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THE HOST-PARASITE INTERFACE

by

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

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Date: 23-9-'77

This is to certify that the work here submitted was carried out by the candidate herself.

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S U M M A R Y

Metacercarial cysts of Bucephalus haimeanus naturally infect Pomatoschistus microps collected from the Tamar Estuary, Devon. Cysts occur throughout the liver, greater concentrations being found around the gall bladder, blood vessels and periphery of the liver. The cyst wall is composed of three layers, an inner granular, middle vacuolated and outer nucleated layer. The inner layer consists of three zones, an inner zone of irregularly-compacted, granular material, a middle densely-compacted zone and an outer moderately-compacted zone. Only small amounts of collagen and very few leucocytes are associated with the cysts.

The cyst wall is carbohydrate in nature containing some free aldehyde groups, galactogen and neutral mucosubstances. The inner layer appears to consist of mainly acidic mucosubstances and weakly acidic sulphomucin. Large amounts of protein, carbohydrate-protein complexes and DNA are also present within the cyst wall.

The formation of the cyst wall and the related changes in the parasite tegument during its metamorphosis from the cercarial to metacercarial stage have been investigated by means of experimental infections into G. flavescens ^P~~E. minutus~~ and Gobius paganellus. The initial, fibrous cyst wall is formed from secretions produced by both the anterior gland cells and the tegument of the parasite. These secretions gradually become compacted against the surrounding hepatic

cells until by the 20th - 30th day post infection the three zoned inner layer, as described above, is formed. Hepatic cells immediately adjacent to this inner cyst wall layer are disrupted by the arrival of the metacercaria and form the middle vacuolated layer. As the metacercaria grows the cyst increases in size causing still further hepatic cells to become flattened and incorporated into the cyst structure forming the outer nucleated layer.

Development of the metacercarial tegument is accomplished by the sequential movement of secretory cell bodies from parenchymal cells into the outer layer. Vesicles of the types V.1 - 5 are released from the outer syncitial layer resulting in an almost complete breakdown of this layer prior to its replacement by the tegument of the fully developed metacercaria. The fully formed metacercarial tegument is characterised by the V.6 - 9 type vesicles, flattened scale-like spines which terminate in five to seven digits and by sensory cilia.

Microdensitometric measurements upon lysosomal β -glucosaminidase did not indicate any change in the staining reactions of the hepatic cells generally due to the presence of the cysts. There is, however, an increase in the staining reaction in the flattened cells of the cyst wall as compared to apparently normal hepatic cells. This is attributed to increases in the amount of enzyme present, kinetic changes or to decreased lysosomal stability resulting in the release of latent hydrolases.

Several enzymes capable of causing cellular breakdown have been demonstrated in the cyst wall, namely, β -glucuronidase, N-acetyl- β -glucosaminidase, alkaline phosphatase, acid phosphatase and a nucleosidephosphatase capable of hydrolysing many substrates. Alkaline phosphatase and nucleosidepolyphosphatases have also been located over the parasite surface possibly indicating a further breakdown of material at this site.

Using electron dense tracers and autoradiographic techniques uptake via the metacercarial tegument of ferritin, ruthenium red, insulin, albumin, glucose, phenylalanine, tryptophan and tyrosine has been demonstrated.

Functional aspects of the cyst wall and the role of the metacercarial tegument in nutrient uptake are discussed in relation to this work.

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INTRODUCTION

In an article dealing with the ~~Schistosoma~~ surface Clegg (1972) remarks, "Perhaps the most striking aspect of the relationship between parasitic worms and their hosts is that they so often succeed in remaining alive long after other foreign invaders would have been destroyed by the immunological reactions of the host." Because these parasites are important pathogens of man they have been extensively studied. The importance of the ~~Schistosoma~~ tegument in relation to its absorptive function (see Pappas and Read, 1975) and its role in avoiding the host's immune response (Smithers and Terry, 1976) is thus known in some detail.

The host-parasite interface between tissue parasites and fish hosts has by contrast received little attention. The recent advent of intensive fish farming and associated disease problems has created some urgency for research into fundamental aspects of fish parasitology in order that effective treatments against disease can be developed. Past research at Plymouth Polytechnic has chiefly been concerned with the response of teleost fish to infection by various pathogens, notably metacercariae (Iddon, 1973; Cottrell, 1975). Matthews (1974b) noted differences in the fish host reaction and in the degree of development of the metacercarial cyst wall with the site of infection. The aim of the present investigation was to make a critical study of the host-parasite interface at the cellular level in order

to determine the degree of metabolic dependence of the parasite on the host. Enzyme histochemical techniques have been employed to shed some light on the functional aspects of the cyst wall; it is the cyst wall and not the metacercarial tegument which is in direct contact with the host tissues and might, therefore, serve similar functions as the Schistosoma tegument.

The parasite selected was Bucephalus haimeanus, the life cycle of which involves the bass, Dicentrarchus labrax, as definitive host, the cockle, Cardium edule, as first intermediate host and the common goby, Pomatoschistus microps, as second intermediate host. This study is concerned with the metacercarial stage encysting in the liver of the goby host. Knowledge of the life cycle made experimental infections possible so that the course of an infection could be followed. Previous work (Matthews, 1973b) has suggested that, in this system, there is only a slight host reaction associated with the thin cyst wall of parasitic material and it, therefore, represents a system ~~in~~ between that of Cryptocotyle lingua which evokes an intense host reaction and Rhipidocotyle johnstonei which lies freely within the host tissues. Gobies are easy fish to catch and plentiful in the Tamar Estuary, Devon, where they have a high infestation rate. This abundance of material makes this a good model for study, however, the extent of any investigation is somewhat limited by the small size of the fish.

REVIEW

The major part of this review is concerned with cysts of metacercariae which occur in fish. A short section deals with the trematode tegument since this is of importance in the formation of the cyst wall.

Metacercarial Cysts. Early work on metacercarial cysts found in fish was mainly concerned with histological descriptions of cyst structure and formation. On the basis of these investigations Hunter and Dalton (1939) classified cysts into two main categories. The first type consisted of two layers, an inner hyaline layer of parasitic origin and an outer cyst elaborated by the host e.g. Posthodiplostomum minimum (Hunter and Hunter, 1940) and Tetracotyle lepomensis (Bogitsh, 1958). The second type were only considered to have a cyst wall of host origin e.g. Clinostomum marginatum (Osborn, 1911; Hunter and Dalton, 1939). More recent work, however, has made it apparent that several other categories of cyst structure exist:-

1. Cyst wall of parasitic origin is secreted which later breaks down e.g. Bucephaloides gracilescens (Matthews, 1974a).
2. Cyst wall of parasitic origin is secreted which later breaks down and is replaced by a host capsule e.g. Proisorhynchus crucibulum (Matthews, 1973a).
3. Cyst wall of parasitic origin e.g. Haplorchis pumilio (Pande and Shukla, 1972), Posthodiplostomum nanum (Asanji and Williams, 1973) and Rhipidocotyle lintoni (Stunkard, 1976).

4. Cyst wall with both parasitic and host components
of which there are many types:-

- i) One layer of parasitic origin and one layer
of host origin e.g. Ornithodiplostomum
ptychocheilus (Hoffman, 1958b), Haplorchis
taichui (Nath, 1973).
- ii) Two layers of parasitic origin and one layer
of host origin e.g. Posthodiplostomoides
leonensis (Asanji and Williams, 1973) and
Ascocotyle chandleri (Lumsden, 1968).
Stictodora lari (Leong and Howell, 1971) can
also be considered to belong to this group
since the parasite secretes a cyst wall which
is encapsulated by host cells. The metacerc-
aria deposits a protein layer on the inner
surface of the cyst wall, following this,
however the original layer of parasitic origin
decays.
- iii) Three layers of parasitic origin and one layer
of host origin e.g. A. leighi (Stein and
Lumsden, 1971a).
- iv) Four layers of parasitic origin and one layer
of host origin e.g. A. pachycistis (Stein
and Lumsden, 1971b).
- v) One layer of parasitic origin and two layers
of host origin e.g. Posthodiplostomum minimum
(Mitchell, 1974) which has an interface zone
of degradation bodies and membranous structures
between the parasitic cyst wall and outer fibrous
capsule.

- vi) Two layers of parasitic origin and three layers of host origin e.g. Holostephanus lühei (Erasmus, 1962).

5. Host capsule only: _

- i) One layer e.g. Clinostomum tilapiae (Asanji and Williams, 1973).
- ii) Compound cyst wall enclosing many metacercariae of Rhipidocotyle septrapillata (Krull, 1974a).

It is evident, from the above list, that a classification based upon the origin and number of cyst wall layers is not satisfactory. Undoubtedly many more permutations of the number of parasite to host cyst wall layers exist.

A more suitable basis for comparison may involve a classification based on the site of encystment and more functional aspects of cyst wall structure. Matthews (1974b) related the degree of host cellular reaction associated with metacercariae of six species of gasterostome with the presence or absence of a cyst wall and with the site of infection in different species of marine fish. R. johnstonei and Proisorhynchus crucibulum are both found in the muscle layers feeding directly on host tissue and initiate an intense host reaction. Neascus flaviatilis (Hughes, 1928), Diplostomum phoxini (Rees, 1955) and B. gracilescens (Matthews, 1974a) similarly feed directly on host tissue but are found in immunologically privileged sites, namely the eyes, brain

and cranial nerves respectively, and initiate no such response. Matthews (1974b) also compared cysts located in the skin, where connective tissue is plentiful, to those in the liver where there is little connective tissue. Only a slight host response might be expected in regions where connective tissue is sparse. Cryptocotyle lingua, found superficially, has a parasitic cyst wall surrounded by a thick, melaninated host capsule (Hsiao, 1941; Chapman and Hunter, 1954). There is considerably less host involvement and no pigmentation, however, connected with the cysts of Bucephalus haimeanus in the liver of the goby. It is interesting to note that in O-group plaice, B. haimeanus encyst in the musculature and here a thick host capsule forms (Matthews, 1973b). Hunter and Hunter (1942) comment on differences in the cyst structure between Crassiphiala bulboglossa which encyst in the skin and Uvulifer ambloplitis found in the deep muscle layers (Hunter and Hamilton, 1941). Further examples of metacercariae located in the skin and muscle layers with thick, often pigmented cyst walls include N. pyriformis and N. longicellis (Chandler, 1951), various opisthorchiids and a plagiorchiid (Rai, 1969).

Relatively few ultrastructural investigations of metacercariae encysting in fish have been performed and these are limited to descriptions of five species of prosostomatous digenean. Descriptions include that of A. chandleri (Lumsden, 1968), A. pachycystis (Stein and Lumsden, 1971b), A. leighi (Stein and Lumsden, 1971a)

and Posthodiplostomum minimum (Mitchell, 1974; Mitchell and Crang, 1976) all of which encyst in either the liver, heart or kidney. In no case has a significant host reaction been reported.

Asanji and Williams (1973) investigated the nature and histochemistry of metacercarial cysts of five species of trematodes of different ecological habitats. They similarly concluded that classification of metacercarial cysts according to the site of encystment was more valuable than one based upon cyst wall composition. The cyst wall, irrespective of its origin always contains simple proteins and carbohydrate or carbohydrate-protein complexes. Further detailed histochemical analyses of cyst wall composition include P. minimum (Bogitsh, 1962), Cyathocotyle bushiensis (Erasmus, 1967a), Stellantchasmus falcatus (Lee and Cheng, 1970), A. pachycystis (Stein and Lumsden, 1971b) and Cryptocotyle lingua (McQueen, MacKenzie, Roberts and Young, 1973). Biochemical analyses of cyst wall composition are limited to Ascocotyle spp. (Lenhoff, Schroeder and Leigh, 1960; Stein and Lumsden, 1971a) and P. minimum (Lynch and Bogitsh, 1962).

Berezntsev (1975) in a study of several helminth larvae commented on the equilibrium that exists between the parasite and host relating this to evolutionary selection in the parasites for the ability to inhibit the protective leucocyte response of the host. Ultrastructural studies upon Stictodora lari (Howell, 1973) in the abdominal cavity of Gambusia affinis, showed that cysts were not encapsulated

for some weeks after infection. Glass beads, implanted into the body cavity, however, were encapsulated after a few days. He suggested that this was due to the incorporation of material of fish origin into the cysts disguising them, or, to the presence of spikes on the initial cyst wall which may be an unsatisfactory substrate for the attachment of cells.

Cottrell has recently reviewed fish immunology including the role of the immune system in disease (Cottrell, 1975). The tissue parasites, R. johnstonei and C. lingua, have been found to initiate an immune response in the plaice (Cottrell, 1976). Excysted metacercariae of P. minimum from a variety of fish exhibit metacercaricidal behaviour when exposed to serum from both infected and uninfected fish (Harvey and Meade, 1969). When heated for 30 min at 56°C serum produced no response. Metacercariae enclosed by intact cysts showed no such activity. No immunity to reinfection has been found in fatheads to Crassiphiala bulboglossa (Hoffman, 1956), or in cyprinid fish to P. minimum (Ferguson, 1943; Hoffman, 1958a). Krull (1934b) reported that older sunfish previously infected with a few U. ambloplitis were refractory to further infection.

The effect of helminth parasitism upon fish serum proteins has been investigated by Meade and Harvey (1969) in Lepomis macrochirus infected with P. minimum. They detected an increase in concentrations of the β and γ -globulin areas but little difference in the α -globulin concentration and a decrease in the albumin level. Similarly, Spall and Summerfelt (1970), also working on P. minimum, found an increase in plasma globulins but also an increase in albumin

levels. These changes were attributed to the homeostatic response of the fish to the nutritional demands of the parasite, altered liver function and to the effects of capillary permeability. Further pathological effects detected included an increase in the rate of excretion and a decrease in haematocrit readings probably due to haemorrhage caused by burrowing and a decrease in erythropoieses.

Other reports upon pathological effects have mostly been based on histological observations and are usually limited to haemorrhage and muscular necrosis (Lee and Cheng, 1970; Hoffman and Dunbar, 1963). Vascular constriction results from infections of Ornithodiplostomum ptychocheilus (Hoffman, 1958b), A. angrense (Sogandares-Bernal and Lumsden, 1963) and A. leighi (Sogandares-Bernal and Lumsden, 1964). Pathological effects due to the presence of Cryptocotyle lingua (McQueen et al, 1973) include epidermal lesions in adjacent tissues, myofibrillar necrosis associated with bacteria possibly introduced by the parasite and a reactive swelling of the intermuscular septa. The effects of migration of *Cercaria* X (Strigeoidea) upon Gasterosteus aculeatus are described by Erasmus (1959).

Deleterious effects of infections upon growth are reported in Eupomotis gibbosus (Krull, 1934b) and the blue-gill, Lepomis macrochirus (Smitherman, 1968). The influence of metacercariae on maturation and fecundity have been investigated by Hubbs (1927) and Holland (1971). In nature, heavy infections could reduce the resistance of fish to other stresses and therefore be a secondary cause of death.

Particularly heavy infections can be the immediate cause of death. Infections of C. lingua have been known to cause deaths in herring (Sinderman and Rosenfield, 1954) and O-group plaice (Steel, 1966; Mackenzie, 1968, 1971).

The so called "self cure" of heavily infected fish by rupture of the body wall, releasing some of the parasites, followed by closure of the wound has been noted in P. m. minimum (Hoffman, 1958a) and O. ptychocheilus (Hoffman, 1958b).

Trematode Tegument. Comprehensive reviews concerning the trematode tegument have been made by Lee (1966, 1972) and Lumsden (1975a). More recent reports include Ubelaker, Specian and Allison (1974), Bennett (1975), Williams (1975), Zdarska (1975), Kuntz, Tulloch, Davidson and Huang (1976), Mitchell and Crang (1976) and Wheeler and Wilson (1976).

The development of the cercarial tegument in C. lingua has been described by Rees and Day (1976) and the changes that occur during metamorphosis of the cercaria to the metacercaria by Day (1976). Matricón-Gondran (1974) also describes the formation of trematode teguments.

A comprehensive review dealing with the importance of helminth teguments in membrane transport has recently been published by Pappas and Read (1975).

MATERIALS AND METHODS

The common goby, Pomatoschistus microps, was collected from stations 1 - 4 (Fig. 1) with the aid of hand nets or seine nets. Liver squashes provided a quick and easy method of determining whether the fish were infected with metacercarial cysts of Bucephalus haimeanus. Over 1 000 fish were examined and all were found to be infected.

MORPHOLOGICAL AND CYTOLOGICAL INVESTIGATIONS

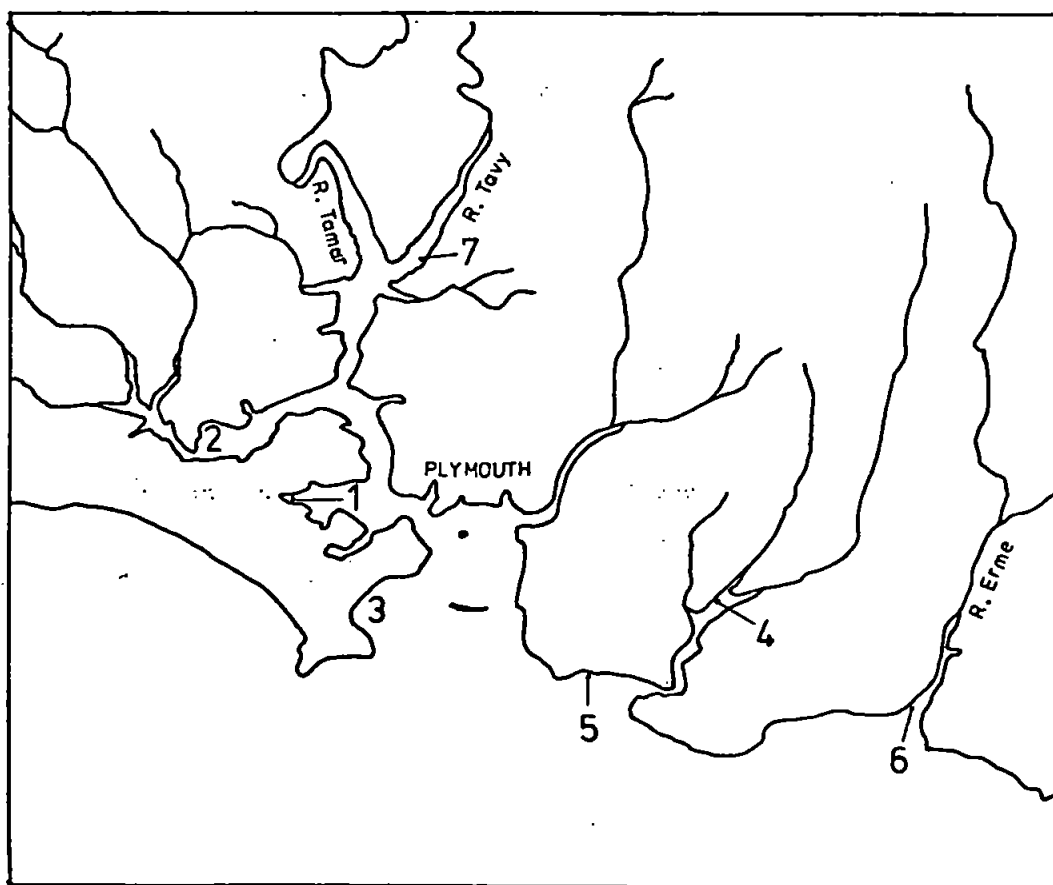
Light Microscopy. For routine histological and histochemical investigations whole infected livers were fixed in Baker's formol calcium (6 h), dehydrated in a graded series of alcohols, cleared in xylene and embedded in paraffin wax (melting point = 56°C). Sections were cut at 7 µm. The staining methods of Papanicolaou (1942, 1957), Van Gieson, Orcein and Verhoeff (Drury and Wallington, 1967) were used to show general structure.

The periodic acid Schiff's technique (PAS) (Pearse, 1968 after McManus) was used to test for polysaccharides with diastase (Gomori, 1952) and pectinase controls. PAS without prior oxidation located free aldehyde groups. Neutral mucopolysaccharides were detected using periodic acid-N, N-dimethyl-p-phenylenediamine (PAD) technique (Spicer and Jarrells, 1961) staining for 7 h, longer staining intervals of 24 h and 48 h being used to distinguish acid mucopolysaccharide. Acid mucosubstances were differentiated by

Fig. 1. Map showing collecting sites in Devon and Cornwall.

Key

1. St. John's Lake
2. River Lynher
3. Cawsand Bay
4. River Yealm
5. Wembury Beach
6. Mothecombe
7. Warleigh Point



the alcian blue stain at pH levels from 0.2 to 2.5 (Pearse, 1968) and by the alcian blue critical electrolyte concentration (CEC) method (Scott and Dorling, 1965) using increasing concentrations of magnesium chloride and also by hyaluronidase digestion (Smyth, 1956). Metachromatic components were tested for with azure A (Pearse, 1968 after Spicer and Warren, 1960) at increasing pH levels and toluidine blue. Mercury bromophenol blue and bromophenol blue procedures (Mazia, Brewer and Alfert, 1953) located total protein and basic protein respectively. The Feulgen test and methyl green - pyronin-Y (Pearse, 1968) test was used to detect DNA and RNA.

For the location of lipid material livers on fixation were transferred to gum sucrose and frozen sections cut at 10 μ m. Total lipids were stained with oil red O (Pearse, 1968 after Lillie, 1944); unsaturated triglycerides, cholesterol esters and fatty acids were distinguished from phosphoglycerides and sulphatides by means of Nile blue sulphate (modified from Cain, 1947 by Adams 1965).

Electron Microscopy. For electron microscope studies, cysts were either excised from the goby livers together with some of the surrounding hepatic tissue or metacercariae were released from the cysts by rupture of the cyst wall. The former specimens were fixed for 2 h in 4% paraformaldehyde/cacodylate fixative at pH 7.4 or in 4% paraformaldehyde/phosphate fixative at pH 7.4 (Burgos, Vitale-Calpe and Téllezde Iñon, 1967) and the latter specimens in 3% glutaraldehyde fixative in cacodylate buffer. The specimens

were washed overnight in the appropriate buffers, post-fixed for 1 h in 1% osmic acid, rinsed in buffer and dehydrated in a graded series of alcohols. Acetone was employed as an intermediate fluid before the material was embedded in either TAAB Araldite or Spurr (Spurr, 1969) resins. Sections (90 nm - 60 nm thick) displaying light gold or silver interference colours were cut using a Porter-Blum MT2B microtome, collected on either coated or uncoated grids and stained with uranyl acetate and lead citrate (Reynolds, 1963; Sato, 1967). The sections were examined in a Philips 300 transmission electron microscope.

For observation in the scanning electron microscope parasites were removed from their cysts, fixed for three days in 3% glutaraldehyde fixative and dehydrated through a graded series of acetone. Parasites were critically point dried using Polaron E 3 000 apparatus with CO₂ as transitional fluid. Cercariae after dehydration in acetone were transferred to amyl acetate and then critically point dried in a Samdri PVT-3. Specimens were examined in a Philips PSEM 500 or SEM 501 or in a Joel P15 or JSEM 35X.

ENZYME CYTOCHEMISTRY

Light Microscopy. For the determination of the following enzymes, livers were fixed in Baker's formol saline and transferred to gum sucrose prior to sectioning: β -glucuronidase (Pearse, 1972 after Hayashi et al, 1964), N-acetyl- β -glucosaminidase (Pearse, 1972 after Hayashi et al, 1965), α -glucosidase (Pearse 1972 after Rutenberg, 1960), alkaline

phosphatase and acid phosphatase (Pearse, 1968 after Burnstone), ATPase (Pearse, 1968 after Wachstein and Meisel), lipase (Pearse, 1972 after Abe et al, 1964) and non-specific esterase (Pearse, 1972 after Holt and Withers, 1952; Holt, 1958). Fresh, frozen material was used to test for succinic dehydrogenase, lactic dehydrogenase and glucose-6-phosphate dehydrogenase (Pearse, 1972). Sections were cut at 10 μ m.

Electron Microscopy. Livers, used in electron cytochemical techniques, were fixed as recommended in the methods listed below and washed in the appropriate buffer. Material was then frozen in Hamilton's freezing mixture on the stage of a Pel Cool apparatus and sectioned at 40 - 60 μ m on a Leitz sledge microtome. The sections were collected in the appropriate buffer before being transferred to the relevant incubation medium. Incubations were performed in solid watch glasses, the sections being transferred using a fine paint brush.

The staining methods used were for alkaline phosphatase (Mayahara, Hirano, Saito and Ogawa, 1967), acid phosphatase (Pfeifer, Poehlmann and Witschel, 1973) and transport adenosine triphosphatase (Na-K-ATPase) (Ernst, 1972a,b).

In addition, nucleosidepolyphosphatase activity was detected using a White and Krivit (1965) modification of the Wachstein and Meisel medium for ATPase as outlined by Hoff and Graf (1966). In this method ouabain (g-strophanthin) and parachloromercuribenzoate (PCMB) were used as inhibitors with ATP as substrate.

In all cases material, after treatment, was dehydrated in a graded series of alcohols and embedded in Spurr (Spurr, 1969) resin using acetone as transitional fluid. Blocks were sectioned as described previously and examined unstained in the transmission electron microscope.

Quantitative Cytochemistry. Quantitative densitometric measurements were made upon β -glucosaminidase. Methods most suitable for quantitative cytochemical work employ unfixed frozen material since fixation of tissue reduces the activity of glucosaminidase (Shannon, 1975) and alters the permeability of lysosomal membranes (Chayen and Bitensky, 1968). To prevent enzyme inhibition by the diazonium coupler, post-coupling methods are usually used. At first freshly excised livers were, therefore, frozen in Analar hexane at -70°C for 1 min and 10 μm cryostat sections cut at -26°C . The haft of the knife was packed with crushed 'dry ice' and the sections collected on glass slides at room temperature. The sections were stored within the cryostat cabinet prior to staining. The incubation medium was that of Moore and Halton (1976) utilising 10% low-viscosity collagen-derived polypeptide (Sigma P5115) as colloid stabiliser with an incubation time of 20 min at 37°C . Post-coupling was achieved using fast red violet LB as diazonium coupler by the method described by Moore and Halton (1976). Using this technique localisation of reaction product within the liver was very poor and was almost completely lost from the cyst wall.

As an alternative 20% polyvinyl alcohol (Sigma P8136) was used as colloid stabiliser (Shannon, 1975) with incubation times from 15 min to 2 h at 37°C. This produced no improvement in localisation of the azo-dye.

Further material was, therefore, fixed in Baker's formol saline, transferred to gum sucrose and then frozen prior to sectioning and staining with the Moore and Halton (1976) method. This gave a better localisation of reaction product within the liver but a very diffuse localisation in the cyst wall.

Owing to the failure of the above staining methods fixed-frozen material was stained using a simultaneous coupling method (Pearse, 1972 after Hayashi 1965) with fast garnet GBC as the azo-dye. This gave a more positive localisation of reaction product.

Quantitative densitometric measurements were carried out using a Vickers M85 scanning microdensitometer. The selected wavelength was 525 nm (obtained from a spectral-absorption curve of the azo-dye produced by the coupling of naphthol AS-BI and fast garnet GBC salt) with a band width of 80 and spot size number 1. 10 to 20 readings were taken on the general liver cells, liver cells immediately adjacent to the cyst wall, and from the cyst wall itself for each of four fish livers.

NUTRIENT UPTAKE

Electron Dense Tracers. To investigate uptake by the tegument of particulate material excysted metacercariae were incubated for 1 h in Young's teleost saline (YTS) (Young, 1933) in which the following tracers were suspended

- 1) 2% ferritin (x 2 recrystallised from horse spleen)
- 2) 2% horseradish peroxidase (HRPO) and 3) 1% ruthenium red (RR) (sonicated for more efficient dispersion). Control metacercariae were either fixed in 3% glutaraldehyde fixative prior to incubation with each of the tracers or incubated in YTS alone. All treatments were carried out at room temperature and in addition ferritin and HRPO incubations were also carried out at 37°C. Following treatment metacercariae were fixed in 3% glutaraldehyde fixative for 30 min. Those exposed to HRPO were stained by the DAB method for peroxidase (Graham and Karnovsky, 1966a,b) using controls in which either 3,3-DAB or H₂O₂ was omitted from the staining medium. The material was prepared for the transmission electron microscope in the normal manner as described above. Sections mounted on carbon coated grids were examined unstained or stained with uranyl acetate and lead citrate. Some sections, of metacercariae exposed to ferritin, were stained with alkaline bismuth subnitrite (Ainsworth and Karnovsky, 1972).

Quantitative Uptake of Glucose. These experiments were initially performed to determine whether an active uptake of glucose occurred and to investigate the concentration of glucose and incubation times required in subsequent autoradiographic studies.

To investigate the quantitative uptake of radioactive glucose in vitro a vial of 50 μ Ci D-glucose-6-C¹⁴ (specific activity = 53.7 mCi/mmol) was made up to 2.5 ml with YTS. Excysted metacercariae were incubated with 100 μ l aliquots of medium. At 30 min, 1 h, 2 h and 3 h time intervals incubations were halted by the addition of Baker's formol saline. Metacercariae were washed with distilled water (about 10 times) until the supernatant had a low count rate of about 50 cpm. Parasites were counted and transferred to counting vials to which 0.5 ml water and 10.0 ml Unisolve 1 (Koch-Light) were added. Count rates were made using a Philips liquid scintillation analyser. Three trials were performed. Control incubations were performed using heat inactivated cercariae to determine the role of diffusion in glucose uptake. A further trial was conducted using encysted metacercariae.

In a second experiment a 50 μ Ci vial of D-(U-¹⁴C) glucose (specific activity = 327 mCi/mmol) was made up to 2.5 ml with YTS and the final glucose concentration adjusted to 100 mg/ml. Metacercariae were incubated in a non-radioactive glucose solution also at 100 mg/ml for 2 h prior to treatment as described above. Three trials were made.

The counts per minute (cpm) were converted into disintegrations per minute (dpm) which were then converted into μ Ci radioactivity and subsequently into μ g glucose. The amount of glucose taken up by each metacercaria was expressed graphically.

Autoradiography. Autoradiographic studies employed D-(U-¹⁴C) glucose in a 50 μ Ci vial (specific activity = 1.72 mCi/mg) made up to 2.5 ml with YTS and the final concentration adjusted to 100 mg glucose per ml by the addition of unlabelled glucose. Excysted metacercariae were incubated for 2 h, fixed in Baker's formol saline, dehydrated and embedded in paraffin wax. Sections, cut at 7 μ m, were hydrated and exposed to Kodak AR 10 stripping film for 21 - 28 days using the stripping film technique for autoradiographic experiments. Slides were developed in D19 at 20°C, 4 min, and fixed in Kodak acid fixer, 10 min, washed in running water and dried.

Another group of metacercariae were incubated for 2 h in D-(U-¹⁴C) glucose (50 μ Ci vial, specific activity = 245 mCi/mmol, made up to 2.5 ml with YTS), fixed in 3% glutaraldehyde and prepared for examination in the transmission electron microscope using methods described previously. Sections were exposed to Ilford I4 gel emulsion for 3 weeks, developed in D19 at 20°C, 2 min, fixed in Kodak acid fixer, 5 min, and washed in distilled water.

Further autoradiographic experiments were carried out using L-3-Phenylalanine - ¹⁴C(U) (specific activity = 513 mCi/mmol, a 50 μ Ci vial made up to 2.5 ml with YTS), L-Tyrosine - ¹⁴C(U) (specific activity = 483 mCi/mmol, a 50 μ Ci vial made up to 2.5 ml with YTS), L-Tryptophan (methylene - ¹⁴C) (10 μ Ci diluted to 1 ml with YTS), iodinated (¹²⁵I) insulin (100 ng insulin in 5 ml phosphate buffer, 5 μ Ci ¹²⁵I) and iodinated (¹²⁵I) human serum albumin

(50 μ Ci/ml, 20 mg albumin/ml). Metacercariae were incubated for 30 min and 2 h in each of the amino acids and for 1 h in the iodinated proteins prior to treatment, as described above, for observation under the light microscope..

INFECTION OF FISH HOSTS

Collection. Fish free from infection were collected from locations thought to be remote from Cardium edule L. Rock gobies, Gobius paganellus L. and two spotted gobies, Gobiusculus flavescens (Fabricius, 1779), were collected from rock pools on Wembury Beach, Devon (Fig. 1). Quinaldine added to the pools temporarily anaesthetised the fish in order that they could easily be found and caught. Samples of the common goby, P. microps, were obtained from Aberystwyth Harbour, Wales. None of the fish examined were found to be infected. Sand gobies, P. minutus (Pallas, 1770), were captured using hand nets from the Erme estuary, Mothercombe, Devon (Fig. 1). On examination a small percentage of fish were found to be infected with B. haimeanus, some containing up to six cysts.

Maintenance. Prior to infection fish were allowed to acclimatise at 10°C for at least one week, and were maintained at this temperature throughout the experiment. A Churchill cooler was used to maintain a constant water temperature (Fig. 2). Sea water was obtained from the M.B.A. where it had been monitored to ensure high quality and constant salinity. Fish were fed every second day on either *Artemia nauplii* or 'Tetra Min' dried fish food.

Infection. Cockles, infected with the sporocyst stage of B. haimeanus were collected from Warleigh point on the R. Tamar, Devon (Fig. 1). Cercariae for experimentation were released by rupture of the sporocyst wall under sea water after removal from host tissues.

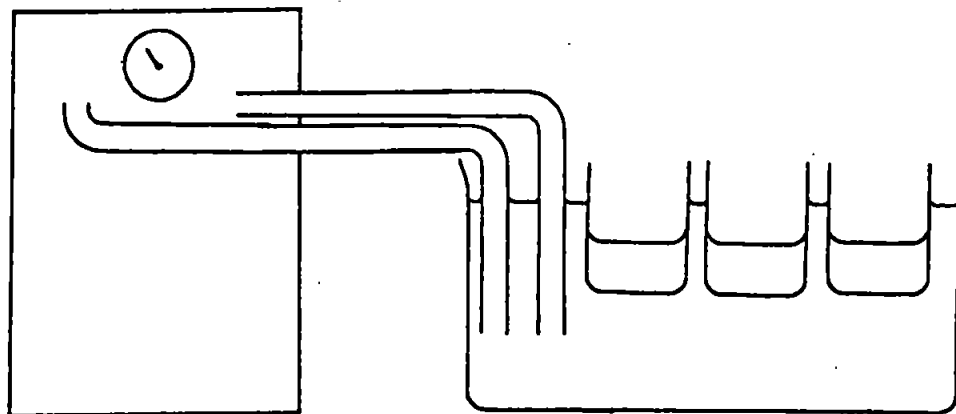
Initially samples of fish from each location were exposed to cercariae in small beakers with sufficient sea water to cover the fish for 4 h at room temperature. 24 h post infection fish were examined for metacercarial cysts.

Further experiments were carried out on G. paganellus and G. flavescens. Gobies exposed to cercariae were killed at intervals over a period of 30 - 40 days. Livers, removed from the fish, were processed for examination under the light and electron microscopes using methods already described.

CHURCHILL COOLER

OUTER TANK CONTAINING FRESH WATER

3 INNER TANKS, EACH DIVIDED INTO
3 COMPARTMENTS, CONTAINING SEA
WATER



- Fig.2. Temperature controlled tank system used for maintenance of fish.

R E S U L T S

Results are considered under the following headings:-
cyst structure and formation, enzyme cytochemistry and nutrient uptake. The structure of the cyst wall is described first, this forming the basis for the consideration of functional aspects including enzyme histochemistry. The cercarial tegument has also been investigated in order to trace the development of the parasite surface and secretory products of significance during attachment, penetration, migration and encystment of the parasite in its fish host. The dynamic aspects of infection are described together with details of infection experiments.

CYST STRUCTURE AND FORMATION

Structure of Cyst Wall

Metacercarial cysts were found throughout the liver, but it was noted that greater concentrations occurred around the gall bladder, blood vessels and periphery of the liver. Cysts had an average diameter of 200 μm . In most instances the cyst wall was in contact with hepatic parenchyma (Pls. 1-1,3) but occasionally a space was present between the two (Pl. 1-2). The cyst lumen contained granular material (Pl. 1-3).

The thickness of the cyst wall varied between 5.5 μm and 1.8 μm and was composed of three layers, an inner granular layer (0.56 μm), a middle vacuolated layer (0.37 μm - 3.82 μm) and an outer nucleated layer (1.18 μm). The middle and outer layers appeared as one layer under the light microscope.

The granular layer was separated into three zones (Pl. 1-1), an inner zone of irregularly-compacted granular material, a thinner evenly-compacted dense zone and a moderately dense outer zone which formed the major component of the layer. Darker areas of more highly-compacted granular material sometimes occurred within the moderately dense zone (Pl. 2-1).

The vacuolated layer contained membrane bound vacuoles (Pl. 1-3). The content, size and shape of these vacuoles varied and some were formed by the fusion of several smaller vesicles (Pl. 1-3). Those vacuoles adjacent to the outer granular zone appeared to be discharging their contents inwards (Pl. 2-1) and similar vacuoles occurred within the cyst cavity. Nuclei were sometimes located in this layer and stacks of membranes (Pl. 1-1) and membrane whorls were often present.

The nucleated layer adjacent to hepatic cells, was often separated from the middle layer by several membranes (Pl. 1-1). It was composed of cells illustrating different degrees of compression. Some of these cells which were considerably flattened had very electron dense, elongated nuclei (Pl. 1-3) and no distinct organelles. Other, slightly flattened electron dense cells were attached to the outer edge of the cyst (Pl. 2-1) which contained many vacuoles several of which were only partially membrane bound. Rough endoplasmic reticulum and glycogen rosettes were plentiful but other types of organelles were absent. Normal hepatic cells, in comparison, had large oval nuclei

with only small peripheral electron dense areas and had large lipid vacuoles in addition to the usual complement of cell organelles. When parasites occurred in groups the cyst dividing one parasite from another had no middle and outer layers.

On the basis of histological tests there was a lack of elastic tissue and only small amounts of connective tissue were present in the cysts. Collagen fibres were abundant around those cysts occurring at the periphery of the liver and adjacent to blood vessels (Pls. 2-1,2). Leucocytes were sometimes observed around cysts located near blood vessels (Pl. 2-2); these cells might be phagocytosing debris from the degenerating cells found at the outer margins of the cysts.

The results of histochemical tests (Table 1) indicated that the cyst wall was carbohydrate in nature containing some free aldehyde groups, galactogen and small amounts of neutral mucopolysaccharide. Most of the carbohydrate present in the inner granular layer appeared to be acid mucopolysaccharide and weakly acidic sulphomucin. There was a generalised strong reaction for proteins including basic proteins. The cyst wall was also PAS positive, diastase fast and showed no metachromasia with toluidine blue. These results suggest the presence of large amounts of carbohydrate-protein complexes. DNA was detected within the cyst wall except where two or more parasites occurred together when the dividing wall was negative, suggesting that only the vacuolated and nucleated layers of the cyst wall contained DNA (Pl. 1-2). No lipid was detected.

Table 1. The Results of Histochemical Tests on the Cyst Wall of Bucephalus haimeanus

Test		Granular Layer	Vacuolated & Nucleated Layers	Inference
PAS		+++ [@]	+++	Carbohydrate Material
Diastase PAS		+++	+++	Absence of glycogen
Pectinase PAS		++	++	Some galactogen
PAS without oxidation		+	+	Free aldehyde groups
PAD	7 h	-	-	} Neutral mucopolysaccharides
	24 h	+	+	
	48 h	+	+(1)	
Alcian blue	pH 0.2	+++	-	} Weakly or strongly acidic sulphomucins
	pH 0.5	+++	-	
	pH 1.0	+++	-	
	pH 2.5	++	-	Carboxylated mucosubstances
Alcian blue CEC	0.1 M MgCl ₂	++	-	Hyaluronic acid, sialomucin and weakly oxidised sulphomucins
	0.2 M	-	-	} Absence of most sulphomucins
	> 0.2 M	-	-	
Hyaluronidase		++	-	Absence of hyaluronic acid and chondroitin
Toluidine blue		+(P)	+(P)	β metachromasia

Table 1. Continued.

Test	Granular Layer	Vacuolated & Nucleated Layers	Inference
Azure A pH 0.5 pH 1.0 > pH 1.0	- + (faint B) + (B)	- + (faint B) + (B)	} No metachromasia. Absence of sialomucin
Oil red O	-	-	No lipid
Nile blue sulphate	-	-	No lipid
Hg BPB	+++	+++	Total protein
BPB	++	++	Basic protein
Feulgen	-	+++	DNA

@ Key

- no reaction
- + weak reaction
- ++ moderate reaction
- +++ strong reaction
- P pale purple
- B blue
- l small black dots in liver cells surrounding cysts

Formation of Cyst Wall

Infection of Fish Hosts

Natural infections were confined to estuarine species of fish namely P. microps and 0-group Crenimugil (= Mugil labrosus (Risso)). The cysts were found chiefly in the livers of these fish.

Four species of goby collected from areas thought to be distant from cockle beds were experimentally exposed to cercariae in an attempt to find a host suitable for use in further infection studies. The percentage success in establishing infections in these fish were as follows:-

<u>P. minutus</u>	100%
<u>G. flavescens</u>	90%
<u>G. paganellus</u>	10%
<u>P. microps</u> (collected from Aberystwyth harbour)	10%

Metacercariae were recovered from the livers of all gobies with the exception of P. microps, collected from Aberystwyth harbour, in which they were in the body musculature. Other species of fish which were experimentally exposed to cercariae include the blenny Blennius pholis L., scorpion fish Myoxocephalus (= Cottus scorpius L. and 0-group sole Solea solea L.. None of these became infected.

Trial experiments, to determine the effect of increasing levels of infection upon the rate of metacercarial development and the host response, were conducted upon P. minutus. These experiments are not reported in full since after their completion a number of P. minutus were found to be naturally

infected with B. haimeanus. The experimental procedure was as follows. Fish were introduced, for 4 h, to vessels containing about 25, 50, 100, 200, 400, 800, and 1600 cercariae. Squashes taken of fish killed three weeks later showed an increase in the numbers of cysts found corresponding to the increase in number of cercariae originally present in the infection vessels. Over 200 cysts occurred in the liver alone of some fish exposed to 1600 cercariae. In these instances although relatively few hepatic cells remained the liver had not disintegrated. Metacercariae were distributed throughout the body including the liver, body cavity connective tissue, gill connective tissue and musculature. Cysts from all groups of fish were of a comparable size and stage of development.

Quantitative microdensitometric readings were performed upon livers of fish from each of the above groups in order to detect metabolic changes in host cells with increasing levels of infection. Measurements of β -glucosaminidase activity were taken in liver cells distant from the cyst, liver cells immediately adjacent to the outer cyst wall and from the cyst wall itself. Differences in enzyme activity between each of these three areas were comparable with those reported later for P. microps naturally infected with B. haimeanus metacercariae. No significant difference, however, was found in readings from each of the groups of fish. In further experiments designed to follow the course of development of the cyst wall fish were not, therefore, exposed to a constant number of cercariae, this being related more to the numbers available at a given time.

Attachment and Penetration

Cercariae, released from the sporocyst into seawater either remained on the bottom of the glass vessel or became suspended in the water with the two furcae extended upwards. Initial contact with the fish host was usually made by the furcae; the cercaria becoming more securely attached at the posterior end by secretions released by the tail stem and furcae (Pl. 3-1). The major sites of attachment were immediately behind the operculum and on the pectoral and pelvic fins. When a strong adhesion had been made the cercaria investigated the surrounding area with its anterior end and penetration organ. Penetration and migration was achieved by means of lytic secretions and the spines (Pl. 3-3). The tail stem was pulled away from the cercarial body and left at the fin surface during penetration. Host tissues were ingested via the ventrally situated mouth. Considerable damage to host tissue was caused by penetration and migration (Pls. 3-3,4), haemorrhages often being apparent in the fins. Histochemical tests and ultrastructural examination of the burrowing parasite showed its structure to be essentially similar to that of the mature cercaria (Pl. 3-2).

Cercarial Tegument

The tegument resembled that of other digeneans in that it consisted of an outer syncytium connected to sub-tegumentary cells via cytoplasmic bridges. The outer cytoplasmic layer rested upon a basement membrane beneath which were circular, longitudinal and diagonal muscle bands.

Four main types of secretory vesicles denoted by V.1 - 4 were present in the tegument (Pl. 4):-

V.1) irregularly shaped vacuoles ($0.43\ \mu\text{m} \times 0.28\ \mu\text{m}$) containing a fibrous material in various states of compaction.

V.2) small electron dense ovoid vesicles ($0.15\ \mu\text{m} \times 0.06\ \mu\text{m}$).

V.3) moderately staining vesicles ($0.19\ \mu\text{m} \times 0.13\ \mu\text{m}$).

V.4) large, lightly staining vesicles ($0.60\ \mu\text{m} \times 0.30\ \mu\text{m}$) susceptible to chatter.

The outer syncytial layer contained mostly V.1 and V.2 secretory cell bodies and a few of type V.4. Tegumental cells contained either the V.1 and V.4 type or the V.3 type of vesicle. V.1 vesicles were most abundant of the four types.

Tegumental cells contained large nuclei, many mitochondria, abundant rough endoplasmic reticulum and ribosomes (Pl. 4).

The cercarial spines present in the anterior portion of the body can be seen in Pls. 5-1, 2. Those on the penetration organ were larger and more densely packed (Pl. 5-3).

The tegument covering the sucker rudiment (Pl. 6-1) was packed with a variety of vesicles including the V.1, 2 and 4 types described from other regions of the tegument. The mouth, central on the ventral surface (Pl. 6-2) opened into a sac-like intestine which extended both anteriorly and posteriorly to the pharynx. The tegument of the pharynx contained electron lucent vesicles ($0.17\ \mu\text{m}$ in diameter)

as well as small, electron dense, ovoid vesicles similar to the V.2 secretory cell bodies of the general body tegument. It was penetrated by many deep channels formed by foldings in the basal plasma membrane. The genital pore is seen in Pl. 6-3.

The bilobed tail stem (Pl. 6-3) had an irregularly convoluted surface due to the many large vacuoles present in the outer tegumental layer of this region (Pl. 7-1). Parenchymal cells of the tail stem were highly vacuolated and disorganised, apparently undergoing breakdown. Two asymmetrical furcae, capable of extension and contraction, originated from two short lateral arms of the tail stem. Large, irregularly shaped vacuoles (Pl. 7-2) present in the tegument of the adjacent surfaces of the furcae gave them a convoluted outer surface. The vacuoles were either of an electron lucent or of a moderately electron dense type (Pl. 7-3). The tegument of the opposite surfaces was more tightly folded (Pl. 7-4) since it contained smaller vesicles (0.32 μ m in diameter) of a fibrous nature. The basal plasma membrane formed many deep channels penetrating the outer tegumental layer; this system being less well developed on the adjacent surfaces of the furcae. Underlying the outer tegumental layer were numerous circular muscle bands and eight longitudinal muscle blocks. The central core of cells contained many mitochondria and were very granular in nature. The paired excretory pores opened ventro-laterally at the proximal end of each furca (Pl. 7-5).

Large numbers of sensory cilia were present over the tail stem (Pl. 6-3) and a few were scattered over the furcae and body surfaces.

Histochemical tests (Table 2) performed upon the cercariae showed the outer tegumental layer of the body to stain a deep magenta upon treatment with PAS. The intensity of staining was reduced in the diastase controls. The cytoplasmic lining of the excretory bladder also stained deep magenta but in the diastase controls was negative. The remainder of the parasite stained a blue-purple. A few small scattered regions of the tegument and some of the tegumental cells stained a blue-green upon treatment with alcian blue at pH 2.5. Alcian blue at pH 1.0 gave an intense reaction in the anterior gland cells of the penetration organ and also throughout the excretory system of the body, tail stem and furcae.

In earlier stages of cercarial development within the sporocyst the tegument differed in that the surface was microvillous (Pl. 8-2) and contained many large nuclei, a granular cytoplasm, small amounts of rough endoplasmic reticulum, mitochondria, golgi and numerous electron light vesicles. The tegument of the developing tail stem and furcae was also microvillous and like that of the body surface (Pl. 8-1). Cilia and spines were absent. The muscle layers were poorly developed.

Table 2. Results of Histochemical Tests upon the Mature Cercaria and Developing Metacercarial Stages of Bucephalus haimeanus.

T E S T	M A T U R E C E R C A R I A			M E T A C E R C A R I A					
				Day 1 - 3		Day 4 - 15		Day 15 - on	
	Outer Tegument	Tegumental Cells	Penetration Gland Cells	Outer Tegument	Tegumental Cells	Outer Tegument	Tegumental Cells	Outer Tegument	Tegumental Cells
Papanicolaou		P - B	B	P	B	P	B	-	-/B
PAS	+++	-	-	+	-	+	-	++	+++
Diastase PAS	++	-	-	-	-	-	-	+	++
Alcian blue									
pH 2.5		++ scattered	-	+	+	+	++	+	++
pH 1.0		++ scattered	+++	+	-	+	-	-	-
Feulgen	ND	ND	ND	-	+++ nuclei	-	+++ nuclei	-	+++ nuclei
Feulgen Control	ND	ND	ND	-	-	-	-	-	-
Methyl-green					+++ P		+++ P		+++ P
- Pyronin-Y	ND	ND	ND	-	some cells	-	some cells	-	some cells
					+++ B		+++ B		+++ B
					nuclei		nuclei		nuclei

Table 3. Results of Histochemical Tests on the Cyst Wall of
Bucephalus haimeanus developing in Gobiusculus flavescens.

T E S T	Day 1 - ~20		Day 30 on	
	Homogenous Layer	Cellular Layers	Homogenous Layer	Cellular Layers
Papanicolaou	P	B	P	B
Van Gieson	-	-	-	-
PAS	+++	+++	+++	+++
Diastase PAS	+	++	+	++
Alcian blue pH 2.5 pH 1.0	+++ } & cyst - +++ } cavity -	-	+++ } & cyst - +++ } cavity -	-
Feulgen	-	Nuclei +++	-	+++
Feulgen Control	-	-	-	+++
Aniline-acetic acid - Feulgen	-	-	-	-
Methyl-green - Pyronin -Y	++ (P)	+++ (B) Nuclei	++ (P)	+++ (B) Nuclei

Key for Tables 2, 3 & 4.

- No reaction
± Diffuse reaction
+ Weak reaction
++ Moderate reaction
+++ Strong reaction
ND Not done
P Pink
B Blue

Table 4. Results of Histochemical Tests on the Cyst Wall of
Bucephalus haimeanus developing in Gobius paganellus.

T E S T	Day 1 - \approx 30		Day 40 on	
	Homogenous Layer	Cellular Layers	Homogenous Layer	Cellular Layers
Papanicolaou	O	G	P	B
Van Gieson	±	-	-	-
PAS	+++	+++	+++	+++
Diastase PAS	+	++	++	++
Alcian blue pH 2.5	+++	-	+++	-
pH 1.0	+++	-	+++	-
Feulgen	-	+++ nuclei	-	+++
Feulgen Control	-	-	-	+++
Aniline-acetic acid - Feulgen	-	-	-	-
Methyl-green - Pyronin-Y	++ (P)	+++ (B) nuclei	++ (P)	+++ (B) nuclei

Development of Metacercarial Tegument and Cyst Formation

Light Microscopy. The most complete sequence of events was obtained for metacercariae encysting in G. flavescens since these were most easily infected. The developmental changes occurring in these fish are, therefore, considered first.

Results of histological and histochemical tests performed are given in Tables 2 and 3. Histological examinations were carried out using the Papanicolaou stain which differentiated an inner, pink homogenous component to the cyst wall (Pls. 9-1,2). An outer, blue staining, cellular layer with elongate nuclei was present in cysts of all ages. No connective tissue was found.

The 24 h old cyst wall stained a magenta colour upon treatment with the PAS test which was only partially lost in the diastase controls. The mature cercarial tegument stained in a similar manner. The metacercarial tegument at 24 h, however, stained only weakly with PAS and negatively in the diastase controls suggesting that the PAS positive material forming the cyst wall was secreted by the tegument. A strong reaction was also given by the cyst wall when stained with alcian blue at pH 1.0 (Pl. 9-4); this component was probably secreted by the gland cells of the cercarial penetration organ which also produced a strong reaction to this stain. The metacercarial surface also stained lightly with alcian blue at pH 1.0. The cyst wall gave an intense staining reaction to alcian blue at pH 2.5 (Pl. 9-3) but only a few cells in both cercarial and metacercarial stages

responded to this stain. There is an increase in the response of the metacercarial parenchymal cells and surface tegument to alcian blue at pH 2.5 as development proceeds. Elongate nuclei in the flattened liver cells immediately adjacent to the cyst wall were stained with both Feulgen (Pl. 10-1) and methyl-green - pyronin-Y.

The staining reactions of the cyst wall remained unchanged until about the 30th day. Cysts at this stage gave an intense homogenous reaction to the Feulgen test (Pl. 10-2) and, unlike those cysts stained in naturally infected fish, also produced a similar reaction in the controls (Pl. 10-3). The reaction, however, was negative in sections treated with aniline-acetic acid indicating the result to be due to the presence of free aldehyde groups.

Metacercariae encysting in G. paganellus differed in that the cyst wall of parasitic origin was often considerably thicker and stained orange, not pink, with the Papanicolaou stain (Table 4). The metacercarial tegument similarly stained orange and not pink. During the early stages of cyst formation small amounts of connective tissue were detectable. As in G. flavescens the cyst wall developed an intense reaction to the Feulgen test.

Electron Microscopy. Metacercariae encysting in G. flavescens and G. paganellus proved difficult to fix for the electron microscope unlike their counterparts in P. microps. A complete picture of the ultrastructural changes which occur in the developing metacercaria was not therefore obtained; gaps being particularly evident in the formation of the final tegument.

On reaching the liver the metacercaria released the V.1 inclusions from the outer tegumental layer forming the hyaline cyst wall (Pl. 11-2). Very few of these vesicles remained in either the outer tegument or tegumental cells of the 24 h metacercaria and by the second day all had been released (Pl. 12-1). Few V.2 vesicles remained in the outer tegument, these having presumably been released during migration or shortly after arrival at the liver.

The tegument initially underwent rapid reorganisation. This was accomplished, in successive stages, by the formation in parenchymal cells of secretory products which then passed into the outer layer. Considerable overlap occurred between each of the stages. Firstly, the V.3 vesicles increased in size ($0.28\text{ }\mu\text{m} \times 0.15\text{ }\mu\text{m}$) and migrated into the syncytial layer (Pl. 11-1). An electron dense granular material was produced in large quantities in the parenchymal cells and this also moved to the surface where it became dispersed throughout the cytoplasm of the tegument and interstitial material of the underlying muscle layers. Production of this granular material reached its peak on the third day (Pl. 13-1). Shortly after the mobilisation of the V.3 vesicles the V.4 secretory cell bodies passed out to the surface (Pl. 12-2). By the third to fourth day they were predominant in the outer tegumental layers (pl. 13-2).

Cells of mesenchymal origin began to produce a new type of secretory cell body, V.5, on the third to fourth day (Pl. 13-3). These vesicles were large ($0.26\text{ }\mu\text{m} - 0.77\text{ }\mu\text{m}$)

wide), irregular in outline and contained loose fibres of material. By the tenth day these had also begun to move into the outer tegument.

The contents of V.3, 4 and 5 secretory bodies were released by the parasite so that by the 15th day a complete breakdown of the outer tegument was underway (pl. 14-1). The basal plasma membrane of the tegument gradually became ill defined enabling the muscle layers to penetrate the outer layer. In some areas the outer tegument was almost completely lost (Pl. 14-2). The cellular breakdown products collected in the cyst cavity which became full of debris (Pl. 15-1). This debris gradually disappeared from the cavity during further development and was apparently re-absorbed by the metacercaria.

The destroyed tegument was then apparently replaced by the new tegument of the fully developed metacercaria. This process, however, was rapid so that some metacercariae had acquired this new surface by the 20th day, and by the 30th day all had developed a new tegument. Development was again accomplished by the formation in parenchymal cells of secretory cell bodies which then passed to the surface via cytoplasmic bridges. The sequence was not determined due to the rapidity of the process, and the inability to obtain successful fixation of material.

Three types of membrane bound vesicle were present in both the tegumentary cells and outer syncytial layer of the fully developed metacercarial tegument:-

V.6) most frequent were slightly elongated, moderately electron dense vesicles ($0.20\ \mu\text{m} \times 0.12\ \mu\text{m}$) (Pls. 16, 17-1).

V.7) spherical secretory cell bodies ($0.20\ \mu\text{m}$ in diameter) containing an irregular secretory product (Pl. 16, 42-1). A few of these vesicles were usually present in tegumentary cells mostly containing the V.6 type of secretory cell body but occasionally a cell was found which contained large numbers of this type of vesicle (Pl. 17-2).

V.8) rod-shaped ($0.20\ \mu\text{m} \times 0.02\ \mu\text{m}$), electron dense vesicles which were orientated perpendicular to the surface when beneath the outer plasma membrane (Pl. 40-1).

In addition, V.9, large electron lucent vesicles ($0.28\ \mu\text{m}$ in diameter) were present within the outer tegumental layer (Pls. 16, 42-1) and were more frequent near the outer surface. Mitochondria were scattered throughout the tegument.

Alternating rows of backwardly pointing spines, $0.28\ \mu\text{m}$ apart, occurred throughout the tegument (Pl. 18-2) with the exception of those regions surrounding the sucker, mouth and posterior pores (Pls. 19-1 - 3). Each spine ($3.50\ \mu\text{m}$ long and $1.40\ \mu\text{m}$ wide) was laterally flattened, the posterior margin being extended as pointed digits which projected above the general level of the tegument. The digits continued distally to the base of the spine as dorsal ridges. Each ridge had a central groove (Pl. 18-2). Areas of greater electron density extending along the length of the spine were associated with each corrugation and with the ventral side of the spine (Pl. 18-3). The concave

bases, which rested upon the internal plasma membrane, also stained more intensely and a similar dark zone occurred opposite this on the underlying sarcolemma (Pl. 18-1).

In section evenly spaced wavy lines ran longitudinally (average periodicity = 8.7 nm) and from the back to the front (average periodicity = 9.6 nm) of the spines (Pl. 18-3). They often changed direction at the sides of the spines to run diagonally. These lines probably represented a protein lattice; a proteinaceous composition was confirmed by histochemical tests (Table 5). The presence of some carbohydrate material was also indicated by the PAS stain.

The basement membrane of the outer tegument was composed of irregularly arranged fibres beneath which were the outer circular and inner longitudinal and diagonal muscle fibres (Pl. 18-1).

Cilia were associated with the genital and excretory pores, tentacles, sucker and mouth and a few were found over the general body surface (Pl. 20-3). In each case the cilium originated from a cytoplasmic bulb, 1.03 μ m in diameter, bound externally by the plasma membrane of the surrounding tegument. The bulb contained numerous membrane bound vesicles and mitochondria, and was attached to the surrounding tegument by septate desmosomes. A collar formed from foldings in the surrounding tegument encircled the cilium at its base. The length of the cilium was variable.

The anterior attachment organ consists of a muscular sucker surrounded laterally and dorsally by seven protrusable tentacles (Pls. 19-1, 20-1). The tegument of the tentacles

and their pits differed from that of the general body surface in that it lacked spines and showed variations in thickness ranging between 1.40 μm and 0.43 μm depending upon its state of contraction. Long necked gland cells which were supported by microtubules, were present in the anterior end of the metacercariae just behind the sucker. The neck of the gland opened into the tentacle pit to which it was attached by septate desmosomes (Pl. 20-2). The granular electron dense secretory product occurred in close packed vesicles (average diameter = 0.52 μm).

The results of histochemical tests performed are presented in Table 5. The outer syncytial layer, tegumentary and gland cells were proteinaceous and rich in carbohydrate material which included glycogen and acid mucopolysaccharide. In addition, the gland cells contained neutral mucopolysaccharide.

Micrographs of the 24 h old cyst showed it to be homogenous and of a fibrous nature extending from the parasite surface to the surrounding hepatic cells (Pl. 11-2). The junction between this fibrous material and the surrounding liver was ill defined (Pl. 11-3); the decaying cells releasing many small vesicles into the hyaline cyst wall layer.

Over the next ten days the fibrous material gradually became more packed against the liver (Pl 12-1) until by the 15th day a distinct inner irregularly-compacted zone, middle densely-compacted zone and outer moderately-compacted zone had formed (Pl. 15-2). This granular cyst wall layer no longer extended from the metacercarial surface to the liver but there was a cavity between the two. Hepatic cells

Table 5. Results of Histochemical Tests on the Metacercariae of Bucephalus haimeanus

Test		Spines	Outer Tegument	Tegumental & Gland Cells
PAS		+ [@]	++	++/+++
Diastase PAS		+	+	++
Pectinase PAS		-	-	++
PAS without oxidation		-	-	-
PAD	7 h	-	-	+
	24 h	-	-	+
	48 h	-	-	+
Alcian blue	pH 0.2	-	-	-
	pH 0.5	-	-	-
	pH 1.0	-	-	-
	pH 2.5	-	+	-/+
Alcian blue CEC	0.1 M MgCl ₂	-	-	+
	0.2 M	-	-	-
	> 0.2 M	-	-	-
Hyaluronidase		-	-	-
Toluidine blue			no metachromasia	
Azure A	pH 0.5	-	-	+ (B)
	pH 1.0	-	-	++ (B)
	> pH 1.0	-	-	++ (B)
Oil red O		-	-	+
Nile blue sulphate		-	± (P)	± (P)
Hg BPB		++	++	+++
BPB		+	+	+++
Feulgen		-	-	++ (nuclei)

[@] Key

- No reaction
- ± Diffuse reaction
- + Slight reaction
- ++ Moderate reaction
- +++ Strong reaction
- B Blue
- P Pink

continued to be incorporated into the cyst wall until by the 20th - 30th day an outer layer of flattened cells was well established (Pl. 21).

INTESTINE AND EXCRETORY SYSTEMS

The intestine and excretory system of the metacercaria were investigated because of their possible involvement in host-parasite interactions. The intestine may be involved in absorption of nutrients obtained from host cells via the cyst wall. The excretory bladder may secrete material which may contribute material to both the fluid within the cyst cavity and the cyst wall itself.

Intestine. The intestine was sac like and extended posteriorally and anteriorally from the pharynx which was situated centrally on the ventral surface.

The gastrodermis (Pl. 22-1) appeared to consist of a single layer of nucleated epithelial cells resting on a basement membrane with a sparse underlying muscle layer. The lateral cell walls, however, were infrequent and often difficult to differentiate. The thickness of the cytoplasmic lining varied considerably due to the highly convoluted nature of the inner and outer plasma membranes. Large amounts of rough endoplasmic reticulum and abundant ribosomes were present. Extending into the gut lumen were numerous lamellae (0.07 μ m thick), formed from extensions of the luminal plasma membrane and consisting of two triple-layered membranes separated by a thin cytoplasmic layer. Although these lamellae remained connected to the gut epithelium at either

end, they frequently inter-connected and branched. Large amounts of deposit were found within the gut and much of this adhered to the outer surface of the lamellae.

Vacuoles ($1.0\text{ }\mu\text{m}$ - $18.0\text{ }\mu\text{m}$ in diameter) were present both within the gut epithelium and the lumen where they remained surrounded by the lamellae. These vacuoles in turn contained cytoplasmic remnants in the form of vesicles bound by a single membrane layer and also numerous electron dense granules (Pl. 22-1). The histochemical tests indicated them to be of a lipid nature. Associated with the vacuoles were electron dense bodies ($0.10\text{ }\mu\text{m}$ - $0.70\text{ }\mu\text{m}$ in diameter) possibly lysosomes.

Excretory System. The excretory bladder was situated posteriorally to the intestine and opened to the exterior by a terminal excretory pore. The cytoplasmic lining of the excretory bladder (Pl. 22-2) had an average thickness of $0.20\text{ }\mu\text{m}$ and occasionally formed finger-like projections into the lumen. It contained nuclei, mitochondria, rough endoplasmic reticulum, numerous ribosomes and a few lipid droplets. Lamellae, similar to those of the gut projected into the lumen and interconnected to form a complex meshwork (Pl. 22-3).

Refractile bodies ($0.60\text{ }\mu\text{m}$ - $1.4\text{ }\mu\text{m}$ in diameter) (Pl. 22-3) found in the lumen were generally in contact with the lamellae. They appeared as concentric spheres of deposit around a single or multiple nucleus and stained positively for calcium, carbohydrate, free aldehyde groups, acid mucopolysaccharide, DNA and alkaline phosphatase (Pl. 22-4).

The flame cells consisted of a nucleated cell containing ribosomes, rough endoplasmic reticulum, large mitochondria and electron lucent vesicles. Cytoplasmic extensions (= ribs of Wilson, 1969) of this cell interlocked with similar extensions from the underlying barrel cell (Pls. 23-1,2). The barrel portion of the protonephridium was formed from cells folded round and joined by desmosomes. Irregular projections were present on the luminal surface of the cell and from the outer surface fine cytoplasmic projections (= leptotriches of Kummel, 1964) extended into the surrounding parenchymal cells (Pl. 23-1). Further elongate projections, or leptotriches, extended from the ribs of both nucleated and barrel cells into the barrel lumen.

Embedded within the nucleated cell were the rootlets of the cilia which extended into the barrel lumen (Pl. 23-1). An intermembranous matrix bound the cilia together.

The tubule (Pl. 23-1) was also formed from cells rolled round and joined by desmosomes. Irregular projections, similar to those of the barrel cell, were present on the luminal surface. The cytoplasm of these cells was granular, contained much endoplasmic reticulum and a few vesicles.

ENZYME CYTOCHEMISTRY

Light Microscopy. The results of the enzyme histochemical tests performed are summarized in Table 6. Intense staining reaction for β -glucuronidase (Pl. 24-1), β -glucosaminidase (Pl. 24-2), alkaline phosphatase, acid phosphatase (Pl. 24-3), ATPase (Pl. 24-4) and a slight reaction for α -glucosidase were obtained within the cyst wall. The visualisation of the reaction product for α -glucosidase was poor compared to the other azo-dye methods used. Lipase, non-specific esterase, glucose-6-phosphate dehydrogenase (G-6-PDH) (Pl. 25-1), succinic dehydrogenase (SDH), and lactic dehydrogenase (LDH) activity was absent from the cyst wall.

The outer tegumental layer of the parasite stained intensely for ATPase and weakly for acid phosphatase, α -glucosidase and SDH. The tegumental cells displayed a very strong ATPase reaction. ATPase activity was also scattered throughout the metacercaria as well as around each of the lipid droplets found in the gut. Strong reactions for β -glucuronidase (Pl. 24-1) and β -glucosaminidase (Pl. 24-2) occurred in the gut. Tests for β -glucuronidase, β -glucosaminidase, alkaline phosphatase, acid phosphatase, α -glucosidase, lipase, SDH (Pl. 25-2) and LDH all produced staining deposit scattered throughout the metacercaria. Esterase activity localised within the nervous system was due to the presence of cholinesterases (Pl. 25-3).

Electron Microscopy. Cytochemical investigations for the location of alkaline phosphatase produced a reaction

Table 6. Results of Enzyme Histochemical Tests on the Metacercaria of Bucephalus haimeanus at the Light Microscope Level

Enzyme Test	Liver	Cyst Wall	Outer Tegument	Metacercaria - General
β -Glucuronidase	+++ [@]	++++	-	++ scattered throughout +++ gut
N-Acetyl- β -glucosaminidase	++++	++++	-	++ scattered throughout ++++ gut
Alkaline Phosphatase	+++	++++	-	++ scattered throughout
Acid Phosphatase	+++	++++	+	+ scattered throughout
Adenosine Triphosphatase	++++	++++	+++	+++ scattered throughout ++++ tegumental cells
α -Glucosidase	+	+	+	++ scattered throughout
Lipase	+	-	-	+ scattered throughout
Non-specific Esterase	-	-	-	++++ nervous system
Glucose-6-phosphate Dehydrogenase	++++	-	-	+ scattered throughout
Succinic Dehydrogenase	+	-	+	++ scattered throughout
Lactic Dehydrogenase	++	-	-	+ scattered throughout

[@] Key

- No reaction
- + Slight reaction
- ++ Moderate reaction
- +++ Strong reaction
- ++++ Very strong reaction

product which mostly appeared as small clusters (0.03 - 0.09 μ m wide) of precipitate. The lead precipitate was absent in controls where substrate had been omitted from the incubation medium (Pl. 28-1). An intense reaction for alkaline phosphatase was obtained within the granular layer of the cyst wall (Pl. 26). Enzyme activity was also located in the middle cyst wall layer around the edges of vacuoles and around each of the individual vesicles, which combined, formed compound vesicles. Small amounts of activity were scattered in the outer cellular layer of the cyst wall.

Reaction product was located in the cyst cavity where it appeared to be surrounding packets of material (Pl. 26). Intense staining occurred around vesicles found within the cavity. These vesicles were either totally or partially membrane-bound with reaction product on both sides of the membrane.

A layer of deposit occurred over the surface of the parasite tegument (Pl. 26). The tegumental cytoplasm was free from precipitate apart from areas surrounding sensory cilia where there was often an intense reaction (Pl. 27-2). Precipitate in a finely granular form was located on the cytoplasmic side of the plasma membrane enclosing the cilia (Pl. 27-3). The majority of vesicles within the tegument were negative for alkaline phosphatase but in some areas a fine granular deposit was associated with all types of vesicle present. The staining deposit was found attached to both layers of the trilaminar membranes surrounding the vesicles (Pl. 27-1).

The parenchyma of the parasite was mostly negative for alkaline phosphatase, although occasional cells had reaction product located in the glycocalyx of the outer plasma membrane. The gut and excretory vesicle were mostly negative but sometimes reaction product was found on the cytoplasmic surface of the membranes surrounding lamellae (Pls. 28-2,3). The refractile bodies in the excretory bladder were strongly positive (Pl. 22-4).

Alkaline phosphatase was located in the bile canaliculi (Pl. 28-4) and along the cell boundaries of a few of the fish hepatic cells (Pl. 28-5).

The fine structural localisation of acid phosphatase produced a granular deposit scattered throughout all control tissues showing a particular association with membranous structures (Pl. 29-1). In test tissues the granular precipitate aggregated to form clusters which varied considerably in size.

Reaction product was located in the vacuoles of the middle layer of the cyst wall (Pl. 30); much of the deposit was associated with the periphery of the vacuoles. Some reaction product was found in vesicles of the outer cellular layer of the cyst wall where the halo-effect typical of lysosomal structures was apparent. The granular cyst wall and cyst cavity were negative.

The metacercarial surface was also free from staining deposit. A few areas of enzyme activity were present in the outer tegumental layer (Pl. 29-2) and large, intensely stained areas were found in the tegumental cells (Pl. 29-3).

Clusters of precipitate occurred around the inner edges of lipid droplets present in the cytoplasmic lining of the gut (Pl. 29-4).

The lysosomes of the general liver cells also stained positively for acid phosphatase.

The ultrastructural location of Na-K-ATPase, or transport ATPase, proved negative. The presence of Na-K-ATPase in the sections would have been indicated by a lack of reaction product in regions of control tissues which, in the test tissue, are positive. Control incubations, however, in which either ouabain had been added (Pls. 31-1,2) or K^+ or Mg^{++} had been omitted produced intense staining with a similar distribution to that of the test incubation. Controls in which substrate had been omitted showed a sparse non-specific deposit (Pl. 32-3).

The test incubations, which employed p-nitrophenyl phosphate (NPP) as substrate, gave intense staining of the granular layer of the cyst wall (Pl. 33). Precipitate was scattered throughout the vacuolated and cellular layers of the cyst wall with greater concentrations occurring at the periphery of the flattened nuclei (Pl. 32-1), while some activity was also present in the cyst cavity.

The outer surface of the parasite was positive (Pl. 33) and considerable amounts of reactivity were scattered throughout the parasite body (Pl. 32-2). Both inner and outer surfaces of vesicles in the outer tegumental layer stained for enzyme activity (Pl. 33); the deposit either being in the form of large, irregular dots or as fine lines associated with both lamellae of the triple

layered membranes enclosing the vesicles. Only small amounts of activity were located in the tegumental or gland cells.

Following the failure to detect a specific transport ATPase further tests were made using a variety of substrates to determine whether the activity was due to an enzyme capable of hydrolysing many nucleosidepolyphosphates. Nucleopolyphosphatase activity was observed in the inner granular layer of the cyst wall, around the vacuoles of the middle layer and scattered throughout the outer cellular layer (Pl. 34-1). Within the cellular layer precipitate was also associated with the periphery of many small spherical inclusions.

The surface of the outer tegumental layer was positive (Pls. 34-1 - 4) and activity was scattered throughout the metacercaria. The cytoplasm of the tegumental cells was strongly positive (Pls. 34-1 - 4) and this activity often spread into the outer tegumental layer via the cytoplasmic bridges (Pl. 34-3). The majority of vacuoles in the outer tegumental layer were negative (Pl. 34-2), however, a few vesicles were found which showed positive staining on the surface of the membrane (Pl. 34-4).

Although little difference in reaction product was observed when ATP (Pls. 34-1 - 4), AMP (Pl. 35-1), GTP (Pls. 35-2,5) and GP (Pls. 36-1,2) were used as substrate, the reaction resulting from hydrolysis of ADP (Pls. 35-3,4) was noticeably reduced, especially over the parasite surface.

The addition of inhibitors to the medium using ATP as substrate produced little alteration in the pattern of

enzyme distribution (Pls. 37-1 - 4), although PCMB reduced the intensity of staining. Stronger reactivity was located in the muscles underlying the tegument when ouabain was present.

Quantitative Cytochemistry. The fish hepatic cell and cyst wall staining reactions for β -glucosaminidase differed according to the staining method used. Hexane-frozen material stained by the post-coupling technique with fast red violet as the azo-dye and collagen derived polypeptide as colloid stabiliser gave a poor localisation of enzyme activity (Pl. 38-1). The majority of staining deposit was found in the hepatic cell lipid droplets with very little deposit occurring in the cell cytoplasm. The cyst wall stained diffusely and similar poor results were obtained for β -glucuronidase, alkaline phosphatase and acid phosphatase when post-coupling methods were employed.

The use of PVA as colloid stabiliser in the incubation mixture resulted in an even greater reduction in reaction product, and both hepatic cells and cyst wall stained a diffuse pink. There was no lysosomal localisation of the azo-dye.

Fixation of livers prior to staining with the post-coupling method gave an intense reaction in the fish hepatic cells (Pl. 38-2) with an improvement in lysosomal localisation. The cyst wall reaction, however, was again very weak and diffuse.

Results obtained with simultaneous coupling techniques on fixed livers were greatly improved. The liver cell staining reaction occurred in small cytoplasmic granules, 0.5 - 2.5 μm in diameter, probably lysosomes (Pl. 38-3). A strong general reaction was obtained within the cyst wall and granules, between 0.5 μm and 10.0 μm in diameter, stained intensely in the middle vacuolated and outer cellular layers of the cyst wall. These granules were probably a mixture of primary and secondary lysosomes. The densitometric measurements performed upon these sections are summarised in Table 7. The readings for the cyst wall were greater and significantly different from those obtained for hepatic cells immediately adjacent to the outer cyst wall and the general hepatic cells. There was no significant difference between the measurements of liver cells adjacent to the cyst wall and those distant from cysts.

Owing to the poor reaction and the absence of lysosomal localisation when hexane-frozen material was employed with a post-coupling staining method, attempts to determine the latency of lysosomal glucosaminidase (Moore, 1976) were unsuccessful.

Table 7. Results of Densitometric Measurements upon Naturally Infected P. microps Livers Stained for β -glucosaminidase

Area	Average Density \pm S.E.	Significance (t-test)
General hepatic Cells	28.55 \pm 1.42	$\left. \begin{array}{l} \text{NS}^{\textcircled{a}} \\ P = .001 \end{array} \right\}$
Hepatic Cells Immediately Adjacent to Cyst Wall	26.10 \pm 0.72	
Cyst Wall	54.88 \pm 2.23	

\textcircled{a} NS = Not significant

NUTRIENT UPTAKE

Electron Dense Tracers. Ferritin molecules adhered to the tegument surface, especially in the pits around the spines, of both living and fixed metacercariae. A few ferritin molecules were freely distributed within the cytoplasm of the outer tegument of living metacercariae incubated at both room temperature (Pl. 39-1) and 37°C. Molecules collected under the inner plasma membrane of parasites incubated at 37°C (Pl. 39-2). Ferritin was not found within the tegument or basement membrane of any of the controls. Microanalysis of these metacercariae yielded little useful information due to the insufficient concentration of iron in the parasitic tissues.

Horseradish peroxidase (HRPO) adhered to the surface of metacercariae (Pl. 40-1) but there was no evidence of uptake by the tegument at either temperature. A similar surface layer was present in the control group which had been fixed prior to incubation with HRPO but was absent in all other controls (Pls. 40-2 - 4).

Ruthenium red (RR) also adhered to the tegument surface but in the form of a reticular meshwork (Pl. 41-1) and as small patches of concentrated material in direct contact with the outer plasma membrane. In some areas RR, adhered to the surface membrane, took on a laminated appearance (Pl. 42-2). Larger areas of concentrated deposit were found, particularly in regions surrounding the spines, where pinocytotic uptake of the RR appeared to be taking place (Pls. 41-1,2). Within the tegument RR was associated with

the membranes of large, electron light vesicles (Pl. 42-1) but deposits located nearer the inner plasma membrane had lost this association with vesicles and occurred freely within the cytoplasm (Pl. 41-3). Small concentrations of RR were located along the cytoplasmic bridges and in tegumental cells. In fixed controls the meshwork of RR was present over the parasite surface but there were no areas of concentrated deposit either over the surface or within the tegument.

Quantitative Uptake of Radioactive Glucose. The relationship between μg glucose taken up by each excysted metacercaria and time is shown in Fig. 3 and Table 8. The values of μg glucose have been corrected for that glucose which entered the control metacercariae passively or which adhered to the surface of these parasites, and also, for that glucose which remained in the final washing solution. After an initial adjustment period uptake was rapid but not linear.

Encysted metacercariae also showed an increase in radioactive glucose count rates over time. Readings, however, were erratic probably due to glucose adhering to hepatic cells remaining attached to cysts and to a build up of glucose in the cyst cavity.

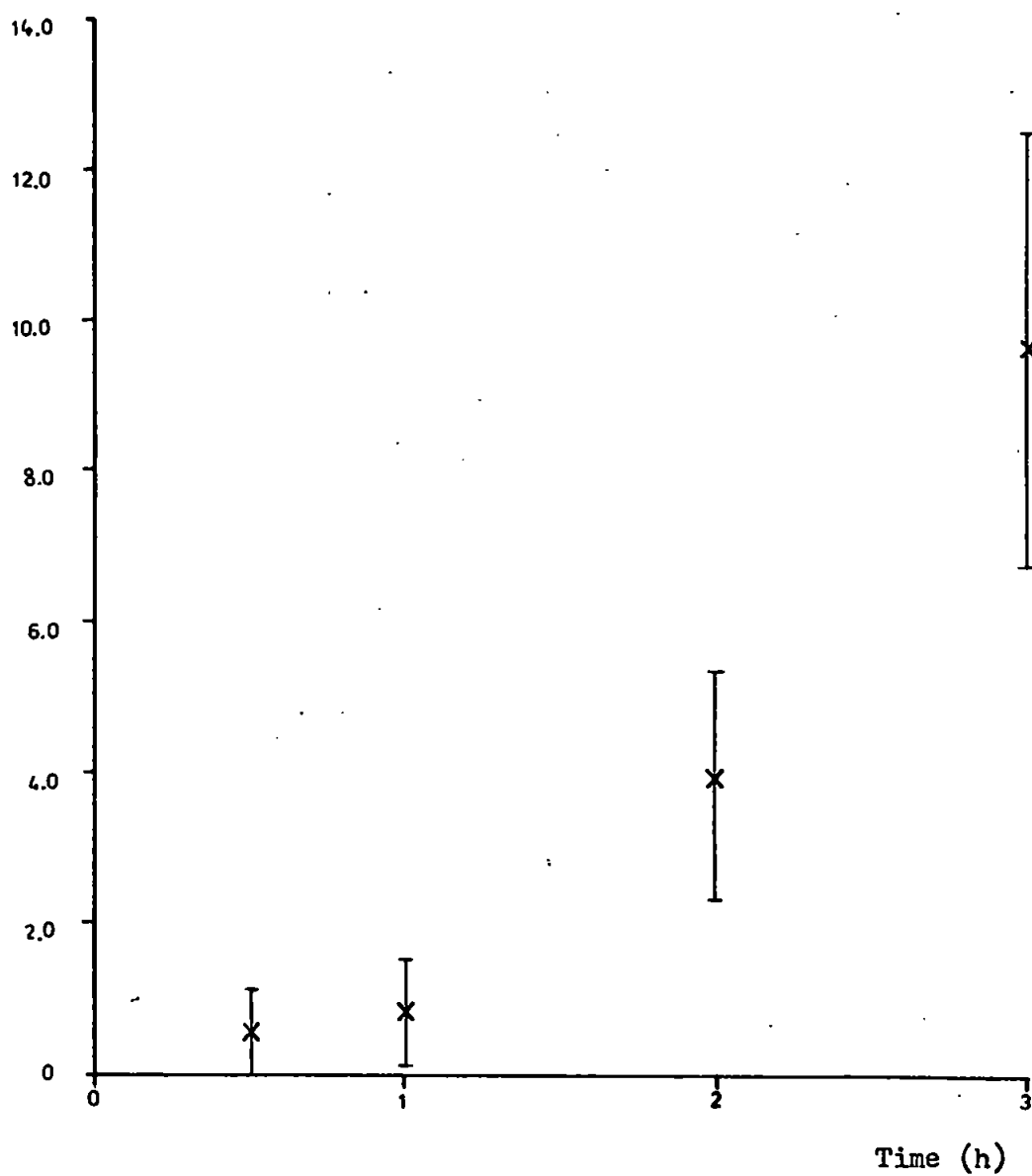
Parasites, preincubated in a non-radioactive glucose solution, also showed an increase in their radioactive glucose content over time. In some cases an average total (i.e. labelled + unlabelled glucose) uptake per metacercaria reached $9.6 \times 10^{-8} \mu\text{g}$ glucose in 4 h. The results were

Table 8. Quantitative Uptake of Radioactive Glucose

Time	Average μg glucose taken up per metacercaria \pm S.D.
$\frac{1}{2}$ h	$0.56 \times 10^{-11} \pm 0.58$
1 h	$0.78 \times 10^{-11} \pm 0.68$
2 h	$3.87 \times 10^{-11} \pm 1.51$
3 h	$9.61 \times 10^{-11} \pm 2.87$

Fig. 3. μg Glucose taken up per parasite against time.

μg glucose
 $\times 10^{-11}$



generally very variable in all three trials and have not been represented graphically.

Autoradiography. In the above experiments incubations using excysted metacercariae and radioactive glucose dispersed in YTS without any supplementary non-labelled glucose were the only ones to give reproducible results. These conditions were therefore employed in the majority of autoradiographic studies.

A considerable accumulation of radioactive glucose occurred in the tegument of light microscope autoradiographs (Pls. 43-1,2) and small amounts of radioactivity were scattered throughout the body. Similarly, radioactive traces were found in the tegumental cytoplasm and underlying muscle layers of parasites prepared for the electron microscope (Pls. 44-1,2). Large amounts of radioactivity were present at the anterior end of the parasites and this was at first thought to be the gut, however, later ultrastructural investigations revealed incorporation of labelled glucose into the secretion bodies of the anterior gland cells (Pl. 44-3).

Experiments employing labelled phenylalanine, tryptophan and tyrosine all produced similar results in that radioactivity was localised in the metacercarial tegument, however, in many cases only small regions of the tegument appeared to be involved in uptake (Pls. 45-1,4). Silver grains were also scattered over all parenchymal cells of the parasites.

The larger protein molecules, insulin and albumin were also localised in the tegument with greater concentrations occurring beneath the basement membrane (Pls. 42-3,4).

DISCUSSION

The cyst wall of Bucephalus haimeanus, naturally infecting Pomatoschistus microps collected from the Tamar Estuary, was found to consist of an inner layer of parasitic origin surrounded by two outer layers of host origin. The results of histochemical tests performed upon the cyst wall support those of Matthews (1973b) in that it contains protein and carbohydrate material some of which is an acid mucopolysaccharide. The further tests carried out reveal the presence of free aldehyde groups, galactogen, neutral mucopolysaccharide, sulphomucin, total and basic proteins, carbohydrate-protein complexes and DNA.

The inner layer surrounding the metacercaria was shown to be composed of a granular matrix, the lumen containing a similar granular material. This finding is in agreement with studies made by Lumsden (1968), Stein and Lumsden (1971a,b) and Mitchell (1974). The inner layer of the cyst wall is secreted by the parasite shortly after arrival at the site of encystment. Gland cells of the penetration organ and the tegument surface are the two main sources of secretion as indicated by the Papanicolaou, PAS with diastase control and alcian blue tests, performed before and after encystment (Table 2), and by electron microscopy. The origin of the cyst wall component which gave an intense staining reaction to alcian blue at pH 2.5 is debatable, since, only a few scattered cells in both cercarial and 24 h metacercarial stages responded to this

stain. This component may result from a secretion which undergoes a biochemical change, thus altering its staining characteristics, after release by the parasite. It is thought unlikely that this alcian blue pH 2.5 positive material could be produced by the hepatic cells in such a short time, less than 24 h, and is not therefore of host origin. Adjacent hepatic cells which later become an integrated part of the cyst wall contribute material to this layer by the inward discharge of vesicles.

The outer and middle layers were not reported by Matthews (1973b) but they can be distinguished from the inner layer, under the light microscope by the presence of nuclei and its failure to stain with alcian blue. Electron micrographs verify the presence of middle and outer layers. These two layers originate from those hepatic cells which are disrupted at the time of arrival of the parasite in the liver and also those which are later compressed by the growth and movement of the metacercaria. Stages in their destruction are exemplified by the dark, partially flattened cells, completely flattened cells and vacuolated cells. Increase in the size of the cyst to accomodate the growing metacercaria probably results from movements of the metacercaria within pushing outwards and from hydrostatic pressure of the fluid within the cyst cavity. Material may be continually added to the inner layer of the cyst wall by the parasite and this is discussed later.

Metacercarial cysts, resulting from experimental infections in *G. flavescens* ~~*P. minutus*~~ and *G. paganellus*, stained in a

similar manner to those naturally infecting P. microps except in their reaction to the Feulgen stain for DNA. Only the flattened nuclei of the cellular layers of the cyst wall stained during the first 30 - 40 days of encystment. About this time the reaction altered to give the intense uniform reaction in the middle and outer layers of the cyst wall as found in natural infections. Unlike the natural infections, however, control sections in which liver nuclei failed to stain also produced this reaction within the cyst wall. Further tests revealed that staining was probably due to the presence of free aldehyde groups. The PAS stain without prior oxidation also revealed the presence of free aldehyde groups in both natural and experimental infections. These free aldehyde groups are probably characteristic of substances formed during one of the stages in the breakdown of cellular components. It was originally thought that in natural infections this Feulgen reaction was due to the breakdown of nuclei and the liberation of nuclear material into the cytoplasm. Neither light nor electron microscope studies showed any further differences to explain why these staining reactions should differ.

Quantitative microdensitometric measurements, taken in hepatic cells distant from cysts, showed that the liver generally was not affected by the presence of the cysts even in heavy infections. This might be expected since after the initial growth phase the metacercaria becomes quiescent and demands upon the host would be very low. Further development of the parasite can only take place in the digestive tract of

the definitive host, the bass. In the present instance fish survived until the end of the experiments with only very few liver cells remaining. Little is known about the function of the liver in fish but it does appear to act as a long term storage organ. A fish when parasitised may be less able to deal with other forms of stress due to a decreased ability to draw upon its energy reserves. Pathological effects in livers of mirror carp, Cyprinus carpio, parasitised with the protozoan, Ichthyophthirius multifiliis are extensive and include devacuolization of the cells in response to hyperplasia of the tissue surrounding the parasites (Hines and Spira, 1974). In gobies a similar loss of lipid vacuoles occurred in the hepatic cells incorporated into the cysts of B. haimeanus. The fate of these lipid stores is not known since both lipase and non specific esterase were absent from this region, however, lipid droplets were found in the guts of metacercariae.

The increase in the staining reaction for lysosomal β -glucosaminidase in the flattened cells of the cyst wall as determined by microdensitometry may indicate either an increase in the amount of enzyme present, kinetic changes or activation of latent hydrolase due to decreased stability of the lysosomes (Bitensky, Butcher and Chayen, 1973) or, to a combination of these. Decreased stability may result in the release of hydrolases causing the autolysis of these cells as seen in electron micrographs. Several enzymes (Table 6)

capable of causing cellular breakdown are present in large quantities in the cyst wall. Ultrastructural localisation of these enzymes shows the inner granular layer and vacuoles of the middle layer to be the most active regions. The release of acid phosphatase from lysosomes into the cell cytoplasm has been linked with cellular lysis in the digestive epithelium of Arion hortensis (Bowen and Davis, 1971).

Many of the basic components of the parasitic cyst wall layer were elaborated in parenchymal cells and then passed to the surface to be released. Lumsden (1975a) has suggested that such cyst walls may be viewed as structures analagous to the surface glycocalyx of other trematodes which is produced in essentially the same manner. In the present instance granular material of similar appearance to that forming the inner cyst wall is distributed throughout the cyst cavity and over the parasite surface. The inner cyst wall may therefore be considered as an extension of the glycocalyx performing a similar function in the hydrolysis of material prior to uptake.

Histological tests and electron microscope studies showed there to be small amounts of collagen around those cysts which occurred at the periphery of the liver or adjacent to blood vessels. In no case were any of the cysts encapsulated by collagenous material. This is in agreement with other metacercarial forms encysting within the liver (Hoffman, 1958a; Lumsden, 1968; Mitchell, 1974). Rai (1969), however, mentions large amounts of connective tissue enclosing isoparorchiid cysts in the liver of an Indian fish.

The localised host response was only slight; occasional leucocytes were found around cysts adjacent to blood vessels. A few accounts (Hunter and Hamilton, 1941; Bogitsh, 1962; Rai, 1969) on metacercarial cysts in fish describe a marked influx of leucocytes associated with the typical immune response whereas electron microscope studies of cysts found in deep organs have described no inflammatory reaction (Lumsden, 1968; Stein and Lumsden 1971a,b; Mitchell, 1974). Stein and Lumsden (1973) considered that the similar surface electronegative charge density to be found on schistosomules and the host's leucocytes may minimize adherence of these cells to the parasite. It is possible that a similar mechanism may be operating here between the inner cyst wall when adjacent to blood vessels and host leucocytes. As mentioned previously the parasitic component of the cyst wall is strongly acidic in nature; acid glycoproteins are thought to be of a low antigenicity compared to neutral glycoproteins (Apfel and Peters, 1970).

Gobies were susceptible to reinfection by metacercariae and do not, therefore, appear to develop an acquired immunity against reinfection. Similarly fatheads, Pimephales p. promelas, and many cyprinid fish (Ferguson, 1943) were susceptible to reinfection by C. bulboglossa and P. minimum respectively. Pomatoschistus microps, collected from Aberyswyth harbour, proved difficult to infect and those parasites that did penetrate encysted in the musculature and not the liver, Matthews (1973b) was able to infect these fish with B. haimeanus obtained from cockles collected from Dovey Estuary, Cardigan

Bay, Wales. This discrepancy may be due to an age resistance operating in the fish. Matthews (1973b) found an age resistance in plaice infected with B. haimeanus as did Krull (1934b) in sunfish, Apomotis cyanellus, infected with U. ambloplitis. Development of immunity by carp to metacercariae was suggested by Zdun and Kulskovskaya (1962). Fish have been found to develop immunity to other forms of parasitic diseases for example against the ectoparasite Epibdella melleni (Nigrelli and Breder, 1934) and the protozoan Ichthyophthirius multifiliis (Hines and Spira, 1974).

Species of Gobiidae were shown to differ in their degree of susceptibility to infection by cercariae of B. haimeanus. P. microps, P. minutus and G. flavescens readily served as hosts but a fourth species, G. paganellus, was much less susceptible and only 10% became infected upon exposure to cercariae. It is interesting that these results are in agreement with the taxonomic grouping of the Gobiidae in which G. paganellus is considered to be more distantly related to the other three species listed above. Matthews (1973a) recorded a similar example of host specificity to another species of gasterostome, Proisorhynchus crucibulum. In this instance intergeneric and interfamilial specificity was exhibited within the Heterosomata. The occurrence of B. haimeanus in O-group mullet and plaice indicates that fish other than gobies are involved in the life cycle. Failure to establish infections in a wide range of other teleost species, however, further suggests a high degree of host specificity unrelated with the estuarine habitat.

Matthews (1973a) has discussed possible factors contributing to host specificity in gasterostomes. He mentions differences in the mucus covering the surface of different species of fish affecting the attachment of the parasites, mechanical barriers due to differences in skin structure and biochemical factors within the host preventing development.

It was originally planned to investigate the permeability of the cyst wall to antibodies using fluorescent antibody techniques. Sufficient blood, however, could not be collected from these small fish to proceed with these experiments. Using indirect fluorescent antibody techniques Cottrell (1976) detected antibody to secretory antigens of R. johnstonei which occurs freely in host tissues. He was unable to detect antibody to Cryptocotyle lingua which is enclosed by a thick cyst wall. This seems to support the suggestion of Stein and Lumsden (1971b) that the cyst wall serves to sequester metacercarial antigens. Reichenback Klinke (1954) also comments upon the advantage to both host and parasite of a cyst wall which partitions the parasite from a potentially destructive immune response by the host. The incorporation of host tissues onto the outside of the cyst may prevent a significant immune response by the host, a phenomenon thought to occur in many host-parasite relationships (Damian, 1964; Howell, 1973; Terry and Smithers, 1975). Fluorescent antibody experiments would have been particularly interesting in the present instance since material from the cellular layers of the cyst wall appears to be able to pass through the inner layer into the cyst cavity, and therefore,

it is probable that antibodies would also be able to pass into the cyst cavity. The cyst wall may, therefore, be of little protective value to the parasite indicating excellent adaptation by the metacercaria to the conditions in the host tissues. Gasterostomes as a group have poorly developed or no cysts. There seems to be a series between metacercariae which encyst freely on vegetation etc. where the cyst acts as a barrier to the environment through those encysting in many species of host e.g. C. lingua where again the cyst acts as a protective barrier to those which only encyst in a narrow range of hosts. Those of the latter group are highly dependent upon the host and finely tuned to the conditions found in the host tissues. The cyst wall no longer acts as a barrier but allows the intertransference of materials. The ability of materials to pass through the cyst wall may explain the failure of metacercariae to mature in plaice; the parasite being less well adapted to the biochemical environment found in this fish compared to that of the goby. This is supported by the fact that the parasites encysting in the liver have a thinner cyst wall compared to those found in the musculature and also die first. R. johnstonei and P. crucibulum are examples of the next stage in the above mentioned series in that they are not enclosed by a cyst wall of parasitic origin but feed directly upon the host tissues. Metacercariae such as B. haimeanus, R. johnstonei and P. crucibulum appear to require a wider range of metabolites from the host than those parasites which are enclosed by more complex cyst walls which only allow small molecules

such as glucose to pass through into the cyst cavity. In this respect B. haimeanus might be considered to be less highly evolved than C. lingua for example.

With reference to the classification of metacercarial cysts as outlined in the review this work would appear to support a functional rather than structural approach. The cyst wall of B. haimeanus has both a parasitic and host component and would therefore be placed in the first of the categories mentioned by Hunter and Dalton (1939). C. lingua is also a member of this category, however, there is clearly a different relationship between host and parasite in each of the two infections. To continue with a comparison of the functional aspects of the cyst wall between these two species; in both cases the cyst wall acts as a barrier to phagocytes but appears to enable antibodies to pass through into the cyst cavity of B. haimeanus whereas in C. lingua it would prevent this from occurring. B. haimeanus obtains a wide range of nutrients from the host but C. lingua only obtains small molecules from the host. The nature of the cyst wall, therefore, enables the parasite to control its immediate environment to a certain extent.

Effects upon growth and fecundity were not investigated although the metacercariae might be expected to inhibit both these processes. Deleterious effects of infections upon growth have been reported in Eupomotis gibbosus (Krull, 1934b), and the bluegill, Lepomis macrochirus, (Smitherman, 1968). Maturation in certain freshwater fish is inhibited by parasitic infections (Hubbs, 1927) although Holland (1971) found that

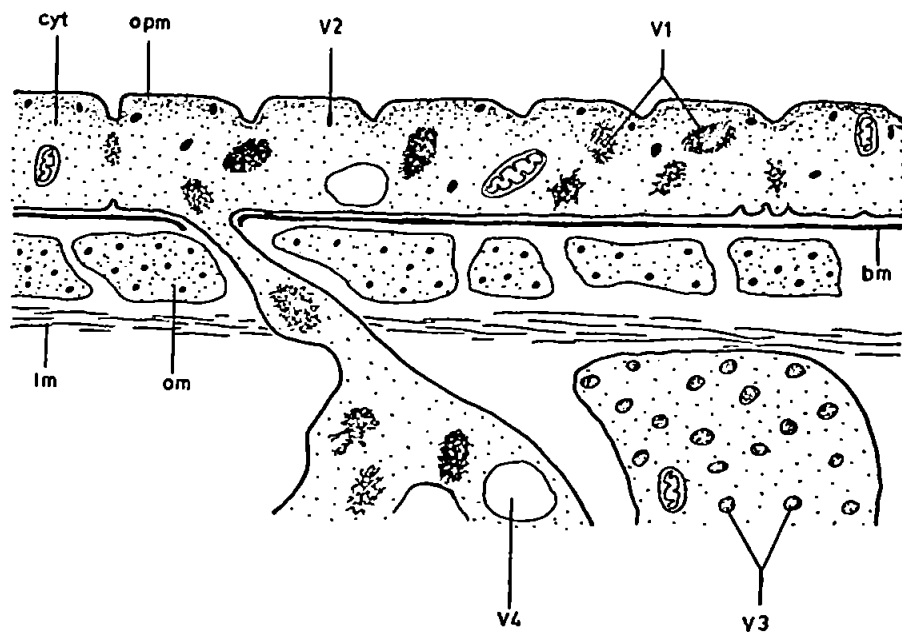
a strigeoid metacercaria had no effect upon the fecundity of Gambusia affinis.

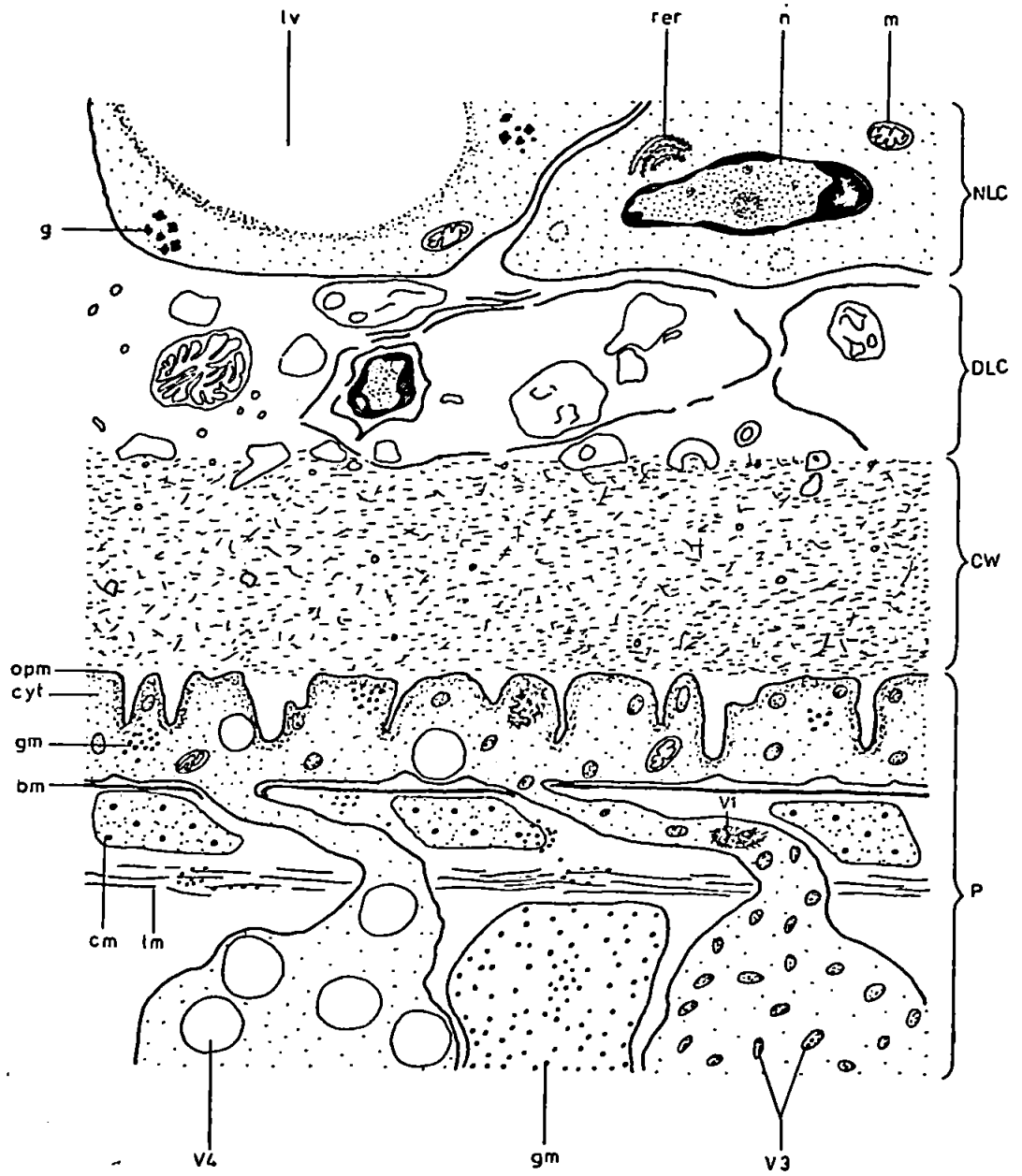
Electron microscope observations showed the tegument to be essentially similar to that in other digeneans studied in that there was an outer cytoplasmic layer connected by cytoplasmic bridges to underlying nucleated cells.

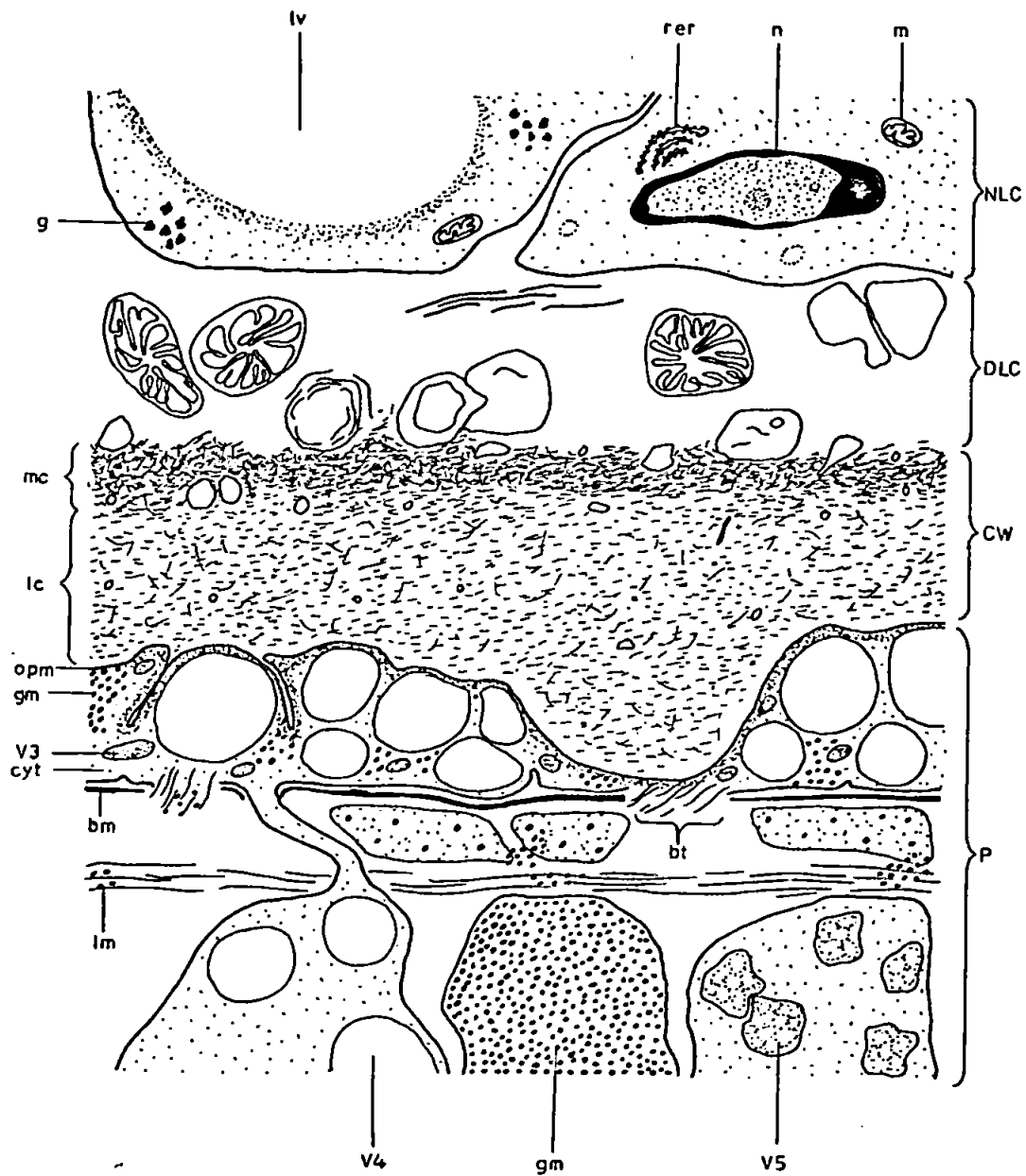
It is evident, by the microvillous nature of the tegument of developmental stages of cercariae that they obtain nutrients from the sporocyst via their surface during growth. The tail stem of the liberated cercariae is highly vacuolated, the contents of these vacuoles being released on contact with the fish to form an attachment between the parasite and host. Many sensory cilia, present on the tail stem and furcae, aid in the location of a suitable attachment site. The tegument of the body of fully developed cercariae is adapted for survival in sea water and the formation of the cyst wall. On arrival at the liver development of the tegument is accomplished by a sequential movement of secretory cell bodies from parenchymal cells into the outer layer. The formation of the metacercarial tegument from the cercarial tegument and the related formation of the cyst wall is summarised in Figs. 4 - 8. Briefly, the fibrous contents of vesicles in the outer tegument are released by the metacercaria to produce the initial cyst wall. These vesicles are replaced by secretory cell bodies from underlying tegumental cells in rapid sequence. The contents of these vesicles are also released into the cyst cavity. Sections taken from several developing

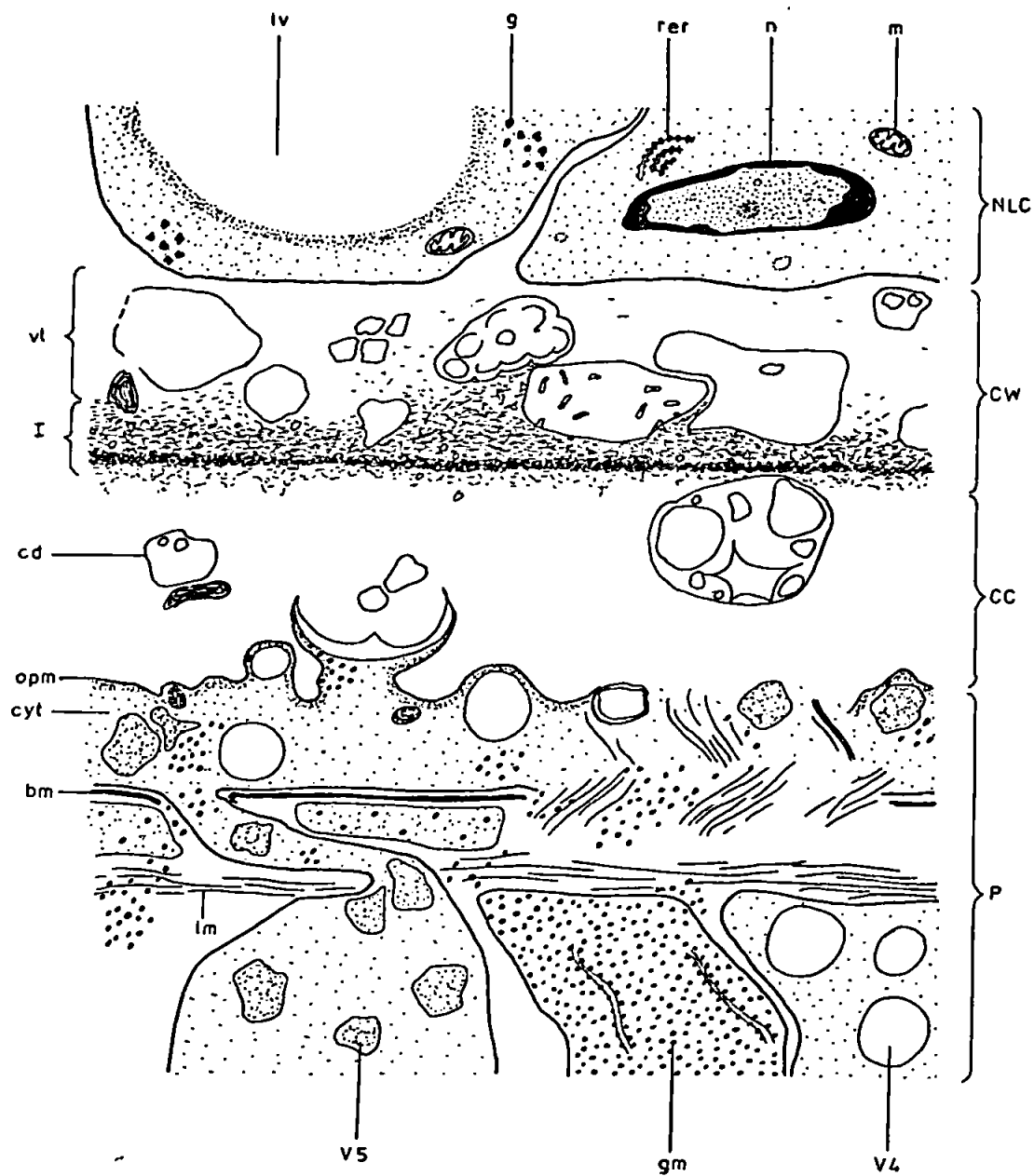
Figs. 4 - 8. Diagrammatic representation of changes occurring in the tegument of Bucephalus haimeanus during its metamorphosis from the cercarial to metacercarial stage and the concurrent formation of the cyst wall.

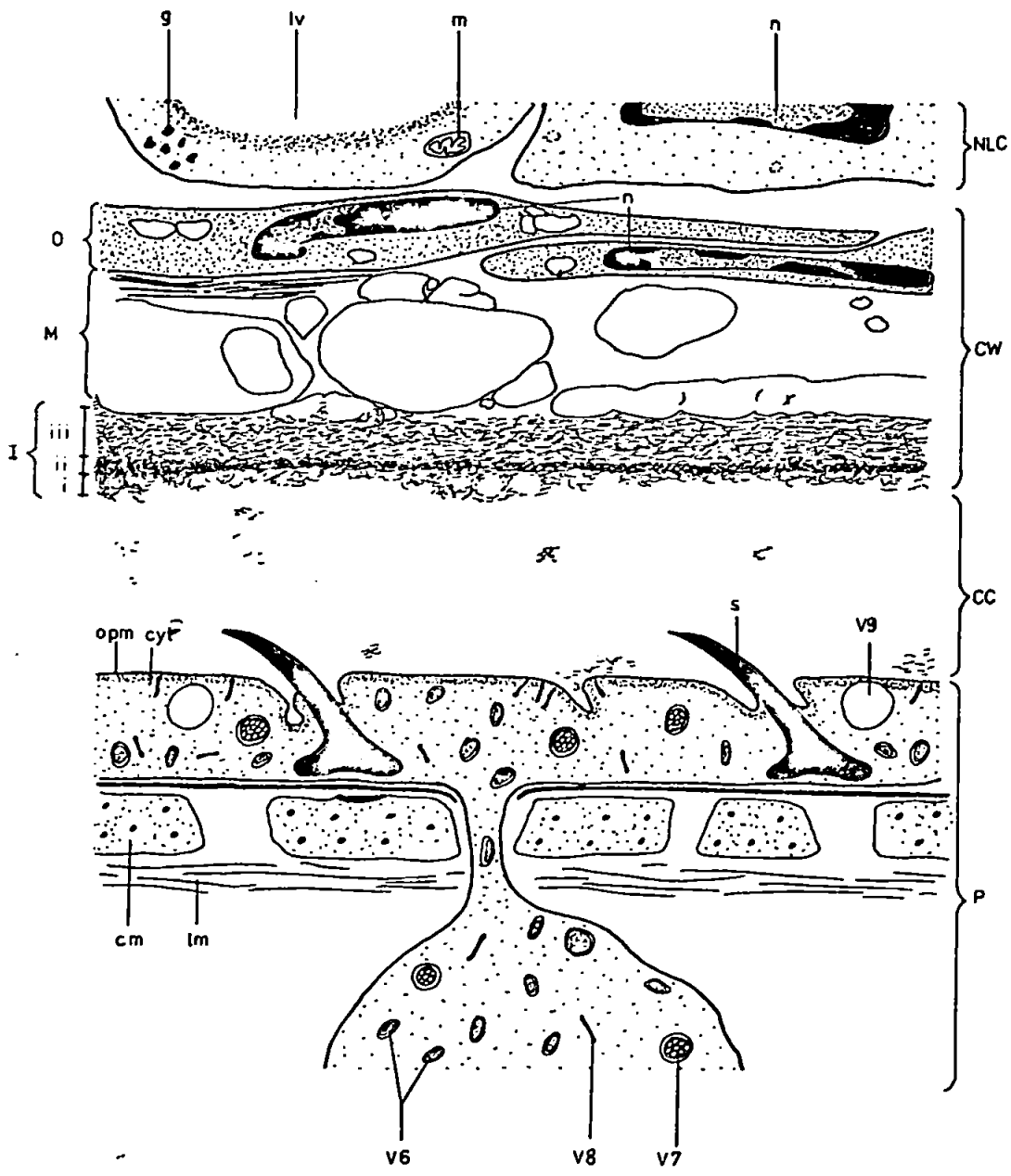
- Fig. 4. Mature cercaria.
- Fig. 5. 1 = 2 day metacercaria.
- Fig. 6. 2 = 10 day metacercaria.
- Fig. 7. 10 = 20 day metacercaria.
- Fig. 8. Mature metacercaria.











metacercariae show an almost complete breakdown of the outer tegumental layer prior to its replacement by the tegument characteristic of the fully developed metacercaria. The development of the tegument and cyst wall in C. lingua is also accomplished by the sequential release of secretion bodies formed in parenchymal cells, from the outer cytoplasmic layer of the tegument (Rees and Day, 1976; Day, 1976).

The folding of the outer plasma membrane of the mature metacercarial tegument might serve to increase the surface area for absorption. The spines present in the outer layer rested upon an internal plasma membrane as in most other trematode species. The protein lattice of the spines differed from those described in detail for H. medioplexus (Burton, 1964) and for S. mansoni (Smith, Reynolds and Von Lichtenberg, 1969) in that the periodicity of the bands was similar in both planes and showed no evidence of broad reinforcement bands. The dark zones present at both the base of the spine and underlying muscle may represent an association between the muscle and spine.

The cilia described were probably sensory and were similar to those described in other trematode species (Erasmus, 1967c; Lyons, 1969; Bibby and Rees, 1971). They were more abundant around the tentacles and posterior pores where they may aid in copulation. Short and long cilia were present which may indicate the presence of different types of sense organ.

Underlying the outer tegument were the muscle blocks embedded in a fibrous interstitial material similar to that

described in Cyathocotyle bushiensis (Erasmus, 1967c) and D. phoxiini (Bibby and Rees, 1971) and not amorphous as in F. hepatica (Threadgold, 1963a,b).

The oval, most frequent type of vesicle, V.6, found in the outer tegumental layer, were of a similar electron density to the tegumental cytoplasm and may contribute to the ground substance of the tegument (Wilson and Barnes, 1974a,b).

V.7 vesicles had an irregular content and sometimes appeared to be on the point of releasing their contents into the cyst cavity. These vesicles may therefore, be the source of a) granular material which is continuously being added to the inner edge of the cyst wall, b) material within the cyst cavity or c) to the glycocalyx (Bogitsh, 1968; Shannon and Bogitsh, 1971). The rod-shaped, V.8, vesicles were also often in contact with the outer plasma membrane and may also contribute material to the glycocalyx.

Large electron light vesicles, V.9, often seen in the tegument may have been produced by pinocytosis as suggested by the tracer studies utilising ruthenium red (RR). When present beneath the outer plasma membrane the RR was located in this type of vesicle. Towards the inner border of the syncytial layer the RR was no longer contained within these vesicles. After passing along the cytoplasmic bridges the RR was dispersed through the tegumental cells. These results agree with those observations of Threadgold (1977). Working on the plerocercoid of Schistocephalus solidus, he also found that the RR appeared to lose its association with the pinocytotic vesicles as it passed through the tegument.

The laminated appearance of RR adhering to the surface in some areas is probably due to the presence of a multilayered plasma membrane in these regions. Multilayered configurations have been reported in postcercarial Schistosoma mansoni tegument (Hockley and McCaren, 1973) where they were thought to result from the stacking of separate unit membranes during rapid elaboration of the plasmalemma.

Uptake of ferritin following a similar sequence of events may explain the presence of a few molecules within the tegument cytoplasm and their collection beneath the basement membrane. The accumulation of ferritin in the fibres of the basement membrane underlying the adhesive organ has previously been demonstrated in Apatemon gracilis minor (Erasmus, 1972). Again there was no evidence of pinocytosis and this was related to the possible occurrence of transmembranosis; a process described by Tanaka (1962). The uptake of ferritin was also observed in Haematoloechus medioplexus (Rothman, 1968). Ferritin was thought to be absorbed by Hymenolepis diminuta (Rothman, 1967); a claim disproved by Lumsden, Threadgold, Oaks and Arne (1970). Ernst (1975) also failed to find evidence of uptake of ferritin as did Bibby and Rees (1971) who also worked on metacercariae found in fish.

Horseradish peroxidase (HRPO), a smaller molecule than ferritin (diameter = 3.5 - 4.0 nm as compared to ferritin which has a diameter = 11 nm) was not taken up by the parasite in the 1 h incubations used. Similarly uptake was not detected by Ernst (1975) but it has been found to occur

in the dorsal tegument of male S. mansoni (Smith et al, 1969) and in Taenia hydatigena (Muthukrishnan, 1975). In the present instance, however, HRP0, like ferritin and RR did bind to the surface of the worm. It is thought that binding to the glycocalyx covering the surface of the worm is a pre-requisite for uptake (Lumsden, 1975b). The amoeba, Chaos chaos, has been found to selectively bind colloidal particles and proteins to its cell surface prior to uptake by pinocytosis (Brant and Pappas, 1960).

The site of pinocytosis was often associated with the indentations of the tegument around the spines possibly indicating the presence of specific binding sites and a concentration of nutrient material prior to uptake.

It should be mentioned that the above electron dense tracers are not normally encountered by the parasite and may be harmful to it, possibly having a deleterious effect upon the parasite surface. For this reason the experiments utilizing iodinated insulin and albumin may provide a better indication of whether the tegument is capable of absorbing large molecules. Autoradiographic experiments employing these molecules were successful in demonstrating uptake by the metacercariae. Uptake again appeared to take place across the tegument with a concentration of material in the basement membrane region.

Glucose is considered to be the prime energy source in digeneans and because of its importance an active absorption might be expected. The uptake of glucose was, therefore, studied using excysted metacercariae and further investigations

involved the detection of enzyme systems which might be involved in uptake. The autoradiographic experiments using radioactive glucose agree with those of Bibby and Rees (1971) on the metacercarial stage of D. phoxini in that the parasite is capable of absorbing dissolved nutrients through its tegument. Further examples of uptake of glucose through the trematode tegument include S. mansoni (Robinson, 1961), Haematoloechus medioplexus (Burton, 1962), F. hepatica (Knox, 1965; Hanna, 1976) S. haematobium (Fripp, 1967) and Philopthalmus megalurus (Nollen, 1968). Measurement of glucose uptake over time in B. haimeanus indicated that an active process was occurring. Unlike D. phoxini (Bibby and Rees, 1971) and the pleurocercoid stage of Schistocephalus solidus (Burns and Walkey, 1975) uptake was not linear over time. This may have been due to an initial adaptation by the metacercariae to the medium followed by a rapid increase in the rate of uptake.

Phosphatases have often been associated with the surface of cells on which active transport of material across the cell surface is taking place (Erasmus, 1968; Threadgold, 1968; Dixon, 1970; Krupa and Bogitsh, 1972). Their presence is not, however, universal in such cells (Ellis, Coertemiller, de Iellis and Kablatsky, 1963) and are not apparently needed for glucose transport in Hymenolepis diminuta (Phifer, 1960). Alkaline phosphatase was present over the tegumental surface of B. haimeanus. Acid phosphatase was absent from the metacercarial surface but was scattered within the tegument.

A Na^+ dependent, ouabain sensitive glucose transport system has been demonstrated in the tapeworm Calliobothrium verticillatum (Fisher and Read, 1971) and in H. diminuta (Gallogly, 1972). Glucose transport in Taenia crassiceps larvae is also Na^+ sensitive (Pappas, Uglem and Read, 1973) and here it was considered that the driving force for glucose transport was the efflux of Na^+ coupled directly to the hydrolysis of ATP through Na-K-ATPase. The system was not, however, sensitive to ouabain and it was thought this may be due to the compartmentalisation of the ouabain within the larvae at a site distant from the Na-K-ATPase. Crane (1962) suggested a Na^+ gradient hypothesis for the transport and accumulation of sugars (Fig. 9) and Fujita and Nakao (1973) visualized a mechanism in which Na-K-ATPase expells Na^+ at the baso/lateral membranes of the cell, this driving active absorption (Fig. 10). Ernst (1972a,b) devised an ultrastructural cytochemical staining method for Na-K-ATPase and located enzyme activity on the baso/lateral membranes of avian salt gland cells. Using the method of Ernst (1972a,b) K-NPPase activity was located on the cytoplasmic side of the apical plasma membrane covering the microvilli and tegument of daughter sporocysts of S. haematobium and S. mansoni (Krupa, Lewis and Del Vecchio, 1975). In the present instance, however, the Ernst method produced a reaction deposit which was not sensitive to ouabain nor to the omission of K^+ or Mg^{++} from the incubation media. Precipitate was located on the outer tegumental surface as well as being associated with the membranes of vesicles

within the cytoplasm. This result gives little support for a Na-K-ATPase driven transport system operating across the tegument of B. haimeanus.

More recently Uglem (1976) has suggested a model for glucose transport in H. diminuta (Fig. 11). There is little evidence for transcellular movement of Na^+ during active transport in H. diminuta as is found in mammalian intestine. In this model, therefore, a baso/lateral distribution of Na-K-ATPase might not be expected.

Also present were enzymes capable of hydrolysing a variety of substrates, namely, ATP, AMP, ADP, GTP AND GP. There may have been one or a few enzymes of broad specificity or many specific enzymes sharing a common site. Bogitsh and Krupa (1971) suggested that areas displaying this kind of nucleosidepolyphosphatase activity were the sites of transport where ions and molecules crossed over into the tegument. There is no nucleosidepolyphosphatase activity present over the surfaces of Megalodiscus temperatus (Shannon and Bogitsh, 1971) or Gorgoderina attenuata (Parkening and Johnson, 1969), neither of which show evidence of tegumental transport. In cestodes it has been shown that the site of hydrolysis of nucleotides and sugar phosphates is close to the loci of mediated transport thus minimising diffusion away from the surface (Dike and Read, 1971; Pappas and Read, 1974) The proximity of these two sites suggests a kinetic advantage for the absorption of the products of nucleotide hydrolysis.

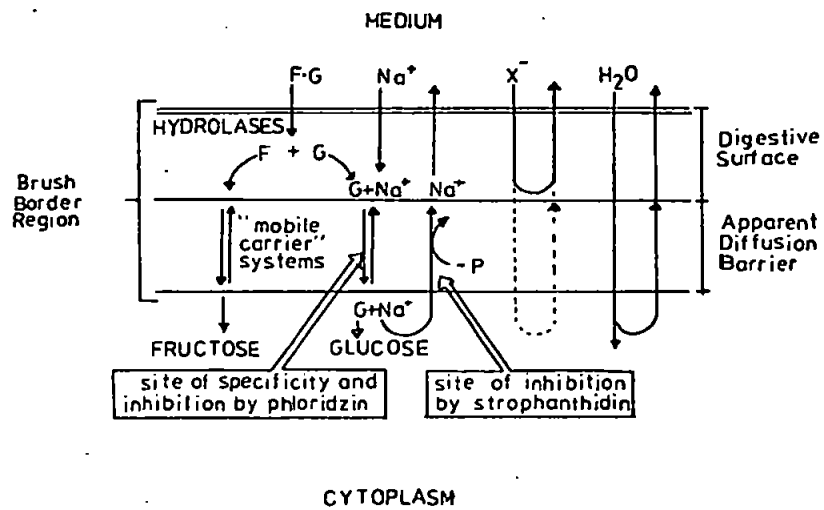
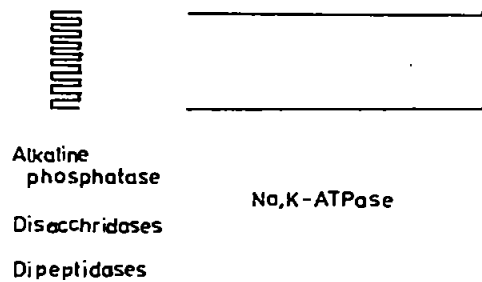
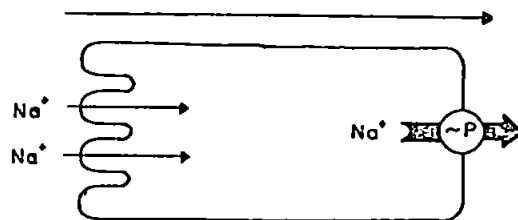


Fig. 9. Hypothesis for mechanism of intestinal active transport of sugars.

Taken from Crane, 1962.



Mutually exclusive distribution of digestive enzymes and ouabain-sensitive ATPase in intestinal mucosal surface membrane.



Transcellular transport of Na⁺ effected by asymmetric distribution of Na⁺ pump activity.

Fig. 10 Taken from Fujita and Nakao, 1973.

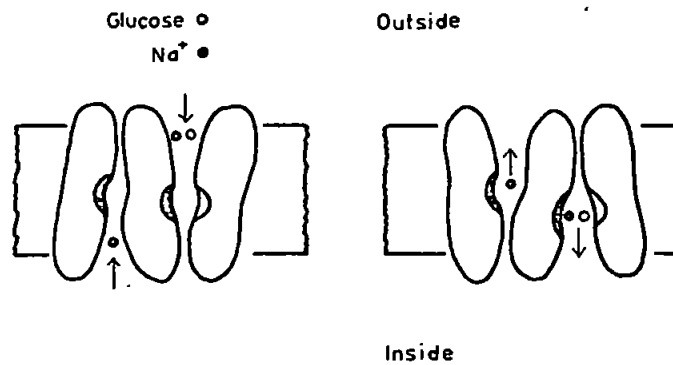
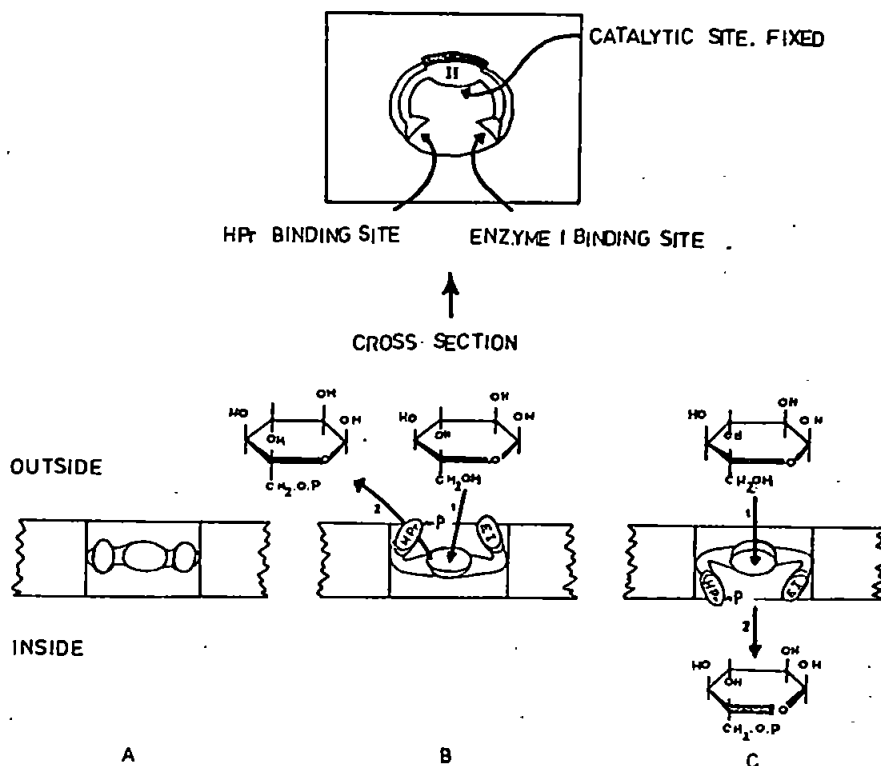


Fig. 11. A two-dimensional model of the glucose transport system in *H. diminuta*. The carrier consists of six subunits of protein forming two structurally separate channels across the membrane. The darkened and outlined areas are the active centers of the channels. The carrier is asymmetric in that glucose, (O) and Na^+ , (●) from the outside are attracted to the active center of one channel while Na^+ alone is attracted to the other. The conformational change in the carrier which transports glucose and Na^+ into the cell through one channel is accompanied by a complementary conformational change which ejects Na^+ from the cell through the other.

Taken from Uglem, 1976.



A. Stripped Enzyme II

B. In vitro assay: HPr and Enzyme I added to outside

C. Transport: HPr and Enzyme I on inside

E I - Enzyme I

E II - Enzyme II

HPr - Heat stable, low M.Wt. protein: functions as P carrier

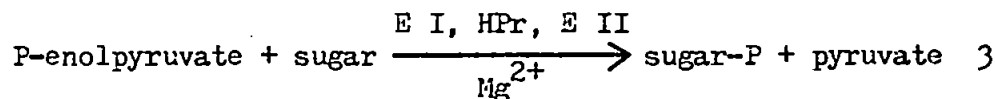
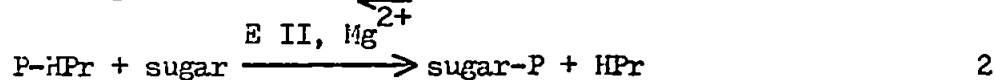
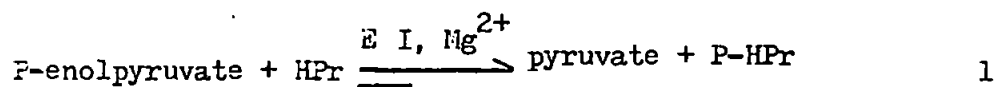


Fig. 12. Schematic model for the mechanism of vectorial (i.e., transport) and nonvectorial phosphorylation of -methylglucoside by isolated bacterial membrane preparations.

Taken from Kaback, 1970.

Glucose transport in S. mansoni was found to be only slightly inhibited by Na^+ and insensitive to ouabain, iodoacetate and phloridzin (Isseroff, Bonta and Levy, 1972). Isseroff et al suggested that a vectorial effect involving 'group translocation' (Kaback, 1970) (Fig. 12) may be operating. Similarly Na^+ had no effect on glucose transport in F. hepatica (Isseroff and Read, 1974) which were also incapable of accumulating monosaccharides against a concentration gradient suggesting that 'facilitated diffusion' was responsible for glucose transport.

Amino acid transport also occurred across the tegument in B. haimeanus and there was some indication that uptake might also occur across the gastrodermis. Amino acid uptake via the tegument has similarly been shown to occur across the tegument of F. hepatica and Fascioloides magna (Isseroff and Read, 1969; Isseroff and Walczak, 1971) and S. mansoni (Senft, 1968; Chappell, 1974; Asch and Read, 1975a). In Fasciola hepatica amino acids appeared to enter by diffusion but in S. mansoni although cysteine was absorbed by diffusion the presence of at least five specific transport systems was suggested to account for the uptake of various other amino acids (Asch and Read, 1975b). The gut also appears to have a role in amino acid uptake in F. hepatica (Thorsell, Björkman and Appelgran, 1966) and male S. mansoni (Senft, 1968), and is the only route of entry in Haematoloechus medionexus (Pappas, 1971).

For completeness the ultrastructure of the intestine and excretory system were described. The gastrodermis

displayed many similarities to those previously described in other digenetic trematodes (Bogitsh, 1975). It consisted of a single epithelial layer with numerous protoplasmic projections providing a large surface area for absorption. Cell boundaries were often difficult to find, but sometimes, adjacent lamellae were seen to adhere to each other with the cellular junction extending down into the epidermal cytoplasm; a structure noted by Thorsell and Björkman (1965) and Davis, Bogitsh and Nunnally (1968) in H. medioplexus. Rough endoplasmic reticulum and ribosomes were plentiful indicating the syntheses and export of proteins. Electron dense inclusions possibly lysosomes bordered and discharged their contents into the lipid droplets present in the gastrodermis. Lipid droplets have been reported to accumulate in the intestine of metacercariae of Leucochloridium constantiae (Fried and Shapiro, 1975). Lipase and non specific esterase activity was lacking. There was no evidence of release of material into the gut lumen or of phagocytosis. The basal membrane did not form invaginations into the gastrodermis, a structure typical of many digenean gut linings (Davis, et al, 1968; Morris, 1968). There was no evidence of a cyclical transformation between absorptive and secretory cell types as in F. hepatica (Gresson and Threadgold, 1959; Thorsell and Björkman, 1965).

The ultrastructure of the excretory bladder was similar to that in Cyathocotyle bushiensis (Erasmus, 1967b) in that it consisted of a cytoplasmic lining containing nuclei, mitochondria, rough endoplasmic reticulum, ribosomes and lipid droplets. The luminal plasma membrane was extended to form

lamellae. The numerous refractile bodies present were similar in composition to those analysed by Chowdhury, Dasgupta and Ray (1955, 1962). Energy dispersive X-ray microanalysis has been performed upon these calcareous corpuscles (Mitchell and Crang, 1976). They consist of concentric layers of deposit layed down peripherally at first (Martin and Bils, 1964). Martin and Bils suggested that mitochondria or cytoplasmic membranes formed the nuclei around which the salts were deposited. The corpuscles may function in CO₂ fixation and Phosphate storage (Von Brand, Weinback and Clagget, 1965) or serve in buffering acids (Martin and Bils, 1964).

The flame cells followed the structural pattern reported for other trematodes by Kummel (1958, 1964), Kruidenier (1959), Lautenshlager and Cardell (1961) Senft et al (1961) and Wilson (1969).

In summary, therefore, host tissues are ingested by the parasite as it burrows through the fish to the liver. Following encystment there is a three fold increase in size of the parasite during the growth of the metacercarial stage, which remains active. This ingested material is a source of energy for these processes but the metacercaria appears to continue to take up nutrients during its encystment and this process is summarized in Fig. 13.

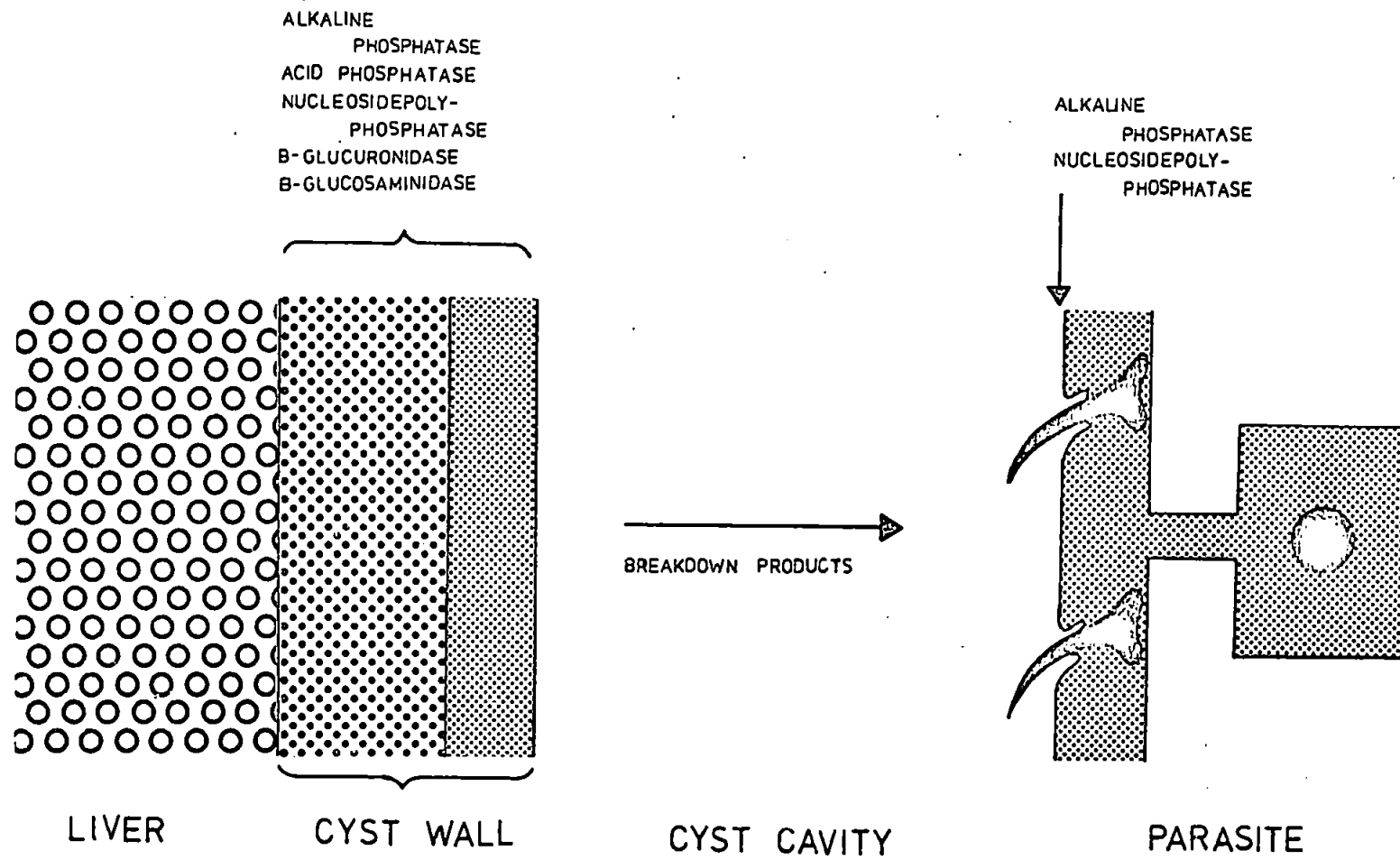
The probable food source is the breakdown products of the decaying liver cells forming the outer layers of the cyst wall. Material was seen to pass from the vacuolated layer of the cyst wall through the granular layer into the cyst cavity. Many enzymes capable of cellular breakdown were demonstrated in the cyst wall.

The presence of several enzymes over the surface of the parasite indicated that nutrients were broken down into still smaller molecules at this site.

Mitochondria and associated SDH activity within the outer tegument indicated that energy requiring processes were occurring. Using electron dense tracers and autoradiographic techniques uptake by the tegument of both particulate material and small molecules in solution has been demonstrated.

A well developed relationship, therefore, seems to exist between host and parasite in which the metacercaria is able to exist for long periods of time in the fish host without directly causing any severe, deleterious effects.

Fig. 13 Summary of Relationships between Fish Liver, Cyst Wall and Metacercarial Tegument.



KEY TO PLATES

AS	Adjacent surface of furca
AZ	Attachment zone
B	Bulb
BC	Barrel cell
BM	Basement membrane
BT	Breakdown of outer tegumental layer
C	Cilium
CB	Cytoplasmic bridge
CC	Cyst cavity
CD	Cellular debris
CJ	Cellular junction
CM	Circular muscle
Co	Collagen
CW	Cyst wall
Cyt	Cytoplasm
D	Desmosome
DLC	Degenerating liver cells
DM	Diagonal muscle
E	Excretory pore
ET	Excretory tubule
F	Furca
Fl	Flame

KEY Continued.

G	Glycogen
GC	Gland cell
GM	Granular material
GP	Genital pore
Gr	Groove
I	Inner layer
	i } zones of inner layer
	ii }
	iii }
Im.Ce	Immature cercaria
In	Intestine
IPM	Inner plasma membrane
L	Liver
La	Lamellae
Le	Leptotrich
LC	Less compacted region of inner cyst wall
LM	Longitudinal muscle
LN	Liver cell nucleus
LV	Lipid vacuole
Ly	Lysosome
M	Middle layer
Ma.Ce	Mature cercaria
MC	More compacted region of inner cyst wall
Mit	Mitochondrion
Mo	Mouth
Mt	Microtubule
Mv	Microvillus

KEY Continued.

N	Nucleus
NC	Nucleated cell
NLC	Normal liver cells
O	Outer layer
OPM	Outer plasma membrane
OS	Opposite surface of furca
P	Parasite
PF	Pectoral fin
PL	Protein lattice
PO	Penetration organ
R	Ribosomes
Ri	Rib
RER	Rough endoplasmic reticulum
RR	Ruthenium red
S	Spine
T	Tegument
TC	Tegumentary cell
Te	Tentacle
TS	Tail stem
V	Vacuole e.g. V.1, V.2 etc.
VL	Vacuolated layer
WBC	White blood cell

PLATE 1.

- 1-1 Electronmicrograph to show the inner layer of cyst wall. Note irregularly-compacted, densely-compacted and evenly-compacted zones. The middle layer is separated from the outer layer by several layers of membranes (arrow). Stacks of membranes (crossed arrow) are present in the middle layer. x 19 250. Stained with uranyl acetate and lead citrate.
- 1-2 Light micrograph of a group of four metacercariae in their cysts in the liver of P. microps. The cyst wall is stained by the Feulgen method for the demonstration of DNA, however, there is no stain in the cyst wall between two adjacent parasites indicating the absence of middle and outer layers in this region. x 390.
- 1-3 Normal liver cells in close apposition to the outer nucleated layer of the cyst. The nucleus of the liver cell has only small, dark peripheral areas as compared to those of the cyst wall. The lumen of the cyst in which the parasite lives contains a fine granular material. x 7 200. Stained with uranyl acetate and lead citrate.

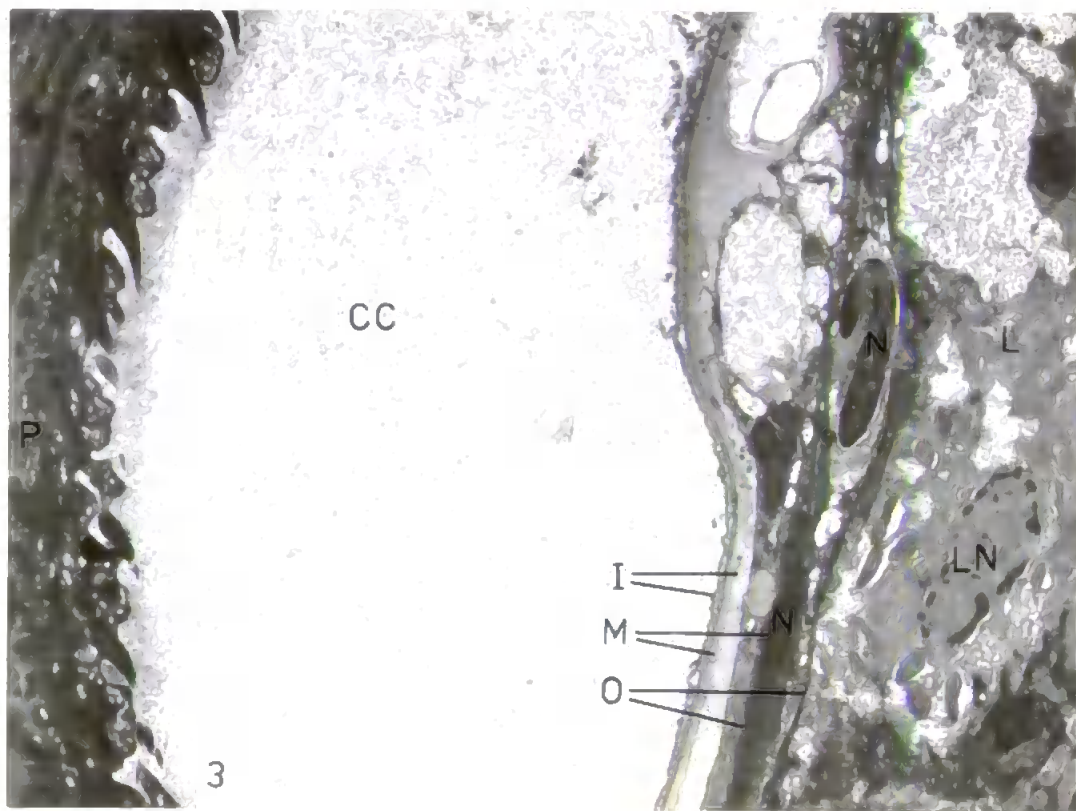
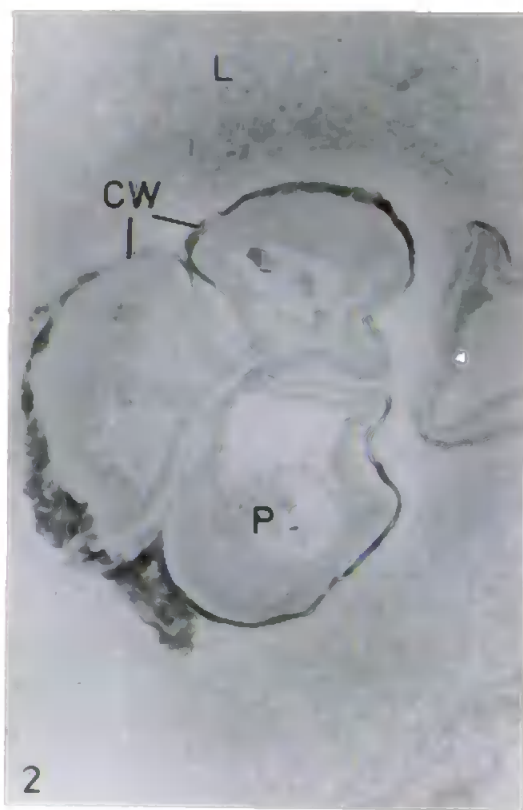
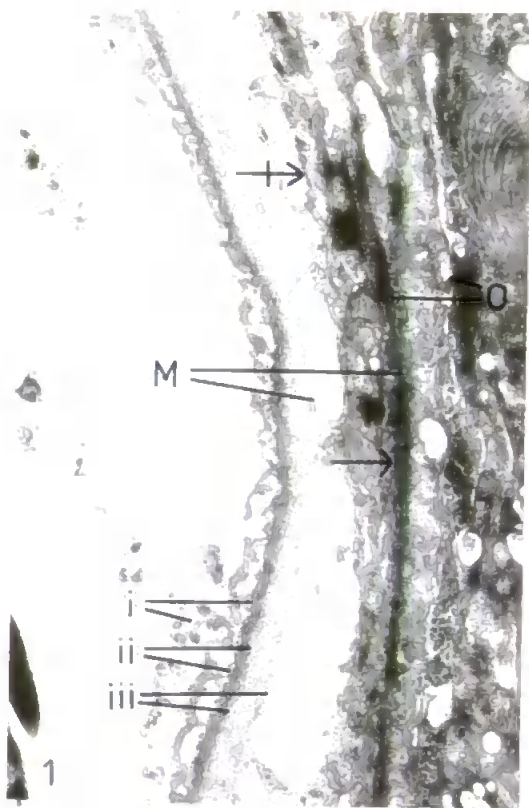


PLATE 2.

- 2-1 Electronmicrograph of the inner homogenous layer adjacent to the lumen of the cyst, consisting of three zones, irregularly-compacted, evenly-compacted and a moderately-compacted zone. Note darker areas in the moderately dense zone. The contents of vacuoles within the degenerating cell are apparently discharged into the inner granular layer. Sparse quantities of collagen fibres are found in the vicinity of regions of the cyst wall which are adjacent to blood vessels. x 12 600. Stained with uranyl acetate and lead citrate.
- 2-2 Electronmicrograph of leucocytes from an adjacent blood vessel, outside the outer nucleated layer of the cyst wall. Collagen fibres are again evident in this portion of the cyst wall adjacent to the blood vessel. x 16 000. Stained with uranyl acetate and lead citrate.

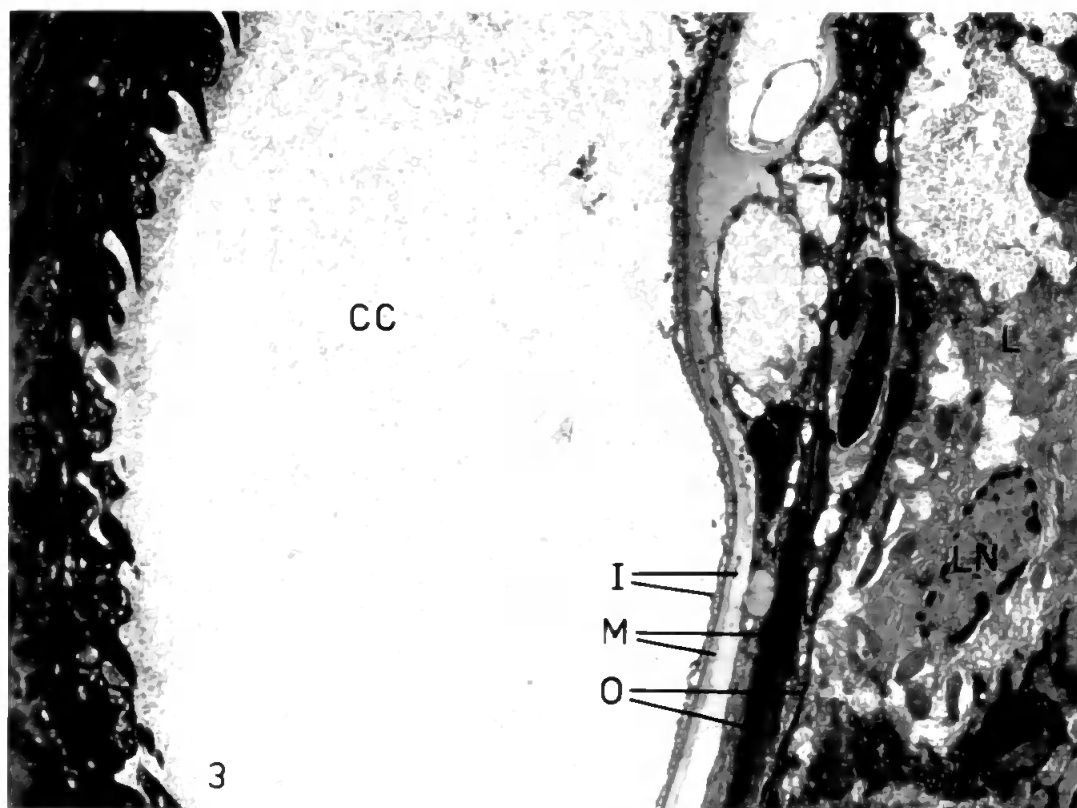
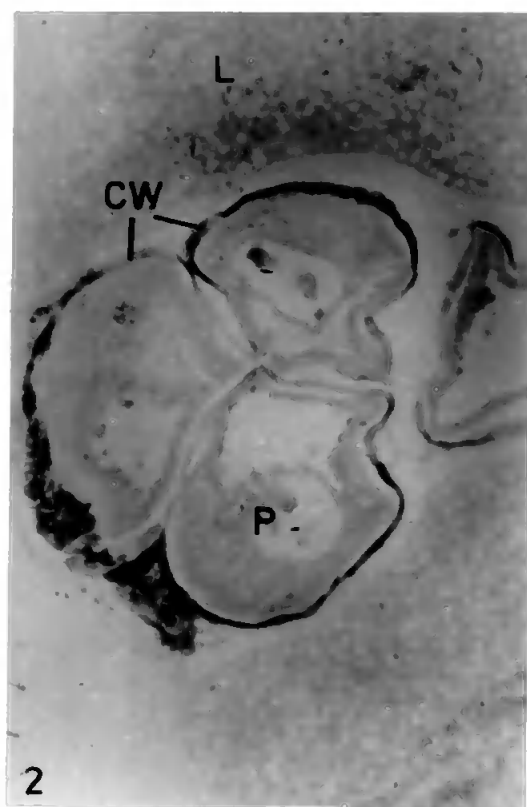
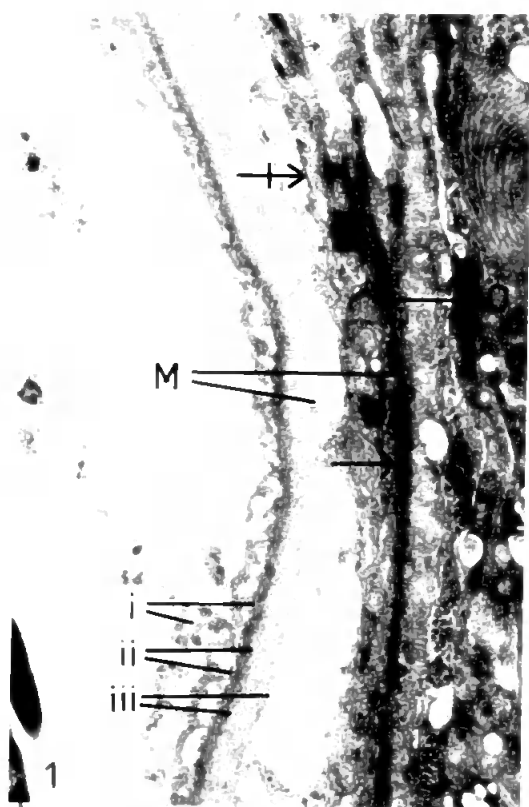


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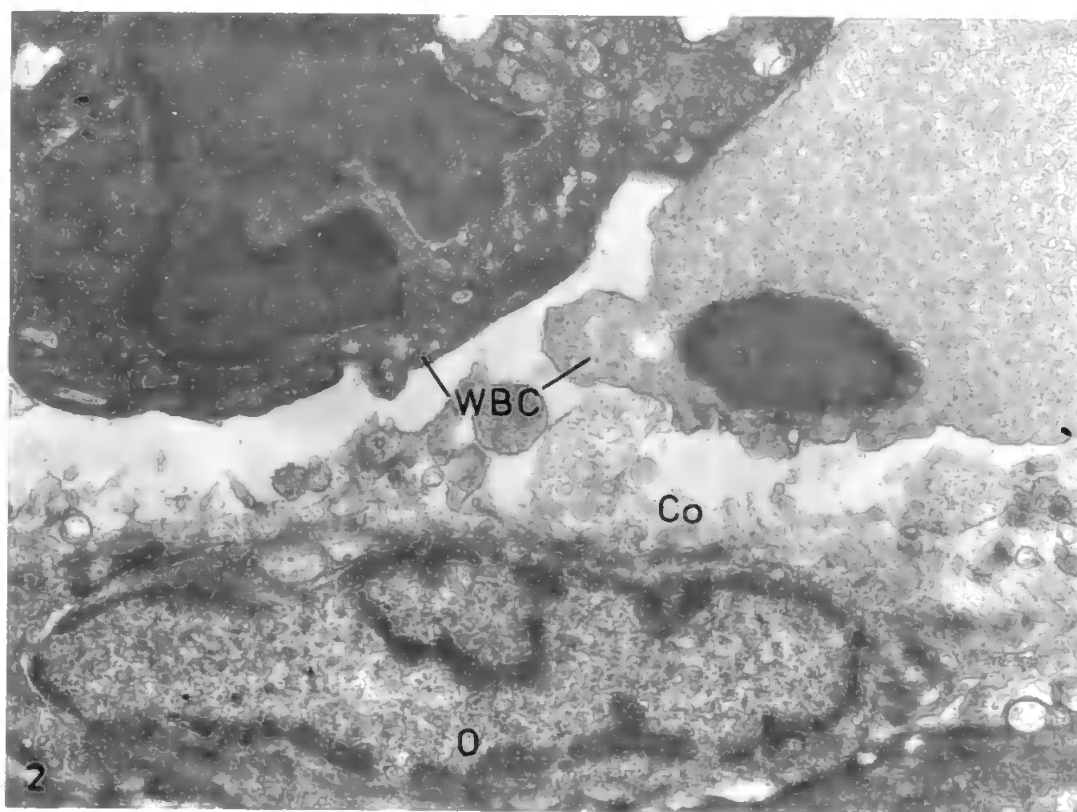
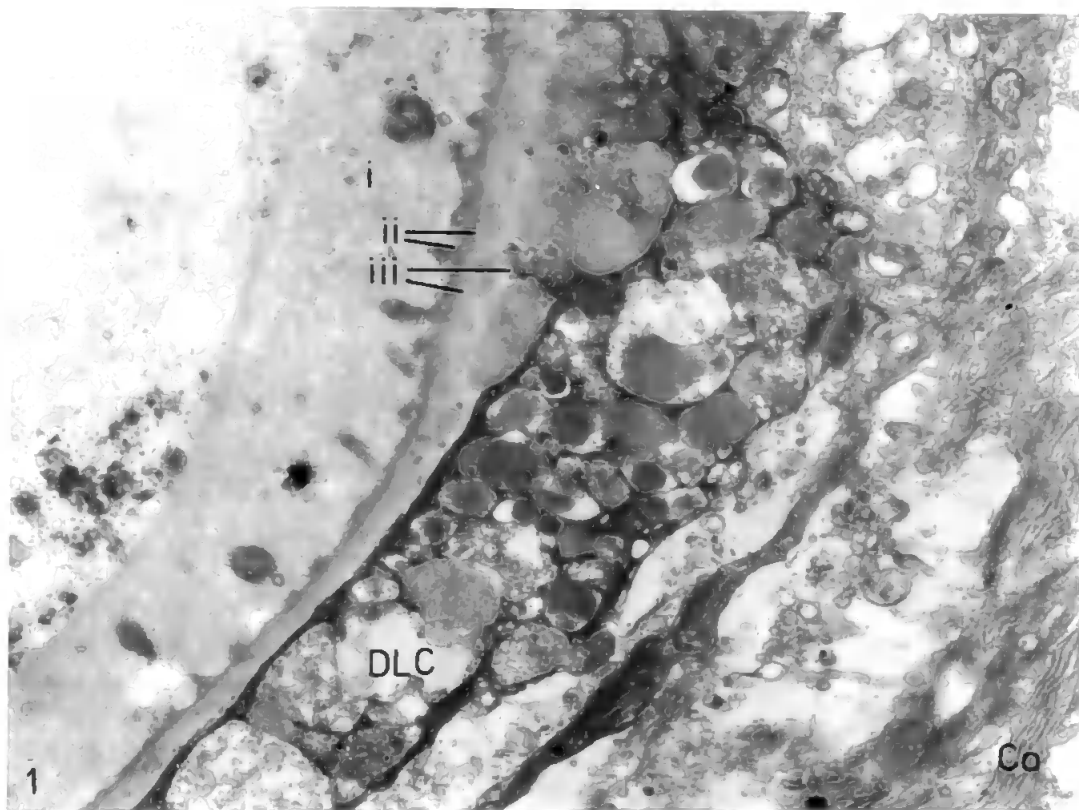


PLATE 3.

- 3-1 Mature cercaria firmly attached to the pectoral fin of Gobiusculus flavescens by secretions released from the cercarial tail stem and furcae. x 3 060. Stained with uranyl acetate and lead citrate.
- 3-2 Electronmicrograph of parasite burrowing through the pectoral fin of G. flavescens. The structure of the tegument is essentially similar to that of the free mature cercaria. x 13 860. Stained with uranyl acetate and lead citrate.
- 3-3 Section through the body musculature of Gobius paganellus containing a cercaria migrating towards the liver. Note damage to surrounding muscle blocks (arrow) caused by the release of lytic enzymes by the cercaria, which is ingesting host tissues. x 1 240. Papanicolaou stain.
- 3-4 Section through body musculature of G. paganellus containing a migrating cercaria, stained by the periodic acid Schiff (PAS) technique. Note PAS positive tegument and damage caused by the cercaria to the surrounding muscle tissue (arrow). x 2 300.

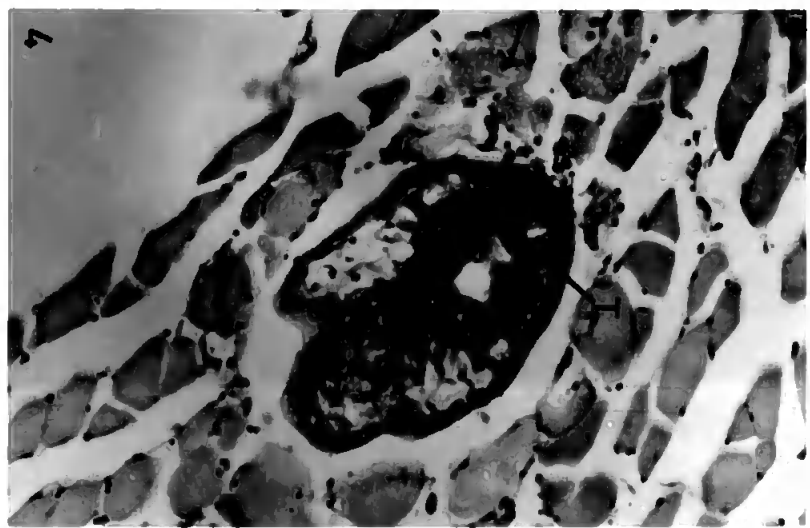
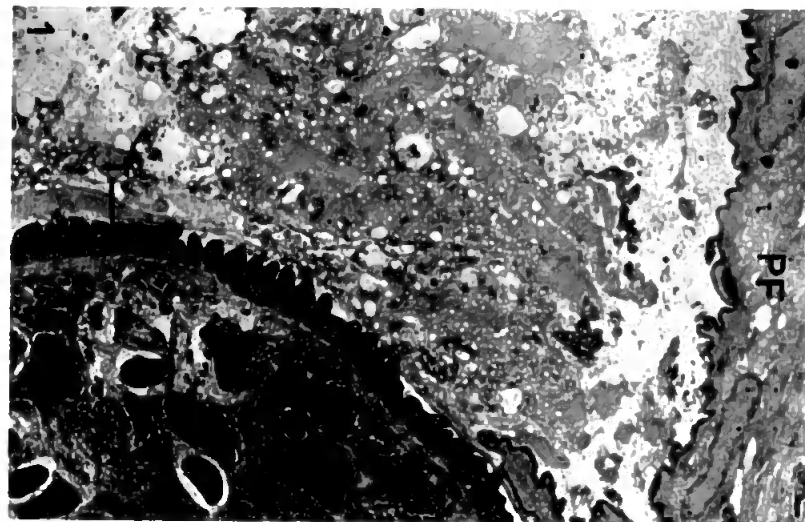
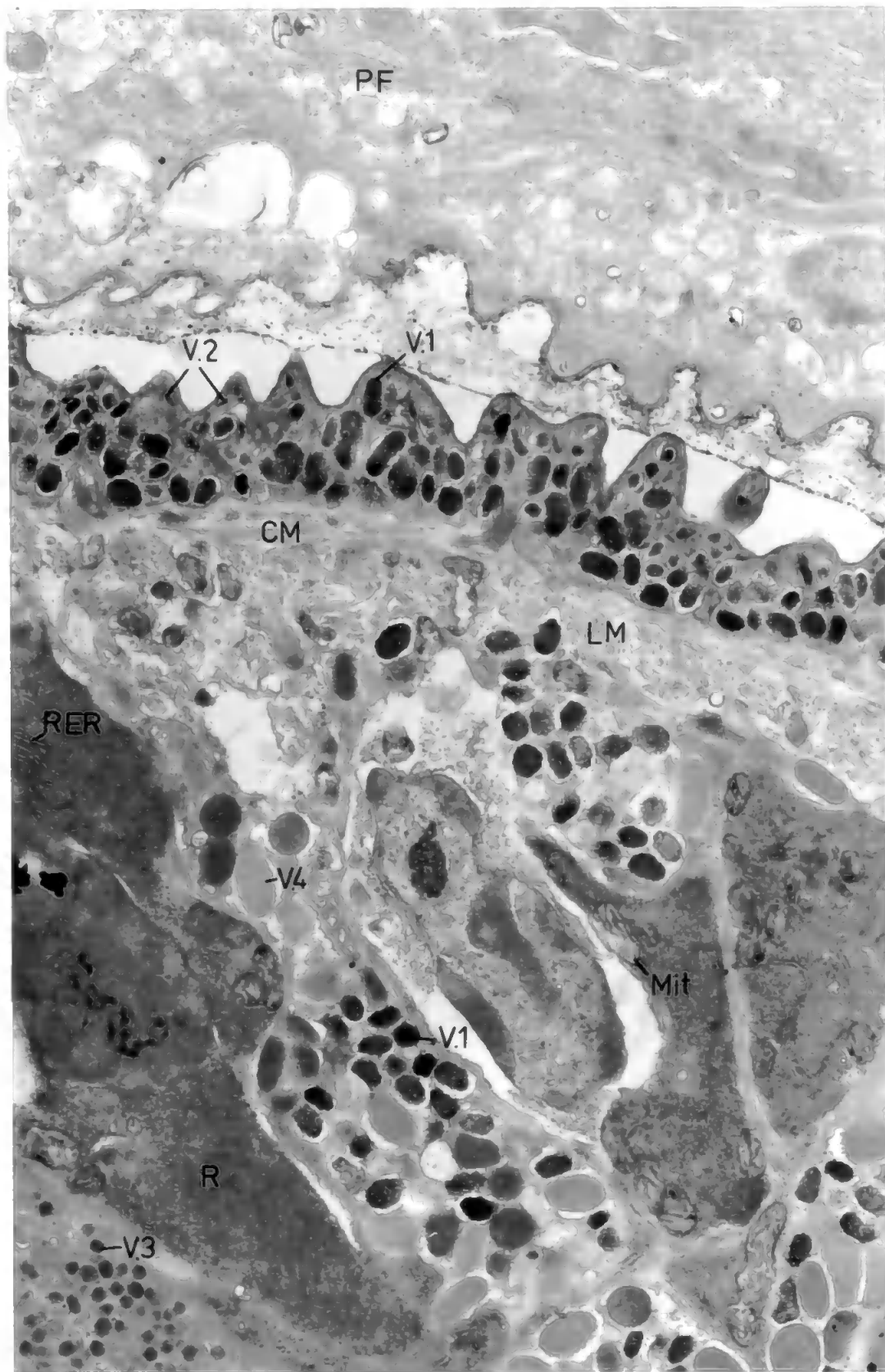


PLATE 4.

Electronmicrograph of cercaria attached to pectoral fin of Gobiusculus flavescens. V.1 and V.2 secretory cell bodies are present in the outer tegumental layer and V.1, V.3 and V.4 vesicles are found within tegumental cells. Note the various degrees of compaction of the V.1 secretory cell bodies. The tegumental cells have large nuclei, abundant ribosomes, rough endoplasmic reticulum and many, large mitochondria. x 17 000. Stained with uranyl acetate and lead citrate.



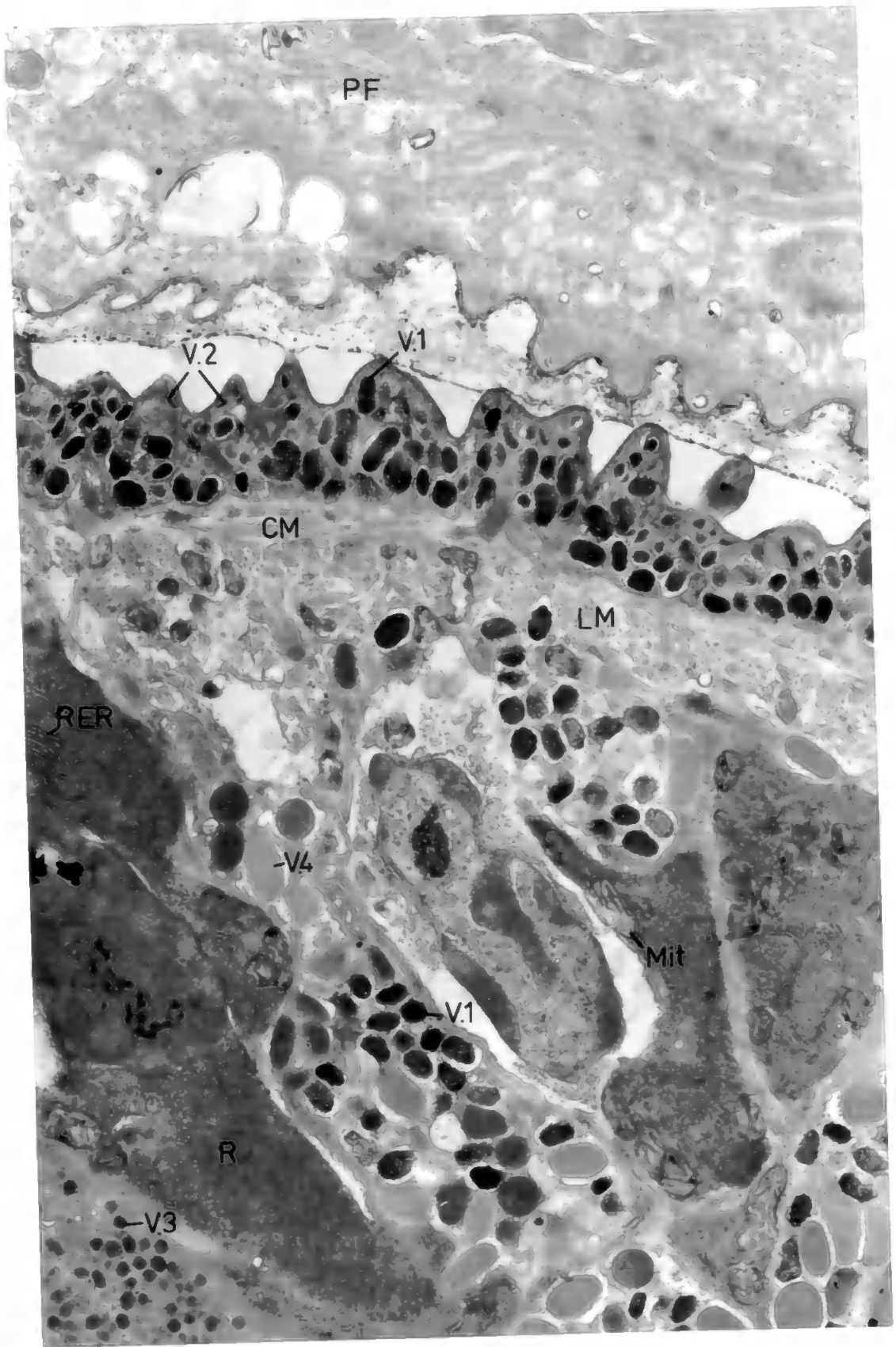


PLATE 5.

- 5-1 Electronmicrograph of a section through the anterior end of a fully developed cercaria showing the spines. x 24 700. Stained with uranyl acetate and lead citrate.
- 5-2 Stereoscan electronmicrograph of spiny anterior end of a fully developed cercaria. The penetration organ is withdrawn. x 22 500.
- 5-3 Stereoscan electronmicrograph of anterior end of cercaria. Note the longer more densely packed spines on the extended penetration organ as compared to those of the general body surface. x 5 000.

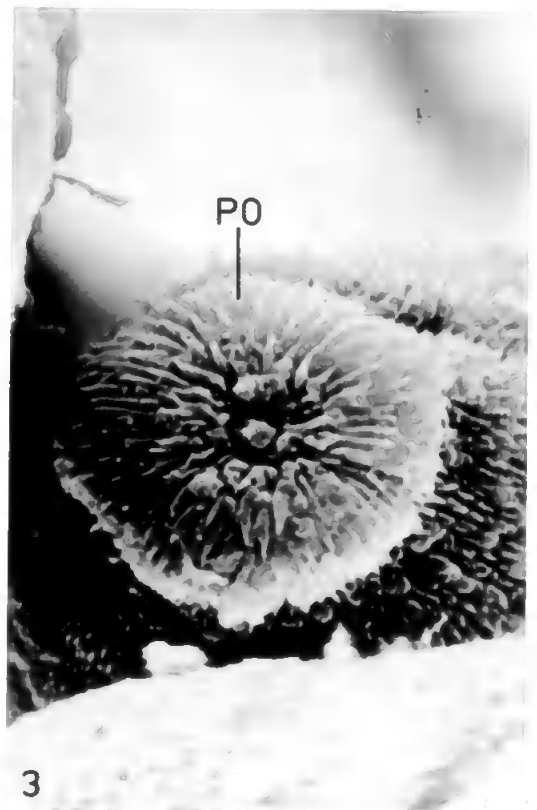
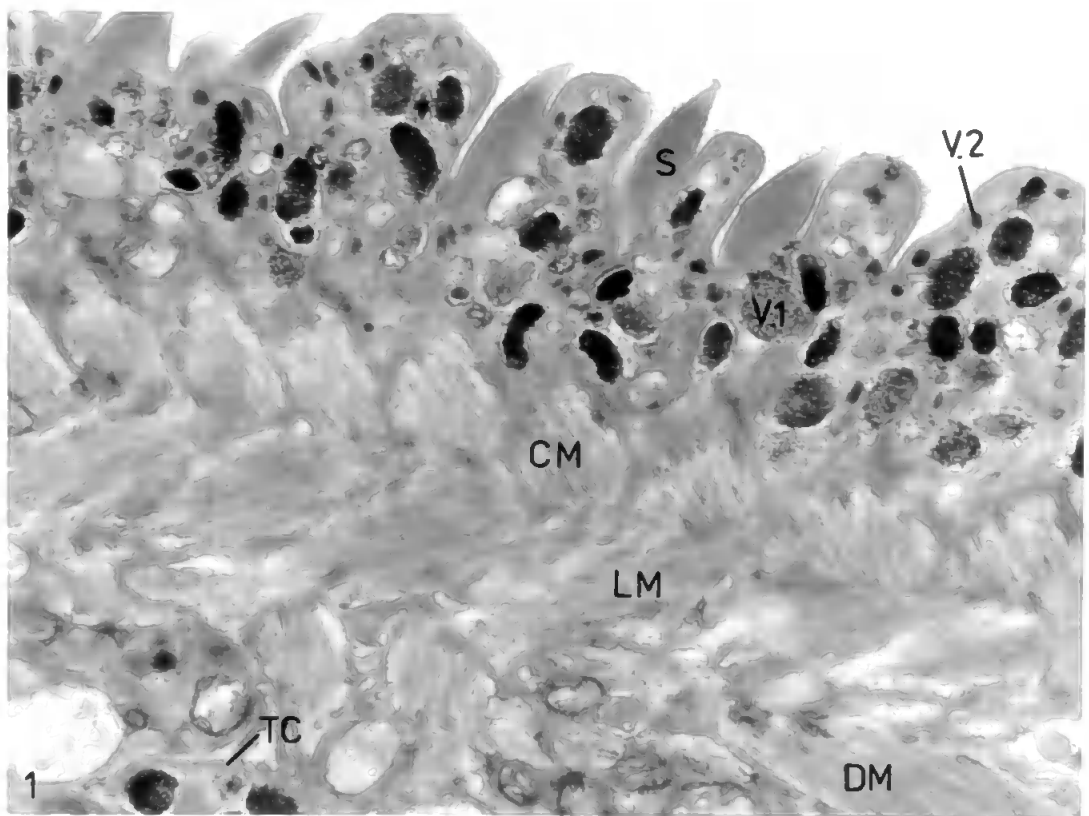


PLATE 6.

- 6-1 Electronmicrograph through the cercarial sucker rudiment containing a variety of vesicles. x 13 200.
Stained with uranyl acetate and lead citrate.
- 6-2 Scanning electronmicrograph of the ventral and central cercarial mouth. The general body surface lacks spines. x 13 000.
- 6-3 Scanning electronmicrograph of posterior end of a cercaria showing its ventral genital pore and highly convoluted tail stem which is well endowed with sensory cilia. x 1 100.

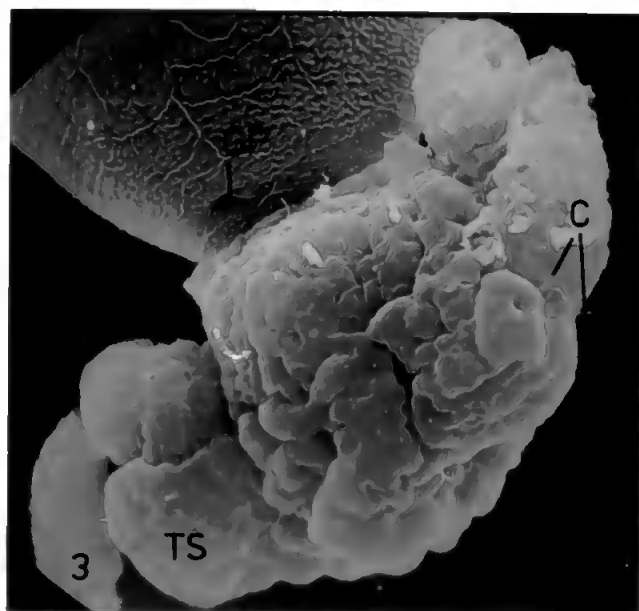
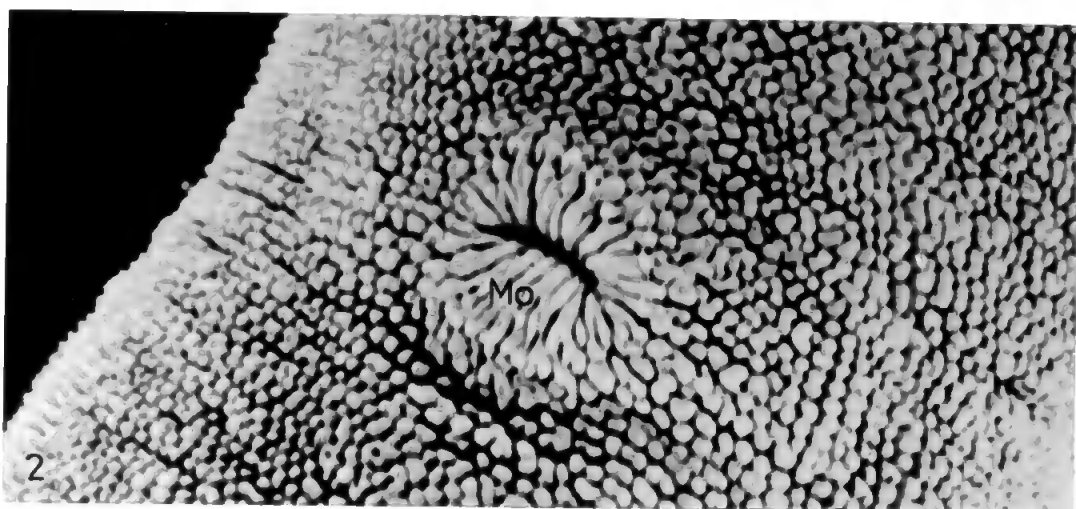
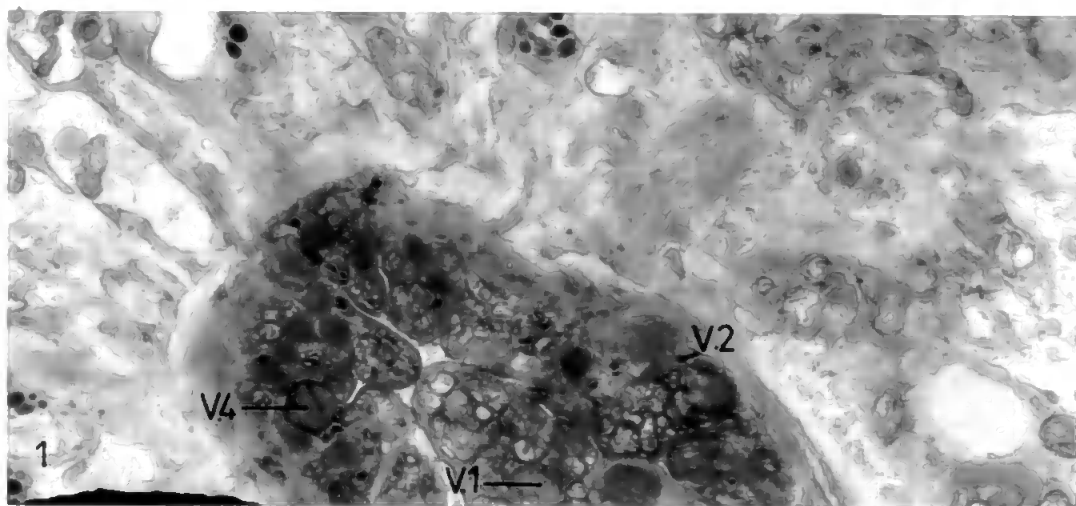


PLATE 7.

- 7-1 Electronmicrograph of cercarial tail stem showing material being released (arrow) by the highly vacuolated tegument. The underlying parenchymal cells are of a disorganised nature. x 9 500. Stained with uranyl acetate and lead citrate.
- 7-2 Furca with longitudinal muscle bands and central core of cells containing many mitochondria and ribosomes. The vesicles of the adjacent surface are large compared to those of the opposite surface which are fibrous in nature. Note the deep channels penetrating the tegument and the presence of sensory cilia. x 7 500. Stained with uranyl acetate and lead citrate.
- 7-3 Electronmicrograph of adjacent surface of furca showing electron lucent and moderately electron dense vesicles present in the tegument. x 10 800. Stained with uranyl acetate and lead citrate.
- 7-4 Stereoscan electronmicrograph of furca showing its asymmetrical nature due to the presence of different types of secretory cell bodies within the tegument. Note sensory cilium on tail stem. x 10 900.
- 7-5 Stereoscan electronmicrograph of excretory pore on ventro-lateral surface of the proximal end of cercarial furca. x 10 900.

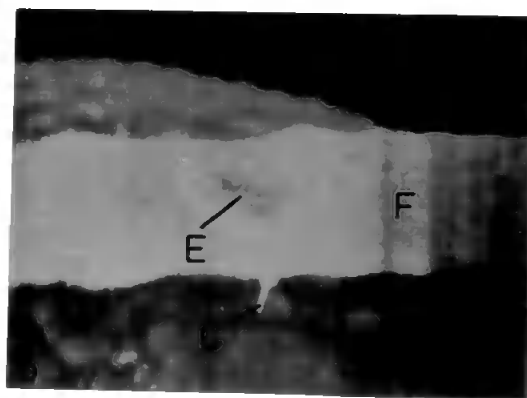
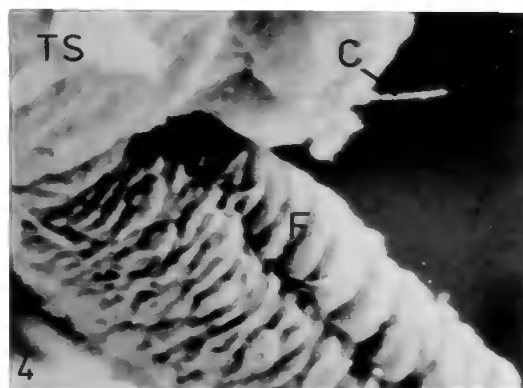
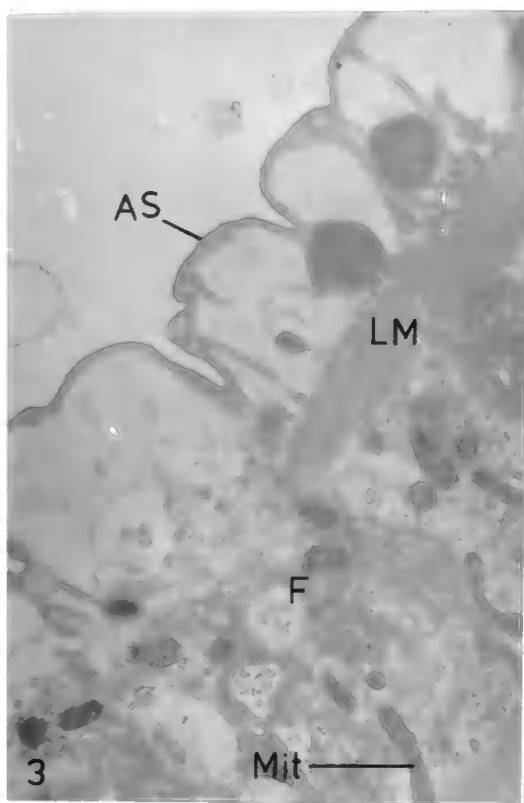
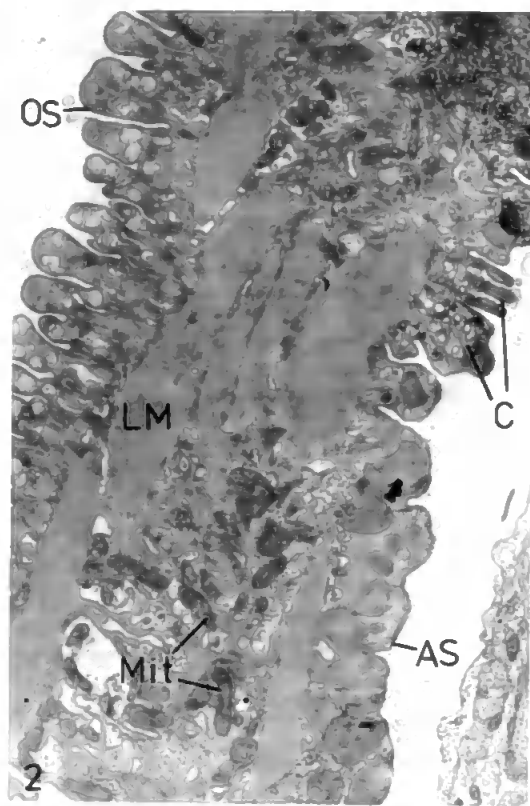
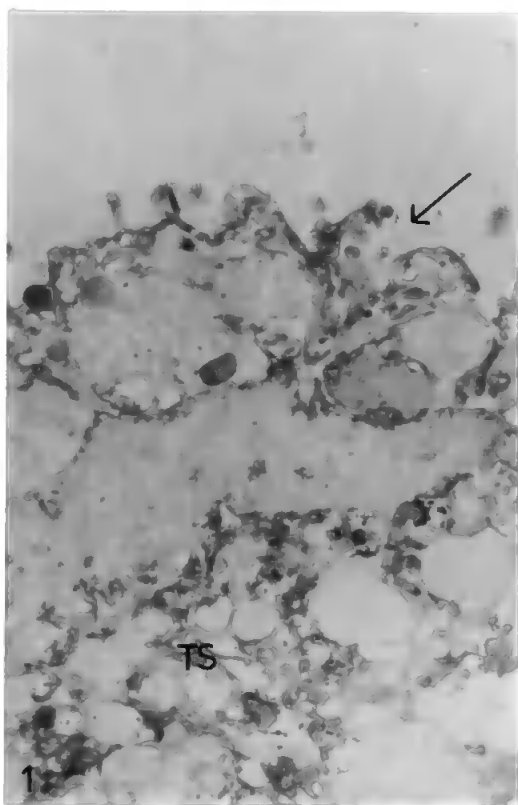


PLATE 8.

- 8-1 Group of cercariae showing microvillous nature of the tail stem and poorly developed furcae of immature stages compared to the highly convoluted tail stem and long furcae of the mature stage. x 170.
- 8-2 Stereoscan electronmicrograph of general body surface of immature cercaria. Short club-shaped microvilli are numerous. x 47 000.

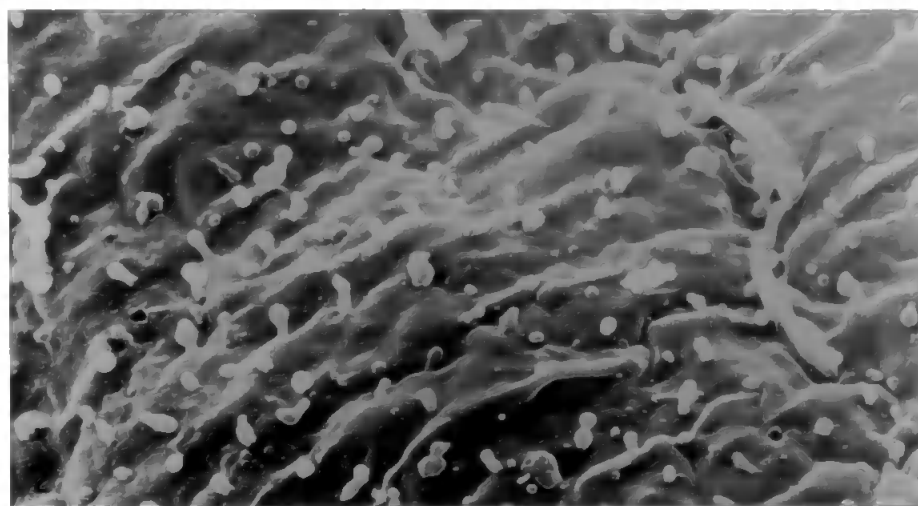
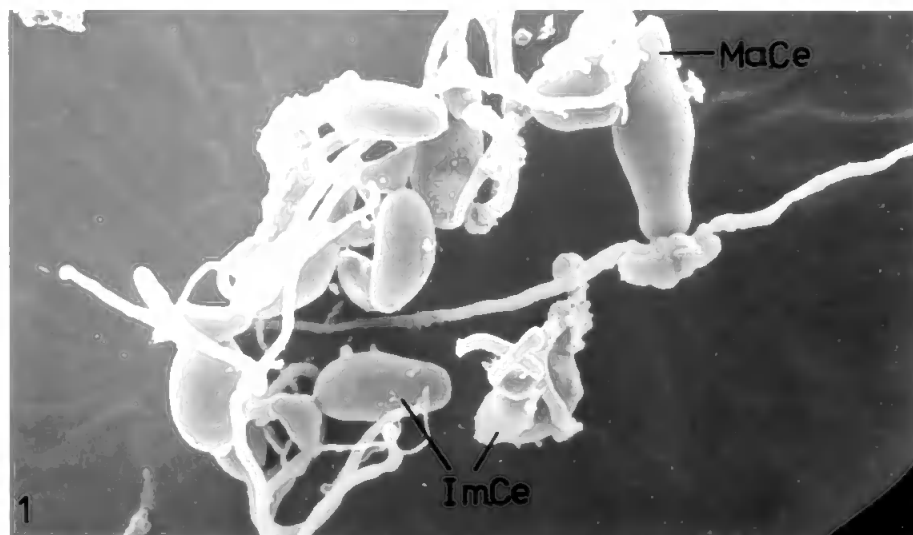


PLATE 9.

- 9-1,2 Light micrographs of 3 day old infections encysted in Gobiusculus flavescens stained by the Papanicolaou technique. Note disorganised, acellular appearance of the metacercarial parenchyma. x 270.
- 9-3 Light micrograph of 24 h old cysts showing strong staining reaction of inner cyst wall to Alcian blue pH 2.5. A few of the metacercarial parenchyma cells stain positively. x 270.
- 9-4 Light micrograph of 24 h cysts showing strong reaction of inner cyst wall to Alcian blue pH 1.0. x 435.

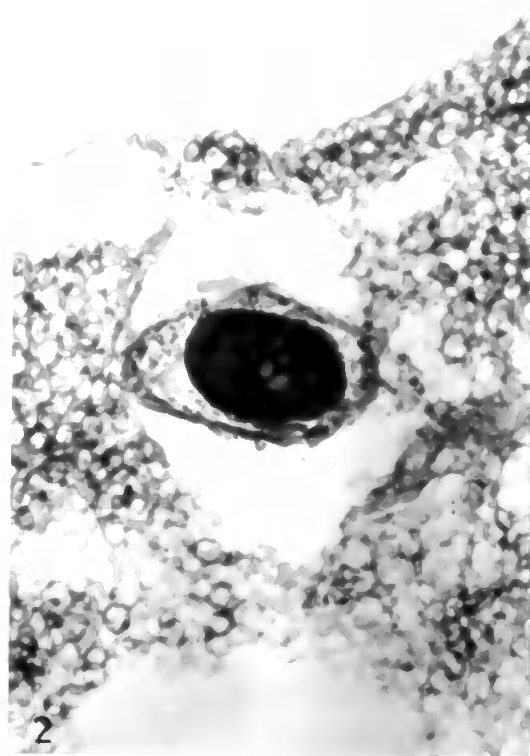
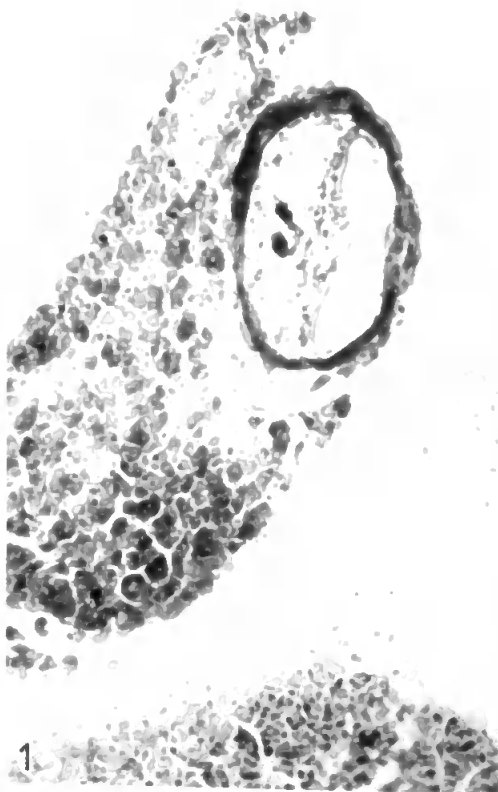


PLATE 10.

Infections of Gobiusculus flavescens with Bucephalus
haimeanus. x 270.

- 10-1 20 day infection stained by the Feulgen technique
for DNA. Nuclei of the hepatic cells, metacercaria
and cyst wall all stain positively.
- 10-2 30 day infection showing intense reaction of the
cyst wall to the Feulgen stain. The hepatic cell
and metacercarial cell nuclei also stain positively.
- 10-3 30 day infection to show intense reaction given
by the cyst wall in control sections (i.e. incubated
in HCl at room temperature) for the Feulgen stain.
The hepatic cell and metacercarial cell nuclei fail
to stain.

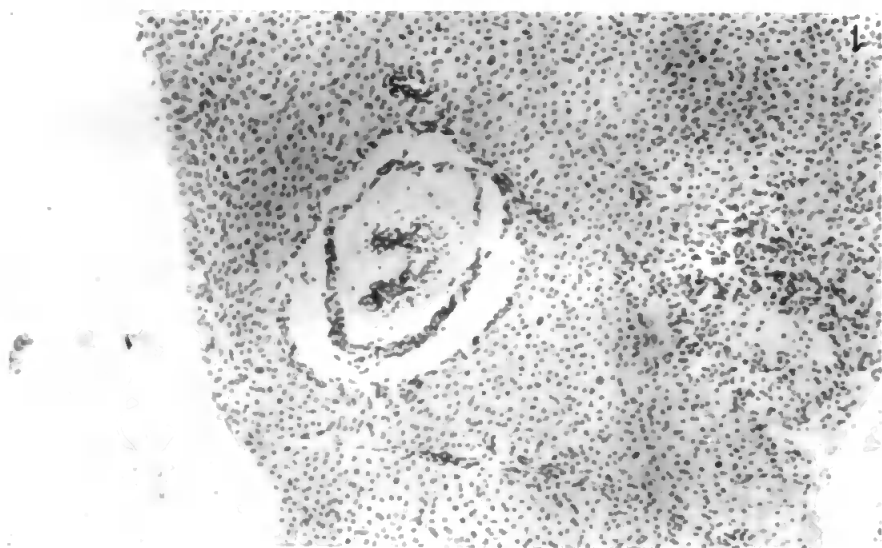
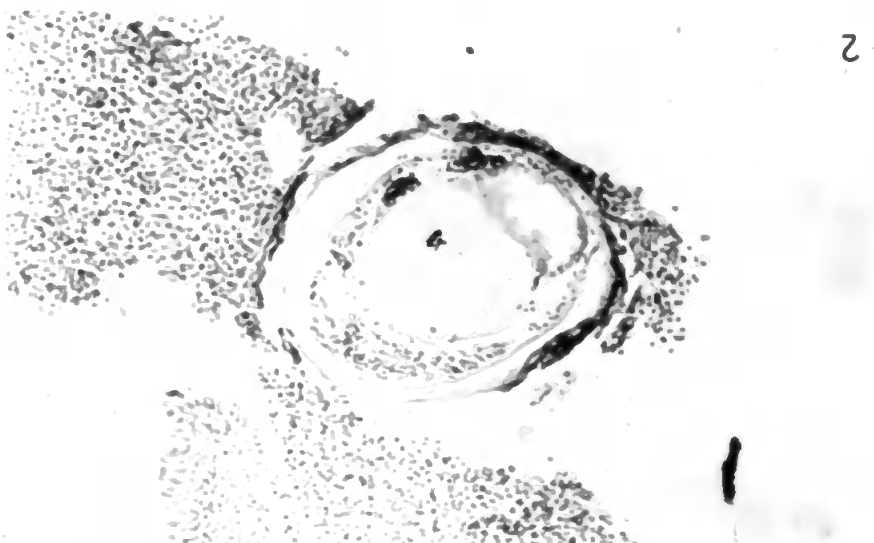
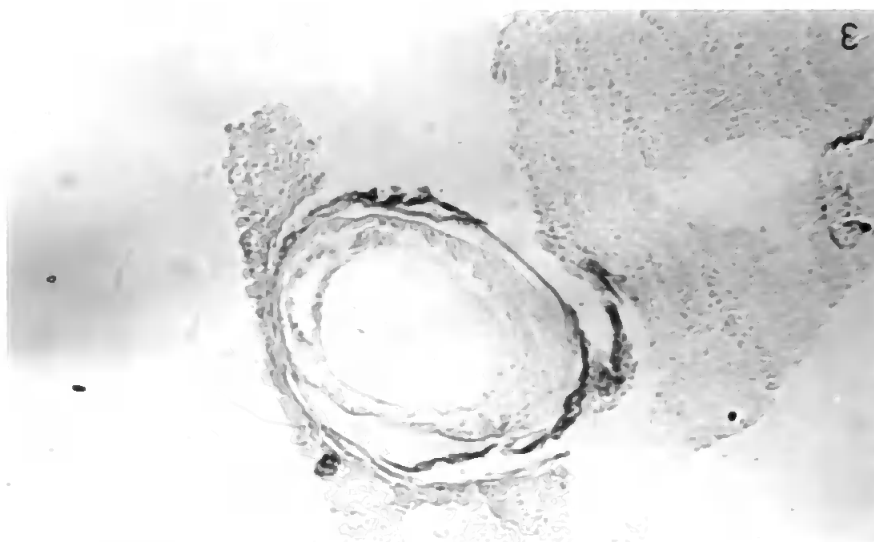


PLATE 11.

24 h infections of Bucephalus haimeanus encysting in livers of Gobiusculus flavescens.

- 11-1 Electronmicrograph showing nature of tegument. A few V.1 vesicles remain in the outer syncytial layer and tegumental cells but no V.2 vesicles remain. V.3 vesicles have migrated into the outer tegumental layer. Small amounts of granular material are present in the outer tegument and underlying muscle blocks. x 20 800. Stained with uranyl acetate and lead citrate.
- 11-2 Electronmicrograph showing fibrous nature of the cyst wall, secreted by the parasite, which extends from the tegument surface to the surrounding hepatic tissue. The liver cells are highly vacuolated and are degenerating. x 8 800. Stained with uranyl acetate and lead citrate.
- 11-3 Electronmicrograph showing ill defined junction between the fibrous cyst wall of parasitic origin and the decaying liver cells which are releasing their contents into the inner cyst wall layer. x 18 700. Stained with uranyl acetate and lead citrate.

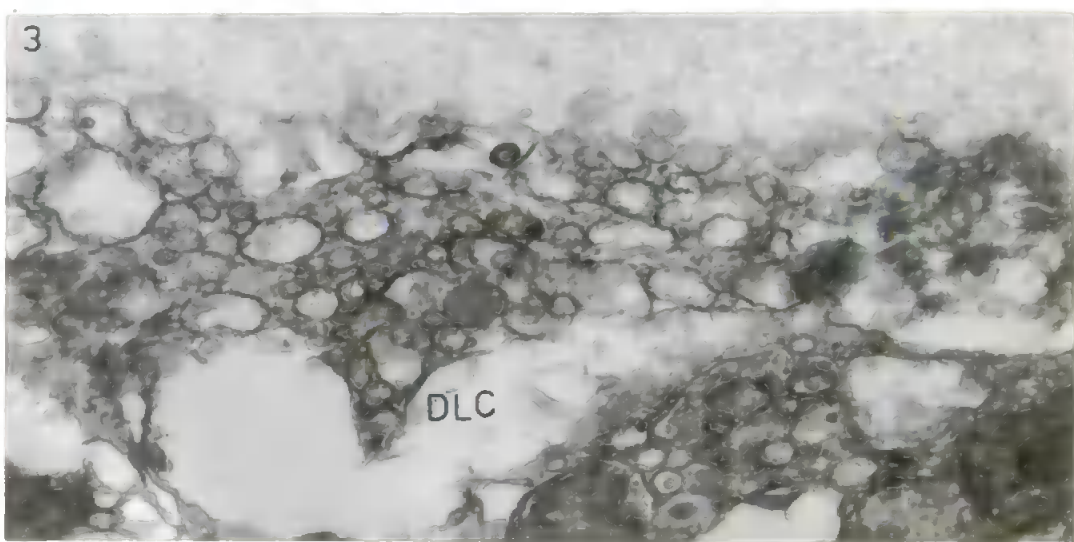
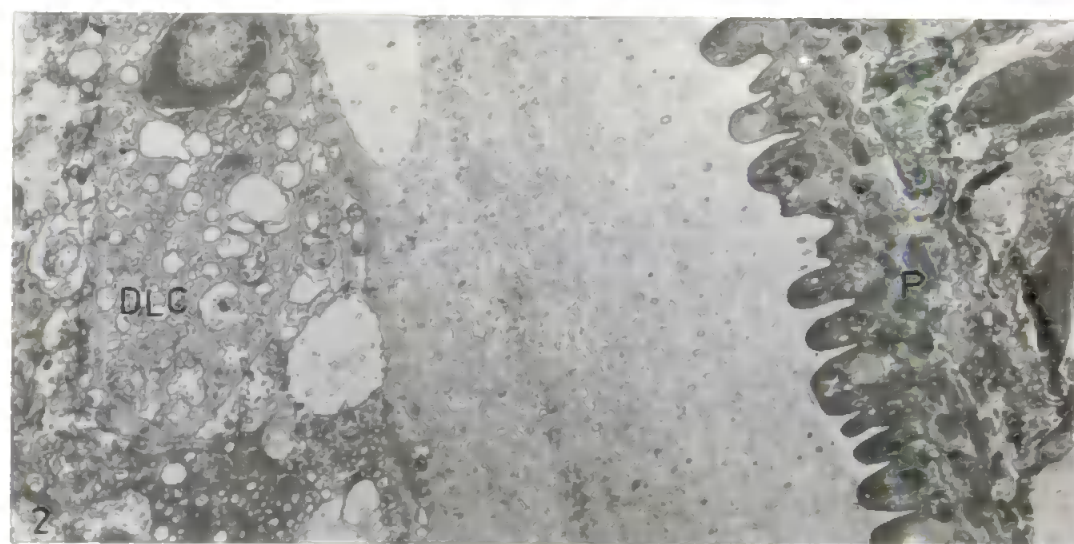
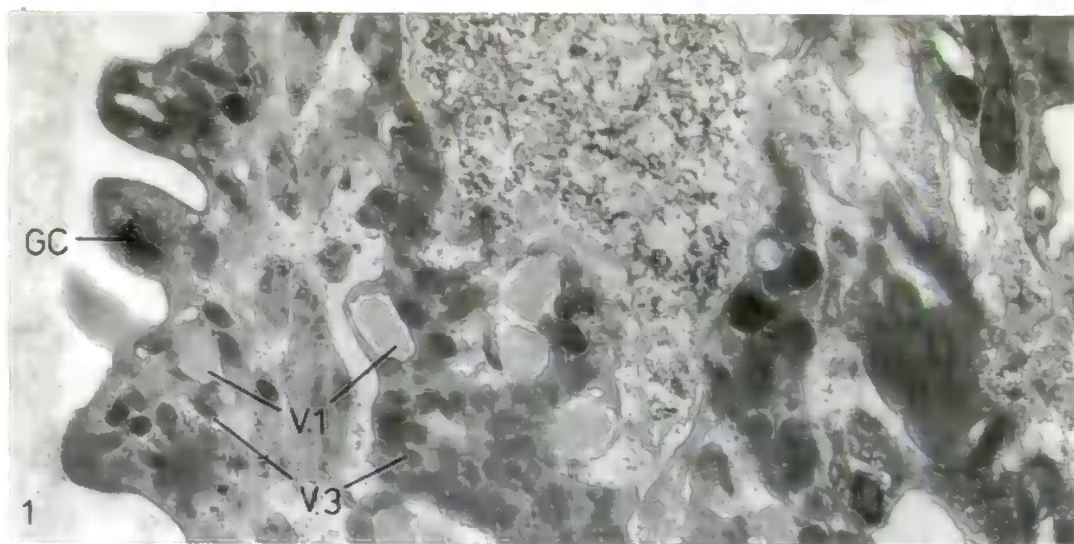


PLATE 12.

2 day infections of Bucephalus haimeanus in the liver of Gobiomorus flavescens. The outer tegumental layer contains many V.3 and V.4 secretory cell bodies. The electron dense, granular material is plentiful in the outer tegumental layer, muscle layers and parenchymal cells. The inner layer, of the cyst wall is compacted against the surrounding hepatic tissue forming a more compacted and a less compacted layer. The degenerating liver cells contain many vacuoles and distended mitochondria.

12-1 x 14 500

12-2 x 16 000

Stained with uranyl acetate and lead citrate.

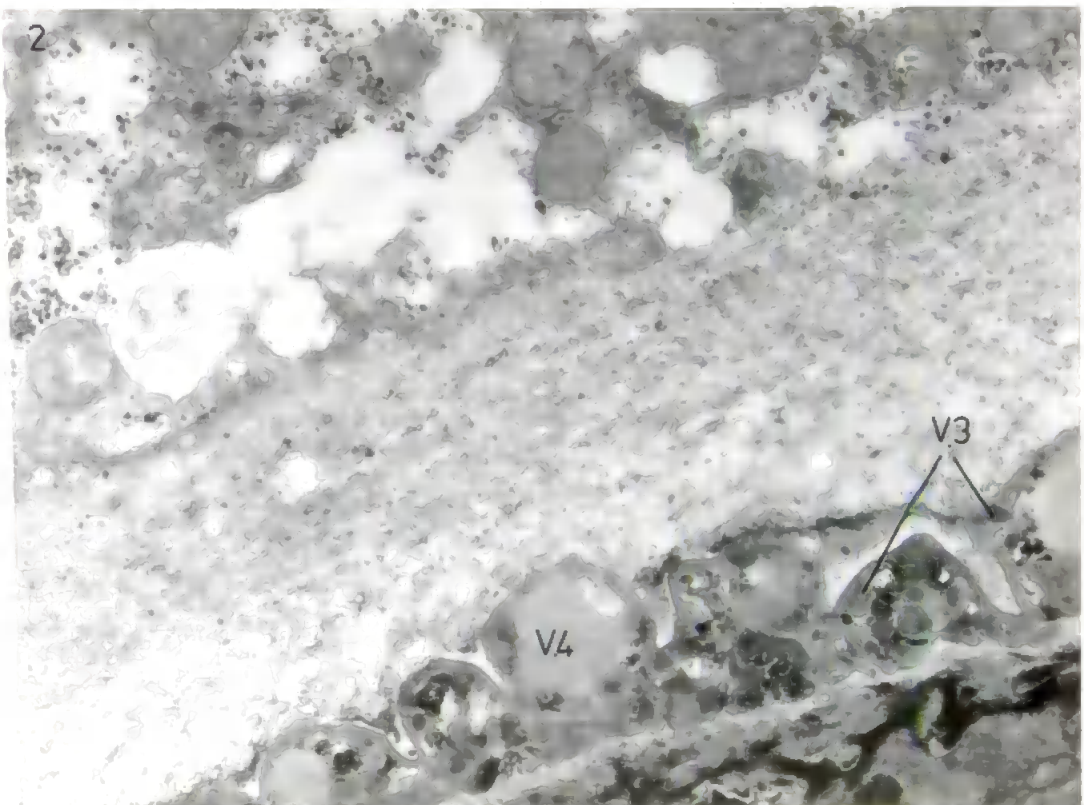
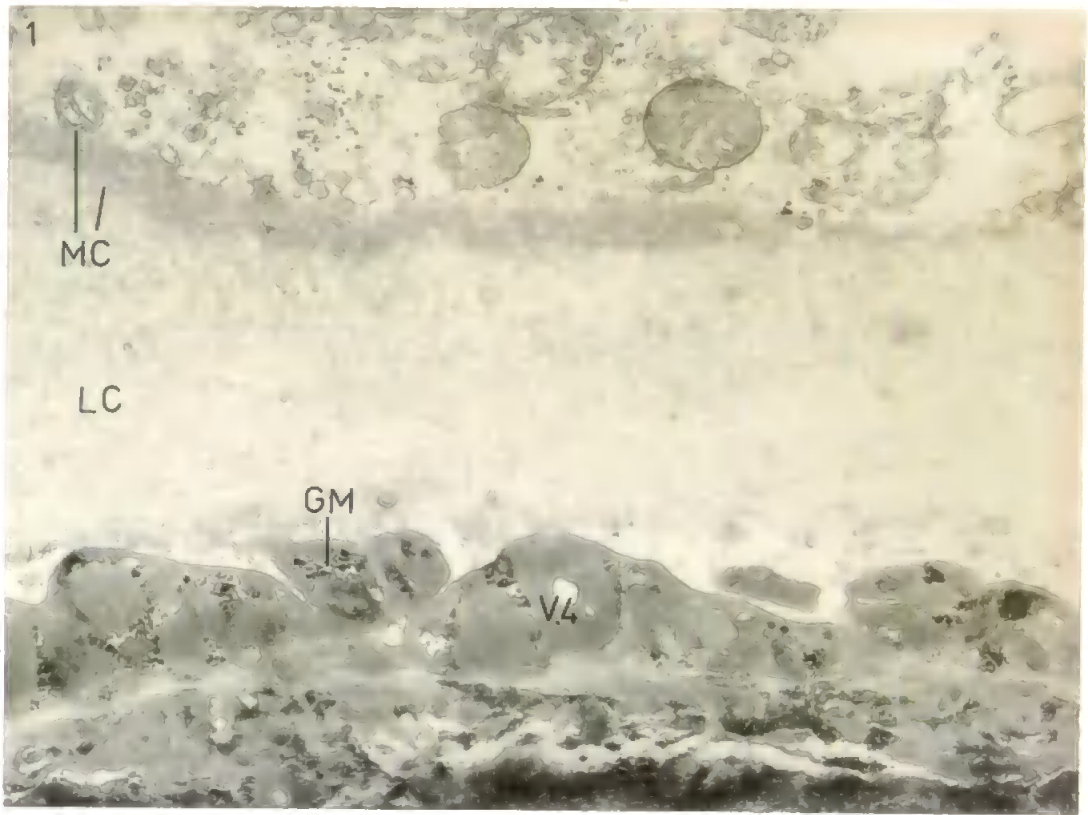


PLATE 13.

- 13-1 Electronmicrograph of 3 day infection in Gobiusculus flavescens showing extensive distribution of granular material in outer tegument and underlying cells. x 12 000. Stained with uranyl acetate and lead citrate.
- 13-2 Electronmicrograph of 4 day infection in G. flavescens showing large numbers of V.4 vesicles in the outer tegumental layer. Many vesicles, originating from the decaying liver cells are scattered throughout the cyst wall of parasitic origin which extends from the metacercarial tegument to the surrounding cells. x 15 000. Stained with uranyl acetate and lead citrate.
- 13-3 Electronmicrograph of 4 day infection in G. flavescens showing the presence of V.3 and V.4 vesicles and granular material in the outer tegumental syncytium and the production of V.5 vesicles in the parenchymal cells. Note the breakdown of the basal plasma membrane of the outer tegumental layer and the infiltration into this layer by the underlying circular muscle. x 11 500. Stained with uranyl acetate and lead citrate.

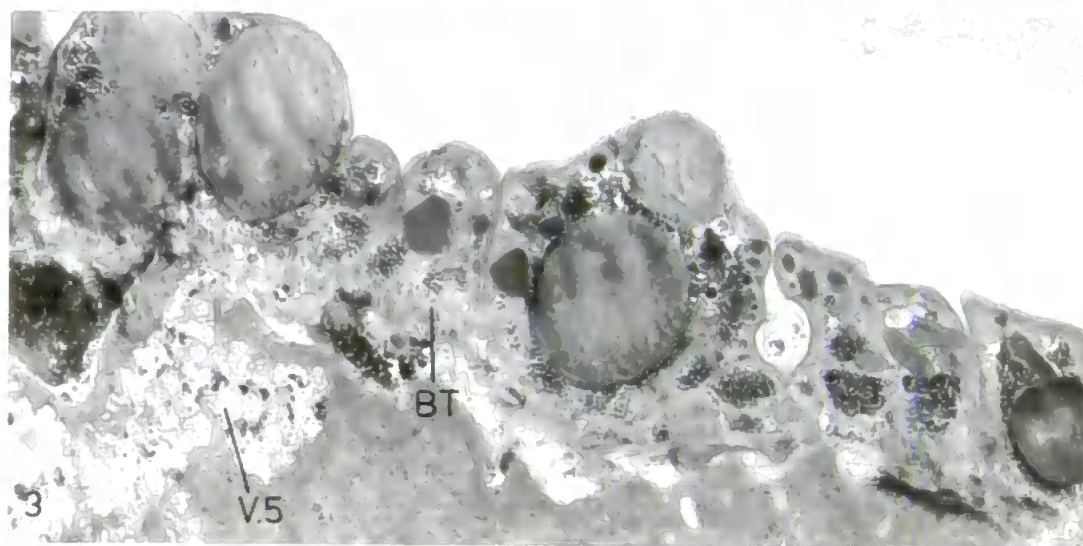
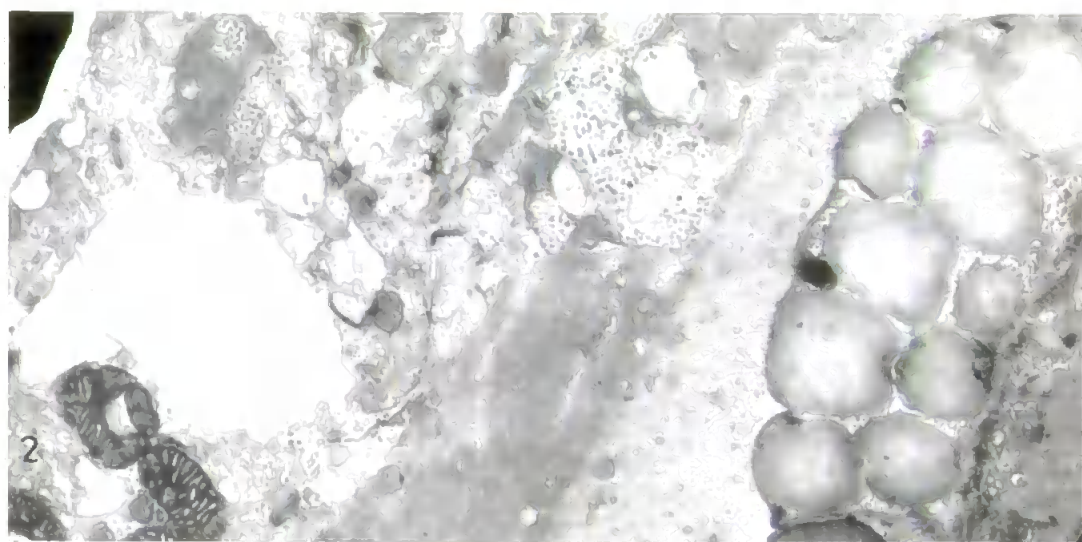
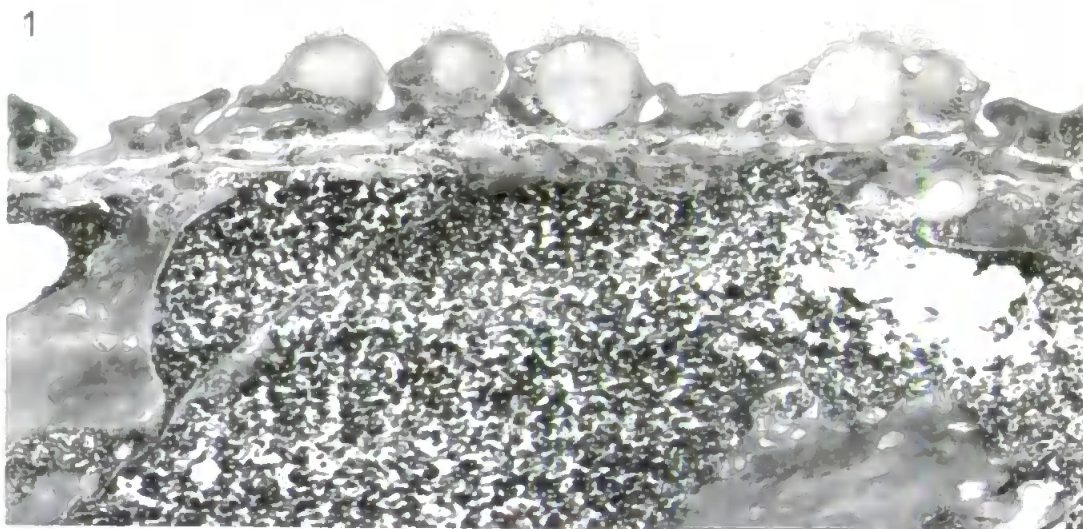


PLATE 14.

15 day post infection in Gobiusculus flavescens.

- 14-1 Electronmicrograph showing the presence of a cyst cavity separating the metacercaria from the cyst wall. V.3, 4 and 5 vesicles are being released by the outer tegumental layer into the cyst cavity with the V.4 and V.5 vesicles being replaced from the underlying tegumental cells. x 10 700.

Stained with uranyl acetate and lead citrate.

- 14-2 Electronmicrograph showing the complete breakdown of the outer tegumental layer and the disorganised nature of the underlying parenchyma. x 13 200.

Stained with uranyl acetate and lead citrate.

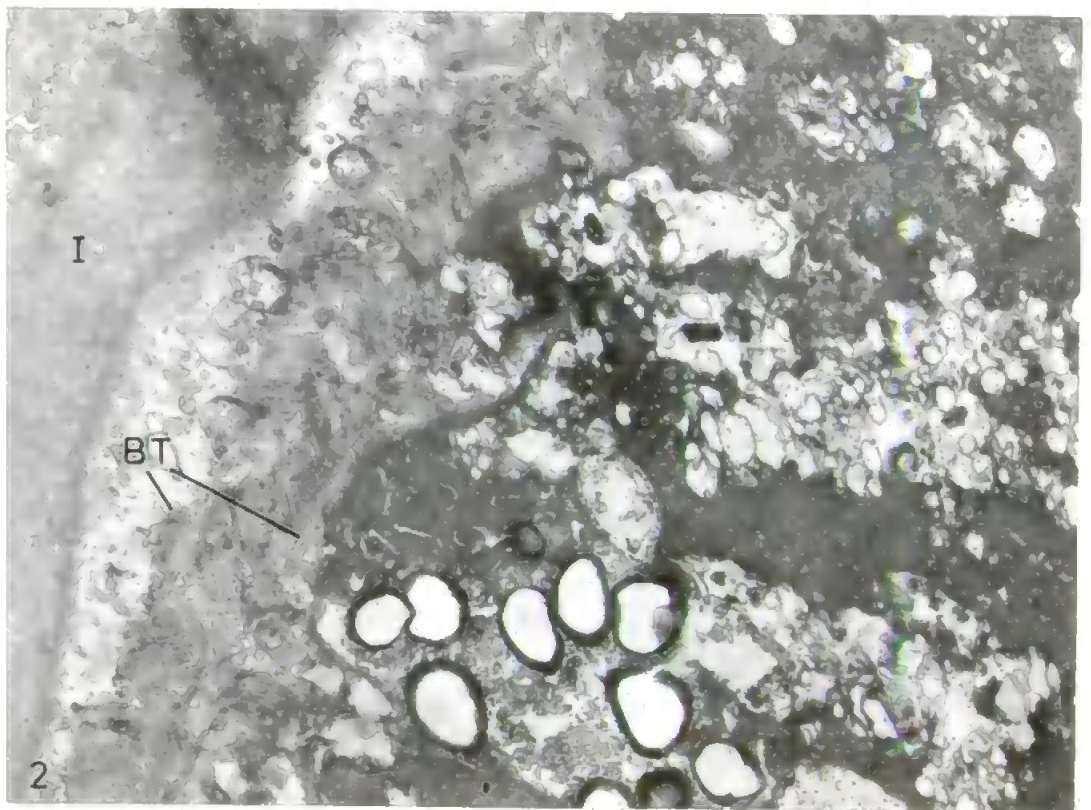
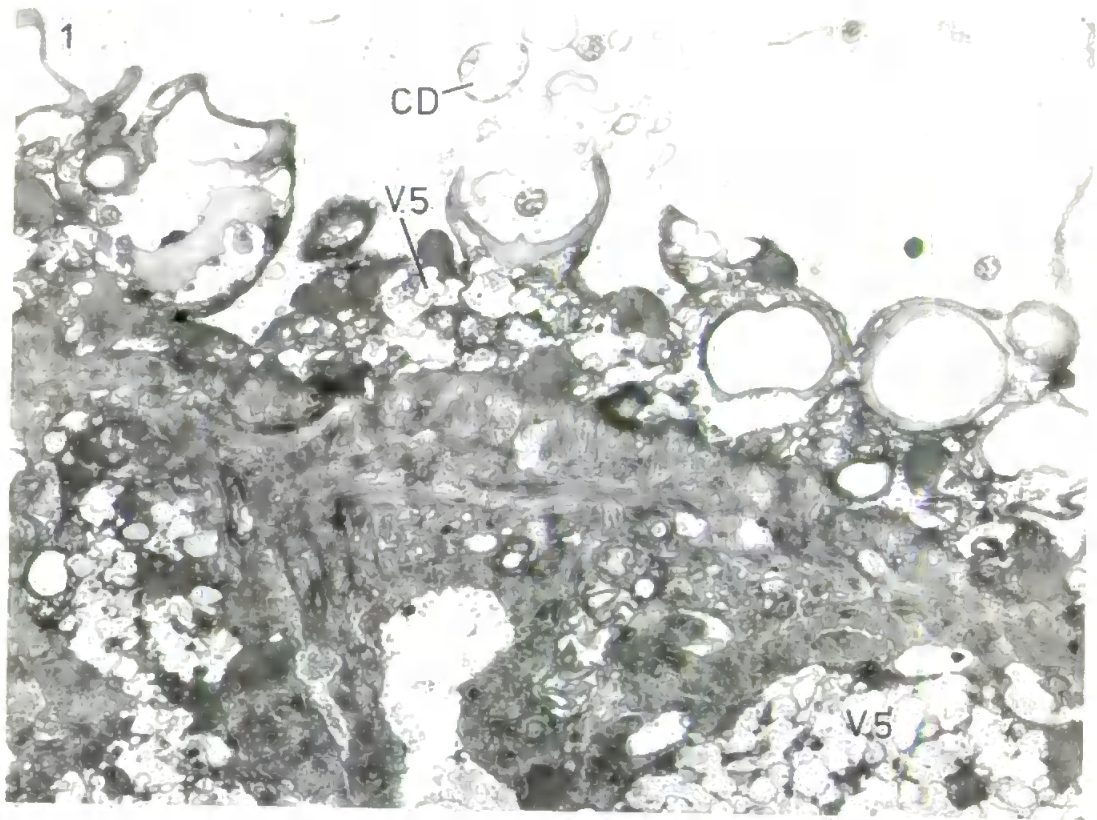


PLATE 15.

20 day post infection in Gobiusculus flavescens.

- 15-1 Electronmicrograph of cellular debris completely filling the cyst cavity. x 3 000. Stained with uranyl acetate and lead citrate.
- 15-2 Electronmicrograph showing the 3-zoned inner layer and middle vacuolated layer of the cyst wall. The cyst cavity contains granular material. x 13 900. Stained with uranyl acetate and lead citrate.

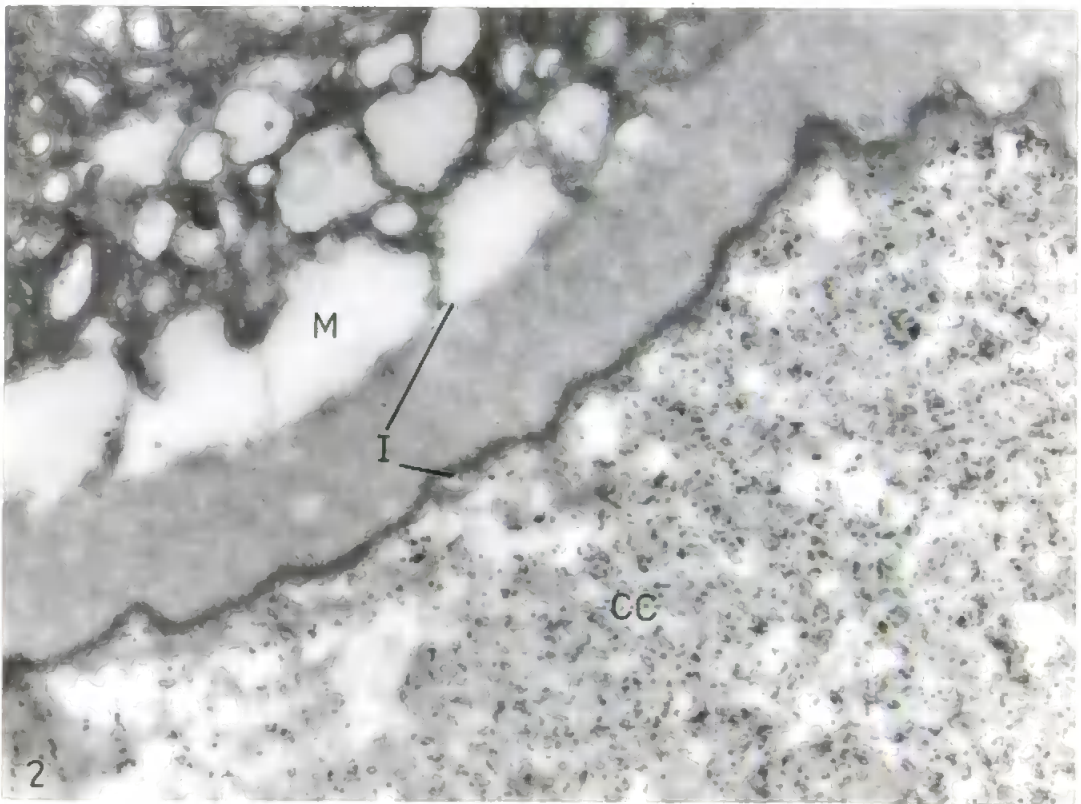
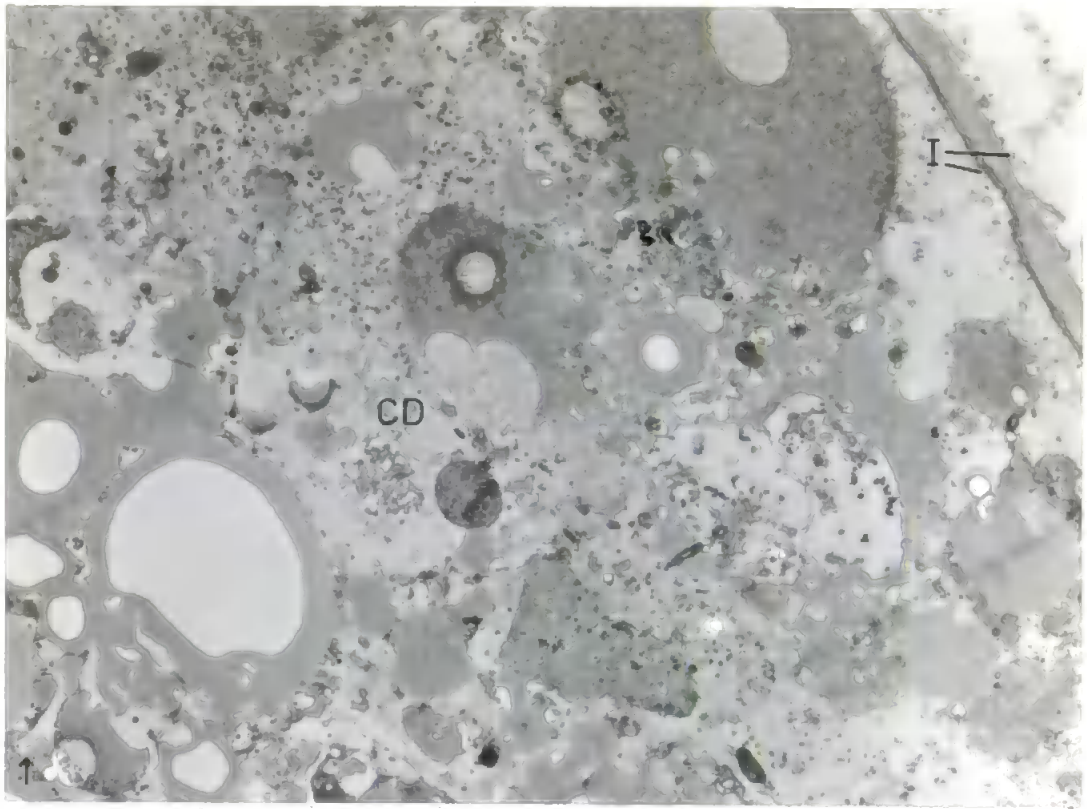


PLATE 16.

Section through anterior portion of a fully developed metacercaria naturally infecting Pomatoschistus microps. The tegumental cells and outer tegumental layer contain V.6, 7 and 8 secretory cell bodies; V.9 vesicles are present in the outer tegument. The elongated gland cells, supported by microtubules, contain an electron dense secretory product.
x 9 100. Stained with uranyl acetate and lead citrate.

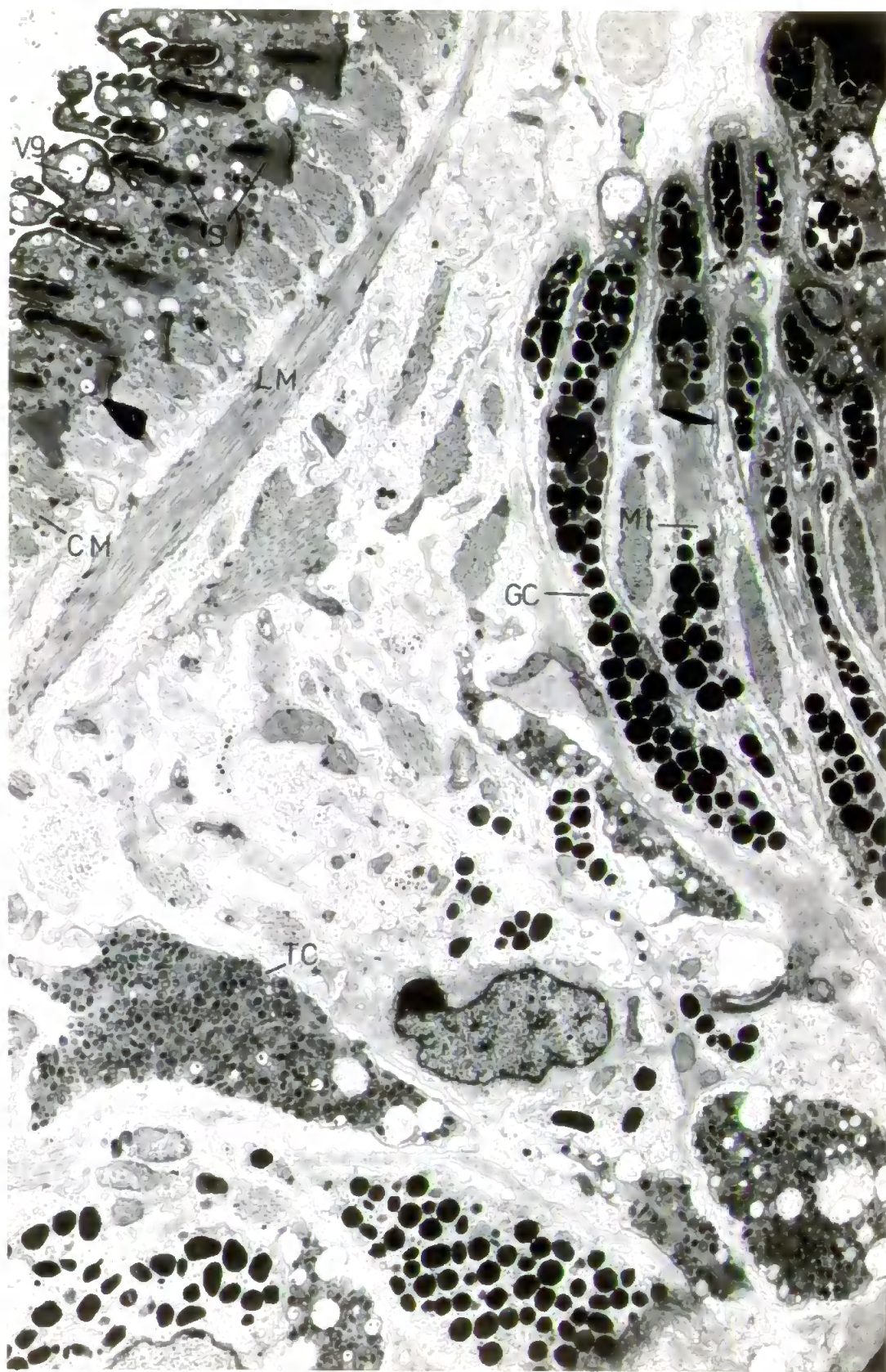


PLATE 17.

- 17-1 Part of PLATE 16 enlarged to show tegumentary cells in greater detail. x 16 200.
- 17-2 Electronmicrograph of tegumentary cell packed with V.7 vesicles; the packeted nature of their contents is apparent. x 38 700. Unstained.

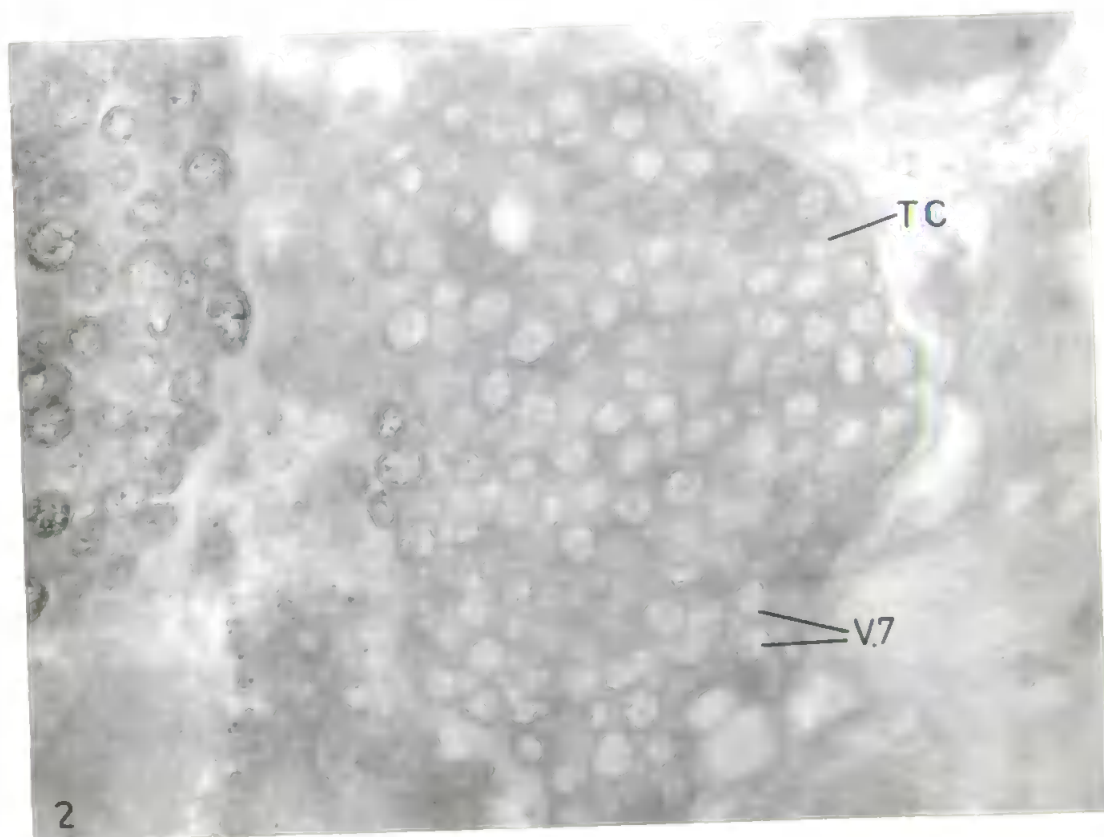
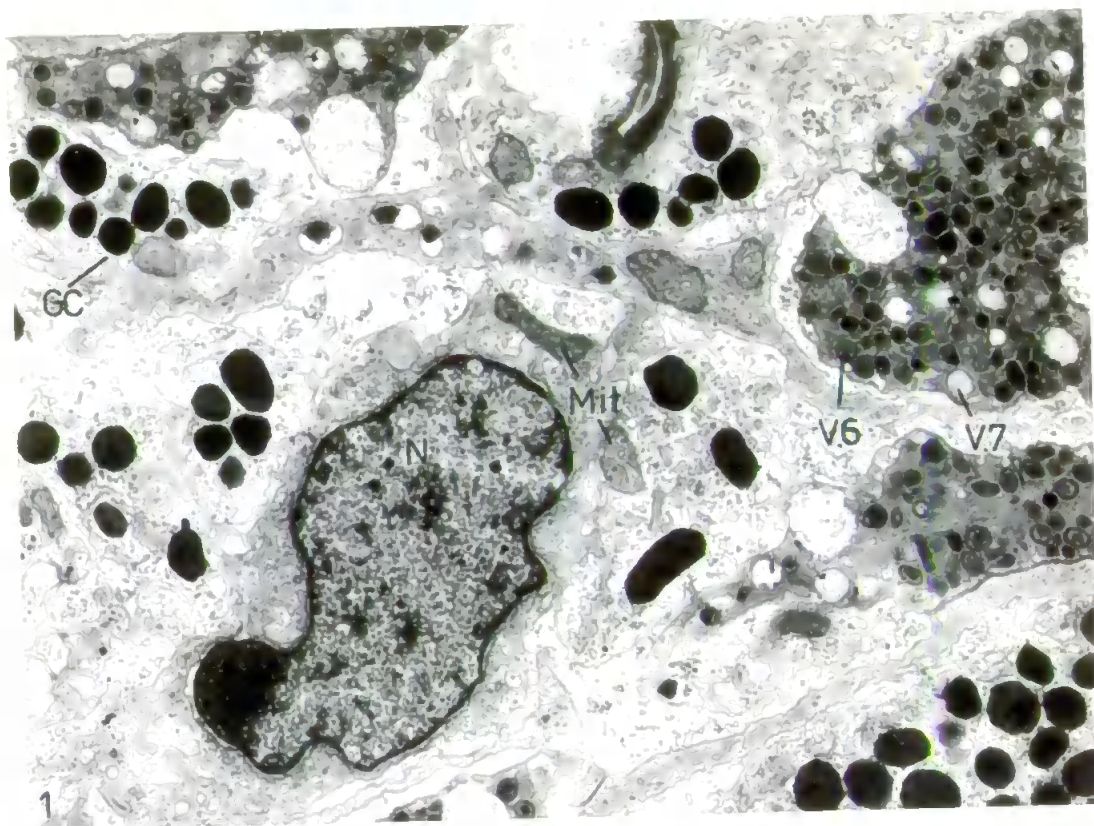


PLATE 18.

- 18-1 Electronmicrograph showing spines resting upon the inner plasma membrane of the outer tegumental layer. At the base of each spine is a dark zone, a similar zone is present on the underlying circular muscle blocks. x 25 000. Stained with uranyl acetate and lead citrate.
- 18-2 Stereoscan electronmicrograph of spines, extending above the general level of the tegument, and showing the grooves in the upper surface of each digit. Note the long and short cilia each of which has a collar, formed from foldings in the outer tegumental layer, surrounding its base. x 16 000.
- 18-3 Cross section of a spine clearly showing the protein lattice. Note the darkened areas of the spine associated with the corrugations in the upper surface and with the back of the spine. x 56 100. Stained with uranyl acetate and lead citrate.

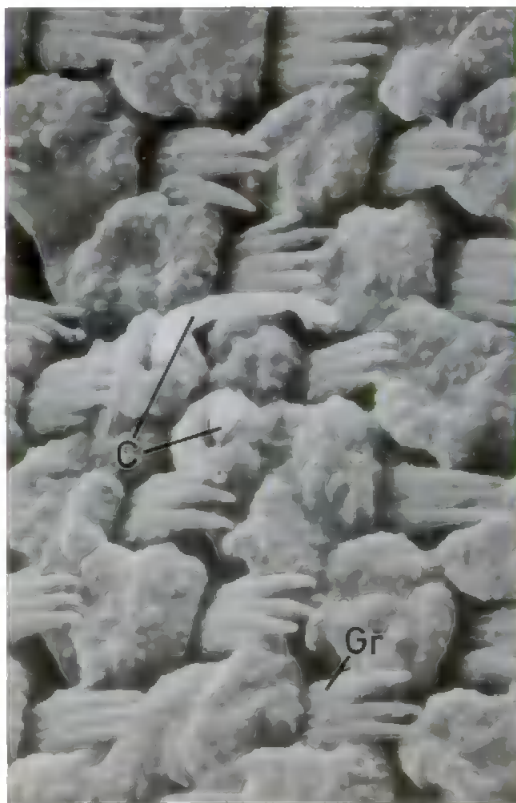


PLATE 19.

- 19-1 Scanning electronmicrograph of anterior end of parasite showing sucker and tentacle pits. x 3 250.
- 19-2 Scanning electronmicrograph of metacercarial mouth. Note the absence of spines in the area surrounding the mouth. x 12 000.
- 19-3 Scanning electronmicrograph of genital pore and excretory pore. Note the presence of many sensory cilia. x 3 250.

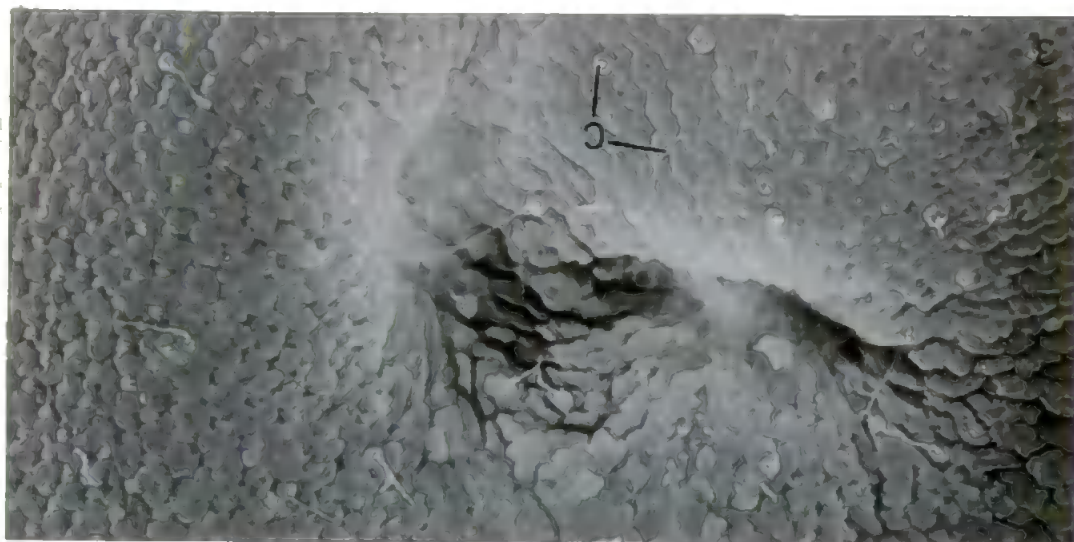


PLATE 20.

- 20-1 Cross section of anterior end of metacercaria showing the seven tentacle pits in three of which the tentacles can be seen. x 800. Papanicolaou stain.
- 20-2 Electronmicrograph of a gland cell, containing a membrane-bound, electron dense secretory product, opening to the exterior. It is attached to the outer tegumental layer by septate desmosomes. x 52 500. Stained with uranyl acetate and lead citrate.
- 20-3 Electronmicrograph of a sensory cilium arising from a bulb which is attached to the surrounding tegument by septate desmosomes and which contains many vesicles and peripheral electron dense patches. The cilium arises from basal rootlets and basal plate. A collar, formed from foldings in the surrounding tegument, encloses the lower portion of the cilium. x 34 400. Stained with uranyl acetate and lead citrate.

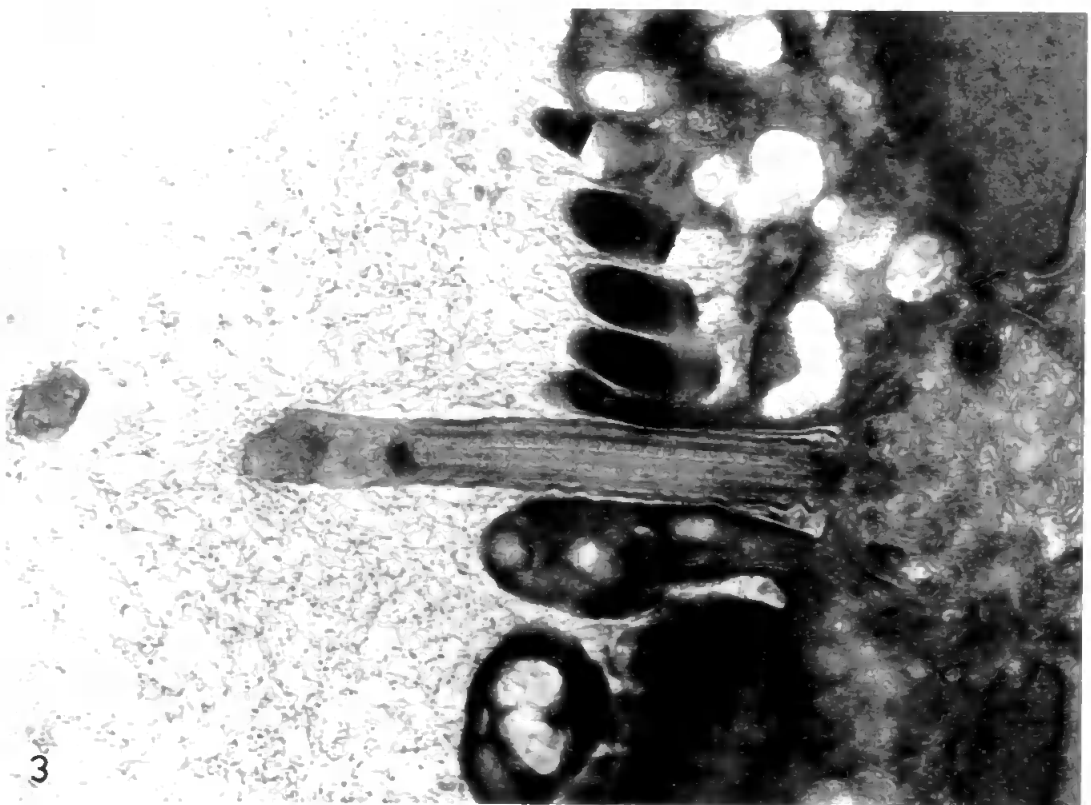
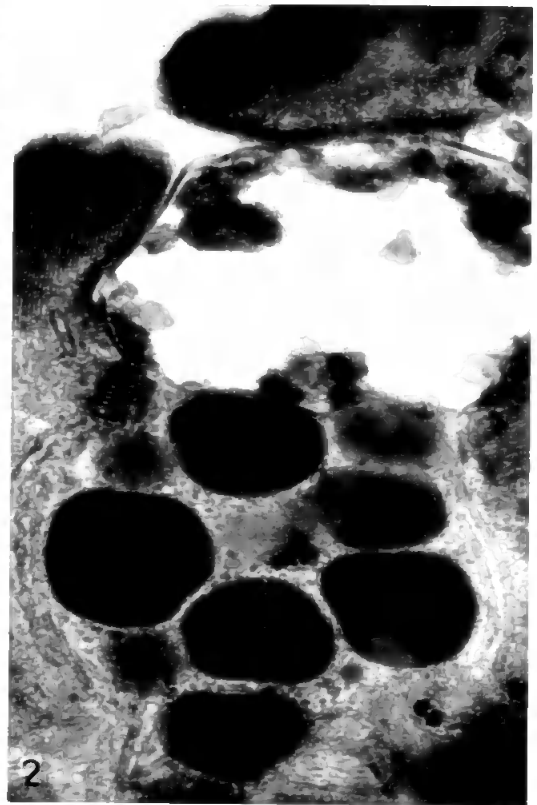


PLATE 21.

Electronmicrograph showing nature of cyst wall, in close apposition to normal liver cells, in a 30 day infection of Bucephalus haimeanus in Gobiusculus flavescens. The inner layer consists of an irregularly-compacted zone, densely-compacted zone and an outer evenly-compacted zone which is adjacent to the middle vacuolated layer. The outer layer is comprised of flattened, nucleated cells. x 12 500. Stained with uranyl acetate and lead citrate.

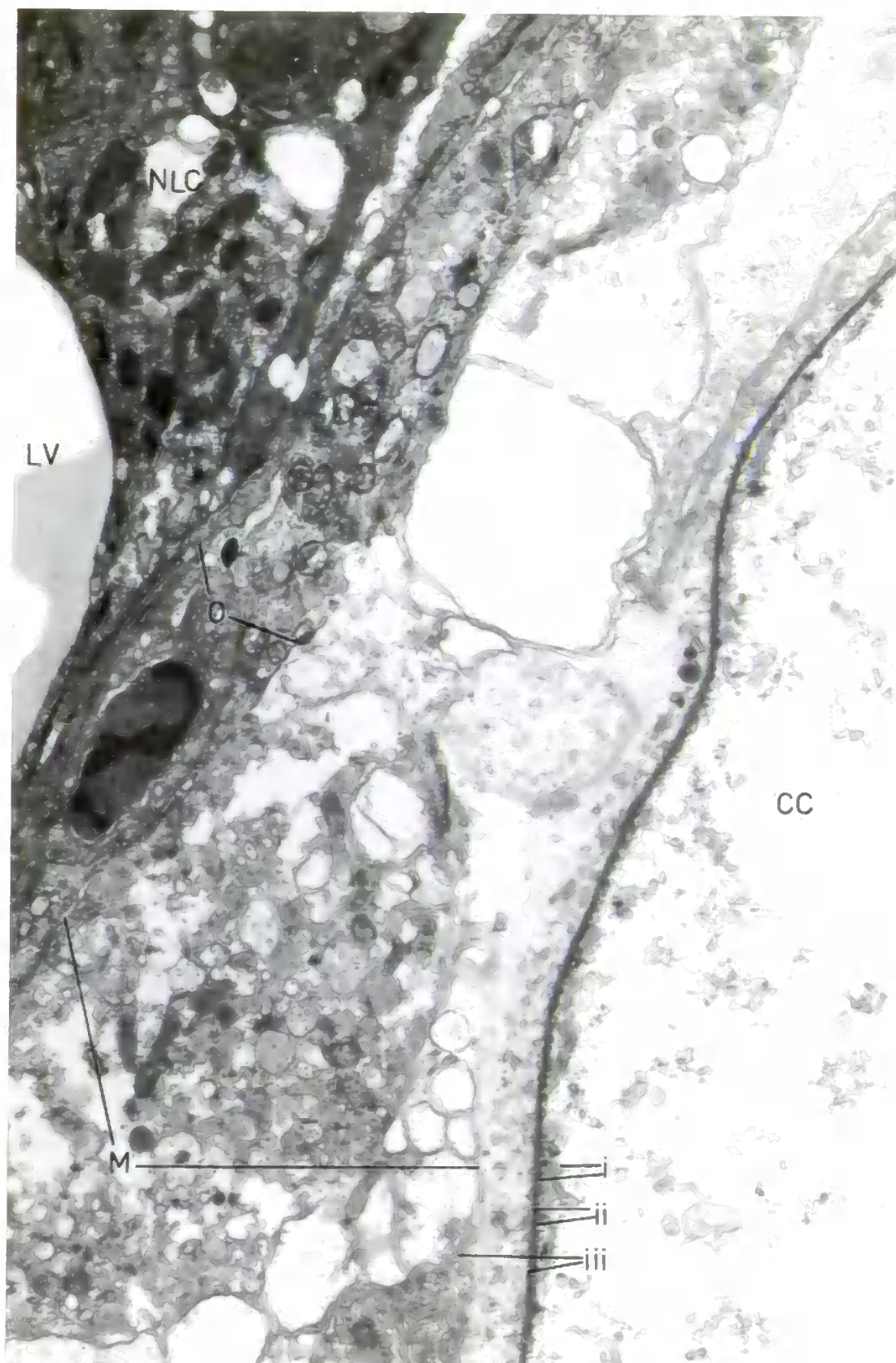


PLATE 22.

- 22-1 Electronmicrograph of gastrodermis showing a cellular junction and the fine lamellae which extend into the gut lumen. Electron dense bodies are adjacent to the lipid vacuoles. x 4 500. Stained with uranyl acetate and lead citrate.
- 22-2 Finger like projection extending into the excretory bladder showing nature of cytoplasmic lining of the bladder. Both lipid vacuoles and lamellae are present. x 14 600. Stained with uranyl acetate and lead citrate.
- 22-3 Electronmicrograph of excretory bladder lumen containing lamellae interconnecting to form a meshwork (arrows). The refractile bodies are formed of concentric layers of deposit around a central nucleus. x 19 500. Stained with uranyl acetate and lead citrate.
- 22-4 Electronmicrograph showing refractile bodies with single or multiple nuclei. x 11 500. Stained for alkaline phosphatase activity.

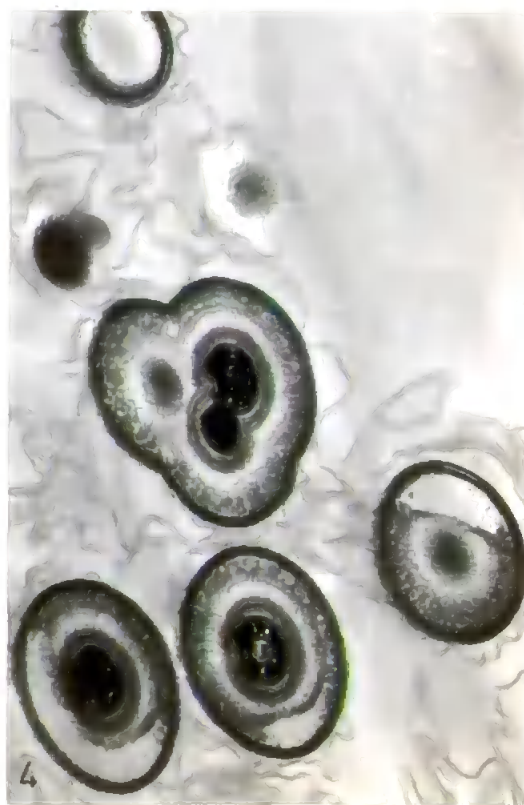
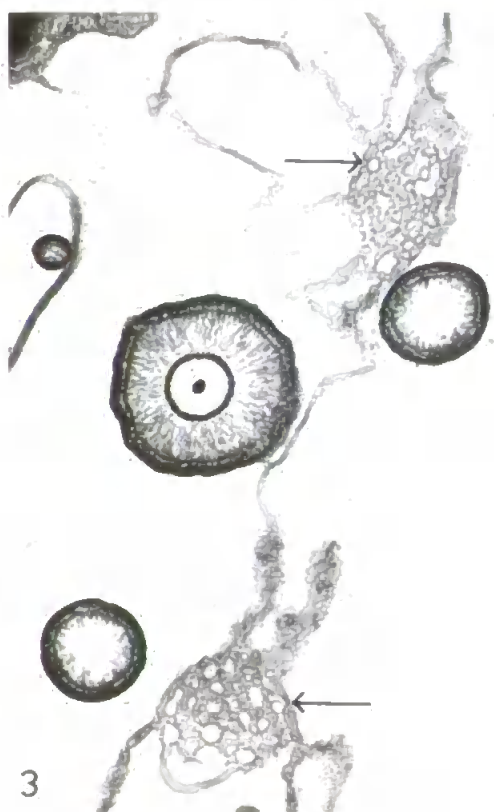
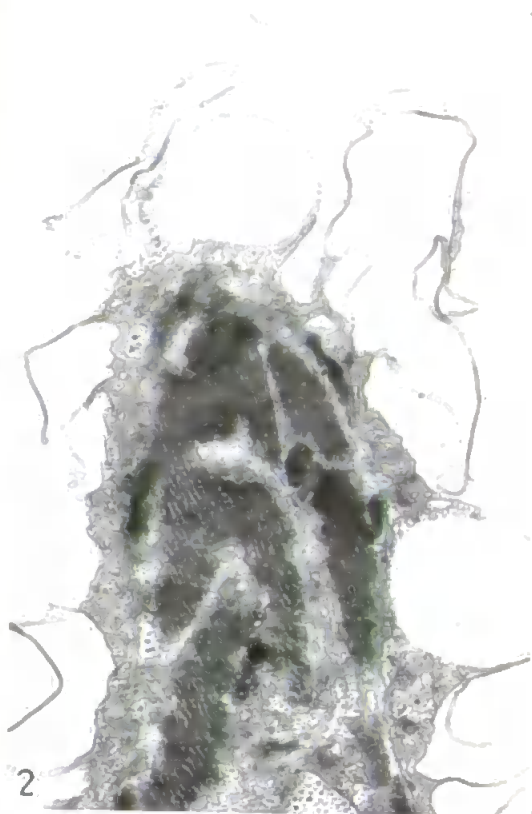
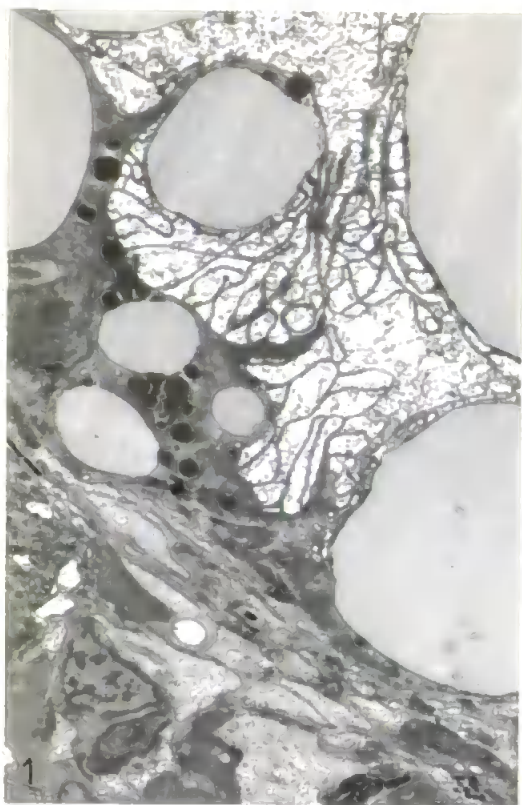


PLATE 23.

- 23-1 Electronmicrograph of a longitudinal section through a flame cell showing it to consist of a nucleated cell, from which the cilia of the flame arise, interlocked by ribs to a barrel formed from cells folded round and joined by desmosomes. The luminal surface of the barrel is irregular in outline and from its outer surface leptotriches extend into the surrounding cells. The excretory tubule is similarly formed from cells folded round and joined by desmosomes. x 13 800. Stained with uranyl acetate and lead citrate.
- 23-2 Electronmicrograph of a transverse section through anterior end of a protonephridium showing interlocking system of ribs. The flame consists of cilia bound by an intermembranous matrix. x 21 600. Stained with uranyl acetate and lead citrate.

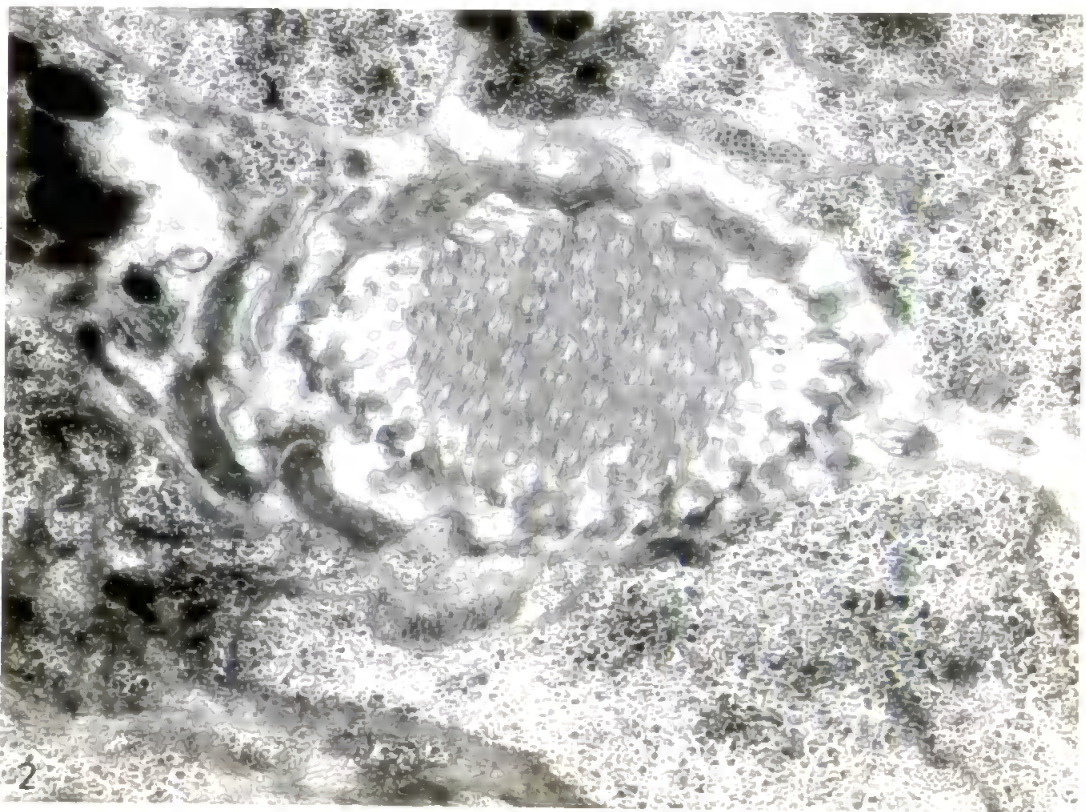
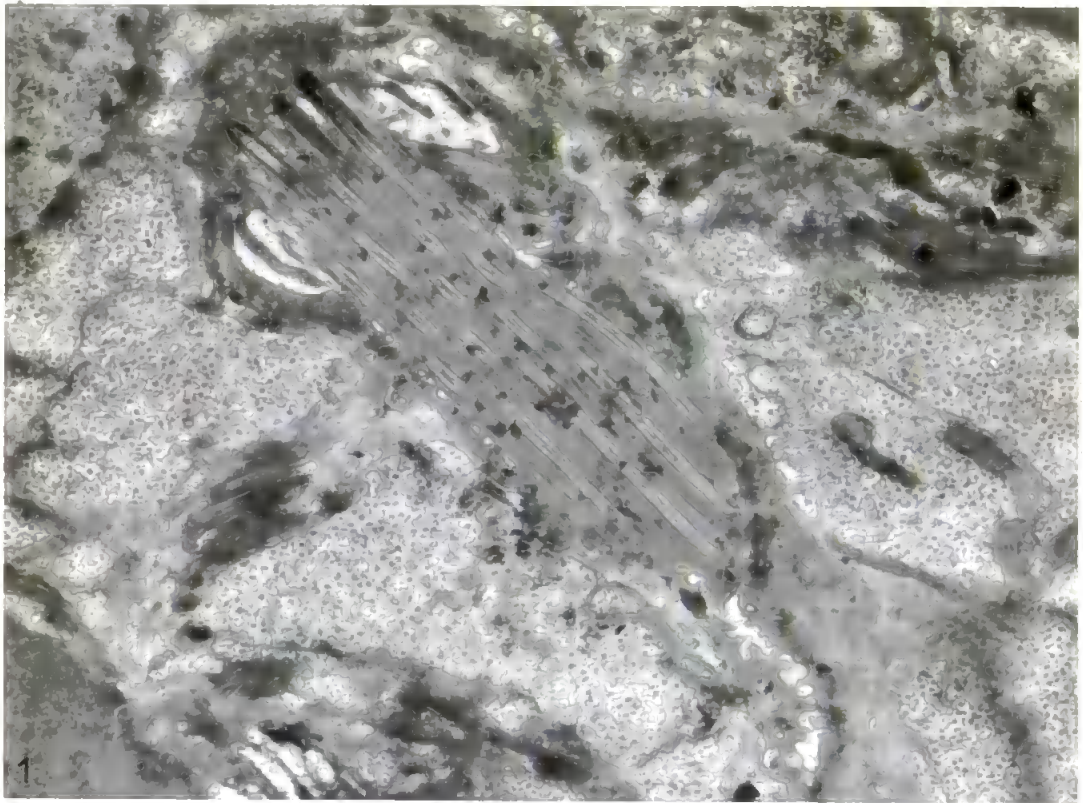


PLATE 24.

- 24-1 Light micrograph showing localisation of β -glucuronidase activity in the hepatic cell cytoplasm, middle and outer layers of the cyst wall and metacercarial intestine. x 830.
- 24-2 Light micrograph showing β -glucosaminidase activity in the hepatic cell cytoplasm, middle and outer layers of the cyst wall and metacercarial intestine. x 830.
- 24-3 Light micrograph showing localisation of acid phosphatase activity in the liver and cyst wall. x 830.
- 24-4 Distribution of ATPase activity in the liver, cyst wall and outer tegumental layer and tegumental cells. x 1 160.

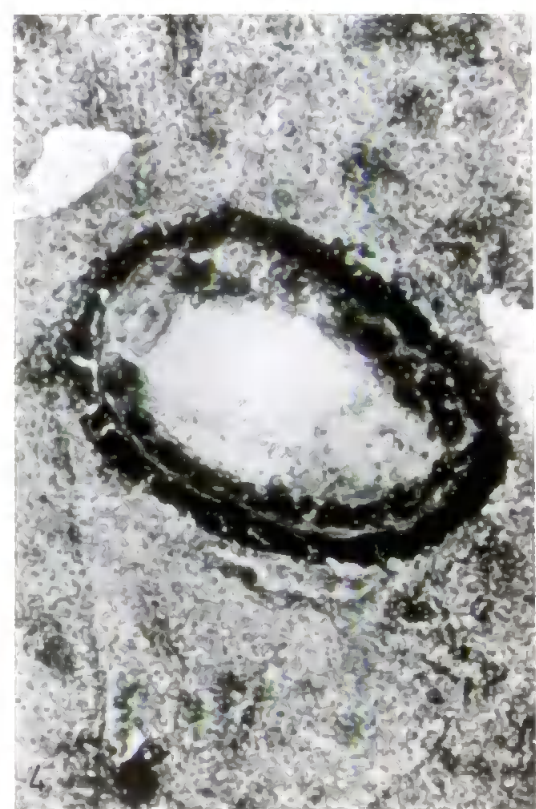
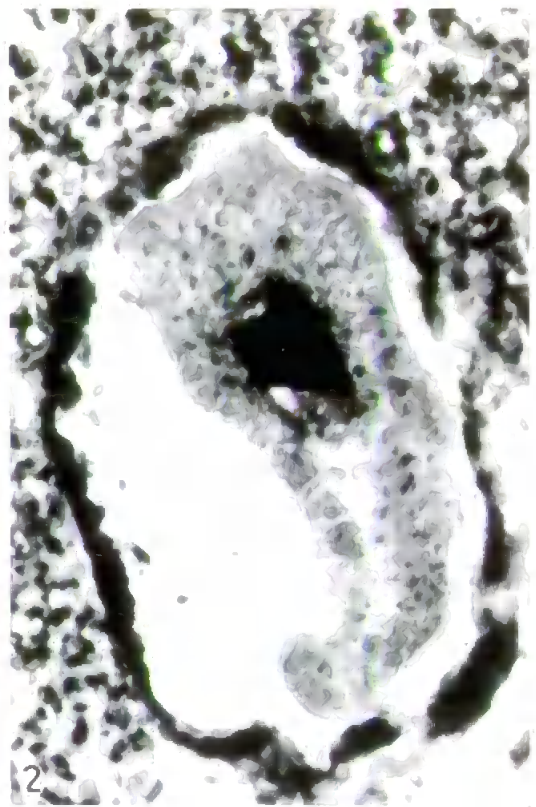
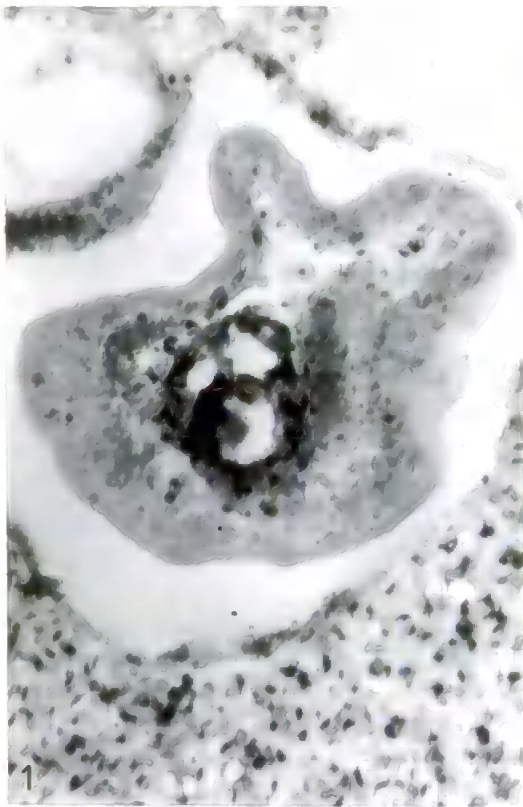


PLATE 25.

- 25-1 Light micrograph showing Glucose-6-phosphate dehydrogenase activity in the liver. Note the lack of activity in the parasite and cyst wall, which cannot be clearly defined. x 1 550.
- 25-2 Succinic dehydrogenase activity is scattered throughout the liver cells and parasite but is absent from the cyst wall. Note the seven tentacle pits at the anterior end of the metacercaria. x 620.
- 25-3 Light micrograph through anterior portion of metacercaria showing positive staining of nervous system for esterase activity. Both cyst wall and liver are negative. x 250.

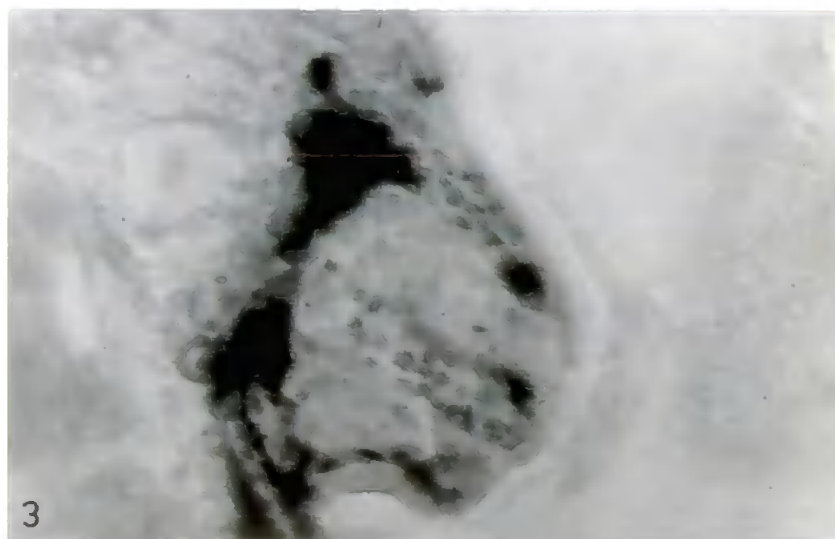


PLATE 26.

Electronmicrograph of alkaline phosphatase activity in the middle and inner layers of the cyst wall, the cyst cavity and over the surface of the metacercaria. A vesicle in the cyst cavity stains intensely. Note the distribution of deposit in the middle layer where it occurs as rings of deposit within the large compound vacuoles.

x 28 000. Not counterstained.

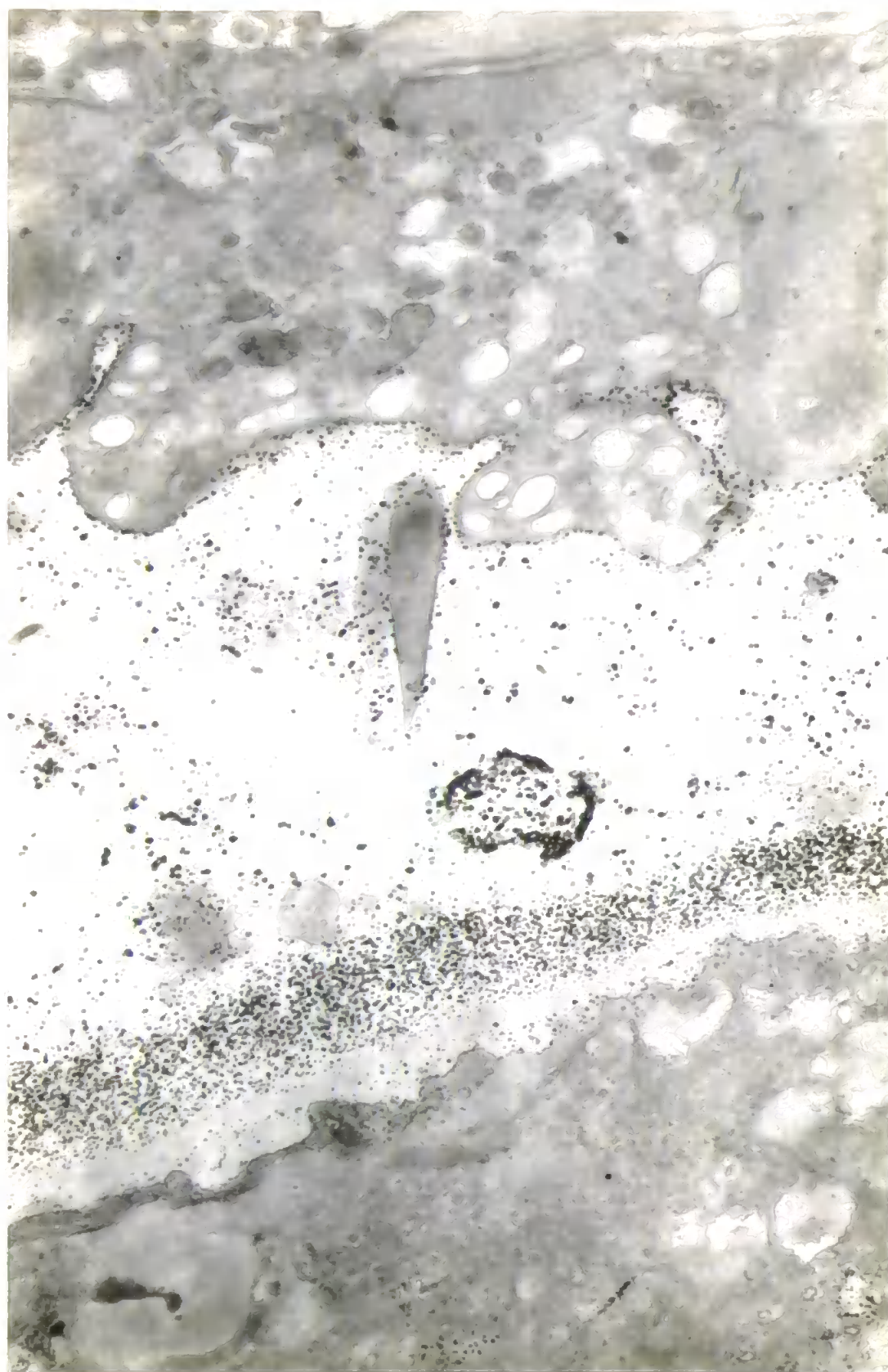


PLATE 27.

- 27-1 Electronmicrograph of a section through part of the metacercarial tegument in which the membranes surrounding the vacuoles stain positively for alkaline phosphatase. x 51 600. Not counterstained.
- 27-2 Electronmicrograph showing intense staining for alkaline phosphatase in outer tegumental layer around a sensory cilium. x 31 200. Not counterstained.
- 27-3 Electronmicrograph showing alkaline phosphatase activity associated with the plasma membrane enclosing a sensory cilium. The outer tegumental surface, cyst cavity and inner and middle layers of the cyst wall stain intensely. x 25 000. Not counterstained.

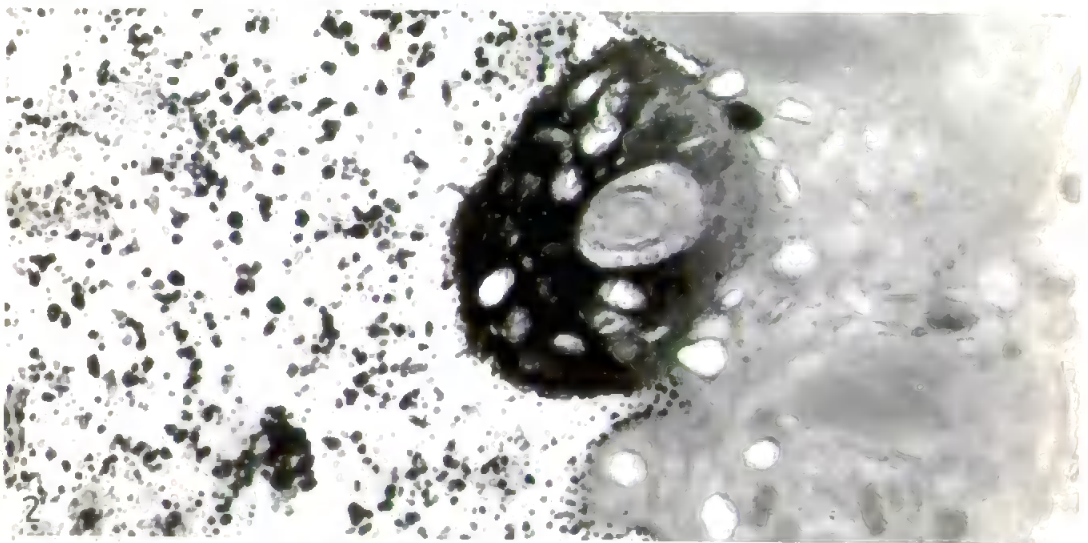
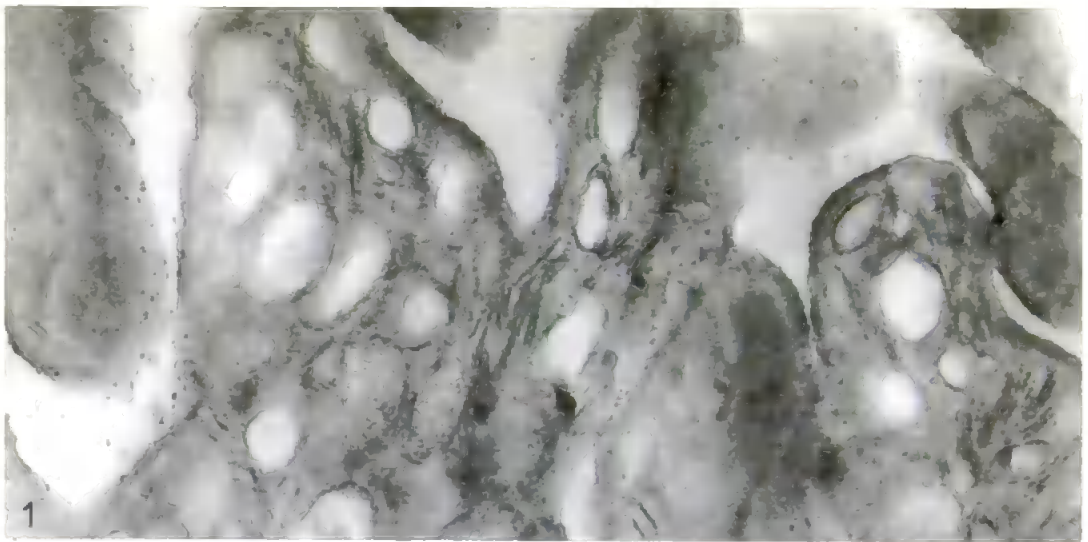


PLATE 28.

- 28-1 Control section for alkaline phosphatase activity incubated in media lacking in substrate. There is no lead deposit. x 11 500. Unstained.
- 28-2 Electronmicrograph of intestine showing small amounts of alkaline phosphatase activity associated with the cytoplasmic side of membranes enclosing lamellae. x 32 400. Not counterstained.
- 28-3 Electronmicrograph of excretory bladder showing small amounts of alkaline phosphatase activity associated with the cytoplasmic side of membranes enclosing lamellae. x 10 000. Not counterstained.
- 28-4 Intense alkaline phosphatase activity associated with the bile canaliculi of Pomatoschistus microps livers. x 11 550. Not counterstained.
- 28-5 Electronmicrograph showing alkaline phosphatase activity along a cellular junction between two hepatic cells in P. microps. x 9 200. Not counterstained.

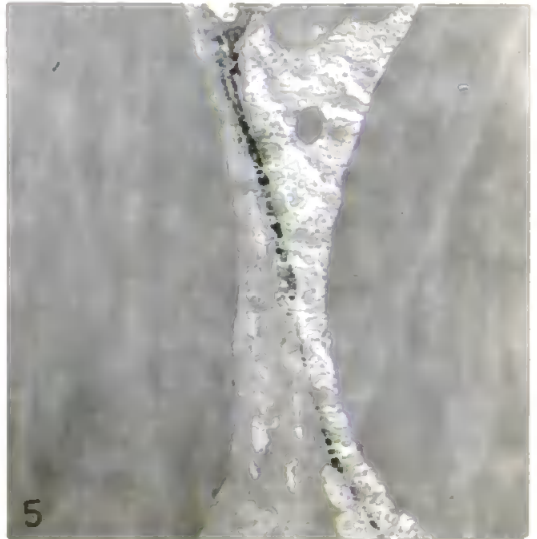
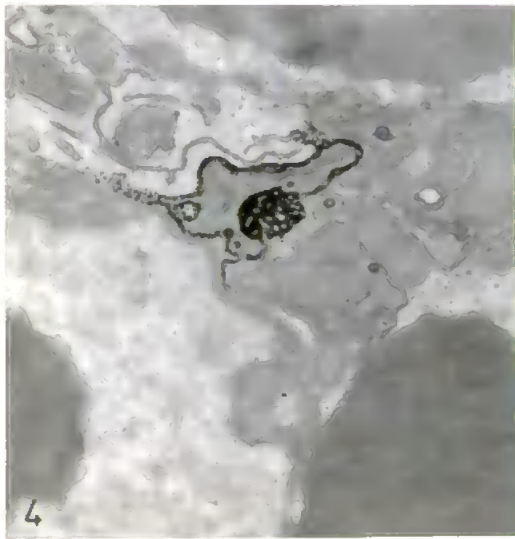
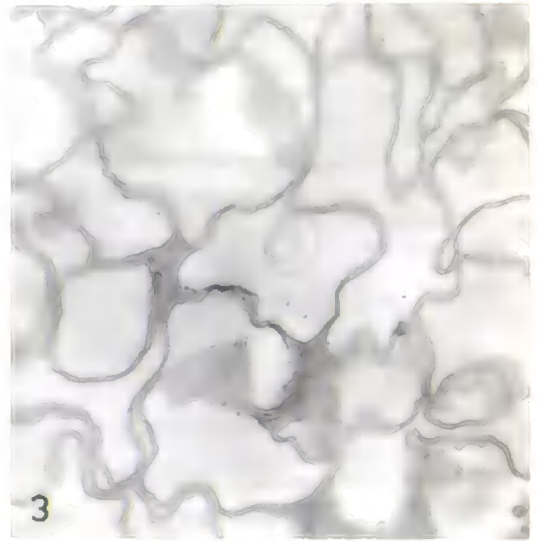
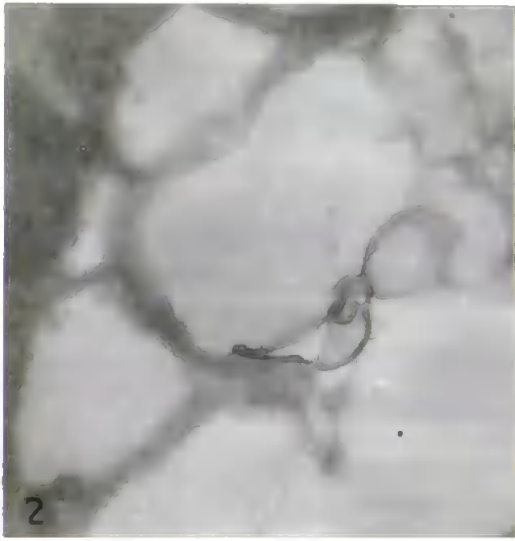
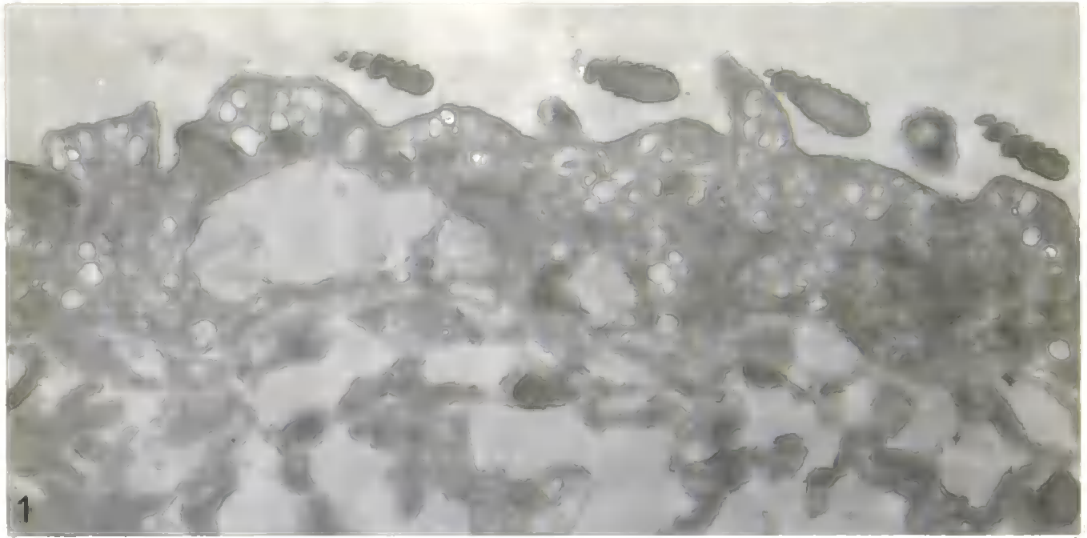


PLATE 29.

- 29-1 Control section incubated in a medium lacking
in substrate for acid phosphatase localisation.
Lead stain is seen to be especially associated with
membranous structures. x 15 600. Not counterstained.
- 29-2 Tegument of metacercarial Bucephalus haimeanus
with acid phosphatase reaction in one vesicle.
Other vesicles are negative. x 25 500. Not
counterstained.
- 29-3 Electronmicrograph showing localisation of acid
phosphatase activity in a tegumentary cell.
x 9 500. Not counterstained.
- 29-4 Acid phosphatase activity in the lipid vacuoles of
the gastrodermis. x 37 800. Not counterstained.

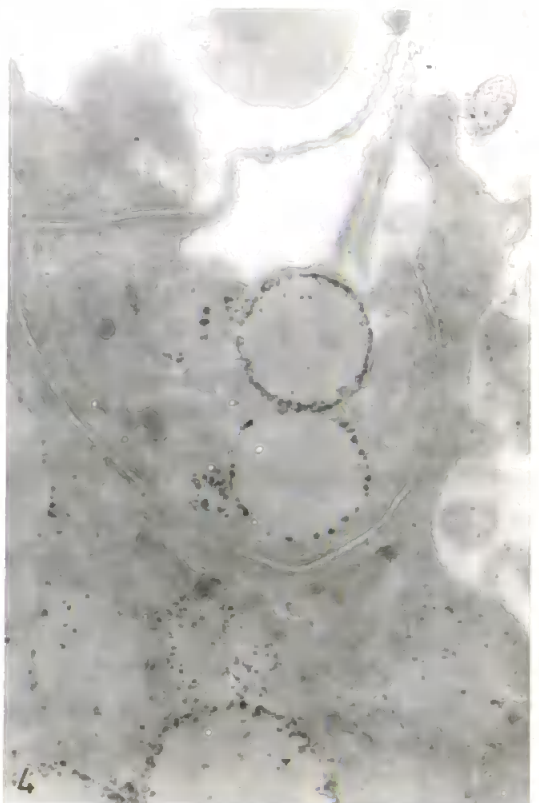
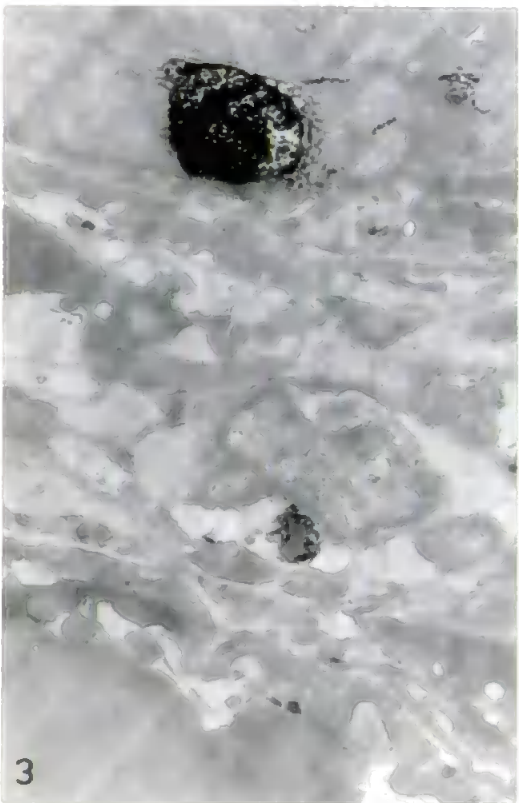
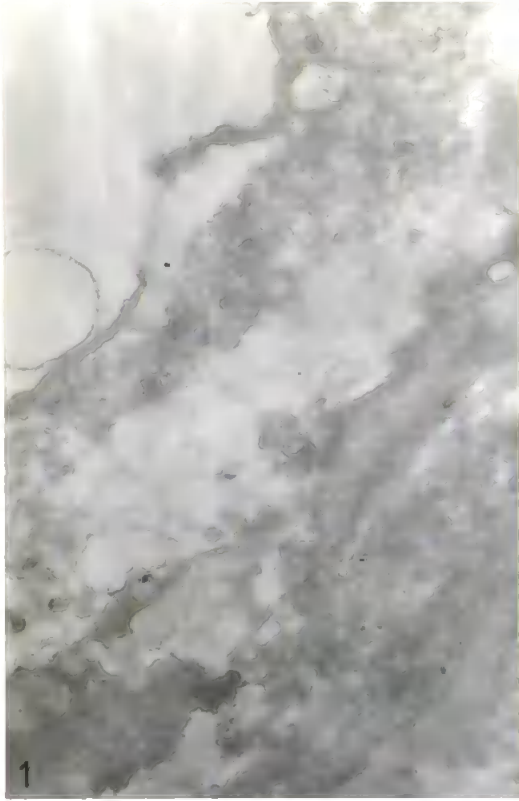


PLATE 30.

Localisation of acid phosphatase activity in Bucephalus
haimeanus encysting in Pomatoschistus microps.

Activity is located in the vacuoles of the middle
layer and lysosomes of the outer layer of the cyst
wall. The inner layer of the cyst wall, cyst
cavity and surface of the parasite are negative.
x 10 400. Not counterstained.

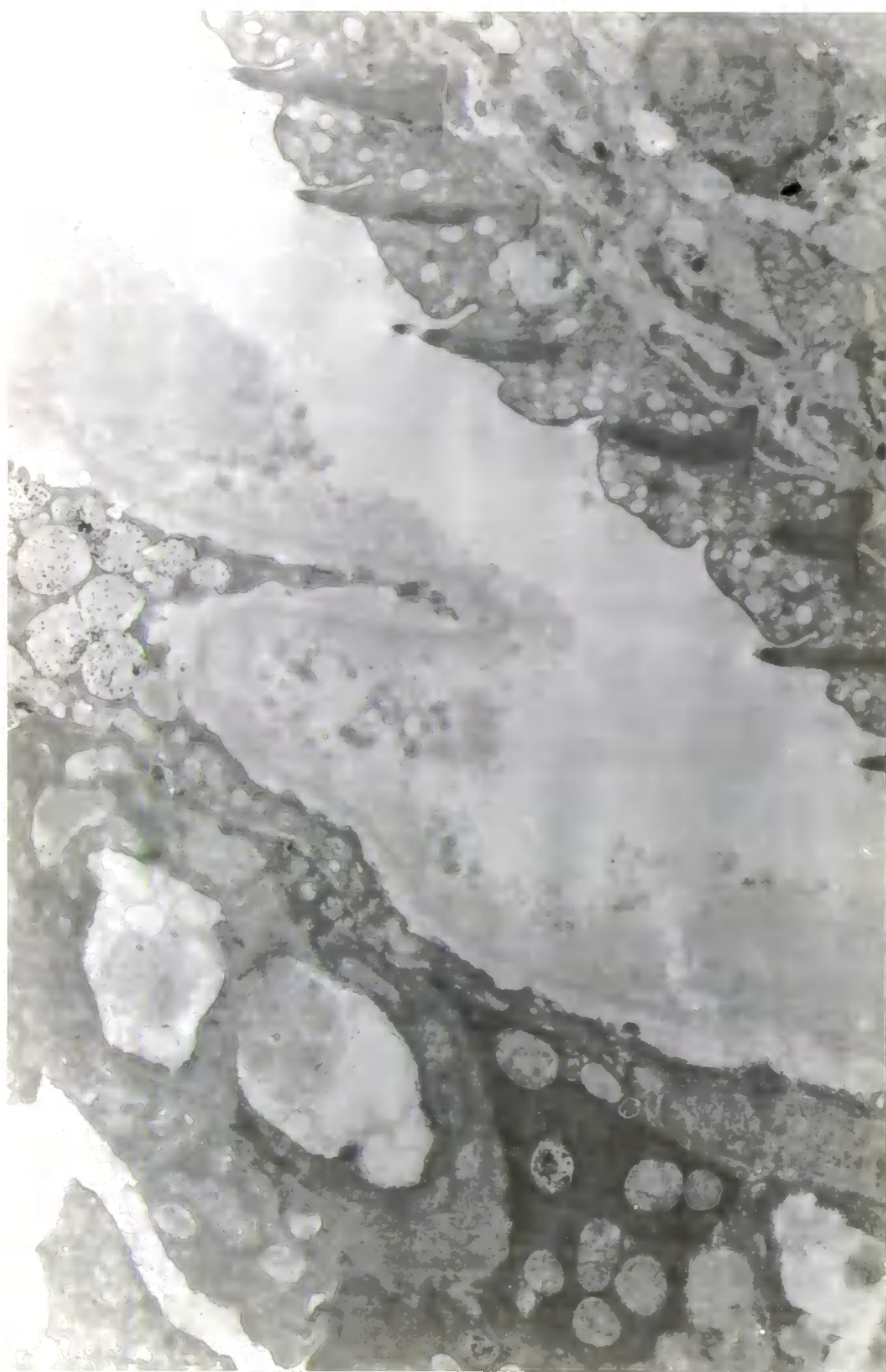


PLATE 31.

Sections through metacercarial tegument and cyst wall incubated in the complete Ernst (1972a,b) medium with the addition of ouabain. Intense activity is present in the tegumental cytoplasm, over the tegument surface and in all layers of the cyst wall. The periphery of the nucleus in the outer cyst wall layer is intensely positive.

31-1 x 14 600.

31-2 x 18 700.

Not counterstained.

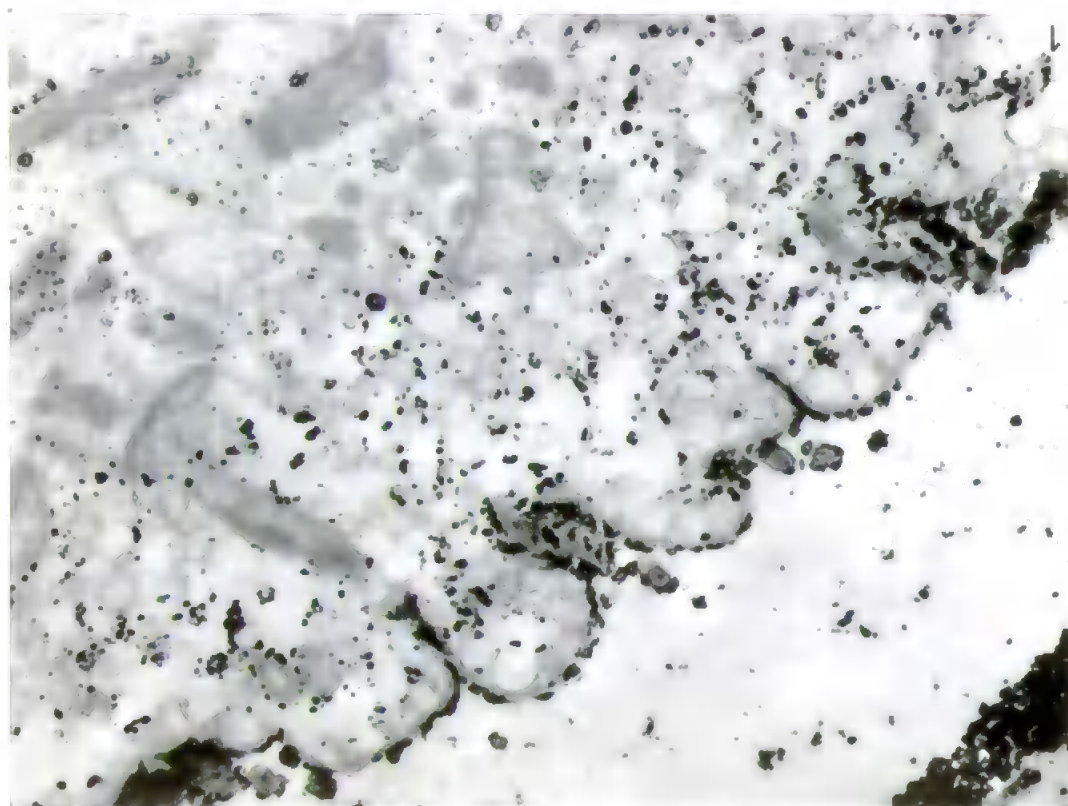
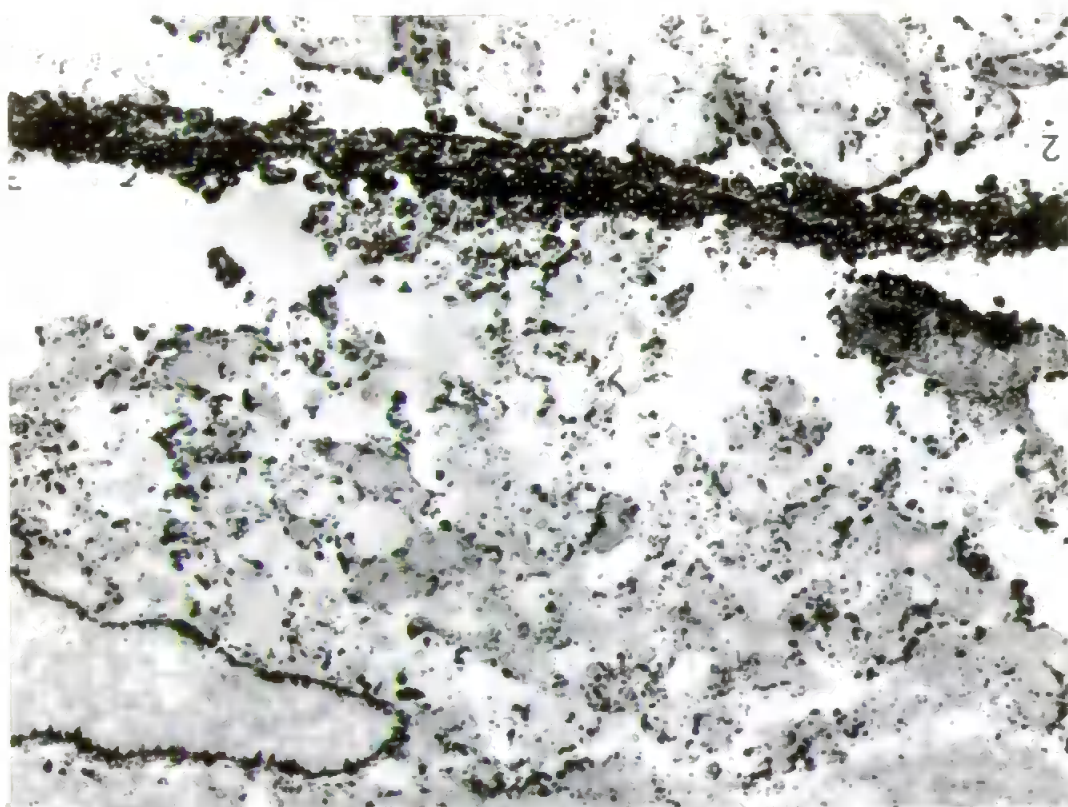


PLATE 32.

- 32-1 Tissue incubated in the complete Ernst (1972a,b) medium for the localisation of Na-K-ATPase. Reaction product is located in the tegument, on the surface of the tegument, and in all layers of the cyst wall. The periphery of a nucleus in the outer cyst wall stains intensely. x 6 800. Not counterstained.
- 32-2 This section is through the anterior of a metacercaria with extended tentacle. The outer surface of the tentacle and the gland cells opening into the tentacular pit mostly lack ATPase activity. Reaction product is associated with the muscle blocks. Note the absence of spines in the tentacle tegument. x 6 400. Not counterstained.
- 32-3 This control section shows a portion of the metacercaria and cyst wall incubated in the Ernst (1972a,b) medium in the absence of NPP. Note the absence of lead deposit in all tissues. x 9 450. Not counterstained.

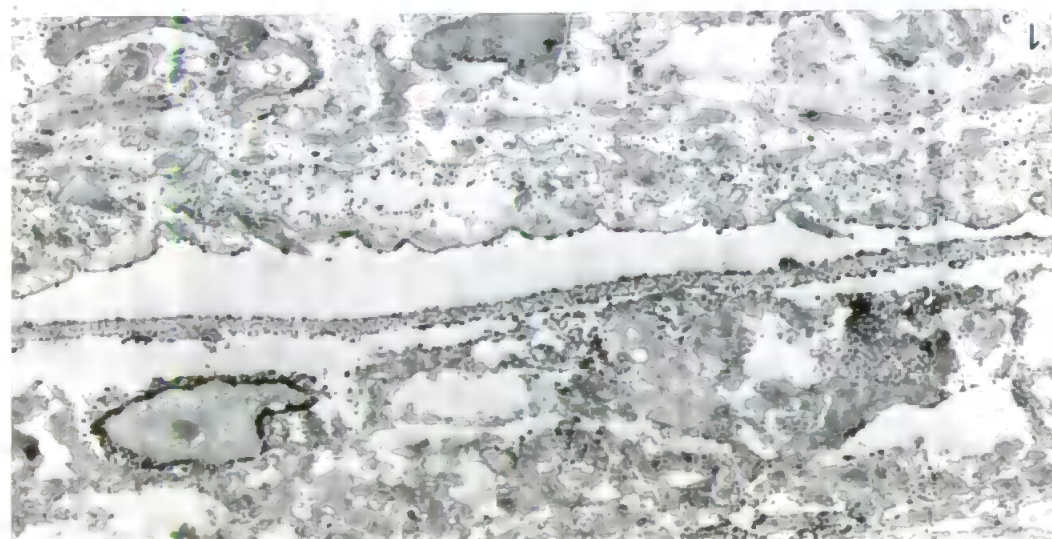
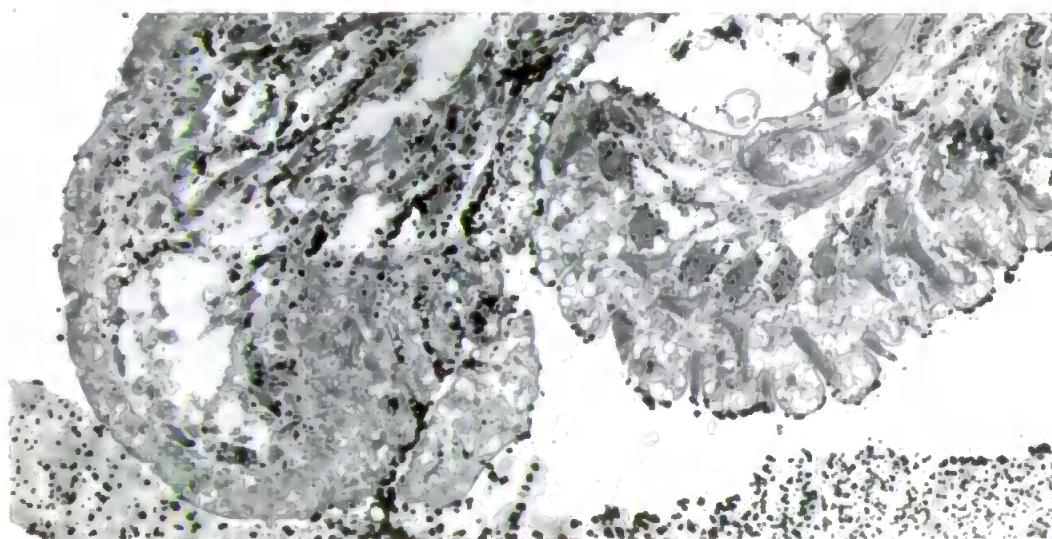
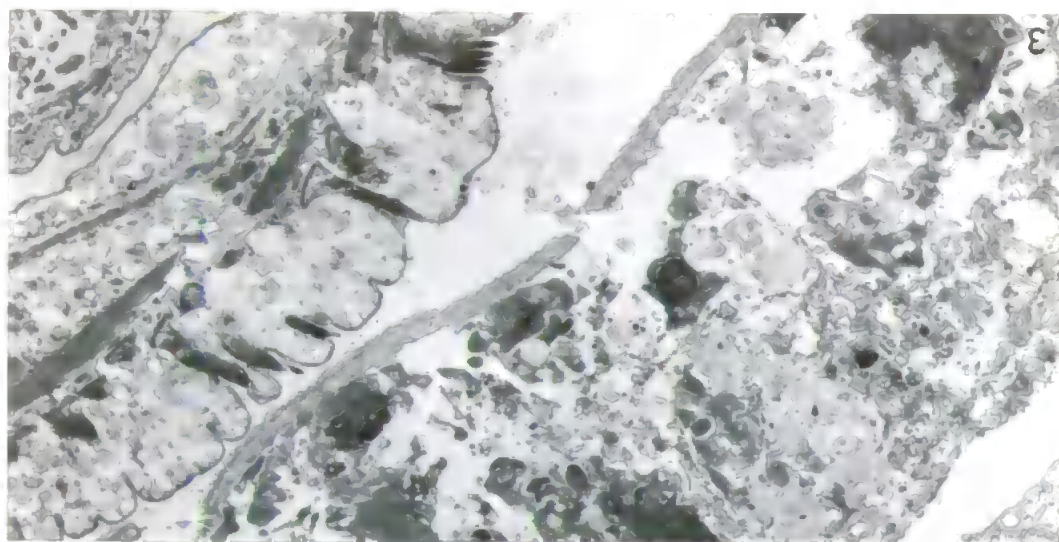


PLATE 33.

Electronmicrograph showing the localisation of reaction product in tissue incubated in the complete Ernst (1972a,b) medium for the location of Na-K-ATPase. Deposit is present in the inner layer of the cyst wall, over the surface of the parasite and in association with the majority of vacuoles within the tegument. x 15 750. Not counterstained.

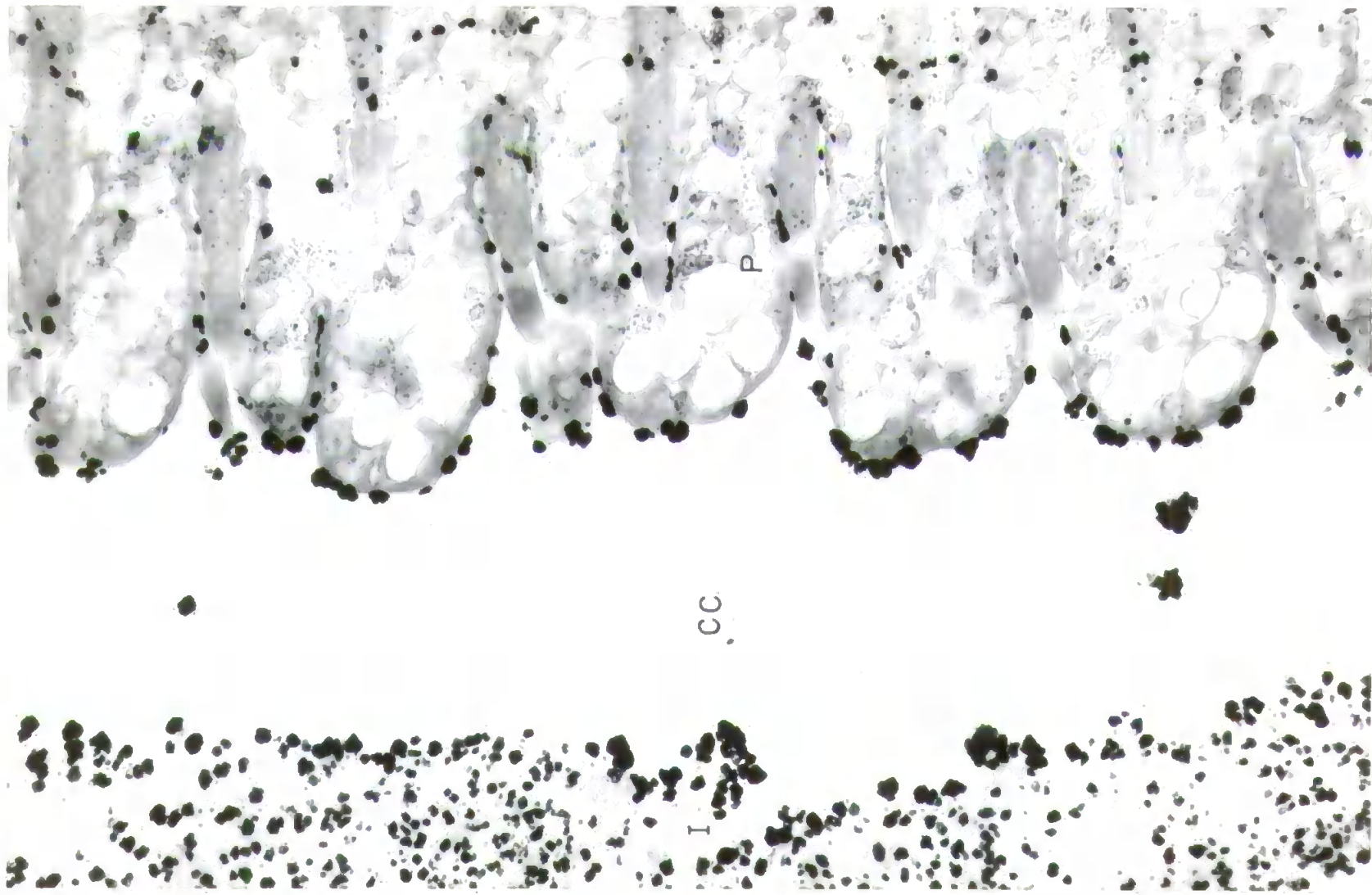
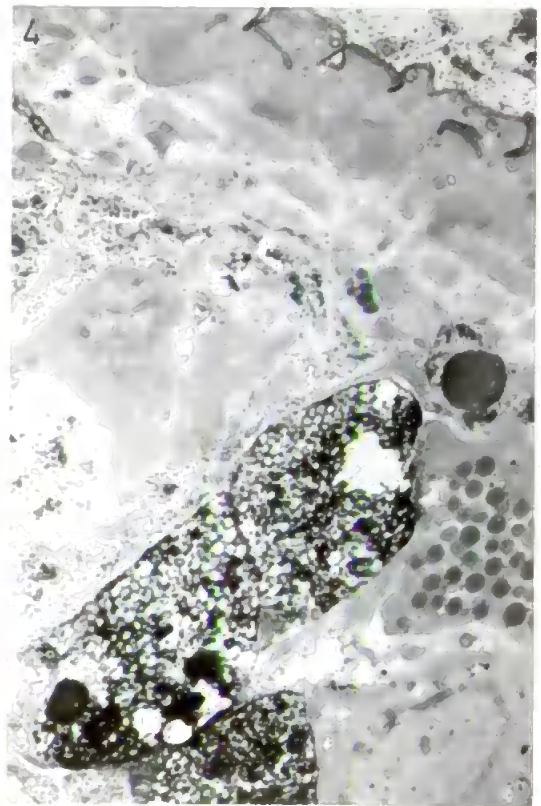
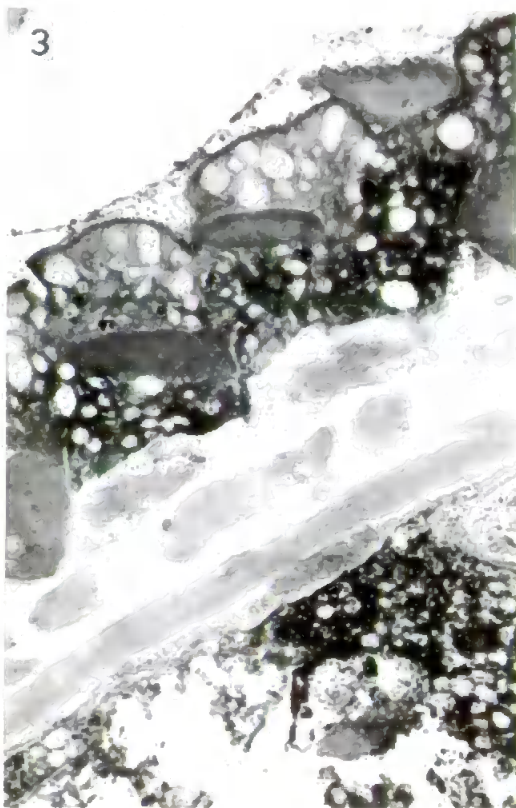
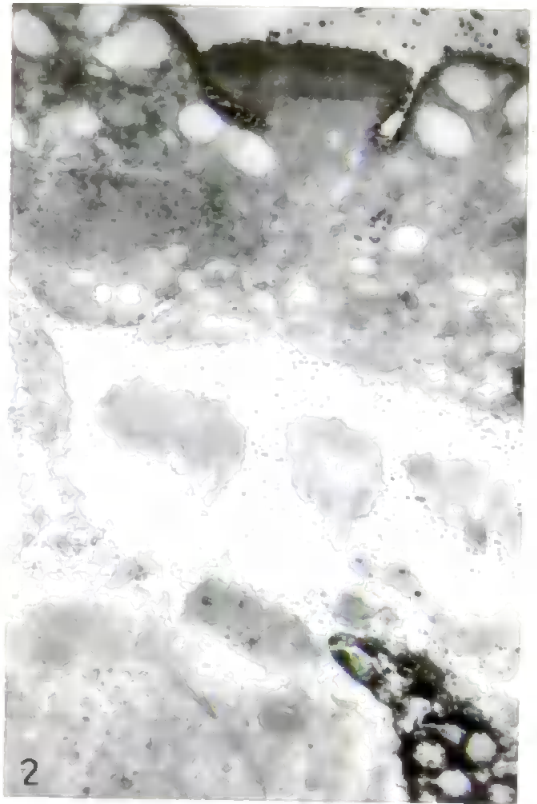
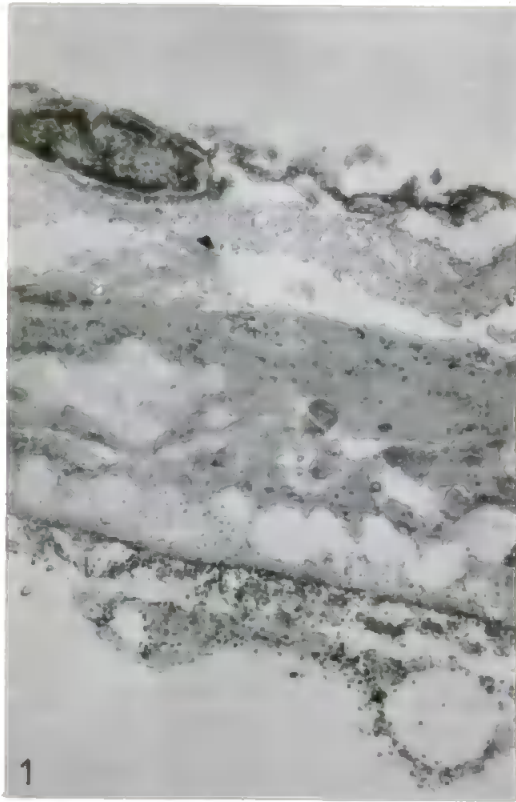


PLATE 34.

- 34-1 Electronmicrograph of cyst wall showing nucleosidepolyphosphatase activity in the irregularly-compacted and densely-compacted zones of the inner layer, vacuoles of the middle layer and the outer layer. Substrate: ATP. x 14 000. Counterstained with uranyl acetate and lead citrate.
- 34-2 Section through metacercarial tegument showing nucleosidepolyphosphatase activity in the cytoplasm of a tegumental cell but which does not extend along the cytoplasmic bridge into the outer tegumental layer. Note activity over surface of parasite. Substrate: ATP. x 22 100. Not counterstained.
- 34-3 Electronmicrograph showing area of metacercarial tegument in which nucleosidepolyphosphatase activity extends into the outer tegumental layer from the underlying tegumental cell. A fine deposit is present in the muscle layers. Substrate: ATP. x 13 500. Counterstained with uranyl acetate and lead citrate.
- 34-4 Section of a portion of metacercarial tegument in which the V.7 secretory cell bodies show nucleosidepolyphosphatase activity. An intense reaction is given by the tegument surface, and tegumentary cell. Note lack of activity in the gland cell. Substrate: ATP. x 6 400. Counterstained with uranyl acetate and lead citrate.



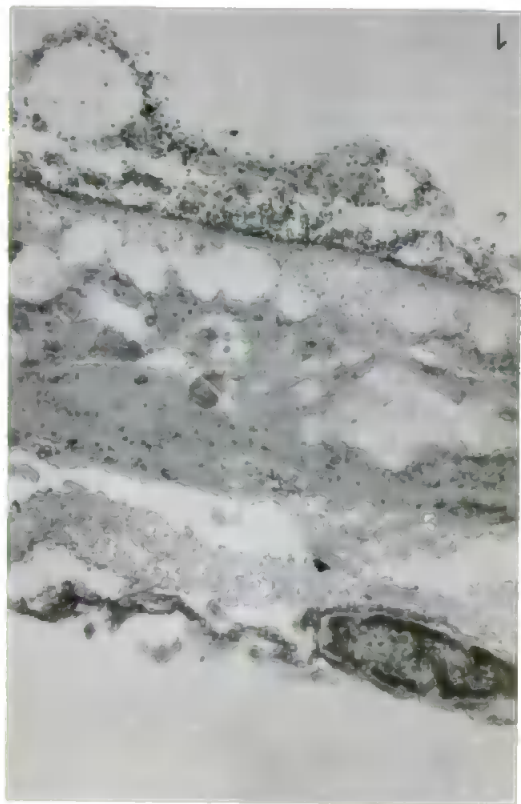
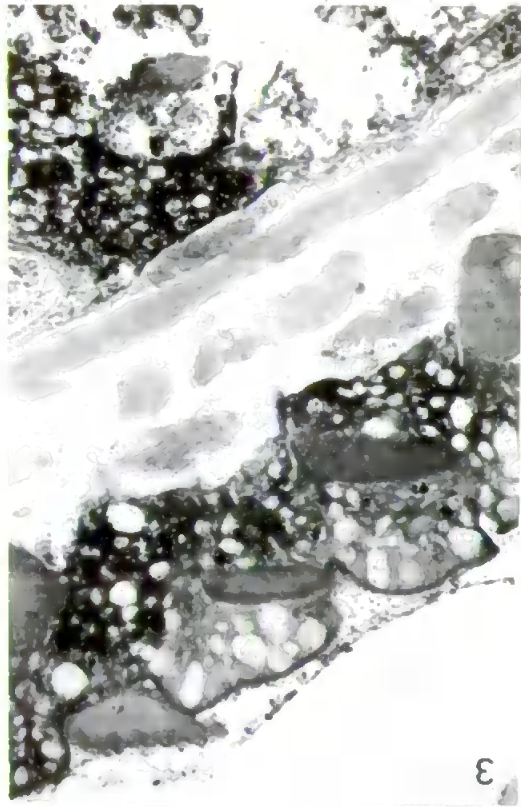
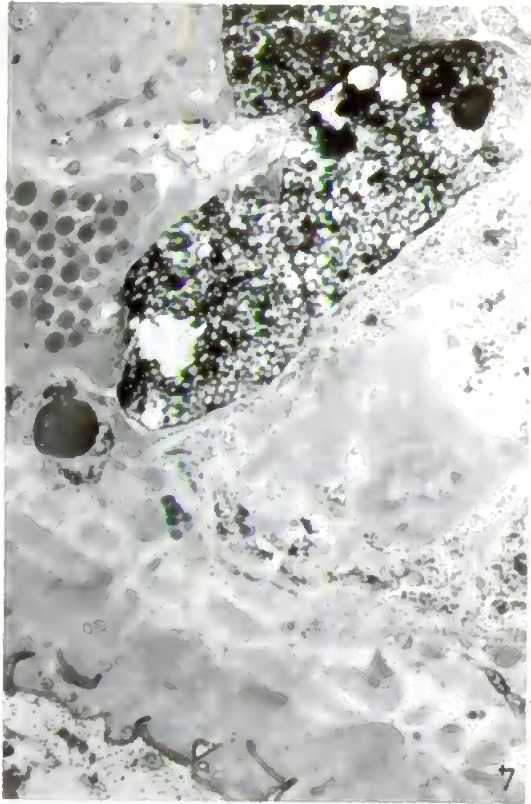


PLATE 35.

- 35-1 Distribution of nucleosidepolyphosphatase activity
in the cyst wall. Substrate: AMP. x 20 400.
Not counterstained.
- 35-2 Distribution of nucleosidepolyphosphatase activity
in the cyst wall. Substrate: GTP. x 11 700.
Not counterstained.
- 35-3 Distribution of nucleosidepolyphosphatase activity
in the cyst wall. Note many small spherical inclusions
in the middle and outer layers of the cyst wall.
Substrate: ADP. x 6 000. Not counterstained.
- 35-4 Section through metacercarial tegument showing
poor nucleosidepolyphosphatase reactivity of outer
surface when ADP is employed as substrate. x 23 800.
Not counterstained.
- 35-5 Intense reaction for nucleosidepolyphosphatase
activity when GTP is employed as substrate. x 28 900.
Not counterstained.

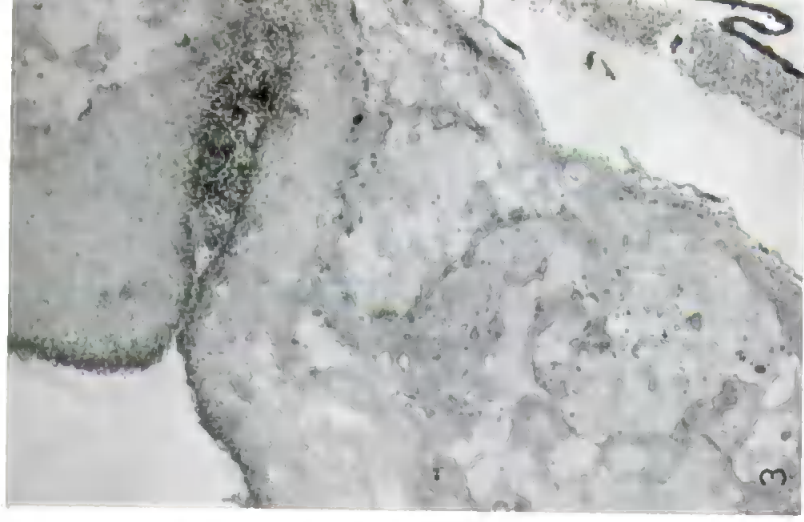
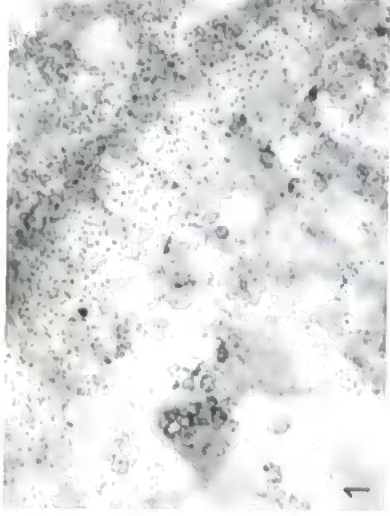
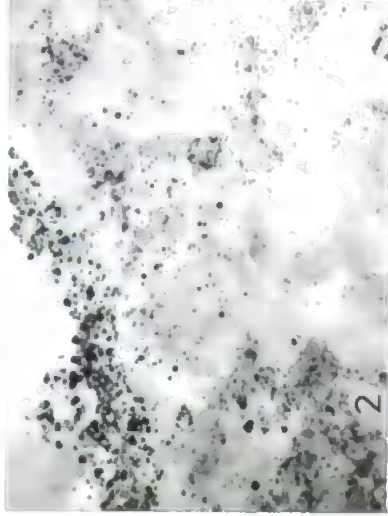
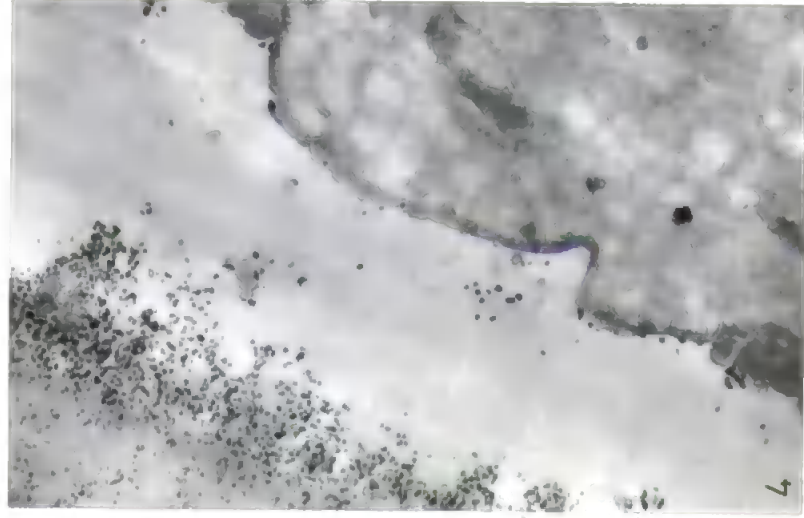
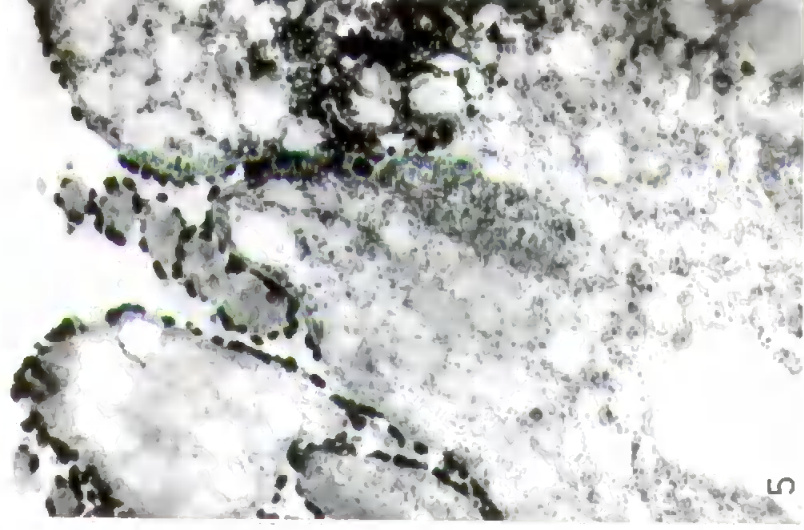


PLATE 36.

36-1 Electronmicrograph showing distribution of reaction product for nucleosidepolyphosphatase over the tegument surface. Substrate: GP. x 51 300.

Not counterstained.

36-2 Electronmicrograph showing nucleosidepolyphosphatase activity in the cyst wall. Substrate: GP. x 32 300.

Not counterstained.

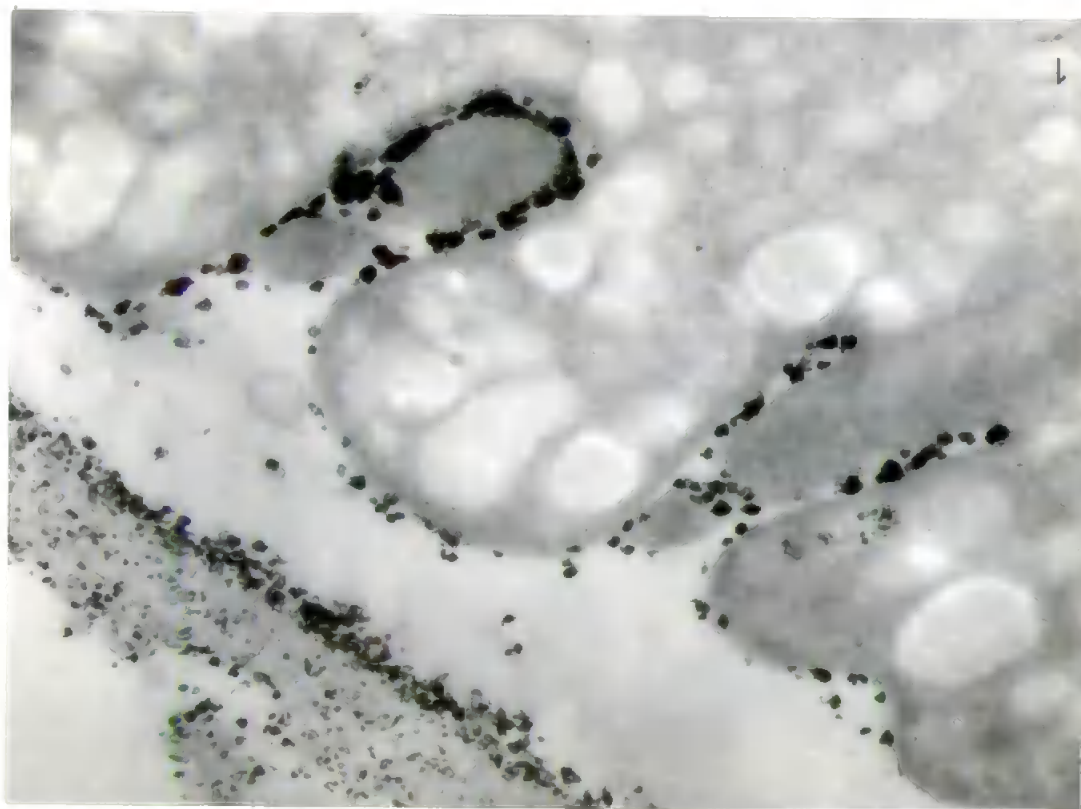
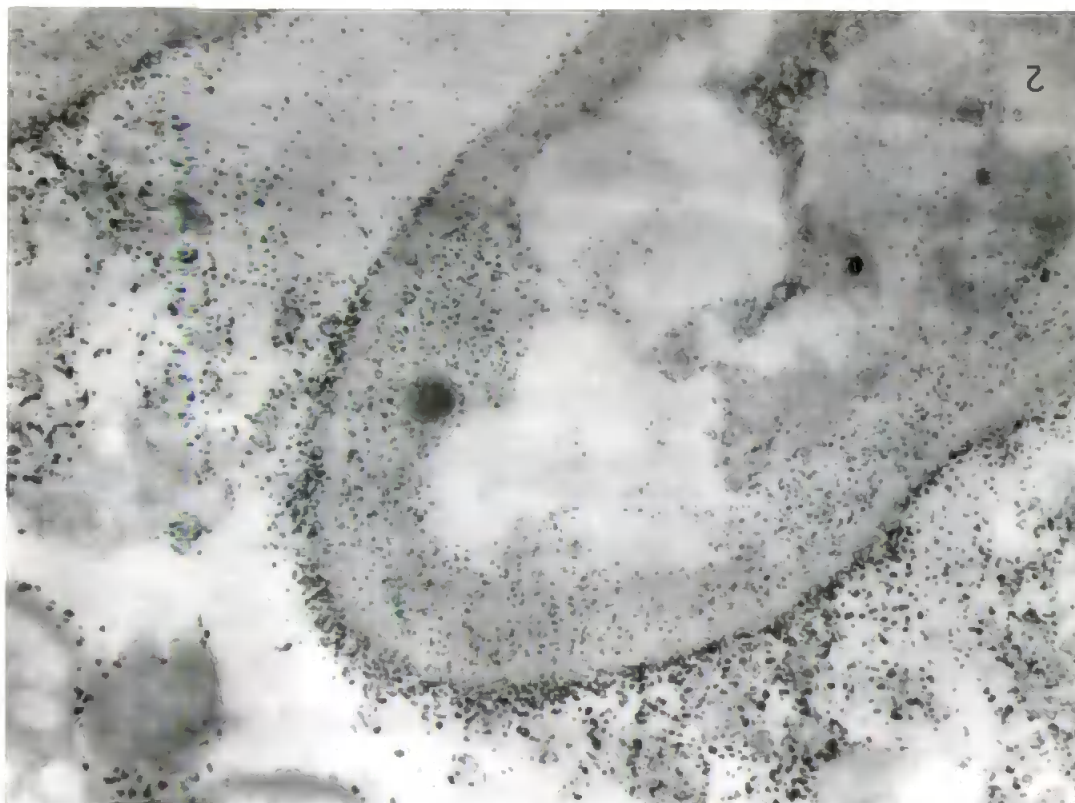


PLATE 37.

- 37-1 Electronmicrograph showing nucleosidepolyphosphatase activity in the inner, middle and outer layers of the cyst wall. Note location of reaction product in the cisternae of stacks of membranes between the middle and outer layer (arrow) and in the middle layer (crossed arrow). Note also the small spherical inclusions in the outer layer. Substrate: ATP + oubain. x 23 800. Not counterstained.
- 37-2 Location of nucleosidepolyphosphatase activity in the tegument surface and in the muscle of the metacercaria of Bucephalus haimeanus. Substrate: ATP + oubain. x 23 400. Not counterstained.
- 37-3 Electronmicrograph showing nucleosidepolyphosphatase activity over the tegument surface. Substrate: ATP + PCMB. x 13 500. Not counterstained.
- 37-4 Electronmicrograph showing nucleosidepolyphosphatase activity over the tegument surface and in the cytoplasm of the outer tegumental layer. Substrate: ATP + PCMB. x 30 600. Not counterstained.

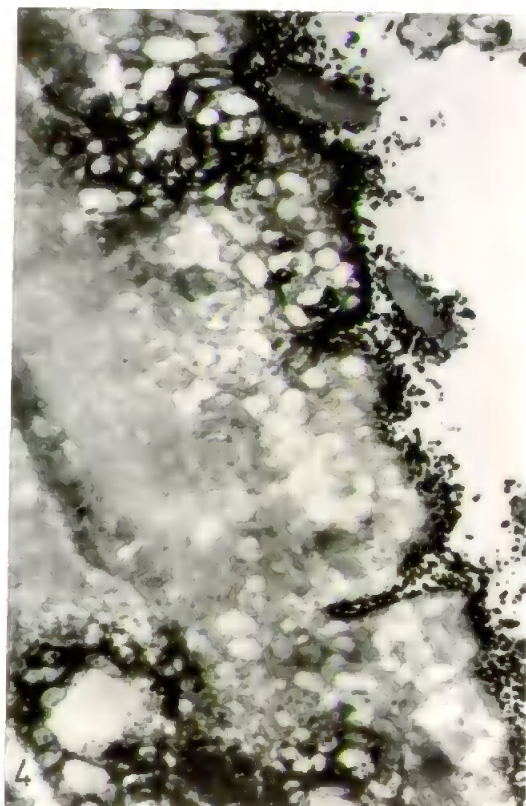
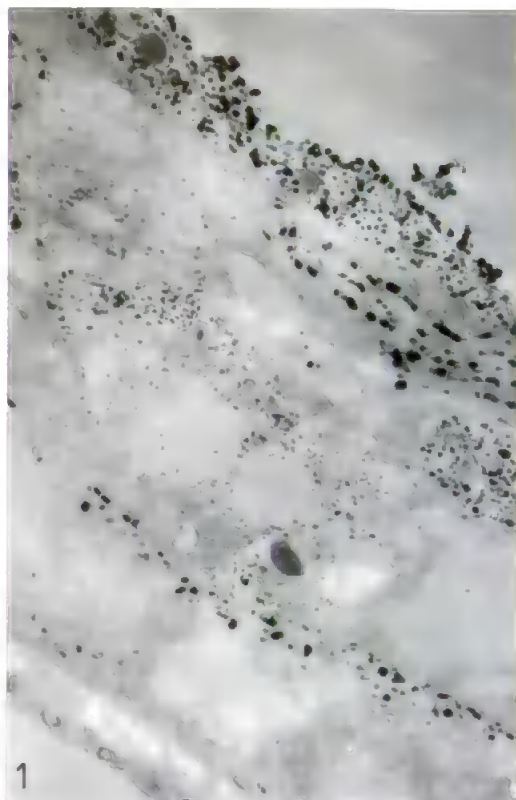


PLATE 38.

- 38-1 Distribution of reaction product for β -glucosaminidase in hexane-fixed tissue stained by a post-coupling method. The majority of reaction product occurs in the lipid vacuoles of the liver and the cyst walls are mostly negative. x 420.
- 38-2 Staining pattern obtained for β -glucosaminidase using fixed material and a post-coupling method. The reaction is intense in the liver cytoplasm but the cyst wall is negative. x 680.
- 38-3 Light micrograph showing intense staining of cyst wall for β -glucosaminidase in fixed tissue stained by a simultaneous coupling method. x 400.

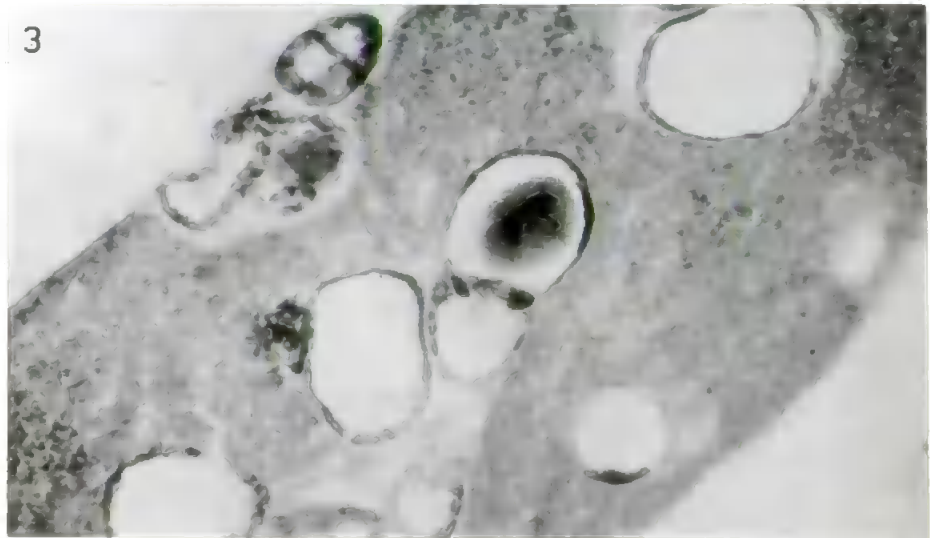
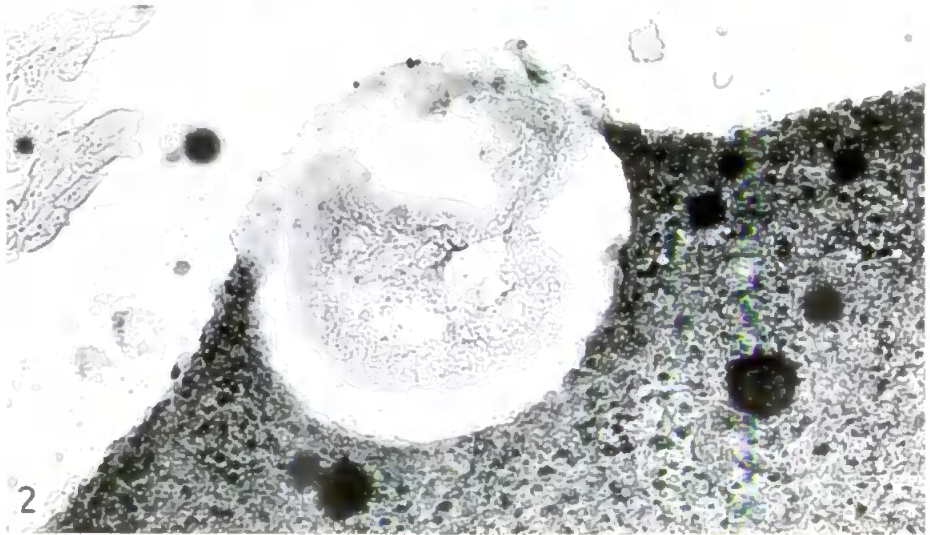
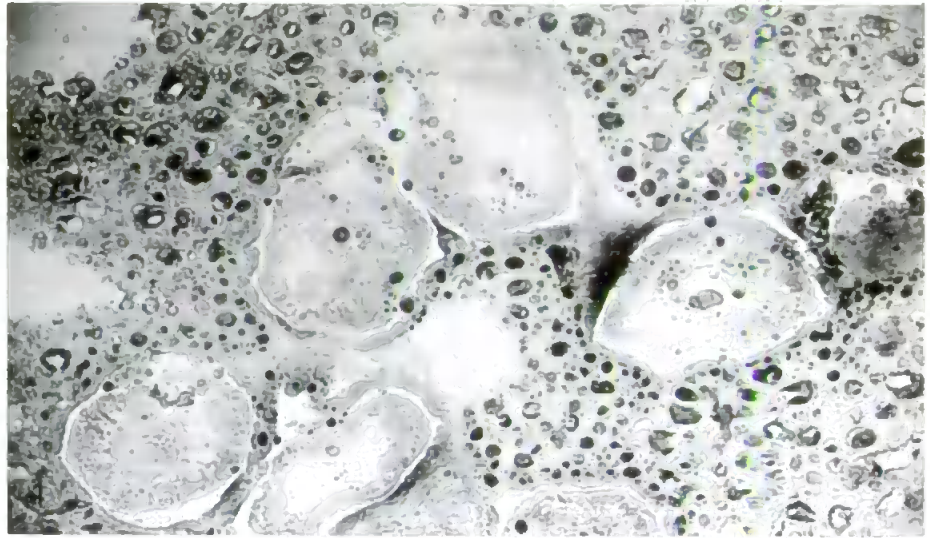


PLATE 39.

- 39-1 Electronmicrograph of section through metacercaria incubated in YTS containing 2% ferritin at room temperature. Note ferritin molecules adhering to tegument surface and in the tegument cytoplasm (arrows). Inset shows ferritin molecules on carbon-coated grid at the same magnification. x 108 500. Section mounted on a carbon-coated grid. Not stained.
- 39-2 Electronmicrograph of section through metacercaria incubated in YTS containing 2% ferritin at 37°C. Note ferritin molecules adhering to surface and within tegument cytoplasm (arrows) and the collection of molecules in the basement membrane region (crossed arrow). Inset shows ferritin molecules on a carbon-coated grid at the same magnification. x 66 500. Section mounted on a carbon-coated grid. Not stained.

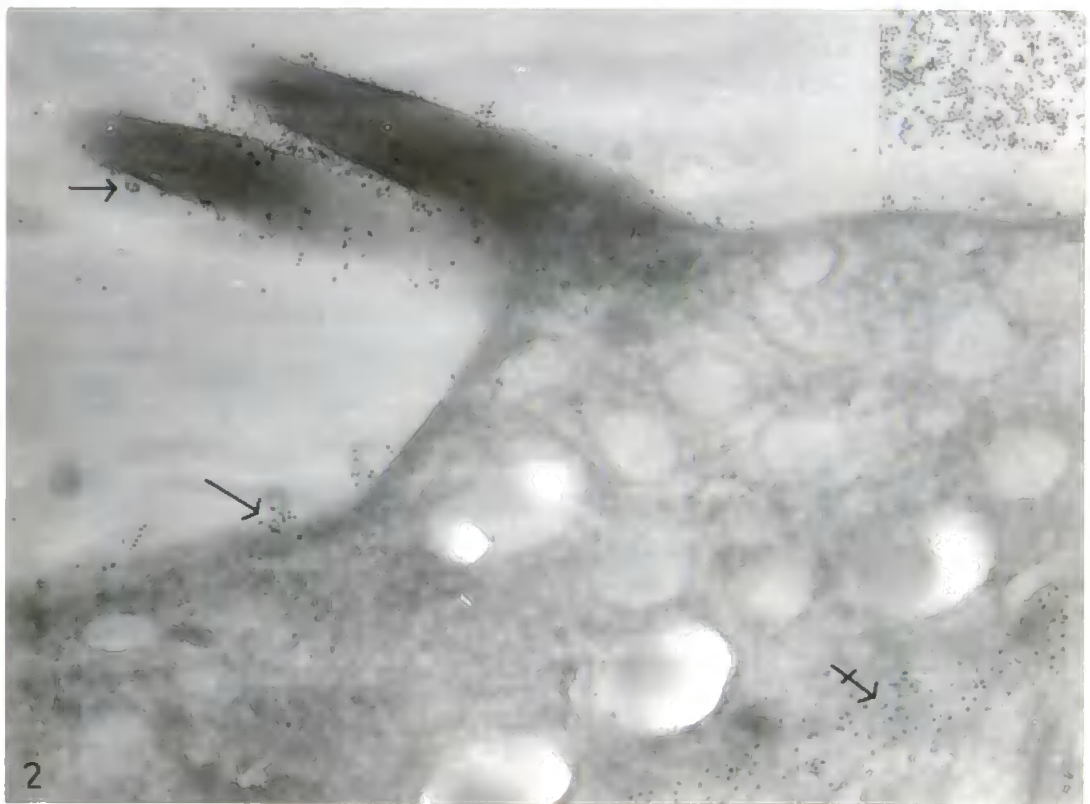
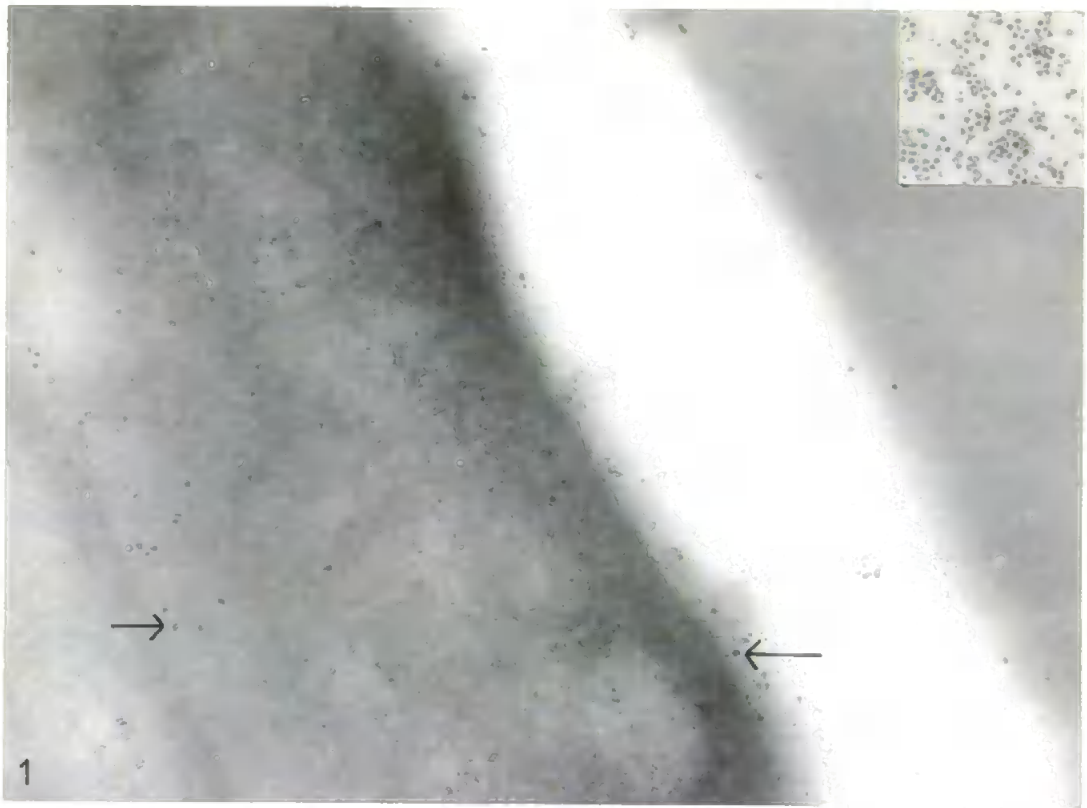


PLATE 40.

- 40-1 Electronmicrograph of metacercaria incubated in YTS containing 2% HRP0 at room temperature. Note adherence of HRP0 to tegument surface but absence in the tegument itself. x 10 800. Not stained.
- 40-2 Electronmicrograph of metacercaria incubated in YTS alone showing lack of reaction product to the DAB method for peroxidase. x 16 600. Not counter-stained.
- 40-3 Electronmicrograph of metacercaria incubated in YTS containing 2% HRP0 and treated by the DAB method for peroxidase using a medium lacking in 3,3-DAB. Note lack of electron dense reaction product. x 10 000. Not counterstained.
- 40-4 Electronmicrograph of metacercaria incubated in YTS containing 2% HRP0 and treated by the DAB method for peroxidase using a medium lacking in H_2O_2 . Note absence of electron dense reaction products. x 7 600. Not counterstained.

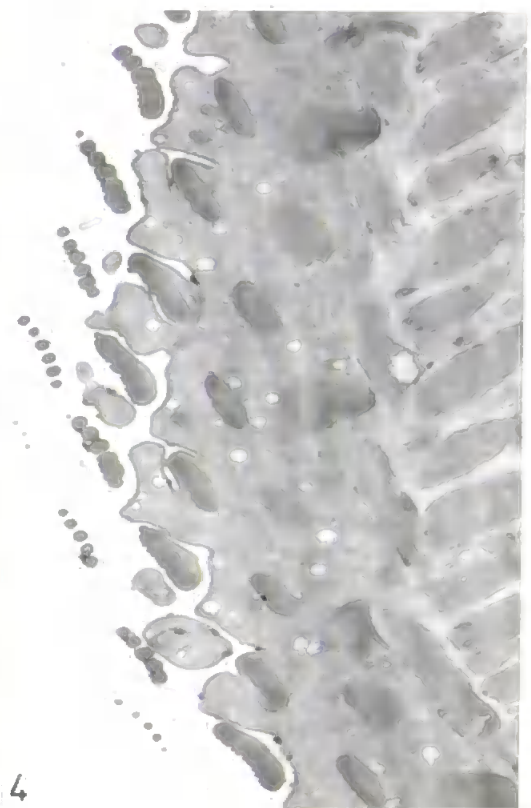
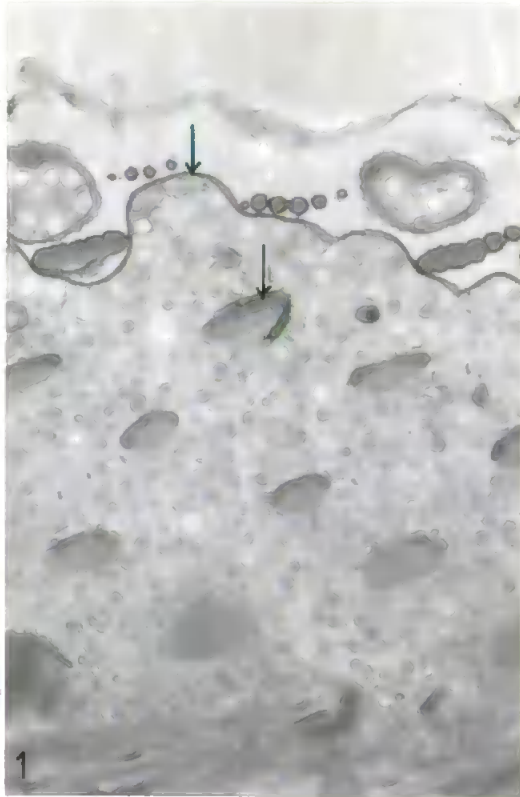


PLATE 41.

- 41-1 Electronmicrograph showing tegument of metacercaria incubated in 1% ruthenium red (RR). Note meshwork of RR over parasite surface and concentration onto outer plasma membrane. x 43 000. Not stained.
- 41-2 Electronmicrograph of section through tegument of metacercaria incubated in 1% RR. A small concentrated patch of RR is apparently being taken up by the tegument by pinocytosis. x 61 600. Stained with uranyl acetate and lead citrate.
- 41-3 Electronmicrograph of section through the basal portion of the outer tegumental layer of a parasite incubated in 1% RR. The RR is not associated with vacuoles but is free in the cytoplasm of both the outer tegumental layer and cytoplasmic bridges. x 29 100. Not stained.

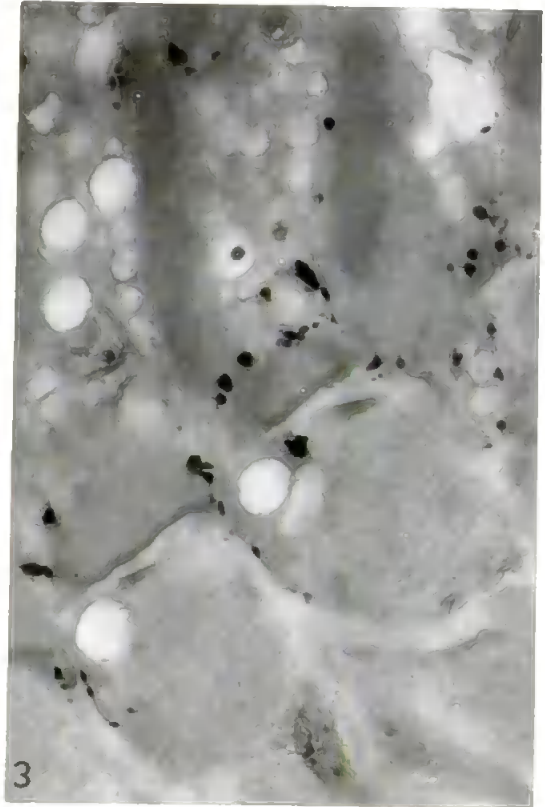
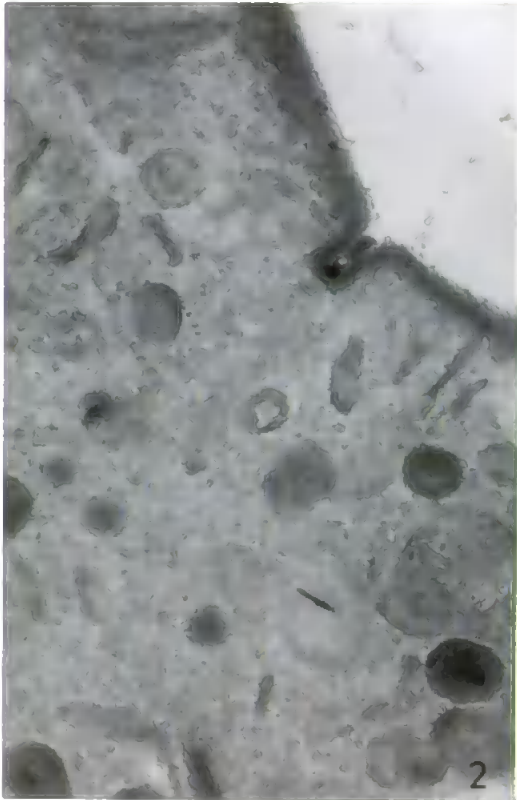
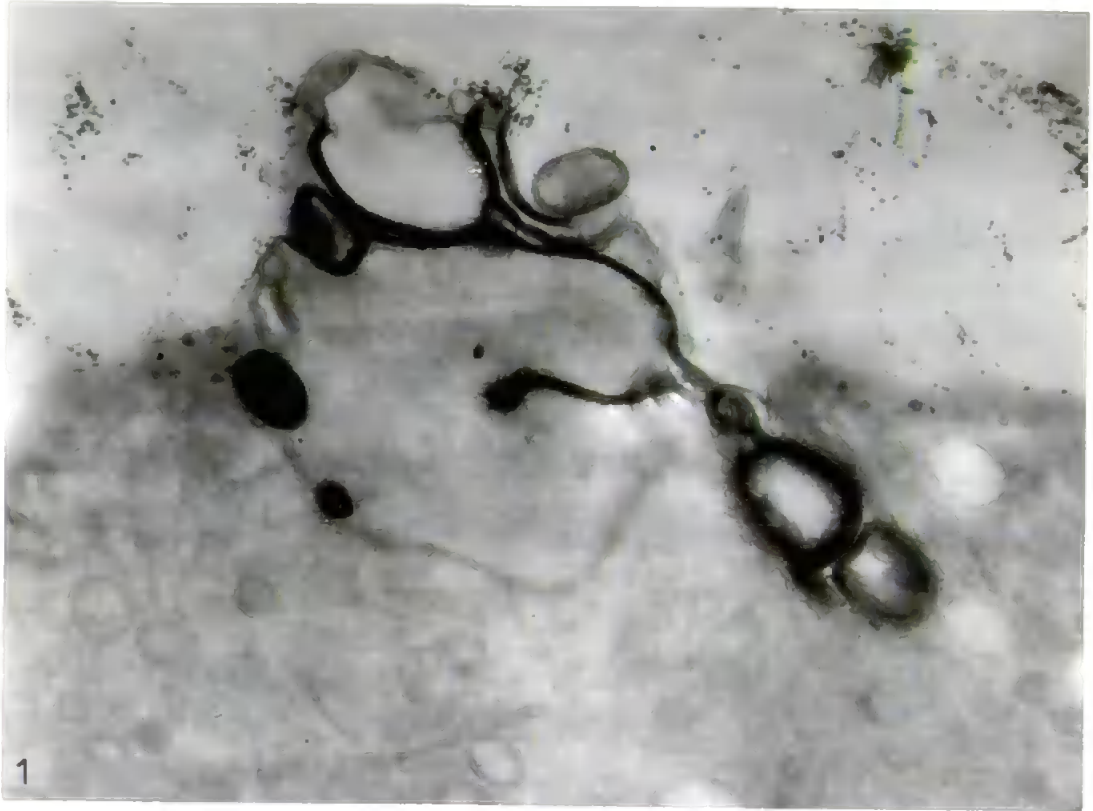


PLATE 42.

- 42-1 Electronmicrograph of tegument of a parasite incubated in RR showing small patches of concentrated RR adhering to the outer plasma membrane, especially in regions surrounding spines. Large electron light vacuoles (V.9) contain RR as small patches either adhering to the inner surface of the vacuole membrane or straddling the vacuole membrane. x 30 100. Stained with uranyl acetate and lead citrate.
- 42-2 Enlargement of part of 42-1 showing laminated appearance of RR adhering to the outer plasma membrane. In the tegument a group of small patches of RR lie freely in the cytoplasm. 79 550.
- 42-3 Autoradiograph of metacercariae incubated in iodinated (^{125}I) insulin. Note concentration of silver grains in the basement membrane region underlying the outer tegument. Small amounts of radioactivity are scattered throughout the metacercariae. x 680. Phase Contrast.
- 42-4 Autoradiograph of metacercariae incubated in iodinated (^{125}I) albumin. Note the patchiness of the concentration of radioactivity in the tegument. x 680. Phase Contrast.

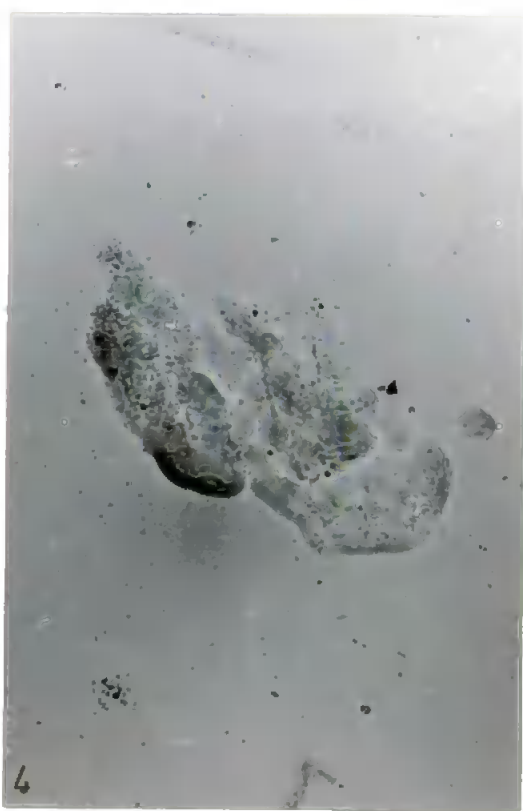
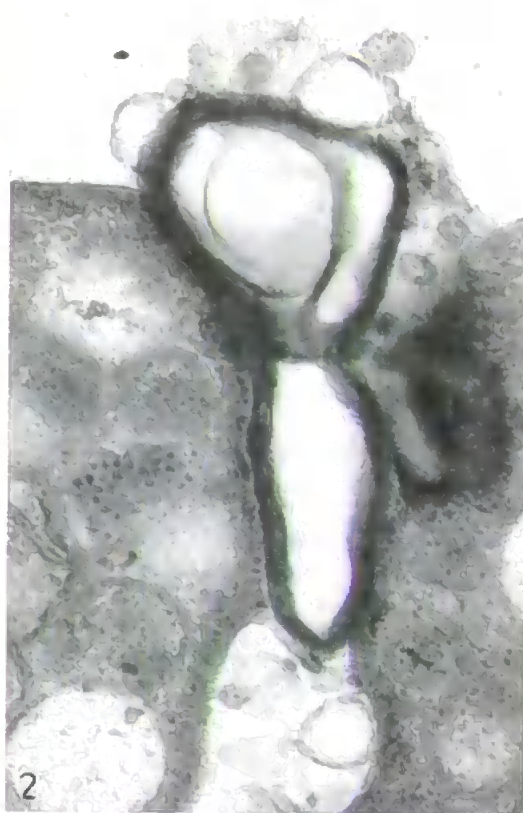
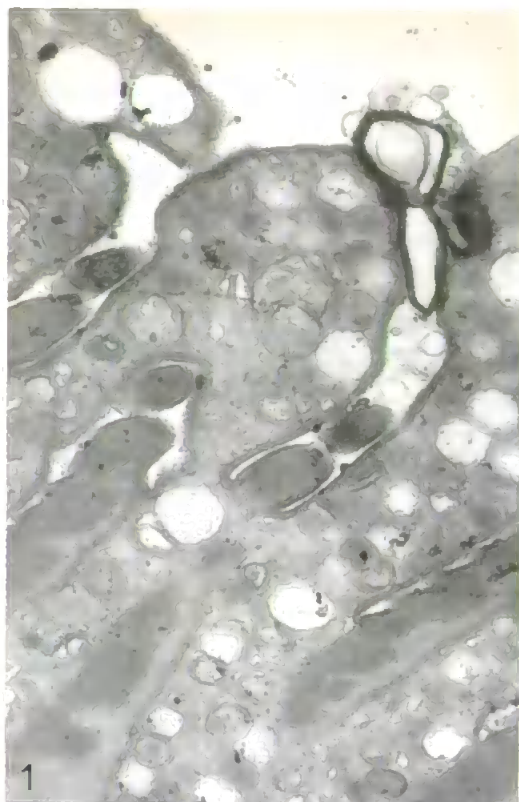


PLATE 43.

- 43-1 Autoradiograph of metacercariae incubated in ^{14}C labelled glucose. Note large concentrations of radioactivity in the tegument and anterior end of the parasites. x 650. Unstained.
- 43-2 Enlargement of central parasite in 43-1. x 1 040.



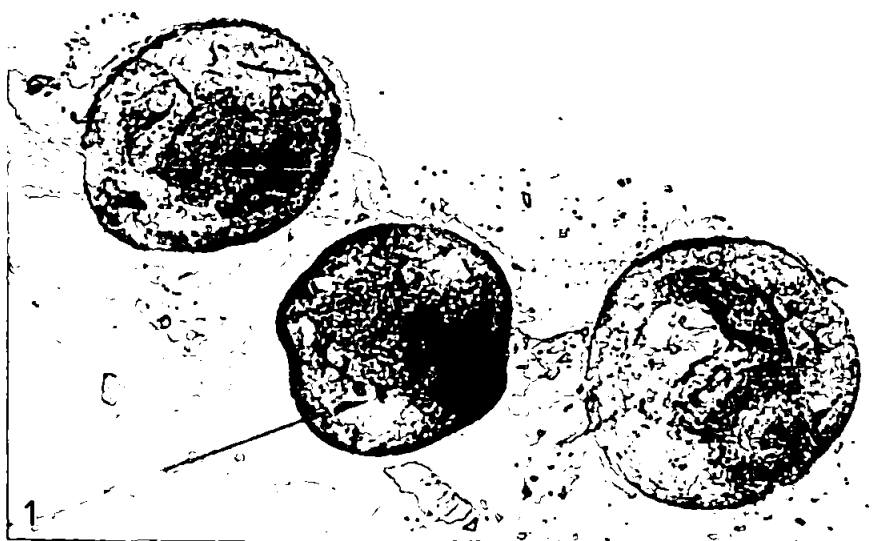


PLATE 44.

Autoradiographs of metacercariae incubated in ^{14}C labelled glucose and prepared for the electron microscope.

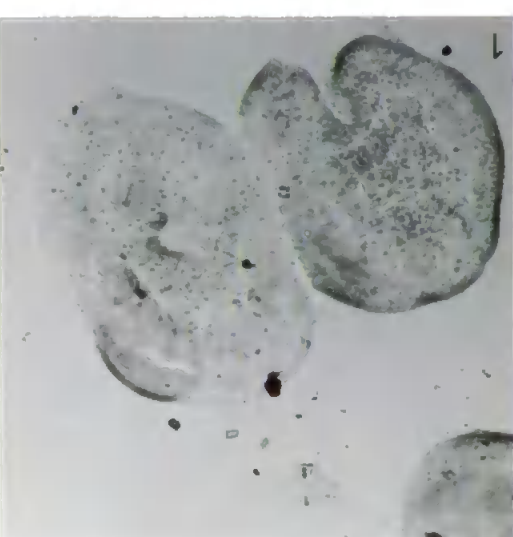
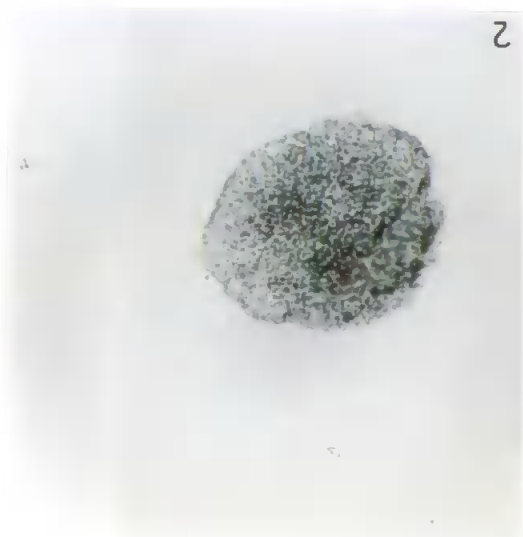
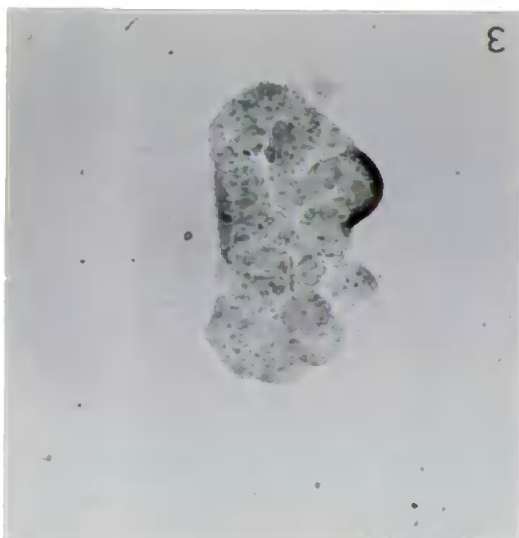
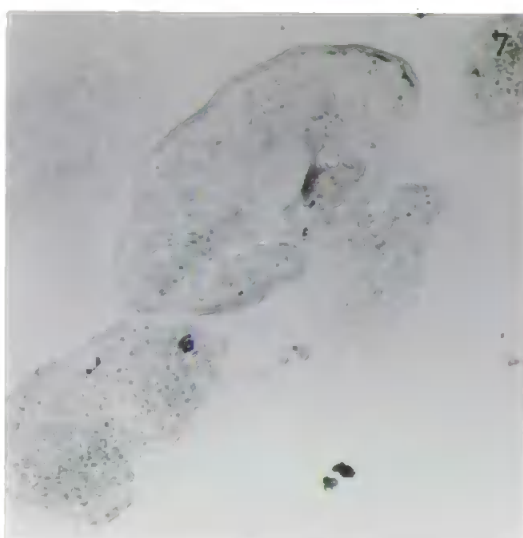
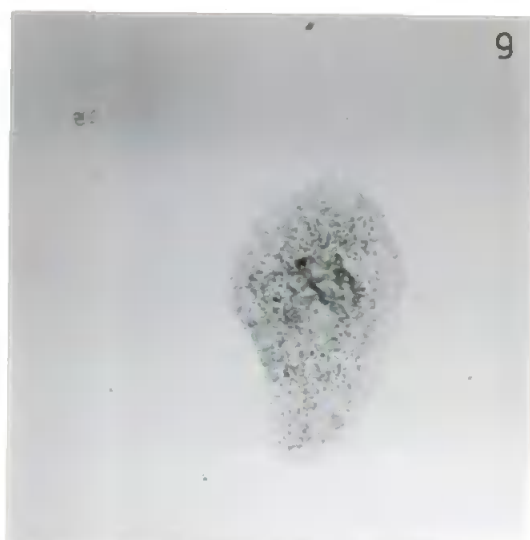
- 44-1 Electronmicrograph showing radioactive trace in the outer tegumental layer. x 14 600.
- 44-2 Electronmicrograph showing radioactivity in the muscle layers underlying the outer tegumental layer. x 15 600.
- 44-3 Electronmicrograph showing large amounts of radioactivity associated with the anterior gland cells of the metacercaria. Note the majority of traces occur over secretory cell bodies. x 10 800.



PLATE 45.

Autoradiographs showing distribution of radioactivity in metacercariae incubated in various labelled amino acids.

- 45-1 L-3-Phenylalanine - $^{14}\text{C}(\text{U})$. $\frac{1}{2}$ h incubation. Note possible concentration of activity in gastrodermis. x 700.
- 45-2 L-3-Phenylalanine - $^{14}\text{C}(\text{U})$. 2 h incubation. x 700.
- 45-3 L-Tryptophan (methylene - ^{14}C). $\frac{1}{2}$ h incubation. Note location of radioactivity in only part of tegument. x 700.
- 45-4 L-Tryptophan (methylene - ^{14}C). 2 h incubation. Note concentration of activity in gastrodermis. x 700.
- 45-5 L-Tyrosine $^{14}\text{C}(\text{U})$. $\frac{1}{2}$ h incubation. Note location of radioactivity in only part of tegument. x 620.
- 45-6 L-Tyrosine $^{14}\text{C}(\text{U})$. 2 h incubation. x 700.



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