Histochemical studies on Acanthocephalan parasite, *Echinorhynchus Veli* infecting the fish *Synaptura Orientalis* (Bl&Sch, 1801)

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ABSTRACT

Echinorhynchus Veli (George and Nadakal, 1978), an acanthocephalid worm infecting the estuarine flat fish, *Synaptura Orientalis*, was collected from the Velilake, Kerala. The parasite was recovered from the intestine of the host fish. The glycocalyx, basement membrane layer, muscles and eggs were found to contain rich stores of glycogen. The radial layer of the tegument showed a moderate reaction while the felt layer only a weak reaction. Intense deposit of protein was demonstrated in the glycocalyx and eggs. The striped layer and muscles showed only moderate reaction. Muscle layer and eggs showed intense deposit, radial basement membrane layers had moderate deposition of tyrosine contain proteins. The glycocalyx and eggs revealed heavy deposit of lipids. Intense alkaline phosphatase activity was noted is in glycocalyx and eggs and moderate acid phosphatase activity was observed in eggs and only mild activity in glycocalyx and muscles.

Key words: *Echinorhynchus Veli* , acanthocephalid, *Synaptura Orientalis,* glycocalyx phosphatase

INTRODUCTION

Histochemistry is quite often defined as the aspect of histology concerned with the identification of chemical components in cells and tissues, thereby revealing their functional anatomy. These studies offer the opportunity to correlate the structure with function of different organs and tissues, thereby broadening the area of investigation. A great deal of chemical studies has been carried out in different parasitic helminths demonstrating carbohydrates, proteins, lipids and enzymes.

A few authors investigated the histochemical localization of polysaccharides in different acanthocephalan species. Among these, the studies on *Macracanthorhynchus hirudinaceus*, *Leptorhynchoides thecatus* and *Echinorhynchus Coregoni* (Von Brand, 1939a, b, 1940), *Pomphorhynchus Bulbocolli*, *Neoechinorhynchus Cylindratus*, and *Neoechinorhynchuse Mydis* (Bullock, 1949b), *Polymorphus Minutes* (Crompton, 1965), *Acanthosentisoligo Spinus* (Sita, 1969), *Moniliformis Dubius* (Rothman and Elder, 1970) and *Echinorhynchus Gadi* (Chu *et al.,* 1977) deserve special mention. The reports of such studies furnished a detailed account of the localisation of glycogen in different tissues of the parasites. Crompton (1963) and Nicholas and Mercer (1965) attempted to demonstrate the presence of glycogen in the acanthocephalan glycocalyx. Changes in the levels of glycogen in ovarian balls, oocytes and shelled acanthors in relation to reproduction and development have been investigated by Von Brand (1940) and Crompton (1965). The dietary role of glycogen in adult acanthocephalans not only as a source of immediate energy but as a carbohydrate reserve has been studied in great detail by Von Brand (1939a, b); Von Brand and Saurwein (1942); Ward (1952); Laurie (1957); Read and Rothman (1958) and Graff and Allen (1963). Amin and Larsen (1989) studied the biochemical profile of *Neoechinorhynchus Cylindratus* and gave an account of the distribution of nucleic acids and histones in tissues and eggs. The major sites of lipid localisation in adult acanthocephalans have been discussed by Beermann*et al.,* (1974) and Parshad and Guraya (1977).

The activities of various enzymes have been studied in a few acanthocephalans. Bullock (1958) reviewed the activity and distribution of alkaline glycerophosphatase. The works of Rothman and Fisher (1964) and Edmonds (1965) offered a better comprehension of the molecular mechanism of the active transport of amino acids in acanthocephala while Armin and Martin (1994) investigated the leucine aminopeptidase (APase) activity histochemically in the body wall of the praesoma as well as metasoma of *P. laevis* with the objective of revealing the sites of amino acid uptake in tissues. The presence of glycolytic enzymes aldolase, triose phosphate dehydrogenase and lactate dehydrogenase in *M. dubius* was reported by Read (1961). In addition to these, Dunagan and Scheifinger (1966) demonstrated six more glycolytic enzymes namely, hexokinase, glucose phosphate dehydrogenase, glutamate dehydrogenase, phosphoglucomutase, phosphoglucoseisomerase and phosphofructokinase in *Macracanthorhynchus hirudinaceus*.

MATERIALS AND METHODS

Live and active worms isolated from the fish intestine were fixed in specific fixatives, processed and paraffin sections of 5 to 7 µm thickness were cut and treated for various histochemical tests.

General Carbohydrates and Glycogen

The worms were fixed in Bouin's fluid. The paraffin sections were dewaxed, re-hydrated and stained for general carbohydrates following McManus Periodic Acid Schiff's (PAS) technique as described by McManus (1948). Some sections were counterstained with iron-haematoxylin. Best's Carmine staining method was applied for the localization of glycogen. Control sections earlier digested with filtered human saliva at 37ºC for three hours (or with alpha amylase for 20 minutes) were similarly stained, and mounted in canada balsam.

Proteins

Worms were fixed in Carnoy's fluid or 10% neutral buffered formalin. For the detection of general proteins, Mercury-Bromophenol blue method (Mazia*et al.,* 1953) was employed. Millon's reaction was applied for the localization of tyrosine containing proteins. As the control, sections treated in 0.5% trypsin for about one hour at 37ºC were used.

Lipids

Worms fixed in 10% neutral buffered formalin were processed and paraffin sections were stained with Sudan Black B (Humason, 1979) and mounted in glycerine. Sections treated with chloroform-methanol in the ratio 1:3 for 24 hours served as control.

Hydrolytic enzymes

To detect the presence of alkaline phosphatase and acid phosphatase, calcium Cobalt Method (Gomori, 1957) and Lead Nitrate Method (Gomori, 1957) were respectively employed.

RESULTS

The results of various histochemical tests are given in Table 1. The glycocalyx and muscle layer of the body wall were rich in carbohydrate (Figure 1). The striped layer and felt layer of the body wall showed moderate reaction and radial layer a mild reaction. Best's Carmine method revealed strong reaction to glycogen in gycocalyx, basement membrane layer

and muscle layer (Figure 2) and weak reaction in felt layer and radial layer. Bromophenol Blue staining revealed intense deposit of proteins in the glycocalyx and moderate reaction in muscular layer (Figure 3). The protein deposit in the tegument showed a decreasing gradation from the surface of the interior. Intense reaction to Millons's reagent was noted in the muscle layer and moderate in radial and basement membrane layers. Glycocalyx, striped and felt layers were free from tyrosine containing proteins. Glycocalyx stained intensely with Sudan Black B. Striped, felt, radial, basement membrane and muscle layers were free from deposition. Eggs were strongly positive to Sudan Black B (Fig. 4). Intense alkaline phosphtase activity was observed in glycocalyx (Figure 5). Moderate reaction was noted in basement membrane and muscle layers and mild activity in other parts of tegument. Moderate reaction was also noted in ovary and eggs. Mild acid phosphatase activity was observed in glycocalyx and muscle layer and moderate reaction in the eggs (Figure 6).

DISCUSSION

Histochemical studies on *E. veli* revealed that the principal sites of carbohydrate and glycogen deposits are the glycocalyx and basement membrane layer. This is due to the fact that glycogen is the primary energy source and is therefore associated with those sites of higher metabolic activity. The present findings are in general agreement with the histochemical observations made by various researchers in different acanthocephalan species. Early investigations on the biochemical composition of *Macracanthorhynchus hirudinaceus* (Von Brand 1939a), *Leptorhynchoides thecatus* and *Echinorhynchus salmonis* (Von Brand 1939b, 1940), *E. gadi, E. salmonis*, *Pomphorhynchus bulbocolli*, *Neoechinorhynchus cylindratus* and *N. emydis* (Bullock, 1949b), *Polymorphus minutus* (Crompton, 1965), *Moniliformis moniliformis* (Wright and Lumsden, 1968), *E. gadi* (Chu *et al.,* 1977) and *N. cylindratus* (Amin and Larsen, 1989) showed that glycogen abounds virtually in all tissues of adult acanthocephalans but principally in the body wall. The glycocalyx is a stabilized system composed of acid mucopolysaccharides, neutral polysaccharides and glycoproteins (Monné, 1959; Wright and Lumbsden, 1968; Beermann *et al.,* 1974; Chu *et al.,* 1977). Presence of carbohydrate containing antigens in the tegument of *Schistosomamansoni* has been demonstrated histochemically as well as by immune-precipitation and affinity chromatography (Hayunga and Sumner, 1986). Shannon and Bogitsh (1971) suggest that in the natural state, the precursors of glycogen and other complex carbohydrates can get into the worm only through the tegument. The tegument is not an inactive zone; instead it is highly active metabolically. According to Barabashova (1971) the tegument of

acanthocephalans is trophic, metabolic, protective and supporting in function. In the present study, the tegument revealed intense to moderate reaction to PAS and Best's carmine staining and implies that it is an important centre of glycogen storage and energy release. In many helminths the body surface plays a major role in integrating the worm's physiology with its micro environment, via nutrient assimilation, regulation of internal chemical pools by selective absorption or secretion mechanisms, and chemosensory activity (Barabashova, 1971). Von Brand (1939a) demonstrated that glycogen is the major stored polysaccharide in *M. hirudinaceus*. He also observed that the body wall of *M. hirudinaceus* contained as much as 80% of glycogen in the entire worm. Worms synthesize glycogen from glucose, fructose, galactose, mannose and maltose absorbed from the host's intestine (Laurie, 1959). It is well known that the anaerobic fermentation requires large deposits of carbohydrates and therefore it is not surprising to find glycogen levels as high as 20-24% of the total worm dry mass (Dunagan, 1964; Starling and Fisher, 1978). Depletion of glycogen during starvation and its subsequent replenishment when placed in nutrient medium emphasizes the significance of tegument as a repository of glycogen (Read and Rothman, 1958; Crompton, 1970, 1972; Körting and Fairbairn, 1972). Miller and Dunagan (1985) reported that glycogen particles are spread over the felt work layer in *Paratenuisentis ambiguus* and Von Brand (1939b) established large concentration of glycogen deposit in *Leptorhynchoides thecatus* and *Macracanthorhynchus himdinaceus*. Read and Rothman (1958) reported that the glycogen stores in adult acanthocephalans turn over dramatically between hosts's feeding periods. The glycogen in the bursa wall of *M. hirudinaceus* was found to be highly water soluble (Von Brand 1939a). He further demonstrated the susceptibility of regimental glycogen to amylase digestion. There are reports of negative PAS reaction from several helminths (Von Brand and Mercado, 1961; Burton, 1962). These variations suggest a highly labile nature of the glycogen, probably due to active metabolic status of the tegument. In *E. veli* there is a uniform deposition of carbohydrates and glycogen in the glycocalyx. It reflects a higher metabolic rate in the glycocalyx making use of the stored glycogen. So the active involvement of the glycocalyx in the absorption of nutrients may be proposed as a possible reason for the increased glycogen deposit in the glycocalyx. *E. veli,* like other acanthocephalans, has no digestive tract so that nutrients are selectively absorbed from the host's intestine through the tegument. In *M. dubius* lactate, acetate and format were reported as the chief end products of carbohydrate metabolism (Laurie, 1957, 1959).

In *E. veli,* the glycocalyx has an intense concentration of proteins and the outer striped zone of the tegument a moderate deposit. The major components of cellular structures are proteins. The protein in the wall of acanthor of *Acanthosentis* sp. was reported to be rich in aromatic and sulphur-containing amino acids (Anantaraman and Ravindranath, 1976). Dezfuli *et al.,* (2000b) identified a protein of about 23 kDa from the cement glands of *P. laevis* males.

But tyrosine containing protein is high in muscular layer and moderate in radial and basement membrane layers. Tyrosine phosphorylation is an essential mechanism in signelling pathways regulating metabolism, growth and development in eukaryotes (Hunter, 1995; Thomas and Brugge, 1997). The intense staining reactions in the muscle layer and moderate reaction in radial and basement membrane layers imply the increased amino acid uptake by these and hence functionally significant. Various amino acids are known to be absorbed through the tegumental surface, but the transport mechanisms can vary according to the type of amino acid (Kumari and Rao, 2009). The amino acids absorbed from the host intestine may be retained in the tegument for shorter or longer duration depending upon the metabolic state of the worm.

The present histochemical study demonstrated intense lipid deposit in glycocalyx and absence in striped, felt, radial, basement and muscle layers throughout the tegument, irrespective of praesoma or metasoma. The role of acanthocephalan body wall in lipid absorption and excretion has been investigated by many researchers. It has been suggested that lipid uptake takes place through the praesoma wall and lemnisci. Electron microscopic studies on *Polymorphus minutus* (Crompton and Lee, 1965) and *Acanthocephalus ranae* (Hammond, 1967) have shown that the praesoma wall and lemnisci contain large amounts of lipid compared to the trunk wall. Crompton and Lee (1965) suggested that lipids may be absorbed through the surface of the praesoma and pass through the lemnisci to the rest of the body whereas Hammond (1967) proposed the reverse process in which the lipids in excess of the metabolic requirements are discharged as waste product through the surface of the proboscis. The fat deposits serve as energy reserve of the worms.

Glycogen and lipid granules are abundantly distributed in the acanthocephalan cuticle (Byung Chai Cho, 1981). Adult acanthocephalans have lipid deposits which in histochemical and cytological studies appear as large and small droplets in virtually all tissues (Von Brand 1939a; Bullock, 1949b; Crompton, 1963; Crompton and Lee, 1965;

Hammond, 1967; Wright and Lumsden, 1968; Byram and Fisher, 1973; Beermann *et al.,* 1974; Chu *et al.,* 1977; Parshad and Guraya, 1977). Dimitrova (1963) reported the topographic distribution of lipids in *M. hirudinaceus* whereas Smith *et al.,* (1979) reported the occurrence of a significant fraction of lipids in its lacunar fluid. Bullock (1949a) found that in several species of acanthocephalids, the most striking regions of fat deposition were the musculature of body wall and around the lacunar channels. He also noticed that the fat distribution is roughly parallel to that of glycogen. Chu *et al*., (1977) also demonstrated a similar trend in *Echinorhynchus gadi*. The co-occurrence of lipids and glycogen suggests the interdependence of lipid carbohydrate metabolism (Dunagan and Miller, 1979).

Phosphatase activity has been associated with accelerated cellular metabolism and transmembrane phosphorylated transfer of substances (Hollis, 1979). Halton (1967a) reports only a slight alkaline phosphatase activity in the ovary of *Fasciola hepatica* and in the vitellaria of trematodes. Physiologically active nature of the uterus is suggested by Halton (1967a,b) who reported alkaline phosphatase activity in the uterus of the monogeneans and digeneans.

In the present study, intense acid phosphate activity has been observed in the glycocalyx and eggs, moderate activity in basement membrane layer and muscles and mild activity in the other parts of tegument. This is in agreement with the histochemical observations in *P. minutus* (Crompton, 1963), *M. hirudinaceus* (Cain, 1970) and *N. cylindratus* (Amin and Larsen, 1989). Intense acid phosphatase activity has been reported in several trematodes (Yamao, 1952; Nimmo-Smith and Standen, 1963; Ma, 1964). Absence of acid phosphatase has been reported in the tegument of *Echinococcus granulous* (Kilejian *et al*, 1961). The role of acid phosphatase again is suggested to be the transmembrane phosphorylated transfer of matabolites.

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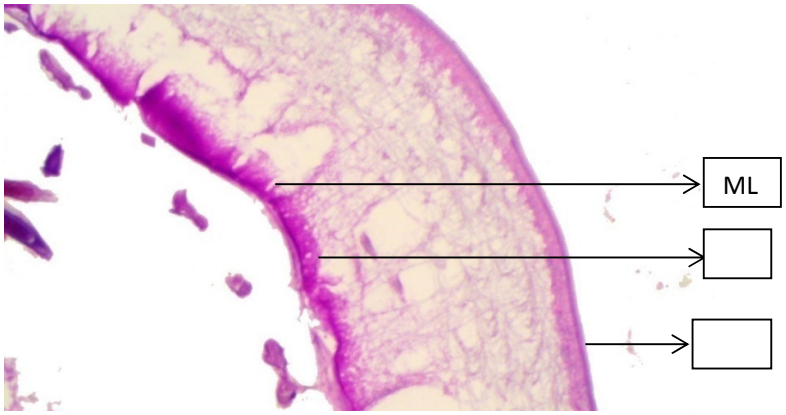
Table-1: The results of the various histochemical tests

Parts of worm	General Carbohydrate	Glycogen	General Protein	Tyrosine	Lipid	AlPase	AcPase
Glycocalyx	+++	+++	+++	0	+++	+++	+
Striped Layer	+	+	++	0	0	+	0
Felt Layer	+	0	+	0	0	+	0
Radial Layer	++	0	+	++	0	+	0
Basement Membrane layer	+++	+++	0	++	0	++	0
Muscle Layer	+++	+++	++	+++	0	++	+
Egg	+++	+++	+++	+++	+++	+++	++

+++ intense reaction, ++ moderate reaction, + mild reaction, 0 negative reaction, AlPase: alkaline phosphatase, AcPase: acid phosphatise

Figure-1: C. S. of *E. veli* showing intense deposit of carbohydrate in the tegument (PAS) (45 x)



ML-Muscle LayerGC- GlycocalyxBL-Basement memberane Layer
RL-Radial LayerE-Egg

Figure-2: C. S. of *E. veli* showing principal deposit of glycogen in the tegument (Best's carmine) (10 x)

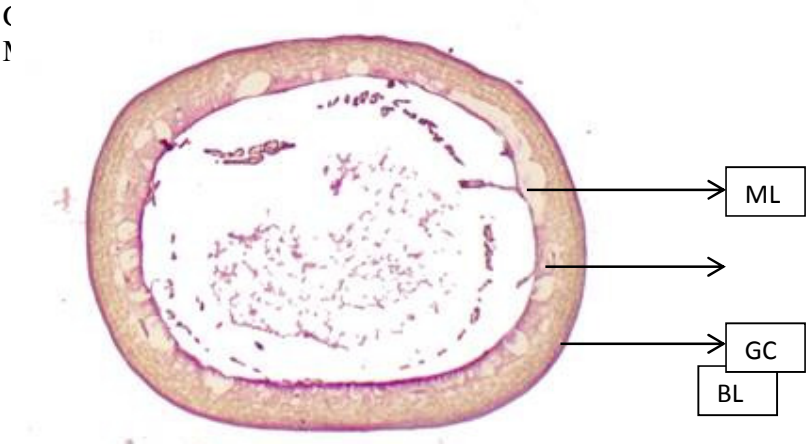


Figure-3: C. S. of *E.veli* showing moderate reaction to protein in the tegument (Bromophenol blue) (45 x)

SL-Striped Layer E-Egg ML-Muscle Layer GC-Glycocalyx

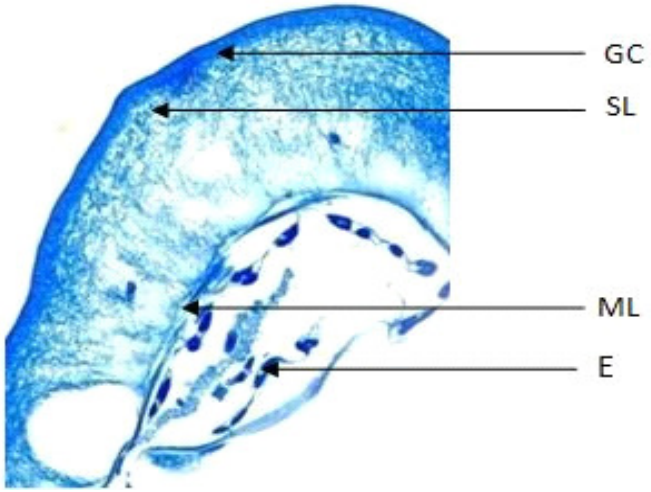


Figure-4: C. S. of *E. veli* showing intense reaction to lipid in the glycocalyx (Sudan Black B) (45 x)

GC-Glycocalyx E-Egg

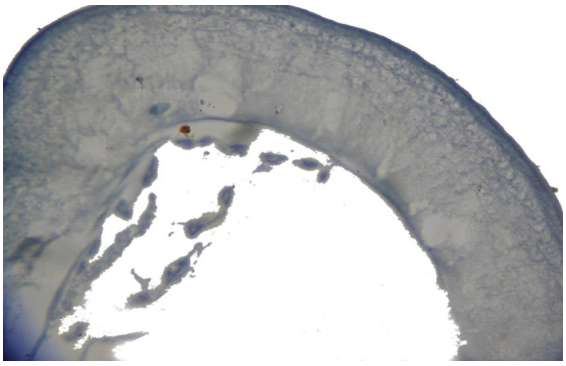


Figure-5: C. S. of *E. veli* showing alkaline phosphatase activity in the tegument (10 x).

ML-Muscle Layer GC-Glycocalyx

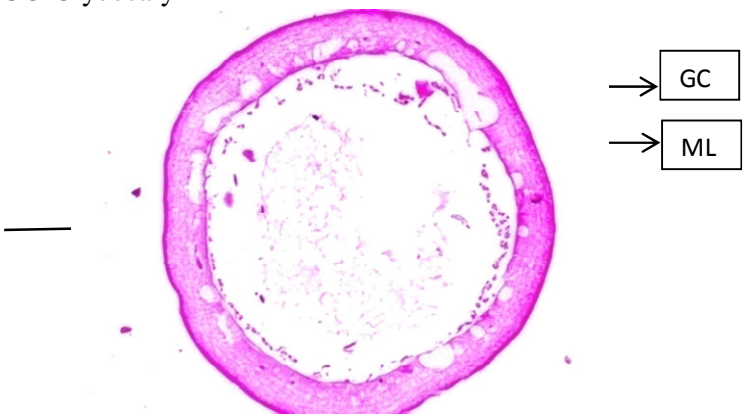


Figure-6: C. S. of *E. veli* showing acid phosphatase activity in the tegument (45 x)

E-Egg MLMusce Layer SL-Striped Layer GC-Glycocalyx