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ULTRASTRUCTURE OF POST-ONCOSPHERAL STAGES OF TAENIA TAENIAEFORMIS AND UPTAKE OF FERRITIN BY METACESTODES OF TAENIA TAENIAEFORMIS

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JOHN A. PICONE

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J. F. Williams

Major professor

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ULTRASTRUCTURE OF POST-ONCOSPHERAL STAGES OF *TAENIA TAENIAEFORMIS* AND UPTAKE OF FERRITIN BY METACESTODES OF *TAENIA TAENIAEFORMIS*

By

John A. Picone

AN ABSTRACT

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ABSTRACT

ULTRASTRUCTURE OF POST-ONCOSPHERAL STAGES OF *TAENIA TAENIAEFORMIS* AND UPTAKE OF FERRITIN BY METACESTODES OF *TAENIA TAENIAEFORMIS*

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An electron microscopic study was made of the sequence of post-oncospheral reorganization changes which occur during the first 10 days of growth of *T. taeniaeformis* in the rat liver. Viable organisms were liberated from host tissues by tryptic digestion. Observations were made on the rate of growth, the process of vesiculation and the development of the tegument and subtegumental tissues. Characteristics of the reorganizational phase included the rapid investment of the entire parasite surface with microvilli, the accumulation of fluid-filled spaces which coalesced to form the central bladder by 4 days, the peripheral migration of cell nuclei and the establishment, by 10 days of age, of a tegumental and subtegumental architecture which was entirely comparable to that previously described in much older metacestodes. Certain changes are discussed in terms of the importance of this early phase of parasite growth in the immunology of the host-parasite relationship.

Metacestodes of *Taenia taeniaeformis* were studied after exposure to ferritin conjugated IgA_{2a} *in vitro*. Uptake occurred by both 10-day-old and 50-day-old live parasites and was inhibited by prior exposure to EDTA. No evidence of adherence or uptake of the conjugated-ferritin was seen in control preparations of formalin-fixed parasites, and unconjugated ferritin was not taken up by live parasites.

Ferritin particles were detected in and on microtriches and within vesicles in the submicrothrix zone. These membrane-bound vesicles were conncected to the external surface in some cases, and the uptake process appeared to resemble pinocytosis in mammalian cells. This may offer an explanation for the incorporation of macromolecules into the bladder fluids in *T. taeniaeformis*.

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I would like to express my appreciation to all those who have been a part of this laboratory. The friendships which were made during the last five years have been invaluable to me in growing as a scientist as well as a person. Special thanks go to Alma M. Shearer for her expert technical assistance.

Lastly, I would like to thank Doctors John Breznak, Harold Miller and Donald Twohy for their advice and support.

DEDICATION

This thesis is dedicated to my parents who have both been a great inspiration to me.

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INTRODUCTION

Cysticercosis and hydatidosis are serious diseases which undermine human and animal health and pose economic problems throughout the world. These infections are caused by the intermediate (metacestode) stages of Taeniid tapeworms which migrate to a number of tissues and organs in suitable vertebrate host species. However, there is little information to date on the early developmental stages of taeniid metacestodes in tissues, despite the fact that this phase in the life cycle is extremely important in the host-parasite relationship. It is during this period that the developing larvae must become resistant to the immune response of the host.

This study was therefore undertaken in an effort to begin to correlate significant changes in the structure of the developing post-embryonic forms of *Taenia taeniaeformis*, with the changing susceptibility of these organisms to host defense mechanisms. The literature review deals with the basic biology and physiology of cestode larvae, the present status of knowledge of cestode morphogenesis and ultrastructure and some aspects of host responsiveness.

LITERATURE REVIEW

Biology of Cestodes

Developmental Aspects of Tapeworm Life Cycles

Tapeworms are metazoan parasites classified in the phylum, Platyhelminthes, class, Cestoda. Adults inhabit the intestinal tract or associated organs of vertebrates and the larval stages are distributed in a variety of invertebrate and vertebrate host tissues.

Although the pattern of many cestode life cycles has been established, most experimental research has centered on the orders Pseudophyllidea and Cyclophyllidea due to their ready availability and medical or veterinary importance. The Pseudophyllideans, with few exceptions are transmitted through two or more intermediate hosts while the Cyclophyllideans pass through only one intermediate host. Post-embryonic reorganization therefore occurs under very different circumstances in these two orders. It will be useful at this point to compare the developmental aspects of each order since ultrastructural observations on the host parasite relationship have been made on organisms in both groups and are referred to later in this review.

A representative life cycle of parasites in the Pseudophyllidea is shown in Figure 1. Eggs from an infected individual are passed in feces and after embryonation in fresh water they hatch to release a free swimming coracidium. This stage must be ingested by an invertebrate, usually a copepod, in which the ciliated layer is lost. The active hexacanth

PSEUDOPHYLLIDEAN

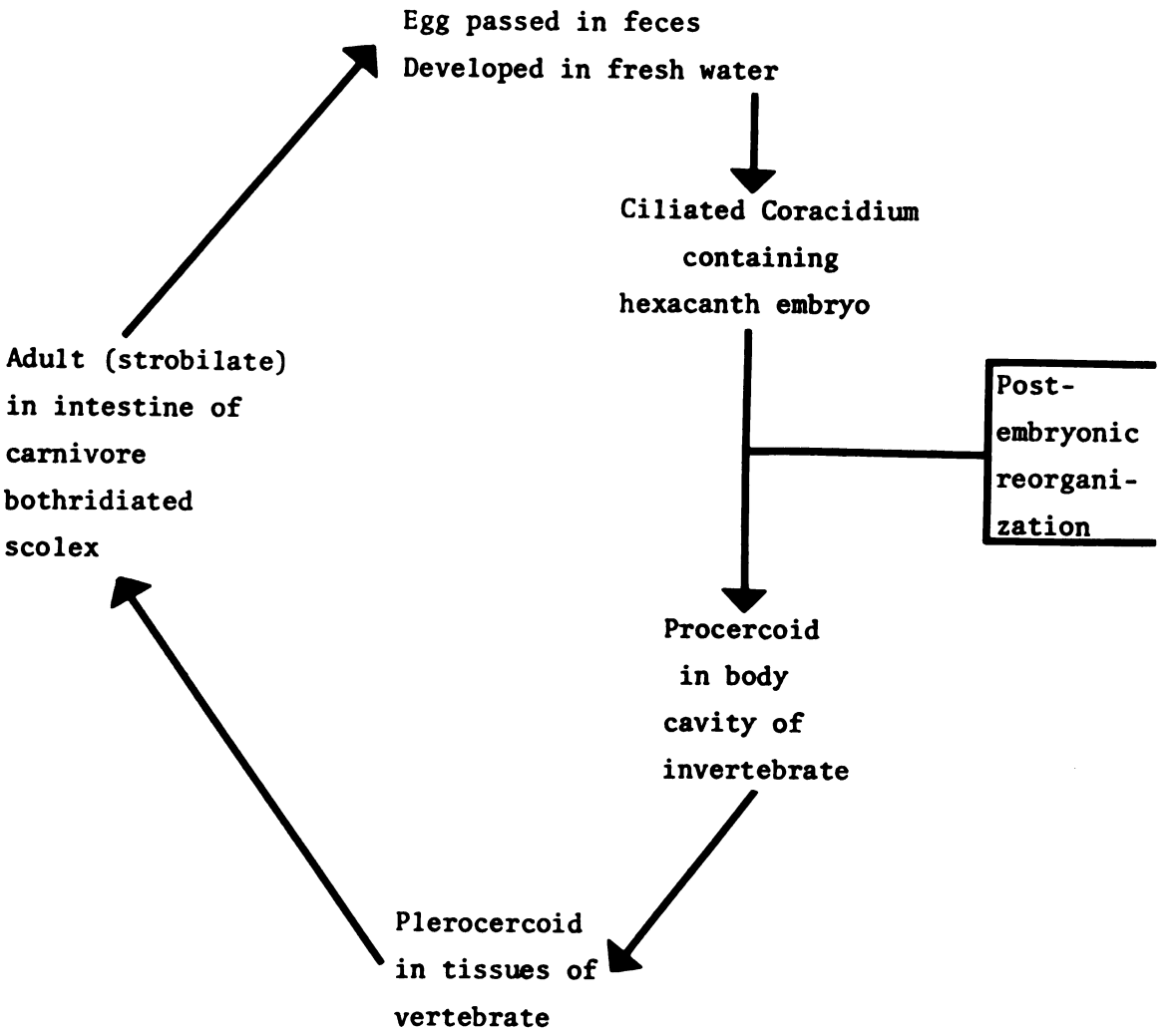


Figure 1.

(6 hooked) "oncosphere" migrates into the body cavity and undergoes post-embryonic reorganization into an elongated procercoid stages. When the infected copepod is eaten by a vertebrate the procercoid penetrates the intestinal wall and migrates to the muscles when development proceeds to a pleroceroid stage. Ingestion of infected tissues by carnivores liberates the plerocercoid which matures in the intestine by strobilization (segmentation). *Diphyllobothrium latum* and *Spirometra mansonoides* are medically important cestodes typical of this order.

In contrast to the Pseudophyllideans where ova must be deposited in water, transimission of Cyclophyllidean cestodes occurs in a terrestrial environment. A typical life cycle is depicted in Figure 2. Eggs of the medically important family, Taeniidae, hatch in the intestine of vertebrates and the hexacanth embryos are activated by poorly defined factors in the intestinal mileu. Activated oncospheres penetrate the intestinal wall and are carried hematogenously to a variety of tissues where they undergo post-embryonic reorganization into fluid filled cystic forms which may be single (cysticercus, strobilocercus) or which may undergo polyembrony resulting in multiple scolices (coenurus, hydatid cyst). In other medically important cyclophyllidean families embryonic reorganization occurs in invertebrate intermediate hosts and results in formation of a non-vesicular cysticercoid.

Consumption of infected tissues by carnivorous vertebrates results in liberation of larvae which attach to the intestinal mucosa and mature by strobilization.

CYCLOPHYLLIDEAN

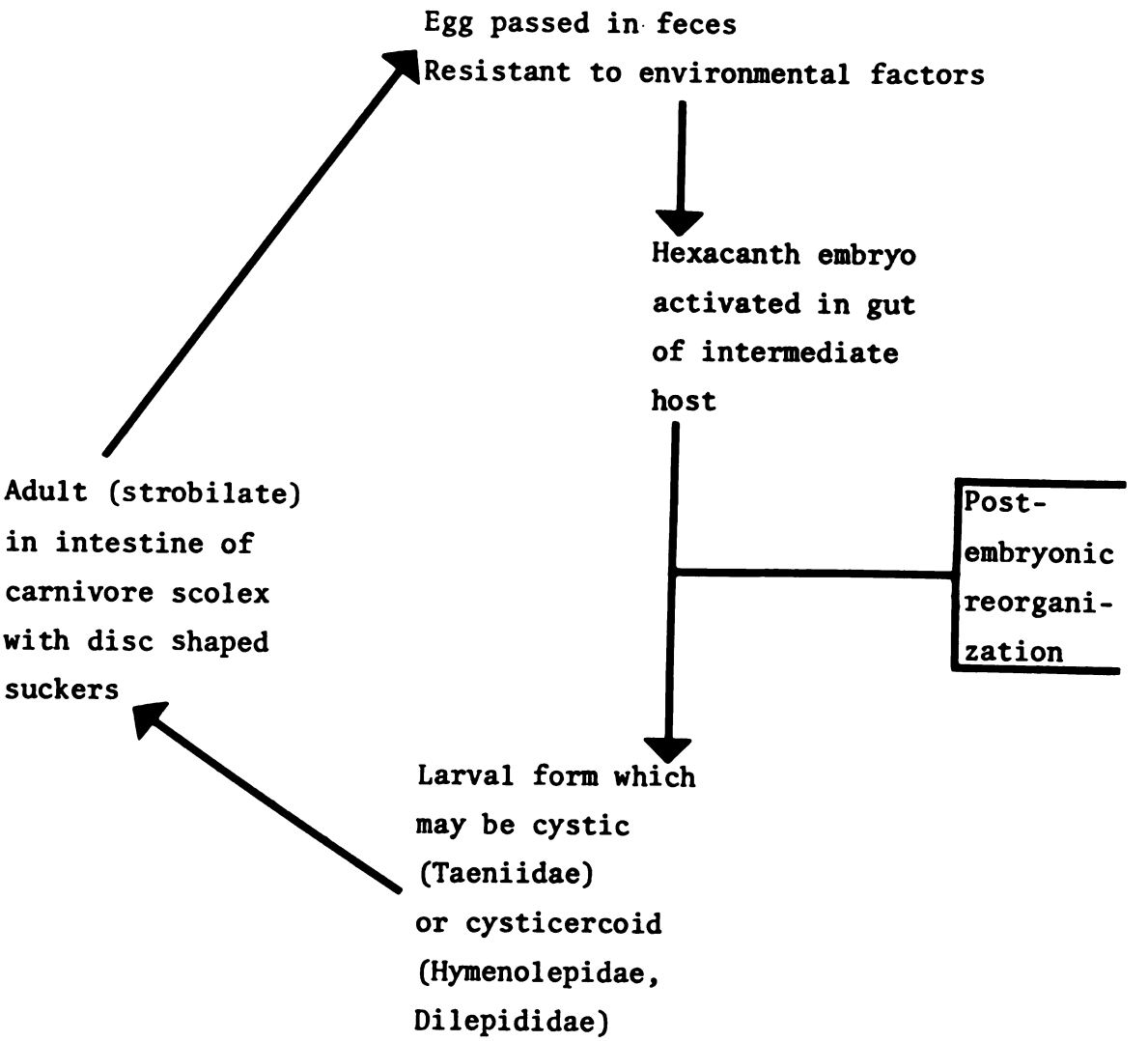


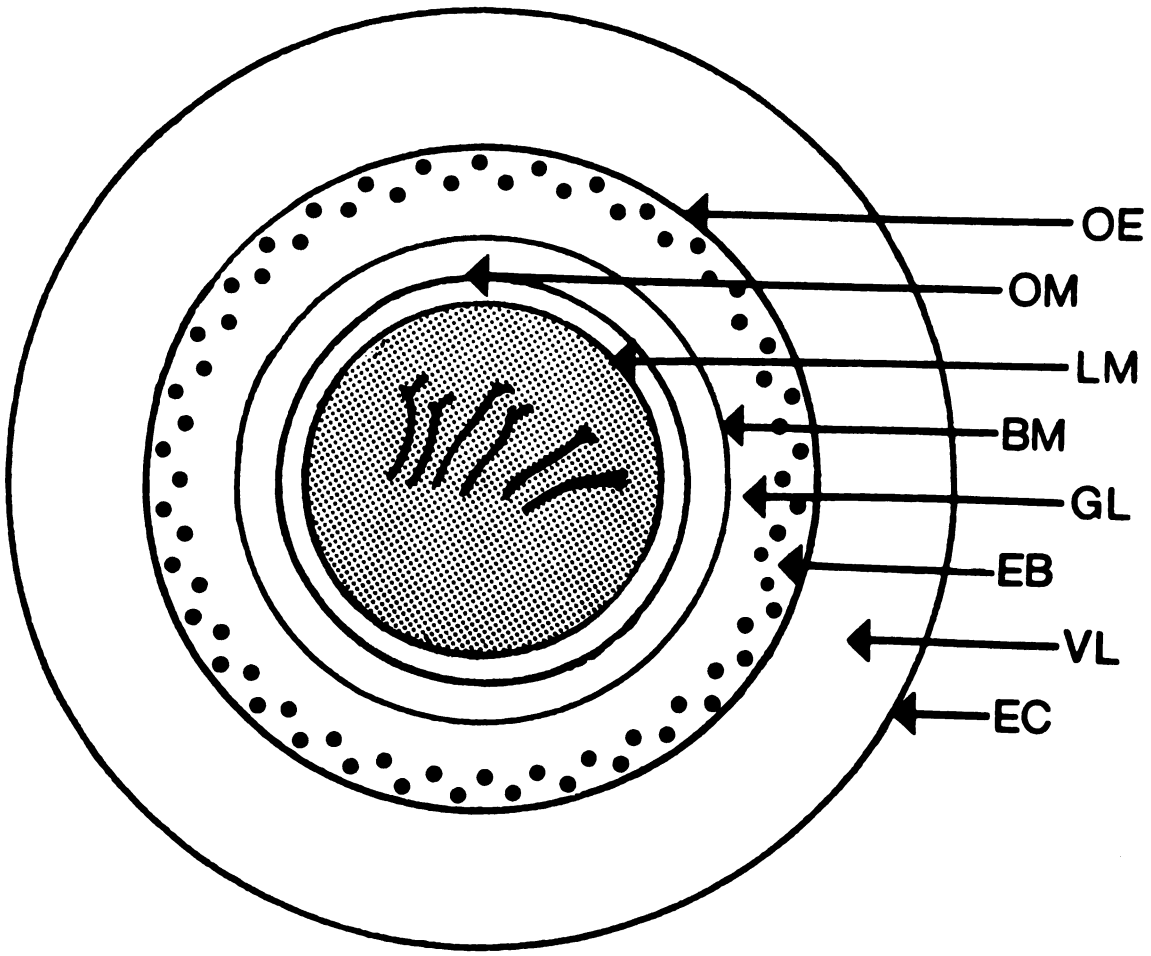
Figure 2.

Formation and Ultrastructure of Eggs

Pseudophyllidean eggs consist of an outer operculated envelope and inner envelope which encloses the hexacanth embryo. However, fully developed Cyclophyllidean eggs are surrounded by a series of protective membranes which confer resistance to chemical and physical agents (Mackie and Parnell, 1967). Embryogenesis and egg formation in the family, Taeniidae, have been thoroughly investigated and since this family includes *Taenia taeniaeformis*, the experimental model used in our laboratory, the characteristics of those processes will be discussed in some detail.

Electron microscopic observations by Morseth (1965) have revealed eight distinct embryonic membranes and layers surrounding taeniid oncospheres. These are depicted diagrammatically in Figure 3. The capsule is the outermost protective layer and is believed to be derived from vitelline cell products (Ogren, 1959). Beneath the capsule is the vitelline layer which contains yolk cells during egg development but these cells usually disintegrate to form a gelatinous layer at maturity (Smyth, 1963). Suspended in this viscous mass is the embryophore delineated by the outer embryophoric membrane described by Inatomi (1962). This contains radially oriented blocks of a keratinaceous material held together by a cement substance (Morseth, 1965). It is this arrangement which is believed to account for the impermeability of the embryophore and its resistance to adverse environments. Light microscopic studies indicate that the blocks are synthesized in the "germinal layer" (Lee *et al.* 1959) which Silverman (1954) and Inatomi (1962) had referred to as the "basement membrane." In electron microscopic studies Morseth

Figure 3.



OE—OUTER EMBRYOPHORIC MEMBRANE
OM—ONCOSPHERAL MEMBRANE
LM—LIMITING MEMBRANE
BM—BASAL MEMBRANE
GL—GRANULAR LAYER
EB—EMBRYOPHORIC BLOCKS
VL—VITELLINE LAYER
EC—EGG CAPSULE

(1965) characterized the oncospheral membrane separate from a limiting membrane around the oncosphere itself, whereas this distinction had not been made in previous light microscopic studies.

In an ultrastructural study of the egg of *T. taeniaeformis* Neiland (1968) correlated his findings on membrane structure and origin with those of Inatomi (1962) and Morseth (1965). Neiland (1968) implied that the "large cellular structure" in the granular layer described by Morseth (1965) might represent nuclei and nucleoli of the embryophore cells. He felt that the "circular bodies" described by Morseth (1965) were really mitochondria and that they were unlikely to be focal points of cement substance deposition as proposed by Morseth. Neiland suggested that the embryophore cell, which is rich in granules resembling ribosomes, is the most probable site of synthesis of blocks and cement substance and also considered that this cell might be responsible for synthesis of the oncospheral membrane.

Ultrastructure and Physiology of Metacestodes

Ultrastructural Characteristics

Most of the ultrastructural observations on metacestodes (tissue dwelling larval forms) have been carried out on mature Cyclophyllidean larvae, particularly on members of the family Taeniidae. It has become clear that the former belief that these parasites possess a "cuticle" or inert covering is no longer tenable. Both larval and adult cestodes have developed a mode of existence whereby there is an interchange of nutrients and excretions through a metabolically active integument which is somewhat analagous both structurally and functionally to the

developing mammalian trophoblast and to the epithelial cells of neonatal mammalian intestine. This interface between host and parasite has begun to attract the attention of physiologists and immunologists because of its pivotal role in survival of the organism.

In mature (i.e. infective) taeniid metacestodes the tegument and subtegumental cells surround a space filled with fluid rich in proteins, some of which are identical to those in host plasma. The ultrastructural features of these forms must be reviewed before discussion of the limited information on the post-oncospheral reorganization processes whereby the microscopic (15-20 μ in diameter) hexacanth embryo develops into a mature taeniid bladderworm.

Smyth (1969) considered that the tegument of larval and adult tapeworms could be divided into two distinct regions; the distal portion and the perinuclear cytoplasm. The free surface of the distal cytoplasm is modified to form multiple tegumental projections called microtriches. These are analagous to the microvilli of mammalian cells but they bear an electron dense tip. Although microtriches have been identified in all ultrastructural studies on cestodes, they are extremely variable in size and shape. For example, Bortoletti and Ferretti (1971) note that the microtriches of strobilocerci of *T. taeniaeformis* are thinner and longer than those of the adult form. In *Multiceps serialis* microtriches are circular in transverse section (Race *et al.* 1965) whereas those in *Echinococcus granulosus* are rhomboid shaped (Jha and Smyth, 1969). Microtriches are bounded by a "double" membrane which is continuous with the limiting membrane of the distal cytoplasm. (Jha and Smyth, 1969) (Race *et al.*). Ultrastructural studies on *E. granulosus* have revealed an inner and outer membrane system in both longitudinal and transverse

sections with discontinuities in the outer plasma membrane and the presence of microtubules in the core of the shaft. The microtubules run parallel to the longitudinal axis of the microthrix and seem to be connected by means of electron dense septa (Jha and Smyth, 1969).

Distributed intermittently between microtriches on the tegumental surface are sensory receptors (Morseth, 1967). These have now been reported in a variety of cestodes (Lee *et al.* 1963). Electron microscopic studies have shown that the sensory endings are bulb-like with elongated distal processes projecting beyond the tegumental surface (Morseth, 1967).

Below the microtriches and sensory endings lies a vacuolated zone of syncytial cytoplasm which contains numerous vacuoles or rhabdiform organelles, each bounded by a double membrane. Mitochondria have been demonstrated within the proximal portion of this zone in *M. serialis* larvae (Race *et al.* 1965), but Lascano *et al.* (1975) found no mitochondria in the vacuolated area of mature cysts of *E. granulosus*.

A basal membrane separates the distal cytoplasm from the perinuclear cytoplasm. Race *et al.* (1965) referred to the perinuclear region as the "parenchyma." The internal plasma membrane forms numerous folds and is common to all tegumental cells in the parenchymal regions. The basal membrane lies above the muscles and is a discontinuous layer through which tegumental cell cytoplasmic bridges pass. Bortoletti and Ferretti (1971) described the basal membrane as an electron transparent area with a few fiber-like structures in 14 day-old larvae of *T. taeniaeformis* but found that these fibers became numerous as larvae matured. Below this membrane they described circular and longitudinal muscle bands

composed of distinct myofibrils, of variable electron density. A comparable muscle layer has been described in other cestode larvae (Collin, 1970; Bortoletti and Ferretti, 1971).

The nucleated portion of the tegumental cells lies below the muscle layers. Numerous cytoplasmic bridges, delimited by a plasma membrane and connecting to the upper layer of the syncytium have been observed in *M. serialis* (Race *et al.* 1965), *T. taeniaeformis* (Bortoletti and Ferretti, 1971) and *E. granulosus* (Lascano *et al.* 1975). Tegumental cell nuclei are surrounded by a small portion of cytoplasm which contains vacuoles, mitochondria, golgi apparatus, and alpha and beta glycogen particles. According to Bortoletti and Ferretti (1971) glycogen is present only in this zone.

Flame cells are distributed in the tegumental cell region. Race *et al.* (1965) described a hexagonal arrangement of cilia in the flame cells, which also contained cellular organelles such as mitochondria, and vacuoles. Flame cells probably function in excretion and in maintenance of water balance (Race *et al.* 1965).

Histochemistry

Early histochemical studies on adult cestodes indicated that the proportions of carbohydrate, lipids and proteins differed from those of most other invertebrate tissues in that there is a high concentration of carbohydrate and a relatively low protein content (Smyth, 1969). In more recent work the distribution of these elements in tissue sections has been studied in some detail especially by Lumsden and his collaborators. Few observations have been made on larval forms, but a review

of histochemical characteristics of parasitic invertebrates has recently been prepared by Lumsden (1975).

Lumsden (1966b) demonstrated that the tegumental matrix of adults of *Hymenolepis diminuta* was largely protein. Radioactive amino acids were incorporated into the subtegumentary cells and subsequently appeared in the submicrothrix layer of the tegument. As a result of this study Lumsden (1966b) suggested that the enzymes and proteins of the tegument are synthesized in the parenchymal cells. He also demonstrated the presence of some non-glycogen polysaccharides which may have been mucopolysaccharides or oligosaccharides conjugated to polypeptides as glycoproteins in the tegument.

A glycocalyx, evidently firmly attached to the plasma membranes covered the surface of several cestodes studied histochemically by Lumsden. In addition the tegument was shown to bear a high density of fixed anions, which he suspected to be acidic glycans, although evidence for the occurrence of sulfated acid mucopolysaccharides has been obtained by Morris and Finnegan (1968) in *Schistocephalus*. Colloidal iron was shown to bind to the surface of adults of *H. diminuta* and this phenomenon was not inhibited by exposure of the parasite to phospholipase, acid or alkaline phosphatase, or ribonuclease (Oaks, 1970). Oaks and Lumsden (1971) incubated adult *H. diminuta* with H^3 labelled hexoses and showed that they also appear in a protein associated with the tegument membrane, and that this material was turned over with a half life of 6 hours, at least *in vitro*.

In one of the few histochemical studies on taeniid metacestodes, Shield *et al.* (1973) demonstrated that the cytoplasm of *T. pisiformis*

contained glycogen, protein, acid mucopolysaccharide and phospholipid. The distribution and location of these coincided with observations of Lumsden (1966b). For example, Shield *et al.* (1973) demonstrated that secretory cells in the subtegument contain strongly acidic mucopolysaccharide, neutral mucopolysaccharide, phospholipid and some alkaline phosphatase.

Waitz (1963) is the only worker who has applied histochemistry to *T. taeniaeformis*. PAS-positive mucosubstances were present in the tegument and subtegumental cells of adults and larvae of *T. taeniaeformis*. The reaction was stronger in the outermost region of the tegument of adults but was uniformly intense in larvae. Both larvae and adults also contained sudan-positive lipid material in the tegument and subtegumental cells (Waitz, 1963). The parenchyma of *T. taeniaeformis* seemed to be the principle storage area for glycogen in both adults and larvae. However, it was shown that parenchymal cells of strobilocerci contained less glycogen than adult worms.

Physiological Characteristics

The physiological significance of microtriches remains controversial, though it seems likely that they serve to augment the surface area available for membrane functions. Beguin (1966) was the first to develop the striking analogy between the intestinal cells and the cestode tegument but although this comparison was based on similarities in structure the physiological basis of the analogy is still incomplete. Some light was shed on adult cestode microthrix function by Rothman (1966) who was able to demonstrate the presence of alkaline phosphatases in the proximal region of microtriches and cholinesterases in the distal

region in adult *Hymenolepis citelli*. He suggested that microtriches provided an extensive surface for membrane associated enzymatic digestion and implied that this function may also be served by microtriches of larval forms. Read (1973) showed that exogenous enzymes could adhere to the surface of the adult cestode tegument and function in contact digestion. Similar processes may occur in larvae and the tegument may provide an increased surface area on which adsorption takes place.

Although Lumsden (1966a) and Jha and Smyth (1969) described vacuoles which may have been indicative of pinocytotic activity at the base of the microthrix layer, no evidence of functional pinocytosis has since been obtained. The origin of host-like proteins within the bladder fluids therefore remains in dispute although the recent demonstration of transport of structurally and functionally intact radio-labelled macromolecules into the bladder fluids of taeniid metacestodes suggests that further work on pinocytosis as a transport mechanism should be pursued (Hustead and Williams, 1977).

A number of workers have studied uptake of small molecules by metacestodes (Pappas and Read, 1973) and presently three uptake mechanisms have been recognized. Some substances evidently enter by a process of simple diffusion which requires no energy expenditure on the part of the organism (Pappas and Read, 1973, 1975). Secondly a transport system has been observed which is similar to diffusion but is stereospecific (Pappas and Read, 1975). Chemicals which are analagous in structure can disrupt or inhibit this process. Finally energy

dependent active transport has been detected against concentration gradients (Pappas and Read, 1975)

Post-oncospheral Stages

The physiology and ultrastructure of the post-oncospheral reorganization phase have received much less attention than the mature metacestode because of difficulties imposed by the microscopic size of the organism at this time. However, activation and migration of the oncosphere is followed by a period of rapid development which represents a crucial stage in the life cycle of many cestodes. In members of the family Taeniidae it has been shown to be very important immunologically because during this phase the protective immune response is stimulated yet the developing larvae are for a short time highly susceptible to immune attack (Campbell, 1938a; and Musoka and Williams, 1975). Reorganization therefore involves the acquisition of resistance to the immune mechanisms of the host.

If the immunologic aspects of interaction between host and parasite are to be clearly understood the structure and development of the early larval stages must be fully characterized. This has not been done at the ultrastructural level for any of the taeniid tapeworms and information is available only from light microscopic studies. However, observations using electron microscopy have been reported for other cestodes and since certain analogies can be drawn these results will be reviewed and compared with current information for the taeniids.

In published descriptions of cestode larvae and post-oncospheral stages by Neiland (1968), and Collin (1970) some similarities in surface

structure have been seen in adult and larval stages. Neiland (1968) described the ultrastructure of activated oncospheres of *T. taeniaeformis* hatched from eggs *in vitro* in artificial intestinal medium. He observed "cytoplasma folds" at the limiting membrane of the oncosphere which may unfold after emergence from the oncosphere, possibly giving rise to a microthrix layer comparable to that on the mature larva. Collin (1970) also identified "cytoplasmic extensions" covering the activated oncosphere of *H. citelli*. He concluded that they were fewer, longer and more randomly distributed than structures on the surface of later post-oncospheral stages which were more like microvilli than microtriches, since they lacked an electron-dense tip. Race *et al.* (1965) and Bortoletti *et al.* (1971) have reported that microvilli were present on the surface of tissue dwelling larval forms but their origin was not studied.

Lumsden, *et al.* (1974), in their studies on the post-oncospheral development of *S. mansonoides*, proposed a mechanism for microthrix development in both larval and adult cestodes. They observed membrane limited granules within the tegumental surface cytoplasm and showed that the matrix of the granules resembled the electron-dense material of microtriches. These were considered to move peripherally and emerge as microthrix tips. This hypothesis was supported by Mount (1970) who demonstrated that the electron-dense rostellar hooks of *Taenia crassiceps* originated from specialized microtriches and subtegumentary granules of a similar material were seen during development.

Collin (1970) identified several cell types, muscle cells and oncospheral hooks below the microthrix zone in early stages of

post-embryonic development in *H. citelli*. Cysticercoids at 3 and 5 days of age were studied in an effort to establish a pattern of morphogenesis which would be applicable to other cestodes. The cell types of 3-day-old cysticercoids were relatively undifferentiated. Most of the cells possessed "electron-opaque nucleoli," electron-pale nuclei and scant cytoplasm filled with free ribosomes. Peripheral muscle fibers were still present at this stage but the muscle system was less extensive than in the earlier oncospheral stage (Collin, 1968). After 5 days the system of muscle fibers had expanded and newly formed circular muscles were evident. In 5-day-old larvae 4 different cell types were distinguished by Collin: an anterior prescolex region of cells with electron pale nuclei which he believed were descendants of the 3-day undifferentiated cestode cell-type; interstitial cells which contained large nuclei and plentiful cytoplasm with differentiated organelles (this cell-type tended to form cytoplasmic extensions which filled the spaces between adjacent cells); myofibers which were typical of those in adult cestode muscles (Lumsden and Byram, 1967); and cells lining the central cavity which were rich in glycogen granules in the alpha form.

In recent years efforts have been made to culture metacestodes *in vitro* in order to characterize physiological and ultrastructural phenomena during the reorganizational phase. Heath (1973a) successfully cultured *T. ovis, T. serialis, T. taeniaeformis, T. hydatigena,* and *T. pisiformis in vitro* from eggs to late cysticerci stages and described the morphological progression in all species. Heath and Elsdon-Dew (1972) noted that activated oncospheres retained their hooks

for up to 2 days post-activation. Developing oncospheres *in vitro* showed rapid cell division, and vesiculation was apparent by 5 days in culture (Heath, 1973a). However the sequence of morphologic changes *in vitro* may not be representative of morphogenesis *in vivo* since parasites do not complete development in artificial culture media even when these are enriched with serum and cells. In addition there is a limit to the conclusions which can be drawn regarding post oncospheral reorganization from light microscope observations on *in vitro* organisms. For example, Heath (1973a) observed a clear halo around 4 and 5-day-old stages of *T. taeniaeformis* and speculated that these might be due to a microvillar layer but ultrastructural studies are required to resolve such issues.

Immunology of Taeniid Metacestode Infections

A marked resistance to superinfection occurs in a variety of experimental taeniiases and there is ample evidence from epidemiologic studies to suggest that protective immune responses are a feature of many of the zoonotic and economically important cestode infections.

Miller and Gardiner (1932) and Campbell (1938a) were the first to examine this phenomenon experimentally. They demonstrated that rats infected with the metacestode stage of *T. taeniaeformis* were resistant to challenge infection. Resistance could be transferred to recipients by serum collected from rats 28 days post infection (Miller and Gardiner, 1932; Campbell, 1938a). Leid and Williams (1974) reinvestigated the protective role of antibody in passively transferring

resistance and showed that during the first several weeks of infection these protective antibodies were exclusively of the $7S\gamma_{2a}$ immunoglobulin sub-class.

Campbell (1938b) observed that larvae of *T. taeniaeformis* became resistant to antibody at approximately six days post-infection. Continued inoculations of immune serum after the parasites had reached this stage no longer had any adverse effect on survival. He suggested that this was due to the formation of a host capsule surrounding the organism in the liver. However, Musoke and Williams (1975) demonstrated that organisms become resistant to the effects of antibody even *in vitro* and they felt that some other mechanism must account for the acquisition of resistance to immunologic rejection. They showed that complement was necessary *in vivo* for immunity to be effective and postulated that local complement depletion by the parasite could contribute to resistance to immunologic rejection. Hammerberg *et al.* (1976) have shown that the worms do indeed release a complement consuming factor, although its physicochemical characteristics have been partially determined (Hammerberg and Williams, 1978).

Richard (1974) attributed the insusceptibility of parasites to immune serum to inherent changes in the larvae. Some alteration in antigenic characteristics would appear to be necessary in light of the recent observations by Coltorti *et al.* (1972), and Hustead and Williams (1977) who found that macromolecules including immunoglobulins had access to the parasites *in vivo* and actually penetrated into cyst bladder fluid.

Since the original proposal involving immunity and the host-parasite relationship by Campbell (1938b), several hypotheses have arisen concerning parasite survival in an immunologically competent host other than those developed by Musoke and Williams (1975) and Hammerberg *et al.* (1976) for *T. taeniaeformis*. Rickard (1974) proposed a hypothesis in which parasites are considered to become coated with specific antibody blocking susceptibility to cell-mediated immunity, in a manner analagous to the enhancing antibody mechanism of tumor survival (Baldwin and Robins, 1976). Varela-Díaz *et al.* (1972) proposed a modification of this hypothesis. They suggested that antibodies are formed against two distinct membrane antigens which are in juxtaposition and that one of the antibodies binds with an antigenic determinant which blocks the action of the lethal antibody.

Damian (1964) considered that survival of parasites might be due to "molecular mimicry." In this hypothesis it is proposed that host immune responses impose selective pressure on "fitness" for survival, and that those parasites survive which are best able to produce host-like antigenic determinants. Capron *et al.* (1968) modified this hypothesis and suggested that host-like antigens are produced as a result of induction though there is no experimental evidence to support this proposal.

Smithers *et al.* (1969) have developed a hypothesis for survival of schistosomes which unlike most others has been well supported by experimentation. They felt that host-like antigens are actually derived from the host by an adsorption process in *Schistosoma mansoni* infections. In electron microscopic studies they showed that the

tegument of adult worms was severely damaged when parasites were exposed to specific antibodies to host antigens. They concluded that the host antigens were in very close association with the tegument of the worm (Smithers, *et al.* 1969).

In studies on the post-cercarial reorganization process McLaren *et al.* (1975) demonstrated that host antigens were acquired very rapidly after skin penetration. These observations correspond to ultrastructural studies of developing schistosomula by Hockley and McLaren (1973). They observed an abrupt change in tegumental characteristics as the parasite became covered by membranes derived from subtegumental vesicles. The authors proposed that these changes were responsible for the acquisition of resistance to immunologic attack since newly formed tegument rapidly absorbed host antigens and no longer bound antibody.

No other attempts have been made to correlate ultrastructural changes with alterations in susceptibility to immunologic attack in metazoan parasites. Characterization of ultrastructural changes in taeniid organisms in post-oncospheral reorganization is a prerequisite to this type of experimentation in the field of cestode immunology.

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ULTRASTRUCTURE OF POST-ONCOSPHERAL STAGES OF *TAENIA TAENIAEFORMIS*

by

J. A. Picone and J. F. Williams

Department of Microbiology and Public Health
Colleges of Osteopathic Medicine
and Veterinary Medicine
Michigan State University
East Lansing, Michigan

SUMMARY

An electron microscopic study was made of the sequence of post-oncospheral reorganization changes which occur during the first 10 days of growth of *Taenia taeniaeformis* in the rat liver. Viable organisms were liberated from host tissues by tryptic digestion. Observations were made on the rate of growth, the process of vesiculation and the development of the tegument and subtegumental tissues. Characteristics of the reorganizational phase included the rapid investment of the entire parasite surface with microvilli, the accumulation of fluid-filled spaces which coalesced to form the central bladder by 4 days, the peripheral migration of cell nuclei and the establishment, by 10 days of age, of a tegumental and subtegumental architecture which was entirely comparable to that previously described in much older metacestodes. Certain changes are discussed in terms of the importance of this early phase of parasite growth in the immunology of the host-parasite relationship.

Metacestodes of *Taenia taeniaeformis* were studied after exposure to ferritin conjugated IgG_{2a} *in vitro*. Uptake occurred by both 10-day-old and 50-day-old live parasites and was inhibited by prior exposure to EDTA. No evidence of adherence or uptake of the conjugated-ferritin was seen in control preparations of formalin-fixed parasites, and unconjugated ferritin was not taken up by live parasites.

Ferritin particles were detected in and on microtriches and within vesicles in the submicrothrix zone. These membrane-bound vesicles were connected to the external surface in some cases, and the uptake process appeared to resemble pinocytosis in mammalian cells. This may offer an explanation for the incorporation of macromolecules into the bladder fluids in *T. taeniaeformis*.

INTRODUCTION

Current concepts of the ultrastructure and function of cestode tegumental membranes have been very thoroughly reviewed by Lumsden (1975). However, most electron-microscopic studies on taeniid tapeworms have been performed on well-developed metacestode forms or adults (Morseth, 1966; Bortoletti and Ferretti, 1971), and little work had been done to date on early post-oncospheral reorganizational stages. Until recently, these early forms were difficult to isolate, but the successes of Heath and Smyth (1970), Heath and Elsdon-Dew (1972), and Heath (1973) in growing a variety of taeniid oncospheres *in vitro* provided these workers with an opportunity for light microscopic observation of the processes of vesiculation and formation of the cysticercus bladderworm and its tegument.

Nevertheless, it seems likely that the sequence of changes which occurs *in vitro* may not be entirely representative of morphogenesis *in vivo*, because parasites do not complete development in serum-enriched culture media and their rate of growth is noticeably slow (Heath and Elsdon-Dew, 1972). Since viable post-oncospheral forms of *Taenia taeniaeformis* can be liberated from livers of infected rats by tryptic digestion (Musoke and Williams, 1975), we have taken advantage of this procedure to study the ultrastructural characteristics of reorganizational events in this species *in vivo* and the results are presented in this paper.

MATERIALS AND METHODS

The strain of *T. taeniaeformis* used in these experiments has been propagated in our laboratory using the methods described by Leid and Williams (1974). Four-month-old cysticerci are dissected from the livers of infected rats and 10-15 larvae are given orally to parasite-free cats. Gravid segments generally appear in the feces no less than 6 weeks later and are collected daily thereafter.

Eggs used in this study were freed from the proglottids by teasing them in saline with dissecting needles. Egg suspensions were given to 21-day-old Spartan (Spb(SD)BR) (Spartan Research Co., Haslett, MI) female rats by gastric intubation. Viable post-oncospheral stages of *T. taeniaeformis* were isolated from livers at 24 hour intervals beginning 1 day post-infection and continuing through day 10. All livers were exposed to 300 ml of .25% trypsin (Difco Laboratories) in BME (Grand Island Biological Co.) for 1.5 hours. Two different procedures were used in preparing livers for trypsinization and for electron microscopy due to differences in size and location of the organisms as they developed within the tissues. Livers containing 1-4 day-old parasites were macerated with a single-edged razor blade and the resulting slurry was digested in trypsin, centrifuged at 1500 g for 10 minutes, washed in BME tissue culture medium, and then fixed in glutaraldehyde s-collidine. Six- to ten-day-old parasites were liberated from the liver by wrapping the organ in a single layer of surgical gauze and squeezing gently until the liver capsule was completely ruptured and the subcapsular tissues containing the parasites had been disrupted. The entire organ was then exposed to trypsin for 1.5 hours. Organisms,

which were extremely fragile, were separated from the digested surface tissue under a dissecting microscope and gently washed in BME before fixation in glutaraldehyde s-collidine buffer. Eight-, nine- and ten-day-old parasites exhibited a squirming activity in the culture medium at this stage. Unfixed organisms prepared by these two digestion procedures were inoculated into the mesenteric veins of normal rats, as described by Musoke and Williams (1975), in order to determine their viability.

All fixed post-oncospheral preparations were subjected to repeated washings in s-collidine sucrose buffer, to osmium tetroxide for one hour, washed in s-collidine buffer and dehydrated up to absolute alcohol. The preparations were then passed through propylene oxide and infiltrated with a 1:2 mixture of propylene oxide and Epon 812 (Luft, 1961) for 12 hours. The pelleted liver digests of days 1-4 were transferred to capsules containing Epon 812 and centrifuged for 15 minutes at 2000 g. Six- to ten-day-old organisms were placed in individual blocks containing Epon 812. All samples were cured at 60°C for 18 hours.

Thick sections (1μ) of the capsules containing tryptic digests of livers from days 1-4 were cut on an LKB 11800 Pyramitome in order to locate parasites for ultrathin sectioning. Additional 1μ sections were cut for light microscopic observations of days 1-10 and stained with toluidine blue until a metallic ring appeared around the periphery of the staining solution. Ultrathin sections were cut on a LKB 8800A Ultratome III and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). Stained specimens were examined on a Zeiss-EM9S-2 Electron Microscope.

OBSERVATIONS

Figures 1-10 illustrate the sequence of post-oncospheral reorganizational changes which are seen by light microscopy in 1μ sections of organisms present in tryptic digests of rat livers on days 1-10 post-infection with *T. taeniaeformis*. At 24 hours (Fig. 1) the parasite appears as a dense mass 15μ x 17μ, but by day 2 discrete cells are seen separated by clear spaces which coalesce over the succeeding days into a central area (Figs. 3-4). There is a rapid increase in size over this period, so that by day 4 the external diameter is 70μ and the peripheral cellular mass varies from 12μ to 22μ in thickness. This vesicular or spherical form is maintained through day 7, at which time the diameter is approximately 280μ, but thereafter more irregular shapes are seen, reflecting the squirming movements of parasites isolated on days 8-10 (Figs. 8-10). Unfixed organisms from each day of infection retained their viability and developed into strobilocerci when inoculated into the mesenteric veins of normal rats.

The ultrastructural characteristics of 24-hr-old parasites are depicted in Fig. 11. Organisms at this stage are typically oval in shape and generally no more than 5 distinct cell nuclei are visible in sections. Extending from the peripheral plasma membrane, or tegument, are many microvillar structures up to .5μ in length and .05μ in thickness. Nuclei, particularly of the peripheral cells, contain dense chromatin clumps and large nucleoli approximately .75μ in diameter. Mitochondria and Golgi complexes are present in the perinuclear cytoplasm and scant areas of muscle fibers are present in the cytoplasm of cells in the submicrovillar region. Electron transparent spaces

Figures 1-10. Sections (1μ thick) of 1-10 day-old metacestode stages of *Taenia taeniaeformis* dispersed by tryptic digestion of infected livers. Toluidine blue stain.

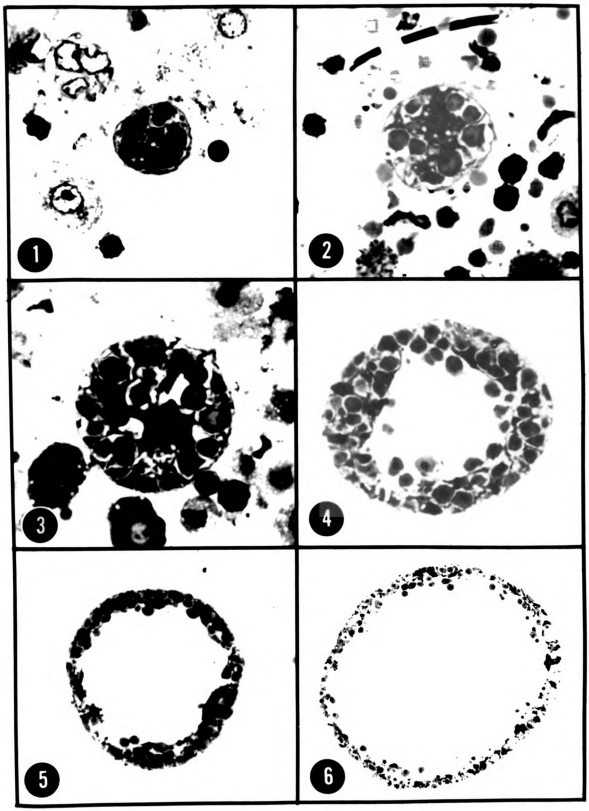
Figure 1. A 24-hr-old metacestode of *Taenia taeniaeformis* approximately 15μ x 17μ consisting of several cells enclosed by a plasma membrane (X1200).

Figure 2. A 2-day-old parasite showing a two-fold increase in cell number and more prominent transparent spaces surrounding the cells (X1200).

Figure 3. Parasite at 3 days post-infection containing up to 24 cells which are approximately 3.65μ in diameter. At this stage, the organism is developing a diffuse communicating system of cytoplasmic extensions (X1200).

Figure 4. A 4-day-old organism exhibiting complete vesiculation resulting in a fluid-filled sac surrounded by a peripheral cellular zone approximately 17μ in thickness (X750).

Figures 5, 6 and 7. At 5, 6 and 7 days old, parasites are showing growth of the fluid-filled bladder, and margination of cells toward the peripheral subtegumental zone (Fig. 5, X456; Fig. 6, X271; and Fig. 7, X205).


1
2
3
4
5
6

Figures 8, 9 and 10. At 8, 9 and 10 days post-infection, organisms assume irregular shapes (Fig. 8, X200; Fig. 9, X266, and Fig. 10, X160).

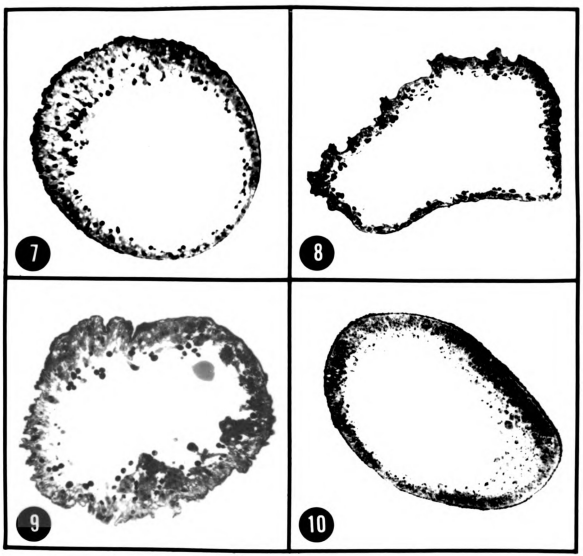

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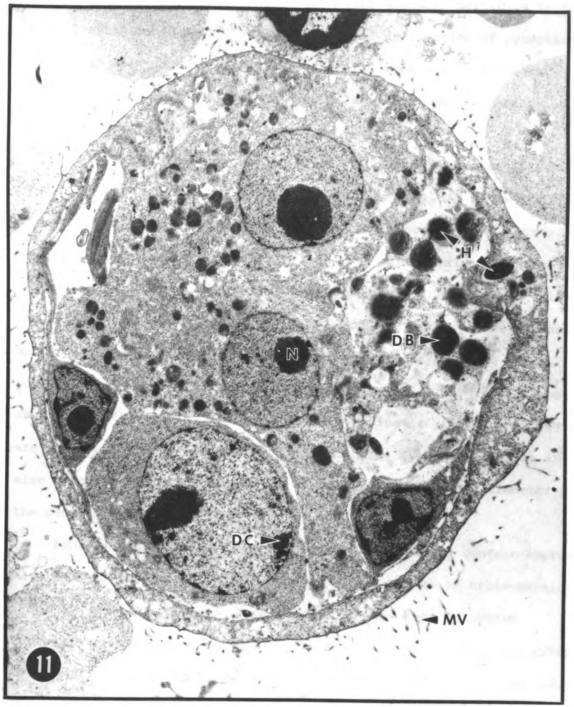
Figure 11. Cross-section of a 24-hr-old metacestode of *Taenia taeniaeformis* showing microvillar structures (MV) extending from the periphery of the tegumental membrane. Nuclei (N) and dense chromatin clumps (DC) are present and electron transparent spaces are distributed throughout the parasite. Dense bodies (DB) occupy a large portion of the cytoplasm. Embryonic hooks (H) are retained in the periphery of the cytoplasm. UrAc and PbCit (X12,420).


H
DB
N
DC
MV
11

surround the cells in some sections. Dense bodies ranging in size from .24μ to .65μ are interspersed with fine granular particles in the central cytoplasmic mass. There is no clear delineation of cytoplasm associated with each nucleus in this central area. In some cases, organisms have an irregular form with a "waist" or narrow connecting area, suggesting that division might be occurring.

By 48 hours, the microvillar layer has become much more dense (Fig. 12) and individual microvilli measure up to 1.54μ in length. There is now an expansion of the electron transparent areas surrounding a markedly increased number of cells in both peripheral and central regions. Cytoplasmic extensions connect perinuclear areas, and 8-16 nuclei are visible in each section. The muscle layer is more extensive and is arranged in the immediate subtegumental zone around a large portion of the periphery of the organism (Fig. 13). Oncospheral hooks are present (Fig. 14). Portions of the cytoplasm in the central region are circumscribed by electron transparent halos (Fig. 12). There are also circular electron transparent vacuoles up to 1.85μ in diameter in the central cytoplasmic zone.

Representative sections of three-day-old parasites contain approximately 24 nuclei, which range up to 3.65μ in diameter in cross-section (Fig. 15) and many of these have discrete perinuclear cytoplasm surrounded by an electron transparent area. In other cases, the cytoplasm is united by continuous communicating extensions or bridges (Fig. 15, 16) and is extremely rich in rough endoplasmic reticulum (Fig. 17). The oncospheral hooks are retained in three-day-old parasites but are not present in subsequent stages (Fig. 16). The microvilli are longer

Figure 12. A 2-day-old metacestode now showing a very distinct microvillar (MV) zone. There is expansion of the electron transparent spaces (ETS) and electron transparent halos (ETH) surround portions of the central cytoplasmic mass. UrAc and PbCit (X5,238).

Figure 13. A 2-day-old organism with distinct muscle fibers (Mu) which are present but only sparsely dispersed beneath the tegumental mebrane. UrAc and PbCit (X13,707).

Figure 14. Retained embryonic hook (H) in a 2-day-old parasite. UrAc and PbCit (X16,383).

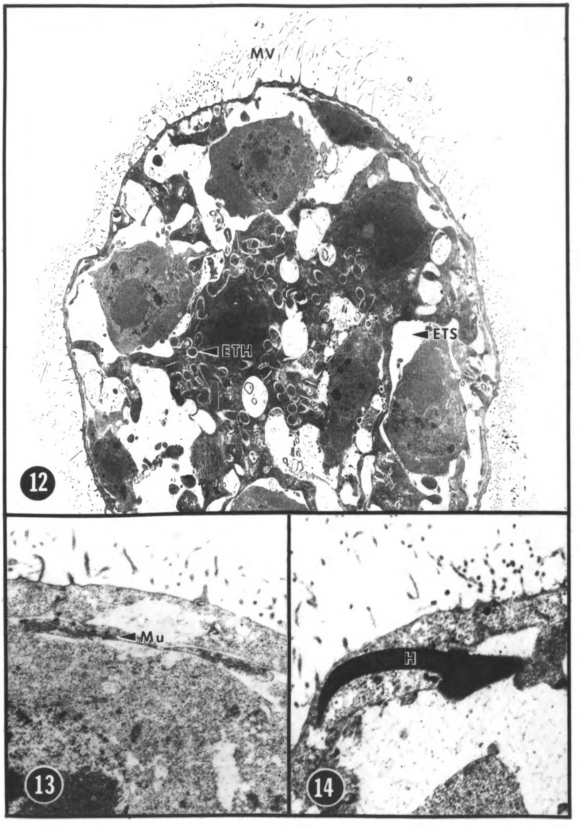
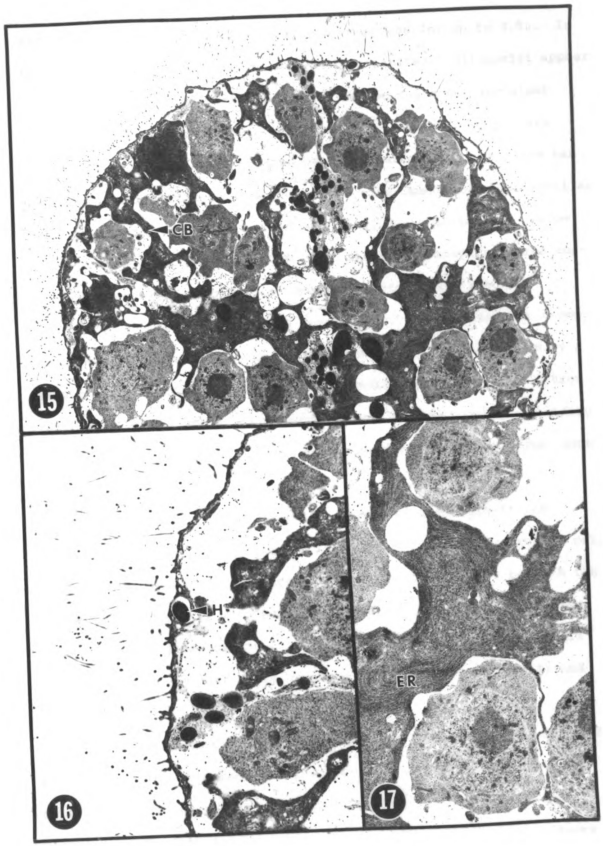

MV
ETH
ETS
12
Mu
13
H
14

Figure 15. A 3-day-old metacestode in which continuous communicating cytoplasmic bridges (CB) extend throughout the organism. UrAc and PbCit (X4,300).

Figure 16. An embryonic hook (H) retained immediately beneath the tegumental membrane in 3-day-old parasites. Hooks were not seen after this stage and may be in the process of extrusion. Lipid-like droplets are present in the subtegumental zone. UrAc and PbCit (X8,779).

Figure 17. A 3-day-old metacestode showing an extensive network of endoplasmic reticulum (ER). UrAc and PbCit (X8,184).


CB
15
IH
16
ER
17

and often extend out from the peripheral membrane for up to 2.0μ. In transverse sections of some three-day-old parasites, cell nuclei appear to be migrating peripherally toward the tegumental zone, and clear areas have begun to coalesce. Accumulations of lipid droplets are present in the cytoplasmic bridges, and the surrounding cytoplasm has a coarsely granular appearance (Fig. 16). The immediate submicrovillar layer is reduced at this stage to less than .2μ in thickness. Below the tegumental membrane (Fig. 16) is an electron transparent area containing irregularly shaped structures which stain very lightly.

By day 4 of post-oncospheral development, the organism has become completely vesiculated and consists of a sac of fluid surrounded by a compact zone of cells bordered by a tegumental membrane, but electron transparent spaces are still present between adjacent cells. Dividing cells are frequently seen, adding to the peripheral subtegumental zone (Fig. 18).

On day 5 there is an increase in volume of the fluid-filled bladder, as well as an increase in the size of peripheral circular cells, to approximately 6.0μ in diameter. The muscle fiber layer beneath the tegumental membrane is more prominent, and has begun to resemble the form typical of later stages of cysticerci. The microvillar zone is very similar to day 5 in depth, numbers of individual microvilli and thickness.

A marked increase in cell numbers occurs between days 5 and 6 and a more extensive cytoplasmic fusion develops connecting the mass of cells in the subtegumental zone (Fig. 20). Both longitudinal and circular muscle fibers are clearly visible (Fig. 21) and these layers

Figure 18. A 4-day-old parasite in which vesiculation is complete and the organism now exists as a fluid-filled bladder (FFB). A circular cell bordering the fluid-filled zone is undergoing division (CD). UrAc and PbCit (X5,652).

Figure 19. Muscular (Mu) zones are present beneath the tegumental membrane of 5-day-old parasites. UrAc and PbCit (X6,000).

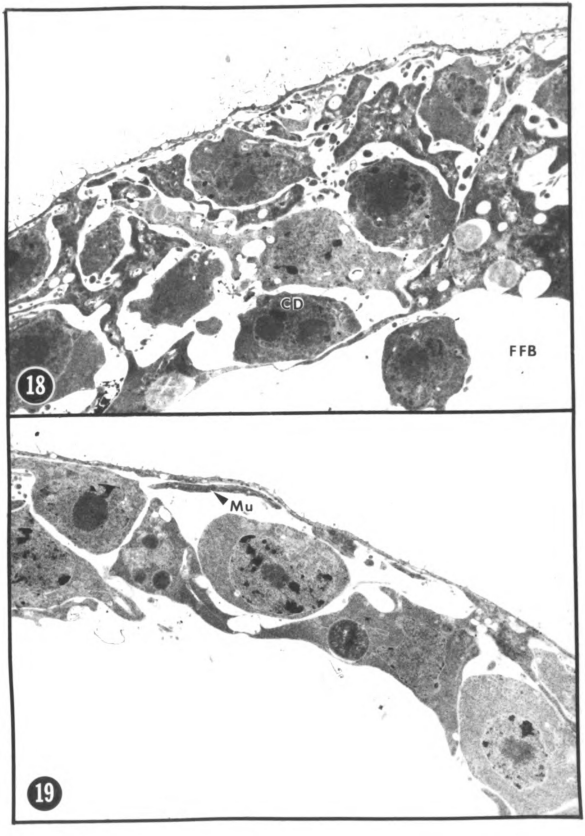
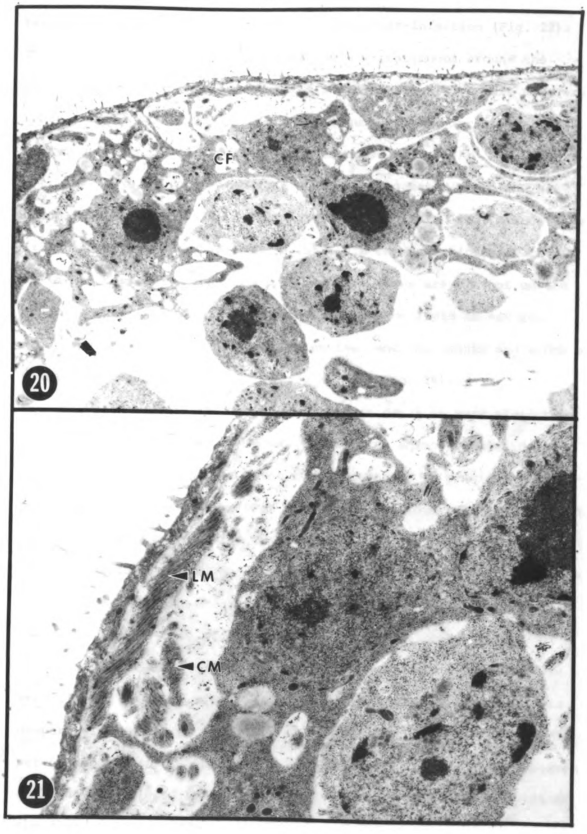

CD
FFB
18
Mu
19

Figure 20. In 6-day-old parasites, the number of cells have increased markedly and cytoplasmic fusion (CF) is extensive. UrAc and PbCit (X4,880).

Figure 21. Longitudinal (LM) and circular muscle (CM) are well developed and complete in the peripheral areas of 6-day-old parasites. UrAc and PbCit (X13,034).


CF
20
LM
CM
21

become strikingly more prominent by 7 days post-infection (Fig. 22). The longitudinal muscle layer, which is now continuous around the periphery of the parasites and the circular muscle layer, are now very closely opposed (Fig. 23). Microvillar numbers have increased and individual microvilli are frequently branched (Fig. 24).

At 8 days, a vacuolated zone below the tegumental membrane has become apparent (Fig. 25). Immediately beneath this area, muscle layers (.29μ to .41μ deep) as well as an intricate communicating network of cells can be seen. Large droplet-like structures are present on the inner aspects of the cell layer and these may be lipid in nature. Branching of microvilli is still extensive, and the length and width of these structures has increased (2.5μ x .1μ) (Fig. 26).

The area beneath the vacuolated zone on day 9 is very similar to that of day 8 (Fig. 27). However, there is an abrupt change in the form of the elements of the brush border compared to previous developmental stages. These structures now have a thickened base approximately .85μ long and .13μ wide from which extends an electron-dense whip-like structure 1.15μ long. Other investigators working with mature cestode stages have generally characterized this structure as a "microthrix" (Morseth, 1966). This microthrix form covers the entire surface of 10-day-old parasites. In other respects, organisms at this time are very similar to day 9 with the exception that the vacuolated zone is deeper. The border of microtriches is evidently more dense with 8-10 per μ compared to 6-8 on day 9. Electron transparent channels between cells have developed by day 10 running from the subtegumental zone to the fluid-filled bladder. Mitochondria are now numerous throughout the cytoplasm in the subtegumental zone.

Figure 22 and 23. A 7-day-old metacestode showing a further increase in the zone of longitudinal (LM) and circular muscle (CM). UrAc and PbCit (X4,503, Fig. 22). UrAc and PbCit (X21,216, Fig. 23).

Figure 24. At 7 days post-infection, there is an increase in the number of microvilli and branching (MB) near the base of these structures is frequent. UrAc and PbCit (X19,030).

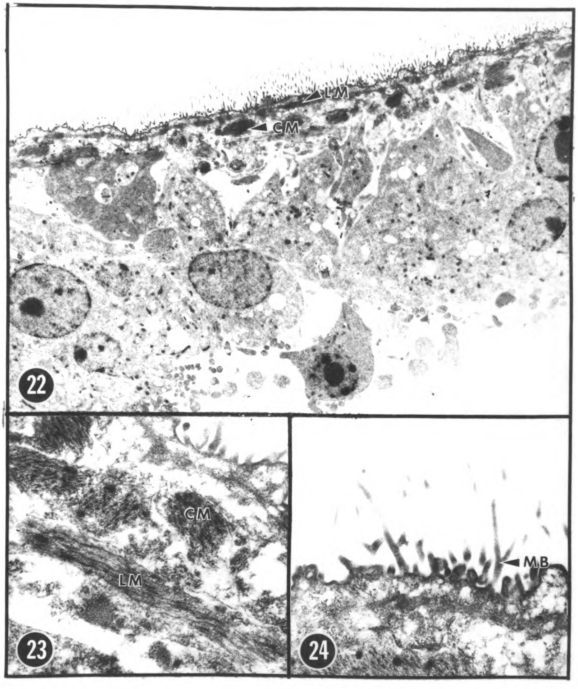

LM
CM
22
CM
LM
23
MB
24

Figure 25. A distinct vacuolated zone (V) has developed beneath the tegumental membrane by 8 days. UrAc and PbCit (X6,400).

Figure 26. Microvillar branching (MB) at 8 days post-infection is frequent but distinct transitional forms leading to the microthrix structure of later days were never seen. UrAc and PbCit (X17,066).

Figure 27. Microvilli on day 9 have a wide base with a whip-like structure extending from it. This structure is now termed a "microthrix" (MT). UrAc and PbCit (X13,442).

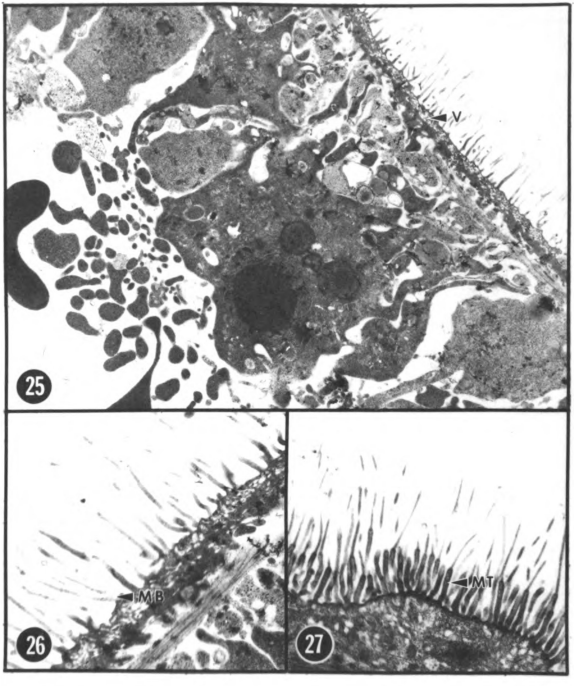
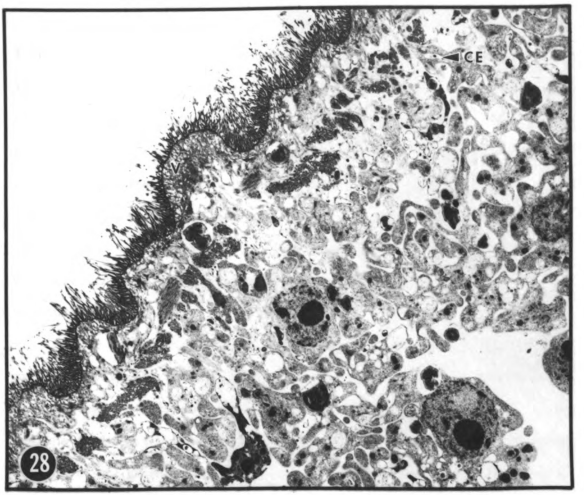

N
25
MB
26
MT
27

Figure 28. At 10 days post-infection, the vacuolated zone (V) has increased in depth and the communicating system of cytoplasmic extensions (CE) is very evident. UrAc and PbCit (X4,500). Mitochondria (inset) are present in the subtegumental cytoplasm.


ICE
28

DISCUSSION

Our observations provide evidence of a remarkably rapid and profound reorganization of *T. taeniaeformis* in host tissue during the first 10 days of life. The changes we have described can be reasonably assumed to occur normally *in vivo* since the parasites isolated in this study were viable, and continued to develop when inoculated into recipient rats. However, the rate of development was not uniform, and on each day there was considerable morphologic heterogeneity. Organisms were therefore selected for illustration which were most representative of the steps which occurred in the transformation from the oncosphere to the 10-day vesicular form.

One of the most prominent changes recognizable after one day of growth *in vivo* was the appearance of many fine microvilli covering the entire surface of the organisms. The manner in which these are formed remains to be determined, but in a previous study on the ultrastructure of eggs of *T. taeniaeformis*, Nieland (1968) showed that newly hatched oncospheres have many "cytoplasmic folds" on the outer tegumental membrane. It seems possible that these may represent stages in the development of the microvillar coat. The dimensions and shape of these tegumental projections in *T. taeniaeformis* on days 1-8 clearly resemble those described by Collin (1970) on 3- and 5-day-old post-oncospheral forms of *Hymenolepis citelli* obtained from the tissues of the intermediate host. They are evidently much shorter than the long microvilli which Heath (1973) suggested may be present on developing forms of *T. taeniaeformis* after 4 days of *in vitro* culture. He observed a halo

effect some 50μ wide around each organism which prevented contact between parasites and red blood cells suspended in the culture medium.

Another characteristic of young parasites revealed by our study was the retention of embryonic hooks through the third day of *in vivo* growth. It is generally considered that these hooks are shed soon after penetration of the intestinal epithelium, but more recent work by Banerjee and Singh (1968) demonstrated that some 1-day-old stages of *T. taeniaeformis* still had hooks. In *in vitro* studies, Heath and Elsdon-Dew (1972) found that hooks were retained for 2 days, although these authors felt that development in culture was generally retarded compared to the rate of reorganization and growth *in vivo*. In their work, early post-oncospheral forms of some taeniid species were shown to divide and, although this was not characteristic of *T. taeniaeformis in vitro*, our observation of sharply "waisted" forms at 1 day post-infection suggests that division may occasionally occur *in vivo*.

By comparison with culture forms, those isolated *in vivo* certainly appeared to undergo the process of vesiculation at a slightly faster rate. By 3 days the clear spaces had begun to coalesce and by 4 days a central fluid-filled cavity had formed, giving the organism a sac-like appearance. This process was delayed by 1-2 days in *in vitro* culture (Heath and Elsdon-Dew, 1972). It is not known at this time whether the fluid is derived from incorporation of host plasma constituents, comparable to the phenomenon observed in older parasites (Hustead and Williams, 1977; Varela-Díaz and Coltorti, 1972) although recent evidence suggests that forms as young as 10 days of age are capable of pinocytosis of ferritin-labelled host globulins (Picone *et al*. 1978).

Clearly, the structural features of metacestode tegument which have been so well characterized in fully-formed larvae (Morseth, 1966; Bortoletti and Ferretti, 1971; Lumsden, 1975) emerge very rapidly in the post-oncospheral reorganization process. The relationship between this peculiar form and the function of the tegument at the interface between parasite and the host is one of the most challenging areas for future study. The first 10 days of growth *in vivo* are particularly crucial because during this time worms become rapidly insusceptible to attack by immune serum *in vivo* (Campbell, 1938; Musoke and Williams, 1975). In primary infections the reorganizing parasites stimulate a brisk antibody-mediated protective response at the same time as they become invulnerable to its effects. The mechanism whereby this comes about is not at all clear. However, recent evidence indicates that the parasites can release a complement-consuming factor into the surrounding medium (Hammerberg and Williams, 1978a, b) and this may prevent fixation of complement on the tegument and the formation of damaging membrane lesions. If this occurs *in vivo*, the extensive surface area provided by the microtriches may be one means whereby quantities of membrane-associated factors can be liberated to consume local complement. Possibly the abrupt change in microvillar form is related to the acquisition of invulnerability and/or changes in antigenicity. Further insight into the functional consequences of the post-oncospheral reorganization may come from investigations on the rate of turnover of membrane components and the characterization of surface antigens in young forms of *T. taeniaeformis* isolated by the techniques which we have used in this study.

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UPTAKE OF FERRITIN BY METACESTODES OF *TAENIA TAENIAEFORMIS*

by

J. A. Picone and J. F. Williams

Department of Microbiology and Public Health
Colleges of Osteopathic Medicine
and Veterinary Medicine
Michigan State University
East Lansing, Michigan

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SUMMARY

An electron microscopic study was made of metacestodes of *Taenia taeniaeformis* after exposure to ferritin-conjugated IgG_{2a} *in vitro*. Uptake occurred by both 10-day-old and 50-day-old live parasites and was inhibited by prior exposure to EDTA. No evidence of adherence or uptake of the conjugated-ferritin was seen in control preparations of formalin-fixed parasites, and unconjugated ferritin was not taken up by live parasites.

Ferritin particles were detected in and on microtriches and within vesicles in the submicrothrix zone. These membrane-bound vesicles were connected to the external surface in some cases, and the uptake process appeared to resemble pinocytosis in mammalian cells. This may offer an explanation for the incorporation of macromolecules into the bladder fluids in *T. taeniaeformis*.

INTRODUCTION

The fluid-filled bladders of taeniid metacestodes are known to contain proteins which are physicochemically and antigenically identical to those present in host plasma (Chordi and Kagan, 1965; Esch and Kuhn, 1971; Coltorti and Varela-Díaz, 1972; Hustead and Williams, 1977). However, the processes by which macromolecules pass through the tegumental membranes of taeniid metacestodes have not been adequately characterized. Recent studies on the translocation of macromolecules across the intact epithelial surface of mammalian gut have involved the use of ferritin-labelling techniques to visualize proteins in the process of absorption by villar cells (Hemmings and Williams, 1976). We have now taken advantage of this procedure to study the uptake of macromolecules by larvae of *Taenia taeniaeformis* and the results are presented in this report.

MATERIALS AND METHODS

Infected rats for this study were prepared as described previously (Picone and Williams, 1978). Ten-day-old parasites were liberated from an infected liver by wrapping the organ in a single layer of surgical gauze and squeezing gently until the liver capsule was completely ruptured and the subcapsular tissues containing the parasites had been disrupted. The entire organ was then exposed to trypsin for 1.5 hr at 22 C. Organisms were separated from the digested surface tissue under a dissecting microscope and washed in BME (Grand Island Biological Co.). Fifty-day-old organisms were removed from infected livers by incising the host capsule with scissors and gently extracting the parasite with a spatula and forceps. All organisms were washed in BME before incubation under the conditions described below.

IgG_{2a} immunoglobulins were prepared from normal rat serum according to the method described by Leid and Williams (1974). Conjugation to ferritin was achieved by the procedure of Andres *et al.* (1967). Briefly, globulins were precipitated from serum with 50% $(NH_4)_2SO_4$ before DEAE chromatography on Cellex-D (Bio-Rad). IgG_{2a} was then purified by tryptic digestion following the method of Nezlin *et al.* (1973). Conjugation to ferritin involved the admixture of cadmium-free ferritin with toluene 2, 4, diisocyanate (Eastman Chemical Co.) followed by addition of the purified immunoglobulin in borate buffer (1.M pH, 9.5). Unreacted immunoglobulin was removed by ultracentrifugal sedimentation of conjugated globulin at 100,000g.

Incubations of BME-washed live parasites were carried out for 1 hr or 2 hr at 37C in ferritin-conjugated IgG_{2a} or ferritin alone. Others

were pre-treated with .04M EDTA for 30 minutes prior to incubation with the conjugate or ferritin alone. Control parasites were either fixed in phosphate buffered formalin for 2 hr prior to exposure to the ferritin preparations, or treated with EDTA before formalin fixation. A final group of parasites was examined after formalin fixation alone.

After incubation, live parasites were then washed in BME and immediately fixed in phosphate buffered formalin (5%). Ten-day-old organisms were placed in petri dishes throughout the remainder of the preparations for electron microscopy. Fifty-day-old organisms were sliced into 4 cm x 1 cm pieces and placed into vials. All parasites were exposed to osmium tetroxide for one hour and dehydrated up to absolute alcohol. After dehydration, specimens were passed through propylene oxide and infiltrated with a 1:2 mixture of propylene oxide and Epon 812 (Luft, 1961) for 12 hours. Ten-day-old organisms and fragments of 50-day-old parasites were processed in individual molds and cured at 60C for 18 hr. Samples were cut on an LKB 8800A Ultratome III and viewed unstained on a Zeiss-EM9S-2 Electron Microscope.

RESULTS

Ferritin particles were only detected in and on live parasites incubated for 2 hr in ferritin-conjugated IgG_{2a}. Ferritin preparations alone were not taken up by live organisms and pre-treatment of parasites with EDTA abolished uptake. Prior fixation in formalin also abolished uptake. EDTA treatment had no observable effects on the parasite surface.

Ferritin-conjugated particles were identified at the surface of the microtriches of both 10- and 50-day-old live parasites, and within vesicles in the subtegumental zone (Fig. 1, 2). At both stages, flask-like pinocytotic vesicles were seen to form at the base of the microtriches with openings on to the surface, and ferritin particles were apparently entering (Fig. 1). Clear evidence of a trilaminar structure to the membrane of the vesicle was also obtained (Fig. 2). Some particles of the conjugate were present on the inner aspect of the membrane of the microtriches, but no vesicles were ever observed forming along the shaft of these structures. Electron-dense particles were never seen in any of the electronmicrographs of either live or dead parasites incubated in unconjugated ferritin.

DISCUSSION

Our results strongly suggest that live developing larvae of *T. taeniaeformis* may take up host proteins by a process analogous to pinocytosis in mammalian cells. Exhaustive examination of dead parasites showed no evidence of adherence or uptake by the ferritin-conjugate, and uptake by live parasites was inhibited by prior exposure to EDTA. However, detection of ferritin in unstained preparations at such high magnification is difficult and it is possible that uptake occurred to some degree even under these conditions. Treatment with EDTA may affect permeability through the release of surface molecules which are involved in the transport system. Leive *et al.* (1968) have shown that EDTA treatment of *Escherichia coli* results in the release of large quantities

of surface lipopolysaccharide. EDTA may also exert an inhibiting effect through its Ca^{++} -chelating activity. The appearance of conjugated ferritin in parasites after 2 hr and not at 1 hr indicates that the process of incorporation is time-dependent, although again it is possible that some uptake occurred to a minor degree at the earlier time.

The association of ferritin particles with the plasma membrane at the base of the microvilli and their appearance in vesicles formed immediately below this layer give rise to electronmicrographs which are strikingly similar to those illustrated by Hemmings and Williams (1976) in their study of the microvillar brush border of rat intestinal epithelial cells. They concluded that uptake by this means was non-selective, although discrimination in favor of homologous globulins may be involved in subsequent secretion into the blood. The incorporation of a variety of isotopically-labelled proteins by metacestodes of *T. taeniaeformis* (Hustead and Williams, 1977) indicates that non-selective incorporation may occur in tapeworm larvae also, but the possibility remains that some degree of selective release into the bladder fluid takes place later.

The observation of ferritin within vesicles by no means resolves the long-standing controversy surrounding uptake of host plasma constituents by taeniid metacestodes. As yet, we have no evidence that molecules entering the subtegumental vesicles actually survive and appear in the bladder, or, if they do, whether this mechanism is quantitatively important in the overall transport process. Further ultrastructural work with isotopically-labelled markers may answer

these questions. Nevertheless, the demonstration of vesicles containing labelled proteins provides grounds for the belief that an active process may contribute to the accumulation of host molecules in parasitic fluids, as opposed to passive diffusion through channels or membrane lesions.

Both active and passive uptake mechanisms for amino acids have been described in cestodes by Pappas *et al.* (1973, 1974), and Esch and Kuhn (1971) detected the uptake of ^{14}C labelled *Chlorella* protein through the tegument of *T. crassiceps*. They suggested that this might occur through a system of "pores" in the membrane. Coltorti and Varela-Díaz (1975) proposed that immunoglobulins passively enter *Echinococcus* bladder fluid as a result of the formation of microfissures in the parasite membrane. However, the recent demonstration of pinocytosis of macromolecules by the tegument of plerocercoids of a pseudophyllidean tapeworm, *Schistocephalus solidus* (Hopkins *et al.* 1978) suggests that the uptake process we have proposed for *T. taeniaeformis* may be representative of that which occurs in other taeniid cestodes.

The occurrence of pinocytotic-like mechanisms at the surface of *T. taeniaeformis* extends the analogy drawn between larval cestode tegument and intestinal epithelial cells by Smyth (1969) on the basis of ultrastructural similarities and such phenomena as Na^{++} -dependent glucose transport. Whether or not other functional characteristics of these surfaces as protein transport systems are common to both remains to be seen, but the technological approaches used in investigations on mammalian gut may be useful in future work on cestodes.

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Figure 1. A 50-day-old metacestode incubated for 2 hr in ferritin conjugated to IgG_{2a}. Arrows indicate ferritin particles. A pinocytotic-like vesicle containing ferritin is present at the tegumental membrane. Unstained (X213,000).

Figure 2. A 10-day-old parasite incubated for 2 hr in ferritin conjugated to $IgG_{2a}2$. Ferritin particles are present in close association with microtriches and the tegumental membrane. A flask shaped vesicle limited by a trilaminated membrane (V) is present with an opening at the tegumental membrane. Unstained (X200,000).

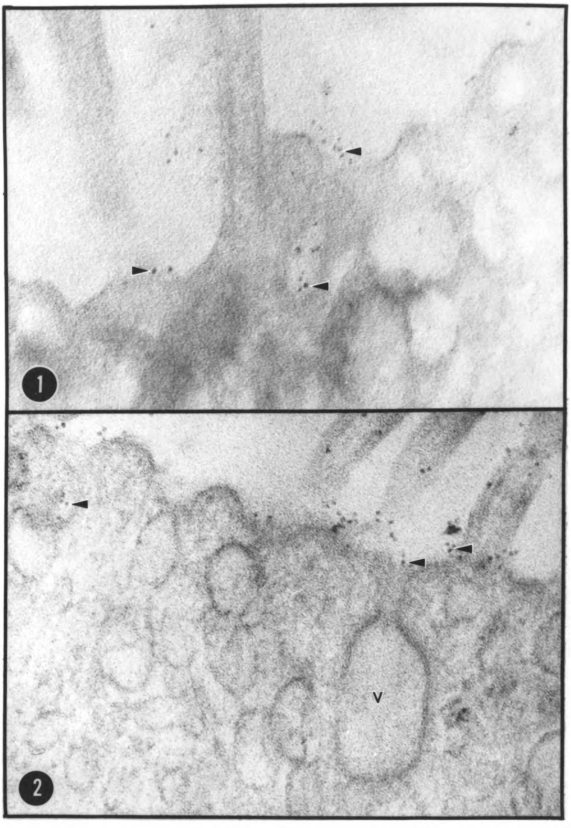
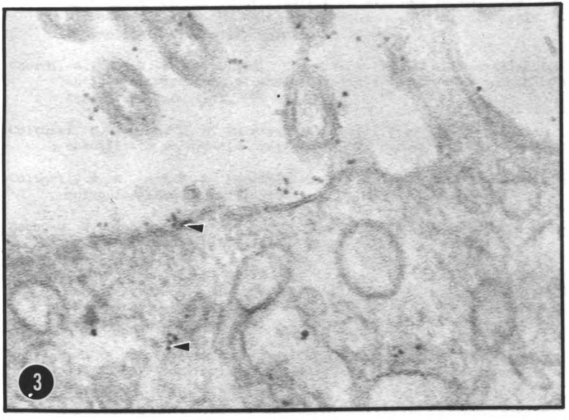

1
2
V

Figure 3. A 10-day-old organism incubated in ferritin conjugated to IgG_{2a} for 2 hours. Ferritin molecules can be seen in and around the microtriches, on the tegumental membrane and in the subtegumental zone. Unstained (X185,600).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