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HISTOCHEMISTRY OF HYDROLYTIC ENZYMES OF VIRGULATE XIPHIDIOCERCARIAE*

Jegdish P. Babu and John E. Hall
Department of Microbiology, West Virginia University, Morgantown, West Virginia 26506

ABSTRACT: Histochemical studies were conducted to localize and determine the roles of hydrolytic enzymes in a virgulate xiphidiocercaria, *Cercaria polypyreta*, before and after penetration of an arthropod intermediate host. Before penetration N-acetyl-β-glucosaminidase was localized in the virgula organ and outer mucoid coat; leucine aminopeptidase was restricted to the flame cells, and an esterase which is inhibited by eserine (cholinesterase) was located in the nervous system. Acetylglucosaminidase activity was weaker after penetration of mayfly naiads (*Litobrancha recurvata*) suggesting that this enzyme is utilized during penetration. No changes were noted in LAP and esterase activities. Sulfhydryl groups and calcium ions were found in the stylet and in the cephalic glands. Acetylglucosaminidase activity was also seen in the virgula organs and outer mucoid coats of 2 other virgulate xiphidiocercariae, *Cercaria apatema* and *C. dolomeda*, and in the mucoid glands of developing *C. apatema* prior to their discharge to form the mucoid coat and virgula organ.

Virgulate xiphidiocercariae such as *Cercaria polypyreta* are larvae of the trematode family Lecithodendriidae and develop within sporocysts in operculate snails. They are armed with stylets, possess cephalic glands, and also mucoid glands which are discharged prior to emergence to form a mucoid reservoir or virgula organ within the oral sucker (Kruidenier, 1951). Ortigoza and Hall (1963) studied the mucoid and cephalic glands of three species of virgulate cercariae and found that they contained mucopolysaccharides and proteins.

While there is literature pertaining to the glandular contents or penetration mechanisms of schistosome cercarie (Lewert and Lee, 1954; Stirewalt and Kruidenier, 1961; Stirewalt, 1973) and strigeoid cercariae (Erasmus and Öhman, 1963; Cheng and Yee, 1968), there is little information regarding cercarial penetration of insects. The present study was designed to examine the role of hydrolytic enzymes and cercarial secretions in the penetration of arthropod cuticle.

MATERIALS AND METHODS

A. Collection of material

C. polypyreta was obtained from naturally infected river snails (*Nitocris dilatatus* Conrad) collected from the Cheat River System in West Virginia. Two related species, *C. apatema* and *C. dolomeda*, were obtained from the same source and used to confirm results obtained with *C. polypyreta* and to study enzyme activity in young, developing cercariae. Mayfly naiads (*Litobrancha recurvata*) were collected from Little Lick Run in Preston County, West Virginia, which is free of operculate snails, and maintained in mud substrate in stream water at 15 C.

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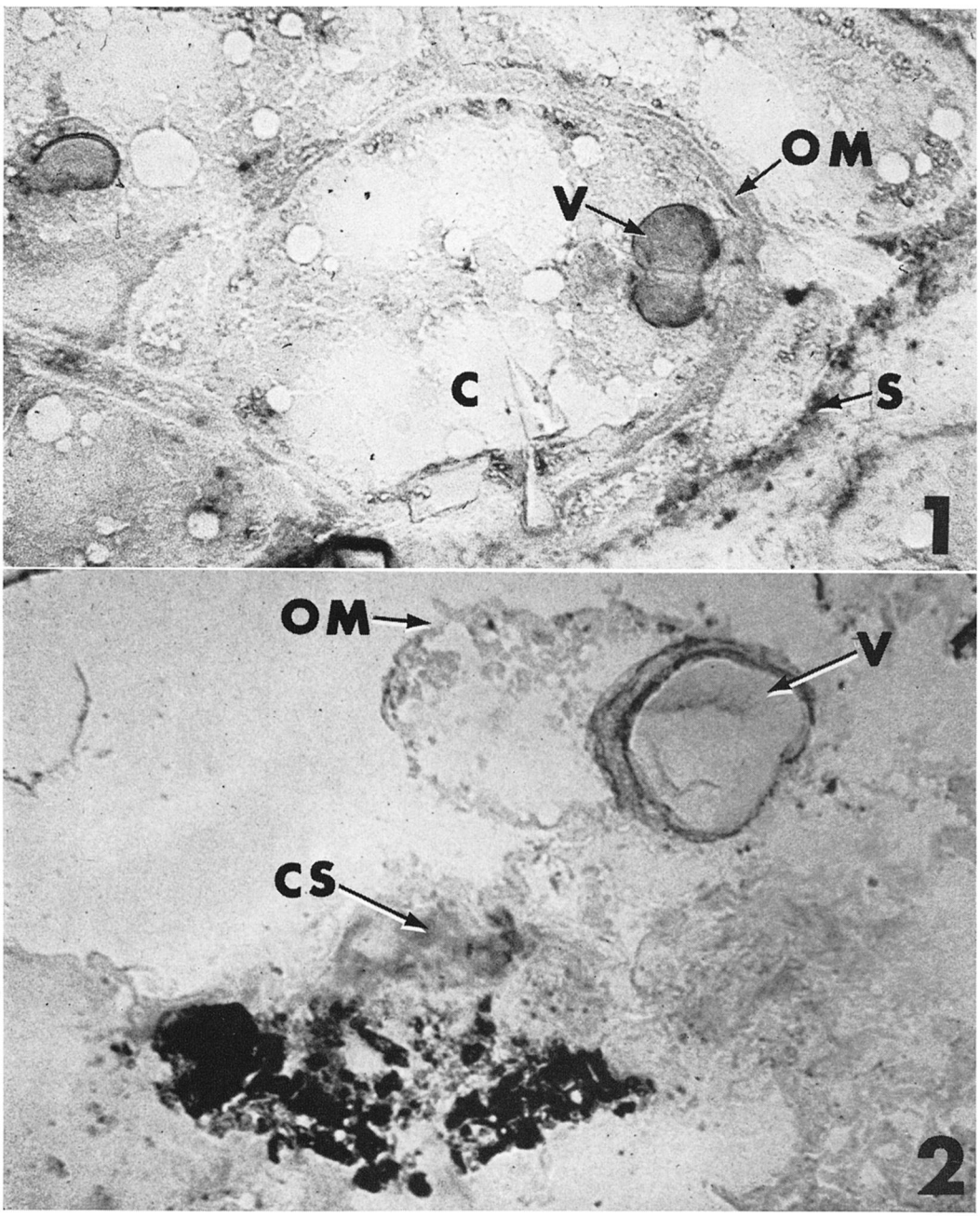
B. Preparative procedures

Enzymes: Sporocysts obtained by crushing infected snails were mounted in OCT medium (Ames Co., Elkhart, Ind.), quick-frozen, and sectioned at 4 to 6 μm on a cryostat. Sections were air-dried for 15 min prior to incubation in substrate.

Histochemical studies were also performed on cercariae in the process of penetration by exposing mayfly naiads to 300 freshly emerged *C. polypyreta* at room temperature, quick-freezing naiads 30 to 60 min after exposure, and sectioning. Sections were stained simultaneously with sporocyst sections for comparison and control.

The following protocols for enzyme localizations were used as described by Pearse (1972): Naphthol AS-BI-Garnet GBC method for N-acetyl-β-glucosaminidase (pH 5.2); α-naphthyl acetate method for esterase (pH 7.4); L-leucyl-4-methoxy-β-naphthylamide method for leucine aminopeptidase (LAP) (pH 7.4); and silver proteinate method for endopeptidase (pH 6.4). Positive control tissues included mouse kidney (glucosaminidase, esterase), mouse small intestine (LAP, endopeptidase), and snail intestine and digestive gland (glucosaminidase, LAP, endopeptidase). Boiling of sections and immersion of sections in incubation medium without substrate were used for negative control.

Calcium or magnesium ions were added as activators for LAP at final concentrations of 1.25×10^{-4} M and 4.2×10^{-4} M respectively. Esterase was inhibited by preincubating sections in a 10^{-5} M concentration of the anticholinesterase agent, eserine. Attempts were made to control endo-



FIGURES 1, 2. Histochemical localization of N-acetyl-β-glucosaminidase in *Cercaria polypyreta*. **1.** Fully developed cercaria within sporocyst. Enzyme activity is seen in the virgula organ (V), outer mucoid coat (OM), and sporocyst wall (S), but not in the cephalic glands (C). × 550. **2.** Metacercaria within hemocoel of mayfly naiad 30 min after exposure. Activity is lost from the mucoid coat region (OM) and activity in the virgula organ (V) is diminished, but the cercarial secretions (CS) are stained. × 380.

TABLE I. *Hydrolytic enzymes of* Cercaria polypyreta.*

Enzyme	Mucoid coat and virgula organ	Cephalic glands	Tegument	Nervous system	Other
N-acetyl-β-glucosaminidase	++	−	−	−	Mucoid glands ++†
Esterase	−	−	+	++	−
After 10^{-5} M eserine treatment	−	−	−	−	−
Leucine aminopeptidase (LAP)	−	−	−	−	Flame cells ++
Endopeptidase	−	++	++	+	Oral sucker ++ Ventral sucker ++ Excretory bladder + Stylet +
After iodoacetic acid, iodoacetamide, trypsin inhibitor, and Trasylol treatment	−	++	++	+	Same
After heat denaturation	−	+	+	+	Same

* ++ = strongly positive, + = positive, − = negative.
† Young *C. polypyreta* were not available, observations on staining of mucoid glands are based on experiments with a related species, *C. apatema*.

peptidase activity by boiling sections for 45 min and by immersing sections in 0.001 M iodacetamide, 40 μg/ml soybean trypsin inhibitor, or the trypsin–chymotrypsin inhibitor Trasylol (FBA Pharmaceuticals, 425 Park Ave., New York, N. Y.) for 1 to 2 hr prior to incubation.

Proteins and calcium ions: Sporocysts were fixed in 10% formalin for 24 hr at 4 C, dehydrated through a butanol–ethanol series, embedded *in vacuo* in Fisher Tissuemat, and sectioned at 4 μm. Sulfhydryl groups were studied by the dihydroxy-6, 6-dinaphthyl disulfide (DDD) procedure (Pearse, 1968) while 4% alcoholic glyoxal-bis-(2-hydroxyanil) (GBHA) was used for calcium ions (Pearse, 1972).

RESULTS

Enzymes

Locations of hydrolytic enzymes in *C. polypyreta* are summarized in Table I. N-acetyl-β-glucosaminidase activity was found in the virgula organs and outer mucoid coats of fully developed *C. polypyreta* (Fig. 1) and two related species, *C. apatema* and *C. dolomeda*. Sporocyst walls were also intensely positive for this enzyme.

Glucosaminidase activity was reduced in the virgula organs and had disappeared from the mucoid coat region of cercariae which had penetrated mayfly naiads, while mucoid secretions released by cercariae during and after penetration stained positively for this enzyme (Fig. 2). Though young developing *C. polypyreta* were not available, glucosaminidase activity was found in the mucoid glands of young *C. apatema* (Fig. 3).

The cephalic glands, cephalic gland ducts, tegument, and oral and ventral suckers of fully developed cercariae stained with silver proteinate, but the nerve ganglia, stylet, and excretory bladder also stained. Heat-denatured sections showed less intense silver proteinate staining but iodoacetamide, iodoacetate, soybean trypsin inhibitor, and Trasylol had no inhibitory effect.

LAP staining was enhanced by either calcium or magnesium ions but was limited to the flame cells and remained stable during penetration of mayfly naiads. Esterase was found in the nerve ganglia, anterior and posterior nerve trunks, and subtegument and was not

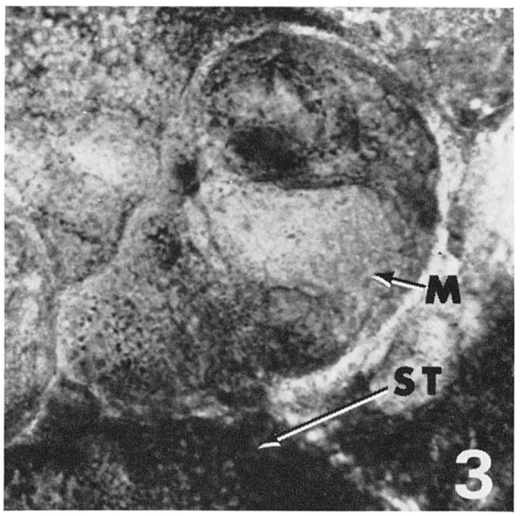


FIGURE 3. Young, developing *Cercaria apatema* stained for N-acetyl-β-glucosaminidase showing enzyme activity in the mucoid glands (M) and adjacent snail tissue (ST). × 490.

altered during penetration. All esterase activity was inhibited with 10^{-5} M *eserine*, and hence represents cholinesterase.

Proteins and calcium ions

SH group staining was located in the cercarial cephalic glands and ducts, in the oral and ventral suckers, and in the excretory bladder wall and tegument. Calcium ion staining was seen in the stylet, cephalic glands, ducts, and flame cells.

DISCUSSION

Kruidenier (1951) studied the behavior of virgulate cercariae in vitro and suggested that the mucoid secretions are used during penetration of the insect intermediate host. Hall and Groves (1963) observed the discharge of metachromatic material from the virgula organ of penetrating xiphidiocercariae but did not test for the presence of enzymes in these secretions. It is now evident that the virgula organ contents are enzymatically active. Since the enzyme present is N-acetyl-glucosaminidase which may be active against the chitin of the insect cuticle, these contents may aid the stylet in penetration. The disappearance of N-acetyl-glucosaminidase from the virgula organ after penetration of mayfly naiads correlates with the loss of mucoid secretions (Hall and Groves, 1963) and may explain the cuticular degeneration associated with attaching cercariae and the diffusion of parasite secretions into the insect cuticle (Hall et al., 1969). The loss of this enzyme from the mucoid coat region correlates with previous observations (unpublished) that the outer mucoid coat is lost during penetration of the second intermediate host. The association of the enzyme with the sticky mucoid substance could hold it at its site of function. These findings conflict with the conclusions of Seitner (1945) and Burns (1961), who believed that the virgula organ plays little or no role in penetration.

The absence of glucosaminidase in the glands of schistosome cercariae (Fripp, 1966) may reflect the fact that this enzyme does not hydrolyze the ground tissue of mammalian skin. The inability of Porter and Hall (1970b) to find glucosaminidase in cotylocercous cercariae which also penetrate insects may reflect more on the peculiarities of the cercarial enzyme rather than on its complete absence.

Most of the silver proteinate staining was not altered by inhibitors and hence was not specific for proteolytic enzymes. The heat-labile staining may represent a proteolytic enzyme but this was not confirmed. Restriction of aminopeptidase activity to the flame cells suggests that this enzyme is limited to an excretory function (Porter and Hall, 1970b), while all esterase activity was associated with the nervous system.

The calcium ions, which have also been found in the cephalic glands or extracts of other cercariae, may activate enzymes (Lewert et al., 1966; Porter and Hall, 1970a; Dresden and Edlin, 1974). The DDD reaction used to demonstrate proteins and as a screening procedure for enzymes (Hall, unpublished) stained cephalic glands with moderate intensity, but the virgula organ, which contained glucosaminidase activity, did not stain.

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RESEARCH NOTE . . .

A Second *Ascaris suum* with Three Uteri

Just over half a century has passed since Chandler (1924, J Parasitol **10:** 208) reported the occurrence of an extra uterus and ovary in a specimen of *Ascaris suum*. This same anomaly was present in a specimen recently collected from a pig in a Montreal abattoir. Although Chandler's sketch was not printed, his written description indicates close similarity to the arrangement shown in Figure 1. In the present case the extra uterus branched from the right side and, *in situ*, it passed anteriorly from its bifurcation for about 0.5 cm before curving back to lie alongside the other viscera. Unfortunately, as can be seen in Figure 1, the normal right ovary broke off during dissection. The worm had apparently enjoyed a normal sex life as there was no sign of a genital girdle (Beaver and Little, 1964, J Parasitol **50:** 128–130) and all three uteri contained fertilized eggs. After photography, the uteri were severed so as to leave 1 cm of each attached to the common uterus and vagina, fixed, and placed in the museum of the Institute of Parasitology. A triple gonad may increase reproductive potential and Chandler felt that an evolutionary tendency to multiplication of uterine branches was suggested by the genus *Polydelphis* with species in reptiles containing four- and six-branched uteri. *Tanqua liera*, also found in reptiles, is another species with four uteri and one of these apparently branches in the opposite direction to the others (Chitwood and Chitwood, An Introduction to Nematology, Section I, Chapter 10, 1940).

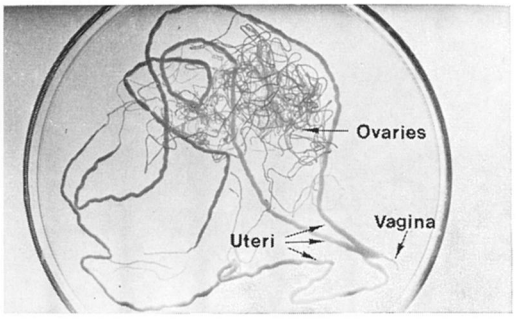

FIGURE 1. Second reported case of *Ascaris* with trifurcated reproductive system.

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R. P. Harpur, Institute of Parasitology, McGill University, Macdonald College Post Office, P.Q., Canada, H0A 1C0