CHAPTER - II

ANALYSIS OF MEMBRANE BOUND MARKER ENZYMES
AND BIOCHEMICAL COMPOSITION OF SURFACE
PLASMA MEMBRANES
INTRODUCTION

Plasma membranes are generally responsible for maintaining a constant internal environment of cell irrespective of changes that may occur outside. The membranes while protecting the cell from variable environment, must allow selective communication with the external surrounding. Plasma membranes are, therefore, associated with a range of transfer systems which enable molecules to pass through the membrane in a specific manner. Beyond this supramolecular level of organisation, the membranes also fulfil an important role as structural support at molecular level. Many of the chemical processes of biological systems involve long series of co-ordinated reactions. Various membrane bound enzymes are also involved in these reactions and they are present in an orderly fashion on the surface membranes. The membrane bound enzymes and the soluble enzymes show some differences in their properties (Harrison and Lunt, 1980). The soluble enzymes exist in a polar hydrophilic environment of high dielectric constant. A variety of molecules including substrates, other metabolites and ions can approach to the soluble enzymes easily. In constrast, the membrane bound enzyme may be largely embedded in a lipophilic region of low dielectric constant, with little opportunity for interaction with small polar molecules. The membrane-bound enzymes exist in a relatively stable microenvironment, the nature of which is determined by the molecular arrangement of the membranes. Changes in membrane composition or structure will modify the environment of the enzyme and thus affect the regulatory system (Harrison and Lunt, 1980). Therefore, to understand the functions of individual membranes in molecular terms, it is necessary to know their precise chemical composition and enzyme systems.
Albert Claude (1946) laid the foundation of present day subcellular fractionation scheme and introduced the idea of characterizing the cell fractions by measuring enzyme activities, and thus paved the way for the identification of marker enzymes. Once an isolated membrane fraction has been identified morphologically, the other studies are required to characterize the membranes. If an enzyme is shown histochemically to be exclusively associated with a certain membrane fraction, it can be used as a primary membrane marker. Among the various enzymes, 5'-nucleotidase, Na\(^+\) - K\(^+\) ATPase and alkaline phosphatase are generally considered as primary marker enzymes for the liver cell plasma membranes.

The occurrence and nature of membrane bound enzymes also throw some light on the functional aspects of the membrane. Therefore, to ascertain the type of transport phenomenon, it is necessary to study the enzyme and its kinetic parameters.

In all of the recent models of membranes, lipids form an integral part of a protein-lipid network, held together in a complex, by non-covalent interactions, and the membranes contain asymmetrically distributed glycoproteins and glycolipids which extend their carbohydrate-bearing portions, mainly sialic acid into the extracellular environment. Membrane lipids are polar lipids which are also referred to as amphipathic, meaning that they incorporate both a hydrophobic tail and a hydrophilic head group within the molecule. The hydrophobic and hydrophilic regions can be bridged by a glycerol moiety, a sphinganine derivative or homologue or, finally, within a sterol molecule.

As far as lipids are concerned, a fundamental role is attributed to the class of phospholipids. The ubiquitous occurrence of phospholipids
as indispensable components of membranes indicates that these substances play a vital role in living cells. Mayer and Schaeffer (1913) observed that the lipid-phosphorus content of most organs did not change markedly under diversified conditions such as overfeeding or inanition. The range of hydrophilic head groups of polar lipids is further extended by the class of glycolipids in which the head group is attached via the glycosidic linkage of a sugar molecule rather than by a phosphate ester bond, as in the phospholipids. In plasma membranes the sugar residues mostly exposed on the outer surface in which the glycolipids are mostly found.

The sterols constitute a third major class of membrane lipids in addition to the phospholipids and glycolipids. Cholesterol is known to cause condensation of phospholipid monolayers. In natural membranes, cholesterol has been shown to act as a stabilizing factor in which increased osmotic fragility follows cholesterol depletion. All these stabilizing and controlling effects have been attributed to the ability of cholesterol to influence the packing of the hydrocarbon chains of phospholipids, possibly by way of an equimolar cholesterol-phospholipid complex.

Membrane lipids which do not fall into one of the three major classes of phospholipids, glycolipid and sterols, are usually minor components. Mono- and di-acylglycerols and free fatty acids are also reported in small amounts in animal membranes. The level of free fatty acids varies with the functional state of the membrane, and it has been suggested that they may be involved in changes in membrane permeability (Harrison and Lunt, 1980).

Besides amphipathic lipid constituents, biological membranes have also been reported to contain minor amounts of non-polar lipids such as
triaclyglycerol and steryl esters of long-chain fatty acids. The location and role of such compounds is not clear, and it is probable that, in many cases, they were observed as artefacts. N-acetylneuraminic acid, also known as sialic acid is a constituent of glycoprotein, glycolipid and ganglioside, occurs at the external surface as the glycocalyx. Most of the carbohydrates of the membranes (e.g. N-acetylgalactosamine, galactose and sialic acid) assembled into a wide variety of oligosaccharide chains linked to protein and lipid components of the membrane by covalent bond, form an integral part of the structure (Singer and Nicolson, 1972).

In helminths, as mentioned earlier, the external surface is highly specialized syncitial layer continuous with the plasma membrane through which absorption, excretion and secretion takes place. The tegument is also engaged with the entire spectrum of the host defence systems and therefore it probably bears the molecular elements responsible for modulation, integration, and regulation of the processes which are essential for the parasite survival.

The aim of the present study is to investigate enzymatic and chemical nature of the surface plasma membranes isolated from the two amphistomes inhabiting liver and rumen of the buffalo. The membranes have been isolated by the treatment of various detergents in order to find out the suitable detergent in which the membrane bound enzymes retain maximum activity. The nature of ATPase has also been monitored by using specific inhibitor as this enzyme plays a vital role in signal transduction as well as in transport mechanisms.

Since the enzyme molecules present in the membrane are influenced by the surrounding chemical components, it is essential to investigate the
chemical composition of the plasma membranes. Among the different constituents, protein and various lipid classes, like, phospholipids, cholesterol, free fatty acids as well as different phospholipid fractions and sialic acid were investigated in the present study. Lastly this study will also provide an opportunity to analyse the influence of microenvironment on the parasite surface as a result of biochemical adaptation in response to inhabiting different habitats.
HISTORICAL REVIEW

The available literature on the membranes of different organisms reveals that in addition to ultrastructural studies, there are many other possible methods for membrane characterization, which include the analysis of membrane bound enzymes as well as the basic biochemical composition of membranes. Such studies not only give information about the chemical configuration but also provide data about their functional role in various physiological activities which are associated with membranes. In recent years these aspects have been comprehensively reviewed by Ragan and Cherry (1986) and Findlay and Evans (1987). But information about the surface plasma membranes of helminth parasites is very limited and confined to only few species of helminths. Mostly parasite membranes have been examined in intact form along with the ultrastructure of the tegument by histochemical and histoenzymological techniques.

The occurrence of enzymes in the trematode tegument has been demonstrated mainly by histochemical techniques and very rarely by biochemical methods. Attempts to identify enzymatic functions of the trematode surface membrane have been directed to cytochemical localization of phosphatases (see review by Lumsden, 1975). Many authors uncritically accept the hypothetical relationship of these phosphatases to membrane transport functions.

In recent years a number of attempts have been made to isolate the plasma membranes of platyhelminths. In order to measure the purity of the isolated membranes, the pellets were analysed by various membrane bound marker enzymes like, 5'-nucleotidase, Na⁺, K⁺-ATPase and alkaline
phosphatases. Beside these primary markers, a number of secondary marker enzymes have also been reported from the isolated surface plasma membranes of *Schistosoma* and *Hymenolepis* spp.

The surface membranes of *S.* *mansoni* have been investigated by many workers. Podesta and McDiarmid (1982) have separated the outward-facing (OFM) and inward facing (IFM) membranes of *S.* *mansoni* by sequential exposure to saponin and characterized them by marker enzymes. The OFM, contained ATPase activity that was stimulated by Mg$^{2+}$ and Na$^+$, but not by K$^+$ or HCO$_3^-$ and was inhibited by Ca$^{++}$ and ethacrynic acid. Further, this enzyme is not affected by ouabain, which indicated that it has similar properties to ATPase of apical membrane of a variety of other epithelial cells. The \((\text{Na}^+ + \text{K}^+) \text{Mg}^{2+}\) ATPase is responsible to regulate the decreasing cell volume. The IFM contained ATPase was stimulated by Mg$^{2+}$, Na$^+$ and K$^+$, and was inhibited by ouabain. This suggests that the IFM ATPase is probably involved in Na$^+$ pump. These authors have discussed the results in the light of transport functions. In another study on *S.* *mansoni*, Noël and Soares De Moura (1986) have investigated the \((\text{Na}^+ + \text{K}^+)\) ATPase of membrane and carcass after the saponin treatment. These authors have suggested that the membrane bound ATPase is ouabain sensitive and a total of 32% activity is confined to the membranes. However, these authors were unable to differentiate the bilayers.

Further, membranes of *S.* *mansoni* isolated by digitonin have also been characterized by membrane bound marker enzymes in both bilayers (McDiarmid et al., 1983). These workers have identified alkaline phosphatase and Na$^+$, Mg$^{2+}$-ATPase in the inner bilayer and concluded that the inner bilayer is analogous to the typical plasma membrane of other animal epithelia.
Whereas, Roberts et al. (1983) have used different membrane disruption solutions and characterized each membrane pellet of *S. mansoni* by using different extrinsic and intrinsic markers. Among the various markers, they observed that alkaline phosphatase was proved to be the most reliable. These authors have also pointed out that freezing and thawing is comparatively most suitable method and over 130 times more alkaline phosphatase activity was noticed. These studies clearly reveal that the activity of membrane bound enzymes seem to be a valid parameter for the characterization of membranes.

Besides the isolated membranes some studies have also been carried out by using cytochemical methods at the level of transmission electron microscopy. These reports also provide the evidence of the presence of ATPase and their functional significance by using certain specific inhibitors. Shaw (1987) and Skuce et al. (1987) have demonstrated (Na⁺ - K⁺ - Mg²⁺) and (Na⁺/K⁺) - ATPase from the surface membranes of *S. mansoni* and *F. hepatica* respectively. Skuce et al. (1987) have assessed the effect of ouabain cytochemically on Na⁺/K⁺ - ATPase and observed that ouabain significantly reduced the overall level of Na⁺/K⁺ - ATPase, which is particularly concentrated along with the invaginations of the apical plasma membranes.

The other parasite which has been most frequently used for the membrane studies is *Hymenolepis diminuta*. In cestodes, the surface plasma membrane is of great physiological value, because it is the only major site of transmembranosis which fulfills the nutritional requirements. In a comprehensive review, Arme and Pappas (1983) have pointed out that in cestodes, various membrane bound enzymes can be used as a parameter
for the characterization of membranes. A number of enzymes have been reported from cestode membranes like alkaline phosphohydrolase, 5'-nucleotidase, monoacyl hydrolase, ribonuclease, Type-1 phosphodiesterase, adenosine triphosphatase, adenyl cyclase, Type-II phosphodiesterase, cyclic adenosine-3', 5'-monophosphate phosphodiesterase, disaccharidase and leucine aminopeptidase.

The available literature on the membrane bound enzymes in isolated membranes of different species of Hymenolepis reveals that among the various enzymes, alkaline phosphohydrolases are the main enzymatic components associated with the brush border (Gamble and Pappas, 1980; 1981a,b; Pappas, 1980; 1981; Pappas and Narcisi, 1982).

Similarly, ATPase activity associated with the surface membranes has also been demonstrated in some other species of cestodes, irrespective of their method of isolation. ATPase activity has been reported in Hymenolepis microstoma (Pappas and Narcisi, 1982), H. diminuta (Pappas and Read, 1974; Khowles and Oaks, 1979; Rahman et al., 1981b; Pappas, 1981) and Cysticercus cellulosae (Sosa et al., 1978). Further, Pappas (1981) and Rahman et al., (1981,b) have investigated the kinetic properties of the membrane bound ATPase of H. diminuta and suggested that Na⁺ and K⁺ do not stimulate the enzyme activity significantly as compared to Mg⁺². Ouabain does not inhibit the enzyme and that ATPase in cestodes is involved in volume regulation during hypotonic stress.

However, it has been pointed out by Pappas (1983) that "it is not known whether the 5'-nucleotidase and ATPase activities of H. diminuta represent distinct enzymes, or whether the two activities simply represent
the sequential hydrolysis of terminal phosphate from the nucleotide by a single enzyme. Analysis of 5'-nucleotidase and ATPase activities in intact worms (Pappas and Read, 1974; Kuo, 1979) and isolated membrane preparations (Pappas, 1981) are equivocal.

Various other biochemical components present in the membranes also play an important role in maintaining a low dielectric constant, which influence the activities of membrane bound enzymes and therefore it is essential to investigate and analyse these substances. Such biochemical components have been widely examined in the membranes of only a few parasites, namely, *H. diminuta*, *Taenia taeniaeformis* and *S. mansoni*.

The membranes of *H. diminuta*, isolated by the treatment of saponin, show 37% and 21.2% lipids in brush border and vesicle rich fractions respectively (Cain et al., 1977). Among the various neutral lipids, cholesterol constitutes a major fraction in brush border as well as in vesicles. In addition to cholesterol, small amounts of glycerides and sterol esters were also observed, whereas, the brush border contained appreciable quantity of free fatty acids. Phosphatidylethanolamine was the most prevalent polar lipid in both fractions, and was particularly abundant (63.4% of total polar lipids). In vesicles, sphingomyelin was also observed.

Mills et al. (1984) have also analysed the lipid and protein of the surface membranes of strobilocerci of *T. taeniaeformis*, isolated after digitonin treatment. They observed that the surface membranes consisted of 52% protein and 32% lipid and the neutral and phospholipids occurred in approximately equal amounts, as 40% in each, and 16% of the lipid was glycolipid. They observed cholesterol as a major neutral lipid while among phospholipids phosphatidylethanolamine and phosphatidylserine were the major fractions of membranes.
In schistosomes, membrane lipids were analysed with respect to different aged worms and it was observed that at least five types of glycolipids were present, out of which four were highly polar (Rogers- and McLaren, 1987). These authors also reported cholesterol and phospholipids by using chromatographic techniques. In the phospholipids, phosphatidylethanolamine or its equivalent Rf value fraction was observed. This study shows that the various lipid fractions are not static but dynamic during the course of parasite development. In addition to this glycolipids were also observed which seem to be highly polar, because they are mostly resolved near the origin.

In addition to lipids and protein composition, some workers have also identified the carbohydrate moieties as well as sialic acid in the membranes, especially in *S. mansoni*. Rumjanek et al. (1979) have examined the membranes of *S. mansoni*, isolated by freezing and thawing procedures and noticed glucose, galactose and mannose as the major monosaccharide components of the plasma membranes. Some workers have characterized the carbohydrates of the membranes of *S. mansoni* through lectin binding as well as by specific staining procedures like ruthenium red (Simpson and Smithers, 1980 and McDiarmid and Podesta, 1984). The sialic acid identified in the glycocalyx of *S. mansoni* shows ruthenium red positive staining, which reveals the existence of a negatively charged external layer of the outer bilayer. The presence of the sialic acid was also confirmed by neuraminidase treatment.

The foregoing review clearly reveals that the relative activities of different membrane bound enzymes and other biochemical components show some quantitative as well as qualitative differences in different parasites.
It is possible that the differences in enzyme activities are influenced by the biochemical components present in the vicinity of the enzyme molecule.

In view of the above facts the present study was undertaken to investigate the membrane bound enzymes and other biochemical components of the surface plasma membranes of amphistomes which have not been examined in the past. This study also provides an opportunity to identify the marker enzymes of the membranes of amphistomes as well as to compare the chemical nature of the two parasites inhabiting two physico-chemically different microhabitats. 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.
MATERIALS AND METHODS

Parasite Collection:

Adult and active Gastrothylax crumenifer and Gigantocotyle explanatum were collected from the rumen and liver respectively of the Indian water buffalo, soon after they were slaughtered at local abattoir. The worms were washed carefully in HBSS at 37 ± 2°C and then transferred into flasks containing MDS (4°C) in order to isolate the surface plasma membranes.

Isolation of Surface Plasma Membranes:

The surface plasma membranes of both amphistomes were isolated separately in three non-ionic detergents. MDS contained 1% Saponin or 0.1% digitonin or 1% Triton X-100 in HBSS (pH 7.3). The flasks containing worms plus MDS were subjected to continuous shaking (90 cycles/min) at 4°C. After 20 min of shaking, the worms plus MDS were transferred into a stoppered tube and vortexed for 2 min. The MDS was then centrifuged at 1500 x g for 15 min and the supernatant was again centrifuged at 16,000 x g for 30 min. The pellet was resuspended in Tris-HCl buffer (pH 7.0) and again centrifuged at 16,000 x g for 1 h. All the centrifugation steps were same as described in chapter I.

Pellet Solubilization and Sonication:

After 1 h of centrifugation at 16,000 x g, the resulting membrane pellets of G. crumenifer and G. explanatum were solubilized in known volume of Tris-HCl buffer (pH 7.0), and then sonicated for 30 sec under chilled conditions by using an ultrasonic disintegrator (Relsonics) fitted with a 5
mm probe. The suspension was then used for the estimation of various membrane bound marker enzymes as well as chemical components like protein, lipids and sialic acid.

Enzyme Assays:

5'-nucleotidase:

5'-nucleotidase (EC 3.1.3.5) activity was determined by the method as described in Sigma Technical Bulletin No. 675 with some modifications. The reaction mixture (total volume 5.7 ml) contained 4.5 ml of 0.05 M Tris-HCl buffer (pH 7.5), 300 \( \mu \) mole substrate (adenosine 5'-monophosphate, Sigma Chemical Co., U.S.A.), to which 0.2 ml of protein sample was added. After 90 min of incubation at 37\(^\circ\)C the reaction was stopped by the addition of 30% TCA. The tubes were centrifuged at 5,000 \( \times \) g, and the supernatant was assayed for phosphorus (Pi).

Phosphorus Estimation:

Phosphorus was estimated by the method of Rouser et al., (1970). The samples were prepared as follows: 0.1 ml of sulphuric acid (concentrated) was added to the samples, which were heated in a boiling water bath for 2 min and then cooled; 0.05 ml of 70% perchloric acid was added and the samples were heated again for 2 min. To these samples, 2 ml of distilled water, 1 ml of 1% ammonium molybdate, 1 ml of freshly prepared 1% ascorbic acid were added. Finally, the test tube contents mixed and heated at 70\(^\circ\)C for 10 min and the absorbance was read at 820 nm. A standard curve was drawn using KH\(_2\)PO\(_4\) (1-10 \( \mu \)g phosphorus). In addition to the blanks, \( \beta \)-glycerophosphate (BCP) controls were also run simultaneously, because AMP also serves as a substrate for non-specific
phosphatases. The reaction mixture contained 200 μmoles of GTP in place of AMP, as the substrate. All other conditions remained the same as described above. The Pi value obtained from these experiments was subtracted from the Pi values obtained from the hydrolysis of AMP and the resultant activity was termed 5'-nucleotidase activity. All glassware used in Pi assays was rendered phosphate-free by soaking in chromic acid and washing in phosphate-free detergent.

Enzyme Unit:

The enzyme activity has been expressed as mg Pi released/mg protein/min.

Acid and alkaline phosphatases:

Acid and alkaline phosphatases were determined by the method of Bergmeyer et al. (1974). For acid phosphatase (EC 3.1.3.2), the assay mixture (total volume 1.1 ml) contained 0.8 ml of 0.05 M acetate buffer (pH 5.0) with 8 mM substrate (p-nitrophenyl phosphate; Sigma Chemical Co., U.S.A.) and 0.3 ml of protein sample. The alkaline phosphatase (EC 3.1.3.1.) activity was determined in a reaction mixture (total volume 1.2 ml) containing 1 ml of 0.05 M glycine-NaOH buffer (pH 9.5) with 5 ml substrate to which 0.2 ml protein was added. In both the enzyme assays the reaction was allowed for 30 min at 37°C and stopped by adding 0.02 M NaOH. The liberated p-nitrophenol was measured at 410 nm. Enzyme activity was determined with reference to a previously calibrated curve of known concentrations of p-nitrophenol (Sigma Chemical Co., U.S.A.).

Enzyme Unit:

The enzyme activity has been expressed as μg pNP liberated/mg protein/min.
Adenosine triphosphatase:

Adenosine triphosphatase (EC 3.6.1.3) (ATPase) activity was assayed by measuring the release of inorganic phosphorus as described by Ryre (1975) with some modifications (Rahman et al., 1981, b; Verna and Frati, 1983). The reaction mixture (total volume 2.2 ml) contained protein samples (40-60 μg protein), 92 mM Tris-HCl buffer, pH 8.5, and 5 mM MgCl₂. The reaction was started after 10 min equilibration at 37°C in a shaking water bath, by the addition of 4 mM Tris-ATP sodium salt (Sigma Chemical Co., U.S.A.). The reaction was stopped after 15 min by the addition of 10% cold TCA. The tubes were centrifuged at 5,000 x g and the supernatants were assayed for liberated Pi, as described above.

Effect of Ouabain on Na⁺/K⁺ and Na⁺/Mg²⁺ ATPase:

The effect of ouabain on Na⁺/K⁺ and Na⁺/Mg²⁺ ATPase has also been monitored. The reaction mixture was essentially the same with the exception that MgCl₂ is replaced by 7.7 mM KCl and 1 mM ouabain. The volume was kept constant and a control without ouabain was also run simultaneously. The remaining steps were the same as described above.

Enzyme Unit:

The enzyme activity has been expressed as mg Pi liberated/mg protein/min.

Estimation of Protein:

The protein content of surface plasma membranes of both amphistomes was estimated by the dye binding method of Spector (1978).
Lipid Analysis

Extraction of Total Lipids:

Total lipid components were extracted by the method of Folch et al. (1957) in organic solvents in a one-phase system.

The sonicated membranes were mixed with chloroform/methanol (2:1), to a final dilution of 20-fold. The mixture was kept overnight at 4°C. The residue was then removed by centrifugation at 400 x g for 5 min. The residue was again re-extracted with chloroform/methanol (2:1) and the supernatants were combined. To the chloroform/methanol extract, 0.2 volumes of 0.88% KCl was added, mixed and allowed to separate into two phases. The lower phase was collected containing most of the lipids. The chloroform in the lower phase was evaporated to dryness in vacuo at 45°C. The dried lipids were then dissolved in known volumes of chloroform and suitable aliquots were taken for the estimation of various lipid classes.

Estimation of Total Lipids:

The total extracted lipids were determined by the method of Zöllner and Krisch (1962). To 1 ml of extracted lipids, 4 ml of concentrated H$_2$SO$_4$ was added. After shaking, the tubes were boiled for 10 min then cooled to room temperature. Suitable aliquots were taken and 4 ml of Zöllner reagent was added. A reagent blank was also prepared simultaneously by using H$_2$SO$_4$ and Zöllner reagent. Both test and blank tubes were kept at room temperature for 30 min. Zöllner reagent contains 13 mM vanillin in 14 M Ortho-phosphoric acid. The colour was read against the reagent blank at 530 nm. The total lipids were calculated from a calibration curve prepared by using a standard lipid of known strength.
Estimation of Lipid Fractions:

1) Total Cholesterol:

Total cholesterol was estimated by the method of Sackett (1925). Suitable aliquots of lipid extract were taken and 5 ml of chloroform was added and shaken vigorously for some time; later 2 ml of ice-cold acetic anhydride and conc. $\text{H}_2\text{SO}_4$ in the ratio of 20:1 was added. This was left in the dark at room temperature. After 15 min the green colour was read against reagent blank at 680 nm. The total cholesterol was calculated from a calibration curve prepared by using known amount of cholesterol in chloroform.

ii) Total Triglycerides:

Total triglycerides were determined by the method of van Handel and Zilversmit (1957). Extracted lipid sample was placed in a stoppered flask containing 4 g Zecocarb 225 and was moistened with 2 ml chloroform. The mixture was shaken intermittently for 1 h at room temperature. Suitable quantity of aliquots were taken into stoppered tubes except in the blank in which chloroform was taken. The tubes were kept at 60-70°C for evaporation of chloroform for 15 min thereafter 0.5 ml of 0.4% alcoholic KOH (w/v) and 0.5 ml of 0.2N $\text{H}_2\text{SO}_4$ were added. Alcohol was evaporated by keeping the tubes for 15 min in a boiling water-bath. After cooling, triglyceride content was determined by periodate oxidation. To the tubes 0.1 ml of 0.5M sodium periodate solution was added and the oxidation was stopped exactly after 10 min by the addition of 0.1 ml of 0.5M sodium arsenite. A yellow colour of iodine appeared which disappeared within few minutes, then 9 ml of 0.24% (w/v) chromatropic acid reagent (in $\text{H}_2\text{SO}_4$ and $\text{H}_2\text{O}$, 2:1, v/v) was added.
and heated for exactly 30 min in a boiling water-bath. The colour was read against reagent blank at 570 nm. The quantity of triglycerides is represented in terms of weight of mustard oil which has been used to prepare a calibration curve as a standard.

iii) Total Free Fatty Acids:

Free fatty acids (FFA) were determined by the method of Lowry and Tinsley (1976). Suitable aliquots of extracted lipids were taken into stoppered tubes and evaporated to dryness. In each tube 5 ml of benzene was added including the blank and the tubes were shaken to dissolve the dried residue. The mixture was warmed slightly and subsequently added 1 ml of cupric acetate pyridine reagent (5% aqueous cupric acetate solution was filtered and its pH 6-6.2 was adjusted with pyridine). The tubes were shaken for 2 minutes and centrifuged for 5 minutes. The lower layer was discarded. The developed blue colour was read against reagent blank at 715 nm. The amount of free fatty acids was calculated with the help of previously calibrated standard curve of palmitic acid.

iv) Total Phospholipids:

Total phospholipids were determined by estimating the phosphorus according to Rouser et al. (1970) as described above. The amount of phospholipid is calculated by multiplying the values of phospholipids found in the sample by factor 25 (Christie, 1982).

v) Fractionation of phospholipids by TLC and their estimation:

Phospholipids were further fractionated by the method of Skipsky et al. (1964) on clean thin layer chromatographic glass plates of 20x20 cm
which were coated with a slurry of silica gel G, containing 13% CaSO₄ as binder. A uniform layer (about 1 mm thick) prepared by vigorously shaking a specific amount of silica gel G with approximately double amount of double distilled water. The plates were left to dry at room temperature for 2 h. They were activated before use in an oven at 110°C for one hour. Known amount of concentrated lipids were applied 2-4 cm from the bottom edge of the plates as spots of 3-4 mm in diameter. The solvent was allowed to evaporate. The chromatoplate was placed in a developing tank of 23 x 23 x 7.5 cm (Griffin & George, Great Britain) containing chloroform: methanol: water (65 : 25 : 4, v/v/v) as the solvent system. The plate was removed and air dried at room temperature for 20 min. Standard of phospholipids (obtained from V.P. Chest Institute, Delhi) was also run simultaneously on the same plate at one side and after developing, the spots of standard phospholipids were visualized with a stream of iodine vapours. The vapours were obtained by keeping iodine crystals in a pasteur pipette. The spots were then scrapped off from the plate by marking the areas parallel to each of the known standard spot by placing a drop of double distilled water. The eluted spots were transferred into the centrifuge tubes containing chloroform and centrifuged. This process was repeated three times so as to obtain complete phospholipid fractions. The areas in which there were no spots, were also removed to be used as blank. After centrifugation the supernatants were taken and their concentrations were determined by the estimation of phosphorus as described above.

Estimation of Sialic Acid (N-Acetyl Neuraminic Acid):

The bound sialic acid (ganglioside) was estimated by the method of Warren (1959). A known volume of surface plasma membrane suspension was
hydrolysed with 1 to 2 drops of 0.01 N H\textsubscript{2}SO\textsubscript{4} for 1 h at 80°C in a water bath. Then 0.1 ml of 0.2 M sodium metaperiodate in 9 M phosphoric acid was added and the tubes were incubated at room temperature. After 20 minutes, 1.0 ml of 10% sodium arsenite in 0.5 M sodium sulphate prepared in 0.1 N H\textsubscript{2}SO\textsubscript{4} was added and the tubes were shaken until the brown yellow colour disappears. Then 3 ml of 0.6% of thiobarbituric acid in 0.5 M sodium sulphate prepared in water was added and the tubes were heated for 15 min in a boiling water bath. The tubes were immediately transferred to ice bath and cooled for 15 minutes. The red colour fades soon after the addition of 4.3 ml of cyclohexanone. The tubes were shaken vigorously with the help of a vortex mixer, then the mixture was transferred to centrifuge tubes. After low speed centrifugation for 5 minutes, two layers separated. Orange colour in the top layer was read at two different wavelengths i.e., 550 and 532 nm. The \textmu m ole of NANA (N-Acetyl Neuraminic acid) were calculated by applying the following formula:

$\text{\textmu m ole NANA per mg protein} = (0.09 \times \text{O.D at 550 nm} - 0.033 \times \text{O.D at 532 nm}) \times 2$

The data were subjected to statistical analysis and the protein, lipid and sialic acid ratios were determined. The values of total lipids and sialic acid are expressed as \textmu mole per mg protein.
RESULTS

The results of the quantitation of primary marker enzymes of the surface plasma membranes isolated after treatment with saponin, digitonin and Triton X-100 of amphistomes under study are summarized in Table 1.

It is evident from these results that the quantitative differences occur in various enzymes of the membranes of *G. crumenifer* and *G. explanatum*. Among these the ATPase activity was maximum in the membranes of both the parasites irrespective of detergents used (Fig. 1.a). Acid phosphatase activity was exceptionally high than the alkaline phosphatase (Fig. 1.b). On comparing the effect of detergents, it was found that 5'-nucleotidase, ATPase and alkaline phosphatase activities were maximum in the membranes isolated with saponin, whereas acid phosphatase was inhibited by saponin. The maximum acid phosphatase activity was noticed in the membranes of both the parasites after digitonin treatment followed by Triton X-100. Thus, it can be surmized from these results that saponin inhibits the acid phosphatase of the membranes of both parasites, while digitonin and Triton X-100 exert inhibitory effects on 5'-nucleotidase, ATPase and alkaline phosphatase systems.

On comparing the enrichment of membrane bound marker enzymes with the enzyme activities of fresh whole worm homogenates, the former exhibited many fold increase than the latter (Table 2, Fig. 1a,b).

On the basis of these results it can be suggested that 5'-nucleotidase could be used as primary marker enzyme for the characterization of surface plasma membranes of amphistomes. In the parasites under study 297 and 315 fold enrichment was noticed in the membranes of *G. crumenifer* and *G. explanatum* respectively. Similarly 86 and 26 fold enrichment of ATPase was
Table 1: Quantitation of membrane bound marker enzymes of membranes of amphistomes after various detergents treatment.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>G. crumenifer ± SEM</th>
<th>G. explanatum ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saponin</td>
<td>Digiton</td>
</tr>
<tr>
<td>5'-nucleotidase *</td>
<td>17.106±0.528</td>
<td>0.516±1.132</td>
</tr>
<tr>
<td>ATPase *</td>
<td>74.912±1.218</td>
<td>6.964±0.286</td>
</tr>
<tr>
<td>Alkaline Phosphatase+</td>
<td>1.131±0.139</td>
<td>0.195±0.019</td>
</tr>
<tr>
<td>Acid Phosphatase+</td>
<td>3.276±0.223</td>
<td>5.232±0.235</td>
</tr>
</tbody>
</table>

Values are expressed as: *μg Pi released/mg membrane protein/minute

+μg pNP released/mg membrane protein/minute

Each value is the mean of five different replicates.
Fig. 1—Marker enzymes in fresh whole worm homogenate (F) and surface plasma membranes isolated with saponin (S), digitonin (D) and Triton X-100 (T) of amphistomes.
(a) 

- **G. crumenifer**
- **G. explanatum**

![Graph showing enzyme activities](image)

- 5'-nucleotidase
- Na\(^+\)/Mg\(^{2+}\) ATPase

(b) 

- Alkaline Phosphatase
- Acid Phosphatase

![Graph showing enzyme activities](image)
Table 2: Enrichment of plasma membrane enzyme activities in comparison with fresh worm homogenate.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>G. crumenifer</th>
<th>G. explanatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FOLD ENRICHMENT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fresh* Homogenate</td>
<td>Saponin</td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>0.0575±0.003</td>
<td>297.48</td>
</tr>
<tr>
<td>ATPase</td>
<td>0.872±0.051</td>
<td>85.9</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>0.1248±0.007</td>
<td>9.06</td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>0.7389±0.029</td>
<td>4.433</td>
</tr>
</tbody>
</table>

*Total enzyme activities in fresh whole worm homogenates.
Values are expressed as: +μg P1 released/mg protein/min, O-μg/mg pN Liberated/mg protein/min.
obtained in membranes of *G. crumenifer* and *G. explanatum* respectively after saponin treatment. Further, in saponin isolated membranes, the enrichment of alkaline phosphatase is greater than the acid phosphatase in both the parasite membranes. It is because of the fact that the total homogenates possess appreciably high quantities of acid phosphatase.

Next to ATPase, the third primary marker enzyme is alkaline phosphatase, which shows appreciably greater degree of enrichment i.e., 9 and 7 folds in saponin isolated membranes in both *G. crumenifer* and *G. explanatum*, respectively (Table 2). Further, similar quantitative differences were also observed in both the parasite's plasma membranes after the digitonin and Triton X-100 treatment.

In order to find out the effects of ouabain on ATPase system of the isolated plasma membranes, the latter were subjected to inhibitor studies. Table 3 shows that ouabain stimulates the ATPase activity, when reaction mixture contained Na$^+$ and Mg$^{2+}$. While ouabain in the same concentration completely inhibits the ATPase activity when the reaction mixture contained Na$^+$ and K$^+$, indicating that Mg$^{2+}$ stimulated and ouabain sensitive ATPase system is present in the plasma membranes of both amphistomes. Further, the level of Na$^+$ and Mg$^{2+}$ dependent ATPase is much higher as compared to Na$^+$ and K$^+$ ATPase.

The results of the major biochemical components of the surface plasma membranes of amphistomes have been summarized in Tables 4 to 9. For the selection of the best detergent, it was considered important to analyse the chemical composition of surface plasma membranes. Therefore, lipid to protein ratio and phospholipid to cholesterol ratio have been calculated and used as a parameters to assess the best detergent.
<table>
<thead>
<tr>
<th>ATPase</th>
<th>G. crumenifer ± SEM</th>
<th>G. explanatum ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>74.01 ± 1.218</td>
<td>58.567 ± 2.249</td>
</tr>
<tr>
<td>(Na⁺/Mg²⁺ dependent)</td>
<td>101.695 ± 1.101</td>
<td>77.519 ± 1.283</td>
</tr>
<tr>
<td>1 mM Ouabain</td>
<td>23.269 ± 0.596</td>
<td>17.328 ± 0.123</td>
</tr>
<tr>
<td>(Na⁺/K⁺ dependent)</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Values are expressed as AIMP released/mg membrane protein/minute. Each value is the mean of five replicates.
Table 4: Protein and lipid contents of surface plasma membranes of amphistomes after various detergents treatment and their ratios.

<table>
<thead>
<tr>
<th>Detergents</th>
<th>G. crumenifer</th>
<th>G. explanatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein (P)*</td>
<td>Lipid (L)*</td>
</tr>
<tr>
<td>Saponin</td>
<td>0.123</td>
<td>0.586</td>
</tr>
<tr>
<td>Digitonin</td>
<td>0.374</td>
<td>1.290</td>
</tr>
<tr>
<td>Tx - 100</td>
<td>0.046</td>
<td>0.199</td>
</tr>
</tbody>
</table>

* Values are expressed as mg/10 worm's surface plasma membranes.

Each value represents the mean of five separate replicates.
Table 5: Molar ratios of phospholipid (P) to cholesterol (C) in the surface plasma membranes after various detergent treatment of amphistomes.

<table>
<thead>
<tr>
<th>Detergents</th>
<th>n</th>
<th>G. crumenifer (P : C)</th>
<th>G. explanatum (P : C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponin</td>
<td>5</td>
<td>1.0510 : 1</td>
<td>0.856 : 1</td>
</tr>
<tr>
<td>Digitonin</td>
<td>5</td>
<td>0.420 : 1</td>
<td>0.395 : 1</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>5</td>
<td>0.70 : 1</td>
<td>0.69 : 1</td>
</tr>
</tbody>
</table>

n = number of replicates; molar ratios were calculated as described by van Deenen (1965).
Table 6: Lipid fractions in surface plasma membranes of amphistome parasites isolated after saponin treatment.

<table>
<thead>
<tr>
<th>Components</th>
<th>G. crumenifer</th>
<th>G. explanatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid</td>
<td>48.3 ± 0.131</td>
<td>54.092 ± 0.226</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>13.378 ± 0.327</td>
<td>18.400 ± 0.452</td>
</tr>
<tr>
<td>Free Fatty Acids</td>
<td>21.248 ± 0.691</td>
<td>22.7265 ± 0.513</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>10.838 ± 0.234</td>
<td>3.314 ± 0.128</td>
</tr>
<tr>
<td>Unknown</td>
<td>6.236</td>
<td>1.468</td>
</tr>
</tbody>
</table>

Values are expressed as mg percent of total lipids ± SEM
Each value is the mean of five separate replicates.
Table 7: Polar and neutral lipid composition of membranes of amphi-stomes isolated after saponin treatment.

<table>
<thead>
<tr>
<th>Parasites</th>
<th>Neutral lipids</th>
<th>Polar Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. crumenifer</em></td>
<td>51.7%</td>
<td>48.3%</td>
</tr>
<tr>
<td><em>G. explanatum</em></td>
<td>45.9%</td>
<td>54.1%</td>
</tr>
</tbody>
</table>

Values are expressed as percent of total lipid.
Table 8: Phospholipid composition of amphistomes tegumental fraction after saponin treatment.

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>G. crumenifer</th>
<th>G. explanatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lysophosphatidylcholine</td>
<td>5.94</td>
<td>3.478</td>
</tr>
<tr>
<td>2. Sphingomyelin</td>
<td>11.574</td>
<td>9.705</td>
</tr>
<tr>
<td>3. Phosphatidylcholine</td>
<td>26.043</td>
<td>25.714</td>
</tr>
<tr>
<td>4. Lysophosphatidylethanolamine</td>
<td>5.362</td>
<td>3.478</td>
</tr>
<tr>
<td>5. Phosphatidylethanolamine</td>
<td>34.255</td>
<td>30.297</td>
</tr>
<tr>
<td>6. Unknown</td>
<td>16.826</td>
<td>27.328</td>
</tr>
</tbody>
</table>

All values are expressed as mg percent of total phospholipids.
Each value represent mean of three separate isolation.
Table 9: Sialic acid composition in surface plasma membranes of amphistomes after saponin treatment.

<table>
<thead>
<tr>
<th>Parasites</th>
<th>Sialic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. crumenifer</td>
<td>7.096 ± 0.726</td>
</tr>
<tr>
<td>G. explanatum</td>
<td>5.524 ± 0.108</td>
</tr>
</tbody>
</table>

Values are expressed as μgm NANA/mg of membrane protein.
Each value is the mean of three separate replicates ± SEM.
It is evident from Table 4 and Fig. 2 that the membranes of both the amphistomes isolated after various detergent treatments exhibited appreciably higher amounts of lipid contents than the protein, however, quantitative differences were noticed in both the worms. Due to non-availability of freeze-drying facility, the results of chemical composition are presented in their respective ratios, instead of expressing on dry weight basis. The results of the molar ratios of phospholipid to cholesterol revealed that the saponin isolated membranes showed nearly equimolar ratio in both the amphistomes under study, while digitonin and Triton X-100 isolated membranes did not show such equimolar ratios in both the worms. In digitonin isolated membranes comparatively greater variations have been observed than the Triton X-100 isolated membranes. Quantitative differences were also noticed in the two amphistomes. (Table 5, Fig. 3).

Since these results clearly reveal that among the various detergents used, the saponin seems to be the best, thus membranes treated with this detergent were used for the analysis of subsequent fractions of lipid and phospholipid.

Major fractions of lipids in surface membranes of *G. crumenifer* and *G. explanatum* are presented in Table 6 and their respective concentrations are shown in Fig. 4. It was found that the levels of phospholipids and cholesterol are higher in *G. explanatum* than *G. crumenifer* while unknown fraction and triglycerol components show reverse pattern. The level of free fatty acids show more or less equal amounts in both the worms with slight variations. In *G. crumenifer* membranes, the level of neutral lipids was greater than the polar lipids, while in *G. explanatum*, a reverse pattern was observed (Table 7).
Fig. 2- Protein : lipid ratio in the isolated membranes of amphistomes after various detergent treatment. (360° assumed as 100 %, actual figures are shown in Table 4).
PROTEIN : LIPID RATIO

- Saponin
- Digitonin
- Triton X-100

G. crumenifer  G. explanatum
Fig. 3—Molar ratios of phospholipids and cholesterol in the isolated membranes of amphistomes after various detergent treatment. (360° assumed as 100%, actual figures shown in Table 5).
PHOSPHOLIPID:CHOLESTEROL
(Molar Ratio)

- Saponin
- Digitonin
- TritonX-100

G.crumenifer  G.explanatum
Fig. 4- Major lipid fractions of surface membranes of amphistomes

PL- phospholipid, CH- cholesterol, FFA- free fatty acids, TG- triglycerides, UNK- unknown.

(360° assumed as 100 %, actual figures shown in Table 6).
G. crumenifer  

G. explanatum
TLC separation of phospholipids reveal the presence of phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylcholine and lysophosphatidylcholine along with sphingomyelin and some unknown components in the surface plasma membranes of amphistomes. Table 8 shows that in the membranes of both the parasites, the phosphatidylethanolamine constitutes the major fraction, while phosphatidylcholine and sphingomyelin were present as second and third major components respectively. Lysophosphatidylethanolamine and lysophosphatidylcholine were found in traces as compared to other constituents. In G. crumenifer, the level of each fraction was found greater, while in the membranes of G. explanatum, the unknown fractions were observed in greater amounts (Table 8 Fig. 5).

Further, the commonly occurring sialic acid moiety of the surface plasma membranes as a carbohydrate component has also been estimated in the isolated surface plasma membranes of parasites under study (Table 9). G. crumenifer shows comparatively higher amounts of sialic acids in their plasma membranes than the G. explanatum membranes and constitutes 7.096 ± 0.726 and 5.524 ± 0.108 μg/mg NANA per mg membrane protein respectively.

On the basis of these results, following protein : lipid : sialic acid ratios were noticed : 1 : 4.76 : 0.007 and 1 : 3.9 : 0.0055 in G. crumenifer and G. explanatum respectively. This suggests that rumen and liver amphistome membranes not only differ in their enzyme activities but also in their basic biochemical compositions.
Fig. 5—Phospholipid fractions in surface membranes of amphistomes

PE— phosphatidylethanolamine
LPE— lysophosphatidylethanolamine
PC— phosphatidylcholine
LPC— lysophosphatidylcholine
SP— sphingomyelin
UNK— unknown.

(360° assumed as 100%, actual figures shown in Table 8).
G. crumenifer  
G. explanatum
DISCUSSION

In the present study an attempt has been made to characterize and monitor the purity and enrichment of the surface plasma membranes of amphistomes under study through the analysis of specific primary membrane bound marker enzymes and biochemical components. As McDiarmid et al. (1983) pointed out that "in the studies of membrane purification it is necessary to have a marker for identifying the purity of the membrane fractions. This is usually accomplished by using enzymes unique to the membranes being isolated". In _S. mansoni_ the outer bilayer is thought to be a specialized secretion of the underlying cells, while the inner bilayer was analogous to the traditional plasma membrane of other cell types having 5'-nucleotidase, alkaline phosphatase and Na⁺, Mg²⁺ ATPase as the marker enzymes (Benedetti and Emmelot, 1968; Waldman and Chepenik, 1980; McDiarmid et al., 1983). The presence of these enzymes in the surface plasma membranes of amphistomes also suggests that they follow the same enzymatic organization as in _S. mansoni_ inner bilayer as well as plasma membranes of other cell types. In a number of other studies on helminth parasites, the effectiveness and suitability of various detergents and the techniques for isolation of membranes have been standardized by analysing membrane bound marker enzymes (Pappas, 1983; Podesta et al., 1986).

In the present study all enzymes including 5'-nucleotidase, ATPase and alkaline phosphatases except acid phosphatase were found significantly high after the treatment with saponin. The presence of appreciable amounts of 5'-nucleotidase activity in the surface membranes of both the amphistomes reveal their important role in conversion of nucleotides to nucleosides. Nucleotides are not known to penetrate the cell membranes (Senft et al., 1973), and may be converted to nucleosides by 5'-nucleotidase before they
can be utilized by the worms and may again be resynthesized inside to nucleotides, possibly by phosphoribosyltransferases. Nucleosides uptake across the tegument has been reported in trematodes (Nollen et al., 1973, 1974).

The digitonin and Triton X-100 produce inhibitory effects on 5'-nucleotidase, ATPase, and alkaline phosphatase. Contrary to this maximum activity of acid phosphatase was noticed in digitonin followed by Triton X-100. In addition to this the quantitative differences were noticed in the membranes isolated from rumen and liver parasites. These differences in the level of enzymes after the various detergents are probably due to their adverse effects on various enzyme systems. The low activity of 5'-nucleotidase in plasma membranes of both the parasites under study after digitonin treatment is probably due to the inhibitory effect of digitonin. Similarly McDiarmid et al. (1983) observed inhibitory effect of digitonin on 5'-nucleotidase activity in the membranes of S. mansoni. Further, inhibitory effects of digitonin on ATPase and alkaline phosphatase have also been noticed during the present study. Such ATPase inhibition by the action of digitonin has also been reported from the membranes of erythrocytes (Lewis et al., 1975).

Like digitonin, Triton X-100 also shows inhibitory effects on the membrane bound marker enzymes. It has been pointed out by Helenius and Simons (1975) that there are a number of protein/enzymes which lose their biological activities when solubilized with this detergent, among which (Na⁺ + K⁺) - ATPase is the one, which is also influenced by Triton X-100. Similar to the present study Podesta et al. (1986) have also detected 5'-nucleotidase, ATPase, alkaline and acid phosphatase in the isolated brush border of S. mansoni after saponin treatment.
Roberts et al. (1983) have used the alkaline phosphatase as a marker for the surface membranes of *Schistosoma* spp. Further, Wheater and Wilson (1976) suggested that alkaline phosphatase could be used as a marker for the tegument membranes. It has been pointed out by Simpson et al. (1981) that 20% of the alkaline phosphatase was located on the surface of *S. mansoni*, while Cesari (1974) estimated 75% of the enzyme from the surface membrane fraction. Similarly, Roberts et al. (1983) observed that 34% of the alkaline phosphatase of total worm homogenate was present in the surface membranes of *S. mansoni*. These findings support our results, in which many fold enrichment of alkaline phosphatase was observed in the isolated surface plasma membranes than the fresh worm homogenate. In helminths, the presence of alkaline phosphatases are often indicative of membrane transport mechanisms (Barrett, 1981).

Acid phosphatase was noticed in appreciable quantities in the surface membranes of both the amphistomes under study. Similarly this enzyme has also been reported from the surface membranes of *S. mansoni* and from the basal and apical plasma membranes of *G. crumenifer* and *G. explanatum* (Watts et al., 1979; Dunn et al., 1987). Regarding its function in the membranes, Threadgold (1968) suggested that the presence of acid phosphatases in plasma membranes are often associated with transmembrane absorption.

The results of the occurrence of ATPase enzyme in the isolated membranes reveal that this enzyme occurs in two forms i.e., Na\(^+/\)Mg\(^{2+}\) and Na\(^+/\)K\(^+\) dependent, the former form is predominant than the latter, as revealed by their normal level of activities. Further, Na\(^+/\)Mg\(^{2+}\) dependent enzyme is stimulated while Na\(^+/\)K\(^+\) dependent form is inhibited by ouabain. Podesta
and McDiarmid (1982) have also observed Mg\(^{2+}\) activated and ouabain sensitive ATPase in the surface membranes of _S. mansoni_. Noël and Soares De Moura (1986) also observed similar results in the membranes of _S. mansoni_ after saponin treatment. Further, McDiarmid _et al._ (1983) observed Na\(^+\), Mg\(^{2+}\), ATPase in the inner bilayers of _S. mansoni_ after digitonin treatment.

It has been shown by membrane transport studies that the surface of helminths, like _H. diminuta_ and _S. mansoni_, possess a polarized epithelial syncitium capable of vectorial transepithelial transport of solutes and water (Brodie and Podesta, 1981; Podesta, 1970; 1977; 1980; 1981; Podesta and Dean, Lyn, 1982a;b). In _S. mansoni_ transepithelial transport is possible only if the limiting membranes of the epithelial layer are asymmetrical with respect to their permeable properties and the enzymes associated with the differential permeabilities. On the basis of these reports and presence of various membrane bound marker enzymes in the surface membranes of the worms under study, it can be suggested that in amphistomes a similar type of mechanism for transmembranosis involving Na\(^+\)/K\(^+\) and Mg\(^{2+}\) - ATPase system may be operative. However, this certainly requires further studies on transport of various substances in these worms.

The effects of detergent on membrane ATPase have also been reported by many workers. It was suggested that generally, below the critical micellar concentration, detergents have little effect on ATPase activity and they even stimulate enzyme and transport activity (Jørgensen, 1975; Skou and Esmann, 1979; Stekhoven and Bonting, 1981; Hokin, 1981). Above the critical micellar concentration detergents often have deleterious effects on the activity of ATPase (Skou and Esmann, 1979; Hokin, 1981; Stekhoven and Bonting, 1981). Saponin, at concentrations below the critical micellar concentration has been
shown to enhance the ATPase activity, while at even higher concentrations the inhibitory effects are not pronounced and therefore, this non-ionic detergent has been widely used to protect the enzyme activity (Skou and Esmann, 1979) and for the isolation of membranes of parasites.

It is evident from the present study that the maximum enrichment of the membranes, as monitored by the marker enzymes, was noticed in saponin isolated membranes, while Triton X-100 and digitonin induced an inhibitory effect on the membrane bound enzymes.

The occurrence of the ATPase in the membranes leads us to suggest that in trematodes ATPase occur in multiple forms in which ouabain sensitive ATPase system is comparatively at low level as compared to ouabain stimulated form. This shows that these parasites have the ability to make use of the ATPase system according to their need and physico-chemical condition of the habitat. At this stage it is difficult to suggest the nature of the transport and the occurrence of Na⁺ pump, which require further studies on kinetics.

From the above discussion it can be concluded that among three detergents used, saponin is the best detergent for the isolation of surface plasma membranes of amphistomes as revealed by the enrichment of the membrane bound marker enzymes. The presence of acid and alkaline phosphatases and ATPase in plasma membranes suggest their possible roles in absorption and transmembrane uptake of various solutes. The presence of 5'-nucleotidase indicate that it may contribute to the metabolic transformation of nucleotides to nucleosides.

The results of the biochemical composition reveal that the surface plasma membranes of both liver and rumen amphistomes possess appreciable
amounts of lipids, protein and sialic acid components like in other cellular membranes. The membranes isolated after the treatment of various detergents show increased level of lipid components than the protein. Generally the plasma membranes contain greater amount of protein than lipid and the ratio of lipid to protein exists approximately as 1:1.5. Since very few reports are available on biochemical composition of helminth surface membranes, therefore it is difficult to ascertain the exact reason of the increased amount of lipid in the surface membranes of amphistomes under study. However, it is generally accepted that membranes from various sources dissociate in detergents and reform after the removal of the detergents (Benedetti and Emmelot, 1965) and the reformed membranes contain only half of the protein found in the original membranes of Mycoplasma laidlawii (Razin et al., 1965). Thus, it can be suggested that the recovery of less protein content in the membranes of amphistomes may be due to the effect of various detergents. In the trilaminate structure of the membranes not all proteins are involved in the structural integrity or physical characteristics (Nystrom, 1973).

The surface plasma membranes of both rumen and liver amphistomes contain protein and lipid components in the ratio of 1:3.4 and 1:2.9 (protein:lipid) respectively when isolated with 0.1% digitonin. Contrary to this, Mills et al. (1984) have obtained 52% protein and 32% lipid in surface plasma membranes from the larvae of T. taeniaeformis and a ratio of 1.6:1 (protein: lipid) was calculated after the treatment with the same detergent in the same concentration. There could be two possible explanations for the differences which exist in protein to lipid ratios in the two amphistomes under study as well as those reported by Mills et al. (1984). The first may be due the influence of habitat and species difference while
the other is probably because of the use of different techniques for the estimation of total lipid contents. Mills et al. (1984) have simply taken the dry weights of membrane lipid aliquots, while during the course of present study the lipid was extracted and estimated by spectrophotometric method.

The lower amount of protein as compared to lipid in the present study may also be due to the loss of peripheral proteins which are believed to be associated with the polar head groups on the outer faces of lipid bilayer and are known to dissociate easily by mild treatment (even by slight change of pH) and generally dissolve in water, in non-aggregated form, while integral proteins can only be isolated by more drastic treatments involving extensive disruption of the membrane by detergents (Harrison and Lunt, 1980). Thus, it can be concluded that the less protein obtained in the present study may be only the integral proteins which are intercalated in the lipid bilayer of the membranes.

Though the ratio of protein to lipid of membranes is more or less similar in all three detergents used in the present study, however, some quantitative differences were noticed among the two parasites as well as in the membranes isolated with different detergents. Recovery of appreciably higher amounts of lipid and protein contents of membranes of both the amphistomes after digitonin treatment indicate that such high amounts of protein and lipid contents might be due to the contamination with other organelles as pointed out in the previous chapter that digitonin induces deep lesions and increased contamination as revealed by SEM/TEM. The recovery of low amount of lipid and protein with Triton X-100 make this detergent unsuitable for the present work and the TEM observation supports this fact because the yield of membrane is low with least contamination.
Comparatively in saponin, the membrane yield is greater with low contamination and appreciable amount of protein and lipid contents were observed.

The amphistomes under study live in a complex habitat where the tegumental surfaces of these parasites play a vital role in parasitic adaptations thus, possibly these worms possess a dynamic surface which changes with time or requirements. The stability of catalytic factors in membranes depend on the existence of precise lipid protein associations which can persist in fluctuating matrix of membranes. The proteins as well as lipid can provide a continuous hydrophobic layer necessary for the high dielectric constant and low permeability of higher molecular weight substances. The appreciable amount of lipid and protein may help the parasite to adjust in the complex habitat, because proteins provide the strength and mechanical stability and lipids are responsible to maintain the fluidity and mutability of plasma membranes (Nystrom, 1973).

In order to assess the detergent suitability the phospholipids and cholesterol contents at the plasma membranes were also estimated following isolation with various detergents. The results of the saponin isolated plasma membranes show nearly equimolar phospholipid and cholesterol ratio than with any other detergent in both the amphistomes. In natural membranes, cholesterol is known to cause condensation of phospholipid monolayers by packing the hydrocarbon chains of phospholipids, possibly by way of an equimolar cholesterol phospholipid complex (Van Deenen, 1965). Similarly, Winkler and Bungenberg de Jong (1941) also visualized cholesterol as stabilizing arrays of the phospholipid molecules in the red cell membrane, which also exhibits a molar ratio of cholesterol to phospholipids closely
to unity. Thus the importance of cholesterol and phospholipids of the membranes led us to investigate these components in further detail, particularly in the saponin isolated membranes.

Among the major lipid components, polar lipids accounted for 48.3% and 54.09% while neutral lipids 51.7% and 45.9% in the surface plasma membranes of *G. crumenifer* and *G. explanatum* respectively. It can be observed from these results that the level of polar lipids are higher in the membranes of *G. explanatum* than *G. crumenifer*, while neutral lipids were found in greater amounts in the surface membranes of *G. crumenifer*. Cain *et al.* (1977) have also obtained 59% polar lipids and 41% neutral lipids in the brush border of *H. diminuta*. The minor quantitative differences in the polar and neutral lipid contents between surface plasma membranes of amphistomes and *H. diminuta*, may be due to differences in their habitats.

Relatively high amounts of free fatty acids were noticed in the surface plasma membranes of both the amphistomes with slight quantitative variations. Similarly, Cain *et al.* (1977) have also obtained rather high amounts of free fatty acids in the brush border fraction of *H. diminuta* and suggested that possibly these fatty acids might have been involved in the process of transtegumental transport (Bailey and Fairbairn, 1968; Lumsden and Harrington, 1966), or they might have released from hydrolysis of triglycerides and/or phospholipids by certain enzymes. Lipases have also been reported from the brush border of the tegument of *H. diminuta* (Bailey and Fairbairn, 1968). The higher amounts of free fatty acids in the surface plasma membranes may also be advantageous to these parasites living in complex habitats since free fatty acids are known to be involved in the changes in membrane permeability (Harrison and Lunt, 1980).
Presence of appreciable amounts of triglycerides in the plasma membranes of amphistomes suggest that these non-polar lipids are either artefacts of the membrane preparations or are in the process of transport. Mills et al. (1984) have also reported the presence of triglycerides in the tegument fraction of T. taeniaeformis. Since, our model of plasma membranes is dependent on amphipathic lipid constituents, the location and role of triglycerides is not clear, therefore any functional aspect of this lipid constituent in amphistome plasma membranes can not be proposed at this stage and further studies are required to find out the molecular arrangements of various components.

The major phospholipid fractions in both the amphistome parasites were phosphatidylethanolamine and phosphatidylcholine. Similar results have also been reported by Oaks et al. (1977) and Mills et al. (1984) in brush border of H. diminuta and T. taeniaeformis respectively, however, species specific differences were noticed in all components. The phosphatidylethanolamine (aminophospholipids) are generally present at the cytoplasmic sides of the plasma membranes, while choline containing phospholipids e.g. sphingomyelin and phosphatidylcholine are present on opposite sides, located externally (Harrison and Lunt, 1980). Since, the phosphatidylcholine constitutes major phospholipid of other biological membranes, phosphatidylethanolamine is known to be intermediate in the synthesis of phosphatidylcholine (Webb and Mettrick, 1973). It has been suggested by Kreier et al. (1966) and Seed and Kreier (1969), that some phospholipids are involved in the immune mechanism. Therefore, occurrence of phospholipids in amphistomes and their possible role in immunity can not be overlooked.
Several investigators favour the idea that phospholipids may transport ions across the membranous lipid barrier. Christensen and Hasting (1940) concluded that crude cephalins can bind Na⁺ and K⁺. In a model system the passage of ions under the influence of membrane constituents have been proposed by many workers. They observed that phosphatidylethanolamine could serve as a carrier between the two aqueous phases effecting a counterflow of K⁺ and Na⁺ (Hoffman et al., 1959; Schulman and Rosano, 1962; and Rosano et al., 1962). According to Van Deenen (1965), the natural membranes act as metabolically active entities. In several ways phospholipids contribute to this dynamic structure. Various enzyme systems located in/or at the membranes involved in many functions like energy-transducing reactions or the active transport of cations which are influenced by the presence of phospholipids. Specifically these lipids themselves play a role as carriers in the active or facilitated membrane transport of sugar and amino acids. Besides these functions a number of workers have also suggested the role of phospholipids in the biosynthesis of protein (Gaby and Sieberman, 1960; Godson et al., 1961; Hunter and Goodsall, 1961; Hendler, 1961).

Like previous studies of Oaks et al. (1977) and Mills et al. (1984) on H. diminuta and T. taeniaeformis, sphingomyelin was also founds in appreciable amounts in the surface plasma membranes of amphistomes which suggests its possible role in reducing the fluidity of the outer membrane by its long-chain saturated fatty acids, and the relatively high content of cholesterol, as mentioned earlier, is known to stabilize the cell membrane. The monoacyl derivatives, lysophosphatidylcholine and lysophosphatidylethanolamine were found as minor components in the present study and in other previous studies on helminths.
The presence of appreciable amounts of sialic acids (N-acetylneuraminic acid) in the surface plasma membrane clearly suggests their possible role in contributing the anionic nature or biological activity of the membranes. Most probably these sialic acid moieties of gangliosides, glycoprotein/glycolipid play an important role in the perception of the stimuli because these are involved in transmission of nerve impulse to synaptic structures of nervous tissues (McIlwain, 1964).

The amount of sialic acid moieties in surface plasma membranes of helminths have been estimated for the first time, although a number of reports are available which suggest the presence of sialic acids especially in surface plasma membranes of *S. mansoni* (Simpson and Smithers, 1980; Samuelson and Caulfield, 1982 and McDiarmid and Podesta, 1984).

The presence of glycocalyx on amphistome surface has already been suggested by Dunn et al. (1987) and from the present study it can be concluded that the surface plasma membranes of amphistomes consist of a complex, hydrated, charged carbohydrate coat (sialic acid) as also reported from *S. mansoni* (McDiarmid and Podesta, 1984). In helminths glycocalyx is primarily involved in the protection from the host immune responses by a variety of mechanisms, including decreased cellular adhesion and also protection from the effects of complement.

Threadgold (1976) has also shown that the glycocalyx of *F. hepatica* is a labile structure, composed of glycoproteins, with projecting side chains of oligosaccharides and gangliosides, both bearing terminal sialic and other acid residues. While Smyth and Halton (1983) have suggested that the carboxyl groups of these acids import a net negative charge to the tegumental surface which accounts for its strong affinity for cationic stains. The glycocalyx coat is also known to play an important role in protection,
absorption and immunological properties of the tegument in trematodes. Like other trematodes, the amphistome can survive for longer period and it might be expected that the 'molecular mimicry' which appears to operate in *Schistosoma* spp. and *Fasciola* spp., would also operate in amphistomes. Hanna (1980a) has proposed the idea that in *F. hepatica*, glycocalyx turnover occurs and the juvenile flukes can slough off host antibody (IgG) linked with surface antigen(s) and the flukes were able to replace the former glycocalyx. Such tegumental turnover induces the antigenic polymorphism during the development of the parasite in the definitive host.

Thus it can be concluded from the foregoing discussion that saponin is the most suitable detergent for the isolation of the surface plasma membranes of amphistomes with appreciable yield and least contamination. Further, the various membrane bound enzymes could be used as markers for monitoring the purity of these membranes.

The quantitative, enzymatic and biochemical differences were noticed in the membranes of liver and rumen amphistomes, which may be a consequence of the parasitic adaptation in physicochemically different habitats.