INTRODUCTION

One problem of animal life is to maintain inside the organism just the proper amount of water. Water content per se is less important than water concentration (osmotic concentration) and this limits the dissolved ions which are critical to life. Osmotic concentration is the total number of moles of solute/liter of solvent; the higher the concentration of solute the greater is the osmotic pressure, lowering of vapour pressure, the elevation of boiling point and depression of freezing point of solution and the osmotic pressure is that pressure necessary to prevent entry of water across the semipermeable membrane. A solution is said to be isosmotic with another if the two are equal in osmotic pressure. Hyposmotic solution is more dilute and hyper is more concentrated. There are two patterns of response to osmotic stress, (1) animals may be osmotically labile, their body fluid concentration may change with the medium (dependent), these are osmoconformers;
other animals are osmotically stable (independent) and, when the medium changes, their internal concentration remains relatively constant, these are osmoregulators. The terms polillosmotic and homeosmotic are sometimes applied to conforming and regulating organisms. Prosser (1984) stated that intestinal cestodes are normally isosmotic to the medium but they can show limited regulation in different media. He also held that excretory organ functions more for ionic than for osmotic regulation and suggested that more accurate data regarding salt and water regulation of various parasitic worms, with and without excretory organs, might reveal variations of regulation.

There are very limited studies on osmotic regulation in trematodes and cestodes. Goll (1966) studied osmotic behavior of Gastrothylax crumenifer and suggested that it attains isotonicity with 0.4% and 0.5% NaCl solution. Siddiqi and Lutz (1966) studied osmotic and ionic regulation in Fasciola gigantica and, assuming normal Tyrode isosmotic to bile, observed that F. gigantica become swollen, inactive and died in a short time in 25% Tyrode and deionized water; in all other media there was a change in the body volume and the worms remained alive for many hours. They concluded that F. gigantica is an osmotic conformer. They also observed excretory vesicle to become enlarged and filled with excretory fluid when
worms were placed in hypotonic media. They concluded that *F. gigantica* produced more urine in response to hypotonic media and thus surmised that the excretory system in trematodes also played a role in osmoregulation. Webster (1970), described *Hymenolepis diminuta*, a cestode parasite of intestine, as osmoconformer and held that the protonephridial canals have no osmoregulatory function. Isseroff and Tunis (1972) observed changes in amino acids of bile in *F. hepatica* infection and found increase of bilary amino acids in most catties. Siddiqi et al. (1975) studied ionic and osmotic behavior of some digenetic trematodes, namely, *Cotylophoron cotylophorum*, *Gastrothylax crumenifer*, and *Gigantocotyle explanaatum*, from the buffalo, *Bubalus bubalis*, and *Isoparorchis hypselobagri* from *Wallago attu* in normal, diluted and concentrated salines and found all these to be osmoconformers. In all species weight gain was quicker than the weight loss and permeability to water was greater than salts, except in *I. hypselobagri*, which was more sensitive to hyperosmotic salines. All species behaved like leaky osmometers and lost Na\(^+\) and K\(^+\) by simple diffusion. *I. hypselobagri* did not lose them as readily. They concluded that differences in ionic and osmotic behaviour in trematodes were due to their water and/or ionic contents. Nizami and Siddiqi (1975) incubated *I. hypselobagri* in aerobic medium and the dialyzed incubate was chemically analyzed. It included amino acids, amines,
amides, ammonia, pyruvic acid, succinic acid, propionic and acetic acid, lactic acid, a-alanine, tyrosine, glycine, a-amino carboxylic acid (amino acids), hemoglobin, nitrogen and reducing materials, acetaldehydes, formaldehyde, formic acid and o-dioxy and oxymethyl were not detected. They concluded that presence of amino groups suggested nitrogen metabolism in these parasites. They may not always be the end products of protein digestion and may have been acquired from the host. There is hardly any work on ionic and osmotic behaviour of monogenean worms. The present author has done some little work on ionic and osmotic behaviour of Neodactylogyrus pharyngocephalus and N. raipurensis and has made microanalysis of their incubates in 40% Hanks saline, through the method of Feigl (1975). The author has also studied qualitatively the amino acid composition of free and protein pools of N. raipurensis vis-a-vis the host gill and serum. The data obtained have been included in this part.

MATERIALS AND METHODS

Glossogobius giuris and Labeo rohita were procured live from Doomer talao and were examined for parasitic infections. Neodactylogyrus pharyngocephalus and N. raipurensis were removed from their gills and were quickly washed twice with the incubating medium. Hanks saline
Taylor and Baker, 1978), ranging from 10-90% and sodium chloride, ranging from 0.1-0.9% were made with distilled water. A counted number of worms were transferred to 5 ml each of the several dilutions of saline and observations made. These worms, being very small and microscopic, did not lend themselves easily to the other methods of weight change analysis (Siddiqi and Lutz, 1966; Goll, 1966; Webster, 1970; Siddiqi et al., 1975).

Worms were incubated in 40% Hanks saline and 0.45% sodium chloride solution, for a period of 12 hours at 22 ± 2° C. The saline incubates were directly used for spot test analysis (Feigl, 1954) to chemically determine the qualitative nature of compounds excreted by the parasites during in vitro incubation. Observations on microanalysis were repeated thrice and the results affirmed.

Counted number of worms were put in different dilutions of Hanks saline for yet another purpose that is to determine their survival time.

Chromatographic analysis of amino acids of free and protein pools of worms was done as follows:

Free Amino acid Extraction: Approximately 25 mg of dried worms/tissues were taken, powdered in a pestle-mortar; homogenized in 2 to 3 ml of 70% ethanol and transferred to centrifuge tube. Homogenate was kept for
about 12 hours at 4°C and allowed complete precipitation of proteins. It was then centrifuged at 3000 rpm for 15 minutes. Supernatent was used for chromatographic analysis of free amino acids.

**Protein Hydrolysates:**

**Acid Hydrolysis:** Protein was extracted from about 20 mg of dried worms/tissues and purified. Acid hydrolysis of this purified protein was then carried out in 6 N HCl at 115°C for 12 hours. The hydrolysate was evaporated to dryness in vacuum evaporator. It was redissolved in a small volume of distilled water and again evaporated to dryness to remove traces of acid. Finally, it was dissolved in 3 ml of 70% ethanol for chromatographic analysis.

**Alkali Hydrolysis:** Purified protein was hydrolyzed in 5 N NaOH for 8 hours at 120°C (Smith and Seakins, 1976) for the detection of tryptophan.

Thin layer chromatography was carried out on glass plates (20x20 cm) using microcrystalline cellulose in appropriate volume of distilled water. Slurry was spread over the glass plates with the help of an applicator. It was allowed to dry at room temperature. Before applying the samples, plates were activated at 60°C for 30 minutes. Samples were applied with the help of a micropipette. Two dimensional chromatogram was developed in a rectangular glass jar (25x25x10 cm) using n-Butanol:acetic acid:water
(4:1:1.5 v/v) and n-Butanol:Pyridine:water (1:1:1 v/v) as first and second solvents respectively. The amino acids were located by spraying 0.2% ninhydrin reagent (in acetone). Identification of amino acids was made by comparing their Rf values with those of standard samples developed under identical conditions.

RESULTS

Data obtained in respect of all these experiments (i) on osmotic behaviour; (ii) spot test analysis; (iii) survival time in 40% Hanks saline and (iv) amino acids of free and protein pools have been included respectively in Tables, 1 to 4.

DISCUSSION

It is obvious from Table-1 that these worms become swollen in dilutions from 10-30% in Hanks saline and 0.1% to 0.3% NaCl and shrink in 60% to normal Hanks saline and 0.6 to 0.9% NaCl. Swelling of the worms was obviously due to uptake of water and shrinkage was on account of water loss. Osmotic behaviours in Neodactylogyrus pharyngocephalus and N. raipurensis n. sp. correspond with Gastrothy/ax crumenifer (Goil, 1966). In all these species the state of approximate isotonicity is attained in 40% to 50% Hanks saline and 0.4% to 0.5% NaCl. Fasciola gigantica was said to be isosmotic to normal Tyrode and host’s bile
(Siddiqi and Lutz, 1966) and was reported to swell, become inactive and died in 25% Tyrode. Cotylophoron ctylophorum and Gastrothylax crumenifer were both sensitive to deionized water; Gigantocotyle explanatum was sensitive to both deionized water and 20% Tyrode. Isoparorchis hypsalobagri, however, was reported insensitive to both these and sensitive to hyperosmotic media (Siddiqi et al., 1975). Siddiqi et al. (1975) opined that the role of habitat was very important in the physiology of trematodes and, the nature of host habitat, to a great extent, determined the permeability of water and salts. Webster (1970) agreed with Siddiqi and Lutz (1966) in that parasites living in a constant environment are osmoconformers and have lost the ability to regulate.

It would be obvious from Table-2 that besides the several intermediate products of carbohydrate metabolism, several amino acids, amides, amines, ammonia and even urea were clearly present in the incubates and eliminated by the worms during osmotic stress. Catabolism of proteins is a normal feature of these parasites. However, their elimination from the body during osmotic stress is perhaps a kind of osmotic regulation. The present author is inclined to disagree with Siddiqi et al. (1975) and Webster (1970) in that these worms have lost the power of osmoregulation. They have limited regulation and when osmotic stress is more severe, the worms swell or shrink and die.
This author's data in Table-3 would clearly suggest that even in 40% Hanks saline, where worms appear normal, there are several that do not survive.

Arme and Whyte (1975) have reported proline to constitute 75% of the protein pool of *Diclidophora merlangi* and speculated on proline involvement in osmoregulation but, subsequently, they abandoned this idea. There is yet no explanation of high percentage of proline in the protein pool of monogenea. This author has studied amino acids of free and protein pools of *N. raipurensis* vis-a-vis host serum and gill (Table-4) to find if they have some individuality in this respect. As luck would have it, the parasite pool of free and protein amino acids almost mimics the host pool qualitatively and so nothing can be said of involvement of individual amino acid in osmoregulation. In the total context, of course, they could be important.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Hanks saline</th>
<th>Parasite state</th>
<th>NaCl solution</th>
<th>Parasite state</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal</td>
<td>Shrinkage</td>
<td>-</td>
<td>Shrinkage</td>
</tr>
<tr>
<td>2.</td>
<td>90%</td>
<td>---</td>
<td>0.9%</td>
<td>---</td>
</tr>
<tr>
<td>3.</td>
<td>80%</td>
<td>---</td>
<td>0.8%</td>
<td>---</td>
</tr>
<tr>
<td>4.</td>
<td>70%</td>
<td>---</td>
<td>0.7%</td>
<td>---</td>
</tr>
<tr>
<td>5.</td>
<td>60%</td>
<td>---</td>
<td>0.6%</td>
<td>---</td>
</tr>
<tr>
<td>6.</td>
<td>50%</td>
<td>Normal</td>
<td>0.5%</td>
<td>Normal</td>
</tr>
<tr>
<td>7.</td>
<td>40%</td>
<td>Normal</td>
<td>0.4%</td>
<td>Normal</td>
</tr>
<tr>
<td>8.</td>
<td>30%</td>
<td>Swelling</td>
<td>0.3%</td>
<td>Swelling</td>
</tr>
<tr>
<td>9.</td>
<td>20%</td>
<td>---</td>
<td>0.2%</td>
<td>---</td>
</tr>
<tr>
<td>10.</td>
<td>10%</td>
<td>---</td>
<td>0.1%</td>
<td>---</td>
</tr>
<tr>
<td>11.</td>
<td>Distilled water</td>
<td>---</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE-2

Chemical analysis of incubates of *Neodactylogyrus pharyngocephalus* (A) and *N. raipurensis* (B) in 40% Hanks saline.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Reaction</th>
<th>Reagents or Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids, amines, amides and ammonia</td>
<td>+ +</td>
<td>Ninhydrin</td>
</tr>
<tr>
<td>Alcohol</td>
<td>+ +</td>
<td>O-dianisidine reagent</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>+ -</td>
<td>Morpholine, Sodium nitroprusside</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>+ +</td>
<td>Chromotropic acid</td>
</tr>
<tr>
<td>Ammonia</td>
<td>+ +</td>
<td>Hessler's reagent</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>+ +</td>
<td>O-hydroxydiphenyl</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>+ +</td>
<td>P-dimethylaminobenzaldehyde, TCA and benzene</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>+ +</td>
<td>P-hydroxydiphenyl</td>
</tr>
<tr>
<td>Formic acid</td>
<td>+ +</td>
<td>Conversion to formaldehyde</td>
</tr>
<tr>
<td>a-Alanine</td>
<td>+ +</td>
<td>Chloramine T, sodium nitroprusside, morpholine</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+ +</td>
<td>a-Nitroso-β naphthol, HNO₃</td>
</tr>
<tr>
<td>Glycine</td>
<td>+ +</td>
<td>H₂SO₄, Chromotropic acid</td>
</tr>
<tr>
<td>a-Amino carboxylic acid (amino acids)</td>
<td>+ +</td>
<td>Ninhydrin, Citric acid, Sodium hydroxide</td>
</tr>
<tr>
<td>Urea</td>
<td>+ +</td>
<td>Fructose, H₂SO₄, Stanous chloride</td>
</tr>
<tr>
<td>Oxaloacetic acid</td>
<td>+ +</td>
<td>Urea, Thiobarbituric acid</td>
</tr>
<tr>
<td>Malate</td>
<td>+ +</td>
<td>NaOH, Urea, Pyridil Chloride, Dimethyl formamide</td>
</tr>
<tr>
<td>Fumarate</td>
<td>- -</td>
<td>NaOH, Urea, Pyridil Chloride, Dimethyl formamide</td>
</tr>
</tbody>
</table>
TABLE-3

Survival of *Neodactylogyrus pharangocephalus* in 40% Hank's saline at 22 ± 2 °C.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Initial number</th>
<th>Survival after Parasites 24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
<th>144 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>34</td>
<td>34</td>
<td>34</td>
<td>13</td>
<td>6</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>53</td>
<td>53</td>
<td>36</td>
<td>11</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>55</td>
<td>53</td>
<td>52</td>
<td>32</td>
<td>8</td>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE-4

Free and Protein amino acids in Necodactylogyrus raipurensis, and in serum and gill of infected Labeo rohita vis-a-vis control.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>M. raipurensis</th>
<th>Host serum</th>
<th>Host gill</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>UI INF</td>
<td>UI INF</td>
</tr>
<tr>
<td>FAA PAA</td>
<td>FAA PAA</td>
<td>FAA PAA</td>
<td>FAA PAA</td>
</tr>
<tr>
<td>Leucine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Valine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methionine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>a-amino-n-butyric acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proline</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alanine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Threonine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Serine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Histidine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ornithine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lysine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cystine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Asparagine</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

FAA = Free amino acids; PAA = Protein amino acids; UI = Uninfected; INF = Infected.
INTRODUCTION

Pathophysiology deals with disturbances in normal physiology, the mechanisms producing these functional abnormalities and the way in which they are expressed as symptoms and clinical signs. It is the reaction pattern between the causative agent and the host. The functional and morphological changes which follow parasitic infections are frequently non-specific, the infective host responding in a limited way to a variety of aetiological agents, for example, the catabolism of proteins, various anemias and the immunopathologic events in several parasitic infections. Soulsby (1976) opined that there is a certain molecular basis and the evolution of this molecular basis occurred as the host and parasites co-evolved. The evolution of host parasite associations have occurred over an enormous span of time and a number of generations of hosts and parasites (Cameron, 1964). The factors which determine the host parasite compatibility for helminths are
largely unknown. Also, the parasite factors which determine pathogenicity of helminths are poorly understood (Sousby, 1976).

In monogenean context, there have been only very few studies on pathology and pathophysiology of infected hosts. Lester (1972) described the attachment of *Gyrodactylus alexanderi* (Mizelle and Kritsky, 1967) to *Gasterosteus aculeatus laurus* and observed that the anchors did not penetrate the epithelial layer and the attachment to the surface membrane of epidermis was effected with the marginal hooks. The anchors were essentially passive structures and the tips of anchors could be moved towards each other or farther apart but no other movements were possible. The host countered the parasite by sloughing off mucous material. This material was found weakly positive for proteins, strongly positive for acid mucopolysaccharides and general mucosubstances. Further, study of this slough revealed that it was a secretion. Skinner (1982) studied gill pathology of fish *Lutjanus griseus* due to *Ancryoccephalus* spp., *Strongylura timucu* due to *A. parvus* Linton, 1940 and *Neodiplectanum wenningeri* Mizelle et Blatz, 1941 on *Gerres cinereus*. He described epithelial hyperplasia and heavy mucous production and the respiratory epithelium was lost in some cases. The side of filament opposite the worm attachment was said to be affected quite often in
similar manner. Besides injury to host tissue the lamellae were reported deflected, adhering to each other, thus reducing gill surface effective for gaseous exchange. In severe cases, when a number of worms attached to the tips of filaments, clubbing and obliteration of gill filament occurred; the affected filaments appeared white and gills were congested with mucous. Additionally, mucous producing cells were concentrated sometimes in several layers at the tips of gill filaments, which have lost their normal structure. All told, fusion of gill lamellae along entire filaments, epithelial hyperplasia and clubbing of lamellae or obliteration of lamellar structure were reported. Cone and Odense (1984) studied pathology of 5 species of Gyrodactylus, namely, G. adspersi Cone and Wiles, 1983, in Tautogolabrus adspersus; G. avalonia Hanek and Threlfall, 1969 in Gasterosteus aculeatus; G. bullatarudis Turnbull, 1956 in Poecilia reticulata; Gyrodactylus spp. in Carassius auratus and G. salmonis (Yin and Sproston, 1948) in Salmo gairdneri, and observed epidermal hyperplasia, with zones of degeneration, and necrosis in the eroded dorsal fins in sections. In some sections they found increased goblet cells. In other sections the marginal hooks were firmly embedded up to 10 um inside the surface epithelium. In four of the five species, namely, G. adspersi, G. avalonia, G. bullatarudis and Gyrodactylus spp., they found attachment as described.
by Lester (1972) and these did not evoke any substantial pathology. G. salmonis, however, was said to lodge its marginal hooks deeply into host tissue and this behaviour was considered unique. They have quoted Moore (1923) who described gyrodactylosis of S. gairdneri. Clinical signs included copious production of skin slimes, particularly on the fins, and a bluish gray discolouration. In advanced stages, fins became frayed and reduced to stubs and with open sores at fin bases. Kawatsu (1978) described hypochromic microcytic anemia in crucian carp from river Asacawa in Diplozoon nipponicum infection. The hemoglobin levels of the host fish were reported depleted and there was a correlation between the number of worms and the extent of depletion of hemoglobin. This resulted in anemia of fish and all haematological indices, MCV, MCHC and MCH were affected. A high percentage of immature erythrocytes was reported. Das and Pal (1987), in a study of Dactylogyrus species infection of gills of cultured carp, described hypertrophy, hyperplasia and fusion of gill lamellae, inhibiting normal physiological function. The entire gill lamellae were said to be transformed into a fused mass. The worms penetrated their hooks into the host tissue, eliciting encapsulation response of the host through hyperplasia of gill epithelial cells and displacement of vascular areas. Schaperclaus (1991) has reviewed the pathology of worms due to monogenean...
infection. In case of vastator dactylogyrosis, branchial epithelium was reported destroyed at places of attachment of worms. Proliferation of branchial epithelium was also reported leading to formation of long thin processes and coalescence of adjoining gill filaments or lamellae (Bauer et al., 1969). This resulted in multilayered epithelial cells. This proliferation was said to follow fish survival from acute infection (Scherbina, 1973). Multilayered branchial epithelium affected gaseous exchange. This also had an effect on the normal blood supply to the gill filaments and their epithelial processes gradually dropped off. The diseased fish consumed considerably less oxygen (Uspenskaya, 1961). Multilayered epithelium was described in extensus dactylogyrosis also. Several epithelial cells changed into mucous cells and this led to excessive secretion of mucous which affected gas exchange. In macracanthus dactylogyrosis, destruction of epithelial, connective and cartilaginous tissues of gill filament all affected the respiratory activity. Further, in lamellatus type, the neutrophils and monocytes increased. Schaperclaus (1991) distinguished gyrodactylosis as skin and gill types. In skin type the fish became spotted, necrotic and dark and covered with bluish green mucous layer. The fins were destroyed leaving only the fin rays. Turbidity in the cornea of the eyes and blindness were also reported to occur. The hematological picture included increased
number of monocytes and agranulocytes, a drop in hemoglobin percentage to 6% and an increase in ESR. In gill gyrodactylosis, the carps were reported to swim below the water surface and thus suffocated. The gills were spotted and filled with blood and the filaments gray. The worms mechanically destroyed gills and extracted blood causing heavy losses in discocotylosis of trout. The pathophysiology during diplozoanosis included destruction of branchial tissue, inflammation and coalescence. Schaperclaus reported mortality of small breams in severe attack of Diplozoon.

It is obvious from the above that there has been no work on pathophysiology of fishes infected with monogeneans in India, except Das and Pal (1987) and there has been no assessment of disease caused by them. This author has studied the pathology of *Channa punctatus*, infected with *Gyrodactylus raipurensis* and pathology and pathophysiology of *Labeo rohita*, infected with *Neodactylogyrus raipurensis* through histochemical methods.

**MATERIALS AND METHODS**

Sections were cut in cryostat at 8 μ thickness, freeze dried and later processed for histochemical determination. Pathology was studied in Haematoxylin and eosin stained sections. The methodology followed for the
several histochemical determinations (Bancroft and Stevens, 1977; Pearse, 1985) in the heavily infected fishes vis-a-vis control are as follows:

**Mercury-bromophenol blue method for Proteins (Bonhag, 1955)**

**Reagents used:**
1. **2% Acetic acid:** 2 ml glacial acetic acid was made to 100 ml with distilled water.
2. **0.5% Acetic acid:** 0.5 ml glacial acetic acid was made to 100 ml with distilled water.
3. **Bromophenol blue stain:** 0.4 g mercuric chloride was dissolved in 40 ml of 2% glacial acetic acid and 25 mg of bromophenol blue was added to it.

**Procedure:** Sections were stained in bromophenol blue solution for 15 min at room temperature, washed twice in 0.5% glacial acetic acid and transferred to butyl alcohol for bluing. They were cleared in xylene and mounted in DPX.

**Result:** Proteins appear deep blue or grayish blue.

**Periodic acid-Schiff’s method for polysaccharides (Mcmanus, 1946)**

**Reagents used:**
1. **Picrate-formalin fixative:** 0.18 g sodium chloride and 0.15 g picric acid were dissolved in 25 ml of distilled water; 56 ml absolute alcohol and 10 ml
of 40% formaldehyde was added to it and the whole was made to 100 ml with distilled water.

2. Periodate solution: 400 mg of periodic acid was dissolved in 15 ml of distilled water; 135 mg of crystalline sodium acetate was dissolved in 35 ml of absolute ethanol separately and both mixed just before use.

3. Reducing rinse: 1 g each of potassium iodide and sodium thiosulphate were dissolved in 20 ml distilled water; when dissolved, 30 ml of ethanol and 0.5 ml of 2N HCl were added to it.

4. Schiff's reagent: 1 g basic fuchsin was dissolved in 200 ml boiling distilled water. After cooling to 50°C, the solution was filtered and 30 ml of 1N HCl and 1 g of potassium metabisulphide was added to it. The resulting solution was allowed to stand in dark for 24 h. 2 g of activated charcoal was added with stirring and the solution filtered.

Procedure: Sections fixed in picrate formalin fixative for 5 minutes; washed in 70% alcohol; treated with periodate solution for 5 minutes; washed again in 70% alcohol and immersed in the reducing rinse for 5 minutes. Sections were stained in Schiff's reagent for 30 minutes, washed in three changes of SO2 water, dehydrated and mounted in DPX.
Result: Red or purple colour indicates a PAS positive reaction.

Alcian blue-PAS method for mucopolysaccharides (Mowry, 1956)

Reagent used:
1. 3% Acetic acid: 3 ml of glacial acetic acid was made to 100 ml with distilled water.
2. 1% Alcian blue: 1 g of alcian blue 8 GX was dissolved in 100 ml of 3% acetic acid.
3. 1% Periodic acid: 1 g of periodic acid was dissolved in 100 ml of distilled water.
4. 0.5% Sodium bisulphide: 0.5 g of sodium bisulphide was dissolved in 100 ml of distilled water.
5. Schiff’s reagent: Prepared as for PAS reaction.

Procedure: Sections were briefly rinsed in 3% acetic acid and stained in alcian blue solution for 2 hours. After staining, the sections were oxidized in 1% aqueous periodic acid for 10 minutes and immersed in Schiff’s reagent for 10 minutes. Finally, the sections were rinsed in three changes of 0.5% sodium bisulphide, washed in running water, dehydrated, cleared and mounted in DPX.

Result: Alcinophillic (periodate-unreactive) mucosubstances blue, alcinophillic (periodate-reactive) mucosubstances bluish-purple, and periodate reactive non-alcinophillic components are red.
Palsmal reaction for phospholipids (Hayes, 1949)

Reagents used:
1. 2% Mercuric chloride: 2 g of mercuric chloride was dissolved in 100 ml of distilled water.
2. Schiff's reagent: Prepared as for PAS reaction.
3. Bisulphite water: 500 mg of potassium bisulphite was dissolved in 100 ml of 0.05 N HCl.

Procedure: Air dried sections were subjected to hydrolysis in 2% mercuric chloride solution for 10 minutes. They were then rinsed in bisulphite water, stained in Schiff's reagent for 10 minutes, washed in tap water and mounted in glycerine jelly.

Results: Phospholipids stain in a diffuse magenta colour.

Alkaline phosphatase (Gomori, 1952 a)

Reagents used:
1. 2% Sodium β glycerophosphate: 2 g of sodium β glycerophosphate was dissolved in 100 ml distilled water.
2. 2% Sodium veronal: 2 g sodium barbiturate was dissolved in 100 ml distilled water.
3. 2% calcium chloride: 2 g calcium chloride was dissolved in 100 ml distilled water.
4. 1% Magnesium sulphate: 1g of magnesium chloride was dissolved in 100 ml of distilled water.
For preparation of incubating medium, 2.5 ml each of 2% sodium \( \beta \) glycerophosphate and 2% sodium veronal, 5.0 ml of 2% calcium chloride, 0.25 ml of 1% magnesium sulphate and 1.25 ml of distilled water were mixed together and final pH adjusted to 9.0 using 0.1 N NaOH.

Procedure: Sections were incubated in incubating medium at 37°C for 1 hour. They were washed thrice in distilled water and treated with 2% cobalt nitrate for 3 minutes. Sections were washed again and transferred to 1% ammonium sulphide. After development of precipitate, washing was repeated. Finally, they were mounted in glycerine jelly.

Results: Sites of enzyme activity brownish-black.

Acid phosphatases (Gomori, 1950)

Reagents used:
1. 0.05 M Acetate buffer (pH 5.0): 70 ml of 0.2 M sodium acetate was added to 30 ml of 0.2 M acetic acid.
2. 3% Sodium \( \beta \) glycerophosphate: 3 g of sodium \( \beta \) glycerophosphate was dissolved in 100 ml of distilled water.

For preparation of incubating medium, 40 ml of 0.05 M acetate buffer, 0.053 g of lead nitrate and 4 ml of 3% solution of sodium \( \beta \)-glycerophosphate were mixed together.
Procedure: Sections were incubated in the medium at 37° C for 1 hour. They were washed with distilled water and immersed in 1% ammonium sulphide for 2 minutes. Washing was repeated and the sections were mounted in glycerine jelly.

Results: Sites of enzyme activity black.

Adenosine triphosphatase (Wachstein et al., 1960)

Reagents used:
1. 0.125% ATP: 125 mg of ATP was dissolved in 100 ml of distilled water.
2. 2% Lead nitrate: 2 g lead nitrate was dissolved in 100 ml of distilled water.
3. 2.5% Magnesium nitrate: 2.5 g of magnesium nitrate was dissolved in 100 ml of distilled water.
4. Tris buffer (pH 7.2): 25 ml of 0.2 M Tris was added to 44 ml of 0.1 N HCl and whole was made to 100 ml with distilled water.

For preparation of the incubating medium, 4 ml each of 0.125% ATP and Tris buffer, 0.6 ml of 2% lead nitrate, 1 ml of 2.5% magnesium nitrate and 0.4 ml of distilled water were mixed together.

Procedure: Sections were incubated in the medium for 1 hour at 37° C. After washing in distilled water they were immersed in 1% ammonium sulphide for 2 minutes. They
were washed again and mounted in glycerine jelly.

Result: ATPase activity - Black.

Tween Method for Lipase (Gomori, 1952 b)

Reagents used:
1. Tris buffer (pH 7.2): 25 ml of 0.2 M Tris was added to 44 ml of 0.1 N HCl and whole was made to 100 ml with distilled water.
2. 5% Tween: 5 g of Tween 60 was dissolved in 100 ml of tris buffer.
3. 2% Calcium chloride: 2 g Calcium chloride was dissolved in 100 ml of distilled water.
4. 2% Lead nitrate: 2 g lead nitrate was dissolved in 100 ml of distilled water.

For preparation of incubating medium, 9 ml of Solution 1, 0.6 ml of Solution 2 and 0.3 ml of Solution 3 were mixed together.

Procedure: Sections were immersed in the incubating medium for 2-8 hours. They were rinsed in distilled water and placed in preheated lead nitrate solution at 55°C for 10 minutes. After washing in distilled water sections were treated with 1% ammonium sulphide for 3 minutes. They were washed again in distilled water and mounted in glycerine jelly.
Results: Lipase activity is indicated by yellow to brown colour.

Copper Thiocholine Method for Acetylcholinesterase (Gerabtstoff, 1959)

Reagents used:
1. Solution A (0.5 M acetate buffer): Prepared as for acid phosphatase.
2. Solution B: 150 mg of acetylthiocholine iodide and 70 mg of cupric sulphate were dissolved in 14 ml of distilled water. They were centrifuged at 4000 rpm for 15 minutes and supernatant used.
3. Solution C: 375 mg of Glycine was dissolved in 10 ml of distilled water.
4. Solution D: 250 mg of cupric sulphate was dissolved in 10 ml of distilled water.

For preparation of incubating medium, 5 ml Solution A, 0.8 ml Solution B, 0.2 ml Solution C, 0.2 ml Solution D and 3.8 ml distilled water were mixed together.

Procedure: Sections were incubated in medium for 1 hour at 37°C. They were rinsed in two changes of distilled water and treated with 2% ammonium sulphide solution for 2 minutes. Finally, they were washed in distilled water and mounted in glycerine jelly.

Results: Cholinesterase activity is indicated by a brown precipitate.
Dehydrogenases (Bancroft and Stevens, 1977)

Reagents used:
1. 0.2 M Tris buffer (pH 7.4): 25 ml of 0.2 M Tris was added to 42.0 ml of 0.1 N HCl and whole made to 100 ml with distilled water.
2. 1.0 M Sodium lactate: 1.25 ml of sodium DL-lactate made to 10 ml with distilled water.
3. 1.0 M Glucose-6-phosphate: 3 g of glucose-6-phosphate was dissolved in 10 ml of distilled water.
4. 2.5 M Sodium succinate: 6.75 g of sodium succinate was dissolved in 10 ml of distilled water.
5. NBT solution: 10 mg of Nitro Blue Tetrazolium salt (NBT) was dissolved in 2.5 ml of distilled water.

Preparation of the incubating medium
Lactate dehydrogenase: 0.9 ml of solution 5 and 0.1 ml of solution 2 were mixed together. 2 mg of NAD was added as coenzyme.
Glucose-6-phosphate dehydrogenase: 0.9 ml of solution 5 and 0.1 ml of solution 3 were mixed together. 2 mg of NADP was added as coenzyme.
Succinate dehydrogenase: 0.9 ml of solution 5 and 0.1 ml of solution 4 were mixed together.
Procedure: Sections were incubated in the appropriate incubating solution for 30 minutes at 37°C. They were transferred to 15% formol saline for 15 minutes to stop the reaction. Sections were washed in distilled water and mounted in glycerine jelly.

Results: Enzyme activity is indicated by purple formazan deposits.

RESULTS

Pathology of *Channa punctatus* due to *Gyrodactylus raipurensis*

Fishes investigated were very heavily infected. Each gill harbouring more than 100 worms. The pathological manifestations of the disease can be made out from microphotograph nos. 1 to 7. Briefly, normal disposition of primary and secondary lamellae is seen in figs. 1 & 2. Necrobiosis of primary and secondary lamellae is visible in figs. 3 & 4. Deposition of collagen material, haemosiderin in area close to attachment site of worms, can be seen in necrotic debris. This necrobiosis is of liquifactive kind. The cytoplasm and several cell organelles are liquefied to pulpy mass. The chromatin material in the nucleus shows all stages from pyknosis (condensation of chromatin material in the form of lumps) to karyolysis (transformation of the chromatin into rod shaped structure) (Fig. 5). Affected fishes also showed hypertrophy of primary and
secondary lamellae (Figs. 5 & 6). Primary lamellae become large and swollen. This hypertrophy is accompanied with enlargement and multiplication of mucous cells, also perhaps increase of chloride cells and their accessory cells (Fig. 7).

Pathology of *Labeo rohita* infected with *Neodactyloerythron ralpurensis*.

*N. ralpurensis* are smaller than *G. ralpurensis* and relatively more numerous in each gill of *Labeo rohita*. Pathology due to *N. ralpurensis* can be assessed from figures 8 to 16.

As in *Channa punctatus*, necrobiosis of gill epithelium is pretty obvious in infected fish vis-s-vis control (Figs. 8 to 10). This necrobiosis is of liquefactive kind. The cytoplasm and several cell organelles are liquefied to a pulpy mass. The chromatin material in the nucleus shows all stages from pyknosis (condensation of chromatin material in the form of lumps) to karyolysis (transformation of the chromatin into rod shaped structure vis-s-vis control (Figs. 11 & 12). This necrosis of epithelial lamellae culminates in the complete dissolution of the gill epithelium (secondary lamellae) leaving behind only filamentous projections (Figs. 9 & 10). Several of these primary lamellae show hyperplasia and hypertrophy of their tips vis-s-vis
control (Figs. 13 & 14). This hypertrophy includes the hypertrophy of epithelial cells (Fig. 15) and hypertrophy of mucous cells (Fig. 16). Biochemical analysis of the affected and control tissues has also been done. The total protein level is increased in infected fishes vis-a-vis control (Figs. 17 to 19). This is because of hyperplasia of epithelial cells at tips of these filaments. The levels of phospholipids were augmented very much in the infected fishes vis-a-vis control (Figs. 20 & 21). Likewise, the infected filaments and their tips showed intense PAS reaction (Fig. 22). This is because of hypertrophy and hyperplasia of mucous cells in the filament and their tips.

Enzyme activities have also been done, histochemically, in the affected tissues. ATPase, which is very much relevant in the ion exchange, particularly in Ca\(^2+\) uptake, is relatively much more in infected tissues, particularly at the tips of filament vis-a-vis control (Figs. 23 & 24). Alkaline phosphatase which is relatively intense and localized in uninfected appears depleted in infected tissues (Figs. 25 & 26). Acetylcholine esterase activity, related with Na\(^+\) uptake and also with the holdfast action, is very much intensified in infected fish vis-a-vis control (Figs. 27 & 28). Both LDH (Figs. 29 & 30) and glucose-6-phosphate dehydrogenase (Figs. 31 & 32).
activity, connected with glucose metabolism and energy cycle, are very much augmented in infected tissue vis-a-vis control. SDH activity, on the other hand, does not show any change as compared from control (Figs. 33 & 34). The hydrolytic enzymes, both lipase (Figs. 35 & 36) and acid phosphatase (Figs. 37 & 38), are very much augmented in infected fish vis-a-vis control.

DISCUSSION

It would be perhaps worthwhile to briefly highlight the gill structure and functions for a proper comprehension of the pathology and pathophysiology of host gill affected by two species of worms, respectively, *Gyrodactylus raipurensis* in *Channa punctatus* and *Neodactylogyrus raipurensis* in *Labeo rohita*. Some of the special features highlighted in the filament (primary lamella) are, two circulatory systems, the arterio-arterial, also called as respiratory for gas transfer, and arterio-venous for supply of nutrients. Piller cells are specific structures common to filament and made up of two parts, the nucleated body forming the pillar and covered with a basement membrane underlying the gill epithelium. These pillar cells constitute a contractile system regulating the blood supply within the lamellae and have smooth muscle myosin and collagen both. Filament epithelium includes five major types of cells, namely, squamous, mucous
(Goblet), chloride, neuroepithelial and nondifferentiated cells. Of these, although the role of mucous cells in osmoregulation is not very clear, their abundance in freshwater fishes suggest that they have a role in controlling the loss of ions or influx of water. Chloride cells, on the other hand, now called as ionocytes, are mitochondria rich and have undoubtedly an important role in ion transport. The lamellar epithelium are concerned with most of the important functions of the gills, namely, gas transfer (elimination of CO₂ mostly), extra renal excretion, ion exchange and regulation of acid-base balance.

Ion-exchange is perhaps the most important function of the gill. The only source of energy for ion transfer across the membrane is ATP; certain ions such as Na⁺, K⁺ and Ca²⁺ are transported through Na⁺, K⁺ (ATPase) and Ca²⁺ ATPase. ATPase catalyses release of inorganic phosphate of ATP in the presence of Mg²⁺ and do not display any strict specificity. These enzymes maintain separation of ions across the plasma membrane ensuring the ionic concentration in the intracellular environment with the external milieu. The mitochondria rich chloride cells have high concentration of ATPases. Besides these cation ATPases, there are anion dependent ATPases for absorption of Cl⁻/HCO₃⁻ from outside through active transport. This exchange through anion dependent ATPase is
Important in acid base regulation in the internal medium. Gills have high permeability to $H^+$ too. This has a marked effect on salt balance.

In the gill metabolism, lactate is identified unequivocally as an important oxidative substance. The fish gill can be identified as lactate consuming and naturally LDH activity becomes important. Although alanine is a poorer oxidative substrate for gill than lactate, it is potentially an important substrate. Glycine and serine are also relevant and the enzyme aminotransferases have important function. The gills have a surprising capacity to synthesize lipids. There is a very high turnover of gill lipids. The membrane lipids are particularly relevant. The high turnover of phospholipids is also known. Gills also have appreciable activity of $\alpha$-glycerophosphate dehydrogenase which catalyses hydrolysis of dihydroxyacetone in to $\alpha$-glycerophosphate. This enzyme is important in $\alpha$-glycerophosphate shuttle and may function to supply $\alpha$-glycerophosphate for triglyceride synthesis. The principal nitrogenous waste of fish is $NH_3$ which is mainly excreted through gills. Gill tissue also have remarkable property to transaminate amino acids.

Considering the importance of the gills in gas transfer, ion-exchange and maintenance of acid base balance in the body, pathology due to infection can impair not
only respiratory but also several vital functions.

*G. raipurensis* parasitizes *C. punctatus.* Several hundreds of these worms are found in the gills of the fish. It would be obvious (fig. 3) that the worms embed firmly in the lamellar epithelium with their anchors and marginal hooks. The anchors can be seen not only to thrust but also to crush tissues. The destruction of tissues and their reduction to almost a pulp like structure follows the attachment and browsing of the parasite and the net result is almost an opaque necrotic homogenate of cytoplasmic and nuclear debris. Extensive haemorrhage due to tearing of arterioles and arteries can safely be inferred from the accumulation of haemosiderin in the necrotic debris. This kind of necrosis is identifiable with liquifactive necrosis (Roberts, 1978). Instances are where the gill filaments are left with filamentous stubs, with hardly any trace of the tissues and the surface epithelium. In the filament and the epithelium where necrosis is not obvious, the cells and tissues show enormous hypertrophy and hyperplasia, particularly at the tips of filament. Hypertrophy and hyperplasia of epithelial and mucous cells are pretty obvious at the swollen tips in sections (Fig.6). Several stages of nuclear degradation, i.e., pyknosis, karyorrhexis (fragmentation of nucleus into several chromatin material) and even karyolysis are all quite
obvious. All this leads one to conclude that hypertrophy of filament and lamellae, following attachment and browsing of parasites, culminates in tissue destruction and necrosis.

In another study of *L. rohita*, infected with *N. raipurensis*, this author has found similar pathology, although not as glaring. *N. raipurensis* are much smaller in size and haptoral armature. Their intensity, however, is much more than in case of *G. raipurensis*. An interesting feature of this infection is that these worms do not anchor themselves firmly into the tissues. They are more mobile, moving from one point of attachment to another. Hypertrophy of lamellar tips, due to hypertrophy and hyperplasia of epithelial, mucous, chloride cells etc., and pyknosis of nucleus, nuclear degradation and even karyolysis are also pretty obvious at filamental tips. Although necrosis of cells and nuclei is rampant hereto and there is cellular and nuclear debris, the liquifactive necrosis, in the form of tissue homogenate, is not that apparent.

This author has also studied, histochemically, some of the important substrates and the key enzymes to be able to attempt physiological correlation with pathological picture. An interesting observation is that despite catabolism of proteins, commonly met with in parasitic infections, augmentation of protein is obvious from the
results given above. This is perhaps because hypertrophy and hyperplasia of the epithelial cells compensates more than adequately the loss of albumin on account of infection. The mucopolysaccharides again show an enormous increase because of hypertrophy and hyperplasia of mucous cells and likewise, the phospholipids. Corresponding enzyme activities have also been studied and the increase in ATPase, acetylcholinesterase, LDH, Glucose-6-phosphate dehydrogenase and lipase activity are all in conformity with the pathophysiological scenario following this infection. These observations are in direct contrast with Lester's (1972) study of Gyrodactylus infection in Gasterosteus, where pathology due to these worms was hardly much, and Cone and Odense (1984), where tissue damage was minimal except in G. salmos which was said to push marginal hooks deep in the epidermis and cause extensive damage to fins and skin. Skinner (1982), however, reported epithelial hyperplasia and heavy mucous production and loss of respiratory epithelium due to injury caused by the hooks of Ancyrocephalus spp. in L. griseus and Ancyrocephalus parvus in Strongyura timucu. In Neodiplecctanum wenningeri on Gerres cinereus, on the other hand, they reported damage to be very little, only mechanical and gill lamellae were deflected. Das and Pal (1987), in their study of Dactylogyrus infection of common carp, observed hyperplasia and hypertrophy of gill
lamellae. The apical margin of lamellae showed hyperplasia and fusion of lamellae. The parasites were said to sink their hooks into the host tissue and the host responded by encapsulation of the parasites by hyperplasia of gill epithelium and displacement of vascular area. In advanced cases they reported fusion of gill lamellae into a fused mass.

Comparing the pathology due to the present worms respectively in *C. punctatus* and *L. rohita* with dactylogyrosis of common carp, as reported by Bauer et al. (1977), and gyrodactylosis, as reported by Bauer et al. (1977) and Schaperclaus (1991), the damage and destruction in the present author's study is more severe. Bauer et al. (1977) noted hemorrhage and hinderence of gas metabolism and respiratory function and decreased levels of ESR in *D. vastator* dactylogyrosis and the damage to respiratory folds, which become multilayered, in *D. extensus* type of dactylogyrosis. In gyrodactylosis of carp, with several species of *Gyrodactylus*, they observed damaged to the fins, skin and formation of a bluish film over the fins and disruption of the respiratory function.

It is pretty obvious from the results and the above discussion that considerable morbidity occurs in *C. punctatus* and *L. rohita*, infected respectively with *G. raipurensis* and *N. raipurensis*. This author has also
studied the incidence and intensity of *G. raipurensis* and *N. raipurensis* in an annual cycle and it would be apparent from Table-3 (page 132) that the incidence is 65% in *L. rohita*. Mean intensities of these worms have also been studied and found to be pretty high. The data obtained from these studies and the pathophysiological picture, as discussed above, lead one to the logical conclusion that there is heavy mortality in these fishes. It is most unlikely that these fishes could resolve the damage due to the disease and mortality is inevitable. In monogenean context, which are ectoparasites, death of affected fishes would not limit the spread of infection (Scott and Anderson, 1984). Crofton's (1971) "lethal level concept", in highly dispersed parasite populations, implicating loss of much larger number of worms with host death, thus regulating the dynamics of parasite population, is not relevant in monogenean context, which are ectoparasites, and the infection can spread by direct contact even after the death of host fish. It is this factor in monogeneans which is responsible for the very high incidence and intensity and rich species composition and colonization of fresh water fishes, in spite of a monogenetic (direct) life cycle.