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Histochemistry of the Miracidial and Early Redial Stage of *Cyclocoelum oculeum* (Trematoda: Cyclocoelidae)[1]

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ABSTRACT: The miracidia of *Cyclocoelum oculeum* each contain a fully formed redia, and histochemical tests can be performed on both stages simultaneously. Glycogen is present in miracidial epidermal plates, penetration glands and apical gland as revealed by positive staining with Best's carmine, Bauer-Feulgen, and PAS and malt diastase lability. The redial esophageal glands were PAS-positive but resistant to digestion. The apical gland is selectively stained by Victoria blue. The miracidial tegument is covered by a thin layer of mucoprotein/polysaccharide as revealed by Alcian blue staining (pH 1 and 2.5) and colloidal iron. The redia, which along enters the snail, does not show the presence of mucoid material on its external surface. Berenbaum's method for bound lipids was positive for the apical gland, flame cells, and the redia. Other lipid stains—Sudan IV, Sudan black B, osmic acid, oil red O—failed to stain either miracidia or rediae. Histochemical tests for proteases, nonspecific esterases, and aminopeptidases were negative. Acid phosphatase activity was observed in epidermal plates, the apical gland, and redial intestine; alkaline phosphatase activity in the epidermal plates; and lipase activity in eggs containing miracidia.

No histochemical studies have been published on cyclocoelids. A comprehensive histochemical study of the miracidium of *Cyclocoelum oculeum* and the redia contained within was undertaken. Previously published histochemical investigations of other species of miracidia (Axmann, 1947; Bogomolova, 1957; Wilson, 1969, 1971; Kinoti, 1971; Buzzell, 1974; Wikel and Bogitsh, 1974) were used as a guide in this study.

Materials and Methods

Gravid *Cyclocoelum oculeum* were removed from the orbits of American coots (*Fulica americana*), placed in previously boiled aquarium water and dissected. Uteri with miracidia and recently hatched miracidia were fixed in 6% neutral formalin, Gendre's fluid, or 3% polyvinyl alcohol. Miracidia allowed to attach to *Physa gyrina* or *Gyraulus hirsutus* were fixed in Gendre's fluid 0.5–1.5 hr post-attachment.

PVA and some formalin-fixed material was frozen and cut at 16 μm with a cryostat. Cryostat sections were stained with oil red O, osmic acid, Sudan IV, and Sudan black B to detect lipids (Humason, 1972). Techniques employed for enzymes were: the Gomori method for alkaline and acid phosphatases, and lipases as reported by McManus and Mowry (1960); the Burstone and Folk technique for aminopeptidases and the procedure for nonspecific esterases after Moloney et al. as outlined by Humason (1972). Attempts to detect proteolytic enzyme activity utilized the method of Fried et al. (1976). Positive and negative controls were used in all enzyme studies. Material not frozen was embedded in paraplast and cut at 5–10 μm on a rotary microtome. These sections were stained with PAS, Best's carmine, Bauer-Feulgen, and Alcian blue at pH 1.0 and 2.5, and colloidal iron to check for polysaccharides and polysaccharide-protein complexes (Pearse, 1960; Humason, 1972). Sections both treated and untreated with malt

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diastase were used with the first three stains to check for glycogen. Proteins were observed using the mercuric bromphenol blue method (Mazia et al., 1953). Bound lipids were tested for after Berenbaum (1954). Victoria blue was used as an apical gland stain (Buzzell, 1974).

Results

Best's carmine and Bauer-Feulgen (using malt diastase-treated sections as controls) indicated large concentrations of glycogen in the epidermal plates, and lower concentrations in the apical and penetration glands (Figs. 1, 2). These areas were also PAS-positive, however, a small amount of PAS-positive material still remained after malt diastase digestion. The esophageal glands were PAS-positive, but malt diastase resistant (Fig. 2). The apical papilla, anterior pit, cytoplasmic ridges, and redia did not stain with PAS. The apical gland is selectively stained with Victoria blue.

Alcian blue pH 1.0 and 2.5 and colloidal iron indicated mucosubstances immediately external to the bases of the cilia. With all three stains the reaction was weak. No such staining for mucosubstances was associated with the redia.

Mercuric bromphenol blue intensely stained the longitudinal and circular muscles of the apical papilla and redia (Fig. 3), moderately stained subepidermal muscles (Fig. 4), apical gland contents (Fig. 5), and flame cells (Fig. 6), and weakly stained subepidermal plates (Fig. 3).

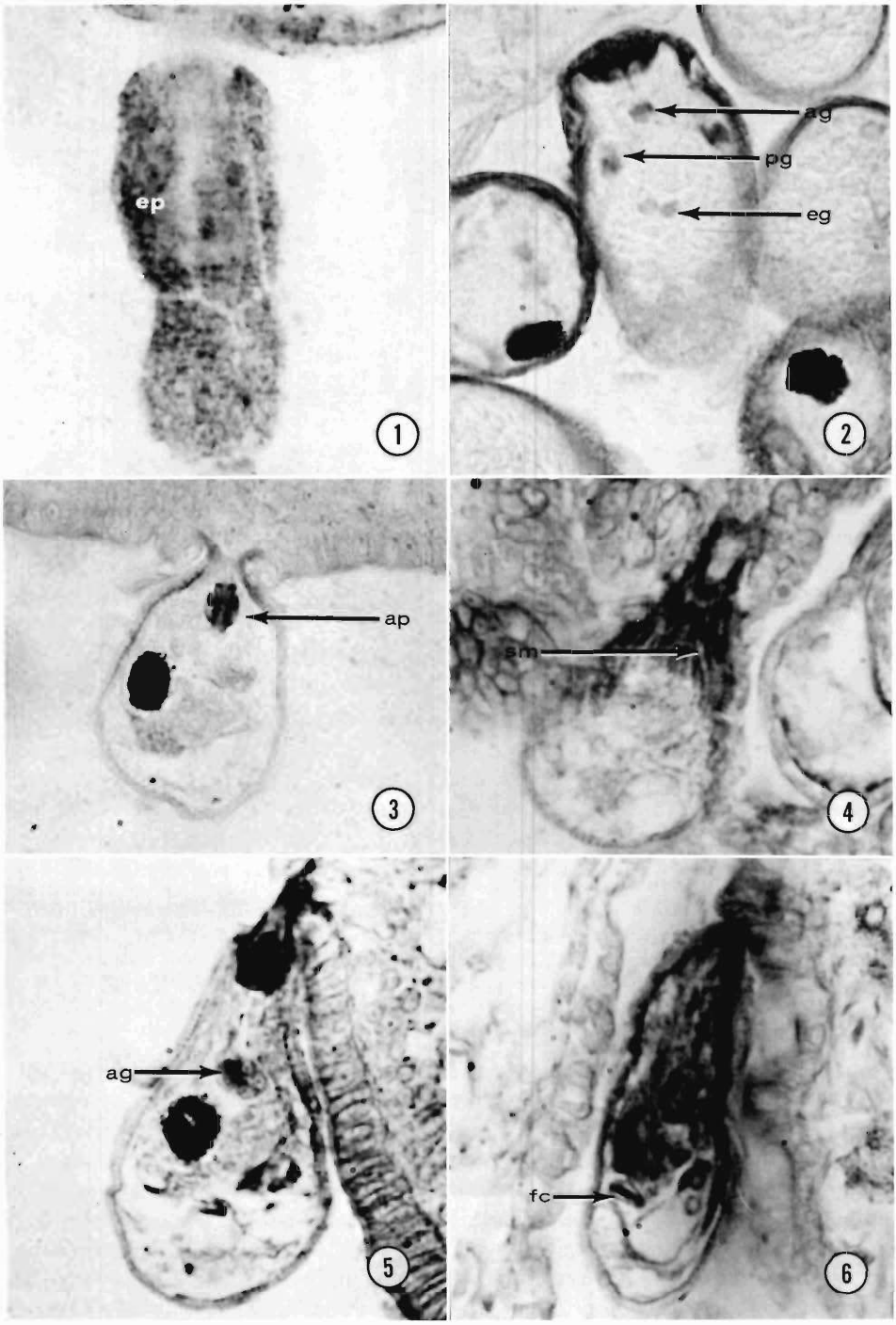
Sudan IV, Sudan black B, osmic acid, and oil red O did not indicate the presence of lipids in either miracidium or redia. However, Berenbaum's acetone Sudan black method for bound lipids revealed large amounts of such lipids in the epidermal plates and apical gland, and lesser amounts in flame cells (Fig. 7). Rediae also stained intensely for bound lipids.

The Gomori acid phosphatase reaction was weak and diffuse in the apical gland (Fig. 8) and epidermal plates, and strong in the redial intestine (Fig. 9). A weak alkaline phosphatase reaction was observed in the epidermal plates and flame cells. The lipase reaction was strong within eggs containing miracidia (Fig. 10), but reaction products for proteases, aminopeptidases, and nonspecific esterases were not detectable in the egg, miracidium, or redia.

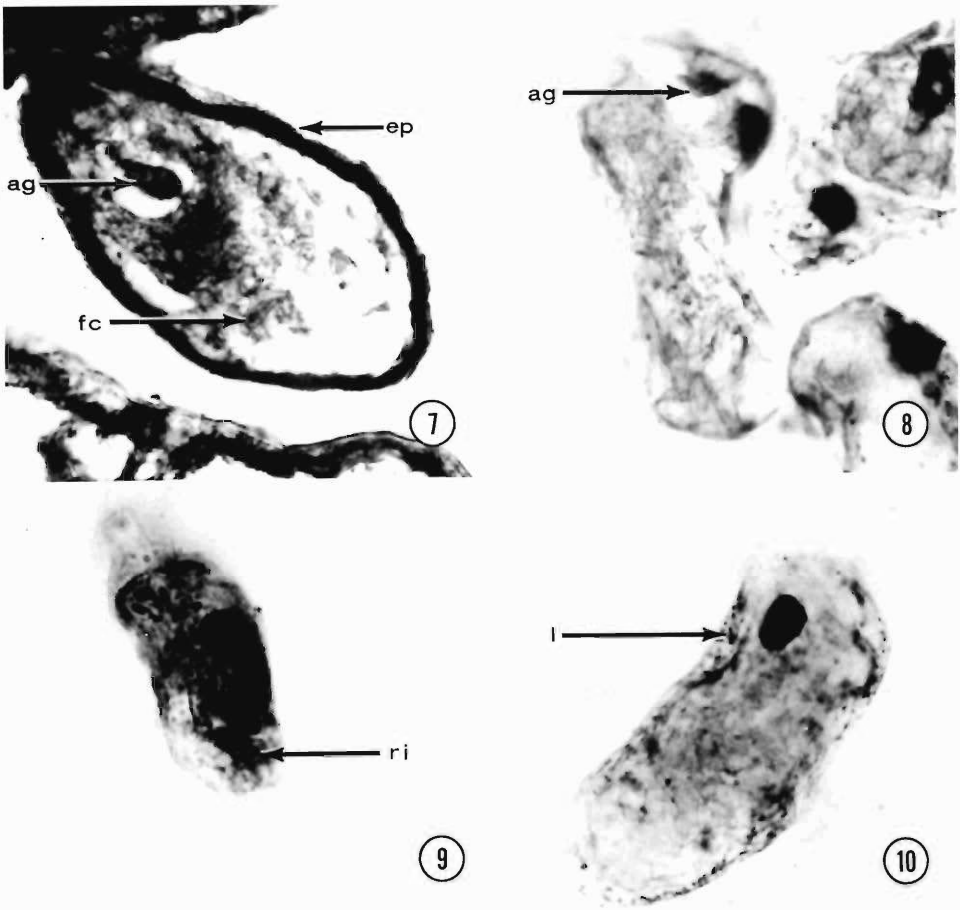
Discussion

Cyclocoelum oculeum miracidia have large concentrations of glycogen in the epidermal plates and lesser amounts in the penetration and apical glands. Various authors have observed glycogen in miracidia. Axmann (1947) observed it in the subepithelium and parenchyma of *Schistosoma mansoni* and *Schistosoma japonicum,* Bogomolova (1957) in parts of the tegument and areas surrounding embryonal cells of *Fasciola hepatica,* Wilson (1969) below the ciliary roots of *F. hepatica,* and Wikel and Bogitsh (1974) in the epidermal plates and penetration and apical glands of *S. mansoni.* The association of glycogen deposits within epidermal plates suggests its use as a substrate for enzymatic reactions required for ciliary movement.

Cyclocoelum oculeum miracidia have apical glands staining with Victoria blue and PAS. This method was developed by Buzzell (1974) to differentiate between the apical gland (Victoria blue positive) and penetration glands (PAS-positive) of



Figures 1–10. ***Cyclocoelum oculeum*** **miracidia and redia. 1. Glycogen present in epidermal (ep) plates after staining with Best's carmine. 2. PAS-positive material is revealed in miracidial penetration glands (pg) and redial esophageal glands (eg). Apical gland (ag) contents stained with Victoria blue. 3. Longitudinal and circular muscles of the apical papilla (ap) stain intensely with mercuric bromphenol**



blue (Hg BPB). 4. Longitudinal and circular subepidermal muscles (sm) stained with Hg BPB. 5. Apical gland (ag) contents stained with Hg BPB. 6. Flame cells (fc) stained with Hg BPB. 7. Bound lipids are observed in epidermal plates (ep), apical gland (ag), and flame cells (fc). 8. A weak diffuse reaction for acid phosphatase in apical gland (ag). 9. A strong reaction for acid phosphatase in redial intestine (ri). 10. Strong reaction for lipase (l) inside egg containing miracidium. All figures 640×, except Figs. 3 and 9 which are 320×.

certain fasciolid miracidia. He found, however, that Victoria blue did not stain apical glands of miracidia of several genera from five other trematode families. The common staining affinities of *C. oculeum* miracidial apical glands and those of fasciolids may reflect chemical and/or functional similarities.

Using Alcian blue as a vital stain for acid mucopolysaccharides Wilson (1969) found positive material on the surface of *F. hepatica* miracidia. He hypothesized that such mucosubstances might protect penetrating miracidia from the host's enzymes. Since the miracidium of *C. oculeum* does not penetrate its host, the extremely weak Alcian blue staining might be a corollary of Wilson's hypothesis. However, the lack of Alcian blue positive mucosubstances on the tegument of rediae recently intruded into snails argues against this for *C. oculeum*.

Muscles of the apical papilla and subepidermis, apical gland contents, miracidial flame cells, epidermal plates, and the redia stained variously with mercuric

bromphenol blue, a general protein stain. Mazia et al. (1953) reported a good correlation between protein concentration and dye binding. The intense staining of apical gland contents indicates the presence of high concentrations of protein. Whether these are structural or enzymatic proteins has not been resolved by the enzymatic histochemical studies performed.

The strong Berenbaum acetone Sudan black staining of the apical gland, flame cells, and epidermal plates indicates high levels of "bound lipids"; i.e., lipids in close association with proteins, carbohydrates, and/or nucleic acids (Berenbaum, 1954). The strong reaction was expected in the epidermal plates because of the proliferation of lipid-protein-rich cytomembranes in these areas. The significance of bound lipids in the lumen of the apical gland is not yet known but may be clarified by ultrastructural studies of the miracidium (Taft, unpublished). Erasmus (1967) noted lipid droplets in the excretory ducts of *Cyathocotyle bushiensis* adults. He speculated that in adults it is an excretory product, but in other stages it may be an energy source. The absence of free lipids in *C. oculeum* miracidia as determined by conventional lipid staining may indicate lipids are not a major energy substrate in this larval stage.

Characterization of miracidial enzymes has proved difficult. Histochemical tests for aminopeptidases, nonspecific esterases, and proteases in *C. oculeum* miracidia and rediae were negative. Lipase was observed within eggs containing *C. oculeum* miracidia, but not in the miracidia or rediae themselves. Lipase is thought to be produced by the miracidium and may be involved in the hatching of the egg. Rogers (1958) isolated hatching fluid consisting of chitinase, lipase, and probably a protease from the eggs of *Ascaris lumbricoides*. Studies by Andrade and Barka (1962) on *S. mansoni* ova containing miracidia demonstrated aminopeptidases, acid phosphatases, and nonspecific esterases in the penetration glands as well as nonspecific esterases and phosphatases in the ovum. These findings are not always consistent. Pepler (1958), for example, working with the same species, found no evidence of nonspecific esterases in *S. mansoni* miracidia. Kinoti (1971) did not observe aminopeptidase in the penetration glands of either *S. mansoni* or *S. mattheei* miracidia.

The presence of alkaline phosphatase in epidermal plates and flame cells, and acid phosphatases in epidermal plates, apical gland, and redial intestine of *C. oculeum* is consistent with previous studies of trematode larvae. Wilson (1971) found a strong acid phosphatase reaction in the epidermal plates and a diffuse reaction in the apical gland of *F. hepatica* miracidia. Numerous authors including Cheng (1964), Probert (1966), and Moore and Halton (1975) have demonstrated acid and alkaline phosphatases in the redial tegument and intestine. The presence of acid and alkaline phosphatase activities in the tegument, gut, and excretory systems of trematodes suggests the presence of active transport systems in these tissues.

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