consisted of a 5 cm length of 0.023" diameter polyethylene catheter tubing passed over a 23 gauge hypodermic needle, and the spore suspension, contained within a 1ml syringe, was attached to this. The catheter tubing was passed into the stomach of the anaesthetised fish and 0.1 ml of spore suspension injected. Intraperitoneal injections were performed using a 25 g needle inserted ventro-laterally approximately 5 mm anterior to the anus. The needle was pushed anteriorly for approximately 5 mm into the body

cavity, ensuring that it ran along the inside of the body wall; this procedure avoided damaging the intestine. 0.1 ml of spore suspension was injected into the peritoneal cavity.

Aseptic removal of liver. Livers were removed aseptically from specimens of *C. labrosus* for the preparation of spore suspensions and tissue cultures. The fish were killed by overdose of MS222, blotted dry and transferred to a laminar flow cabinet. Here they were surface sterilised by swabbing with ethanol and the body cavity opened to expose the liver. The liver was removed whole, avoiding causing damage to the intestine and consequent loss of sterility.

Microgemma hepaticus : Identification and removal of parasite stages

Screening for infection. Infected fish were externally indistinguishable from un-infected fish; individuals were therefore dissected and their livers examined. Screening was performed by visual observation, squash preparations, smear preparations and histological sectioning. Superficial infections were visible to the naked eye as white spots in the liver. Deeper foci were observed by squashing portions of liver tissue between microscope slides. Microsporidiosis was confirmed by maceration of pieces of infected tissue in Young's teleost saline (Young, 1933) on a microscope slide. These preparations were examined for the presence of phase-bright microsporidian spores using x40 and x100 objectives. Smear preparations were made from portions of liver tissue, air dried, fixed in methanol and stained with Giemsa. Histological methods, including wax and cryostat sectioning are described in the appropriate section (page 33).

Extraction of spores. Spores of *M. hepaticus* were extracted from

infected livers using the potassium hydroxide homogenisation technique described by Weidner (1972). All procedures were performed under aseptic conditions. O-group mullet were killed by overdose of MS222, their livers removed and stored in Young's teleost saline; up to 30 livers were stored in this way prior to processing. The livers were transferred to a 10 ml Jencons glass tissue homogeniser containing 10 ml of 0.1 m potassium hydroxide and homogenised for 10 minutes. The homogenate was filtered through gauze and centrifuged at 650 g for 10 minutes. The supernatant was discarded and the pellet washed 3 times by resuspension and centrifugation in distilled water. The pellet was finally resuspended in sterile sea water containing 200 units penicillin and 200 µg streptomycin/ml. Spore suspensions were stored in the dark at 4°.

Spore viability. After extraction, spores were tested for filament extrusion by treatment with saturated iodine water or 0.88 m hydrogen peroxide. 10 µl of the spore suspension were placed on a microscope slide and covered with a 22 x 22 mm coverslip. 5 µl of the exsporulation inducing agent were allowed to run underneath the coverslip. After approximately 5 minutes many spores were observed with extruded polar filaments. Such a test revealed the extrusion mechanism of spores to be intact; however a true test of viability would have to include infection experiments.

Spore suspension concentration. The concentration of spores in a suspension was determined by haemocytometer counting of samples; at least 1000 spores were counted before calculation of the total number of spores in the suspension. The concentration was adjusted as required by dilution, or centrifugation and resuspension in a smaller volume.

Preparation of reagents used in investigation of filament extrusion

Biological extracts. Extracts were prepared from the stomach wall or intestinal wall of 0-group *C. labrosus*. One stomach or intestine was placed in a glass tissue homogeniser containing 2 ml of Young's teleost saline, and homogenised for 10 minutes. The homogenate was centrifuged at 1000 g for 10 minutes and the supernatant used as the extract. Bile was taken directly from the gall bladder of freshly dissected 0-group mullet by puncture of the wall and removal of the contents with a finely-drawn pasteur pipette. The mammalian bile salt sodium tauroglycocholate (Sigma) was dissolved in Young's teleost saline to form a 1% solution.

Enzyme preparations. The enzymes pepsin, trypsin and chitinase (Sigma) were dissolved in Dulbecco's saline (Flow Laboratories) to form 1% solutions. The pH of the solution was adjusted to pH 2 for pepsin, pH 7.6 for trypsin and pH 6 for chitinase by the dropwise addition of 0.1M hydrochloric acid and 1M tris (hydroxymethyl) methylamine (Sigma).

Oxidising agents. Serial dilutions of the oxidising agents dehydroascorbic acid (Koch-Light Ltd), iodine water, hydrogen peroxide, sodium hypochlorite and potassium iodide were prepared in distilled water in microtitre plates. The range of concentrations employed was dependent on the solubility of the test reagent. The concentrations of iodine water and sodium hypochlorite cannot be expressed conventionally as iodine has a very low solubility, and sodium hypochlorite cannot exist out of solution. In consequence, the concentration of iodine water is expressed as % saturated, and that of sodium hypochlorite as the % of free chlorine present in the solution. The reagents were prepared in 10 µl quantities at twice

the required concentration. The concentration was returned to that required in experiments by the addition of 10 µl of spore suspension in distilled water.

Pretreatment media. A range of pH from 1-10 was prepared by adjusting the pH of aliquots of Dulbecco's saline (Flow Laboratories) using 0.1M hydrochloric acid and 1M tris (hydroxymethyl) methylamine. Sodium chloride and potassium chloride solutions were prepared at concentrations of 1M , 2M and 5M in distilled water.

Sterilisation of instruments, glassware and solutions

Sterile materials were required in the extraction of spores and cell culture. Instruments, glassware and inorganic solutions were sterilised by autoclaving for 15 minutes at 10 lbs/in^2. Sea water and culture medium components not purchased sterile were passed through a syringe mounted millipore filter of pore size 0.22 µm.

RTG cell culture

Source of cells. An established cell line, RTG-2, was obtained from the M.A.F.F. fish disease laboratory, Weymouth and routinely maintained in 75 cm^2 plastic tissue culture flasks (Nunc). These cells were descended from one of the earliest established cell lines prepared from fish tissues (Wolf and Quimby, 1962).

Growth medium. The medium used in maintenance of RTG cells was BHK 21, Glasgow's modification of minimal essential medium, supplemented with tryptose phosphate broth and foetal calf serum (FCS). The complete medium composition is presented in Table 1.

Table 1. The composition of 100 ml of BHK 21 growth medium for RTG cells.

MEDIUM COMPONENT	SUPPLIER	VOLUME
BHK 21 (10x concentration)	Gibco	8 ml
Sodium bicarbonate (7.5%)	Flow Laboratories	0.37 ml
L-glutamine (200 mM)	Flow Laboratories	0.5 ml
Tris (hydroxymethyl) methylamine (1M)	Sigma	1.88 ml
Hydrochloric acid (0.1M)	BDH	1 ml
Distilled water		68.25 ml
SUPPLEMENTS		
Tryptose phosphate broth	Flow Laboratories	10 ml
Foetal bovine serum	Flow Laboratories	10 ml
	TOTAL VOLUME	100 ml
ANTIBIOTICS		
Pencillin/Streptomycin 10,000 units/ml	Flow Laboratories	2 ml

Subculture. Subculture was necessary at 14 day intervals with cultures maintained at 22°. The medium was poured off and replaced with 4 ml of trypsin 0.5%/EDTA 0.02% (Flow Laboratories). The flask was allowed to stand at 18°-22° until the cells began to detach from the growth surface, at which stage they were completely detached by agitation and pipetting. The cells were then transferred to 60 ml of fresh culture medium which was divided between three new 75 cm^2 flasks. The new flasks were allowed to stand for 2 hours prior to transfer to the incubator, to facilitate cell attachment, and then

incubated at 22°. Confluency was attained 7 days after subculture.

Suspension cultures. Cells were removed from stock flasks with trypsin/EDTA as at subculture. The resulting suspension in trypsin/EDTA was centrifuged at 100 g for 10 minutes at 4° and the pellet resuspended in 10 ml of BHK 21 medium. The concentration of cells in the suspension was determined by haemocytometer, and adjusted to the level required in experiments. Cell suspension for experiments were prepared in 5 ml bijou bottles in a shaking water bath at 22°.

Coverslip cultures. RTG cell monolayers were grown on 13 mm diameter glass coverslips for use in infection experiments. The coverslips were stored in absolute ethanol, and flamed immediately before use. Single coverslips were placed on the bottom of 5 ml plastic bijou bottles (Sterilin). The bottles were then inoculated with 2 ml of cell suspension containing 2 x 10^4 cells and centrifuged at 100 g for 5 minutes. The cultures were incubated at 22° and became confluent after 3 days.

Examination of cultures. Coverslip cultures were prepared for light microscopy by air drying, fixation in methanol and staining with Giemsa. Suspension cultures were prepared for examination by light and electron microscopy. For light microscope studies cells were deposited on microscope slides using a Shandon "Cytospin" cytocentrifuge. The cytocentrifuge chambers were assembled using 2 absorbent cards. 1 ml of culture containing approximately 10^6 cells was poured into each chamber and centrifuged at 400 rpm for 10 minutes. The microscope slides were then separated from the absorbent cards and stained as coverslip cultures with Giemsa.

Cells were fixed for electron microscopy using the same

reagents as for mullet liver fixation (Histology, page 34). The cells were pelleted by centrifugation and fixed by resuspension in glutaraldehyde (2½ hours). Postfixation was in osmium tetroxide, and dehydration in an alcohol series. The final cell suspension in absolute ethanol was transferred to BEEM capsules contained within glass centrifuge tubes. These were centrifuged at 650 g for 10 minutes and the pellets treated with propylene oxide for 5 minutes. Infiltration was performed with Spurr's resin as with liver tissue with the infiltration time reduced to 30 minutes per resin concentration.

Primary culture of *C. labrosus* liver cells

Source of material. Cultures were prepared from the livers of uninfected 1-group *C. labrosus* collected from St. Johns lake during the summer months. Sufficient material for culture was obtained from two livers.

Culture media. Two culture media were used in primary culture of liver cells. These were BHK 21 (Gibco) and Leibovitz L-15 (Flow Laboratories), modified for use with marine cells by the addition of 1.35 ml of 26% sodium chloride solution per 100 ml of medium (Sigel and Beasley, 1973b). BHK 21 was used in the formulation presented in Table 1. Leibovitz L-15 was used as purchased, with the addition of L-glutamine, tryptose phosphate broth, foetal bovine serum and antibiotics as with BHK 21.

Tissue dissociation and culture procedure. The liver cells were dissociated by trypsin digestion. Livers were removed aseptically and stored on ice in Dulbecco's saline without Mg^{++} or Ca^{++} (PBSA-Flow Laboratories) containing 10% FCS. Before processing the livers

were immersed in Dakin's solution (0.4% sodium hypochlorite + 0.8% sodium bicarbonate in 6 mM hydrochloric acid) to ensure surface sterility, and washed in two changes of PBSA containing 200 units/ml of penicillin and 200 µg/ml of streptomycin. The tissue was then chopped finely and transferred to 25 ml of 0.5% trypsin solution (Flow Laboratories) at 20° and continuously stirred by magnetic stirrer. At 20 minute intervals the trypsin was poured off into large centrifuge tubes and replaced with a fresh aliquot. 2.5 ml of FCS was added to each tube to prevent further enzyme action. This procedure was repeated until the tissue had completely disintegrated. The first aliquot, comprising mainly fat droplets, was discarded, and the remainder centrifuged at 400 g for 15 minutes at 4°. The supernatant was removed and the cells pooled in a small volume of culture medium; this was then diluted to 100 ml with culture medium and seeded into five 75 cm^2 plastic tissue culture flasks (Nunc). The flasks were incubated at 15°, 20° and 25°, and the medium replaced after 24 hours to remove debris after the cells had stuck.

Histology

Fresh material. Pieces of infected liver were macerated in Young's teleost saline and examined directly under phase-contrast using X10, X40 and X100 objectives (Zeis Standard Microscope) Cryostat sections were prepared using a Bright FS/FAS cryostat, after freezing of tissue with liquid nitrogen or solid carbon dioxide. For freezing in liquid nitrogen the liver was placed on a hollow aluminium stub and immersed in liquid nitrogen for approximately 30 seconds. For carbon dioxide freezing the liver was placed in a drop of glycerine solution on the stub (Bright cryo-m-bed) and frozen in a solid carbon dioxide

generating unit for approximately 30 seconds. Frozen material was transferred to the cold shelf of the cryostat and allowed to equilibrate to cryostat temperature for 30 minutes before sectioning. Sections were cut at 10 µm thickness, attached to warm microscope slides and fixed in formol saline. Staining was by Price's or Wolbach's Giemsa techniques (Luna, 1968).

Wax histology. Whole livers from 0-group mullet were fixed for 8 hours in Bouin's fixative, washed for 8 hours in running water to remove picric acid, dehydrated in an alcohol series, cleared in chloroform and embedded under vacuum in paraplast. Sections were cut at 6-8 µm using a Leitz rotary microtome and attached to slides coated with egg albumen. After dewaxing and rehydration the sections were stained with Mallory's triple stain (Bradbury, 1973). The method was modified in several respects. Slides were first immersed in lithium carbonate solution for 2 minutes, to remove picric acid. They were then stained with Mayer's haemalium (20 min), blued in lithium carbonate, placed in acid alcohol (5 seconds) and reblued in lithium carbonate. The slides were then immersed in acid fuchsin (Mallory's solution A) for 5 seconds, washed in four changes of distilled water and placed in phosphomolybdic acid (Mallory's solution B) for 30 seconds. After washing in distilled water the slides were transferred to aniline blue/orange G (Mallory's solution C) for 65 seconds and rinsed in distilled water. The sections were then rapidly dehydrated, cleaned in xylene and mounted in DPX. Hepatocytes stained red, collagen blue and microsporidian spores orange-brown.

Electron microscopy. Fish required for electron microscope studies were killed within 3 hours of capture, their livers rapidly removed

and placed in 3% glutaraldehyde (EMscope) in 0.1(m) sodium cacodylate buffer, pH 7.2. Foci of microsporidian infection were dissected out in tissue blocks measuring approximately 1 mm^3, transferred to fresh 3% glutaraldehyde and fixed for 2½ hours. The blocks were post-fixed in 1% osmium tetroxide (EMscope) in sodium cacodylate buffer, dehydrated in an alcohol series, 5 minutes per step, and embedded in Spurr's resin (EMscope). Infiltration was performed as follows: propylene oxide 5 minutes; 3 parts propylene oxide : 1 part Spurr's resin 4 hours; 2 parts propylene oxide : 2 parts Spurr's resin 8 hours; 1 part propylene oxide : 3 parts Spurr's resin 12 hours, pure Spurr's resin 12 hours. The tissue blocks were transferred to fresh resin in BEEM capsules and polymerised at 70° for 8 hours.

Blocks were sectioned using glass knives on Reichert Ultracut and LKB mk III ultramicrotomes. Semi-thin (1 µm) sections were mounted on glass slides and stained on a hotplate with 1% methylene blue or 1% toluidine blue for light microscopy. Ultra-thin sections (gold and silver interference colours) were mounted on 200 mesh copper grids, stained with saturated uranyl acetate (10 min) and lead citrate (Reynolds, 1963) for 10 minutes and examined using Philip's EM 200 and EM 300 transmission electron microscopes.

Photography

Live fishes. Individual fishes were confined in new 25 cm^2 tissue culture flasks which had their necks removed, containing millipore filtered sea water. These conditions gave the necessary optical clarity and freedom from suspended particles. The flask was placed on a black background and illuminated for photography with two small electronic flashguns held at an angle of approximately 45° either side of the flask at a distance of approximately 50 cm. Photographs

were taken with a Zenza Bronica 2¼" square SLR camera using the standard lens mounted on a single extension tube and stopped down to minimum aperture. Film used was Ilford Pan-F, rated at 50 A.S.A and developed in Microphen, and many pictures were taken to give an adequate selection free from subject movement and stray reflections.

Macrophotography. Close-up photographs of mullet liver were taken using a Zeiss "Tessovar" photomacrographic microscope, on 35 mm Ilford Pan-F film rated at 50 A.S.A. and developed in Microphen. Dissected fish were pinned out as required in dishes containing clear white wax, and completely covered with Young's teleost saline. Specimens were evenly illuminated with a Zeiss two source fibre optic illuminator, and a range of exposures taken, of ¼, ½, 1 and 2 seconds, for each photograph required. Extreme care was taken with the location and setting up of the microscope and in exposing photographs owing to the high sensitivity of the apparatus to extenal vibration.

Photomicrography. Photographs of histological sections and fresh material were taken on a Zeiss standard microscope fitted with "Neofluor" lenses and Zeiss MC63 automatic attachment camera, with M35 camera back. Phase contrast and bright field images were recorded on Pan-F film rated at 50 A.S.A. and developed in Microphen.

EXPERIMENTS AND RESULTS

The results are presented in five main sections. The first section, entitled *Chelon labrosus*, includes details of the seasonal occurrence, size range and liver anatomy and histology of the host fish. The second deals with the ultrastructural characterisation of the parasite, *Microgemma hepaticus*. Section three covers the effects of *M. hepaticus* on the host and describes the structure and distribution of infected cells and the host response. The fourth section, transmission, presents the methods used and results obtained in infection experiments on *C. labrosus*; the final section, exsporulation and the infection process *in vitro*, includes experiments to determine conditions required for exsporulation of *M. hepaticus*, infection experiments performed using RTG cell cultures and a description of the progress of primary cell cultures derived from *C. labrosus* liver.

Chelon labrosus

Seasonal occurrence and size range. Juvenile mullet (Fig. 1) were caught only during the period September to May at St. Johns lake. The average length of these increased from 3 cm in September to 5 cm in the following May. '1' and '2' group *C. labrosus*, and also the golden grey mullet, *Liza auratus*, were only occasionally found in St. Johns lake during the summer months, the fish collected at this time measuring approximately 10 cm in length. Post-larval *C. labrosus* measuring 1.5 - 2 cm in length were rarely caught, their first appearance being in low numbers in late May. These fish may have been rare separations from the main body of '0' group mullet, as they were not present in the estuary during the summer months.

Anatomy and histology of the liver. It was necessary to study the circulatory and biliary systems of the liver to investigate possible routes of infection and pathological effects of the parasite.

The liver is located within the peritoneal cavity, being closely applied anteriorly to the wall of the pericardium (Fig. 2). Two distinct lateral lobes originate from the main bulk of the liver, a large left lobe which extends posteriorly overlying the stomach, and a smaller right lobe. In addition, there are two smaller anterior lateral dorsal lobes (Fig. 3).

The hepatic blood system is basically the same as in other vertebrates in that blood enters by the hepatic portal system and hepatic artery and leaves by the hepatic vein. Particular attention was given here to the arrangement of the hepatic portal system as this, bringing blood directly from the digestive tract might well be the route of infection of the parasites following exsporulation in the intestine. In the mullet, blood entered the liver from the digestive tract via three vessels; a large vessel collected blood from the intestine, entering the liver in the median posterior region and two smaller vessels entering the left lobe collected blood from the pyloric caecae and the stomach. In addition, connections were made directly via minor vessels into the left lobe where this was in close proximity to the intestine. Blood passes from the liver via two short hepatic veins which leave either side of the anterior median region. These pass directly into the pericardium.

Studies of liver histology revealed a simple structure with no organisation into portal and lobular regions. Vessels were arranged randomly in the liver parenchyma. Hepatic arteries were narrow vessels with thick walls composed of cuboidal endothelial cells (Fig. 4). Presumably capillary branches of the hepatic artery join

into sinusoids ensuring a mixture of oxygenated and portal blood. Bile ducts were of greater diameter (Fig. 3) with prominent columnar endothelial cells, and were supported externally by concentric layers of collagen. Large branches of the hepatic portal vein (Fig. 5) were variable in outline and had collagenous support. In contrast hepatic veins were more or less circular and thin-walled (Fig. 6). The origin of sinusoids from hepatic portal veins and their entry into the hepatic veins were clearly seen (Figs. 5, 6). Sinusoids were lined by flattened endothelial cells and bounded by cords of hepatocytes two cells thick (Fig. 7).

Ultrastructural studies indicate the presence of rounded electron-lucent 'light' hepatocytes and stellate, highly electron dense 'dark' hepatocytes (Fig. 9). Cells of intermediate density were also present. Light cells measured 14 - 20 µm in diameter, and possessed single, rounded nuclei, diameter 4.5 µm. The cytoplasm contained lipid droplets, glycogen granules, endoplasmic reticulum and mitochondria, these measuring 0.33 µm in diameter and 1.2 - 2.4 µm in length with evenly spaced tubular cristae. The intermediate density hepatocytes contained more densely packed organellae; the only distinguishable organellae in dark hepatocytes were lipid droplets. It appeared that variation in glycogen content was responsible for the density variations. Bile ductules were present between adjoining hepatocytes, measuring approximately 2 µm by 4 µm, and were completely filled with microvilli. Sinusoids (Fig. 8) were circular in transverse section (diameter 9 µm), with a lining of thin, flattened endothelial cells separated from the surrounding hepatocytes by the space of Disse. Microvilli from the hepatocytes projected into this space. Numerous large mitochondria with densely packed cristae were present in the cytoplasm of these cells adjacent to the sinusoid.

Description of *Microgemma hepaticus* gen.n.sp.n.

The following events in the life cycle of *M. hepaticus* are those described from observation of infected cells within the liver. There is no evidence of a developmental sequence outside the liver, for example in the lamina propria of the intestine immediately following exsporulation. Stages described below from liver xenomas were clearly grouped into merogonic and sporogonic stages. A particularly clear sequence of sporogenesis was observed.

Merogony. Merogonic stages were identified as multinucleate plasmodia of variable size and shape, ranging from spherical 5 µm in diameter (Fig. 10) to elongated, measuring 15 µm x 5 µm (Fig. 12). Nuclei were 1.5 µm in diameter and were occasionally paired, although the diplokaryotic state was not confirmed. Up to eight nuclei were observed in sections of large meronts. The cytoplasm was uniform, fibrillar in appearance with rough endoplasmic reticulum and scattered groups of ribosomes. Nuclear division was synchronous within a meront (Fig. 12). Dumbell shaped telophase nuclei were observed (Fig. 14). Cell division was by plasmotomy, giving rise to multinucleate daughters, and occurred in both small and large meronts (Figs. 10-12).

A notable feature of merogonic stages was their complete enclosure by membranes (Figs. 10-12). The membranes appeared to be single (Fig. 13) and resembled membranes of the xenoma endoplasmic reticulum, being frequently associated with ribosomes. The meront-enclosing membrane was often surrounded by cisternae and vesicles of smooth endoplasmic reticulum (Figs. 10, 13).

Sporogony. The onset of sporogony was recognised by the presence of electron-dense blebs on the outside of the plasmalemma and the

absence of surrounding membrane systems (Figs. 15, 16). The extramembraneous blebs measured 40 nm in thickness and were composed of three layers : outer electron dense (10 nm thick), middle electron-lucent (10 nm) and inner electron-dense (20 nm). Sporonts were more or less rounded cells approximately 6 µm in diameter containing 6-8 nuclei (diameter 1.7 µm). The cytoplasm was similar to that of the meront but with greater development of endoplasmic reticulum.

Sporogony proceeded by budding, the first stage being the production of single uninucleate buds (Fig. 17). The process accelerated with the formation of multiple buds resulting in a variety of divisional stages. These included several separate buds on the same sporont (Fig. 18); multiple buds at a single site (Fig. 19) and multiple buds on a cellular protrusion (Figs. 20, 21). Buds carried a greater density of extramembraneous thickenings than the parent sporont, and internally possessed rough endoplasmic reticulum arranged around the nucleus. The parent sporonts were frequently highly vacuolated (Figs. 18-20). The budding process eventually resulted in the complete breakdown of sporogonial plasmodia into various combinations of buds, including rosettes (Fig. 22), pairs (Fig. 23) and chains (Fig. 24). Sporogonic division was concluded by the separation of these bud groups into individual sporoblasts. Nuclear division was infrequently observed during sporogony; however occasional division figures were observed in sporonts (Fig. 21) and in separated buds (Fig. 25).

In one host cell, a series of uni- and bi-nucleate parasite cells, with sparse extramembraneous thickenings, were observed that were completely unlike normal sporogonic stages, most notably in their remarkable development of endoplasmic reticulum (Figs. 26, 27).

The reticulum was expanded and contained discontinuous electron dense material, and may have been connected with the plasmalemma in some examples. In addition, the reticulum development occasionally led to internal partitioning, or possible necrosis, of the cell. The cells were associated with host membranes, in some cases being surrounded by several layers of smooth membrane.

Nuclear division. Nuclear division was by acentriolar pleuromitosis and appeared identical in merogonic and sporogonic nuclei. Spindle plaques were depressions in the nuclear envelope 0.19 µm in diameter (Fig. 28). The membranes of the nuclear envelope were thickened and electron dense at the plaque, the inner membrane measuring 12 nm and the outer 5 nm in thickness. External to the spindle plaque were three oval polar vesicles having double membranes, and internally tubular spindle fibres, diameter 19 nm, radiated from the plaque to the chromosomes. In transverse sections close to the spindle plaque up to 16 spindle fibres were counted (Fig. 29). Telophase nuclei took up a dumbell shape characteristic of microsporidian division (Fig. 14). Spindle fibres passed across the isthmus connecting the two new nuclei. No evidence of a sexual process was obtained - synaptonemal complexes, indicating meiotic division, were not observed in any stage examined.

Sporogenesis. Early sporoblasts were uninucleate (nucleus diameter 1.6 µm) with incompletely thickened cell membranes (Fig. 25). The cytoplasm contained endoplasmic reticulum and ribosomes as in sporogonial buds. The first stage of sporogenesis was the completion of the sporoblast wall by further secretion and fusion of extramembraneous material (Fig. 25). The wall at this stage (Fig. 32) was composed of an outer electron dense layer (15 nm in

thickness), middle electron-lucent layer (10 nm) and inner electron-dense layer (22 nm). As development progressed a fourth electron-lucent layer (17-44 nm) was deposited at the innermost region adjacent to the cell membrane.

Development of the spore organellae began with the formation of the precursor of the polar sac and polar filament (Fig. 31). This was identified as an oval sac (1.01 x 0.37 µm) lateral to the nucleus containing electron-dense material anteriorly and laterally and an electron-dense core terminating in a region of higher electron density. Posterior to this sac were numerous membranous vesicles derived from the endoplasmic reticulum and a cluster of electron-dense bodies. Some sections of sporoblasts at approximately this stage of development revealed no filament material (Figs. 30, 33), but possessed a large vacuole containing electron-dense floccular material aggregated in certain parts of the vacuole. A notable feature of these sporoblasts was the presence of electron-dense blebs, diameter 9 nm, on the nuclear envelope. Development progressed with the migration of the polar sac away from the nucleus to the anterior pole of the sporoblast (Fig. 32). The anterior portion of the sac expanded and flattened to form the precursor of the anchoring disc, the polar filament developing as an extension of the posterior region. The electron-dense anterior termination of the core of the polar sac formed a link between the polar filament and anchoring disc. Filament development progressed with the formation of the straight part, or manubrium, the lengthening end of the filament being enclosed by an electron-dense, granular structure. Another notable feature formed at about this stage was the perinuclear membrane system, a complex system of smooth membranes closely applied laterally to the nucleus (Figs. 34-36). On entering

the posterior third of the sporoblast the polar filament formed coils. At this stage filament formation was associated with a reticular membrane system which enlarged and became more electron-dense as the filament lengthened. Coils of polar filament formed at the periphery of the system, usually singly but occasionally in small groups (Fig. 38). Other important organellae developing at this stage were the posterior vacuole (Fig. 35) and a spherical electron-dense inclusion (diameter 0.9 µm) in the posterior third of the sporoblast (Figs. 41-43). As development progressed a variety of configurations of the filament forming system were observed. In some cases the system was highly reticular (Fig. 37); in other rare cases, regular cisternae were formed, terminating at either end in polar filament coils (Figs. 38, 39) or smaller elements of polar filament structure (Fig. 45). Other organellae became involved in filament formation. The electron-dense inclusion was frequently observed intimately associated with filament forming membranes (Figs. 38, 42). The perinuclear membrane system became expanded and electron-dense (Figs. 38, 41) and was associated with both the electron-dense inclusion and filament coils. Elements of the filament forming system were frequently observed penetrating into the posterior vacuole (Figs. 37-42). In older sporoblasts clumps of reticular material accumulated in the posterior vacuole.

Development of the polaroplast began with the appearance of membrane bound sacs 0.25 µm in diameter, radially arranged around the anterior part of the manubrium and containing discrete electron-dense material arranged in rings or clumps (Fig. 43). In some cases the sacs contained rounded bodies with granular inclusions (Fig. 44). In later stages the sacs were formed more posteriorly and the anterior sacs flattened to form lamellae (Figs. 45, 46). The membranes of

these were evidently in intimate association with the outer layer of the polar filament. In some sporoblasts the sacs were observed at the extreme ends of the cisternae (Fig. 46). As development progressed the cisternae increased in number and became compressed anteriorly to form lamellar polaroplast. The saccate form was rarely observed at this stage.

An unusual structure observed in one sporoblast (Fig. 36) was a tubular invagination of the cell membrane and inner layer of the sporoblast wall (diameter 0.1 µm) terminating in a vesicle with discrete contents. The small size of this organelle may have prevented its observation in other sections of sporoblasts. Aberrant forms of sporoblasts were observed in one infection, with the presence of bi- and tri-nucleate forms (Figs. 47, 48). Earlier developmental stages in this infection appeared normal.

Spore structure. At light microscope level, spores were highly refractile under phase-contrast, pyriform in shape and measured 4.2 µm x 2.4 µm in greatest breadth. A less refractile vacuole occupied the posterior third of the spore (Fig. 89). Spores stimulated to exsporulate were not refractile, and extruded filaments measured 57 µm in length (Fig. 90). At ultrastructural level, spores were of much higher electron density than sporoblasts, internal structures frequently being difficult to distinguish (Fig. 49). The spore wall was thickened to 110 nm, the electron-lucent endospore measuring 80 nm, and the exposure, which had indistinct layering compared to that of sporoblasts, 30 nm. The straight part of the polar filament ran posteriorly at an angle of approximately 45° to the long axis of the spore to the coiled part in the posterior third of the spore. Six to nine coils were present, having a four-layered structure in transverse sections : an outer trilaminate membrane-like

layer (12 nm thick); a second electron dense layer (12 nm); third electron-lucent layer (9 nm) and an electron-dense core (61 nm diameter), total filament diameter being 94 nm (Fig. 52). Filament structure was similar at the manubrium, but slightly thicker at 10 nm. At the insertion on the anchoring disc (Figs. 50, 51), the filament was swollen into a bulb 163 nm in diameter, the greatest increase in thickness being in the central core, this measuring 93 nm in diameter. The anchoring disc was concavo-convex in shape with a central anterior protrusion and was composed of five or six layers of differing electron density arranged perpendicularly to those of the filament. The outer layer was membranous and continuous with the outer layer of the polar filament (Fig. 50). This layer extended from the edges of the anchoring disc to form a cisterna with electron-dense contents closely applied to the outer layer of the polaroplast (Fig. 51). Immediately anterior to the anchoring disc the endospore was reduced in thickness to 16 nm.

The polaroplast, which occupied the anterior third of the spore, was lamellar in organisation, the saccate form observed in sporoblasts being absent (Figs. 46, 49, 50, 51, 53). Polaroplast lamellae were trilaminate in structure, having thin outer membranes and a thick central membrane, measuring 10 nm in overall thickness (Fig. 53). The lamellae were tightly packed anteriorly, resulting in an electron-dense/electron-lucent banded appearance, but were more loosely arranged posteriorly. The inner termination of the lamellae was on the manubrium; however, their relationship to the outer membrane of the manubrium was unclear (Figs. 51, 53). Transverse sections showed that the polaroplast was not arranged uniformly around the manubrium, but had a lobed or petal-like structure (Fig. 54).

The posterior vacuole occupied the posterior third of the spore and was irregular in outline with no clear bounding membrane (Fig. 49). The single spore nucleus was frequently indistinct, but where visible occupied the mid-region of the spore and was of lower electron density than the cytoplasm (Fig. 53). The nucleus was spherical (diameter 1.2 µm) or compressed into a concave shape between the polaroplast and posterior vacuole. Spore cytoplasm was of high electron density and contained large numbers of ribosomes. Ribosomes were observed attached to the outer layer of the polar filament and ensheathing the polaroplast (Figs. 53, 54). The electron-dense body observed in sporoblasts was rare in spores, and the perinuclear membrane system was not observed.

Aberrant forms of spores were occasionally observed in the form of double spores (Fig. 55) or triple spores, each component containing disorganised spore organellae.

Effects of *Microgemma hepaticus* on the host, *Chelon labrosus*

The invasion of a host cell by *M. hepaticus* results in host cell hypertrophy and the formation of an integrated host/parasite system, an example of the xenoma parasitic complex as described by Weissenberg (1922, 1968, 1976). Xenomas encountered in the liver of *C. labrosus* were at various stages of development. Most were either active, containing developmental stages and spores, or degenerate, containing only spores and associated with a host inflammatory granulation response. In addition, a series of cells believed to be very early stages in xenoma development were observed from one fish. The distribution and structure of active xenomas, the early stages of xenoma development and the host response are described below.

Xenoma structure and distribution. Active xenomas were more or less spherical in shape measuring up to 500 µm in diameter and having a slightly irregular outline (Fig. 56). In histological sections xenomas were present at all depths of the liver (Fig. 57). An important feature, however, was the association of xenomas with hepatic portal vein branches (Fig. 58), bile ducts (Fig. 59) and the host capsules surrounding metacercariae of the trematode *Bucephalus haimeanus* (Fig. 60). Spores were never observed in the metacercaria itself; however spores were frequently observed penetrating into the lining cells of bile ducts (Fig. 59).

Ultrastructural studies revealed that superficially the xenoma cytoplasm was thrown into microvillar processes 90 nm in diameter which formed a border region of variable thickness, from 0.7 µm - 2.7 µm (Figs. 61-66). Numerous vesicles were present in the cytoplasm immediately below this layer (Fig. 64). In some cases adjacent hepatocyte membranes were broken down, and the xenoma peripheral vesicles contained glycogen and debris from the damaged hepatocytes (Fig. 64). Immediately beneath the microvillar layer was a band 20 - 25 µm thick consisting of tightly packed mitochondria (Figs. 61, 62). These measured 0.23 µm in diameter and 1.2 µm - 3.6 µm in length and were of abnormal appearance, possessing sparse, expanded cristae and an expanded outer envelope. Numerous vesicles and cisternae of smooth endoplasmic reticulum were also present in this band. The host cell nucleus was hypertrophied and reticulate and positioned at the inner part of the mitochondrial band encircling most of the central region of the xenoma (Fig. 62). The nucleoplasm contained numerous spherical, electron-dense nucleoli (Fig. 65). The central part of the xenoma (Fig. 67) was occupied by developmental stages and spores of *M. hepaticus* and host cell organellae including

rough and smooth endoplasmic reticulum, glycogen granules, ribosomes and scattered mitochondria of similar appearance to those of the mitochondrial band.

Early stages of xenoma formation. Early xenomas (Figs. 68-75) were small rounded cells (diameter 3.25 - 5.6 µm) infected with intracellular parasites; these were observed in Giemsa stained smears of the liver of an 'O'-group mullet 2 cm in length taken from St. Johns lake in late May. The cells possessed weakly staining cytoplasm, one or two indistinct nuclei and strongly staining parasitic stages. Parasite stages had blue cytoplasm and circular red nuclei (diameter 0.9 µm). Several forms of the parasite were present, including single uninucleate forms, diameter 1.1 µm (Fig. 68), paired uninucleate forms (Fig. 69), groups of uninucleate forms and multinucleate plasmodia (diameter 3 µm) containing up to six nuclei (Fig. 70). These were sometimes vacuolated. Plasmodia and groups of uninucleate forms frequently occurred within the same host cell, with dividing forms also being observed (Fig. 72). However, the relation of these stages to those already described from large active xenomas at ultrastructural level is unclear. The host cells themselves were frequently arranged in tight groups of two, three or more (Figs. 73, 74, 75).

Host response. No host response was observed around active xenomas. Degenerate xenomas contained only densely packed spores, no developmental stages or host cell organellae being present. The structure of the degenerate xenoma was determined by the progress of the inflammatory tissue response. Initial stages included the infiltration of lymphocytes and fibroblasts (Fig. 76) and the deposition of a thin layer of collagen around the xenoma (Fig. 77). The response progressed with the formation of capillaries and

cellular proliferation within the xenoma, forming a granuloma. Granulomas (Fig. 58) possessed a necrotic inner zone, an outer zone of phagocytosed spores and a dense surrounding layer of small cells including lymphocytes and fibroblasts. Necrosis was occasionally observed in adjacent liver tissue, particularly where bile ducts were involved in the infection (Fig. 78). Xenomas at the periphery of the liver were additionally infiltrated by melanocytes, and were eventually detached into the body cavity (Figs. 77, 79).

Ultrastructural studies revealed the inner part of degenerate xenomas to be composed of necrotic macrophages and the debris from spores destroyed by phagocytosis (Fig. 80). The outer, surrounding layer was composed of tightly packed lymphocytes including monocytes (Fig. 82) and heterophils (Figs. 83, 84). Tracts of collagen fibres were formed in the region (Fig. 80). Spores were destroyed by repeated phagocytosis and cell necrosis, eventually resulting in the reduction of the xenoma to a region of phagocytic cells each containing a small number of spores in electron-dense phagocytic vacuoles (Fig. 85).

Transmission in vivo

Observations were undertaken on host fish to investigate the site, seasonality and host age dependence of *Microgemma hepaticus*. Incidence of infection was calculated from examination of fish dissected for the preparation of spore suspensions. Two infection experiments were performed; the first to investigate suitable methods of introduction of spores to *Chelon labrosus*, and the second to investigate the effects of temperature on transmission.

Observations on host fish. The earliest observation of 0+ mullet in St. Johns lake was made in the second week in September. Fish

were collected at this time already had patent infections in the liver. The fish remained in the estuary until late May, the incidence of infection through this period being 38.5% (Table 2). The occasional '1' group and '2' group specimens of C. labrosus and Liza auratus obtained during the summer months were uninfected.

Table 2. Incidence of hepatic microsporidiosis in two species and age groups of mullet.

Species	Age Group	Number examined	Number infected	Incidence
C. labrosus	'0' years	90	35	38.5%
C. labrosus	'1','2' years	8	0	0
L. auratus	'1','2' years	15	0	0

Experiment 1. To investigate the infection process of M. hepaticus in C. labrosus using four methods of spore administration.

Fifty '0' group mullet ranging in size from 3 cm to 4 cm were divided into five groups, A-E, each containing 10 fish, maintained at 18°. Spores were administered to groups A-D by the use of four different routes, as follows:-

Group A 10^5 spores introduced orally by stomach tube.

Group B Fish fed Artemia previously exposed to a spore suspension.

Group C Fish fed on chopped, heavily infected liver.

Group D 10^5 spores injected intraperitoneally.

Group E was maintained as a control group. One fish was removed from each group at weekly intervals post infection and screened for infection using Giemsa smears of small intestine and

liver. The smears were carefully examined using x40 and x100 objectives. Material removed after week 5 was additionally examined using cryostat sections stained with Giemsa.

Established xenomas from natural infections were present in the livers of 13 of the fish, including three of the controls. In the experimental groups, two types of infections possibly resulting from experimental procedures were observed. Two extracellular multinucleate bodies (Fig. 86) were present in the intestinal smear of one fish from group A two weeks post infection. At five weeks post infection host cells containing spores were observed in the liver smear of another fish from group A (Figs. 87, 88). Similar cells were observed from week seven onwards in liver smears of fish from other groups. The full results are presented in Table 3.

The results remain inconclusive. The rarity of the intestinal stages makes their origin uncertain; larger numbers would have been expected if they were the result of the infection experiments performed here. In only one case were the liver stages observed in the absence of natural infections; they may well have been the results of spore phagocytosis from established infections.

A second experiment was performed to investigate the effects of temperature on transmission, using the stomach tube method of spore administration.

Experiment 2. To investigate the effects of temperature on transmission of *M. hepaticus* to *C. labrosus*.

Three experimental groups, A, B and C, of 10 'O' group *C. labrosus* each, were established and maintained at temperatures of 12.5°, 18° and 25° respectively. These were exposed to spores of *M. hepaticus* administered in a single dose of 8×10^5 spores

Table 3. To investigate the infection process of *M. hepaticus* in *C. labrosus* using four methods of spore administration.

DAYS AFTER EXPOSURE	SITE	EXPERIMENTAL GROUP				CONTROL
		A	B	C	D	E
7	INTESTINE	O	O	O	O	O
	LIVER	N	O	O	O	N
14	INTESTINE	E	O	O	O	O
	LIVER	O	N	O	N	O
21	INTESTINE	O	O	O	O	O
	LIVER	O	O	O	O	O
28	INTESTINE	O	O	O	O	O
	LIVER	O	O	O	O	N
35	INTESTINE	O	O	O	O	O
	LIVER	E	O	N	O	O
42	INTESTINE	O	O	O	O	O
	LIVER	O	O	O	O	O
49	INTESTINE	O	O	O	O	O
	LIVER	N/E	O	N/E	O	O
56	INTESTINE	O	O	O	O	O
	LIVER	O	N/E	O	N/E	O
63	INTESTINE	O	O	O	O	O
	LIVER	N/E	O	O	O	O
70	INTESTINE	O	O	O	O	O
	LIVER	O	O	N/E	O	N
% infected		50	20	30	20	30

O = No infection present

E = Possible experimental infection

N = Established natural infection

introduced by stomach tube. One fish was removed weekly from each group and screened for infection as in Experiment 1. The results are presented in Table 4.

Table 4. To investigate the effects of temperature on transmission of *M. hepaticus* to *C. labrosus*.

DAYS AFTER EXPOSURE	SITE	EXPERIMENTAL GROUP		
		A	B	C
7	INTESTINE	O	O	O
	LIVER	O	O	N
14	INTESTINE	O	O	O
	LIVER	O	O	O
21	INTESTINE	O	O	O
	LIVER	N	O	N
28	INTESTINE	O	O	O
	LIVER	O	O	O
35	INTESTINE	O	O	O
	LIVER	N	N/E	O
42	INTESTINE	O	O	O
	LIVER	O	O	N
49	INTESTINE	O	O	O
	LIVER	O	O	O

O = No infection

E = Possible experimental infection

N = Established natural infection

No stages were present in intestinal smears. Intracellular liver stages were only observed in one fish, after 35 days at 18°, which had a heavy established natural infection.

Exsporulation and the infection process in vitro

Three experiments were performed to investigate factors triggering exsporulation of M. hepaticus in vitro, using biological extracts, oxidising agents and changes in the physico-chemical characteristics of the treatment media. Three experiments were then performed to establish the parasite in RTG cell culture, the progress of the experiments being investigated using Giemsa techniques and electron microscopy. All experiments were performed using spores extracted from a single batch of mullet.

Experiment 3. To investigate the effects of biological extracts on triggering exsporulation of M. hepaticus in vitro.

Aliquots of suspension containing 3.55×10^5 spores were centrifuged at 650g for 10 min, the supernatant removed and the spores resuspended in 100 µl of the following extracts : freshly prepared extracts of the mullet digestive tract, mullet bile, the mammalian bile salt sodium tauroglycocholate and selected commercial enzyme preparations. Digestive tract extracts were prepared from the stomach and intestinal wall. Mullet bile was taken directly from the gall bladder, and sodium tauroglycocholate (Sigma) was prepared as a 1% solution in Young's teleost saline. These reagents were incubated with the spores for two hours at 20° in a shaking water bath. Enzyme preparations were made up at 1% concentration in tris/HCl buffered Dulbecco's saline (Flow Laboratories), the following enzymes being used : pepsin (Sigma) pH 2; trypsin (Sigma) pH 7.6 and chitinase (Sigma) pH 6. Pepsin and trypsin were incubated with spores in a shaking water bath for 2 hr at 20°. Chitinase, owing to the low activity of the preparation, was incubated for 48 hr at 20°. The results are presented in Table 5.

Table 5. The effects of fish extracts, bile salts and enzymes on exsporulation of *M. hepaticus* at 20°.

Reagent	% Exsporulation	Effects on spores (+ = phase darkening)
stomach wall extract	0	-
intestinal wall extract	0	-
mullet bile	0	+
1% sodium tauroglycocholate	0	+
1% Pepsin pH 2	0	-
1% Trypsin pH 7.6	0	-
1% Chitinase pH 6	0	+

From the results (Table 5), none of the reagents induced filament extrusion. Changes in the appearance of the spore contents (phase-darkening) were observed in mullet bile, 1% sodium tauroglycocholate and chitinase. In addition, damage to the spore wall was caused following chitinase treatment.

Experiment 4. To investigate the effects of inorganic and organic oxidising agents on exsporulation of *M. hepaticus in vitro*.

A total of six oxidising agents were selected for investigation, including two biological agents, dehydroascorbic acid and reduced cytochrome C, and the inorganic agents iodine water, hydrogen peroxide, sodium hypochlorite and potassium iodide. With the exception of cytochrome C, 10 µl of spore suspension containing 3.55×10^4 spores was added to serial dilutions of each agent to give final concentrations as shown in Table 5. Cytochrome C was used at

0.2% final concentration. Experiments were incubated at 20° for 20 minutes and examined under phase-contrast.

The results table (Table 6) shows that each of the reagents investigated, with the exception of cytochrome C, induced filament extrusion. The most effective reagents were iodine water and hydrogen peroxide (Figs. 89, 90), the percentage of exsporulated spores increasing to a maximum of 20% in 50% saturated iodine water. A notable feature was the inhibitory effect of saturated iodine water. In sodium hypochlorite there were signs of structural damage at high concentrations, and a little exsporulation at low concentrations.

Experiment 5. To investigate the effects of various pretreatments on exsporulation of *M. hepaticus* in the cell culture medium BHK 21.

Spores were subjected to one of fourteen pretreatments prior to immersion in BHK 21, the cell culture medium selected for maintenance of RTG cells. The pretreatments were designed to investigate pH and osmolarity as factors in priming spores for exsporulation. Aliquots of spore suspension were centrifuged, the supernatant removed and the spores resuspended in the pretreatment medium. A range of pH's from 1-10 was established using tris/HCl buffered saline and spores were maintained in one of these for 45 minutes. In addition the direct effect of HCl at a concentration of 0.1M and also the alkalis tris (1M) and potassium hydroxide (0.1M) was investigated. Osmolarity was adjusted by using a range of concentrations of potassium and sodium chloride giving concentrations from 1 to 5 molar. Following treatment for 45 minutes spores were resuspended in BHK 21. Control spores underwent pretreatment in teleost saline. The results

Table 6. The effects of oxidising agents on exsporulation of *M. hepaticus*.

Nature of Agent	Agent & concentration	% Exsporulation	Effects on spores (+ = phase darkening)
Biological	Dehydroascorbic Acid		
	0.01M	2%	-
	0.005M	2%	-
	0.003M	2%	-
	0.0015M	<1%	-
	0.0008M	0	-
Biological	Reduced Cytochrome C		
	0.2%	0	-
Inorganic	Iodine Water		
	100% saturated	0%	-
	50%	20%	-
	25%	15%	-
	12.5%	10%	-
	6.2%	5%	-
	3.13%	2%	-
	1.57%	0%	-
Inorganic	Hydrogen Peroxide		
	0.88M	15%	-
	0.44M	10%	-
	0.22M	11%	-
	0.11M	5%	-
	0.050M	2	-
	0.025M	0	-
	0.012M	0	-
Inorganic	Sodium Hypochlorite		
	7.5% free Cl_2	0	+
	3.75	0	+
	1.88	0	+
	0.94	0	+
	0.47	0	-
	0.24	0	-
	0.12	0	-
	0.06	0	-
	0.03	0	-
	0.015	<1%	-
	0.008	<1%	-
	0.004	<1%	-
Inorganic	Potassium Iodide		
	1M	<1%	-
	0.5M	<1%	-
	0.25M	<1%	-
	0.125M	0	-
	0.063M	0	-

(Table 7) indicate both low pH and high osmolarity to be a possible factor in inducing exsporulation in the culture medium. Of particular interest in osmolarity experiments was the failure of complete exsporulation at high osmolarity - polar filaments were only partially everted.

Table 7. The effects of various pretreatments on exsporulation of *M. hepaticus* in BHK 21 medium.

Pretreatment reagent	% exsporulation in BHK 21	Changes in spore appearance
Tris/HCl buffered saline pH 1	<1%	-
pH 2	<1%	-
pH 3	0	-
pH 9	0	-
pH 10	0	-
0.1 M HCl	0	-
1 M Tris	0	-
0.1 M KOH	0	-
1 M KCl	<1%	-
1 M NaCl	<1%	-
2 M KCl	2%	-
2 M NaCl	2%	-
5 M KCl	2%	+
5 M NaCl	2%	+

Establishment of host cell/parasite system. Attempts were made to establish cultures derived from mullet liver for use in infection experiments. Although these proved to be impractical, the progress of cultures is described. Three experiments were then undertaken to investigate the invasion of RTG cells *in vitro* with *M. hepaticus*.

In the first of these, experiment 6, spores were exposed to the cells in monolayers on coverslips (Fig. 91), assessment of results being based on direct examination of the Giemsa-stained monolayer. In two further experiments, experiments 7 and 8, spores were added to RTG cells maintained in suspension. These were assessed in one of two ways, experiment 7 by light microscope examination of cytospin preparations (Fig. 92) and experiment 8 by electron microscopy. In all three experiments spores were pretreated with saline buffered to pH 2 (as in experiment 5). To compensate for low exsporulation rates a ratio of spores to cells of 10:1 was employed.

Progress of primary cultures of mullet liver cells. In mullet liver cell cultures, cell growth was observed in cultures maintained at 20°, and followed a similar pattern in both BHK 21 and Leibovitz L-15 culture media. The cells attached to the flasks within one day. Cell types present were hepatocytes, recognised by their large size and prominent lipid inclusions, and small fibroblastic cells. Hepatocytes did not multiply; they spread over the growing surface over a period of three days, and disintegrated. The fibroblastic cells multiplied and attained patchy confluency in L-15 medium after 7 days, and complete confluency in BHK 21 cultures after 12 days. Subculture at this stage was unsuccessful, with no cell growth occurring afterwards. Fibroblastic cells survived in unsubcultured flasks for 18 days.

Experiment 6. To investigate the invasion of RTG cells by *Microgemma hepaticus* in monolayer cultures.

Four confluent RTG cell coverslip cultures were located monolayer upwards in flat-bottomed bijou bottles (Sterilin)

containing 2 µl of BHK 21 medium. To each of these, 3.55 x 10^4 spores were added following pretreatment for 45 min in saline buffered to pH 2; the spores were transferred in a 10 µl volume of the pretreatment medium. In addition four more cultures were inoculated with 10 µl of the pretreatment medium as controls. Following inoculation of spores, all cultures were centrifuged at 1000g for 30 min to ensure rapid sedimentation of spores onto the cell monolayers. The cultures were then incubated for periods of 30 min, 24 hr, 72 hr and 7 days at 20°. At the termination of each culture the coverslip was removed, fixed in methanol and stained with Giemsa for examination. A control culture was examined at the same time.

Intracellular inclusions suspected of being early stages of parasite development were present in the 30 minute and 72 hour cultures. The stage observed in the 30 minute culture was circular in outline with a diameter of 1.6 µm, the cytoplasm staining a more intense blue than that of the RTG cell (Fig. 93). The nucleus was enlongated but irregular in outline measuring approximately 0.5 µm x 1 µm. A larger stage was observed in the 72 hour culture (Fig. 94), the inclusion being more or less oval in shape, measuring 3 µm x 2.2 µm, and containing 12 nuclei. Although these inclusions were clearly demonstrated within the RTG cells it must be stated that only one stage was present in each of the cultures.

Experiment 7. A light microscope investigation of the invasion of RTG cells by *M. hepaticus* in suspension cultures.

Three suspension cultures, each containing 10^6 RTG cells in 1 ml of BHK 21 medium were inoculated with 10^7 spores pretreated in saline at pH 2, the spores being introduced in 10 µl of the

pretreatment medium. Three further cultures were inoculated with 10 µl of the pretreatment medium as controls. The cultures were then incubated at 20° in a shaking water bath for periods of 1 hour, 1 day and 3 days post inoculation. At the termination of each test the entire contents of a culture were deposited onto a single slide by means of the cytospin. The cells were then examined following fixation and Giemsa staining. No evidence was found in this or any other preparation that cells had been invaded.

Experiment 8. An ultrastructural investigation of the invasion of RTG cells by *M. hepaticus* in suspension cultures.

4.6×10^7 spores pretreated with saline at pH 2 were added in 100 µl of pretreatment medium to 5 ml of RTG cell suspension containing 2.2×10^6 cells. The culture was centrifuged for 5 min at 500 g in order to bring RTG cells and spores into close proximity, and then allowed to stand for 30 min. The pellet was then resuspended and the culture incubated overnight at 20° in a shaking water bath before fixation for electron microscopy. An electron micrograph of RTG cells is shown in Fig. 95.

Electron microscope investigations indicated that the culture process was successful in stimulating exsporulation, empty spore cases (Fig. 97) and extruded filaments being observed. Extruded filaments were occasionally seen passing through RTG cells in sections (Fig. 96); however the discharged sporoplasms were not located. Discharged spores were of similar shape and dimensions to intact spores, but contained only loose membranes clumped beneath the exit of the polar filament (Figs. 97, 98). The extruded filament was attached at the anterior pole of the spore case, with amorphous electron-dense material deposited around its exit from the spore.

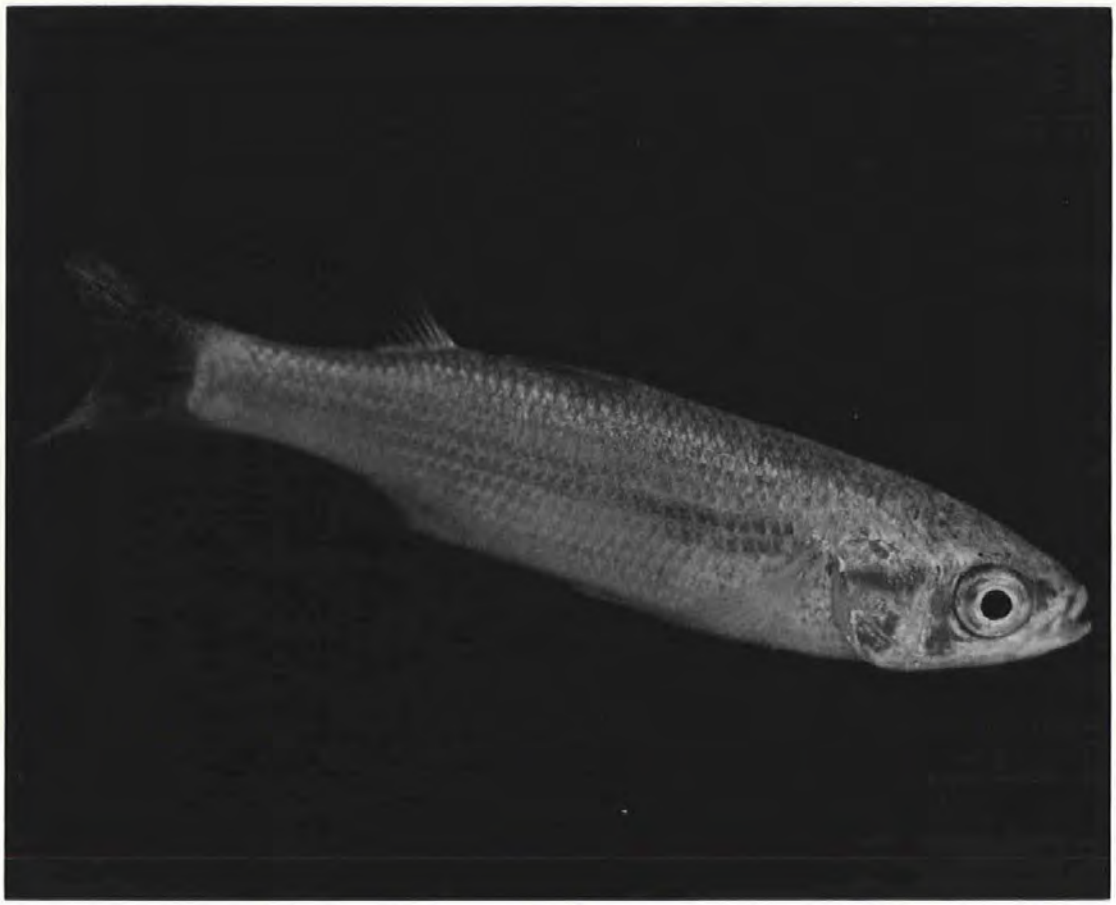
Extruded filaments were tubular (diameter 0.11 µm) with electron-dense walls 25 nm thick and a hollow lumen having a diameter of 60 nm (Fig. 99). The inner part of the wall consisted of an electron-dense membrane. There was no such clear termination at the outer part. Filaments passing through RTG cells were surrounded by a membrane separated from the filament by an electron lucent space (Fig. 96). Degenerating spores were occasionally observed within phagocytic vacuoles in the RTG cells (Fig. 96).

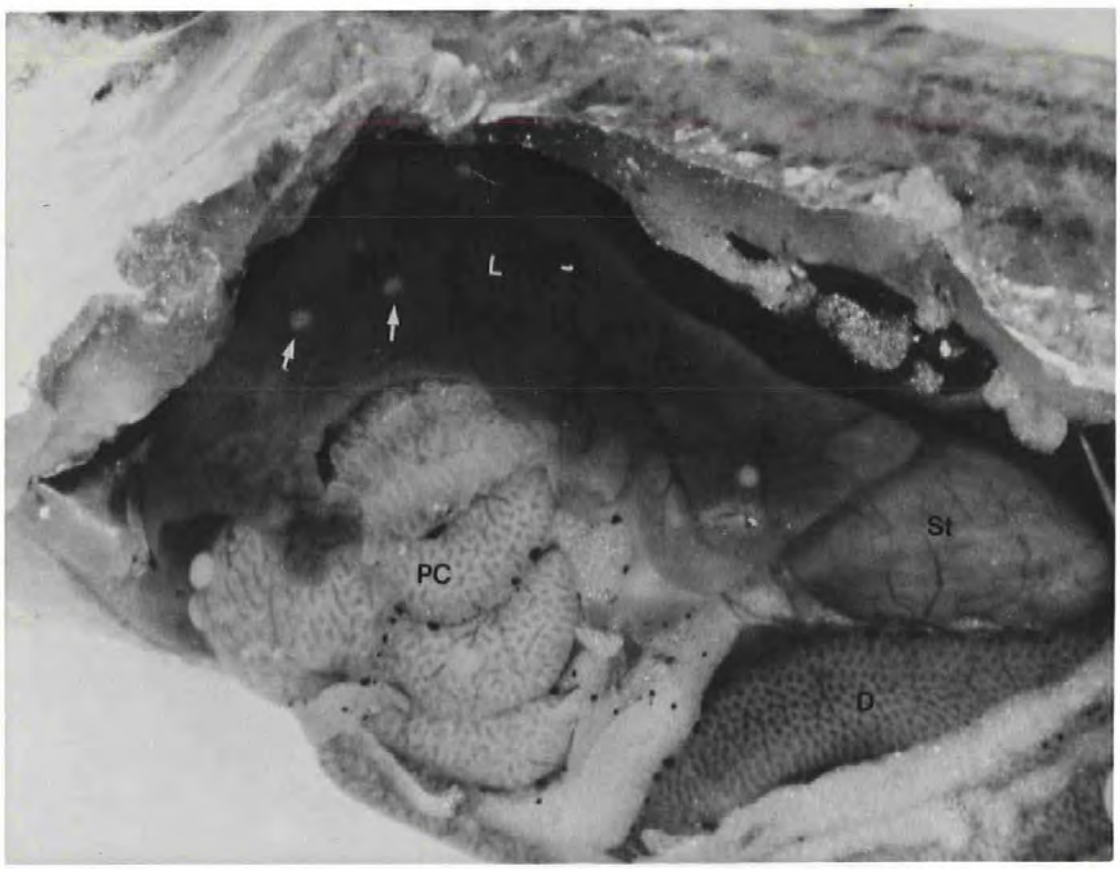
FIGURES 1-99

Figs. 1 and 2. Appearance and internal organs of *Chelon labrosus*.

Fig. 1. 'O'-group *Chelon labrosus*. x7

Fig. 2. Internal organs of *C. labrosus*. x18. D, duodenum; L, liver; PC, pyloric caecae; St, stomach; arrows, xenomas of *Microgemma hepaticus*.

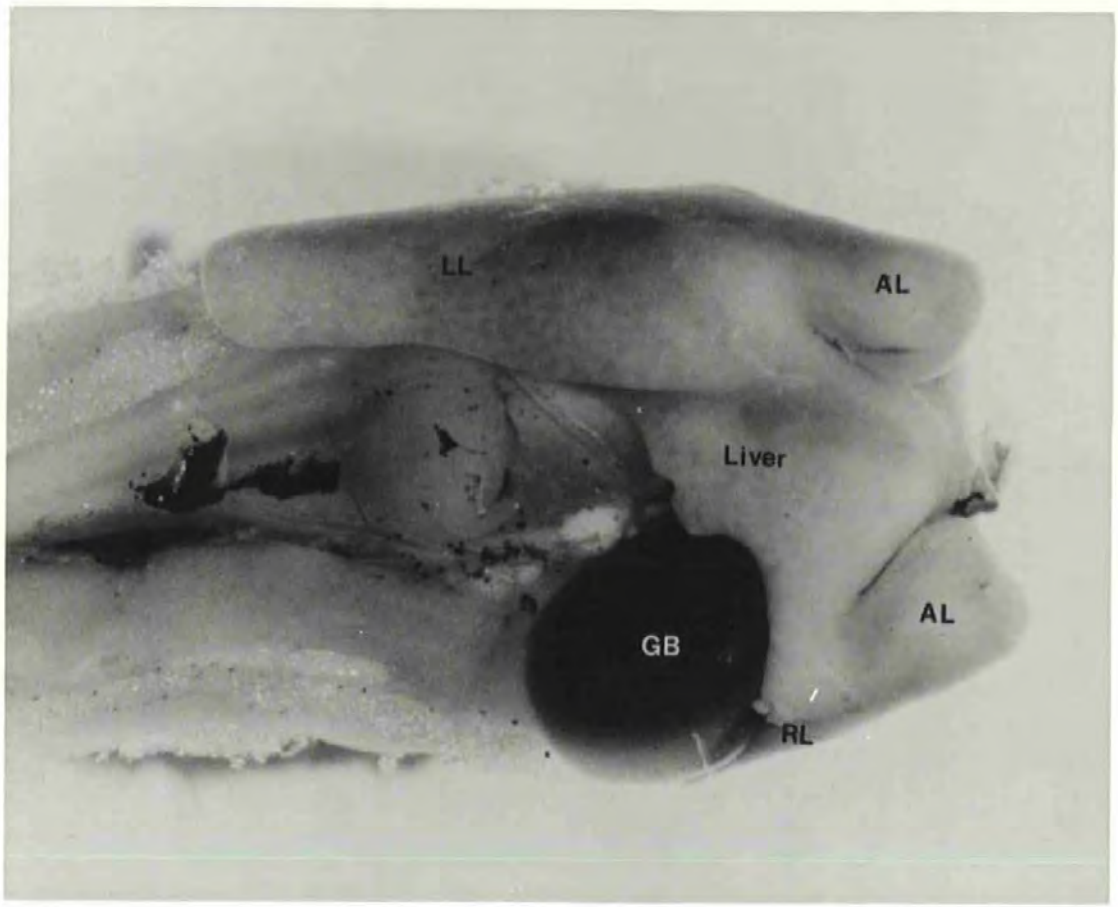


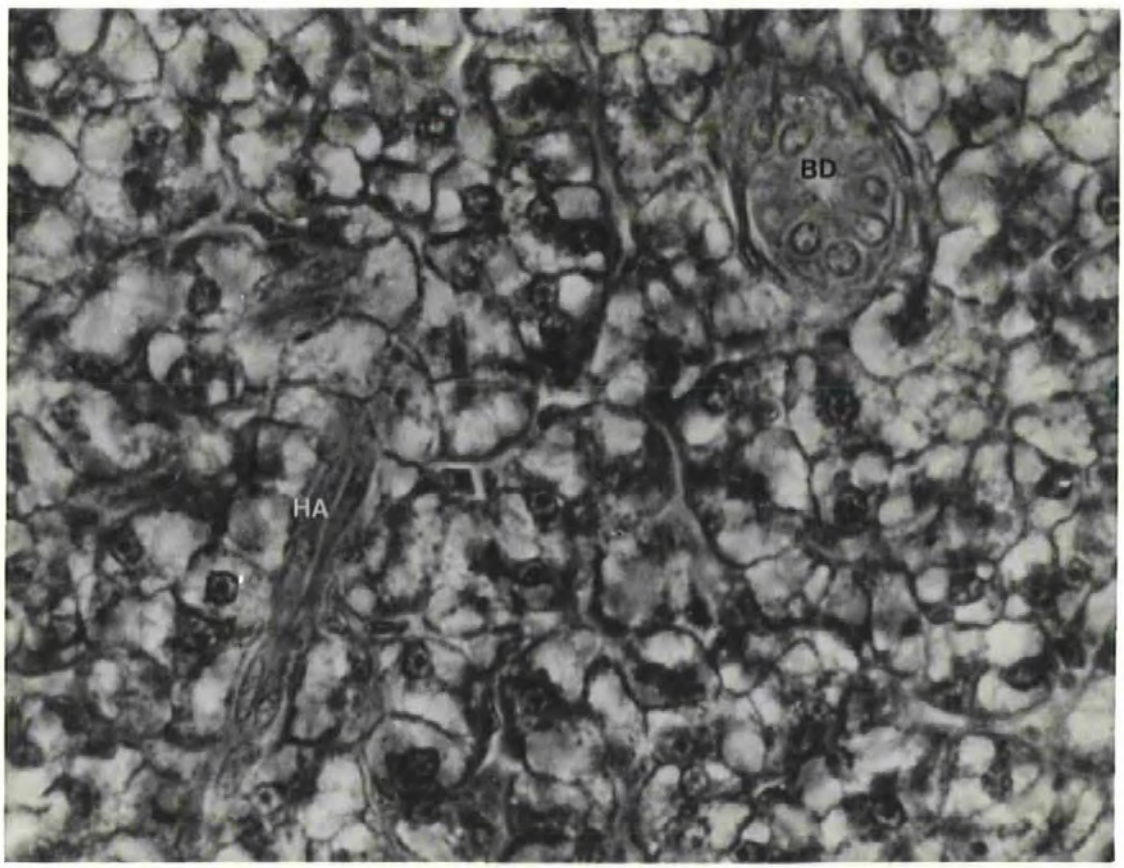

L
PC
St
D

Figs. 3 and 4. Internal organs and liver histology of *C. labrosus*.

Fig. 3. Internal organs of *C. labrosus* - dorsal view. x18. AL, anterior lobe; LL, left lobe; RL, right lobe; GB, gall-bladder.

Fig. 4. Histological section of liver showing bile duct and hepatic artery. x3100. BD, bile duct; HA, hepatic artery. Mallory's triple stain.

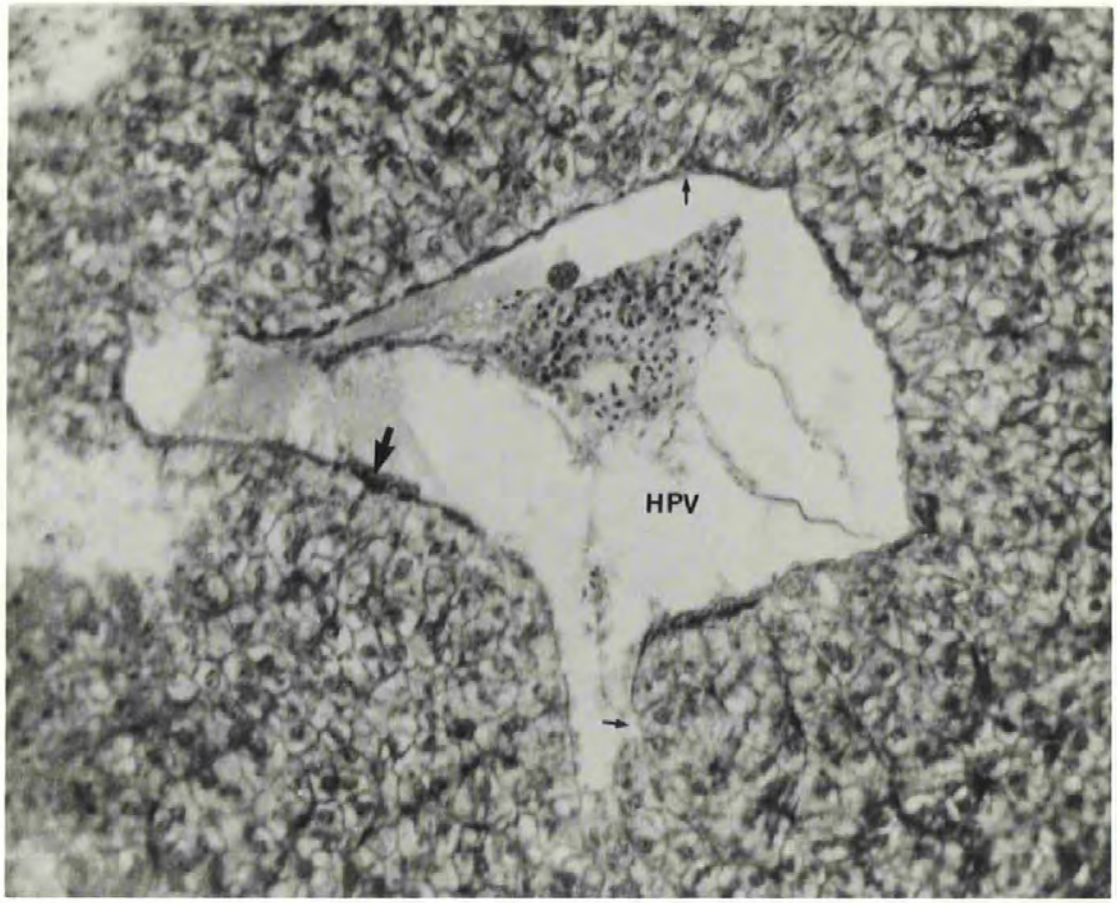

LL
AL
Liver
AL
GB
RL

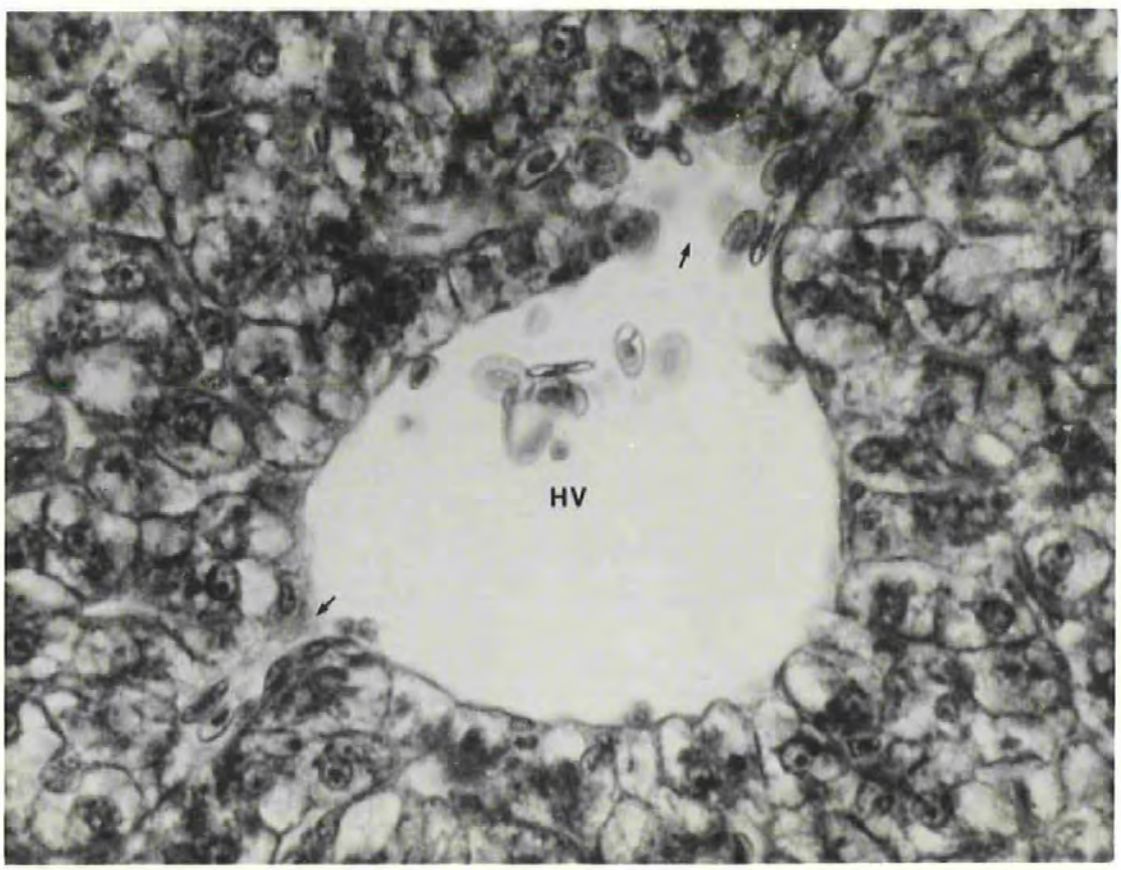

BD
HA

Figs. 5 and 6. Histology of the liver of *C. labrosus*.

Fig. 5. Hepatic portal vein. x1410. HPV, hepatic portal vein; large arrow, collagenous support; small arrow, junction of sinusoid with vein. Mallory's triple stain.

Fig. 6. Hepatic vein. x3700. HV, hepatic vein; arrows, junction of sinusoids with vein. Mallory's triple stain.

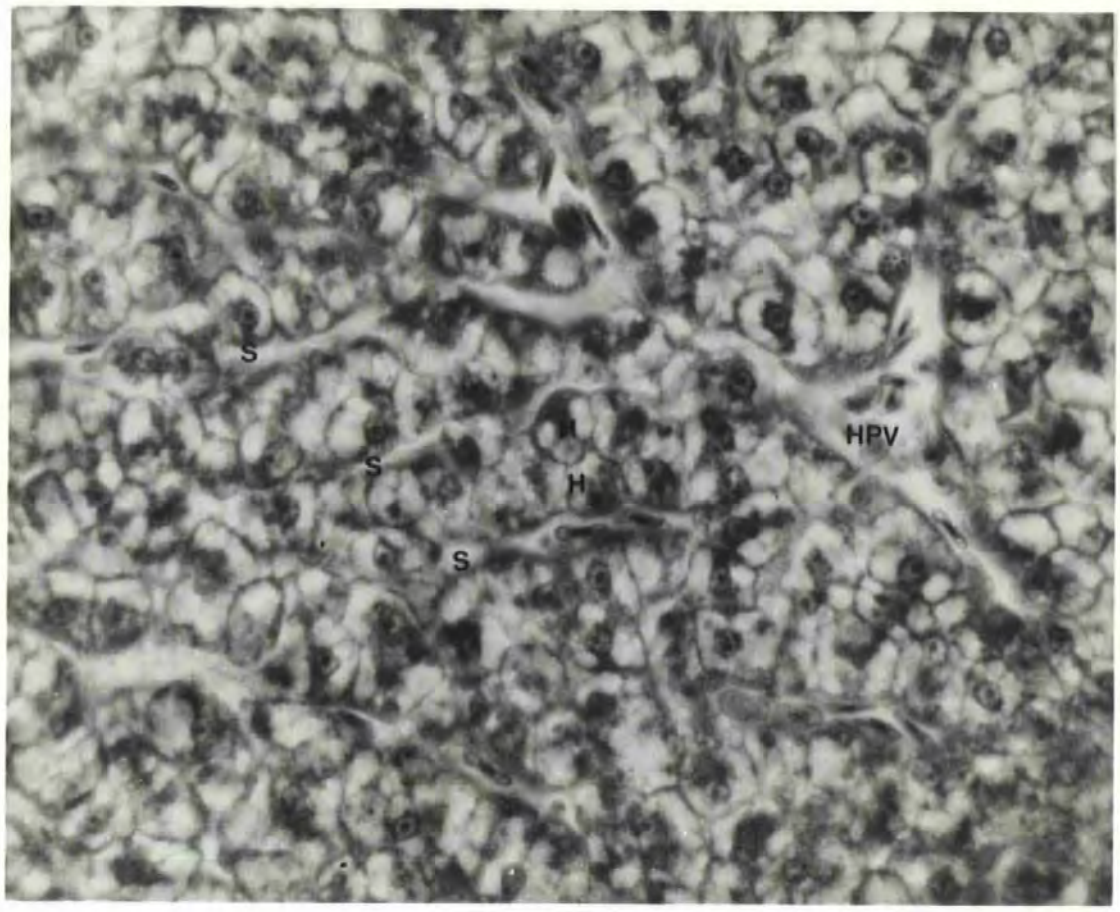

HPV


HV

Figs. 7 and 8. Histology of the liver of *C. labrosus*.

Fig. 7. Liver parenchyma - sinusoids and hepatocyte cords. x3100. H, hepatocyte; HPV, hepatic portal vein; S, sinusoid. Mallory's triple stain.

Fig. 8. Electron micrograph of transverse section of sinusoid. x10,300. E, endothelial cell; M, mitochondrion; MvP, microvillar processes; SD, space of disse.


S
S
S
H
HPV

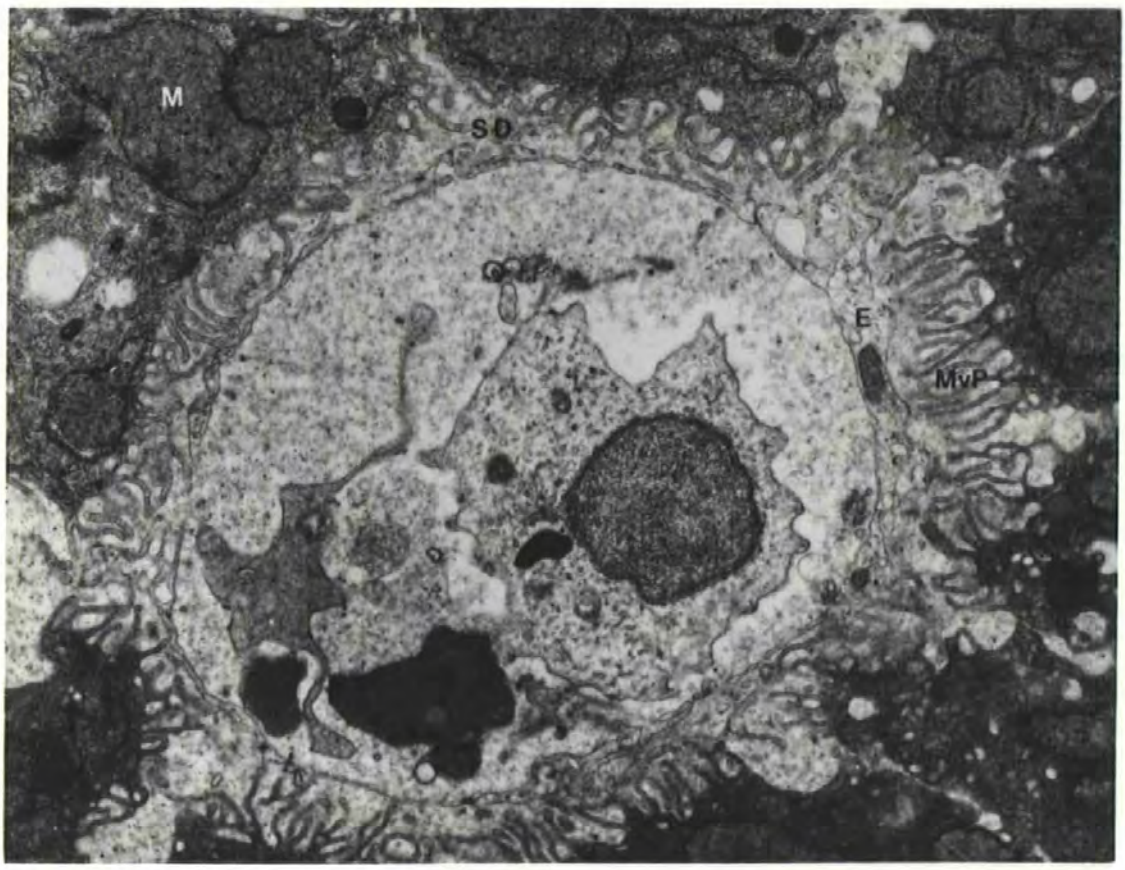
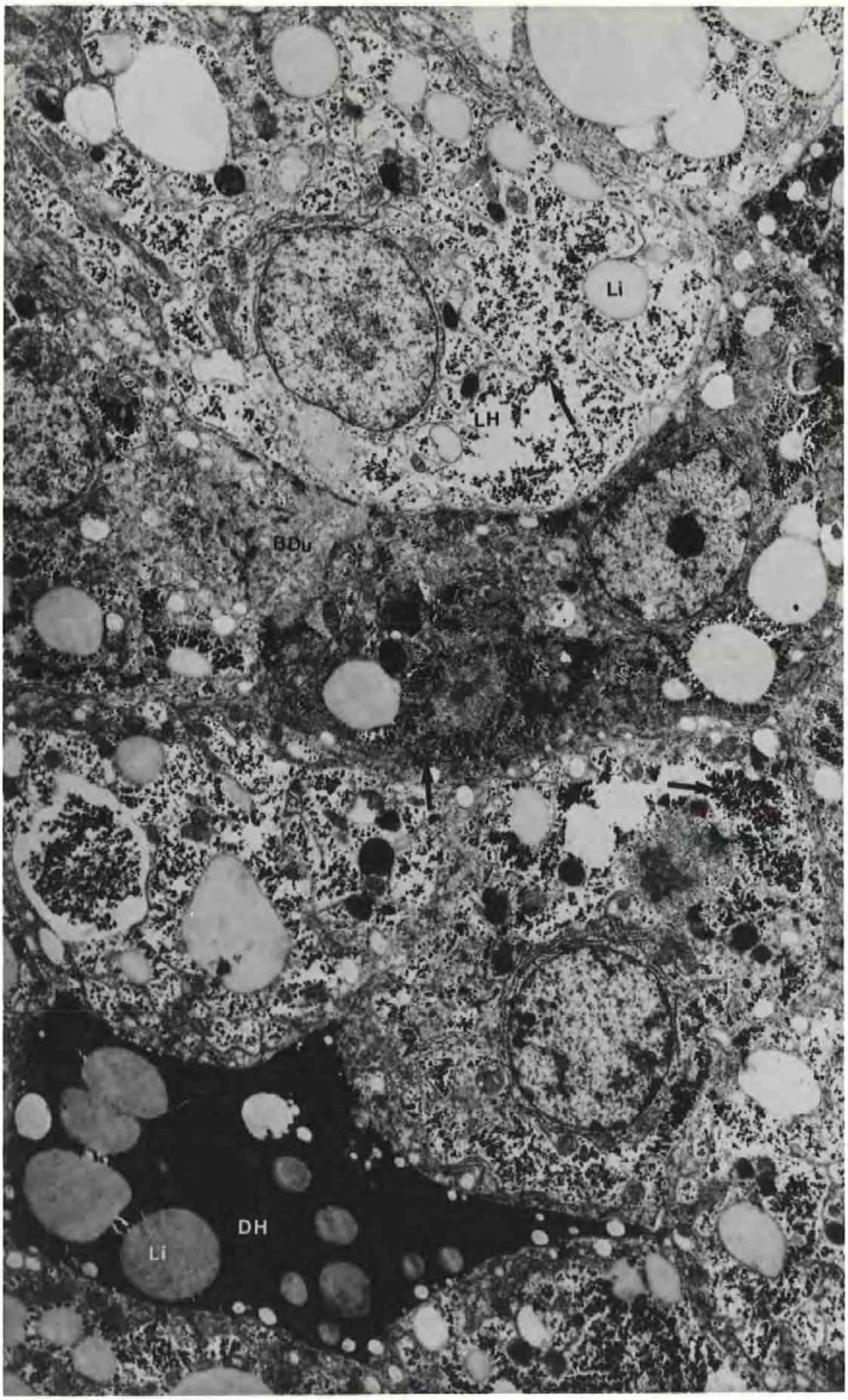

M
SD
E
MvP

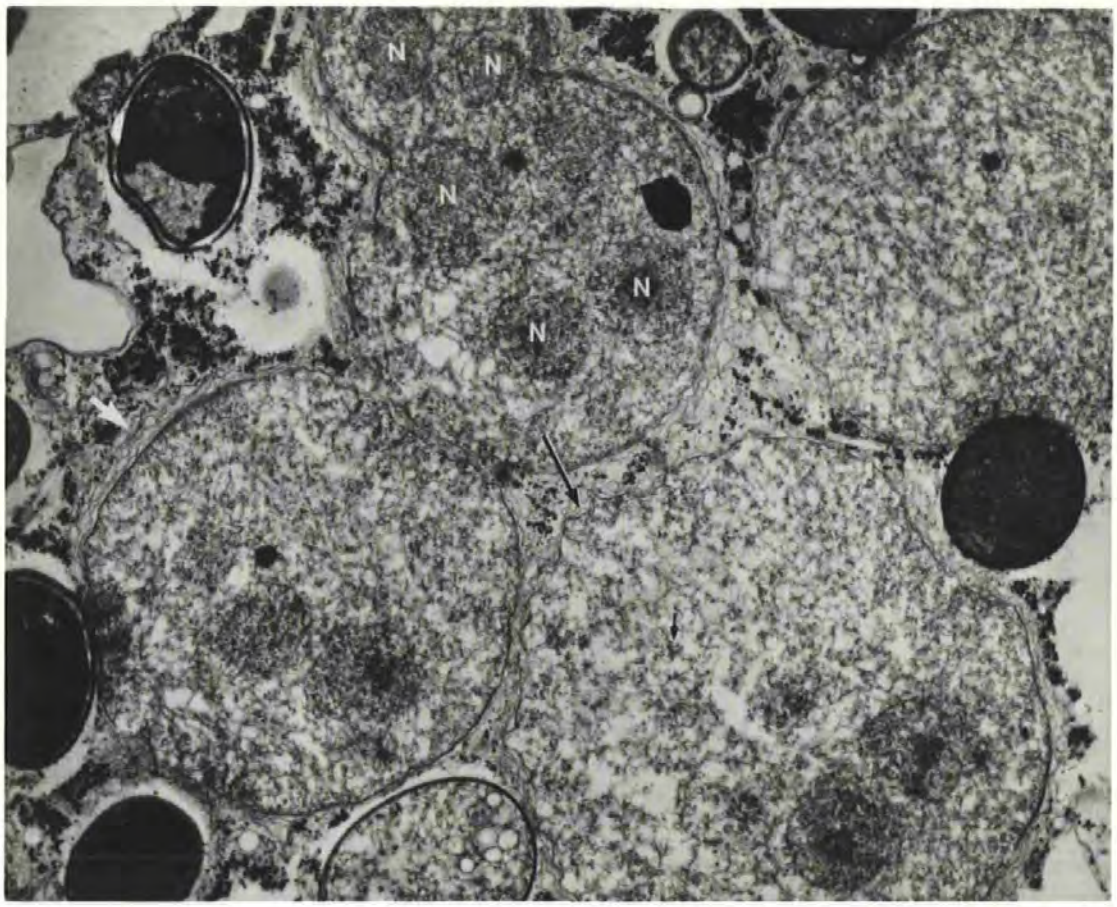
Fig. 9. Electronmicrograph of portion of liver parenchyma. x6400. BDu, bile ductule; DH, dark hepatocyte; LH, light hepatocyte; Li, lipid droplet; arrowed, glycogen.

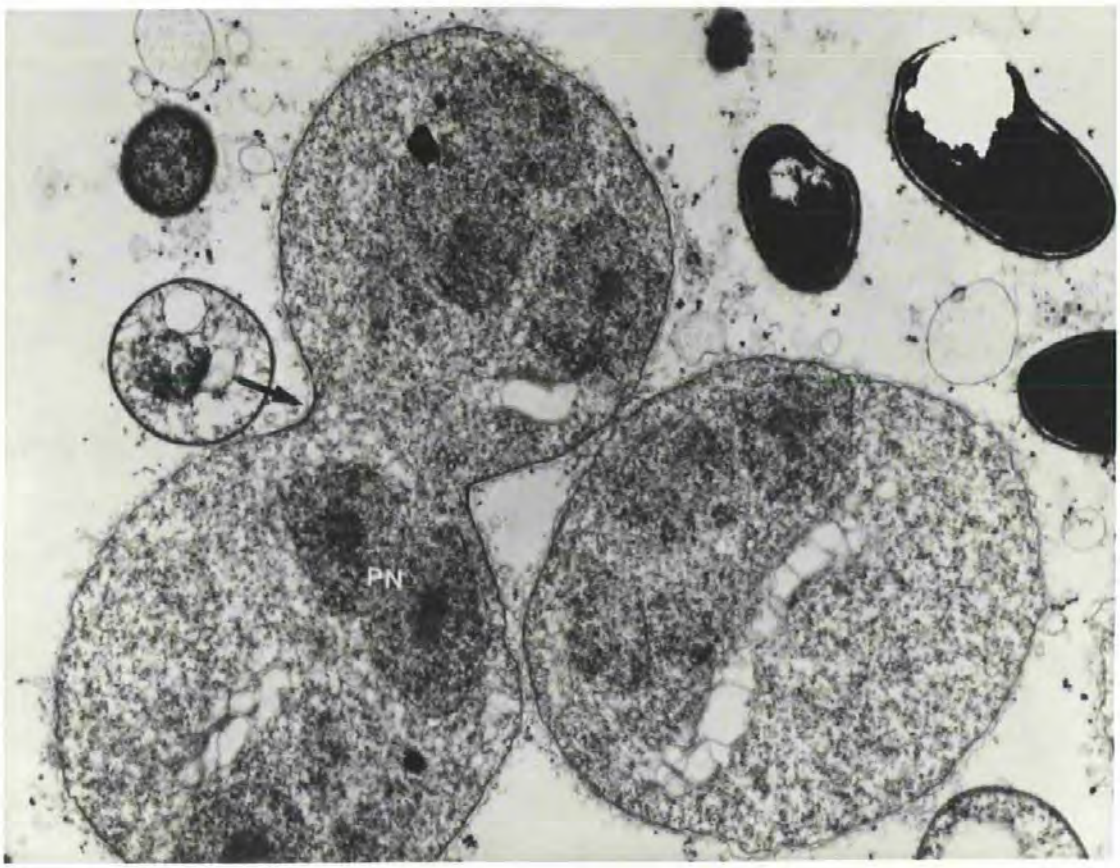

Li
LH
BDu
DH
Li

Figs. 10 and 11. Vegetative developmental stages of *Microgemma hepaticus*.

Fig. 10. Electron micrograph of meronts of *M. hepaticus*. x11,100. White arrow, smooth endoplasmic reticulum; short arrow, ribosome clusters; long arrow, meront enclosing membrane.

Fig. 11. Electron micrograph of dividing meronts of *M. hepaticus*. x12,040. PN, paired nuclei; arrow, constricting meront enclosing membrane around dividing meront.

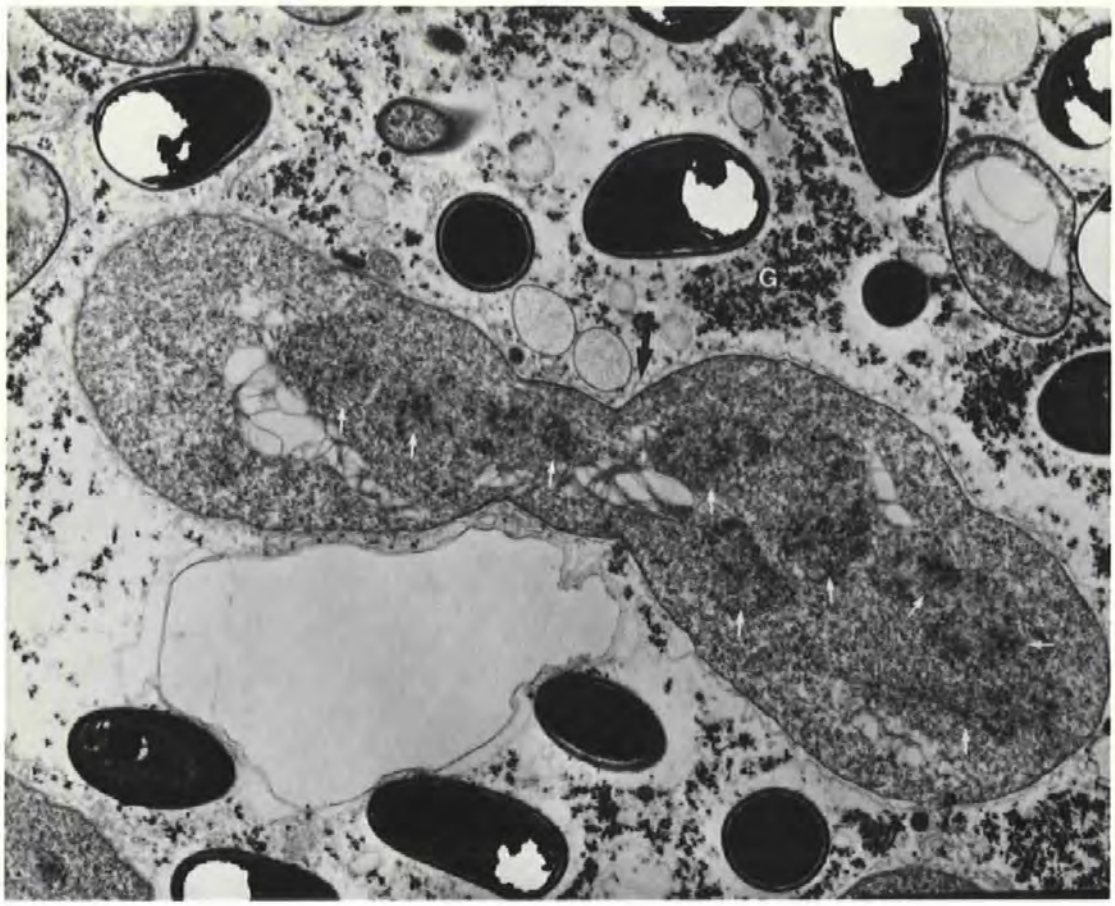

N
N
N
N
N

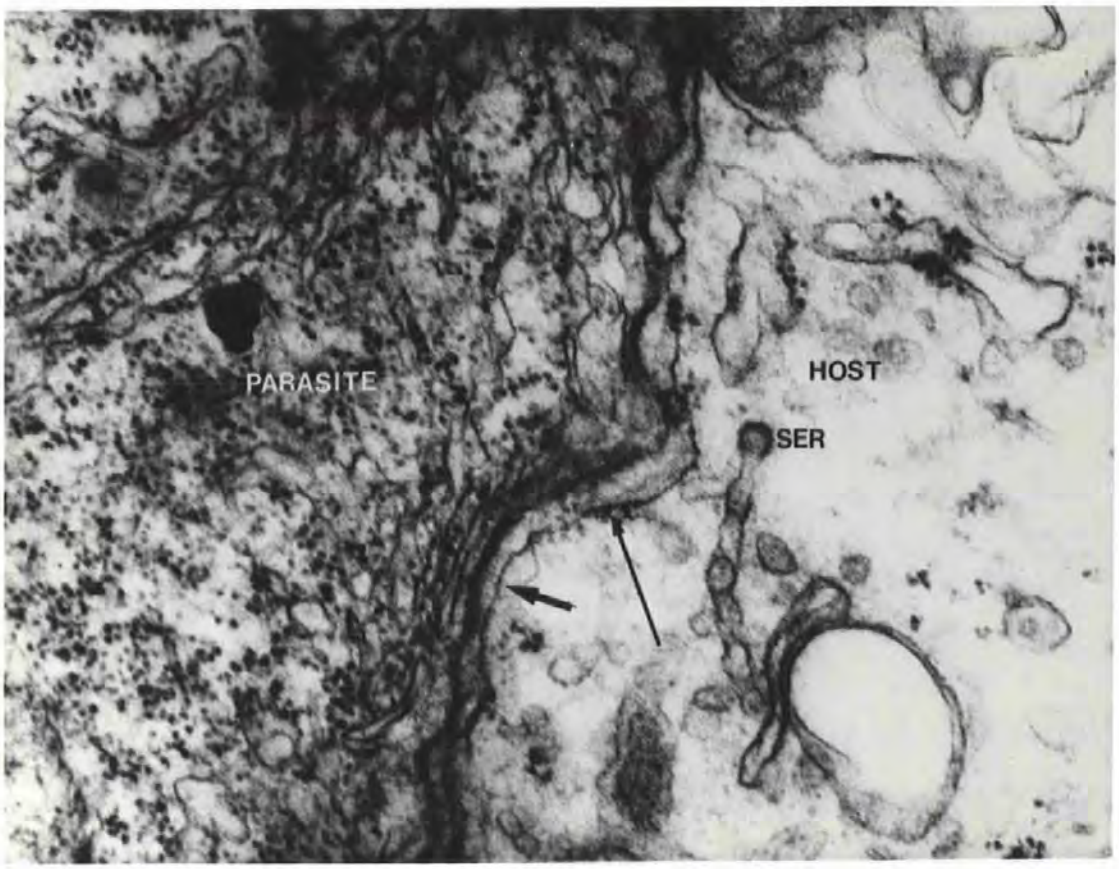

PN

Figs. 12 and 13. Features of meronts of Microgemma hepaticus.

Fig. 12. Electron micrograph of large merogonial plasmodium. x9400. Arrows, nuclei with condensed chromatin, indicating division.

Fig. 13. Electronmicrograph of meront/host cell interface. x72,000. Broad arrow, meront enclosing membrane; thin arrow, ribosomes attached to meront enclosing membrane. SER, Smooth Endoplasmic Reticulum.

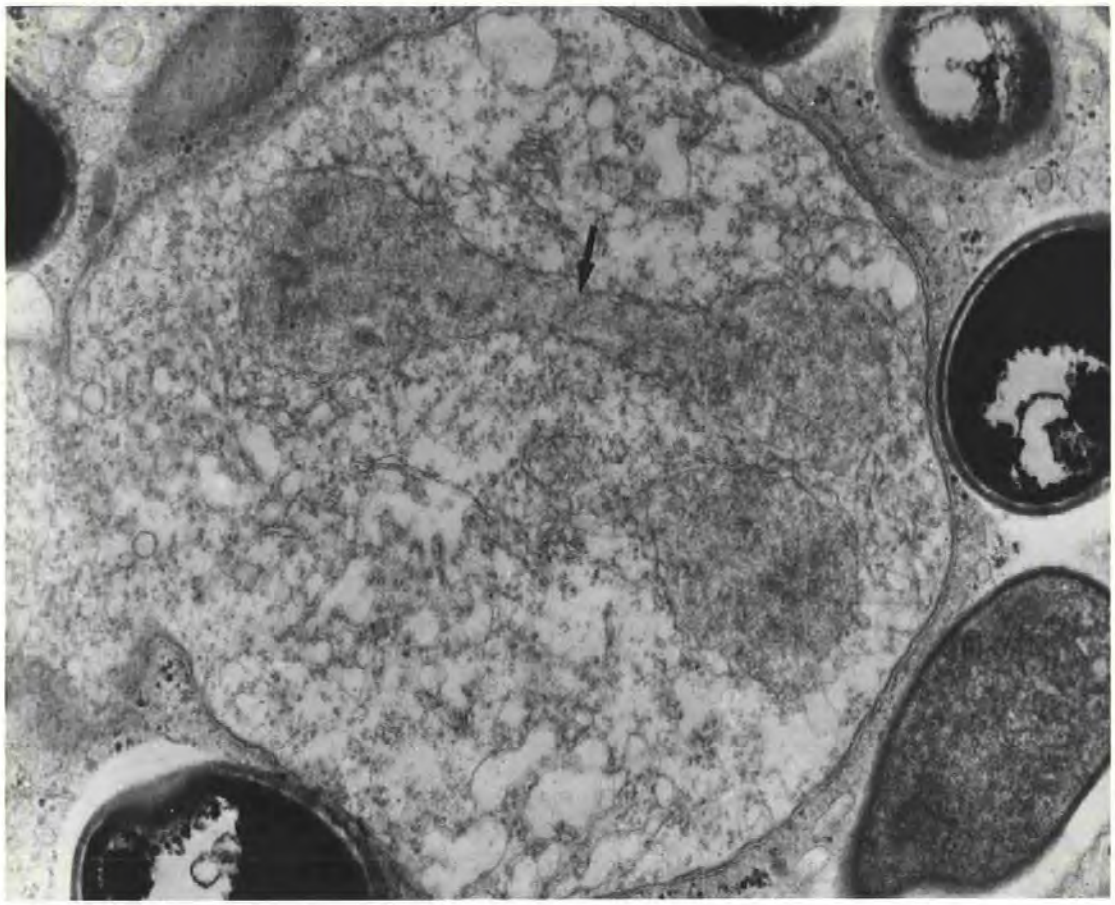

G

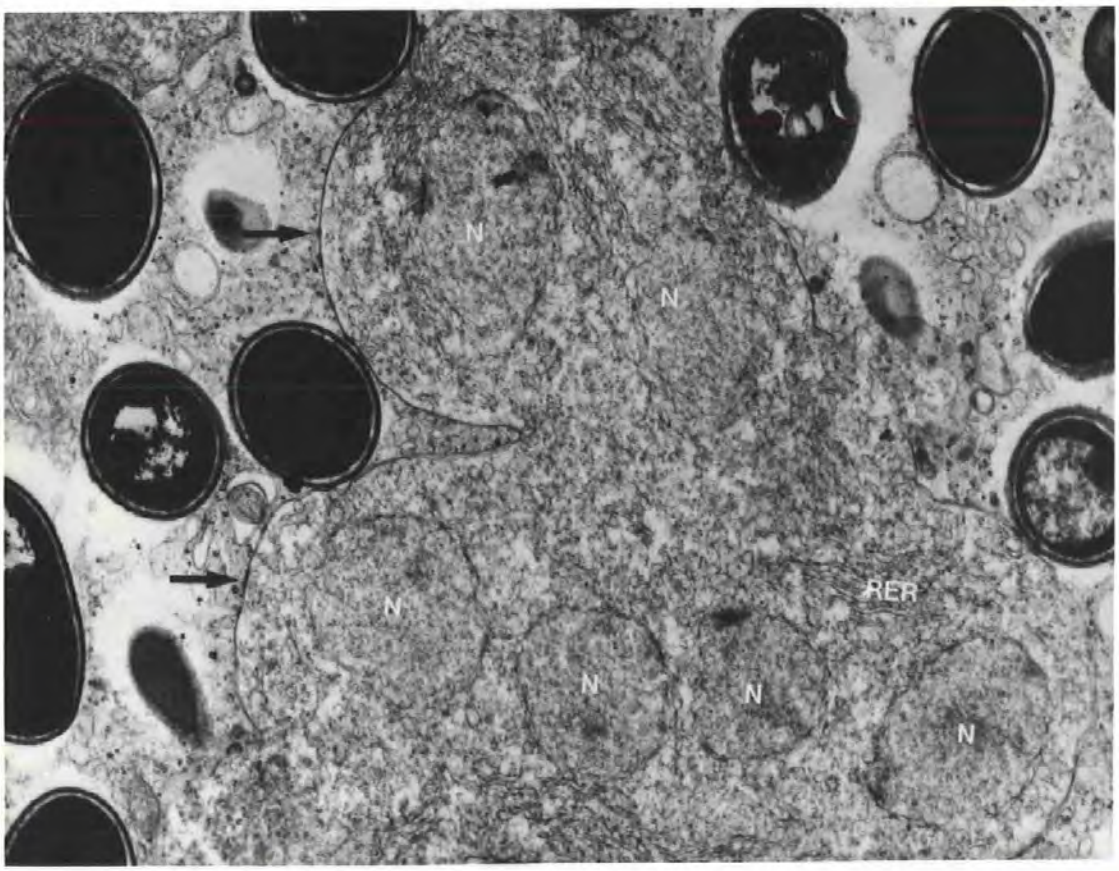

PARASITE
HOST
SER

Figs. 14 and 15. Meront nuclei and early sporogony of *M. hepaticus*.

Fig. 14. Electron micrograph of meront telophase nucleus. x20,500. Arrow, spindle fibres passing across isthmus of dividing nucleus.

Fig. 15. Electron micrograph of early sporogonic stage of *M. hepaticus*. x13,200. N, nucleus; RER, rough endoplasmic reticulum; arrow, extramembraneous thickenings.

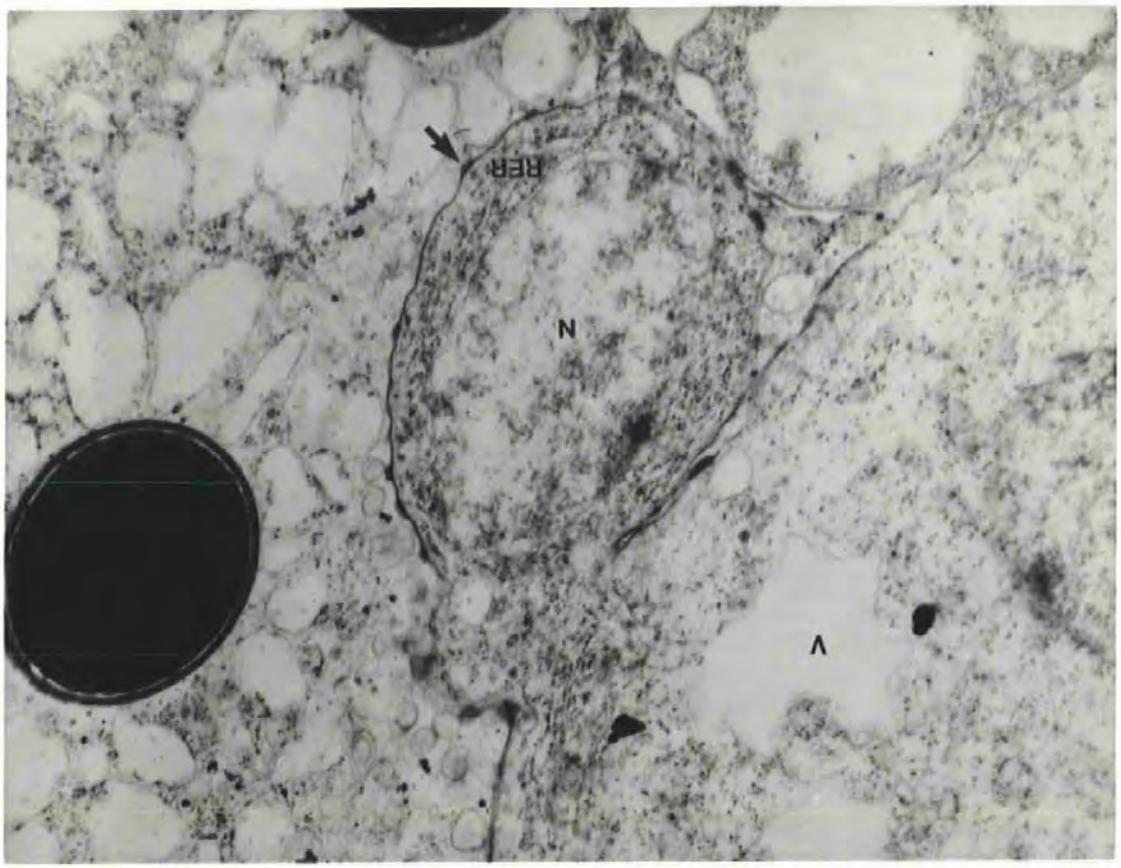


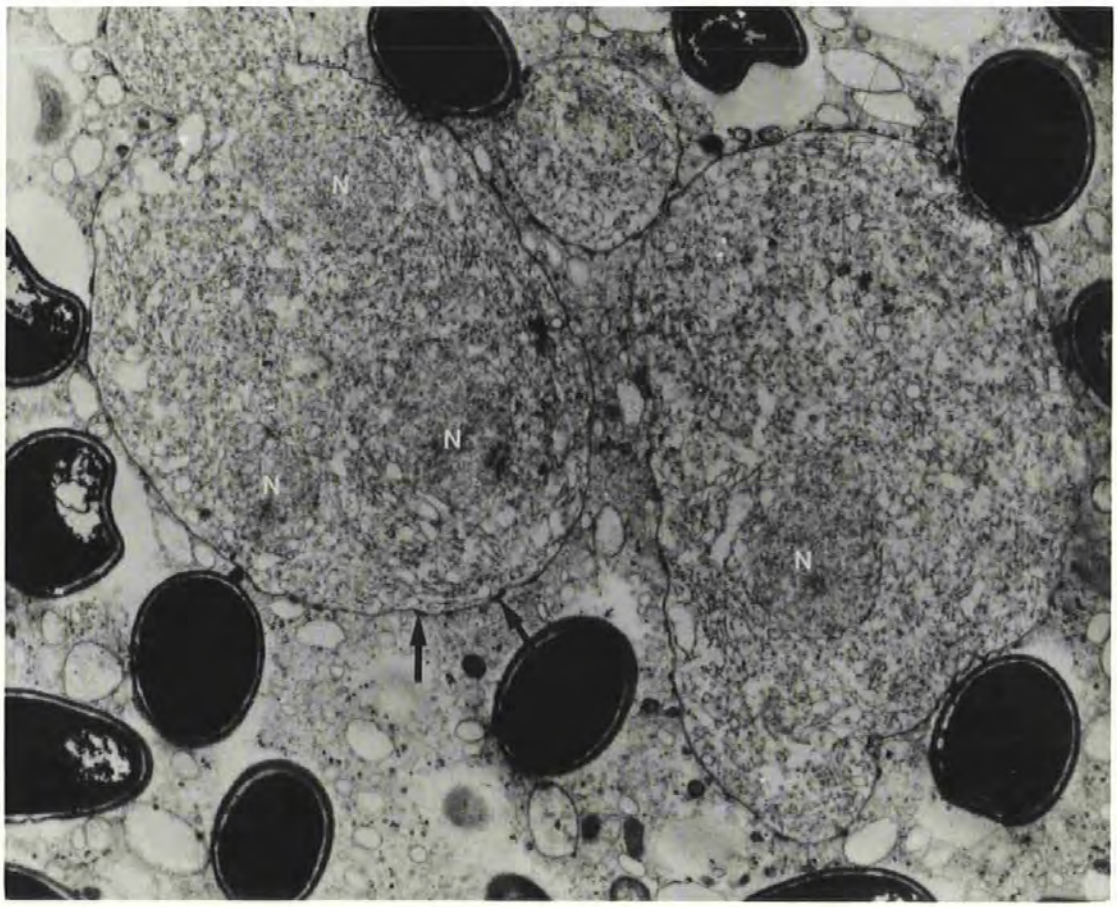

N
N
N
RER
N
N
N

Figs. 16 and 17. Stages of sporogony of *M. hepaticus*.

Fig. 16. Electron micrograph of early sporonts. x8,800. N, nucleus; arrow, extramembraneous thickenings.

Fig. 17. Electron micrograph of single sporont bud. x42,500. N, nucleus; V, vacuole; arrow, extramembraneous thickenings.

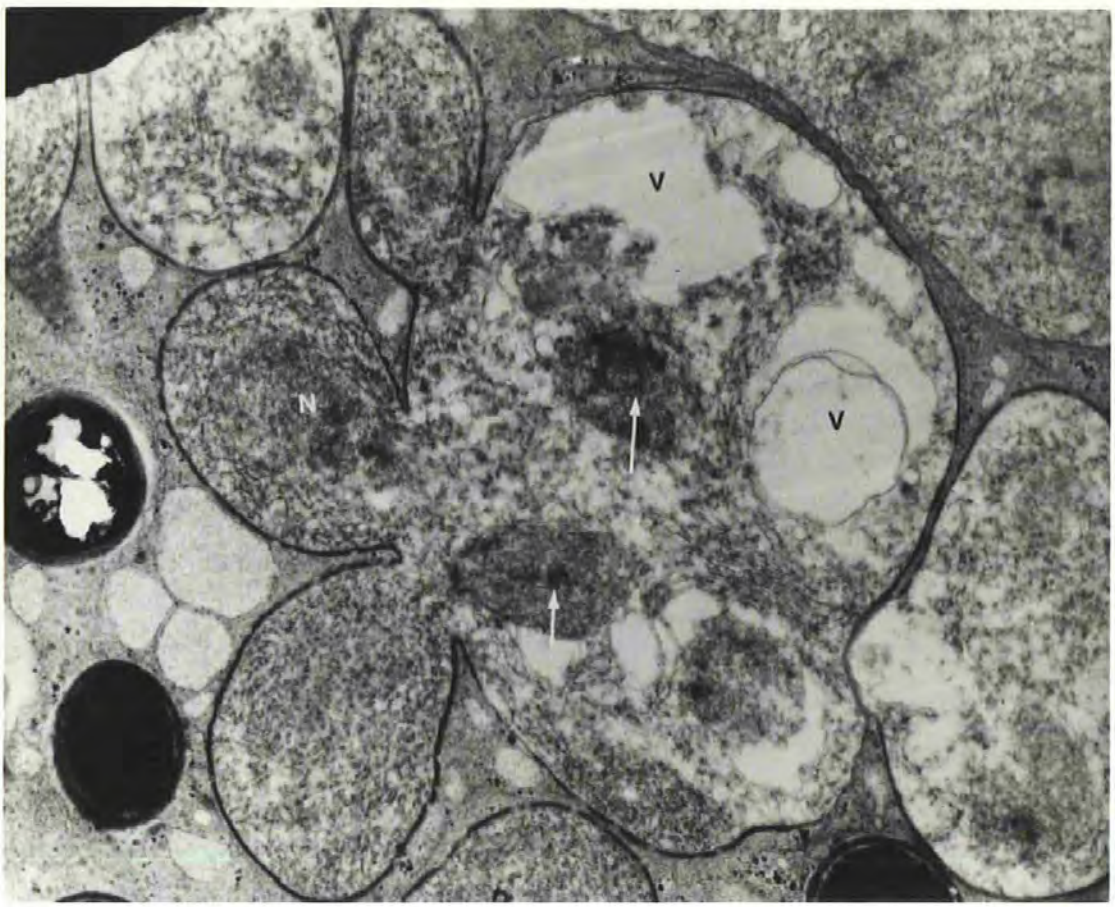

RER
N
V

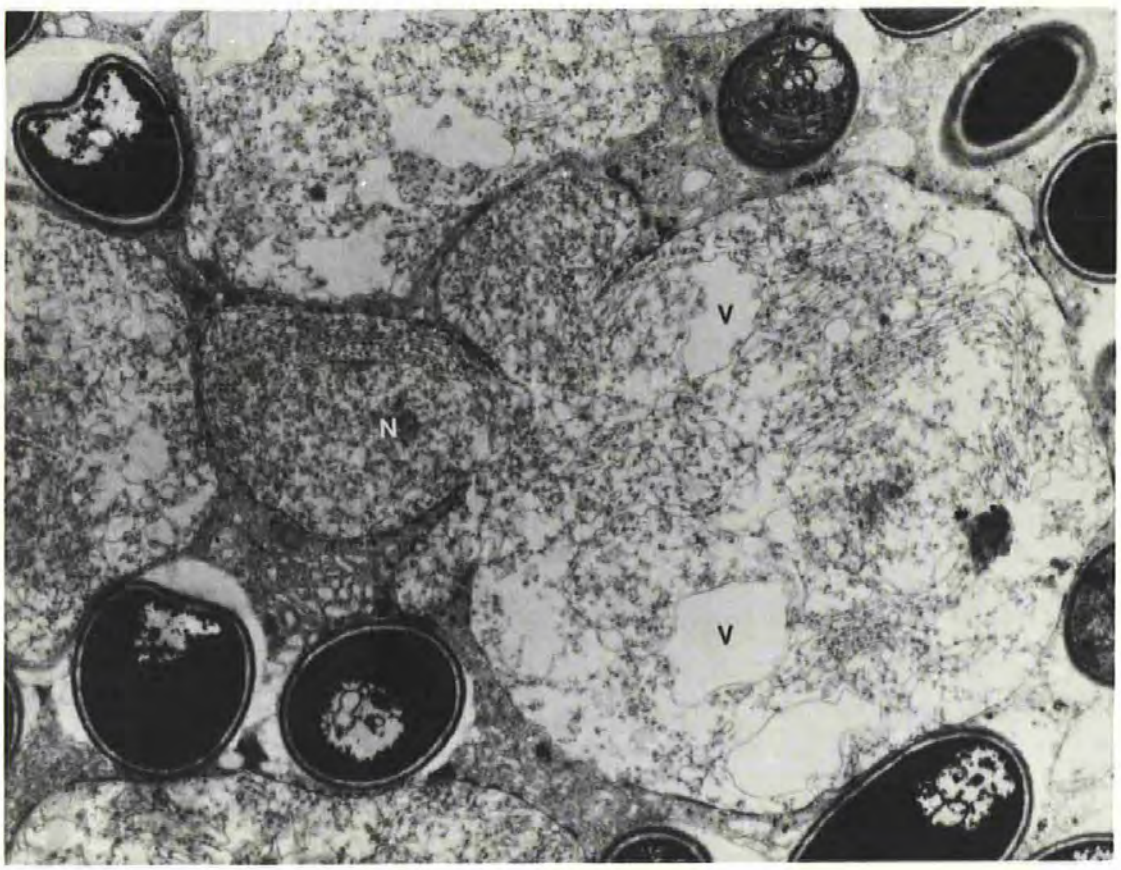

N
N
N
N

Figs. 18 and 19. Sporogonic divisions of *M. hepaticus*.

Fig. 18. Electron micrograph of sporont with 3 separate buds. x14,400. N, nucleus; V, vacuole; arrow, dividing nuclei.

Fig. 19. Electron micrograph of sporont with 3 connected buds. x15,500. N, nucleus; V, vacuole.

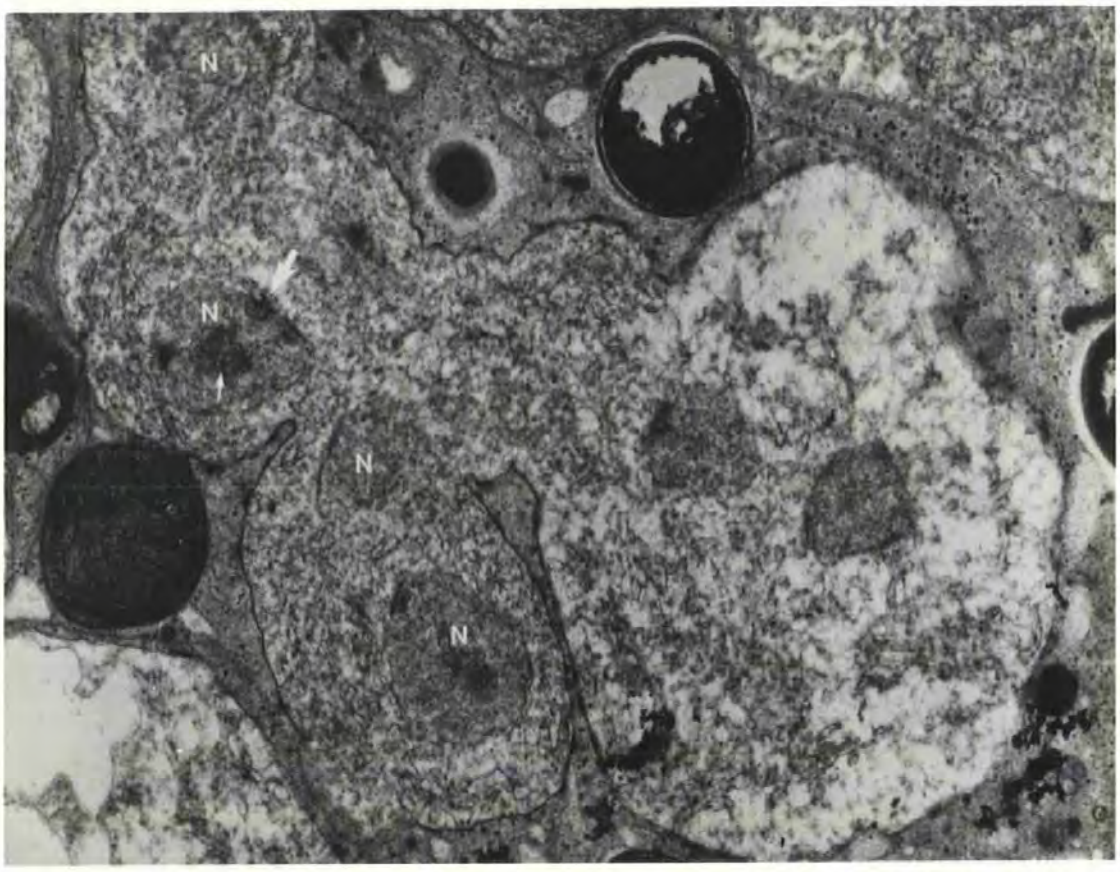

V
N
V

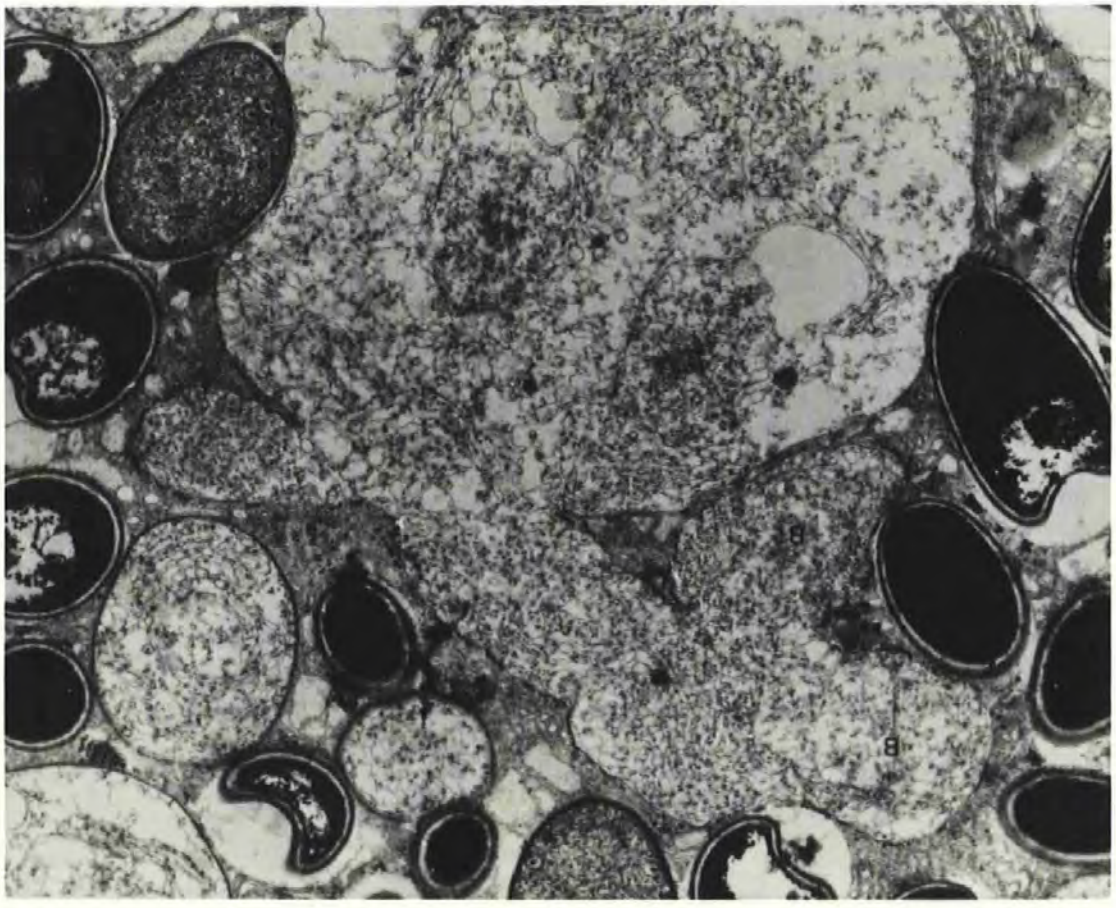

V
N
V

Figs. 20 and 21. Sporogonic division of *M. hepaticus*.

Fig. 20. Electron micrograph of sporont with buds on a cytoplasmic protrusion. x12,400. B, sporont bud.

Fig. 21. Electron micrograph of sporont with large bud containing 4 nuclei. x14,300. N, nucleus; broad arrow, spindle plaque; thin arrow, chromosome on spindle fibre.

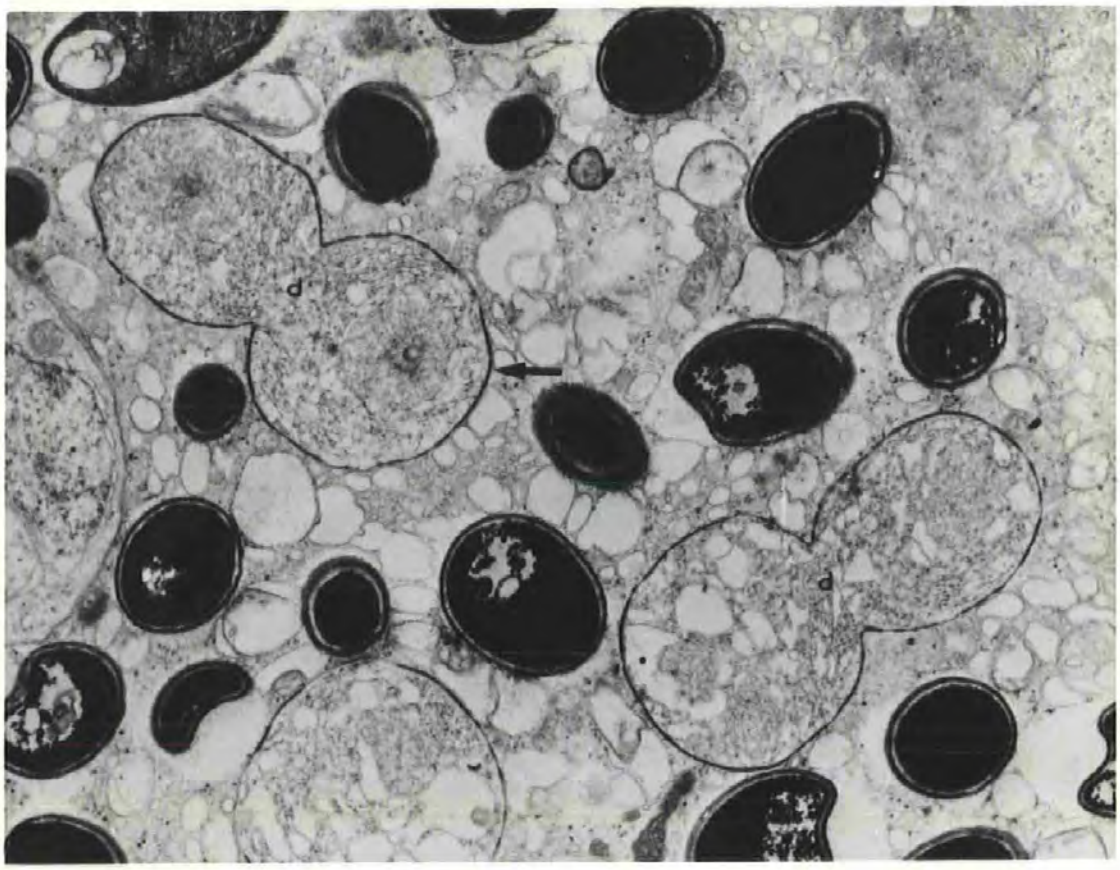

N
N
N
N

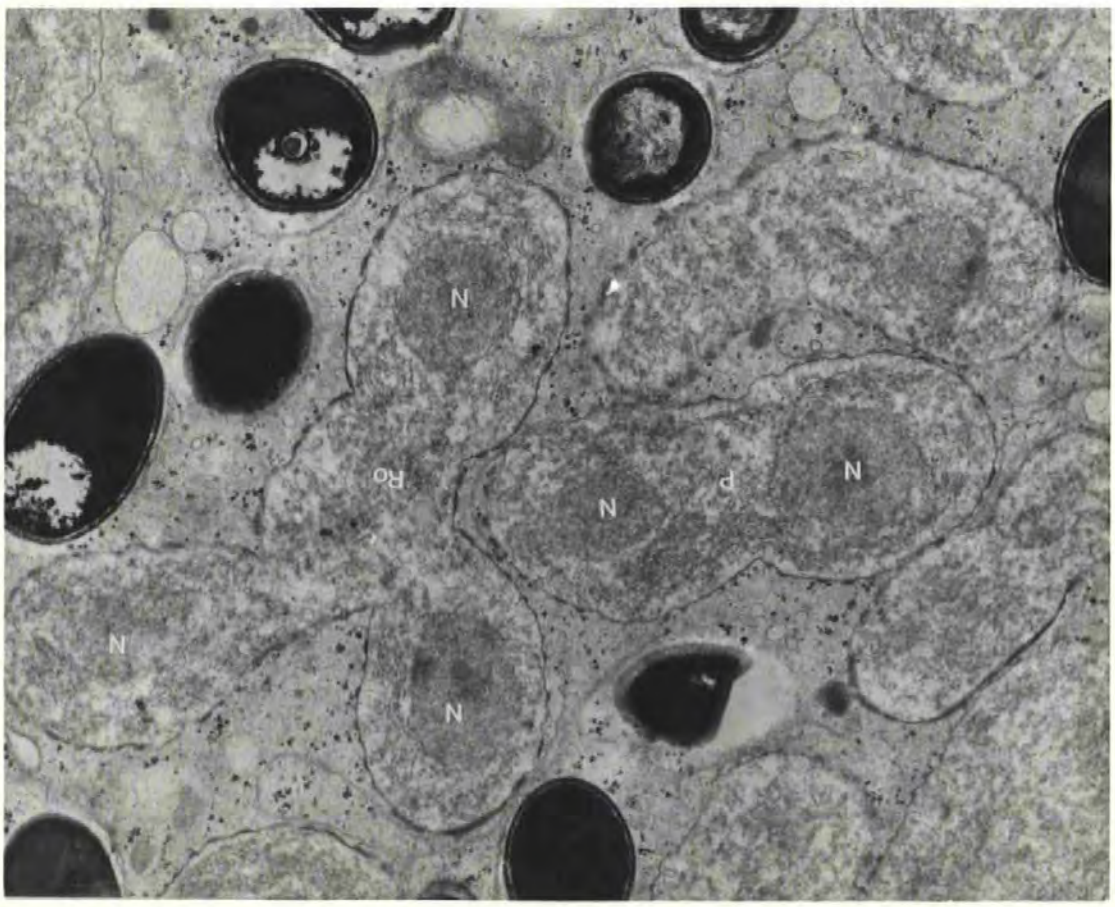

B
B

Figs. 22 and 23. Arrangements of sporoblasts resulting from the fragmentation of sporogonial plasmodia of *M. hepaticus*.

Fig. 22. Electron micrograph of rosette and paired arrangements of sporoblasts. x11,900. Ro, rosette of sporoblasts; P, paired sporoblasts.

Fig. 23. Electron micrograph of sporoblast pairs. x12,800. P, paired forms; arrow, fusing patches of extramembraneous material.

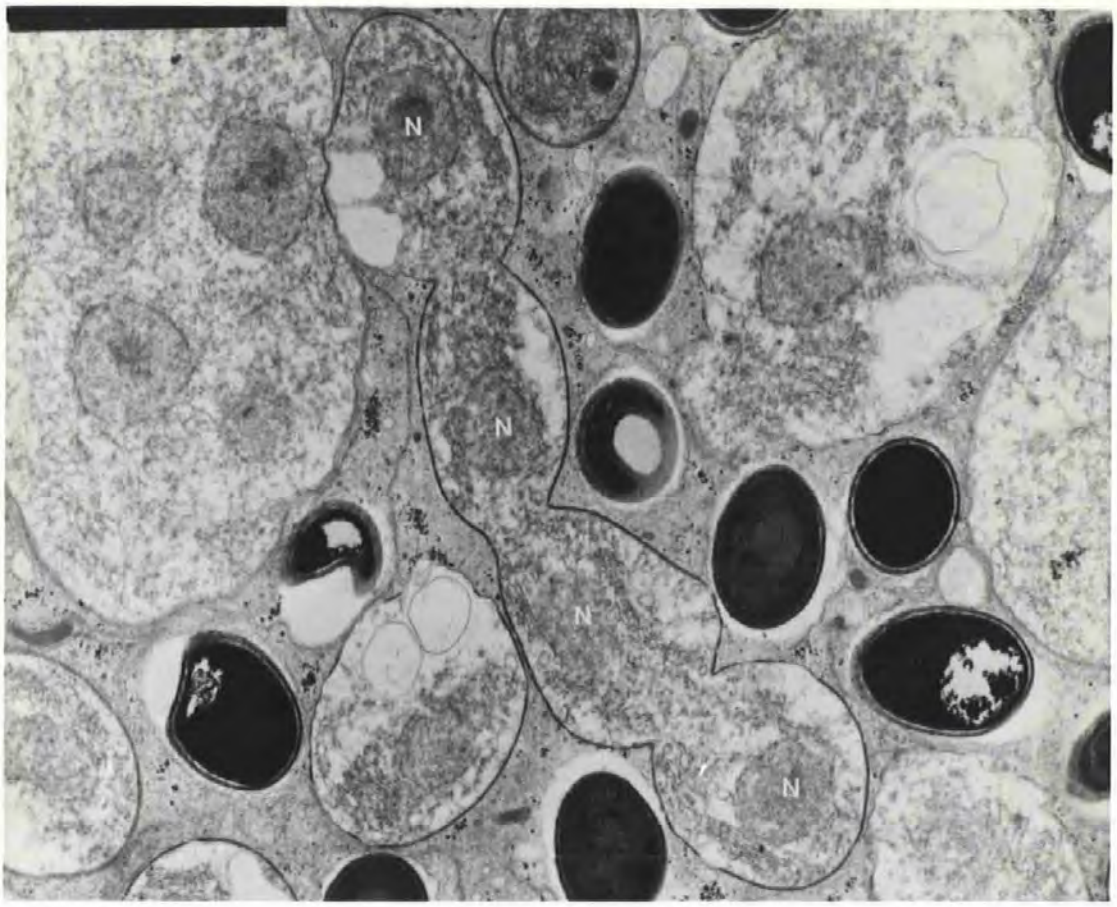

d
d

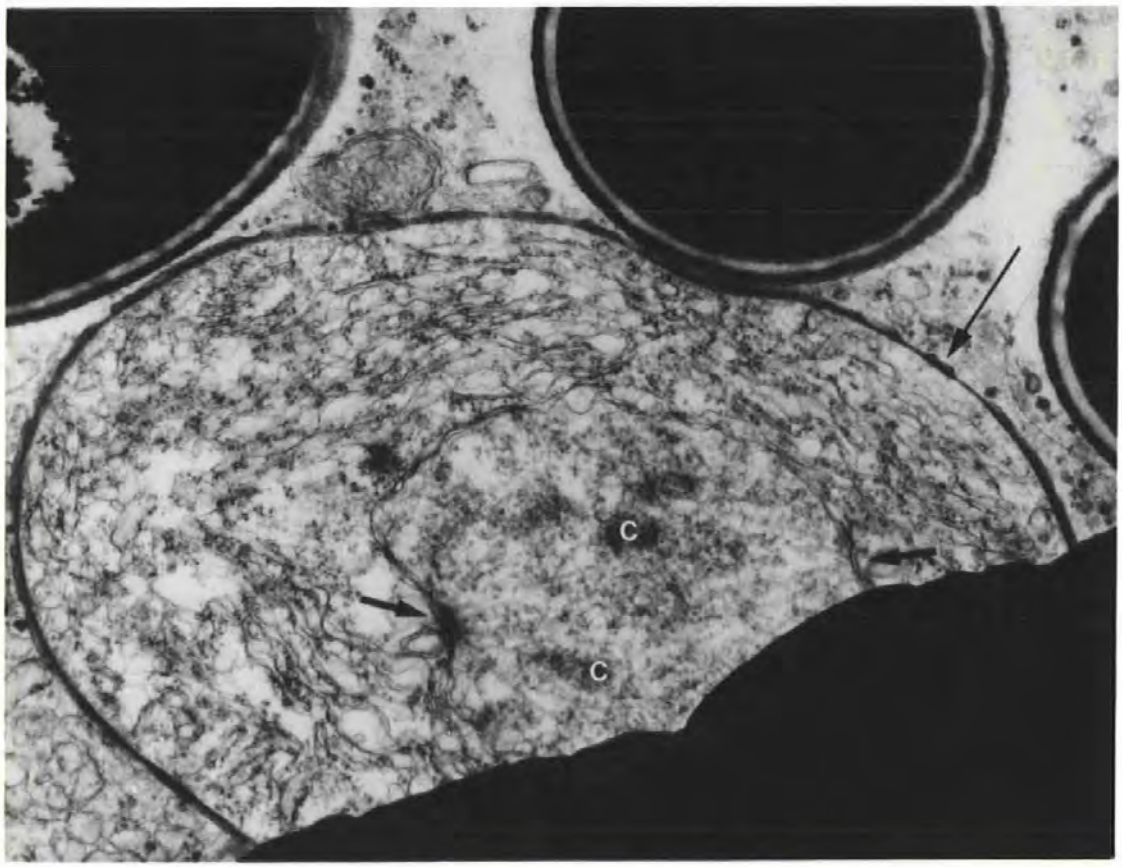

N
Ro
N
p
N
N
N

Figs. 24 and 25. Features and arrangements of sporoblasts of *M. hepaticus*.

Fig. 24. Electron micrograph of chain of sporoblasts. x11,400. N, nucleus.

Fig. 25. Electron micrograph of sporoblast with dividing nucleus. x32,200. C, chromosome; broad arrow, spindle plaque; thin arrow, extramembraneous thickening.

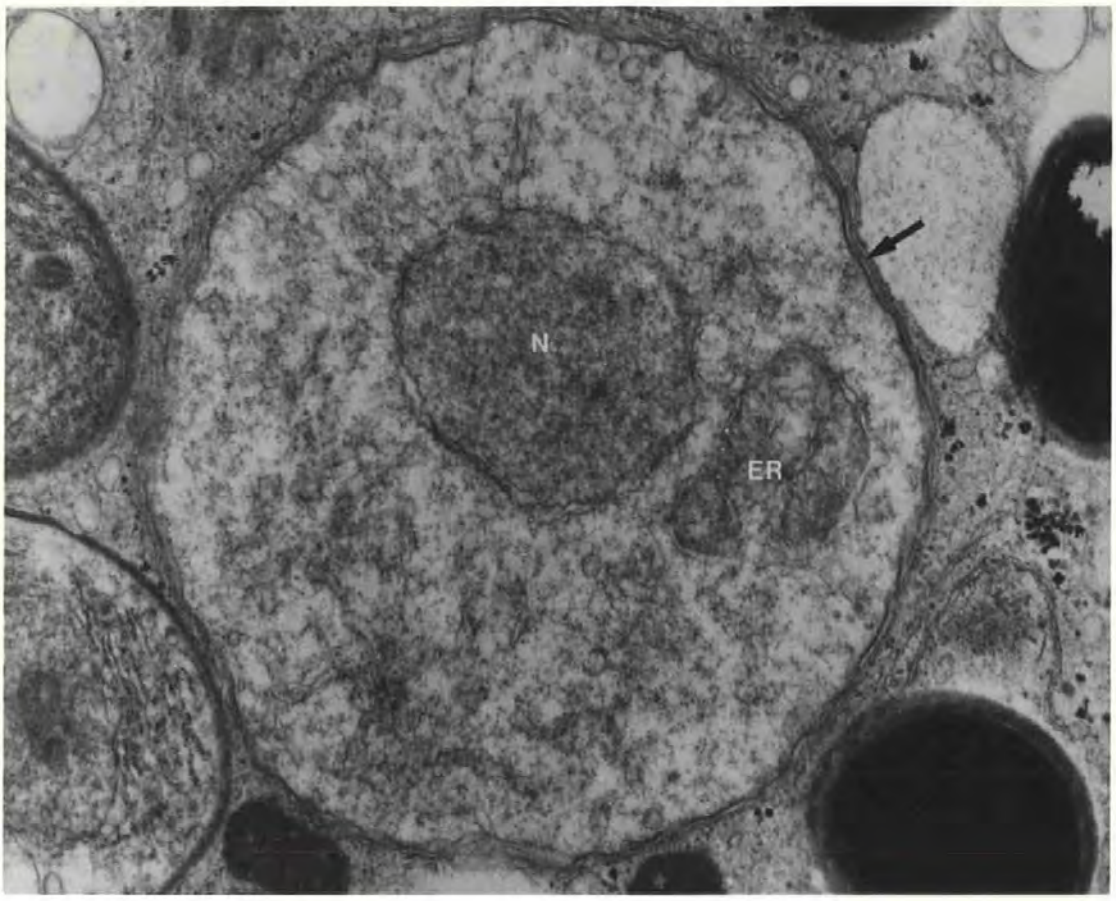

N
N
N
N

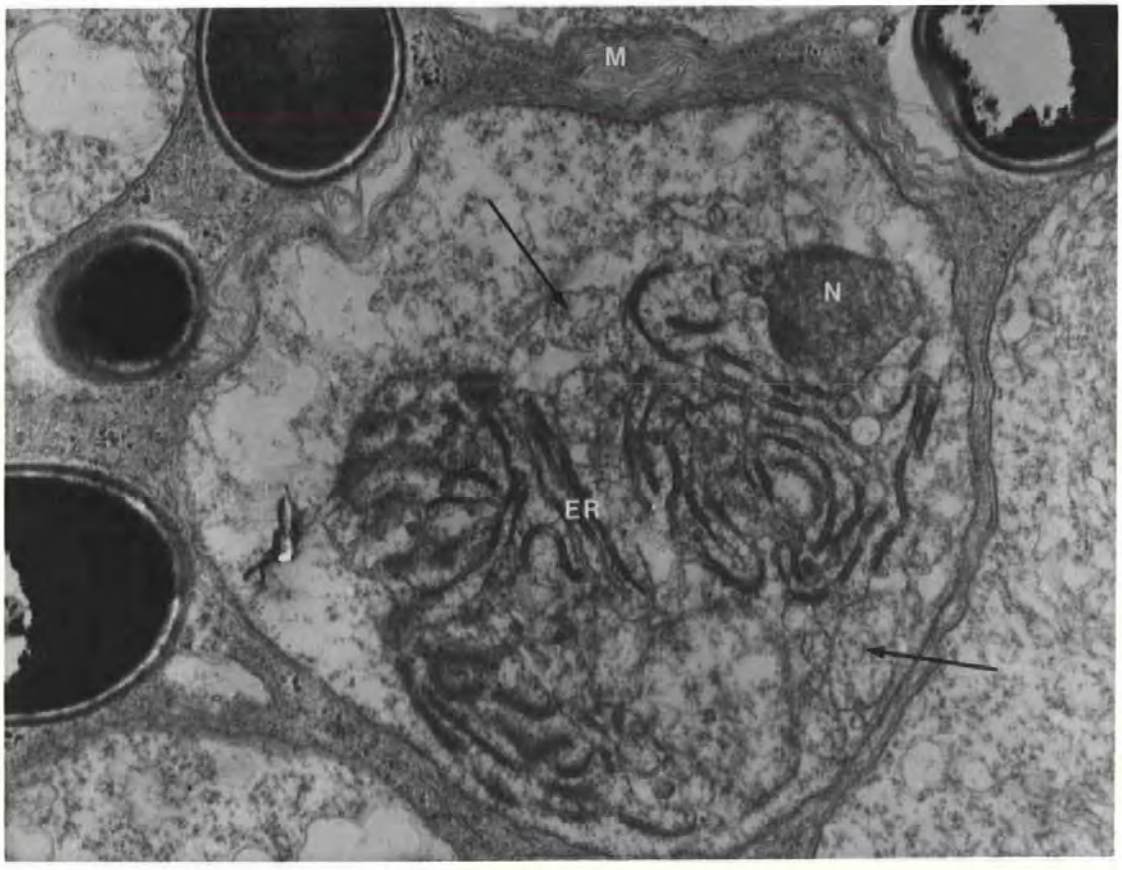

C
C

Figs. 26 and 27. Unusual developmental stages of *M. hepaticus*.

Fig. 26. Electron micrograph of unusual stage with enclosing membrane and expanded endoplasmic reticulum. x24,200. ER, expanded endoplasmic reticulum; arrow, enclosing membrane.

Fig. 27. Electron micrograph of unusual stage having greatly expanded endoplasmic reticulum. x21,100. M, enclosing membrane; arrows, intracellular membrane partitions.

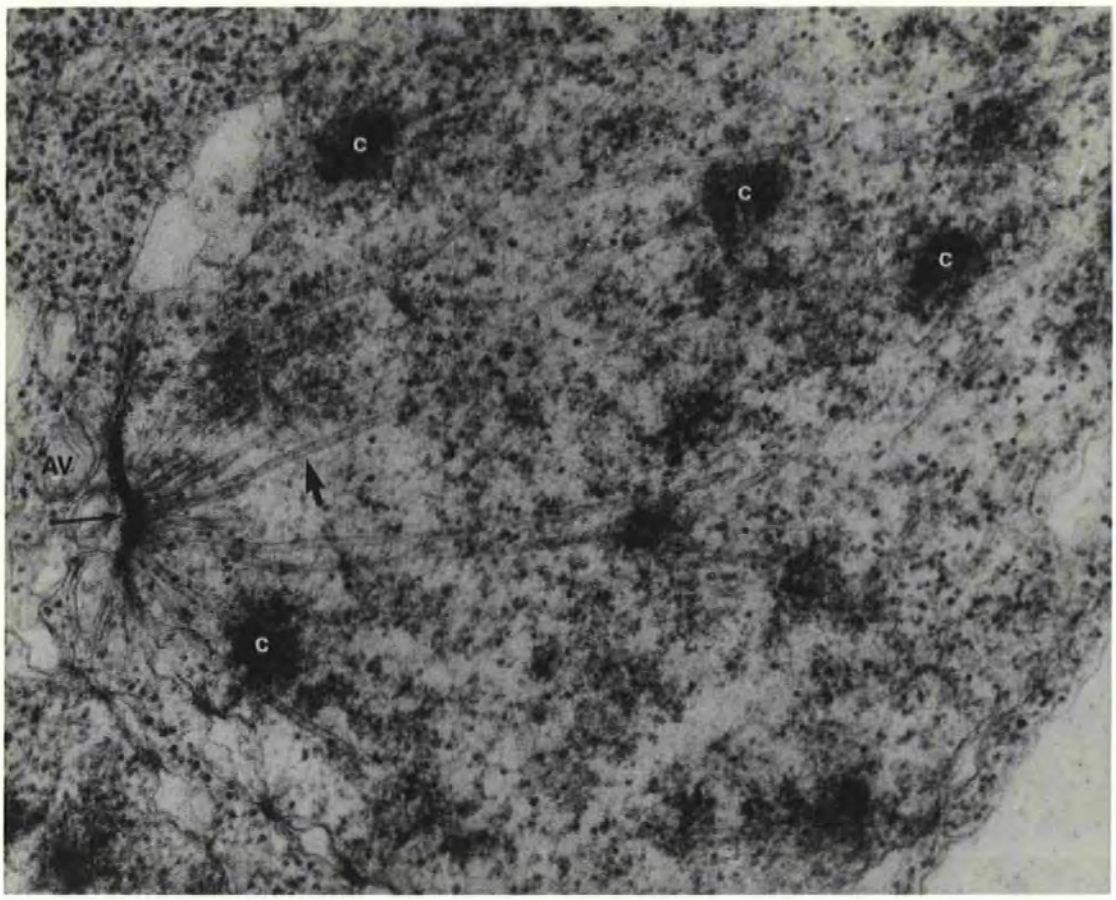

N
ER

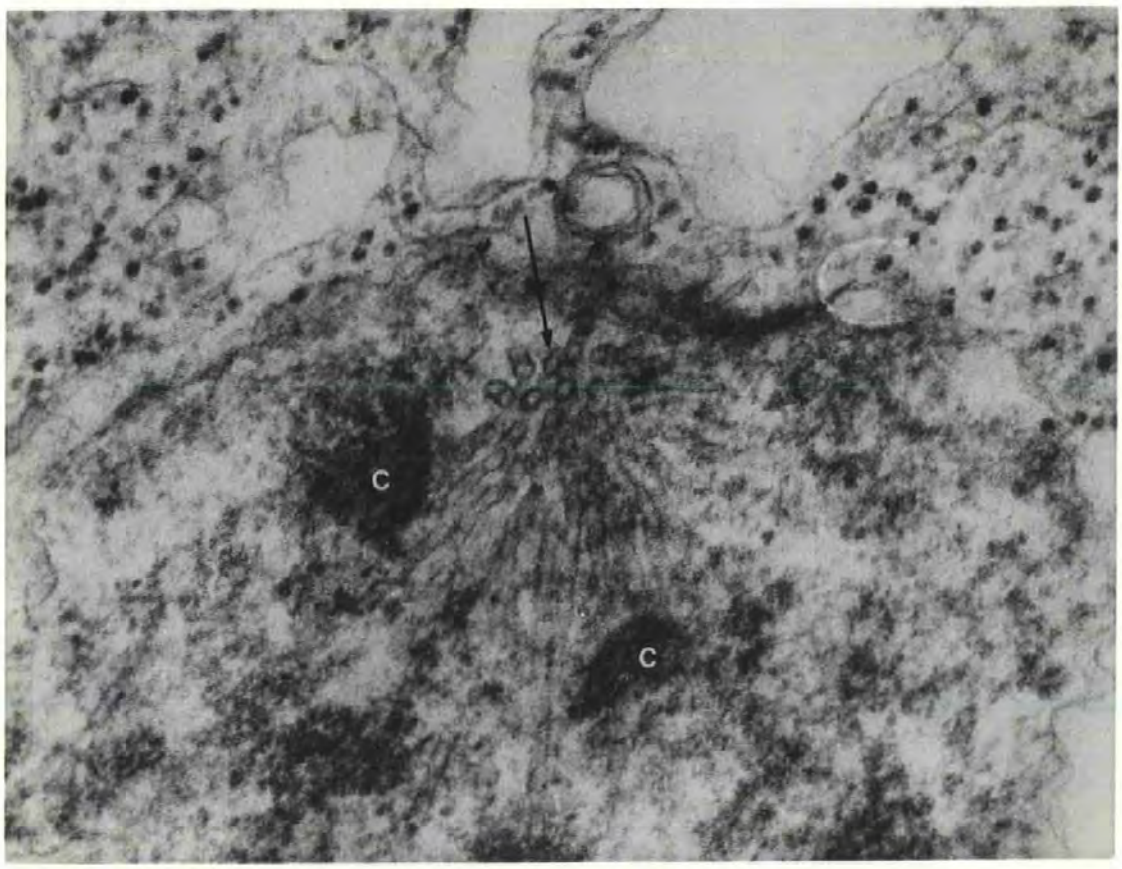

M
N
ER

Figs. 28 and 29. Features of nuclear division in *M. hepaticus*.

Fig. 28. Electron micrograph of longitudinal section of spindle plaque and spindle fibres. x64,000. AV, accessory (= polar) vesicles; C, chromosome; thin arrow, spindle plaque; broad arrow, spindle fibre.

Fig. 29. Electronmicrograph of transverse section of spindle fibres adjacent to spindle plaque. x117,000. C, chromosome; arrow, spindle fibre.

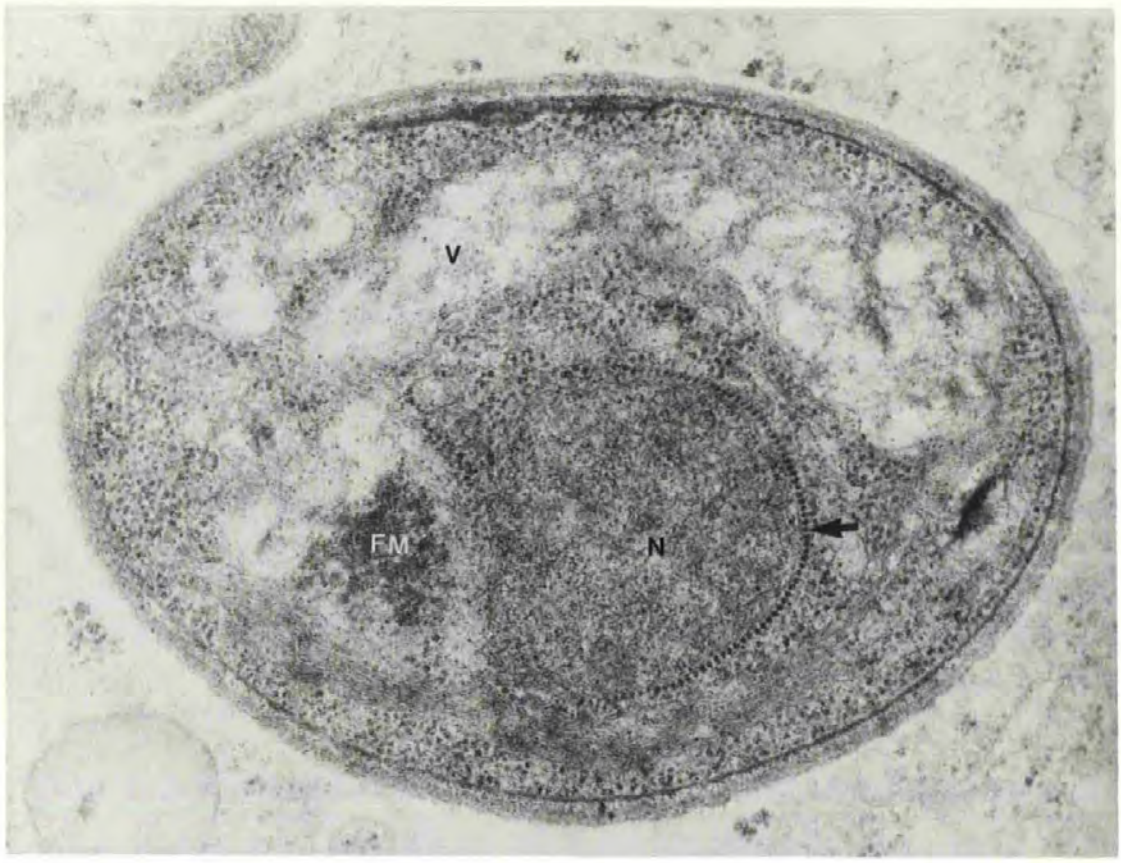

c
c
c
AV
c

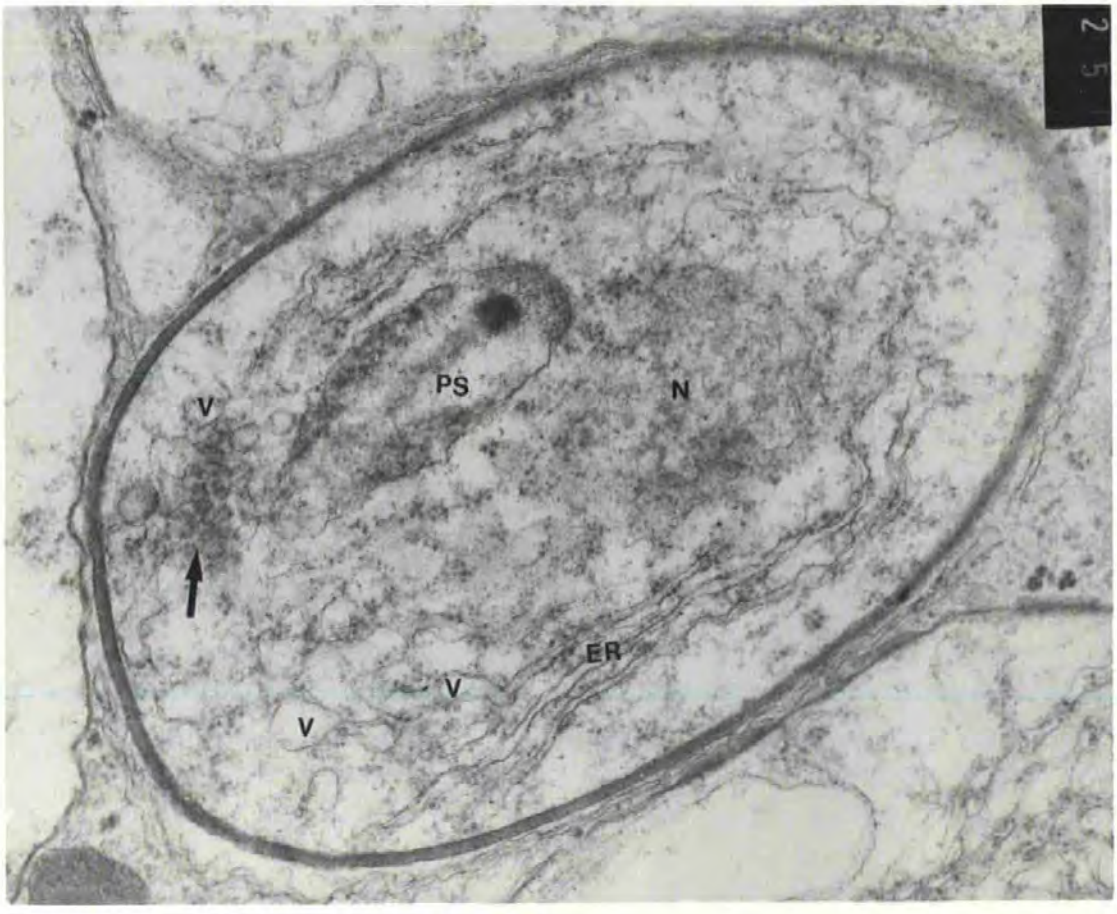

C
C

Figs. 30 and 31. Ultrastructure of sporoblasts and sporogenesis in *M. hepaticus*.

Fig. 30. Electron micrograph of oblique section of sporoblast. x55,700. FM, floccular material; N, nucleus; V, vacuole; arrow, blebs on nuclear membrane.

Fig. 31. Electron micrograph of early stage of sporogenesis : sporoblast with polar sac. x52,400. ER, endoplasmic reticulum; N, nucleus; PS, polar sac; V, electron-lucent vesicles; arrow, electron-dense vesicles.



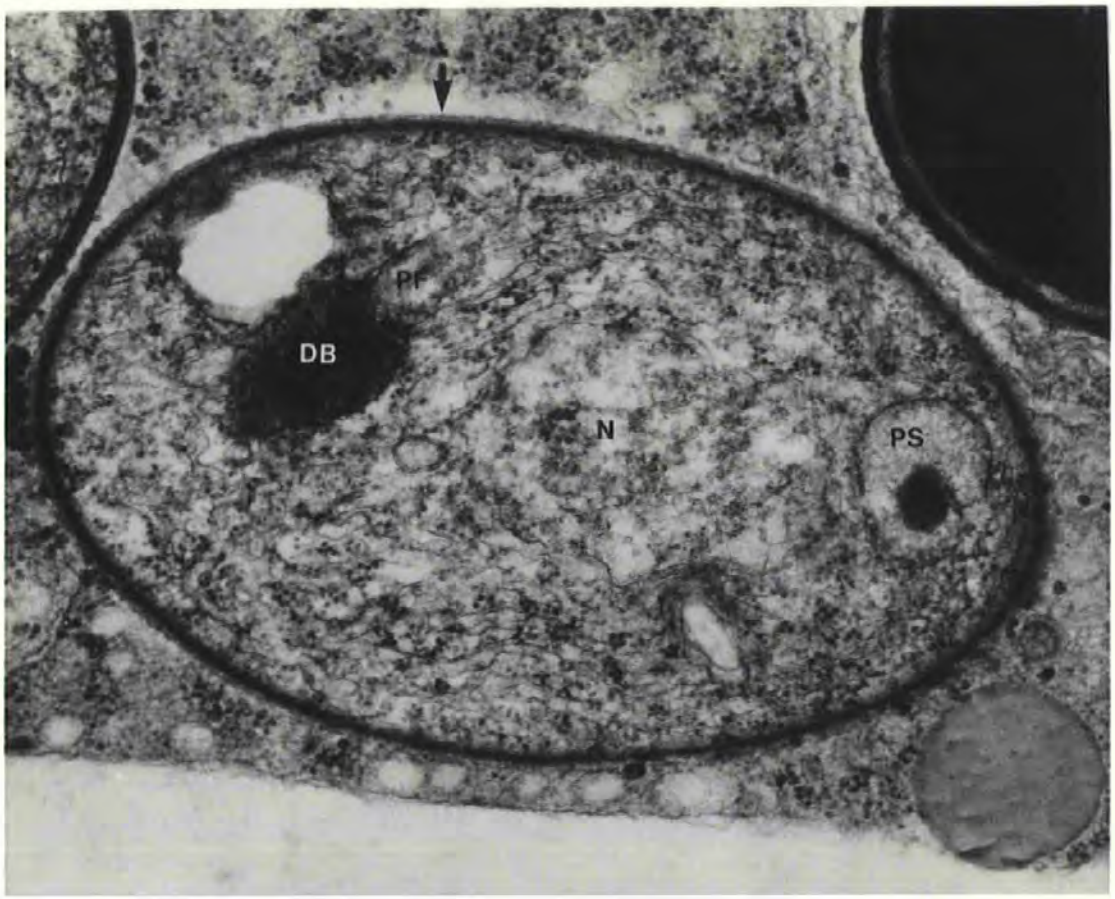


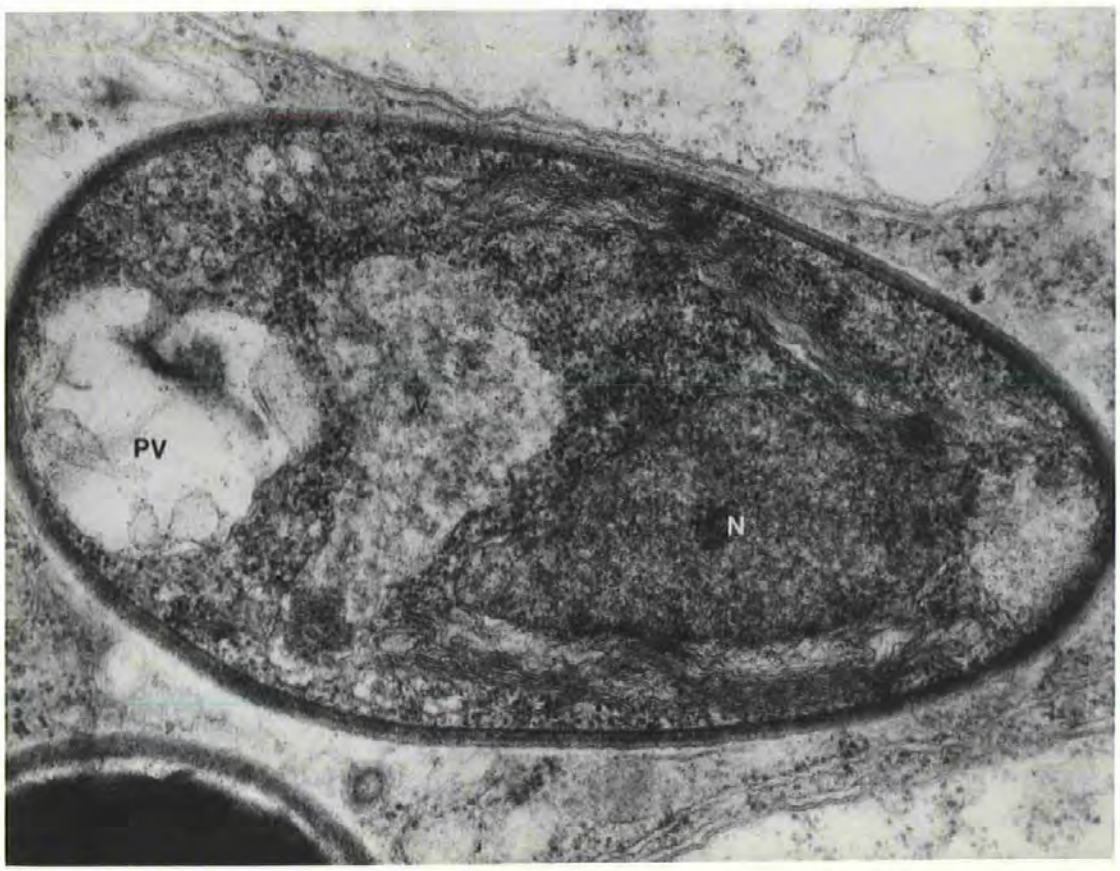
25

Figs. 32 and 33. Sporogenesis in *M. hepaticus* : development of the manubrium and perinuclear membrane system.

Fig. 32. Electron micrograph of sporoblast with elongating manubrium. x50,000. DB, dense body; N, nucleus; PF, manubrium of polar filament; PS, polar sac.

Fig. 33. Electron micrograph of sporoblast with perinuclear membrane system. x54,100. N, nucleus; PV, posterior vacuole; V, vacuole.

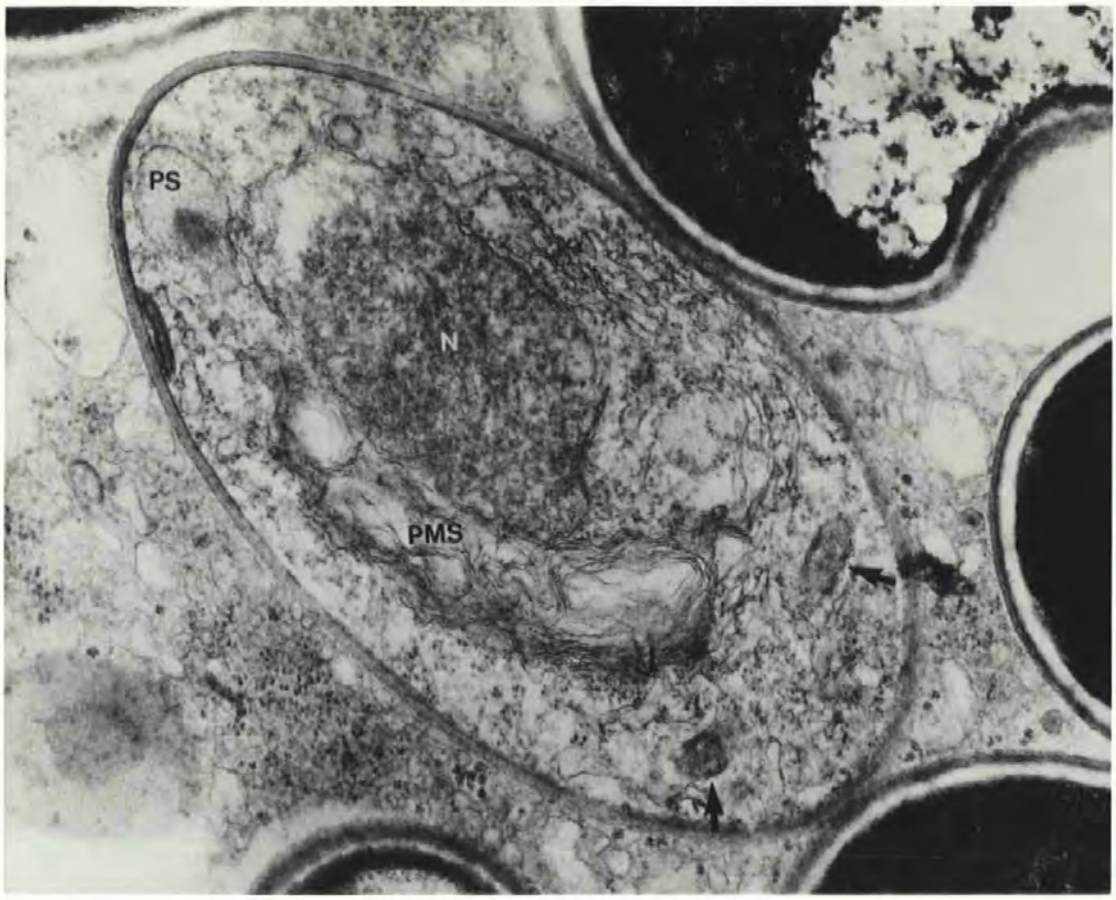

PF
DB
N
PS

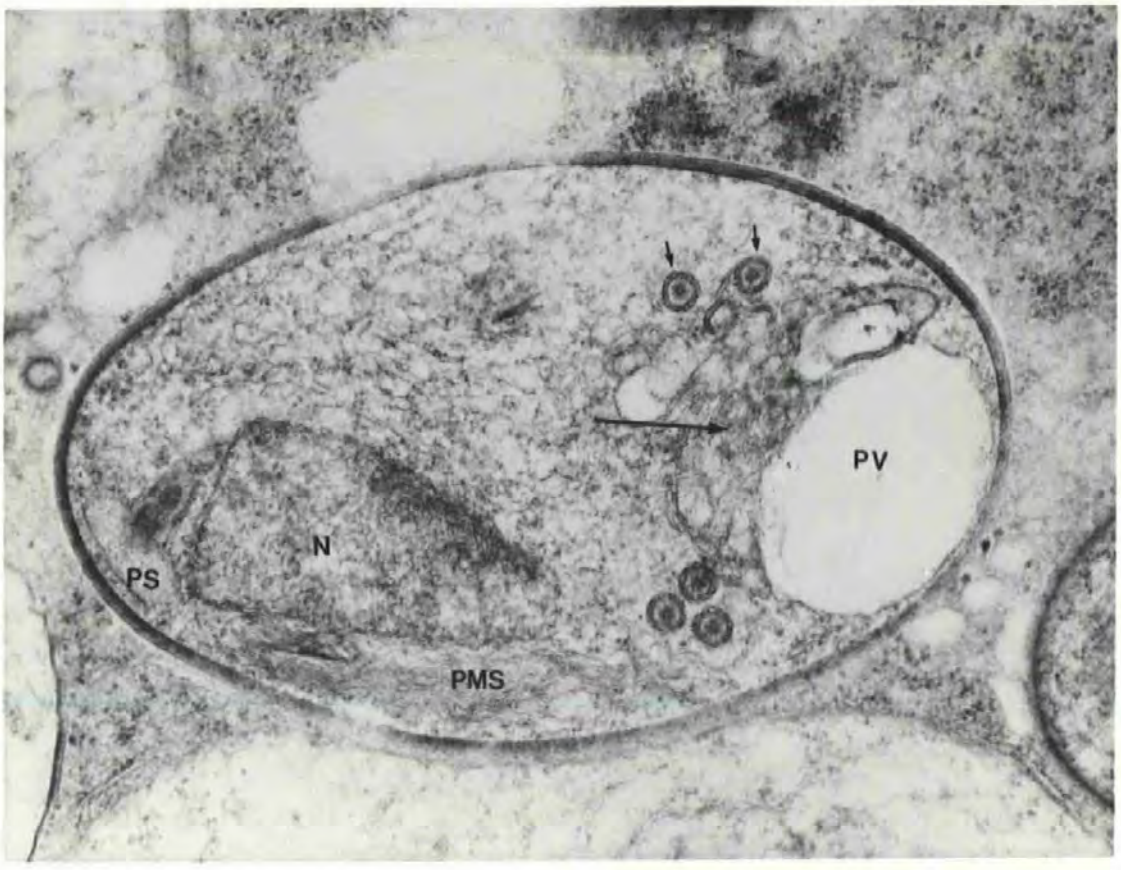

PV
N

Figs. 34 and 35. Sporogenesis in M. hepaticus : development of the coiled part of the polar filament.

Fig. 34. Electron micrograph of sporoblast showing the formation of the first coil of polar filament. x42,500. N, nucleus; PMS, perinuclear membrane system; PS, polar sac; arrow, first coil of polar filament.

Fig. 35. Electron micrograph of sporoblast showing the reticular filament forming system and coils of polar filament. x36,300. N, nucleus; PMS, perinuclear membrane system; PS, polar sac; large arrow, filament forming system; small arrows, coils of polar filament.

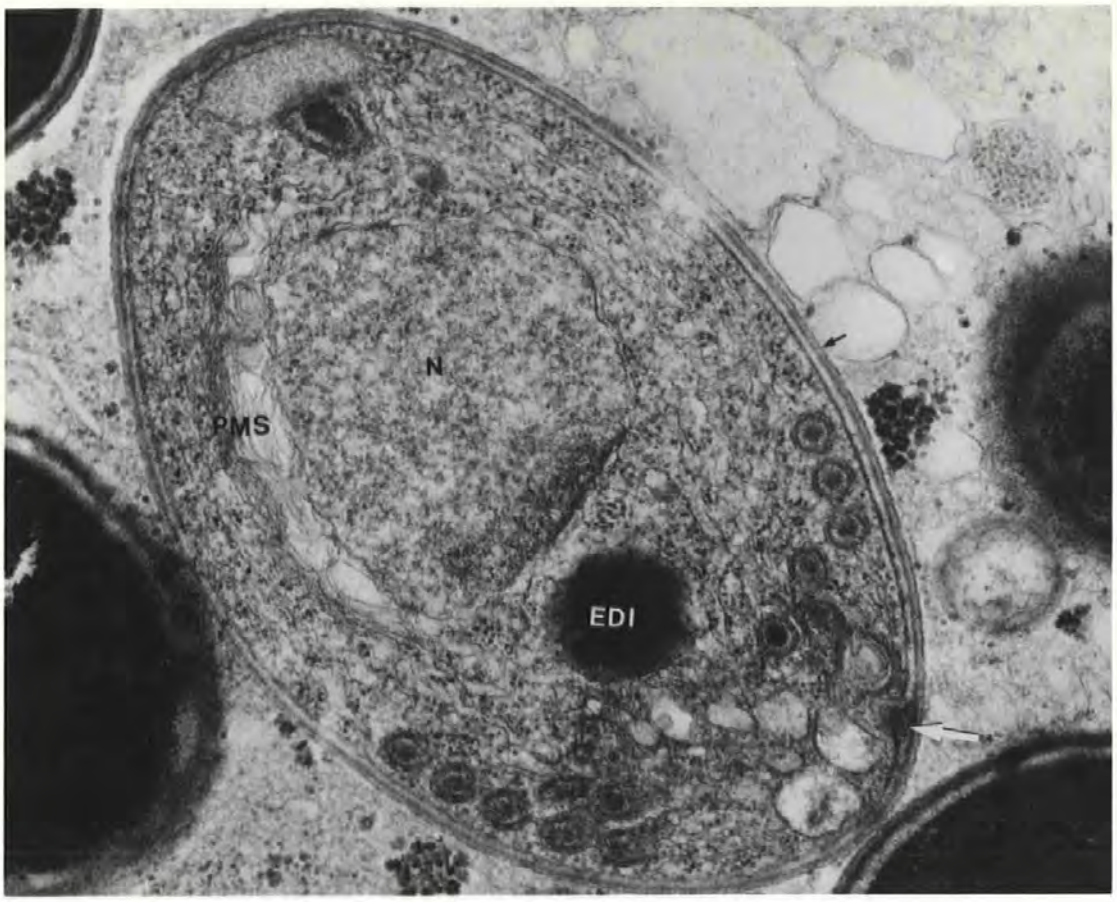

PS
N
PMS

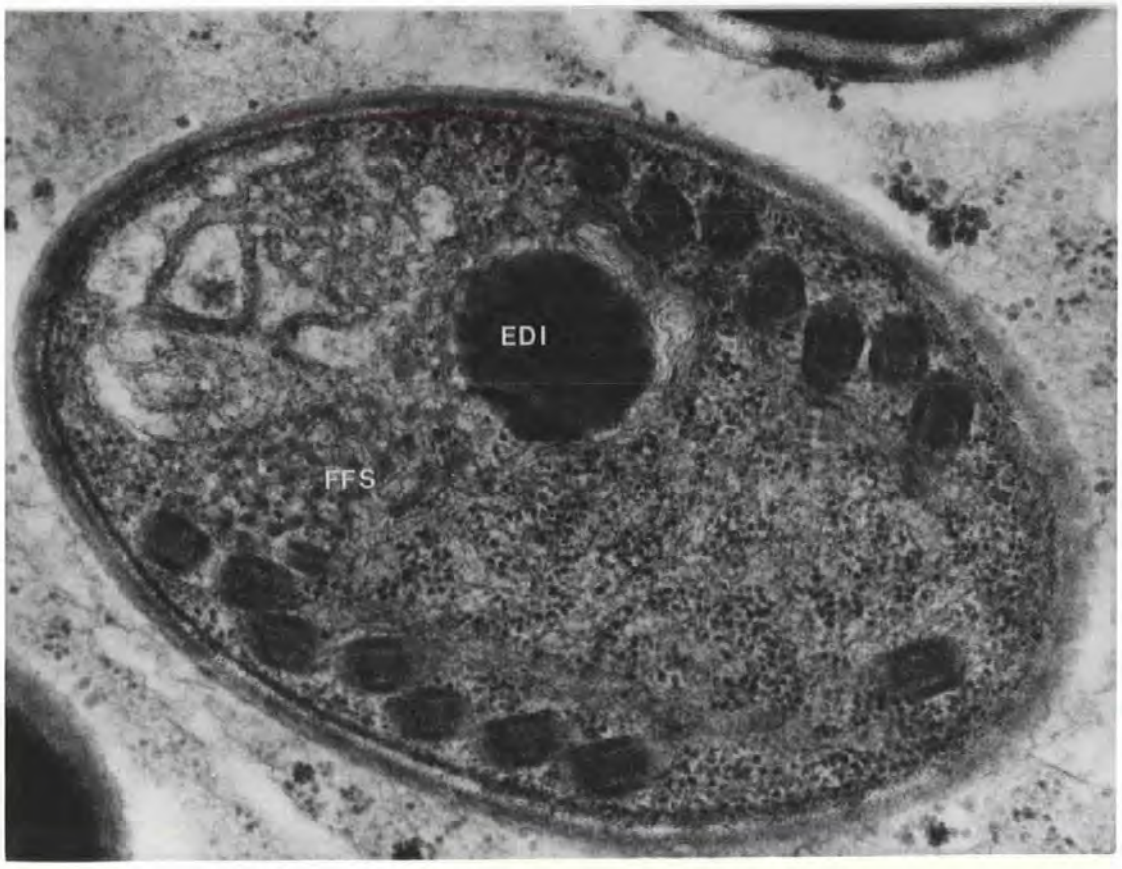

PV
N
PS
PMS

Figs. 36 and 37. Sporogenesis in *M. hepaticus* : features of sporoblast synthetic systems.

Fig. 36. Electronmicrograph of sporoblast showing connection between developing endospore and cytoplasm. x44,900. EDI, electron-dense inclusion; N, nucleus; PMS, perinuclear membrane system; arrow, tube connecting endospore with cytoplasm.

Fig. 37. Electron micrograph of sporoblast showing large, reticular filament forming system. x60,700. EDI, electron dense inclusion; FFS, reticular filament forming system.

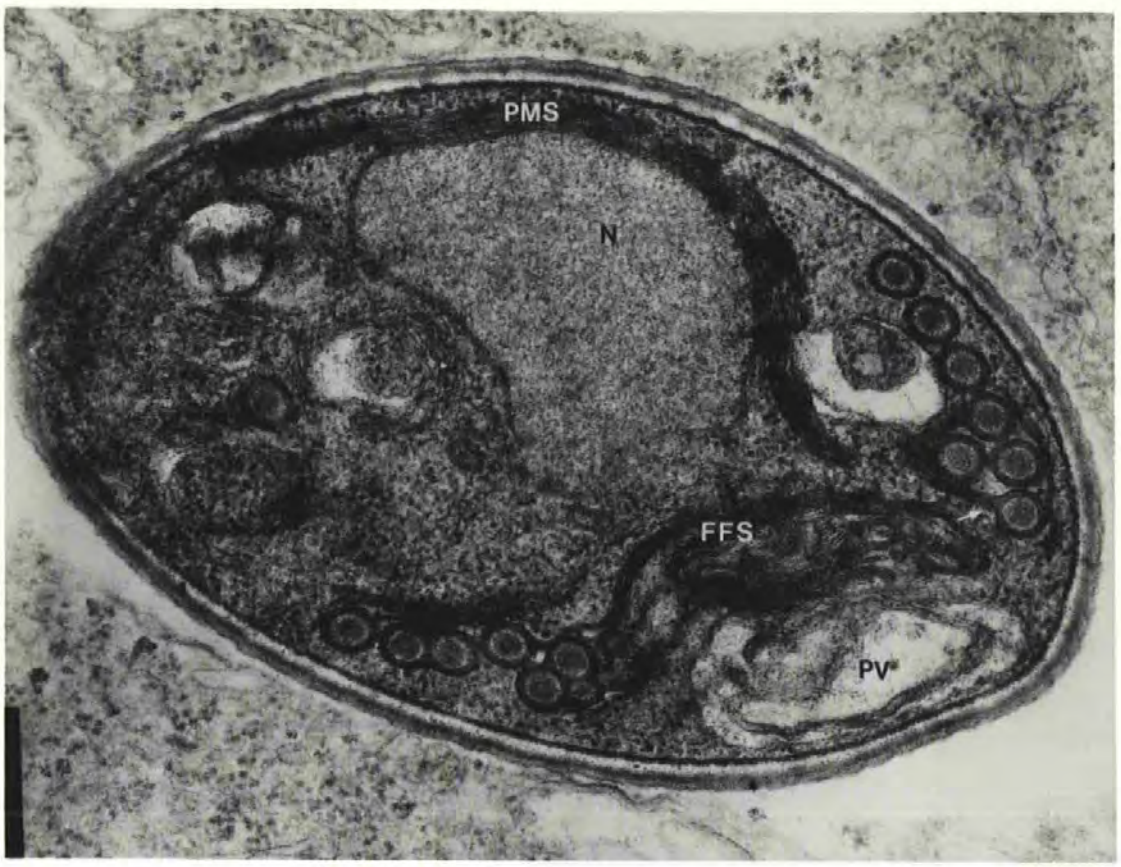

N
PMS
EDI

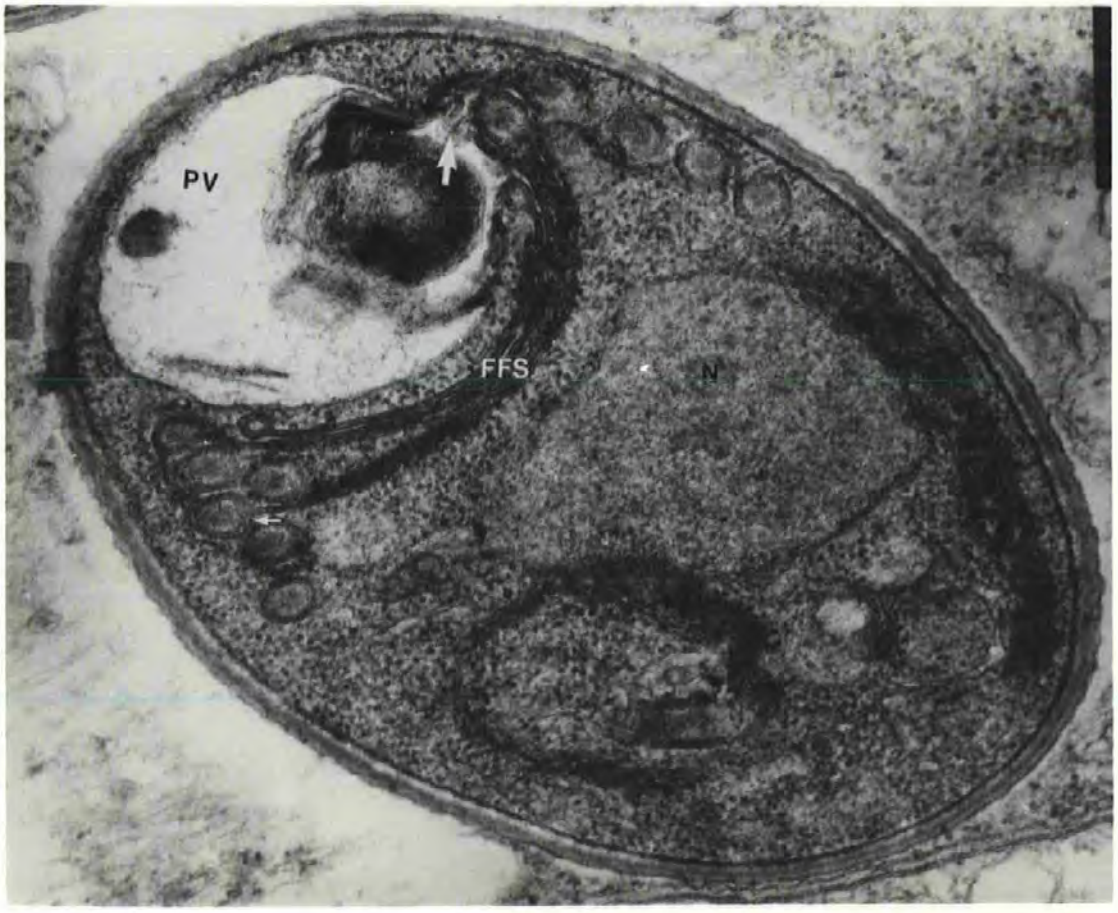

EDI
FFS

Figs. 38 and 39. Sporogenesis in M. hepaticus : features of filament forming systems.

Fig. 38. Electron micrograph of sporoblast showing the relationship between the prinuclear membrane system and the filament forming system. x60,000. FFS, filament forming system; N, nucleus; PMS, perinuclear membrane system; PV, posterior vacuole.

Fig. 39. Electron micrograph of sporoblast with lamellar arrangement of filament forming system. x59,200. FFS, filament forming system; PV, posterior vacuole; arrow, intrusion of polar filament into posterior vacuole.

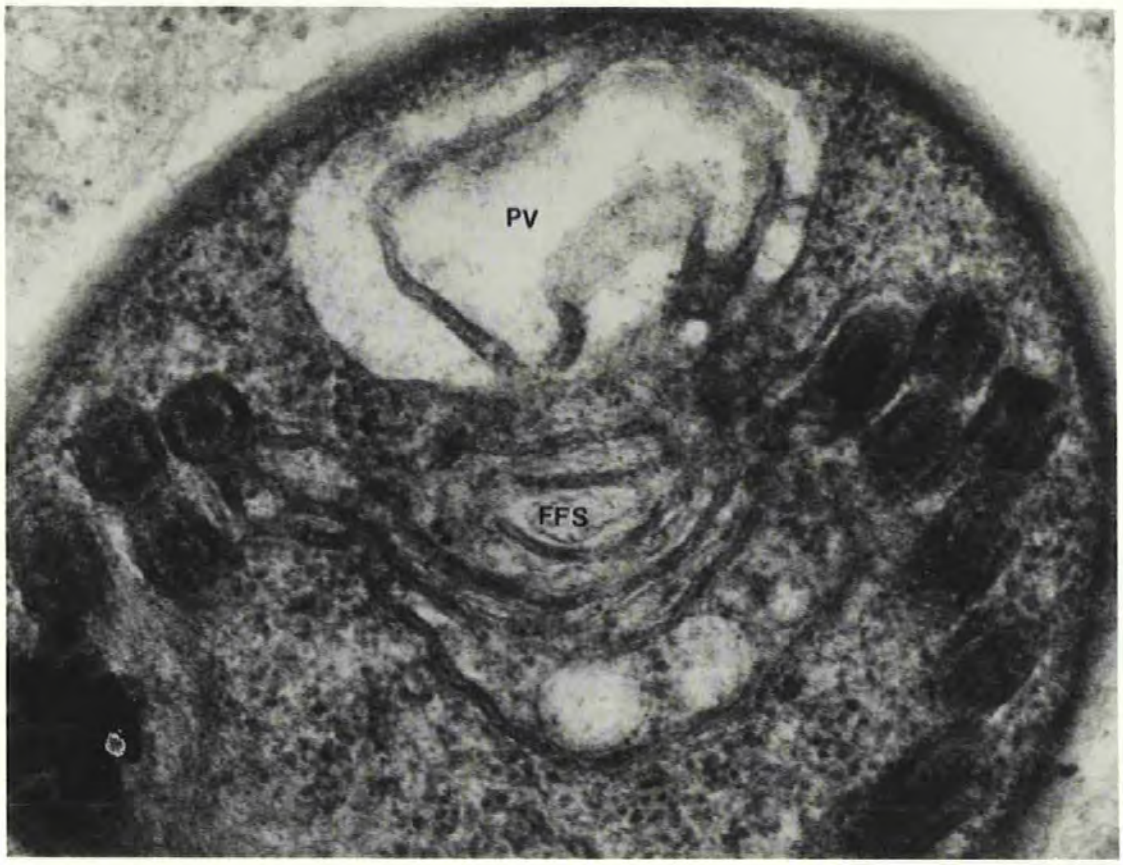

PMS
N
FFS
PV

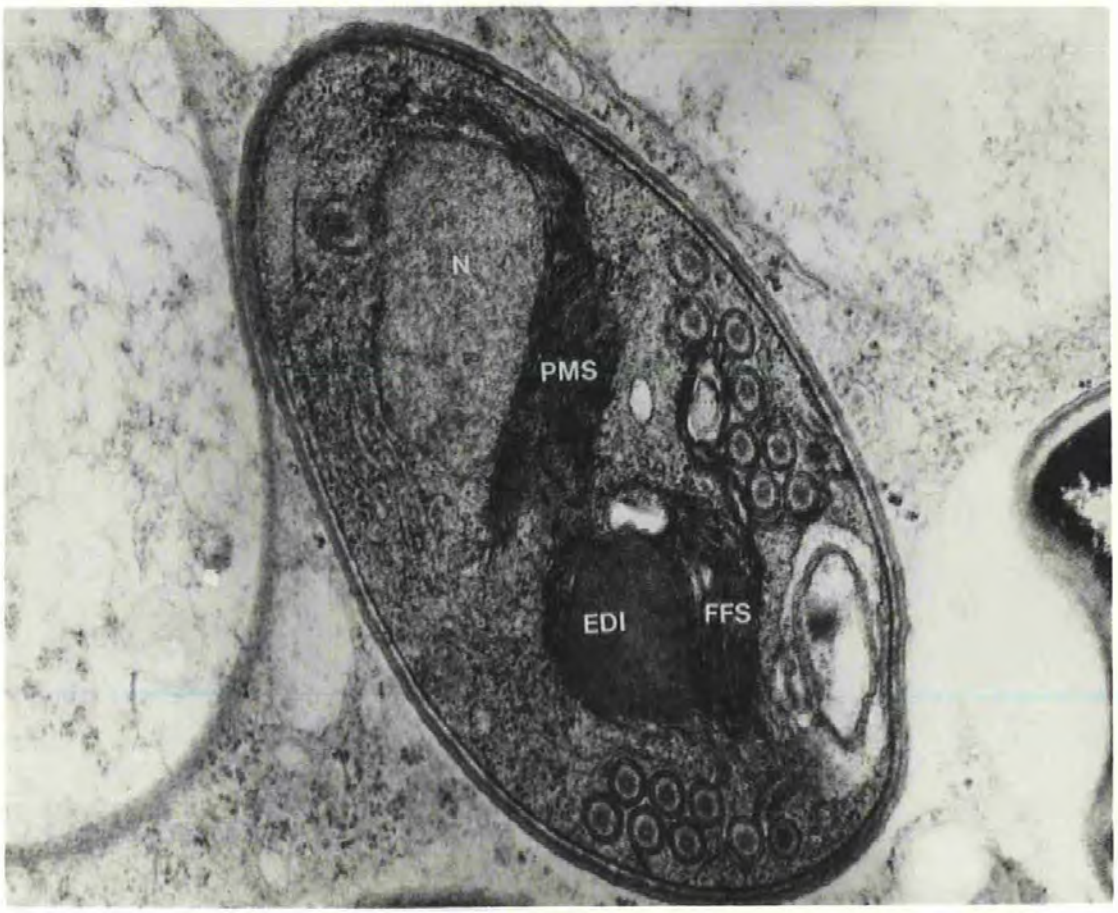

PV
FFS
N

Figs. 40 and 41. Sporogenesis in M. hepaticus : features of filament synthetic systems.

Fig. 40. Electron micrograph of sporoblasts having almost lamellar filament forming system intruding into posterior vacuole. x103,100. FFS, filament forming system; PV, posterior vacuole.

Fig. 41. Electron micrograph of sporoblast showing highly developed perinuclear membrane system and filament forming system. x56,000. EDI, electron dense inclusion; FFS, filament forming system; N, nucleus; PMS, perinuclear membrane system.

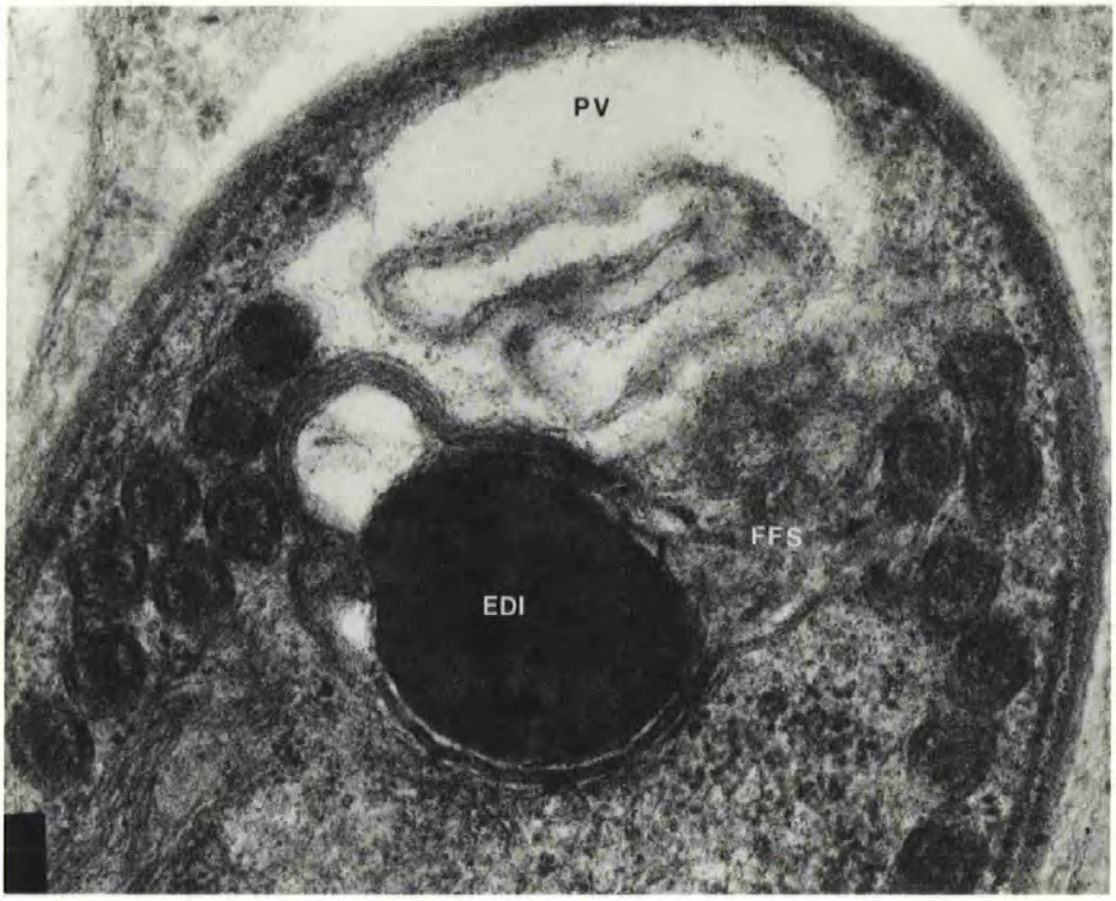

PV
FFS

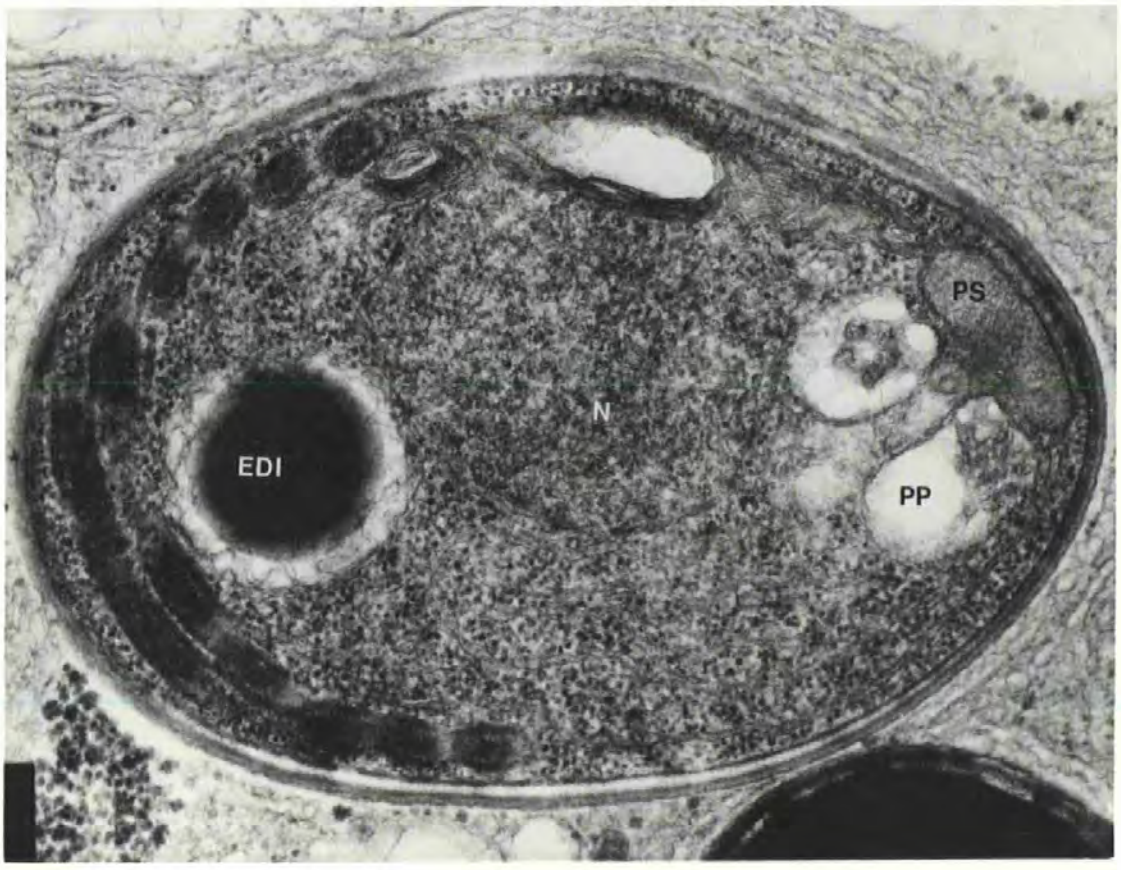

N
PMS
EDI
FFS

Figs. 42 and 43. Sporogenesis in M. hepaticus : features of filament forming systems and the onset of development of the polaroplast.

Fig. 42. Electron micrograph of sporoblast showing the associaton of the filament forming system and posterior vacuole. x91,900. EDI, electron dense inclusion; FFS, filament forming system; PV, posterior vacuole.

Fig. 43. Electron micrograph of sporoblast showing the initial stages of polaroplast development. x56,000. EDI, electron-dense inclusion; N, nucleus; PP, polaroplast; PS, polar sac.

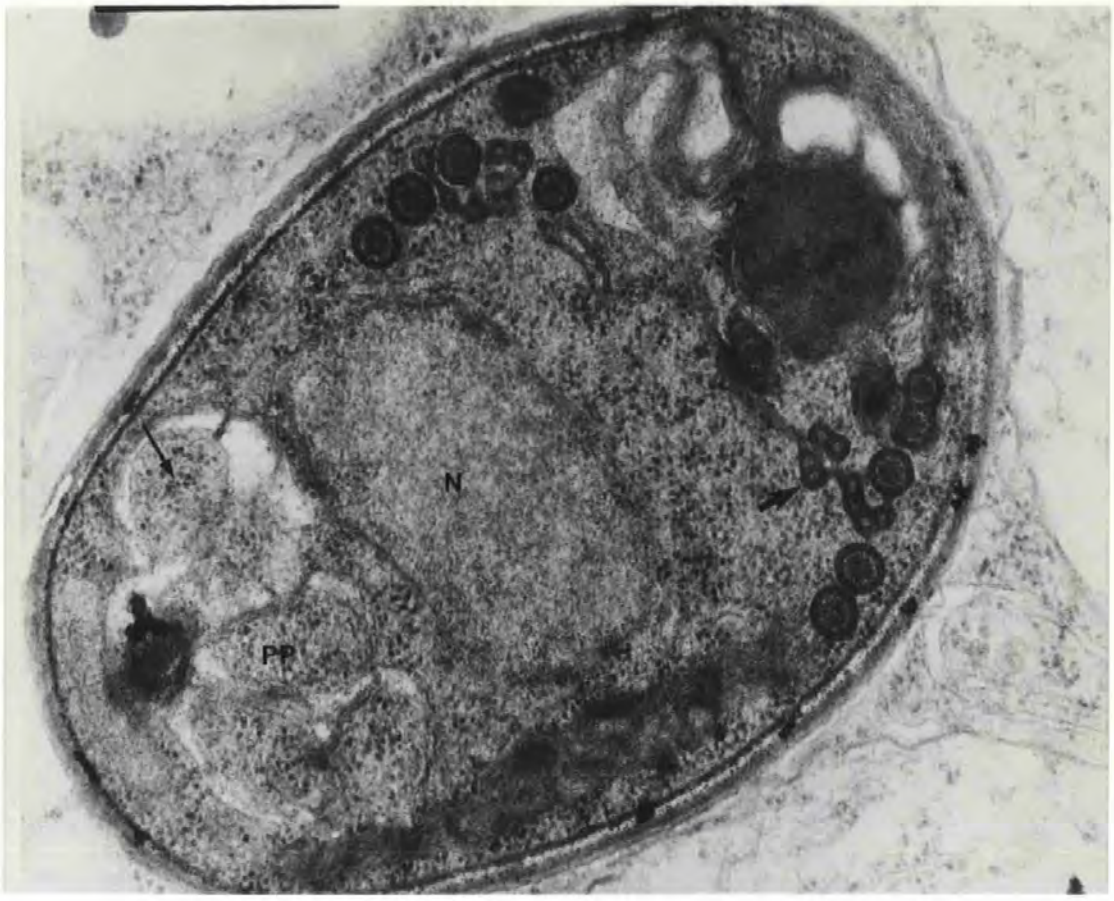

PV
FFS
EDI


PS
N
EDI
PP

Figs. 44 and 45. Sporogenesis in *M. hepaticus* : development of the polaroplast.

Fig. 44. Electron micrograph of sporoblast showing expanded sacs with granular contents in polaroplast region. x55,500. N, nucleus; PP, polaroplast; broad arrow, small components of polar filament; thin arrow, granular material in polaroplast.

Fig. 45. Electron micrograph of sporoblast with polaroplast sacs flattening to form lamellae. x38,000. LP, lamellar polaroplast; SP, saccate polaroplast; N, nucleus.


N
PP

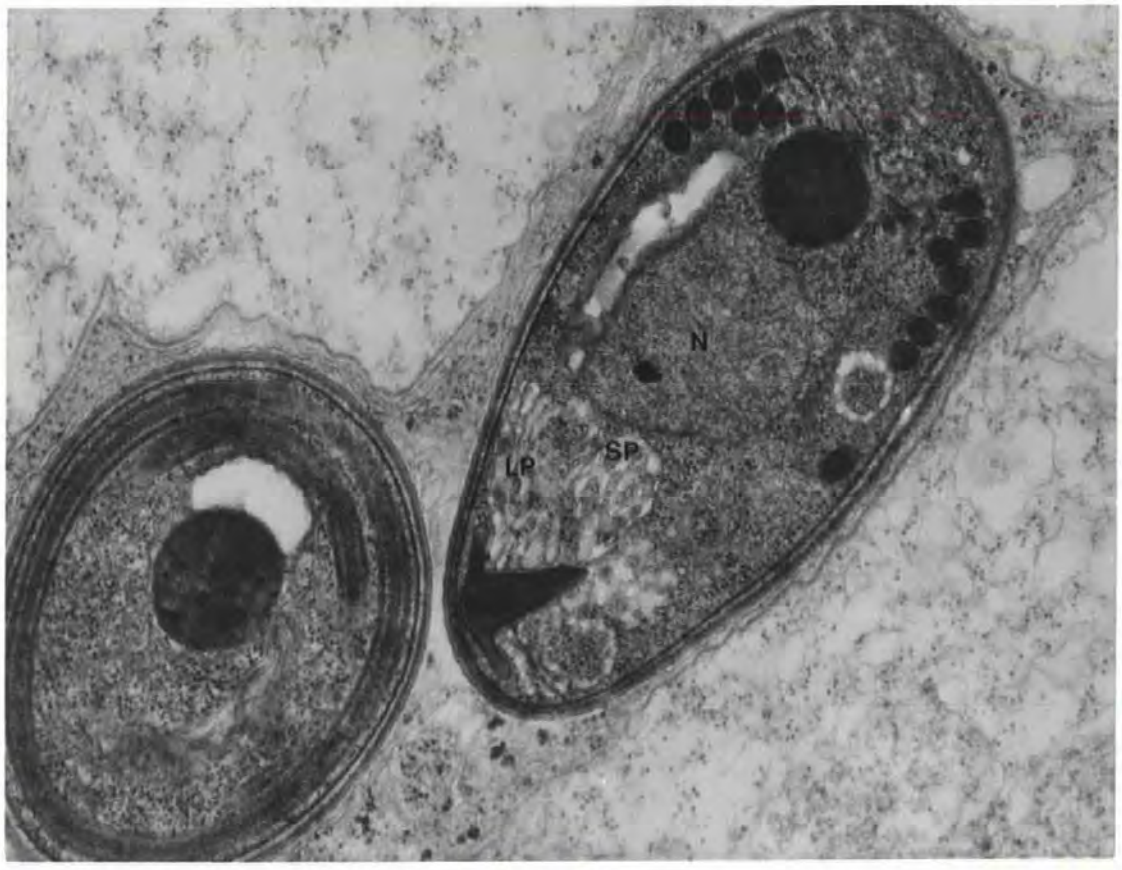
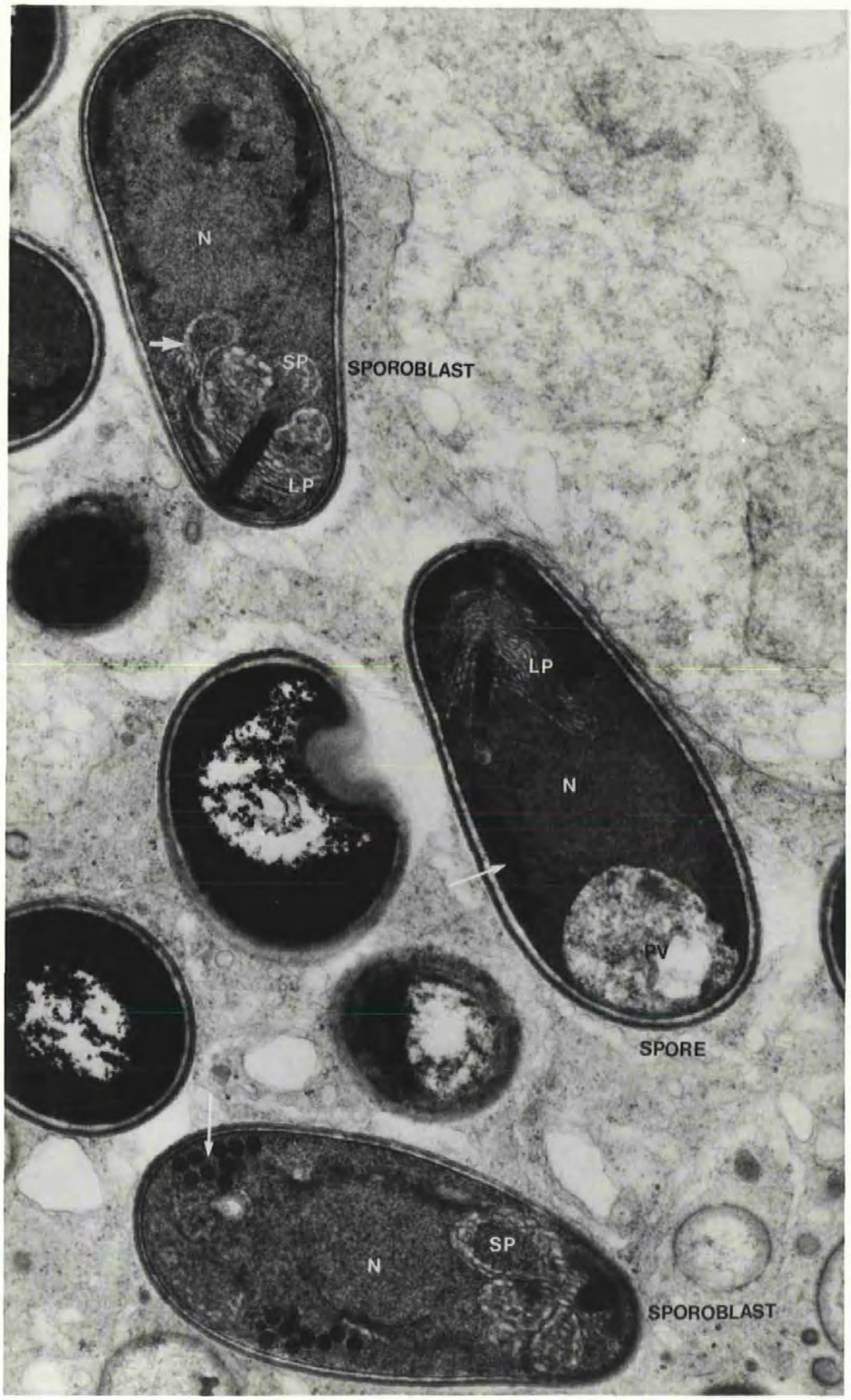

N
LP
SP

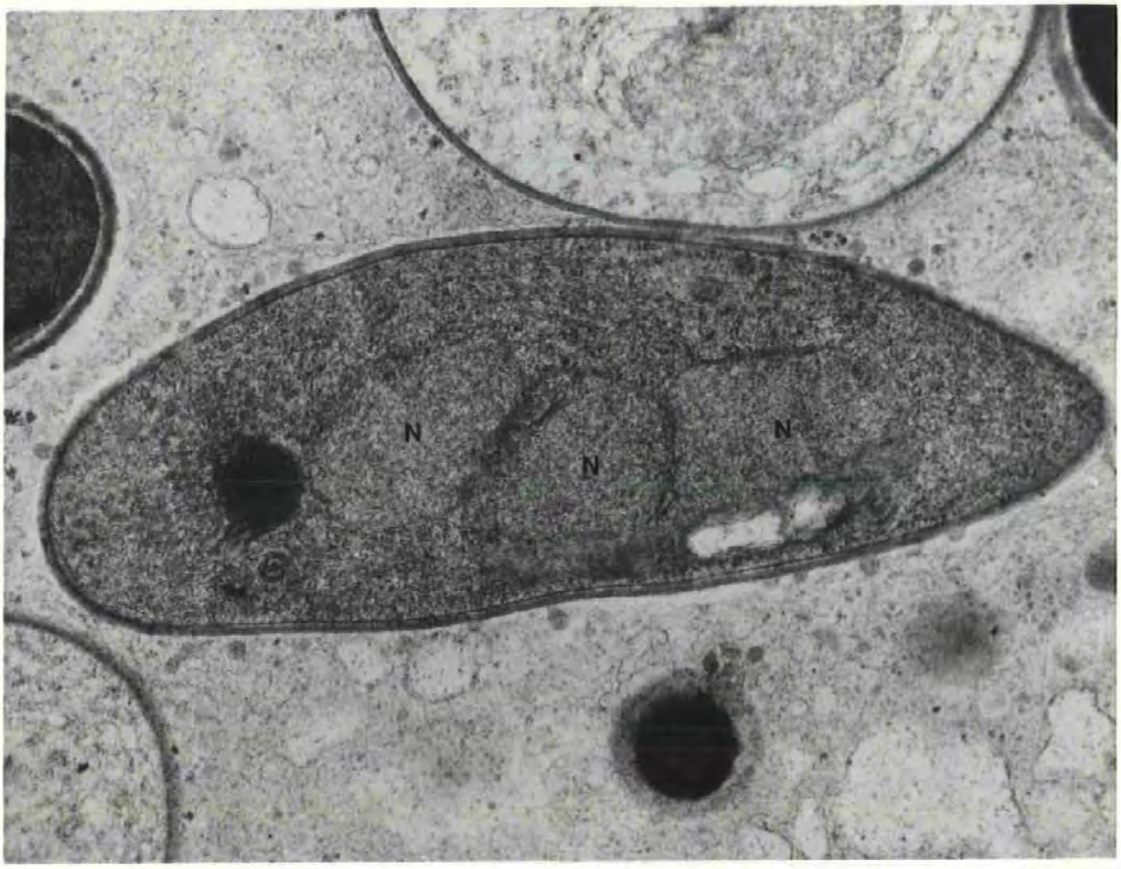
Fig. 46. Electron micrograph of a group of stages of *M. hepaticus* including sporoblasts and a spore, showing stages in polaroplast formation. x28,400. N, nucleus; LP, lamellar polaroplast; SP, saccate polaroplast; broad arrow, saccate polaroplast at termination of lamellar polaroplast; thin arrow, polar filament coils.


N
SP
SPOROBLAST
LP
LP
N
PV
SPORE
SP
N
SPOROBLAST

Figs. 47 and 48. Aberrant sporoblasts of *M. hepaticus*.

Fig. 47. Electron micrograph of sporoblast with two nuclei. x30,500. N, nucleus.

Fig. 48. Electron micrograph of sporoblast with three nuclei. x31,200. N, nucleus.


N
N
N
G

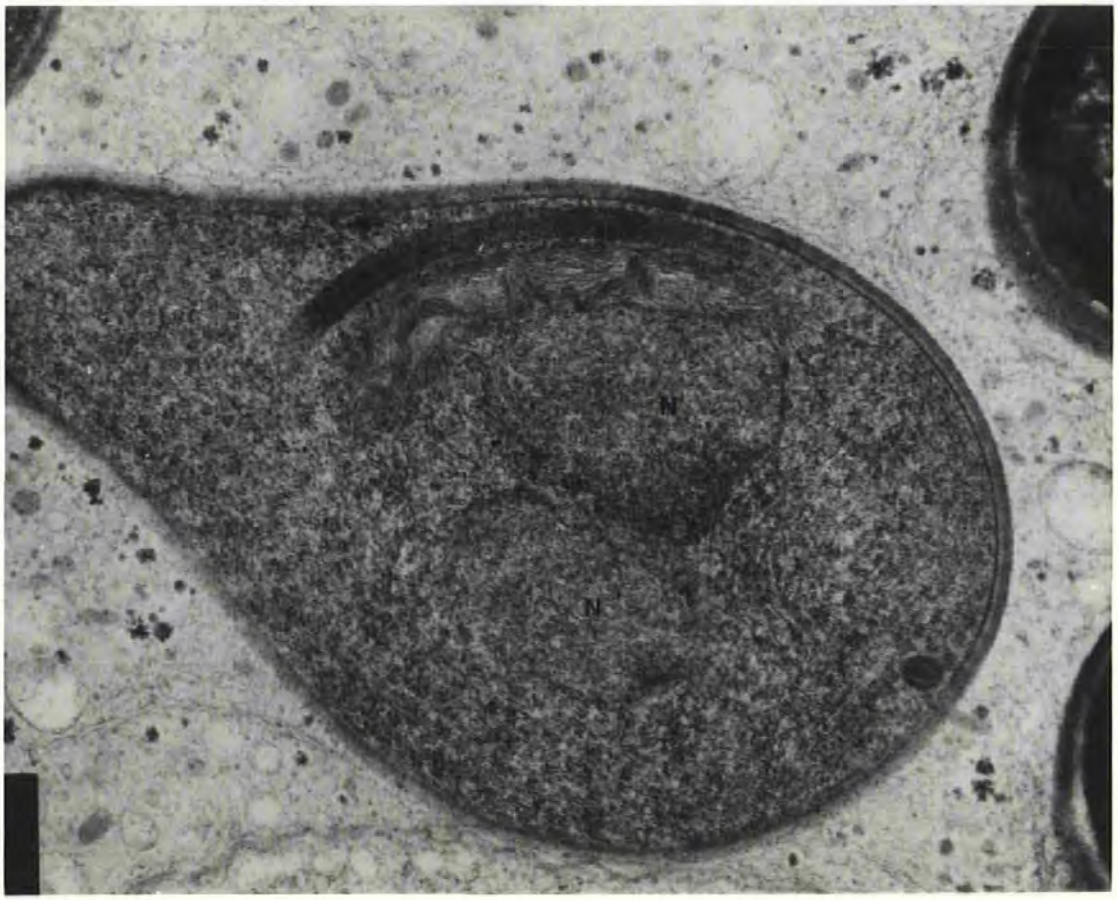
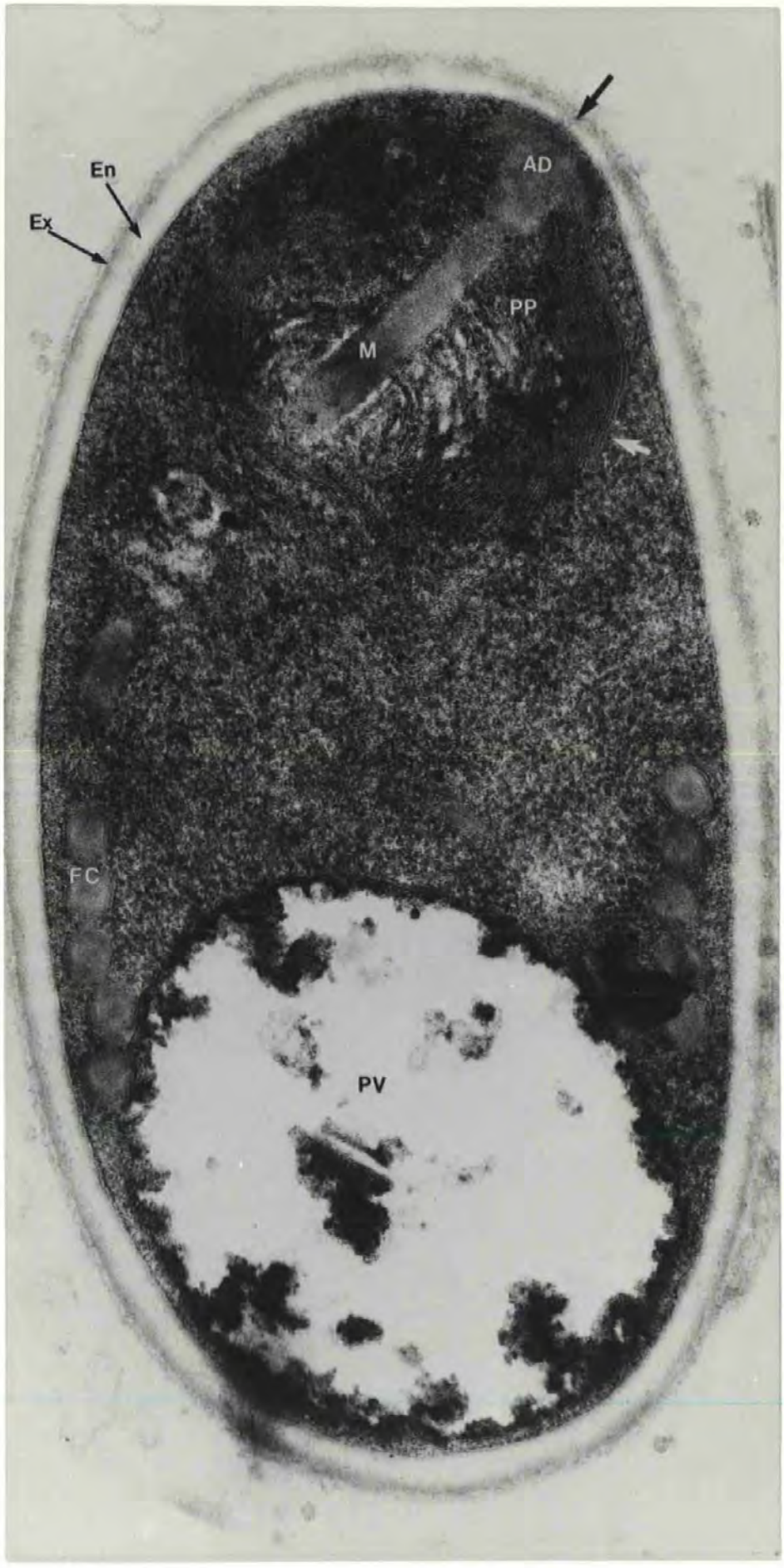

N
N

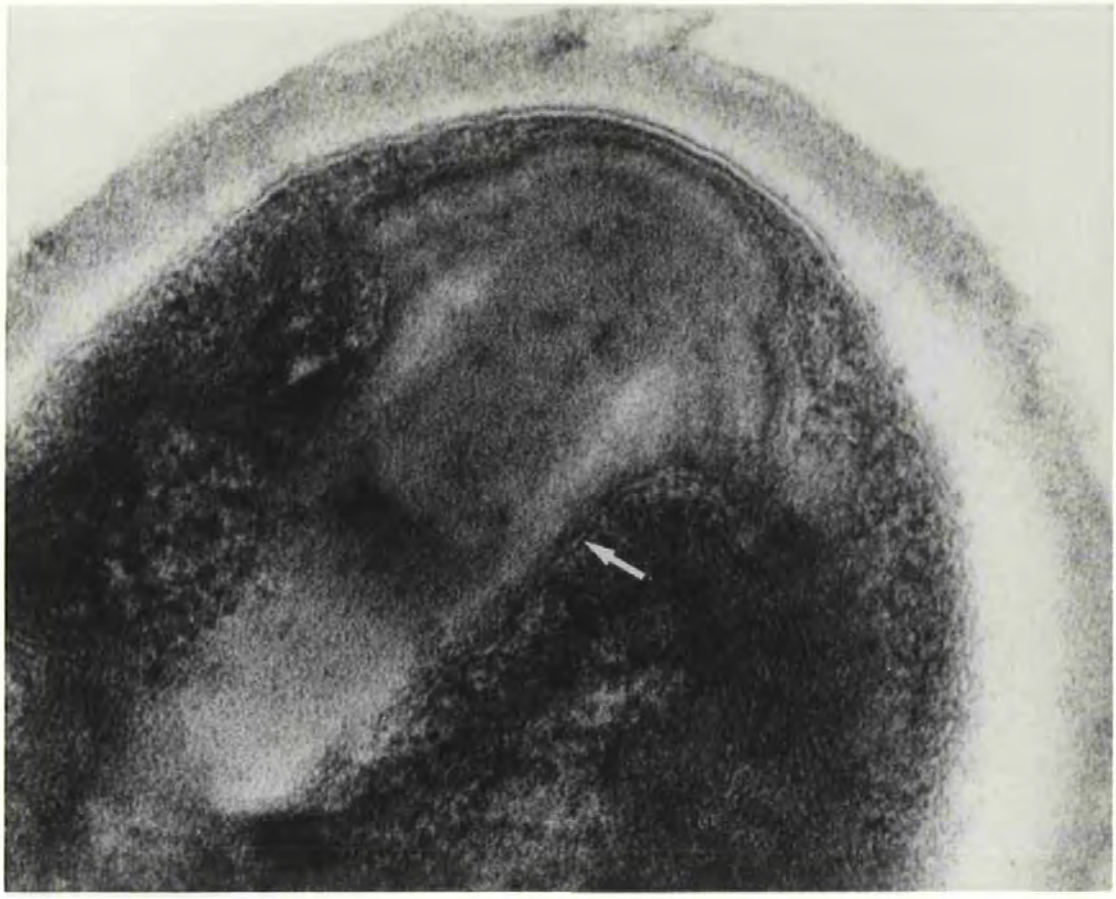
Fig. 49. Electron micrograph of a longitudinal section of the spore of *M. hepaticus*. x82,000. AD, anchoring disc; En, endospore; Ex, exospore; M, manubrium; PP, polaroplast; PV, posterior vacuole; black arrow, thinned endospore adjacent to anchoring disc; white arrow, lateral extension of anchoring disc.

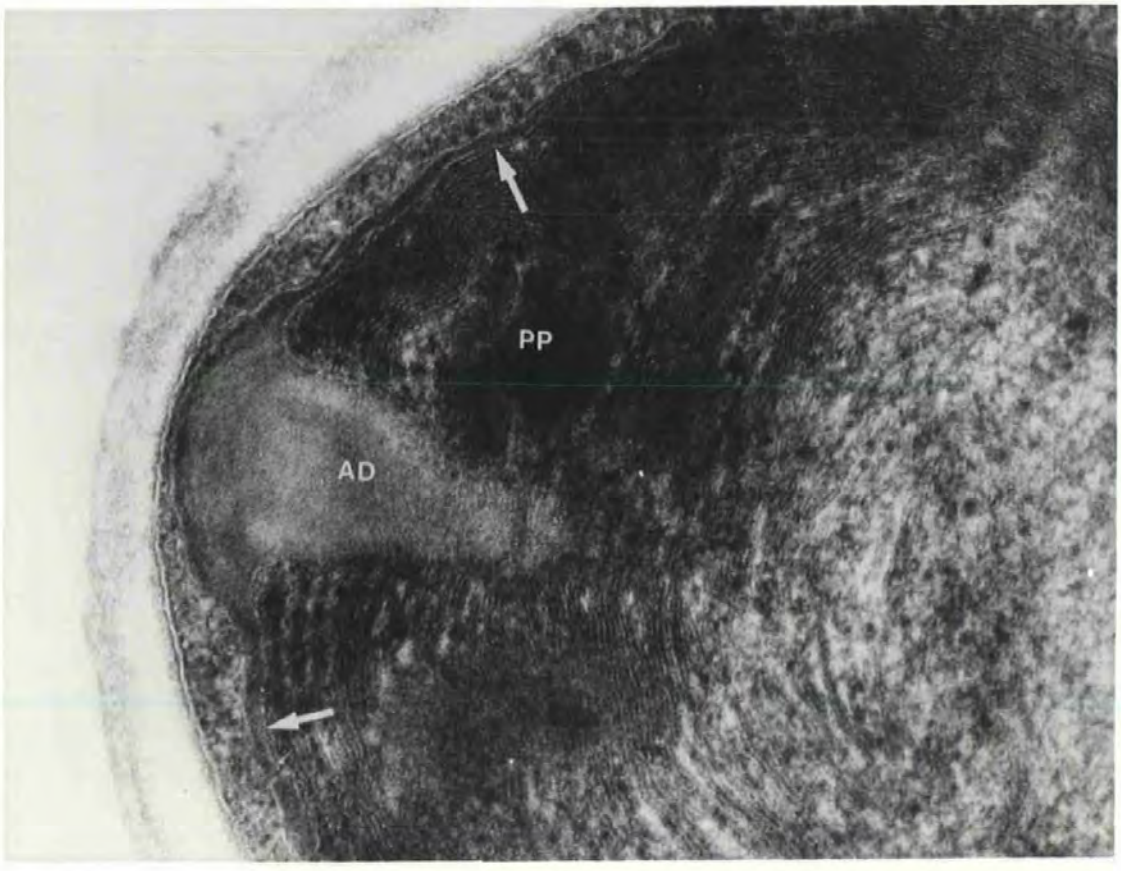

En
Ex
AD
PP
M
FC
PV

Figs. 50 and 51. Features of spore structure of *M. hepaticus*.

Fig. 50. Electron micrograph of spore showing manubrium and anchoring disc with anterior protrusion. x186,000. Arrow, outer layer of manubrium.

Fig. 51. Electron micrograph of spore showing the anchoring disc and polaroplast. x178,000. AD, anchoring disc; PP, polaroplast; arrow, lateral extension of anchoring disc.

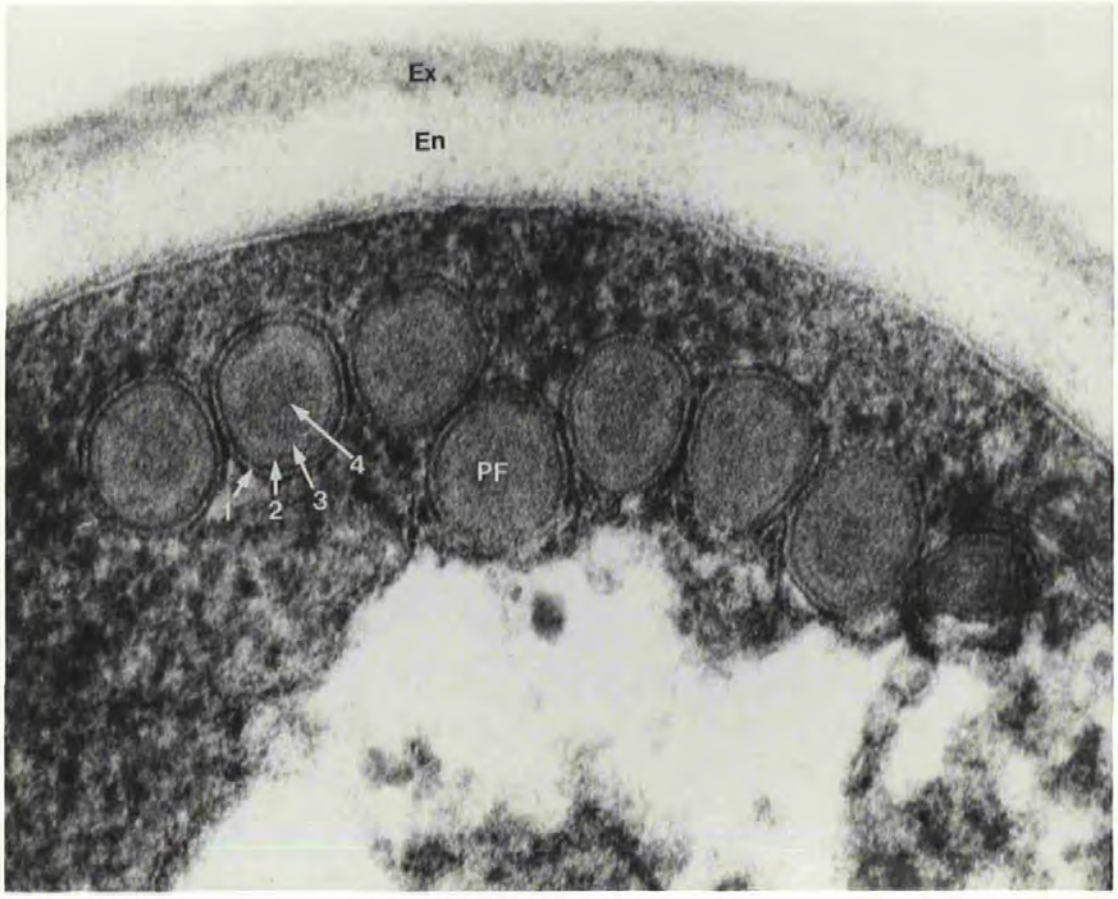


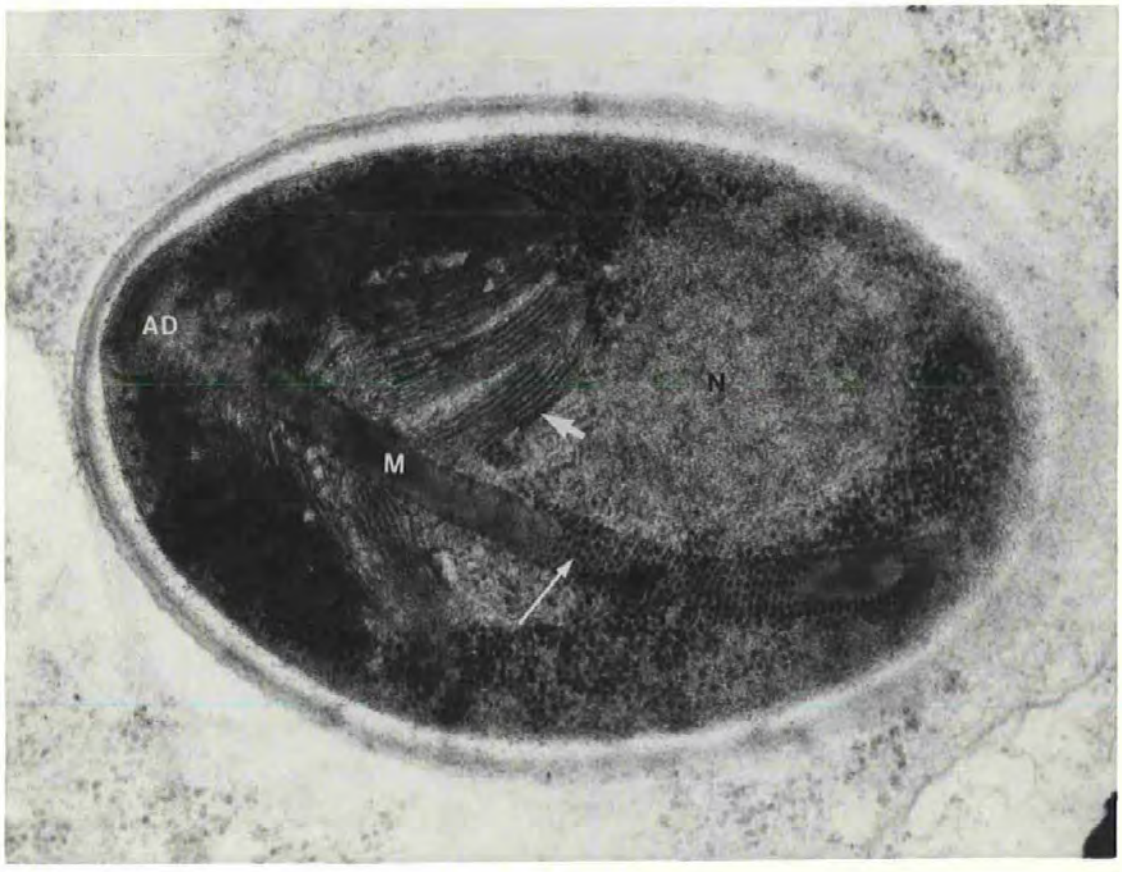

PP
AD

Figs. 52 and 53. Features of spore structure of *M. hepaticus*.

Fig. 52. Electron micrograph of polar filament coils and the spore wall. x197,000. En, endospore; Ex, exospore; PF, polar filament; arrows 1-4, layers of polar filament.

Fig. 53. Electron micrograph of oblique section of a spore showing the manubrium, polaroplast and anchoring disc. x64,600. AD, anchoring disc; M, manubrium; N, nucleus; broad arrow, polaroplast lamellae; thin arrow, ribosomes attached to manubrium.

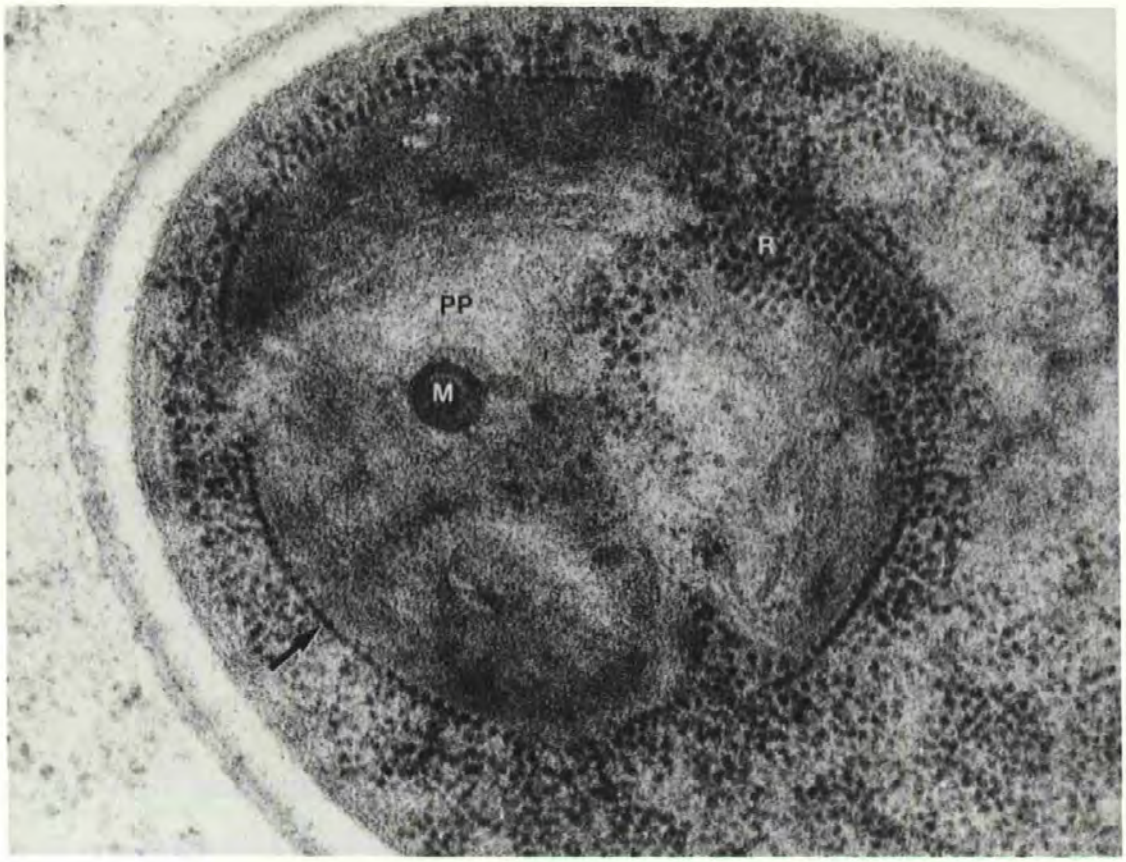

Ex
En
PF
1
2
3
4

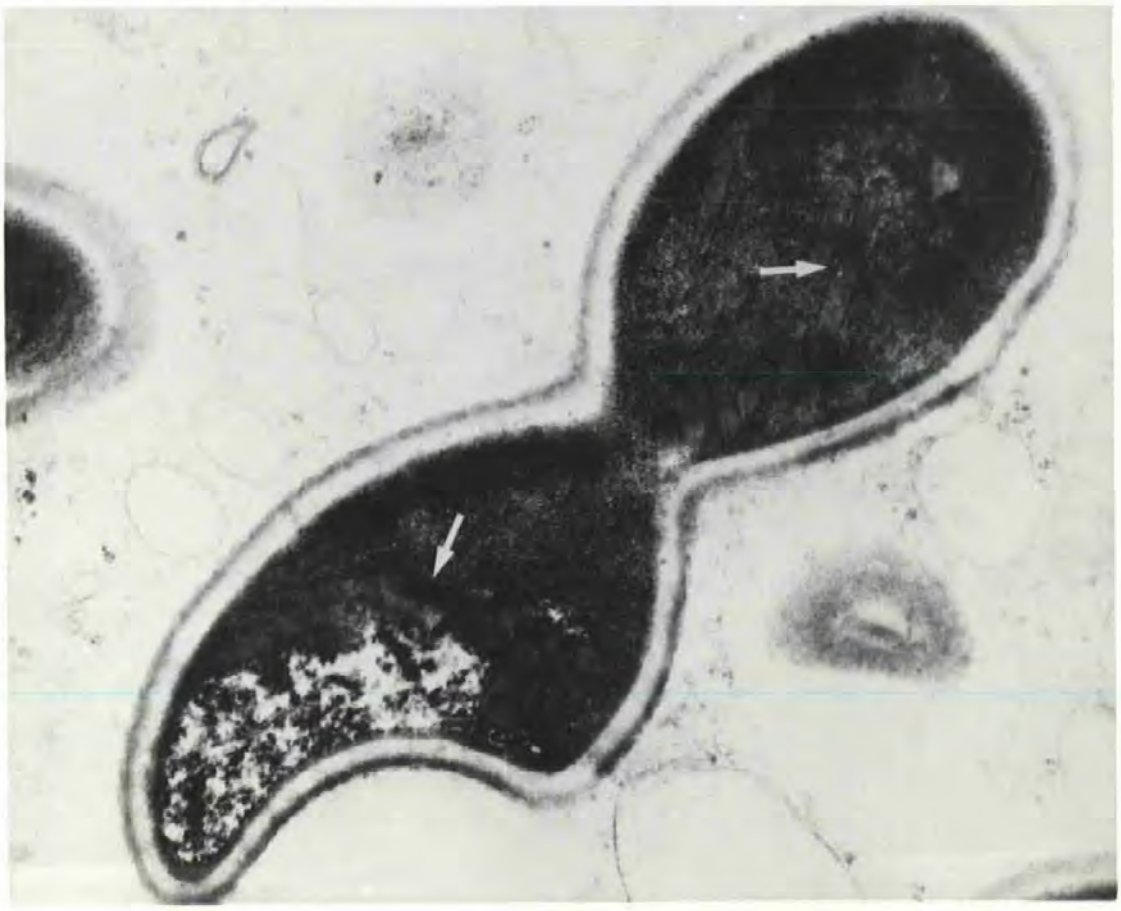

AD
N
M

Figs. 54 and 55. Features of spore structure of *M. hepaticus*.

Fig. 54. Electron micrograph of transverse section of spore in the polaroplast region. x92,500. M, manubrium; PP, polaroplast; R, sheet of ribosomes associated with polaroplast; arrow, ribosomes attached at periphery of polaroplast.

Fig. 55. Electron micrograph of abnormal double spore. x35,500. Arrow, polar filament coils.

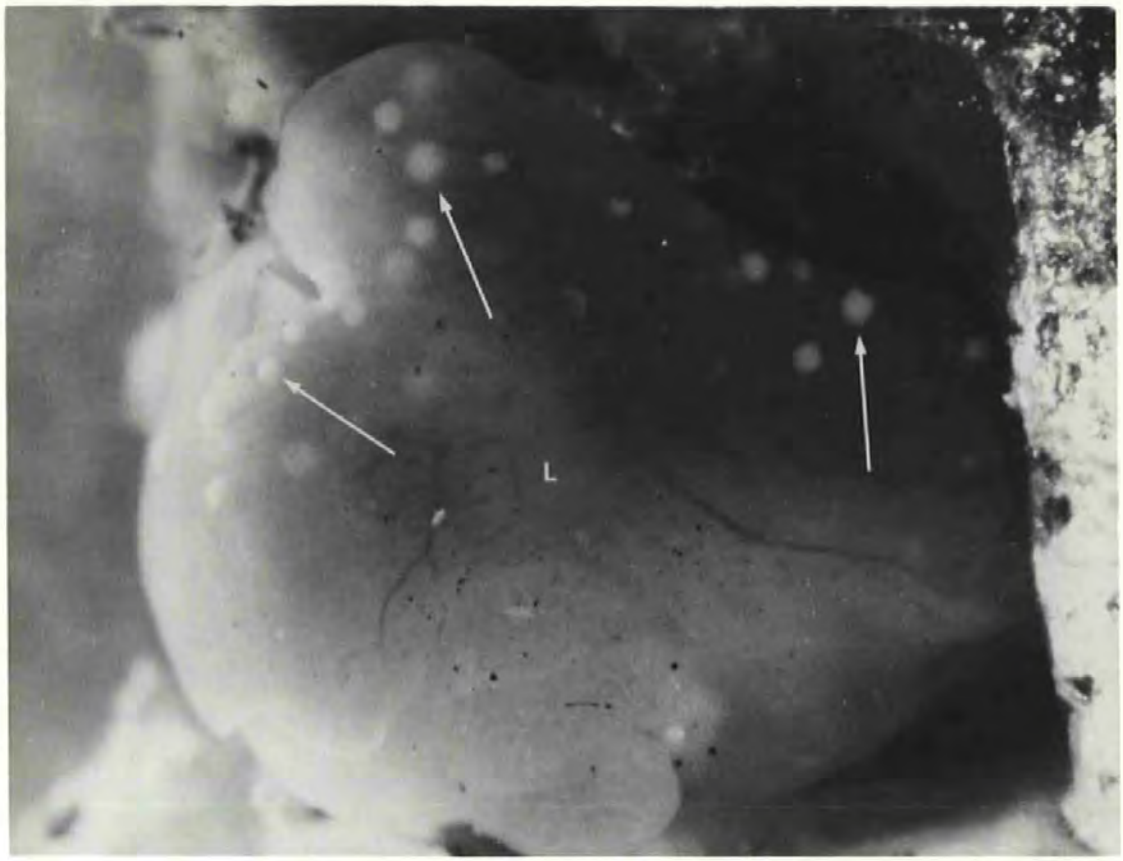

R
PP
M

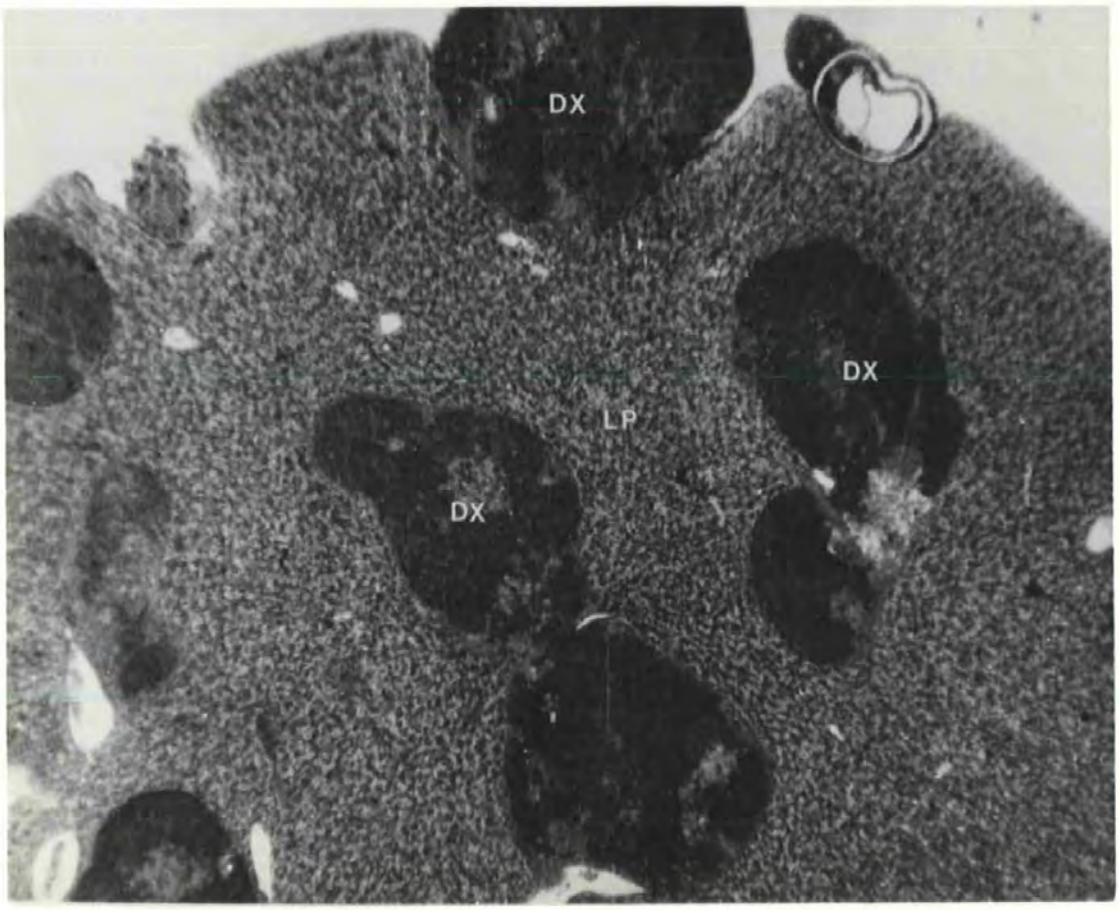


Figs. 56 and 57. Distribution of xenomas of *M. hepaticus* in the liver of *C. labrosus*.

Fig. 56. Photograph of surface of heavily infected liver. x24. L, liver; arrow, xenomas of *M. hepaticus*.

Fig. 57. Low power micrograph of section of infected liver. x280. DX, degenerate xenomas.

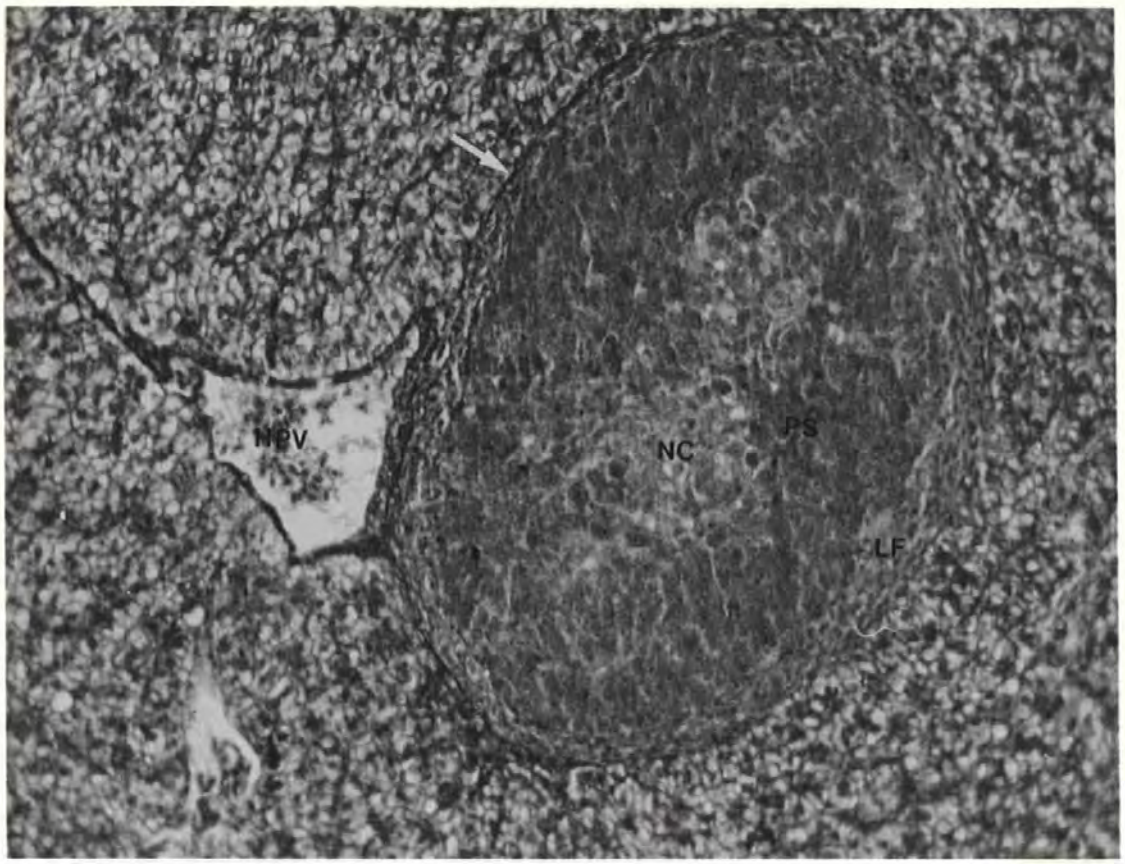

L

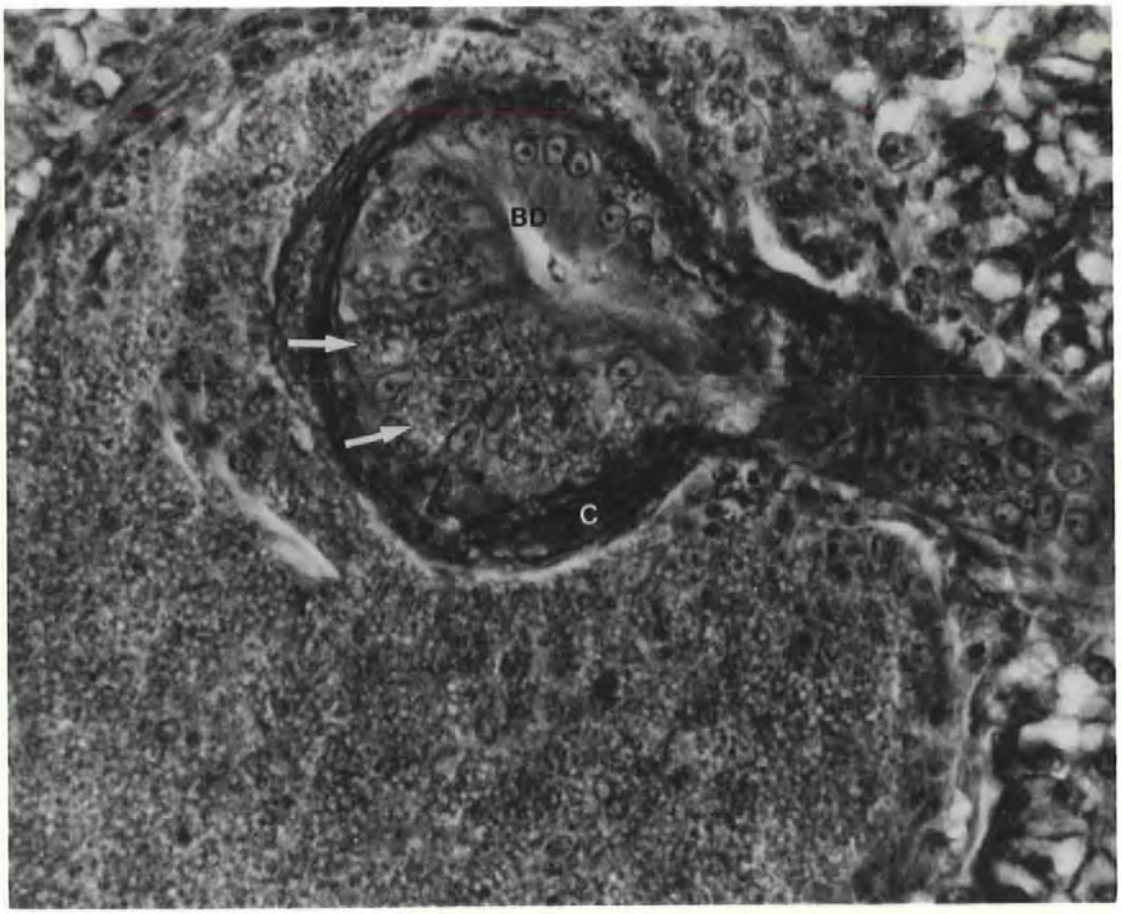

DX
DX
LP
DX

Figs. 58 and 59. The association of M. hepaticus with liver vessels.

Fig. 58. Old xenoma (granuloma) associated with hepatic portal vein. x1015. HPV, hepatic portal vein; LF, lymphocytes and fibroblasts; NC, necrotic centre of granuloma; arrow, collagen capsule; PS, phagocytosed spores.

Fig. 59. Granuloma associated with bile duct. x2790. BD, bile duct; C, collagen; arrow, phagocytosed spores in cells of bile duct.

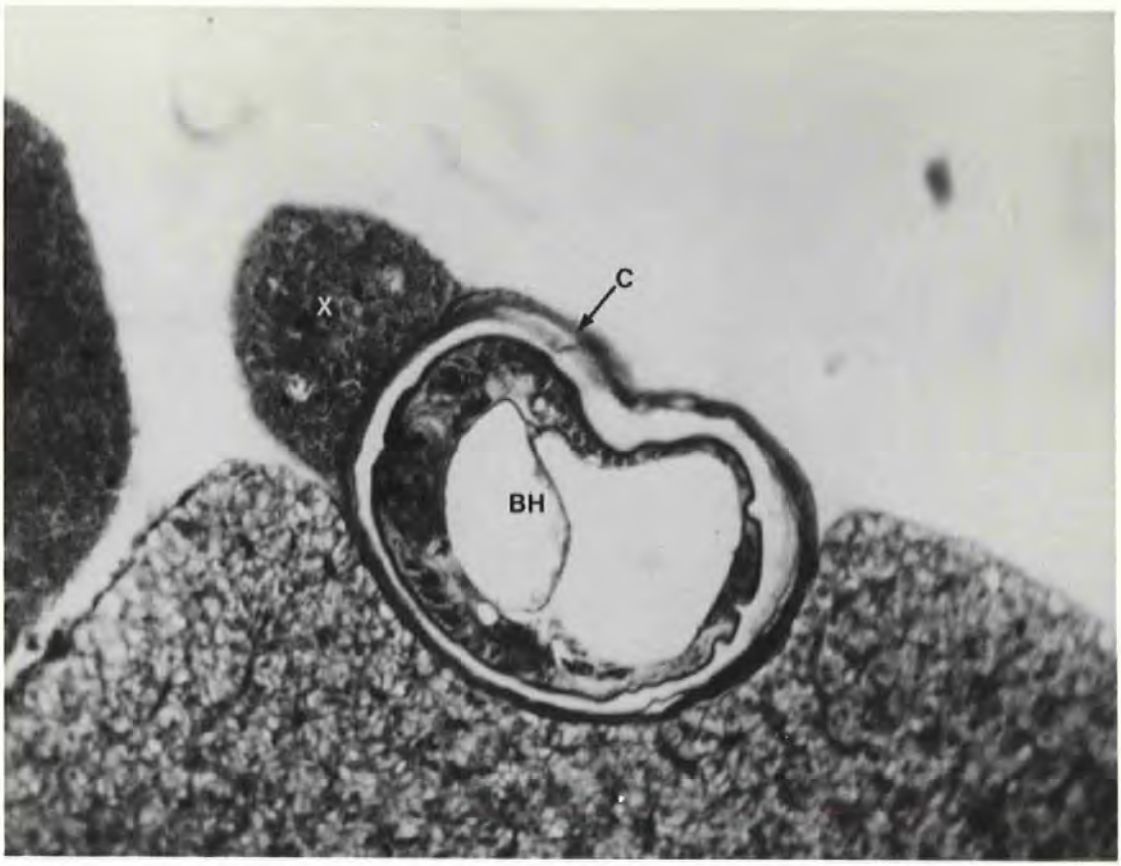

NPV
NC
PS
LF

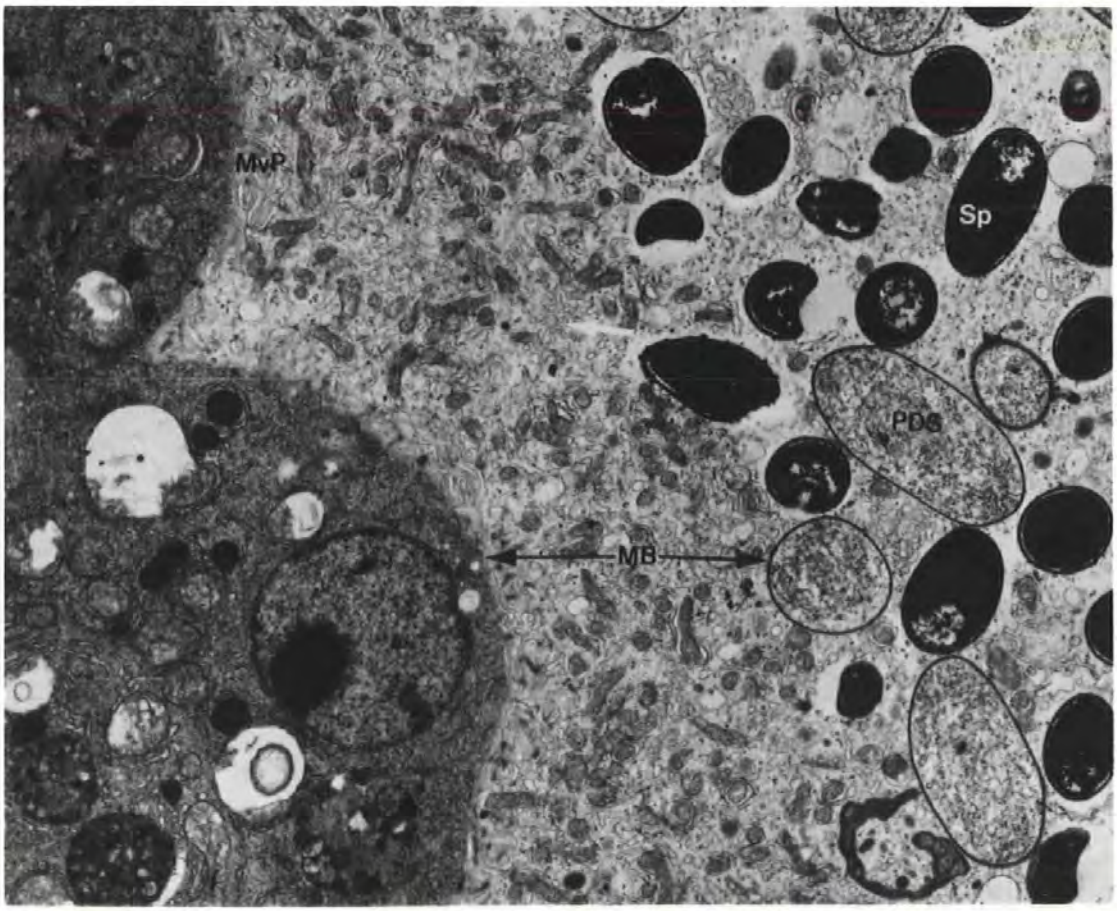

BD
C

Figs. 60 and 61. Distribution and structure of xenomas of *M. hepaticus*.

Fig. 60. Granuloma associated with the host capsule of *Bucephalus haimeanus* metacercaria. x1016. BH, *B. haimeanus*; C, capsule; X, xenoma (granuloma)

Fig. 61. Electron micrograph of peripheral part of active xenoma. x7,500. MB, mitochondrial band; MvP, microvillar processes; PDS, parasite developmental stage; Sp, spore; white arrow, smooth endoplasmic reticulum.

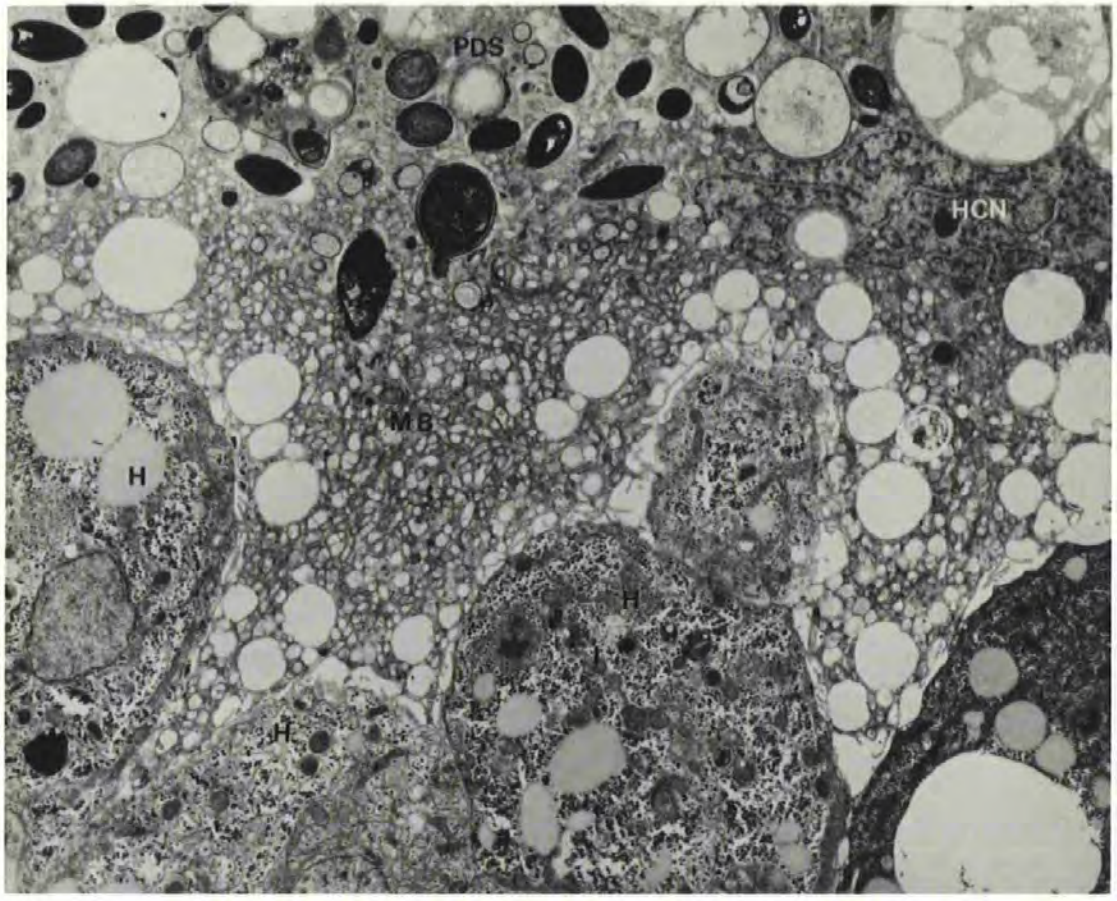

X
C
BH

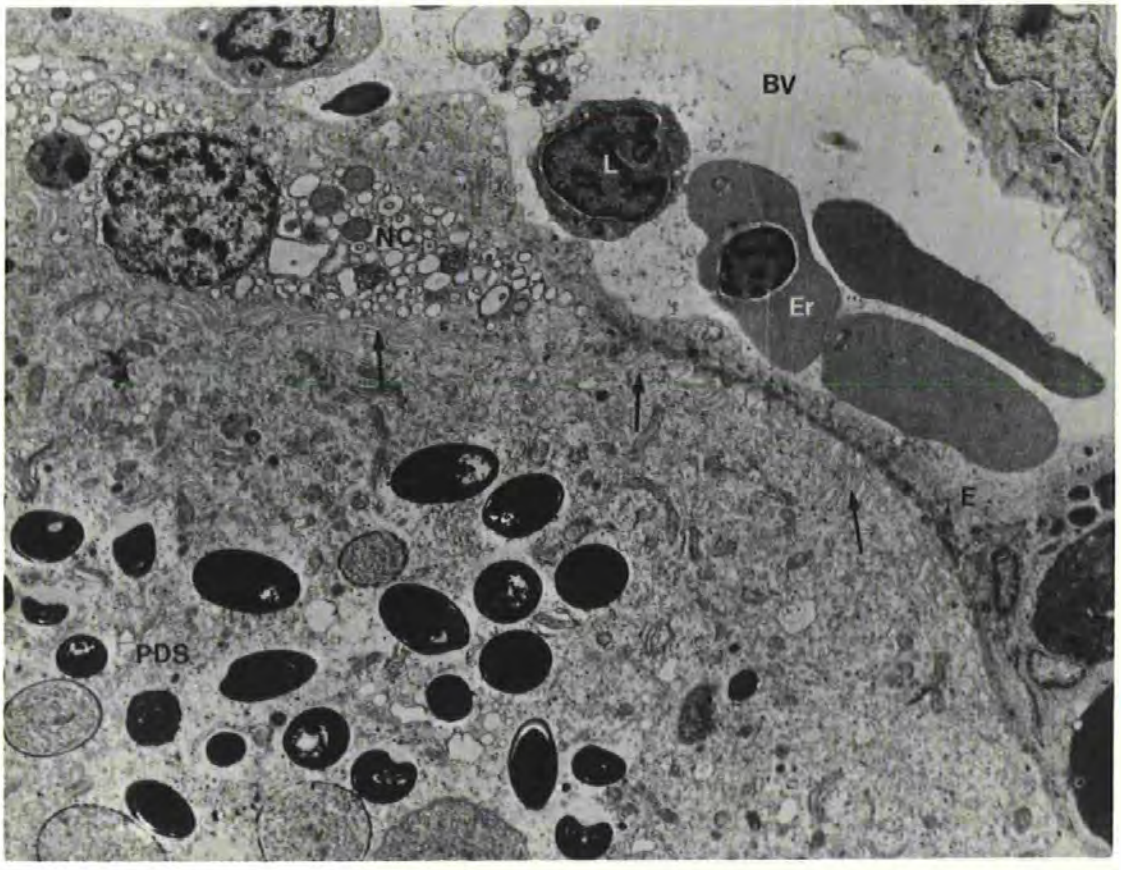

MvP
Sp
PDB
MB

Figs. 62 and 63. Ultrastructure of the xenoma of *M. hepaticus*.

Fig. 62. Electron micrograph of periphery of xenoma, showing relationship with surrounding hepatocytes. x3,300. H, hepatocyte; HCN, host cell nucleus; MB, mitochondrial band; PDS, parasite developmental stages.

Fig. 63. Electron micrograph of the periphery of a xenoma positioned adjacent to a blood vessel. x3,200. BV, blood vessel; E, endothelial cell; Er, erythrocyte; L, lymphocyte; NC, necrotic cell; arrows, microvillar processes.

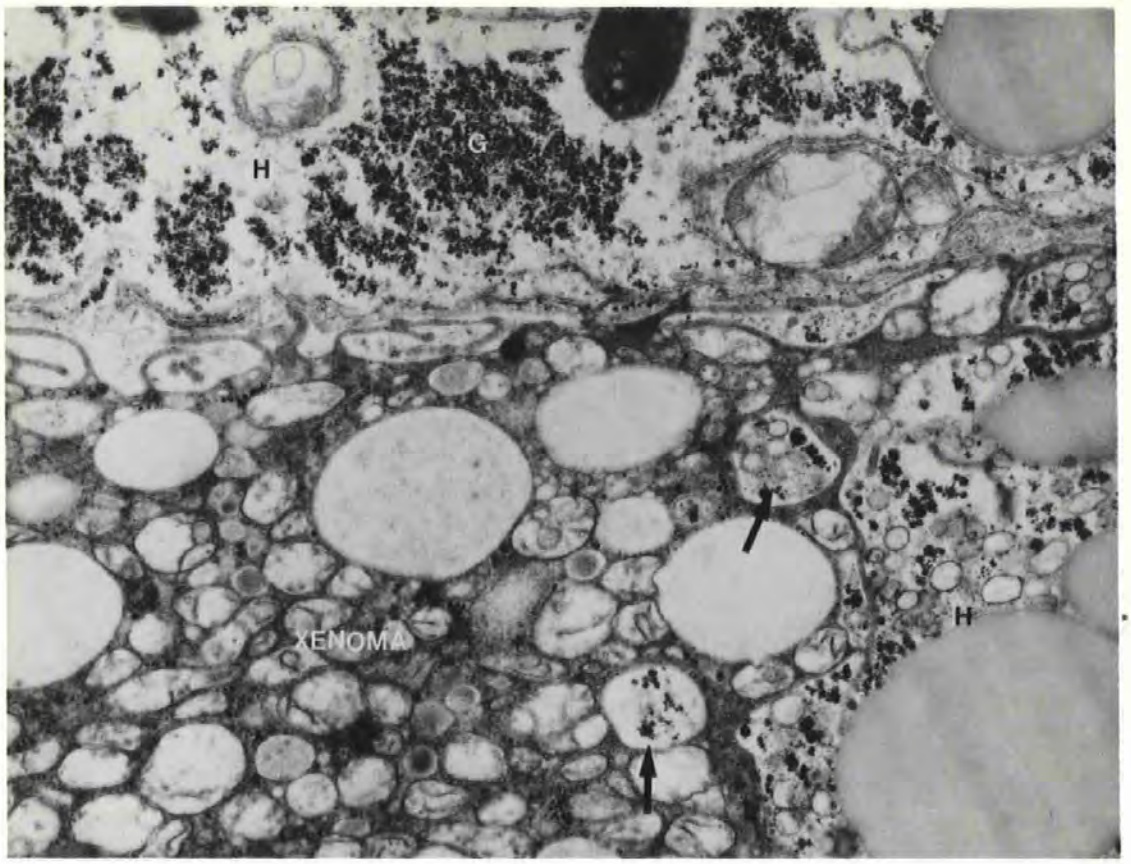

PDS
HCN
MB
H
H
H

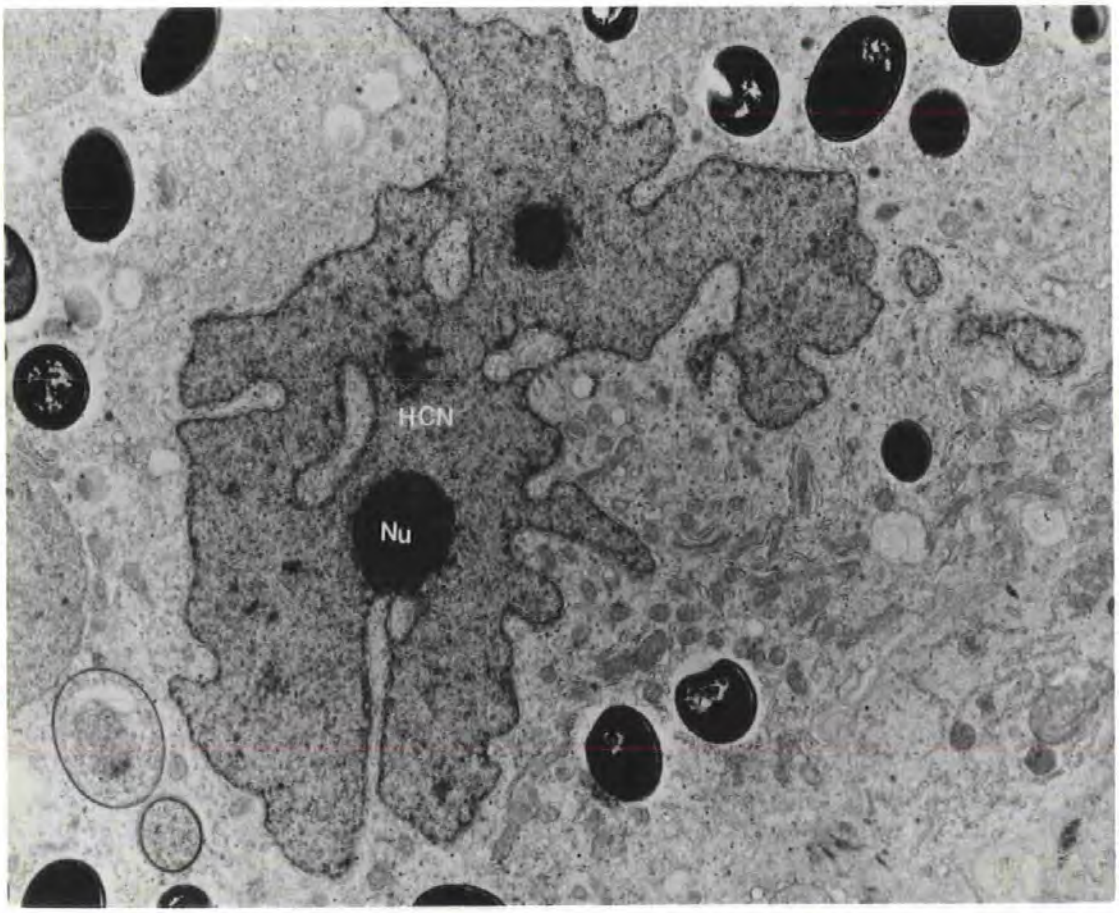

BV
L
NC
Er
E
PDS

Figs. 64 and 65. Ultrastructure of the xenoma of *M. hepaticus*.

Fig. 64. Electron micrograph of xenoma periphery, showing phagocytic vacuoles. x15,070. G, glycogen; H, hepatocyte; arrow, material from degenerate hepatocyte in phagocytic vacuole.

Fig. 65. Electron micrograph of the xenoma host cell nucleus. x21,000. HCN, host cell nucleus; Nu, nucleolus.

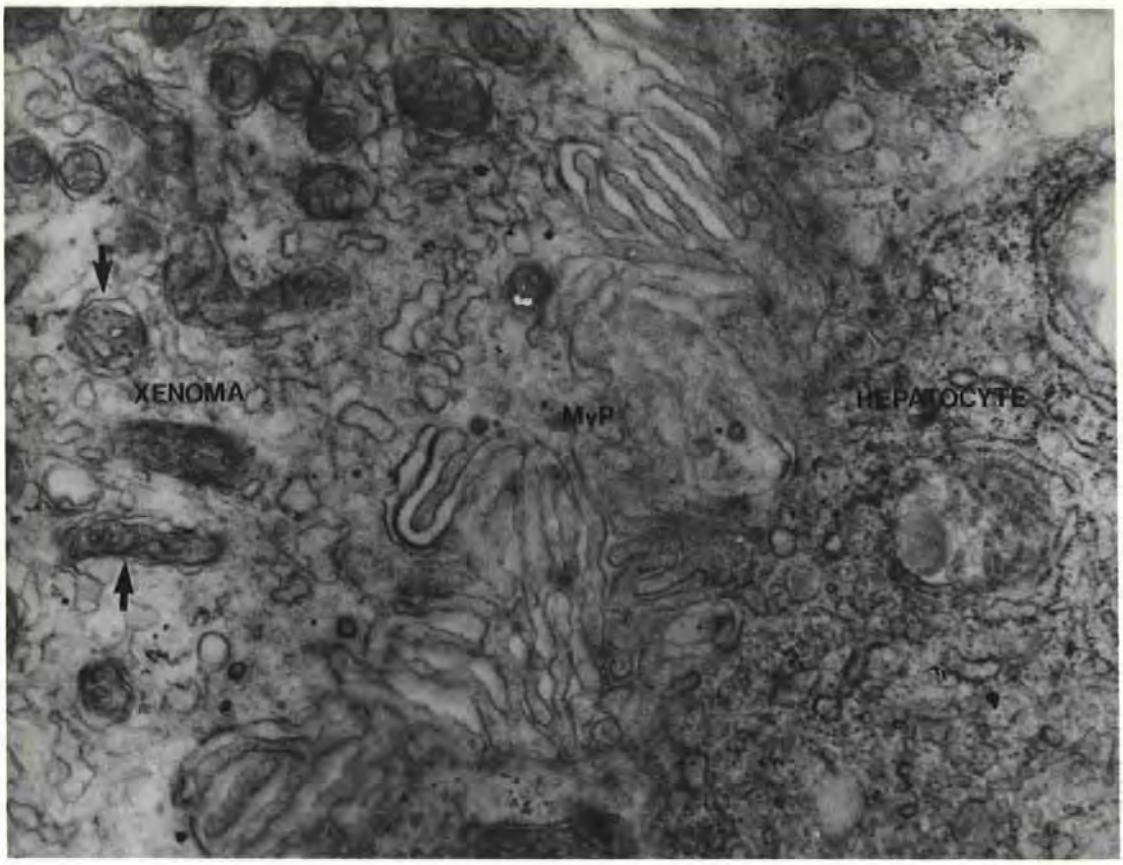

H
G
XENOMA
H

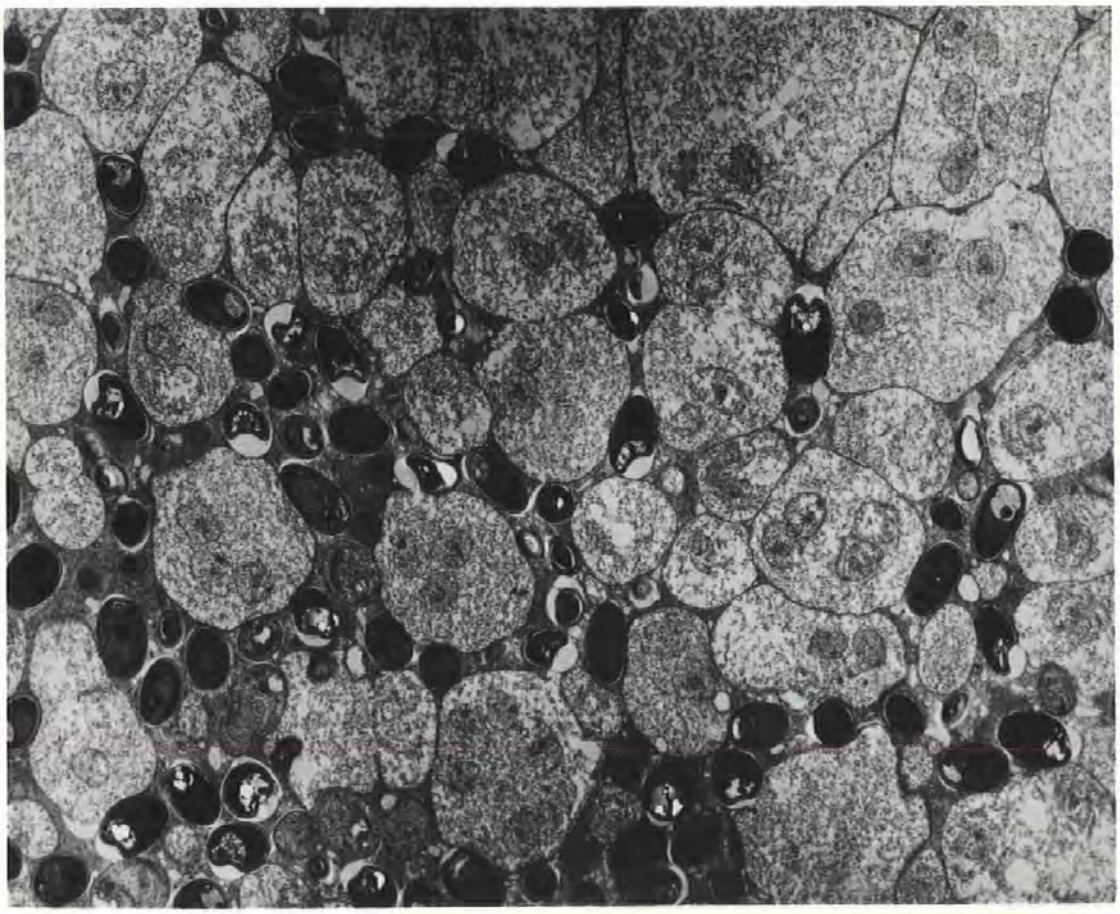

HCN
Nu

Figs. 66 and 67. Ultrastructure of the xenoma of *M. hepaticus*.

Fig. 66. Electron micrograph of peripheral part of xenoma, showing the cell membrane thrown into microvillar processes. x27,300. MvP, microvillar processes; arrow, mitochondria of xenoma mitochondrial band.

Fig. 67. Electron micrograph of central portion of xenoma, showing the random arrangement of the various parasite stages. x4,060.


XENOMA
MyP
HEPATOCYTE



Figs. 68-75. Early stages of xenoma development of M. hepaticus, from smear preparation of the liver of a post-larval specimen of C. labrosus. Giemsa staining.

Fig. 68. Micrograph of cell containing a uninucleate parasite stage. x6,480. n, nucleus; black arrow, parasite cytoplasm; white arrow, host cell nucleus.

Fig. 69. Micrograph of cell containing two uninucleate parasite stages. x7,200. n, nucleus; white arrow, host cell nucleus.

Fig. 70. Micrograph of cell containing a multinucleate plasmodium. x7,200. n, nucleus; white arrow, host cell nucleus.

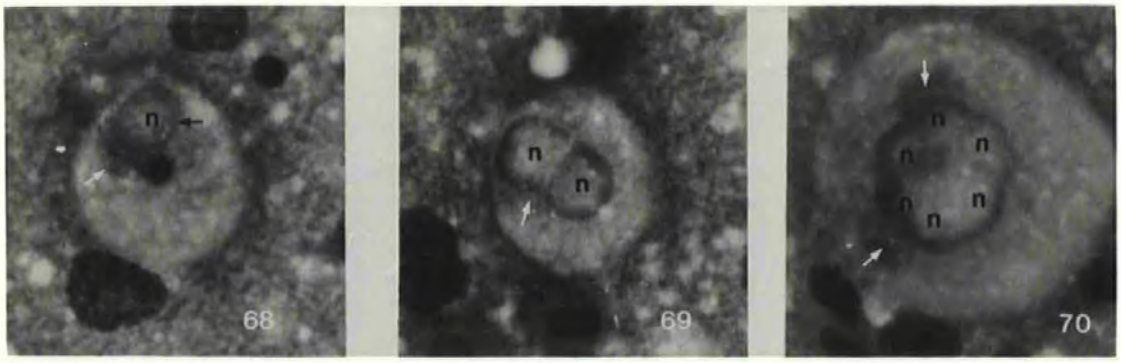
Fig. 71. Micrograph of cell containing uninucleate and multinucleate parasite stages. x7,200. mp, multinucleate plasmodium; u, uninucleate cell; white arrow, host cell nucleus.

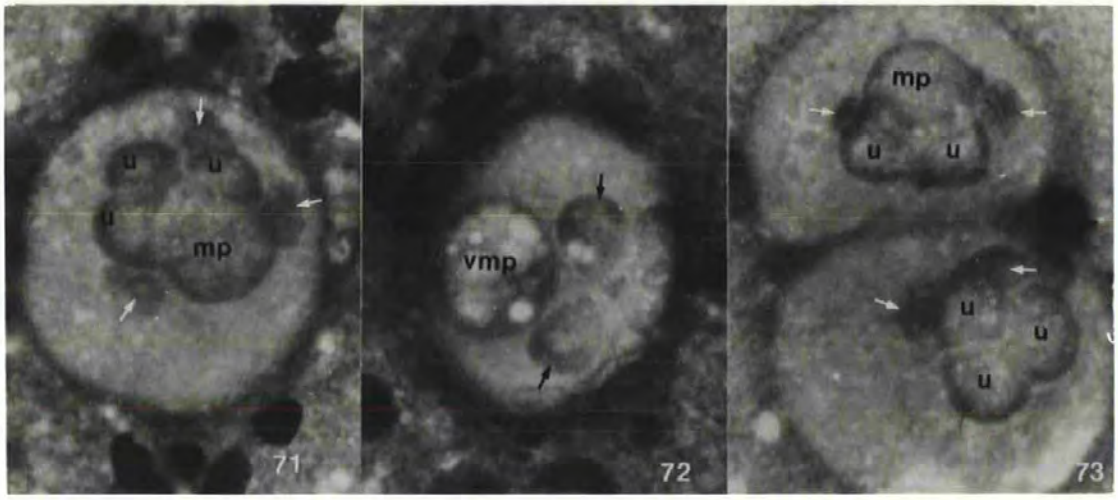
Fig. 72. Micrograph of cell containing a multinucleate, vacuolated plasmodium and dividing stages. x7,200. vmp, vacuolated multinucleate plasmodium; arrow, dividing stage.

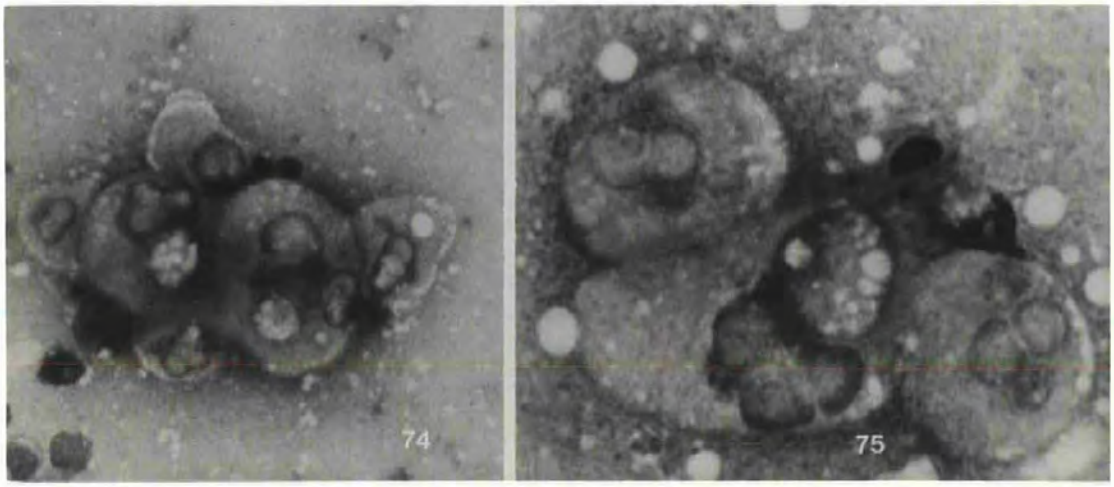
Fig. 73. Micrograph of a pair of infected cells. x7,050. mp, multinucleate plasmodium; u, uninucleate cell; arrow, host cell nucleus.

Fig. 74. Micrograph of a cluster of infected cells. x2,880.

Fig. 75. Micrograph of a cluster of infected cells. x6,330.


n
68
n
n
69
n
n
n
n
n
70

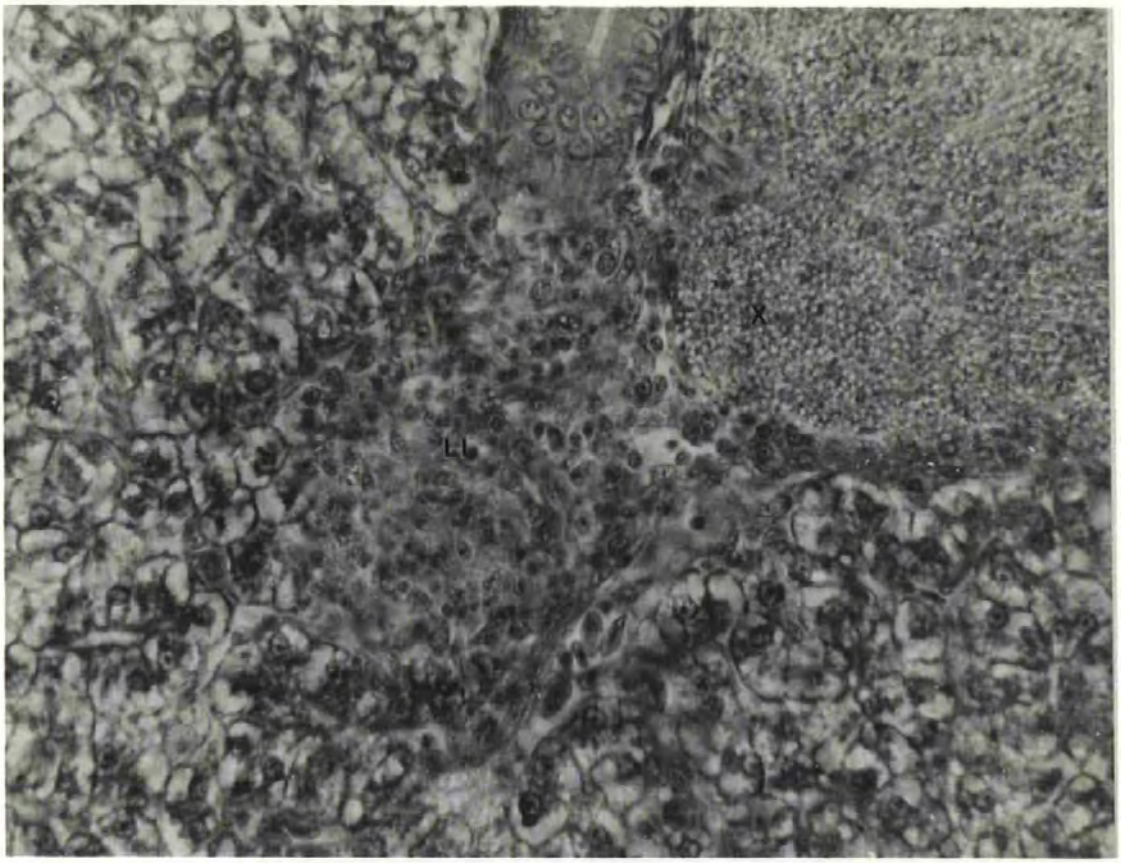

u
u
u
mp
71
vmp
72
mp
u
u
u
u
u
73

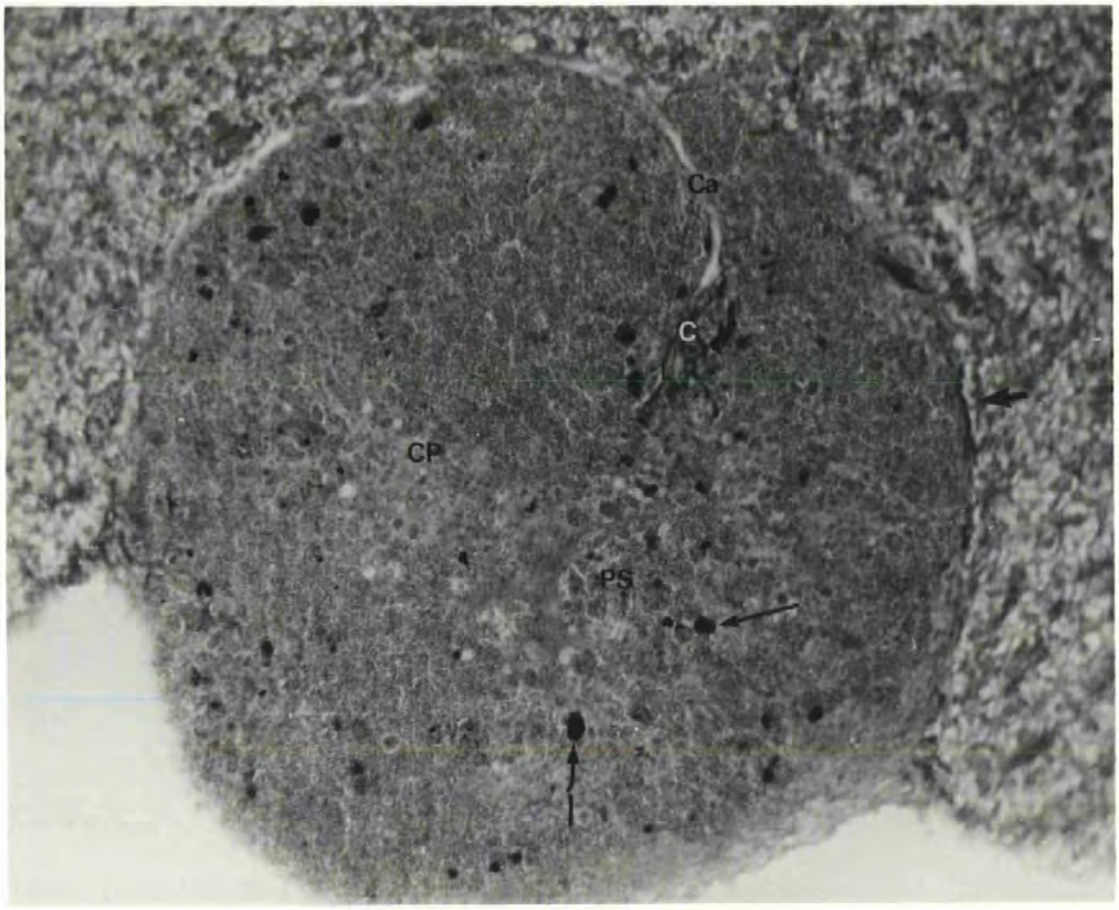

74
75

Figs. 76 and 77. Host response of C. labrosus to M. hepaticus : infection response to degenerate xenomas and granuloma structure.

Fig. 76. Micrograph of lymphocyte infiltration into affected region of liver. x960. Li, lymphocytes; X, xenoma. Mallory's triple stain.

Fig. 77. Micrograph of granuloma formed from a degenerate xenoma. x940. C, collagen; Ca, capillary; CP, cell proliferation; PS, phagocytosed spores; broad arrow, collagenous capsule; thin arrow, melanin. Mallory's triple stain.


X
LL


Ca
C
CP
PS

Figs. 78 and 79. Host response to, and effects of *M. hepaticus* : infection in the liver of *C. labrosus*.

Fig. 78. Micrograph of large degenerate infection associated with bile ducts, showing necrotic regions. x530. Ne, necrotic areas; arrow, bile ducts. Mallory's triple stain.

Fig. 79. Micrograph of granuloma at periphery of liver being broken off into the body cavity. x1,440. Mallory's triple stain.


Ne

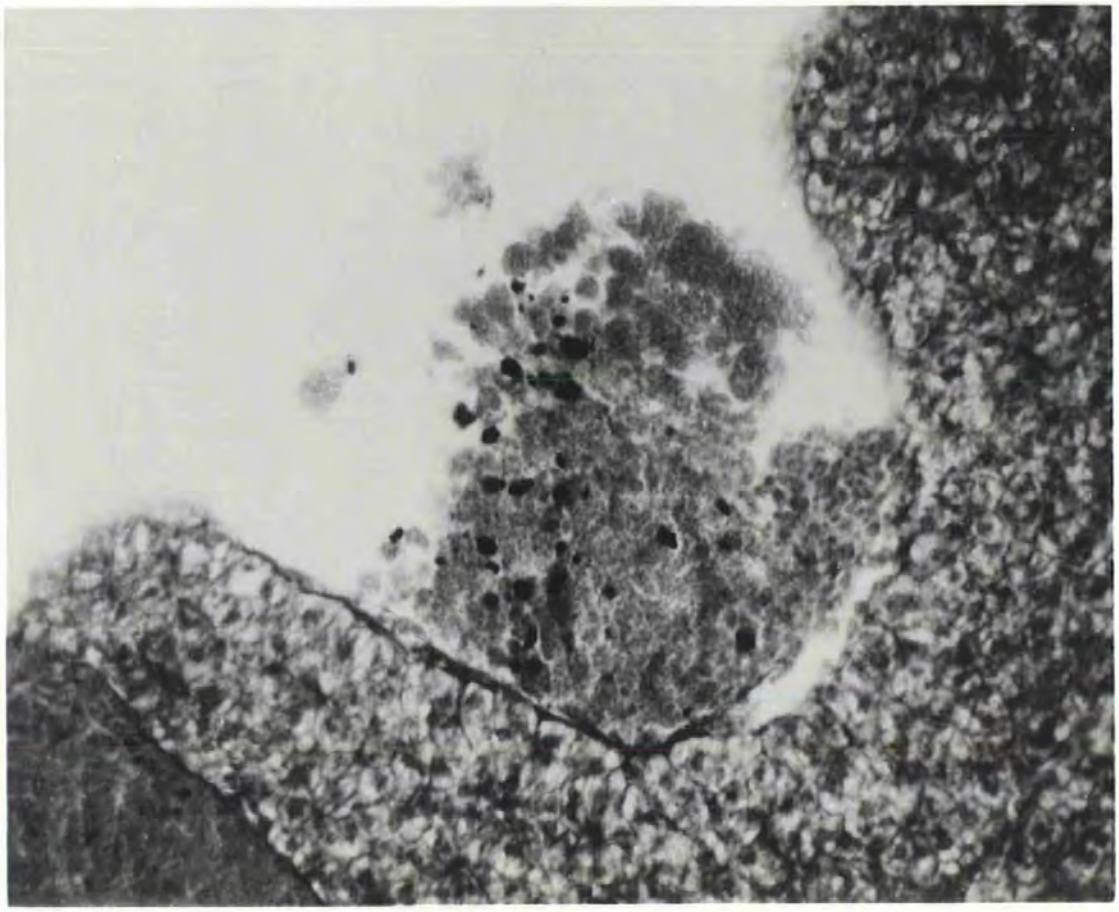
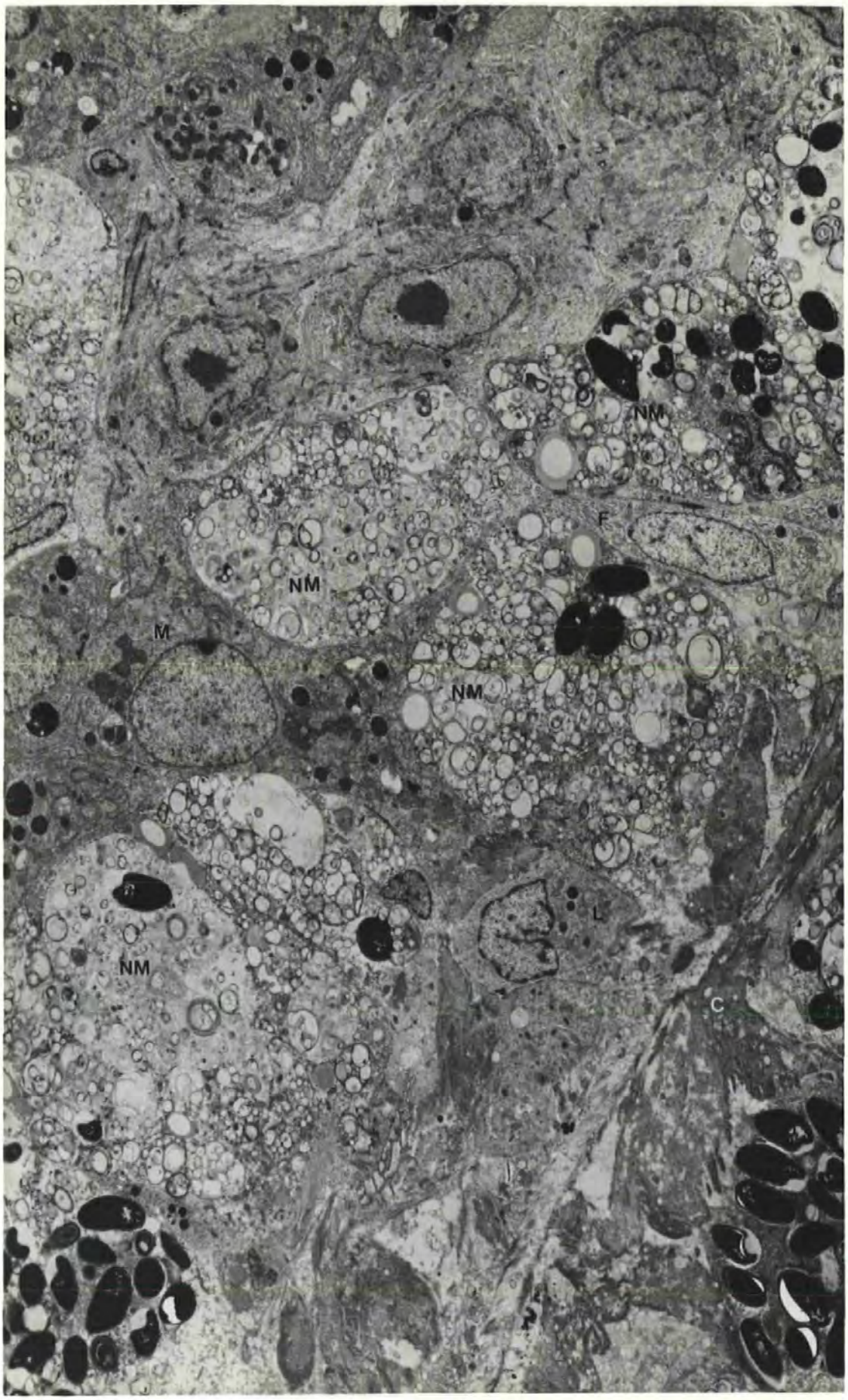


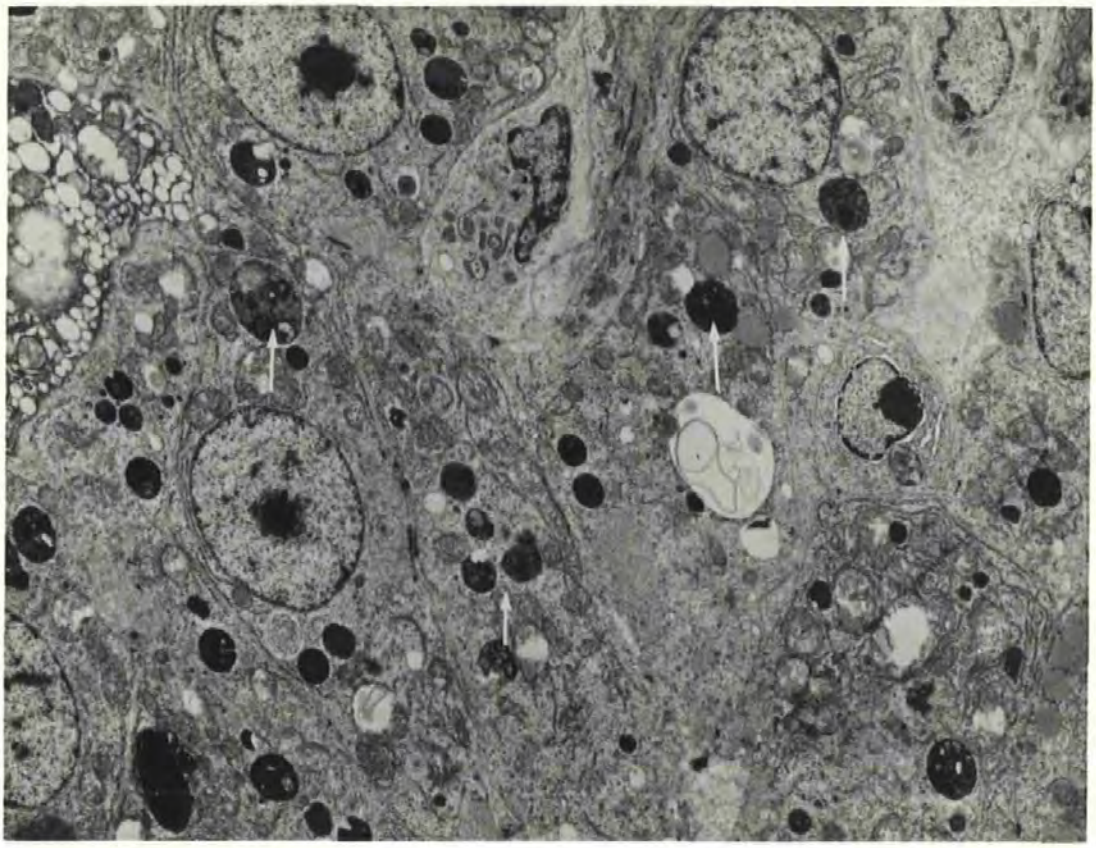
Fig. 80. Electron micrograph of necrotic centre of granuloma formed from *M. hepaticus* infection. x4,350. C, collagen; F, fibroblast; L, lymphocyte; M, macrophage; NM, necrotic macrophage.

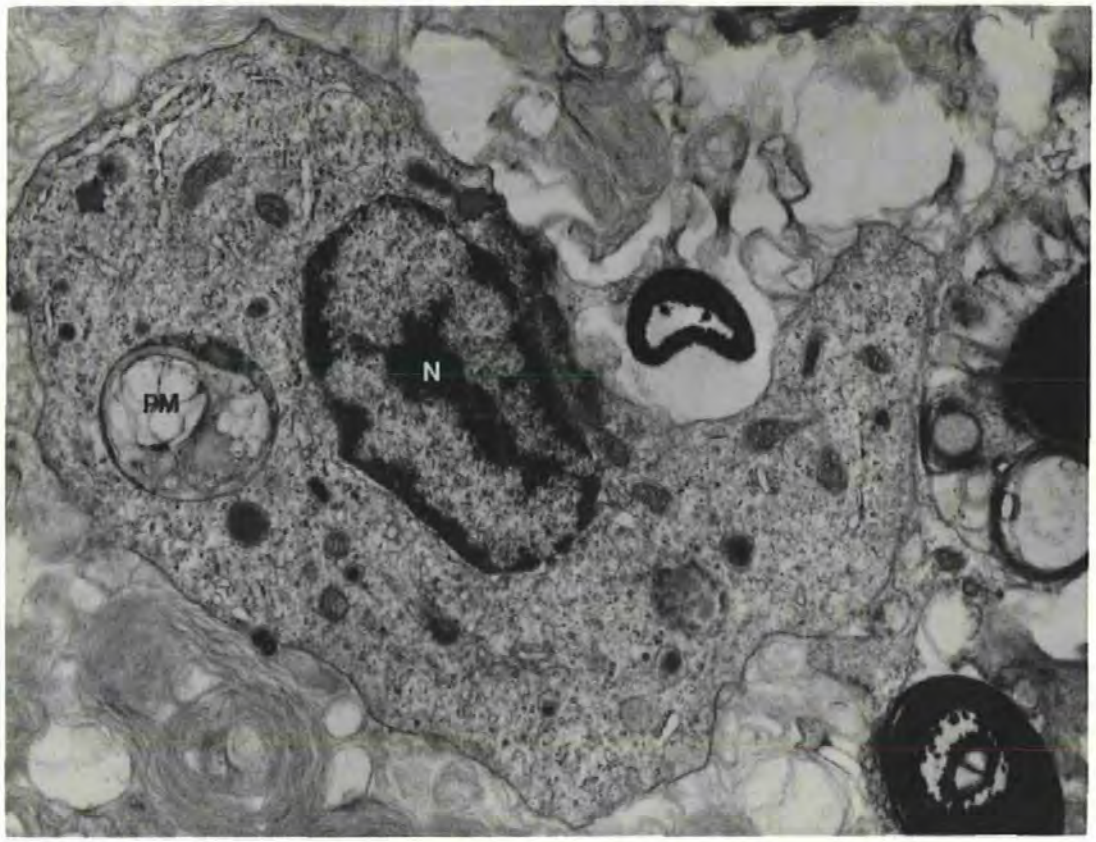

NM
F
NM
M
NM
L
NM
C

Figs. 81 and 82. Lymphocytes of the host response of C. labrosus to infection with M. hepaticus.

Fig. 81. Electron micrograph of the band of leucocytes at the periphery of granuloma. x5,130. Arrowed, phagocytic vacuoles.

Fig. 82. Electron micrograph of a monocyte from the granuloma. x14,500. N, nucleus; PM, phagocytosed material.

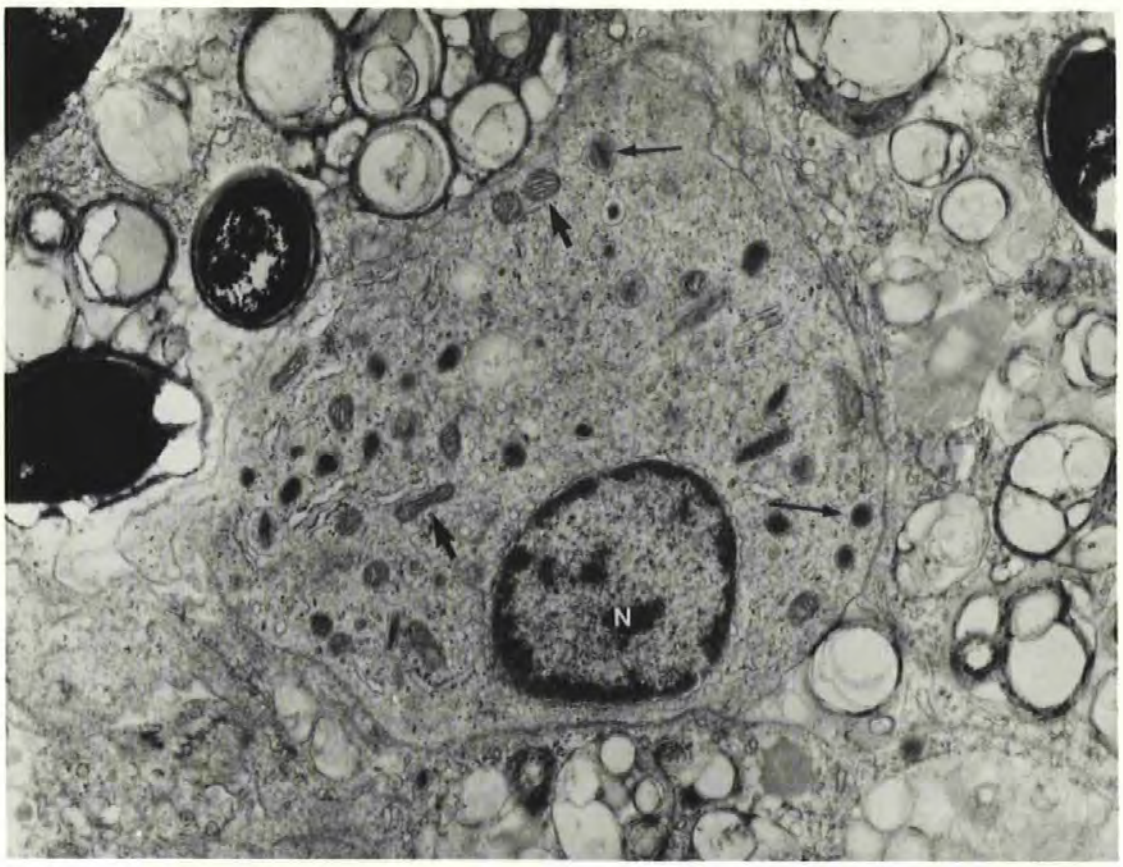



N
PM

Figs. 83 and 84. Lymphocytes from granuloma of *M. hepaticus* in *C. labrosus*.

Fig. 83. Electron micrograph of a heterophil. x14,600. N, nucleus; thin arrow, granules; broad arrows, mitochondria with longitudinal cristae.

Fig. 84. Electron micrograph of a heterophil. x14,600. N, nucleus; thin arrow, granules; broad arrows, mitochondria with longitudinal cristae.


N

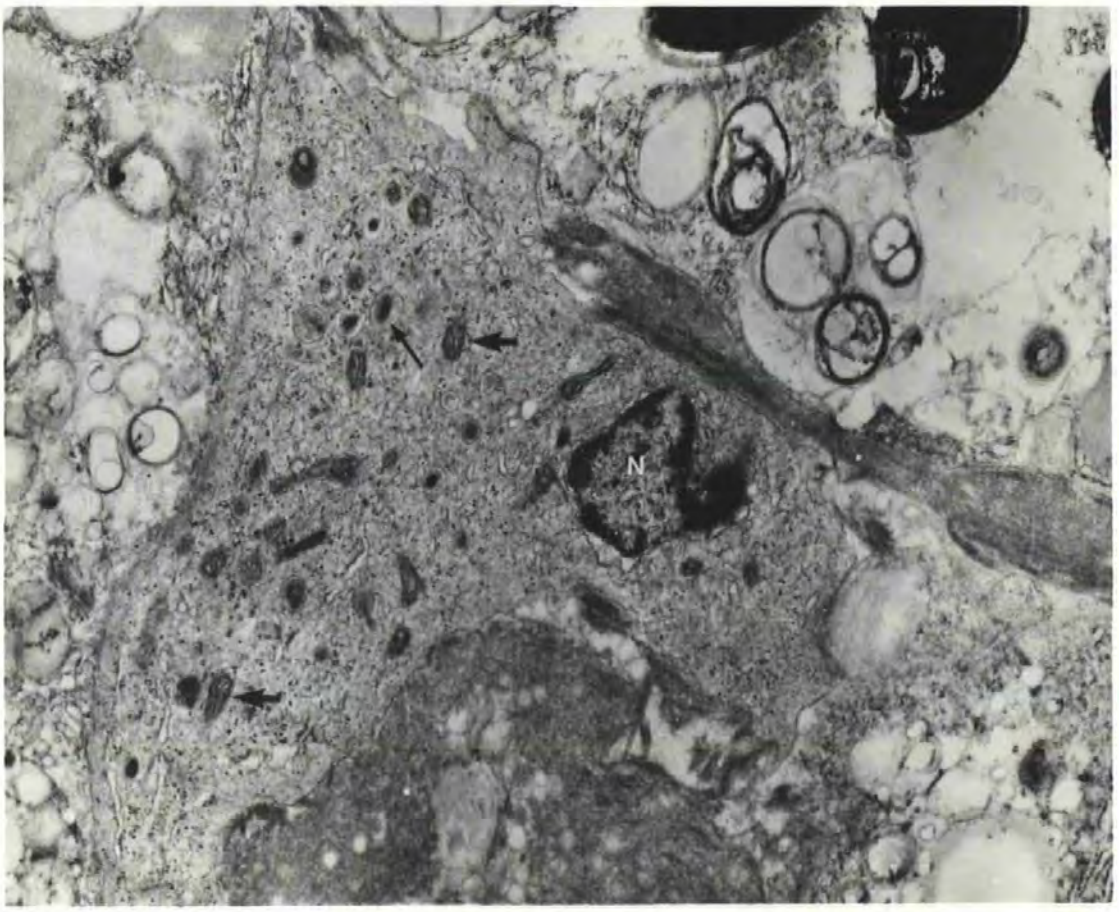
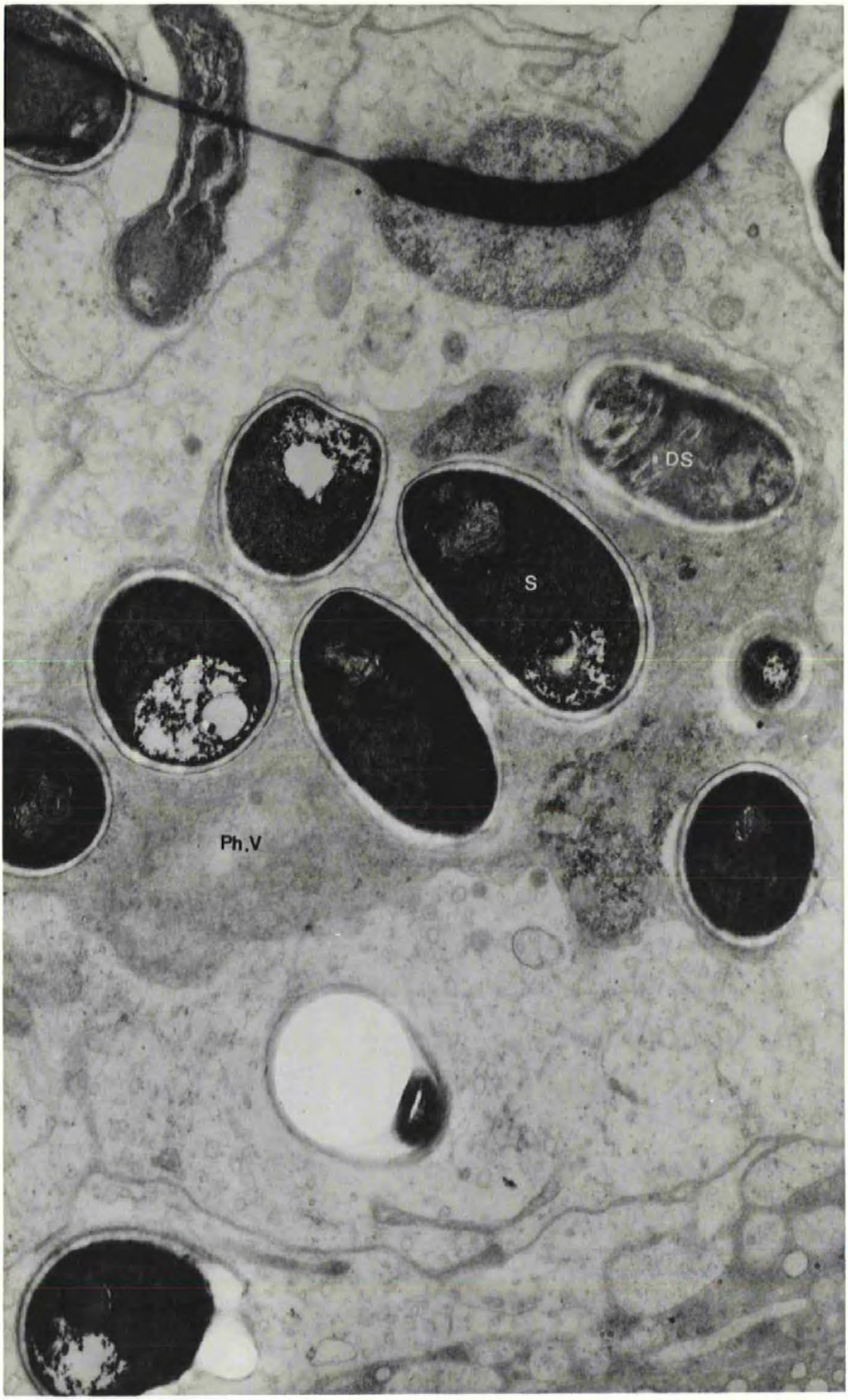

N

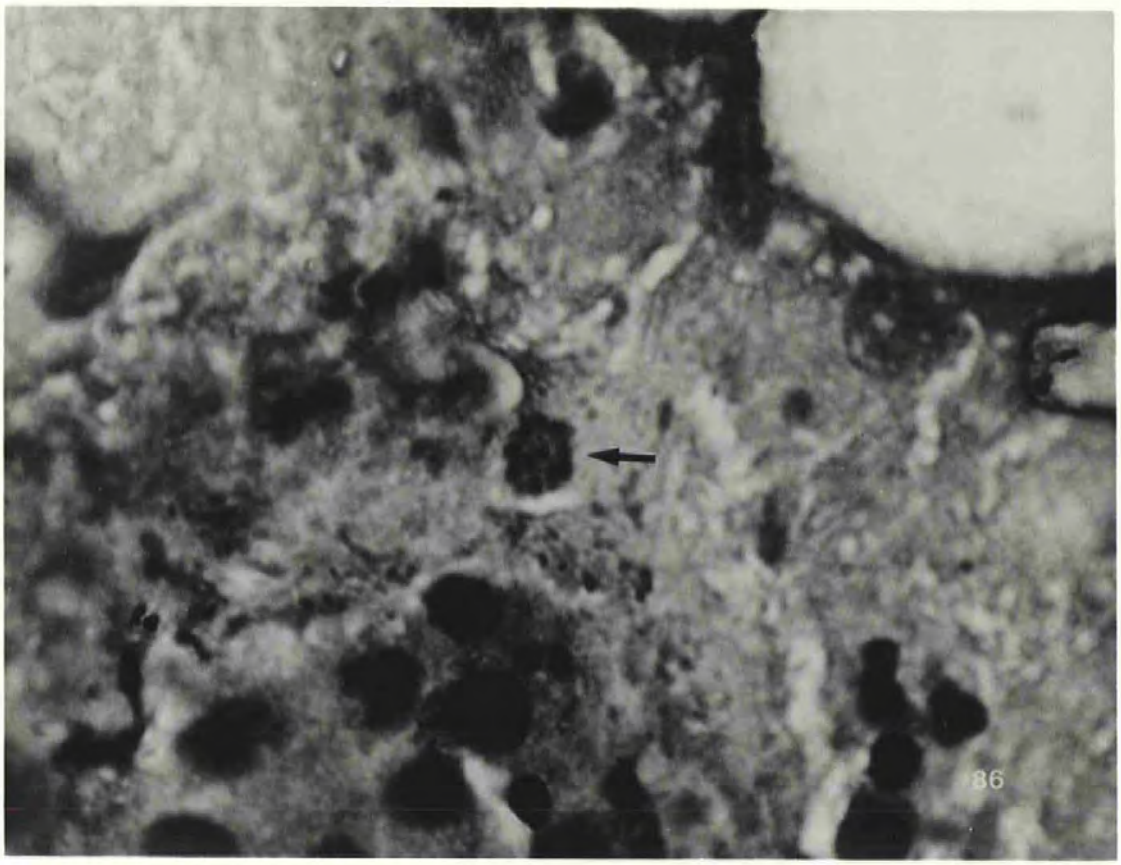
Fig. 85. Electron micrograph of phagocytic vacuole containing spores and degenerate spores of *M. hepaticus*. x20,700. DS, degenerate spore; S, spore; PhV, phagocytic vacuole.

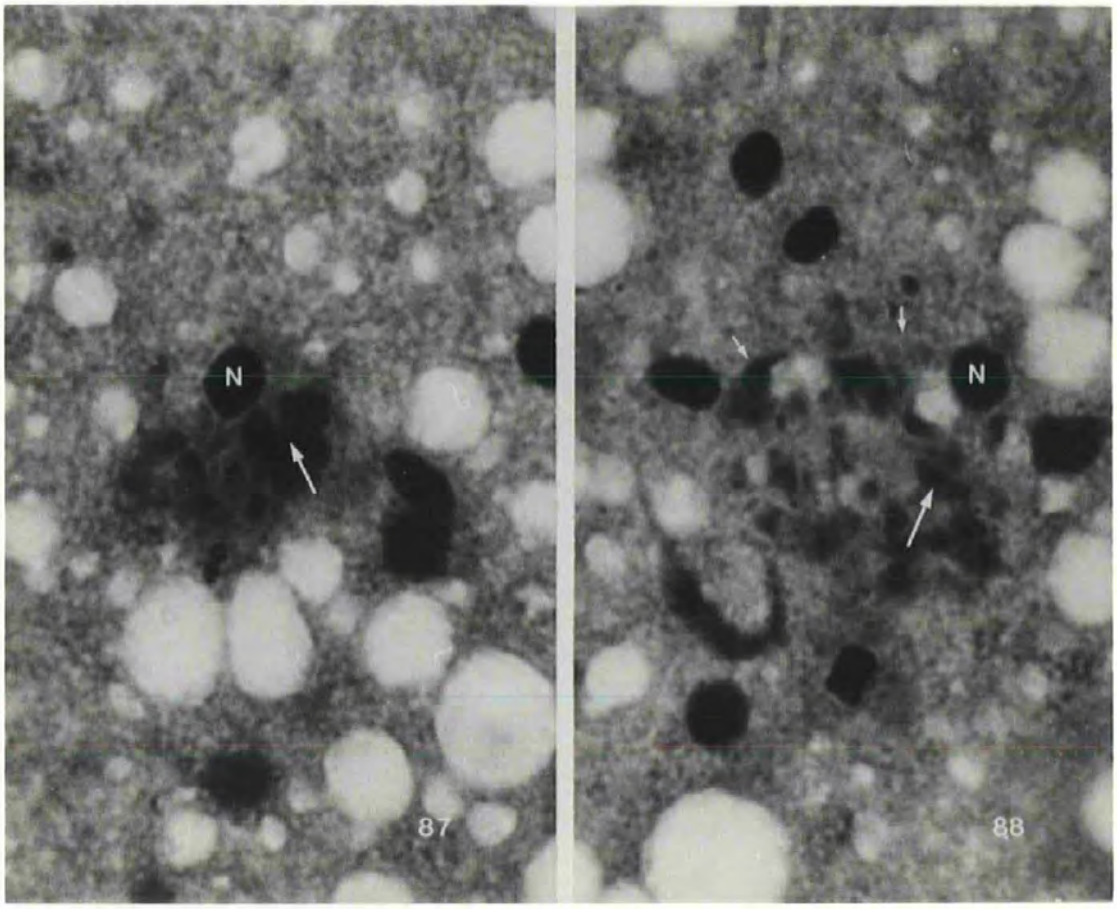

DS
S
Ph.V

Figs. 86-88. Intestinal and liver stages observed after experimental infections of *C. labrosus* with *M. hepaticus* (Experiment 1).

Fig. 86. Micrograph of smear preparation of duodenal wall 14 days post-infection. x9,300. Arrowed, multinucleate body. Giemsa staining.

Figs. 87 and 88. Micrographs of smear preparation of liver 35 days post-infection. x10,800. N, nucleus; short arrow, cell boundary; long arrow, phagocytosed spores. Giemsa staining.

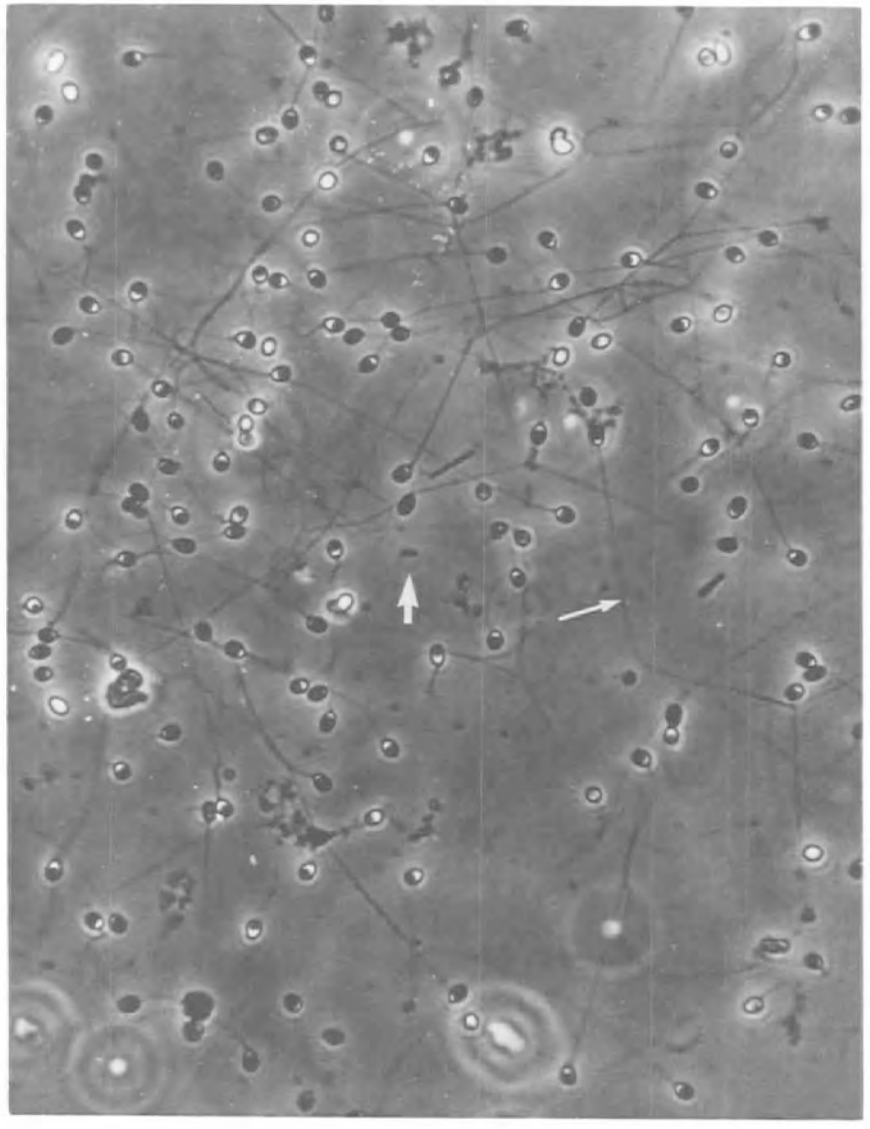

86

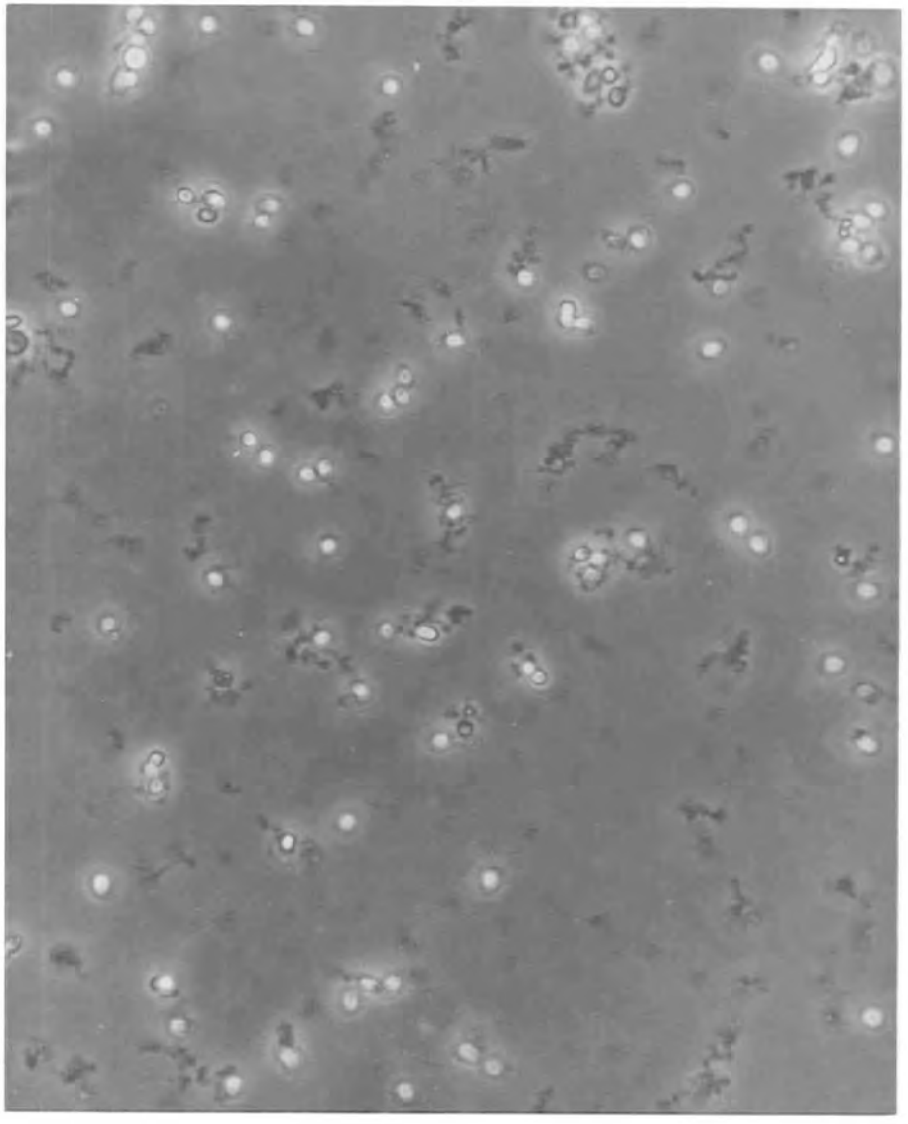

N
87
N
88

Figs. 89 and 90. Fresh spores of *M. hepaticus*.

Fig. 89. Micrograph of spores before treatment with hydrogen peroxide. x3,200. Phase contrast.

Fig. 90. Micrograph of spores stimulated to exsporulate by treatment with hydrogen peroxide. x3,200. Long arrow, extruded filament; short arrow, sporoplasm. Phase contrast.

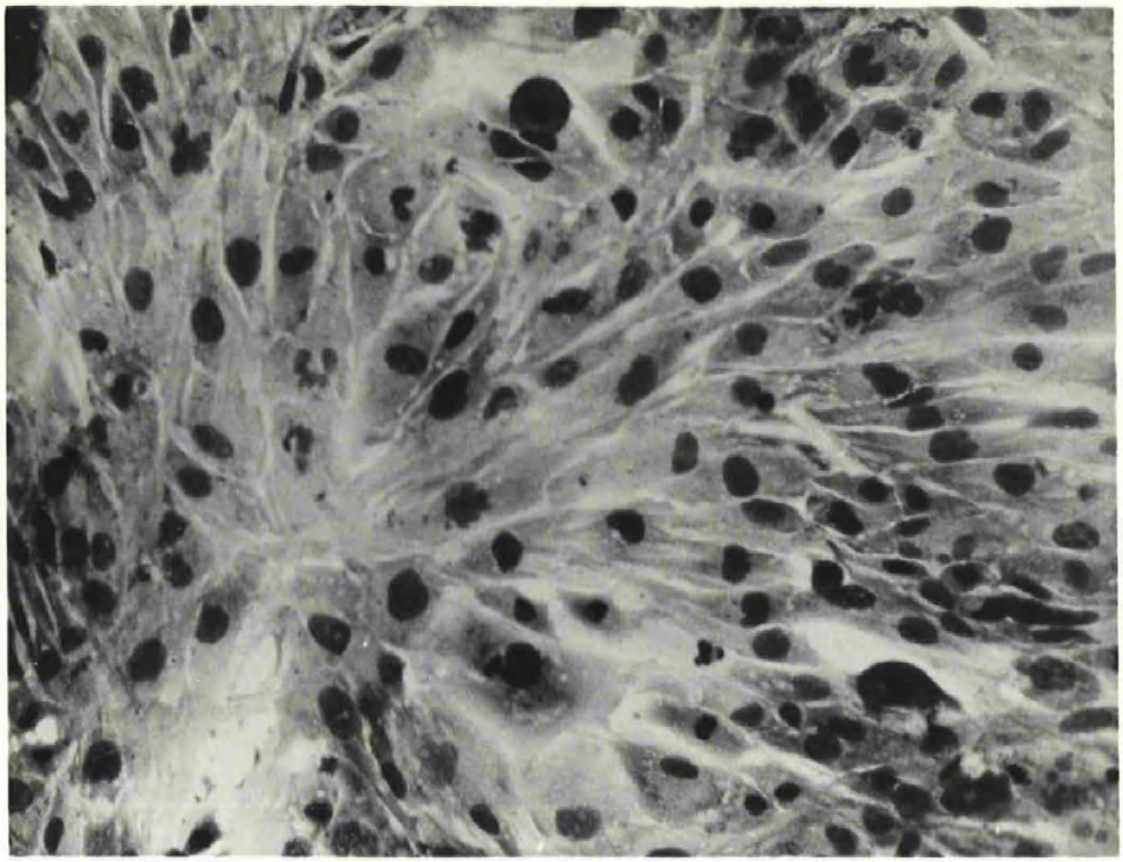


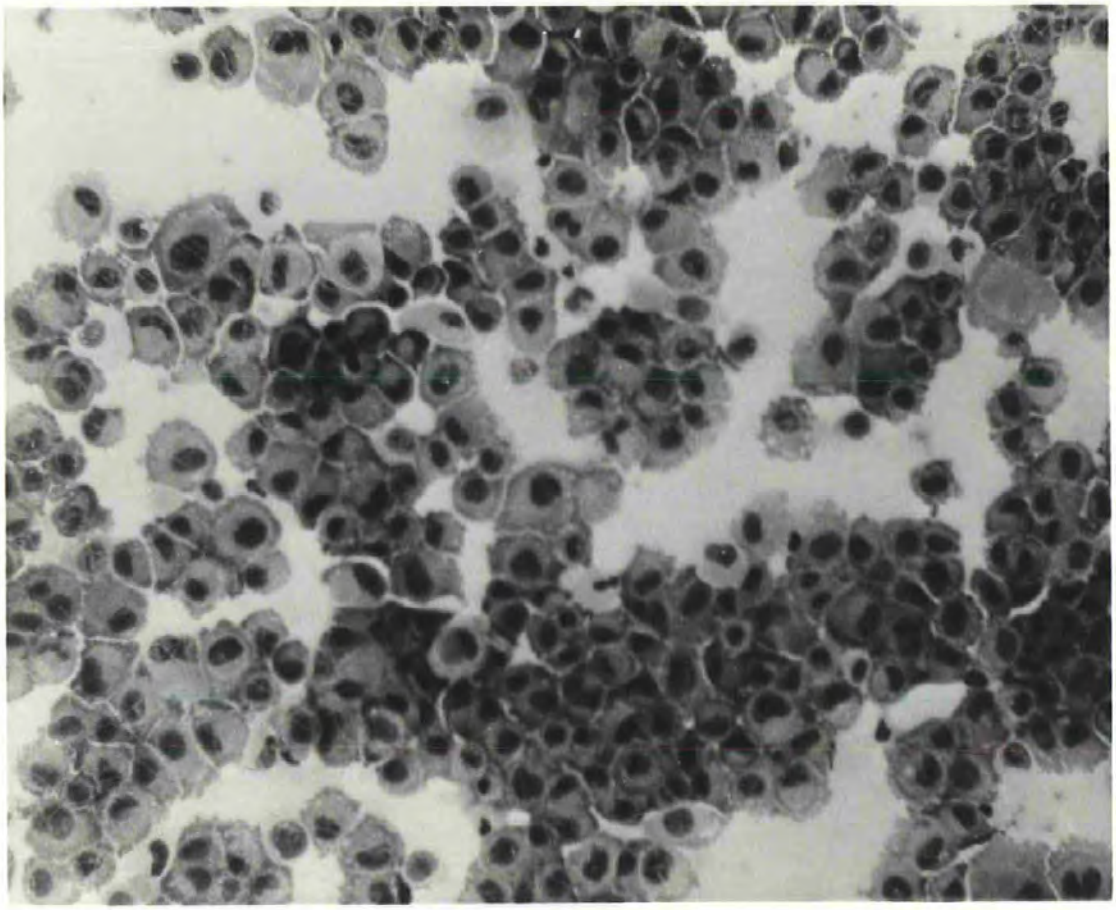


Figs. 91 and 92. RTG cell cultures.

Fig. 91. Micrograph of RTG cell monolayer. x1,110. Giemsa staining.

Fig. 92. Micrograph of RTG cell cytospin preparation from cell suspension. x1,100. Giemsa staining.

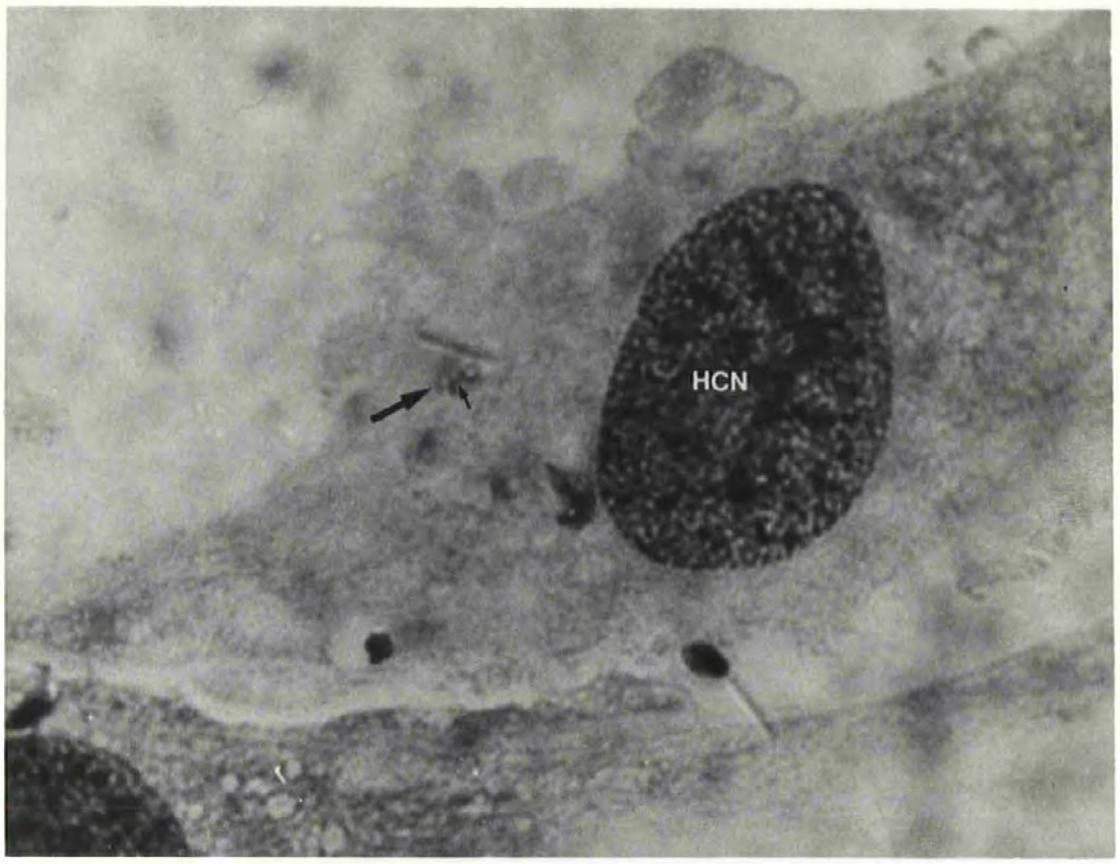


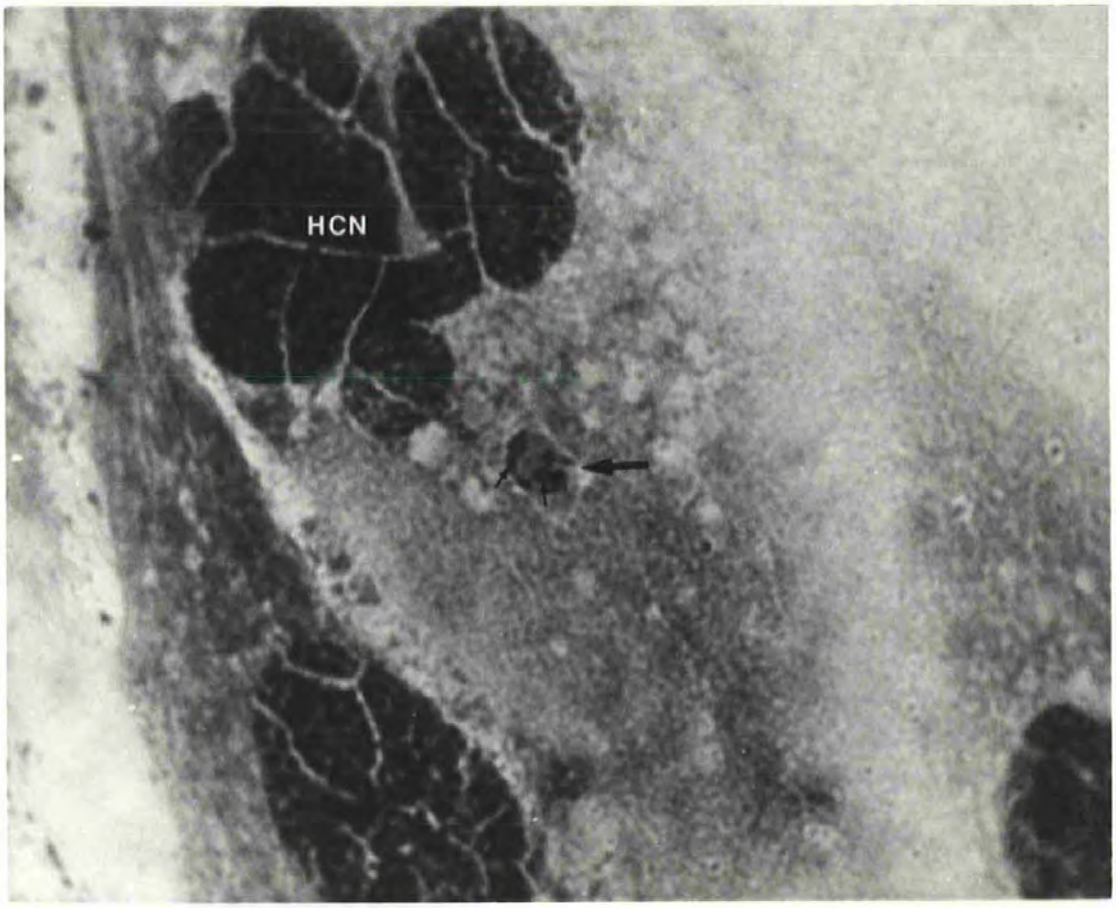


Figs. 93 and 94. RTG cell cultures infected with *M. hepaticus*. (Experiment 6).

Fig. 93. Micrograph of RTG cell containing injected sporoplasm. 30 min post-infection. x10,200. HCN, host cell nucleus; large arrow, sporoplasm ; small arrow, sporoplasm nucleus. Giemsa staining.

Fig. 94. Micrograph of RTG cell containing possible plasmodial stage. 72 hr post-infection. x10,200. HCN, host cell nucleus; large arrow, plasmodium; small arrows, nuclei.

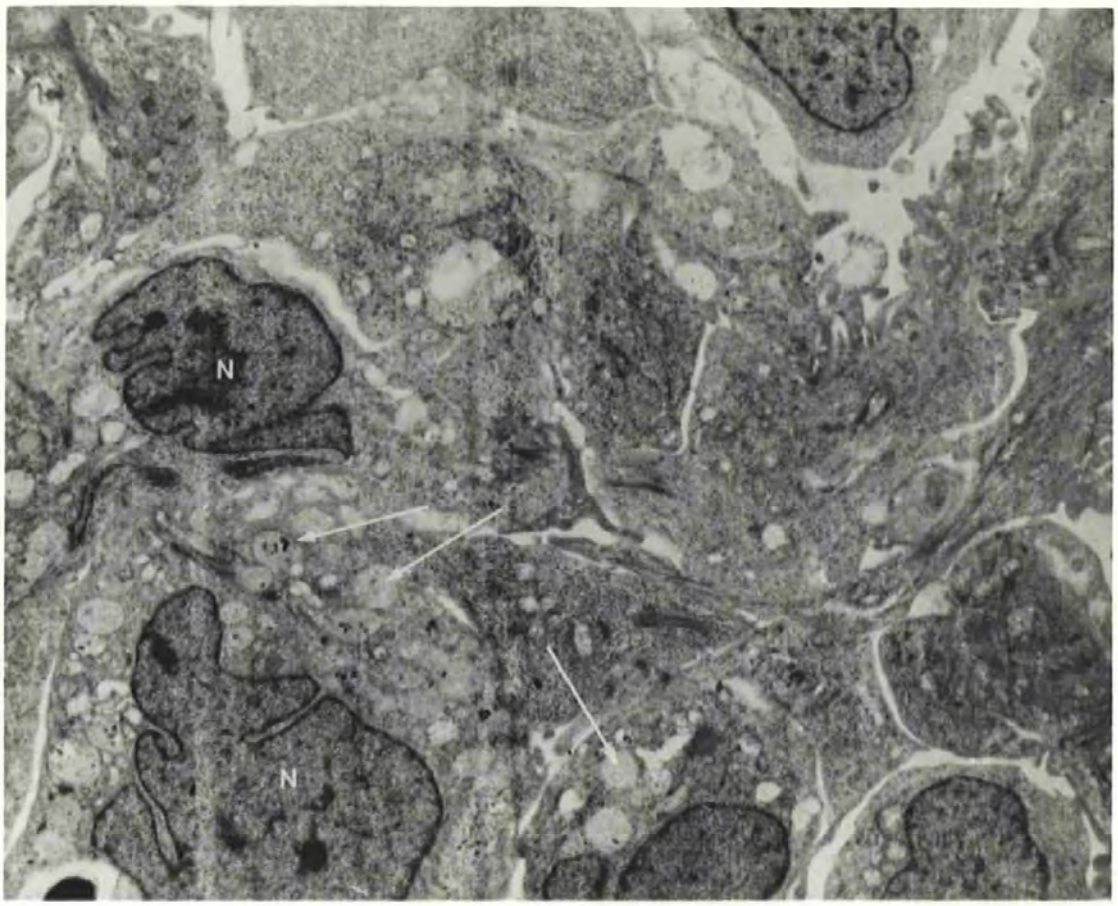

HCN

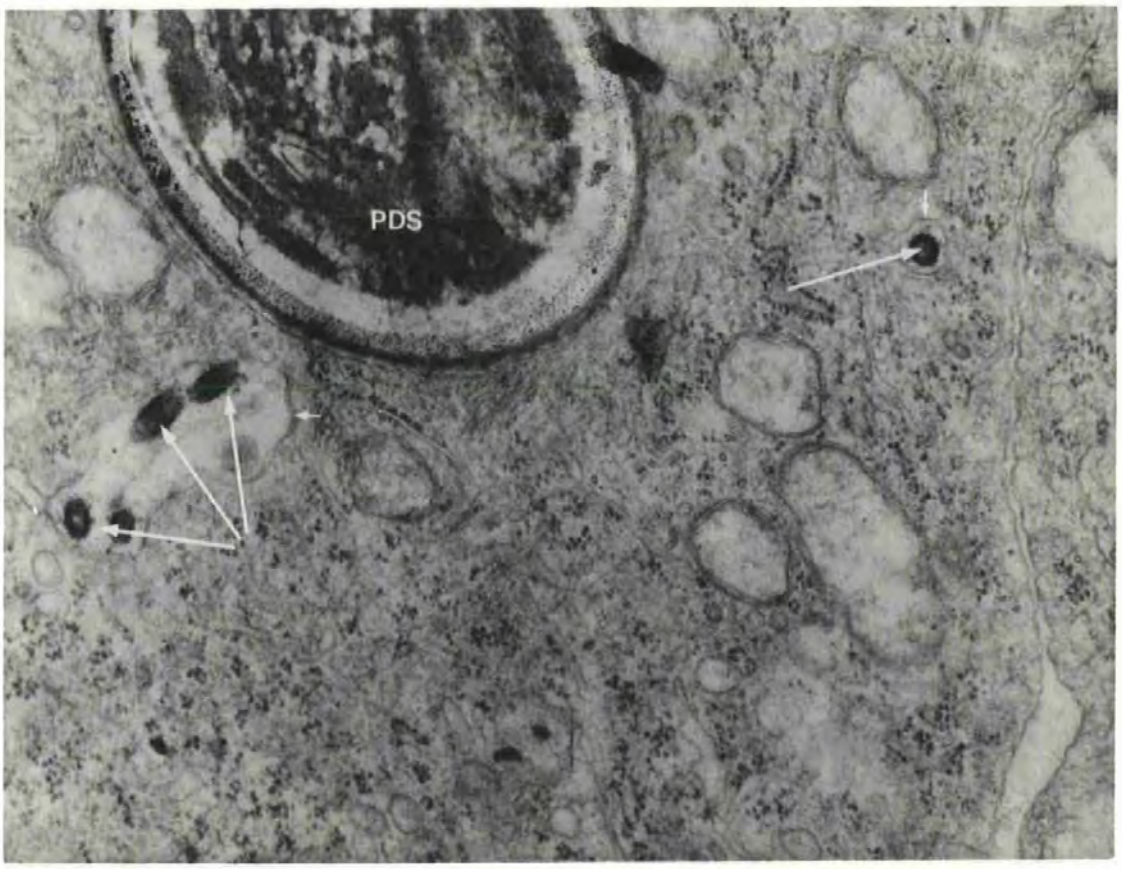

HCN

Figs. 95 and 96. RTG cell suspension cultures inoculated with spores of *M. hepaticus*.

Fig. 95. Electron micrograph of pellet of RTG cells. x7,170. HCN, host cell nucleus; N, nucleus; arrows, phagocytic vacuoles.

Fig. 96. Electron micrograph of RTG cell having extruded filaments of *M. hepaticus* passing through cytoplasm. x40,500. Large arrow, extruded filament in RTG cell; small arrow, filament surrounding membrane; PDS, Phagocytosed degenerate spore.

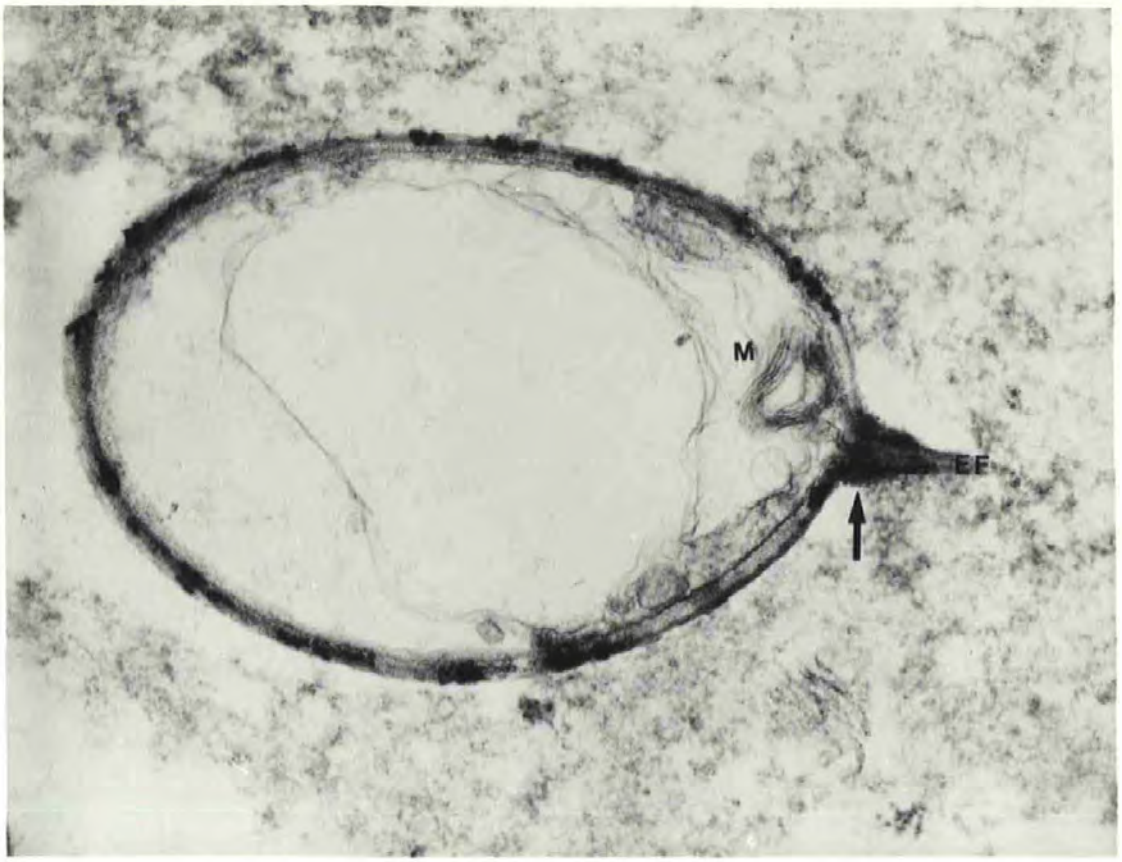

N
N


PDS

Figs. 97 and 98. Empty spore cases of *M. hepaticus* following filament extrusion.

Fig. 97. Electron micrograph of empty spore case. x32,250. EF, extruded filament; M, loose membranes within spore case; arrow, electron-dense material around base of extruded filament.

Fig. 98. Electron micrograph of extruded spore, with prominent residual membranes. x46,530. EF, extruded filament; M, residual membranes.


M
EF

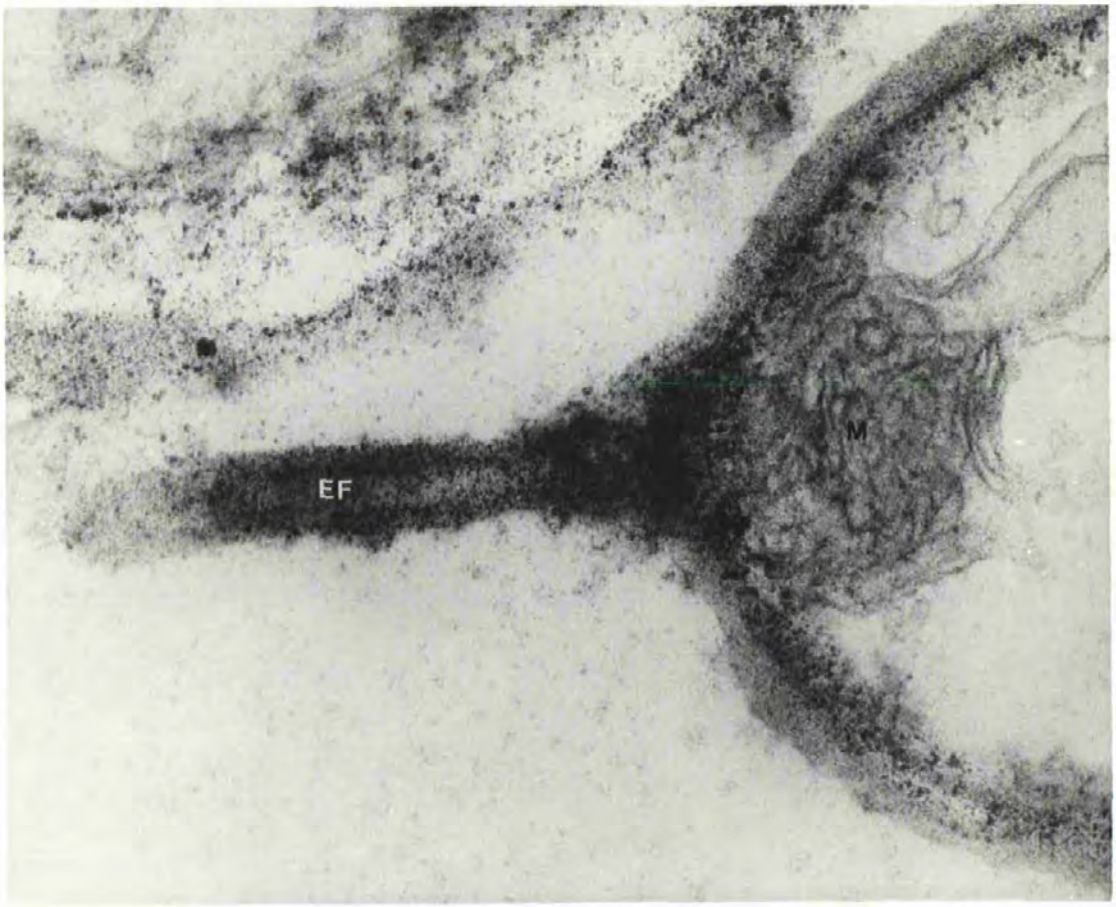
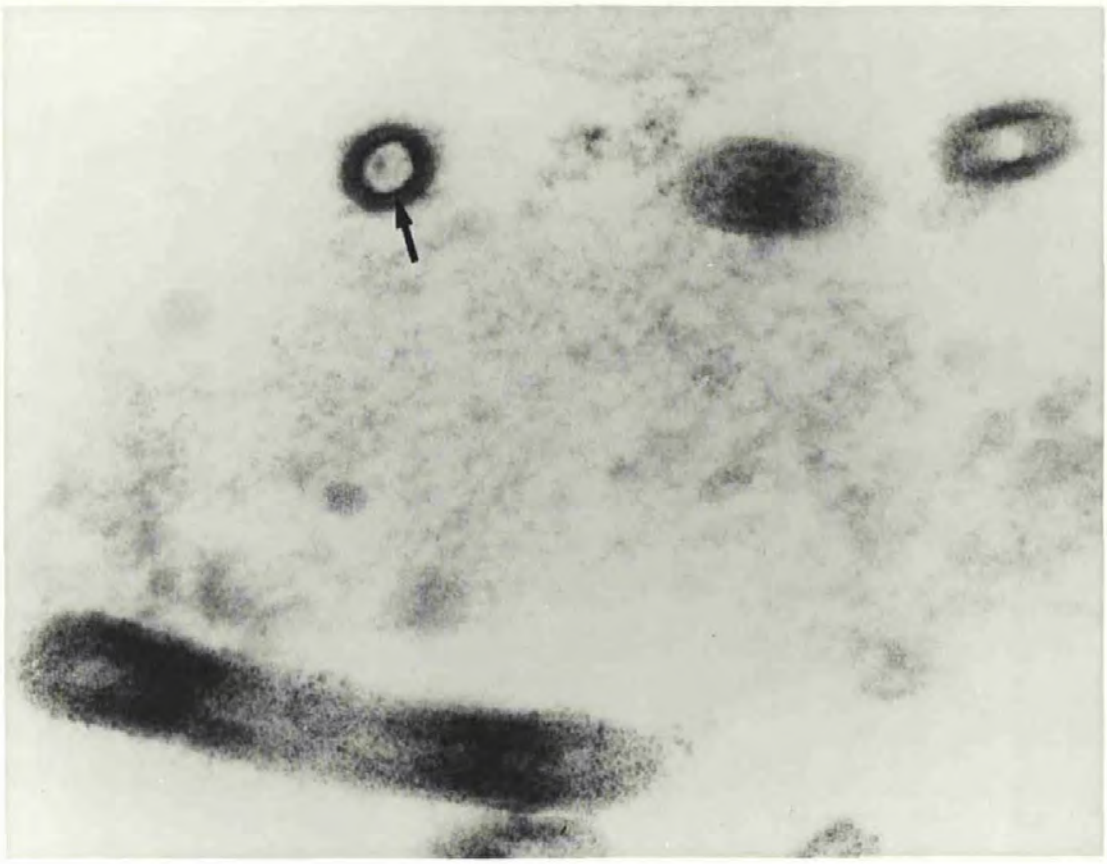

EF
M

Fig. 99. Electron micrograph of extruded polar filaments of *M. hepaticus*. x109,200. Arrow, membrane at inner surface of extruded filament.



DISCUSSION

Life history of *Microgemma hepaticus*. It is generally considered that microsporidia are transmitted directly from fish to fish, either as free spores or via invertebrate transport hosts (Weissenberg, 1968; Weidner, 1973; McVicar, 1975; Olson, 1976; Scarborough and Weidner, 1979). Attempts here, however, to experimentally infect '0'-group mullet by administration of spores *per os* suggest that more complex factors than simple ingestion of spores may be involved in transmission of *M. hepaticus* to *C. labrosus*. One of the problems involved in experimental infections is the dependence upon fishes obtained from the wild and thus the uncertainty of obtaining fish naive to the disease to be investigated. In consequence, although the rare intestinal forms observed in experimentally infected fishes could be interpreted as multinucleate microsporidian stages (Fig. 86) it is not possible to state that these were the result of the infection procedures. In previous successful experimental infections of fish via the oral route (Weissenberg, 1968; McVicar, 1975; Olson, 1976; Scarborough and Weidner, 1979; Dykova and Lom, 1979), fish became heavily infected as a result of infection procedures. It is probable, therefore, that the intestinal stages encountered here were the results of natural infections with protozoan parasites, and not experimental procedures. The spore containing cells encountered in the livers of some fishes (Figs. 87, 88) could be interpreted as early stages of infection; however, there were no obvious changes induced in the "host" cells and they did not resemble early stages observed in wild infections (Figs. 67-75). It seems likely, therefore, that spore containing cells in the livers of experimental fishes were phagocytes containing spores from old, degenerate infections. Phagocytosis of spores is a feature of the host response

of mullet and has been reported in the host responses of many other fish species to microsporidian infection (reviewed by Dykova and Lom, 1980).

In assessing the failure to experimentally infect fish in the present study it is necessary to discuss parasite viability, host susceptibility, environmental conditions and the possibility of cyclical development involving a second intermediate host.

The viability of spores extracted from mullet livers is likely to be affected by three factors: the progress of the host response in destruction of spores and spore age; the extraction technique used in the preparation of spore suspensions; and the conditions and length of time of storage of spore suspensions. Electron microscopy revealed spore damage caused by the host response in old infections. However, spore suspensions used in experiments were prepared from fish obtained in the autumn and winter, when relatively few degenerate infections were encountered. A proportion of spores may have been non-viable owing to host responses, but the majority would be expected to be unaffected. The techniques used in the extraction of spores (Weidner, 1972) resulted in suspensions of spores having identical appearance to those observed in fresh squash preparations; they also responded similarly to artificial exsporulating agents. The technique was used by Matthews and Matthews (1980) in the successful infection of turbot with Tetramicra brevifilum, and thus results in viable spore suspensions. Mullet were also fed here with fish liver containing M. hepaticus with no infections resulting, even with these untreated spores. Little information is available on the storage of fish microsporidian spores, although Olson (1976) achieved infections with Glugea stephani using spore suspensions stored for one month at room temperature, and Dykova and Lom (1978) with Glugea

anomala after "several months" storage at 4°C. Certain microsporidia of insects have been shown to retain viability in sterile chilled suspension for several months (Kramer, 1970; Maddox, 1973; Oshima, 1973) or even years (Undeen, 1983). It seems likely that the storage conditions used here for M. hepaticus would similarly not effect spore viability.

Experimental factors governing the effectiveness of transmission of M. hepaticus are the method of administration of spores, and the temperature at which the fish were exposed to the infection. The methods of spore administration used here have been used successfully in several experimental infections of fish (Weissenberg, 1968; McVicar, 1975; Olson, 1876; Dykova and Lom, 1978; Scarborough and Weidner, 1979). The importance of temperature in transmission has been reported by McVicar (1975) and Olson (1976), who noted that fish had to be exposed to spores of Glugea stephani at temperatures below 15°C if transmission was to occur. In the present study 12°C was selected, this being related to sea temperatures expected to prevail during exposure of mullet to the disease in the natural environment.

A further possibility is that the fish used in experiments were insusceptible to further infection. High susceptibility of juvenile fishes and low susceptibility of adults is a common observation (Stunkard and Lux, 1965; Legault and Deslile, 1967; Weidner, 1973; Scarborough and Weidner, 1979) and it may be that the young mullet here, although frequently carrying active infections, were insusceptible to further infection. It might be postulated that insusceptibility of older fishes is due to protective acquired immunity; however such a mechanism seems unlikely. There was evidence that the infection was only antigenic when old xenomas

degenerated and parasite stages were exposed. As all active parasite stages are intracellular, newly infected cells would probably not be recognised. More likely causes of insusceptibility are innate factors, which could be changes in intestinal physiology or structure resulting in either a loss of the stimulus for filament extrusion or the prevention of the extruded filament from penetrating suitable host cells. Shehadeh et al. (1972) reported dietary change in the Egyptian grey mullet Mugil cephalus from larval feeding on zooplankton to juvenile and adult feeding on plant material. Such a change, if present in C. labrosus, could result in changes in intestinal physiology and the possible loss of the stimulus for exsporulation of M. hepaticus. In support of this view is the report of Weidner (1973) who demonstrated that intestinal conditions of 4-6 year old examples of host species of Glugea stephani and Glugea hertwigi would not stimulate filament extrusion. Alternatively, if filament extrusion was stimulated, filament penetration to suitable host cells could be prevented mechanically by increased intestinal wall thickness, mucus secretion or by a changed distribution of suitable host cells. These could be reduced in number or positioned differently, reducing the probability of penetration by the polar filament, or could be absent altogether. The susceptible cells could be embryonic in nature, such as the mesenchyme cells of Weissenberg's (1968) infections of stickleback larvae with Glugea anomala, and thus could be less common or absent in older fishes.

The advantage to the parasite of only infecting juvenile fish can be discussed in environmental and population terms. Samples of juvenile mullet from St. Johns lake indicate a population infection rate of 38%. On bringing live specimens back to the laboratory it was frequently found that those with heavy infections were amongst

the first to die, suggesting that these were more vulnerable to stress situations. It is likely therefore that the incidence of infection in the 'O'-group population is considerably higher than the 38% observed in samples. Heavy mortalities due to microsporidian infection in juvenile fish have been noted by many workers in the past (Haley, 1953; Stunkard and Lux, 1965; Weidner, 1973; Olson, 1976; Scarborough and Weidner, 1979) and similar intensities of infection in non-lethal infections by Summerfelt and Warner (1970). In all these reports, infections were present at low prevalence in the adult population. McVicar (1975) and Takvorian and Cali (1981) also observed low prevalence in adult hosts. Few adult mullet have been examined here, chiefly because of difficulty in netting. It thus remains possible that a similar age-related distribution of M. hepaticus is present in the wild population of C. labrosus with the rare adult infections not being detected owing to the small number of adult fish screened for infection. However, in view of the fact that spores can only be released on the death of the host fish, it is of advantage to the parasite to infect the juvenile population, owing to the natural high mortality rates in this age group. M. hepaticus infection may even contribute to larval mortality as liver function in these hosts could be impaired by small numbers of xenomas. Infections of adult fishes, even if possible, would be disadvantageous as these would be lost to the parasite population. The low death rates of adult fishes and the progress of the host response would result in a high probability of spore destruction before host death.

After their release on the death of the host, spores must be ingested by new hosts. In view of the small size of spores, it seems unlikely that the ingestion of free spores in the environment is the

major mode of infection; more likely is the concentration of spores in filter feeding prey organisms of the target hosts. The effectiveness of crustacea as transport hosts for fish microsporidia has been shown in experimental infections by Weisenberg (1968), Olson (1976), Dykova and Lom (1978) and Scarborough and Weidner (1979). In the case of M. hepaticus, such an arrangement would tend to direct spores towards the larval mullet. Kuo and Shehadeh (1972) showed that larval mullet feed exclusively on zooplankton, and would thus tend to ingest more spore carrying transport hosts than the largely herbivorous juveniles and adults (Shehadeh et al., 1972). These latter would still tend to ingest such hosts accidentally, along with their more usual algal food, but would presumably be exposed to infection to a lesser extent than the larval fish.

Once M. hepaticus is established in the new generation of larval mullet, host to host transmission is easily explained by spore release following host mortality. However, there must be an initial input of M. hepaticus into the new host population. Spores cannot persist in fish hosts over the period between the presence of the susceptible, probably larval, mullet owing to their destruction by the host response, and must therefore persist in the environment. With the spawning of C. labrosus in April and May (Bagenal, 1973) and the presence of fish from September until May at St. Johns lake, it can be assumed that spores have to persist in inshore waters at least from May, when the fish leave, until September, when the next generation arrives. In the absence of successful transmission of M. hepaticus experimentally, viability was assessed on the basis of spore integrity and filament extrusion. Although detailed longevity experiments were not performed, spores of M. hepaticus maintained integrity and ability to exsporulate for at least four months. As

previously discussed other microsporidian species have been shown to remain viable for longer periods under experimental conditions. It thus seems feasible that spores of M. hepaticus from infections of one generation of larval mullet could persist in the environment and provide the initial source of infection for the following generation of larvae. Cerkasova and Vavra (1972) detected a low level of respiratory activity in microsporidian spores, and hence a gradual decline in spore viability might be expected with time. However, it is probable that in a suitable region for transmission, such as the estuarine nursery habitat, a small input of viable spores is all that would be required to establish M. hepaticus in the new generation.

Observations suggest that M. hepaticus is primarily a parasite of larval or juvenile fishes, is probably transmitted via transport hosts, and spores persist in the environment between the appearance of generations of larval mullet. Thus, ideal sites are those where larval fishes tend to congregate, mortalities resulting in the release of spores and the accumulation of spores in the benthic environment within filter feeders and amongst general debris. Such sites can be regarded as being primed with M. hepaticus every winter in preparation for the appearance of the new generation of fishes the following year. It might be considered therefore that nursery grounds might form the ideal transmission sites, with a high mortality rate and a good chance of spore release and the exposure of a susceptible population to infection. Although St. Johns lake is a nursery area for the post-larval fish, experimental studies on transmission suggest that even at this stage fish were becoming refractory to infection. This might be due to experimental technique, as discussed earlier; alternatively it might indicate that the fish become infected before their arrival in the St. Johns

area. As previously noted, spawning occurs in the vicinity of the Scilly Isles during April and May (Bagenal, 1973) . Under experimental conditions it has been shown that mullet eggs and larvae are free-floating (Kuo and Shehadeh, 1972); and thus in the wild are probably pelagic. Dispersal eastwards from the spawning sites would therefore be dependent on tidal currents. The distance moved up channel by larval fishes is therefore related to time after spawning, and hence to fish age. Assuming this, larval fishes in more western waters are likely to be younger than those found in the Plymouth area. As discussed previously, two factors are believed to operate in reducing fish susceptibility with age, namely changes in feeding behaviour and intestinal physiology, and possible changes in intestinal structure. Whilst changes in feeding behaviour from zooplanktonic feeding to algal feeding may reduce exposure to spores, this is probably not the major factor operating, as accidental ingestion of spores must be a common occurrence amongst algal feeding mullet at a site like St. Johns. The innate factors may, however, explain the apparent insusceptibility of fishes at St. Johns, if it is assumed that the average time taken for fishes to migrate from the spawning grounds to the nursery grounds in the Plymouth area is greater than the time required for the innate resistance factors to develop. A further factor is that the density of potential hosts is probably reduced the further the larvae are dispersed from the spawning grounds. Although the effects of host density changes may be reduced by shoaling behaviour, by the time the fish arrive in the Plymouth area age related reduction of host susceptibility coupled with reduced host density probably results in the cessation of parasite transmission. The rare examples of larval mullet encountered at St. Johns can probably be explained by chance

acceleration of the passive migration process resulting in the occasional appearance of recently infected younger fishes.

Passive persistence of spores in the environment may seem a rather haphazard method of parasite transmission when susceptible hosts are only present for a short period each year, although the enormous reproductive capacity of M. hepaticus probably overcomes this. It is, however, tempting to speculate that M. hepaticus may persist in the environment as a parasite of other hosts. Hepatic microsporidiosis has been observed in North Cornwall in juvenile turbot (Matthews pers. comm.) and in cottids, rocklings and wrasses in the Plymouth area (Stubbs pers. comm.). Certain fish microsporidia have been reported from several host species, for example Glugea stephani from a variety of pleuronectid flatfishes (Stunkard and Lux, 1965; McVicar, 1975; Olson, 1976) and Pleistophora hyphessobryconis from various aquarium fishes (Schaperclaus, 1941). In addition, Weidner (1973) showed that infections of Glugea stephani and G. hertwigi were possible in gammarid crustacea and suggested that these acted as reservoirs of infection and initiated yearly epizootics amongst juvenile fishes. It thus seems possible that various species act as hosts to M. heapticus and a range of fishes become infected when they happen to enter parasite-rich areas at a susceptible age. A more intriguing speculation would be that a genuine developmental phase occurs outside of the mullet host, in another species, resulting in a genuine two-host life cycle. Two cycles have been shown to occur amongst dimorphic insect-infecting microsporidia (e.g. Keller et al., 1966; Hazard and Weiser, 1968; Pilley, 1976; Andreadis, 1983). It could be that the developmental sequence described here from C. labrosus represents only one half of the life cycle of M. hepaticus,

and that the spores are only infective to an as yet unidentified second host. Further speculation is difficult, but such a host could be another fish species, or perhaps an invertebrate. Experimental infection systems would be required to investigate the interrelationships of the microsporidian infections present in the inshore environment.

On ingestion of spores, it is assumed that factors in the digestive tract of the fish host initiate exsporulation, with filament extrusion and injection of sporoplasms into the epithelium or lamina propria. Although these factors remain unknown *in vivo*, it is probable that physico-chemical factors are involved as *in vitro* filament extrusion was induced by strong oxidising agents, rapid pH change from acid to neutral conditions and rapid decrease in osmolarity. Physico-chemical characteristics have also been implicated in the exsporulation of insect microsporidia, with the stimulatory effects of alkali metal ions and pH (Ishihara, 1967, 1969; Undeen, 1978, 1983). The influence of alkali metal ions appears to be different in fish microsporidia, however, as external calcium is inhibitory to exsporulation in *Glugea hertwigi* (Weidner and Byrd, 1982). Investigation of reports of the requirements of other parasitic groups for the triggering of the infection process indicates the tendency for individual groups to utilise a particular set of trigger factors : thus microsporidia tend to respond to physico-chemical characteristics such as pH and metal ions; metacercariae of trematodes to bile salts, redox potentials and CO_2 tension (McDaniel, 1966; Dixon, 1966; Smyth and Halton, 1983 review); cestodes to enzymes and bile salts (Smyth, 1969 review) and nematodes to redox potentials and CO_2 tension (Rogers, 1966 review). One might expect, therefore, *M. hepaticus* to be more responsive to

factors such as pH, osmolarity and oxidising agents than to biochemical or enzymatic factors.

It has long been known that oxidising agents will induce exsporulation of microsporidia (Thelohan, 1895; Kudo, 1918; Oshima, 1927, 1937; Lom and Vavra, 1963). Experiments here indicate that the level of exsporulation induced is approximately proportional to the oxidising strength of the solution; the inhibition of exsporulation by high concentrations of iodine water and sodium hypochlorite is probably due to toxicity. Investigations were performed using biological oxidising agents in attempts to find suitable triggers that may be equivalent to those present in the host. Exsporulation rates obtained using dehydro-ascorbic acid were similar to those induced by equivalent concentrations of hydrogen peroxide; however the levels of dehydroascorbic acid necessary to produce a reasonable response would not be present in the natural situation. It would appear from the work of many authors that oxidising agents, notably iodine water and hydrogen peroxide are non-specific stimulators of exsporulation, both insect and fish parasites responding to treatment. However, it is unlikely that oxidising conditions are the true triggers of exsporulation in the wild state, a view supported by Lom and Vavra (1963) who suggested that the specific stimulus for exsporulation within the host intestine was substituted by hydrogen peroxide. Comparison of these observations with the work of Weidner and Byrd (1982) may indicate that treatment with oxidising agents substitutes for the natural trigger in causing displacement of calcium from the polaroplast.

In view of the effectiveness of alkali metal ions in exsporulating insect microsporidia (Ishihara, 1967, 1969; Undeen, 1978, 1983) similar effects might be expected with *M. hepaticus*.

However, in the solutions of sodium chloride and potassium chloride used as pretreatment media no exsporulation occurred; subsequent filament extrusion can be ascribed to osmotic effects. The sensitivity of insect microsporidia to these conditions emphasises the precise tailoring of a parasite transmission stage to its host environment; the conditions are presumably absent in the fish intestine, or masked by stronger stimuli, and are thus not utilised by M. hepaticus. This view is supported by Weidner and Byrd (1982), who showed that external calcium was inhibitory to exsporulation of the fish parasite Glugea hertwigi, and other metal ions were inert.

As previously stated, bile salts, pepsin and trypsin act as triggers for the excystment of many helminth metacercariae, including those from fish hosts (McDaniel, 1966; Dixon, 1966; Smyth and Halton, 1983 review); none of these were successful in exsporulating M. hepaticus. However, phase darkening of spores following treatment with mullet bile and mammalian bile salts indicates that the spore is permeable to bile components, even if these are not sufficient to induce filament extrusion. The effects of chitinase on the spore wall confirms the presence of chitin in fish microsporidian spores (Erickson and Blanquet, 1969). Chitinase has been demonstrated in the intestine of the grey mullet Mugil cephalus as a product of the intestinal flora (Hamid et al., 1979) and in the sea bream Acanthopagurus schlegedi (Sera and Okutani, 1968) and would thus be a potential trigger here. However, as with the other enzymes tested, chitinase was not directly involved in the exsporulation process, although if as suggested by Lom and Vavra (1963) and Undeen (1978) exsporulation follows a weakening of the polar cap it is conceivable that the presence of chitinase might aid the exsporulation process.

The small amounts of exsporulation encountered following the

pretreatment routines may be the indicators of a larger real response to the various pretreatment regimes. Weidner and Byrd (1982) showed that external calcium ions were inhibitory to exsporulation of the fish parasite Glugea hertwigi. The concentration here of calcium chloride in the second treatment media (cell culture medium) was 2.4 mM, a concentration at which Weidner and Byrd obtained a reduction in spore hatching from approximately 45% to 10%.

The stimulation of exsporulation of M. hepaticus following pretreatment in acid media has not been described from other species, although Hashimoto et al. (1976) demonstrated increased exsporulation rates of Pleistophora anguillarum in acid media. The response to a reduction in pH may be related to conditions within the host gut. pH change from acid conditions in the stomach to more alkaline in the intestine has been described in the cottid Cottus gobio (Western, 1971) and the eel Anguilla anguilla (Clarke, 1981) and could thus be involved in the exsporulation of certain microsporidia. In contrast to these reports Ishida (1935) reported the absence of gastric glands and peptic activity in adults of the Mugilidae in general, suggesting that low pH may not be a feature of the gut of C. labrosus. However, as previously discussed dietary change occurs between larval and juvenile/adult mullet (Shehadeh et al., 1972) and thus it is probable that gut conditions change also; suitable pH conditions for exsporulation of M. hepaticus may well be present in the larval mullet intestine. Stimulation of exsporulation by acid conditions or change from low to higher pH is contrary to the situation in invertebrate infecting microsporidia, which exsporulate following treatment in alkaline media (Undeen and Alger, 1971; Weidner, 1972; Weidner and Trager, 1973) or an immersion in alkaline media of high potassium ion concentration (Ishihara and Sohi, 1966; Ishihara,

1967, 1969; Undeen, 1975, 1978, 1983), these conditions being similar to those of the insect intestine. The potassium ion concentration was shown to be the exsporulation trigger; pH was the major mediating factor. It is likely that pH similarly represents a mediating factor rather than the trigger for exsporulation of M. hepaticus.

The stimulation of exsporulation by pretreatment in media of raised osmolarity has not previously been reported. Of particular interest in the response of M. hepaticus was the incomplete exsporulation produced following preincubation in the higher strength media. Incomplete exsporulation has been described in spores exposed to oxidising solutions adjusted to high osmolarity (Oshima, 1927, 1937; Lom and Vavra, 1963). The exsporulation of M. hepaticus following treatment in 1M and 2M sodium and potassium chlorides may have been caused by ion diffusion from the pretreatment media resulting in the increase of spore osmolarity and the triggering of exsporulation following the osmotic entry of water in the test medium. The partial exsporulation after treatment in 5M solutions may also be explained in this way if applied to the exsporulation hypothesis of Undeen (1978), who suggested a two stage process for microsporidian filament extrusion. The first stage was the rupture of the polar cap following pressure increase caused by polaroplast expansion; the second stage was the osmotic ingress of water resulting in the final extrusion of the filament and sporoplasm. The incomplete exsporulation of Oshima's (1927, 1937) and Lom and Vavra's (1963) experiments can be explained by the absence of osmotic pressure differences between the spore contents and medium preventing the second phase of exsporulation. The reverse may be true here with M. hepaticus. Preincubation may raise the osmolarity of the spore to

such a level that immersion in media of low osmolarity results in sufficient osmotic pressure to prevent polaroplast expansion, but to rupture the polar cap, resulting in partial exsporulation. The consequent disruption of spore structure may not allow out of sequence polaroplast expansion and exsporulation would proceed no further. The effect of osmolarity change in stimulating exsporulation of M. hepaticus may have analogies with the situation in the wild. Williams et al. (1970) demonstrated that the intestinal lumen of the cod Gadus morhua was hypotonic to sea water. If this was also the case in C. labrosus spores would undergo a slight reduction in osmolarity on ingestion by the host. Such a mechanism could play a part in the prevention of exsporulation within host tissues, with spores not being primed until immersed in sea water, at a higher osmolarity. However, as with chitinase and pH changes, osmolarity changes, if involved in the exsporulation process, probably act as a mediating influence rather than as the true stimulus for exsporulation.

In attempts at in vitro infections, primary cultures of liver cells were thought appropriate in view of the specificity of M. hepaticus to this organ. To the best of my knowledge, there have been no previous successful attempts to establish any cells of C. labrosus in culture. It was necessary therefore to proceed using standard techniques for the growth of marine fish cells in general, according to Sigel and Beasley (1973a,b). Although C. labrosus is an estuarine fish, the young spend most of their lives in high salinity sea water. It seemed appropriate therefore to raise the osmolarity of culture media to that of marine teleost fish tissues as proposed by Sigel and Beasley (1973). The physiological and blood serum conditions of teleosts are similar to those of terrestrial

vertebrates (Wolf and Quimby, 1976) allowing the use of conventional media adjusted for osmolarity. BHK 21 medium was chosen as it has been successfully used in the growth of many fish cell cultures (Williams pers. comm.). In primary cultures of liver tissue a number of cell types might be expected, including hepatocytes, endothelial cells from sinusoid linings, fibroblasts from the walls of vessels, blood cells etc. Only hepatocytes were clearly recognisable after tissue dissociation. The failure of these to survive for more than three days, although adhered to culture vessels, was probably the result of their over-specialisation and low proliferative potential. The identity of the small fibroblastic-type cells could not be determined, and although they grew to confluency, they did not survive subculture. At this point, primary cultures were abandoned in favour of established cell lines, as previous work had suggested that intracellular protozoa, including coccidia and microsporidia, although confined to particular cells *in vivo*, show a low degree of specificity for cells in culture (Strout *et al*., 1965; Doran and Vetterling, 1967; Ishihara, 1968a; Undeen, 1975; Shadduck, 1976; Smith *et al*., 1982). It seemed appropriate therefore to use RTG2 cells for further studies. The extremely low level of infection of cultured RTG cells with *M. hepaticus* suggests that the correct stimulus for filament extrusion was not present in the culture medium. The exsporulation methods used were chosen because they were the only ones available that would induce some degree of exsporulation whilst being compatable with the cell cultures. The low pH treatments were easily neutralised in cell culture medium; however low exsporulation rates had to be accepted in the experiments.

In assessing the success of cell invasion, all inclusions of

RTG cells other than the normal organellae were suspect stages of M. hepaticus. It was obvious, however, from the low percentage exsporulation value that infected cells would be most likely to be seen at the onset of infection rather than later when they might be obscured by the growth of the cultures. Nucleated cell inclusions described here are therefore considered to be early invasive and developmental stages of M. hepaticus, a view supported by the following observations. The RTG cell line had been passaged many times without the introduction of pathogens, and thus the presence of rare inclusions in experimental cultures and their absence in controls is most likely to be due to M. hepaticus infection. The rarity of stages supports their origin from microsporidian infections, as with only about 1% exsporulation in culture medium a very few spores would be expected to be in the right position and orientation to pierce cells with their polar filament. The timing and structural organisation of the stages is also consistent with microsporidian infection. The stage observed after 30 minutes would be a freshly injected sporoplasm, the nuclear appearance of which was probably the result of chromatin condensation as described by Ishihara (1968b) and Weidner (1972) in sporoplasms immediately after their inoculation into cells. The stage encountered after 72 hours was a plasmodium with 12 nuclei, consistent with the vegetative stages observed in vivo. These results indicate that inoculation of cells occurs within 30 minutes of the introduction of primed spores into cultures, and that vegetative development progresses by four to five nuclear divisions during a 72 hour period. There are no reports of the development of fish microsporidia in vitro; however in successful cultures of invertebrate microsporidia spore production begins between 60 and 72 hours post infection (Ishihara and Sohi,

1966; Undeen, 1975). Differences might be expected owing to the different culture conditions, these reports being of cultures grown at a higher temperature, or may reflect differences in growth rate between insect and fish microsporidia, as the latter generally take two to three months to form xenomas (Weissenberg, 1968; Matthews and Matthews, 1980). Alternatively, it is possible that the intracellular environment of RTG cells is unsuitable for the growth of M. hepaticus. There was no evidence of any cytological changes in the host cell at the 72 hour stage, and this may support the suggestion that RTG cells are unsuitable hosts. Cellular changes might be expected in a host/parasite system destined to become a xenoma. It is possible that M. hepaticus, and other xenoma forming microsporidia, have a much narrower range of suitable host cells than the insect microsporidia, and Encephalitozoon cuniculi, previously investigated in culture.

The membrane bound spaces surrounding cell-penetrating polar filaments encountered here has previously been reported by Weidner (1972), and similar structures were shown in micrographs presented by Ishihara (1968b). Weidner showed that cytoplasmic flow occurred from the host cell outwards along the polar filament and suggested that this was the cause of the cytoplasmic space. Cytoplasmic flow only occurred in cells that could be successfully infected; cells which did not produce this response were lysed. The presence of spaces around filaments in RTG cells may therefore indicate that these can at least be injected with sporoplasms without suffering damage; however as discussed above this may not be adequate to ensure successful infections with xenoma-forming microsporidia.

Xenoma. Infection of host cells with Microgemma hepaticus results

in the formation of a host/parasite complex similar to that described as a "complexe xenoparasitaire" by Chatton (1920) or xenoma by Weissenberg (1922, 1949). These structures are characteristic of a wide range of microsporidia, including Glugea (Weissenberg, 1922; Berrebi and Bouix, 1978), Ichthyosporidium (Sprague and Vernick, 1974; Sprague and Hussey, 1980), Tetramicra (Matthews and Matthews, 1980), Loma (Morrison and Sprague, 1981a,b,c; 1983), Mrazekia (Janiszewska et al., 1979) and some Nosema species (Spelling and Young, 1983), and have been recorded in a wide variety of hosts, including fish, annelids, leeches and insects. In general organisation the xenoma of M. hepaticus resembles those of Mrazekia and Tetramicra, with the surface being thrown into microvilli. There is some evidence to suggest that the degree of microvillar development in these species may be dependent on nutrient availability at the site of infection. M. hepaticus has short microvilli, sometimes sparsely arranged, and inhabits the nutrient rich liver. T. brevifilum (Matthews and Matthews, 1980) has larger, anastomosing microvilli and has to compete with muscle cells for available nutrients. Mrazekia lumbriculi has possibly the greatest microvillar elaboration (Janisewska et al., 1979) and inhabits the annelid coelom, a site very low in nutrients. Differences in xenoma structure can occur within a species, depending on their site within the host. In Glugea atherinae, xenomas from the body cavities are larger and thinner-walled than those from the intestinal wall (Berrebi and Bouix, 1978) although are the same in internal structure (Berrebi, 1979). This difference may similarly be related to nutrient availability at the two sites. In Glugea species, specialisation of the xenoma plasmalemma takes the form of pinocytic channels rather than microvillar processes (Weidner, 1976; Lom

et al., 1979). This can probably be explained by the presence of a wall external to the plasmalemma in Glugea, the other species being bounded by simple cell membranes. The presence of vacuoles containing glycogen and cell debris suggested the active uptake of nutrients by phagocytosis. Pinocytosis and the formation of large numbers of pinocytic vesicles in the peripheral cytoplasm is a feature of Glugea xenomas (Weidner, 1976; Lom et al., 1979) and is the major method of nutrient uptake in these species. This was not the case here; there was no evidence that the xenoma caused disintegration of the surrounding hepatocytes, and phagocytosis seems to be a response to chance disintegration of adjacent cells. The arrangement of mitochondria at the cell periphery is consistent with an active site of nutrient uptake, and in the absence of mitochondria in microsporidia must be providing for the energy requirement of both host and parasite components of the xenoma. Similar concentrations of mitochondria occur in xenomas of Glugea species (Berrebi, 1979; Lom et al., 1979; Weidner, 1976), Ichthyosporidium giganteum (Sprague and Vernick, 1974) and Nosema herpobdellae (Spelling and Young, 1983); however the zone in M. hepaticus appears to be particularly well developed. In contrast, xenomas of T. brevifilum (Matthews and Matthews, 1980) and Loma species (Morrison and Sprague, 1981a,b,c; 1983) show no such zonation, although the number of mitochondria is also raised. Such differences are difficult to explain, as the nutrient and general energy demand of xenomas might be expected to be similar. However, mitochondrial variations could be related to the immediate environment of the xenoma and may reflect the amount of active feeding undergone at various sites in the body. The association of meronts with mitochondria, as previously described by Berrebi (1979), Matthews and Matthews (1980) and Canning (1981)

was not observed here. It may be that the greater elaboration of the mitochondrial band in M. hepaticus renders this association unnecessary. However, these observations are consistent with the view, discussed later, that meronts occupy a privileged site within the host cell. Mitochondrial increase occurs in non-xenoma forming microsporidia (Sprague and Vernick, 1971; Canning, 1981) and in infections of other intracellular protozoa (e.g. Eimeria; Scholtyseck, 1973), but in neither case is the level of increase comparable with that in xenoma formation. In the case of Eimeria, this is probably due to the smaller size of foci of infection and the relative simplicity of the host/parasite interaction. In Eimeria, the parasites can in addition contribute to energy requirements using their own mitochondria. Some coccidia show a similar ability to microsporidia in enlarging their host cells, diameters of 200-450 µm being described by Melhorn and Frenkel (1980). However, the interaction with the host cell is different, as all parasite stages are enclosed within a single large parasitophorous vacuole. In general, manipulation of the host cell is limited to its hypertrophy to surround the enlarging parasitophorous vacuole, and specialisation of the vacuolar membrane and the thin layer of host cell cytoplasm into a cyst wall (Toxoplasma, Hammondia). In Besnoitia infections (Frenkel, 1977; Melhorn and Frenkel, 1980) a greater degree of manipulation occurs with the induction of hypertrophy and hyperplasia of the host cell nucleus, possibly indicating a closer approach to the xenoma type of host/parasite relationship. However, the degree of integration of parasite and host cell components encountered in the microsporidia does not occur in these other groups.

In M. hepaticus xenomas, the host cell nucleus undergoes considerable enlargement, becoming reticulate with some

fragmentation. This is similar to the situation in other species, including Glugea spp. (Weissenberg, 1968; Sprague and Vernick, 1968; Berrebi, 1979; Canning et al., 1982), Tetramicra brevifilum (Matthews and Matthews, 1980), Loma spp. (Morrison and Sprague, 1981a,b,c; 1983) and Nosema herpobdellae (Spelling and Young, 1983). The involvement of the host cell nucleus in the parasite's life cycle is not clear. The proximity of stages to the nucleus led some to suggest a continuum between it and the parasite population (Sprague and Vernick, 1968), a view not now generally accepted. There is evidence, however, that the parasite can manipulate host nuclei. Pavan et al. (1969) demonstrated that alterations in microsporidian-infected dipteran salivary gland cells were related to chromosomal changes caused by the microsporidian. Matthews and Matthews (1980) suggested that the nuclear arrangement provided scope for DNA replication, and retained a high surface to volume ratio for transfer of materials between nucleus and cytoplasm. The increase in size and shape of nuclei in M. hepaticus xenomas must be in response to the presence of microsporidian infection, and these may well be involved in the control of the xenoma itself.

One of the original aims of the project was to detect the target cells for the infection. Unfortunately, without being able to develop a laboratory system, it was not possible to detect infections at stages where the host cell was identifiable. The association of xenomas with liver capsule, blood vessels, bile ducts and host capsules surrounding metacercariae suggests that the host cell may well be a component of the connective tissue rather than the specific liver cells such as hepatocyte, endothelial, epithelial etc. Xenomas of other fish-infecting microsporidians appear to be involved with connective tissue; Glugea anomala, Glugea hertwigi and Glugea

stephani in the intestine and other sites (Weissenberg, 1922, 1968; Stunkard and Lux, 1965; Scarborough and Weidner, 1979) and Tetramicra brevifilum in muscle connective tissue (Matthews and Matthews, 1980). Connective tissue is present within the liver at sites of infection with M. hepaticus, and it seems reasonable to suggest that M. hepaticus parasitises this tissue. Weissenberg (1968) suggested that a connective tissue macrophage or histiocyte was the target cell for G. anomala infections and such a cell could well be involved here. A cell of this nature is likely to be one of the few suitable cell types common to the site of the initial infection in the intestine and the site of xenoma formation in the liver. It is assumed that the intestine of the fish is the initial site of infection and migration to the liver occurs via the blood system. The mechanism of transfer of the infection from the intestine to the liver is not known; however it seems probable that this occurs within a host cell. The transfer of microsporidian infection around the body has been reported as intracellular in fish hosts by Weissenberg (1968) and in invertebrates by Weidner (1970). As previously discussed, microsporidian stages in the liver only appear to be antigenic when the host cell is ruptured; a naked sporoplasm, therefore, seems unlikely to survive movement to the liver. The migration itself probably occurs via the hepatic portal vein. This is the simplest available route, and requires only entry to the portal system; further movement could be passive, in the blood flow.

Species of microsporidia infecting fishes illustrate all degrees of tissue specificity within their host. M. hepaticus occurs only in the liver, whereas T. brevifilum (Matthews and Matthews, 1980) is confined to the skeletal muscle, and Loma species (Morrison and Sprague, 1981a,b,c; 1983) to the pilar system of the gills. In

contrast, Glugea species show a lower degree of site specificity (Weissenberg, 1968; Scarborough and Weidner, 1979). It might be relevant that the delicate microvillar surface of Microgemma hepaticus and Tetramicra brevifilum is more sensitive to the physiological environment of their respective host organ systems as opposed to the xenoma of Glugea which has a thick fibrous capsule. The alternative view is that these parasites show a high degree of cell specificity and the host cell type is confined to the host tissue. In view of the suggestion that these species are all involved with connective tissue cells, it is likely that the former situation applies here. This would indicate a difference in the relationship with the host cell between M. hepaticus and T. brevifilum, and Glugea species. In Glugea, xenoma formation follows the infection of the host cell, whereas in M. hepaticus and T. brevifilum this requires the additional step of migration to the final site of infection. The significance of such a mechanism may lie in the avoidance of competition with other parasites. For example, in the mullet heavy infections of the myxosporidean Myxobolus exiguus (Pulsford and Matthews, 1982) frequently occur, resulting in considerable intestinal disruption which would presumably lead to competition at the site for space and nutrients. The possession of a physiological trigger for xenoma formation would lead to a separation of habitats and consequently the reduction of competition between species.

Most studies of xenomas are confined to late infections where sporogony is well advanced. It is at this stage that the parasite is most easily detected and foci of infection clearly visible to the naked eye. The detection of young xenomas of Microgemma hepaticus in wild fish was fortuitous owing to the rarity of very young fish, the

absence of any detectable signs of infection and the dependency on microscopic screening. Nevertheless, stages were observed here (Figs. 68-75) which resemble type I xenomas described by Weissenberg (1968) in experimental infections of Glugea anomala. These probably represent relatively unmodified host cells, with slight hypertrophy, but possessing several distinct host nuclei and containing only vegetative stages of the parasite. A distinctive feature here was the occurrence of groups of xenomas at similar stages of development. It is possible to speculate that these represent cells which have been induced to multiply by the primary infection. This feature would be of considerable advantage to the parasite by allowing multiple xenoma formation from a single spore infection. The ability of the host cell nucleus to divide in xenomas has already been shown and discussed, and cell division as a result of microsporidian infection has been described by Weiser (1976b) in simulids infected with Polydispyrena simulii. On this basis Weiser proposed two types of xenoma, the neoplastic, as above, which induces cell division, and the syncitial where infected cells fuse and form multinucleate plasmodia. Although this division was based mainly on insect infecting species, the Glugea xenoma was included in the neoplastic category on account of "neoplastic" nuclear division within the xenoma. Microgemma hepaticus may provide examples of both forms of neoplastic xenoma, with the possible stimulation of cell division at an early stage, and nuclear division in the later classical fish cell xenoma.

The eventual host response to xenomas followed the general pattern as described by Dykova and Lom (1980) in their review of fish responses to microsporidian infection. Granuloma formation resulted in the destruction of spores by phagocytosis and the eventual

elimination of the focus of infection. The containment of spores in electron-dense phagocytic vacuoles in macrophages is in agreement with other reports (Matthews and Matthews, 1980; Dykova and Lom, 1980). An unusual feature of the response here was the ejection of degenerating peripheral xenomas into the body cavity. This effect is likely to be caused by the normal isolation of the granuloma by encapsulation; lacking further links with the liver itself the spore mass becomes pushed into the body cavity. A further notable feature of the response to M. hepaticus xenomas in comparison to that of Dykova and Lom (1980) was the absence of weakly reactive stages and pressure atrophy of surrounding cells, which in other species leads to the formation of a collagen capsule. The absence of any noticeable response to intact xenomas suggests that the xenoma is not recognised as a foreign antigen until the spores themselves are exposed at the onset of xenoma necrosis. The absence of tissue damage around xenomas here is probably due to the soft and spongy nature of liver tissue reducing pressure effects as the xenomas enlarge. This view is supported by the occasional observation of necrotic hepatocytes where these are trapped between the xenoma and blood or biliary vessels; pressure effects are therefore dependent on the presence of adjacent solid structures. Detectable pathological changes were not caused around the individual xenomas, and moderate parasite burdens were apparently easily tolerated. As noted earlier, however, there was a tendency for heavily infected individuals to be amongst the first to die after capture. The effects of heavy infections are liable to be twofold; firstly the disruption of blood and bile flow by the occlusion of vessels, and secondly greatly increased pressure atrophy effects on hepatocytes trapped between the xenomas themselves. Thus for a given size of

liver there is likely to be a critical intensity of infection where liver function is severely impaired. This critical intensity will be less the smaller the host fish, and as discussed previously is more likely to be achieved in the recently hatched larval fishes believed to be the main hosts of the infection.

Structure and Development of *Microgemma hepaticus*. The stages in merogony described here, namely the multinucleate plasmodia of various sizes, have been described in other species of microsporidia (Vavra, 1976a). The distinctive feature concerning merogony of *M. hepaticus* was the absence of clearly defined phases, such as uninucleate and cylindrical forms (Weissenberg, 1911, 1913, 1968; Debaisieux, 1920; Lom and Corliss, 1967) suggesting a more continuous cycle of merogonic development, based on the plasmodial forms. In this respect it differs from *Pleistophora hyphessobryconis* (Lom and Corliss, 1967) which has an early uninucleate meront population, *Encephalitozoon* (Sprague and Vernick, 1971; Canning, 1981) in which merogonic division is by binary fusion alone, and *Nosema* (Weidner, 1970; Canning *et al.*, 1983; Spelling and Young, 1983) where this is also the usual case, although small plasmodia are occasionally observed. Whether such diversity of merogonic forms is of significance in the various developmental cycles is uncertain. The occurrence of uninucleate stages in some species could be involved in movement of the infection within a host cell (e.g. *Pleistophora hyphessobryconis* along muscle fibres) or in transmission of infection from one cell to another, as in Ishihara's (1969) "secondary invasive form" of *Nosema bombycis*, and as suggested by Lom and Corliss (1967) in *P. hyphessobryconis*. In xenoma forming species intra- or extra-cellular movements may be unnecessary, and in the

case of M. hepaticus nuclear division within large plasmodia may simply be the most efficient method of vegetative development. This form of development is not universal amongst xenoma-forming microsporidia, however; large plasmodia do occur in xenomas of Glugea infections (Weissenberg, 1968; Berrebi, 1979; Canning et al., 1982), but not in Nosema herpobdellae infections (Spelling and Young, 1983).

The diplokaryotic state is not here considered to be a feature of Microgemma hepaticus. Although paired nuclei were occasionally seen in electron micrographs, these lacked the characteristic flattening and thickening of opposed membranes (Vavra, 1965, 1976a; Weidner, 1970; Spelling and Young, 1983; Canning et al., 1983).

The structural organisation of the merogonic stages was typical of that described in other species (Vavra, 1976a) and is in agreement with the view of a non-specialised cell destined for rapid multiplication. Observations on nuclear division confirmed the mechanism of acentriolar pleuromitosis (Hollande, 1972) and are therefore consistent with that of species already described. Small discs have been detected on the nuclear membrane external to the spindle plaques of some microsporidia (Loubes and Maurand, 1975; Berrebi, 1979; Canning and Nicholas, 1980; Morrison and Sprague, 1981c). These are not present in M. hepaticus. Polar vesicles, however, were present and resemble those described by Youssef and Hammond (1971) and Batson (1982). Although no function has been attributed to these spindle-plaque associated structures, their variation in number and appearance may at some stage prove useful in taxonomy. Studies were not made on chromosomes; however it was feasible to count the number of spindle fibres in transverse sections of dividing nuclei. It is tentatively suggested that, on the basis

of one spindle fibre per chromosome, the diploid number of this species is sixteen. From past records of chromosome studies there appears to be no consistency in chromosome number, with eight reported in Glugea weissenbergi (Sprague and Vernick, 1968), six in Nosema herpobdellae (Spelling and Young, 1983), and five in Nosema algerae (Canning and Sinden, 1973).

In the present study all stages recognised as meronts were clearly enclosed within parasitophorous vacuoles limited by a single host membrane. A similar situation has been described for two other species of microsporidia occurring in marine fishes, namely Tetramicra brevifilum Matthews and Matthews (1980) and Microgemma dunkerli (Stubbs, pers. comm.). Although the origin of the host cell membrane is not clear, it is here suggested that this represents a membrane of the endoplasmic reticulum and therefore the meronts are enclosed within this system. This would suggest a more intimate association with host cell organellae than previously thought. There are clear advantages to M. hepaticus in this site. The most rapidly growing stage of the parasite is placed in the host cell's protein synthetic system, and thus has a ready supply of materials. The meronts are also directly connected to the host cell nucleus, a link of possible major significance in control of the host cell and xenoma formation. Recently, Glugea meronts have been shown to be completely surrounded by cisternae of endoplasmic reticulum (Berrebi, 1979; Loubes et al., 1981; Canning et al., 1982). In addition, association of endoplasmic reticulum with meronts has been reported in numerous species (Szollosi, 1971; Weidner, 1970; Youssef and Hammond, 1971; Canning, 1981; Canning and Hazard, 1983). Microgemma hepaticus appears to have progressed a stage further in the relationship of its meronts with the host cell. The merogonic

stages would therefore appear to be occupying prime positions within the host cell.

Progression from meront to sporont was not clearly demarcated in M. hepaticus, and there was evidence to suggest that a sporogonic stage could be formed directly from a merogonial plasmodium. This is contrary to development in certain other species having large multinucleate vegetative phases. In Glugea for example, sporogonial stages develop from a uninucleate population (Weissenberg, 1913; Debaisieux, 1920; Berrebi, 1979; Canning et al., 1982; Loubes et al., 1981). Although the advantage to M. hepaticus of this pattern of development is not clear, it may be that the apparent efficiency of the plasmodial merogonic multiplicative system is carried through to the sporogonic stage. The transformation directly from meront to sporont without an intervening phase may be more rapid than the Glugea form of development, resulting in faster spore production. This could be of advantage in transmission of M. hepaticus amongst larval fishes. Susceptible fishes are only available for a short period; a rapid developmental cycle would therefore be essential for transmission amongst the larval mullet population. This may be assisted by the site of the parasite within the liver, which is particularly rich in stored, accessible nutrients.

A further distinctive feature of M. hepaticus was the absence of an additional membrane in sporogonic stages, these lying free in the host cell cytoplasm. This is similar to the situation in Tetramicra brevifilum (Matthews and Matthews, 1980) and Microgemma sp. (Stubbs, 1984) and differs from Glugea and Pleistophora species (Weissenberg, 1913; Debaisieux, 1920; Canning et al., 1979; Canning and Nicholas, 1980; Canning and Hazard, 1983). The

functional significance of the presence or absence of the sporophorous vesicle in the life cycle of microsporidia is unclear. However, there is a tendency for the spores of pansporoblastic fish-infecting species to be released initially as clusters within their pansporoblast walls (Lom and Corliss, 1967; Canning et al., 1979). In contrast, the spores of Microgemma hepaticus, Tetramicra brevifilum (Matthews and Matthews, 1980) and Microgemma sp. (Stubbs, 1984) will be released singly on the degeneration of the xenoma. The greater size of pansporoblast spore packages may make spores more likely to be ingested by new host fishes. Alternatively, the packaging of spores may ensure multiple infections in new hosts; these are likely to be of advantage to some non-xenoma forming parasites, where the host cells may perhaps not have the parasite producing capacity of the xenoma type host-parasite complex. M. hepaticus, T. brevifilum and Microgemma sp., possibly having greater proliferative potential, may not be so dependent on initial multiple infections. The recent observation that the classical xenoma forming Glugea species are in fact pansporoblastic (Canning et al., 1982) does not support this suggestion. However the pansporoblastic capsule of Glugea species is vestigial compared with Pleistophora species, and could perhaps represent a reduction in the elaboration of pansporoblast walls following evolution of the xenoma form of host/parasite complex. This would therefore reflect phylogenetic rather than functional aspects of the life cycle.

Pansporoblastic membranes are not homologous with the endoplasmic reticulum enclosure of meronts in M. hepaticus, which is lost at the onset of sporogony. This indicates a change in the meront's cellular environment, and may act as the stimulus for the onset of sporogonic development. Several authors have suggested that

sporogonic development begins after a change in the environment within the host cell (Ishihara, 1969; Sprague and Vernick, 1971; Vavra et al., 1972; Maurand and Vey, 1973; Vavra, 1976b), resulting in the reduction of nutrient supply to the parasite. In the case of M. hepaticus, the loss of enclosing membranes may indicate the onset of host cell necrosis and as such would act as a suitable trigger for the formation of resistant transmission stages. Some reports have shown that a change from mitotic to meiotic division occurs at the onset of sporogony in certain species (Loubes, 1976, 1979; Vivares and Sprague, 1979). Synaptonemal complexes, indicative of meiotic division, were not present in merogonic or sporogonic nuclei of M. hepaticus, and meiosis is therefore not a factor in sporogony here. The life cycle of M. hepaticus is asexual.

Cytological features of early sporonts were little changed from those of meronts, with the exception of extramembraneous material and increase in endoplasmic reticulum. This is in agreement with the situation in other species (Vavra, 1976 review; Canning et al., 1979; Canning and Nicholas, 1980) and might be expected in view of the high degree of cell differentiation about to occur. What were considered to be unusual, possibly abnormal, sporogonic stages showed possible association between plasmalemma and endoplasmic reticulum. It cannot be concluded here, however, that this is of functional significance or could be found in other stages. It is probable that the extramembraneous material is a product of the golgi apparatus, in common with the activity of this organelle in other cells. The involvement of the golgi in formation of extramembraneous material has been suggested by Canning and Sinden (1973), Canning and Nicholas (1974) and Canning et al. (1979).

The polysporoblastic nature of M. hepaticus does not resemble

that described in other genera of microsporidia, the main distinction here being the random budding resulting in the production of a great variety of forms. It is suggested that not too much reliance should be placed on brief investigations of sporogony where this feature is considered as a taxonomic criterion. In support of this view are recent studies by Stubbs (pers. comm.) on another hepatic microsporidian from the cottid.

The formation of sporoblast buds of M. hepaticus begins simply, by the evagination of the cell membrane enclosing a peripheral nucleus. The formation of multiple clusters of buds appears to proceed by two processes; some followed the formation of buds from adjacent nuclei resulting in separate, adjacent buds. The formation of clusters of sporoblasts on cytoplasmic protrusions can be explained if division is initiated by a partial plasmotomy. A group of nuclei are pinched off in a plasmotomy type division, but before separation of the parts condensation of the cytoplasm occurs around the nuclei, resulting in the typical appearance of a cluster of sporoblasts at the end of a cytoplasmic protrusion. The formation of chains and rosettes of sporoblasts is probably dependent on the particular arrangement of nuclei at this stage. It is of interest that the various groupings of sporoblasts encountered in M. hepaticus have been individually described in various microsporidian genera; rosettes of sporoblasts in Tetramicra brevifilum (Matthews and Matthews, 1980) and Nosema vivieri (Vinkier et al., 1970, 1971), chains in Ameson species (Loubes et al., 1977; Vivares and Sprague, 1979) and paired sporoblasts in numerous genera including Encephalitozoon, Glugea and Nosema (Sprague and Vernick, 1971; Sprague, 1977d). Possibly the nearest equivalent to the variable process in M. hepaticus is in certain division stages of sporogonial

plasmodia of Pleistophora and Glugea species. In Pleistophora species sporoblasts are formed by stepwise division of a large plasmodium, the final stages of which take up a variety of bud-like arrangements of sporoplasts (Canning et al., 1979; Canning and Nicholas, 1980). Glugea species proceed in similar fashion, although the final division products undergo a further binary fusion (Weissenberg, 1913; Debaisieux, 1920; Canning et al., 1982). The way in which sporogonial division proceeds may be dependent on the size of the sporogonial plasmodium in any given species. In those having small plasmodia, e.g. T. brevifilum (Matthews and Matthews, 1980), Ameson species (Loubes et al., 1977; Vivares and Sprague, 1979) and Nosema vivieri (Vinckier et al., 1970) division is achieved simply, by simultaneous separation of sporoblasts resulting in consistent, characteristic division stages. In species having large plasmodia, e.g. Pleistophora and Glugea species, simultaneous division may not be possible and so division proceeds by a stepwise process until the division products are of a size where fragmentation to single parts can occur. Microgemma hepaticus may be an example of this type, where the stepwise fragmentation of sporogonial plasmodia proceeds by sequential budding of sporoblasts before the final fragmentation of the plasmodium itself.

Nuclear division occurred during the sporogonic phase, but was asynchronous and not as frequently seen as in meronts. It seems likely that each sporont nucleus underwent a single division; assuming this, sixteen sporoblasts could have resulted from the largest, octonucleate, sporont encountered in ultrathin sections. Only monomorphic spores were produced as in Tetramicra brevifilum (Matthews and Matthews, 1980) and Microgemma dunkerli (Stubbs, pers. comm.). The paired and multinucleate spores occasionally encountered

were anomalous forms, probably the result of late sporont nuclear division. Dimorphic spores have been described from fish species; in Pleistophora spp. these were distinguished on the basis of size (Canning et al., 1979; Canning and Nicholas, 1980) and in Spraguea lophii on the basis of morphology and development (Loubes et al., 1979). The significance of dimorphism has been discussed in relation to insect infecting species (Kellen et al., 1966; Hazard and Weiser, 1968; Andreadis, 1983). Dimorphic spores of insect species are responsible for normal insect to insect transmission and in transovarial transmission to the following generation. Transovarial transmission has not been demonstrated in the dimorphic fish infecting species, and in these infection is separate from the gonads. The formation of two types of spore may nevertheless indicate alternative life cycles of the parasite, one spore form being involved in direct fish to fish transmission and the other requiring the infection of an intermediate host before becoming infective to new fish hosts.

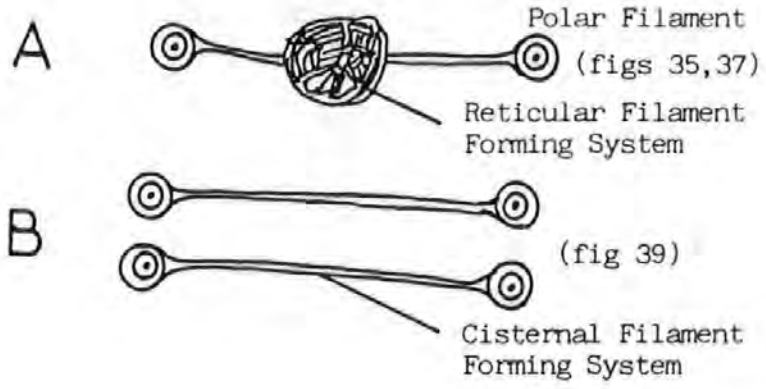
Sporoblasts, immediately after budding from the sporont, showed little internal differentiation other than the initial extramembraneous material which was already evident in the sporont stage and subsequently forms the exospore. Endospore development was progressive through the remaining sporogenesis period. Although the origin of the endospore is unknown, it might be significant that a single connection was observed in an ultrathin section between this layer and internal membranous structures. This appears to be the first record of such a connection between the spore wall and cytoplasm in microsporidia. In support of this view is the report of Liu and Davies (1972a) who detected vesicles associated with the cell membrane in Thelohania bracleata using freeze-fracture techniques.

The polar sac and rudiment of the manubrium of the polar filament adjacent to the nucleus were the first signs of cellular organisation of the sporoblasts. A possible earlier stage of development in microsporidian sporoblasts has been described by Vavra (1965), Vinckier et al. (1971), Jensen and Wellings (1972), Vinckier (1975) and Loubes et al. (1979, 1981), in which they identified a membranous sac and aggregation of electron-dense vesicles closely applied to the nuclear membrane. Further development in general fits the common microsporidian pattern, the polar sac migrating to the anterior pole of the sporoblast, the sac becoming progressively flattened and extending around the anterior third of the cell to form the anchoring disc. It was fortunate that the present study covered a particularly comprehensive sequence of events in sporogenesis. From this sequence it has been possible to put forward a more detailed view of the differentiation of structures within the spore. It is here considered that the early polar filament is assembled by the coalescence of vesicles, as described by Vinckier et al. (1971), Jensen and Wellings (1972), Vinckier (1975) and Loubes et al. (1979, 1981). In the present study, however, a second electron-lucent type was clearly identified and its possible involvement is here described for the first time. Sprague and Vernick (1974) believed all elements in sporogenesis were of golgi origin. The present study suggests that these electron-lucent vesicles were pinched from the edges of cisternae positioned laterally to the nucleus which resemble classical golgi structures. However, these are in the position occupied by endoplasmic reticulum in early sporoblasts and may well be endoplasmic reticulum. It is tempting to speculate here that the internal electron-dense structures of the early anchoring disc and polar filament are derived from electron-dense golgi vesicles and the

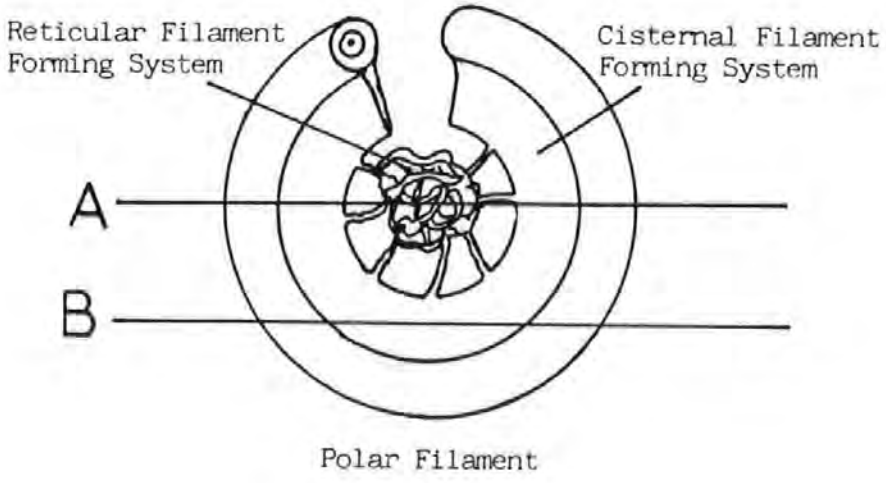
surrounding membrane and electron-lucent parts from endoplasmic reticulum derived electron-lucent vesicles. Jensen and Wellings (1972) and Liu and Davies (1972a) simularly implicated both endoplasmic reticulum and golgi in filament formation. The elongation of the filament in association with a granular electron dense structure has not previously been reported, and is here thought to represent an intermediate stage between the coalescence of vesicles and complex reticular filament forming systems. This structure could well represent a portion of the reticulum which has become compacted. The elongation of the filament into the posterior coiled arrangement occurs in association with a complex reticular and cisternal system, with the filament coils forming at the periphery. This arrangement has been frequently reported in other microsporidia (Vavra, 1976 a review; Vernick _et al_., 1979; Morrison and Sprague, 1981c, 1983; Canning _et al_., 1982; Batson, 1983). The term "reticulum golgienne", or golgi reticulum, was used to describe this structure by Takizawa _et al_. (1975) and supported subsequently by Vernick _et al_. (1979). A further stage of development is described here for the first time which shows a much simpler structure consisting of a stack of cisternae containing polar filament at their extremes. An explanation of the relationships between these two systems in filament formation is proposed in Figure 100. The arrangement observed will depend upon the sectional plane, that passing through opposing loops of filament and membrane and excluding the golgi reticulum being less likely. Following completion of filament formation much membranous material remains in the posterior region of the sporoblast. It is thought that the golgi reticulum remains within, and possibly contributes to, the posterior vacuole. Membranes and debris of the golgi reticulum have been described in

Figure 100. Structure and interrelationships of reticular and lamellar filament forming golgi systems in sporoblasts of Microgemma hepaticus.

Arrangements of filament forming systems observed in sections



Postulated arrangement of systems and section planes required to form the above structures: diagram of single coil of filament spiral.



Assuming the cisternal filament forming system to be of small size compared to the reticular filament forming system, section plane A would be expected to occur frequently; section plane B would be rare.

the posterior vacuole of late sporoblasts and spores of other species (Schubert, 1969; Pakes et al., 1973; Sprague and Vernick, 1974; Morrison and Sprague, 1981a,c; 1983). Using the above structural hypothesis, the filament forming system can be divided into two functionally distinct parts, the central golgi reticulum concerned with the synthesis of materials and the spiral cisterna which assembles and lays down the filament coils, including its outer membrane. Other cellular systems, such as the membrane systems associated with the nucleus and electron-dense inclusion body would be linked to the golgi reticulum which would modify their product and pass it to the filament forming cisterna. This relationship of synthetic systems is entirely consistent with classical golgi function (Beams and Kessel, 1969; Anderson et al., 1970). The possibility of endospore material being passed to the outside from membranes within the sporoblast was earlier discussed. A further feature in support of this view was the proximity of this structure to the golgi reticulum. More than one filament coil was occasionally found in the periphery of cisternae. It is not easy to explain this on the above hypothesis other than through the occasional malformation. Detailed reports of the formation of the coiled part of the filament are rare, probably the most comprehensive being that of Berrebi (1979) who suggested the development of polar filament of Glugea atheriae in a spirally arranged sac, single coils being laid down at the termination of this structure. Jensen and Wellings (1972) observed clumps of filament contained in large sacs, and speculated a similar method of filament deposition. Several other authors have reported filament formation in clumps contained in membranous sacs of golgi origin (Sprague and Vernick, 1969; Weidner, 1972; Sprague and Vernick, 1974). In addition, Takvorian and Cali

(1983) include micrographs of similar appearance of those of Berrebi (1979) but do not comment upon them. The basic similarity of microsporidian spores would suggest that development occurs along broadly similar lines, and the spiral cisternae of M. hepaticus and the larger aggregations in other species probably represent different levels of development of the same golgi system. This may be related to the number of filament coils in the mature spore, large amounts of filament material being formed in large spiral aggregations and smaller quantities in small cisternae as in M. hepaticus. The two systems are functionally identical, with reticular golgi systems passing material to sacs or cisternae for final assembly and deposition as filament coils.

Differentiation of the polaroplast complex occurred late in the development of the polar filament, the first prominent feature being the appearance of the sacs. The origin of the polaroplast from vesicles positioned around the manubrium has been described by Jensen and Wellings (1972), Canning and Sinden (1973), Liu and Davies (1972b), Vinckier (1975) and Loubes et al. (1979), the process proceeding as here by the flattening of the vesicles to form polaroplast lamellae. The result of development in this manner is the appearance of the mature polaroplast as cisternae arranged in petal-like formation around the manubrium as shown clearly in freeze-etch preparations by Liu and Davies (1972a, 1972b) and suggested in transverse sections here. No clearly defined system was present to account for the initial development, in common with previous reports. Lom and Corliss (1967), followed by Sprague et al. (1968) and Weidner (1971) suggested that the polaroplast was derived from the endoplasmic reticulum on the basis of its appearance in spores. This view was superseded when Sprague and Vernick (1969) suggested

that most of the spore components, including the polaroplast, were derived from golgi membranes, a view accepted by most subsequent authors (Jensen and Wellings, 1972; Canning and Sinden, 1973; Sprague and Vernick, 1974; Vavra, 1976 a review). However, structural evidence of a link with the golgi system has proved difficult to demonstrate. A hypothesis for the formation of polaroplast is presented here which explains the absence of a formative system. This encompasses previous ideas on the golgi origin of the sacs, explains the various appearance of the developing polaroplast and the intimate relationship of the polar filament and polaroplast membranes. At the time the polaroplast initially appears, polar filament development is well advanced, with the anchoring disc and manubrium occupying their final positions and several coils present in the posterior part of the sporoblast. Most authors from Vavra (1965) onwards accept that the filament is derived from the golgi apparatus. Sprague and Vernick (1974) indicated that the outer part of the filament, including the outer membrane, was of golgi origin, this being confirmed here with M. hepaticus. The manubrium therefore represents a branch of the main cell synthetic system and is the only such system present in the anterior part of the sporoblast. It therefore seems reasonable to suggest that the early saccate form of the polaroplast is formed from the outer, golgi, membrane of the polar filament. The sacs would initially form as small evaginations of the membrane, these then swelling into balloon-shaped sacs arranged radially around the manubrium, linked to the filament by a narrow neck. Liu and Davies (1972) showed just such balloon-shaped sacs in their freeze-etch preparations (their figs. 2 and 7), although the connection with the polar filament was not commented on. Subsequent flattening of these sacs would result

in the formation of cisternae with expanded, saccate ends, as in Figure 46, assuming that the inner part near the attachment to the polar filament has little opportunity for expansion. This hypothesis also accounts for the continuity of polaroplast and polar-filament membranes reported in mature spores (Weidner, 1970; Vavra, 1976a). Connections between these membranes were not resolved in spores of M. hepaticus, although they were clearly in intimate association; this appears to be due to the close packing of the lamellae in the section obscuring detail, rather than the absence of this linkage.

The spore of M. hepaticus is in general representative of microsporidian spore structure as reviewed by Vavra (1976a). The high electron-density of spores here was apparently due to the increase in density of ribosomes in late sporogenesis, as reported in other species (Lom and Corliss, 1967; Liu and Davies, 1972b; Weidner, 1972). The striking aggregation of ribosomes on cytoplasmic structures, here the nucleus, polar filament and polaroplast, has been previously described by Schubert (1969), Vinckier et al. (1971), Canning and Sinden (1973), Desportes (1976) and Larrson (1980). In the case of M. hepaticus it was likely that this aggregation was caused by the great density of ribosomes leading to the coating of all structures, rather than being actively associated with the organellae. The presence of large numbers of ribosomes indicates a high potential for protein synthesis and may be connected with the takeover and control of the host cell immediately after infection. The densely-packed polaroplast lamellae showed a smaller periodicity than in previous reports (Lom and Corliss, 1967; Stanier et al., 1968; Schubert, 1969). This may be related to the degree of packing of lamellae within the individual spores, or alternatively to the effects of various fixation regimes on the osmotically sensitive

lamellae. The separation of polaroplast membranes in threes confirms the trilaminate structure of the lamellae (Weidner, 1970; Lom and Corliss, 1967). This structure can be explained if two of the original flattened sacs are pressed together, opposing membranes being closely applied and giving rise to the typical appearance of two thin membranes arranged at either side of a central thicker membrane. The function of such an arrangement is not immediately clear. However, the strengthened central membrane could serve to limit lateral expansion of the polaroplast lamellae at exsporulation, resulting in a longitudinal piston-like expansion to give directionality to the pressure increase. The concentric layered structure of the polar filament has frequently been reported. However, the layering was not as marked as in some reports, with only five being present enclosed by a membrane-like layer; this is in contrast with the 13 described by Schubert (1969) and 10 by Canning et al. (1979). As with the polaroplast this could be a specific difference, or may be the result of varying fixation and staining methods. Owing to the functional similarity of microsporidian spores, it seems likely that the latter applies. The slight variation of filament diameter was probably the result of the differing system involved in its formation. Such variations have frequently been reported (e.g. Kudo and Daniels, 1963; Stanier et al., 1968; Matthews and Matthews, 1980) and the degree of variation used as a taxonomic character (Canning and Vavra, 1977); for taxonomic purposes here the filament diameter should be regarded as uniform, or isofilar, as variations are slight. The occurrence of a dense membranoid layer at the inside of extruded filaments, and the absence of external membranes, confirms that the filament is turned inside-out in the extrusion process (Schubert, 1969; Vernick et al.,

1969; Lom, 1972). The outer layer of extruded filaments, described by Weidner (1972) as the glycoprotein sheath, is probably the outer electron-dense layer of unexsporulated spores. The fate of the inner core of the filament is not known. Several authors have suggested that this serves a stiffening function during filament extrusion (Lom and Corliss, 1967; Sprague and Vernick, 1969; Schubert, 1969; Lom, 1972) and this view is accepted. It seems likely also that some component of the inner part of the filament also serves as a lubricant in the mechanically demanding extrusion process. The continuity of polar filament and anchoring disc is well established (Vavra, 1976a), and particularly clearly demonstrated in M. hepaticus was the intimate association of the lateral extensions of the disc with the polaroplast. The functional significance of this is not clear; the anchoring disc could be involved in mediating polaroplast expansion at exsporulation, or it may simply be in the only position available to it in the anterior region of the spore consistent with an anchorage function. Connection between the anchoring disc and spore wall has not been demonstrated in intact spores; however this may not be necessary as the anchoring disc could serve as a flange, preventing the filament from pulling out of the spore; connection between filament and spore wall may thus not be necessary. In argument against this, in M. hepaticus and other species (Ishihara, 1968b; Vernick et al., 1969; Weidner, 1972) the extruded filament is clearly firmly attached to the spore case. The central anterior protrusion of the anchoring disc of M. hepaticus may be evidence of a link with the spore wall in unexsporulated spores; it is certainly difficult to envisage such a link being formed at the moment of filament extrusion. The thinning of the endospore immediately over the anchoring disc is a common observation (Canning and Nicholas,

1974; Percy et al., 1982) and provides a weak point for the rupture of the spore wall at exsporulation. The weakening of the spore wall in this region is carried to greater levels in some species, Larsson (1981) showing in SEM preparations a definite ring structure in this region indicating the presence of a weakened cap in the spore coat.

Taxonomy and evolution. The erection of a new genus, namely Microgemma, for the hepatic microsporidian from C. labrosus is clearly necessary on the basis of comparative morphology of features usually employed in the separation of genera (Canning and Vavra, 1977; Vavra et al., 1981) and in comparison with genera described by Sprague (1977d) and Weiser (1979) and by Loubes et al. (1979), Matthews and Matthews (1980), Canning and Nicholas (1980), Morrison and Sprague (1981a), Canning et al. (1982) and Canning and Hazard (1982). Before being able to place this species in a taxonomic system, it is necessary to recap on the main diagnostic features of this parasite. The full generic and species description of Microgemma hepaticus is set out at the end of this section; a synopsis of the generic diagnosis is as follows. Features of the genus Microgemma are: isolated nuclei at all stages of development; asexual developmental cycle; multinucleate plasmodial merogonic stages, which divide by plasmotony to form multinucleate daughters; merogonic stages are enclosed in host cell endoplasmic reticulum, and pass directly to the sporogonic phase without an intermediate cell population; sporogonic stages are in direct contact with the host cell cytoplasm; sporogony is polysporoblastic, by an exogenous budding process being followed by multiple budding and fragmentation of the plasmodium; development within a cell hypertrophy tumour; members of the genus are parasitic in marine fishes.

Sprague (1977d) lists seven species of microsporidia parasitic in fish liver, from ten hosts. In addition to M. hepaticus, two further species have been reported, Pleistophora sp. by Paperna et al. (1978) parasitic in the metacercarial cyst wall of Heterophyces heterophyces in the mullet Mugil cephalus, and Microgemma dunkerli (Stubbs pers. comm.) from the liver of Taurulus bubalis. A comparison of spore sizes given for these hepatic species is presented in Table 8.

Although comparison of spore size is viewed with caution due to differing methods of handling of material, spores of M. hepaticus and M. dunkerli are distinctly different from those of other speices. It is unlikely, therefore, that Microgemma spp. have been previously observed and assigned to an established genus, although it is probable that when further information becomes available many of the previously reported species may be rearranged into new or different genera. The possibility exists that the liver species have been wrongly placed in the genera Pleistophora and Glugea, there having been a tendency amongst early workers to assign species to a small number of genera, particularly Pleistophora and Glugea in fish infecting species. More recent work indicates that Pleistophora species are muscle parasites (Schaperclaus, 1941; Lom and Corliss, 1967; Canning et al., 1979; Canning and Nicholas, 1980; Canning and Hazard, 1982), and Glugea species are primarily parasites of the connective tissue of the intestine (Weissenberg, 1911, 1913, 1922, 1968; Stunkard and Lux, 1965; Berrebi and Bouix, 1978; Scarborough and Weidner, 1979; Canning et al., 1982). It is unlikely, therefore, that representatives of these genera would be specific liver parasites. Further research at ultrastructural level is necessary before the true generic affiliations of the liver parasites

Table 8. Hepatic microsporidia of fishes (after Sprague, 1977d; modified to include new records).

Microsporidian	Host	Spore size (µm)	Reference
Microgemma hepaticus	*Chelon labrosus*	4.2 x 2.4 µm	Ralphs, 1984
Microgemma dunkerli	*Taurulus bubalis*	4.2 x 2.4 µm	Stubbs (pers. comm.)
Glugea depressa	*Julius vulgaris*	4.5-5 x 3 µm	Thelohan, 1895
Glugea ovoidea *Nosema ovoideum* *Microsporidium ovoideum*	*Motella tricirrata* *Cepola rubescens* *Mullus barbatus*	2.5 x 1.5 µm	Thelohan, 1895 Raabe, 1936 Sprague, 1977c
Pleistophora sp.	*Rhombus triacanthus*	2.5 µm x "a little less"	Linton, 1901
Pleistophora labrorum *Ichthyosporidium giganteum*	*Crenilabrus melops*	6 x 3 µm	Le Danois, 1910 Sprague, 1977c
Octosporea machari *Glugea machari*	*Dentex vulgaris*	2.3-4 x 0.8-1.5 µm	Jirovec, 1934 Sprague, 1977c
Glugea caulleryi	*Ammodytes lanceolatus*	-	Van den Berghe, 1940 (loc. cit. Sprague, 1977c)
Glugea pseudotumefasciens	*Brachydanio rerio*	"oval"	Pflugfelder, 1952
Pleistophora sp.	*Liza ramada*	3.7 x 1.7	Paperna *et al.*, 1978

can be established. Morrison and Sprague (1981a,b,c; 1983) have recently reassigned Pleistophora salmonae Putz, Hoffman and Dunbar, 1965 and Nosema branchiale Nemeczek, 1911 to the new genus Loma on the basis of ultrastructural studies, and it is anticipated that similar transfers of hepatic microsporidia may be made to the genus Microgemma. Already a further species has been added to the genus, namely M. dunkerli (Stubbs, pers. comm.), a common species in the liver of cottids. The possibility of M. dunkerli and M. hepaticus being synonymous cannot be overlooked on the basis of comparative morphology of currently available information. However, the differing environments, estuarine and marine, make separate species possible, and it is the opinion here that they should remain separate.

Examination of other reports for generic affiliations of Microgemma reveals a single genus from fish hosts, Tetramicra Matthews and Matthews, 1980, having similar features of development included in the generic diagnosis. Similarities between the two genera are: the relationship of meronts and sporonts with the host cell; monokaryotic development throughout; probable asexual life cycle; development mainly in plasmodial form; and the structure and organisation of the xenoma. Differences between the genera are: the presence, in Tetramicra, of clear intermediate stages between merogony and sporogony; the fixed tetrasporoblastic sporogony process; details of spore structure; and the restriction of Tetramicra to the musculature. The genera are thus clearly distinct; however sufficient similarities exist between them to suggest that Tetramicra and Microgemma should be placed in the same family. Matthews and Matthews (1980) suggested the family Tetramicriidae to contain the genus Tetramicra, and if the family diagnosis is modified

to replace "tetrasporoblastic development" with "polysporoblastic development", Microgemma could also be placed in this family. Alternatively, both genera could be placed in the family Unikaryonidae Sprague 1977, as both fulfil the requirements of Sprague's (1977b) diagnosis, namely sporulation in direct contact with the host cell cytoplasm, polysporoblastic development and uninucleate sporoblasts. The other genera included in this family are Unikaryon Canning et al., 1974, Nosemoides Vinckier 1975 and Perezia Leger and Duboscq 1909. It has been suggested that Perezia may be synonymous with Ameson, and would thus not belong in the family (Ormieres et al., 1977; Vavra et al., 1981). The remaining genera are hyperparasitic, Unikaryon in trematodes and Nosemoides in gregarines. Certain features of development of Microgemma and Tetramicra might argue against their inclusion in the Unikaryonidae, as the established genera of the group do not possess meront-enclosing membranes and are not xenoma forming. It has been argued that xenoma formation is more an expression of the host cell type than of the parasite itself (Berrebi and Bouix, 1978; Loubes et al., 1981; Canning et al., 1982). It is the opinion here that the parasite must have a significant effect on the development and organisation of the host parasite complex, and some evidence in support for this view may be in the variety of xenoma forms encountered, e.g. Microgemma, Tetramicra (Matthews and Matthews, 1980); Glugea (Canning et al., 1982) and Mrazekia (Janisewska et al., 1978). An important point, however, is that the host cell must be capable of hypertrophy in the first place; it is possible that cell types capable of the degree of hypertrophy encountered with fish cells are not present in trematodes. The gregarine cell similarly may not be capable of the necessary re-organisation. The

non-expression of the xenoma is thus not necessarily an indication that the fish-infecting and trematode/gregarine infecting genera are unrelated. The differing hosts of Unikaryon and Nosemoides compared to Microgemma and Tetramicra might also be held against their inclusion in the same family; however, the occurrence of related genera in invertebrate and vertebrate hosts has already been clearly established (Vavra et al., 1981; Canning and Hazard, 1982). In consequence, while it is tempting to place the marine genera Microgemma and Tetramicra in their own, separate, family it is the belief here that they can usefully be included in the family Unikaryonidae. However, in recognition of their similarities, and in particular their different hosts from the other members of the family, it is proposed to link the genera Microgemma and Tetramicra at sub family level, in the sub family Tetramicriinae. Tetramicriinae (named after the earlier genus Tetramicra Matthews and Matthews (1980) is diagnosed as having features of the Unikaryonidae Sprague 1977b, but developing in xenomas within marine fishes. Comparison of the hosts and parasites within the family Unikaryonidae leads to speculation on the evolution of this branch of microsporidian parasitism; this lends support to the linkage of Tetramicra and Microgemma. In addition, with the recent emphasis given to life cycles and nuclear behaviour in the microsporidia (Loubes et al., 1976; Canning and Vavra, 1977; Loubes, 1979), the linkage of monokaryotic, asexual genera may prove to be of use in future taxonomic systems.

Sprague (1977c), in a highly speculative article, suggested the origin of microsporidian parasitism in the ancient annelid/gregarine system. The platyhelminths, as another ancient group, could also be regarded as a potential original host group for microsporidian

infection. Several reports have been made of microsporidia parasitic in trematodes (review Canning, 1975) and Canning et al. (1983) noted that 16 species had been named. Of particular interest here are reports of infections of encysted metacercariae themselves (Canning, 1975; Stubbs pers. comm.) and the report of Paperna et al. (1978) of a species parasitic in the cyst wall of encysted metacercariae in the liver of the mullet Mugil cephalus. It is possible to speculate that infection was initially confined to the primitive encysted metacercaria, spread to the fish cells most intimately connected with the original host, and then to other structures of the affected organ having similar cells to those of the metacercarial capsule. A problem with this interpretation is that the protozoan would initially be taken into the host as a parasite of the penetrating cercaria; the microsporidian would therefore have to evolve new transmission mechanisms if it was to infect fishes without the presence of helminths. An alternative evolutionary route would be the development of fish parasites from hyperparasitic microsporidia of adult intestinal flukes, present-day representatives of which include Unikaryon allocreadi and Nosema gigantica (Canning and Madhavi, 1977). These authors indicated that infection of the trematode occurs within the hosts intestine; the microsporidian, therefore, already has the ability to exsporulate in a fish host. Penetration of fish intestinal cells rather than the normal host cells must be a common event, and transfer of infection from trematodes to fish as major hosts would simply require the adaptation of the parasite to fish intracellular conditions. This could result in the intestinal wall form of fish parasitism as encountered today in the Glugea species (e.g. Weissenberg, 1968; Sprague and Vernick, 1968; Berrebi and Bouix, 1978; Scarborough and Weidner, 1979). As

previously discussed, the branching out of infection from this site could be the result of avoidance of competition in the intestinal wall with the evolution of site-specific triggers for development.

Higher taxonomy of the microsporidia, although completely revised by Sprague (1977b) and Weiser (1977), remains problematical. In Sprague's (1977b) system the family Unikaryonidae was placed in the suborder Apansporoblastina along with the families Glugeidae, Courgourdellidae, Caudosporidae, Nosematidae and Mrazekidae. Subsequently, Canning et al. (1982) have shown that the type species Glugea anomala is pansporoblastic, and the family was removed from the suborder. Examination of the remaining families, and the genera Encephalitozoon and Spraguea left after the removal of Glugeidae, reveals a hetergeneous group with no apparent natural linkage other than the absence of pansporoblastic membranes. Coudosporidae, Nosematidae and Mrazekidae are diplokaryotic; Courgourdellidae and Unikaryonidae are monokaryotic; the genus Encephalitozoon is monokaryotic; and Spraguea is dimorphic (Loubes et al., 1979). These observations tend to support the view of Canning et al. (1982) that it was doubtful whether the two suborders Apansporoblastina and Pansporoblastina could be maintained with the occurrence of dimorphic genera having both forms of development. Weiser's (1977) classification has received less attention than that of Sprague (1977b), and is based on different criteria; the classical forms are divided into two orders, the Pleistophoridida for monokaryotic species, and Nosematidida for diplokaryotic species. However, as with Sprague (1977b), dimorphic species, having both mono- and diplokaryotic sporogony sequences, are difficult to fit into the classification. Certain dimorphic species were grouped into the family Ambylosporidae Weiser 1977, contained in the monokaryotic

order Pleistophoridida. In contrast, the dimorphic genus Variomorpha is contained in the order Nosematidida for diplokaryotic families. Such conflicting arrangements, as in Sprague (1977b), seem unlikely to reflect true phylogenetic relationships, and as indicated by Canning et al. (1982) and Canning and Hazard (1982) a different approach to microsporidian taxonomy seems necessary.

The recent interest shown in meiosis, and thus a sexual phase, in certain microsporidia (Loubes et al., 1976b; Vavra, 1976a; Hazard et al., 1979; Loubes, 1979; Vivares and Sprague, 1979; Canning and Hazard, 1982) suggests that future taxonomic systems might be better based in their fundamental divisions, on details of the life cycle rather than structural features. Five basic forms of life cycle can be distinguished amongst the classical forms of microsporidia, and these are compared in Table 9. In all cases, meiosis where present occurs at the onset of sporogony. In diplokaryotic genera, the parts of the diplokarya separate before meiosis (Loubes, 1979).

A notable feature of these cycles is the absence of a form having monokaryotic merogony and diplokaryotic sporogony. This could have implications in the evolution and taxonomy of the group, as it may indicate that the diplokaryotic state is an earlier evolutionary stage than the monokaryotic, and that ancestral forms had diplokaryotic merogony. Evolutionary routes could therefore be suggested from dimorphic or Thelohania-type forms. Canning and Vavra (1977) suggested that the various monomorphic genera may have evolved from dimorphic types by suppression of one or other of the life cycle phases. The origin of the five life cycles can be explained by elaboration of this hypothesis. Species with diplokaryotic merogony and monokaryotic sporogony indicate a suppression of asexual stages;

Table 9. Nuclear behaviour in Microsporidian life cycles (after Canning and Vavra, 1977; Loubes, 1979).

Merogony	Sporogony	Meiosis	Examples	References
Diplokarya	Diplokarya	-	*Nosema* *Ichthyosporidium*	Sprague, 1977c Loubes, 1979 Sprague & Vernick, 1974 Canning *et al.*, 1983
Isolated Nuclei	Isolated Nuclei	-	*Pleistophora* *Glugea* *Unikaryon* *Tetramicra* *Microgemma*	Canning & Nicholas, 1980 Canning *et al.*, 1982 Canning *et al.*, 1974 Matthews & Matthews, 1980
Diplokarya	Isolated Nuclei	+	*Thelohania* *Tuzetia* *Polydispyrena*	Loubes, 1979 Loubes, 1979 Canning & Hazard, 1982
Isolated Nuclei	Isolated Nuclei	+	*Baculea* *Gurleya* *Microsporidium babrodesmi*	Loubes, 1979 Loubes, 1979 Loubes *et al*, 1976; Sprague, 1977c.
Diplokarya	Isolated Nuclei and Diplokarya	+ -	Dimorphic:- *Parathelohania* *Variomorpha* *Stempellia* *Ambylospora*	 Hazard & Weiser, 1968 Pilley, 1976 Hazard & Fukuda, 1974 Hazard *et al.*, 1979

those with diplokaryotic merogony and sporogony a suppression of sexual stages. Species having monokaryotic cycles throughout require further explanation, and it appears that the key to these would be in the separation of diplokaryon nuclei at the onset of sporogony. Suppression of vegetative stages at this stage could result in a monokaryotic, sexual cycle; monokaryotic merogony would then be a secondary elaboration. Monokaryotic, asexual genera would be the result of substitution of meiotic division with mitosis. An alternative evolutionary pathway could be based on the suggestion that dimorphism

is a secondary specialisation rather than the ancestral state. The ancestral form in this case would have diplokaryotic merogony, a meiotic division, and monokaryotic sporogony as in Thelohania etc. (Table 9). Species having asexual diplokaryotic life cycles would be the result of an early onset of sporogony with complete suppression of sexual stages. Dimorphic cycles would be created by early spore development and incomplete suppression of later stages. Monokaryotic forms require the suppression of diplokaryotic stages, elaboration of vegetative stages and suppression of meiosis as described above. There is no evidence as to which evolutionary route is the most likely, although it might be significant that the latter system requires fewer individual evolutionary steps from the ancestral form. However, whatever the precise relationship, it would appear possible that the life cycle types could have been derived from an ancestral form. There may, therefore, be justification for a new taxonomic system based on the life cycles themselves (Table 9), and to treat the five life cycle types as five main taxonomic groupings. Subdivisions within groups would then be on the various structural and sporogonic features currently used (Sprague, 1977b; Canning and Vavra, 1977; Vavra et al., 1981). It should be stressed, however, that the number of life cycles in which meiosis and dimorphism have been investigated is small, and it is conceivable that a number of genera already described have sexual phases in their life cycles which are as yet unrecognised. Equally, a number of insect species, and possibly even fish species, may be dimorphic and the two parts of the life cycle regarded as different species. Further work along the lines of Canning et al. (1982), Canning and Hazard (1982) and Canning et al. (1983) in determining features of developmental cycles of type species at ultrastructural level, in the light of modern

developmental knowledge, is necessary before a new and reliable taxonomic system can be proposed.

Specific, generic and subfamily diagnosis.

Microgemma hepaticus gen. n. sp. n

Locality: St. Johns lake, Tamar Estuary, Cornwall, England.

Host and Site: *Chelon labrosus* (RISSO 1826) juveniles; liver.

Lesion: Spherical xenoma (granuloma in old infections) up to 500 µm in diameter, visible as white spots in the liver.

Vegetative stages: with characters of genus (see below).

Sporulation stages: with characters of genus (see below).

Spore: Pyriform, dimensions 4.2 µm x 2.4 µm at widest; posterior vacuole occupies posterior third of spore; polar filament isofilar, 7-10 coils, 57 µm long when extruded; polaroplast lamellar.

Microgemma gen. n. Nuclei unpaired at all stages of merogony and sporogony; developmental cycle asexual; meronts are multinucleate plasmodia which divide by plasmotomy to form multinucleate daughters; merogonic stages enclosed by single host membranes and are probably contained within the host endoplasmic reticulum; multinucleate meronts enter the sporogonic phase directly, with no recognisable intermediate cell population; sporogony in direct contact with host cell cytoplasm (meront enclosing membrane lost at onset of sporogony); sporogony polysporoblastic, division beginning by single exogenous budding and accelerating by multiple budding and fragmentation of the plasmodium.

Tetramicriinae subfam. n. Parasites of marine fishes; developmental characteristics compatible with family Unikaryonidae Sprague 1977b;

development occurs within cell hypertrophy tumour (xenoma) which lacks an external capsule and has a microvillar surface; stages of development are spread randomly through the xenoma cytoplasm.

All experimental procedures applied to live fishes during the course of this study were performed under Home Office Licence, Number SW1 3979.

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