STUDIES ON THE HISTOCHEMISTRY OF PARAMPHISTOMES OF SHEEP AND CATTLE

THESIS

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In fulfillment of the requirement for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

ZOOLOGY (PARASITOLOGY)

By

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***No**: F. PGZ/Ph.D./Thesis/07*
***Date**: 30. 11. 2007*

Certificate

This is to certify that the Thesis entitled ***"Studies on the Histochemistry of Paramphistomes of Sheep and Cattle"*** submitted to the University of Kashmir for the award of the Degree of *DOCTOR OF PHILOSOPHY IN ZOOLOGY*, is the original research work of ***Mr. Perviz Ahmad Dar***, a bonafide Ph. D. Research Scholar of the Department, carried out under our supervision. The thesis has not been submitted to this University or to some other University so far and is submitted for the first time. It is further certified that this thesis is fit for submission for the degree of Doctor of Philosophy (Ph. D.) in Zoology and the candidate has fulfilled all the statutory requirements for the completion of the Doctoral Programme.

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Sheep and cattle constitute one of the premier economic group, providing animal protein which is indispensable in regular staple diet of common man in our country, especially in native state of Jammu and Kashmir. The present study, which deals with the histochemistry of Paramphistomes of sheep and cattle, was undertaken from January 2004 to January 2007. Since there has been no detailed study on the histochemistry of helminths particularly Paramphistomes in Kashmir valley, the present study is a step towards bridging the gap. Sheep and cattle like other animals develop diseases due to variety of parasitic infections, resulting in huge economic losses. Fascioliasis and Paramphistomiasis rank high in significance of helminth diseases of sheep and cattle because they cause heavy mortality and morbidity in young cattle and sheep. Various species of Paramphistomes such as *Paramphistomum cervi, Paramphistomum microbothrium, Paramphistomum skrajabini, Cotylophoron cotylophorum, Gastrothylax crumenifer* and

Gigantocotyle explanatum have been incriminated as aetiological agents of Paramphistomiasis. In order to develop various control measures to these helminths we should have a clear understanding of host parasite relationship and a thorough understanding of the host parasite relationship can not be realized until a careful study is made on the biochemical nature of the parasite and its host. Histochemical study helps us to investigate qualitatively the biochemical pattern of different tissues in cellular architecture. The present work demonstrated the presence of general proteins, glycogen, lipids, cholinesterase, acid phosphatase, alkaline phosphatase and calcium in *Cotylophoron cotylophorum* collected from cattle in comparison with *Paramphistomum cervi* collected from sheep.

In order to have a clear understanding and background information about the work done related to the various aspects of the present study, a comprehensive survey of the literature was conducted. Literature survey revealed that most of the studies on the histochemistry of digenetic trematodes have been restricted to some model species like *Fasciola hepatica* and *Schistosoma mansoni.* Many other equally important species of trematodes like Paramphistomes from the point of their veterinary importance have been neglected. Literature survey also revealed that histochemistry is still a fledging field of research primarily so in Kashmir with no information on the histochemistry of helminths. Hence the present work entitled "Studies on the histochemistry of paramphistomes of sheep and cattle" was undertaken.

Mature worms of *Cotylophoron cotylophorum* and *Paramphistomum cervi* were collected from the rumen of cattle and sheep respectively, slaughtered at local abattoirs and analysed for demonstration of various biochemical constituents. The various histochemical methods which were followed during present endeavour include: Mercury Bromophenol Blue for general proteins, Best's carmine for glycogen, Sudan Black B for lipids, Myristylcholine for cholinesterase, Lead Acetate for acid phosphatase,

Calcium Cobalt for alkaline phosphatase and Alizarin Red S for calcium. Paraffin sections were used for the demonstration of proteins, glycogen, lipids and calcium; while as frozen sections were used for the demonstration of enzymes.

Observations of the present study have been divided into two parts. The first part deals with the description of the two amphistome species which formed the subject of the present endeavour and the second part includes the histochemical study. Histochemical distribution of general proteins in *Cotylophoron cotylophorum* and *Paramphistomum cervi* was ubiquitous. High concentration of proteins was observed in the tegumental muscles, oral sucker, acetabulam, ovary and vitellaria. Moderate amount of proteins was observed in tegument, gut caeca and parenchyma in both the species under study. Staining reaction for glycogen revealed large amount of glycogen deposits in parenchyma, oral sucker, acetabulam and vitellaria of *Cotylophoron cotylophorum* as well as *Paramphistomum cervi*. In the present study moderate amount of glycogen was present in tegumental muscles, tunica of ovary and ovary. However glycogen was present in small amount in tegument, tunica of testes and testes in both the species of paramphistomes. Most conspicuous sites for the presence of lipids were excretory ducts and vesicles in both the species of paramphistomes. Histochemical distribution of lipids revealed few sudanophilic lipid granules in parenchyma which were more in the vicinity of the intestinal caeca and subtegumental regions. Moderate amount of lipids were present in tegument and subtegumental muscles. The staining reaction for lipids revealed weak to moderate reaction in gastrodermis, vitelline cells, suckers, ovary and testes.

Distribution in the tissues of *Cotylophoron cotylophorum* and *Paramphistomum cervi* revealed moderate activity for cholinesterase in the muscles of oral sucker, acetabulam and pharynx. Moderate to intense reaction was observed in gut and tegumental musculature. Tunica of

testes and tunica of ovary of both these species revealed moderate activity. Weak activity of cholinesterase was recorded in tegument, parenchyma and vitellaria. Histochemical distribution of acid phosphatase revealed its positive activity in almost all the tissues of *Cotylophoron cotylophorum* and *Paramphistomum cervi*. Muscles of pharynx, its tegument as well as tegument lining the mouth and acetabulam showed intense reaction for acid phosphatase. Similar reaction for acid phosphatase was recorded in the intestinal caecae and various muscle layers of acetabulam in both the species. Vitelline cells and their secretory products were also acid phosphatase positive. Histochemical localization in the tissues of *Cotylophoron cotylophorum* and *Paramphistomum cervi* revealed substantial amount of alkaline phosphatase in almost all the tissues. Intense reaction for alkaline phosphatase was recorded in intestinal caeca, excretory vesicle, tunica of testes and ovary in both the species under study. Moderate reaction for alkaline phosphatase was recorded in oral sucker, acetabulam, vitellaria, tegument and sub tegument; while as parenchyma of both the species showed weak reaction for alkaline phosphatase. In the present study the histochemical distribution of calcium revealed moderate amount of calcium deposits in tegument, vitellaria, intestinal caeca, oral sucker and acetabulam of *Cotylophoron cotylophorum* and *Paramphistomum cervi*. Parenchyma, testes, ovary and excretory vesicles of both these species showed weak reaction for calcium.

As per our observations and research done by various workers, it is revealed that there is no significant difference in the histochemical distribution of various biochemical substances in the tissues of two amphistomes under study. This may be due to the fact that both these species are present in the same habitat i.e., rumen of the host. However differential histochemical distribution of various biochemical substances in different tissues of both the amphistomes showed considerable difference. The current study demonstrated high concentration of proteins

in those organs which are metabolically more active and are involved in synthetic activities. However moderate amount of proteins in caecal lining and contents is expected from dietary origin. Intense reaction for glycogen in the parenchyma can be explained by the fact that parenchyma of trematodes serves as a storage organ for energy reserves and trematodes mostly use glycogen as energy metabolite. Large amount of glycogen was observed in the reproductive organs during the present study. The reason for this may be that trematodes produce enormous amount of eggs and these eggs need sufficient amount of reserve food for the development of the embryo. Lipids were present in the tegument, caeca and reproductive organs, besides the excretory ducts and vesicles. Thus it was concluded that lipids are not exclusively the end products of carbohydrate metabolism in trematodes but they play their role in various metabolically active tissues and organs.

During the present study cholinesterase was localized in musculature of body wall, suckers, digestive system and reproductive system of the amphistomes under study. Presence of cholinesterase in the musculature of these organs suggested its role in contractility. Presence of alkaline phosphatase in the metabolically active tissues is explained by the fact that this enzyme is associated with the membrane transport of carbohydrates constituting the major source of energy for these parasites. Acid phosphatase was observed in the tissues which were associated with absorption, secretion and excretion and is postulated to be associated with these functions. The present study revealed the presence of calcium in those organs which are associated with higher muscular activity, which is explained by the fact that calcium plays very important role in muscle contraction.

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List of Abbreviations used

AC	Acetabulam
CM	Circular muscle
ChE	Cholinesterase
DV	Delimited vesicle
EX	Excretory duct
EV	Excretory vesicle
GD	Gastrodermis
GL	Gastrodermal lining
GS	Genital sucker
IC	Intestinal caeca
LS	Longitudinal section
NSE	Nonspecific esterase
OS	Oral sucker
OV	Ovary
PC	Parenchyma
PH	Pharynx
RM	Radial muscle
TE	Testes
TG	Tegument
TM	Tegumental muscle
TS	Transverse section
ST	Subtegument
VT	Vitellaria
VR	Vitelline reservoir

The domestication of animals occurred during the hunting and gathering phase of human civilization. Domestic ruminants such as sheep and cattle are among the first animals to be domesticated by man to obtain mutton, wool, leather, and milk.

India ranks sixth among the countries of the world with respect to sheep population. India is at the top with fourteen percent of cattle population. Sheep are for the most part docile, non aggressive, gregarious creatures. From husbandry perspective, the most important behavioral characteristic of sheep are their tendency to flock. Sheep dung is a natural source of organic fertilizers with nitrogen and potassium contents double than cattle, so sheep manure is preferable for increasing the fertility of soil. The cow serves as the primary source of milk, the only dietary animal protein for a majority of Indians. In fact cow was so important to early

people of Central Asia that wealth was measured in numbers of cattle. From the early days to present, the cow has continued to be the servant of man. India is occupying first position regarding milk production among the developing countries of the world. India happens to be one of the largest exporters of leather goods made from cattle skin. Hides, horns and hooves are used to prepare cattle feed and fertilizers. Cattle are used by Indian farmers in ploughing and in harvesting agricultural products and transporting it to the market.

Jammu and Kashmir is primarily an agricultural state and animal rearing is one of the major sources of economy to farming community. Our state is situated in the north $32^0.17"$ and $36^0.59"$ north latitude and $72^0.26"$ and $80^0.30"$ east of longitude with total area of 2,22,236 km^2. The climate is variable from subtropical (Jammu plains) to temperate (Kashmir), to temperate cold but arid (Ladakh region). The valley of Kashmir is a bowl shaped basin of Western Himalayan range. Thus practically, the valley is cut off from the Ladakh province in north and from Jammu in south. Named as the “Paradise on the Earth”, it has earned its name due to heavenly features that it possesses. The soil topography, geoclimate, natural meadows and highland pastures of valley are naturally conducive for sheep and cattle rearing. In our state 70% of total population lives in rural areas (1991 census) whose main occupation is agriculture, farming and rearing of livestock. Its contribution to

state economy according to preliminary estimates has been calculated to Rs. 1127 crores (1991- 1992).

There are various diseases which are a major set back to sheep and cattle industry. There are other practices which contribute to low wool, milk and meat production for this industry. The main contributing factors include large animal population with largely diminishing grazing areas and consequent over stocking, poor nutritional standards and traditional husbandry practices. Among diseases viral, bacterial and parasitic diseases are very important in retarding the progress of this industry. The first two i.e., viral and bacterial diseases are easily diagnosed by their clinical signs but parasitic infections when less in number or in early stage are with out clinical signs and thus act as one of the major causes of production loss. Faizal (1999) reported 1/3rd growth retardation in ruminants due to helminth infections.

Almost 300 species of helminths parasitize livestock in India. These belong to three classes of helminths viz. Trematoda, Cestoda and Nematoda. The higher incidence of parasitic infections in domestic animals in a grazing system lowers productivity, leading to important economic losses. The parasite – infected animals increase their metabolic rate and reduce the amount of metabolic energy used for production, as the parasites use their nutrients, damage some vital organs and cause animal to become susceptible to other pathogenic agents (Sykes *et al.*, 1992).

Pathogenecity of these helminth parasites is varied with different intensity. Nematodes are more pathogenic in adult stages. Cestodes are far less pathogenic than nematodes and trematodes, as they only compete for food with host in less numbers but may block the intestinal lumen in heavy infections.

Trematodes are usually more pathogenic in immature stages, feeding vigorously on mucosa in duodenum and other parts of the body. Trematodes are known to infect spectrum of hosts and cause considerable damage to both poikilothermic as well as homeothermic animals, hardly sparing any organ system. The survival of the parasites is influenced by the general biotic factors associated with the micro and macro environments as well as by the intimate physiological and immunological interactions between the parasite and the host which forms the basis of host-parasite relationship. Among the trematode infection, Fascioliasis and Paramphistomiasis rank high in significance of helminthic diseases of sheep and cattle. Various species of paramphistomes such as *Paramphistomum cervi*, *Cotylophoron cotylophorum*, *Gastrothylax crumenifer*, *Paramphistomum microbothrium* and *Gigantocotyle explanatum* have been incriminated as aetiological agents of Paramphistomiasis.

Kashmir is full of meadows and pastures. It is surrounded on all sides by mountains which almost appear to touch the skies. These mountains are covered by lush green grass and over flow with

water streams and springs. These streams and springs contain plenty of snails which serve as intermediate hosts of amphistomes. In early spring, sheep and cattle are allowed to graze in pasture lands and other grazing grounds available in every village. But in the middle of spring and early summer these animals are sent to meadows, pastures and far away forests. These are reared by "Bakerwals" and "Gujars" who are a nomadic race. In the middle of spring they go along with their livestock to these meadows and pastures. They return to the warmer places in autumn. It is at these sites that the live stock get the infection of various diseases, one of them being Paramphistomiasis which is caused by eating grass contaminated with metacercarial stage of different species of amphistomes.

Paramphistomes are parasitic in the alimentary canal of many ruminants. Mature parasites are especially prevalent in the reticulum and rumen. They are usually thick, short (4 – 12 mm. long), fleshy, maggot like worms. These stomach flukes have a complex life cycle which requires an intermediate host for completion. The intermediate hosts are aquatic snails belonging to genus *Helisoma*, *Planorbis*, *Lymnaea* etc. These are small, flat snails from 2 – 5 mm. in diameter. These snails are found in permanent and temporary water courses, irrigation channels, swamps, dam edges and depressions. They are normally found attached to vegetation in these habitats. Adult flukes live in the rumen and reticulum of cattle, sheep and goats. The eggs are passed in faecal

mater. Larvae then hatch in a wet environment and infect the intermediate host – Planorbid snails. Larval development is completed in the snail, and the next stage, the cercaria, leave the snail and attaches to vegetation where it encysts (metacercaria). When the vegetation is grazed by ruminants, the immature fluke excysts and attaches itself to the walls of the small intestine. It later migrates to the rumen and reticulum to become egg producing adult. In light infections, young flukes migrate to the rumen within 4 – 6 weeks and normally no clinical symptoms occur. Egg production begins soon after the fluke enter the rumen.

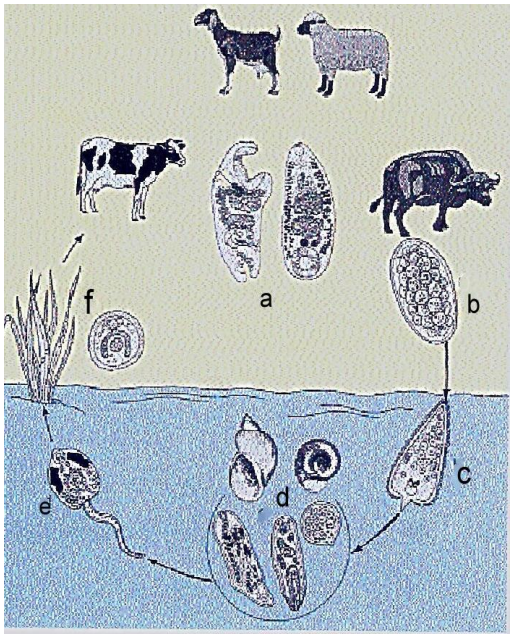


Fig 1.1. Graphic representation of life cycle of Paramphistomum: **a.** Adult fluke found in rumen and reticulum of ruminants pass eggs in manure; **b.** Egg hatchs in wet environment; **c.** Miracidium comes out of the egg; **d.** Larval development in Planorbid snail; **e.** Cercaria comes out from snail; **f.** Cercaria encysts on grass to form metacercarial, where it is eaten by grazing ruminant.

Mature paramphistomes rarely produce clinical symptoms (Horak, 1967; Dube *et al.*, 2003), however immature migrating parasites have been reported causing serious disease and even the death of their hosts by burying themselves in the sub mucosa of duodenum and feeding on the epithelial cells of Brunner's glands which results in anorexia, polydypsia, profuse diarrhoea, a drop in plasma protein concentration and anemia (Buttler and Yeoman, 1962; Boray, 1969; Singh *et.al*, 1984).

A thorough understanding of the physiological aspects of host-parasite relationship can not be realized until a careful study is made on the biochemical nature of parasite and its host. Our knowledge in the field of biochemistry is increasing day by day. The parasite biochemists are employing latest experimental techniques which provide increasingly more accurate results. Most of the findings of biochemical and physiological nature are based on total homogenates because the isolation of different organs and organ system is impossible. The results thus obtained may not provide possible clues to the biological significance of a particular substance in relation to specific organs and organ systems.

To overcome this difficulty to some extent, the biochemical and physiological studies should be supplemented by histochemical studies. The histochemical studies help us to investigate qualitatively the biochemical pattern of different tissues in cellular architecture. Recent advances in the field of histochemistry have

made this task possible. Specific staining techniques give exact location and intensity, thus revealing the degree of involvement of the substances in biochemical reaction in the organ. Histochemical studies will increase the knowledge of worm physiology, which could further lead to deeper understanding of the well recognized host parasite interactions and such information according to Rolfe *et al.* (1994) would be valuable in designing control measures that are efficient and economical.

In recent years much attention has been focused on the histopathology of helminth parasites. Although studies have been made on the histochemistry of various digenetic trematodes, however most of these studies have been restricted to some model species like *Fasciola hepatica* and *Schistosoma mansoni*. Many other equally important species of trematodes like Paramphistomes from the point of view of their veterinary importance have remained more or less neglected. Besides, literature survey reveals no information on histochemistry of Paramphistomes of ruminants in Kashmir valley. To fill this void, a comprehensive work covering the histochemical localization of proteins, lipids, glycogen, calcium, alkaline phosphatase, acid phosphatase and cholinesterase in *Paramphistomum cervi* collected from sheep in comparison with that of *Cotylophoron cotylophorum* of cattle was under taken.

Sheep and cattle, like other animals, develop diseases due to variety of parasitic infections. Fascioliasis and Paramphistomiasis rank high in significance of helminth diseases of sheep and cattle because they cause heavy mortality and morbidity in young ones. A thorough understanding of physiological aspects of the host-parasite relationship can not be realized until a careful study is made on the biochemical nature of the parasite and its host. Most of the work has been done on the effect of parasite on host's haematology and biochemistry and a very little attention has been paid to the changes in the parasite brought about by host. Histochemical studies help us to investigate qualitatively the biochemical pattern of different tissues in cellular architecture. The valuable contributors towards the development of histochemistry are those of Gomori (1952), Gurr (1958) and Pearse (1972). Reports

on the histochemical localization of biochemical constituents in helminths, particularly in paramphistomes are scanty. A brief account of literature on the histochemical localization of various biochemical components in helminths related to present study has been critically reviewed and summarized below.

Von Brand and Mercado (1961) studied glycogen histochemicaly in *Fasciola hepatica*. They found that glycogen granules of various sizes were present in the parenchymal cells, muscular organs like suckers and the cirrus pouch. They also found that glycogen deposition occurs only in non-contractile parts of muscle cells. Vitelline cells and uterine ova also showed large amount of glycogen deposition.

Crompton (1963) studied body wall of *Polymorphus minutus* morphologically and histochemically and concluded that lipoprotein is one of the main structural compounds. Distribution of two enzymes, non- specific esterase and alkaline phosphatase was found through out the body of animal.

Watiz and Schardein (1964) studied histochemical localization of various components including lipid, glycogen, acid phosphatase, alkaline phosphatase and several other components in *Hymenolepis nana*, *H. diminuta*, *Hydatigera taeniaeformis* and *Dipylidium caninum*. They reported species differences in the distribution of alkaline phosphates, lipids, glycogen; while as no species differences in distribution of other substances. They reported moderate level of lipid in cuticle, uterine, testicular and ovarian structures. Larger

quantities of lipids were reported in sub-cuticular cells and parenchyma. Varying amounts of glycogen were reported in parenchyma, excretory duct lining, ovary and testes. Acid phosphatase was reported to be present in heavy amounts in cuticle and less of the enzyme activity in sub-cuticular cells. All other structures were essentially non-reactive for acid phosphates. All four species examined were reported with high alkaline phosphatase activity in cuticle and slight in parenchyma and parenchymal cells.

Schardein and Watiz (1965) studied histochemical localization of non-specific (simple) esterase and cholinesterase in four species of Cyclophyllidean cestodes, *Hymenolepis diminuta*, *H. nana*, *Dipylidium caninum* and *Hydatigera taeniaeformis*. They found that these enzymes were primarily localized in the cuticle and nervous system, but with quantitative and qualitative differences from species to species.

Bogitsh (1967) studied histochemical localization of some enzymes in cysticercoids of the two species of *Hymenolepis*. He reported that intermediate cell layers of the cysts surrounding the larvae of *Hymenolepis diminuta* and *H. microstoma* showed acid phosphatase activity and this activity was optimal at pH 5.0. Alkaline phosphatase activity was reported to be associated with tegument with pH range of 6.0-9.0. The author hypothesized that former enzyme could be involved in the excystation of the cysticercoids. Bogitsh and Shannon (1971) demonstrated acid phosphatase activity in *Schistosoma mansoni* and *Schistosomatium*

douthitti while carrying out cytochemical and biochemical observations on the digestive tracts of digenetic trematodes. They reported acid phosphatase activities in the esophageal gland cells of *Schistosoma mansoni, Schistosomatium douthitti* and in gastrodermis of *Schistosoma mansoni*. At the electron microscope level they reported acid phosphatase activity in esophageal gland cells of both species in cytoplasmic vesicles. They also reported that acid phosphatase activity was associated with the infoldings of basal plasma membranes of esophagus and gastrodermis. They hypothesized that this enzyme was involved with membrane transport.

Fripp (1967) studied the histochemical localization of Acetylcholinesterase and pseudocholinesterase activity in adult *Schistosoma haematobium*, *S. mansoni* and *S. rodhaini*. The researcher observed strong esterase activity in the central nervous system of both sexes.

Halton (1967) studied the distribution of carboxylic esterase activity in adult *Fasciola hepatica* and three distinct esterases were localized. Acetylcholinesterase activity was found in the tegument and neuro-muscular tissues, non specific esterases in the reproductive structures and resistant type indoxyl esterase occurred in gut epithelium.

Krvavica *et al*. (1967) studied histochemical localization of acetylcholinesterase and butyrylcholinesterase in liver fluke (*Fasciola hepatica*) and its developmental stages. They reported large

amount of acetylcholinesterase in the muscles of pharynx, cirrus, the cerebral ganglion and the nerves of fluke. Acetylcholinesterase activity was demonstrated in different organs of larval stages also.

Barry and Thomas (1968) studied histochemical distribution of seventeen enzymes in adult liver fluke. According to their findings, the tegument which was thought to be responsible for absorption of food and excretion of waste materials, the gut was better equipped enzymatically of these functions. Gulcose-6 phosphatase was present in small amounts and β-hydroxybutyrate dehydrogenase was completely absent which they explained by the fact that glycogen was supplied by the liver of host.

Thorpe (1968) carried out comparative histochemistry of immature and mature stages of *Fasciola hepatica* and demonstrated lipid, hydrolytic and oxidative enzymes. He reported that lipid droplets were present in caecal cells, excretory ducts and parenchymal cells of adult flukes but immature fluke contained more amount of lipid than in the adult. Mature *Fasciola hepatica* was reported to show positive reaction for alkaline phosphatase, acid phosphatase, succinic dehydrogenase, tetrazolium reductase, cytochrome oxidase, lactate dehydrogenase and glutamate dehydrogenase. However no detectable cytochrome oxidase or glutamate dehydrogenase activity was reported in immature flukes.

Shield (1969) worked on the histochemical identification of cholinesterases in tape worms - *Dipylidium caninum*, *Echioncoccus*

granulosus and *Hydatigera taeniaeformis* and studied the nervous system in these cestodes by esterase techniques.

Porter and Hall (1970) used histochemical techniques to study the glandular contents of a Cotylocercous cercaria of *Plagioporus lepomis*. They used histochemical techniques to demonstrate carbohydrates, proteins, lipids nucleic acids and metal ions including calcium. They reported presence of acid micro-polysaccharide, protein and sudanophilic substances (lipid) in mucoid glands located dorsally. The caudal glands were reported to contain micro-polysaccharide, protein, calcium and sudanophilic material. They also discussed the possible functions of these glands, with relation to their contents.

Davis and Bogitsh (1971a) while carrying out cytochemical and biochemical observations on the digestive tract of digenetic trematodes demonstrated Arylsulfatase (acid hydrolase) activity in the gastrodermis of *Gorgoderina attenuata* and *Haematoloechus medioplexus*. They reported that its activity is localized in the cytolysomes and micro vesicles as well as on invaginations of basal plasmalemma and membranous remnants in the lumen and its activity was operative over a pH range of 4.2 - 7.2. The same authors (1971b) carried out cytochemical and biochemical observations on the digestive tracts of digenetic trematode *Gorgoderina attenuata*. They reported that the gastrodermis had a basal lamina (muscular) and the luminal surface was extended as digitiform cytoplasmic extensions. Cytoplasm demonstrated an

extensive system of endoplasmic reticulum, Golgi areas, and numerous mitochondria. They also reported three types of membrane - delimited vesicles (DV) designated as DV_1, DV_2 and DV_3. According to them DV_1 and DV_2 vesicles demonstrate acid phosphatase activity and were interpreted as lysosmes and cytolysomes.

Trimble and Bailey (1971) carried out studies on histochemical localization of acid and alkaline phosphatases in *Aspidogaster conchicola* (Trematoda: Aspidobothrea). For acid phosphatase, they reported intense reaction in haptor and gut, moderate reaction in sub-tegument, pharynx, testis and eggs. Weak reaction in vitellaria, while no reaction on body tegument, tunica of testis, cirrus and uterus. For alkaline phosphatase intense reaction was reported in haptor, body tegument, sub tegument, tunica of testes, cirrus and vitellaria; moderate reactions in eggs; weak reaction in testes and no reaction in gut, pharynx and uterus.

Parshad and Guraya (1976) analyzed immature and mature stages of the sheep amphistome *Cotylophoron cotylophorum* histochemically for their lipids. They reported that excretory ducts of the immature worms were the common sites for the accumulation of neutral lipids (Triglycerides) and phospholipids which showed very sparse distribution at the corresponding sites in adult. They also reported that phospholipids and lipoproteins observed in the tegument of the adult were not seen in the immature forms. They reported that intestinal caeca of both forms showed the presence of

diffused and granular lipids which were relatively less in immature. They also discussed the significance of these differences in the lipid contents of the immature and mature forms. The same workers (1977) carried out histochemical localization of proteins, lipids, carbohydrates, acid and alkaline phosphatases in Ovarian balls of *Centrorhynchus cervi* (Acanthocephala). They reported intense to moderate reactions for lipids, proteins and negative reaction for glycogen in oogonia and growing oocyte. For alkaline phosphatase, negative reaction was observed in both oogonia and growing oocyte. However they reported moderate to intense reaction for acid phosphatase in growing oocyte. The same researchers (1978a) carried out morphological and histochemical studies on oocyte atresia in *Centrorhynchus cervi* (Acanthocephalan). They carried out histochemical localization of different tissue constituents including proteins, lipids and nucleic acids in growing and mature oocyte. They reported marked differences in nucleic acid distribution for which more intense reaction was observed in growing oocytes than mature ones. No such marked difference was observed in other constituents. The same investigators (1978b) studied phosphatases of four helminth species; *Ascaridia galli*, *Centrorhynchus cervi*, *Cotylophoron cotylophorum* and *Raillietina cesticillus* calorimetrically. They reported that the optimum pH for acid phosphatase activity was 5.4, 4.5, 4.7 and 5.0 in *A. galli*, *C. cervi*, *R. cesticillus* and *C. cotylophorum*, respectively. The optimum pH for alkaline phosphatase activity was 9.1, 9.5, 8.7 and 8.4 in *A. galli*, *C. cervi*, *R. cesticillus* and *C. cotylophorum* respectively. They also reported that

in *A. galli* and *C. cotylophorum* acid phosphatase showed more activity than alkaline phosphates where as the later was reported more active in *R. cesticillus* and *C. cervi*. The same authors (1978c) made morphological and histochemical studies on the digestive system of amphistome *Cotylophoron cotylophorum*. They reported intense reaction for proteins in caecal cells (both in apical and basal region), brush border epithelium and luminal contents. For glycogen they reported no reaction in caecal cells and brush border epithelium but moderate reaction in luminal contents. They observed moderate reaction in caecal cells, intense reaction in brush border epithelium but no reaction in luminal contents for acid phosphatase. They also reported moderate to weak reaction in caecal cells but no reaction in brush border epithelium for alkaline phosphatase.

Patil and Rodgi (1976) studied histochemical localization of non-specific esterase activity in *Paramphistomum cervi* recovered from sheep. They reported moderate esterase activity in oral sucker and that too restricted to the outer part of this organ. Acetabulum was reported to exhibit weak to moderate activity. The activity of esterase was reported to be more pronounced along the length of the caeca with slightly more intense reaction in caecal contents. Esterase activity in the parenchyma appeared in the form of small granules which were uniformly distributed throughout the tissue with slightly more granules accumulated in cells surrounding the caeca. They also reported moderate activity in epithelial layer of

testes and ovary. The vitellaria were reported to be negative for this enzyme activity.

Mandawat and Sharma (1978) histochemically demonstrated acetyl and butyryl cholinesterase in different tissue of *Paramphistomum cervi*. They reported intense activity of acetylcholinesterase in tegumental musculature, pharynx, gut musculature, tunica of testes, uterus, vitellaria and Mehli's gland, while as moderate to weak activity in lymph channels, excretory canals, excretory bladder, and tunica of ovary. Negative reaction for acetylcholinesterase was reported in tegument, posterior sucker and gut epithelium. For butyrylcholinesterase intense reaction was reported in tegumental musculature, pharynx, gut musculature tunica of testes, uterus and vitellaria, while as moderate to weak activity in tegument, excretory canals, excretory bladder and Mehli's gland. They reported negative activity for butyrylcholinesterase in pharynx, lymph channels, parenchyma and gut epithelium.

Gupta and Agarwal (1979) studied phosphatase system (alkaline and acid phosphatase) in *Gastrothylax crumenifer* (Trematoda). They used king and Wooton method for measuring enzyme activity. They reported maximum acid phosphatase activity in tissue extract of *Gastrothylax crumenifer* at pH 5.0 and that of alkaline phosphatase at pH. 10.0.

Maki and Yanagisawa (1979) carried out a study on acid phosphatase activity demonstrated by intact *Angiostrongylus contonensis* with special references to its function in general. They

reported some monophosphate esters (glucose-1- phosphate, glucose-6- phosphate, α and β-glycerophosphate, P- nitrophenyl phosphate, adenosine-5- phosphate (AMP), guanosine-5- phosphate, cytidine-5- phosphate, uridine-5- phosphate and thymidine-5-phosphate) were hydrolyzed to varying degree, while other esters including adenosine triphosphate and adenosine diphosphate were hydrolyzed to a low or negligible degree. They reported that possible functions of phosphatases in helminths could be the hydrolysis of phosphate esters prior to absorption of the products of hydrolysis, in other words, phosphatases function as intrinsic digestive enzymes. Same investigators (1980) carried out histochemical studies on the acid phosphatase of body wall and intestine of adult filariae in comparison with other parasitic nematodes. Filariae examined were *Litomosides carinii*, *Brugia pahangi* and *Dirofilaria immitis;* other parasitic nematodes used for comparision were *Angiostrongylus contonensis*, *Ascaris lumbricoides* and *Trichuris muris*. They reported that four species of nematodes inhabiting host body fluid viz. three species of filarial worms and *Angiostrongylus contonensis* had intense to moderate acid phosphatase activity in body wall, where as intestinal nematodes such as cuticle of *Ascaris lumbricoides*, *Trichuris muris* and *Ancylostomum caninum* do not showed any activity for it. They also reported intense acid phosphatase activity in the intestine of *Ascaris lumbricoides* while as weak to moderate activity of acid phosphates in the intestine of other nematodes.

Roy (1979) localized certain phosphatases by histochemical techniques in various tissues of a pigeon cestode, *Raillientina johri*. The author reported presence of acid phosphatase, alkaline phosphatase and adenosine triphosphatase (ATPase) in almost all structures (tegument, sub-tegumental muscles, sub-tegumental cells, excretory canal, testes, sperm ductules, vas deferens, cirrus sac, cirrus, ovary, reaceptaculum, seminis, vagina, vitelline gland cells, oocysts, uterus, embryonated eggs). Alkaline phosphatase activity was reported to be absent in parenchyma, spermatocytes, spermatids and spermatozoa and more intense in the tegument of mature and gravid proglottides. The same researcher (1980a) worked on the histochemical localization of non-specific esterase (NSE), acetylcholinesterase and pseudocholinesterase (ChE) in various tissue of a cestode *Raillietina* (*Raillietina*) *Johri* obtained from the intestine of pigeon. He reported localization of NSE in the rostellum, suckers, hooks, tegument, sub-tegumental muscle, excretory canal, cirrus sac, vagina and eggs. Acetylcholinesterase besides being localized in nerves was also visualized in all most all structures as in case of NSE, except hooks, excretory canal and eggs. ChE was reported to be present in nerves, vas deferens, cirrus sac and vagina. The same investigator (1980b) carried out studies on distribution of non-specific esterase (NSE), acetylcholinesterase (AchE) and pseudocholinesterase (ChE) in *Ceylonocotyle scoliocoelium* a bovine amphistome using cytochemical techniques. He reported NSE and AchE activity in almost all tissues of the parasite. He also reported that some structures like sub-tegumental

muscles, vitelline cells, prostate gland cells and secretory vesicles showed NSE activity but not AchE. On the other hand structures like sub-tegumental cells, pars muscular and oviduct exhibited AchE activity but not NSE. He also reported that ChE activity was present only in pharynx, ovary and posterior sucker. The author (1980c) studied distribution and functional significances of alkaline phosphatase and acid phosphatase, adenosine triphosphatase, 5-nucleotidase, glucose-6-phosphatase, thiamine pyrophosphatase and nucleoside diphosphatase histochemically in various tissues of a bovine amphistome *Ceylonocotyle scoliocoelium*. He reported intense reaction for acid phosphatase in anterior and posterior sucker, pharynx, tegument, sub-tegumental muscles, gut, tunica of testes, testes, seminal vesicles, tunica of ovary, ovary, oviduct, uterus, vitelline cells, while as moderate reaction for alkaline phosphatase in all these tissues. Both these enzymes were reported to show no reaction in sub-tegumental cells, ootype and Mehli's gland.

Sharma and Mandawat (1979) studied the histochemical distribution of acid mucopolysaccharide in different tissues including tegument, sub-tegumentry cells, gut epithelium, parenchyma, pharynx, lining of sucker, excretory system, uterus, ovary, testis, prostate gland, Mehli's gland, vitellaria and nervous system of *Paramphistomum cervi* (Trematoda). They reported that acid mucopolysaccharide activity was more intense in the organs which are concerned with secretion, like prostate gland, Mehli's

gland, vitelline gland, sub-tegumentry cells, lining of excretory canal, uterus and gut epithelium. Sharma and Sharma (1981) carried out histochemical studies on neurosecretory cells of *Ceylonocotyle scoliocoelium*. They reported that neurosecretory cells stained intensely with mercuric bromophenol blue indicating synthetic activity of cells. Cells reacted weakly to Sudon Black B. These cells also showed positive reaction for mucopolysaccharides. For various enzymes they reported that neurosecretory cells contained significant amount of esterases, namely non specific esterase (NSE), acetylcholinesterase (AchE), and butyrylcholinesterase (BchE). Non specific alkaline and acid phosphatases were reported to be present in moderate quantities. Sharma *et al*. (1981) used histochemical techniques to study the chemical composition of Mehli's gland secretion in *Ceylonocotyle scoliocoelium*. They reported intense reaction for glycogen in Mehli's gland secretion. Moderate reaction was reported for lipids and proteins. Mehli's gland secretion was reported to exhibit intense reaction for alkaline phosphatase and moderate reaction for acid phosphatase, acetylcholinesterase and butyrylcholinesterase. Sharma and Ratnu (1982) while carrying out morphological and histological architecture of the lymph system of *Orthocoelium scoliocoelium*, also studies histochemical localization of various constituents like glycogen, lipids, proteins, calcium carbonate, alkaline phosphatase, acid phosphatase, aectylcholinesterase and butyrylcholinesterase in the walls of lymphatic vessels and lymphatic fluids. Sharma and Hora (1983) used histochemical

techniques to study the chemical composition of esophageal gland secretion of *Orthocoelium scoliocoelium* and *Paramphistomum cervi*. In non-enzymatic histochemical reactions they reported negative reaction for glycogen in esophageal gland, gastrodermis and brush border. For simple lipids intense reaction was reported in esophageal gland, moderate reaction in gastrodermis and weak reaction in brush border. For non-specific proteins intense reaction was reported in esophageal gland and gastrodemis and moderate reaction in brush border. In enzymatic histochemical reactions they reported moderate activity for alkaline phosphatase in esophageal gland, gastrodermis and brush border. For acid phosphatase intense reaction was reported in esophageal gland and brush border while as moderate activity in gastrodermis. For nonspecific esterase esophageal gland and gastrodernis was reported to show intense activity and brush border was reported to show moderate activity. On the basis of histochemical studies they discussed the role of esophageal glands in the digestive physiology of these two amphistomes. Sharma and Hanna (1988) examined tegument of *Orthocoelium scoliocoelium* and *Paramphistomum cervi* using histochemical techniques and electron microscopy. On the basis of histochemical distribution of acid phosphatase, alkaline phosphatase, non-specific esterase, cholinesterase and succinate dehydrogenase at light microscope level, two distinct regions were recognized an outer and an inner zone. They reported moderate acid phosphatase activity in the distal region of subsyncytial zone and relatively strong activity in tegumental cells. For alkaline

phosphatase moderate but diffused and poorly localized reaction was reported in surface syncytium of tegument near to plasma membrane. Small reaction products were also reported in tegumental cells and musculature. For acetylcholinesterase, intense reaction in subsyncytial zone and moderate reaction in muscle tissue and tegumental cells was reported. The reaction product for non specific esterase was reported to be distributed in the subsyncytial zone of tegument, longitudinal muscle fibers and tegumental cells.

Kanwar and Kansal (1980) carried out cytochemical studies on the prostrate glands of trematodes, *Paramphistomum epiclitum* and *Paradistomoides orientalis*. They reported that pear shaped prostrate gland cells in these trematodes were so arranged that their broader ends containing nuclei, were away from the central lumen. The cytoplasm secretion granules and globules were present. They reported that these granules stained blue with Sudan black B and acid haematein tests revealing the presence of phospholipids. These granules also stained blue with mercuric bromophenol blue and therefore contain proteins.

Sathyanarayana and Anantaraman (1980) carried histochemical studies for localization of peroxidases in tissues of *Gastrothylax crumenifer* (Trematoda: Paramphistomidea). They reported very strong reaction of peroxidase in cuticular region, gastrodermal cells and ventral pouch. In parenchyma, positive

reaction was reported around digestive tract. Weakest activity was reported in oral sucker region.

Saxena (1980) studied acid and alkaline phosphatase in *Aspiculuris pakistanic*a (Nematode). The author reported intense reaction for acid and alkaline phosphatase in intestine (basal, epithelial layer and bacillary layer), ovary and uterus but moderate to weak reaction in musculature, esophagus and excretory canals. Negative reaction was reported for acid phosphatase in cuticle and hypodermis but intense to moderate reaction for alkaline phosphatase in these two structures. Moderate reaction for acid phosphatase was reported in testis and ovary but no reaction for alkaline phosphatase.

Sharma and Sharma (1980) studied histochemical localization of proteins, lipids, glycogen, nucleic acids and acid phosphatases and their relative importance during spermatogenesis in germ cells of *Ceylonocotyle scoliocoelium* (Trematoda: Digenea). They reported intense to moderate reaction for proteins, lipids, and glycogen in spermatogonia, primary spermatocytes, spermatids and cytoplasmic residual mass. Intense reaction was also reported by them in all the above mentioned stages of spermatogenesis except cytoplasmic residual mass which showed weak reaction. Sharma (1984) studied histochemical localization of ATPase and succinate dehydrogenase (SDH) in various tissues of *Ceylonocotyle scoliocoelium* maintained *in vitro* in medium for 15 days and compared the results with normal activities of these enzymes in tissues of worms freshly

collected. He reported low activities of enzymes in the tissues of cultured flukes and suggested that it was due to low metabolic rate. He also reported that enzymes intensity was tissues specific.

Venkatanarsaiah (1981) demonstrated cholinesterase histochemically in nervous system, tegumental and sub-tegumental musculature of the haptor and in the pharyngeal bulb of the Oncomiracidium of *Priceamultae* (Monogenea). According to him presence of cholinesterase in the nervous system was attributed to neurotransmission.

Haque and Siddiqi (1982) worked on histochemical and electophoretic studies on phosphatases of four species of trematodes viz. *Gigantocotyle explanatum* from liver and *Gastrothylax crumenifer* from rumen of water buffalo and *Echinostoma malayanum* and *Fasciolopsis buski* from small intestine of pig. They reported that both alkaline and acid phosphatases were present in tegument, gastrodermis, suckers, testes, ovary, eggs, vitellaria and uterus but in the parenchyma and execratory ducts only alkaline phosphatase activity was observed by them.

Baqui and khatoon (1982) studied the histochemical changes in the adult worms caused by Suramin and Levamisole in rat-*Steria cervi* system and reported notable alteration in the histochemistry of parasite in respect of protein, glycogen and alkaline phosphatase.

Choubisa and Sharma (1983) histochemically demonstrated cholinesterase in the nervous system of Stregeoid metacercaria *Tetracotyle lymnaei*. They reported non specific esterase activity in

the entire nervous system, sub-tegumentary cells, fore body gland cells and lappets. They reported no reaction in the alimentary canal. Oral and posterior suckers were reported to reveal strong esterase activity. Adhesive organs were reported to show only weak activity and caecal cells of fully mature metacercaria revealed moderate activity.

Farooq and Farooqui (1983) studied histochemical localization of esterases in *Avitellina lahorea* (Cestoda) intestinal parasite of sheep and goats. They reported that nonspecific esterases were present in the sucker muscles, post acetabular ganglia, nerve trunks, tegument, excretory canals, cirrus sac, vagina, uterus and the inner membrane of the embryophore. Acetylcholinesterase was also reported in all the above organs including vas deferens and sperm ducts, but was absent from excretory canals and eggs. They also reported that intensity of acetylcholinesterase and acetyl thyocholinesterase was weak compared to NSE. Same authors (1984) histochemically localized non-specific and specific phosphatases in different tissues including tegument, parenchyma, nerves, testes, ovary, uterus, paruterine organ, egg pouches, eggs, muscles, cirrus sacs, vagina, excretory canals and reproductive ducts of *Avitellina lahorea*, an intestinal, parasite of sheep and goats. They reported large quantities of acid phosphatase, alkaline phosphatase and adenosine triphosphatase in almost all these organs except parenchyma, where they reported moderate amounts of acid phosphatase and no alkaline phosphatase. Reproductive

ducts were reported to show moderate amounts of alkaline phosphatase.

Gupta *et al.* (1983) carried out histochemical studies on the oocapt gland cells of Paramphistomes. They studied histochemical localization of various components including protein, glycogen, lipids and mucopolysaccharides in oocapt gland cells. They reported moderate amount of protein in nucleus and duct of the oocapt gland and body of the oocapt gland showed weak reaction for proteins. Strong reaction was reported for glycogen in body and duct of oocapt gland while as negative reaction for glycogen in nucleus. They also reported moderate reaction for lipids in body and duct while as negative reaction in nucleus of oocapt gland for lipids. Gupta *et al.* (1987a) carried out histochemical studies on egg shell formation in *Paramphistomum cervi* (Digenea: Paramphistomatidae). They reported that the newly formed egg shell stained lightly with mercuric bromophenol blue, ninhydrin-schiff and chloramine T-schiff, revealing the presence of proteins containing both free and bound NH_2 groups. Test for phenol and phenoloxidase were reported completely negative. The egg shell was reported to stain moderately with alkaline tetrazolium revealing the presence of both -SH groups and S-S linkages. Strong reaction was reported with performic acid Schiff and positive reaction with performic acid alcian blue revealed the presence of keratin. They also reported that egg shell do not stained positively for carbohydrates and lipids with the various histochemical tests employed in this study. Gupta *et al.* (1987b)

carried out studies on the histochemistry of immature, maturing and mature vitelline cells of *Paramphistomum cervi*. They carried out histochemical localization of different components like proteins, glycogen, lipids, amino acids and phenols in the above mentioned cells. For proteins they reported intense reaction in immature and maturing vitelline cells. For glycogen and lipid intense reaction was reported in all the three types of cells.

Leflore and Bass (1983) carried out observations on morphology and hydrolytic enzyme histochemistry of excysted metacercariae of *Himasthla rhigedona* (Trematoda: Echinostomatidae). They reported that reactions for alkaline phosphatase occur throughout the excretory system while as for acid phosphates in the gut, oral and ventral suckers. Reactions for non-specific esterases and cholinesterases were reported through out nervous system, in the gut and in the oral and ventral suckers.

Gupta and Sinha (1984) carried out studies on acid and alkaline phosphatase biochemically and histochemically in *Haplorchoides ritae*. They reported acid and alkaline phosphatase activity in cuticle, oral sucker, testes, ovary uterus, vitellaria and eggs.

Haseeb *et al. (*1984) carried out histochemical lipid studies on *Schistosoma mansoni* adults maintained *in situ* and *in vitro*. They reported that males contain neutral lipid mainly in the parenchyma and tubercles, while as females contain neutral lipids in vitellaria. They also reported that neutral lipids were released from tubercles

of both paired and unpaired males maintained *in vitro*. Sudan black B staining for total lipids was reported positive in tubercles, parenchyma and vitellaria.

Dunn *et al.* (1985) carried out ultrastructure and histochemical studies on lymph system in three species of amphistome viz., *Gigantocotyle explanatum*, *Gastrothylax crumenifer* and *Srivastavaia indica* from the Indian water buffalo *Bubalus bubalis*. They reported weak to moderate histochemical reactions for carbohydrates in some lymphatic vessels in all the three trematodes. For general proteins, intense reaction in *Gastrothylax* and moderate reaction in other two trematodes was reported. Moderate reaction was reported for lipids in all the three trematodes. Dunn *et al.* (1987a) carried out ultrastructural and histochemical studies on the foregut and gut caeca of *Gigantocotyle explanatum*, *Gastrothylax crumenifer* and *Srivastavaia indica*. They used histochemical methods for the localization of carbohydrates, general proteins, lipids, acid phosphates, non specific esterases, DNA, RNA, haemoglobin, succinate dehydrogenase and adenosine triphosphatase in gut caeca (lumen, microvilli and epithelium) and esophagus and esophageal cells of these trematodes. They reported that fine structure and histochemistry of gut was similar in *Gigantocotyle explanatum*, *Gastrothylax crumenifer* and *Srivastavaia indica*. Dunn *et al.* (1987b) carried out ultrastructural and cytochemical observations on the tegument of paramphistomes - *Gigantocotyle explanatum*, *Srivastavaia indica* and *Gastrothylax*

crumenifer. Histochemical tests for general proteins, lipids, acid phosphatases, non-specific esterases, succinic dehydrogenase, adenosine triphosphatase, DNA, RNA, haemoglobin and ferric iron on the tegument and tegumental cells of the above mentioned trematodes were performed by them. Moderate reaction for carbohydrates and proteins was reported in all the three species. For lipids moderate reaction was reported in tegumental cells. Weak to moderate reaction was reported for acid phosphatase in tegumental syncytium and tegumental cells in *G. crumenifer* and *S. indica* but no results in *G. explanatum*. Negative results for non-specific esterase were reported in *G. crumenifer* and *S. indica* but no results in *G. explanatum*. With their results, they concluded that tegument was largely protective in function and had limited absorptive potential.

Arfin and Nizami (1986) used histochemical techniques to determine chemical nature of egg shell/capsule of some *Cyclophyllidean* cestodes. They reported moderate to intense reaction for basic proteins in testes, ovary, vitelline glands, uterus and egg shell by using Bromophenol blue method.

Fujino and Ishii (1986) carried out comparative histochemical studies of glycosidase activity in *Clonorchis sinensis*, *Eurytrema pancreaticum*, *Fasciola hepatica*, *Dipylidium caninum*, *Hymenolepis nana*, *Ascaris suum*, *Toxocara canis*, *Ancylostoma caninum*, *Trichuris vulpis* and *Dirofilaria immitis*. They reported variation in enzyme distribution and intensity among species and also between

trematodes and nematodes and no marked positive reaction of these enzymes in cestodes.

Mishra and Tandon (1986) used histochemical techniques to visualize nervous system in *Olveria indica*, a rumen Paramphistome. On the basis of esterase localization they described complete nervous system in *Olveria inidica*.

Rajvanshi and Mali (1986) carried out studies on biochemistry and histochemistry to analyse alkaline and acid phosphatase in digenetic trematode, *Pegosomum egretti*. They reported that the optimum pH for acid phosphatase was 5.0 and for alkaline phosphatase 10.0. Histochemical localization of acid and alkaline phosphatase revealed difference in enzyme activity in various tissues, like epidermis, gut, vitellaria, eggs, ventral sucker, tunica of testes , testis, ovary and prostate gland. Intense reaction for acid phosphatase was reported in gut, vitellaria, eggs and prostate gland.

Rao and Krishna (1986) studied histochemical localization of malate dehydrogenase activity in tissues of *Gigantocotyle explanatum* (Trematoda: Digenea). They reported intense malate dehydrogenase activity in tegument, vitellaria and eggs. Moderate activity was reported in musculature, gut, nerves, excretory canal, uterus, testes and Mehli's gland.

Saxena *et al*. (1986) studied distribution pattern of different hydrolytic enzymes including acid phosphatase and alkaline phosphatase in various body parts of *Setaria cervi*. They reported

that intestine of *S. cervi* exhibited higher levels of these enzymes than the genital tract and body wall.

Wajihullah *et al.* (1986, 1990) carried out studies on histochemical distribution of glucose phosphatase, succinic dehydrogenase, glutamate and malate dehydrogenase in *Setaria cervi*, *Diplotriena tricuspis* and *Oesophagostomum columbianum*.

Mackinnon (1987) carried out histochemical localization of proteins, lipids, glycogen and nucleic acids in the oogonia and oocytes in the Trichostronglid nematode *Heligmosomoides polygyrus*. He reported that small granules in cytoplasm of oocytes stained strongly for proteins and lipids. He also reported very little staining for glycogen in both oogonia and oocytes.

Breckenridge and Nathanael (1988) carried out studies on vitelline glands in the commensal temnocephalid *Paracaridinicola platei* using histochemical techniques. They used histochemical techniques to detect various tissue components like carbohydrates (glycogen), proteins, lipids and other tanning precursors. They reported the presence of tanning precursors namely protein, phenols and phenolase in the vitelline glands of *Paracaridinicola platei* (Platyhelminth).

Sukhdeo *et al.* (1988) carried out studies on the histochemical localization of acetylcholinesterase in the cerebral ganglia of *Fasciola hepatica*. They reported acetyl cholinesterase activity in the cell bodies and extra-cellularly in the neuropile of the cerebral ganglia of the adult *Fasciola hepatica*. They also reported that the

reaction product of acetylcholinesterase reaction was found around the somatic cell membranes and extracellular space between closely apposed nerve processes in the neuropile.

Kishore and Sinha (1989) carried out histopathological and histochemical observations on *Microsomacanthus coliaris* (Hymenolepididae: Eucestoda) infection in small intestine of domestic ducks. They used histochemical techniques for the detection of glycogen, proteins, lipids and mucin. They reported presence of heavy glycogen reserves in the sections of cestode and found intense PAS and bromophenol positivity in the section of cestodes. They reported negative reaction for mucin.

Kulkarni and Deshmukh (1989) studied in detail the histochemical distribution of lipids in the parasitic nematode *Trichuris muris* using Sudan black B method. They found cuticle, muscles of the body wall, esophagus, brush border of intestine, reproductive organs - testes, ovary, Oviduct and uterine wall containing eggs to be rich in lipids.

Ramakrishna *et al.* (1989) carried out studies on the demonstration of nervous system in whole mounts of *Moniezia expansa* and *Moniezia benedeni* based on the histochemical distribution of cholinesterase. Besides the presence of acetylcholinesterase and non specific esterase in nerves system, they also reported their presence in reproductive organs.

Abidi and Nizami (1991) carried out a comparative study of the protein content of ten different species of helminths including

Gigantocotyle explanatum, *Fasciola hepatica, Gastrothylax crumenifer*, *Fischoederius elongatus*, *Orthocoelium scoliocoelium*, *Calicophoron calicophorum*. *C. cauliorchis*, *Paramphistomum epiclitum*, *Stilesia globipunctata* and *Avitellina lahorea*. They found that all the amphistomes as well as other trematodes and cestodes occupying same or different habitats show wide intra-Specific variations in their protein content. They suggested that these biochemical differences might be attributed either to the individual metabolic state of the parasite or due to the influence of the host physiology leading to the biochemical variations and adaptations of the parasites as in the case of pouched amphistomes.

Brennan *et al.* (1992) worked on ultrastructural and histochemical studies of lymph system of *Gastrodiscoides hominis* (Paramphistoma: Digenea). They carried out histochemical test for localization of general carbohydrates, general proteins, lipids, DNA, RNA and haemoglobin on the lymphatic fluid of the above mentioned trematode. They reported that lymph matrix contained high amounts of proteins and neutral lipids while carbohydrates were generally absent. They also reported presence of haemoglobin in the lymph system.

Mattison *et al.* (1992a) carried out ultrastructural and histochemical studies on the digestive tract of juvenile *Paramphistomum epiclitum*. They carried out histochemical tests of esophagus and intestinal caeca for localization of carbohydrates, general proteins, acid mucopolysaccharides, melanin, DNA, RNA

haemoglobin and ferric iron. They reported that esophagus displayed staining reaction similar to the tegument, while as lumen was reported to show faint evidence of amylase, susceptible carbohydrates. According to them general proteins and acid mucopolysaccharides displayed faint staining in lumen of both the esophagus and caeca. They reported faint staining for amylase resistant carbohydrates in caecal epithelium and moderate staining for general proteins and mucopolysaccharides. They also carried out enzyme histochemical tests on the esophagus and intestinal caeca of juvenile *Paramphistomum epiclitum* for various enzymes including acid phosphatase and alkaline phosphatase. Moderate activity for acid phosphatase was reported in esophagus and weak activity for alkaline phosphatase. In intestinal caeca they reported moderate enzyme activity for both acid phosphatase and alkaline phosphatase. Same investigators (1992b) carried out ultrastructural and histochemical studies on proto-nephridial system of juvenile *Paramphistomum epiclitum* and *Fischoederius elongatus*. They carried out histochemical tests on protonephridial tertiary ducts of newly excysted *Paramphistomum epiclitum* for general carbohydrates, general proteins, acid mucopolysaccharides and melanin. They reported moderate to intense reaction for carbohydrates in syncytium and lumen of tertiary ducts. For general proteins faint staining was reported in the syncytium and no reaction in lumen. They also carried out histo-enzymological tests on protonephridial ducts of juvenile *Paramphistomum epiclitum* and *Fischoederius elongatus* for various enzymes including acid and

alkaline phosphatase. They reported intense activity for alkaline phosphatase in syncytium of *Paramphistomum epiclitum* and *Fischoederius elongatus*, while no reaction for alkaline phosphatase, in the lumen of these parasites. Moderate activity for acid phosphatase was reported in syncytium of *Paramphistomum epiclitum* only and no reaction in *Fischoederius elongatus*. Same researchers (1992c) carried out ultrastructural and histochemical studies on lymph and parenchyma of juvenile paramphistomes, *Paramphistomum epiclitum* and *Fischoederius elongatus*. They reported intense staining for general proteins and only faint staining for glycogen in rumen stages of *Paramphistomum epiclitum.* Faint staining was also reported for DNA and RNA moderate to intense staining for lipid bound sulphydryl and disulphide groups was reported. Enzyme histochemical tests were also carried out by them on lymph and parenchyma of juvenile *Paramphistomum epiclitum* and *Fischoederius elongatus* for various enzymes including acid phosphatase and alkaline phosphatase. They reported moderate reaction for acid phosphatase and alkaline phosphatase in lymph of *Paramphistomum epiclitum* and *Fischoederius elongatus* but weak activity in parenchyma of both the trematodes. Same authors (1994) carried out studies on histochemistry and ultrastructure of the tegument of juvenile paramphistomes *Paramphistomum epiclitum* and *Fischoederius elongatus*. Histochemical tests were carried out for localization of general carbohydrates proteins, acid mucopolysaccharides, melanin, DNA, RNA, haemoglobin, ferric iron and various enzymes including acid phosphatase and alkaline

phosphatase in the tegument of above mentioned trematodes. They reported moderate to intense staining for carbohydrates (amylase resistant) in the syncytium and intense in the cytons and pigment cells. For general proteins they reported faint to moderate staining in sycnytium and pigment cells. They also reported intense staining for general proteins in the cytons where carbohydrates were not detected. In histo-enzymological studies they reported that phosphatases exhibited only faint activity in the syncytium and moderate activity in the tegumental cytons.

Johal and Joshi (1992) histochemically observed localization and distribution of proteins and carbohydrates in female reproductive system of *Trichuris ovis*, a nematode parasite of sheep. They reported that oocytes accumulate both proteins and carbohydrates during their migration down the ovary as well as in the fertilization chamber. According to them during shell formation carbohydrates were used in the formation of chitinous layer where as proteins were stored in the form of yolk. They also reported that uterine epithelium secreted proteins which were deposited in the outermost layer of the egg shell. Johal (1995) carried out studies on histochemical localization of proteins, carbohydrates lipids and nucleic acid in *Oesophagostomum columbianum* during oogenesis. The author reported presence of negligible amount of protein and carbohydrates in oogonia where as oocytes showed progressive increase in concentration of these two metabolites. The researcher reported that in mature ova, proteins formed the main bulk of egg

yolk while the glycogen was used in the formation of outer envelops of the egg shell. Lipids were reported to be restricted to oolemma and nuclear region in oocytes but were incorporated in large quantity in the egg yolk of fertilized ova. Johal and Shivali (1996) carried out histochemical observations on the body wall of *Trichuris ovis*. They reported that the cuticle of *Trichuris ovis* was enveloped by a thin membranous epicuticle having carbohydrates, acid mucopolysaccharides and lipids as its main constituents. The latter two were responsible for the resistant nature of the cuticle and make it a selectively permeable entity. The protein present in the contractile part of the muscle cells was of collagenous type where as proteins along with RNA were observed in cortical layer, hypodermis and non-contractile part of the muscle cell suggesting that these layers of the body were metabolically active. Johal and Jatindar (1998) carried out histochemical study on the intestinal epithelium of *Oesophagostomum columbianum*. They used mercuric bromophenol blue (for proteins), periodic acid Schiff (for carbohydrates), Best's carmine (for glycogen) Sudan Black B (for lipids) and Methyl green and Pyronin Y (for nucleic acids) methods for histochemical study. They found that the intestinal epithelium is rich in carbohydrates, glycogen and proteins. Lipids were reported to form main structural elements of basal lamina enclosing intestinal epithelium.

Fried *et al.* (1995) used thin layer chromatography and histochemistry to analyze neutral lipids in the intramolluscan larval

stages of *Leucochloridium variae* and in tissues of the snail host *Succinea ovalis*. In the histochemical tests they reported the presence of neutral lipid droplets in the suckers, parenchyma, and excretory system of the encysted metacercariae. The residual snail tissue was reported ORO (oil red o) negative by them.

Humphries and Fried (1996) made histochemical and histological studies on the excysted metacercarial and cercariae of *Echinostoma revolutum* and *Echinostoma trivolvis*. They found general staining of body with PAS, Alcian blue, Toluidine blue, thionine, bromophenol blue, Oil Red O, Alizarin red and silver nitrate in excysted metacercariae and cercariae of both echinostome species.

Arsac *et al.* (1997) studied histochemically alkaline phosphatase activity of *Echinococcus multilocularis*. They used Gomori's method for detection of alkaline phosphatase in different stages of the developing worm. They reported alkaline phosphatase activity in the excretory ducts of 8 to 11 day old strobila and in the tegument of mature proglottis of 16 day old worm.

Sampour (2001) studied the chemical nature of the egg shell of *Haploporus benedenii* (Haploporidae: Digenea) using histochemical tests. He found that egg shell was produced by phenolic compounds in vitelline globules within vitelline cells and consisted of a guinone-tanned protein, together with some stabilization with S-S bonds.

Humiczewska (2002) by using histochemical and cytometric methods studied the enzymes responsible for membrane transport (alkaline phosphatase, adenosine tri phosphatase and 5-nucleotidase) in developing sporocyst of *Fasciola hepatica*. He subjected tegument, parenchyma as well as the germ balls to the histochemical analysis at various periods of growth and development of sporocyst. He found that the most active metabolism occurred in the germ balls of sporocysts on 8th and 15th day of development, which was associated with intensive proliferation and subsequently differentiation of embryos with in the germ balls.

Cokugras (2003) reviewed the structure and physiological importance of butyrylcholinesterase (Cholinesterase). He reported that animal cholinesterase was widespread enzyme present in cholinergic and non-cholinergic tissues as well as in plasma and other body fluids. BChE preferentially acts on butyrylcholine, but also hydrolyses acetylcholine. Its activity was more important in scavenging of organophosphate and carbamate inhibitors, in regulating cholinergic transmission in the absence of acetylcholinesterase and in activation of some drugs such as cocaine, aspirin, amitriptyline, bambuterol and heroin.

Zurawski *et al.* (2003) carried out cytochemical studies on the neuromuscular system of the deporpa and juvenile stages of *Eudiplozoon nipponicum* (Monogenea: Diplozoidae). By using histochemical localization they demonstrated the neuronal

pathways in whole mount preparation of the unpaired diporpae and freshly paired juvenile stages of *Eudiplozoon nipponicum*.

Ghosh *et al.* (2005) carried out studies on lipid classes and fatty acid composition of a digenetic trematode, *Paramphistomum cervi* and compared it with the fatty acid composition of its host, *Capra hircus*. They found that the total lipid content of *Paramphistomum cervi* was 1.23% of the wet weight of the tissue. Percentage of lipid classes of *Paramphistomum cervi* recorded were 39.04% (neutral lipid), 21.23% (glycolipid) and 39.73% (phospholipid). Palmitic (saturated) and oleic (unsaturated) acids were predominant fatty acids in both the parasite and the host. They assumed that the parasite was completely dependent on the host for fatty acids; however parasite increased the amount of some of the fatty acids by chain elongation process.

Humiczewska and Rajski (2005) studied the effects of the presence of sporocysts, rediae and cercariae of *Fasciola hepatica* on the lipid content in the digestive gland of *Lymnaea truncatula* as well as on lipid levels in tissues of the parasites. Lipids were examined by means of histochemical and cytophotomertric techniques. They found that the snail's digestive gland lipid level was almost halved in 20 days past infection, a more than 80% reduction was visible after the subsequent 40 to 60 days. They suggested that the reduction of lipids in the digestive gland of the infected snail points at mobilization of lipid's energy reserves to compensate for the deficiency of carbohydrate used by the parasites.

They also found that parasite tissue such as tegument, pharynx, suckers and germ balls show considerable lipid contents and were metabolically active. From this they concluded that lipids were used as energy source by developmental stages of this parasite.

Kemmerling *et al.* (2006) studied the activity, location and molecular forms of acetylcholinesterase (AChE) in different stages of development of *Mesocestoides corti* (cestode) from larvae to adult forms of this endoparasite. They suggested that AchE is a molecular marker of nervous system in Platyhelminthes. The change in molecular forms of this enzyme and increase in its activity during development from larvae to adult worm may reflect the more complex nervous system necessary to adjust and coordinate the movement of a much biggest structure.

Literature survey reveals that histochemistry is still a fledgling field of research primarily so in Kashmir. Some work regarding histochemistry with respect to fish fauna of Kashmir has been done by Channa (1979), Raina (1983) and Tiku (1983) who have histochemically investigated the absorption of iron and lipid in few freshwater teleosts. But no information is available on the histochemistry of helminth parasites including Paramphistomes in Kashmir. Hence the present work entitled *"Studies on Histochemistry of Paramphistomes of Sheep and Cattle"* was undertaken in order to make a beginning of this type of a study which could prove a beacon light for future researchers.

Since scanty work has been carried out on the histochemistry of helminths in general and no work in this part of the globe in particular and as such a detailed methodology was not available in a consolidated form; whatever little is present, it is in a scattered form. So it was felt necessary to give a detailed account of the methodology adopted for present study.

3.1. Collection of material

The live amphistomes were collected from rumen of freshly slaughtered sheep and cattle in local abattoirs. Worms were carefully removed with the help of fine forceps, placed in normal saline (0.75%) and washed thoroughly. The worms were fixed in the suitable fixatives depending up on the component to be studied.

3.2. Fixation and Fixatives

The worms were fixed so as to preserve permanently the tissues and cells in as life like state as possible and to leave them in a condition which facilitated differential staining with dyes and other reagents.

Various fixatives used for localization of different components during the present study are summarized below.

Constituent	Fixative used	Time of fixation
Protein	Carnoy's fixative	2 – 6 hours
Glycogen	Carnoy's fixative /Absolute alcohol	1 – 4 hours
Lipid	Cobalt calcium formol	24 hours
Calcium	Absolute ethanol	2 – 4 hours
Cholinesterase	4% cold neutral formalin	1 – 4 hours
Alkaline phosphatase	4% cold neutral formalin	1 – 4 hours
Acid phosphatase	4% cold neutral formalin	1 – 4 hours

The aforesaid fixatives were prepared as under:

3.2.1. Carnoy's fixative

Absolute alcohol	:	60 ml.
Chloroform	:	30 ml.
Glacial acetic acid	:	10 ml.

The tissues fixed in Carnoy's fixative were directly transferred to dehydrating solvent (70% alcohol).

3.2.2. Cobalt calcium formal

Cobalt nitrate	: 1 gm.
10 % calcium chloride	: 10 ml.
Commercial formalin	: 10 ml.
Distilled water	: 80 ml.

The tissues fixed in cobalt calcium formol were washed in running water so as to remove all the traces of formalin and then transferred to dehydrating solvents.

3.2.3. 10 % buffered formalin

Commercial formalin	:	10 ml.
Distilled water	:	90 ml.
Sodium acid phosphate (monohydrate)	:	0.4 gm.
Disodium phosphate (anhydrous)	:	0.65 gm.

3.2.4. 10 % neutral formalin

Commercial formalin	:	10 ml.
Distilled water	:	90 ml.

The above mixture was kept in a container containing a layer of marble chips ($CaCO_3$) which neutralizes formic acid produced by the formalin.

3.2.5. Absolute ethanol

Tissues fixed in the said fixative were directly transferred to the clearing agent.

3.3. Preparation of specimens for histochemical studies

For histochemical localization of various components paraffin sections and frozen sections were used.

3.3.1 Preparation of specimens for getting paraffin sections

Paraffin sections were used for histochemical localization of proteins, glycogen, lipid and calcium and for getting paraffin sections following steps were employed.

3.3.1.1. Pre embedding treatment

Specimens fixed in formalin were thoroughly washed in running water for 1-2 hours so as to remove all the traces of formalin. Such specimens were then dehydrated in ascending grades of alcohol viz. 30%, 50%, 70%, 90% & absolute alcohol.

Specimens fixed in Carnoy's fixative and preserved in 70% alcohol were dehydrated by passing through 90% and absolute alcohol.

Dealcoholization was done by two changes in xylene.

3.3.1.2. Infiltration

After clearing in xylene, the material was infiltrated with paraffin wax, by keeping it first in a mixture of xylene and wax in

the ratio of 1:1 for half an hour at 40 – 42 ^{0}C in an oven and finally two changes in pure wax (melting point 56 – 58 ^{0}C) maintained in a molten state in an oven, for 2 – 4 hours.

3.3.1.3. Embedding

Embedding of infiltered material was done in rectangular paper boats. Fresh embedding paraffin wax was used to fill paper boats to the brim. The paper boat was placed on and between glass slides to facilitate handling. The bottom layer of paraffin was allowed to solidify by keeping the paper boat in cold water, while the surface layer was kept melted by touching it occasionally with heated spatula or forceps. The material was properly oriented in the wax with heated forceps. The boat was kept in water for 15 – 60 minutes. The blocks were stored in a cold place for further processing.

The blocks were removed from the paper boats and after trimming them properly, each block was fixed to the block holder. The sections, 5 -8 microns thick, were cut with the help of a rotatory microtome (Weswox model). Continuous ribbons of the material were cut and passed over a ribbon carrier. These ribbons were placed in section trays in order in which they were cut.

PARAFFIN WAX EMBEDDING SCHEDULE

Solution/ Reagent	**Time**
Material fixed in formalin fixative	2 hrs – overnight
Washed in running water	1 – 2 hrs
30% alcohol	20 – 30 minutes
50% alcohol	20 – 30 minutes
70% alcohol	2 hrs – overnight
90% alcohol	20 -60 minutes
Absolute alcohol 1st change	20 – 30 minutes
Absolute alcohol 2nd change	20 – 30 minutes
Absolute alcohol + Xylene (1:1)	20 – 30 minutes
Pure xylene 1st change	20 – 30 minutes
Pure xylene 2nd change	20 – 30 minutes
Xylene saturated with paraffin wax	20 – 30 minutes
Molten paraffin wax 58 ^{0}C 1st change	30 minutes – 2 hrs
Molten paraffin wax 58 ^{0}C 2nd change	30 minutes – 2 hrs

3.3.1.4. Affixation of paraffin sections

The cleaned slides were coated with thin film of Mayer's affixative, whose composition and preparation are as under:

Mayer's Affixative (Mayer's albumin)

Egg albumin	:	50 ml.
Glycerol	:	50 ml.
Sodium salicylate (preservative)	:	1 gm.

The three ingradients were thoroughly mixed together and filtered.

Preboiled distilled water was used as floating medium for sections with this fixative. The ribbons of sections were divided into strips of the correct lengths, which were then placed, with shiny side down, in rows on the smeared slides. Enough floating medium (water), to float all the sections free of the slide, was added from one end of the slide. The slides were carefully transferred to a Hot plate, which flattened the sections. Additional floating medium was added whenever necessary. When the sections flattened and straightened, the slides were removed from the hot plate and excess fluid drained away. The slides were kept overnight for complete drying.

3.3.1.5 Processing of paraffin sections

The paraffin sections were then further processed under following steps.

a) Deparaffination / Dewaxing

The removal of paraffin was accomplished by submerging the slides in pure xylene. Two changes were given, each for about 5 minutes.

b) Removal of xylene

Xylene was removed from the sections by processing the slide through two changes of absolute alcohol for about 5 minutes in each change.

Further processing of sections was done according to the methods employed for detection of different components.

3.3.2. Preparation of specimens for getting frozen sections

For histochemical localization of various enzymes frozen sections were used. For this purpose material was fixed in cold (chilled Formalin), for 1- 4 hours in deep freezer and washed thoroughly with running water. Some times fresh material immediately after collection, without fixation, was as such used for sectioning. The frozen sections 8 – 10 microns thick were cut at – 20 ^{0}C by using ***Leica CM 3050 S*** cryostat. The frozen sections were fixed on clean glass slides, dried in air at room temperature and processed immediately according to the procedure employed for the detection of different enzymes.

3.4. Methods used for the histochemical localization of different constituents

The various methods which were used in the present study for the histochemical localization of different constituents are as under.

Constituent localized		Method employed
1. Proteins	:	Mercury Bromphenol Blue (Bonhag, 1955).
2. Glycogen	:	Best's Carmine (Best, 1906)
3. Lipid	:	Sudan Black B (Mc Manus, 1946)
4. Calcium	:	Alizarin Red S (Dahl, 1952)
5. Cholinesterase	:	Myristoylcholine method (Gamori, 1952)
6. Alkaline phosphatase	:	Calcium Cobalt method (Gamori, 1952)
7. Acid phosphatase	:	Lead Acetate method (Lake, 1965)

These methods employed for the localization of various components are summarized below.

3.4.1. Bromphenol blue method for proteins (Bonhag, 1955)

This staining method was introduced by Durrum (1950) for demonstration of proteins on filter paper spots. It was adopted as a general stain for protein by Mazia *et al.* (1953). These authors stated that preparations stained by their procedure followed Beer-Lambert laws and that the amount of dye bound was proportional to amount of protein over a wide range. Proteins are stained deep clear blue colour.

Solutions and material

1% Mercuric chloride and 0.05 % Bromphenol Blue in 2 % aqueous acetic acid.

Method proper

Paraffin sections were brought to water through xylene and descending grades of alcohol, stained in the staining solution for 1-2 hours at room temperature and rinsed in 0.5 % acetic acid. The stained sections were then transferred directly into Tertiary Butyl alcohol solution, cleared in xylene and mounted in DPX.

3.4.2. Best's Carmine method for glycogen (Best, 1906)

This staining technique demonstrates glycogen by bond formation between OH group on glycogen, and H atoms of carminic acid. Thus glycogen deposits appeared red.

Solutions and materials

i) Ehrlich's Hematoxylin

Acetic acid glacial	:	10 ml.
Alcohol, Absolute	:	25 ml.
Hematoxylin crystals	:	2 gm
Glycerin	:	100 ml.
Water, distilled	:	100 ml.
Potash alum	:	10 gm.

Glacial acetic acid and alcohol were mixed and to it hematoxylene crystals were added.

When dissolved additional 75 ml. of absolute alcohol and glycerin were added. Distilled water was heated and to it potash alum was added. Then this solution was added to hematoxylene solution. The mixture was kept in light (with occasional admission of air) until it acquired a dark red colour.

ii) Carmine stock solution

Carmine	:	2 gm.
Potassium carbonate	:	1 gm.
Potassium chloride	:	5 gm.
Distilled water	:	60 ml.

iii) Staining solution

Stock carmine solution	:	10 ml.
95 % alcohol	:	15 ml.
Conc. Ammonia	:	15 ml.

Iv) Differentiating solution

Absolute Alcohol	:	60 ml.
Distilled water	:	40 ml.
Conc. Ammonia	:	a few drops.

Method proper

The paraffin sections were de waxed in xylene and treated with 1% celloidin in Absolute ethanol / ether equal parts and dried in air. After this sections were brought to water through different

grades of alcohol and stained in Ehrlich's hematoxylene for 5 minutes. The sections were rinsed and differentiated rapidly in 1% acid alcohol. Subsequently sections were then rinsed in water and stained in freshly prepared Best's carmine staining solution for 5-10 minutes. The stained sections were differentiated in Best's differentiating solution without previous rinsing for 5-60 seconds. The sections were then washed in 80% alcohol and dehydrated in absolute alcohol. Finally sections were cleared in xylene and mounted in DPX.

3.4.3. Sudan Black B method for lipids (Mc Manus, 1946)

Sudan Black B is a diazo dye and these dyes are soluble in lipids, particularly in phospholipids. Sudan Black B was introduced into Histochemistry by Lison and Dagnelie (1935). Lipids stain black or blue if present in sufficient quantity. Even a brownish black stain may be an indication of the presence of lipids.

Preparation of solutions and reagents

i) Saturated solution of Sudan black B

It was prepared by adding excess of the dye to 70% alcohol in a tightly stoppered bottle followed by vigorous shaking. The whole solution was kept over night incubated at 37 ^{0}C. Some more dye was added in the morning and kept continuously in incubator at 37

^{0}C. The solution was shaken several times during the course of day and filtered before use.

ii) Glycerin jelly

The composition of Glycerin jelly used was

Gelatin	:	10 gm.
Distilled water	:	60 ml.
Glycerin	:	70 ml.
Concentrated Phenol	:	1 gm.

The glycerin jelly was prepared by dissolving gelatin in water, heating just to dissolve the gelatin. Then glycerin and phenol was added.

Method proper

The sections, after bringing to 70% alcohol through xylene and grades of alcohol were stained in Sudan Black B for 1 – 4 hours. Excessive dye was removed by rinsing quickly in 70%alcohol. The sections were then washed in water and counter stained in 1% aqueous Neutral Red for 1 minute. Finally sections were again washed in water and mounted in Glycerin jelly.

3.4.4. Alizarin Red S method for Calcium (Dahl, 1952)

This technique was evolved by Dahl (1952) and mechanism of Alizarin staining was investigated by Pucutler *et al.* (1969). They

found that salt formed between calcium (deposits) and Alizarin Red S appears orange red in tissues and this reaction is sensitive to pH.

Solutions

i) Staining solution

Alizarin Red S	:	0.5gm
Distilled water	:	45 ml.
1: 100 dilutions of 28% NH_4OH	:	5 ml.

The pH was maintained at 6.3 – 6.5.

ii) Acid Ethanol

Concentrated HCl	:	1 part
95% Ethanol	:	10,000 parts.

The sections mounted without adhesive were brought to water through xylene and grades of alcohol and stained in Alizarin Red S solution for 2 minutes. After washing the stained sections thoroughly in distilled water, followed by a rinse in acid alcohol for differentiation, sections were then dehydrated by passing them through ascending grades of alcohol. Finally the sections were cleared in xylene and mounted in Cedar wood oil.

3.4.5. Myristylcholine method for cholinesterase (Gamori, 1952)

The substrate here used was higher fatty acid of choline. On hydrolysis the fatty acid combines with cobalt to give a fine granular

white precipitation at the site of enzyme action. This white precipitate was made visible by subsequent treatment with yellow ammonium sulphide resulting in the formation of dark brown or black precipitate of cobalt sulphide at site of enzyme action.

Solutions and reagents

i) Stock solution I

0.1 M Vernol Acetate buffer, pH 7.6 : 50 – 100 ml.

0.1 M Cobaltous Acetate : 30 – 50 ml.

Distilled water was added to make up 300 ml.

To it 1 gm. each of $CaCl_2$, $MgCl_2$ and $MnCl_2$ was added. A crystal of Thymol was added to this solution and stored at 4 ^{0}C.

ii) Stock solution II

0.02 M Myristyl choline in distilled water.

A crystal of Thymol was added and stored at 4 ^{0}C.

For use 1 ml. of stock solution II was added to 50 ml. of stock solution I, previously heated to 37 ^{0}C.

Method proper

Frozen sections 8 – 10 microns thick were mounted on clean slides and incubated for 1- 4 hours in the working solution of Myristyl choline at 37 ^{0}C. The sections were then washed in running water and treated with dilute Yellow Ammonium Sulphide for 1 – 2 minutes. This was followed by brief washing in water and counter

staining in 1% eosin. Finally sections were again washed in water and mounted in glycerin jelly.

3.4.6. Calcium Cobalt method for Alkaline phosphatase (Gamori, 1952)

It is based upon the principle that Calcium phosphate deposition takes place at the site of enzyme action when sections are incubated with an organic phosphate ester in presence of calcium ions, usually at pH 9. Calcium phosphate is changed to cobalt phosphate by treating with cobalt nitrate and finally cobalt phosphate is changed into cobalt sulphide by the treatment of ammonium sulphide, which appears black or brownish black in tissues.

Preparation of solutions and reagents

i) Incubating medium

3% Sodium-β-Glycerophosphate	:	10 ml.
2% Sodium diethyl barbiturate	:	10 ml.
Distilled water	:	5 ml
2% Calcium chloride	:	20 ml.
5% Magnesium sulphate	:	1ml.

ii) 2 % cobaltous acetate : 50 ml.

Method proper

Frozen sections 8 – 10 micron thick were mounted on clean slides without any adhesive, dried in air at room temperature for 1-2 hours and incubated in Incubating solution for ½ - 4 hours at 37 ^{0}C. The sections were then washed in running water and treated with 2% Cobaltous acetate solution for 3 – 5 minutes. After this the sections were again washed in water and given the treatment of dilute yellow ammonium sulphide for 1 – 2 minutes. Again washed in water and counter stained in aqueous 1% Eosine. Finally the sections were washed in water and mounted in glycerin jelly.

3.4.7. Lead Acetate method for Acid phosphatase (Lake, 1965)

Frozen sections were incubated in a medium containing Sodium-β-Glycerophosphate, an organic substrate for this enzyme and lead acetate. Lead phosphate deposition occured at the site of enzyme action which was made visible by subsequent treatment with ammonium sulphide, resulting in the formation of black or dark brown precipitate of lead sulphide at site of enzyme action.

Solutions and reagents

i) Standard Gamori medium:

31.5 mg of Sodium-β-Glycerophosphate in 5ml. 0.1 M acetate buffer at pH 5.0

5 ml. of 0.008 M Lead Acetate was added before use.

Method proper

Frozen sections mounted on clean glass slides were incubated in Gomori medium for 1-30 minutes at 37 ^{0}C, washed well in tap water and treated with buffered (pH 7-8) dilute Ammonium sulphide for 1-2 minutes. Sections were then washed in water and counter stained in 1% aqueous Eosine for 3-5 minutes. Finally sections were again washed in water and mounted in Glycerin jelly.

3.5 Preparation of whole mount specimen for study

3.5.1. Fixation

The worms collected from the rumen of freshly slaughtered sheep and cattle were fixed separately in Carnoy's fixative. The worms were washed thoroughly in distilled water and placed between two slides, Carnoy's fixative was allowed to run between the slides with the help of dropper and some additional weight was placed over the slides to ensure proper fixation of the specimens. The specimens were kept as such for 10 – 30 minutes. After fixation the specimens were washed 3 - 4 times in 70% alcohol, so as to remove all traces of fixative.

3.5.2. Preservation

After washing in 70% alcohol, the worms were preserved in 70 % alcohol.

3.5.3. Staining

The worms were stained in Aceto-alum Carmine (aqueous stain). Since the stain used was aqueous, the worms were brought to water by passing through the descending grades of alcohol – 50% and 30% alcohol, before staining.

Composition of Aceto-alum Carmine

Carmine powder	:	5 gm.
Glacial acetic acid	:	5 ml.
Potash Alum	:	5 gm.
Distilled water	:	200 ml.

The specimens were kept for 5 – 10 minutes in stain and the exact staining was checked by transferring the specimens in distilled water and observing the gross appearance. If under stained, it was returned back to stain till properly stained. In case of over stain, they were destained by using acid water.

Composition of Acid water

Hydrochloric acid (HCl)	:	1 ml.
Distilled water	:	100 ml

3.5.4. Dehydration

After proper stain was taken by the specimens they were washed in distilled water. The specimens were then dehydrated

gradually by passing through ascending grades of alcohol (30%, 50%, 70%, 90% and absolute alcohol). In lower grades they were kept for 3 – 5 minutes. In absolute alcohol, two washings were given each of 10 minutes duration to ensure complete dehydration.

3.5.5. Dealcoholization

After complete dehydration the specimens were transferred to xylene for dealcoholizaton and clearing. In xylene two washings were given each of 10 - 15 minutes duration to ensure complete dealcoholization.

3.5.6. Mounting

After clearing, the worms were mounted in the desired amount of DPX (Dextrin Plasticized Xylene) on clean glass slides and covered by cover slips.

3.6. Photomicrography

Photomicrography of slides was done under digital microscope ***Bx60 F-3 Olympus*** by using *Olympus* ***DP 12*** **camera**.

4.2.1 Histochemical distribution in tissues of *Cotylophoron cotylophorum*

The tissues of *Cotylophoron cotylophorum* were subjected to various histochemical tests for the localization of following biochemical components.

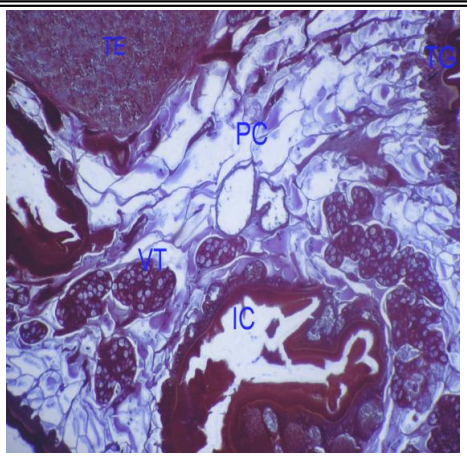
4.2.1.1 General proteins (Table 4.1, Photomicrographs 4.1- 4.8)

In the present study distribution of general proteins in the tissues of *Cotylophoron cotylophorum* was ubiquitous. Intense blue colour was observed in the tegumental muscles, oral sucker, acetabulam, ovary, testes and vitellaria, which indicated the high concentration of proteins in these organs. Intensity of blue colour in the tegument, gut caeca and parenchyma was low, which revealed the presence of moderate amount of proteins in these organs. The marked histochemical reaction for general proteins in well defined organs of *Cotylophoron cotylophorum* is presented in Table 4.1. Photomicrographs 4.1-4.8 show histochemical distribution of general proteins in various tissues of *Cotylophoron cotylophorum.*

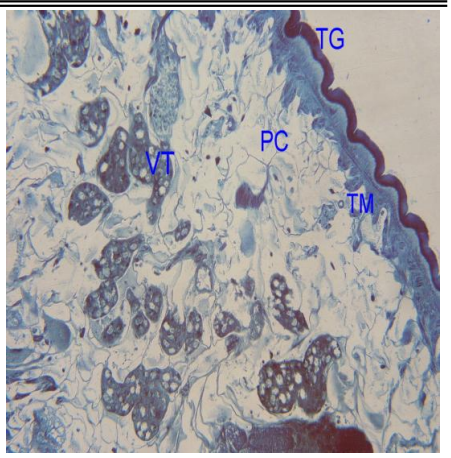
Table 4.1: Statement of staining reaction for general proteins in the tissues of *Cotylophoran cotylophorum*

Tissue/ Organ	Distribution of proteins
Tegument	++
Tegumental muscles	+++
Anterior sucker	+++
Acetabulam	+++
Gut caeca	++
Testes	+++
Ovary	+++
Parenchyma	++
Vitellaria	+++

+ Weak activity, ++ Moderate activity, +++ Intense activity.



Pmg. 4.1



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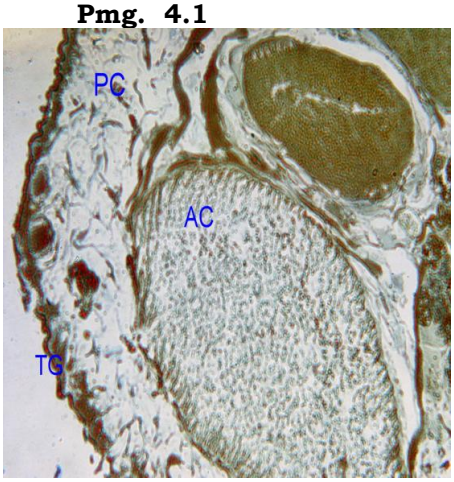

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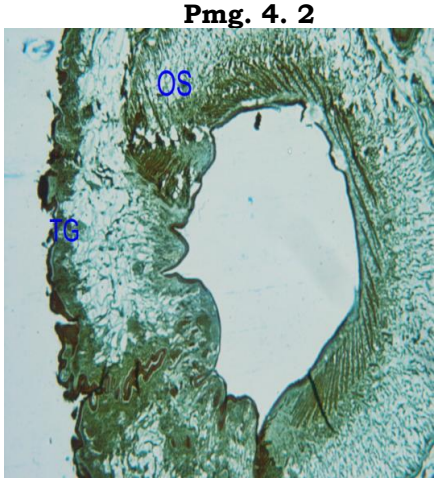

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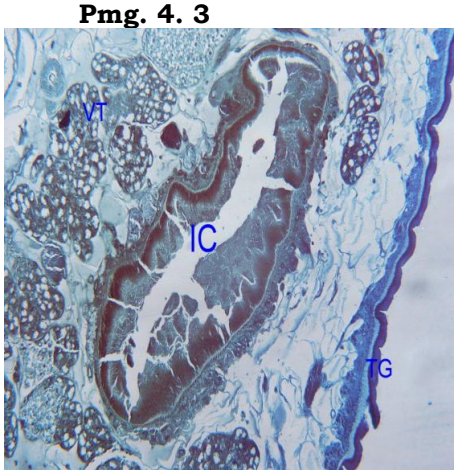

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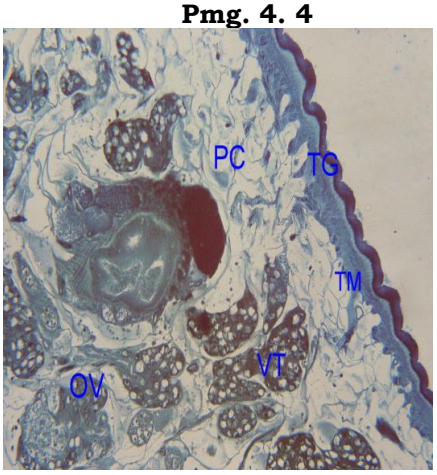
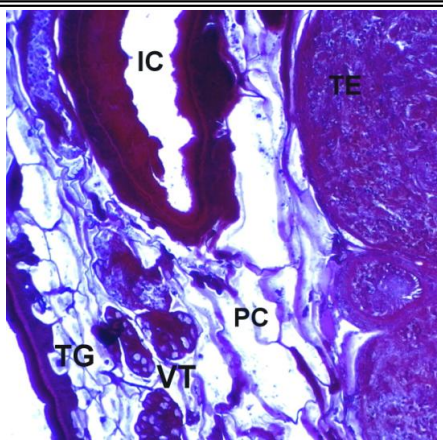


Pmg. 4. 6



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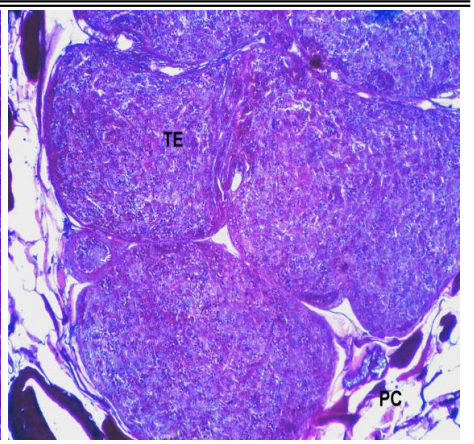


Pmg. 4.8

Pmg. 4.1. & 4.2. L.S passing through IC, TG, TM, VT and PC.x200. **Pmg. 4.3**. LS passing through AC.x200. **Pmg. 4.4**. TS passing through OS.x200. **Pmg. 4.5**.LS passing through the IC.x200. **Fig.4.6.** LS passing through OV.x200. **Pmg. 4.7 & 4.8** LS passing through, VT, TG, PC & TE.x400.

4.2.1.2. Glycogen (Table 4.2, Photomicrographs 4.9-4.12)

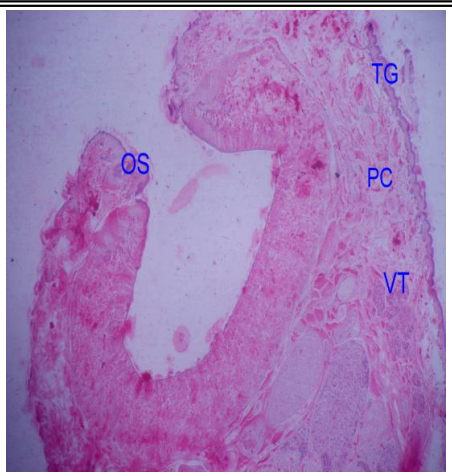
In the current study intense red colour was observed in the parenchyma, oral sucker, acetabulam and vitellaria of *Cotylophoron cotylophorum*. The intense red colour revealed large amount of glycogen deposits in these organs. Tegumental muscles, tunica of ovary and ovary were stained lightly with Best's carmine, indicating the presence of moderate amount of glycogen in these structures. The present study also revealed small amount of glycogen in tegument, tunica of testes and testes which was observed in the form of diffused red colour in these tissues. The statement of

staining reaction for glycogen in different tissues/organs of *Cotylophoron cotylophorum* is presented in Table 4.2. Photomicrographs 4.9-4.12 depict histochemical distribution of glycogen in different tissues of *Cotylophoron cotylophorum.*

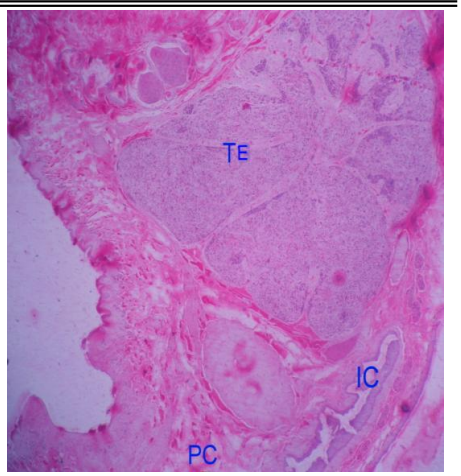
Table4.2: Intensity of staining reaction for Glycogen in the tissues of *Cotylophoron cotylophorum*.

Tissue/ Organ	**Distribution of Glycogen**
Tegument	+
Tegumental muscles	++
Anterior sucker	+++
Acetabulam	+++
Gut caeca	+
Tunica of testes	+
Testes	+
Tunica of ovary	++
Ovary	++
Parenchyma	+++
Vitellaria	+++

+ Weak activity, ++ Moderate activity, +++ Intense activity



Pmg. 4.9



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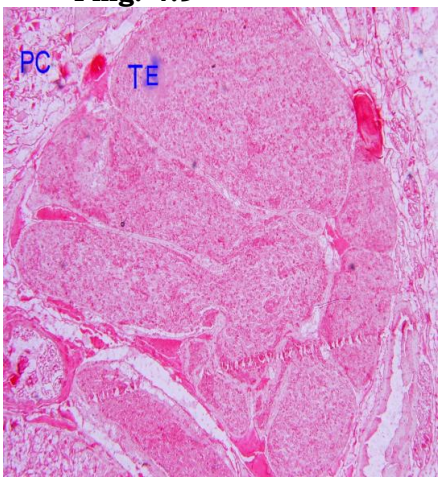

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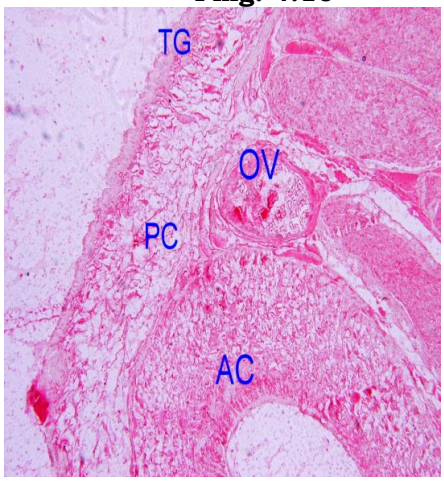
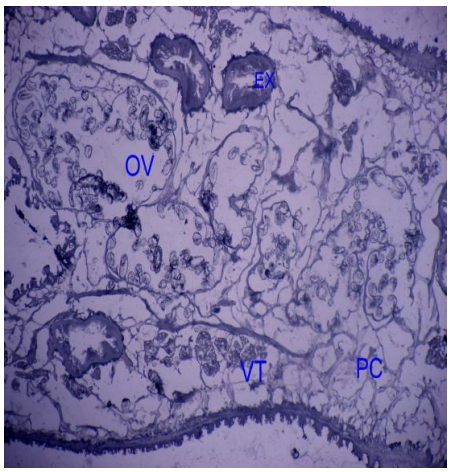


Pmg. 4.12

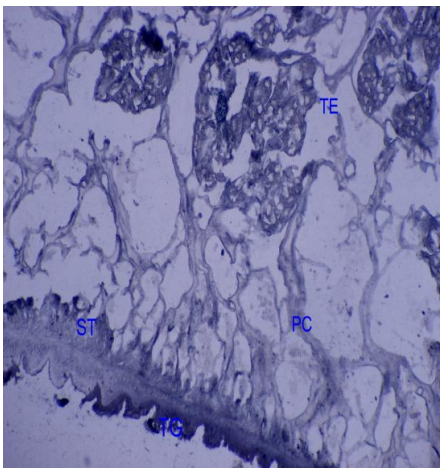
Pmg. 4.9. LS passing through the OS, TG, VT and PC.x200. **Pmg. 4.10.** LS passing through TS and IC.x200. **Pmg. 4.11.** LS passing through TS.x400. **Pmg. 4.12** LS passing through OV and AC x400.

4.2.1.3. Lipids (Table 4.3, Photomicrographs 4.13-4.18)

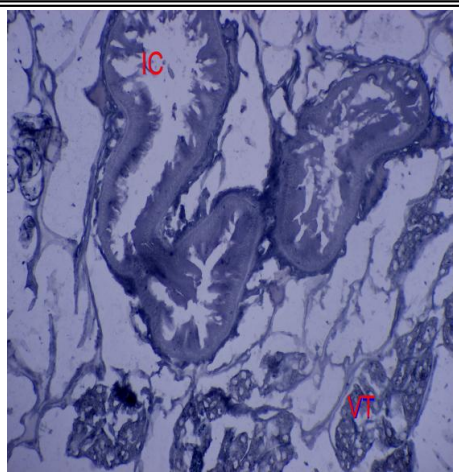
In the present study the most conspicuous sites for the presence of sudanophilic lipids in the tissues of *Cotylophoron cotylophorum* were excretory ducts and vesicles. Parenchyma showed a few sudanophilic lipid granules, but the lipids were relatively more in the vicinity of the intestinal caecae and subtegumental regions. The intestinal caeca itself showed positive reaction for lipids. Tegument and subtegument musles stained deeply with Sudan black B, revealing the presence of moderate amount of lipids. Weak to moderate reaction for lipids was observed in gastrodermis, vitelline cells, suckers, ovary and testes. The marked histochemical reaction for lipids in well defined organs of *Cotylophoron cotylophorum* is presented in Table 4.3. Photomicrographs 4.13-4.18 depict the histochemical distribution of lipids in various tissues of *Cotylophoron cotylophorum.*



Pmg. 4.13



Pmg. 4.14



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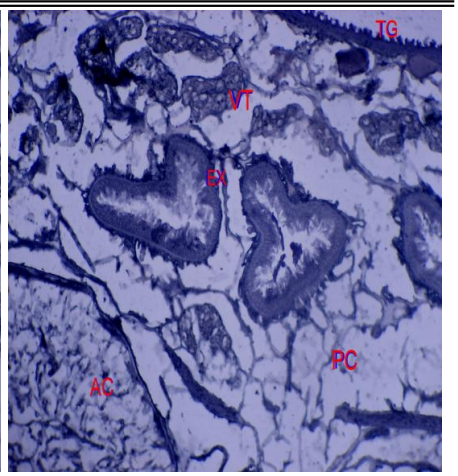

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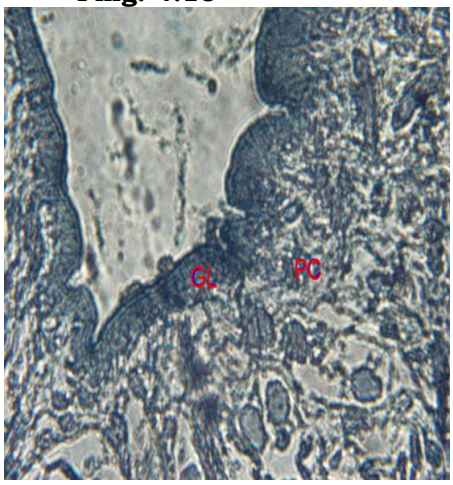

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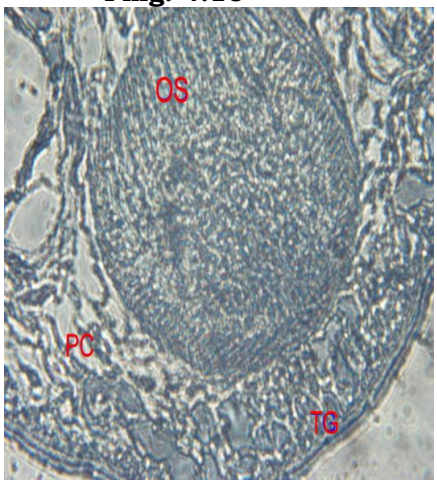


Pmg. 4.18

Pmg. 4.13. LS passing through OV, EX, VT and PC.x200. **Pmg. 4.14**. LS passing through TS, TG and STx200. **Pmg. 4.15**. LS passing through IC.x200. **Pmg. 4.16** LS passing through EX and AC x200. **Pmg. 4.17** LS through GL x 400. **Pmg. 4.18** LS showing oral sucker OS x200.

Table 4.3: Statement of staining reaction for lipids in the tissues of *Cotylophoran cotylophorum*

Tissue/ Organ	Distribution of lipids
Tegument	++
Tegumental muscles	++
Oral sucker	++
Acetabulam	++
Intestinal caeca	+
Excretory duct	+++
Tunica of testes	+
Testes	++
Tunica of ovary	+
Ovary	++
Parenchyma	+
Vitellaria	++

+ Weak activity, ++ Moderate activity, +++ Intense activity.

4.2.1.4. Cholinesterase (Table 4.4, Photomicrographs 4.19-4.26)

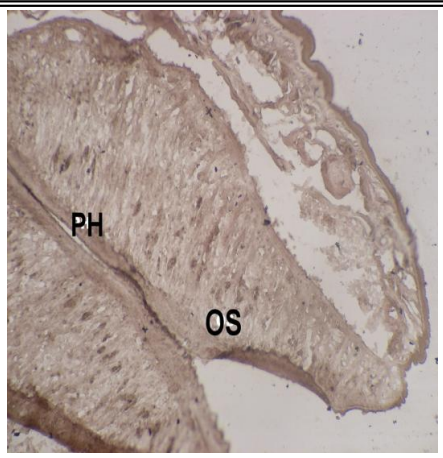
In the present observation, muscles of oral sucker, ventral sucker and pharynx showed moderate activity for cholinesterase. Gut musculature and tegumental musculature showed moderate to intense activity for cholinesterase. Cholinesterase activity was

moderate in tunica of testes and tunica of ovary. Weak activity of cholinesterase was recorded in the tegument, parenchyma and vitellaria. The statement of staining reaction for cholinesterase in different tissues/organs of *Cotylophoron cotylophorum* is presented in Table 4.4. Photomicrographs 4.19- 4.26 depict the histochemical distribution of cholinesterase in various tissues of *Cotylophoron cotylophorum.*

Table 4.5: Statement of Intensity of Cholinesterase activity in the tissues of *Cotylophoran cotylophorum*

Tissue/ Organ	**Cholinesterase Activity**
Tegument	+
Tegumental muscles	+++
Pharynx	++
Oral sucker	++
Acetabulam	++
Intestinal caeca	+++
Tunica of testes	++
Tunica of ovary	++
Parenchyma	+
Vitellaria	+

+ Weak activity, ++ Moderate activity, +++ Intense activity.



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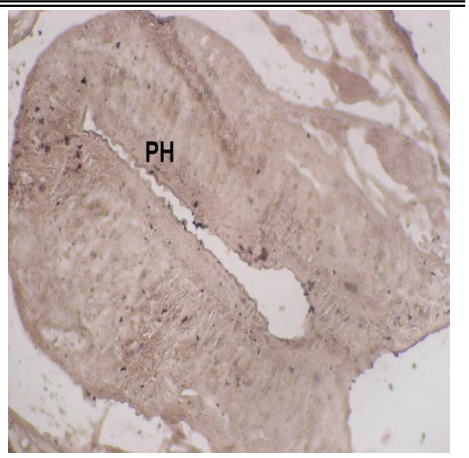

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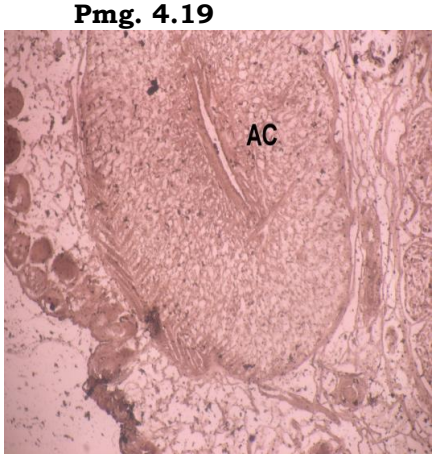

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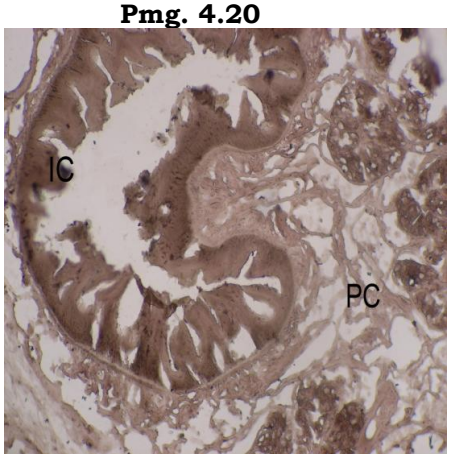

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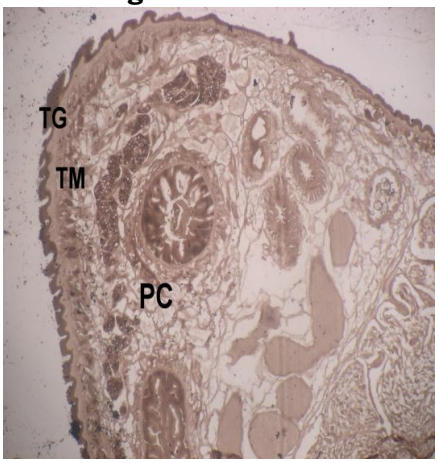

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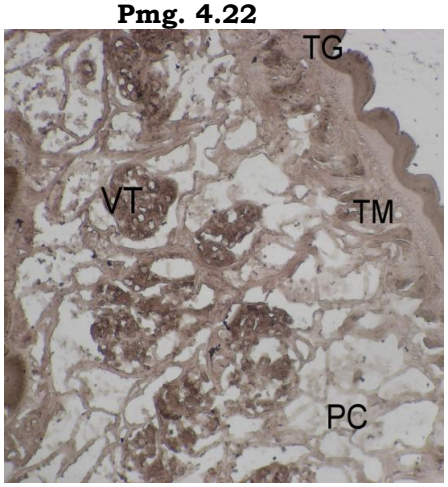

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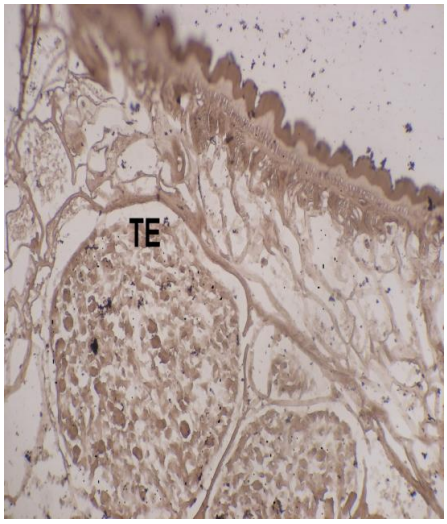

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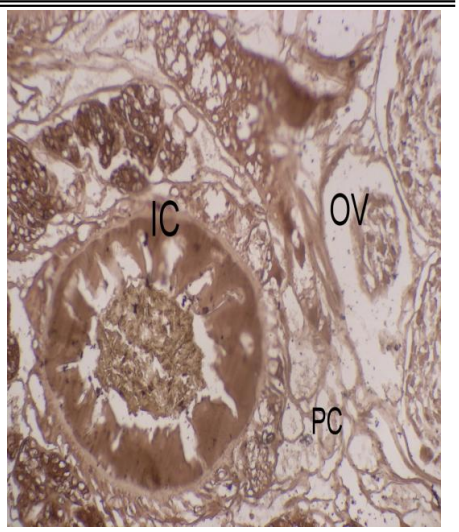


Pmg. 4.26

Pmg. 4.19. and 4.20. LS passing through OS and PH.x200. **Pmg. 4.21**. LS passing through AC.x200. **Pmg. 4.22**. LS passing through the ICx200. **Pmg. 4.23** LS passing through TG and TM.x100. **Pmg. 4.24** LS showing TG, TM and VT.x200. **Pmg. 4.25** LS passing through TE.x200. **Pmg. 4.26**. LS passing through OV.x200.

4.2.1.5. Acid phosphatase (Table 4.5, Photomicrographs 4.27-4.34)

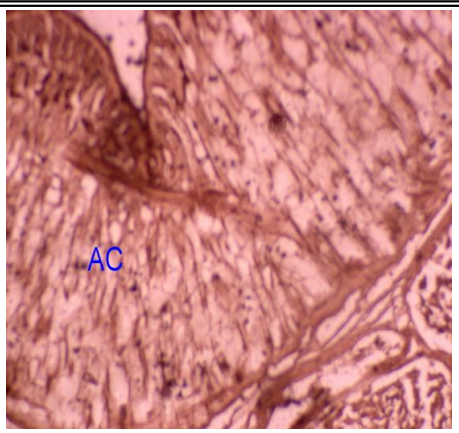
In the present study, acid phosphatase activity was observed in almost all tissues of *Cotylophoron cotylophorum*. Intense enzyme activity was observed in the muscles of pharynx, its tegument as well as tegument lining the mouth and posterior sucker. Various muscle layers of posterior sucker showed moderate to high enzyme activity. Luminal surface of the gut was also intensely positive, where as its outer muscle layer exhibits activity of comparatively

less intensity. Vitteline cells as well as their secretory products were found acid phosphatase positive in the present study. The marked histochemical reaction for acid phosphatase in well defined organs of *Cotylophoron cotylophorum* is presented in Table 4.5. Photomicrographs 4.27-4.34 depict the histochemical distribution of acid phosphatase in different tissues of *Cotylophoron cotylophorum*.

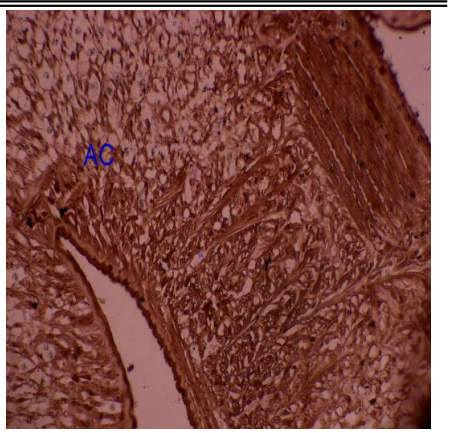
Table 4.5: Intensity of Acid phosphatase staining reaction in the tissues of *Cotylophoran cotylophorum*

Tissue/ Organ	**Acid phosphatase Activity**
Tegument	++
Tegumental muscles	+++
Pharynx	+++
Oral sucker	+++
Acetabulam	+++
Intestinal caeca	++
Tunica of testes	+++
Testes	+++
Tunica of ovary	+++
Ovary	+++
Parenchyma	++
Vitellaria	+++
Excretory vesicles	++

+ Weak activity, ++ Moderate activity, +++ Intense activity.



Pmg. 4.27



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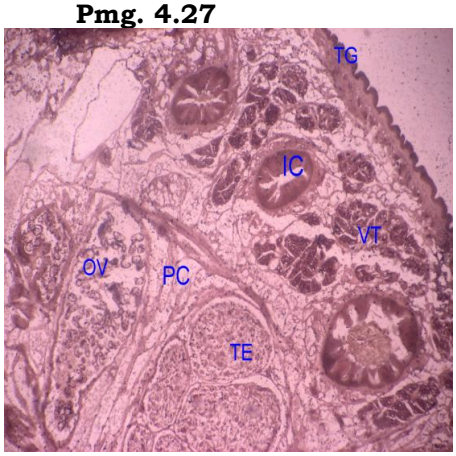

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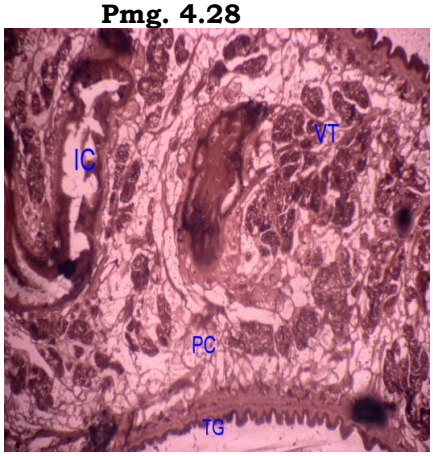

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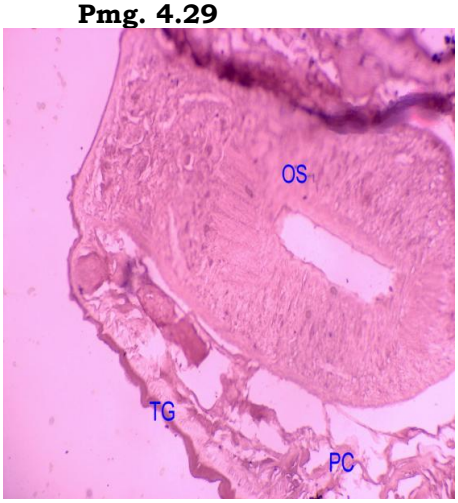

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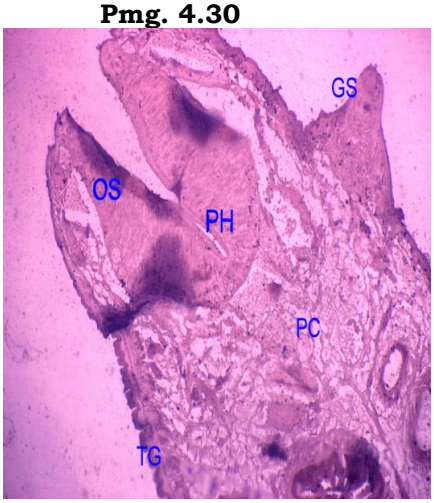

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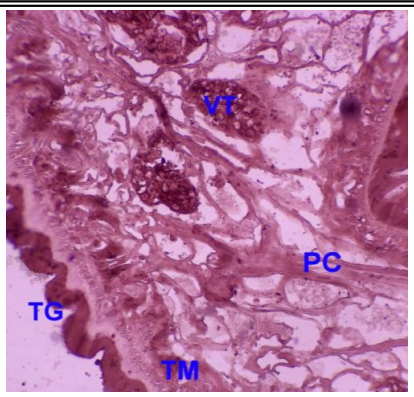

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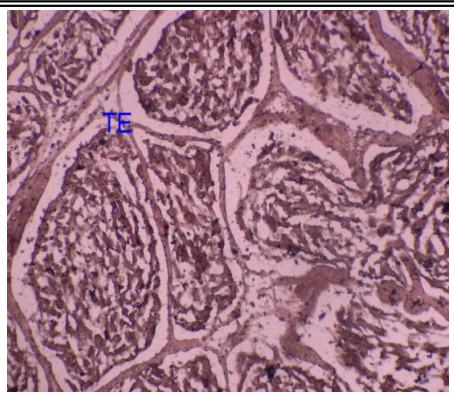


Pmg. 4.34

Pmg. 4.27 & Pmg. 4.28. TS passing through AC.x400. **Pmg. 4.29**. LS passing through TE and OV.x200. **Pmg. 4.30**. L.S passing through IC, VT, PC and TG.x200. **Pmg. 4.31.** & **4.32.** LS passing through OS, PH and GS.x200. **Pmg. 4.33** LS through TG, TM, PC and VT.x400. **Pmg. 4.34.** LS passing through TE.x400.

4.2.1.6. Alkaline phosphatase (Table 4.6, Photomicrographs 4.35-4.40)

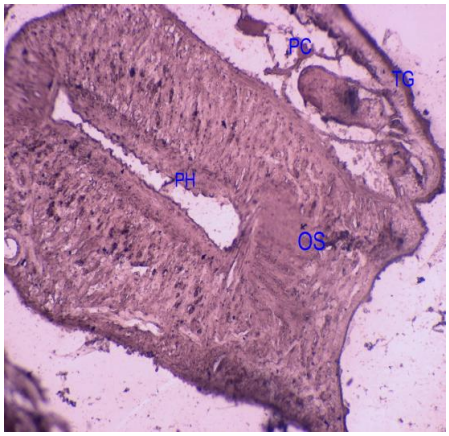
In the present study substantial amount of alkaline phosphatase was observed in the studied structures of *Cotylophoron cotylophorum*. Intense reaction for alkaline phosphatase was observed in intestinal caeca, excretory vesicle, tunica of testes and ovary. Moderate reaction for alkaline phosphatase was observed in oral sucker, acetabulam, vittelaria, tegument and subtegument of *Cotylophoron cotylophorum*. Parenchyma showed weak reaction for alkaline phosphatase in the present study. The statement of staining reaction for alkaline phosphatase in different tissues/organs of *Cotylophoron cotylophorum* is presented in Table 4.6. Photomicrographs 4.35–4.40 show the histochemical

distribution of alkaline phosphatase in various tissues of *Cotylophoron cotylophorum*.

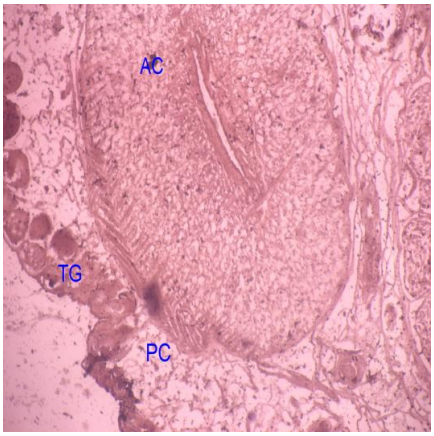
Table 4.6: Intensity of Alkaline phosphatase staining reaction in the tissues of *Cotylophoran cotylophorum*

Tissue/ Organ	Alkaline phosphatase Activity
Tegument	++
Tegumental muscles	++
Pharynx	++
Oral sucker	++
Acetabulam	++
Intestinal caeca	+++
Tunica of testes	++
Tunica of ovary	++
Parenchyma	+
Vitellaria	++
Excretory vesicles	+++

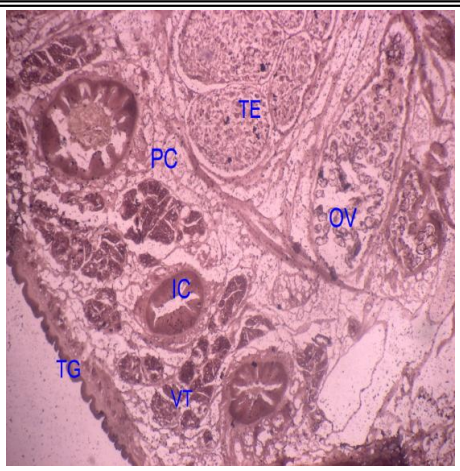
+ Weak activity, ++ Moderate activity, +++ Intense activity



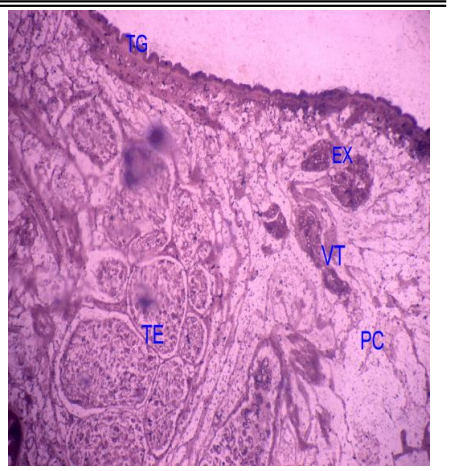
Pmg. 4.35



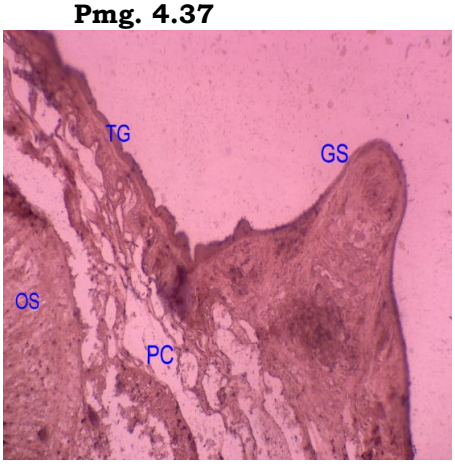
Pmg. 4.36



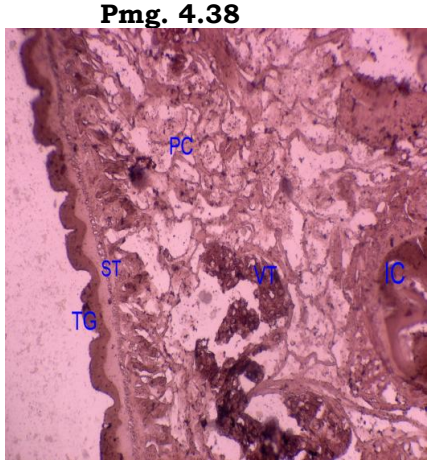
Pmg. 4.37



Pmg. 4.38



Pmg. 4.39



Pmg. 4.40

Pmg. 4.35 LS through OS and PH.x200. **Pmg. 4.36** LS through AC.x200. **Pmg. 4.37** & **4.38** LS through TE, OV, TG, EX, VT and PC.x200. **Pmg. 4.39** LS through GS x200. **Pmg. 4.40** LS through IC, SB and TG.x200.

4.2.1.7. Calcium (Table 4.7, Photomicrographs 4.41-4.46)

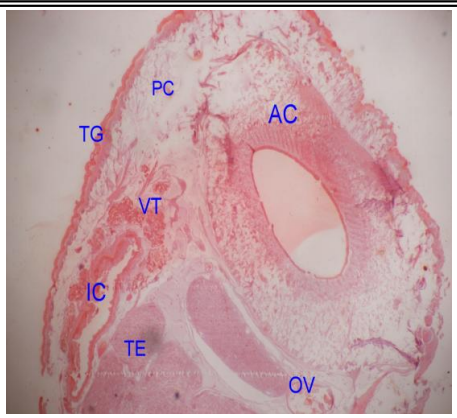
In the present study tegument, vitellaria, intestinal caeca and suckers revealed moderate amount of calcium deposits which appeared in the form of deep orange red colour. Diffused orange red

colour in the parenchyma, testes, ovary and excretory vesicles of *Cotylophoron cotylophorum* revealed weak reaction for calcium. The marked histochemical reaction for calcium in well defined organs of *Cotylophoron cotylophorum* is presented in Table 4.1. Photomicrographs 4.41 – 4.46 show the histochemical distribution of calcium in different tissues of *Cotylophoron cotylophorum*.

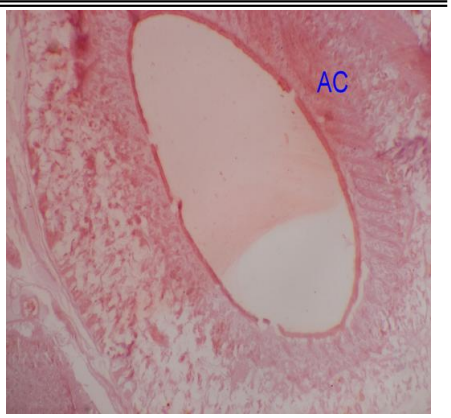
Table 4.7: Statement of staining reaction for calcium in the tissues of *Cotylophoran cotylophorum*

Tissue/ Organ	**Distribution of Calcium**
Tegument	++
Tegumental muscles	+
Pharynx	-
Oral sucker	++
Acetabulam	++
Gut	++
Tunica of testes	+
Tunica of ovary	+
Parenchyma	+
Vitellaria	++
Excretory vesicles	+

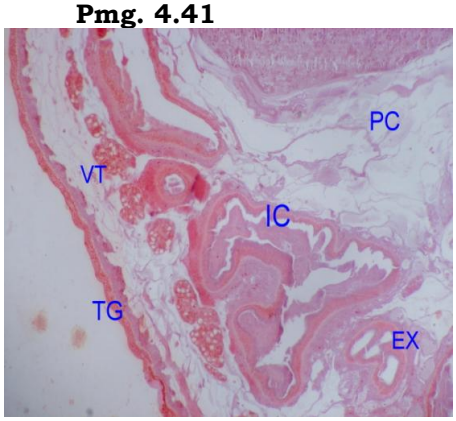
+ Weak activity, ++ Moderate activity, +++ Intense activity



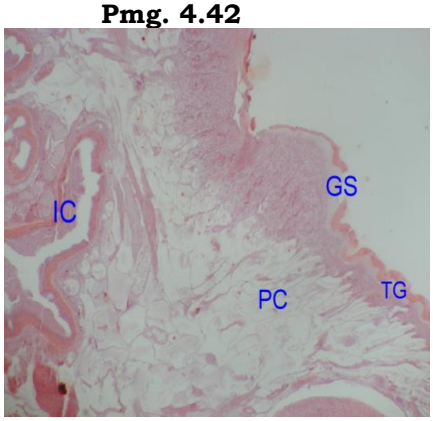
Pmg. 4.41



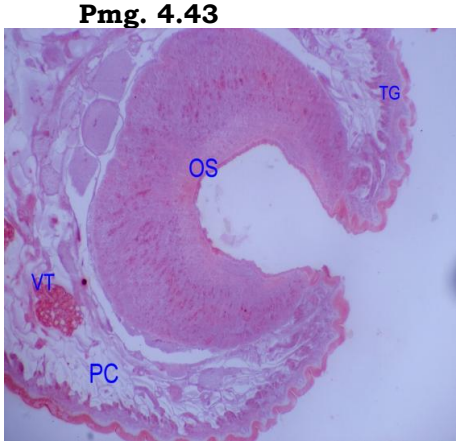
Pmg. 4.42



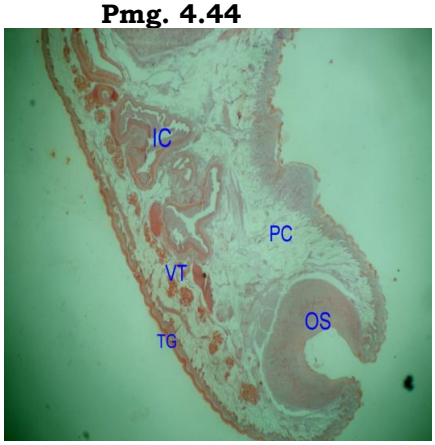
Pmg. 4.43



Pmg. 4.44



Pmg. 4.45



Pmg. 4.46

Pmg. 4.41 LS through AC, TE, OVx200. **Pmg. 4.42** TS through AC.x400. **Pmg. 4.43** & **4.44** LS through IC, TG, VT and GS.x200. **Pmg. 4.45 & 4.46** LS through OS, VT and IC.x200,x100.

The present study was undertaken to study histochemistry of two amphistome species from two different hosts viz., *Paramphistomum cervi* from rumen of sheep and *Cotylophoron cotylophorum* from rumen of cattle. Since both the species were subjected to various histochemical tests for the localization of general proteins, glycogen, lipids, cholinesterase, acid phosphatase, alkaline phosphatase and calcium; the chapter has been accordingly divided into seven headings as under:

5.1. General proteins

5.2. Glycogen

5.3. Lipids

5.4. Cholinesterase

5.5. Acid phosphatase

5.6. Alkaline phosphatase

5.7. Calcium

The seven headings refer to the different aspects as have been worked out during the present endeavor and for clear understanding, the observations made during present study are discussed as under.

5.1. General Proteins

The physiological significance and ubiquitous distribution of proteins make them an integral part of every biological activity. In the present study moderate amount of proteins were observed to be distributed evenly in various tissues of both the species of amphistomes under study.

High concentration of proteins was observed in vitellaria, suckers and organs of reproduction. Similar results were observed by most of the workers (Breckenridge and Nathanel, 1988; Gupta *et al.*, 1983; Gupta *et al.*, 1987b; Sharma and Sharma, 1980; Sharma *et al.*, 1981). Gupta *et al.* (1987b) reported intense reaction for general proteins in vitellaria of *Paramphistomum cervi*. Sharma and Sharma (1980) reported high concentration of proteins in the testes of *Ceylonocotyle scoliocoelium*. Similarly Gupta *et al.* (1983) observed high concentration of general proteins in oocapt of *Paramphistomum cervi*. High concentration of proteins in these organs suggests that the protein metabolism is probably more active in these tissues and is involved in synthetic activities. High concentration of proteins in gonads and other reproductive organs can be correlated with high fecundity of these parasites, as Alam and Nizami (1984) suggested

that the accumulation of higher amounts of protein is in anticipation of enormous egg production.

Moderate amount of general proteins was observed in gut caeca, tegument and parenchyma. Similar results have been observed by other investigators in different trematodes (Dunn *et al.*, 1987a, b; Sharma and Hora, 1983; Mattison *et al.*, 1992a; Parshad and Guraya, 1978c; Mattison *et al.*, 1994). Sharma and Hora (1983) reported moderate amount of proteins in gastrodermis and brush border epithelium of two rumen amphistomes– *Orthocoelium scoliocoelium* and *Paramphistomum cervi*. Parshad and Guraya (1978c) reported similar results for general proteins in the digestive system of *Cotylophoran cotylophorum*. Mattison *et al.* (1994) reported moderate amount of proteins in the tegument of juvenile paramphistomes. Dunn *et al.* (1987b) while working on the tegument of three species of paramphistomes reported the presence of moderate amount of proteins in all the three species.

Mattison *et al.* (1992c) reported moderate amount of general proteins in the parenchyma of juvenile paramphistomes. The presence of moderate amount of proteins in the caeca indicates that they might be derived from host during active feeding as supported by Cheng (1963). Since the trematodes are either haematophagous or tissue feeders, many proteolytic enzymes have been reported from the intestinal caeca (Smyth and Halton, 1983).

Therefore, the protein deposition in the caecal lining and content may be expected from dietary origin.

5.2. Glycogen

The distribution of glycogen in the two species of amphistomes studied was more or less the same. Glycogen was observed to be intensely present in the parenchyma, vitellaria and cells of suckers, moderate to weak activity was observed in other organs. The parenchyma of trematode serves as a storage organ for energy reserves in trematodes and trematodes mostly use glycogen as energy metabolite. Glycogen has been reported to be most common stored polysaccharide evenly distributed in high quantities throughout the parenchyma of trematodes and has been associated with the sites of metabolic or musculature activity (Von Brand, 1979; Erasmus, 1972). Substantial amount of glycogen in parenchyma of newly excysted *Paramphistomum epiclitum* was reported by Mattison *et al.* (1992c).

In the present study intestinal cells and male reproductive organs of both the species of paramphistomes under study showed weak reaction for glycogen. These results are in agreement with those of Parshad and Guraya (1978c) in *Cotylophoran cotylophorum* and Sharma and Sharma (1980) in *Ceylonocotyle scoliocoelium*. However intense reaction for glycogen was observed in female reproductive organs particularly in vitellaria. These results are in

agreement with those of Hanna (1976), Kanwar and Agarwal (1977) and Gupta *et al.* (1987b). Since these trematodes produce enormous number of eggs and these eggs should contain sufficient amount of reserved food for the development of the embryo, this seems to be the reason for the presence of large amount of glycogen in female reproductive organs. Gupta *et al.* (1987a) also made the same interpretation that glycogen is used as food reserve during embryogenesis in trematodes.

Von Brand (1979) has reviewed the previous work and stated that the vitelline cells of *Fasciola hepatica* and Fasciolide species contain large amount of glycogen, while no polysaccharide could be demonstrated histochemically in Schitosomes. Probert *et al* (1972) reported the localization of glycogen in *Fasciola gigantica* which is almost similar to present results. Occurrence of glycogen in many other trematode species has been reviewed in many texts (Von Brand 1979; Chappel 1980; Barett 1981; Smyth and Halton, 1983) and the results are in agreement with the present study.

5.3. Lipids

In the present study lipids were demonstrated histochemically in *Cotylophoron cotylophorum* and *Paramphistomum cervi* and the most conspicuous sites for the presence of sudanophilic lipids were excretory ducts and vesicles. Some investigators believe that parasites (trematodes), particularly adult ones are incapable of

utilizing lipids as a source of energy (Reznik 1968; Voogt 1972; Gupta *et al*. 1974) rather that lipids are the end products of carbohydrate metabolism and have expressed a view that trematodes accumulate lipids as "ballast" component which is either excreted or packed as a product under going no further decomposition. *Fasciola* and other trematodes were found to secrete via the excretory system cholesterol and its esters, triglycerides, free fatty acids and phospholipids (Reznik 1968; Voogt 1972). The apparent inability of these parasites to degrade lipids is questionable by some workers. For example, Mendlowitz *et al*. (1960) and Erasmus (1972) reported the presence of lipid degredating enzymes (lipases and esterases) in these parasites and they concluded that neutral lipids could constitute energy resources in them. Other authors (Barrett, 1981; Barret and Saghir, 1999; Swideriski and Mackiewicz, 2004) are of the opinion that at least some parasites, particularly their free living stages and those living in intermediate hosts, can utilize lipids as source of energy.

Moderate reaction for sudanophilic lipids was observed in the tegument of *Cotylophoron cotylophorum* and *Paramphistomum cervi*. Similar results were reported by Dunn *et al*. (1987b) in the tegument of three species of Paramphistomes – *Gigantocotyle explanatum*, *Srivastavaia indica* and *Gastrothylax crumenifer*. The presence of sudanophilic lipids in the tegument of other trematodes has also been demonstrated (Lee,1966; Lee, 1972; Erasmus, 1967).

Humiczewska and Rajski (2005) reported considerable lipid content in tegument of developmental stages of *Fasciola hepatica*. The lipids present in the tegument may be involved in the transport of lipophilic substances because the tegument of trematodes helps in the transport of nutritive substances although to a lesser extent and according to Parshad and Guraya (1976) the tegument of adult trematode is both protective and active surface.

Sudanophilic lipids were observed in the caecal wall and lumina of *Cotylophoran cotylophorum* and *Paramphistomum cervi*. The present results are in agreement with those of Sharma and Hora (1983), who reported weak to moderate reaction for sudanophilic lipids in gastrodermis of *Paramphistomum cervi* and *Orthocoelium scoliocoelium*. Similar results were also reported by Parshad and Guraya (1976) in *Cotylophoron cotylophorum* and Dunn *et al.* (1987a) in *Gigantocotyle explanatum*, *Gastrothylax crumenifer* and *Srivastavaia indica*. Lipids observed in the gut of two amphistomes under study may be the metabolic products or these contents may be of dietary origin, as also suggested by Parshad and Guraya (1976) and Harris and Cheng (1973). But according to Pearse (1972) although Sudan Black B is mostly employed as a lipid stain, it may also stain lipoproteins, thus the positive reaction for Sudan Black B in gastrodermis may indicate the presence of lipoproteins. Staining for total lipids was positive in parenchyma and vitellaria and the results are in agreement with those of Gupta

et al. (1987b) in *Paramphistomum cervi* and Haseeb *et al.* (1984) in *Schistosoma mansoni*.

Weak to moderate activity for sudanophilic lipids was observed in reproductive organs and associated glands. These results are in agreement with those of Sharma and Sharma (1980); Sharma *et al.* (1981); Gupta *et al.* (1983); Kanwar and Kansal (1980) and Breckenridge and Nathanael (1988). Numerous authors support the view that adult trematodes transfer lipid to forming eggs, parthenogenetic forms transfer lipids to the developing embryonic spheres (Becker, 1980; Smyth and Halton, 1983; Yavorskiy, 1989) and which may serve as the reserve food for the developing embryo. In the present study the suckers of the amphistomes under study showed moderate to weak activity for sudanophilic lipids.

From the present study it seems that the parasite tissues which are metabolically active like tegument, pharynx, suckers and reproductive organs sudanophilic lipids are present there. Humiczewska and Rajski (2005) reported considerable amount of lipids in the tegument, suckers, pharynx and germ balls of developing stages of *Fasciola hepatica* and supports the fact that parasite tissues which are metabolically active show considerable amount of lipids.

5.4. Cholinesterase

Cholinesterase has been demonstrated and localized in the musculature of body wall, suckers, digestive system, reproductive

system and nervous system of the species under study and its distribution is more or less similar in both the species. Cholinesterase in neuromuscular system of *Fasciola hepatica* has been reported by Chance and Mansour (1953), Kravica *et al.* (1967) and it is cholinergic as has been reported by various workers (Taylor & Radie, 1994, Dan *et al.*, 1999, Ekholm, 2001). Kemmerling *et al.* (2006) points to cholinesterase as molecular markers in the development of nervous system. Moderate activity of cholinesterase in the oral sucker indicated its role in the ingestion of host tissue as speculated by Becejae *et al.* (1964). Acetabulam exhibits weak to moderate reaction for esterase activity particularly in outer lining of the acetabulam there by suggesting its attachementary function as has been reported to certain trematodes by Nizami *et al.* (1977). Activity of cholinesterase was very weak in tegument except at the boundaries of suckers and musculature of tegument where its activity is moderate, therefore suggesting that its tegument does not play major role in metabolite transport (Dunn *et al.*, 1987b). However presence of cholinesterase in the tegumental musculature suggests its role in contractility (Mandavat & Sharma, 1978). The activity of cholinesterase was more pronounced in and along the length of caeca with slightly more intense reaction in caecal contents and present observation clearly indicated that caecum was more active for esterase activity than integument, therefore suggesting that caecum is more involved in the absorption and

transfer of metabolites into the worm (Sharma & Hora, 1983). Moderate activity of cholinesterase was recorded in the tunica of testes and ovary where it may help in discharge of sperms and eggs and propel them in ductules (Farooq & Farooqui, 1983). Esterase activity in the parenchyma appears to be deposited in the form of small granules which were uniformly distributed throughout the tissue with slightly more granules accumulated in the cells surrounding the caeca, which suggests that the parenchymal cells probably help in the supply of nutrients to neighboring tissues.

5.5. Acid Phosphatase

Acid phosphatase has been generally detected histochemically by many workers in the sites where absorption, secretion and excretion occur and are postulated to be related with these functions.

In present study, acid phosphatase was observed to be present in higher intensity in anterior and posterior suckers. Similar results were reported by Haque and Siddiqi (1982) in four different trematodes – *Gastrothylax crumenifer*, *Gigantocotyle explanatum*, *Echinostomum malayanum* and *Fasciola buski*. Our results are also in agreement with those of Roy (1980c) in *Ceylonocotyle scoliocoelium* (amphistome) and Trimble *et al.* (1971) in *Aspidogaster conchichola*.

Intense acid phosphatase activity was observed in the tegumental cells and moderate activity in subsyncytial zone (zone

between surface syncytium and circular muscles). These results are in agreement with those of the other workers (Roy, 1980c; Haque and Siddiqi, 1982; Dunn *et al.*, 1987b; Sharma and Hanna, 1988; Mattison *et al.*, 1994). Acid phosphatase in the tegument and in the caecal epithelium, their role in digestion and absorption has been speculated on from time to time (Van Brand, 1979; Rajvanshi and Mali, 1986). Hora and Sharma (1980) reported that higher levels of phosphatase are required for the up take of larger amount of glucose in amphistomes. But Sharma and Hanna (1980) while working on ultrastructure and histochemistry of tegument of *Orthocoelium scoliocoelium* and *Paramphistomum cervi* reported that acid phosphatase activity in subsyncytial zone are associated with the extensions of parenchymal cells and trabaculae from tegumental cells. They also reported that mitochondria are absent from the tegument of amphistomes and indicated that high energy intermediates are not available in sufficient concentrations to power extensive active transport. Thus large quantities of amino acids and other metabolites are not taken up across the tegument of the rumen amphistomes. However Dunn *et al.* (1987b) in *Gastrothylax crumenifer* and Mattison *et al.* (1994) in Juvenile Paramphistomes reported that adequate ATP for tegumental function is procured from the parenchyma because there is apparently intimate association between the syncytium and parenchyma in adult amphistomes. Thus tegument plays a role in transportation and absorption of nutritive materials though to a limited extent.

Luminal surface (apical membrane of gastrodermis) of gut caeca was intensely positive for acid phosphatase, where as its outer muscle layer (basal membrane of gastrodermis) exhibited activity of comparatively less intensity. Similar results were reported by Dunn *et al.* (1987a) while working on three paramphistome species – *Gigantocotyle explanatum*, *Gastrothylax crumenifer* and *Srivastavaia indica*. Results of present study are also in agreement with other workers (Bogtish, 1972; Haque and Siddiqi, 1980; Roy, 1980c; Rajvanshi and Mali, 1986; Sharma and Hora, 1983). Acid phosphatases are involved in the hydrolytic and transport processes (Smyth and Halton, 1983). The more intense reaction for acid phosphatase in the anterior region of gastrodermis in the paramphistomes depicts a greater commitment to secretory and or absorptive process in this region (Dunn *et al.*, 1987a). Besides, the presence of enzyme at the basal lamina of gastrodermis indicates its involvement in transport of substances to the parenchyma (Parshad and Guraya, 1978c). The tight junctions between the gastrodermis and under lying parenchyma represent important sites of precursor transport between these two systems and the parenchyma beneath the gut, which is rich in mitochondria and lysosomes, plays a role in the processing of nutrients in transit between gut and deeper tissues which is also supported by Dunn *et al.* (1987a).

In the present study moderate amount of acid phosphatase activity was observed in the parenchyma of *Cotylophoron*

cotylophorum and Paramphistomum cervi. Similar results were observed by Mattison *et al.* (1992c) in the parenchyma of paramphistomes – *Paramphistomum epiclitum* and *Fischoederius elongatus*. The presence of this enzyme in the parenchymal tissue itself indicates that parenchyma plays role of transport system in this animal and is not merely a packing or storage tissue. This is also supported by the fact that there are tight junctions between the basal lamina of gastrodermis and parenchyma on one side (Parshad and Guraya, 1978c) which are sites of transport of nutritive substances to parenchyma and on the other hand parenchyma establishes contact with the syncytium of tegument to meet the nutritive requirements of the tegument (Dunn *et al.*, 1987b).

Intense reaction for acid phosphatase was observed in the vitellaria in the present study. Presence of acid phosphatase in the vitelline cells has been reported by a number of workers (Guraya, 1970; Rodgi *et al.*, 1976; Sharma, 1976; Roy, 1980c; Haque and Siddiqi, 1982). Roy (1980c) reported intense reaction for acid phosphatase in vitellaria of bovine amphistome *Ceylonocotyle scoliocoelium*. Haque and Siddiqi (1982) reported intense to moderate activity in four different trematodes - *Gigantocotyl explanatum*, *Gastrothylax crumenifer*, *Echinostomum malayanum* and *Fasciolopsis buski*. Vitelline glands are important in the egg shell formation and in mature paramphistomes the vitellaria fill almost two third of the body and about 32 vitelline cells are

incorporated into each egg (Gupta *et al.*, 1987b; Breckenridge and Nathanael, 1988). Role of acid phosphatase in the vitelline glands is in transportation and formation and secretion of secretory granules in vitelline cells ((Sharma, 1976; Roy, 1980c; Breckenridge and Nathanael, 1988).

Reproductive system, including gonads – testes and ovary, showed intense reaction for acid phosphatase in both the amphistome species under study. These findings are in agreement with those reported in many other trematodes including paramphistomes (Probert and Lwin, 1974; Sharma 1976; Tandon and Misra, 1978; Roy, 1980c; Rajvanshi and Mali, 1986). The heavy reaction for acid phosphatase in these organs occurs because they are metabolically highly active.

5.6. Alkaline Phosphatase

It is commonly known that alkaline phosphatase is the enzyme functionally associated with membrane transport and its role mostly lies in participation in active transport of nutrients and metabolites through the cellular membranes (Smyth and Halton, 1983). Diverse and multilateral functions are attributed to alkaline phosphatase. In particular, it is assumed that alkaline phosphatase takes part in the regulation of NADP levels, in proliferation and differentiation of cells and also in the regulation of cell membrane

dimensions (Sawicka, 1980; Kierek-Jaszezuk, 1981). Similarly diverse functions are attributed to alkaline phosphatase in parasites. Smyth and Halton (1983) associated the level of alkaline phosphatase activity with the synthesis of cytoplasm proteins and with the cell growth. On the other hand, Dum and Yashino (1988), Pujol and Cesari (1990), Cesari *et al.* (1991) and Lewis and Strand (1991) while studying alkaline phosphatase in *Schistosoma mansoni* discovered an antigenic character of this enzyme. Alkaline phosphatase is also a sensitive indicator of viability of the developing embryos of *Schistosoma mansoni*, and lack of alkaline phosphatase activity in the egg is a first sign of their death (Giboda and Zdarska, 1994).

In the current study, substantial amount of alkaline phosphatase was found in the studied structures of *Cotylophoron cotylophorum* and *Paramphistomum cervi* which is probably associated with intense transport of carbohydrates constituting the major source of energy for parasite.

Moderate reaction for alkaline phosphatase was observed in the suckers of the amphistomes during this study. The reaction products are localized all along the tegumental surfaces of suckers which are in direct contact with the host tissue and may have glandular activity. The distribution is more or less similar to that

reported by Haque and Siddiqi (1982) in *Gastrothylax crumenifer* and by Roy (1980c) in *Ceylonocotyle scoliocoelium*. The presence of phosphatases in the suckers indicates that they may be involved in carbohydrate metabolism and absorption of nutrients as well as in dissolving host tissue at the host – parasite interface for extracorporeal digestion which is also supported by Ohman (1966).

Moderate to intense reaction was observed in the tegument and sub tegument for alkaline phosphatase in *Cotylophoron cotylophorum* and *Paramphistomum cervi* and the results are in agreement with those of Tremble *et al.* (1971), Roy (1980c), Sharma and Hanna (1988) and Mattison *et al.* (1994). Mattison *et al.* (1994) reported moderate to weak activity for alkaline phosphatase in the tegument of Juvenile paramphistomes. Similar results for alkaline phosphatase were reported by Sharma and Hanna (1988) in the tegument of *Orthocoelium scoliocoelium* and *Paramphistomum cervi*. It is suggested that phosphatases are related to transport mechanism, the identical distribution of enzymes in the tegument and gut suggests that nutrients are absorbed through both gut and tegument (Haque and Siddiqi, 1982). Mattison *et al.* (1994) also reported that the presence of moderate amount of phosphates in the tegumental syncytium suggests that at least part of these paramphistomes nutritive requirement is met by transtegumental absorption.

Present study has revealed the presence of alkaline phosphatase in high concentration in the gut caeca. These findings are in agreement with those of Parshad and Guraya (1978c) in *Cotylophoron cotylophorum*; Haque and Siddiqi (1982) in *Gastrothylax explanatum* and *Gastrothylax crumenifer*; Roy (1980c) in *Ceylonocotyle scoliocoelium* and Mattison *et al.* (1994) in juvenile paramphistomes. Alkaline phosphatase in the gut indicates its involvement in the digestive physiology of parasite probably by the process of dephosphorylation and phosphorylation.

Moderate to high amount of alkaline phosphatase was demonstrated in organs of reproductive system. Intense reaction was observed in the tunica of testes and ovary, while moderate to weak reaction in the testes and ovary. These findings are in agreement with those reported in *Aspidogaster conchicola* (Trematoda) by Trimble *et al.* (1971); in *Fasciola hepatica*, *Schistosoma mansoni* and many other trematodes (Probert and Lwin 1974; Sharma, 1976; Tandon and Misra, 1978). In trematodes, organs which are metabolically highly active such as gonads, must rely on surrounding parenchyma as pool of nutrients (Trimble *et al.*, 1971), the presence of alkaline phosphatase at these sites facilitates the transport of raw materials in to the reproductive organs (Rajvanshi and Mali, 1986).

In the present endeavour vitellaria revealed moderate activity for alkaline phosphatase in both amphistomes under study. The results are in agreement with most of the workers (Gupta *et al.*, 1987; Roy, 1980c; Guraya, 1970; Rodgi *et al.*, 1976; Sharma, 1976) in various trematodes. Vitelline glands are important in egg shell formation and the raw materials such as sugars, fatty acids and amino acids needed for this purpose are transported by the activity of alkaline phosphatase (Haque and Siddiqi, 1982; Rajvanshi and Mali, 1986; Gupta *et al.*, 1987b). The present study has also revealed the presence of alkaline phosphatase in parenchyma and excretory ducts. Weak reaction was observed in parenchyma. Similar results were reported by Mattison *et al.* (1992c) in *Paramphistomum epiclitum* and *Fischoederius elongatus*; Roy (1980) in *Ceylonocotyle scoliocoelium*; Probert *et al.* (1972) in *Fasciola gigantica*; Haque and siddiqi (1982) in various trematodes including *Gastrothylax crumenifer*. Just like in other organs, the presence of alkaline phosphatase in the parenchymal tissue indicates its involvement in the phosphorylation of absorbed substances which might ensure there retention. Presence of alkaline phosphatase in the excretory vesicles suggests that the enzyme facilitates the absorption and dislodgement of metabolic end products.

5.7. Calcium

Both the species showed moderate reaction for calcium in tegument, vitellaria, intestinal caeca and suckers. Parenchyma, testes, ovary and excretory canals showed weak reaction for calcium. Since calcium ions play important role in the muscle contraction, suckers are highly muscular and these muscles need to undergo regular contraction for the continuous attachment with the host tissue, this may be the reason for the presence of considerable amount of calcium in these organs. Calcium ions are of vital importance for the contraction of muscles. In the absence of calcium ions, the regulatory proteins inhibit the interaction of actin and myosin. When calcium ion concentration is as high as 10^{-6} M, it binds to troponin C. When calcium ion binds to tropomyosin C, tropomyosin moves away from the groove and exposes the actin binding site of the myosin protein, making it possible for the actin myosin interaction and the resulting contraction to take place. Similar justification may be suggested for the presence of calcium in tegumental muscles, which help in the elongation and shortening of the worm and caecal muscles which helps in peristalsis. Literature on this aspect was not available, reflecting towards the need for the further studies on this and many other aspects in these parasites.

Conclusion

The present study was carried out to demonstrate histochemically the presence and distribution of proteins, glycogen, lipids, cholinesterase, alkaline phosphatase, acid phosphatase and calcium in *Paramphistomum cervi* of sheep in comparison with *Cotylophoron cotylophorum* of cattle. From this study it can be concluded that there is no significant difference in the histochemical distribution of various biochemical substances in the tissues of two amphistomes under study. This may be due to the fact that both these amphistomes are present in the same habitat that is, rumen of the host. However differential histochemical distribution of various biochemical substances in different tissues of both the amphistomes showed considerable difference. Present study revealed that high concentration of proteins is present in those organs which are metabolically more active and are involved in synthetic activities. Moderate amount of protein in caecal lining and content may be expected from dietary origin. Since parenchyma of trematodes serves as a storage organ for energy reserves and trematodes mostly use glycogen as energy metabolite, this is the possible reason for intense reaction for glycogen in parenchyma of these trematodes. In the present study, it was observed that reproductive organs particularly vitellaria revealed intense reaction for glycogen. The reason is that trematodes produce enormous

amount of eggs and these eggs should contain sufficient amount of reserve food for the development of the embryo. During the present study the most conspicuous sites for the presence of lipids were excretory ducts. Most of the authors are of the opinion that trematodes particularly adult ones are incapable of utilizing lipids as a source of energy and are end products of carbohydrate metabolism. However in the current study moderate reaction for lipids was observed in the tegument, caeca and reproductive organs. Thus it can be concluded that lipids are not exclusively the end products of carbohydrate metabolism but they play their role in various metabolically active tissues/organs.

Cholinesterase is regarded as molecular marker of nervous system and is cholinergic. During the present study cholinesterase was localized in musculature of body wall, suckers, digestive system and reproductive system of the amphistomes under study. Presence of cholinesterase in the musculature of these organs suggests its role in contractility.

Alkaline phosphatase is the enzyme functionally associated with membrane transport. Present study revealed the presence of alkaline phosphatase in suckers, tegument, parenchyma, caeca, vitellaria and reproductive organs and the possible reason for this is that the alkaline phosphatase in the metabolically active organs is

associated with the intense transport of carbohydrates constituting the major source of energy for these parasites.

Acid phosphatase has been generally detected in site where absorption, secretion and excretion occur and are postulated to be associated with these functions. The present study revealed presence of acid phosphatase in sufficient amount in suckers, tegumental cells, intestinal caeca, vitellaria, reproductive organs and parenchyma. Presence of acid phosphatase in tegument and caeca suggests it role in absorption and hydrolytic processes. In vitelline cells acid phosphatase plays role in secretion of secretory granules in vitelline cells.

During the present study moderate reaction was observed in suckers, tegument, caeca and vitellaria for calcium. Since these organs are associated with higher muscular activity and for the muscle contraction calcium ions play important role, this may be the reason for the presence of calcium in these organs.

Besides this, from the present study it can be concluded that in *Paramphistomes* the majority of nutrients are absorbed through gut but atleast some part of their nutritive requirement is meant by transtegumental absorption. The parenchyma of the paramphistomes showed positive reaction for most of the biochemical constituents localized in the present study, from which

it can be concluded that parenchyma in these trematodes is not merely a storage organ but it serves as a transport system in them.

As more and more facts are becoming known about the biochemistry and physiology of trematodes, ever increasing efforts are being made to develop the anthelmintics by rationale approach rather than experimental approach. This can only be achieved if we know more and more about the nature and properties of biochemical components and enzyme systems of the parasite as well as their host. It is hoped that the results of this study will prove to be useful as a positive contribution in the vast field of parasite biochemistry and physiology. This should also help others in carrying out more purposeful studies with ever increasing new ideas and techniques.

Conclusion

The present study was carried out to demonstrate histochemically the presence and distribution of proteins, glycogen, lipids, cholinesterase, alkaline phosphatase, acid phosphatase and calcium in *Paramphistomum cervi* of sheep in comparison with *Cotylophoron cotylophorum* of cattle. From this study it can be concluded that there is no significant difference in the histochemical distribution of various biochemical substances in the tissues of two amphistomes under study. This may be due to the fact that both these amphistomes are present in the same habitat that is, rumen of the host. However differential histochemical distribution of various biochemical substances in different tissues of both the amphistomes showed considerable difference. Present study revealed that high concentration of proteins is present in those organs which are metabolically more active and are involved in synthetic activities. Moderate amount of protein in caecal lining and content may be expected from dietary origin. Since parenchyma of trematodes serves as a storage organ for energy reserves and trematodes mostly use glycogen as energy metabolite, this is the possible reason for intense reaction for glycogen in parenchyma of these trematodes. In the present study, it was observed that reproductive organs particularly vitellaria revealed intense reaction

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biochemical constituents localized in the present study, from which it can be concluded that parenchyma in these trematodes is not merely a storage organ but it serves as a transport system in them.

As more and more facts are becoming known about the biochemistry and physiology of trematodes, ever increasing efforts are being made to develop the anthelmintics by rationale approach rather than experimental approach. This can only be achieved if we know more and more about the nature and properties of biochemical components and enzyme systems of the parasite as well as their host. It is hoped that the results of this study will prove to be useful as a positive contribution in the vast field of parasite biochemistry and physiology. This should also help others in carrying out more purposeful studies with ever increasing new ideas and techniques.

The present study was undertaken to demonstrated histochemically general proteins, glycogen, lipids, cholinesterase, acid phosphatase, alkaline phosphatase and calcium in different tissues of paramphistomes of sheep and cattle. The facilities available in the Department were very limited for histochemistry and the present work was done in a moderately equipped laboratory, therefore, only a few aspects were investigated. Still many problems have remained unsolved due to lack of facilities which will form the future line of research particularly by using the modern tools and techniques. Following are a few suggestions for carrying out future studies in the field of histochemistry of Paramphistomes of ruminants in this region.

1. Carbohydrates form the chief energy source in the trematodes. In view of the importance of carbohydrates in helminths, any difference in their carbohydrate metabolism and that of their hosts might be usefully exploited in helminth control. Therefore future studies should be carried on the

histochemical localization of enzymes of carbohydrate metabolism especially enzymes of glycolysis which will include hexokinase, phosphoglucomutase, phosphoglucoisomerase, aldolase, glyceraldehydes-3-phosphatase, dehydrogenase, phosphoglycerate kinase, pyruvate kinase and lactate dehydrogenase.

2. *In vitro* studies in combination with histochemistry should be carried out on the effect of various chemicals on the glycolytic enzymes. The inhibitory effect may block glycolytic pathway and deprive the parasite of its ATP production. Such chemicals can be used as anthelmintics against Paramphistomes.

3. The alternative pathway of glucose catabolism is pentose pathway. Its main function is to provide NADPH and C_5 and C_7 sugars for synthetic reactions. Histochemical studies should be carried out to demonstrate key enzymes of this pathway, viz. glucose-6-phosphatase dehydrogenase and 6-phosphogluconate dehydrogenase fallowed by the *in vitro* effect of various anthelmintics on these enzymes in Paramphistomes.

4. Amino acids commonly found in proteins, also occur as free acids. There are a number of amino acids which are never found as constituents of proteins but play important metabolic roles. Therefore it is important to study both free

and bound amino acids in Paramphistomes, inhibiting different environments, both histochemically and biochemically to find out any significant variation in amino acids.

5. In trematodes like paramphistomes where major metabolic activities are directed towards egg production, the emphasis on the protein and hence amino acid biosynthesis must be considerable. If we are able to find some differences in the enzymes involved in amino acid biosynthesis by the parasite and host, we can selectively check the synthesis of amino acids in the parasite. Thus, these amino acids would not be available for incorporation in the egg proteins, and hence propagation of a species could be checked.

6. Carbohydrates form the major and possibly the sole energy source of trematodes. However the importance of lipids can not be over looked, these being structural and functional constituents. These are important components of membrane which are in constant stage of dynamic equilibrium. Also these are incorporated into eggs and are important energy reserves in free living stages of trematodes. Thus if we are able to selectively inhibit lipid biosynthesis in these parasites, lipids would not be available for incorporation into eggs, and we can check the propagation, if not eliminate the parasites. Further, lipid biochemistry and histochemistry will indicate

the host specificity, which can be exploited for *in vitro* growth or life cycle studies. Besides, such studies in different trematode groups may help in revealing phylogenetic relationship or adaptation of parasites.

7. Histochemical localization of digestive enzymes especially proteases and selective purification of isomers of proteases by SDS-PAGE (sodiumdodisulphide polyacrylamide gel electrophoresis) should be carried out. Studies should be made to elucidate inhibition of proteases by immunoglobulins raised in rabbit against these proteases, which will serve as effective tool for controlling these trematodes.

8. Study of alterations induced in the absorptive surfaces (and neuromuscular system) of the parasites by the *in vitro* incubation with anthelmintics drugs is of prime importance, particularly in view of repeatedly reported resistant strains of parasitic helminths. The mechanisms by which the drugs act on these absorptive surfaces are quite obscure. During their absorption, anthelmintics are expected to induce some cellular and chemical changes in the absorptive surfaces. Keeping in view these facts, the morphological and histochemical effect of *in vitro* incubation with different anthelmintics on the various organ systems of Paramphistomes should be investigated.

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