ULTRASTRUCTURAL AND HISTOCHEMICAL STUDIES OF THE DIFFERENTIATING PLEROCERCOID CUTICLE OF *SCHISTOCEPHALUS SOLIDUS*.

by

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ABSTRACT

To date, most studies of the cestode cuticle have been carried out on adult worms and in no instance has an attempt been made to correlate ultrastructural and histochemical observations. The present study was designed to obtain information on both the histochemical composition and the ultrastructure of the plerocercoid cuticle of the pseudophyllidean cestode Schistocephalus solidus (Müller, 1776) and to study any changes which might occur during plerocercoid differentiation.

Initially, this work has demonstrated that the plerocercoid tegument of S. solidus is of the same fundamental type as that of previously described cestodes. An outer anucleate but cellular region (the cuticle) is connected by cytoplasmic tubes to nucleated tegumental cells lying in the parenchyma.

It has been shown that the pore canals of S. solidus are different from any described for other cestodes and it is suggested on morphological grounds that they may function in a secretory capacity. In addition, the pore canals provide a direct connection between certain parenchymal cells and the external medium.

Papilla-like cuticular processes on the cuticular surface have been described and both adhesive and absorptive functions are suggested for these structures. It has been

shown that the cuticular processes are not present in the smallest forms studied but rather appear at about the same time as the worm becomes infective.

Changes in the histochemical and ultrastructural composition of the cuticular matrix have been described. It is probable that increased plerocercoid growth is accompanied by a change in cuticular composition from a more proteinaceous state to one which is largely carbohydrate.

A surface sulphomucin-basic protein complex has been described and partially characterized. Possible functions have been suggested for this layer.

RNA as well as acid and alkaline phosphatases were found in the cuticle and the possible significance of these substances in cuticular synthetic activities is considered.

It is suggested that plerocercoid cuticular differentiation may consist of two phases: (1) an initial phase in which various mechanisms are elaborated to allow survival in the vertebrate intestine, and (2) a second phase in which the plerocercoid is modified to permit prolonged growth in the stickleback coelom.

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INTRODUCTION

It has recently become apparent that the cestode cuticle is not the non-cellular secreted layer of classical description, but rather is part of an actively metabolizing integument. The ultrastructural studies of Kent (1957), Rothman (1963) and Threadgold (1962, 1965) have shown that the cestode "tegument" consists of two portions: (1) an outer anucleate layer (the cuticle) bounded by a plasma membrane and possessing numerous microvillus-like microtriches, and (2) an inner cellular layer joined to the outer portion by numerous cytoplasmic tubules. These two layers are separated by an acellular subcuticular membrane. It is not yet certain whether the outer anucleate portion of the tegument is syncytial or compartmentalized (Threadgold, 1962, 1965).

The above studies have provided a morphological framework for the results of biochemical, physiological and histochemical studies on cestodes. A number of workers (see Read and Simmons, 1963) have demonstrated that cestodes possess the ability to selectively absorb materials from their environment and since cestodes lack all traces of a digestive cavity this function must be performed by the cuticle. Histochemical studies (Bogitsh, 1963; Lee et al., 1963; Rothman and Lee, 1963; and Waitz and Schardein, 1964) have shown the presence in the cuticle of lipids, protein, polysaccharides,

and such enzymes as acid phosphatase, alkaline phosphatase and those associated with terminal oxidation.

To date most studies of the cuticle have been carried out on adult worms of groups other than the pseudophyllideans and, in no case has an attempt been made to correlate ultrastructural and histochemical data. The present study was designed to obtain information on the histochemical and ultrastructural composition of the differentiating plerocercoid cuticle of the pseudophyllidean cestode Schistocephalus solidus (Müller, 1776).

The growth of the plerocercoid of S. solidus takes place in the second intermediate host, the three-spined stickleback (Gasterosteus aculeatus). The worm is located in the coelomic cavity and the weight of plerocercoids in one fish may total up to 1/2 or more of the host's body weight (Dogiel et al., 1961). Somatic growth is apparently completed in the plerocercoid stage, which is unique among cestodes in that it is completely segmented (Smyth, 1946).

When the worm enters its final host, any one of several species of piscivorous birds, it rapidly reaches sexual maturity and is passed in the faeces within 4-6 days. An increase in temperature to that of the bird host is the only stimulus necessary to induce differentiation of the genitalia (Smyth, 1946). The transition from the larval to the adult form is accomplished solely through tissue differentiation which is supported by endogenous reserves accumulated during

plerocercoid development (Hopkins and McCaig, 1963). The complete life cycle of S. solidus and the morphology of the larval stages are described in Appendix A (Taken from Clarke, 1954).

MATERIALS and METHODS

A. Source of Material.

Plerocercoids were obtained from the coelom of the three-spined stickleback, Gasterosteus aculeatus, taken from local brackish waters. The sticklebacks were decapitated and the worms were removed immediately from the host's coelom and weighed to the nearest .0005 gm on an electric balance.

B. Preparation for Electron Microscopy.

Entire worms were placed in Palade's fixative at pH 7.2, 6% glutaraldehyde buffered to pH 7.2, or 4% paraformaldehyde and sliced into thin segments with a razor blade, before being transferred to fresh fixative for 1-2 hours. Glutaraldehyde and paraformaldehyde-fixed tissue was post-fixed in osmium for 1½ hours. Tissue fixed in Palade's fixative or glutaraldehyde was embedded in Epon 812 (Luft, 1961) or Araldite (Luft, 1961), while that fixed in paraformaldehyde was embedded in Maraglas (Spurlock et al., 1963).

Sections were cut with glass knives on a Porter-Blum ultramicrotome and stained with lead citrate or lead acetate and uranyl acetate. Photomicrographs were taken with an Hitachi HU-11A and an Hitachi HS-7S at magnifications of 2,000 to 100,000.

C. Preparation for Light Microscopy.

The worms used for the majority of the histochemical and morphological observations were fixed for 24 hours in buffered formol-saline while those worms used for enzyme studies were fixed for 24 hours at 4°C in acetone. In addition, Carnoy's fluid, formol-alcohol, formol-calcium and cetyl pyridinium chloride/formalin were employed when required for specific techniques and for comparative purposes. Specifically, material fixed in Carnoy's fluid was required for the Biebrich scarlet method (see page 30) and formol-calcium fixation was used with the Sudan black B method for bound lipids. Material fixed in Carnoy's fluid or in the formalin-containing fixatives was dehydrated in alcohol, cleared in xylene and embedded in paraffin at 52-59°C. Material fixed in cold acetone was dehydrated in acetone at room temperature, cleared in benzene and embedded in paraffin at 52°C. Paraffin sections were cut at 5-10 μ. Formalin-fixed frozen sections were also used for some of the lipid tests.

D. Preparation for Morphological Studies.

For histological examination, sections were stained with one of the following: Harris's alum hematoxylin, Mallory's triple stain, Heidenhein's hematoxylin, Ehrlich's hematoxylin or Weigert's hematoxylin, according to methods described by Culling (1963) and Ford (1958).

E. Preparation for Histochemical Studies.

The histochemical techniques utilized are listed below according to the material to be demonstrated, i.e., (1) carbohydrates, (2) nucleic acids, (3) enzymes, (4) proteins and (5) lipids. Where no references are given, standard procedures as described in Pearse (1961) have been followed.

(1) Tests for carbohydrates:

a. Periodic acid Schiff (PAS) (Culling, 1963).

b. PAS after digestion with 0.1% malt diastase for 30 minutes at 37°C.

c. PAS after acetylation (and saponification) (Culling, 1963).

d. Combined alcian blue-PAS method (AB-PAS) (Culling, 1963).

e. AB-PAS after acid hydrolysis to remove sialic acid (Culling, 1963).

f. 0.3% AB in 3% acetic acid.

g. 0.3% AB after digestion with 0.05% testicular hyaluronidase in 0.1M phosphate buffer (pH 5.5) at 37°C for 2 hours.

h. Toluidine blue (TB). Various stain concentrations (0.1% - .005%), pH's (4.5 - 7) and solvents (30% ethanol, acetate buffer or distilled water) were employed.

i. 0.1% aqueous TB after digestion with testicular

hyaluronidase.

j. 0.1% azure A (AA) in 30% ethanol (Barka and Anderson, 1963).

k. 0.2% AA (pH 1.5, pH 2.5, pH 4.0) (Szirmai, 1963).

l. Best's carmine.

m. Methylene blue extinction (Barka and Anderson, 1963).

n. Aldehyde fuchsin-alcian blue (AF-AB) (Spicer and Meyer, 1960).

o. AF-AB after methylation (and saponification) (Culling, 1963).

p. AB-AF (Spicer and Meyer, 1960).

(2) Tests for nucleic acids:

a. Methyl green-pyronin Y (MGP) (Culling, 1963).

b. MGP after digestion with 0.1% pancreatic ribonuclease at 37°C for 1 hour.

c. Toluidine blue (TB) after digestion with pancreatic ribonuclease.

d. Feulgen method for DNA.

e. MGP or TB after extraction with 4% trichloroacetic acid at 90°C for 15 minutes (Culling, 1963).

(3) Tests for enzymes:

As a control for all the enzyme tests, sections were incubated in a medium in which distilled water had been substituted for the substrate.

a. Azo-coupling technique for alkaline phosphatase with the diazonium salts Fast blue RR and Red violet (Culling, 1963).

b. Gomori's lead sulphide technique for alkaline phosphatase (Culling, 1963).

c. Naphthol AS-BI phosphate method for acid phosphatase with the diazonium salt Fast red.

d. Gomori's lead sulphide technique for acid phosphatase (Culling, 1963).

e. Azo-coupling technique for non-specific esterase (Culling, 1963).

(4) Tests for proteins:

a. Biebrich scarlet for basic proteins (Spicer, 1962).

b. Aqueous bromphenol blue for basic proteins (Johri and Smyth, 1956).

c. Mercuric bromphenol blue for general proteins (MBPB) (Mazia et al., 1953).

d. Mercury orange (RSR) reaction for -SH groups. A control technique was not used with this reaction.

(5) Tests for lipids:

a. Lillie and Ashburn's isopropanol oil red O method (Culling, 1963).

b. McManus's Sudan black B method (SBB) for compound lipids in paraffin sections.

c. Sudan black B method for neutral lipids in frozen sections.

RESULTS OF LIGHT MICROSCOPY

A. Morphological Observations.

(1) Fixation

Formol-saline, formol-calcium and 10% formalin containing 0.5% cetyl pyridinium chloride (CPC/F) were found to be the most satisfactory fixatives for morphological observations - but, formol-calcium fixation has certain limitations for histochemical studies (see Culling, 1963; p. 37). CPC/F fixation gave the best preservation of mucins and would be the fixative of choice for future investigations of mucopolysaccharides.

Plerocercoids fixed in Carnoy's fluid contracted to less than 1/2 of their original length with a resultant distortion of cuticular structure and, in most instances, a separation of the cuticle and subcuticular layers. Formol-alcohol fixation also produced some cuticular distortion although not as much as did Carnoy's fluid. Material fixed in formol-alcohol did appear to be more sensitive to ribonuclease digestion than did that fixed in other formalin-based fixatives. Acetone-fixed material also exhibited considerable separation of the cuticle and subcuticular structures (see Figure 20). In some instances this made the exact localization of enzyme sites difficult.

(2) Morphology of the Differentiating Plerocercoid.

Light microscope examination of S. solidus plerocercoids has demonstrated a progressive change in cuticular morphology correlated with an increase in size. The smallest forms studied (1-5 mg) showed no obvious segmentation and a very thin ($< 4\ \mu$) cuticle with no marked variation in thickness was present (Figure 1). A surface layer which stained with most morphological stains less intensely than the basal cuticular region and an obvious microthrix border could be discerned. The cells of the subcuticular parenchyma were spindle shaped and oriented at right anles to the cuticular surface. Long projections from some of these cells (probably, in these small worms, developing muscle fibres) appeared to cross the entire worm. Very thin layers of circular and longitudinal muscle fibres were observed immediately below the subcuticular membrane. A layer of longitudinal muscles, which would in older worms separate the subcuticular and medullary parenchyma, could also be seen. The subcuticular membrane was very thin and difficult to observe in these small worms so that ultrastructural observations were necessary to confirm its existence. The parenchymal nuclei were quite large and spherical and contained a single nucleolus. Only a few calcareous corpuscles were observed scattered throughout the parenchyma. While, at these magnifications, the cytoplasmic tubules which connect the parenchymal and cuticular portions of the tegument were visible,

their very small size made it impossible to assign them to specific cells.

Plerocercoids of 10 mg gave evidence of segmentation and a somewhat thicker cuticle, but one which was still of uniform thickness. By 20-30 mg the characteristic pattern of the differentiated plerocercoid cuticle had developed. The superficial portion of the cuticle now was significantly thicker than the velum and a microthrix border of greater density and depth was present. Small cuticular protrusions or "cuticular processes" could be observed on the superficial portion of the cuticle. Internally, serially repeated genital rudiments could be seen.

Pore canals became visible with the light microscope only in worms between 10 and 20 mg, although ultrastructural studies have provided evidence for their existence in worms as small as 1 mg. In larger stages, the pore canals appeared as a tube which penetrated the cuticle and ended just above the subcuticular membrane. The base of the pore canal was formed by an extension of a cell lying in the subcuticular parenchyma with as many as six pore canals receiving processes from a single parenchymal cell. The electron microscope also revealed the presence of droplets, possibly of a secretory nature, in the basal region of the pore canals (Figure 31).

The fundamental pattern seen in the 10-20 mg plerocercoid was preserved throughout further growth and

development. A plerocercoid of more than 200 mg had a superficial cuticular thickness of 30-40 μ but the velum showed little increase during development so that at a weight of 200-250 mg it was only 3-4 μ thick. The phenomenon of spatially localized growth of the cuticular matrix may be directly related to the greater number of tegumental cells connected to the superficial cuticle. The thin velar region of the cuticle had few tegumental cells underlying it and these cells were positioned parallel to the velum surface (Figure 7).

The microthrix border increased in depth during development, reaching at least 25 μ, as was particularly evident in sections through the extreme anterior and posterior segments. The cuticular processes also increased in size with plerocercoid growth so that in very large worms they attained a height of 2-4 μ. The pore canals were larger and in greater number per segment in worms of larger size. In many cases they took on a flask-like or hourglass shape (Figure 2). In some instances the base of the pore canal became enlarged, bulging against the subcuticular membrane without, however, penetrating it. Although no quantitative data have been collected on this point, it did appear that pore canals were more numerous in the anterior part of the superficial cuticle. Certainly, large numbers of pore canals were frequently observed in this region (Figure 7), in many instances grouped together in a small area of the cuticle.

No attempt has yet been made to determine whether a constant ratio exists throughout growth between the number of pore canals and the area of the cuticular surface. Although cuticular processes have never been observed in the velum, pore canals were seen in this region with the light microscope in plerocercoids of more than 200 mg.

B. Histochemical Results.

(1) Distribution of Carbohydrates.

The PAS reaction is based on the fact that certain tissue elements yield aldehydes as reaction products after oxidization by periodic acid. These aldehydes may then be demonstrated with a Schiff reagent. According to Hotchkiss (1948) any substance with the following properties will give a positive result with the PAS method:

a. The substance must contain the 1:2 glycol grouping, or the equivalent amino or alkyl-amino derivative, or the oxidation product CHOH-CO.

b. It must not diffuse away in the course of fixation.

c. It must give an oxidation product which is not diffusible.

d. Sufficient concentration must be present to give a detectable final colour.

With the use of controls and supplementary staining techniques it is possible to distinguish between the wide variety of substances demonstrated by this method.

In all the worms tested, material that was PAS positive and diastase labile was assumed to be glycogen. The results of the Best's carmine method were identical to those obtained with the PAS reaction, thus providing confirmation of this interpretation. Glycogen was found in varying quantities (based on relative intensities of reaction) in all parts of plerocercoids except in the cuticle and subcuticular membrane. Quantities of glycogen, in muscle tissue, were deposited among the muscle fibers. In worms of 7-20 mg, dense concentrations of glycogen were detected in the cortical and medullary parenchyma; the density generally being dependent on the cellular mass of any region. Glycogen was also present in the subcuticular parenchyma and subcuticular muscle layers with the greatest concentrations in the region of the tegumental cells. Although the glycogen concentrations of the cortical and medullary parenchyma remained relatively constant throughout growth, little glycogen could be demonstrated in the tegumental cells in worms of more than 20 mg. But, large quantities of glycogen were concentrated in the rest of the subcuticular parenchyma. On the basis of reaction intensity no marked gradients in glycogen concentration could be detected over the anterior-posterior axis.

A glycogen extraction from whole plerocercoids (KOH

digestion, ethanol precipitation method) also revealed the presence of large quantities of this substance. A sample of 15 plerocercoids of all stages of development was found to contain 16.1% glycogen (wet weight). This figure is identical to that of S. solidus published by Hopkins (1950).

Following diastase digestion a marked PAS response was retained in two regions of the cuticle, the subcuticular membrane and the basal organelles of the pore canals. In addition, most stages examined had an extracuticular coat of PAS positive material which was concentrated in the region of the microthrix border, clearly external to the cuticular surface. The PAS reactivity of the cuticular matrix varied considerably from almost entirely negative in small and medium sized (7-150 mg) plerocercoids to very positive in large (> 200 mg) forms. In worms between 7 and 80 mg the bulk of the cuticular matrix was PAS negative or only faintly positive. All, however, had a clearly positive layer of PAS positive material about 1½ μ below the cuticle surface. This layer was most sharply delineated externally, but internally the reactivity decreased over a distance of 1-2 μ until it matched that of the general matrix. In larger worms, i.e. 90-200 mg, the PAS positive layer appeared at the cuticular surface in the superficial region and included the cuticular processes. The PAS reactivity rapidly decreased internally beyond the first 2-3 μ of the cuticular surface. There were indications that the PAS positive layer remained below the cuticular surface in the velum and in the extreme

posterior region of the superficial cuticle in worms over 90 mg. The reactivity of the cuticular matrix also increased with size. The cuticle of a 138 mg plerocercoid was PAS positive only at the surface but worms of 204 and 263 mg showed an intense general cuticular response to the PAS stain. The 263 mg worm was intensely positive in its response so that little or no difference in reactivity between the surface layer and the general cuticular matrix was observed.

The PAS positive response of the subcuticular membrane was definite and constant in all stages (Figure 3). A marked PAS positive response was given by the basal organelles of the pore canals. This response was particularly obvious where the cuticular matrix was PAS negative but a positive response was given by the cuticle surface surrounding the external portions of the canals. In the smaller worms the tegumental cells were only faintly PAS positive but this reactivity increased with size of worm and a relatively intense PAS positive response was given by the tegumental cells in worms of more than 200 mg.

Since substances other than carbohydrates may give a positive PAS reaction, the source of cuticular PAS reactivity was controlled by acetylation. The PAS reactivity of 1-2 glycol groups (carbohydrates) is removed by treatment with a mixture of acetic anhydride and pyridine (acetylation). Substances that retain their PAS reactivity after acetylation are assumed to be of lipid character (Barka and Anderson, 1963).

The restoration of hydroxyl groups and hence the restition of PAS staining may be accomplished by treatment with 0.1 N KOH for 45 minutes at room temperature.

After 8 hours acetylation, PAS reactivity was totally removed from all plerocercoid tissues except the calcareous corpuscles of formol-saline fixed worms. A total restitution of PAS reactivity resulted from saponification for 45 minutes at room temperature. These results confirmed the carbohydrate nature of all cuticular and parenchymal substances, except the calcareous corpuscles, demonstrated by the PAS reaction.

Alcian blue is a polybasic copper phthalocyanin derivative which interacts with carboxyl and sulfate groups both in vitro and in tissue sections (Quintarelli and Dellovo, 1965). When used in conjunction with hyaluronidase digestion it is one of several useful histochemical methods for the identification of acid mucopolysaccharides. Testicular hyaluronidase specifically removes hyaluronic acid and chondroitin sulphate A and C but does not hydrolyse chondroitin sulphate B (see Curran, 1964; p. 195).

The bulk of the cuticular matrix of S. solidus plerocercoids was unreactive with Alcian blue although a faint response appeared in worms of more than 200 mg. There was, however, an intensely reactive alcianophilic layer discernable in worms of from 10 to 118 mg in the same superficial location as the PAS positive layer described above. In the 118 mg worms the Alcian blue reaction was most

intense in a narrow layer under the cuticular surface but it appeared to be continuous with the Alcian blue positive region superficial to this layer. In all worms of more than 118 mg the positive Alcian blue response included only the cuticular surface to a depth of about 2 μ. The reactivity of the cuticular surface was not affected by incubation in testicular hyaluronidase. The tegumental cells of all sizes of worms gave a light positive response and the calcareous corpuscles were intensely reactive. Both the lumena and the basal organelles of the pore canals were unreactive with Alcian blue.

The areas of the cuticle which reacted with Alcian blue could be determined with greater exactitude when this stain was combined with the PAS method or with aldehyde fuchsin. When these methods were employed it became apparent that there was a layer immediately beneath the cuticular surface which stained selectively with Alcian blue. In small, undifferentiated forms this layer composed about 1/2 of the cuticular matrix (Figure 4). The cuticular surface, in the small forms, was intensely positive with both the PAS and aldehyde fuchsin methods while the basal region was only lightly positive. In larger worms the bulk of the cuticular matrix was unreactive with Alcian blue though it did give a light positive reaction with both PAS and aldehyde fuchsin. There was, however, an Alcian blue positive layer immediately beneath the cuticular surface (Figures 5 and 6). In worms of 100 mg the Alcian blue positive region also extended into

the bulk of the cuticular matrix in the anterior portion of the superficial region (Figure 3). A layer of apparently extracuticular Alcian blue positive mucins could also be observed covering the velum and the extreme posterior tip of the superficial cuticle (see Figure 12). It is interesting to note that acid hydrolysis, to remove sialic acid, caused a significant dimunition in Alcian blue reactivity of the matrix in two out of three worms examined.

The methylation procedure was employed to block the staining of both sulphated mucopolysaccharides (by desulphating them) and non-sulphated acid mucopolysaccharides (by esterifying the carboxyl groups). After methylation the reactivity of the cuticular matrix to the aldehyde fuchsin-Alcian blue method was totally removed. However, the cuticular surface remained at least partially reactive with aldehyde fuchsin after methylation. This resistance to methylation is characteristic of certain sulphated acid mucopolysaccharides (Spicer, 1960).

Subsequent saponification endowed the cuticle with an even greater reactivity than existed previously. Both the aldehyde fuchsin and Alcian blue stains reacted intensely with the cuticle.

Toluidine blue and azure A produce a metachromatic response in the presence of oriented negative surface charges. Although many different substances can give a metachromatic response, the technique is considered diagnostic for acid

mucopolysaccharides when applied to tissue sections, and when the metachromasy is intense and alcohol resistant, it may be taken as firm evidence for the existence of sulphated acid mucopolysaccharides (Barka and Anderson, 1963).

Azure A will, above pH 3, form a metachromatic precipitate with all acid mucopolysaccharides, including hyaluronic acid, but if the pH is lowered below this point the carboxyl group dissociation is decreased and thus hyaluronic acid staining is extinguished. However, the sulphated acid mucopolysaccharides contain both carboxyl and sulphate groups and at a pH of 1-2 the sulphate group is still dissociated so the disaccharide will bind one dye molecule. Hence, azure A metachromasia at pH 1.5 may be regarded as specific for sulphated acid mucopolysaccharides.

Although the above interpretation is theoretically sound, Szirmai (1963) has observed differential metachromasia of chondroitin sulphate when stained with azure A at low pH's. He attributes this result not to the presence of a non-sulphated mucopolysaccharide but to a competitive effect of the cationic groups of proteins, relatively increasing by lowering the pH.

The results of the metachromatic staining procedures applied to sections of various sized worms varied considerably with the techniques used. When sections stained with toluidine blue or .1% azure A in 30% ethanol were examined in distilled water, the entire cuticular matrix gave a brilliant metachromatic response. After sections were air-dried and cleared in xylol

this metachromatic response was reduced to the β type. However, the more superficial portion of the cuticle (not including the surface) of 24 and 44 mg worms became orthochromatic after this treatment while in a 123 mg worm the entire cuticle, including the cuticular processes, remained metachromatic (β) (Figure 13). If sections of this same 123 mg worm were dehydrated in alcohol prior to clearing and mounting, the superficial portion of the cuticle became orthochromatic while the rest of the cuticle gave a metachromatic response. This orthochromatic staining of the superficial portion of the cuticle was observed, after alcohol dehydration, in sections of 10 worms of from 10 to 150 mg. However, there was a thin layer of metachromasy at the extreme cuticular surface in all instances, and the basal organelles of the pore canals and the subcuticular membrane were orthochromatic (see Figure 2). Many sections also possessed a layer which was external to the cuticular processes and which gave a metachromatic response. It is probable that the areas which were orthochromatic with toluidine blue are the same as those which reacted most intensely with alcian blue.

Sections stained with toluidine blue after hyaluronidase digestion showed significantly diminished cuticular metachromasia (Figures 13 and 14). After this treatment some areas of the cuticle became completely nonreactive and the positive response which remained likely resulted from the presence of cuticular RNA (see page 26 and Figures 15 and 16).

When sections stained with azure A at pH 4.0 were examined in distilled water, the entire cuticle displayed a γ metachromasy. This response was reduced to the β form after air drying and clearing, and following alcohol dehydration only a thin surface layer of β metachromasy remained. Sections stained with azure A at pH 1.5 showed no reactivity of the cuticular matrix but did display a thin, intensely staining surface layer which remained visible after alcohol dehydration (See Figures 9, 10 and 11). This reactivity was located in only the most superficial region of the cuticle and included the surface, but not the matrix, of the cuticular processes. In some instances the superficial reactive layer extended over as much as 2/3 of the length of a pore canal but the basal portions of pore canals were totally unreactive in all cases.

Cuticular reactivity with azure A at pH 1.5 was not diminished by incubation in testicular hyaluronidase. In fact, the reactivity of the general cuticular matrix was more intense in the hyaluronidase treated sections than in the controls in 1/3 of the sections observed. In those instances where azurophilia was increased by digestion, the reactive areas were extended to include all of the cuticular matrix except the most superficial 1-2 μ, in addition to the previously observed layer on the cuticular surface.

The methylene blue extinction point of the cuticle was found to lie between pH 3.6 and 4.1. At pH 3.6 a faint

cuticular response could be determined in a water mount and at pH 4.1 the entire cuticle stained intensely. A methylene blue extinction point within the above range approximates that which would be expected from the presence of a non-sulphated acid mucopolysaccharide such as hyaluronic acid. After sections were cleared and mounted a faint surface response could also be detected in sections incubated at pH 2.6 and 3.2 which would be expected if sulphate groups were present in the macromolecules located at the cuticular surface.

In view of the preceeding results it would appear that at least three separate layers of acid mucins can be detected in the cuticle:

a. A surface layer of sulphated acid mucopolysaccharides.
b. A layer immediately beneath the surface which reacts intensely with alcian blue and which may contain sialic acid.
c. A third layer which, in larger worms, composes the bulk of the cuticle, contains carboxyl groups, and may be composed of hyaluronic acid.

(2) Distribution of nucleic acids.

Methyl green-pyronin staining preceded by ribonuclease digestion was used for the identification of RNA. RNA was assumed to be present in those structures which:

a. were pyronin positive before ribonuclease digestion,

b. did not stain with pyronin after ribonuclease digestion.

The Feulgen test was used for the identification of DNA and since only nuclei of definite cells were positive no further controls were carried out. No Feulgen positive response was observed in any portion of the cuticle. Some portions of the cuticle were positive for methyl green when methyl green and pyronin stains were applied separately (Davenport, 1960) but this response was not given by the combined methyl green-pyronin method of Jordan and Baker (Culling, 1963).

The bulk of the cuticular matrix, the tegumental cells and the cells of the parenchyma were pyronin-positive in all worms studied. In addition, two bands of intensely pyronin positive material could be detected near the cuticular surface. The first band was composed of extracuticular mucin-like material located within the bounds of the microthrix border and was most commonly observed in smaller worms. The second band of pyronin positive material was immediately below the cuticular surface in worms of less than 100 mg but included the cuticular surface and cuticular processes in all worms larger than this. Although the pyronin positive response of the cuticular matrix, the tegumental cells and the parenchymal cells was largely removed by digestion with ribonuclease, the positive staining characteristics of the superficial bands were not affected by this enzyme treatment (Figures 17 and 18).

Toluidine blue staining proved to be a more sensitive indicator of RNA'ase activity than did the methyl green-pyronin method. Extraction with hot trichloroacetic acid removed the bulk of the cuticular toluidine blue metachromasia and all parenchymal metachromasia except that of the calcareous corpuscles. Very little effect on pyronin staining was observed when the extraction was followed by the methyl green-pyronin stain. The effect of RNA'ase treatment was similar to hot trichloroacetic acid in that the toluidine blue metachromasia of the cuticle was significantly reduced, but not totally removed (Figures 15 and 16).

(3) Distribution of enzymes.

Both alkaline and acid phosphatase appear to be present in the cuticle of all sizes of worms. Incidentally, the azo-coupling techniques allowed accurate localization of phosphatases but the Gomori methods were inferior in this respect. The entire cuticle plus the tegumental cells were reactive for alkaline phosphatase and in the larger sexually mature forms the uterine walls were reactive (Figure 19). The reactivity of the pore canals was slight and variable though the lumen was intensely reactive with the fast blue technique. Although little variation in alkaline phosphatase reactivity existed through the depth of the cuticle there was an enhanced activity in the velum and in the anterior portion of the superficial cuticle (Figure 19).

The tegumental cells in these same areas also demonstrated increased activity.

With the Gomori method for acid phosphatase the cuticular reaction was confined to the most superficial and the more basal regions and acid phosphatase activity was present in the lumen of the pore canals (Figure 20). This latter reaction site could not be related to reactivity of any particular tegumental cells and where precipitates were observed in the tegumental region their appearance indicated that they represented diffusion artefacts rather than actual enzyme sites. A positive response for acid phosphatase was also given by the testes of sexually mature forms. The positive reaction of calcareous corpuscles obtained in all worms does not indicate the presence of acid phosphatase as this reaction was also given by control sections. The extreme variability of the results of the Gomori method for acid phosphatase made difficult the detection of any gradients of activity over the length of the cuticle. In some areas there was little or no cuticular reaction while in others the reaction was so dense that no meaningful localization would be obtained. Despite these handicaps it could be observed that the region of most reproducible activity included the velum and the anterior superficial cuticle. It was in these same regions that the basal portion of the cuticular matrix also reacted consistently.

The naphthol AS-BI phosphate method for acid

phosphatase indicated a more widespread occurrence of this enzyme than did the Gomori method and the results appeared to mimic those given by the azo-coupling method for alkaline phosphatase. The distribution of cuticular acid phosphatase reactivity was similar in each segment to that described for alkaline phosphatase since an intense activity could be seen in the velum and the anterior superficial cuticle region. In addition, a less intense reaction was seen throughout the entire cuticular matrix and in the tegumental cells. No sites of esterase activity could be detected in any portion of the worms but this result must be regarded as tentative pending the application of more sensitive procedures.

(4) Distribution of proteins.

The mercuric brom-phenol blue method is a general protein stain while the aqueous method is supposedly specific for basic proteins (Johri and Smyth, 1956). The aqueous brom-phenol blue method proved to be non-specific and the results were judged unreliable as all portions of the plerocercoid were stained with equal intensity. The intensity of cuticular staining varied with plerocercoid size when the mercuric brom-phenol blue method was applied. In small worms both the microthrix border and the cuticular matrix stained intensely while the pore canals appeared as empty channels and the subcuticular membrane gave a light but positive response. As worm weight increased there was a progressive reduction in

the response of the cuticular matrix so that in worms of more than 150 mg only a faint positive response could be observed. The cuticular surface was, however, intensely positive in all stages examined, a response which was particularly obvious in those larger stages in which the general matrix responded weakly. (See Figure 8.) In the largest stage examined (263 mg) the reaction extended inward from the cuticular surface to a depth of 1-2 μ and included the walls of the pore canals. The most intense reaction, though, was at the cuticular surface. Extra-cuticular mucin-like material found in the region of the microthrix border also gave a positive response to the mercuric bromphenol blue method (i.e., general protein stain). The entire plerocercoid gave a light positive response to the Millon reaction (specific for tyrosine) but no localized staining could be detected.

When the staining is carried out at a pH of 9-10 Biebrich scarlet is specific for nucleohistones and histone-like basic proteins (Spicer, 1962). Evidence has also been presented (Spicer, 1962) for the presence of strongly basic, histone-type, protein (mucihistone) in sites containing acid mucopolysaccharides. An attempt to identify basic proteins in formalin-fixed plerocercoids with Biebrich scarlet at pH 9.5 was unsuccessful. Only the calcareous corpuscles reacted consistently and although a portion of the cuticule stained lightly this can not be regarded as significant

in the absence of a nuclear reaction. When the same staining method was applied to Carnoy-fixed material a vivid orange-red response was given by the nuclei and by the cuticular surface. The most intense nuclear response was given by the testes and ovaries. This response was of a dense red nature and included the entire nuclear structure. The response of the tegumental cell nuclei was equally vivid but of a finely granular nature. The cuticular surface stained brilliantly over a narrow band which covered both the velum and superficial cuticle and which extended completely into the pore canals. A positive response may also have been given by the basal organelle of the pore canal but this could not be determined with certainty as almost all of the cuticle had separated from the subcuticular tissue in the Carnoy-fixed material. The calcareous corpuscles were also reactive, particularly in the membrane surrounding the corpuscle and in the corpuscle-cell nucleus.

A general positive response to the Mercury orange reaction for protein sulphydryl groups was given by all tissues. The only indication of local concentrations was observed in the cuticular surface of a 263 mg plerocercoid but this was not regarded as significant and no further controls were carried out for this reaction.

(5) Distribution of lipids.

The Sudan black B and oil red O methods were applied

to frozen sections in order to reveal the presence of any concentrations of neutral lipids in the cuticle. All plerocercoid tissues responded to these stains in a moderately intense but non-specific manner. The vitellaria did, however, give a granular brilliant red response with the oil red O method, indicating the presence of free lipids in these organs.

Where formalin-fixed, paraffin-embedded tissue is used, positive results with the Sudan black method signify "formol-fixed" lipoprotein whose lipid component is usually phosphatide or cerebroside (Pearse, 1961). The cuticular matrix was positive with this method, particularly in worms of less than 90 mg (see Figures 21 to 23). The most intensely positive results were given by the cuticle of a 10 mg plerocercoid while the cuticle of plerocercoids of 150 mg was only faintly positive. In all formol-saline fixed worms examined the calcareous corpuscles were positive (see Figure 22) but all parenchymal tissues, the subcuticular membrane and the pore canals were negative or only faintly positive.

Although the cuticular response did not appear to be localized, the previously described surface layer of the cuticle was clearly visible in sections stained with Sudan black. The visibility of the surface layer appeared to be more a result of different refractive properties than of stain affinity.

It should be noted that formol-calcium is a preferred

fixative for the Sudan black method for bound lipids. Much more vivid staining did result when tissue was fixed in formol-calcium rather than in formol-saline or formol-alcohol (see Figure 24).

RESULTS OF ELECTRON MICROSCOPY

(1) Morphology of differentiated plerocercoids.

As described above (see Figure 2) the cuticular matrix appears with the light microscope as a homogeneous layer, relatively thick in the superficial regions and much thinner in the velum, penetrated at intervals by pore canals, possessing a surface region of papilla-like "cuticular processes" and microtriches and terminated basally by a subcuticular membrane which is 2-3 μ thick.

Electron microscopy revealed a much more heterogeneous morphology (see Figure 33). The superficial cuticle was thrown into a series of folds or cuticular processes which were continuous with the general cuticular matrix (Figures 25 and 33). The cuticular processes were typical of all plerocercoids from 18-300 mg but none could be observed in plerocercoids of less than 10 mg (see Figure 35). The cuticular processes varied in height from about 0.7 to 4 μ and were spaced at intervals of 1-3 μ. Although considerable variation in size and spacing of cuticular processes was observed in individual plerocercoids, the processes from a specific region were strikingly uniform in this respect. No data have been collected on variation in cuticular processes but it would appear at this time that the largest processes were found on the anterior portion of the superficial cuticle in the region overlapped by the velum (i.e., the same

region demonstrating intense enzyme activity). In some instances the bases of the cuticular processes were constricted giving the structures a flask-like shape (Figure 25). The matrix of the cuticular processes was continuous with that of the general cuticle and microtriches originated from the adjacent cuticular surfaces. An unusual type of cuticular process was observed on the posterior segments of a 94 mg plerocercoid. These cuticular processes were loosely organized and surrounded by quantities of a mucous-like material. Light microscope observations have indicated that large, loosely organized cuticular processes of this type may be characteristic of the more anterior and posterior segments. No cuticular processes have been observed in the velar region of any specimen.

The cuticular microtriches were present in both the velum and the superficial regions of the cuticle (Figure 25). The velum microtriches were shorter and appeared more rigid than those of the superficial cuticle. The microtriches of S. solidus differed in some respect from those of Hymenolepis diminuta (Rothman, 1963), Dipylidium caninum (Threadgold, 1962) and Proteocephalus pollanicoli (Threadgold, 1965). They were much longer, reaching lengths of 10 μ and more and the electron dense distal half did not lie at an oblique angle to the proximal portion as described for P. pollanicoli (Threadgold, 1965). In S. solidus the surface plasma membrane covered the entire microthrix (Figures 27 to 34). The basal region

contained a dark core possibly formed by a ring of twenty fibers (see Figure 26). The distal portion was ovoid rather than spherical or angular and had a central zone of closely packed fibers surrounded by a zone of electron lucid material (see Figure 27). As yet no projections have been observed passing from the base of a microthrix into the cuticular matrix.

The cuticular matrix was composed of many closely packed vesicular structures, each of which was bounded by a double membrane. These vesicles were of two general types: a large ovoid type with a membraneous electron lucid interior and a smaller rod-like type with an electron dense interior. Both types of vesicle may be seen in Figure 28. In some areas the vesicles were arranged in stacks while in other areas they were randomly oriented, but there was no evidence of any regional concentration of one type or the other within the cuticular matrix of large plerocercoids. There was, however, a greater concentration of the rod-like vesicles in the cuticular processes than in the cuticular matrix. It would also appear at this time that the rod-like vesicles were characteristic of small plerocercoids and the ovoid type appeared later in development. Little could be determined about the morphological nature of the cuticular ground substance, but the cuticular vesicles appeared to occupy more of the matrix volume in the larger plerocercoids. No evidence of layering of the cuticular matrix could be seen

in electron micrographs.

Mitochondria were generally located in the basal region in larger plerocercoids while in early developing forms the mitochondria were located throughout the cuticle, were larger and had a swollen appearance. Other mature cuticular inclusions included myelin figures (see Figure 29), small pinocytotic-like vesicles and occasional membraneous channels.

No evidence has been obtained which would support the hypothesis that the cuticle is subdivided into compartments in the manner of a typical epithelium. This is in contrast to the results obtained by Threadgold (1962) in a study of D. caninum, but in accordance with Threadgold's (1965) observations on the tegument of P. pollanicoli.

The ultrastructure of the pore canals of S. solidus is illustrated in Figures 29, 30 and 31. Microtriches arose from the cuticular surface bounding the sides of the canals but not from the terminal region. A common feature of the terminal region of pore canals observed was an aggregation of electron lucid droplets (see Figure 31), and in some instances, the lumen of the pore canals contained a mucous-like substance (Figure 30). Unlike D. caninum (Threadgold, 1962), no membraneous structures could be observed crossing the lumen of S. solidus pore canals.

The cuticle was connected, at intervals, to the parenchymal portion of the tegument by cytoplasmic tubules. These tubules, which were vacuolated or solid, contained

numerous mitochondria (Figure 32). A basal plasma membrane could not be detected butthe basal region of the cuticle was penetrated, to varying depths, by numerous small channels which were continuous with the substance of the subcuticular membrane.

Flame cells which were commonly observed in the parenchyma resembled those described for other helminths (Rothman, 1963; Pantelouris and Threadgold, 1963). Each "flame" contained about 80 cilia, each of which displayed the 9 + 2 filament pattern typical of these structures.

Although no attempt was made to differentiate between parenchymal cell types, certain features were commonly observed. A typical nuclear structure was seen in glutaraldehyde-fixed and uranyl acetate stained tissue (Figure 37). The chromatin was recognisable as dense granular material located around the nuclear periphery and a double nuclear membrane with pores was evident. In some instances ribosomes could be seen on the nuclear membrane and in at least one instance the outer nuclear membrane appeared continuous with the endoplasmic reticulum. Granular endoplasmic reticulum was found in all parenchymal cells observed. In Figure 37 the portion of the granular endoplasmic reticulum located along the plasma membrane had ribosomes only on the internal membrane. Figures 38 and 39 illustrate a type of endoplasmic reticulum observed in the parenchyma of the two plerocercoids. This type of endoplasmic reticulum is also found in protein-secreting glandular cells of vertebrates (Fawcett, 1966).

(2) Morphology of undifferentiated plerocercoids.

Plerocercoids of less than 5 mg had a cuticular depth of approximately 3 μ and a subcuticular membrane about .2 μ thick. The microtriches were identical to those seen in larger plerocercoids and attained a length of at least 10 μ. The cuticular surface showed no indication of cuticular processes. Some micrographs showed the presence of many small surface invaginations, resembling pinocytotic vesicles. Large vesicles (about ½ μ in diameter) could also be seen in the cuticular matrix; usually in the outermost 1/3 of the cuticle (Figure 35). These vesicles did not have a structured interior and their electron density was identical with that of the extracuticular area. They were, however enclosed by a double membrane and appeared to be of mitochondrial origin (see Figure 34). Myelin figures could also be seen scattered evenly throughout the cuticular matrix in much greater numbers than observed in any of the larger plerocercoids. Structures resembling the myelin figures of the cuticle were observed in the cytoplasm of the tegumental cells and in the cytoplasmic tubules connecting these cells to the cuticle.

The rod-like electron dense vesicles appeared to predominate in the cuticle of small plerocercoids, in some instances to the almost total exclusion of the larger electron lucid type. Although these results are tentative it did appear that the cuticle of very small plerocercoids was characterized by the presence of these rod-like vesicles,

with the electron-lucid type appearing later in development. Examination of the cuticle of a 22 mg plerocercoid revealed the presence of larger numbers of rod-like vesicles in the cuticular processes than in the adjoining matrix. A greater number of the rod-like vesicles than of the electron-lucid type was also visible in the velum of the worm. As described previously, the cuticular processes of much larger plerocercoids also seemed to contain a high proportion of the rod-like vesicles.

Numerous mitochondria were observed throughout the cuticle of the small plerocercoids and although some concentration at the base existed it was not as marked as in larger forms. In addition, many large membraneous structures resembling mitochondria were located in the basal region of the cuticle of a 1.5 mg plerocercoid. The exact nature of these structures has not, however, been determined.

No pore canals have yet been found in plerocercoids of less than 5 mg but several structures resembling the basal organelles have been observed in the cuticle of 1 and 4 mg plerocercoids. This may indicate that the pore canals are in the process of formation in these small worms.

(3) Observations on embedding and fixation techniques.

No differences could be detected between material embedded in epon and that embedded in araldite and both were judged satisfactory. Tissue fixed in osmium displayed

a typically granular nature in electron micrographs but it is not possible at this time to state whether this result was a property of the fixation or a result of faulty staining. Figure 39 illustrates the granular appearance of osmium fixed material. Material fixed in glutaraldehyde and post-fixed in osmium (see Figure 38) did not display this artefact but in all other respect gave identical results. Figures 38 and 39 show the essentially identical morphology of mitochondria and endoplasmic reticulum when tissues were fixed with the two different procedures.

In contrast, fixation in paraformaldehyde and embeddment in Maraglas resulted in a morphology significantly different from that described above (see Figure 33). Detail of cuticular membranes was much clearer but the general contrast was less. The plasma membrane surrounding microtriches could be very clearly seen but the proximal fibrous core and the fibres of the distal portion were very indistinct. There also is reason to believe that paraformaldehyde fixation made visible a layer, possibly mucoprotein, attached to the surface plasma membrane (see Figure 36). Improved mucoprotein fixation in the paraformaldehyde fixed tissue would possibly explain the presence of a reticular content in the electron lucid vesicles. The presence of cuticular mucoprotein components is indicated by the results of histochemical tests (see page 24).

(4) Observations on staining techniques.

Although most of the material observed was stained with lead citrate, considerable difficulty was experienced with contamination, a problem more likely the result of errors in technique than of stain properties (see Pease, 1964, page 224). The most satisfactory results were obtained with use of uranyl acetate and lead acetate since this technique produced excellent staining of membraneous components and a particularly vivid chromatin staining (see Figure 37).

DISCUSSION

These results, as do those of previous workers, suggest the hypothesis that the cestode tegument is an actively metabolizing cellular tissue of unique structure and composition. It would appear that the tegument is composed of an outer syncytial envelope which encloses the worm and is directly connected by cytoplasmic bridges to nucleated regions in the parenchyma.

Threadgold (1962) has remarked that the cestode tegument resembles a "peculiarly everted gut" and although it would be a mistake to dwell excessively on this analogy, certain similarities of structure and function are apparent. The cuticle must, of necessity, function as an absorptive organ in the same manner as the intestinal epithelium and conversely it must also protect itself from the action of digestive agents. There is, though, no indication that the cuticle is capable of producing such agents (Read and Simmons, 1963), a fact which must be considered in any comparison of the two tissues.

The cestode microtriches have been compared to microvilli and it has been hypothesized (Threadgold, 1962; Rothman, 1963; Race et al., 1965) that they may perform similar absorptive functions. These workers also suggest a function, involving an interdigitation of the dense distal ends of the microtriches with the microvilli of the host's

cells and effecting the worm's position. Threadgold (1962) has suggested that the microtriches may have an abrasive function and thus, destroying host cells, provide high nutrient concentrations in the immediate area of the parasite. The long microthrix typical of the superficial portions of S. solidus would seem to offer no advantage for any of these functions. The relatively great increase in length of the electron dense distal portion would sacrifice rigidity for no gain in absorptive area. It may be that the prime function of the microthrix border of S. solidus is the creation of a microenvironment which prevents the dispersal of a protective mucin coat. Support for this hypothesis is found in the observation that a material, considered (on the basis of its morphology and from histochemical evidence) to be a mucopolysaccharide was frequently demonstrable in micrographs, as shown in Figure 33. It should be emphasized that I do not consider this mucin coat to be identical to the thin surface layer of sulphated mucoprotein which has been demonstrated histochemically. Threadgold (1965) described inward projections of the bases of some microtriches and remarked that they were morphologically reminiscent of ciliary basal bodies. A single micrograph of the cuticular surface of S. solidus published by McCaig and Hopkins (1965; page 268) showed an apparent continuation of the central core of microthrix fibers into the cuticular matrix, although the point was not noted by the authors.

The cuticular processes represent a feature peculiar to S. solidus among the cestodes described thus far. Structures resembling the cuticular processes have been described for P. pollanicoli (Threadgold, 1965) but they are apparently neither as numerous nor as structurally significant as those reported herein. The cuticular processes of S. solidus are not fully developed until the plerocercoid has reached an infective size (at least 10 mg; Hopkins and McCaig, 1963) and they may have an adhesive function in the final host. Since S. solidus has no functional holdfast organs the cuticular processes could function in this manner by interdigitating with intestinal folds in the final host. In addition, it would be difficult to describe a function for them in the stickleback coelome. Finally, it is possible that these superficial structures may increase the surface area for absorption in a manner analogous to intestinal villi.

The ultrastructure of the pore canals of S. solidus described herein differs markedly from that reported by Threadgold (1962) for D. caninum and Rothman (1963) for H. diminuta. At variance with my observations for S. solidus, the pore canals of D. caninum apparently lie between sections of tegument, have no microtriches originating in the lumen and terminate on the subcuticular membrane (Threadgold, 1962). The pore canals described by Rothman (1963) are obviously unrelated morphologically to those of S. solidus and possibly represent structures similar to the electron lucid cuticular vesicles (see page 35). That all three types of "pore canals"

are related is unlikely and a clarification of what actually constitutes a pore canal must await further study.

On the basis of morphological evidence the pore canals of S. solidus may be considered as secretory. The intense PAS reactivity and the toluidine blue orthochromasia displayed by the basal organelles would indicate that the droplets observed with the electron microscope are composed of neutral (non-acid) mucopolysaccharides. In addition, the histochemical analysis demonstrated that the bases of the pore canals represent the only areas of the cuticular surface which are free of oriented negative charges and are in close proximity to the parenchyma. As such, these areas would offer less interference to the uptake of certain large molecules which might otherwise be excluded by cuticular acid mucopolysaccharides. There is, however, the further possibility that the pore canals are actually the sites of secretion of the cuticular sulphated acid mucopolysaccharides. The manner in which the cuticular surface stains with azure A at pH 1.5 and with aldehyde fuchsin indicates the presence of a thin layer of sulphated acid mucopolysaccharide. The resistance of this layer to digestion with testicular hyaluronidase could be explained by the presence of chondroitin sulphate B, although biochemical analyses would be necessary to obtain a definite identification. Biebrich scarlet staining has shown that strongly basic histone-like proteins are also found at the cuticular surface and through the depth of

the pore canals. Spicer (1962) has shown that this type of protein is often associated with acid mucopolysaccharides. Spicer (1962) has also indicated that the weak azurophilia of certain intestinal goblet-cell sulphomucins may be due to masking of sulphate esters by combination with a basic moiety. A sulphomucin-basic protein complex of this type might explain the lack of azurophilia displayed by the basal organelles of the pore canals. Further strength for this hypothesis is provided by the observation that pore canal reactivity with Biebrich scarlet may have included the basal organelle. A complex of this type would explain the variable metachromasy displayed by the most superficial 1-2 μ of the cuticular matrix of many plerocercoids. However, further work will be necessary to clarify this possibility.

If the superficial portion of the cuticular matrix does contain a sulphomucin-basic protein complex it is apparent that free sulphate groups are found only on the extreme surface of this layer. Monne (1959) has demonstrated the presence of acid mucopolysaccharides on the cuticular surfaces of several tapeworms and has suggested a possible role as host enzyme inhibitors for these substances. Crompton (1963) has detected a similar layer of acid mucopolysaccharides on the surface of the acanthocephalan worm Polymorphus minutus. Bogitsh (1963) also assigns a protective function to the layer of acid mucopolysaccharides observed on the cuticle of Hymenolepis microstoma. However, Waitz and

Schardein (1964) do not report this acid mucopolysaccharide coat in their study of H. nana, H. diminuta, Hydatigera taeniaeformis and Dipylidium caninum. If the acid mucopolysaccharide coat of S. solidus does in fact have a protective function it is of some interest that this coat is already present in the plerocercoid which exists in the colelomic cavity of the stickleback rather than in the intestinal habitat of the mature form.

It may well be that the inhibitory activity of the mucoprotein layer varies with the pH of the surrounding medium, i.e., from approximately neutral in the stickleback coelomic cavity to acid in the bird intestine.

If such a variation in the enzyme inhibitory activity of the sulphomucin-basic protein complex did exist, this could also explain the concurrent existence of such a complex with surface cuticular enzymes. It is probable that the cuticular enzymes (acid and alkaline phosphatases, and possibly others) are functional during the growth of the plerocercoid in the stickleback coelome, at a pH where the sulphomucin-protein complex might not exhibit inhibitory action. Since nutrients are not absorbed during the short adult life of the worm the negation of any absorptive functions of these enzyme systems by mucoproteins could be tolerated.

It would, perhaps, be profitable to mention some other properties displayed by polyelectrolytes of the type possessed by the cestode cuticle. At acid pH's ($<$ pH 6)

polyacids such as polyacrilate exist in a relatively contracted form due to the numerous hydrogen bonds present between the carboxylic groups. If some of the acidic groups are ionized by the addition of a base, repulsive forces develop between the charged carboxyl groups and the chain tends to unfold and lengthen (Katchalsky, 1964). Such a phenomenon would probably enhance the inhibitory activity of a polyacid by increasing the number of exposed charged groups, an increase which would be dependent on the surrounding pH. Basic and acid polyelectrolytes also may have separate enzyme inhibiting activities (Katchalsky, 1964). For example, the action of pepsin is inhibited by basic polymers and restored by the sulphomucin heparin. On the other hand, the activities of hyaluronidase and ribonuclease are inhibited by polyacids and their activity is restored by polybases (Katchalsky, 1964).

The histochemical properties of the bulk of the cuticular matrix indicate that this region also contains significant quantities of acid mucopolysaccharides - probably hyaluronic acid. It is likely that hyaluronate, probably associated with protein, forms the cuticular ground substance within which the cuticular vesicles are located. A hyaluronate matrix could function in both structural and physiological capacities. The large hydrodynamic specific volume and accompanying turgescence characteristic of hyaluronate would endow the cuticle with a certain rigidity

as well as providing a control over random diffusion of large molecules. It has also been hypothesized (see Rogers, 1961) that hyaluronate (in addition to other mucopolysaccharides) may function as an exchange resin and regulate the flow and concentration of cations.

This is to my knowledge the first demonstration of acid mucopolysaccharides in the cuticular matrix. Waitz (1963) could detect no toluidine blue metachromasia in the cuticle of adults and larvae of Hydatigera taeniaeformis; but he did speculate that the observed orthochromatic staining below pH 4.0 might indicate the presence of an acid mucopolysaccharide.

Various phosphatases have been reported from many types of helminths (Ma, 1964). In the majority of cases both acid and alkaline phosphatases have been detected though, in some instances (Yamao, 1952; Lee and Tachell, 1964), no significant acid phosphatase levels could be shown. Alkaline phosphatase appears to be an almost universal constituent of the helminth coat. Studies by Erasmus (1957a, 1957b) on the phosphatases of Taenia pisiformis, Moniezia expansa and Cysticercus tenuicollis agree closely with the results presented for S. solidus. It should be borne in mind that a spectrum of phosphatase isozymes may exist and all may be demonstrated by one histochemical reaction. Ma (1964) has described three acid phosphatase isozymes from the tissue of Clonorchis sinensis.

A great deal of speculation has centered on the role of helminth cuticular phosphatases. Bullock (1949, 1958)

has claimed that phosphatases in the acanthocephalan cuticle are concerned with the absorption of foodstuffs or with the elimination of waste products. Erasmus (1957a, 1957b) suggests that acid and alkaline phosphatases may be involved in the release of phosphate for carbohydrate metabolism or perhaps for phosphorylated passage of materials through the cuticle. Lewert and Dusanic (1960) have shown that the inhibition of alkaline phosphatase activity in Schistosoma mansoni by a diamino-dibenzyl-alkane is correlated with a decrease in in vitro glucose uptake. Similar work has been done by Ponz and Larralde (1952) using phlorizin as an enzyme inhibitor. However, Jervis et al., (1956) have demonstrated that glucose uptake is inhibited by much lower concentrations of phlorizin than are necessary for the inactivation of alkaline phosphatase. Similar results published by Phifer (1960) indicate that phosphates are not necessary for glucose metabolism. The results of this study do not justify any further comment on the functions of cuticular phosphatases.

The distribution of cuticular lipids in S. solidus agrees substantially with that described by Waitz (1963) for H. taeniaeformis. Since the Sudan black staining of the S. solidus cuticle presumably indicates the presence of sphingolipids, the observed decrease in stain intensity in large plerocercoids may reflect a decrease with growth in the quantity of cuticular membranes. Another possibility is that a substantial lipid component may be complexed with

cuticular proteins in the rod-like cuticular vesicles since the decrease in lipid staining with growth is concomitant with that of the protein stain and the quantity of rod-like vesicles decreases with an increase in plerocercoid size. A further indication that the rod-like vesicles may contain a proteinaceous substance is found in the observation that my high resolution micrographs reveal the presence of a lattice-like matrix resembling that seen in certain crystalline cellular inclusions (see Fawcett, 1966).

If, as suggested above (see Page 40), the electron lucid cuticular vesicles contain mucopolysaccharides the increase in number of these vesicles in larger worms would explain the increase in cuticular PAS staining. It should be emphasized that since hyaluronic acid does not give the PAS reaction (Barka and Anderson, 1963) the change in cuticular reactivity must reflect a change in some other cuticular carbohydrates. An increase in the cuticular PAS response of adult H. taeniaeformis over that of larval forms was reported by Waitz (1963) although this increase was confined to the surface region. However, the increase in cuticular carbohydrate composition reported herein must be regarded with some caution since the intense PAS reactivity displayed by the cuticle of larger worms did not consistently occur when further specimens were examined.

This report is apparently the second to indicate the presence of RNA in the cestode cuticle (Rothman, personal

communication). However, Bogitsh (1963) did not find any cuticular RNA in H. microstoma after methyl green-pyronin staining and Waitz (1963) did not attempt to detect RNA in H. taeniaeformis. The presence of cuticular RNA poses certain questions on the nature of cuticular synthetic activities. Lumsden (1965) has shown autoradiographically that labelled amino acids are incorporated in the tegumental cells and then transported to the cuticle. It would be of interest to determine to what extent, if any, synthesis can occur in the cuticle. On morphological grounds extensive cuticular synthetic activities would seem unlikely since there is no indication of ribosome-bearing cuticular organelles and the cuticular mitochondria are comparatively few and located basally in large plerocercoids. It should also be noted that the glycogen deposits, which presumably serve as an energy source for synthetic activities are located at some distance from the cuticle.

On the basis of the available information it seems likely that the cuticle is not active synthetically but rather receives its cellular components from the tegumental cells. It may be, however, that the cuticular mitochondria are involved in the production of ATP necessary for the active transport of nutrients into the tegument from the external medium.

Histochemically and morphologically the cuticle of small plerocercoids appears to be in a state of transition. At the ultrastructural level the cuticle of 1-4 mg plerocercoids

is characterized by large numbers of mitochondria, many of which appear to be in a state of degeneration. The contents of the cuticular vesicles appear to be primarily proteinaceous, in agreement with the histochemical characteristics of the worm. In these small worms no cuticular processes can be seen and there are indications that pore canals are in the process of formation (see Page 39). The cuticular carbohydrate constituents appear to consist of a basal concentration of hyaluronic acid, a layer of sialic acid-containing mucin and a surface layer of sulfomucins.

With progressing development (> 10 mg) the cuticle becomes elaborately structured as the cuticular processes and pore canals are developed. The mitochondria are now basally located and large numbers of electron lucid vesicles are present. As shown by histochemical examination this differentiation is accompanied by a compaction of surface sialomucins and an increase in the PAS reactivity of the matrix. The increase in PAS reactivity is accompanied by a dimunition in the lipoid-protein content and may be related to the change in the relative concentrations of cuticular vesicles.

It must be assumed that many of the above changes are, in some manner, involved in the preparation of the plerocercoid for the short adult existence in the bird gut. The problem of interpreting these changes is complicated, however, by the fact that the ultimate cuticular structure is

only attained long after the plerocercoid is infective.

It may be that the differentiation of the plerocercoid is divided into two phases; (1) an initial period of transition from the procercoid, during which the defensive mechanisms required for survival in the final host are elaborated, and (2) a much longer phase in which the plerocercoid is modified to permit prolonged growth and survival in the stickleback coelom. It would appear from the present study that the major morphological changes occur during the second post-infective phase. Clearly, a more detailed study of the morphological and histochemical events accompanying the first phase of differentiation is required before any judgement of this hypothesis can be made.

SUMMARY

1. This work demonstrates that the plerocercoid tegument of Schistocephalus solidus consists of an outer anucleate but cellular region (the cuticle) which is connected by cytoplasmic tubes to nucleated tegumental cells lying in the sub-cuticular parenchyma.
2. The pore canals of S. solidus have been shown to differ in structure from any previously described for other cestodes. The pore canals of S. solidus allow certain parenchymal cells a direct connection with the external medium. It is suggested on morphological grounds that these cells may function in a secretory capacity.
3. Papilla-like cuticular processes on the cuticular surface have been described and both adhesive and absorptive functions are suggested for these structures. It has been shown that the cuticular processes are not present in the smallest forms studied but rather appear at about the same time as the worm becomes infective.
4. Changes in the histochemical and ultrastructural composition of the cuticular matrix have been described. It is probable that increased plerocercoid growth is accompanied by a change in cuticular composition from a more proteinaceous state to one which is largely carbohydrate.
5. A surface sulphomucin-basic protein complex has been described and partially characterized. Possible functions

have been suggested for this layer.

6. RNA as well as acid and alkaline phosphatases were found in the cuticle and the possible significance of these substances in cuticular synthetic activities is considered.

7. It is suggested that plerocercoid cuticular differentiation may consist of two phases:

   (1) an initial phase in which various mechanisms are elaborated to allow survival in the vertebrate intestine, and

   (2) a second phase in which the plerocercoid is modified to permit prolonged growth in the stickleback coelom.

LIST OF ABBREVIATIONS

(1) Abbreviations for structures:

BF - basal fibrils of microthrix

CP - cuticular process

DP - distal portion of microthrix

ED - electron-lucid droplets of pore canal

ER - endoplasmic reticulum

F - fibrous apical portion of microthrix

M - mitochondrion

MB - myelin figure

MU - mucous-like material

MV - cuticular vesicles presumed to be of mitochondrial origin

N - nucleus

NL - nucleolus

PC - pore canal

PM - plasma membrane

SM - subcuticular membrane

V - velum region of cuticle

VR - vacuolated region of cytoplasmic bridge between external and internal levels

(2) Abbreviations for fixatives used in light microscope studies:

FS - formol-saline

FC - formol-calcium

FA - formol-alcohol

CPC/F - .5% CPC in 10% formalin

Acet. - cold acetone

All weights are expressed in mg, eg. 'FS 138' indicates that the micrograph is of a 139 mg worm which has been fixed in formol-saline.

The abbreviations for stains are given in section E of materials and methods (pages 4-7).

PLATE I

Figure 1. Carnoy-fixed. 5 mg TB. 850X. The orthochromatically-staining surface layer and the non-staining microthrix border are visible. The portion shown is typical of the cuticle of very small worms.

Figure 2. FS 138. AA 4.0. 850X. The entire cuticle is metachromatic with the most intense reaction at the cuticular surface. The basal organelle of the pore canal is clearly orthochromatic. Note also the cuticular processes and the difference in the thickness of the superficial and velar portions of the cuticle.

Figure 3. FC 101. AB-PAS. 225X. Note the relatively intense alcian blue staining in the anterior portion of the superficial cuticle. The subcuticular membrane is PAS positive.

Figure 4. FA 26. AB-PAS. 1,365X. The region shown includes portions of two segments. In worms of this weight there is little difference in the cuticular thickness of the velum and the superficial portion. Three cuticular layers are indicated. I and III are PAS positive while II is AB positive.

Figure 5. FS 90. AF-AB. 135X. The superficial AF positive layer and the AB positive layer can be seen.

Figure 6. FS 212. AF-AB. 850X. The AB positive layer is difficult to distinguish as a separate entity in worms of this size.

Figure 7. FS 65. TB. 1,080X. Several pore canals can be seen receiving processes from parenchymal cells. Note also the orthochromatic surface layer and the difference in orientation of the tegumental cells beneath the velum and beneath the superficial cuticle.

Figure 8. FS 138. MBPB. 850X. Note the relatively intense staining of the surface and of the matrix immediately beneath the sub-surface layer. The bulk of the cuticular matrix has stained lightly with this method.

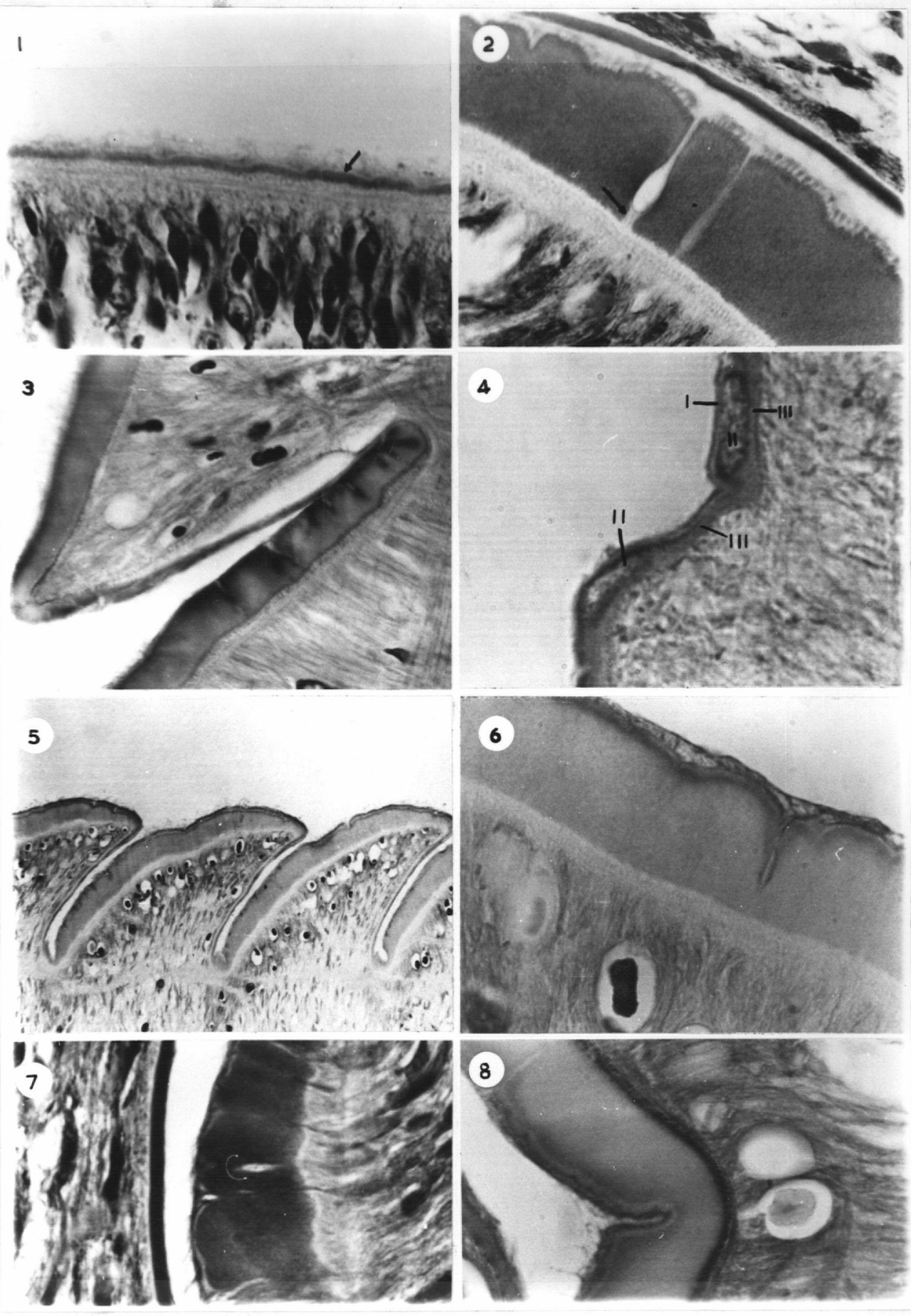
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PLATE II

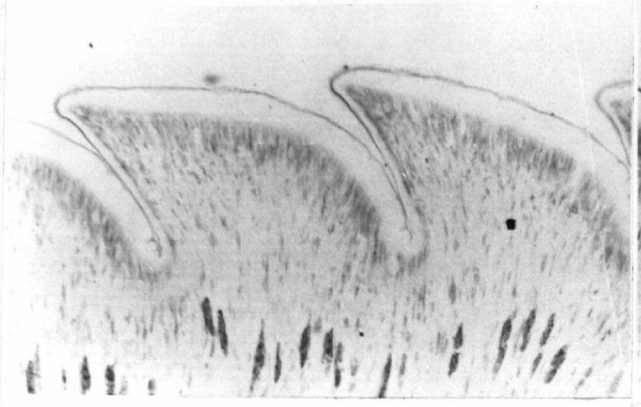
Figures 9, 10 and 11. CPC/F 171. AA 1.5, 2.5 and 4.0. 108X. At pH's of 1.5 and 2.5 (Figures 9 and 10) only the surface of the cuticle reacts with azure A. When the pH is raised to 4.0 (Figure 11) the entire cuticular matrix reacts with the stain.

Figure 12. FS 110. AA 4.5. 340X. At this pH the entire cuticle is strongly metachromatic. The indicated extracuticular mucin reacted consistently with alcian blue when that method was applied.

Figures 13 and 14. FS 123. TB and TB after hyaluronidase digestion. 108X. Treatment with hyaluronidase removed most but not all of the cuticular metachromasia.

Figures 15 and 16. CPC/F 171. TB and TB after RNA'se digestion, 85X. When RNA'se digestion was employed the bulk of the cuticular metachromasia was removed (compare with Figure 14). The reactivity of the surface was not, however, impaired.

9



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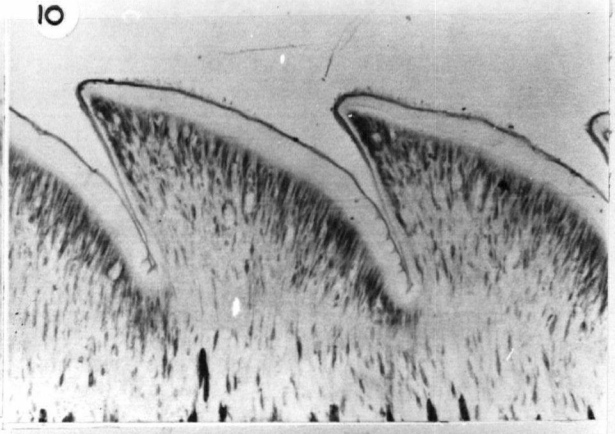

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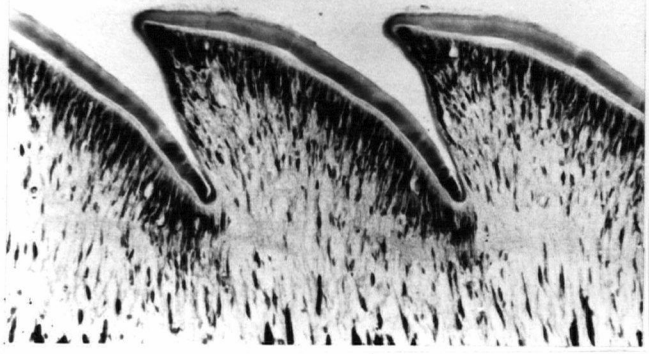

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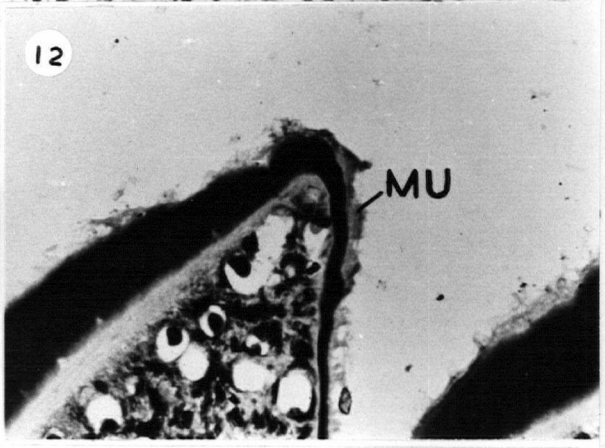

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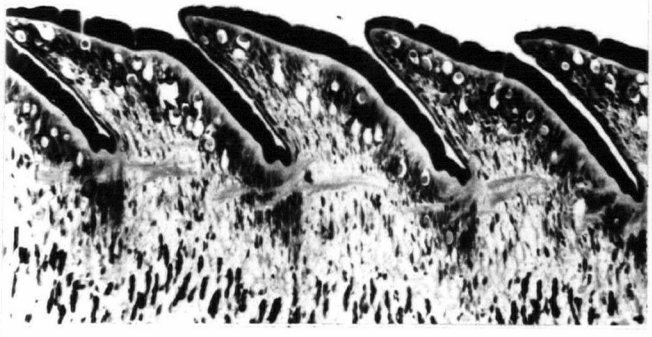

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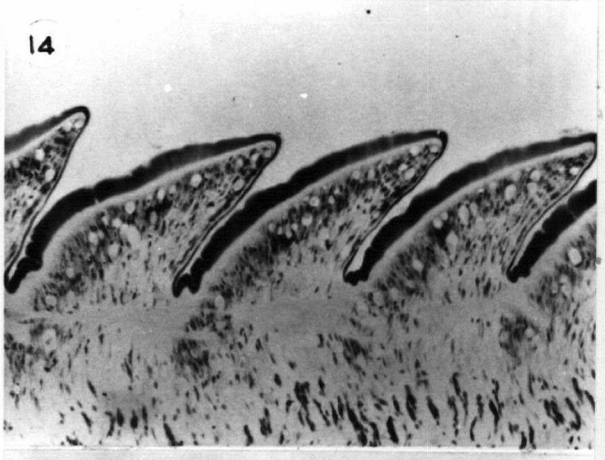

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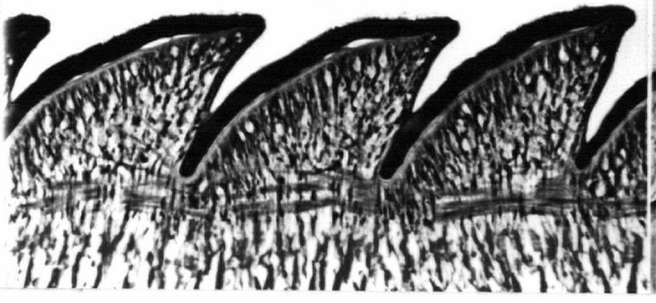

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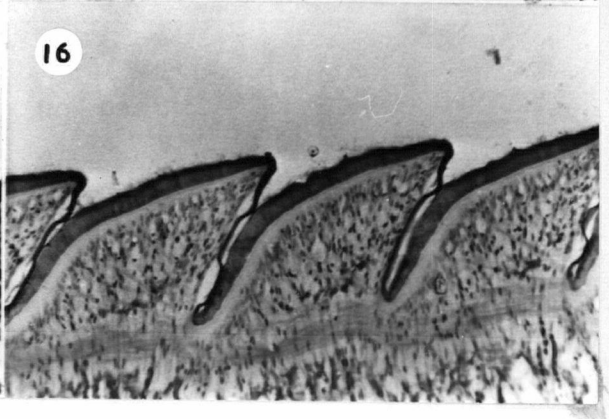


PLATE III

Figures 17 and 18. FS 110. MGP and MGP after digestion with RNA'se. 108X.

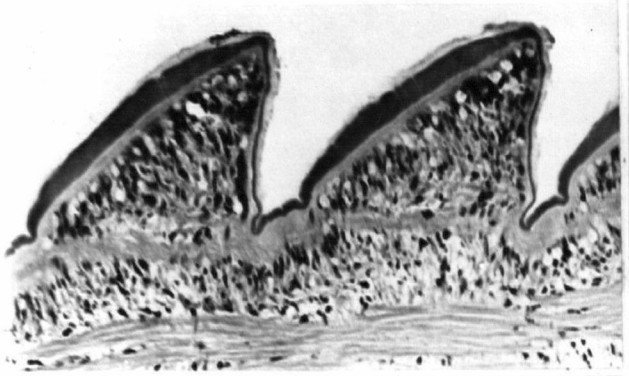
Figure 19. Acetone-fixed mature worm. Azo-coupling method for alkaline phosphatase. 108X.

Figure 20. Acetone-fixed mature worm. Gomori method for acid phosphatase. 108X.

Figures 21, 22 and 23. FA 30, FS 35, and FS 90. SBB method for bound lipids. 85X. Note the decreased lipid staining with increased size. Also note the intense staining of the calcareous corpuscles in FS-fixed worms.

Figure 24. FC 101. SBB. 85X. A comparison of this micrograph with that in Figure 23 will emphasize the improvement in lipid staining which resulted from formol-calcium fixation.

17



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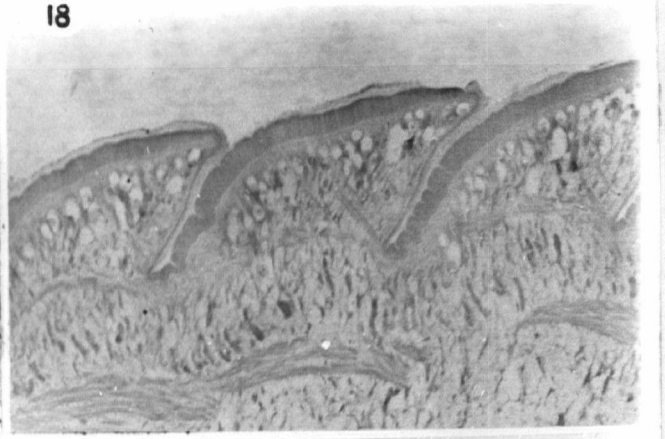

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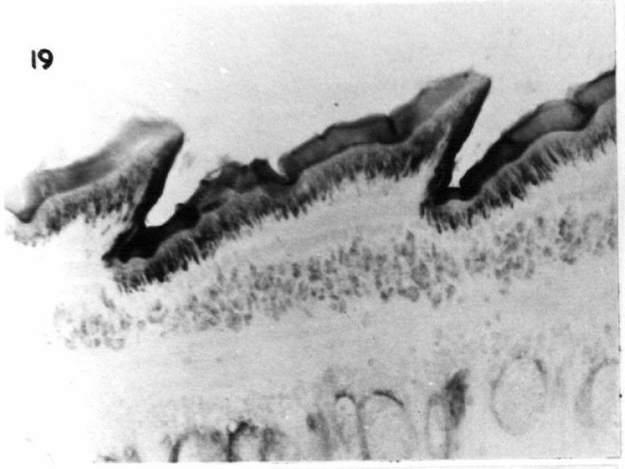

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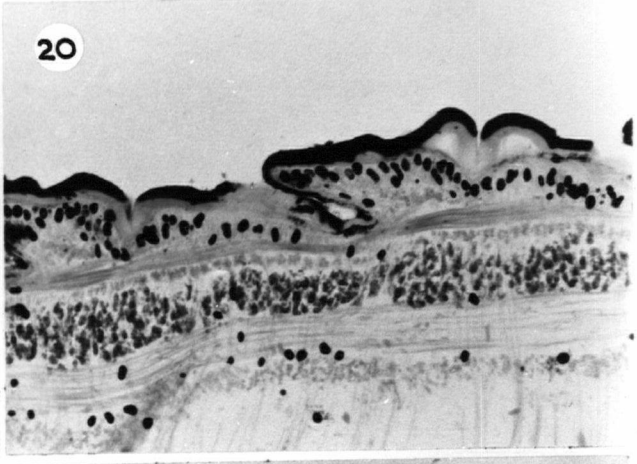

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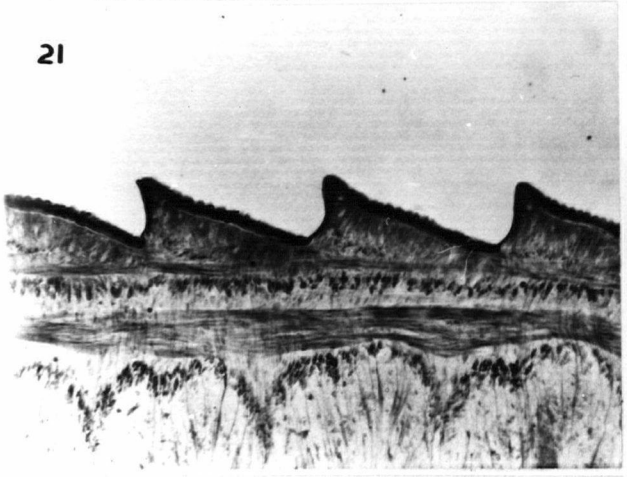

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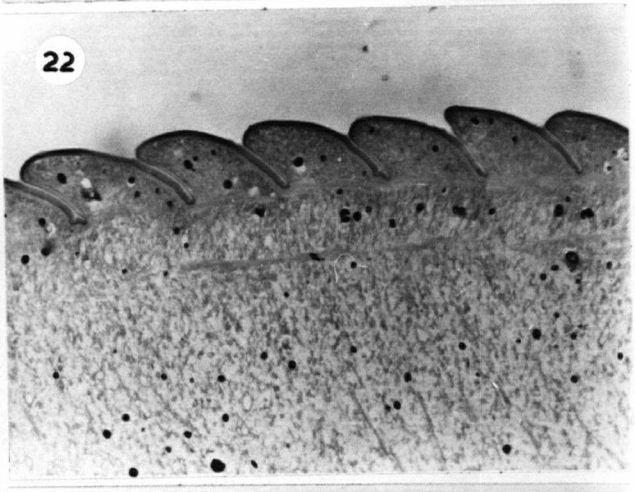

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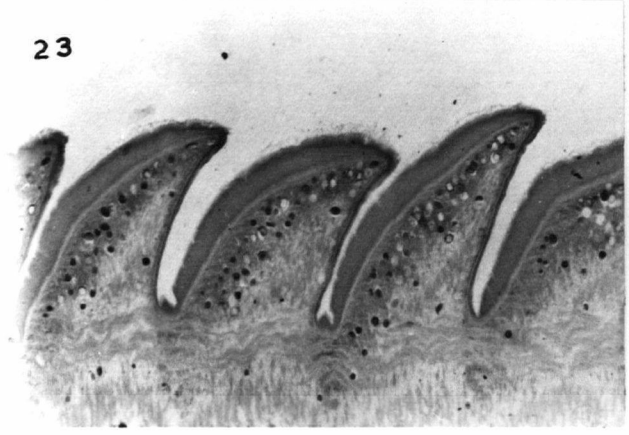

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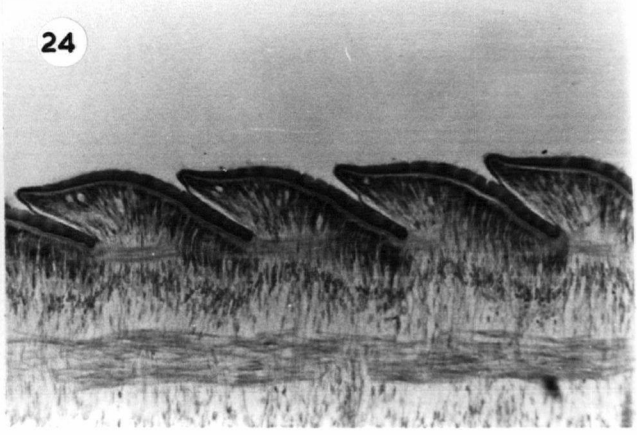


PLATE IV

Figure 25. Section of cuticular surfaces of velum and superficial region of a 22 mg plerocercoid. Note absence of cuticular processes at velum surface and presence of mucous-like material. Glutaraldehyde fixed, O_sO_4 post-fixed (Glut.-O_sO_4). Embedded in Araldite (Ar). 22,000X.

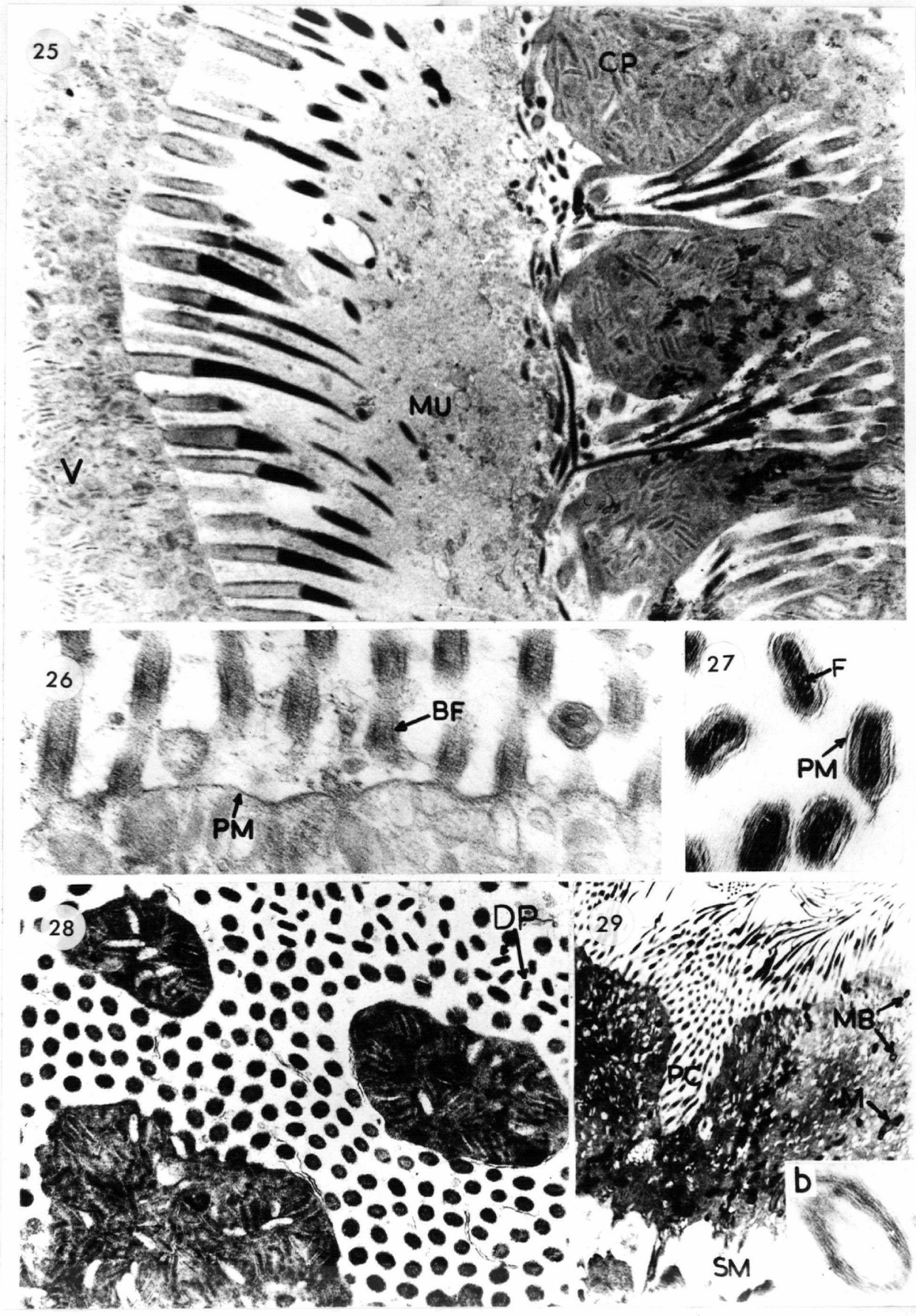
Figure 26. Cuticular surface of a 10 mg plerocercoid showing surface plasma membrane and fibrillar core of basal portion of microtriches. O_sO_4 fixed. Embedded in Epon (Ep). 53,000X.

Figure 27. Section through distal portion of microtriches of a 100 mg plerocercoid illustrating electon-dense fibrous interior, outer electron-lucid region and external plasma membrane. Paraformaldehyde fixed, O_sO_4 post-fixed. (Par-O_sO_4) Embedded in Maraglas (Mar). 127,000X.

Figure 28. Oblique section of the surface of a 136 mg plerocercoid illustrating cuticular vesicles and microtriches. Note circular shape of basal portion of microtriches. Glut.-O_sO_4. Ar. 24,000X.

Figure 29. Section through the cuticle of a 94 mg plerocercoid showing portions of a pore canal, myelin figures and cuticular mitochondria. Note aggregation of electron-lucid droplets at base of pore canal and the microtriches originating from the walls. Glut.-O_sO_4. Ar. 6,000X.

B. Photographic enlargement of a myelin figure from the cuticle of a 10 mg plerocercoid. 57,000X.


25
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b

PLATE V

Figure 30. Basal region of a pore canal from an 18 mg plerocercoid showing electron-lucid droplets and mucous-like material in lumen. Glut.-O_sO_4. Ar. 12,000X.

Figure 31. Detail of the electron-lucid droplets observed in the pore canal of a 27 mg plerocercoid. O_sO_4. Ep. 35,000X..

Figure 32. Section through the subcuticular membrane of a 10 mg plerocercoid illustrating the vacuolated regions of the cytoplasmic bridges. Note that the mitochondria of the cytoplasmic bridges are larger than those of the cuticle. O_sO_4. Ep. 13,000X.

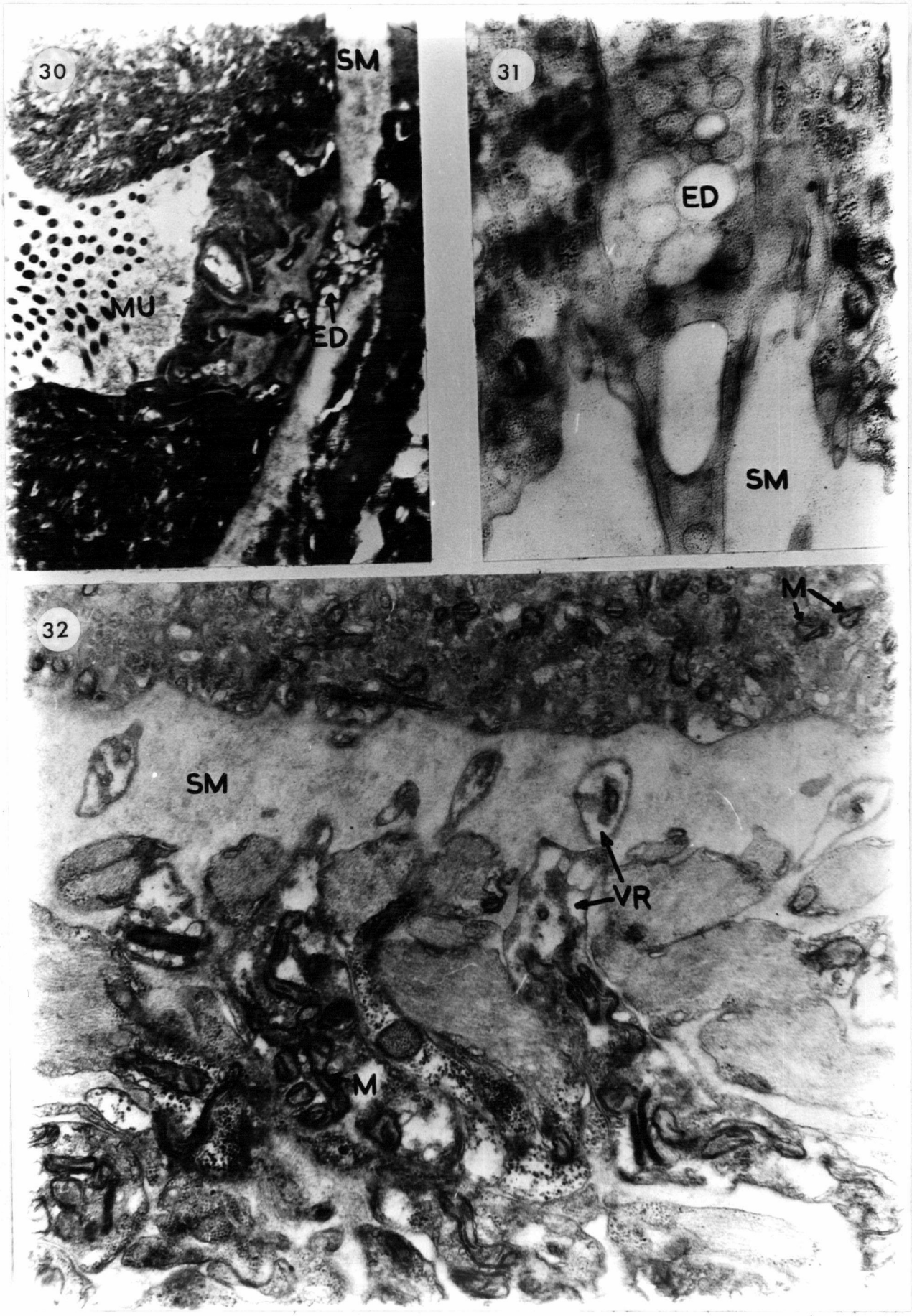
30
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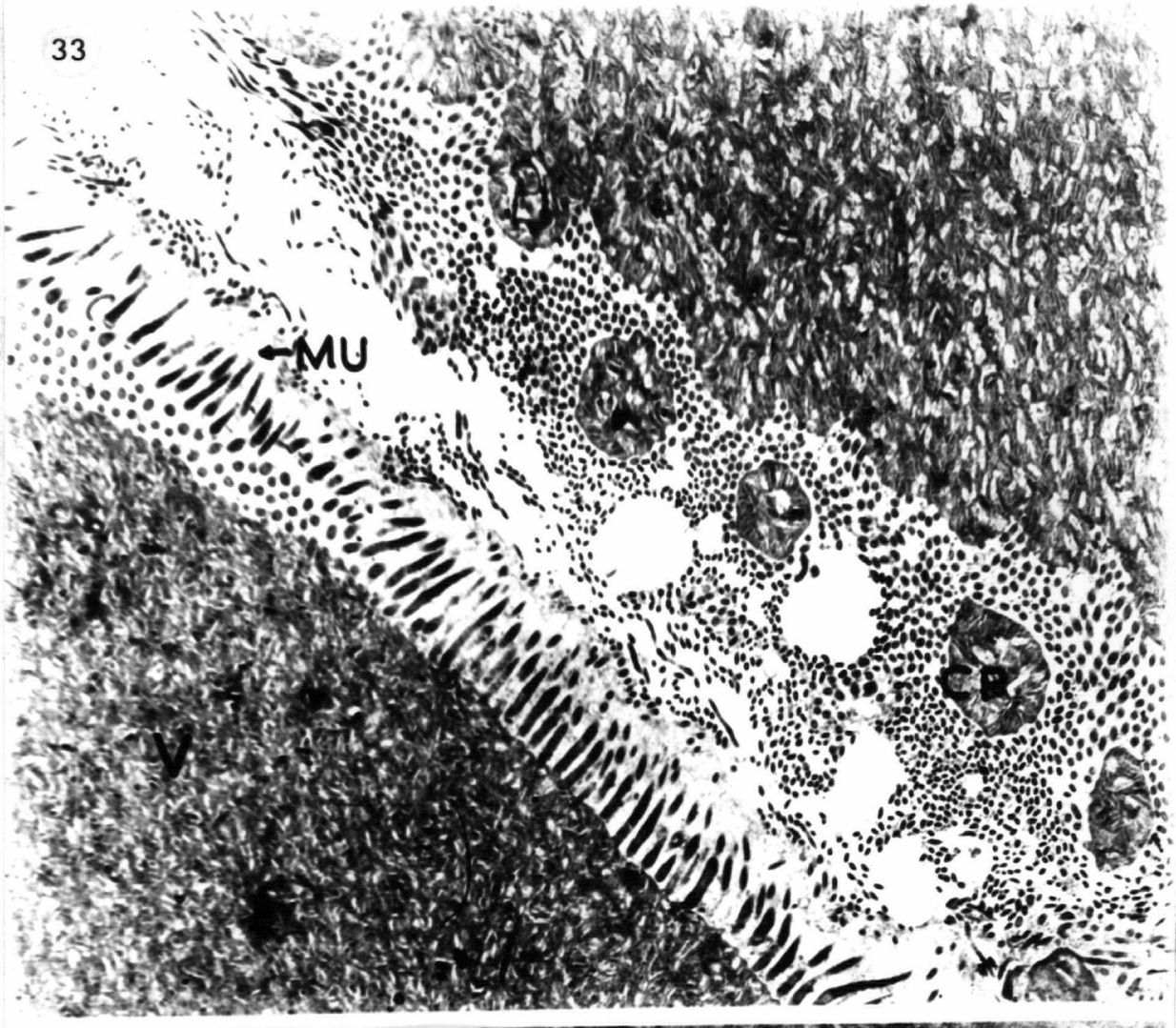
PLATE VI

Figure 33. Section through velum and superficial cuticle of a 100 mg plerocercoid. Note the cuticular vesicles and the presence of extracuticular mucous-like material among the velum microtriches. Par.-OsO_4. Mar. 12,000X.

Figure 34. Basal region of the cuticle of a 1 mg plerocercoid showing detail of vesicles believed to be of mitochondrial origin. Glut.-OsO_4. Ep. 23,000X.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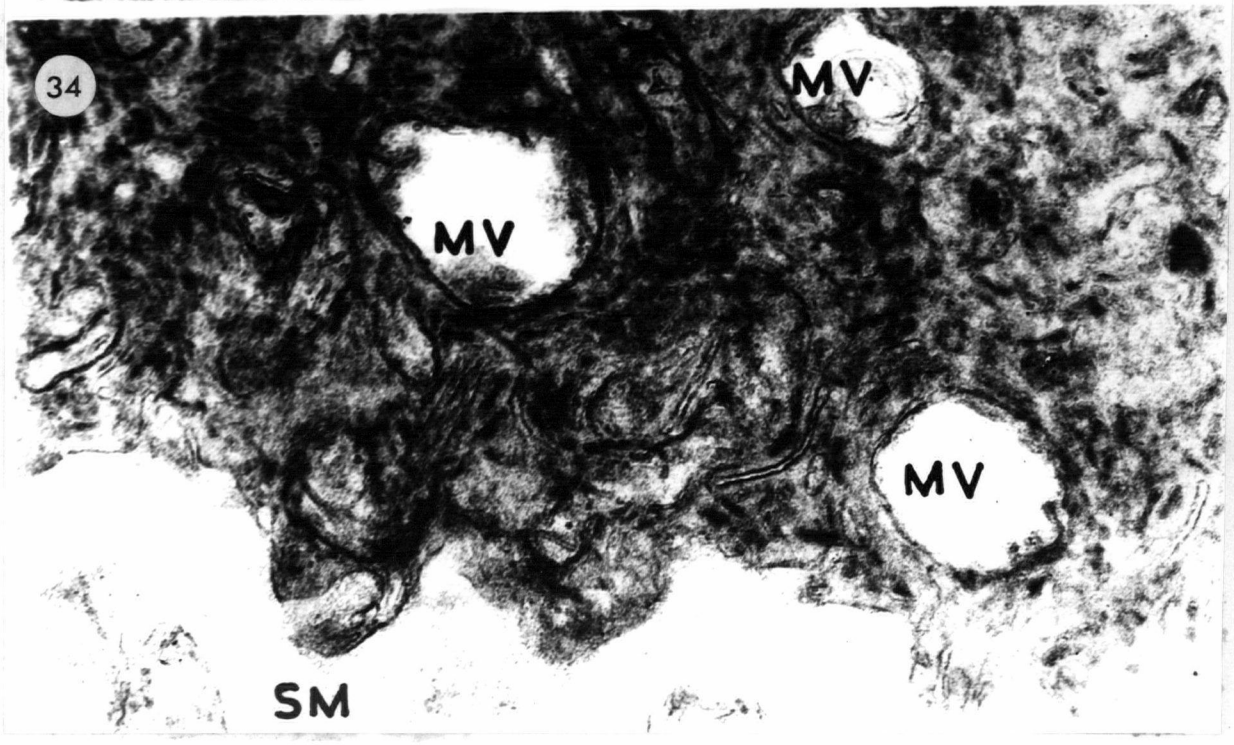
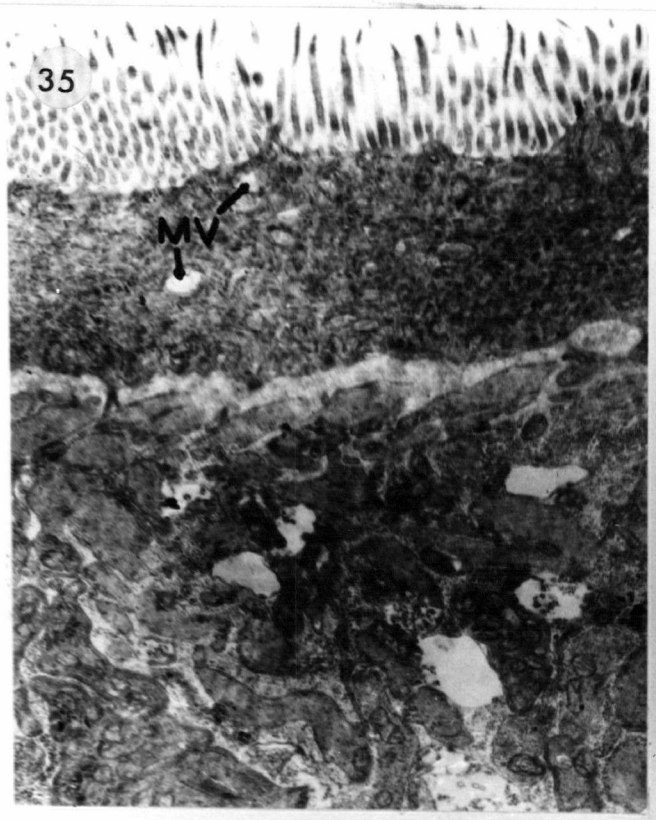

34
MV
MV
MV
SM

PLATE VII

Figure 35. Section through the cuticle of a 1 mg plerocercoid in which large vesicles can be seen in the cuticle. Note the absence of cuticular processes. Glut.-O_sO_4. Ep. 9,000X.

Figure 36. Detail of a microthrix. Note the fibrous nature of the electron-dense distal portion and the presence of a plasma membrane. Material that is assumed to be part of a mucoprotein surface coat can also be seen. Par.-O_sO_4. Mar. 135,000X.

Figure 37. Detail of typical parenchymal cells. Note the extensive endoplasmic reticulum and the typical nuclear structure. 4 mg plerocercoid. Glut.-O_sO_4. Ep. 18,000X.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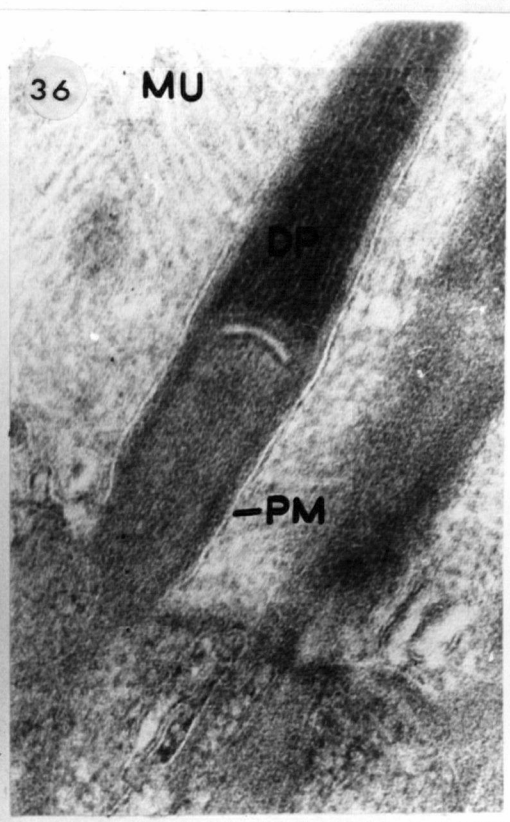
MV


36
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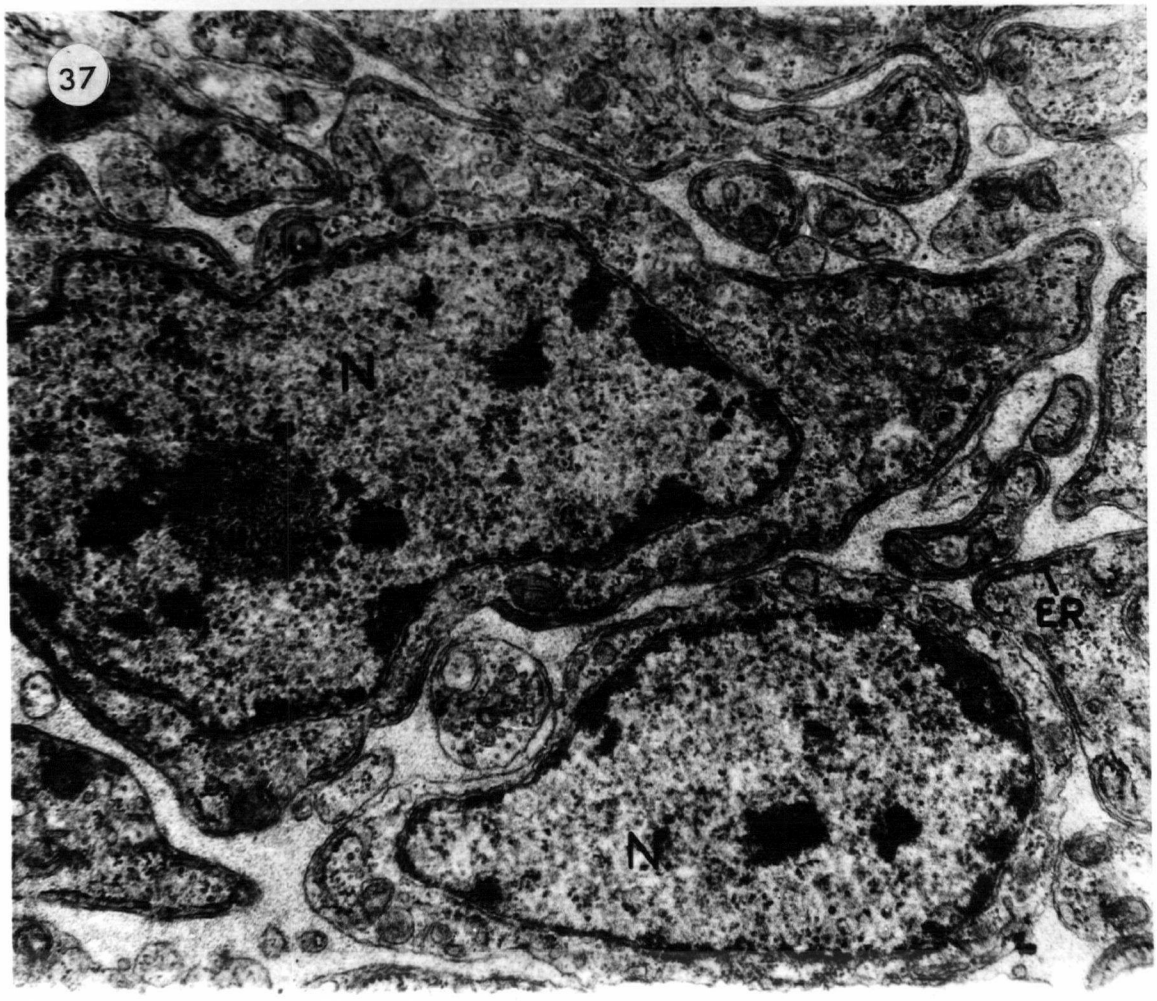
37
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PLATE VIII

Figure 38. An unusual endoplasmic reticulum formation in cells of the parenchyma of a 136 mg plerocercoid. Glut.-O_sO_4. Ar. 35,000X.

Figure 39. Cellular organelles from a 161 mg plerocercoid. Compare the morphology of the endoplasmic reticulum in this figure with that shown in Figure 38. The granular nature of the background was commonly encountered when O_sO_4- fixed tissue was examined. O_sO_4. Ep. 16,000X.


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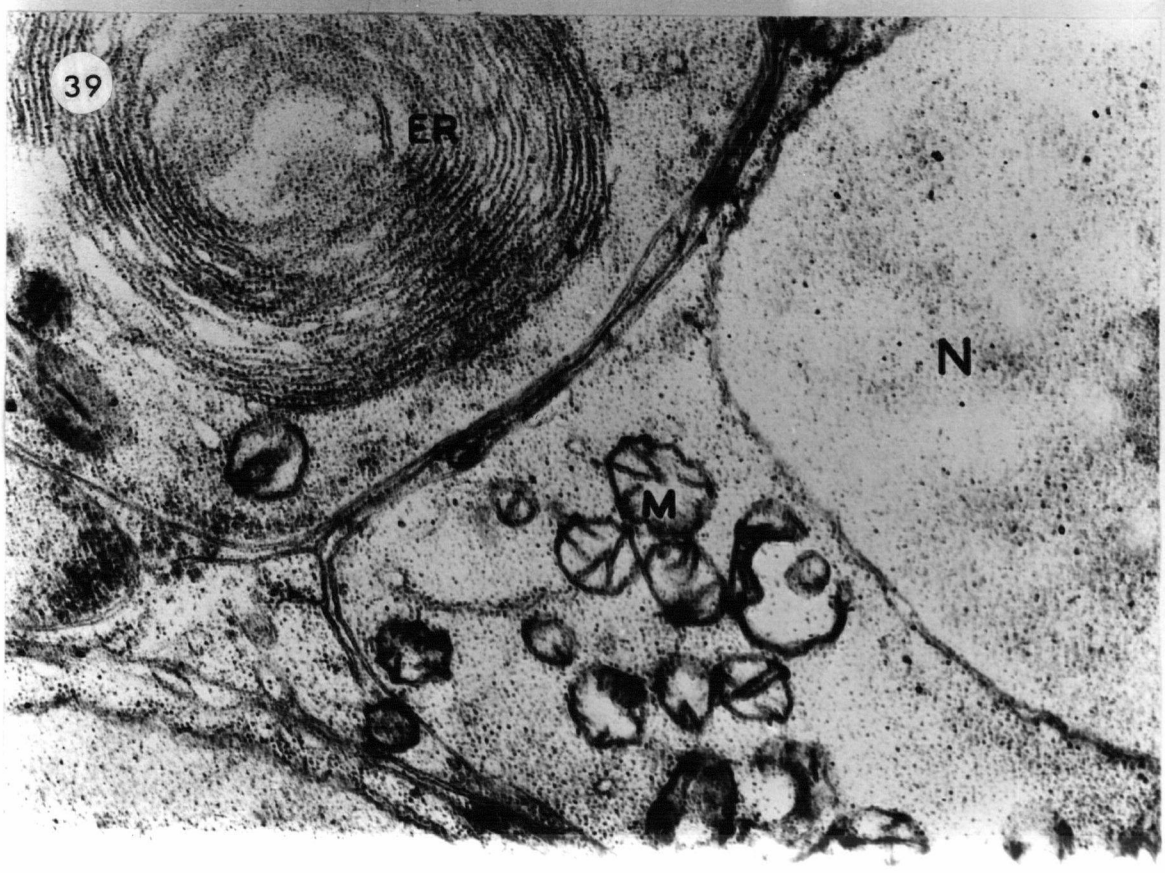
M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APPENDIX A: Outline of the life cycle of *Schistocephalus solidus*.

1. About 20,000 eggs may be shed by an adult worm.

2. Egg with developing coracidium nearly ready to emerge.

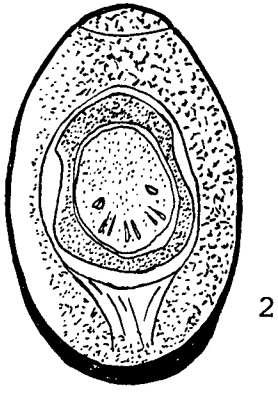
3. Free swimming coracidium with inner oncosphere. The coracidium may emerge within 2-4 weeks after eggs are shed.

4. *Cyclops* is infected by the coracidium. The ciliated layer of the coracidium is lost and a procercoid develops. One hatch of eggs may infect *Cyclops* at intervals over a period of 5-6 months. The infective procercoid form is reached in about 3 or 4 weeks but may survive in *Cyclops* for at least 3 months.
   a. Posterior region of *Cyclops* infected with procercoids.
   b.-f. Stages in the development of the procercoid.

5. Sticklebacks are infected after ingesting *Cyclops*. The cercomer is lost and the plerocercoid develops. The plerocercoid becomes infective at a weight of 10-15 mg but may attain a weight of more than 500 mg.
   a.-c. Various sized plerocercoids showing development of genitalia and proglottids.

6. Genitalia differentiate and eggs form within 2-4 days after the stickleback is ingested by the bird host.

1.



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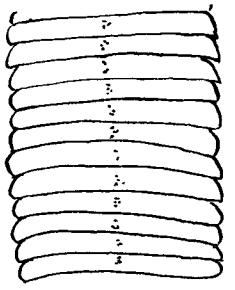

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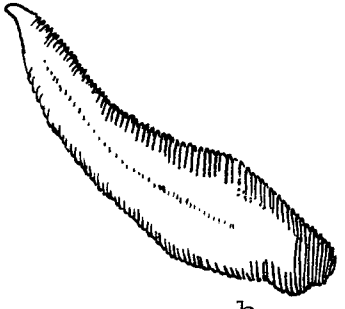
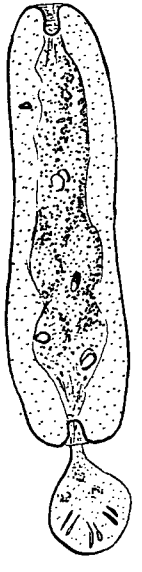


b.

5a.



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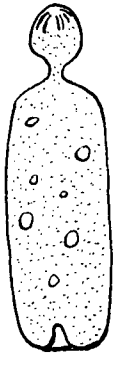
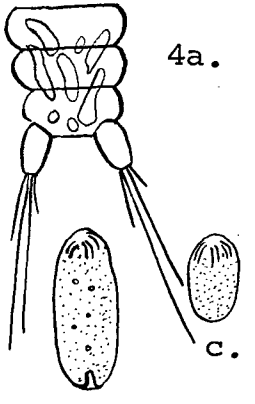


e.



4a.

d.

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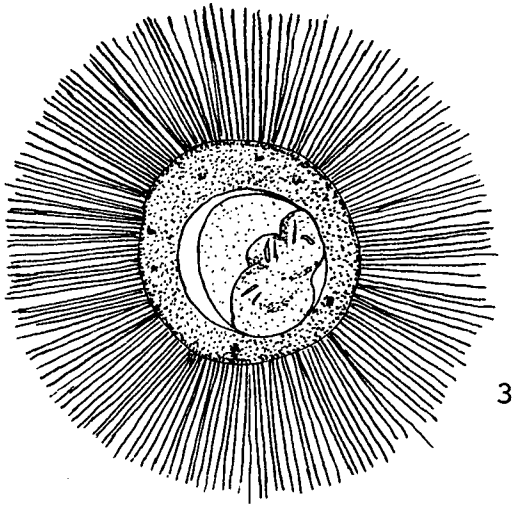
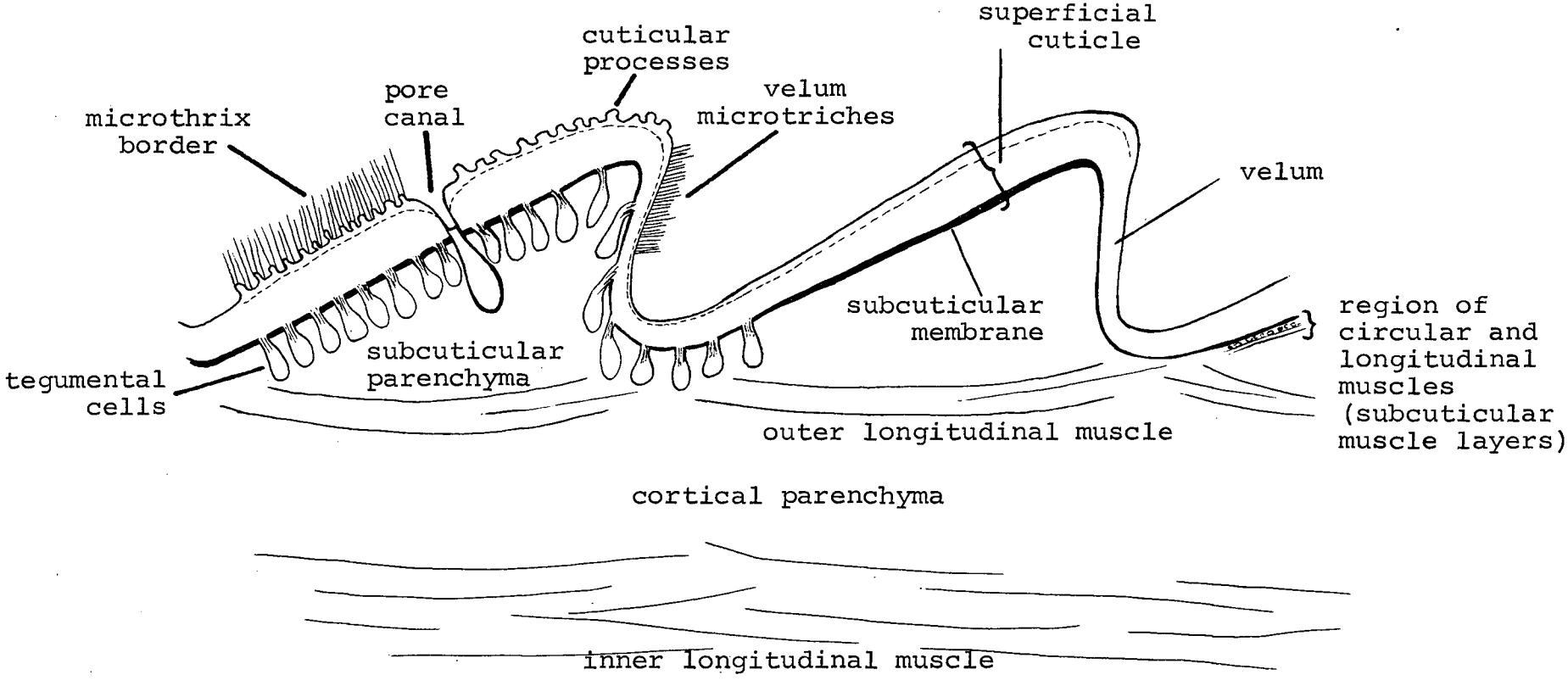


3.

APPENDIX B: Diagram of the structures and regions of Schistocephalus solidus discussed in the text.



The dotted line indicates the approximate location of the alcian blue positive cuticular layer.