ABSTRACT

In the present thesis some aspects of surface plasma membranes and metabolism of amphistome parasites of water buffalo, Bubalus bubalis have been investigated. In order to make our study comparative two amphistomes, Gastrothylax crumenifer and Gigantocotyle explanatum inhabiting buffalo rumen and liver respectively were selected.

The review of literature reveals that the surface plasma membranes are mainly investigated in Schistosoma spp. and Hymenolepis spp. and occasionally in other parasites, although surface plasma membranes play an important role in the host-parasite relationship.

The present study was undertaken to investigate the similarities and dissimilarities in the surface membranes and in various metabolic processes of amphistomes inhabiting two different habitats. This study will also provide informations about the differences which might exist as a result of biochemical adaptation and niche segregation. The main emphasis was given to membrane characterization and its associated metabolic activities which provide basic data for host-parasite interaction, since surface of parasites serve as a main target for immunological and pharmacological control. Besides this, seasonal reproduction and the associated biochemical changes were also investigated, which undoubtedly can be used for the development of a time based control programme.

These studies were carried out in a moderately equipped laboratory and therefore, many aspects could not be investigated due to lack of facilities.

The salient features of the results obtained in the present study are summarized below:
Chapter - I deals mainly with the isolation of surface plasma membranes of *G. crumenifer* and *G. explanatum*. As a first step a technique for the isolation of surface plasma membranes of amphistomes to a greater purity was standardized. Three non-ionic detergents were used and the treatment time was standardized. Emphasis was focussed to obtain maximum yield of the membrane with least subcellular contamination. The yield was monitored by protein recovery and the contamination was checked by lactic and succinic dehydrogenases activities for cytosolic and mitochondrial contaminations respectively. Transmission electron microscopy has been used to confirm the basic structural organization of the surface plasma membranes of amphistomes and also the degree of contamination. The effect of various detergents and the degree of lesions caused by detergents was monitored by scanning electron microscopy of the worm's carcasses.

The results indicate that for appreciable yield and greater purity of surface plasma membranes saponin treatment for 20 min is optimum. In addition to this the vortexing time and the centrifugation protocol adopted in the present study was also found significant. It was noticed that the isolation procedure was suitable for both the amphistomes understudy.

Chapter - II deals with the analysis of membrane bound marker enzymes and chemical nature of surface plasma membranes of two amphistomes in order to characterize the surface plasma membranes isolated with different detergents. For the present study primary membrane bound marker enzymes like 5'-nucleotidase, ATPase, acid and alkaline phosphatases were quantitated in order to confirm the best detergent. Further, analysis of the enzyme levels and the chemical composition of the surface membranes will be useful to assess the differences/similarities among two amphistomes inhabiting two physicochemically different microhabitats. Since, it is expected that due to
inhabiting different microenvironments, the surface membranes may develop some chemical changes as a result of niche segregation and biochemical adaptation.

The results indicate that the membranes isolated with saponin possess appreciably greater quantities of enzyme activities than any other detergent used in both *G. crumenifer* and *G. explanatum*, thus indicating that saponin is the best detergent for the isolation of membranes.

Presence of many fold enrichment of primary membrane bound marker enzymes in the surface plasma membranes of both amphistomes indicate that these enzymes could be used as optimum markers in order to characterize the surface membranes of other amphistomes. The results indicate that in the surface membranes of both the amphistomes two ATPase systems viz. Mg$^{2+}$ stimulated (Na$^+/Mg^{2+}$ ATPase) and ouabain sensitive (Na$^+/K^+$ ATPase) were operating simultaneously, where former form was predominant than the latter.

In addition to enzyme studies, the suitability of detergent was also assessed by calculating the phospholipid to cholesterol ratios. The results indicate that only saponin isolated surface membranes show equimolar ratios of phospholipid to cholesterol which is essential to maintain the fluidity of the bilayer.

Besides this some of the major lipid fractions like free fatty acids and triglycerides were also analysed in two amphistomes under study. TLC separation of phospholipids reveal the presence of phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, lysophosphatidylethanolamine and sphingomyelin and some unidentified phospholipids. In both the amphistomes membranes the level of free fatty acids and phosphatidylethanolamine were found maximum, suggesting their important role in maintaining the membrane permeability.
The presence of sialic acids in the membranes of amphistomes indicate anionic nature and biological activity of the membranes as well as in the electrogenic nature of the surface of the parasites.

Such quantitative biochemical and enzymatic differences in the membranes of liver and rumen amphistomes may be a consequence of parasitic adaptation.

Chapter III deals with the similarities and dissimilarities in the polypeptide profile and antigenicity of surface plasma membranes of *G. crumenifer* and *G. explanatum*, investigated by using gradient polyacrylamide SDS - slab gel electrophoresis and double immunodiffusion (DID) techniques. A total of 33 and 34 distinct membrane protein bands were observed in *G. crumenifer* and *G. explanatum* respectively. Among which 17 polypeptides were found common in both the amphistome's membranes under study. The similarities in the protein profile of surface plasma membranes of both the amphistomes indicate some fundamental homologies in between two different species. The common peptides may be of cross-reactive values among different species, while the presence of peculiar polypeptides are of great significance for the development of immunodiagnostic techniques.

The surface membranes of two amphistomes did not show any similarity in the precipitation pattern, when the heterologous antigen - antibody (raised in rabbits) reactions were analysed. The results indicate that *G. explanatum* posses two major antigenic components while rumen amphistome posses only a single antigenic component. The non-reactivity of heterologous membrane antigen and antisera suggest that both the amphistomes posses different antigenic moieties in their surfaces, it might be the influence of different habitats. The heterogeneity in the polypeptides may be of obvious importance for the diagnostic and protective measures. The results of the DID indicate that there are some antigenic components are present in membranes but this require further
characterization and their role in immunoprotection. Due to the limitations of specificity and sensitivity of DID technique, it was not possible to comment on these aspects.

However, further studies are required to ascertain the antigenic nature of surface plasma membranes by using more specific and sensitive methods like radio-iodination labelling, ELISA and immunoblotting.

Chapter - IV deals with the biochemical changes associated with reproductive behaviour of amphistomes. Seasonal changes in the glycogen, protein, DNA, RNA and lipid contents were investigated in *G. crumenifer* and *G. explanatum*. These biochemical changes in these two amphistomes can be directly correlated with the morphometric changes in reproductive organs and the production of eggs with respect to seasonal variations. In liver amphistomes no definite reproductive cycle was recorded and hence all biochemical components showed minor fluctuations with minor changes in gonadal recrudescence and regression, whereas in *G. crumenifer* the protein and lipid contents increases exactly during the egg production phase with broad single peaks corresponding to the recrudescence of gonads while the glycogen, DNA and RNA levels build up just prior to the peak level of protein and lipid contents.

Analysis of the total lipid fractions of rumen and liver amphistomes also reveal the seasonal changes and monthly changes were observed in the levels of cholesterol, phospholipids and free fatty acids in both the amphistomes, but the triglyceride fluctuations were not so pronounced. In *G. crumenifer*, the maximum levels of total lipid content and lipid fractions build up simultaneously whereas in *G. explanatum* the lipid fractions increases as the level of total lipid decreases. TLC separation of various phospholipids like phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylcholine, sphingomyelin also show marked seasonal variations in both the amphistomes.
The results indicate that the fluctuations in the biochemical components in the rumen amphistomes are associated with the egg production cycle. This data could be further exploited for the development of an effective time based chemotherapeutic control programme.

Chapter-V deals with the uptake of glucose which has been investigated in *G. crumenifer* and *G. explanatum* in order to find out the differences which might exist as a result of physiological adaptations in different habitats. The glucose uptake studies were performed by using different parameters. Autoradiography was used to find out the actual route for glucose entry while scintillation techniques were carried out in order to check the mode of transport kinetics in two amphistomes. In addition to this various ions, inhibitors and ionophores were also used to detect their effects on the uptake of glucose in *G. crumenifer* and *G. explanatum*. Simultaneously the effect of inhibitors/ionophores on the motility of amphistomes was also monitored by using isometric transducer.

The results of autoradiography reveal the presence of transtegumental uptake of glucose in both the amphistomes. The appearance of silver grains in other organs indicate that a functional and efficient carbohydrate metabolism is operative. Kinetic studies showed that the uptake of glucose in both *G. crumenifer* and *G. explanatum* takes place via a mediated process involving a diffusion component. However, velocity of uptake and affinity for glucose differ in two amphistomes. The liver amphistomes show less affinity for glucose than rumen amphistomes.

The *Na⁺ - K⁺ ATPase* and glucose uptake inhibitors namely ouabain and phloridzin showed inhibitory effects on glucose uptake in both the amphistome. These effects were concentration dependent. These results indicate that in amphistomes the uptake of glucose involve both facilitated and active transport which is ouabain and phloridzin sensitive.
Further, it was noticed that the glucose uptake require simultaneous presence of both Na$^+$ and K$^+$ ions. However, in absence of K$^+$ ions (0.145 M NaCl) no uptake was noticed by these amphistomes this may be due to the counter flow, since instead of uptake, the glucose leakage was noticed.

The higher concentrations of mobile ion carriers monensin (Na$^+$ ionophore and valinomycin (K$^+$ ionophore) also showed inhibitory effects on glucose uptake in both the worms. Due to high specificity of these ionophores for Na$^+$/K$^+$ ions, it probably alters the ion gradients across the bilayer. As result the ATPase system become inactive and the glucose uptake was inhibited.

The effect of inhibitors and ionophores have also been investigated on the motility of amphistomes. The results indicate that both Na$^+$ and K$^+$ ionophores produced complete flaccid paralysis but the effects were more rapid in monensin than valinomycin in both the worms. While ouabain produced an increase in muscle tone which very quickly lead to complete spastic paralysis. On the other hand phloridzin does not show any pronounced paralytic effect and only disturbed activity was observed. The quick effects produced by ouabain indicate that in these amphistomes, the Na$^+$ pump is acting electogenically.

Since, monensin is widely used as an animal feed supplement for meat producing animals and promote more favourable fermentation of cellulose in bovine rumen, therefore this ionophore can be used as a potential agent to control these infections.

Chapter-VI deals with the glycogen utilization and excretory/secretory products of G. crumenifer and G. explanatum. Interestingly it was noticed that G. explanatum utilizes endogenous glycogen continuously with the increasing starvation time while in G. crumenifer no such gradual pattern of endogenous glycogen depletion was noticed and an increase followed by the decrease in glycogen levels was observed. This increase and decrease was continuous upto
60 h of starvation, indicating that the rumen amphistomes have active gluconeogenesis. Changes in the pH and redox potential (Eh) were more rapid in *G. explanatum* than *G. crumenifer*. These results indicate the release of excretory products during *in vitro* incubation of worms.

Among various excretory products, appreciable amounts of glucose leakage were also observed. The rate of glucose leakage was more in *G. explanatum* than *G. crumenifer*, which might be the influence of habitat. Pyruvate and lactate have also been analysed following *in vitro* incubation of amphistomes in Hank's medium with or without glucose at optimum physiological temperature. In presence of glucose the worms excrete more lactate and the rate was greater in *G. explanatum* than *G. crumenifer*, whereas in the absence of glucose *G. crumenifer* does not excrete lactate. The pyruvate excretion is also influenced by the presence or absence of glucose in medium.

Both the amphistomes also excrete/secrete various lipids when incubated in non-nutrient media and the rate of excretion is again high in *G. explanatum*. The hydrolysis of various phosphate esters by intact worms *in vitro* clearly indicate that these amphistome excrete/secrete various phosphomonoesterases. However, the degree of hydrolysis varies in different incubating media as well as in two amphistomes.

The role of these phosphomonoesterases in glucose uptake has also been verified. It was observed by using molybdate ions as inhibitor of phosphomonoesterases that for the uptake of glucose the phosphatases are not involved.

Thus, it can be concluded from the work presented in this thesis covering the area of surface plasma membranes and some metabolic processes of amphistomes that the habitat of the parasite play an important role. Therefore, the similarities and dissimilarities are the result of parasite
adaptation and niche - segregation. Further, the seasonal reproductive behaviour of these parasites as well as the quantitative differences in metabolic activities could be further exploited for the development of time based control programme through immunological and pharmacological measures.