GLYCOPROTEIN SYNTHESIS AND SIGNAL TRANSDUCTION

IN SCHISTOSOMA MANSONI

A   thesis   presented   for   the   degree   of

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by

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ABBREVIATIONS

The abbreviations used in this thesis are those recommended in the Instructions to Authors the Biochemical Society (1986), with the following additions:

A_{492}	Absorbance reading at 492nm.
BPB	Bromophenol blue.
BSA	Bovine serum albumin.
ConA	Concanavalin A.
EDTA	Ethylenediamine tetra - acetic acid (di- sodium salt).
ELISA	Enzyme linked immunosorbent assay.
FCS	Foetal calf serum.
FITC	Fluorescein isothiocyanate.
FRAP	Fluorescence recovery after photobleaching
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline.
PI	Phosphatidylinositol
PNA	Peanut agglutinin.
PZ	Praziquantel
SDS	Sodium dodecyl sulphate.
TCA	Trichloroacetic acid.
TEM	Transmission electron microscopy.
TEMED	N,N,N',N',-tetramethylenediamine.
TM	Tunicamycin.
TM-1,-2,or-3	Analogues of tunicamycin.
WGA	Wheat germ agglutinin.

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Chapter six: General discussion , conclusion and future works

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Summary

Schistosomes are metazoan parasites which live within the blood vessels of man and his livestock. Infection results in a debilitating disease known as schistosomiasis or bilharzia. Over 200 million people in numerous tropical and sub - tropical countries are believed to harbour the parasite and effective long - term control has proved difficult. During my three years of research, I have concentrated on glycoprotein synthesis and signal transduction in S. mansoni .

Chapter three describes the effect of Tunicamycin (TM) on S. mansoni. TM is a nucleoside antibiotic which inhibits the transfer of GlcNAc - 1 - P from UDP - GlcNAc to dolichyl - P to form dolichyl - PP - GlcNAc. S. mansoni can synthesize N - linked glycoproteins and this synthesis can be inhibited by TM. However, the degree of inhibition by TM of glycosylation in S. mansoni is only about 15 - 30 % at 10 μg / ml. A possible explanation for this low inhibition is that the pathway for synthesis of N - linked glycoproteins in S. mansoni is not very active. Most of the glycoproteins which are present in S. mansoni contain O - linked oligosaccharides. However, N - linked glycoproteins appear to play a important role in maintaining the stability of the membrane in S. mansoni. Treatment with TM results in numerous changes in the membrane of the outer tegument in S. mansoni. These changes were detected by alteration in lectin binding, antibody binding, glycoprotein shedding, lateral diffusion of lipid and uptake of 2 - amino (1 - 14 C) Isobutyric acid. Although the structure of the heptalaminate membrane was unaffected by TM, large vacuoles could be found in 4 week adult worms after treatment with the drug. All of these changes in the membrane seem to affect the development of schistosomula in the host. TM inhibited schistosomula development in mice. In chapter 4, inositol phosphate metabolism, which is a major signalling pathway that utilizes the inositol lipids as part of a signal transduction mechanism in mammalian cells was measured in different life - cycle stages of S. mansoni.. The activity of this metabolic pathway is too low to be

measured in the schistosomula stage. However, the activity of this metabolic pathway increases with the development of the parasites. The effects of several ligands which were known to affect the surface membrane of S. mansoni were measured. Only praziquantel was shown to increase PI turnover, neither human serum or polylysine had any effect. In chapter 5, protein phosphorylation during transformation from cercariae to schistosomula is also analysed. Many proteins are found to be phosphorylated and some of these phosphorylated proteins were stable to KOH treatment. In addition, human serum and ultraviolet.(U.V) irradation show different effects on protein phosphorylation. This means that they may act on different protein kinases. In drawing together the results of chapter 4 and 5, it could be concluded that signal transduction systems are present in S. mansoni. However, the mechanisms of these systems are not as clear as in mammalian cells.

Chapter 1: General introduction

General Introduction

1.1.1. General introduction to the life cycle stages of S. mansoni

Schistosomiasis is a debilitating tropical parasitic disease caused by a digenetic trematode worm of the genus Schistosoma, which lives within the blood vessels of man and his livestock. Archaeological records in China and Egypt show that schistosomes (blood flukes) have been parasitic companions of man for at least 3500 years. There are now 76 countries in which schistosomiasis is endemic, with more than 600 million people at risk of infection and some 200 million infected.

Three of the many species of schistosome are an important cause of human infection: Schistosoma mansoni , which is responsible for intestinal schistosomiasis in Africa, parts of Latin America and the Caribbean; S. haematobium, which is responsible for urinary schistosomiasis in Africa and the Middle East; and S. japonicum, which causes intestinal schistosomiasis in China.

S. mansoni is the species of schistosome to be described here. The life cycle of S. mansoni, like the other two species, must be completed in two different hosts, invertebrate and vertebrate. A diagrammatic representation of the life cycle is shown in Fig.1.1. Eggs are released from the body of the final host via faeces. These eggs are mature when released and, on contact with fresh water, hatch to liberate the first larval stage, the miracidium. This small, ciliated organism has limited powers of survival and must come into contact with the correct species of snail if the cycle is to continue. When contact is made, the miracidium penetrates into the snail,and transforms into the sporocyst. Within its body, permanently embryonic cells divide and differentiate to form a second generation of sporocysts. In these, similar cells divide and differentiate to form the next larval stage, the cercaria. The cercaria is the stage infective to the vertebrate host. It leaves the snail and becomes temporarily free-living. Infection of the vertebrate host occurs by direct penetration of the skin. After successful penetration of a person's skin, the cercaria sheds its tail and transforms to a schistosomulum, which migrates via the bloodstream to the lung and from there to the liver. Following

Fig.1.1. Life cycle of schistosome in snail and man. (based on S. mansoni., Reproduced from Wakelin, 1984).

Transforms into sporocyst

Second generation sporocyst releases cercaria

Penetrate into snail

Hatch in fresh water to release miracidium

Cercaria penetrates human skin

Eggs pass to external world in faeces

SNAIL

WATER

MAN

Transforms into schistosomulum

Migrates from skin to lungs, then to liver. Matures, pairs (♂ x ♀) and pair moves to mesenteric veins

Adults live in mesenteric vein in permanent copula. Eggs laid into vessels, move through gut wall to lumen

growth and maturation in this organ, the adult male and female worms pair and move into the veins of the mesenteries. The complete cycle, from egg release to full sexual maturity, may take five weeks.

1.1.2. The morphology of S. mansoni

Fig.1.2. shows the principal features of adult worms and cercariae of S. mansoni.

Cercariae consist of two parts, which are the body and tail. A strong muscular terminal oral sucker and a weaker ventral sucker (acetabulum) are present on the head.

The schistosomulum is derived from the cercarial head, and develops over some four weeks into the adult worm.

At maturity, the lateral margins of the male worm curve ventrally and overlap to form a gynaecophoric canal encompassing the female. The pairing of male and female of S. mansoni occurs in the portal vein. The females are longer (20mm) and more slender than males.

1.1.3. Schistosomiasis

Schistosomiasis is a chronic and insidious disease, producing long-term, debilitating pathology. When the worms mature and begin to produce eggs, the host must adjust to a heavy, new antigen burden, yet, only few infected persons develop an acute febrile illness at that time. This phase begins about two months after first cercarial exposure. Most infected humans have only minor early symptoms or none at all. They may continue in apparent good health during the subsequent chronic phase of schistosomiasis. Eventually, five or more years into infection, there develops fibrovascular pathology with portal fibrosis and hypertension and with congestive splenomegaly. Those conditions are difficult to resolve completely.

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Fig. 1.2. The principal features of adult worm (a) and cercariar (b) of

S. mansoni

(a). adult worm
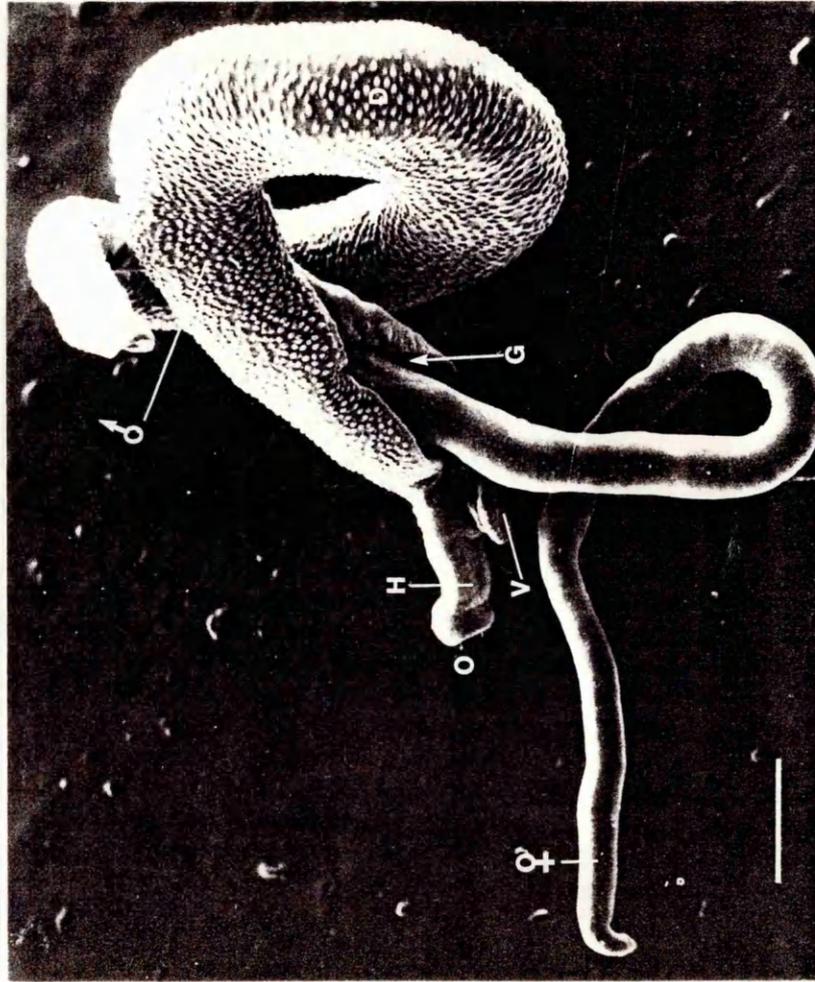
(b). cercariae

Figure 2. A pair of adult male (♂) and female (♀) *Schistosoma mansoni* as viewed by scanning electron microscopy. *H*. head; *V*. ventral sucker; *G*. gynaecopic canal; *D*. dorsal surface of body; *O*. oral sucker. Bar, 250 μm.

4
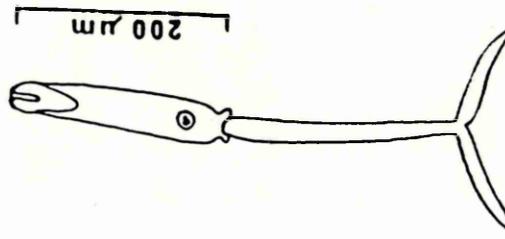
1.2. Immunity to schistosomes

There is extensive evidence for immunity to schistosome infection in man or in experimental animals. However, this immunity against reinfection is never complete.

1.2.1. Evasion of immunity by adult worms, while developing larvae are killed

Adult worms can live in the host for many years. These adult worms are themselves not directly pathogenic, but they are strongly immunogenic. Antigenic material is released from the tegument, from the intestine, and also released during metabolism. A variety of antibody responses is made to these antigens, including marked reaginic responses.

The early schistosomula which develop from a challenge infection seem the most important targets for the immune system.(Wakelin, 1984). In in vitro studies, a variety of potential effector mechanisms to damage and destroy schistosomula have been examined.(Table 1.1). As can be seen, almost all involve cooperative interactions between cells and antibodies. However, in vitro killing is effective only if the target schistosomula are young. After only a few days, their susceptibility is lost and effector mechanisms have little effect.(Wakelin, 1984).

The operation of these mechanisms in vivo suggests that the resistance is thymus - dependent, but this dependency may occur at several levels, e.g. in antibody formation, in myeloid cell responses or in cell-mediated immunity. The incompleteness of the protection after immunization may be due to (a) variability in the efficacy of local (skin, lung or liver) responses in the host, or (b) variability in the surface or metabolic properties within the challenge parasite population (Jones & Kusel 1989). This variability (b) may result in differences in the responses of each individual parasite to the effects of the host's immune system.

Table.1.1. Cells and antibodies known tobe effective in the _in vitro_ killing of schistosomula larvae.(Reproduced from Wakelin, 1984)

Cell	Antibody isotype	Host species
Neutrophil	IgG	Rat
Eosinophil	IgG	Man
Eosinophil	IgG2a	Rat
Eosinophil	IgG	Mouse
Eosinophil	IgE	Rat
Macrophage	IgE	Rat
Monocyte	IgE	Man, Baboon.

1.2.2. The mechanism of concomitant immunity

The adult worms remain unharmed by the immunity which they themselves engender, However, this resistance is clearly directed against the migrating larvae of the challenge infection. The term "concomitant immunity" has been used to to describe this situation. In comparison with "vaccine immunity" which develops following exposure to irradiated cercariae, this type of immunity is associated with the adult stage of the parasite, and with egg-induced pathology which is not induced by radiation - damaged schistosomula during their curtailed migration. This type of immunity (concomitant immunity) can cross the species barrier rather than being species specific.(McLaren and Smithers, 1987). How do the older worms of an established infection circumvent the host's immune response? Various mechanisms have been proposed,including the acquisition by the older worm of a coating of host-derived molecules that mask parasite antigens, the simple loss of expression of parasite antigens from the outer of the two parasite tegumental membranes, and the development of a tegument that is refractory to immune attack.(Butterworth 1987). Although evidence for each of these mechanisms has been obtained, it is likely that more than one may operate. The nature of these phenomena still are not well understood.

The schistosome surface membrane is seen as a strategically important interface between host and parasite. Some changes in morphology and biochemical composition take place in this tegument during development from schistosomulum to adult worm. These developmentally regulated changes may enable the adult worm to resist the immune response mounted by the host. A number of recent studies have shown that carbohydrate components linked to protein or lipid contribute significantly to the antigenicity of the schistosomulum surface and may play important roles in the development of immunity. The abundant carbohydrate epitopes may be responsible for inducing the non-species specific concomitant immunity. Therefore, studies in glycoprotein synthesis will yield valuable information. On the other hand, the study of membrane signalling will give some valuable information about the mechanisms by which the parasite responds to an immunologically hostile environment.

1.3. The tegument of S.mansoni

1.3.1. The function of the tegument of S. mansoni

A characteristic feature of all stages of schistosome in the vertebrate host is that there is a continuous thin layer of cytoplasm 2um in depth forming a tegument around the parasite. It has been shown that the tegument of S. mansoni has protective, absorptive and secretory functions.

Although it is known that S. mansoni possesses two absorptive surfaces i.e. the gut epithelium and external tegument, the dramatic proliferation of the tegument and the resulting increase in surface area are consistent with a major absorptive function by the tegument. During the development of S.mansoni, numerous low molecular weight organic components are taken up. The results from methionine uptake by larval and adult Schistosoma mansoni show that two kinetically distinguishable systems exist. One is saturable and the other appears to be a system depending on simple diffusion (Chappell,1974) .

This unique surface membrane of S. mansoni is likely to be a major factor in its remarkable powers of survival. After penetration, the trilaminate tegumental membrane of the invading cercaria is transformed into a double outer membrane. The extreme rapidity with which the new double outer membrane is completed indicates that this unique surface configuration is an essential prerequisite for subsequent migration.

1.3.2. Changes in the morphology of the outer membrane of the tegument of S.mansoni at different stages in the life cycle

Ultrastructural observation shows that the tegument of cercariae is a single, continuous, cytoplasmic structure over the body and tail. The outer membrane is trilaminate. A 1-2 μm thick, fibrillar glycocalyx covers the outer membrane of cercariae. Fig 1.3. summarizes changes in the tegument of schistosomula up to 3hr after penetration of the vertebrate host.

Thirty minutes after penetration, the outer membrane of the schistosomulum is

Fig. 1.3. Changes in the tegument of schistosomula after penetration of the vertebrate host for 30 min and 3 hr. (Reproduced from Hockley and McLaren, 1973).

Trilaminate outer membrane

Thin surface coat

Cercarial spherical body

Connection to subtegumental cell

Vacuoles with two trilaminate limiting membranes

Vacuole with additional membraneous contents in subtegumental cell

Microvillus with trilaminate membrane

Cast microvillus

Surface coat

Dense stacks of membrane

Heptalaminate membrane

Vacuoles with two trilaminate limiting membranes

Large membraneous vacuole joined to outer membrane

Large membraneous vacuole

Heptalaminate membrane

Fold in tegument: two fused penta-or heptalaminate membranes

Pentalaminate membrane

Fused tri- and pentalaminate membranes

Vacuole on surface of tegument

Large membraneous vacuole

Tightly packed membraneous body

Elongate body

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similar to that of the cercaria in that it is trilaminate. Little of the surface coat remains.

1hr after penetration, parts of the tegument have a multilaminate outer membrane and there are microvilli with a trilaminate membrane and a thin, surface coat. There is no distinct surface coat on the heptalaminate membrane. The tegument contains large vesicles with a heptalaminate limiting membrane. Some large vesicles open to the tegumental surface.

In the 3hr schistosomulum, the tegument is almost entirely heptalaminate. The tegument contains large vesicles, as well as smaller membranous bodies and elongate bodies.

Compared with 3hr schistosomula, the outer membrane of 4 and 7 day old schistosomula is mostly 7 layered and more regularly and deeply pitted. Another type of inclusion body which has a trilaminate limiting membrane and homogeneous contents is present.

Fig.1.4. shows the tegument of the adult worm. The surface of the adult worm is extremely irregular and pitted. The outer membrane usually consists of 7 layers giving the membrane a thickness of about 17 nm. The tegumental cytoplasm contains a number of bodies. Certain of the bodies are sometimes seen to fuse with the outer membrane and unroll to cover the surface.

1.3.3 Changes in biochemical composition of the outer membrane of S. mansoni at different stages in the life cycle

The surface of the schistosome, despite its unusual morphology, is in terms of overall chemical composition, similar to other eukaryotic cell membranes (Stein and Lumsden,1973. Snary et al.1980. Rumjanek and McLaren,1981). It contains proteins, lipid and carbohydrates. These surface molecules also undergo radical changes during parasite development.

Fig. 1.4. The tegument of adult worm. (Reproduced from McLaren, 1980).

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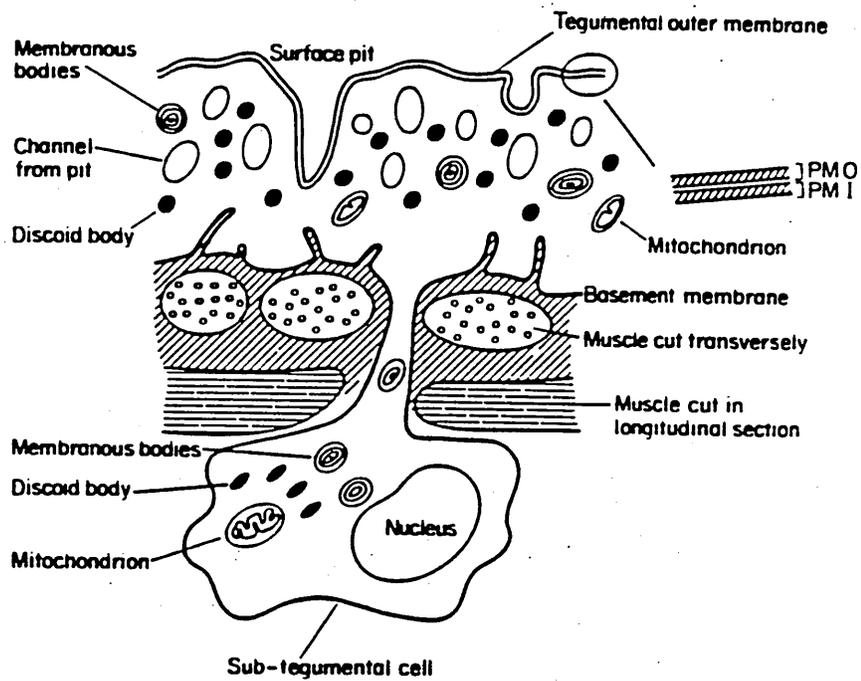
Lipid:

The surface membrane of adult worms has a greater cholesterol: protein ratio than that of the newly transformed parasite (Kusel et al, 1981) and the highest levels of unsaturated fatty acids are in the adult worm. Kusel et al. (1981) found that amphotericin B, and vitamin A alcohol damaged the outer membrane of the schistomomula surface but did not damage the adult worm surface as assessed by ^{51}Cr release. Viall,et al .(1985) using several radioactive lipid precursors compared the lipid metabolism at different stages of the schistosome life cycle. The most striking aspect of this study is the relatively high metabolic activity in 11 day old worms and the lower but sustained activity at 15 days and at the schistosomula stage. By comparison, biosynthetic activity in adult S. mansoni is very low. At the schistosomulum stage and in male, female or paired worms, glycerol and oleate incorporation into neutral lipids is greater than into phospholipids, whereas in 11 and 5 day old worms, incorporation into the phospholipid is greater.

Using a fluorescent lipid probe which is able to insert itself into the parasite surface membrane, fluorescence recovery after photobleaching studies have also indicated that there are changes in biophysical properties of the parasite's lipid bilayer during development. The cercarial outer membrane is mainly in the gel phase. (Foley.et.al. 1988). In schistosomula up to the lung-worm stage, the external leaflet of the outer bilayer exhibits a considerable fraction of immobile lipid presumed to be organized into some domain-like structure. In the adult stage the equivalent leaflet is like the host cell plasma membrane: highly fluid and has apparently lost some of the domain structure. However, it has also developed a totally immobile inner bilayer or bilayers.(Foley,1986; Kusel and Gordon. 1989). The large proportion of lipid immobilized as indicated by the FRAP results of Foley et al (1986) is certainly not a characteristic of the plasma membranes of most host cells which surround the parasite in the bloodstream.

Protein:

Surface-labelling studies of S. mansoni have shown that cercariae have a simple labelling pattern in comparison to schistosomula. Transformation of cercariae to schistosomula results in the loss of low molecular weight material which may be the glycocalyx, Many new protein bands can be detected on schistosomula after transformation. (Snary et al.1980). Freeze fracture studies have also demonstrated that both the number and the distribution of intramembranous particles (IMP) alter as maturation progresses. Migration of schistosomula from the skin to the lung is accompanied by an increase in IMP size (McLaren et al.1978). Table 1.2. is a compilation from 28 publications dealing with the the surface composition of the newly transformed (3h) schistosomula. Evidence points to the expression of macromolecules pre-formed during cercarial development, rather than synthesised de novo during transformation. The majority of surface molecules appear to be glycosylated (Samuelson and Caulfield,1982). Different culture conditions appear to regulate expression of some proteins. For example, the 45KDa molecule (a doublet) has been implicated as a lipid receptor (Rumjanek et al, 1983). It only becomes accessible to labelling reagents after culture of schistosomula in medium containing human serum.

The majority of proteins found on schistosomula appear to be present throughout the remaining part of the developmental cycle; however, adult male worms have only low levels of these antigens. Iodination and lectin binding studies also have shown that the number of surface proteins in direct contact with the exterior appears to decrease gradually as the schistosomula develop. The process through which these proteins become unavailable for detection is thought to be due to the acquisition of host molecules, including neutral lipids, by the parasites. This leads on to a general masking of the parasite's proteins.

Table.1.2. Macromolecules expressed on the surface of the 3-h schistosomula of S. manson **(** Reproduced from Wilson, R.A. 1987)

MW(kDa)	References
>150	Dissous et al. (1985), Yi et al. (1986)
105[a]	Taylor et al. (1981), Samuelson and Caulfied (1982)
92-97	Simpson et al. (1983), Knight et al. (1984)
80[a]	Taylor et al. (1981), Samuelson and Caulfield (1982)
66-68[a]	Taylor et al (1981), Simpson et al. (1983)
53-54[a]	Samuelson and Caulfield (1982), Smith and Clegg (1895)
44-46[a]	Taylor et al. (1981), Samulson and Caulfield (1982)
40-41[a]	Dissous et al. (1981), Samuelson and Caulfield (1982)
38[a]	Simpson et al. (1983), Dissous et al. (1985)
37	Dissous et al. (1981,1985)
32	Knight et al. (1984), Dissous et al. (1985)
27-28[a]	Taylor et al. (1981), Samuelson and Caulfield (1982)
24	Taylor and Butterworth (1982), Taylor et al. (1984)
22[a]	Samuelson and Caulfield (1982), Simpson et al. (1983)
19-20[a]	Samuelson and Caulfield (1982), Simpson et al. (1983)
16-17[a]	Samuelson and Caulfield (1982), Simpson et al. (1984)
14-15	Taylor et al, (1981), Simpson et al. (1983)

[a] Glycosylated; see Samuelson and Caulfield (1982).

Carbohydrates:

Carbohydrates in schistosomes have been found associated with proteins, lipids and as polymers in the glycocalyx. The carbohydrates on the surface of different stages of <u>Schistosoma mansoni</u> have been studied by histochemistry (Wheater and Wilson,1976); by the binding of lectins to the tegumental surface membrane (Stein and Lumsden,1973; Wilson and Barnes,1977; Simpson and Smithers,1980); by the immunoprecipitation of radiolabelled tegumental glycoproteins (Hayunga et al 1979; Hayunga et al 1982); and by lectin affinity chromatography (Hayunga and Sumner 1986b).

From lectin binding experiments, it appears that internal-D-mannosyl residues, terminal D-galactose and N-acetyl-D-glucosamine are present as the exposed oligosaccharides of newly transformed schistosomula and adult worms. Neither terminal N-acetyl-D-galactosamine nor N-acetyl-D-galactosamine subterminal to sialic acid could be detected on schistosomula although this latter sequence is present on adult worms. The lung worm binds less ConA, RCA, WGA and PNA per unit area than the 2h schistosomula. (Simpson et al 1983). In addition, lung stage schistosomula can be shown to possess sialic acid and have totally lost their ability to bind fucose binding protein (FBP). In both these respects they are similar to adult worms.

In addition, MacGregor (1989) has indicated that a peanut agglutinin-binding glycoprotein of molecular weight about 170 kDa could be detected on the worm surface in large amounts at four weeks post-infection. However, this glycoprotein was shown to be absent from pre-liver worms.

All these results show that the exposed carbohydrates, which represent an important component of the surface of <u>S. mansoni,</u> alter as the parasite matures and new glycoproteins appear to be synthesised during development. The importance of the glycoprotein fraction in immunopathology is indicated by the fact that only this fraction is capable of eliciting a T - cell response (Carter and Colley, 1979). ELISA assays using the isolated 170 kDa. glycoprotein showed the presence of antibodies to it

in serum from infected mice and from humans infected with S.mansoni and S. haematobium. Therefore,it is important to define which changes in synthesis of carbohydrate epitopes occur during the development of the parasites.

1.4. Glycoprotein

Glycoproteins are a kind of protein containing covalently linked carbohydrate chains as part of their molecular structure. (Olden,K. et al., 1978). Such molecules are found in fungi, green plants, viruses, bacteria and animal cells. Glycoproteins are common components of animal cell surfaces,and are also commonly found as constituents of lysosomes and among the products exported by the cell. Cell surface glycoproteins have been shown to play important roles in cell-cell adhesion, differentiation, recognition, regulation, modulation of protein receptors and as mediators of immunological specificity. The secreted products function as enzymes, hormones, immunoglobulins and serum transfer factors (Olden, et al 1981).

The carbohydrate chains of glycoproteins are called the oligosaccharide side chains. The oligosaccharide linkage in glycoproteins can be through covalent attachment to either asparagine (N-linked) or to serine or threonine (O-linked). The structures of two kinds of linkages are shown in Fig.1.5.

Many cell surface glycoproteins contain either N- or O-linked oligosaccharide or both types. These two types of structures differ in composition and are synthesized by distinct pathways. The biosynthesis of N-linked oligosaccharides, although involving a more complex series of reactions than O-linked biosynthesis, is somewhat better understood. (Cummings, et al. 1983). In addition, the N-linked glycoproteins are considered to confer biological specificity at cell surfaces (Elbein. 1987). Therefore, the biosynthesis of N-linked glycoproteins will be considered in detail as follows.

1.4.1. Structures of N-linked oligosaccharides

N-linked oligosaccharides are divided into three main categories termed high mannose, hybrid, and complex. Fig.1.6. shows typical examples of these

Fig. 1.5. Two major types of carbohydrate - peptide linkages.

(a). N - glycosidic linkage between asparagine and N - acetylglucosamine.
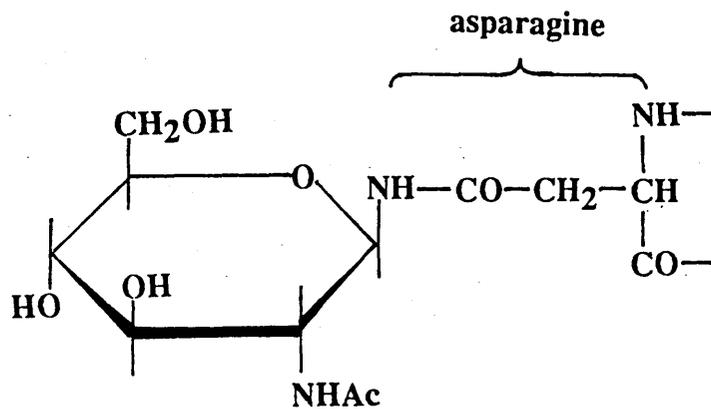
(b). O - glycosidic linkage between serine or threonine and N - acetylglucosamine.
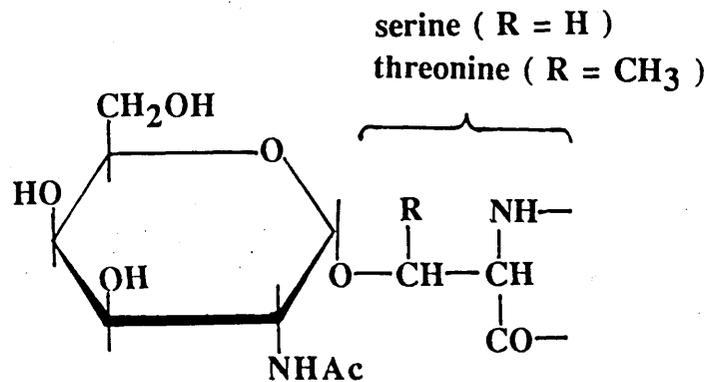
Fig.1.6. Structures of the major types of asparagine - linked oligosaccharides. (Reproduced from Kornfeld and Kornfeld 1985.)

Complex   **Hybrid**   **High mannose**
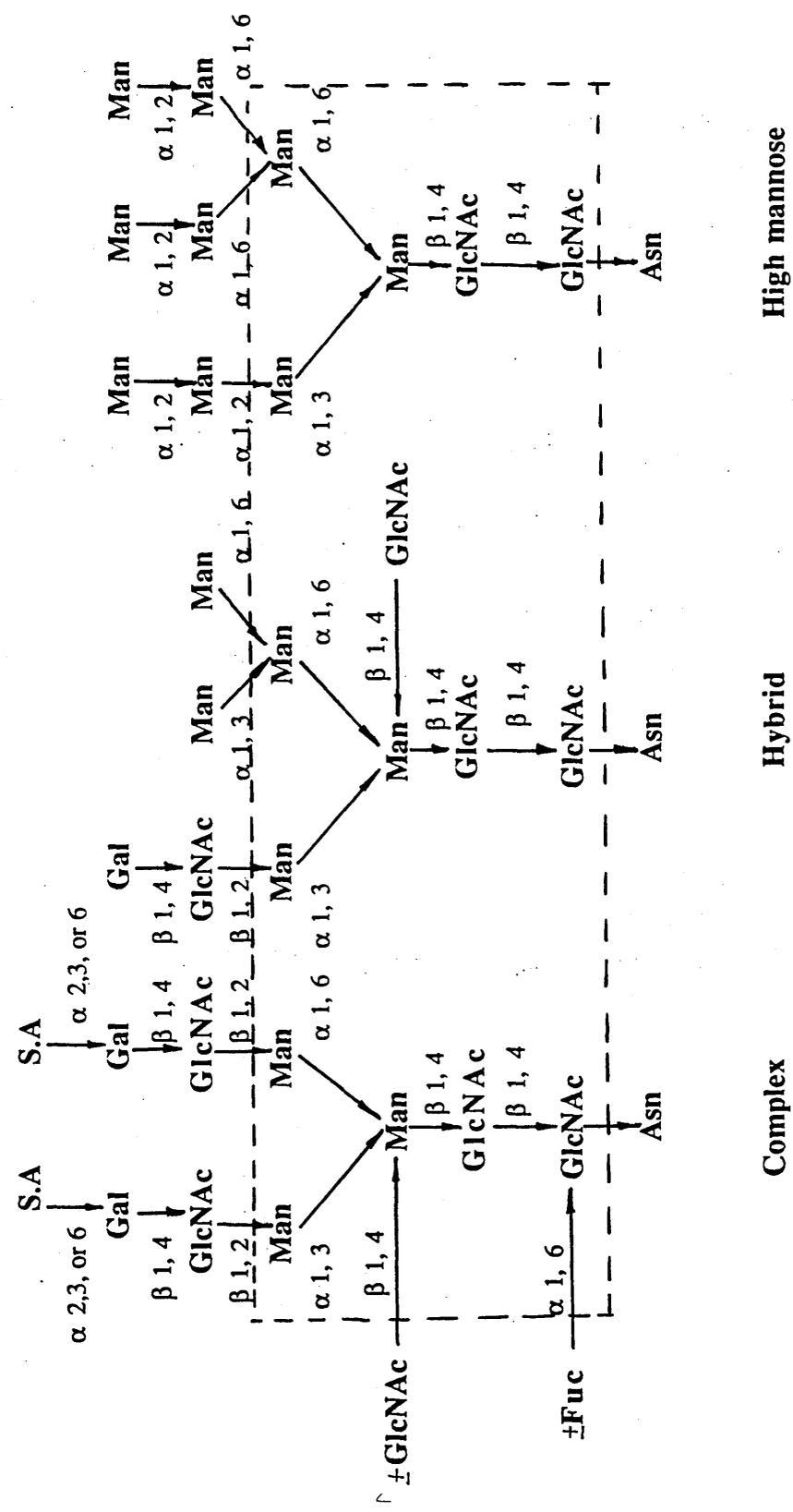
The boxed area encloses the pentasaccharide core common to all N - linked structures

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oligosaccharides. They all share the common core structure Manα1-3 (Manα1-6) Man β 1-4 GlcNAcβ 1-4 GlcNAc-Asn, contained within the boxed area in Fig.1.6. but differ in their other branches. All of the N-linked oligosaccharide structures have the common pentasaccharide core structure because they all arise from the same lipid-linked oligosaccharide precursor

1.4.2. Biosynthesis of N-linked glycoprotein

The steps in the synthesis of the lipid-linked oligosaccharide precursors are shown in Fig.1.7. The first step in the pathway is the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylglucosamine to dolichyl phosphate. This can then be elongated by the addition of one N-acetylglucosaminyl, nine mannosyl, and three glucosyl residues. After elongation is complete, the oligosaccharide is transferred from the lipid to the growing polypeptide chain, within the lumen of the endoplasmic reticulum. The large oligosaccharide is then processed in a fashion dependent on whether it is to become hybrid, complex or high mannose. The enzymatic pathway of oligosaccharide processing is shown in Fig.1.8. This processing begins in the rough endoplasmic reticulum (ER) and continues as the protein moves through the Golgi membranes. A glycoprotein with a Man$_9$ (GlcNAc)$_2$ structure is produced by removing all three glucose residues by two different glucosidases(I and II) that are located in the endoplasmic reticulum. The route from high-mannose structure to hybrid and complex chains involves the removal of 4-6 mannose by specific α-mannosidases that reside in the ER and Golgi apparatus, and addition of GlcNAc and other sugars by Golgi-bound glycosyl transferases to produce an array of oligosaccharide structures. Finally, the newly synthesized glycoproteins are transported to their final destination.

The biosynthesis of the N-linked glycoproteins thus involves a number of metabolic pathways. The inhibition of any of these pathways will affect the final product. Tunicamycin which is an inhibitor of lipid-linked saccharide formation will be discussed here.

Fig. 1.7. Reactions of the lipid - linked saccharide pathway leading to the formation of Glc_3 Man_9 $(GlcNAc)_2$ - pp - dolichol. (Reproduced from Elbein, 1987).

GDP-Man

α1,6 Man Trans.   α1,2 Man Trans.

α1,3 Man Trans.   α1,2 Man Trans

Man-GlcNAc-GlcNAc-PP-Dol

GDP-Man

GlcNAc-GlcNAc-PP-Dol

UDP-GlcNAc

GlcNAc-PP-Dol

UDP-GlcNAc

Dol-P

Mevalonic   Dol-PP

HMG-CoA

Man
Man-Man-Man   Man-GlcNAc-GlcNAc-PP-Dol

Man-P-Dol

Glc-P-Dol

Man-Man
Man-Man-Man
                Man-GlcNAc-GlcNAc-PP-Dol
Glc-Glc-Glc-Man-Man-Man
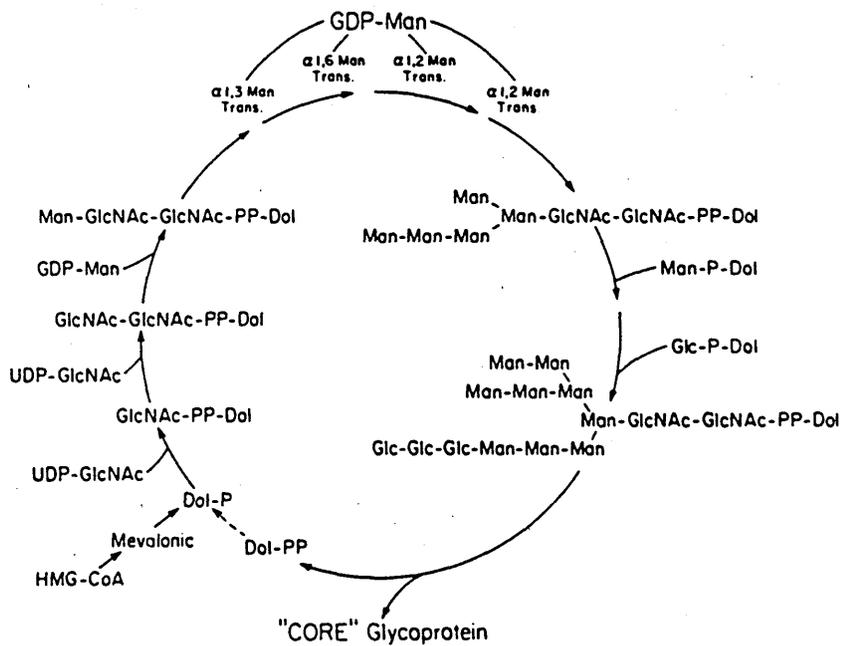
"CORE" Glycoprotein

Fig. 1.8. Reactions involved in the processing of the oligosaccharide chains of N - linked glycoprotein. (Reproduced from Elbein, 1987).

$Glc_3\ Man_9\ GlcNAc_2$ - PP - Dol

Protein

$Glc_3\ Man_9\ GlcNAc_2$ - Asn - Protein

Glc

$Man_9\ GlcNAc_2$ - Protein

High - Mannose Structure

Man

Man
    Man
Man

Man Glc NAc_2 - protein

Man

UDP - GlcNAc

Man
    Man
Man

Man Glc NAc_2 - protein

Glc NAc - Man

Man

Man

Man Glc NAc2 - Protein

Glc NAc - Man

UDP - GlcNAc
UDP - Gal
CMP - Neu NAc

Neu NAc - Gal - Glc NAc - Man

Man Gla NAc2 - protein

Neu NAc - Gal - Gla NAc - Man

21
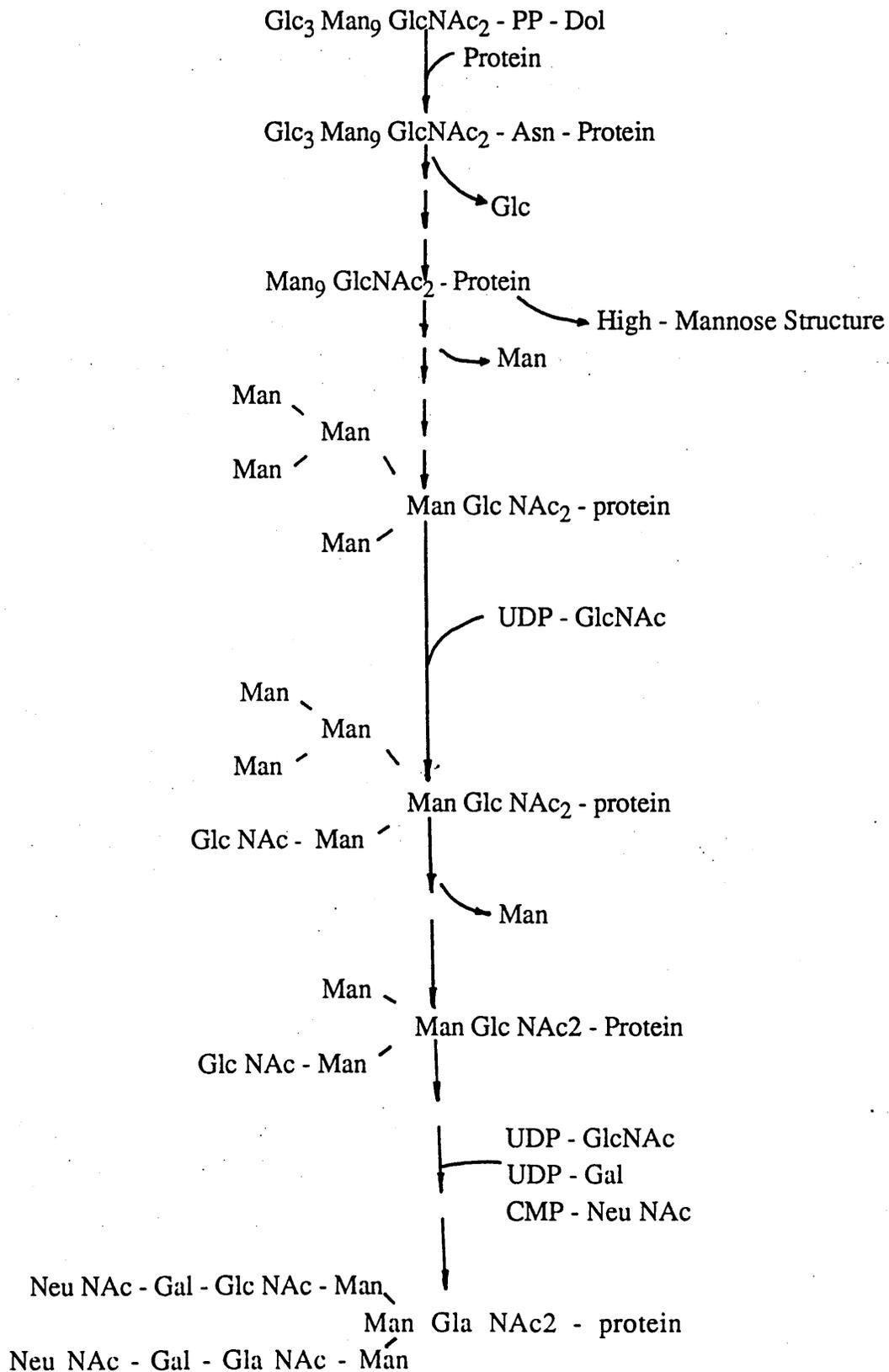
1.4.3. Inhibitor of N-linked glycosylation-Tunicamycin

Tunicamycin was first identified by its antiviral activity against enveloped viruses and isolated from *Streptomyces lysosuperificus* by Takatsuki et al, (1971)

The structure of tunicamycin is shown in Fig.1.9. Tunicamycin is a nucleoside antibiotic which is composed of uracil, a fatty acid, and two glycosidically linked sugars. The sugars are N-acetylglucosamine and an unusual 11-carbon aminodeoxydialdose, called tunicamine.

The site of action of TM is in the first step of the lipid linked saccharide pathway.i.e. the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P to form GlcNAc-PP-Dol (Tkacz and Lampen,1975), thus preventing the formation of any lipid intermediates.

It is suggested that the mechanism of action of tunicamycin is to act as a tight-binding, reversible inhibitor; it may be a substrate-product transition state analogue. Heifitz et al.(1979) have suggested that there are two classes of TM binding sites on the membranous elements of the cell. The high-affinity sites are represented by UDP-GlcNAc: dolichylphosphate GlcNAc-1-phosphate transferase, while low affinity sites are characterised by non-specific binding of TM to lipid. The fatty acid component of TM probably interacts with the membrane. Kuo and Lampen (1976) demonstrated that phosphatidylcholine or phosphatidylserine can prevent binding of TM to yeast protoplasts and block antibiotic inhibition. TM has very precise structural requirements for expression of inhibitory effects upon protein glycosylation. Small variations of structure can lead to loss of its inhibitory effects (Hashim and Cushley 1986). It does not interfere with either sugar or nucleotide metabolism and is not metabolized under either in vitro or in vivo conditions.

In addition , TM may inhibit protein synthesis, (Hickman et al. 1977, Duksin and Bornstein 1977, Struck et al. 1978, Olden et al. 1978) , but not in all cell systems. Furthermore, it is not known whether TM inhibits the synthesis of all proteins, or whether it only inhibits synthesis of certain kinds of proteins.

Fig. 1.9. The structure of tunicamycin. (Reproduced from Hashim and
Cushley. 1987.).
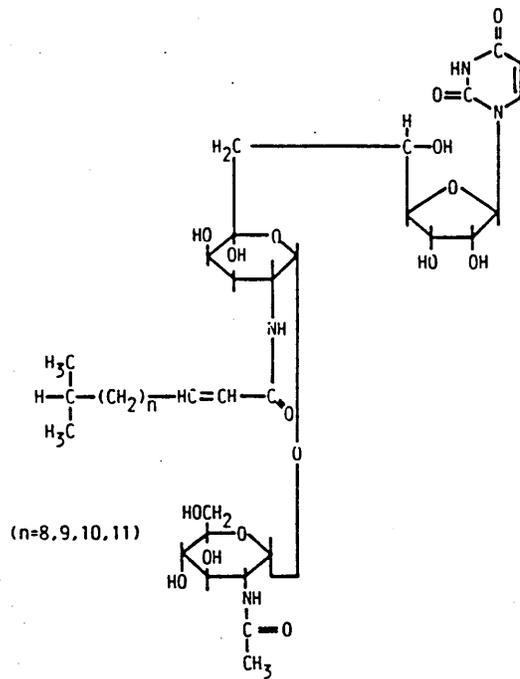
TM itself is not without cytotoxic effects. It appears to inhibit synthesis of cellular RNA and DNA so that when a long-term exposure to the drug is required, side effects must be noted.

1.4.4. Glycosylation in S.mansoni

Biochemical studies have revealed that the human blood fluke S. mansoni contains numerous glycoproteins. The glycoproteins obtained by in vitro metabolic labelling with hexose precursors are clearly synthesized by the parasites. Some recent experiments have indicated that many of the schistosome antigens in the infected host are glycoproteins,(Norden and Strand.1984) and it has been shown that the carbohydrate moieties themselves are antigenic.(Weiss et al, 1986).

1.4.4.1. The role of carbohydrate epitopes

It has been shown by Gryzch et al (1985) that a single carbohydrate epitope can mediate immunity to schistosomiasis in rats. They produced a monoclonal anti-idiotype antibody which corresponds to a carbohydrate epitope present on the Mr 38 kDa and >200 kDa surface antigens. Subsequently, a number of studies have reported that the abundant carbohydrate epitopes may be responsible for inducing the non-species specific concomitant immunity (induced by normal infection), whereas polypeptide epitopes are more likely to be involved in the species-specific immunity induced by vaccination with irradiated cercariae. Under conditions of antibody excess, sera from chronically infected mice bound at a two-to three fold higher level to the surfaces of schistosomula of S.mansoni than did sera from vaccinated mice. Treatment with reagents that selectively removed or modified carbohydrate residues indicated that most of the binding by antibodies in chronic infection serum was to specific carbohydrate epitopes not recognized by vaccine serum. There is also evidence that much of the cross-reaction between the schistosomulum surface and the egg is due to shared carbohydrate epitopes (Kelly, 1987). These epitopes are not species-specific and it is suggested that the production of antibodies recognizing such carbohydrate epitopes may

be a factor in cross-specific concomitant immunity.

On the other hand, carbohydrate specific antibodies have been shown to block immune effector function in vitro, although their in vivo relevance is not clear.

1.4.4.2. Carbohydrate composition of glycoproteins synthesized by schistosomes

Carbohydrate composition of glycoproteins in S.mansoni have been characterized by metabolic labelling using hexose precursors or lectin affinity chromatography. The most abundant monosaccharides present in schistosomes were found to be mannose, galactose, glucose, N-acetylglucosamine and N-acetylgalactosamine. When the parasites were metabolically labelled with hexose precursors, it was found that the radiolabelled saccharides were incorporated into at least 10 tegumental glycoproteins. In comparing the SDS profiles obtained using different labelled precursors, several peaks are detected by metabolic labelling with more than 1 precursor. A 41kDa -43 kDa component is detected upon labeling with galactose, mannose and N-acetylgalactosamine. Likewise, galactose, glucose, glucosamine and N-acetylgalactosamine are each incorporated into a 210 - 220 kDa. glycoprotein.

In addition, when surface glycoproteins on S.mansoni adult worms were studied using lectin affinity chromatography, analysis of the sugar eluates by SDS-PAGE showed that many of the glycoproteins reacted with more than one lectin.

All these findings strongly indicate that glycoproteins contain a complex arrangement of carbohydrates.

1.4.4.3. The structure of carbohydrate moieties in glycoprotein synthesized by schistosomes

The glycoproteins synthesized by S.mansoni appear to contain both types of linkage, i.e. O-linked and N-linked.

In 1987, Nyame et al first demonstrated that S.mansoni adult males synthesized glycoproteins containing at least two types of O-linked oligosaccharide. The carbohydrate moieties were found to be simple mono- and disaccharides lacking sialic acid residues. The O-linked disaccharide was identified as galactose- N-acetylgalactosamine, which represents a minor fraction of the O-linked oligosaccharides and appears to be similar in structure to that made in vertebrates. The major O-linked sugar in schistosome glycoproteins is terminal O-linked GlcNAc.

Thereafter, in 1988, Nyame et al reported that high-mannose-type N-linked oligosaccharides also exist, in glycoproteins synthesized by S.mansoni adult male worms. The high mannose type chains range in size from $Man_7GlcNAc_2$ to $Man_9GlcNAc_2$ and appear to be similar in structure to those synthesized by mammalian cells.

Although some limited information is available on the structure of schistosome glycoproteins in their completed forms, little is known about the actual processes of glycoprotein synthesis by the parasite.

1.5. Signal systems:

1.5.1. Stimulus and response in biological systems

One characteristic of living organisms is that they are able to exchange materials and chemical/electrical information with their environment and with each other. The ability of cells to respond to stimuli from their environment is of fundamental importance to a variety of cellular processes including cell growth, development, differentiation and behaviour. With respect to S.mansoni, it undergoes a complex life cycle involving different larval, intermediate and adult stages, all of which have a different morphology, live in extremely different environments and express different functions. After shedding from the molluscan intermediate host, the way in which schistosome cercariae locate their mammalian host is still poorly understood. However, the changes in cercarial swimming behaviour and penetration into host skin

can be triggered by chemical stimuli. There is no evidence for cell division or growth as defined by increase in mass or nitrogen content (Lawson and Wilson,1980a,b) until arrival in the liver. The initiation of parasite growth after arrival in the liver may be under endogenous control by ecdysteroids (Nirde et al,1983) and these hormones may in addition modulate the processes of tegument membrane turnover (Torpier et al, 1982). The fact that many adaptive changes are made during these transitional stages suggests that the parasite must be capable of receiving and processing information from its environment not only for a variety of cellular processes to occur, but also to determine whether a host or host environment is suitable. In particular, the parasite must respond to stimuli emanating from the host's immune system.

Considerable advances have been made in the study of molecular mechanisms of signal transduction in a wide variety of cellular systems. However, little comparative work has been done on signal transduction in parasites.

1.5.2. Signal transduction systems in animal cells

The mechanism for transduction of extracellular signals into intracellular events in the animal cell can be generalized to include the following prerequisites:

Ligand (signal) - Receptor (membrane) - Second messengers (cAMP, Ca^{2+}, inositol trisphosphate) - Amplification (protein kinase/phosphatase : protein substrates) - response element (biochemical physiological, behavioural)

The receptors on the cell surface function as molecular antennae, detecting external information (for example, hormones, growth factors, neurotransmitters or other biologically active substances.). The signal is transduced and amplified into the second messengers which control many cellular processes such as metabolism, secretion, contraction and cell growth.

1.5.2.1. cAMP and inositol trisphosphate in cellular signal transduction

There are two known second messenger systems in animal cells. The role of cyclic AMP as a second messenger in signal transduction is perhaps the best

understood. Adenylate cyclase which produces cAMP from ATP, is controlled by the coupling of a receptor to this enzyme through a GTP-binding protein called Gs located at the cytoplasmic side of the plasma membrane. Once a receptor is occupied it undergoes a conformational change which is transmitted to Gs causing a loss of GDP and enhanced affinity for GTP acting from within the cell. In this way, the adenylate cyclase can be activated and catalyse the formation of cyclic AMP from adenosine triphosphate (ATP). cAMP will bind to the regulatory subunit of a protein called A-kinase, liberating the catalytic component which can phosphorylate specific proteins that regulate a cellular response. The activity of the Gs-GTP complex is controlled by an enzyme called GTPase which hydrolyses GTP to guanosine diphosphate(GDP); Gs-GDP can no longer induce adenylate cyclase to form cAMP.

The other major signalling pathway is that which utilizes the inositol lipid as part of a transduction mechanism (Berridge, and Irvine,1984). Fig.1.10. The key feature of this receptor mechanism is that an inositol lipid located within the inner leaflet of the plasma membrane is hydrolysed to generate second messengers.(Berridge,1987,). When an agonist binds to an external receptor (Ri) it stimulates phosphatidylinositol 4,5-bisphosphate ($PtdIns4,5P_2$) phosphodiesterase (PDE) to initiate the hydrolysis of $PtdIns4,5P_2$) into two second messengers,which are diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). PDE is controlled not only by the occupation of an appropriate receptor but also by the presence of a GTP-binding protein as in the mechanism used to link receptors to adenylate cyclase (Gompert,1983. Gilman,1984). G-proteins are a family of cell membrane proteins involved in transducing information across the plasma membrane. Because the putative G-protein of the inositol lipid receptors seem to differ from that described previously, it has been abbreviated to Gp with the subscript referring to phospholipid (Cockroft and Gomperts 1985). Gp serves as a coupling agent to relay information from the receptor to the PDE (Haslam & Davidson 1984; Cockroft & Gomperts 1985: Taylor and Merritt 1986).

DG acts within the plane of the membrane to stimulate protein kinase C which

Fig. 1.10. The proposed role of inositol trisphosphate and diacylglycerol in cellular signal transduction. (Reproduced from Houslay 1987)
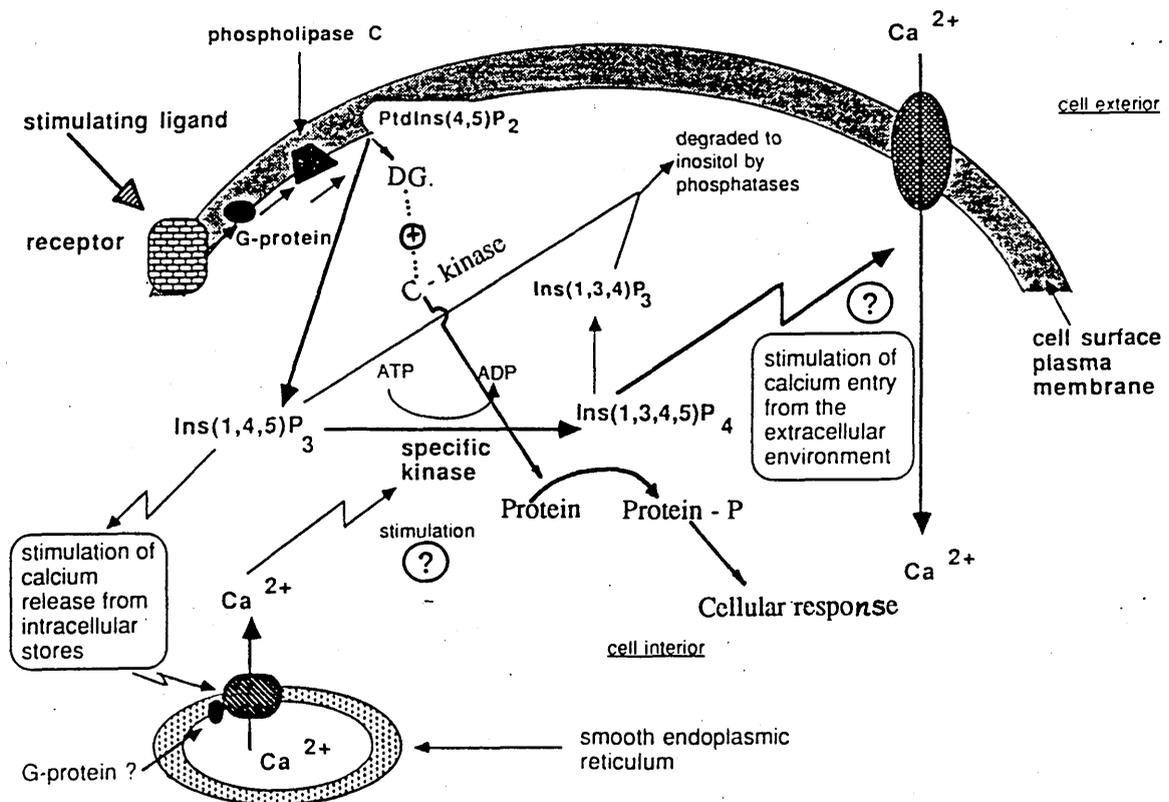
phosphorylates various target proteins within the cell (Nishizuka.1983). The water-soluble IP_3 released to the cytosol functions as a second messenger to mobilize calcium from the endoplasmic reticulum (ER) (Berridge & Irvine 1984). Phosphatidylinositol breakdown and Ca^{2+} mobilization are often associated with the control of long-term effects such as cell growth and proliferation.

Each of the initial second messengers (DG and IP_3) formed by hydrolysing $PtdIns4,5P_2$ can be metabolized by two separate pathways. A simplified scheme for inositol lipid metabolism is shown in Fig 1.11. The DG can either be recycled back to reform PtdIns or it can be the substrate of DG lipase resulting in release of arachidonic acid. The IP_3 can also be metabolized along two separate pathways. Along one pathway it is sequentially dephosphorylated to free inositol which can be reused to synthesize PtdIns. The alternative pathway results in the generation of additional inositol polyphosphates which may yield other important signal molecules. It has been suggested that IP_4 may regulate the entry of external calcium (Irvine and Moor 1986). The formation of PIP and PIP_2 requires ATP for phosphorylation.

1.5.2.2. Role of protein phosphorylation in signal transduction

Protein phosphorylation / dephosphorylation is a very widespread type of regulation in signal transduction. Recently, various protein kinases have been described which respond to a variety of different effectors.(Hemmings et al., 1989). When ⅍ neurotransmitter or hormone activates a specific receptors on the cell surface, initiating a sequence of biochemical reactions, protein phosphorylation is usually involved, leading to an alteration in the physiological properties of the target cell. There may be either direct activation of a receptor-associated protein kinase or an alteration in the level of a second messenger, which in turn regulates a specific protein kinase or phosphoprotein phosphatase. Thus, protein phosphorylation has the potential for a large amplification of the initial signal and represents a final common pathway in the molecular mechanism through which neurotransmitters and hormones produce many of

Fig. 1.11. Inositol lipid metabolism
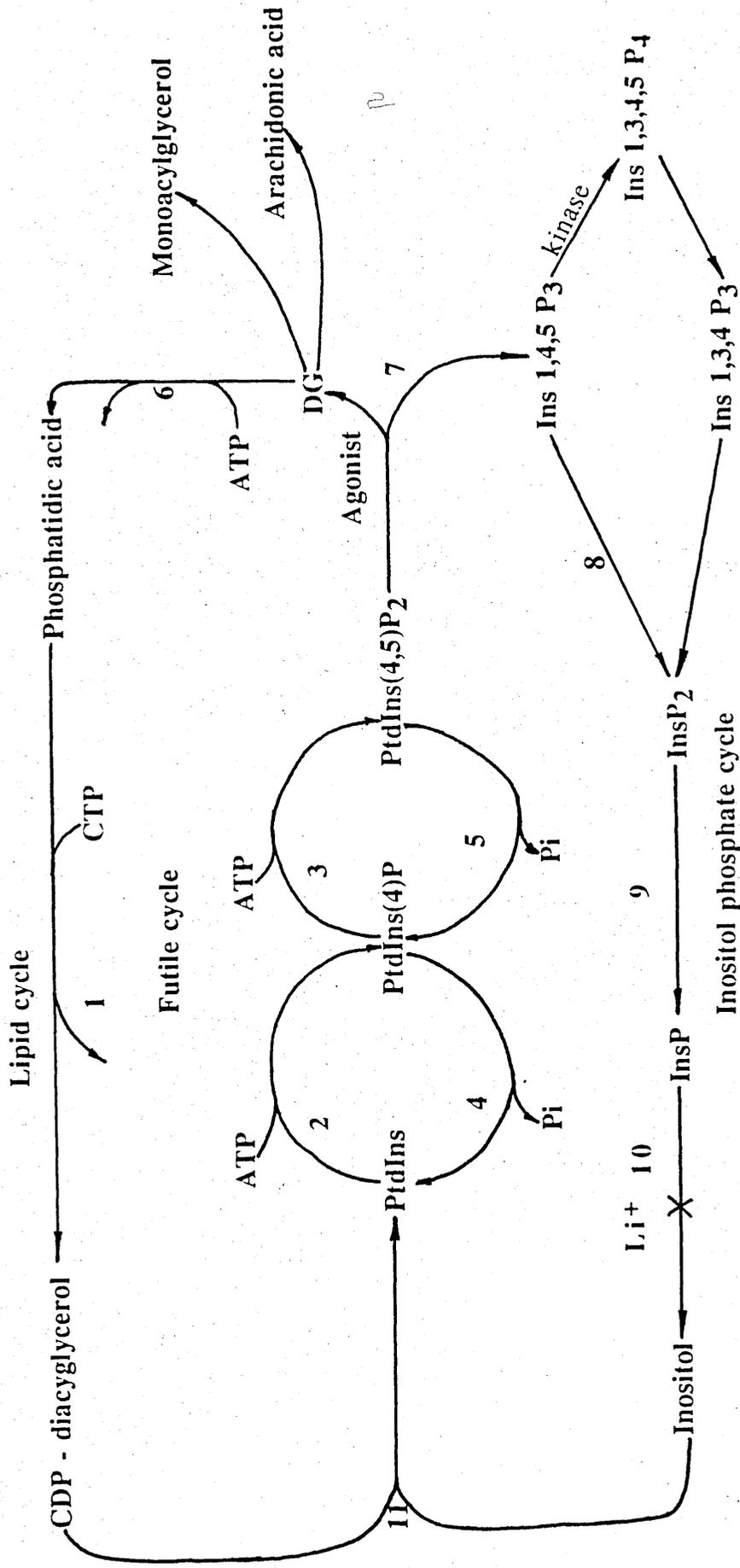
1.CTP phosphatidate cytidyl transferase; 2. Phosphatidylinositol kinase; 3. PtdIns (4)P kinase; 4. PtdIns (4) phosphomonoesterase; 5. PtdIns (4,5) P_2 phosphomonoesterase; 6. Diacylglycerol kinase; 7. PtdIns (4,5) P_2 phosphodiesterase; 8. InsP3 phosphatase; 9. InsP2 phospatase 10. InsP phosphatase; 11. CDP-diacylglycerol inositol phosphatidate transferase. DG: diacyglycerol

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their biological effects in target cells. In the nervous system, many proteins have been shown to be regulated by phosphorylation, including neurotransmitter receptors, ion channels, cytoskeletal protein, synaptic vesicle-associated proteins, enzymes, and various regulatory proteins. Direct evidence for a role of protein phosphorylation in mediating signal transduction in neurons (as well as a variety of other cell types) has been obtained from studies involving the intracellular injection of purified protein kinases or specific protein kinase inhibitors into identified cells. These studies have demonstrated an obligatory role for cAMP-dependent protein kinase (cAMP-PK), cGMP-dependent protein kinase, Ca^{2+} / CaM-dependent protein kinaseII, and Ca^{2+} / diacylglycerol-dependent protein kinase in regulation of a variety of physiological responses of neurons to extracellular signals. Thus, microinjection of each of these specific protein kinases has been shown to mimic or potentiate the actions of extracellular signals and their respective intracellular messengers, which indicates that activation of the protein kinase is sufficient to generate the physiological response. Moreover, in some cases, the microinjection of specific protein kinase inhibitors has been shown to prevent the physiological responses to extracellular signals and their intracellular messengers, indicating that activation of the protein kinase is necessary for the response.(Hemmings. et al 1989).

Protein phosphorylation involves the covalent modification of key substrate proteins by phosphoryl transfer, which in turn regulates their functional properties. The most common modifications involve esterification of phosphate to serine and threonine. However, there are instances where the tyrosine,hydroxyl group is phosphorylated. Phosphotyrosine (P.Tyr) is about 0.05% of the phosphorylated amino acids within the cell. P.Tyr was first detected in hydrolysates of viral transforming protein labelled by incubation of immunoprecipitates with radioactive ATP (Eckhart, et al ,1979., Hunter. et al 1980., Witte er al 1980.). The phosphorylation of proteins at tyrosine is often one of the earliest measurable features in cells transformed by certain viruses or treated with growth factors or hormones. In addition, the half-life of the phosphate on tyrosine is less than 30min (Sefton et al.1980), ideal

for a regulatory signal. It is also interesting that most protein-tyrosine kinases (PTK)
are associated with the plasma membrane and thus are in a position to transduce signals
from the outside of the cell. A clear prerequisite for a multicellular organism is a means
of cell-cell signaling and the PTK nature of many of the growth factor receptors may be
pertinent here.

1.5.3. Signal transduction in S.mansoni

Although some investigations of basic regulatory processes in parasites are being
carried out, the field is still a neglected one. It has been shown that adenylate cyclase
activity is present in the tegument of S.mansoni, and this activity is modulated by
neurotransmitters, e.g. serotonin.(Mansour. 1979). The co-operation in the surface
membrane of receptors and adenylate cyclase involves the mediation of GTP-binding
proteins. There are distinct differences between the developmental stages of the
parasite with regard to adenylate cyclase activity and 5HT responsiveness. It has been
shown that cercariae have low total cyclase activity and almost no responsiveness of the
enzyme to serotonin . During development from the newly transformed schistosomula
to the adult stage, the 5HT-stimulated activity of adenylate cyclase increases, with the
adult stage having the highest adenylate cyclase activity. The full response to
stimulation by 5HT develops during the first four days when schistosomula are
cultured in vitro. (Kasschau and Mansour, 1982). The observed differences in
adenylate cyclase activity do not seem to be due to the absence of the G-protein. A
serotonin-activated adenylate cyclase in S.mansoni is activated in the first 48-96hr of
the schistosomula life cycle, which corresponds closely to the time of complete skin
penetration (Wilson and Lawson 1980, Bickle 1982) and arrival in the vascular
system where serotonin is present. Therefore, it is feasible that a serotonin-coupled
adenylate cyclase is important as a regulatory system for the control of cyclic AMP in
the parasite. It has been reported that serotonin activates three key enzymes in the
regulation of metabolism in a closely related parasite, Fasciola.hepatica: glycogen

phosphorylase,(Mansour et al, 1960) phosphofructokinase (Mansour, and Mansour, 1962) and cyclic AMP-dependent protein kinase (Gentleman et al 1976). Cyclic AMP has also been reported to be important for the development of trypanosomes (Mancini and Patton 1981) and malarial parasites (Kaushal 1980). The development of S.mansoni from the free living cercarial form to the adult parasites involves considerable change in the milieu. In addition to the need for the regulation of its metabolism, therefore, cyclic AMP may be required for the development of the parasite in its mammalian host.

Recently, Podesta.et al (1987) have reported that in S. mansoni, serotonin and C3b can, respectively stimulate synthesis of the apical syncytial plasma membrane (APM) and overlying envelope (E). C3b is probably operating through a Ca^{2+} dependent response sequence leading to increased synthesis of E, because C3b but not 5HT accelerated net inflow of Ca^{2+}. 5HT also stimulated phosphorylation of all APM phosphoproteins. However, the in vitro protein phosphorylation reaction in response to 5HT and cAMP were quantitatively and qualitatively different,suggesting that 5HT may be operating through a different second messenger system.

The role of calcium and inositol trisphosphates as second messengers in S.mansoni is an open question. PI as a component of phospholipids is found in different life cycle stages of S.mansoni. Some drugs such as actinomycin-D, fenfluramine, and U.V-irradiation all show an effect on phospholipid synthesis. (Wales 1989). Praziquantel (PZ) which is a drug broadly effective against trematodes and cestodes (Harnett. 1988) can increase Ca^{++}-fluxes in schistosomes. Although it may be linked to the muscular contraction of schistosomes, PZ has also been shown to increase parasite antigen exposure in adult worm in vivo (Brindley and Sher 1987) and in vitro. (Harnett and Kusel 1986). The question is how this drug specifically influences the parasite's behaviour and changes in its metabolism.

Protein phosphorylation in schistosomes has also been examined by Kalopothakis et al (1987). They find that protein phosphorylation systems are

present in the tegumental membranes at high levels, and suggesting that the complementary transmembrane signalling processes and generation of second messengers may also occur.

Therefore, the regulatory processes in <u>S.mansoni</u> deserve further study.

Aims of the thesis:

The surface of S. mansoni seems to play an important role in the parasite's interaction with the host immune system. The aims of my thesis are concentrated on two fields: (1). features of glycoprotein synthesis and (2), features of signal transduction in S. mansoni.

(1). Various types of carbohydrates are found to be associated with schistosome tegument and the presence of these carbohydrate epitopes on different schistosome glycoconjugates appears to have a qualitative effect on the antibody response to the schistosomular surface. In addition, carbohydrate specific antibodies have also been shown to block immune effector function in vitro. Therefore, a detailed knowledge of carbohydrate structure and biosynthesis is required. TM was chosen to study the synthesis and function of glycoproteins in S. mansoni.

TM is a potent inhibitor of asparagine-linked (N-linked) protein glycosylation. It can inhibit the growth of gram-positive bacteria, yeast, and fungi and usually induces morphological changes. It has reported that it can affect the certain parasites, e.g. Leishmania donovani (Lovelace and Gottlieb 1985). Here, the effects of TM on S. mansoni will be studied in the following stages:

a. Effect of TM upon glycoprotein synthesis at different life cycle stages in S. mansoni.

b. Effect of TM on the outer membrane of S. mansoni. The effects include changes in lectin binding, antibody binding, lateral diffusion of macromolecules in the surface membrane and ultrastructure of outer membrane.

c. Effect of TM on the the growth of S. mansoni and antibody production.

(2). The surface of S. mansoni also appears to respond to a variety of ligands and external signals, including irradiation. However, the mechanisms underlying responses to signals at the membrane of S. mansoni are not clear. Therefore, it is quite important to examine signal transduction systems which are important in S. mansoni. The following experiments have been carried out:

a. Measurement of the inositol phosphate metabolism pathway at different life cycle stages of S. mansoni.

b.Determination of the effect of different ligands on the inositol phosphate metabolism pathway.

c.Measurement of changes in protein phosphorylation during transformation and after irradiation, human serum stimulation and TM treatment.

Chapter 2: Materialsand methods

2.1. Materials

2.1.1. The parasites

The life cycle of a Puerto Rican strain of <u>Schistosoma mansoni</u> is maintained in the Dept. of Biochemistry, University of Glasgow.

2.1.2. Animals

BALB/c mice were obtained from the Biochemistry / Physiology Animal House, University of Glasgow.

2.1.3. Chemicals:

Absolute ethanol (A.R.) was obtained from James Burroughs Ltd., Essex, England. Phenol was obtained from Formachem, Strathaven, Scotland. Bromophenol blue, Coomassie Brilliant Blue R 250: George T. Gurr Ltd., London, England. Ethylenediamine tetra-acetic acid (EDTA) and 2-mercaptoethanol: Koch-Light Laboratories, Colnbrook, England. Ecoscint scintillation fluid: National Diagnostics, New Jersey, U.S.A. . Methanol, Chloroform : May and Baker Ltd. , Dagenham, England. Molecular weight standards for SDS-PAGE Gel and ConA-sepharose 4B were from Pharmacia Ltd. , Milton Keynes, England. N,N,N',N'-tetramethylethylene diamine (TEMED) Phenylmethylsulphonyl fluoride(PMSF), N-tosyl-L-Lysine chloromethyl ketone (TLCK), L-1-tosylamide-2-phenyl-ethylchloromethylketone (TPCK), Triton X 100 and Tween 20, a - methyl - D - glucoside, O-phenylene diamine (OPD), Cacodylic acid, Bovine serum albumin (BSA), Fluoresceinated (FITC)- ConcanavalinA, FITC-Peanut agglutinin, FITC-Wheat germ agglutinin were obtained from Sigma Chemical Co. , Poole, England. 5(N-octadecanoyl)-aminofluorescein (C_{18}-Fl) was from Molecular Probes, Ltd., U.S.A. Iodogen(1,3,4,6-tetrachlor-3a,6b-diphenylglycoluril): Pierce Chemical Co. (U.K.) ltd., Cambridge, England. All other chemicals were obtained from the British Drug House (BDH) Ltd,Poole,England. Penicillin-streptomycin (10,000units/ml), L-

Glutamine 200mM (100X), BHK 21 Medium (10X), Sodium Bicarbonate 7.5 % were produced in the UK by Gibco Limited PO Box X 35, Paisley, Scotland. Foetal calf serum (FCS) was obtained from Gibco-BRL, Paisley, Scotland.

2.1.3.1. Inhibitor

Tunicamycin was purchased from Boehringer Mannheim GmbH, Mannheim, W-Germany. Analogues of tunicamycin were kindly provided by Dr. W. Cushley. (Dept. of Biochemistry, University of Glasgow.)

2.1.3.2. Immunochemicals

FITC-labelled rabbit antibody against mouse IgG and Anti-mouse IgG peroxidase conjugate were from Sigma Chemical Co. Ltd., Poole, England.

2.1.3.3. Radiochemicals:

D - [2,6-^{3}H] - mannose (54Ci / mmol), ^{35}S - Methionine (800 Ci / mmol). L -[4,5-^{3}H] - Leucine (131.5 Ci / mmol), 2 - amino [1-^{14}C] Isobutyric acid (59 mCi / mmol), ^{3}H - Tunicamycin (110.17 Ci / mmol), [^{3}H] - inositol (mCi /ml), [^{32}P] - orthophosphate (10 mCi /ml) were all from Amersham International plc., Amersham, England.

2.1.3.4. Photographic materials

Kodak X-Omat S film was obtained from Kodak, London, England. Plastic intensifying-screen holders were purchased from Anthony Monk Ltd., Sutton-In-Ashfield, England.

2.1.4. Solutions

1. Phosphate Buffered Saline (PBS) (pH 7.4)

	g/L	mM
NaCl	80	140
KCl	0.20	2.7
KH_2PO_4	0.20 ,	1.5
Na_2HPO_4	1.15	8.1

2. Proteinase inhibitor cocktail stock solution (X100)

PMSF:	1.0M
TLCK:	2.0mg/ml
TPCK:	0.5mg/ml

The inhibitors were prepared, at the above concentrations, in absolute ethanol and mixed. The 100X concentrated stock solution was stored at - 20°C.

2.1.5. Incubation media

1. Glasgow's modification of Minimal Essential Medium (GMEM) (500 ml) :

Dist-H_2O :	425ml
BHK-21(10X) :	50ml
7.5 % $NaHCO_3$:	15ml
L-glutamine [200mM(100X)]:	5ml
Pen/Strep (10,000 units/ml):	5ml

to pH 7.4 with 5 M NaOH.

2. Dulbecco's modification of Minimal Essential Medium (DEME) without inositol.

3. RPMI 1640 medium.

4. 1X Minimum Essential Medium Eagle(Modified) without phosphate.

These are all from (Gibco) - BRL.

2.2. Methods

2.2.1. Biological methods

2.2.1.1. Heavy infection of mice

Mice were anaesthetized using 1.0ml/100g body weight of 10% (v/v) Sagatal in distilled water/ ethanol (9:1 v/v). The anaesthetized mice were shaved to remove abdominal hair and a metal ring was then placed on each abdomen. 200μl H_2O containing approximately 500 - 1000 cercariae was added to each ring. Mice were then left undisturbed for 30 min to allow cercarial penetration of skin.

2.2.1.2. Preparation of schistosomula by mechanical transformation and skin transformation (Colley and Wikel. 1974; Clegg and Smithers 1972 .)

Cercariae freshly shed from snails were transferred to a universal bottle and concentrated on ice for 30 min . Water was removed until approximately 1 ml remained and 9 ml of GMEM, pre-warmed at 37^0C, was added. The cercariae were transformed into schistosomula by passing 15 times through a "21Gx11/2"needle fitted to a 10 ml syringe. The transformed schistosomula were then allowed to sediment under gravity. The tail-rich supernatant was decanted. After resuspension in GMEM (pH7.4), the schistosomula were allowed to sediment again. This procedure was repeated three times, before the schistosomula were used.

Skin-transformed schistosomula were prepared by allowing cercariae to penetrate isolated pieces of ventral skin from Parkes mice. The schistosomula were collected in GMEM medium.

2.2.1.3. Recovery of lung stage worms (Clegg. 1965)

Mice were anaesthetized 8 days after a heavy (1000 cercariae) infection. Then the animals were dissected to expose the abdominal and thoracic cavities. An incision was made in the portal vein and citrate-saline was pumped into the heart to remove all blood. The lungs were taken out and cut into small pieces. 10ml GMEM medium was

added. The suspension was incubated at 37^0C for 3hr. This crude lung suspension was then filtered through muslin. The suspension was spun at 500 rpm for 1 min in a MSE Minor bench centrifuge. The supernatant was discarded . The pellet was washed twice in GMEM medium.

2.2.1.4. Perfusion of animals for recovery of adult worms (Smithers and Terry, 1965)

Mice infected with 150 cercariae 3-6 weeks previously were killed using chloroform ($CHCl_3$). The animals were dissected to expose the abdominal and thoracic cavities. An incision was made in the portal vein and citrate-saline was pumped into the heart. The flow of fluid though the circulation forced the worms out though the incision in the portal vein. The worms were collected in a glass dish and washed 3 times using GMEM medium before use.

2.2.2. Measurement of protein

The concentration of protein was measured by the Folin phenol method (modified from Lowry et al 1951)

Stock solution:

Solution A: 2% Na_2CO_3 in 0.1 N NaOH.

Solution B: 0.5% $CuSO_4 \cdot 5H_2O$.in 1% sodium citrate.

Alkaline copper solution was made up by mixing 50 parts solution A with 1 part solution B. 1.5 ml of alkaline copper solution was added to each tube containing a unknown protein solution, mixed and incubated for 10 min at 37^0C. 0.3ml of Folin reagent previously diluted 1:1 with H_2O was then added, mixed and incubated for 10 min at 37^0C. The colour was read at 750 nm on the spectrophotometer. (Ultrospec II U.V./ Vis- from LKB Instruments Ltd.).

A standard curve was made using a BSA stock solution (1mg/ml). The protein concentration in the sample was deduced from this standard curve.

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2.2.3. Biosynthetic labelling

2.2.3.1. Biosynthetic labelling of glycoproteins

1). Short term labelling: Prior to labelling, parasites were incubated in the presence or absence of tunicamycin (TM) for 3hr at 37^0C. Parasites were then resuspended in RPMI-1640 medium containing sodium pyruvate as an energy source in place of glucose. ^3H-mannose was normally obtained in 50% (v/v) ethanol solution and therefore had to be lyophilized and resuspended in labelling medium, prior to use. A dose of 25 μCi of [^3H] -mannose was then added and incubation was carried out for 2-6 hr at 37^0C in 5% carbon dioxide.

2). Long term labelling: Parasites were incubated in RPMI 1640 medium containing 25 μCi [^3H] - mannose with TM or without TM under 5% carbon dioxide for 21 hours.

2.2.3.2 Biosynthetic labelling of protein

After the parasites were treated with TM for 3hr, they were then incubated using EMEM medium without methionine. 25μCi [^{35}S] - Methionine was added into each sample and they were incubated for 2hr at 37^0C.

2.2.3.3. Measurement of radioactivity incorporated into protein

After the labelling period, the parasites were washed 3 times using GMEM medium. One ml PBS was added to each sample and the parasites were homogenized. 0.1ml 100% trichloroacetic acid (TCA) was added to precipitate the protein. The sample was kept in ice for 30 min and centrifuged for 5 min on MSE bench centrifuge. The supernatant was discarded. The pellet was dissolved in 200ul sample buffer [2.2.4.1. (1.g)] containing 8M urea. To measure the incorporation of radioactivity, an aliquot was transferred to Ecoscint for scintillation counting.

2.2.4. Analysis of biosynthetically labelled protein

2.2.4.1.Separation of labelled proteins by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

SDS-Polyacrylamide gel electrophoresis was performed according to the discontinuous buffering method described by Laemmli (1970)

1. Stock solutions:

a. 50% Acrylamide (w/v) [48.75% (w/v) acrylamide, 1.25% (w/v) N;N'-methylene bis acrylamide], stored in dark at 4°C.

b. 1M Tris-HCl buffer, (pH 8.8)

c. 1M Tris-HCl buffer, (pH6.8)

Solutions b,c, were kept at 4°C

d. 10% SDS

e. Stock running buffer: (5x) (pH 8.3-8.5)

    0.125M Tris

    0.965M Glycine

    0.5% SDS

Solutions d,e were kept at room temperature.

f. 1.5% Ammonium persulphate (APS) (freshly prepared).

g. Sample buffer:

10% SDS (w/v) , 10% 2-mercaptoethanol , 50 %sucrose (w/v) , 0.005% Bromophenol blue in 0.125 M Tris-HCl (pH 6.8).

h. 0.1% Coomassie blue stain solution:

    0.1% Coomassie blue

    50% methanol

    5% acetic acid

i. Destain solution:

  25% methanol

  10% acetic acid

2. Separating gel preparation:

10% and 15% separating gels were prepared from stock solutions as follows:

	Final Conc. of polyacrylamide	
	10% (ml)	15% (ml)
Acrylamide Stock (50% w/v)	8.0	12.0
1M Tris-HCl buffer pH 8.8	15.0	15.0
H_2O	15.7	11.7
	degas and add	
10% SDS	0.40	0.40
1.5% APS	0.90	0.90
TEMED	50ul	50ul

The gel solution was poured between the glass plates of the gel mould, overlayed with distilled water to ensure that the surface of the gel was flat and then allowed to polymerise for 45 min

3. Stacking gel preparation

5% stacking gel was prepared from stock solutions as follows:

	Vol (ml)
Acrylamide Stock (50% w/v)	1.00
1M Tris-HCl buffer pH 6.8	1.25
H_2O	7.30
10% SDS	0.10
1.5% APS	0.35
TEMED	20ul

The water at the top of the separating gel was removed and stacking gel solution

was poured.

4. Sample preparation

The sample treated with TCA was dissolved in 200ul of reducing sample buffer [2.1.4.1.(1.g)] containing 8M urea and incubated at 65°C until dissolved.

Schistosomula and worm homogenates were dissolved in sample buffer [2.1.4.1.(1.g)] and heated at 100°C for 3 min.

5. Electrophoresis conditions

All sample were electrophoresed either for 2-4 hours at a constant current of 50 mA or overnight at 5-10 mA

6. Stain and destain

The gel was stained with 0.1% Coomassie blue for 1 hr at 37°C. The gel was then destained until the background became colourless.

2.2.4.2. Identification of radiolabelled protein
1. Autoradiography

The gel was washed several times to remove acetic acid and dried on a piece of Whatman 3 MM paper under vacuum refrigeration conditions. The gel was subsequently exposed to X-ray film at -70°C.

The film was developed automatically, by a Kodak X-Omat processor, model ME-3.

2. Gel-slicing and liquid scintillation counting

After being dried, each track of the gel was then cut into 5mm slices. 0.5 ml of 20% H_2O_2 was added to each slice in a scintillation vial. The vials were incubated overnight at 37°C to elute protein. A 9ml volume of Ecoscint was added into the

samples for scintillation counting.

2.2.5. Analysis of ^{3}H-labelled surface proteins

2.2.5.1. Extraction of membrane proteins from parasites

^{3}H-labelled parasites were resuspended in 1.0ml of 0.02 M Tris-HCl buffer (pH 7.4) containing 0.5M NaCl, 1mM $CaCl_2$, 1mM $MnCl_2$, 0.5% SDS and 10mM proteinase inhibitors. The suspension was incubated at 37°C for 30 mins to extract tegumental surface membrane proteins. The suspension was then centrifuged for 5 min, at 13,000rpm. The supernatant and worm body fraction were stored at -20°C.

2.2.5.2. Affinity chromatography

Affinity chromatography was performed according to the system described in Johnstone and Thorpe (1985).

Starting buffer : 0.02M Tris-HCl buffer pH 7.4 containing 0.5M NaCl,

1mM $CaCl_2$, 1mM $MnCl_2$ and 0.05% SDS

A 2.00ml gel of ConA-Sepharose 4B was packed into a 1x5 cm column, and washed with 10 times the column volume of starting buffer to remove preservative. The diluted membrane extract (2.2.5.1) was applied to the ConA-sepharose column, at a flow rate of 0.2 ml / min. After collection of the initial or 'fall through' peak, the column was washed with 20mM starting buffer using an LKB Fraction Collector to collect each fraction until the count of radioactivity was very low. The bound glycoprotein was then eluted with 10.0ml 0.2M α-methyl-D-glucoside containing 0.05% SDS solution. The volume of each elution fraction was 0.6 ml. 10 μl aliquots from each fraction were taken and the radioactivity was counted. Finally, the column was cleaned with 10.0 ml starting buffer.

2.2.5.3. Analysis of fraction eluted by α-methyl-D-glucoside from column

All fractions eluted by α-methyl-D-glucoside containing radioactivity were combined, dialyzed against distilled water overnight and dried under vacuum refrigeration conditions. The dried samples were resuspended in 200 μl sample buffer, electrophoresed on 10% SDS PAGE gel (2.2.4.1) and their radioactivity profiles were determined by gel slicing.(2.2.4.2).

2.2.6. Electrophoretic transfer and lectin overlay

2.2.6.1. ConA labelling with ^{125}I (Fraker,P.J. and Speck,J.C. 1978)

Con-A was labelled with Na^{125}I using Iodogen. The reaction mixture consisted of Na^{125}I, 1mg ConA, 10mM KI in 0.5M PBS buffer, (pH 7.4). The reaction was allowed to proceed for 15 min, in a glass tube containing Iodogen dried down from dichloromethane solution. Unbound ^{125}I label was removed using a Pharmacia PD-10 Sephadex column.

2.2.6.2. Electrophoretic transfer

Transfer buffer (pH 8.3):

> 25 mM Tris
>
> 192 mM Glycine
>
> 20% v/v Methyl alcohol
>
> 0.02% SDS

a. Proteins were separated by SDS-PAGE

b. Proteins were transferred from gel to nitrocellulose sheets by the western blot method.(Renart,T.,et al 1979. Towbin,H., et al. 1979)

In a shallow tray, a sandwich of gel / nitrocellulose was prepared as follows:

> Scotch Brite pad
>
> Filter paper
>
> Gel

Nitrocellulose paper

Filter paper

Scotch Brite pad

All were inside a transfer cassette. The sandwich was totally submerged in transfer buffer and care was taken to remove all air bubbles. The cassette was then placed in the transfer tank with nitrocellulose paper nearest the anode. The transfer was effected by either 400 mA for 3-3.5 hr or 40 mA overnight.

2.2.6.3. Lectin overlay

0.1% Amido Black (Naphthalene Black):

0.1% amido black

45% methanol

10% acetic acid

Lectin overlay buffer:

20 mM Tris

0.12 M KCl

1 mM $MnCl_2$

1 mM $CaCl_2$

1.0% Tween 20 (w/v)

0.05% sodium azide

pH to 8.0 with HCl

a. After transferring, the nitrocellulose paper was removed and a test strip was cut and stained in 0.1% amido black solution for 1-2 min. The test strip was then destained.

b. The remainder of the nitrocellulose sheet was washed on a shaker for 3hr in five changes of lectin overlay buffer.

c. The nitrocellulose sheet was shaken in 100ml lectin overlay buffer containing 1-$2x10^6$ cpm $[^{125}I]$-ConA for 1.5 hr.

d. The nitrocellulose sheet was washed in lectin overlay buffer 5-6 times until little

[^{125} I] was detectable in the buffer using a hand monitor. It was dried and autoradiography was carried out.

2.2.7. Time course experiments on binding of 3H- Tunicamycin

Fresh schistosomula were incubated at 37^0C with [^{3}H]-TM (10 μCi) in RPMI-1640 medium containing 10% FCS. At various intervals, the schistosomula were collected by centrifugation at 2000 rpm on a MSE bench centrifuge for 1 min. The parasites were washed 5 times and 100μl GMEM medium was added. 5μl suspension was collected and the number of schistosomula was counted. 5μl of solution was taken to measure [^{3}H]-TM binding to whole parasites. The remaining sample was centrifuged and parasites were resuspended in 200μl 0.2% Triton X-100 in 50 mM Tris-HCl buffer and kept on ice for 10 min. They were then vortexed for 30 seconds and centrifuged at 6500 rpm for 10 min. The supernatants and worm bodies were added to scintillation vials containing 4.5 ml of Ecoscint and counted in a Beckman liquid scintillation counter.

2.2.8.Measurement of substrate uptake by S. mansoni

Prior to labelling, parasites were incubated in the presence or absence of TM for 3hr. The parasites were then resuspended in different labelling media. Different labelled substrates were added to the incubation mixture for 45min-2h. After labelling, the parasites were washed with GMEM medium and homogenized in PBS. 5μl of homogenate was taken to measure total radioactivity. 100μl of homogenate was taken to precipitate protein by adding 10μl of 100% TCA. The resulting precipitate and supernatant were added to scintillation vials containing 4.5 ml of Ecoscint and counted in a Beckman liquid scintillation spectrometer.

2.2.9. Analysis of viability of parasites after TM treatment

2.2.9.1. In vitro experiments

Schistosomula after treatment with TM for 3h were washed three times using GMEM medium containing 5% FCS and resuspended in 2.00 ml GMEM medium with 5% FCS. The schistosomula were then cultured at $37^{o}C$ under 5% CO_2. The parasites were examined under light microscopy, and the numbers of dead schistosomula were counted after different intervals of culture.

2.2.9.2. In vivo experiments

Schistosomula after incubation with or without TM for 3hr were washed and resuspended in fresh GMEM medium. The number of schistosomula in the suspension was counted. 0.1ml of the suspension of treated or control schistosomula was then injected subcutaneously into BALB/c mice. All mice were were perfused 5 weeks after infection and surviving worms were counted.

2.2.9.3. Determination of antibody levels in mice injected with normal or TM treated schistosomula

1. Collection of anti-parasite antisera

Starting 1 week after infection, blood was collected weekly by bleeding the mice from the tail vein. Blood was allowed to clot at $4^{o}C$ overnight. The serum was collected after centrifugation on a bench Eppendorff centrifuge (MSM Microcentaur, from Beckman Instruments, Ltd), at 6500 rpm for 10 min and was stored at $-20^{o}C$.

2.Preparation of adult worm antigen

Crude adult worm antigen was prepared from freeze-dried worms kindly supplied from the World Health Organization. 2-3 mg of dried worms were re-hydrated by suspending in 1.00ml of PBS at $4^{o}C$ for 30 min and then homogenized. The homogenate was dialysed against 3000ml PBS at $4^{o}C$ overnight. The suspension was then subjected to centrifugation at 10,000 rpm for 10 min at $4^{o}C$. The supernatant

was stored in aliquots at $-20^{\circ}C$ until use.

3.Enzyme-linked Immunosorbent Assay (ELISA)

Solutions:

Washing buffer: 0.5% (w/v) Tween 20 in PBS

Blocking buffer: 2.0% (w/v) BSA and 3.0% (v/v) normal Goat serum in PBS.

Citric phosphate buffer: 17.9 ml of 0.1M citric acid and 32.1ml of 0.2M Na_2HPO_4 were mixed and the total volume was made up to 100 ml to give a solution of pH6.0.

Substrate solution: 4mg O-phenylene diamine was added to 1.00 ml of 0.1%(v/v) hydrogen peroxide and 9ml of citric phosphate buffer (pH 6.0).

Each well of a 96-well microtitre plate (Dynatech Micro Elisa Systems) was coated with 100 μl adult worm antigen (1mg protein in PBS) and was then covered and left overnight at $4^{\circ}C$. The plate was washed three times with washing buffer and was blocked with blocking buffer (100μl / well) for 30 min at room temperature to block the unreacted sites in the wells. After washing 3 times, different serial dilutions of antiserum were then added to the plate, followed by incubation at $37^{\circ}C$ for 2hr. The plate was washed 3 times before addition of 100μl of second antibody (anti-mouse IgG peroxidase conjugate, 1:1000 dilution in PBS) to each well. The plate was left at $37^{\circ}C$ for 2 hr and subsequently washed 3 times. 100μl of substrate solution was added to each well and incubated in the dark for 15-20 min. Reaction was stopped by adding 50μl of 2 M H_2SO_4 to each well and the colour was measured at 492 nm using a Titretek Multiskan Spectrophotometer.

2.2.10. Fluorescence microscopy

2.2.10.1. FITC-lectin labelling of parasites

The parasites collected following treatment with TM for 3hr or 21hr were washed three times and labelled with FITC-lectin for 30 min at 37^0C in $200\mu l$ GMEM medium containing $50\mu g$ FITC-lectin/ml. The parasites were then washed 3 times using GMEM medium. The parasites were resuspended in 20-25 μl GMEM and $10\mu l$ 0.2% carbachol was added to immobilize the worms. Finally, the labelled parasites were mounted on slides for examination under the fluorescent microscope (Leitz Ortholux I).

2.2.10.2. Analysis of FITC-ConA release from labelled parasite

Live or 1% formaldehyde fixed parasites were incubated with FITC-ConA for 30min at 37^0C. The labelled parasites were washed three times and cultured in GMEM medium containing TM ($10\mu g$ / ml) with or without 5% FCS for 21hr at 37^0C under 5% CO_2. Control specimens were incubated in GMEM medium with or without 5% FCS. The parasites after culture were washed three times using medium. The FITC-ConA remaining on the surface of parasites was examined under fluorescent microscope (Leitz Ortholux I).

2.2.10.3. Incorporation of fluorescent probes

Schistosomula and 3 week worms were incubated in 1 ml of GMEM medium with $10\mu g$ 5 (N-octadecanoyl) - aminofluorescein (C_{18}-Fl) for 10 min at room temperature. The parasites were then washed extensively to remove any free probe. The labelled parasites were examined by placing them within a square of silicone grease with 100 μl of GMEM medium containing $50\mu l$ of the paralysing agent carbamyl choline.

2.2.10.4. Indirect fluorescent antibody method

The parasites which had been incubated with TM for 3hr or 21hr were incubated in 200µl GMEM medium containing normal serum or antiserum for 30 min at 37°C. The parasites were washed 3 times with medium and 100µl of FITC-labelled rabbit antibody against mouse IgG was added. A further 30 min incubation at 37°C followed. The parasites were washed and resuspended in 40µl GMEM medium containing 10µl carbachol (0.2%). Finally, the parasites were placed on slides for examination under the fluorescent microscope (Leitz Ortholux I).

Normal serum or antiserum was used at 1:10 dilution. FITC-rabbit antimouse-IgG was employed at a 1:100 dilution.

2.2.10.5. Measurement of fluorescence intensity

Fluorescence was quantified by photon counting from an area of approximately 100 μm^2 using a fluorimeter attached to a Leitz Ortholux I microscope. The result are expressed in arbitrary units (mean + standard deviation). The count rate was corrected for background, autofluorescence and dark current.

Fluorescence photo-micrographs were taken with a standard camera attachment of a Leitz Ortholux II fluorescence microscope, and Ektachrome 1600 film.

2.2.10.6. Fluorescence Photobleaching Recovery

Measurement of the two-dimensional lateral diffusion of C18-Fl and succinylated FITC-ConA was made by the fluorescence photobleaching recovery method(Axelrod, et al. 1976.; Rees, et al 1984). A low-powered, water-cooled argon ion laser, and a X 40 water-immersion objective lens were used throughout. The 1 / e^2 radius of the focussed laser spot at the sample was calculated to be 2.2µm (Foley et al. 1986). The lateral diffusion coefficient (D_L) was calculated from the kinetics of the recovery curve (Axelrod et al. 1976), and the percentage recovery of fluorescence was based on the estimated signal at infinite time after photobleaching, as calculated from a computer-fitted curve.

2.2.11. Electron microscopy (Hockley and McLaren 1973)

Solutions:

i. 0.2M Cacodylate buffer (pH 7.4):

> 4.28g Na cacodylate
>
> 5.4 ml 0.2M HCl

made up to 100 ml with distilled water, and pH checked.

ii. 2% Fixation solution:

> 4.00 ml 25% glutaraldehyde
>
> 12.5 ml of 0.2M Na cacodylate
>
> 0.5 ml of 0.2M Ca acetate

pH was adjusted to 7.4 with HCl, and the volume was then made up to 50 ml with H_2O.

iii. Rinsing buffer:

> 24 ml of 0.2M Na-cacodylate
>
> 1.00 ml 0f 0.2M Ca-acetate

pH was adjusted to 7.4 with 0.2M cacodylic acid and the volume was then brought up to 100 ml with 0.13M sodium sulphate.

After incubation with TM, parasites were washed 3 times in cacodylate buffer and then exposed to:

a. 2% fixation solution for 4h at $4^{O}C$ in a fume cupboard, followed by washing 3 times in rinsing buffer. The sample were kept in this buffer overnight at $4^{O}C$.

b. The parasites were fixed in 1% osmium tetroxide in 100mM Na-cacodylate buffer, pH7.4, for 2hr at $4^{O}C$. The worms were washed 10 times in deionised water.

c. The worms were further fixed in 0.5% aqueous uranyl acetate containing 45mg/ml sucrose for 1.5h at $4^{O}C$ in darkness.

After several washes in deionised water, the worms were dehydrated in a graded ethanol (10-100%). The samples were then kept in 100% ethanol until they were

processed for transmission electron microscopy by Dept. of Pathology, Western Infirmary, Univ. of Glasgow by Dr. I. More and Mr. T. Downie.

2.2.12. Measurement of the production of inositol phosphates in S. mansoni

2.2.12.1. Labelling of parasites with [^{3}H]-inositol

The parasites were labelled in DMEM medium containing [^{3}H]- inositol at 2μCi / ml at 37°C, under 5% CO_2 for 18hr. The labelling medium was removed and parasites were rinsed five times with fresh GMEM medium.

2.2.12.2. Stimulation by different ligands

The labelled parasites were resuspended in 1.00ml GMEM medium and incubated with Li^+ (10mM). Different ligands were added to stimulate inositol phosphate production. The control group was incubated with DEME medium alone. Ahter stimulation, the parasites were washed and homogenized in 0.24 ml PBS. 0.94ml $CHCl_3$:MeOH (1:2) was added to the homogenate and mixed rapidly. The samples were left to stand for at least 10min at room temperature.

2.2.12.3. Extraction of lipid

0.31ml $CHCl_3$ and 0.31ml H_2O were added into each tube containing parasites, mixed rapidly and centrifuged at 2000rpm in a MSE bench centrifuge for 5min. The upper phase containing water-soluble extracts and the lower phase containing lipid-soluble extracts were collected. The volumes of the two phases were measured.

2.2.12.4. Assays of total inositol phosphates in upper-phase

0.8ml of upper phase was removed into a fresh tube and 4.0ml dist.H_2O was added to decrease the ionic strength. These samples were applied to 2.0ml of gel of Dowex 1x8-formate . The column was washed with 1).2 x 5ml H_2O; 2). 2 x 5ml

5mM sodium tetraborate/60mM ammonium formate; 3). 2 x 5.0ml H_2O. The inositol phosphates were then eluted with 3 x 5.0ml of 1.2M ammonium formate / 0.1M formic acid. A 1.0ml portion of each fraction was taken for liquid-scintillation counting.

2.2.13. Assay of protein phosphorylation in S.mansoni
2.2.13.1. Labelling parasites with [^{32}P]-phosphate

Parasites were incubated in phosphate-free medium containing $100\ \mu$Ci [^{32}P]-phosphate for periods indicated. The parasites were then washed four times with GMEM medium and centrifugated four times.

2.2.13.2. Preparation of samples for SDS-PAGE

Washed parasites radiolabelled with [^{32}P]-phosphate were homogenized in PBS. The homogenates were dissolved in sample buffer.

2.2.13.3. Assay of protein phosphorylation which is stable to KOH treatment

The labelled proteins were separated by SDS-PAGE (2.2.4.1). The gel was then stained by Coomassie blue and destained. The SDS-gel was incubated in 1N KOH plus 5mM NaH_2PO_4 at 55°C for 1.5hr. During the incubation period, the medium was changed once. For the control, a gel was incubated in 5mM NaH_2PO_4 under the same conditions. The gels were then transferred into destain solution for 30min and washed with H_2O 3 times. Finally, the gels were dried on a piece of Whatman 3MM paper under vacuum refrigeration conditions and autoradiography performed as usual.(2.2.4.2)

Chapter 3: Effect of tunicamycin on different life cycle stages of S. mansoni

3.1. Glycoprotein synthesis in different life cycle stages of Schistosoma mansoni

Several studies have demonstrated that the human blood fluke S. mansoni contains numerous glycoproteins. Some workers investigated surface glycoproteins on S. mansoni. (Hayunga and Sumner 1986a,b) by using lectin affinity chromatography or metabolic labelling methods which involved using radioactive hexoses. However, this study was only performed on adult worms, and not on immature forms. The results from lectins labelled with ^{125}I or conjugated with fluorescein indicated that modification of the exposed carbohydrates of S. mansoni occurs during maturation of the parasites in vivo (Simpson et al. 1983). Here, a ^{3}H-mannose labelling method to determine the change in the pattern of glycoprotein synthesis at different life cycle stages of S. mansoni has been used. The reason mannose was chosen was due to the fact that many glycoproteins have carbohydrate side-chains in which mannose is commonly present.

3.1.1. To determine whether TCA affects the glycoprotein profile in SDS PAGE

During some experiments, the total proteins of 24 day worms were precipitated by 10% TCA after ^{3}H-mannose labelling. It is known that TCA can solubilize some glycoproteins. Therefore, firstly it had to be determined whether the TCA had an influence upon the glycoprotein profile in the sample. After labelling, the parasite sample was separated into two parts. In one part of the sample TCA was used to precipitate total protein and then the pellet dissolved in sample buffer. The other part was dissolved in sample buffer without treatment with TCA. The results are shown in Fig.3.1. From this figure, it is clearly shown that there are no major differences between the two samples. Clearer peaks are in TCA treated homogenates.

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Fig. 3.1. To determine whether TCA had an influence upon glycoprotein radioactivity profile of SDS-PAGE
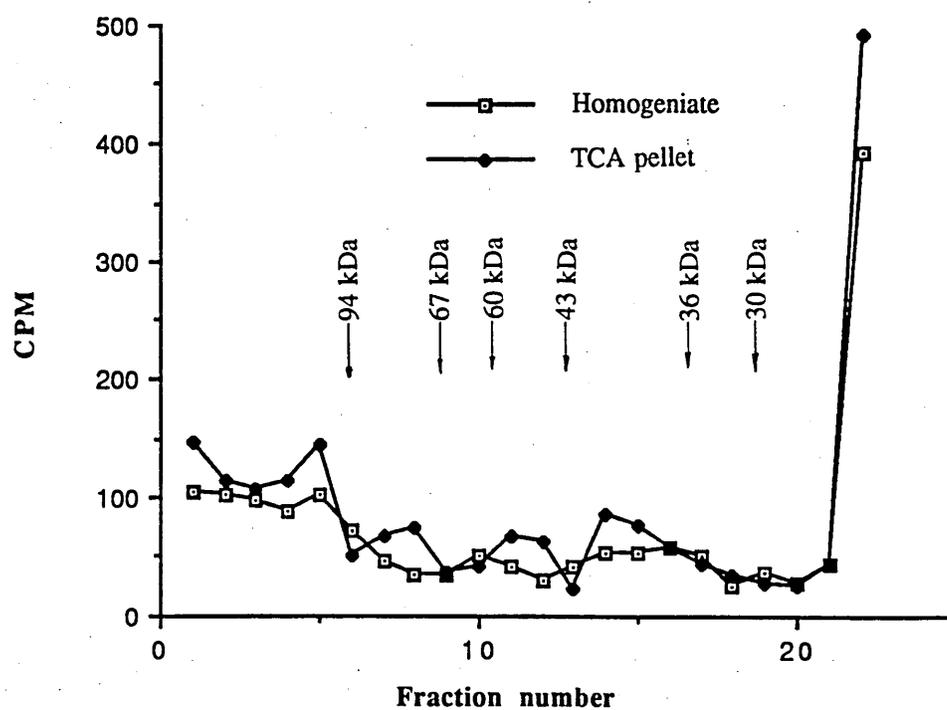
24 - day worms incubated in RPMI-1640 medium containing 25 μCi ^{3}H-mannose / ml for 4hr at 37°C. The conc. of SDS-PAGE Gel is 10%.

3.1.2. Determination of the changes in glycoprotein synthesis at different growth stages of S.mansoni, by metabolic labelling using ^{3}H-mannose

The changes in glycoprotein synthesis at different growth stages of S.mansoni have been determined by metabolic labelling using ^{3}H-mannose. The result of this experiment is illustrated in Fig.3.2. In cercaria and schistosomula stages, there is no significant incorporation detectable after slicing and counting the gel except near the dye front, but some incorporation can be determined in 3 week old and adult worms after 4hr incubation. In all cases, the labelled ^{3}H-mannose was found in a prominent peak co-migrating with the dye front. After cercariae were transformed into schistosomula, the amount of radioactivity in this part was greatly increased. In order to know what components are present in this peak, a 15% SDS-PAGE was prepared instead of a 10% SDS-PAGE gel. In 15% SDS-PAGE, this very highly radioactive, rapidly- migrating fraction did not appear at the dye front (see Fig.3.3). This peak could be some glycolipid or low molecular weight glycopeptides.

3.1.3.ConA overlay

In the metabolic labelling experiment, after a short time incubation with ^{3}H-mannose, there is no significant incorporation in cercariae and schistosomula. Therefore, the glycoproteins at different stages of S. mansoni were characterised by SDS-PAGE followed by ConA overlay and autoradiography. After autoradiography, ConA was bound to most of the glycoproteins of 3 week, 4 week and 6 week worms. (results not shown) When a comparison was made with the autoradiography pattern, there was only a slight difference between growth stages in low molecular weight material, which may be due to glycopeptides and glycolipids.

3.1.4.Affinity chromatography

The surface glycoproteins on S. mansoni labelled metabolically using radioactive hexoses as precursors were analysed by lectin affinity chromatography. Tegumental

Fig.3.2. SDS-PAGE (10%) profile of ^{3}H-mannose labelling of different life stages of S.mansoni

Cercariae

94 kDa

CPM

Fraction number

Schistosomula

43 kDa

CPM

Fraction number

3 week worms

adult worms
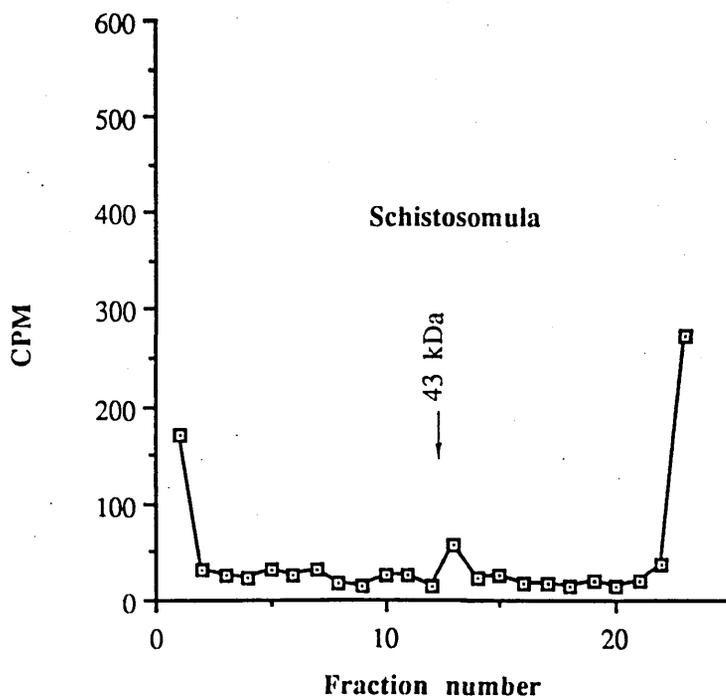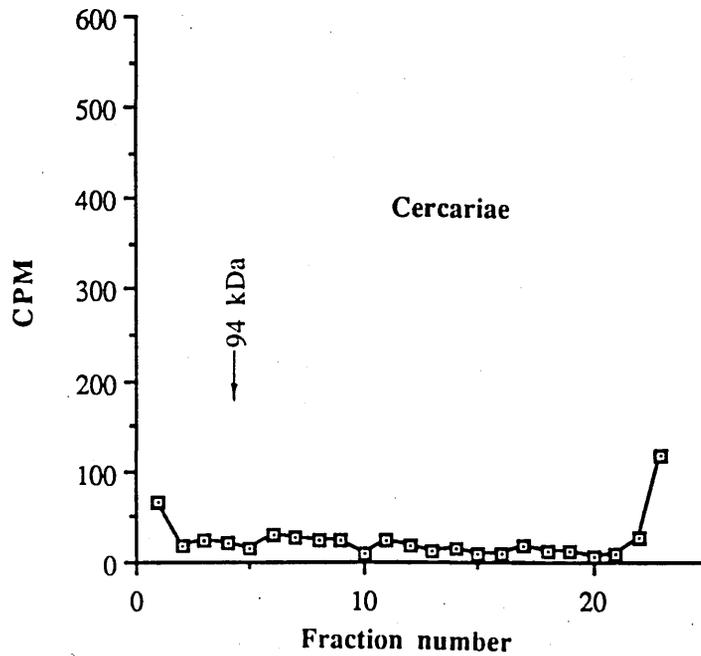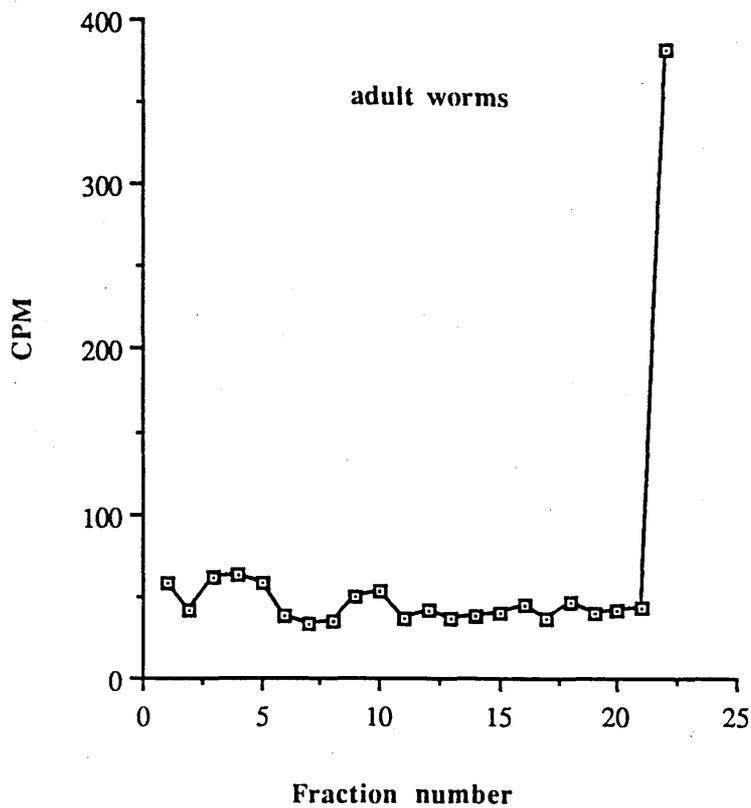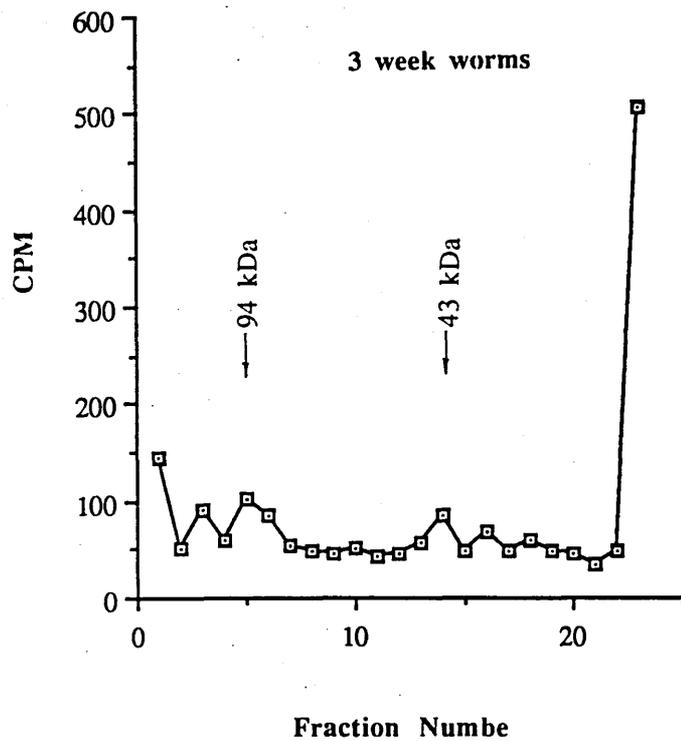
Cercariae were incubated in Cu^{++} - free water containing 25 μCi ^{3}H-mannose / ml for 2hr. The other parasite stages were incubated in RPMI-1640 medium containing 25μCi ^{3}H-mannose / ml for 4hr. The gel was cut into 5mm slices to count radioactivity.

Fig.3.3. A determination of those components present at the dye front of 10% SDS - PAGE using 15% SDS-PAGE
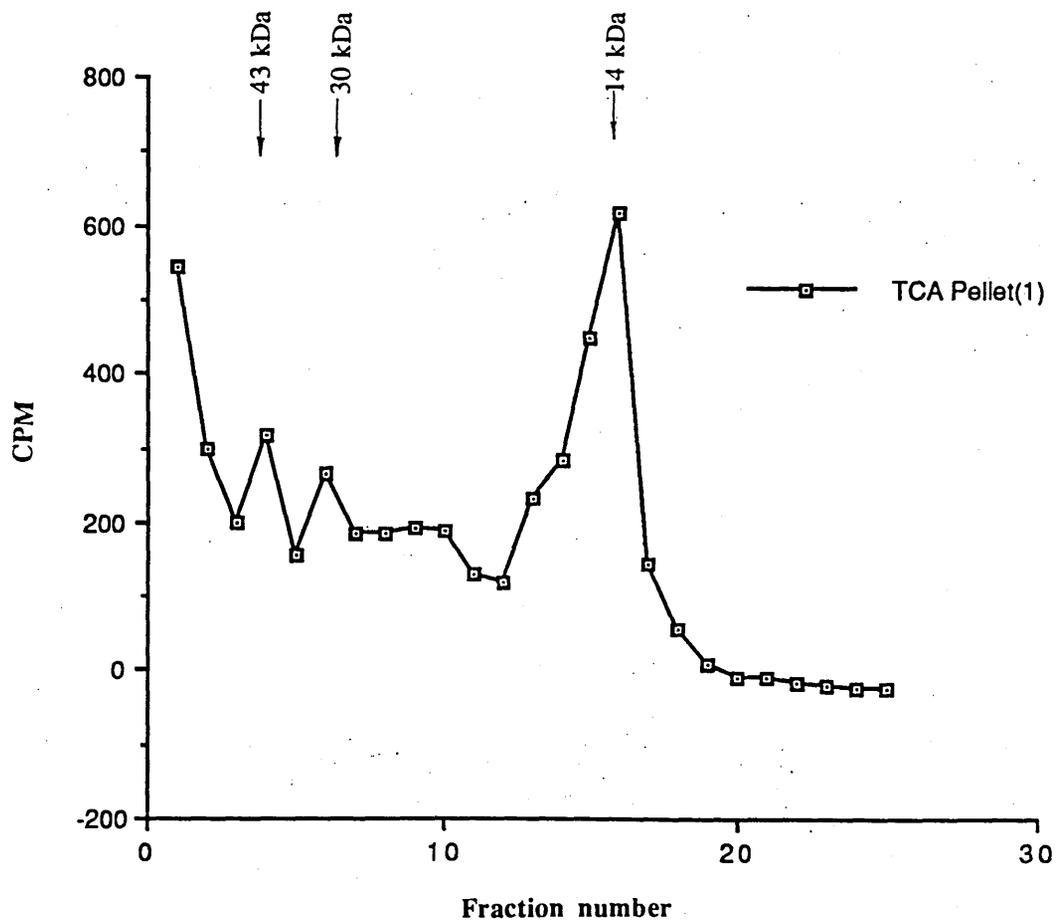
24 - day worms incubated in RPMI-1640 medium containing 25µCi ^{3}H-mannose at 37°C for 4hr. The gel was cut into 5mm slices to count radioactivity.

material was extracted with SDS, then applied to ConA-Sepharose. The profile obtained is shown in Fig.3.4. Most of the proteins passed through the column, and were not bound to it. They are in peak one. Peak two represents material bound by the column. It was eluted by 0.2 M α-methyl-glucoside. The result when peak two was analysed by SDS-PAGE is shown in Fig.3.5.b. Most of the c.p.m. are recovered in the peak at about mol. wt.60 kDa. In addition, there was a very light peak between mol. wt. of 330 kDa and 94 kDa. However, after affinity chromatography, there is no very high radioactive count at the dye front. In contrast, extracts of whole worms before affinity chromatography have a very high radioactive count at the dye front (3.5 a), possibly due to glycolipid and glycopeptides. This material does not appear to bind to ConA or it can not be eluted.

3.1.5. Discussion:

All parasite stages of S.mansoni express complex surface carbohydrates and the exposed carbohydrates on the surface undergo an important modification during maturation in vivo as shown by lectin binding. The way in which the parasite makes these changes can be explained by considering that the changes may be due to the exchange of glycosylated molecules with the host.(Simpson et al 1983), since the lectin binding profile exhibited by adult worms is indistinguishable from that exhibited by the erythrocytes of their host (Simpson et al 1983). However, another reason for the alteration of carbohydrates may be due to active modifications mediated by the parasite's biosynthetic functions. Although Nyame et al (1987, 1988 a) studied the structure of O-linked and N-linked oligosaccharides synthesized by adult male schistosomes, the metabolic events associated with the synthesis of glycoproteins, oligosaccharides and glycolipids in the schistosome are still not very clear. In the metabolic labelling experiments described here, it was found that significant amounts of ^{3}H-mannose were not incorporated into proteins of cercariae and newly transformed schistosomula. The prominent peak co-migrating with the dye front was however

Fig.3.4. Affinity chromatography with ConA - Sepharose
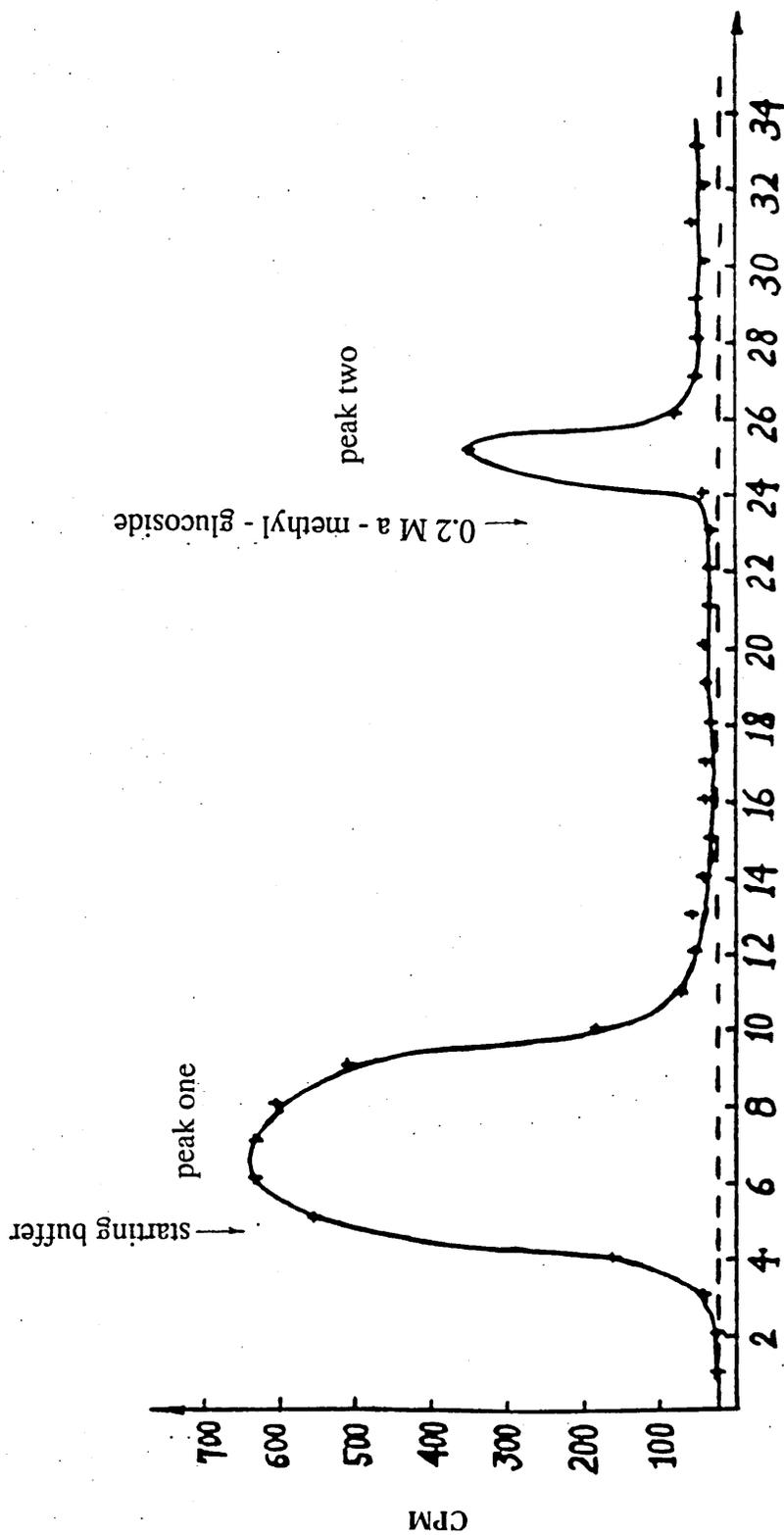
Column bed volume: 2.00 ml. Flowing rate: 0.6 ml / 3mins. Sample volumn: 3.00 ml of diluted membrane extract solution containing 0.05 % SDS. Elution solution was 0.2 M α - methyl - D - glucoside containing 0.05 % SDS. 10μl was taken out from each fraction to count.

Fig.3.5. Comparison of whole worm tegumental material extract of labelled adult worms (a) and α - methyl - glucoside elute of this material on a ConA column (b).

(a). SDS -PAGE analysis of whole worm tegumental material extract.
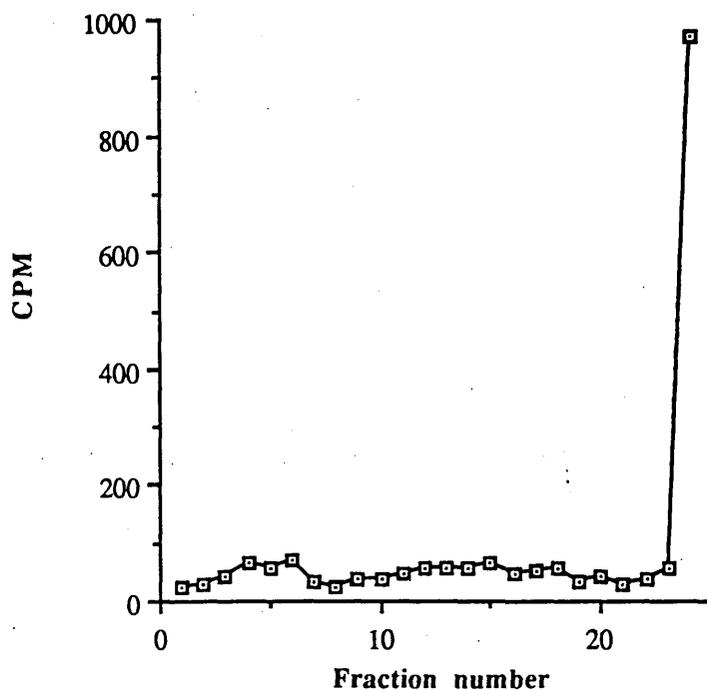
The preparation of the whole tegumental extract is seen in section 2.2.5.1.

(b). SDS - PAGE analysis of the α - methyl - glucoside eluates from concanavalin A - Sepharose 4B columns.
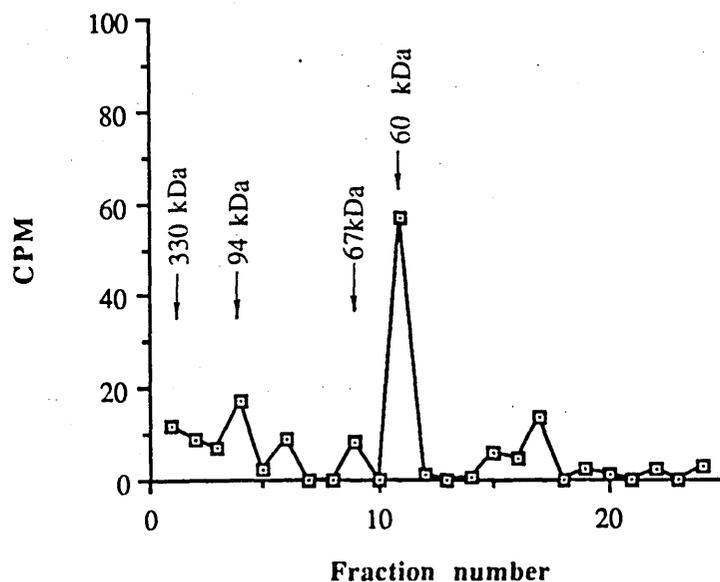
The preparation of the sample is seen in section 2.2.5.1

heavy labelled. Although it is known that newly transformed schistosomula bind ConA strongly, it seems that these mannosyl residues of exposed oligosaccharides of schistosomula do not represent newly synthesised glycoproteins of the schistosomula but are pre-formed during cercarial development. The reason for this may lie in the fact that there is little biosynthetic activity at this stage of growth of the parasite, with membrane manufacture occurring later.

With the maturation of parasite, a quite significant amount ^{3}H-mannose is taken up and incorporated by 3 week old worms. This could reflect the fact that this stage is metabolically very active. The growth of the parasites is initiated after arrival in the liver. The glycosyl transferase activity was found to be associated with the surface membrane of adult worms, which are capable of utilizing most of the sugars detected on the schistosome (Simpson. and Smithers 1980; Simpson et al 1983). MacGregor and Kusel (1989) have also shown that a peanut agglutinin-binding glycoprotein of molecular weight about 170 kDa could be detected on the worm surface in large amounts at four weeks post-infection whereas this glycoprotein was absent from pre-liver worms. Therefore, it is not surprising that ^{3}H-mannose is incorporated into adult worms.

The surface glycoproteins on S. mansoni labelled metabolically using ^{3}H-mannose were analysed by ConA-Sepharose. Most of the labelled proteins did not bind to the column and were in peak 1. A few labelled proteins bound to the column and could be eluted from it as a sharp peak (peak 2) by 0.2M α-methyl-glucoside. When peak two was analysed by SDS-PAGE, there was no very highly radioactive material at the dye front. It could be that this material is still bound to the column or else does not bind to the column. Previous studies (Cummings and Kornfield. 1982., Ogata, et al 1975., Krusius et al 1976) have shown that ConA-Sepharose has different affinities for different oligosaccharides structures. Studies on the binding of oligosaccharides and glycoproteins to ConA-Sepharose have indicated that at least two nonsubstituted or 2-O-substituted α-mannosyl residues are required. Fig.3.6 shows

Fig.3.6. Structures of Asn - linked oligosaccharides.(Reproduced from Schachter et al 1983)

COMPLEX TYPE GLYCOPEPTIDES
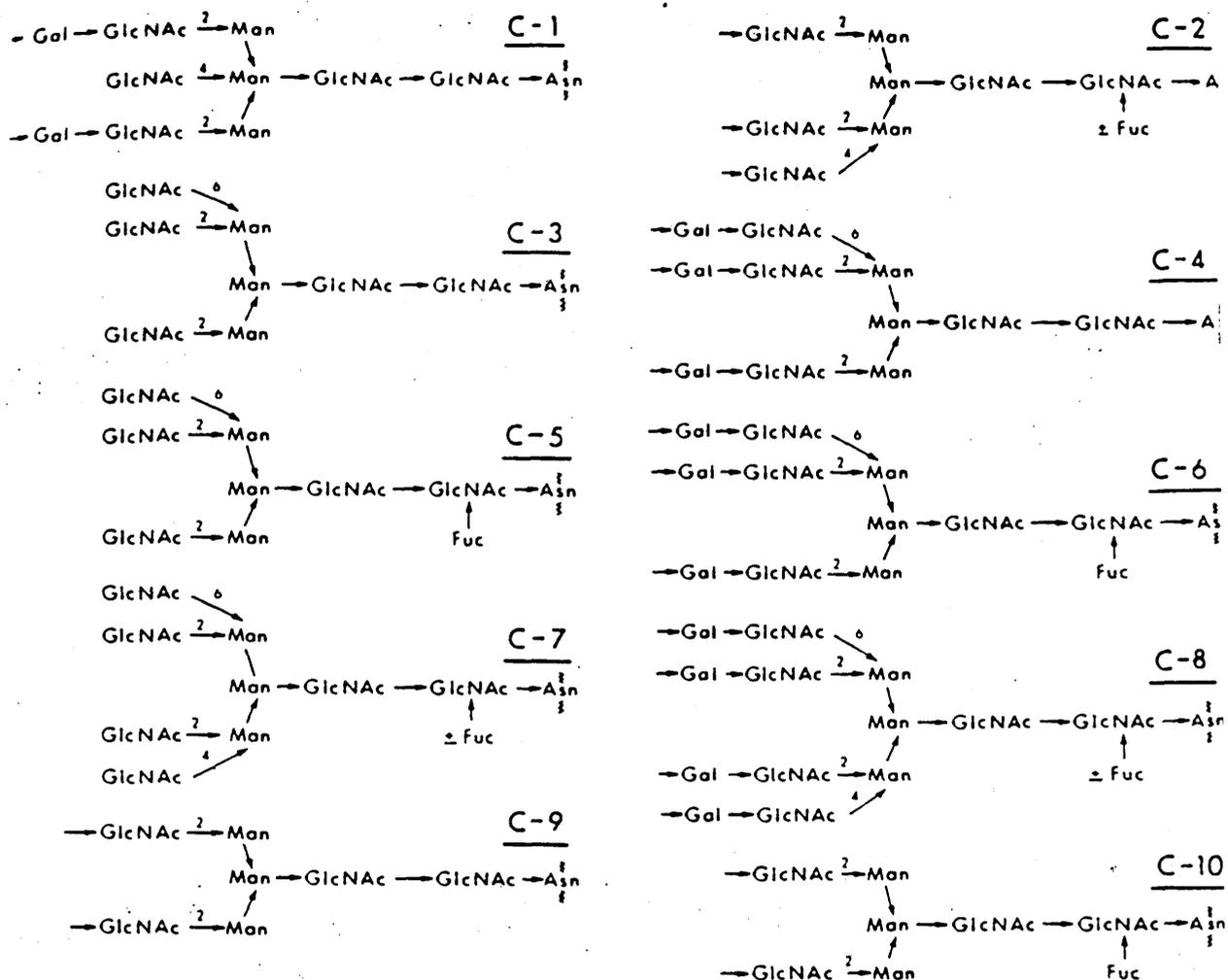
HIGH MANNOSE TYPE GLYCOPEPTIDES
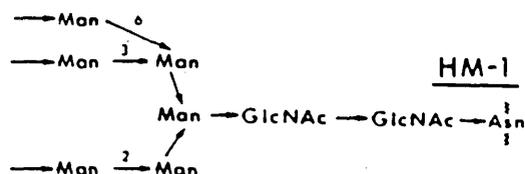
HYBRID TYPE GLYCOPEPTIDES
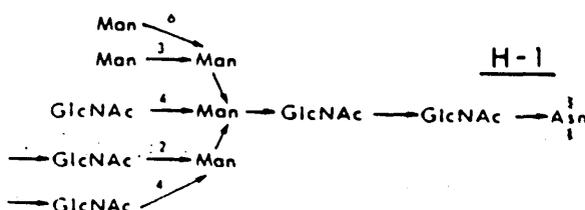
the structure of N-linked oligosaccharides. Glycopeptides having two or three peripheral branches are called ' biantennary ' and ' triantennary ' glycopeptides. ConA-Sepharose interacts with very low affinity with most O-linked oligosaccharides and complex-type bisected biantennary, tri- and tetra- antennary N-linked oligosaccharides and these can be eluted with buffer alone. However, ConA-Sepharose interacts with relatively high affinity with many of the complex-type non-bisected biantennary N-linked oligosaccharides (Cummings and Kornfield, 1982., Ogata et al. 1975., Krusius et al 1976) and these can be eluted from it. C_9 and C_{10} biantennary glycopeptides containing two 2-O-substituted α - mannose residues shown in Fig.3.6. will be bound and other will not. The high mannose type glycopeptides consist of only mannose and N-acetylglucosamine. Since the glycopeptides in this case contain a large cluster of α-mannosyl residues, which are frequently α 1 - 2 linked or branched to form non-reducing α-mannosyl residues, the glycopeptides of this type are highest affinity for ConA Sepharose and need a much higher methyl α-glucoside concentration for their elution. The complex type glycopeptides contain galactose and fucose in addition to mannose and N-acetylglucosamine. The number of mannose residues in glycopeptides of this type is small. Moreover, α-mannosyl residues are usually substituted by N-acetylglucosamine, which is the starting point of side chains. Therefore, it is suggested that this type of glycopeptide shows different degrees of affinity with ConA Sepharose according to the number and mode of attachment of side-chains to α-mannosyl residues in the core structure. The affinity chromatography result, (Fig. 3.5) could confirm that the glycoproteins in S.mansoni contain different oligosaccharides structures. Nyame et al (1988a) have analysed the high mannose asparagine - linked oligosaccharides synthesized by S. mansoni and shown that this type of glycoproteins can be eluted from ConA - Sepharose with 100 mM α-methylmannoside. Thus the very highly radioactive material at the dye front may contain complex - type bisected biantennary, tri- and tetra- antennary N-linked oligosaccharides, since a very high concentration of methyl α-glucoside for the elution was used in my experiment.

In all cases, the prominent peak co-migrating with the dye front appears to be rapidly and highly labelled. This very highly radioactive material could be some low molecular weight glycopeptides. When the same sample was run on 15% SDS-PAGE, this very highly radioactive, rapidly - migrating fraction did not appear at the dye front

3.2. Effect of tunicamycin on glycoprotein and protein synthesis in S. mansoni

3.2.1. Effect of TM on glycosylation in S.mansoni

5 week adult worms were incubated with different concentrations of TM for different times at $37^{\circ}C$ prior to labelling for 2.5 hr with ^{3}H-mannose. The level of ^{3}H-mannose incorporation into total acid-precipitable glycoprotein was assessed. These results are illustrated in both Fig.3.7. and Table.3.1. which show the data from two individual experiments. It was found that there was a great difference between a lymphocyte cell line and adult worms. It appears that the lymphocyte cell line is more sensitive to TM under the same experimental conditions. When the incorporation of ^{3}H-mannose into total TCA - precipitable material was inhibited by 86%-92% in lymphocytes, there was only 15%-30% inhibition in the adult worms. Even with the increased concentration of TM (15μg/ml) and incubation time (6hr), the extent of inhibition was never large.

In schistosomula, the inhibition of ^{3}H-mannose incorporation into total acid-insoluble glycoprotein was about 24% after incubation with TM for 3hr and labelling with ^{3}H-mannose for 3hr.

The time of parasite incubation with TM and labelling with ^{3}H-mannose was increased up to 24hr and the samples analysed by SDS-PAGE. TM (Conc.10μg / ml) appears to inhibit synthesis of some glycoproteins, although the incorporation of ^{3}H-mannose into total TCA-precipitable protein was still only inhibited by 27%. The results of two individual experiments are shown in Fig.3.8 (a),(b). The molecular weights of these glycoproteins are about 60 kDa, 38 kDa, 32 kDa and < 10 kDa near

70

Fig.3.7. **Effect of TM on incorporation of ^{3}H-mannose into adult worm protein**
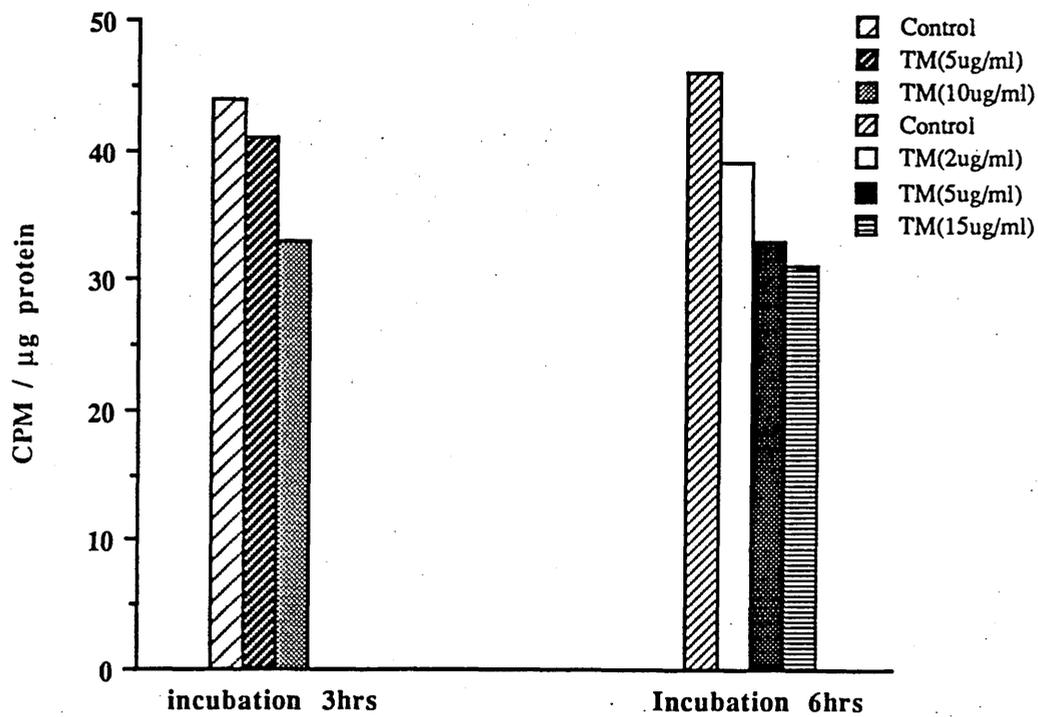
Prior to labelling, parasites were incubated in presence or absence of TM for 3 or 6 hr at 37°C. Then, parasites were resuspended in RPMI-1640 medium. 25μCi of ^{3}H-mannose was added into the incubation medium. At the end of labelling period, the worms were washed and homogenized, 10% TCA was added to form a precipitate and the samples were counted for radioactivity.

Fig.3.8. Effect of TM on ^{3}H-mannose incorporation into adult worms after 24h incubation

(a). Experiment 1

(b). Experiment 2
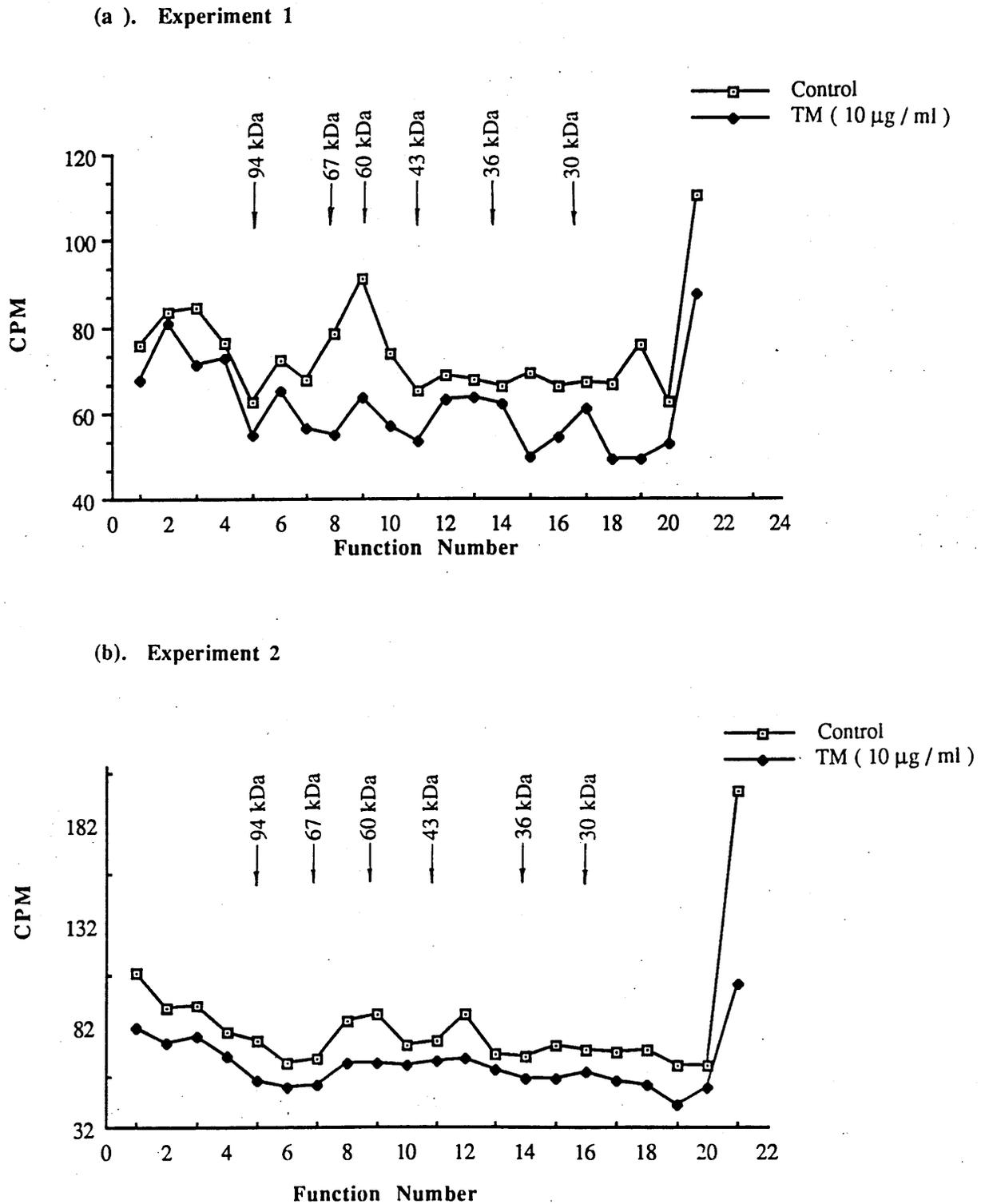
Worms were incubated in RPMI-1640 containing 5% FCS, 10μg / ml TM and 25μCi ^{3}H-mannose for 24hr at 37°C. The Conc. of the gels is 10%. The gels were cut into 5mm slices to count radioactivity.

Table.3.1. Effect of TM on 3H-mannose incorpoeation into total-precipitable glycoprotein in S.mansoni (5 week adult worms) and a mouse lymphocyte cell (MOPC-3K)

	S.mansoni				Lymphocyte Cell (MOPC – 3K)		
TM Conc.(µg/ml)	0	10	5	2	0	5	2
TCApp Counts/10µl	1454	1539	1613	2044	2501	234	371
Protein(µg)/10µl	35	51	43	58	38	45	41
Counts/protein(µg)	42	30	37	35	65	5.2	9.0
Inhibition(%)	/	29	12	17	/	92	86

dye front. There is a little difference in the kinds of glycoprotein inhibited by TM between the two experiments. However, some inhibition of incorporation into 60 kDa protein was seen in both experiments.

When schistosomula transformed by skin penetration were incubated with TM (10μg/ml) and labelled for 24 hr, the amount of ^{3}H-mannose incorporated into peaks of molecular weight 43 kDa and at the dye front was decreased.(See Fig3.9)

3.2.2. Protein synthesis by different life cycle stages of S. mansoni in the presence or absence of tunicamycin

Although TM appeared to inhibit glycosylation after 3, 6 hr treatment and inhibited some kinds of glycoprotein synthesis in S.mansoni after 24hr treatment, several studies have suggested that glycosylation and protein synthesis are linked in such a way that when glycosylation is blocked, the synthesis of certain proteins is prevented (Elbein,1987). Protein synthesis at different life cycle stages of S.mansoni that had been incubated with TM for 3hr was therefore measured. Different life cycle stages of S.mansoni were incubated with TM for 3hr. The data are presented in Fig.3.10. It is apparent that the percentage of inhibition of ^{35}S-Met incorporation into acid-insoluble protein at different development stages of S. mansoni was between 1-24%. However, it was found from TCA soluble radioactivity that there was very high inhibition of methionine uptake, especially for 22 day worms and 40 day worms. (Fig. 3.10, control and TM total) The inhibition of methionine uptake was about 58-68%. Thus, it appeared that TM affected uptake of substrates by S. mansoni. TM might be affecting the properties of the parasite surface membrane and some amino acid transport systems.

3.2.3. Discussion

Earlier studies by Rumjanek et al (1978, 1979) reported that homogenates of adult S. mansoni contained glycosyl transferase which transferred mannose, glucose,

Fig.3.9. Effect of TM on ^{3}H-mannose incorporation into skin transformed schistosomula after 24hr incubation with this drug.
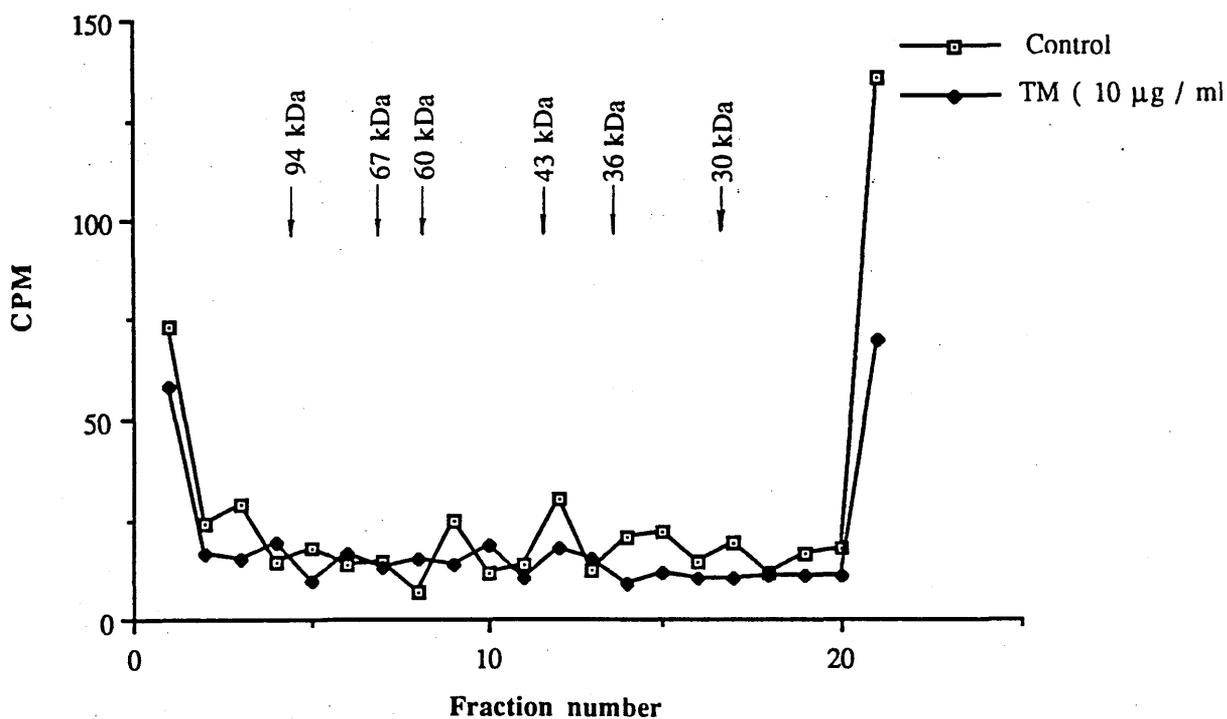
Skin tranformed schistosomula were incubated in RPMI-1640 medium (1% FCS) containing 25μCi ^{3}H-mannose and TM (10μg / ml) for 24hr at 37°C. The conc. of SDS-PAGE is 10%. The gel was cut into 5mm slices to count radioactivity.

Fig.3.10. Effect of TM on incorporation of 35S - methionine into protein
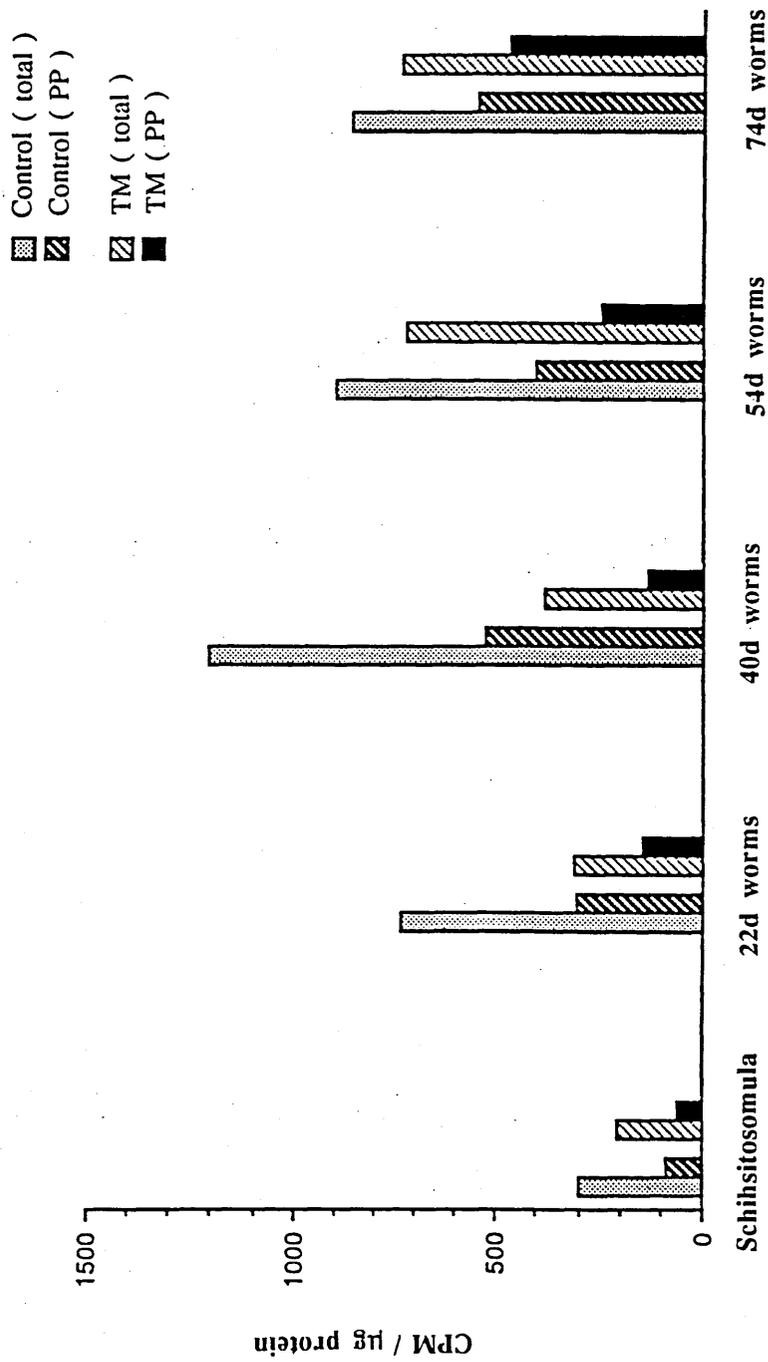
Prior to labelling, parasites were incubated with TM (10μg / ml) for 3hr. Then, the parasites were resuspended in EMEM modified medium without Met. 25μCi 35S-Met was added into each sample and incubated for 2hr at 37°C. At the end of labelling period, the parasites were washed and homogenized. 10μl was taken out to count total radioactivity. 10μl was taken out to precipitate protein by 10% TCA and count for radioactivity in precipitated protein.

and galactose from GDP-mannose, UDP-glucose and UDP-galactose respectively to a lipid acceptor. The lipid acceptor is believed to be an intermediate in the synthesis of the parasite's glycoproteins and in the biosynthesis of oligosaccharides and glycolipids. The experiments presented in this section demonstrate that the pathway for biosynthesis of N-linked glycoprotein in S. mansoni appears to be similar to that in mammalian cells. TM can affect glycosylation in adult worms.

The pathway for biosynthesis of N-linked glycoproteins in mammalian cells is shown in 1.2.4. TM, as a inhibitor of lipid-linked saccharide formation is a useful tool to distinguish N-linked and O-linked glycoproteins. The site of action of TM is in the first step of the lipid linked saccharide pathway, thus preventing the formation of any lipid intermediates and inhibiting the N-linked glycosylation of newly synthesisd proteins. In contrast to N-linked glycosylation, O-linked oligosaccharide biosynthesis in animal cells is initiated by the addition of GalNAc residues to Ser or Thr residues. (Cummings. et al 1983). O-linked glycosylation is unaffected by TM treatment.

The degree of inhibition by TM of glycosylation in S. mansoni is not as high as that in lymphocytes. When the adult worms were preincubated for 30min with TM and labelled for 2.5hr with ^{3}H-mannose in the presence of TM, the incorporation of ^{3}H-mannose into trichloracetic acid-insoluble fractions was decreased by 15-30%, whereas under the same experimental conditions, the incorporation of ^{3}H-mannose was inhibited almost 89-95% in lymphocyte cells. With the increased concentration of TM (15 μg/ml) and incubation time (6hr), the extent of inhibition did not change. A possible explanation for the present data is that the pathway for synthesis of N-linked glycoproteins in S. mansoni is not very active. Most of the glycoproteins which are present in S. mansoni might contain O-linked oligosaccharide. Recent studies from Nyame. et al (1987, 1988a) have reported that S. mansoni contains two types of glycoproteins, O-linked and N-linked glycoproteins. Another explanation for the lack of TM inhibition in S. mansoni is that there may be a slight difference in the structure of the TM - sensitive enzyme in parasites as compared to lymphocytes. This might affect the extent of action of this antibiotic.

In order to know whether TM inhibits synthesis of certain kinds of glycoproteins, the adult worms and skin transformed schistosomula were labelled for 24hr with ^{3}H-mannose in presence or absence of 10 μg/ml of TM and samples were examined by SDS-PAGE. In schistosomula, decreased incorporation of ^{3}H-mannose was found only at the dye front. However, ^{3}H-mannose incorporation was decreased in the peaks of molecular weight about 60 kDa, 43-36 kDa, 36-30 kDa and the dye front part in adult worms. The result of ConA Sepharose shows that these different peaks comprised glycoproteins (Fig. 3.5) which could contain different structures of N-linked oligosaccharide. The glycoproteins of molecular weight about 60 kDa in peak two (Fig. 3.5) may have complex-type non-bisected biantennary N-linked oligosaccharide, or high mannose N-linked oligosaccharide, since they bound to ConA Sepharose and were eluted by 0.2M α-methyl-glucoside. Molecules at the dye front may have complex-type tri- and tetraantennary and bisected biantennary N-linked oligosaccharide, Taking these results together, it could be concluded that S.mansoni can synthesize N-linked glycoproteins. This synthesis can be partially inhibited by TM. The structure of the N-linked oligosaccharides has been shown to be different in schistosomula from that in adult worms.

The effect of TM on protein synthesis at different life cycle stages of S. mansoni was also measured, using ^{35}S-Methionine. Protein synthesis (i.e. ^{35}S-Methionine incorporation into TCA precipitable material) was inhibited between 1-24% at concentration of TM 10 μg/ml. However, it was found that there was very high inhibition of methionine uptake, specially in 22 day and adult worms. Studies on methionine uptake by larval and adult S.mansoni (Chappell , 1974) showed that all stages of the parasite have two kinetically distinguishable systems for absorption, one which is saturable and a second which appears to be simple diffusion. Sodium, temperature and pH all have an effect on the rate of methionine uptake in both larval and adult schistosomes. The difference in response to temperature (Chappell, 1974) by juveniles and by adults might be best explained in terms of physical properties of the

tegument. The effect of TM on methionine uptake by schistosomes may also result from a change in the properties of the tegument.

I conclude that in schistosomula and adult worms althouh TM does significantly affect protein synthesis, Part of its effect could be due to an inhibition of amino acid transport.. In order to study the consequences of inhibition of glycosylation by TM, we studied the surface of parasites by lectin binding, antibody binding and FRAP.

3.3. Effect of TM on outer membrane of S. mansoni at different stages in the life cycle

3.3.1. Effect of TM on FITC-lectin binding to S.mansoni

Lectins are proteins that can agglutinate erythrocytes and other cell types. Lectin binding is usually quite specific, each lectin only binding to certain carbohydrate receptors. Therefore, lectins have provided a powerful tool in the analysis of carbohydrates and have been used to gain information on the nature of the carbohydrates of many cell surfaces. In the present experiment, ConA, WGA and PNA are chosen. They are specific for D-mannose, D-glucose; N-acetyl-D-glucosamine and D-galactose, respectively.

3.3.1.1. Binding of FITC-ConA to newly transformed schistosomula

Quantitation of FITC-ConA binding to freshly transformed schistosomula that had been treated with TM for 3hr or 24hr is shown in Table 3.2.and Fig.3.11. In all cases, there was a significant increase in ConA binding to TM treated schistosomula, as compared with normal schistosomula. This increased binding was greatly reduced by 0.1 M α-methyl-glucoside. When parasites were incubated with TM for longer times, the amount of ConA bound to TM treated parasites was reduced by FCS in medium, suggesting that FCS may prevent this effect of TM on the parasite surface.

Table.3.2. The binding of FITC-ConA to fresh schistosomula after TM treatment for 3hr

Expt.No.	Units of Fluorescence(X±S.D)(n)			
	Normal	TM(20µg/ml)	TM(10µg/ml)	TM(5µg/ml)
1.	22.0±8.9(10) (5.6±1.1)(10)	80.6±25.4(10) (11.6±4.2)(10)	77.1±58.6(10) (10.1±3.0)(10)	45.9±13.1(10) (7.1±3.4)(10)
2.	13.2±4.6(15) (3.8±1.9)(11)	47.9±23.6(15) (5.7±3.6)(10)	40.4±27.1(19) (3.8±1.2)(10)	23.7±11.1(27) (6.6±5.9)(16)
3.	45.0±8.9(39) (13.5±3.9)(20)	87.4±26.3(77) (25.3±5.5)(25)	77.4±26.0(35) (21.0±3.4)(24)	65.4±29.9(33) (19.5±6.7)(20)

Mechanically transformed schistosomula were incubated with TM at different conc. in GMEM medium for 3hr at 37°C, washed 3 times, incubated in FITC - ConA at 50µg / ml for 30 min at 37 °C, washed and mounted.
All values in parentheses are the binding in the presence of 150 mM of the appropriate competing monosaccharide.
All P values are at < 0.001(compared with normal).

Fig.3.11. Effect of TM on FITC-ConA binding to schistosomula after 24hr incubation with TM

Experiment 1

Significance: 0.000   0.000
(T - test)

Experiment 2

Significance: 0.000   0.394
(T - test)
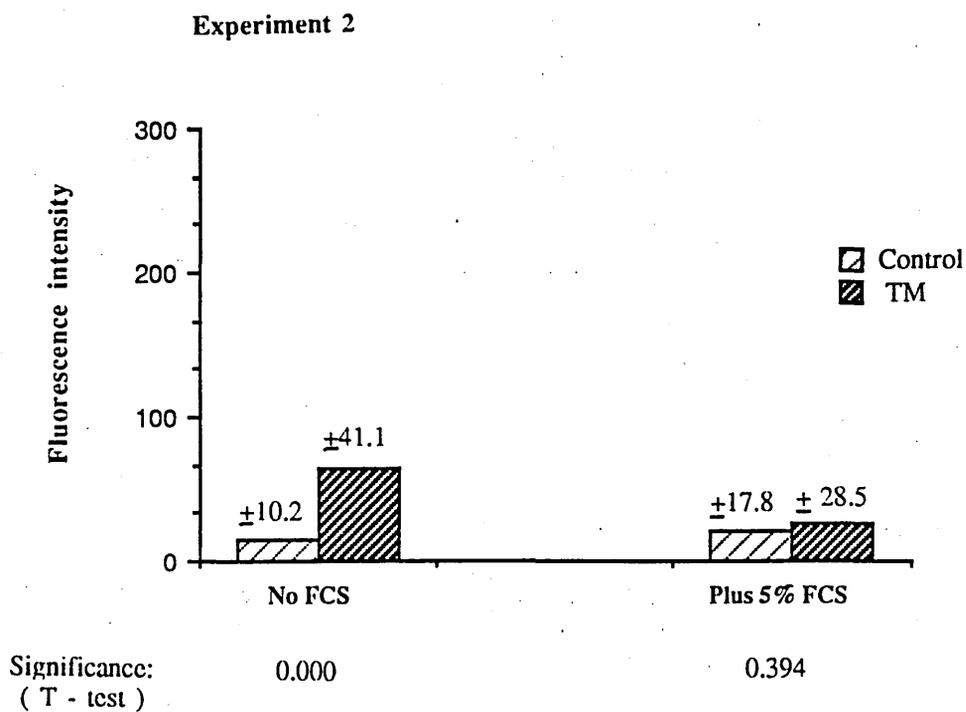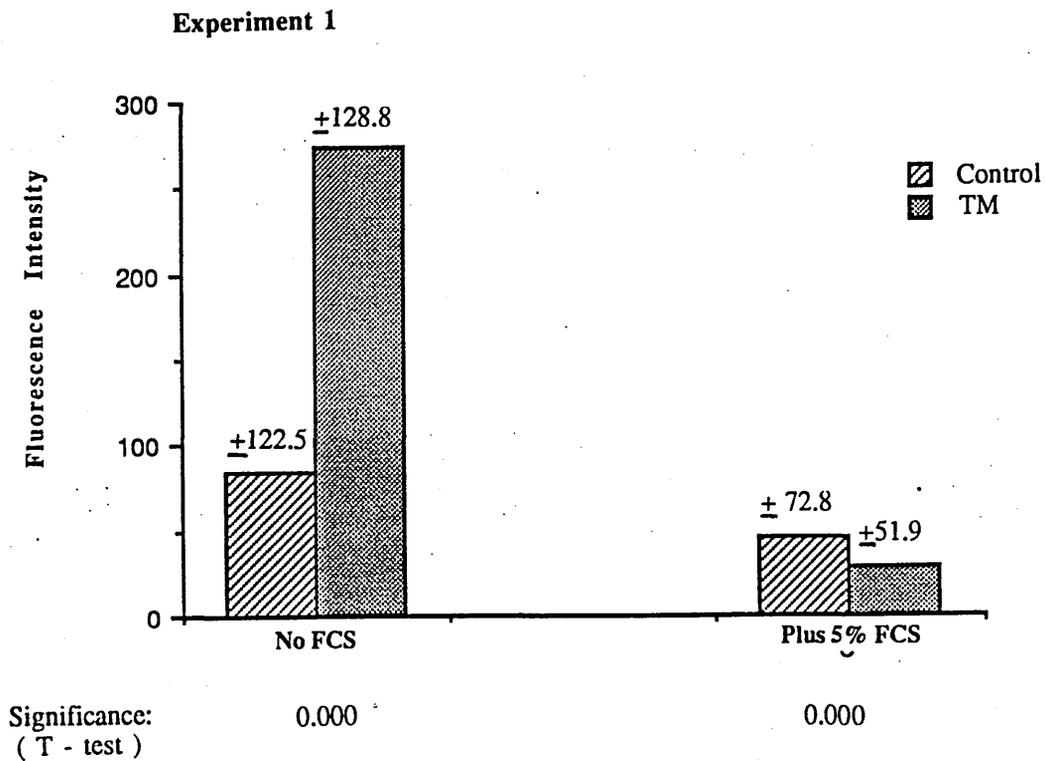
Mechanically transformed schistosomula were incubated in GMEM medium containing 5% FCS or without FCS in presence of 10 μg TM / ml or in absence of TM for 24hr at 37°C, respectively. After incubation, parasites were labelled by FITC-ConA at conc. of 50 μg/ml for 30 min, washed and mounted.

3.3.1.2. Binding of FITC-PNA and WGA to newly transformed schistosomula

To investigate whether this increase in ConA binding was a general phenomenon for increased lectin binding after TM treatment, the binding of FITC-PNA and WGA were investigated. Fig.3.12 (b). and Fig.3.13 (b) are fluorescent photographs of fresh mechanically transformed schistosomula labelled with FITC-PNA and WGA, respectively, after incubation with TM for 3hr. Fig.3.12.(a) and Fig.3.13 (a) are bright - field micrograph of the same schistosomula, respectively. Fig.3.14 (a,b,c,d,) show the bright - field micrographs and fluorescent photographs of normal schistosomula labelled with FITC - PNA and WGA, respectively. It was seen that there was a very large increase in fluorescence after TM treatment. The fluorescence intensity was also measured.(see Table3.3). The intensities of binding of FITC-PNA and WGA to TM treated schistosomula are similar to that detected using FITC-ConA. However, the increase compared to untreated parasites is higher for PNA and WGA binding than for ConA binding. The binding of WGA was not reduced in presence of excess of the corresponding sugar (Conc. 150 mM). Fig. 3.15. (a,b,c,d,) show the fluorescent photographs of normal and TM treated schistosomula labelled with FITC - PNA and WGA in presence of excess of the corresponding sugar (Conc. 150 mM) respectively. This may be due to the special properties of WGA. It is known that WGA binding is partly due to a non-specific charge interaction.(Simpson and Smithers, 1980).

3.3.1.3. Binding of FITC-ConA to lung Worms, 22 day worms and 54 day adult worms after TM pretreatment for 3hr

As the parasite matures, the density of surface carbohydrate on the schistosomula decreases.(Simpson et al 1983). When the binding to this stage of parasites was assayed, those not treated with TM were less fluorescent, as compared with fresh schistosomula, (see table.3.4.) After these parasites were pretreated with TM for 3hr, however, TM showed a significant effect on ConA binding to lung worm and 22 day worm. To 54 day worm, there appears to be an increase in ConA binding when TM

Fig.3.12. Light microscopy of TM treatment schistosomula labelled with FITC - PNA.

(a). Bright - field micrograph of TM treatment schistosomula.

(b). Fluorescence micrograph of the same schistosomula.
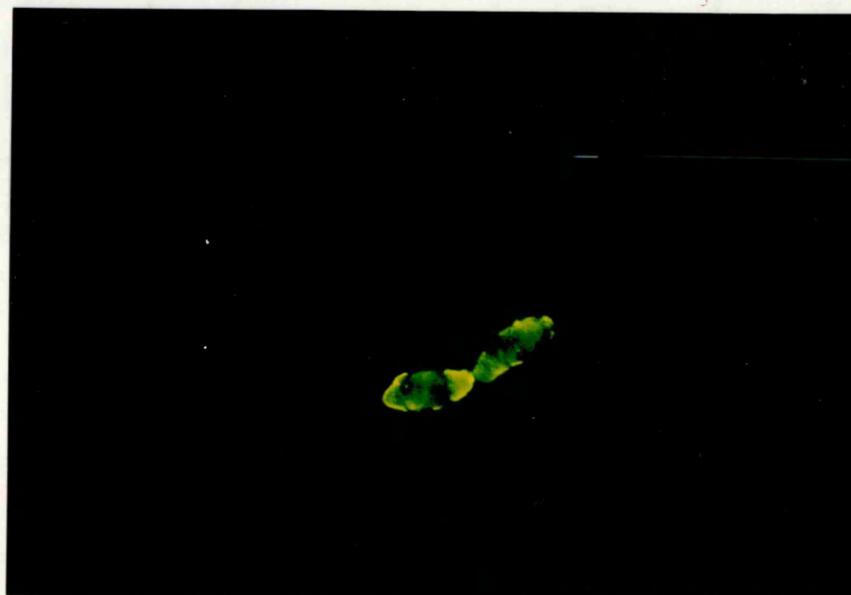
Mechanically transformed schistosomula were incubated in GMEM medium containing TM (10μg/ml) for 3hr at 37°C. After incubation, the parasites were labelled with FITC - PNA for 30min, washed and mounted.

Fig.3.13. Light microscopy of TM treatment schistosomula labelled with FITC - WGA.

(a). Bright - field micrograph of TM treatment schistosomula.
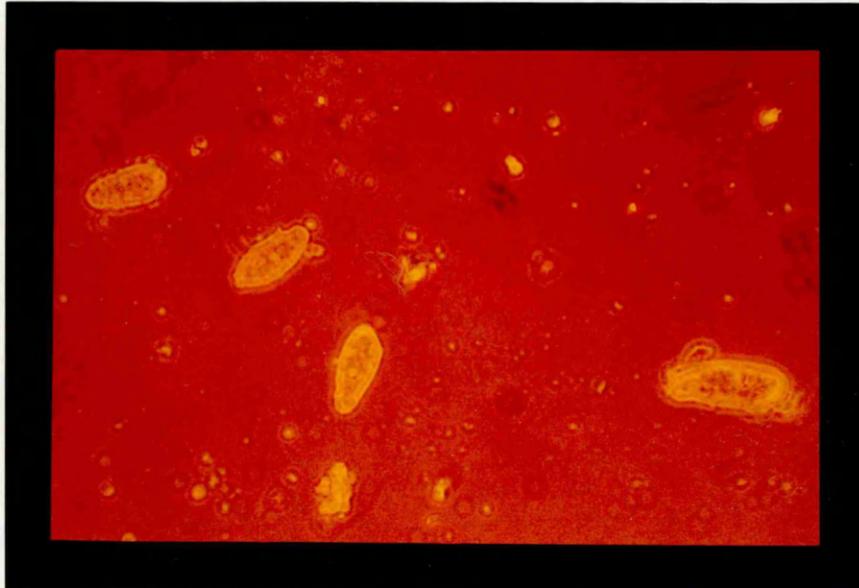
(b). Fluorescence micrograph of the same schistosomula.
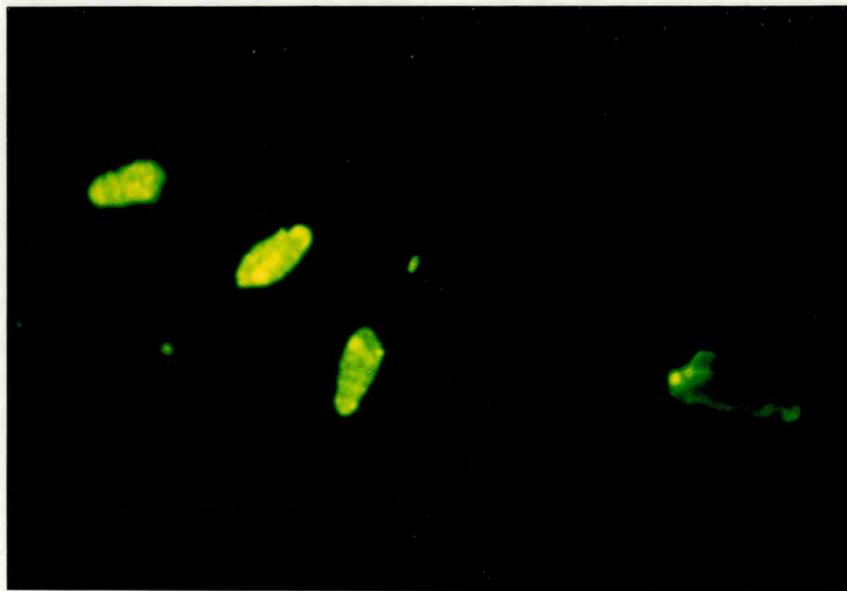
Mechanically transformed schistosomula were incubated in GMEM medium containing TM (10μg/ml) for 3hr at 37°C. After incubation, the parasites were labelled with FITC - WGA for 30min, washed and mounted.

Fig.3.14. Light microscopy of normal schistosomula labelled with FITC - PNA and FITC - WGA, respectively.

(a). Bright - field micrograph of normal schistosomula labelled with FITC - PNA.

(b). Corresponding fluorescence micrograph of the same schistosomula.
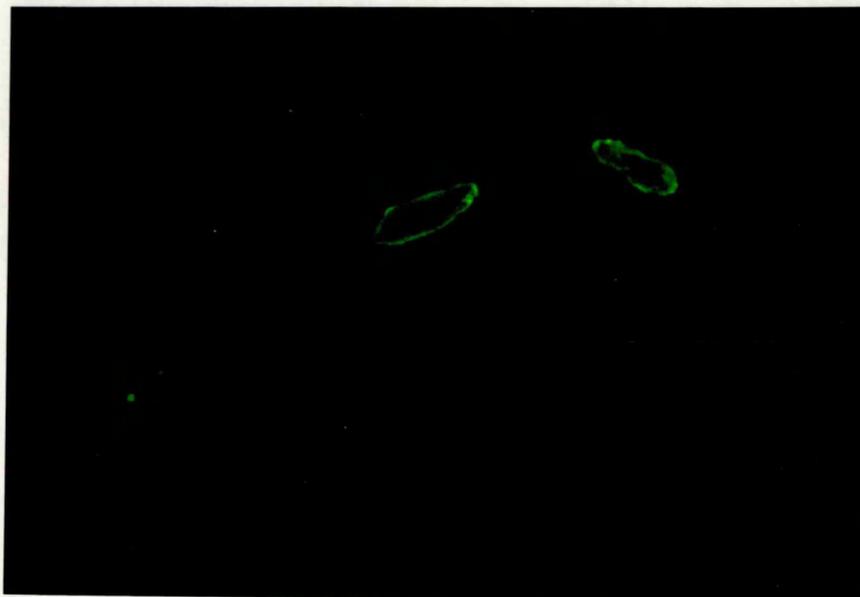
(c). Bright - field micrograph of normal schistosomula labelled with FITC - WGA.
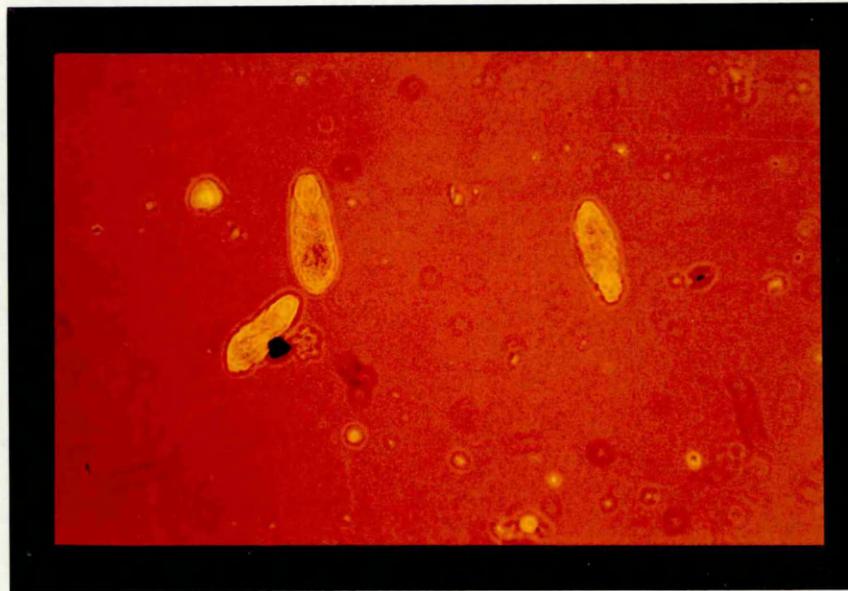
(d). Corresponding fluorescence micrograph of the same schistosomula.
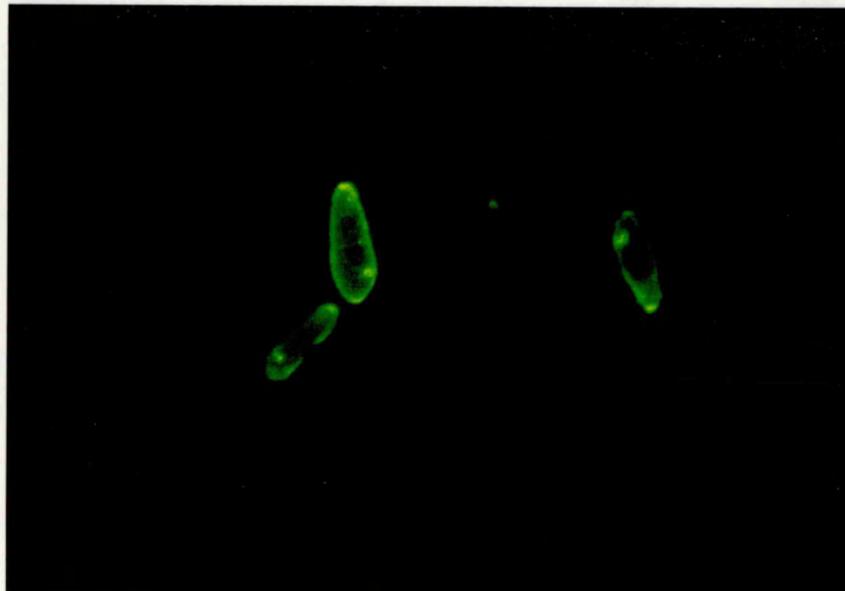
Mechanically transformed schistosomula were incubated in GMEM medium for 3hr at 37°C. After incubation, the parasites were labelled with FITC - WGA for 30min, washed and mounted.

Fig.3.15. Fluorescence micrograph of normal schistosomula and TM treatment schistosomula labelled with FITC - PNA and FITC - WGA in presence of excess of the corresponding sugars, respectively.

(a). Normal schistosomula labelled with FITC - PNA.
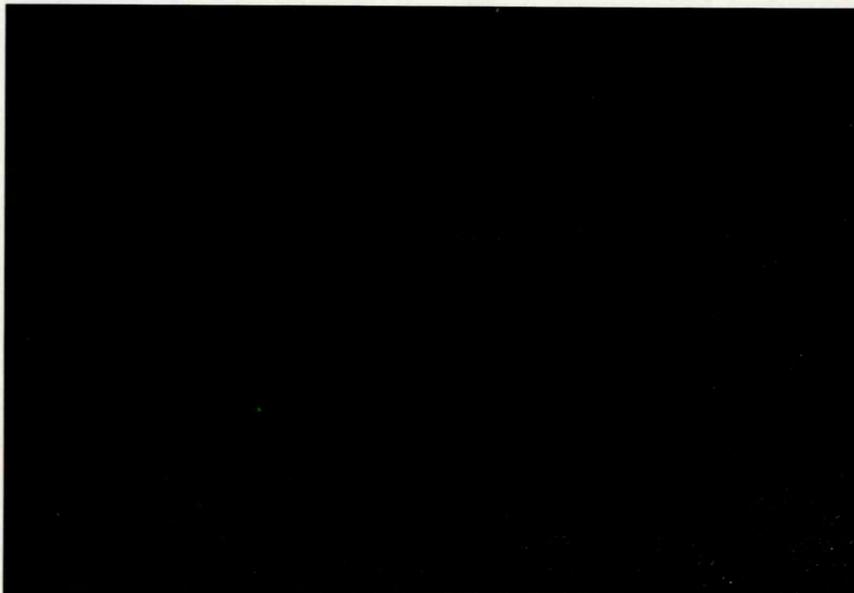
(b). TM treatment schistosomula labelled with FITC - PNA.
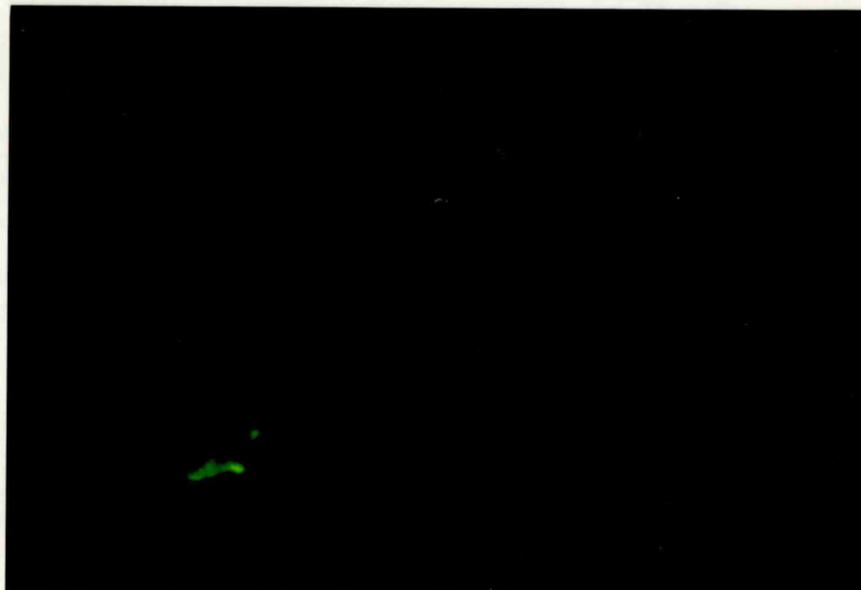
(c). Normal sehistosomula labelled with FITC - WGA.
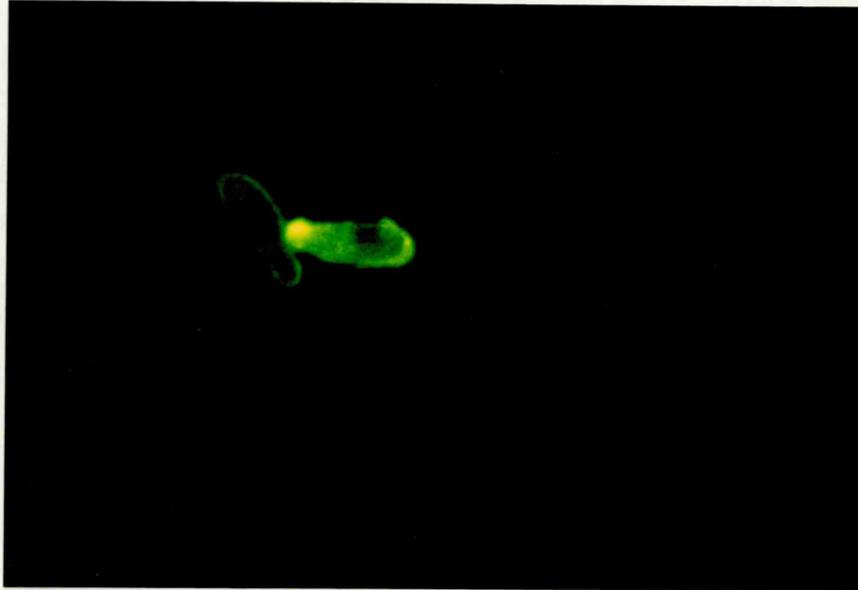
(d). TM treatment schistosomula labelled with FITC - WGA.
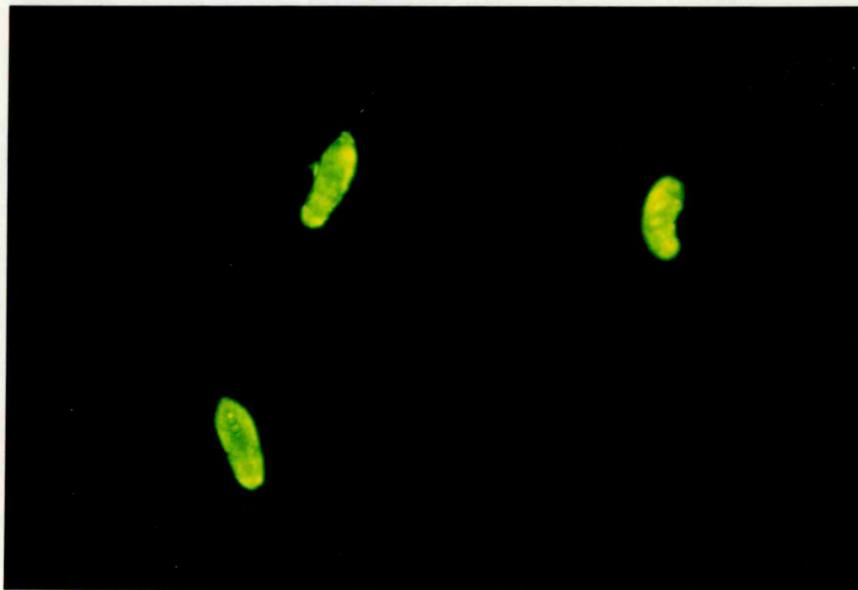
Mechanically transformed schistosomula were incubated in GMEM medium containing TM (10µg/ml) for 3hr at 37°C. In control group, schistosomula were incubated in GMEM medium for 3hr. After incubation, the parasites were labelled with FITC - PNA and FITC - WGA for 30min in presence of excess of the corresponding sugars, respectively, washed and mounted.

Table.3.3. The binding of FITC-PNA and WGA to fresh schistosomula after TM treatment for 3hr

Lectin.	Units of Fluorescence (X±S.D) (n)				
	Normal	TM(20µg/ml)	TM(10µg/ml)	TM(5µg/ml)	
PNA	29.4±9.1(67) (2.5±0.9)(30)	264.1±13.1(34) (13.5±6.4)(17)	153.5±58.0(57) (15.1±15.8)(24)	174.1±51.4(34) (6.5±4.7)(12)	
WGA	70.9±19.1(44) (52.4±17.7)(49)	417.3±155.5(17) (343.1±197.5)(25)	315.6±155.8(57) (264.9±99.1)(20)	207.6±94.7(38) (167.2±87.9)(38)	

After treatment with TM for 3hr, the parasites were incubated in FITC - PNA and WGA in GMEM medium
for 30 min at 37°C, respectively, washed 3 times and mounted.
All values in parentheses are the binding in the present of 150mM of the appropriate competing monosaccharide.
All the the values of P are at 0.000 (compared with normal)

89

Table.3.4. The relative binding of FITC-ConA to lung worm, 22day worm and 54day worm after TM treatment for 3hr.

TM Concentration	Relative fluorescence $(X \pm S.D)$ (n)					
	lung stage worm	P	22 day worm	P	54 day worm	P
Control	2.5±2.3(40)		12.8±4.2(10)		5.6±3.8(6)	
5µg/ml	(n.d)		42.6±25.4(13)	0.002	6.6±1.2(7)	0.49
10µg/ml	19.3±17.2(29)	0.000	(n.d)		5.3±2.1(7)	0.86
20µg/ml	(n.d)		22.6±12.0(14)	0.04	10.2±3.3(7)	0.04

Different stages of parasites were incubated in different conc. TM in GMEM medium for 3hr at 37°C, washed 3 times, incubated in FITC - ConA 50µg / ml for 30 min at 37 °C, washed and mounted.

n.d.: not done

90

Conc.is 20 μg/ml, however, at Conc. of 10 and 5 μg/ml, the ConA binding does not increase.

3.3.1.4. Binding of FITC-ConA, PNA to adult male and female worms after pretreatment with TM for 24hr

After the worms were incubated with TM for 24hr, male and female worms became separated. The binding of the above two FITC-lectins to males and females was measured. Four regions from head to tail in each worm were counted. There were no significant differences between these parts. Therefore, the mean value for fluorescence of four parts was used to describe the binding of ConA or PNA in one worm as given in Fig.3.16. or Fig. 3.17. There was a significant increase in PNA binding to males that had been pretreated with TM either in medium without 5% FCS or with FCS, However, TM did not affect PNA binding to females.(see Fig. 3.16.)

Fig.3.17 shows the ConA binding experiment. It appears that TM treatment only increased in ConA binding to males when the medium was free of FCS. In no other situation was any difference in binding observed.

3.3.1.5. Effect of TM on FITC-ConA labelled glycoprotein shedding from surface membrane of Schistosomula

Membrane turnover is one of the very important properties of the surface of S.mansoni. Experiments attempting to measure membrane turnover indicated that the outer bilayer had a half-life of 2-3 hr in adult worms (Wilson and Barnes, 1977. Zhou and Podesta,1989). Kusel and Mackenzie (1975) have also detected surface protein turnover using a double labelling technique. In contrast, although schistosomula appeared to have a more stable membrane, most ligands including complement receptors, nonphysiological haptens such as TNP, and carbohydrates in general are released from the schistosomula, with a half-life of 10-15hr. Samuelson and Caulfield (1982) have reported that both covalently labelled glycoproteins and glycolipids are

Fig.3.16. Effect of TM on FITC-PNA binding to adult worm after incubation with TM for 24 hr
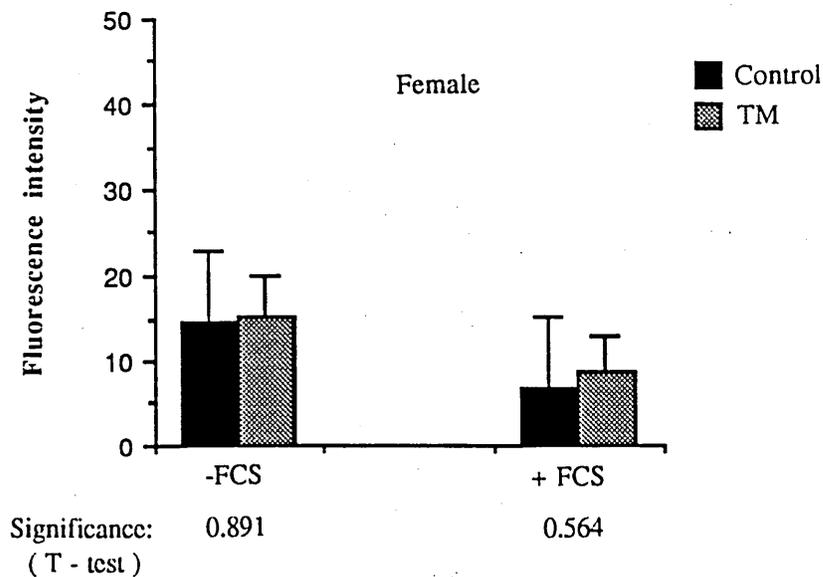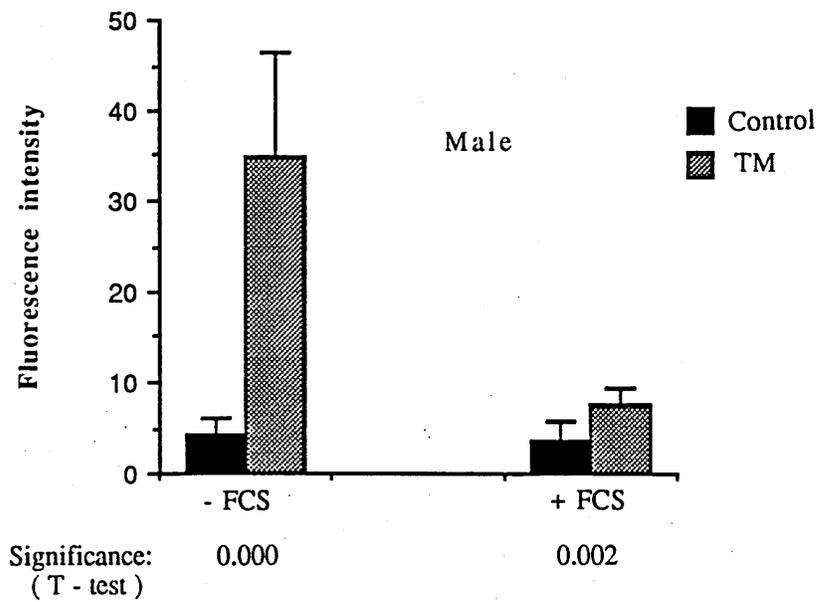
Adult worms were incubated in GMEM medium with FCS or without FCS in presence of or in absence of TM for 24hr at 37°C, washed 3 times, labelled with FITC - PNA for 30 min at 37°C, washed and mounted.

Fig.3.17. Effect of TM on FITC-ConA binding to adult worm after incubation with TM for 24hr
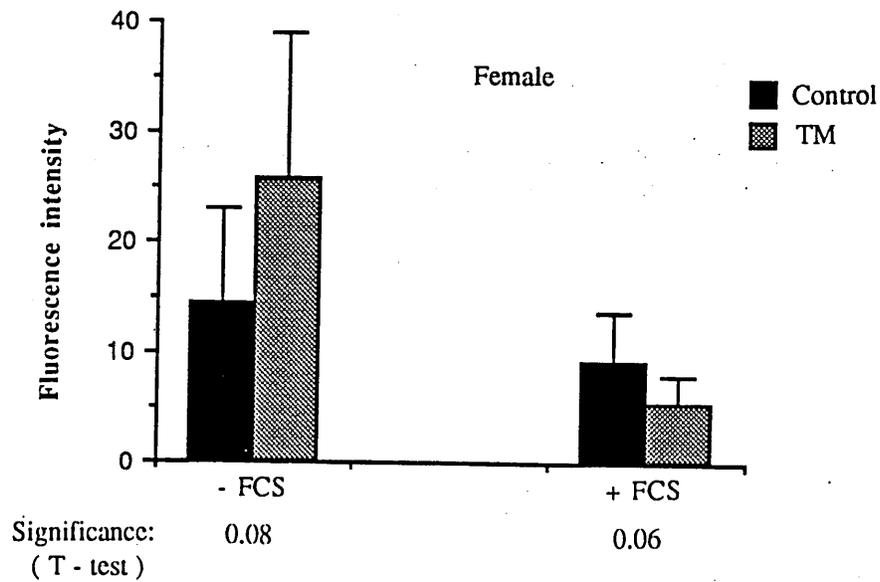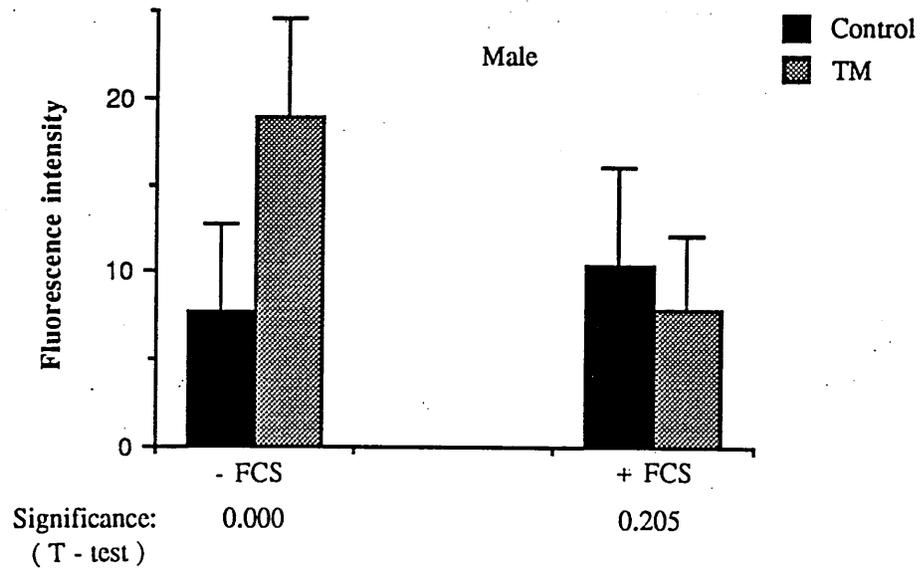
Male

Control
TM

Significance: 0.000   0.205
(T - test)

Female

Control
TM

Significance: 0.08   0.06
(T - test)

Adult worms were incubated in GMEM medium with FCS or without FCS in presence of or in absence of TM for 24hr at 37°C, washed 3 times, labelled with FITC - ConA at 50 µg / ml for 30 min at 37°C, washed and mounted.

lost from the surface of newly transformed schistosomula of S. mansoni with a half time of 10-15 hr in culture in defined medium.

After about 24 hr treatment with TM, it appears that there is inhibition of some glycoprotein synthesis in the adult worm from the results of metabolic studies (Fig.3.8), but TM treatment still increases ConA binding to schistosomula. There could be several reasons for this increase in ConA binding. One reason for increased ConA binding may be the effect of TM on membrane dynamics in schistosomula. We tested this possibility in the following way: newly transformed schistosomula of S. mansoni were labelled with FITC-ConA for 30 mins followed by incubation with TM (10µg/ml) in presence of 5% FCS or in absence of 5% FCS for about 20hr to study the loss of the surface membrane of these parasite in vitro. Fig.3.18 shows the results of two experiments. The loss of surface labelled glycoproteins was quantified by comparing the fluorescence intensity of freshly labelled schistosomula and cultured schistosomula. The data shows the fluorescence intensity of freshly labelled schistosomula in the right hand column. After 21hr incubation, the remaining fluorescence on the schistosomula was measured. It was found to be reduced to 20%-50% of the control when the culture medium was without FCS. The fluorescence intensity of normal cultured schistosomula is significantly less than that in TM treated cultured schistosomula. This indicated that while most surface-FITC-ConA labelled proteins are being lost, TM inhibits the loss of some of these labelled proteins from the schistosomula surface.

Because the FITC-ConA binding to schistosomula is not covalent, the loss of fluorescence in cultured schistosomula may be by the sloughing of FITC-ConA from their surface binding sites. In order to control for this possibility, the newly transformed schistosomula were fixed by 1% formaldehyde for 30min before labelling with FITC-ConA. Fixed schistosomula were labelled with FITC-ConA for 30 min and followed by 21hr incubation. The fluorescence intensity in these cultured parasites, both normal and TM treated, did not decrease.(see Table.3.5). This indicates that the

Fig.3.18. Effect of TM on shedding of FITC-ConA labelled surface glycoproteins by fresh schistosomula.

Experiment 1
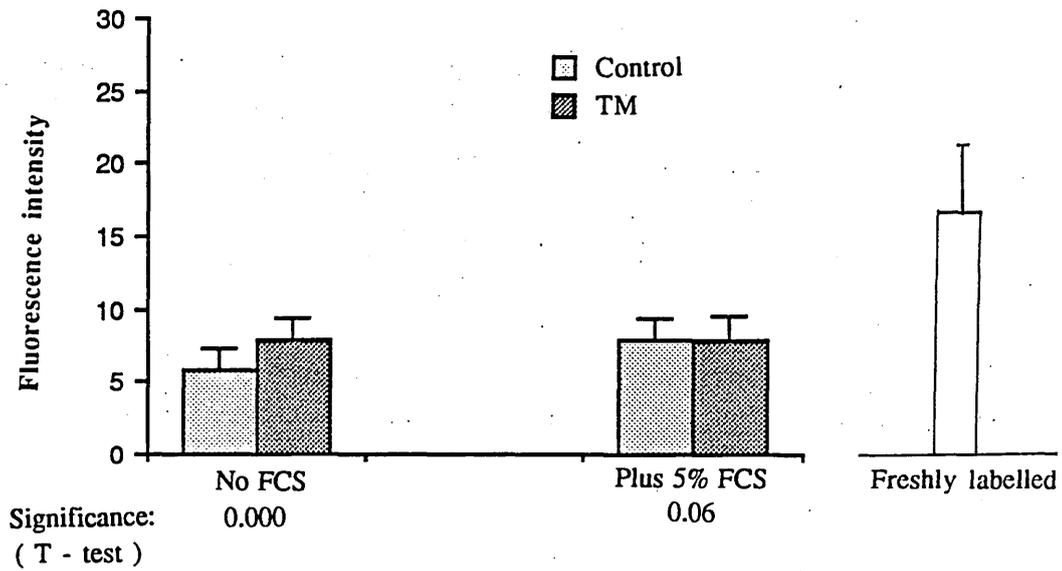
Significance: (T - test)   No FCS 0.000   Plus 5% FCS 0.06

Experiment 2

Significance: (T - test)   No FCS 0.000   Plus 5% FCS 0.685
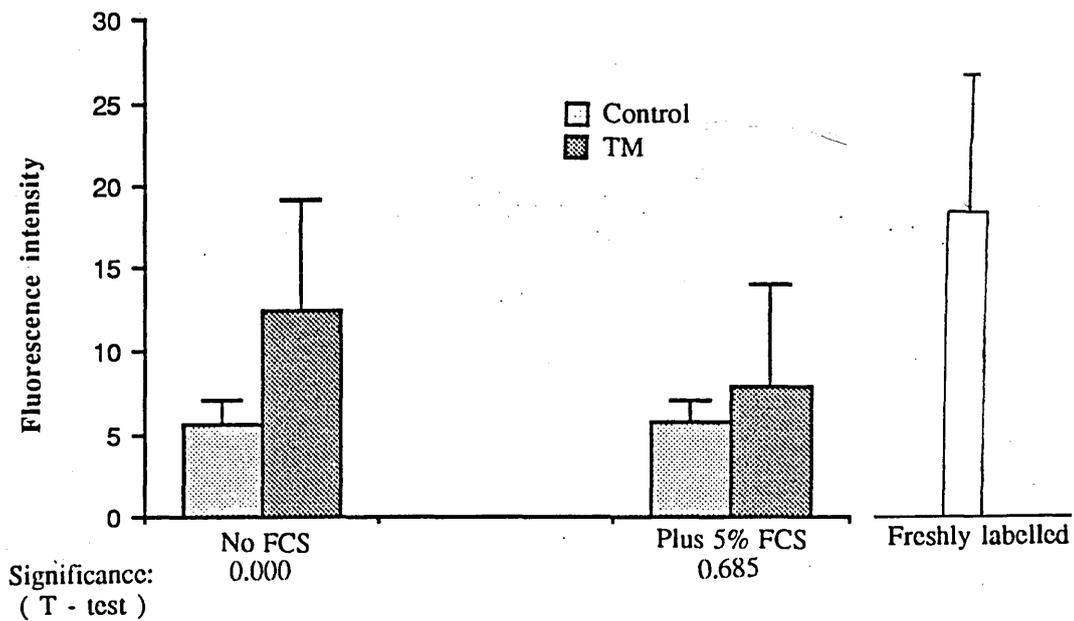
Newly mechanically transformed schistosomula labelled with FITC-ConA for 30min, followed by culture for about 21hr in GMEM medium with or without TM and FCS. At the end of the culture, the fluorescence intensity was measured again.

Table.3.5. Effect of TM on FITC-ConA labelled glycoprotein shedding from live and fixed schistosomula

Exp.No.	Parasites	Fluorescence intensity before incubation	Incubation medium	Intensity after incubation		P
				Normal	TM treated	
1.	live parasite	17.8±7.2	without 5% FCS	11.6±4.1	13.0±5.8	0.268
			containing 5% FCS	12.5±0.8	9.8±4.5	0.01
	1%HCHO fixed	4.0±1.0	without 5% FCS	7.9±1.0	10.9±1.7	0.000
			containing 5% FCS	5.8±0.8	6.0±0.8	0.232
2.	live parasite	19.2±4.9	without 5% FCS	11.4±4.1	14.2±5.6	0.01
			containing 5% FCS	9.4±6.1	10.9±7.3	0.432
	1%HCHO fixed	6.0±2.2	without 5% FCS	10.5±1.9	11.7±2.2	0.032
			containing 5% FCS	8.1±2.8	7.5±1.9	0.29

All values + standard error of the mean

Before FITC-ConA labelling of schistosomula, some parasites were fixed with 1%HCHO for 30min, then the fixed and live parasites were incubated with FITC-ConA for 30min. After washing, the labelled parasites were cultured for 21hr. The fluorescence intensity remaining on the parasites was counted

decrease in fluorescence is due to loss of a complex of FITC-ConA bound to surface glycoprotein by shedding into the medium. and not release of FITC - ConA molecules alone from the parasite surface. However, it is not clear why the fluorescence intensity was increased after a 21 hr incubation of fixed parasites.

3.3.2. Effect of TM on parasite antigens at the surface of S. mansoni

Schistosomulum surface antigens have been analysed for the most part by surface labelling and immunoprecipitation techniques that have demonstrated a complex set of protein and glycoprotein antigens.(Omer - All et al, 1986). During the development of the parasite, the parasite antigens are thought to be masked, or disguised. The changes in parasite antigens after treatment with TM were detected by means of the binding with different antisera. Two sera have been chosen in this experiment. One serum is from infected BALB/c mice. The other serum is from rabbits infected with irradiated cercariae.

3.3.2.1. The binding of infected mouse serum to schistosomula treated with TM for 3 hr or 24hr

The surface of newly transformed schistosomula is antigenically highly complex. After 3hr treatment with TM, the binding of antibody to these parasites did not appear to change greatly. However, in one experiment the binding of antibody to TM treated parasite appeared to show a decrease which was statistically significant. This was not reproducible in two other experiments, where there was no difference between normal and TM treated schistosomula. If the schistosomula were treated with TM for 24hr, the binding of anbtibody to treated parasites demonstrated a little increase but this was not statistically significant.(see Table.3.6.)

Table.3.6. The binding of antibody from infected mouse serum to live schistosomula at varying times after TM treatment

Expt.No.	Time of TM treatment	Relative fluorescence (X ± S.D)		P
		Normal	TM treated	
1.	3hr	52.4±14.9 (0.7±0.3)	36.0±17.7 (0.9±0.5)	0.000
2.	3hr	85.7±15.7 (0.9±0.3)	81.9±13.7 (1.0±0.5)	0.4
3.	3hr	76.2±13.4 (0.7±0.3)	78.6±18.8 (1.1±0.3)	0.5
4.	24hr	41.4±8.4 (n.d)	45.1±19.9 (n.d)	No

After treatment with TM for different times, the parasites were incubated in GMEM medium containing normal serum or antiserum for 30 min at 37 °C, washed three times, incubated with second antibody for 30 min at 37 °C, washed and mounted. Values in parentheses are the binding of normal serum to schistosomula.

n.d: not done

3.3.2.2. Comparative binding of infected mouse serum and serum from rabbits infected with irradiated cercariae to normal and TM treated schistosomula following culture for different time periods

Newly transformed schistosomula were incubated with TM (10μg/ml) for 3hr, followed by culture for 20hr or 41hr. At the end of the different incubation times, the binding of the two sera to these parasites was measured (see Table.3.7). After 3hr TM treatment, the two sera bound to TM treated parasites to the same extent as to normal parasites. When TM treated parasites had been incubated for 20hr, the binding of infected mouse serum to those parasite was measured in two experiments. In one experiment, the TM treated parasites bound more antibody than the controls, while in the other experiment, the treated and control parasites did not differ significantly in the amount of antibody bound.

The binding to TM treated parasites by the rabbit antiserum was increased both after 21hr and 41hr incubation.

3.3.2.3. Effect of TM on antibody from infected mice serum binding to 3 week adult worm

Three-week-old parasites have a unique surface topography, an unusual distribution of intramembranous particles in the double outer membrane, and are the stages in which susceptibility to killing by an immune effector mechanism directed against acquired host determinants can first be detected (McLaren,1980; McLaren and Terry,1982). After TM treatment for 3hr, it was found that TM did not greatly increase lectin binding to this parasites stage. The surface membrane antigenicity in this stage of the parasite after TM treatment has been measured. Fig.3.19 shows the binding of antibody from infected mouse serum to normal and TM treated parasites. No difference can be seen between normal and TM treated parasites

Table.3.7. Comparative binding of infected mouse serum and serum from rabbits infected with irradiated cercariae to normal and TM treated schistosomula

Expt.No.	Time after culture	Relative fluorescence (X ± S.D)					
		Infected mouse serum			Infected rabbit serum		
		Normal	TM treated	P	Normal	TM treated	P
1.	0hr	85.7±15.7	81.9±13.7	0.4	44.7±6.7	42.1±8.4	0.23
	20hr	124.2±22.6	198.6±73.1	0.002	95.9±29.5	219.6±33.9	0.000
2.	0hr	76.2±13.4	78.6±18.8	0.573	(n.d)	(n.d)	
	20hr	59.0±20.1	51.7±9.2	0.382	34.4±11.2	47.9±13.9	0.000
3.	41hr	(n.d)	(n.d)		101.2±22.0	118.5±27.9	0.014

Schistosomula treated with TM for 3hrs, then cultured for different times. At the end of each culture time, parasites were taken neut to determine binding by the two antisera.

n.d: not do.

Fig.3.19. Effect of TM on antibody from infected mouse serum binding to 3 week adult worms after TM treatment for 3hr.

Significance: 0.319
(T - test)
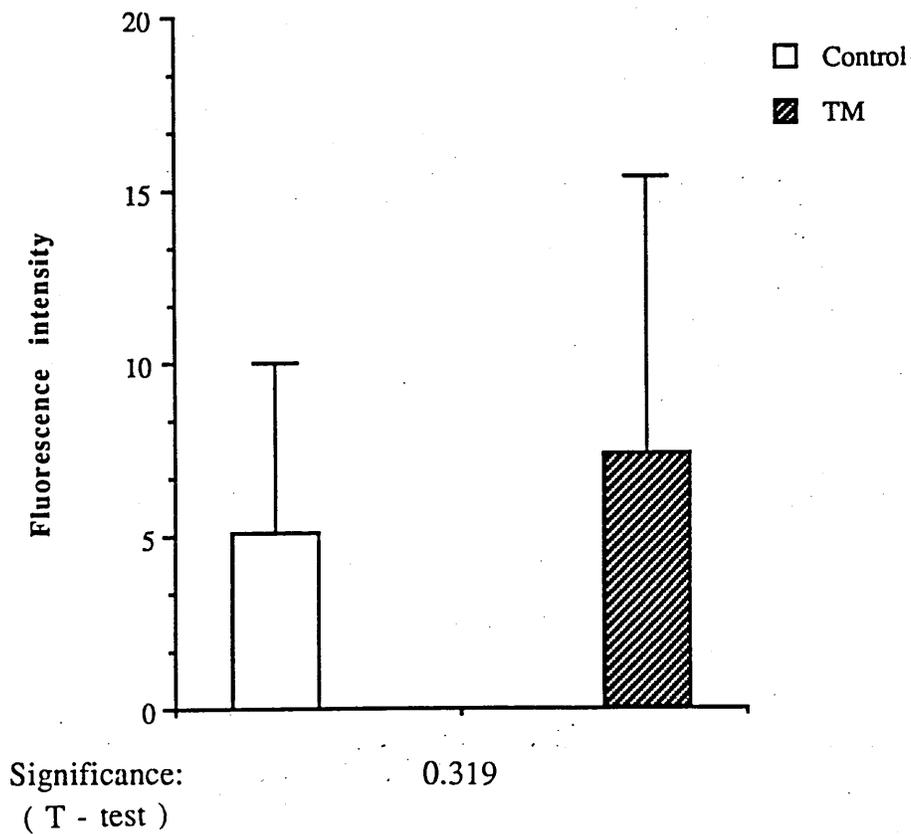
After TM treatment for 3hr, the parasites were incubated in GMEM medium containing antiserum for 30 min at 37°C, washed 3 times, incubated with second antibody for 30 min at 37°C, washed and mounted.

3.3.3. Effect of three analogues of TM on FITC-ConA binding to Schistosomula

Hashim and Cushley (1987) have reported that minor modifications to the structure of tunicamycin lead to the loss of the biological activity of the antibiotic. In our present experiment, three analogues of tunicamycin (TM-1,TM-2,TM-3), each with minor a modification in structure in different regions of the molecule, have been employed to study their effects on lectin binding. A double labelling (FITC-ConA and Hoechst 33258) technique has been used in each experiment. Hoechst 33258 (H33258) is a fluorescent chemical which can bind to DNA. It is used to assess the degree of membrane damage induced by a treatment. The parasites were treated with TM and the three analogues of TM for 3hrs. The parasites were then labelled with FITC-ConA, firstly, then the parasites were labelled with H33258. The data for lectin binding indicated that FITC-ConA binding to treated parasites was increased to the greatest extent by normal TM and to a small but significant degree by TM-3. Neither of TM-2 or TM-1 analogues had increased FITC-ConA binding.(Fig.3.20(a)).

Normal TM and an analogue of TM,TM-1, result in an increase in uptake of H33258, compared with normal schistosomula (Fig.3.20 (b)). However, TM-1 did not increase FITC-ConA binding. In order to test the hypothesis that an increase in FITC-ConA binding observed in treated parasites reflects membrane damage, the degree of correlation between the intensity of fluorescence of FITC-ConA and H33258 was investigated statistically.

Fig. 3. 21 (a, b, c, d) shows the correlation between the FITC ConA reading and H33258 reading on individual schistosomula. The coefficient of correlation was very low in TM,TM-1 and TM-2 treated parasites, the r = 0.261, 0.356, 0.124 respectively, which was not significant at the level of > 0.05. Although the coefficient of correlation was 0.577 in TM -3 treated parasites, it was still not significant at the level of > 0.05. These indicated that there was no good correlation between FITC-ConA and H33258 binding. TM and its analogues caused an increase in the amount of

Fig.3.20. Effect of three analogues of TM on FITC-ConA binding to schistosomula (a) and uptake of H33258 (b)

(a)

Fluorescence intensity

- ■ Control
- ▨ TM
- ▨ TM-1
- ▨ TM-2
- ☐ TM-3

FITC - ConA

(b)

Fluorescence intensity

- ■ Control
- ▨ TM
- ▨ TM-1
- ▨ TM-2
- ☐ TM-3
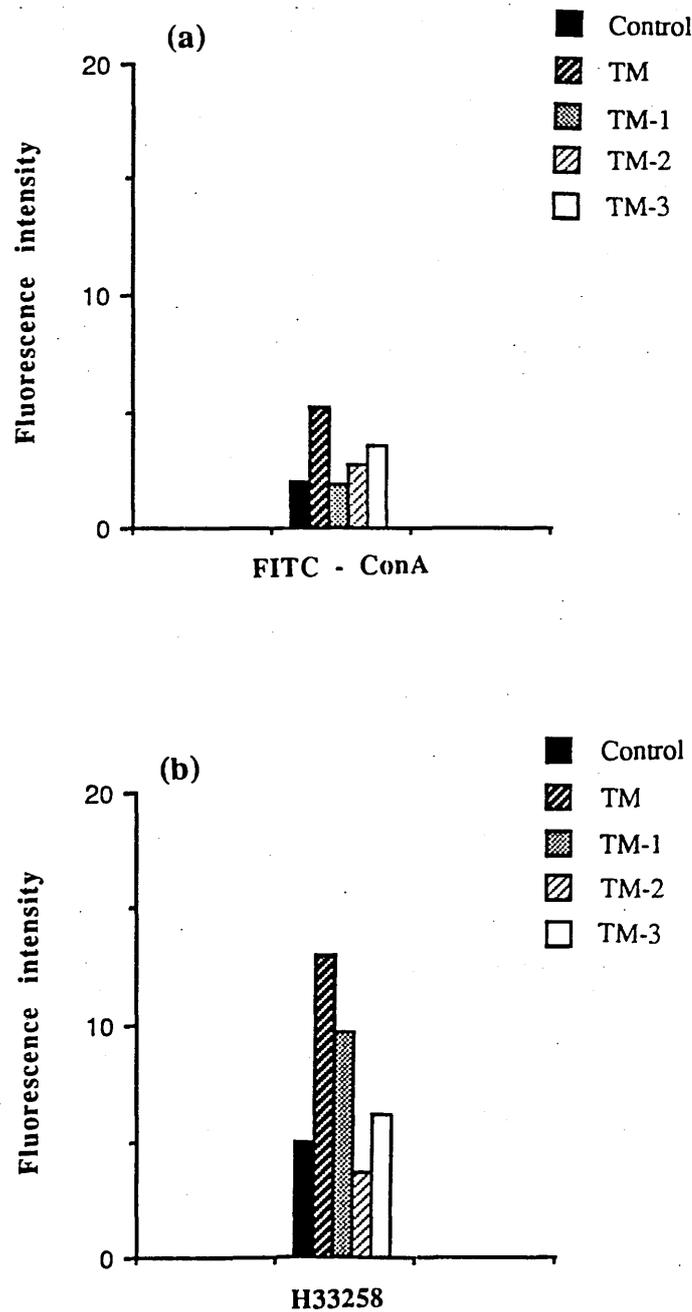
H33258

After treatment with TM and three analogues of TM for 3hr, the parasites were incubated in FITC - ConA in GMEM medium for 15 min, H33258 was added to each sample and incubation continued for 15 min at 37°C, then the parasites were washed and mounted.

Fig. 3.21. Correlation between the uptake of FITC - ConA and the uptake of H33258

FITC - ConA

d).

e).
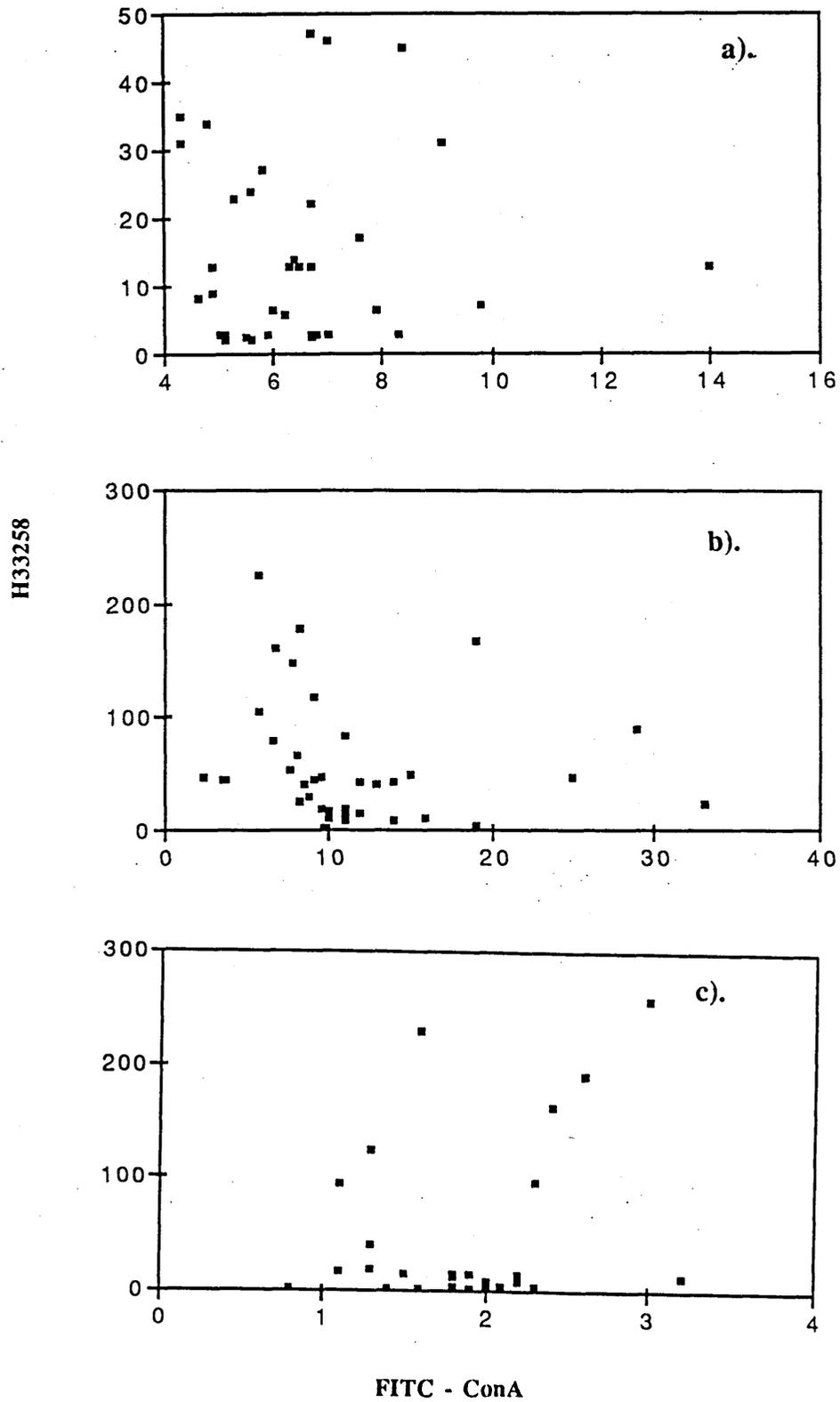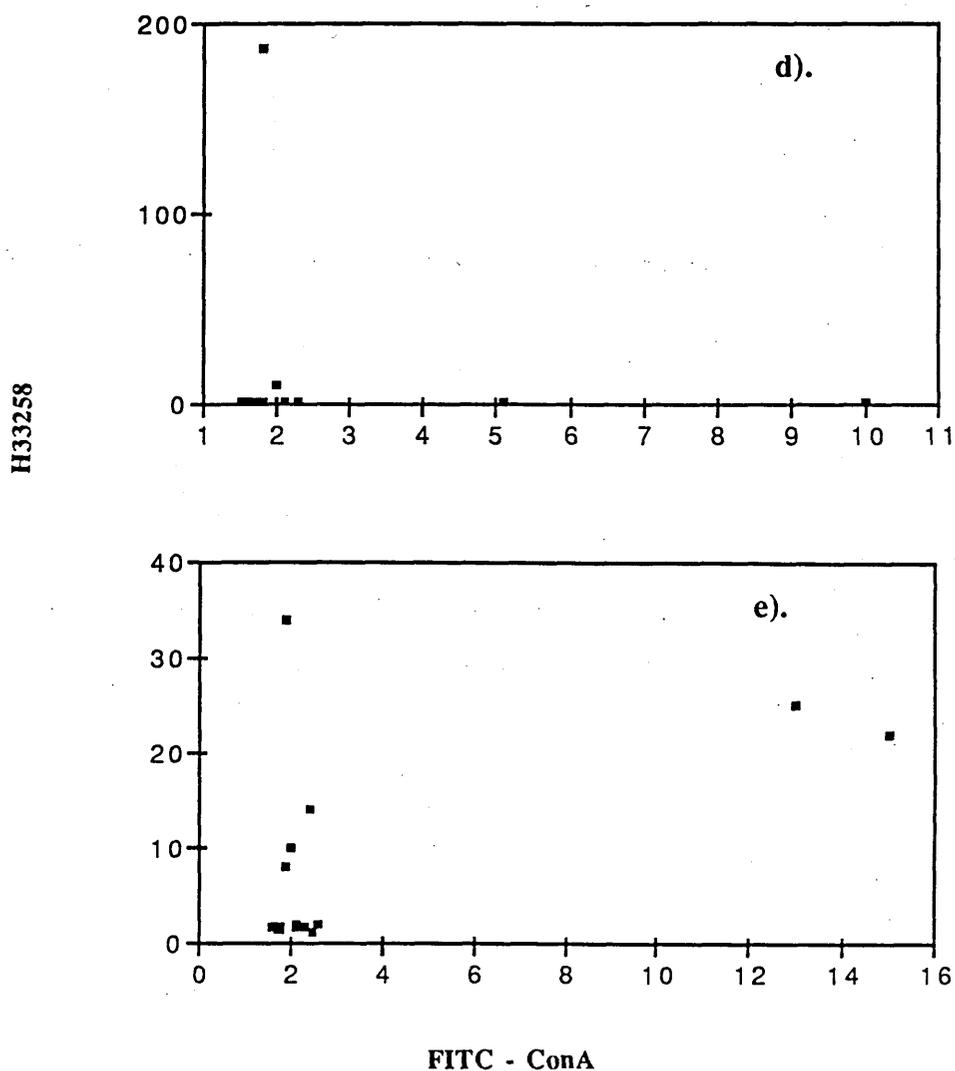
FITC - ConA

After treatment with TM and three analogues of TM for 3hr, the schistosomula were incubated in FITC - ConA in GMEM medium for 15 min, H33258 was added to each sample and incubation continued for 15 min at 37°C, then the parasites were washed and mounted. Each point represents readings on a single schistosomulum. (a). Control group, r = 0.016. (b). TM treatment group, r = 0.261. (c). TM-1 treatment group, r = 0.356. (d) TM-2 treatment group, r = 0.124. (e). TM-3 treatment group, r = 0.577.

uptake of H33258, which did not result from membrane damage, but may be due to a change in the permeability of the membrane.

3.3.4. Effect of TM on S. mansoni uptake of substrate

The changes in permeability of the membrane between normal and TM treated parasites were investigated by using the uptake of a labelled amino acid [α - amino(1-^{14}C) isobutyric acid)]. This amino acid is not a normal amino acid. It can be taken up by the worm, but not incorporated into protein. The result of this experiment is given in Fig.3.22. It was found that TM increased the uptake of this amino acid compared with the control group. Therefore, TM may affect the surface membrane of S mansoni to change the permeability and increase the uptake of this amino acid.

3.3.5. Change in biophysical properties of surface membrane in S.mansoni after TM treatment

Mobility of the lipid and protein components of biological membranes is an important property of membranes. The fluidity of the schistosome membrane changes during the development of this parasite from larva stage to adult worm. The results of the previous experiments show that TM increases lectin binding, increases rabbit antibody binding but only after 24h incubation, decreases glycoprotein shedding and inhibits parasite development in the mouse.(section 3.3.7) We next asked the question. Does the TM change the biophysical properties of membrane in S. mansoni. ? The mobility of lipid and protein in the surface membrane of S. mansoni after TM treatment has been measured using Fluorescence Recovery After Photobleaching technique (FRAP).

Fluorescence recovery after photobleaching is a technique in the study of lateral diffusion of biomembrane components. FRAP provides two measures of diffusion: first, the fraction of the component that is free to diffuse (percent recovery %R), based on the estimated signal at infinite time after photobleaching and second, the diffusion coefficient (D_L) of that fraction, calculated from the kinetics of the recovery curve.

Fig. 3.22. Effect of TM on 2-amino (1-^{14}C)isobutyric acid incorporation into different life cycle stages of S. mansoni

Legend:
- Control (Total)
- Control (Sup)
- Control (PP)
- TM (Total)
- TM (Sup)
- TM (PP)

Y-axis: CPM / µg protein

X-axis groups:
- Schistosomula (Control, TM)
- 54 day worms (Control, TM)
- 74 day worms (Control, TM)
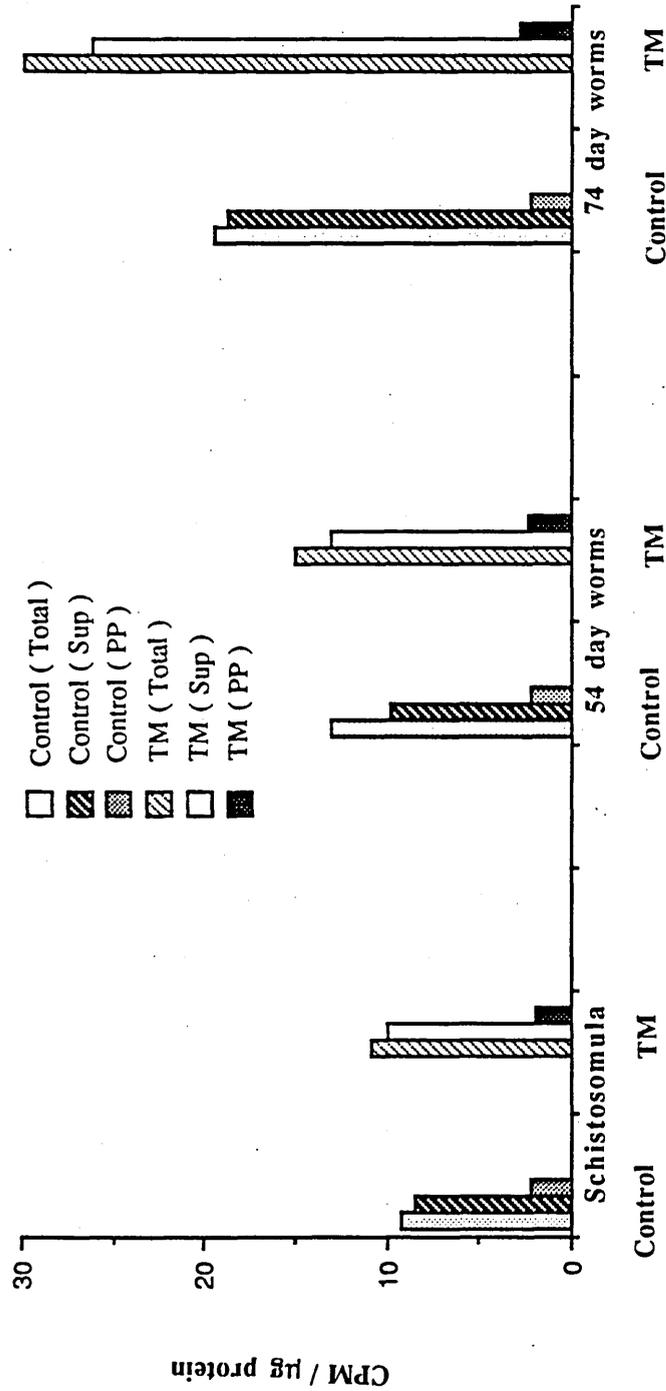
The parasites were incubated with TM (10µg / µl) for 3hr. Then , 1.25 µCi 2-amino (1-^{14}C)-isobutyric acid was added with further incubation for 2.5hr. The radioactivity in different parts were counted.

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Examination of the kinetics of recovery reveal information on the properties of the membrane.

3.3.5.1 Effect of TM on lipid analogue (C_{18}Fl) diffusion in surface membrane of S.mansoni

C_{18}-Fl is a lipid analogue that does not only insert into S. mansoni surface but is also confined to the outer monolayer. After treatment with TM ($10\mu g/ml$) for 3hr, the worms were incubated with C_{18}-Fl for 30 min. Table.3.8. summarizes the results of FRAP experiments on schistosomula and Fig.3.23. illustrates representative FRAP curves from these parasite. The data from experiment 1 show that the percentage recovery and the rate of lateral diffusion (D_L) both altered significantly after TM treatment. TM increased the percentage recovery. On the other hand, TM decreased the rate of lateral diffusion (D_L). In the second experiment, TM only altered the D_L, not percentage recovery. The variability between different experiments could be due to the fact that cercariae were shed from different batches of snails. This problem will be discussed later.

Table.3.9. summarizes the result of FRAP on 3 week worms. The rate of lateral diffusion (D_L) was very variable both between experiments and within experiments, as revealed by the high standard errors, and satisfactory estimates could not be generated from this data. However, no significant differences were found between control and TM - treated groups in percentage recovery. TM appears to affect the membrane lipid fluidity in schistosomula, but not 3 week worms.

3.3.5.2. Effect of TM on Succinylated FITC-ConA Diffusion in the Surface Membrane of S. mansoni

Adult worms were incubated with TM for 24 hr in GMEM medium containing 5% FCS. After incubation, parasites were labelled with FITC-ConA. Table.3.10. shows the result. There is great variability within an experiment. It seems that TM did

Fig.3.23. FRAP curves of C_{18} - Fl in the surface membrane of S. mansoni schistosomula

Filename : stm3

K-WU schist tunicomycin
%-bleach = 60.9   %-recovery = 95.73   T-half.sec = 16.89
Beam um = 2.2   Gamma-2D = 1.22199   D1 = 8.7543   10-10 cm2/sec
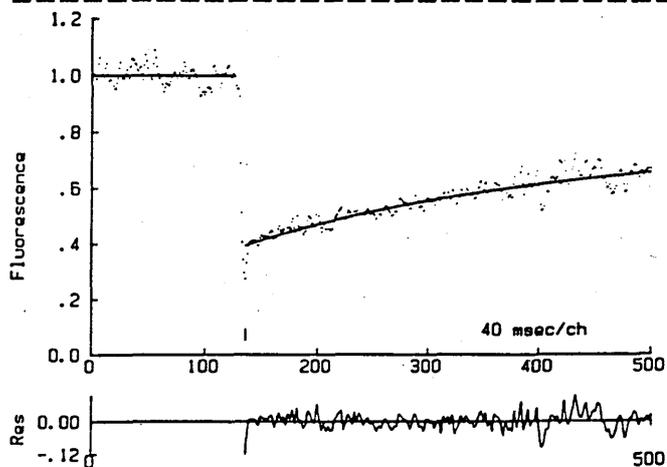
Filename : sc3

K-1 WU schist control
%-bleach = 72.2   %-recovery = 68.55   T-half sec = 4.78
Beam um = 2.2   Gamma-2D = 1.32893   D1 = 33.640   10-10 cm2/sec
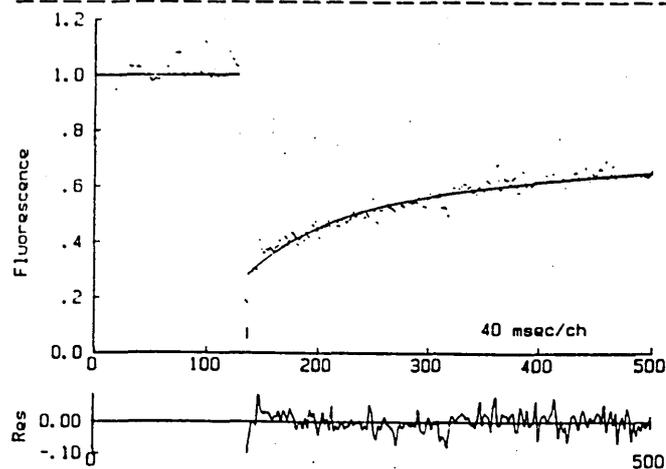
Table.3.8.   Effect of TM on C_{18}-Fl diffusion in the surface membrane of <u>S.mansoni</u> schistosomula

Expt. No.	% recovery			$D_L \times 10^{-10} cm^2 sec^{-1}$		
	Normal	TM treated	P	Normal	TM treated	P
1.	56.4±10.5(5)	85.0±10.2(5)	0.003	37.4±11.2(5)	12.0±6.9(5)	0.005
2.	58.7±15.3(11)	61.1±11.2(15)	0.66	28.1±16.1(11)	25.7±10.2(15)	0.04

After treatment with TM for 3hr, the schistosomula were incubated in C_{18} - Fl in GMEM medium for 15 min at room temperature, washed three times and mounted.

The values of D_L and percentage recovery are given as mean + standard deviation, the numbers in parentheses are the number of measurements for each experiment, one measurement/parasite

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Table.3.9 .   Effect of TM on C_{18}-Fl diffusion in the surface membrane of 3 week adult worms

Expt. No.	% recovery			$D_L \times 10^{-10} cm^2 sec^{-1}$		
	Normal	TM treated	P	Normal	TM treated	P
1.	22.0±7.1(4)	35.0±9.5(3)	0.14	54.0±16.9(3)	560.9±174.7(2)	
2.	51.7±12.2(6)	56.0±27.4(7)		49.1±36.6(4)	13.9±3.5(6)	

After treatment with TM for 3hr, the 3 week worms were incubated in C_{18} - Fl in GMEM medium for 15 min at room temperature, washed 3 times and mounted.
The values of DL and percentage recovery are given as mean + standard deviation, the numbers in parentheses are the number of measurements for each experiment, one measurement/parasite.

Table.3.10 . Effect of TM on succinylated FITC - ConA diffusion in surface membrane in S. mansoni

Expt. No.	% recovery			$D_L \times 10^{-10} cm^2 sec^{-1}$		
	Normal	TM treated	P	Normal	TM treated	P
1.	79.3± 8.4 (7)	66.4±16.1 (10)	0.06	1885.1±2480 (7)	1668.9 ±1224 (8)	0.668

After treatment with TM for 3hr, the schistosomula were incubated in succinylated FITC - ConA for 30min at 37oC, washed and mounted.

The values of DL and percentage recovery are given as mean + standard deviation, the numbers in parentheses are the number of measurements for each experiment, one measurement/parasite.

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not affect percentage recovery and D_L. The very rapid diffusion coefficients suggest that the succinylated ConA is binding to loosely associated glycoproteins or glycolipids.

3.3.6. Ultrastructural observations on the outer membrane of fresh schistosomula and adult worm after treatment with TM

(1). 3hr normal and TM treated Schistosomula

Figures 3.24, a) to c) show the surface of normal schistosomula cultured in GMEM medium for 3hr after mechanical transformation. As described by Hockley and Mclaren (1973) , the outer surface is almost entirely heptalaminate (c). The tegumental bodies, consisting of tightly-packed membranous whorls, contribute to and renew this heptalaminate surface.(d).

The surface of 3hr TM treated schistosomula appears identical to the normal one (Fig. 3.25, a, b, c, d). The structure of the double outer membrane and tegumental cytoplasm are intact.

(2). 24 hr normal and TM treated Schistosomula

At 24 hr, the structure of the outer membrane is clearly heptalaminate in both normal and TM treated parasites whether the medium contains FCS or does not contain FCS.(Fig.3.26; Fig. 3.27,a -e; Fig. 3.28,a - b; Fig. 3.29, a - c.). However, the distribution of electron density in some areas of the heptalaminate membrane does not appear as a continuous layer in TM treated parasites.

(3). 4 four week normal adult worms and TM treated worms

After the worms had been incubated with TM (10μg/ml) for 3hr, the parasites surface were examined by electron microscopy. The structure of the heptalaminate membrane was unaffected.(Fig.3.30,a,b.; Fig. 3.31,a,b). Some slight differences could be seen in the size of vacuoles. Large vacuoles could be found in 4week adult worms after incubation with TM for 3hr, but not in normal worms. The appearance of big vacuoles in the tegument was a sign of membrane damage.(Popiel and Erasmus. 1984)

Fig. 3.24. a) - d). The surface of normal schistosomula, cultured in GMEM medium for 3hr after transformation.

S: spine.

M: muscle layers.

MB: membranous bodies.

hm: Heptalaminate membrane.

a.

MB
m
S

1 μm

b.

S

1 μm
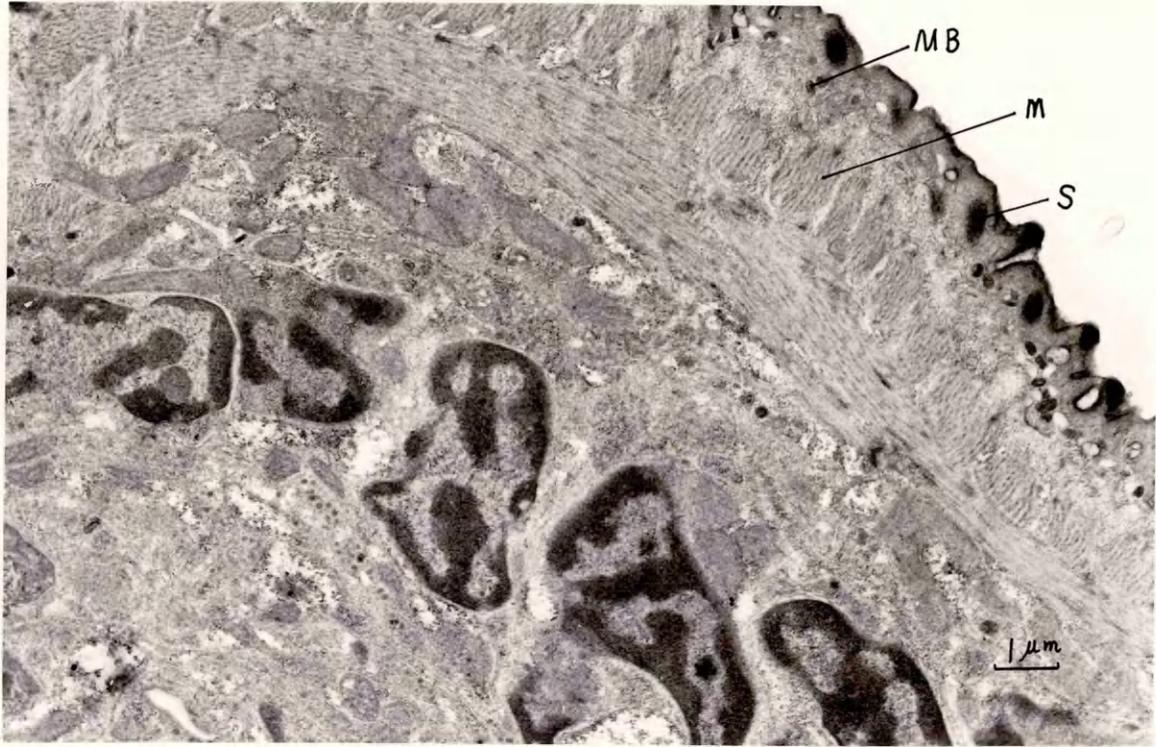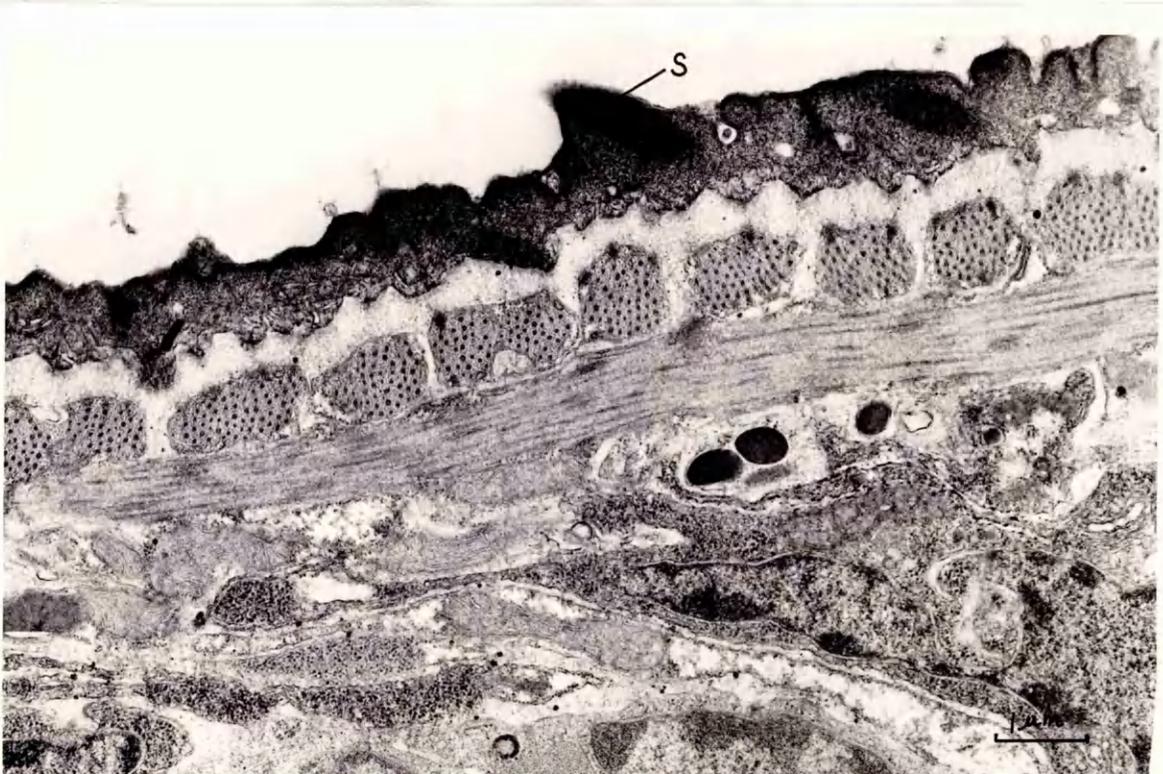
c.
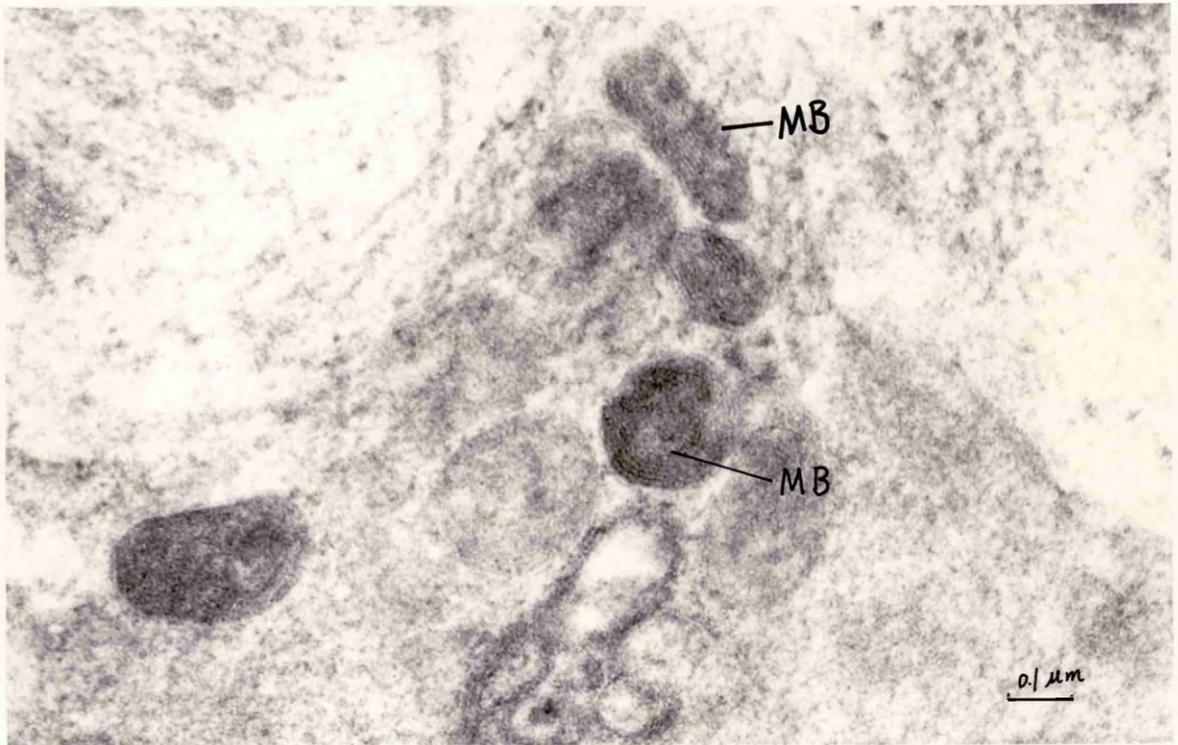
d.
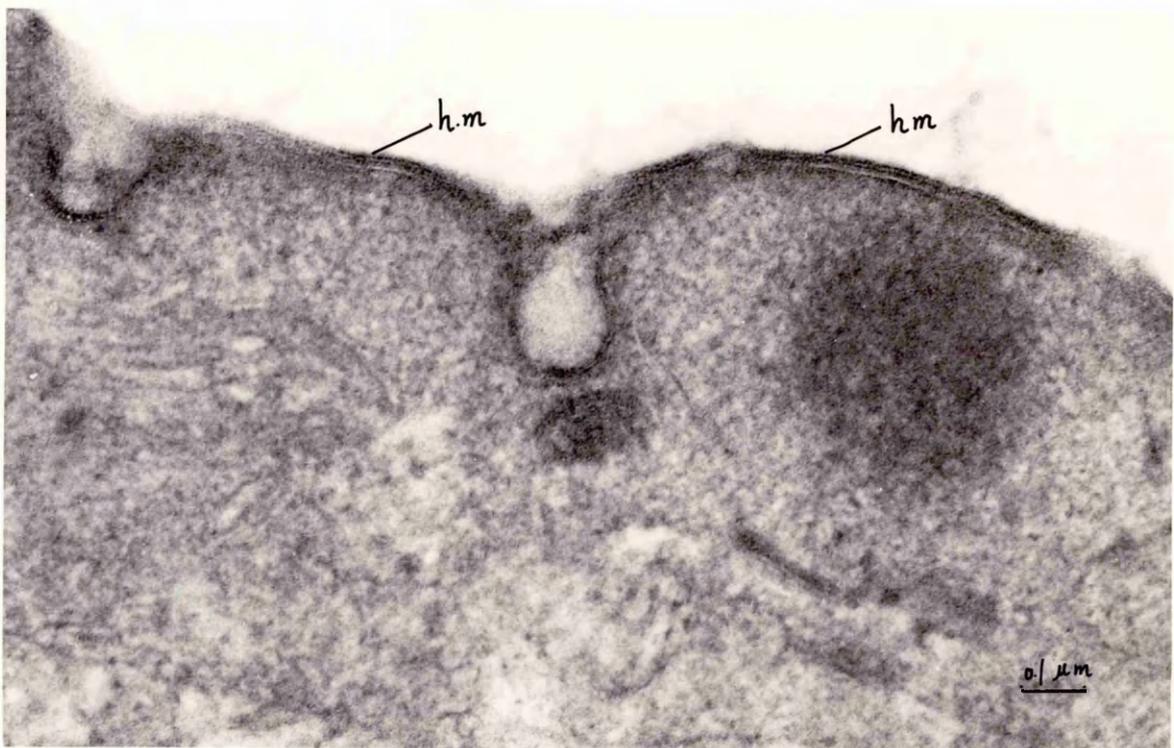
Fig. 3.25. a) - d). The surface of schistosomula treated with TM for 3hr

S: spine.

MB: membranous bodies.

hm: Heptalaminate membrane.

a.

b.

c.

hm

0.1 μm

d.

1 μm
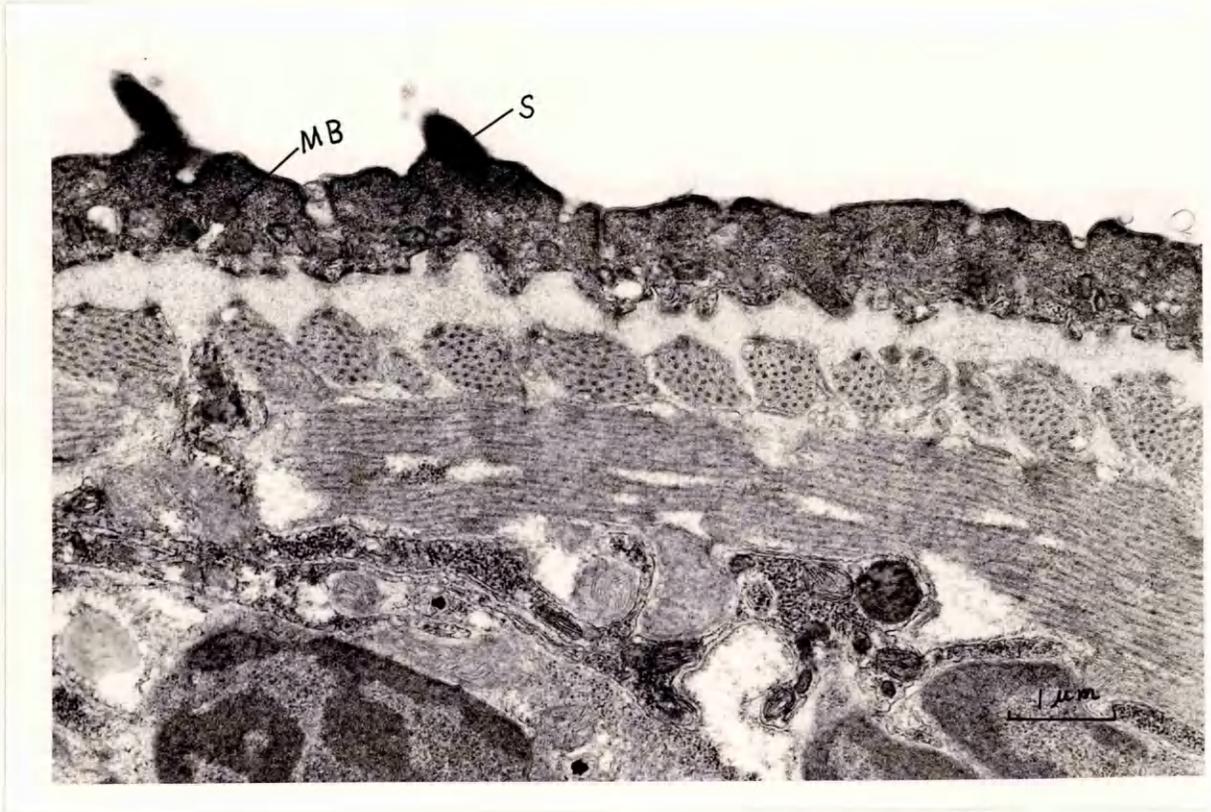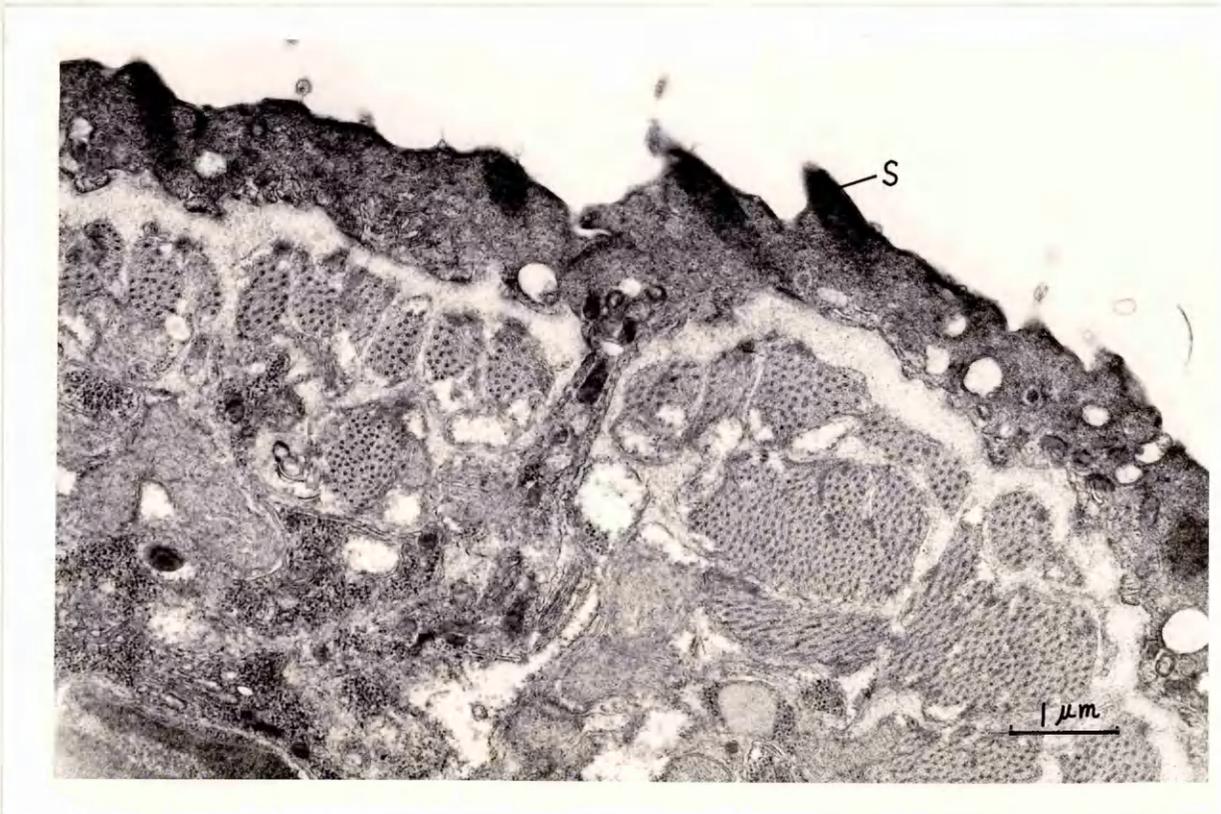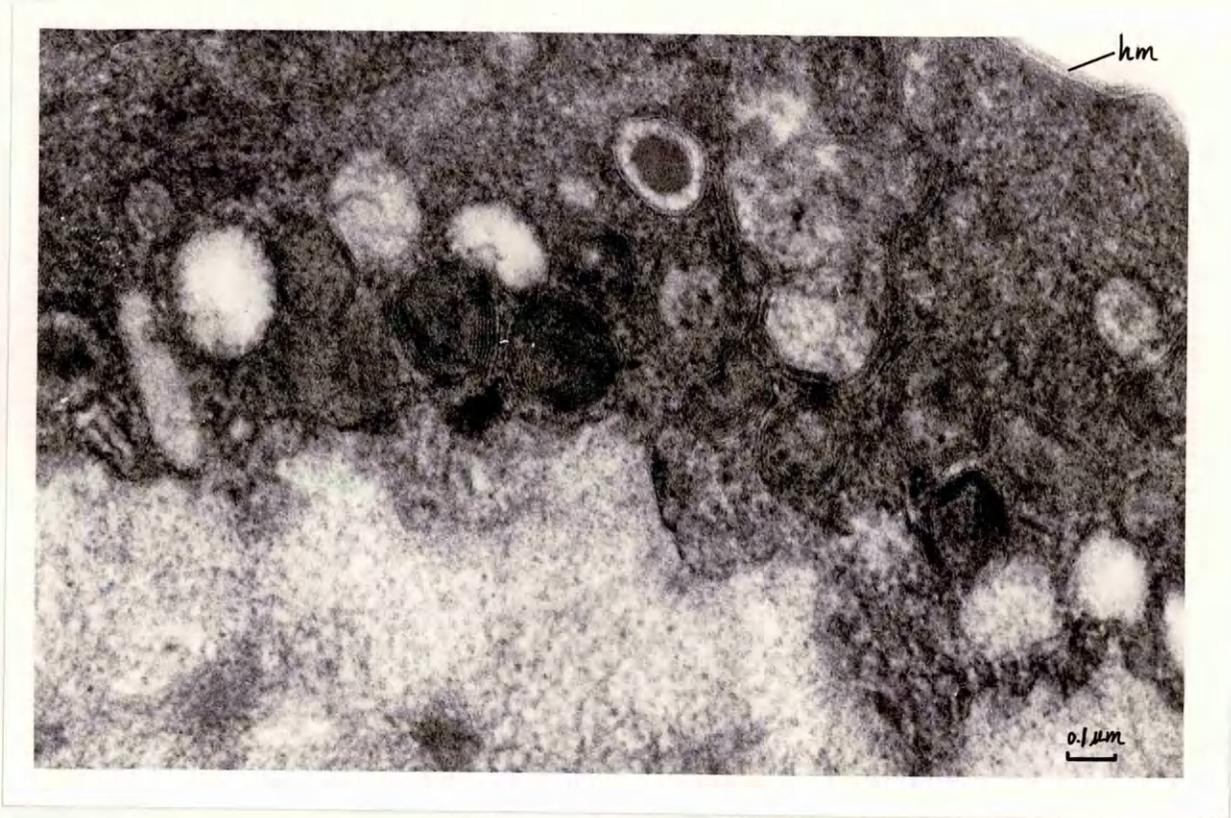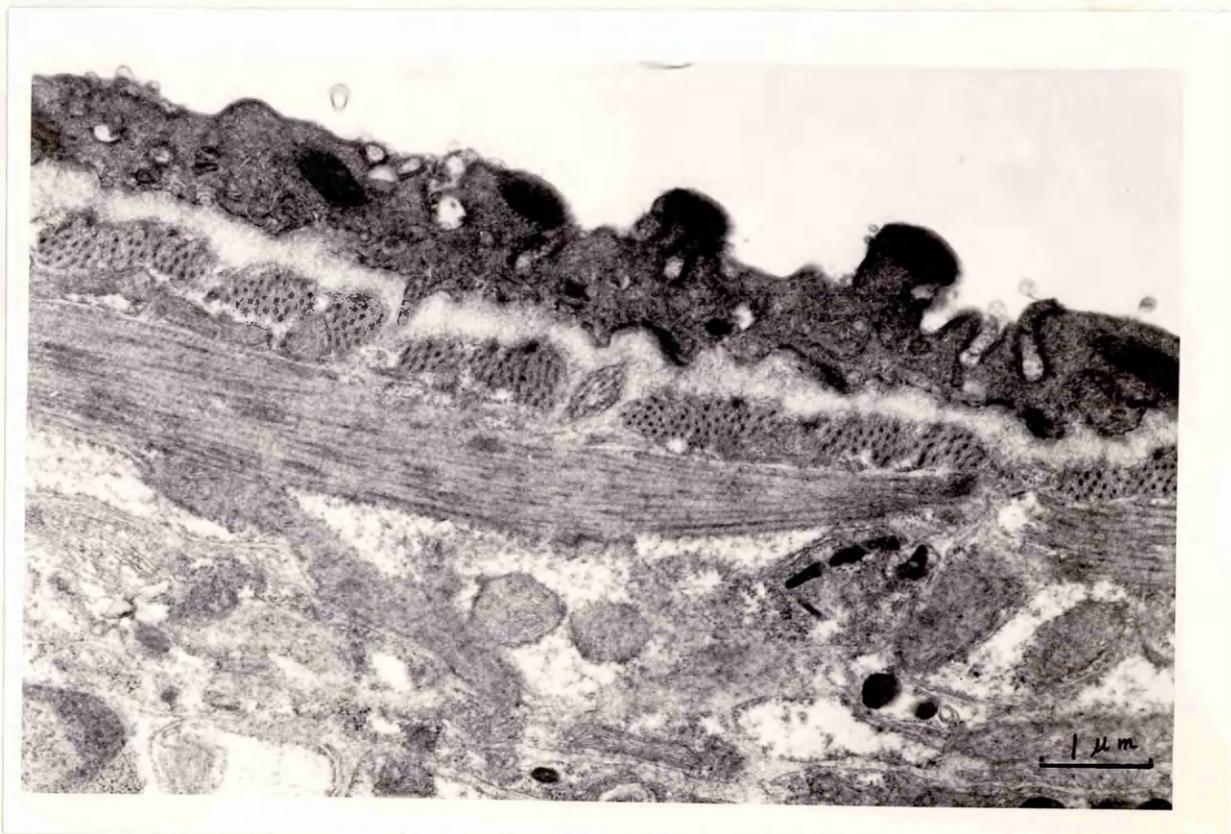
Fig. 3.26. The surface of schistosomula , cultured in GMEM medium without FCS for 24hr.

hm: heptalaminate mambranes
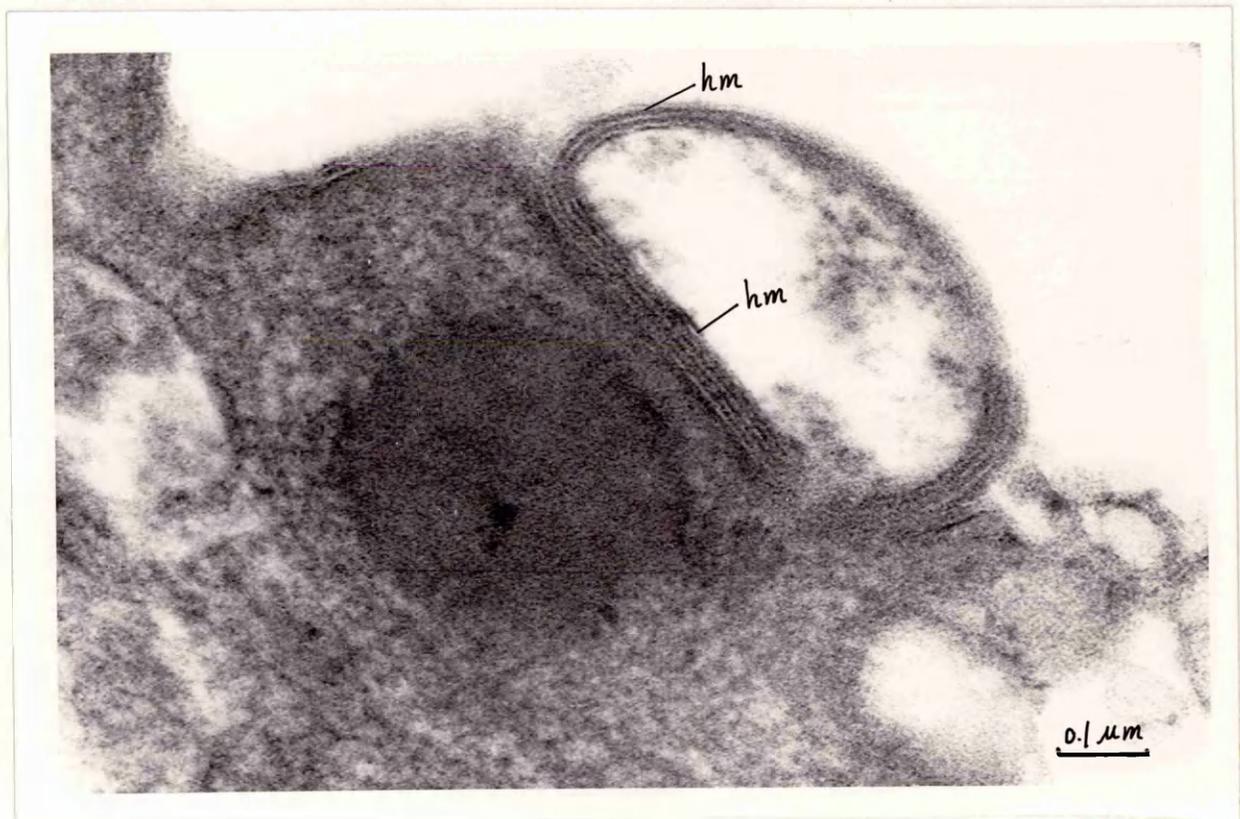
Fig. 3.27 a) - e). The surface of schistosomula, cultured in GMEM medium containing TM without FCS for 24hr

TM did not affect the structure of heptalaminate membrane, but the distribution of electron density in some areas of the heptalaminate membrane does not appear as a continuous layer.

MB: membranous bodies.

hm: Heptalaminate membrane.

a.

hm

0.1 um

b.

MB

0.2 um

C.

0.5 μm
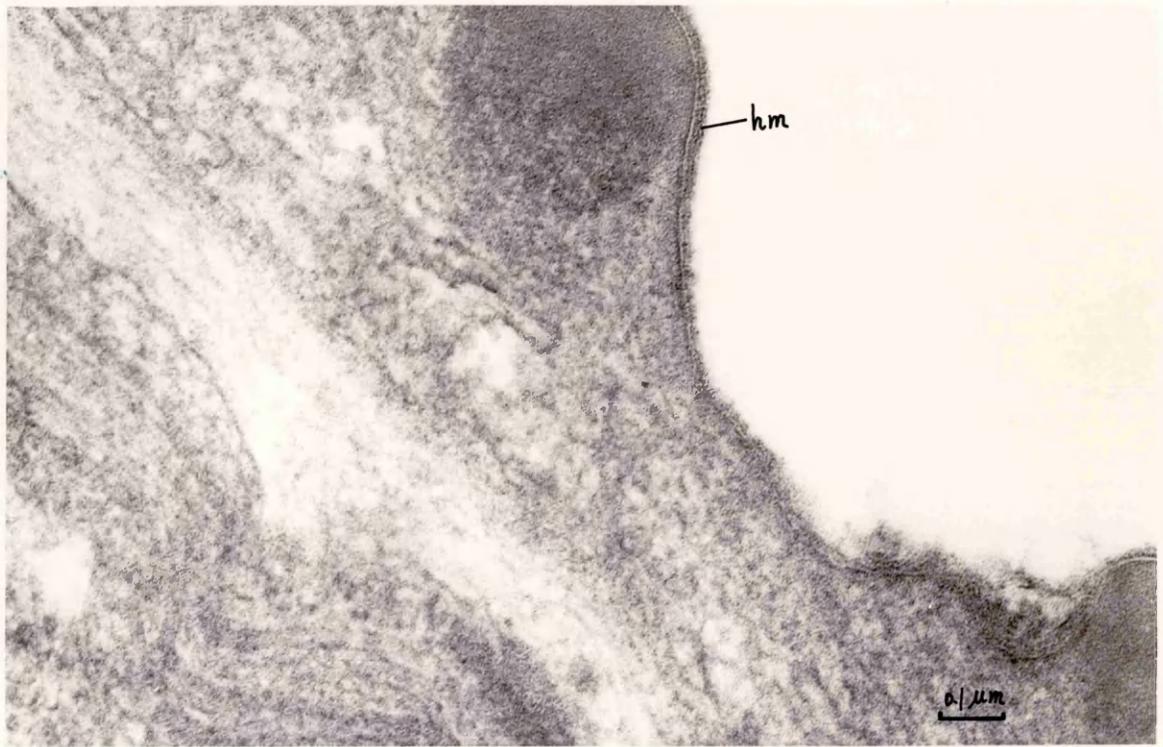
d.
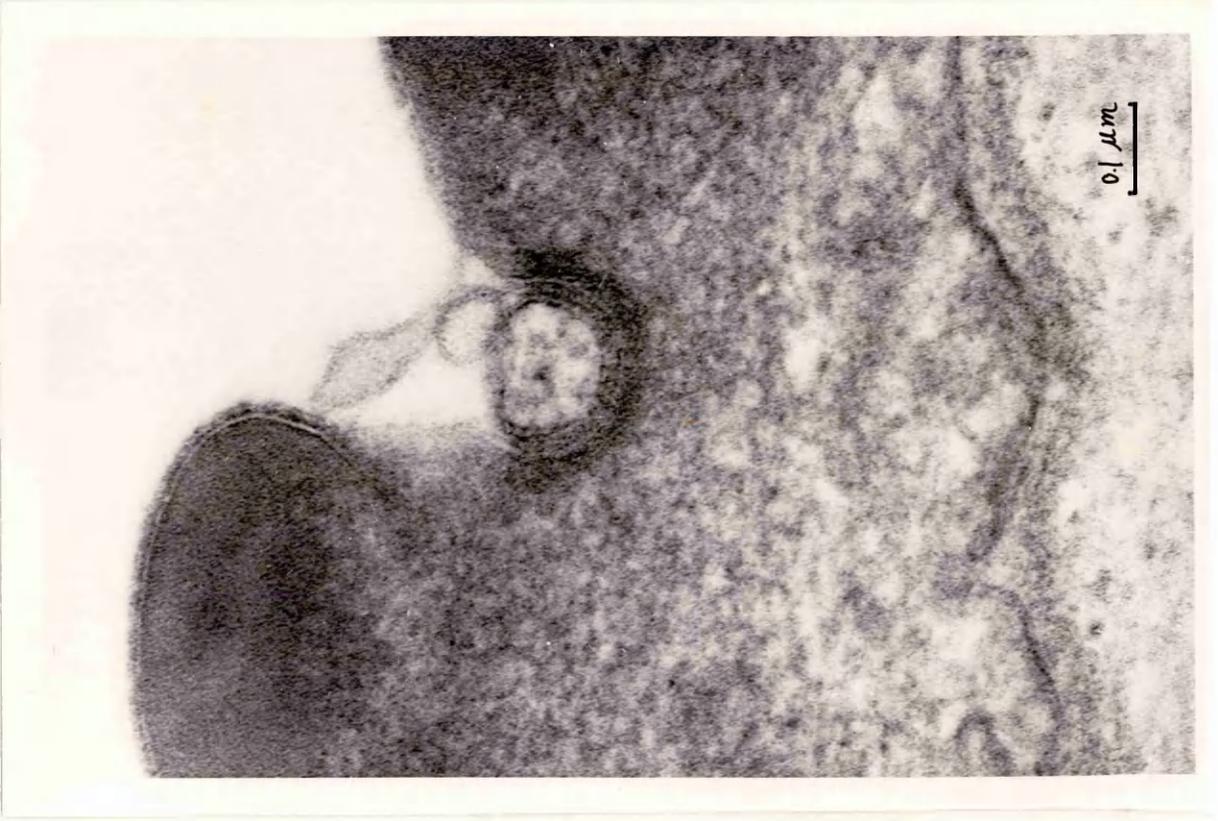
e.

Fig. 3.28. a) - b). The surface of normal schistosomula, cultured in GMEM
medium containing FCS for 24hr.

The structure of the outer double membrane is well preserved.

hm: Heptalaminate membrane

a.

hm

b.

hm

0.1 μm
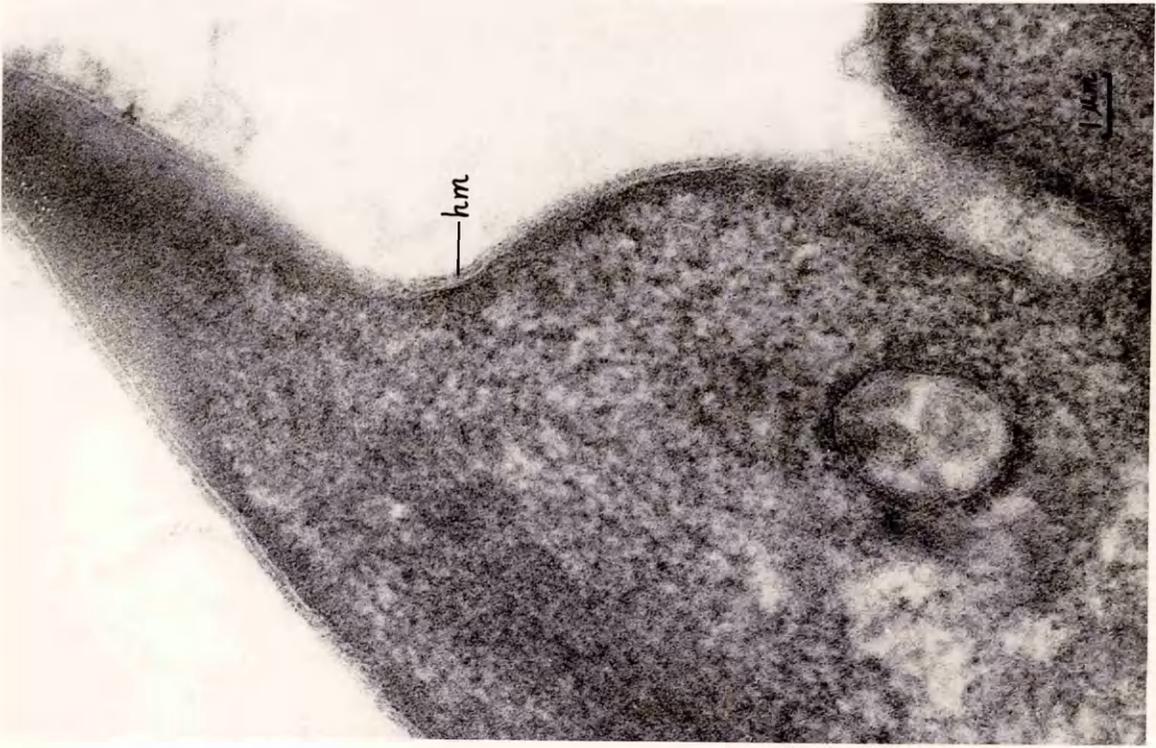
Fig. 3.29. a) - c). The surface of schistosomula, cultured in GMEM medium containing TM and FCS for 24hr.

The heptalaminate membrane did not seem to be affected by TM. But, the distribution of electron density in some areas of the heptalaminate membrane did not appear a continuous layer. (well shown in pp.119 c)

hm: Heptalaminate membrane.

a.

b.

hm

0.2 μm

0.2 μm

c.

0.1 μm
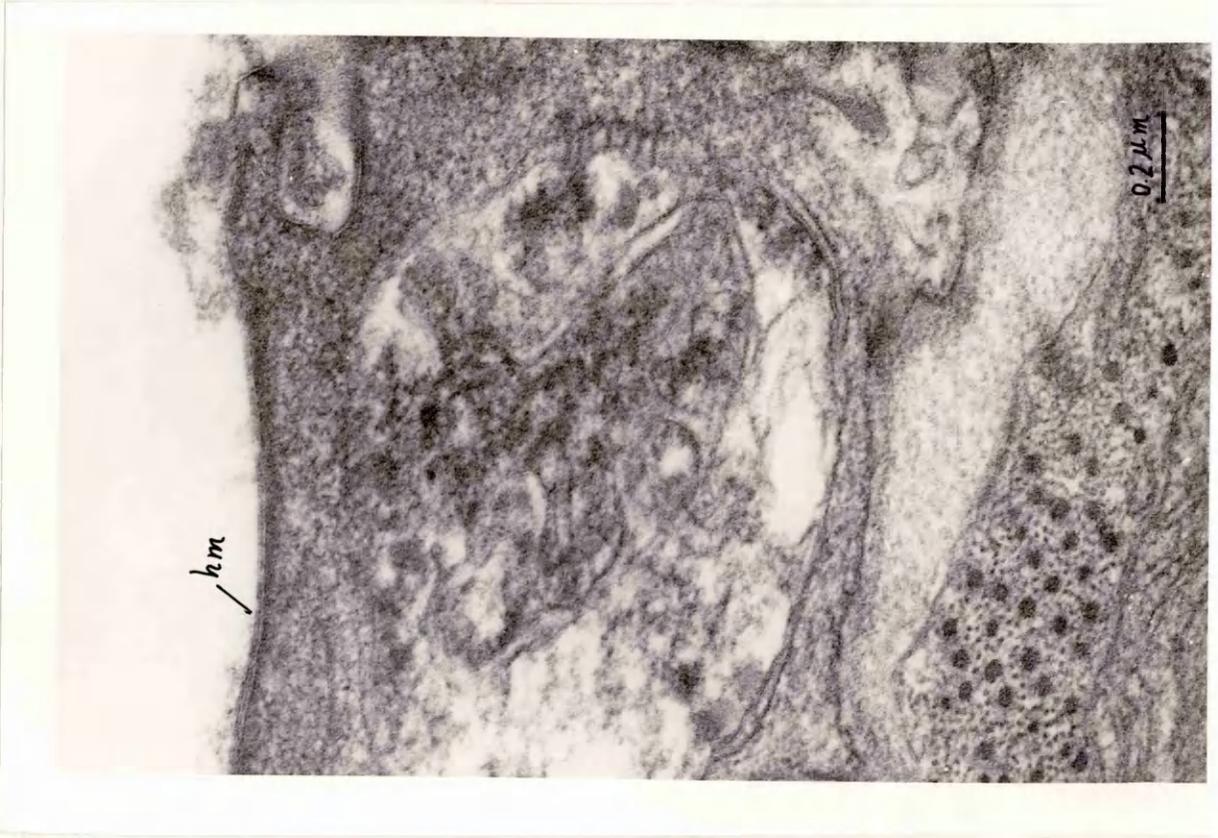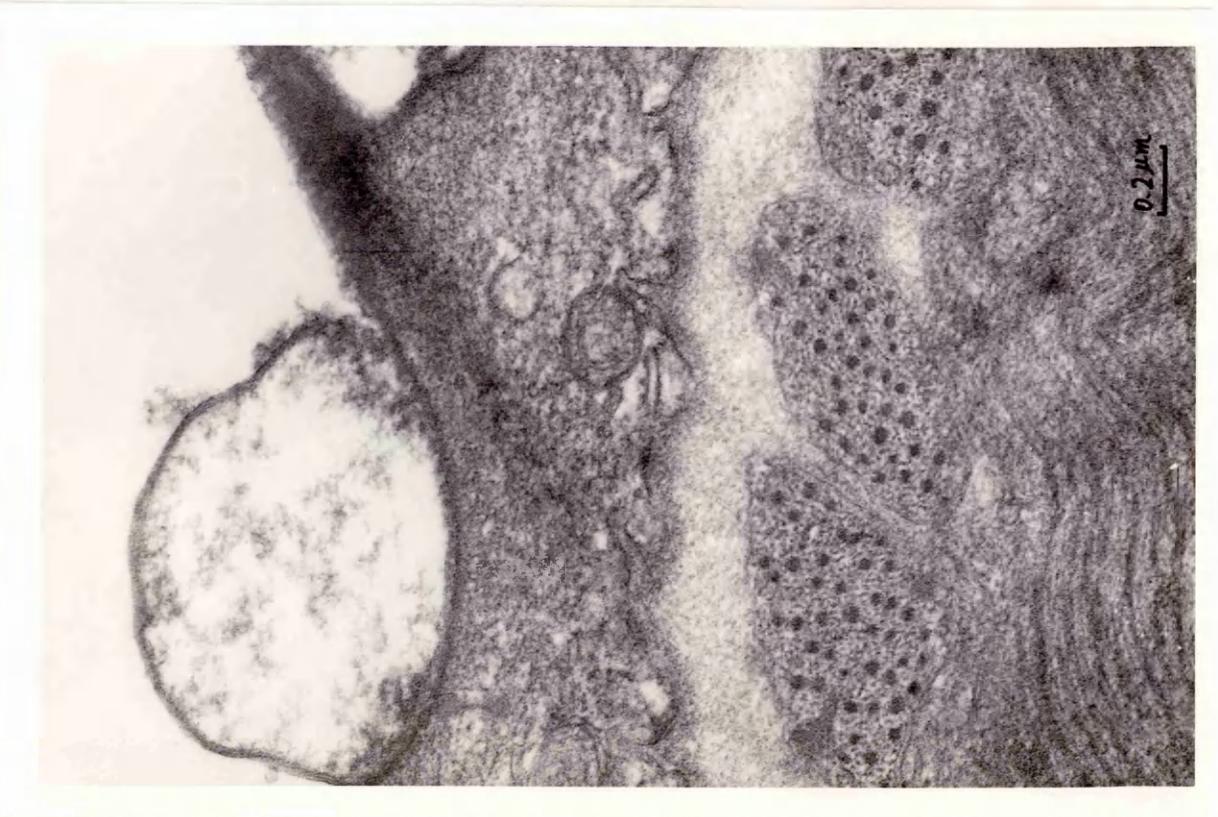
Fig. 3.30 a) -b).   The surface of normal 4 week adult worm

hm: Heptalaminate membrane.

DB: Discoid body.

Vac: Vacuole

a.
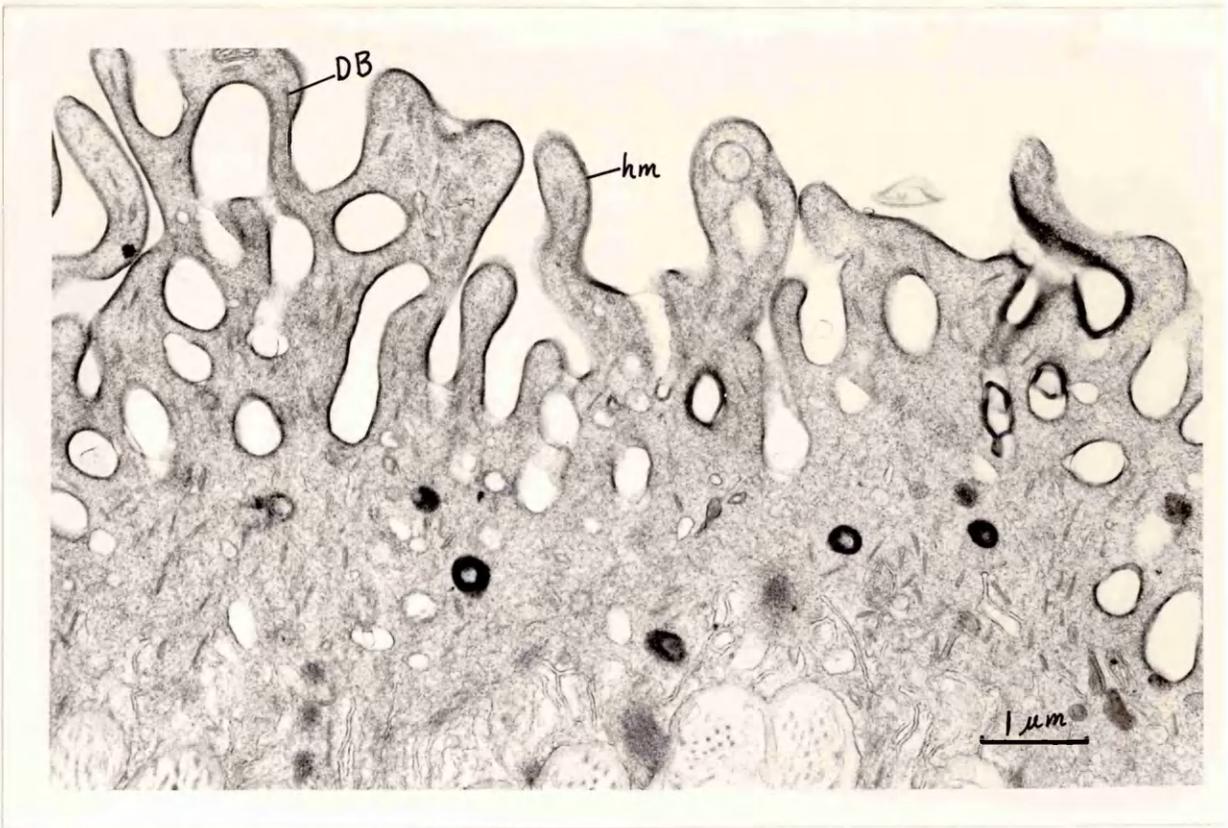
b.
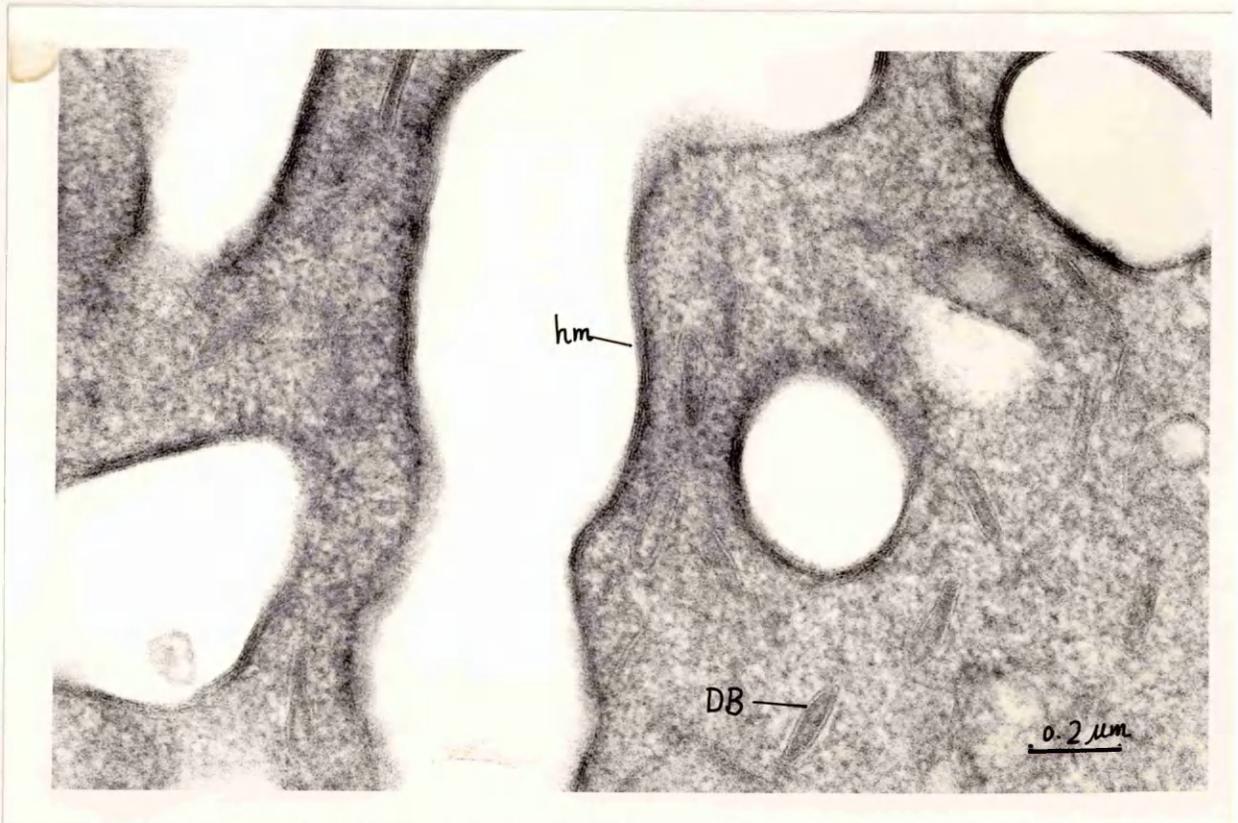
Fig. 3.31 a) - b). The surface of 4week adult worms after TM treatment for 3hr.

Large vacuoles could be found in 4 week adult worms after TM treatment for 3hr. No changes were observed in the discoid bodies.

hm: Heptalaminate membrane.

DB: Discoid body.

Vac: Vacuole

a.

DB

hm

large vacuole   large vacuole

1 μm

b.

hm

DB

0.2 μm
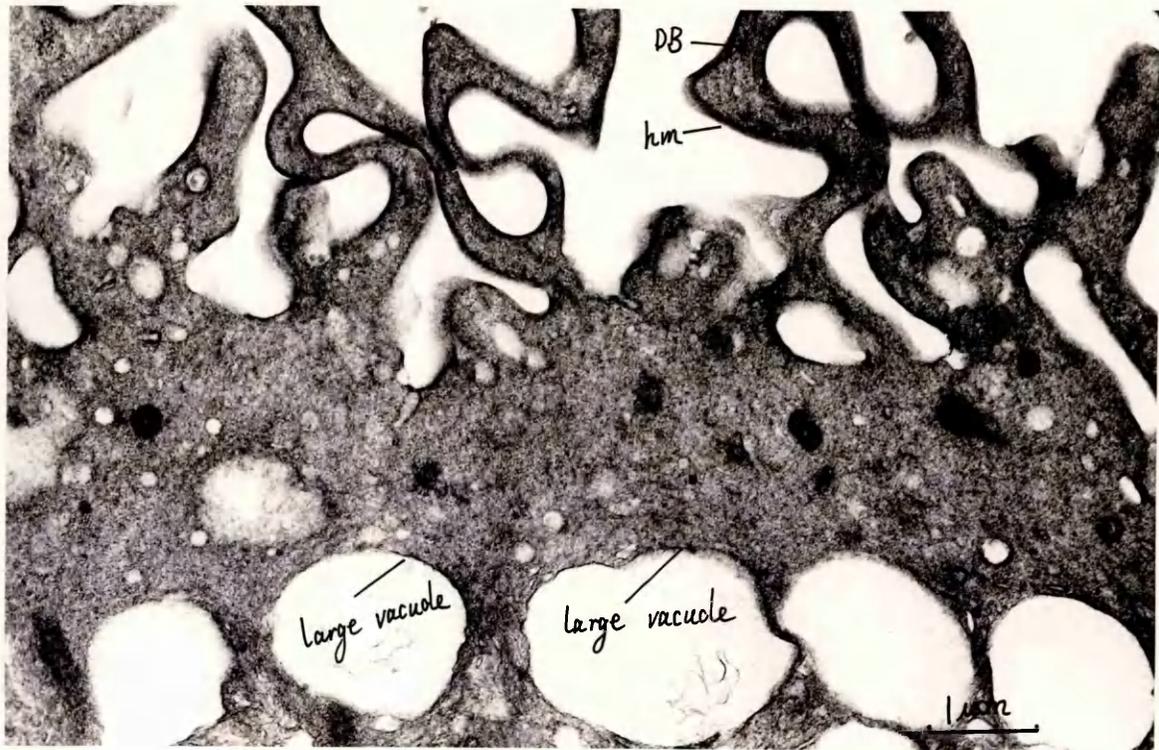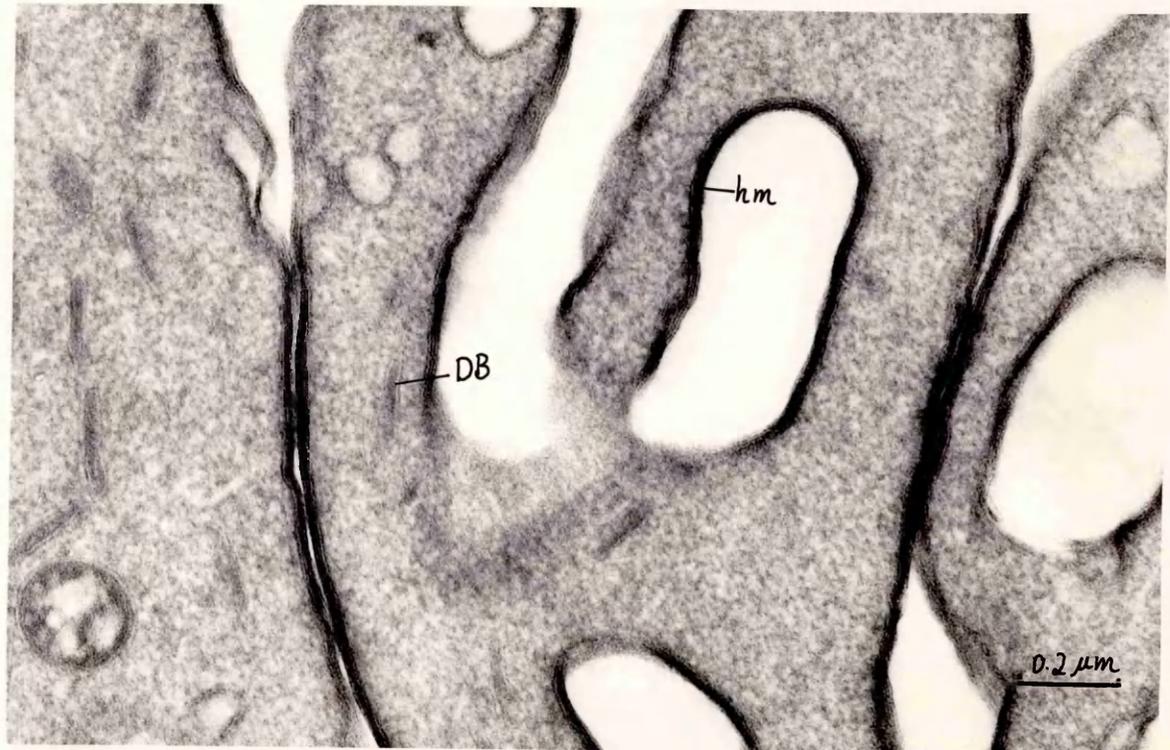
3.3.7. Effect of TM on viability of parasite in vivo and in vitro

Having determined that TM inhibits the synthesis of some glycoproteins of schistosome after 24hr treatment, and changes some properties of the surface membrane of larval parasites, we undertook to determine the effect of TM on viability of parasites in vivo and in vitro.

3.3.7.1. Effect of TM on viability of the schistosomula in vitro

Newly mechanically transformed schistosomula were treated with TM (10μg/ml) for 3hr, and cultured in 5ml GMEM medium plus 10% fetal calf serum at 37°C, 5% CO_2. The number of viable schistosomula remaining in culture were visually determined at different times up to two days after transformation.(see Table.3.11). After 41hr of culture, TM treated, mechanically-transformed schistosomula seemed to have a reduced mobility. TM treated larva stage parasites died at 72-96hr.

3.3.7.2. Effect of TM on viability of the schistosomula in vivo

The viability of schistosomula treated with the same dose of TM (10μg/ml) was assessed in vivo by using them to infect BALB/c mice subcutaneously. The numbers of surviving schistosomula are shown in Table 3.12. Comparison of the number of parasites recovered from mice 5 week after infection showed that TM had a significant inhibitory effect. No adult worms were recovered from the group of mice infected with TM treated schistosomula 5 weeks after infection.

In addition, the level of antibody in these mice was determined by ELISA. The level of antibody was quite low in those mice infected with treated TM schistosomula. However, in the group of mice infected with normal schistosomula, 5 weeks after infection, the antibody appeared to be very high (see Fig.3.32 a, b). This result demonstrated that schistosomula treated with TM may died at a relatively early stage of development in mice.

Table.3.11. Survival in culture of normal and TM-treated mechanically transformed schistosomula

Experiment 1.

Schistosomula	Normal	TM treated
Hours after transformation	Percentage of schistosomula dead	
6hrs	0.2%	1.6%
21hrs	0.6%	1.0%

Experiment 2.

Schistosomula	Normal	TM treated
Hours after transformation	Percentage of schistosomula dead	
6hrs	0.9%	1.3%
19hrs	2.3%	2.4%
23hrs	2.3%	3.2%
41hrs	3.2%	3.9%

After schistosomula had been incubated with TM (10µg/ml) for 3hrs, the parasites were washed and cultured in 5ml GMEM / 10% hi FCS at 37^0C in the presence of 5% CO_2.

Table.3.12 .Viability of TM-treated schistosomula in vivo

Experiment 1.

Experimental group	Normal	TM treated
Mouse No.	1, 2, 3, 4, 5.	1, 2, 3, 4, 5.
Worms surviving.	1 36 33 38 36	0 0 0 0 0
Mean ± SD	28 ± 15	0 ± 0

Experiment 2.

Experimental group	Normal	TM treated
Mouse No.	1, 2, 3, 4, 5.	1, 2, 3, 4, 5.
Worms surviving.	0 44 21 21 9	0 0 0 0 0
Mean ± SD	19 ± 16	0 ± 0

In experiment 1., each mouse in both control and TM treated groups was injected subcutaneously with 500 schistosomula.

In experiment 2., each mouse was injected subcutaneously with 300 schistosomula in control group and 320 schistosomula in TM group.

Newly mechanically transformed schistosomula were treated TM (10 µg / ml) for 3hrs at 37^0C in GMEM medium plus 5% FCS. They were then concentrated by centrifugation and injected subcutaneously into BALB/c mice. All mice were perfused 5 weeks after infection and surviving worms counted.

Fig.3.32. Measurement of antibody in mice infected with normal schistosomula (a) and TM treatment schistosomula (b)

(a)
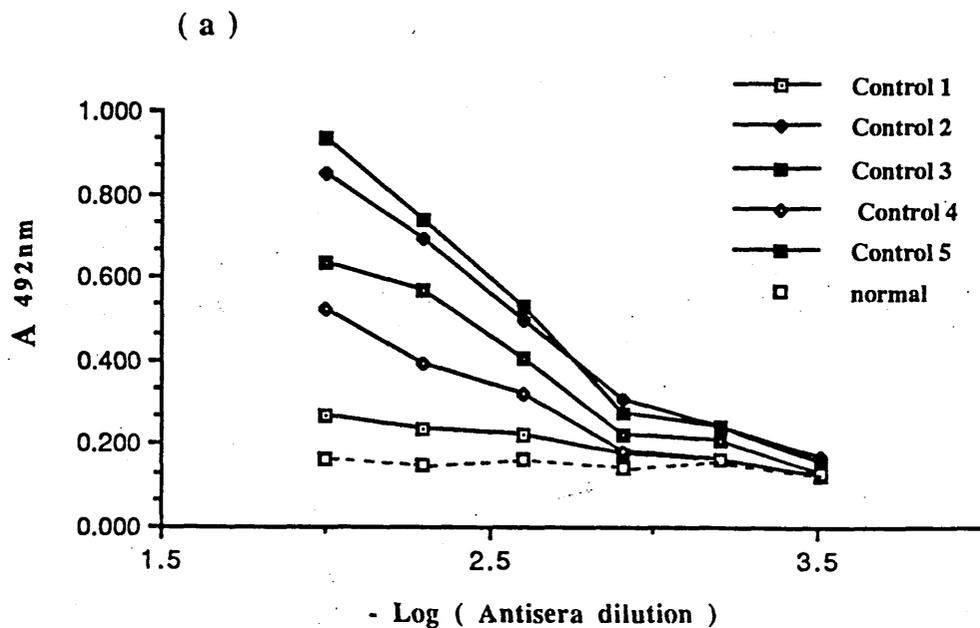
Each mouse was injected subcutaneously with 500 normal schistosomula.

(b)
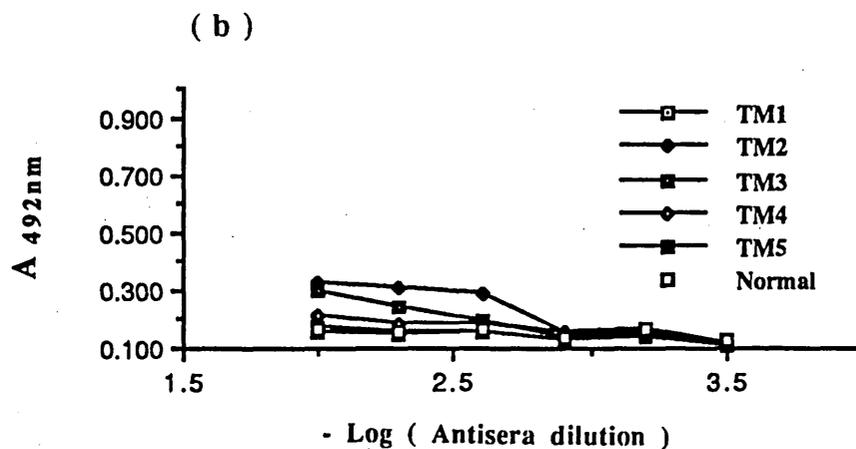
Each mouse was injected subcutaneously with 500 TM treatment schistosomula.

3.3.8. The Binding of ^{3}H-tunicamycin to fresh schistosomula and adult worms

In order to gain further insight into the action of TM, the binding of ^{3}H-TM by fresh schistosomula and adult worms was examined. The parasites were incubated with ^{3}H-TM at 37^0C. At various intervals, the parasites were collected and the radioactivity bound to them was measured. As shown in Fig.3.33 and Fig.3.34, TM binding to schistosomula was affected by the time of incubation. After 2hr incubation, the amount of TM binding to intact schistosomula appeared to increase greatly. Compared with schistosomula, tunicamycin bound to intact adult worms more quickly than to schistosomula. The curves for TM binding to schistosomula and adult worms both tend towards saturation after 3hr incubation. ^{3}H-TM can diffuse into the body tissues of both schistosomula and adult worm after incubation for more than one hour, since the body counts have increased.

Kuo and Lampen (1975) had reported that TM binding to bacterial protoplasts was complete within one minute and it was found that bound ^{3}H-TM was associated with the particulate membrane fraction (20,000g; 20 min pellet). The ^{3}H-TM binding to schistosomula takes 3hr to reach saturation. What is the distribution of ^{3}H-TM bound on surface of S.mansoni? Is the TM binding by schistosomes specific? These questions have been addressed by using ^{3}H-TM plus unlabelled TM. Fig.3.35. describes the experimental result when ^{3}H-TM was incubated with unlabelled TM. When unlabelled TM was added to the incubation medium, there was little affect on ^{3}H-TM binding to worm body tissues. However, ^{3}H-TM binding to the schistosomula tegument was decreased by the addition of unlabelled TM. This implies that there are some specific sites on or near the surface of worm for TM, but much non-specific binding.

3.3.9.Discussion

The importance of surface carbohydrates is stressed by the fact that S. mansoni

Fig.3.33. ^{3}H-TM binding to fresh schistosomula
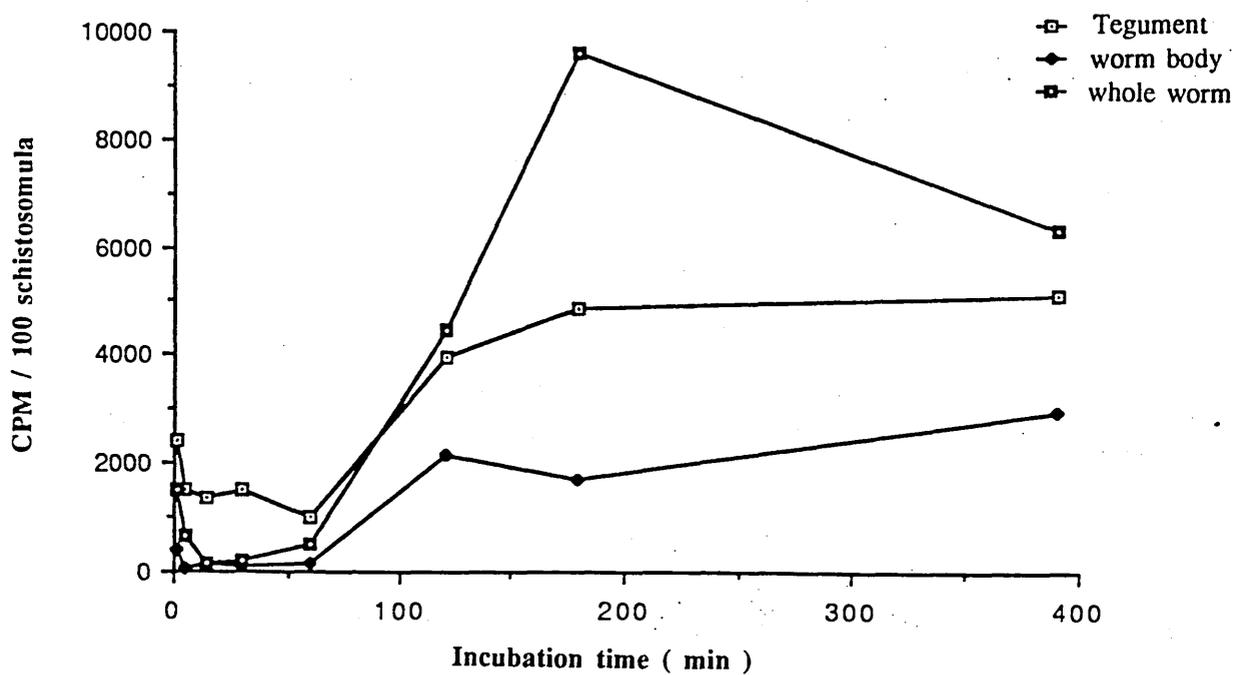
Newly mechanically transformed schistosomula were incubated with ^{3}H-TM for different times up to 3hr. Then, the labelled parasites were treated with 0.2% Triton X-100 to remove the tegument. ^{3}H -TM conc. is 10 μCi / ml

Fig.3.34. ^{3}H-TM binding to adult worm
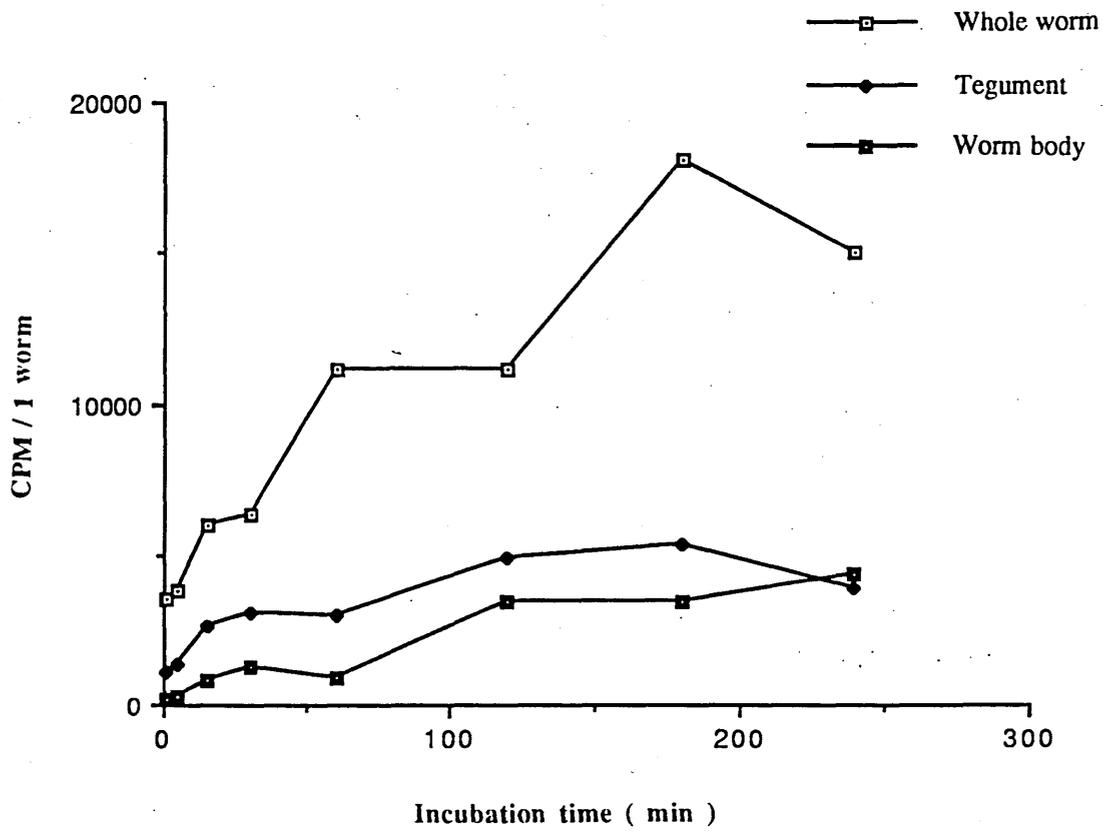
Adult worms were incubated with ^{3}H-TM for different times up to 3hr. Then, the labelled parasites were treated with 0.2% Triton X-100 to remove the tegument. ^{3}H - TM conc is 10 μCi / ml.

Fig. 3.35. Effect of unlabelled TM on ^{3}H-TM binding to schistosomula
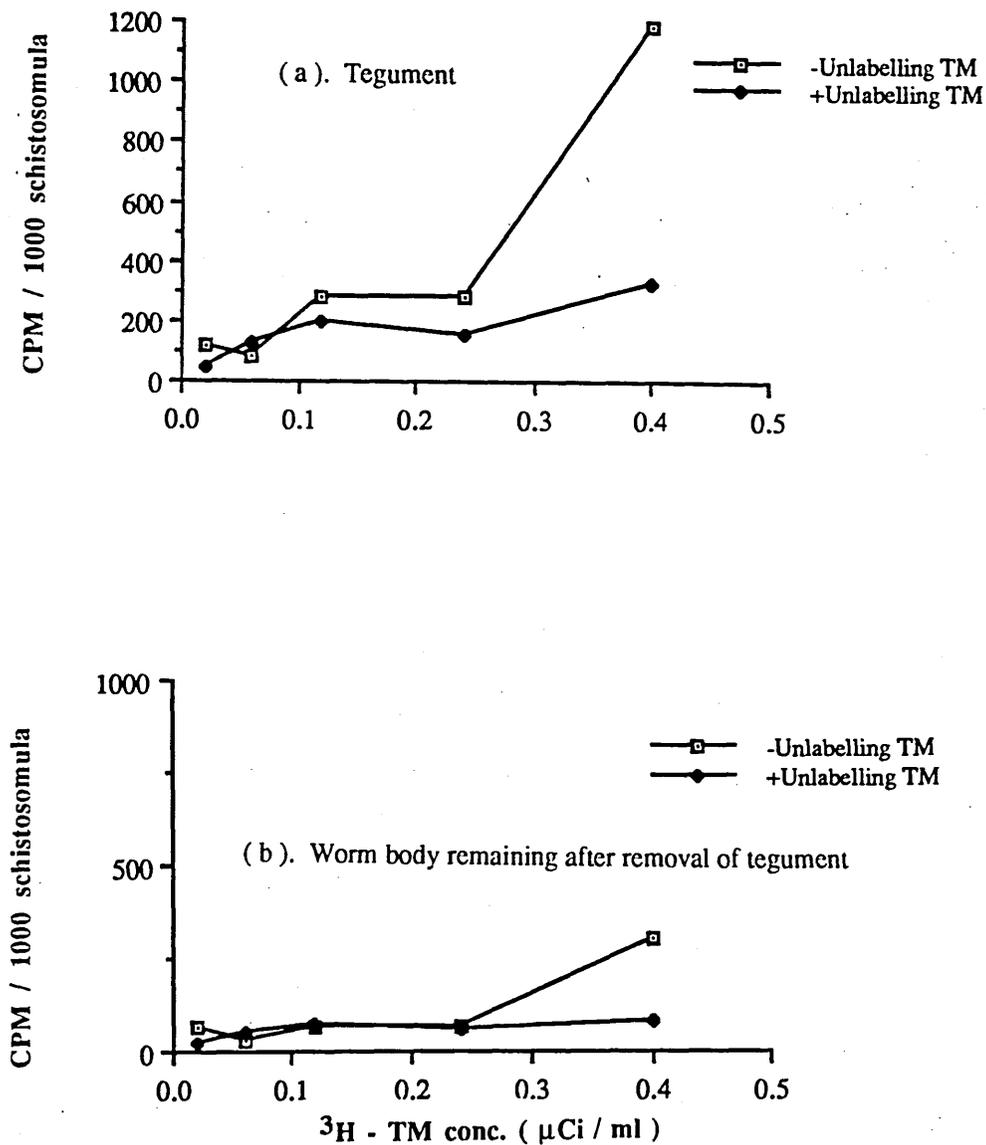
Schistosomula were incubated with ^{3}H - TM at different conc. plus unlabelled TM at 50 μg / ml for 2hr at 37 °C. In control group, schistosomula were incubated with 3H - TM for 2hr alone. Then, the labelled parasites were treated with 0.2% Triton X - 100 to remove the tegument.

is invested with a syncytial tegument about 4.0 nm in the thickness. The outer tegumental membrane of cercariae consists of a single bilayer. A 1-2 μm thick glycocalyx coat covers the outer membrane of cercariae. After the cercariae penetrate the skin of the vertebrate host, they lose their tails and the glycocalyx is shed. The outer tegumental membrane rapidly changes to a double bilayer consisting of two closely apposed lipid bilayers. Robertson and Cain (1985) have identified glycosaminoglycans (GAGS) at the parasite surface in the later stages of development. The glycosaminoglycans might interact with the extended chains of membrane glycoproteins and glycolipids to form a loose and possibly incomplete glycocalyx (McDiarmid and Podesta, 1984). It is possible that the carbohydrates create a microenviroment.(Kusel and Gordon, 1989). This structure may be an outer cytoskeleton and it has been therefore suggested that the outer cytoskeleton is associated closely with the membrane by both ionic and hydrophobic bonding. This carbohydrate structure could be quite variable in its density and the number of negative charges. This may explain very variable results. (for example, Fig. 3.11.)

To investigate these surface structures, we have used a variety of lectins. Lectins can bind specifically to saccharides on the surface of cells.

Quantitation of FITC-ConA binding to different life cycle stages of S. mansoni that had been treated with TM (Conc. of TM from 5μg-20μg / ml) for 3hr showed a significant increase in binding (except for 54 day adult worms) as compared with normal parasites (Table.3. 2; Table. 3. 4.). At the schistosomula stage, the binding of FITC-PNA and WGA was also investigated. The relative intensities of binding of FITC-PNA and WGA to TM treated schistosomula are similar to that detected using FITC-ConA. As compared to normal parasites, the increase observed is larger for PNA and WGA binding than for ConA binding. The increase in lectin bound after TM treatment could reflect changes in the surface membrane or the tegument.

The increased binding by WGA was not reduced by the corresponding sugar. It seems that this binding is not specific. Simpson and Smithers (1980) demonstrated

that the large amount of WGA binding is due to an electrostatic interaction between the negatively charged schistosome surface and WGA rather than specific binding. Thus, the negative charge at the surface of the parasites seemed to be increased after TM treatment. How TM can increase the negative charge is not understood. However, the increased binding by ConA and PNA was greatly reduced in presence of an excess of corresponding sugar (Conc. 150 mM). This means that binding by these lectins was specific. PNA is a galactose binding lectin and requires not only a terminal galactose residue, but also that galactose be conjugated to N-acetyl-D-galactosamine and PNA binding is often considered diagnostic for this sequence.(Lotan, et al. 1975). Although ConA has a broad specificity, its binding is usually attributed to the presence of internal α-D-mannosyl residues in exposed oligosaccharides. However, inhibition studies indicate that 2-deoxy-D-glucose, 2-O-methyl-D-glucose and N-acetyl-D-Glucosamine exhibit almost the same inhibitory activity as D-glucose. Thus, glucose, and other sterically related sugars may be involved in the binding of ConA. Recently, Nyame et al (1987, 1988a,b) have been the first to study systematically the structure of N- and O-linked oligosaccharides derived from schistosomular and adult worm glycoproteins. A number of unusual features of the schistosome derived O- and N-linked oligosaccharides were found. There are for instance, terminal O-linked N-acetylgalactosamine and an O-linked disaccharide, galactose- N-acetylgalactosamine and other major types of simple O-linked sugar chains containing the unusual structure terminal O-linked N-acetylglucosamine linked to peptide. Another unusual feature of these oligosaccharides was the absence of sialic acid. Their results also suggest the possibility that O-linked oligosaccharides are highly clustered on the glycopeptides. Thus, the increased binding by PNA and ConA could be related to the exposure of these sugar residues. TM only affects N-linked glycosylation, not O-linked glycosylation. TM shows very little effect on glycosylation in S. mansoni. The very small inhibition of N-linked glycosylation may affect the structure of outer cytoskeleton and result in exposure of O-linked oligosaccharides. On the other hand, the increased negative charge on the surface membrane may also affect the structure of the outer

cytoskeleton and result in exposure of some oligosaccharides.

Other reasons for the increased ConA binding could be considered. TM contains a N-acetyl-D-Glucosamine in its structure. The ^{3}H-TM experiment shows that TM can bind to the surface membrane of schistosomula and adult worms. However, it is not clear how the TM binds to parasite. Heifitz et al (1979) have suggested that there are two classes of tunicamycin binding sites on the membranous element of the cell. The high-affinity sites are represented by UDP-GlcNAc: dolichyl phosphate GlcNAc-1-phosphate transferase, while low-affinity sites are characterised by non-specific binding of tunicamycin to lipid. TM may bind non - specifically to lipid and not to high - affinity sites on the parasite surface. Thus, the presence of TM on the surface membrane of parasites may result in binding of the drug to ConA, and this might account for the increase in total fluorescence intensity. The results with TM analogues have shown that ConA binding to treated schistosomula was increased by normal TM and by TM-3; neither of the TM-1 or TM-2 analogues gave an increase in ConA binding. The structures of three analogues of TM is shown in Fig.3.36. One difference in three analogues is that TM-1 and TM-2 analogues have saturated structures in their respective aliphatic tails. Previous studies (Kuo and Lampen, 1976) have demonstrated that the binding of TM to yeast protoplasts can be inhibited by the inclusion of phospholipid in the incubation medium. Furthermore, the added phospholipid could inhibit TM binding only if an unsaturated structure was present. Neither TM-1 nor TM-2 analogues have the unsaturated structure in their respective aliphatic tails, and this particular modification might be expected to result in an inability of the analogues TM-1 and TM-2 to bind to parasites. However, in Fig. 3.20. was shown that TM - 1 causes increased permeability in to S. mansoni. Therefore, it must bind to the parasites. This makes it likely that the increase in ConA binding is due to some effect induced directly by the drug (e.g. inhibition of glycosylation).

When the schistosomula were incubated with TM for 24hr, quantitation of FITC-ConA binding was still higher for TM treated parasites than for the normal

Fig.3.36. Structures of tunicamycin analogues. (from Drs. Hashim and Cushley 1987.).

TM-3

TM-2

TM-1

133
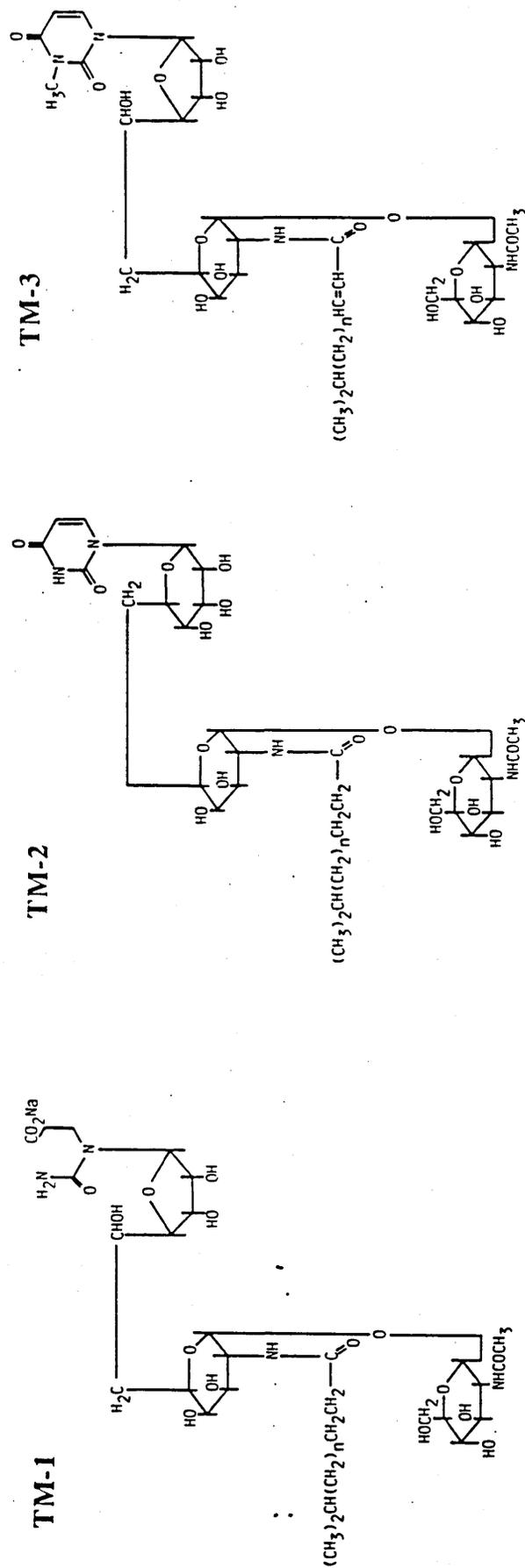
parasites. The amount of ConA bound to TM treated parasites was reduced by FCS in medium, suggesting that FCS prevents the effect of TM on the parasites surface. It has been reported that FCS has a stimulatory effect on protein turnover of membrane proteins of schistosomula (Cordeiro et al. , 1984), presumably by providing precursors and, above all, lipids for membrane manufacture. Thus, TM seems also to be affected by membrane dynamics in schistosomula. In schistosomula, most ligands, including glycolipids, glycoproteins, and complement receptors are released from parasites with a half-life of 10-15 hr (Rumjanek 1987). When newly transformed schistosomula were labelled with FITC-ConA for 30 mins followed by incubation with TM in the presence of 5% FCS or in absence of 5% FCS for 21 hr, the remaining fluorescence on normal cultured schistosomula is significantly less than in TM treated cultured schistosomula. If labelled, fixed schistosomula were cultured in present of 5% FCS or in absence of 5% FCS for 21 hr, the fluorescence intensity in these cultured parasites, both in normal and TM treated, did not decrease. This indicates that the decrease in fluorescence is due to loss of a complex of FITC-ConA bound to surface glycoprotein by shedding into the medium. TM reduced the rate of membrane turnover, thus perhaps increasing in the density of carbohydrate on the surface. The lectin ConA has been found to inhibit the turnover of high molecular weight proteins, an effect which is probably related to its ability to stabilize the tegument of the schistosomula by interlinking membrane glycoproteins through their carbohydrate moiety (Cordeiro, 1984). Why does TM inhibit membrane turnover ? The reduced membrane turnover is probably related to its unsaturated structure, its aliphatic tails binding to membrane lipid and stabilizing the tegument in some way. There is evidence (Kusel and Gordon 1989) that the proteins in the surface of the parasites seem to be much less available to a lipophilic nitrene than are proteins in the erythrocyte membrane, which suggests the presence of very powerful lipid-protein interactions within the parasite surface. FCS in the medium may inhibit TM binding to the parasites and prevent its effect on membrane turnover. A second reason for a reduction

in membrane turnover by may be that TM inhibits fusion of newly formed vesicles. A third reason may be related to changes in the composition of surface carbohydrates, altering the structure of the outer cytoskeleton, since TM inhibits synthesis of N - linked glycoproteins.

As well as lectin binding, other properties of the outer tegumental membrane were also measured.

Schistosomulum surface antigens have been analyzed by surface labelling and immunoprecipitation techniques that have demonstrated a complex set of protein and glycoprotein antigens.(Omer - All et al, 1986)

The effect of TM on surface antigens of S. mansoni was analysed by means of the binding of two different sera. One serum is from infected BALB/c mice. The other serum is from a rabbit infected with irradiated cercariae. The work of Bickle and Ford (1982) suggested that antibodies from chronically infected mice and mice immunized with irradiated cercariae do recognize different sets of epitopes. Omer-ali et al (1986) have demonstrated that antibodies from chronically infected mice specifically recognize schistosomulum surface epitopes of which approximately 60 % are carbohydrate in nature. Moreover, the result of 50 mM sodium metaperiodate treatment of fixed parasites suggests that the carbohydrates recognized by antibody from mice infected with irradiated cercariae are different in chemical composition from those recognized by antibody from chronically infected mice. TM did not affect the binding of antibody from infected mouse serum to schistosomula or to 3 week worms after 3hr or 24hr treatment with TM. TM was also unable to change the binding of serum from a rabbit infected with irradiated cercariae to schistosomula treated with TM for 3hr. However, if the schistosomula were treated with TM for 3hr, followed by culture for 20hr or 42hr, the binding of serum from the rabbit infected with irradiated cercariae to these parasites was increased in two independent experiments. The absence of an effect of TM on binding by infected mouse serum suggested that these antigens may contain O-linked glycoproteins. The increase in binding by the rabbit serum possibly results from some surface antigens being exposed, or to the reduced rate of membrane turnover.

However, it must take some time to expose these epitopes.

The lateral diffusion of macromolecules in the surface membrane is an important biophysical property of membranes. It is known that during cercarial transformation the surface lipid changes from an immobile to a mobile state (Kusel and Gordon 1989). The changes in the diffusion of macromolecules in surface membrane during parasite growth could be related to the increase in susceptibility of the parasite to damage. The lateral diffusion of lipids and glycoproteins in the parasite surface after TM treatment was measured by the fluorescence recovery after photobleaching technique. TM did not change the lateral diffusion of glycoproteins. However, the drug did affect the lateral diffusion of lipid in schistosomula. When the diffusion of C18-Fl in the outer membrane of schistosomula was measured by FRAP after TM treatment there was a significant reduction in the rate of lateral diffusion (D_L). Thus, TM affects the lipid diffusion. The decrease in lipid diffusion in the surface of schistosomula may alter the rate of membrane turnover.

The effect of TM on the ultrastructure of S. mansoni was also investigated. No membrane damage was seen by transmission electron microscopy, when the schistosomula and 4 week adult worms were incubated with TM for 3 hr. The structure of the double membrane was unaffected. The tegumental inclusions were normal. One slight difference can be seen in TM treated 4 weeks adult worms. There was a large degree of vacuolation in TM treated worms. The appearance of big vacuoles in the tegument is a sign of damage to the membrane.(Popiel and Erasmus 1984). When the schistosomula were incubated with TM for 24hr, the ultrastructure of the outer membrane was clearly heptalaminate in both normal and TM treated parasites, whether or not the medium contained FCS. However, the heptalaminate surface of TM treated parasites has unusually dense regions of membrane. This unusual ultrastructure of the heptalaminate surface may be related to blocking of glycosylation by the drug or to insertion of the lipophilic drug. It also might have been expected to see some changes in the discoid granules, since they contain glycoproteins and may fuse

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continuously with surface.(Kusel and Gordon, 1989). But no changes were observed.

All of these results have demonstrated that TM does affect the outer tegumental membrane of S. mansoni . These changes in the surface membrane may affect the viability of parasites in mice. After treatment with TM for 3hr, the schistosomula were injected subcutaneously in BALB/c mice. No adult worms were recovered from mice 5 weeks after infection. The level of antibody in these mice was quite low by ELISA test. The low level of antibody in these mice may be due to the fact that schistosomula died at a relatively early stage of development in mice. Or the antibody stimulated by TM treated schistosomula may not be recognised by normal parasite antigens. Overall, the mechanism of action of TM on the surface membrane of S. mansoni seems to be complex. Possible mechanisms of the effect of TM on the surface membrane of S. mansoni are summanized in Fig. 3.37. Some changes in the surface membrane can be measured immediately after the drug treatment, but some negative results were obtained, perhaps due to membrane dynamics in the schistosome. These changes in the surface membrane could affect membrane functions involved in receiving and processing information from its environment and could inhibit the development of parasites in mice.

Fig. 3.37. Possible mechanisms of effect of TM on surface membrane of _S. mansoni_
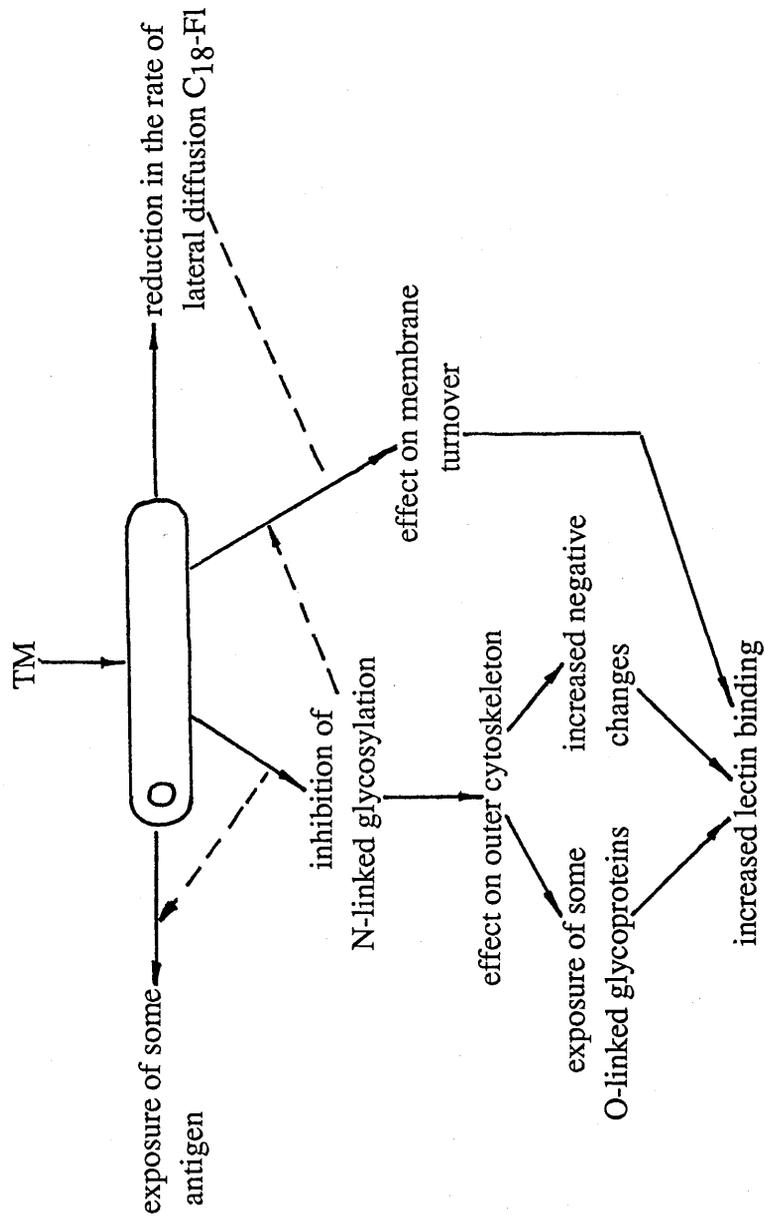
Chapter 4: Inositol phosphate metabolism in <u>S. mansoni</u>

4.1. Introduction:

Changes in growth rate, organogenesis and metabolism occur during maturation of <u>Schistosoma mansoni</u> in the mammalian host. The surface membrane is therefore likely to receive signals from the host. In the TM experiments, the drug appears to inhibit some kinds of glycoprotein synthesis after a long incubation, although there is no great effect on glycosylation after the parasites are treated with this drug for a short time. Some surface membrane properties of the parasite are changed by TM treatment. Serum in the medium gives some protection against this drug. Despite the apparent protective effect of serum, schistosomula treated with TM for 3hr were inhibited in their development in the mouse. It therefore seems that some changes in membrane properties can affect the viability of schistosomula in the host. One possible reason is that these changes in surface membrane properties have an effect on signalling systems in parasites. These changes may influence the parasites' responses to signals from the host, particularly those emanating from the host's immune system. It is therefore of paramount importance to design a rational method to define and characterize the components of signal transduction mechanisms at the surface of parasites.

Biochemical mechanisms of signal transduction in mammalian cell plasma membranes include activation of cyclic AMP production, stimulation of phosphatidylinositol (PI) breakdown and receptor and membrane phosphorylation. It has been reported that <u>S.mansoni</u> possesses an adenylate cyclase system (Kasschau and Mansour, 1982) and GTP-binding proteins which are part of the trans-membrane signalling system (Mansour and Mansour, 1989). However, the pathway of PI turnover has not been studied in <u>S. mansoni</u>. It has been shown that a wide variety of neurotransmitters, hormones, growth factors and many other biologically active substances can stimulate the turnover of inositol phospholipid and this is a signal transduction mechanism.

As a component of phospholipids, PI is found in different life cycle stages of <u>S. mansoni</u>. It has been shown (Wales, 1989) that some drugs such as actinomycin-D,

fenfluramine, and U.V-irradiation all show an effect on phospholipid synthesis. Therefore, it will be very interesting to investigate whether inositol phospholipid turnover can be demonstrated in S. mansoni or not. In this chapter, the phosphatidyl inositol pathway that produces IP_3 as second message has been measured at different life cycle stages of S.mansoni.

4.2. The identification of PI and its breakdown using metabolic labelling

The inositol phosphate cycle in mammalian cell systems has been shown before (Fig.1.11). This metabolic pathway can be measured by using ^{3}H-inositol as a label. In the following experiments with the parasites, different life cycle stages were prelabelled with ^{3}H-inositol for about 20hr. Then the labelled parasites were extracted by $CHCl_3$:MeOH (1:2). The total inositol phosphate can be separated from other components in the water phase by using ion exchange chromatography on Dowex 1x8-formate columns. The results of experiments are summarized in Fig.4.1. which shows that PI turnover occurs in S. mansoni. However, inositol phospholipid metabolism is very slow in the early schistosomula stage and only a small amount of total inositol phosphate has been produced. During development of parasites to maturity, the production of total inositol phosphate increases quickly, indicating that the rate of PI turnover at these stages is much higher than at the schistosomula stage.

4.3. Effect of human serum on PI turnover.

It is known that serum and tissue factors may well affect the parasite's development. Human serum is known to bind to the parasite surface and has the ability to modify the biochemical and immunological properties of the schistosomula surface (Rumjanek and Mclaren 1981). Therefore, the effect of human serum on PI turnover was tested. Prelabelled parasites were stimulated by 10% human serum for 30min in the presence of 10mM Li^+. Li^+ inhibits the monophosphatases and causes

Fig.4.1. Production of total inositol phosphates at different life cycle stages of S.mansoni
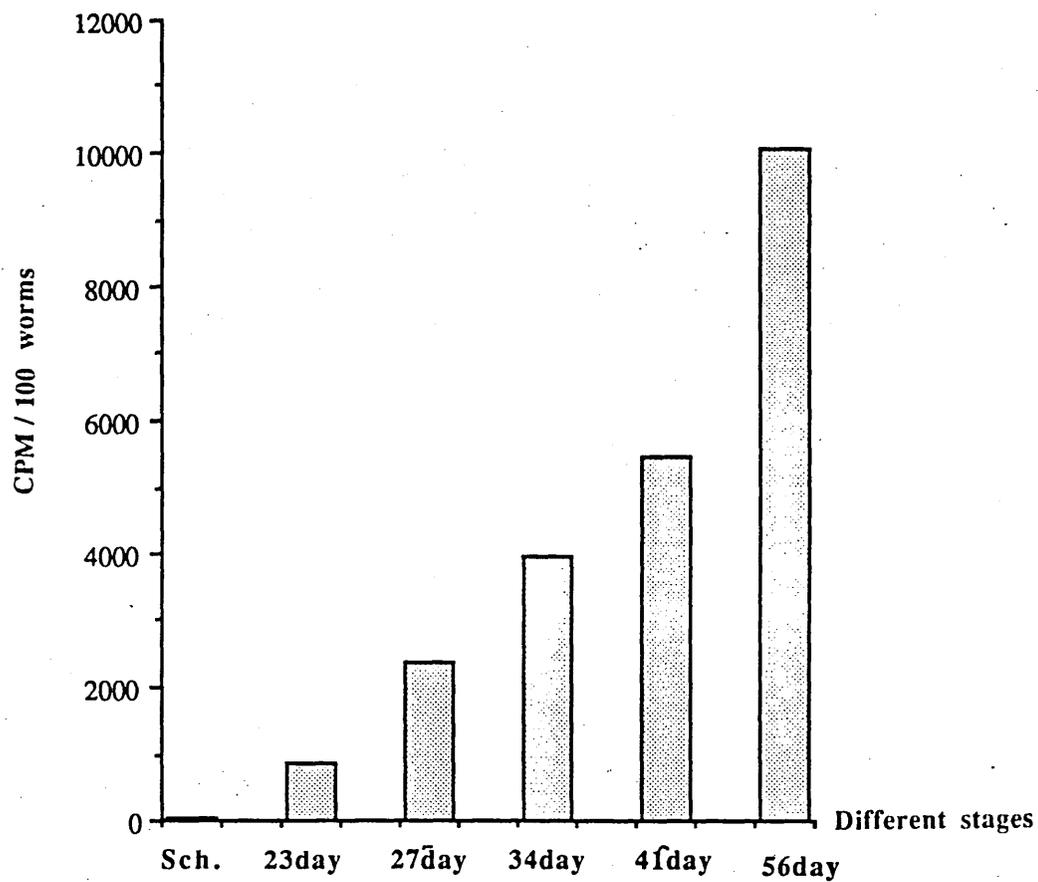
The parasites were prelabelled with ^{3}H-inositol for 20hr. After incubation, the parasites were extracted by using CHCl$_3$:MeOH(1:2). 0.8 ml of the water phase was loaded on 1.0 ml 1x8-formate columns. Total inositol phosphate was eluted by using 3 X 5.0 ml 1.2M ammonium formate / 0.1M formic acid. 1.0 ml eluted solution was taken out to count.

Sch.   Schistosomula

accumulation of the monophosphate fraction. When different life cycle stages of S. mansoni were prelabelled with ^{3}H-inositol, the accumulation of radioactivity in the inositol phosphates was proportional to the breakdown of PIP_2. The results of these experiments are illustrated in Fig.4.2. Human serum did not cause accumulation of the monophosphate fraction of schistosomula. However, stimulation of 23 to 32 day old worms by 10% human serum resulted in an increase in the production of inositol phosphates. The extent of increase is about 20-30%. When the parasites developed into the adult worm stage (81 days), the production of inositol phosphates was a little higher in stimulated parasites than in normal parasites.

We concluded from this that when measurements were made with the intact parasite, no evidence for greatly increased turnover was found.

4.4. Effect of Praziquantel on PI turnover

Praziquantel (PZ) is a drug broadly effective against trematodes and cestodes (Harnett. 1988). It is known that this drug can act on the surface membrane of S. mansoni, and induces muscle contraction, tegumental disruption and increased Ca^{++}-fluxes. PZ has also been shown to increase parasite antigen exposure in adult S.mansoni in vivo and in vitro. Harder et al (1987) have reported that PZ and Ca^{++} can affect bilayer-isotropic-hexagonal transition of model membranes. Therefore, the effect of PZ on PI turnover in S. mansoni was measured.

Fig. 4.3. summarizes the results of the effect of PZ on PI turnover at different life cycle stages of S. mansoni. PZ seems to affect the inositol phosphate pathway in 32, 34 and 41 day old worms. The amount of total inositol phosphate produced was greatly increased by using PZ treatment for 30min at $10\mu g/ml$ in the presence of Li^+ (10mM) on worms of two ages. However, when the drug concentration was increased to $20\mu g/ml$, the production of total inositol phosphate was no different from normal.

4.5. Effect of polycation on PI turnover

Table. 4.1 indicates the effect of different molecular weight polycations on PI

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Fig.4.2. Effect of human serum on PI turnover at different life cycle stages of S.mansnoi
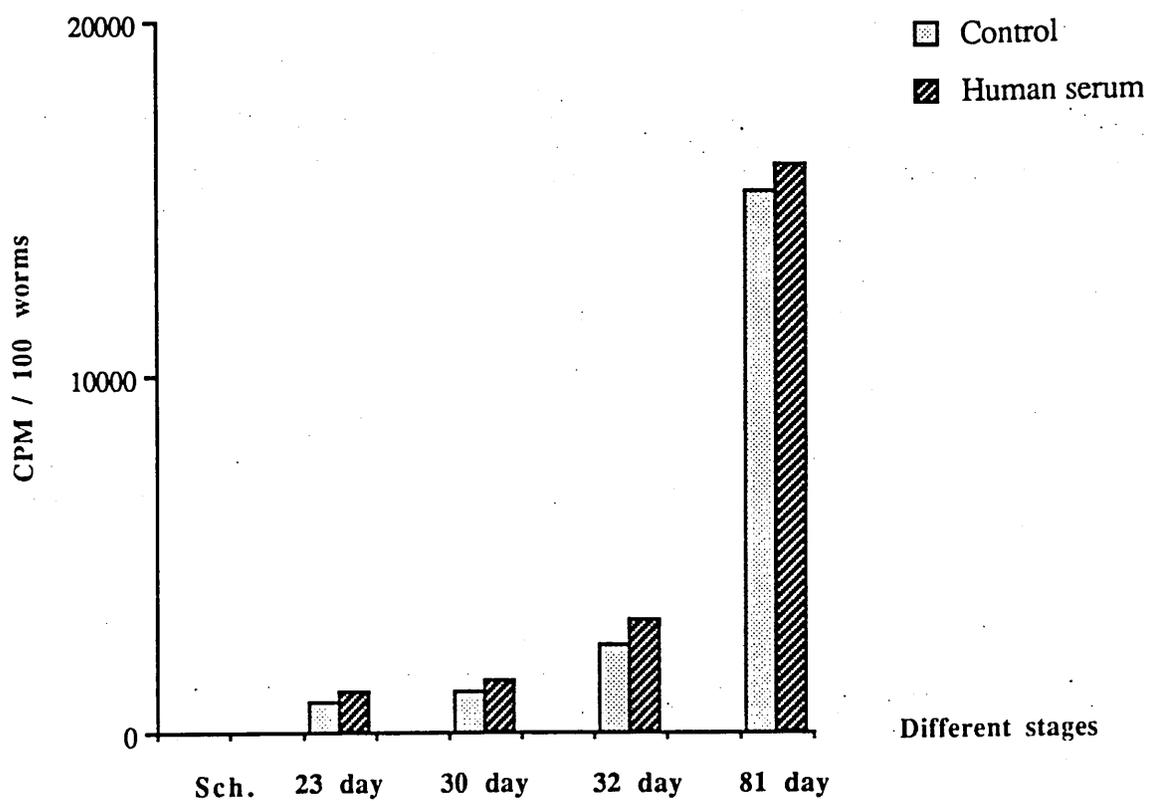
After parasites had been prelabelled with ^{3}H-inositol for 20 hr, the parasites were incubated with Li^+ (10 mM) for 30 min. Then, 10% human serum was added to stimulate. After end of stimulation, the parasites were extracted by $CHCl_3$:MeOH (1:2). 0.8 ml of water phase was loaded on 1.0 ml 1X8-formate columns. Total inositol phosphates were eluted by 3 X 5.0 ml 1.2M ammonium formate / 0.1 M formic acid. 1.0 ml eluted solution was taken out to count.

Fig.4.3. Effect of PZ on PI turnover at different life cycle stage of S. mansoni
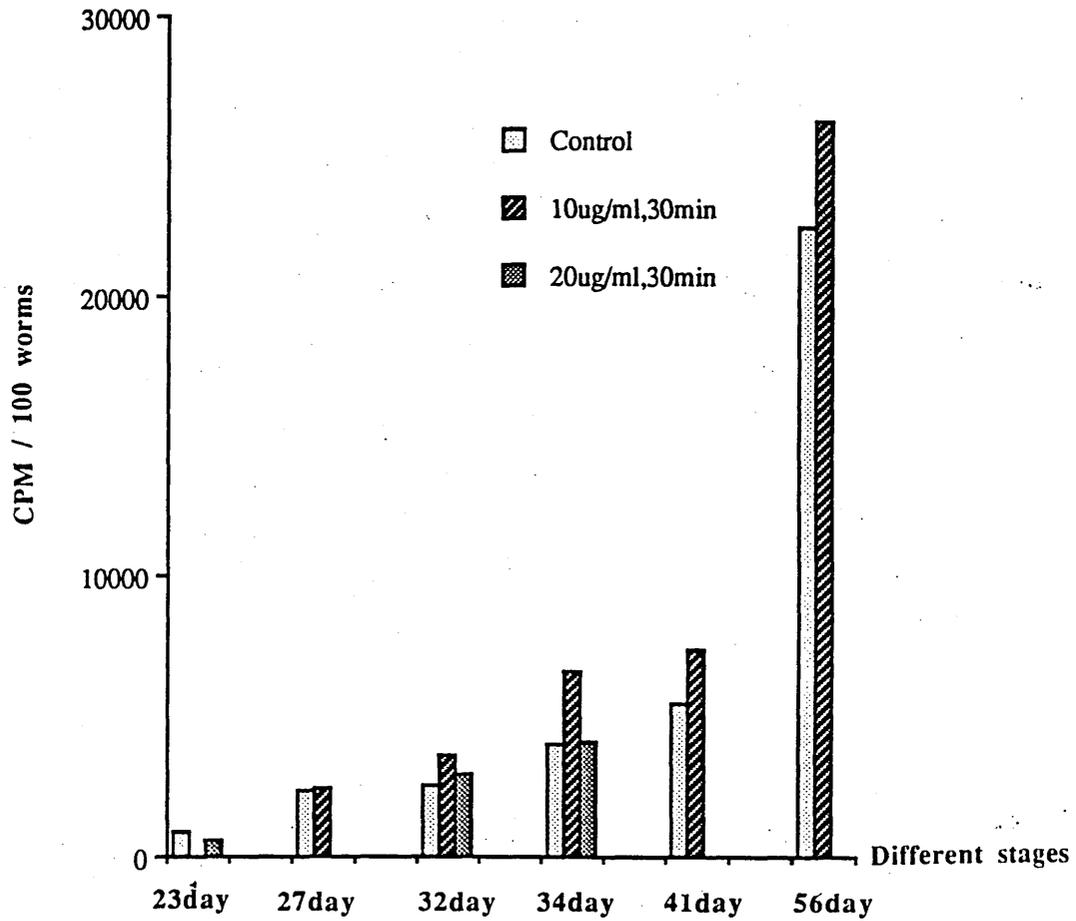
Parasites prelabelled with ^{3}H-inositol for 20 hr were incubated with Li^+ (10 mM) for 30 min. Then, different Conc. of PZ were added to stimulate. After end of stimulation, the parasites were extracted by $CHCl_3$: MeOH (1:2). 0.8 ml of water phase was loaded on 1.0 ml 1 X 8 - formate column. Total inositol phosphates were eluted by 3 X 5 ml 1.2M ammonium formate / 0.1 M formic acid. 1.0 ml eluted solution was taken out to count.

Sch.: Schistosomula

Table.4.1. Effect of Poly-L-Lysine on PI turnover in <u>S. mansoni</u>

Parasite stages	Molecular weight of PLL	Conc. of PLL	Total inositol phosphates (CPM/100 worms)	
			Control	Stimulation
Schistosomula	100,00-300,00	40ug/ml	108	98
43 day worm	38000	20ug/ml	8780	9150
	38000	100ug/ml	8780	8780
	100,00-300,00	20ug/ml	8780	6300
	100,00-300,00	100ug/ml	8780	6420
56 day worm	38000	20ug/ml	10075	11700
	38000	100ug/ml	10075	11100
	100,00-300,00	20ug/ml	10075	9950
	100,00-300,00	100ug/ml	10075	10700

Parasites prelabelled with ^{3}H-inositol for 20 hr were stimulated by different molecular weight of poly-l-lysine (PLL) and different Conc. of PLL for 30 min in present of Li$^+$ (10 mM). After end of stimulation, the parasites were extracted by CHCl$_3$: MeOH (1:2). 0.8 ml of water phase was loaded on 1.0 ml 1 X 8 - formate column. Total inositol phosphates were eluted out by 3 X 5.0 ml 1.2 M ammonium formate / 0.1 M formic acid. 1.0 ml eluted solution was taken out to count

turnover in adult worms. It was found that the polycations did not greatly affect the inositol phosphate pathway. The low molecular weight polycations at low concentration caused a slight increase in the amount of total inositol phosphate in treated parasites. However, the high molecular weight polycation seems to slightly decrease the amount of inositol phosphates.

4.6. Discussion:

Rapid progress has been made in understanding inositol lipid metabolism as a signal transduction mechanism . A wide variety of neurotransmitters, hormones, growth factors and many other biologically active substrances are known to provoke the turnover of phosphatidylinositol (PI) in their target tissues. There are four biochemical cycles concerned with the role of the inositol lipid in signal transduction: the lipid and inositol phosphate cycles ultimately unite to synthesize phosphatidylinositol, which is fed into the phosphatidylinositol / PtdIns (4,5) P2 futile cycles. Binding of certain hormones, neurotransmitters and growth factors to their specific cell surface receptors leads to the hydrolytic cleavage of PIP_2 to generate inositol 1, 4, 5 - trisphosphate (IP_3) and sn - 1, 2 - diacylglycerol (DAG). These two molecules are both second messengers : IP_3 stimulates the release of Ca^{2+} from intracellular stores and DAG is the physiological activator of protein kinase C. Myo-[2- (n) - ^3H] inositol has been commonly used in both animal and plant systems to investigate phosphatidylinositol turnover. In this work we have concentrated on the inositol phosphate cycle. In this pathway, Li^+ inhibits the monophosphatases and cause accumulation of the monophosphate fraction. Thus, when cells have been prelabelled with [^3H] inositol, the accumulation of radioactivity in the inositol phosphates is proportional to the breakdown of PIP2 and thus a measure of activation of receptors. Dowex - formate can be used to determine the production of total inositol phosphates.

The level of inositol phosphates in different life cycle stages of S. mansoni was

measured first. (see Fig. 4.1). The amount of total inositol phosphates is too low to be detectable at the schistosomula stage. The level of inositol phosphates in later stages is greatly increased. It seems that the pathway of inositol phospholipid metabolism is present in S. mansoni. The marked increases in the activation of inositol phospholipid metabolism may be required for the development of the parasite in its mammalian host. In order to know the importance of the pathway of inositol phospholipid metabolism in S. mansoni, some ligands were chosen for testing which have known effects on surface membranes of S. mansoni. Lipoprotein components of human serum are known to bind to the parasite surface and have the ability to modify the biochemical and immunological properties of the schistosomula surface (Rumjanek et al 1983). Polycations also have been shown to act on the membrane of parasites. However, neither of these ligands caused significant accumulation of the inositol phosphate fraction. Praziquantel (PZ) is a drug broadly effective against trematodes and cestodes (Harnett. 1988). When the different stages of parasites were treated with PZ for 30min at $10\mu g/ml$ in the presence of Li^+ (10mM), the amount of total inositol phosphate produced was increased in 32, 34 and 41 day old worms. Although the precise way in which PZ kills schistosomes and other helminths is not understood, it has been known that PZ can change the flux of divalent cations. The uptake of Ca^{2+} by male S. mansoni is rapidly increased by the drug, whilst that of K^+ is reduced (Pax et al., 1979). However, people consider that changes in the flux of divalent cations are linked to the muscular contraction of schistosomes. There is no indication of any effect on neurotransmitters. However, PZ affected inositol phospholipid metabolism. Irvine and Moor (1986) have provided indirect evidence which suggests that IP_4 might control Ca^{2+} entry across the plasma membrane. They have used sea urchin eggs in their approach to the problem of identifying a function of IP_4. In view of those findings they deduced that IP_3 elicited full activation by virtue of its ability first of all to mobilize intracellular Ca^{2+} by a direct action on the IP_3 receptor associated with the endoplasmic reticulum. Then, IP_3 could complete its stimulatory effect by being

converted to IP_4, allowing plasma membrane Ca^{2+} gates to be opened. Whether the rapid increase in uptake of Ca^{2+} in S. mansoni by the drug is related to IP_4 is not clear, because inositol phosphates have not been separated and analysed It is also reasonable to speculate that changes in the flux of divalent cations may function to activate other factors that affect the PI turnover. (Tang et al 1988)

Chapter 5: Protein phosphorylation in <u>S. mansoni</u>

5.1 Introduction:

The interplay between phosphorylation and dephosphorylation of cellular proteins is recognized as a major mechanisms by which intracellular events are controlled by extracellular signals. In normal cells, phosphoserine makes up a large proportion of all phosphate in protein, phosphothreonine accounts for about 10%,while phosphotyrosine is about 0.05% (Sefton et al, 1980). However, the phosphorylation of proteins at tyrosine residues is often one of the earliest measurable features in cells transformed by certain viruses or treated with growth factors or hormones. Binding of epidermal growth factor to mammalian cells leads to the phosphorylation of tyrosine residues of EGF-receptor.(Ushir and Cohen, 1980). In addition, the rarity of tyrosine phosphorylation may reflect the fact that the substrates of the PTKs are themselves nonabundant proteins whose functions are regulatory. Tyrosine phosphorylation could be reserved for the regulation of signalling mechanisms rather than of metabolic processes per se. It has been shown that schistosome membrane proteins can be phosphorylated both in the living parasite and in isolated membrane fractions (Kalopothakis 1987). Differences in protein phosphorylation at different life cycle stages of S. mansoni were studied using a ^{32}P labelling method. The phosphorylation of tyrosine residues was also measured. The phosphorylation of tyrosine residues is stable to treatment with base (Hunter and Sefton 1980). Therefore, it can be distinguished from the phosphorylation of other residues.

5.2. Time course for ^{32}P-phosphate-labelling in fresh schistosomula

In order to find a time suitable for labelling parasites, fresh mechanically transformed schistosomula were incubated for varying time periods in a medium containing 100uCi [^{32}P] phosphate. The experimental results are shown in Fig. 5.1(a) and (b). In Fig. 5.1(a), the SDS-PAGE was not treated with 1N KOH after electrophoresis. Therefore, the total phosphorylated polypeptides were detected. It seems that polypetides were more heavily phosphorylated after longer incubation of

Fig.5.1. **Time course of ^{32}P - labelling of fresh schistosomula.**

Fresh mechanically transformed schistosomula were incubated for varying time periods in a medium containing 100 μCi [^{32}P] phosphate. The labelled parasites were dissolved in sample buffer and run on an SDS - PAGE.

L: Lane.

Lane 1: incubation with [^{32}P] phosphate for 1hr.

Lane 2: incubation with [^{32}P] phosphate for 3hr.

Lane 3: incubation with [^{32}P] phosphate for 6hr.

(a). The gel was not treated with KOH for 1.5hr.

(b). The gel was treated with 1 N KOH for 1.5hr after running on SDS - PAGE.

a.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

— 36

— 30

L :   1   2   3

b.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

— 36

— 30

L :   1   2   3
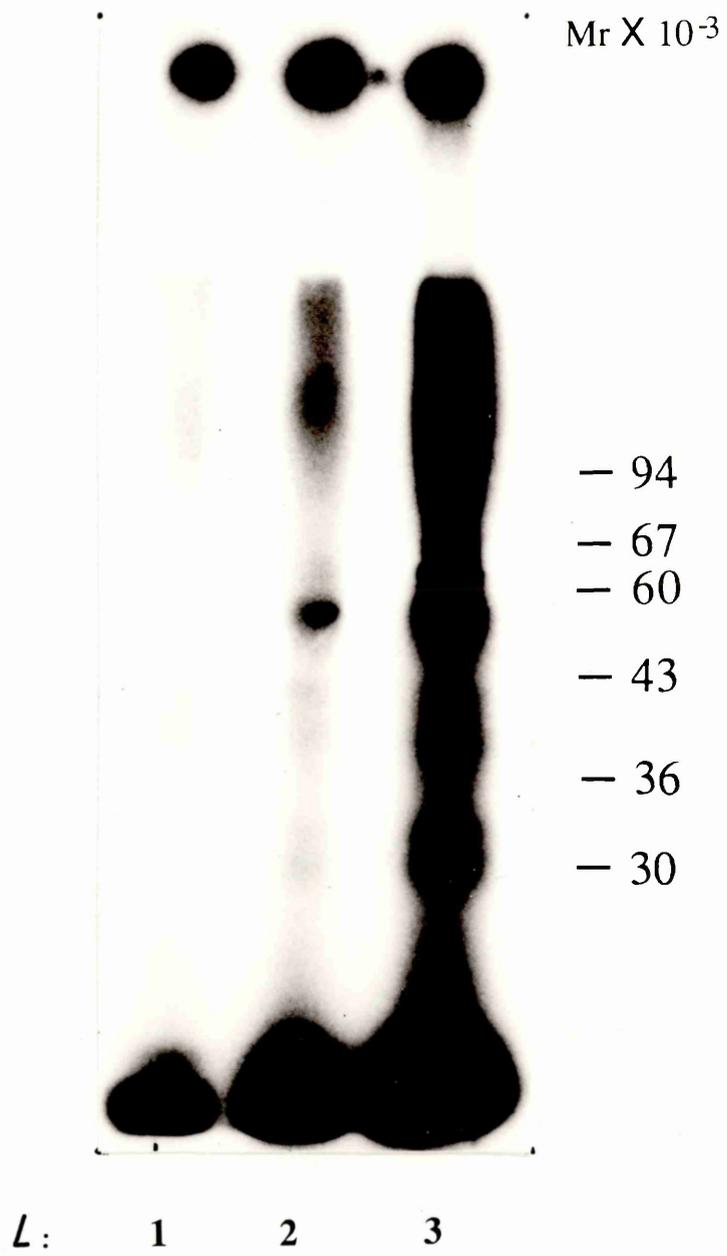
parasites. However, the patterns of the phosphorylation in parasites cultured for periods from 3hr to 6hr were similar.

When the SDS-PAGE was treated with 1N KOH for 1.5hr after electrophoresis, it can still clearly be seen that some bands of phosphorylated polypeptides are detected in parasites which were incubated for 3hr or 6hr. However, the phosphorylation of polypeptides was too slight to see when the parasites were incubated for 1hr.(Fig. 5.1.b.). Therefore, 3hr was chosen as a time suitable for labelling in parasites.

5.3. Distribution of phosphoproteins in cercariae, mechanically and skin transformed schistosomula.

From cercariae to schistosomula, the parasite undergoes big changes, involving a different morphology, life in environments of different osmolarity and expression of different functions. The fact that many adaptive changes are made during this transformation stage suggests that the parasite must be capable of receiving and processing information from its environment. Therefore, the number and size distribution of phosphorylated polypeptides in cercariae, mechanically and skin transformed schistosomula have been evaluated.

The representative results of a series of experiments on phosphorylated proteins in these parasites is depicted in Fig 5.2(a). In cercariae, very few proteins have been phosphorylated. However, after cercariae were transformed into schistosomula, the number of phosphorylated polypeptides was increased. Many phosphorylated polypeptides were located in the 220-94 kDa, 67-30 kDa ranges and at the dye front. Mechanically and skin transformed schistosomula had similar complements of phosphorylated polypeptides.

When the SDS-PAGE was treated with KOH, no bands of phosphorylated proteins were left in the cercariae sample. However, a few bands of phosphorylated proteins can be clearly identified in the mechanically and skin transformed schistosomula samples. The patterns of those proteins in both samples were similar.(see Fig. 5.2 b). It seems that these phosphorylated proteins are more stable to base.

Fig.5.2. Distribution of phosphoproteins in cercariae, mechanically and skin transformed schistosomula.

Lane 3 is cercariae, Lane 2 is mechanically transformed schistosomula, Lane 1 is skin transformed schistosomula. Lane 4 is adult worm. The parasites were incubated with ^{32}P - phosphate for 3hr. Labelled parasites were dissolved in sample buffer and run on SDS - PAGE.

L: Lane.

(a). The gel was not treated with KOH for 1.5hr.
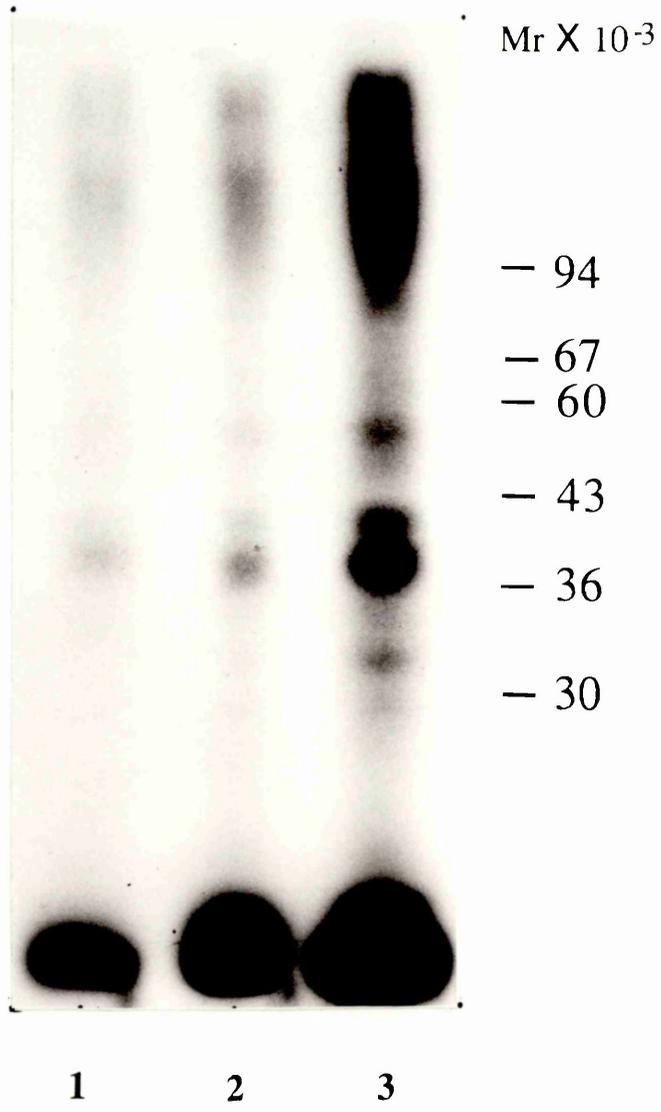
(b). The gel was treated with 1 N KOH for 1.5hr after running on SDS - PAGE.

Mr X 10⁻³

— 94
— 67
— 60
— 43
— 36
— 30

3   2   1
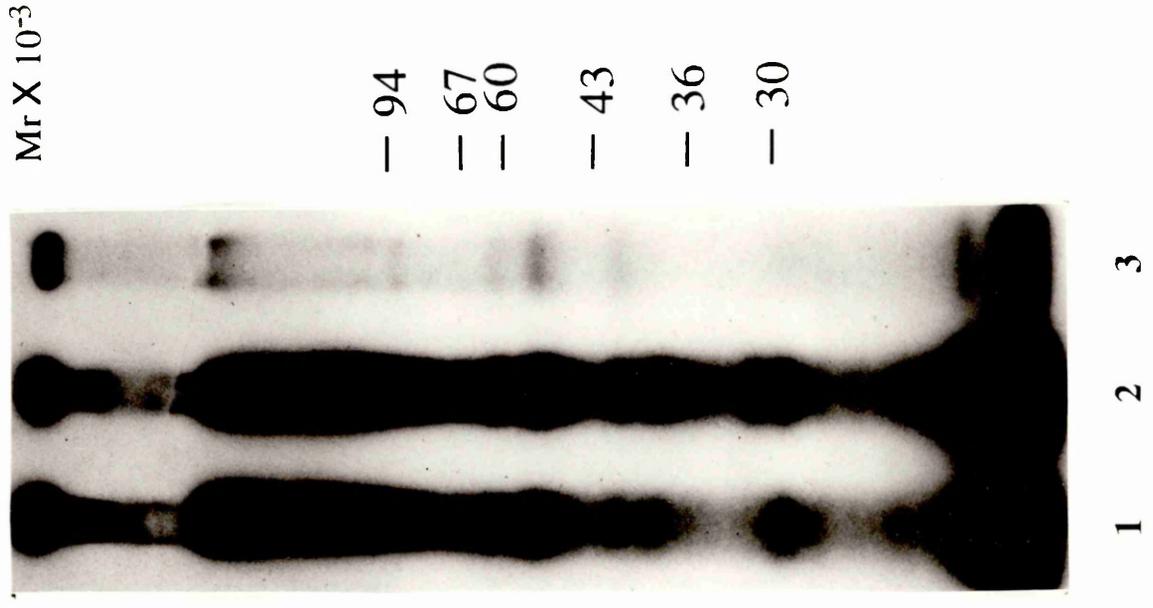
Mr X 10⁻³

— 94
— 67
— 60
— 43
— 36
— 30

4

a.

Mr X 10⁻³

94
67
60
43
36
30

b.
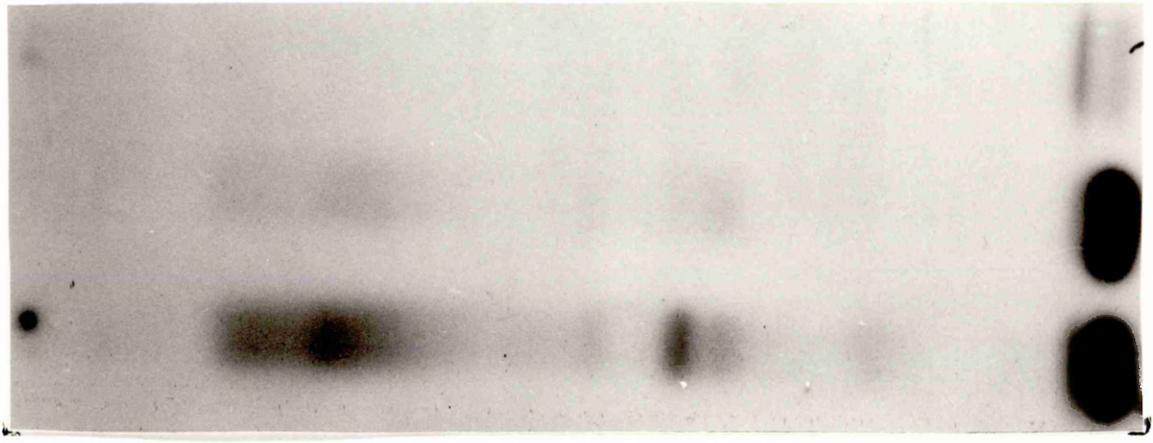
They may contain phosphotyrosine rather than phosphothreonine or phosphoserine. The nature of these phosphorylated proteins has been investigated in the following experiment.

5.4. The effect of culture time in vitro on protein phosphorylation in schistosomula

It has been reported that covalently labelled glycoproteins and glycolipids are lost from the surface of newly transformed schistosomula when these parasites are cultured in vitro. The effect of culture time in vitro on protein phosphorylation has therefore been studied.

After labelling of newly transformed schistosomula with [^{32}P]-phosphate for 3hr, the labelled parasites were divided into two parts. One part was a control group which was kept at $-70^{o}C$. The other part was incubated in GMEM medium containing 5% FCS for 24hr. Fig. 5.3(a) indicates that after culturing labelled parasites for 24hr, the phosphorylated proteins of the parasites are slightly changed, compared with control parasites. One phosphorylated protein with molecular weight of about 67 kDa is lost. Another new phosphorylated protein appears. The molecular weight of this new protein is about 38kDa. After treatment of the SDS-PAGE with KOH, this phosphorylated protein was still present. (see Fig. 5.3(b)). The result shown in Fig. 5.3 (a,b) is from one experiment only

However, as a result of the variability between cercariae shed from different batches of snails, sometimes this result can not be repeated in every experiment. The results from five experiments are summarized in Table. 5.1. This new phosphorylated protein could be newly synthesized and phosphorylated during culturing or a presynthesised protein may be phosphorylated. This band is not phosphorylated by U. V. - irradiated parasites. It is known that UV irradiation can inhibit synthesis of new proteins in parasites (Wales 1989).

When normal (non - irradiated) schistosomula are incubated for longer periods

Fig.5.3. The effect of culture time in vitro on protein phosphorylation in schistosomula

Fresh mechanically transformed schistosomula were incubated with ^{32}P - phosphate for 3hr. The labelled schsistosomula were divided into 2 groups. One was cultured in GMEM medium plus 5% FCS for 21hr (lane 2) and the other was kept at -70 ^{o}C. Next day, samples from both groups were dissolved in sample buffer and run on SDS - PAGE.

L: Lane.

(a). The gel was not treated with KOH for 1.5hr.

(b). The gel was treated with 1 N KOH for 1.5hr after running on SDS - PAGE.

a.

Mr X 10⁻³

— 94

— 67
— 60

— 43

← 38 36

— 30

L: 1   2

b.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

←38 — 36

— 30

L: 1   2
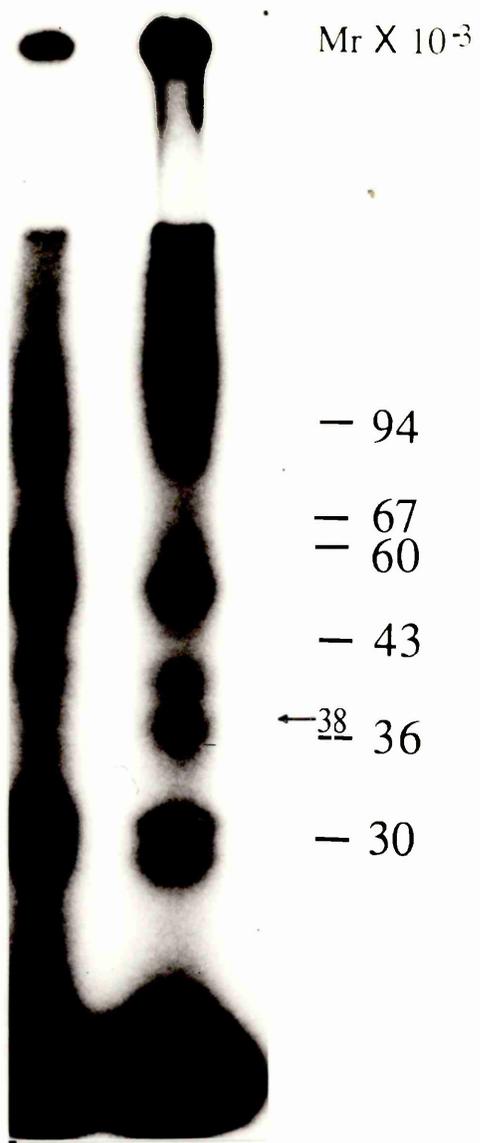
Table. 5.1. The appearance of 38 kDa protein in five times experiments

Different treatment \ 38 kDa protein \ Source from	Gel was treated with KOH		Gel was not treated with KOH	
	Culture time		Culture time	
	0hr	21 - 24hr	0hr	21 - 24 hr
Fig. 5.3	-	+	-	+
Fig. 5.4	+	+	+	+
Fig. 5.5	+	+	+	+
Fig. 5.6	-	+	+	+
Fig. 5.7	-	+	-	+

" - " means not present.

"+" means present.

in vitro and then labelled with [^{32}P] - phosphate for 3hr, most of the phosphorylated proteins show increased phosphorylation and seem to be more stable to KOH treatment. (See Fig. 5.4 a,b).

5.5. Effect of irradiation on phosphorylation of proteins in schistosomula

Three experiments have indicated that irradiation did not have a significant effect on protein phosphorylation in schistosomula incubated with ^{32}PO$_4$ for 3hr. There is also variability between experiments, and the result did not appear to repeat well. (see Fig. 5.6.a. lane2 and 4; Fig. 5.7. a. lane 1 and 2). However, when labelled irradiated parasites were incubated for 24hr, protein phosphorylation was decreased, compared with normal parasites. (see Fig. 5.5. a. lane 2 and 3; Fig. 5.6 a lane 1 and 3; Fig. 5.7a. lane 3 and 4). Phosphorylated proteins in irradiated parasites seem more unstable to base. The treatment of SDS - PAGE with KOH for 1.5 hr seems to remove all of the phosphorylation. (see Fig. 5.5b. Fig. 5.6b. Fig. 5.7b).

In addition, the band of molecular weight about 38 kDa did not appear in irradiated parasites during the culture period of 24 hr. Thus, this protein could be newly synthesized or phosphorylated by the parasites.

5.6. Effect of human serum and TM on protein phosphorylation in schistosomula

It is known that human serum can have an effect on surface membranes of schistosomula and result in some expression of new antigens, one of which is the LDL receptor. Incubation in human serum seems to increase protein phosphorylation and did not result in any major changes in the pattern of phosphorylated proteins.(see Fig. 5.8.a.b.).

In mammalian signal systems, some receptors are glycoproteins. (Jacobs et al. 1979). Therefore, there may be some relation between protein phosphorylation and

Fig.5.4. The distribution of phosphorylated proteins in schistosomula after 24hr incubation.

Fresh mechanically transformed schistosomula were divided into 3 groups. Sample one (lane 3) was labelled with ^{32}P - phosphate for 3hr and kept at -70 ^{o}C. Sample two (lane 2) was labelled with ^{32}P - phosphate for 3hr and cultured in GMEM medium plus 5 % FCS for 24 hr. Sample three (lane 1) was cultured in GMEM medium plus 5 % FCS for 24 hr and then labelled with ^{32}P - phosphate for 3hr. All labelled parasites were dissolved in sample buffer and run on SDS - PAGE.

L: Lane.

(a). The gel was not treated with KOH for 1.5hr.

(b). The gel was treated with 1 N KOH for 1.5hr after running on SDS - PAGE.

a.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

— 36

— 30

L: 1   2   3

b.
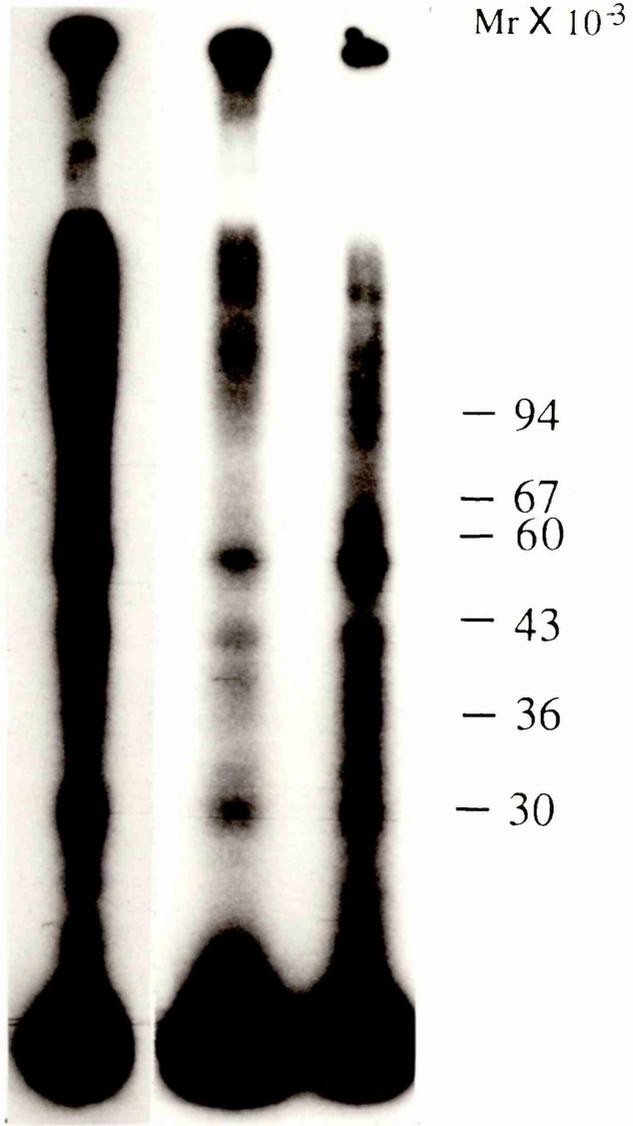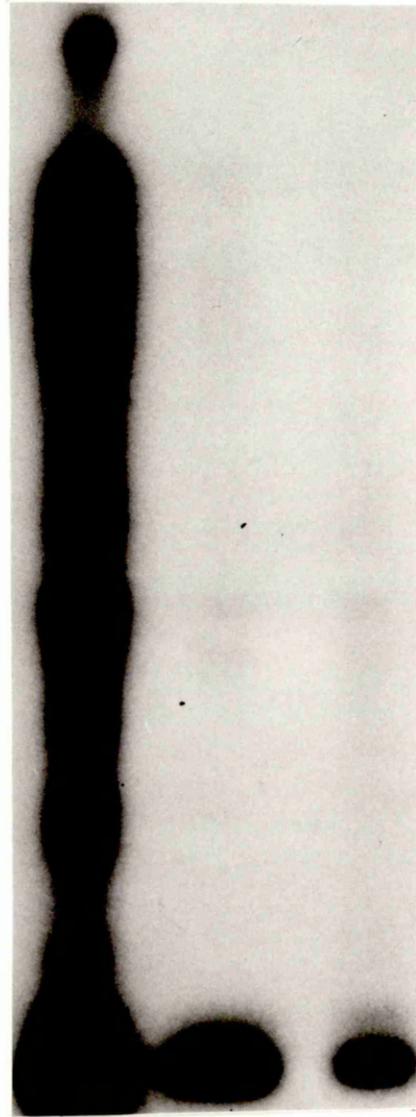
Mr X 10^{-3}

— 94

— 67
— 60

— 43

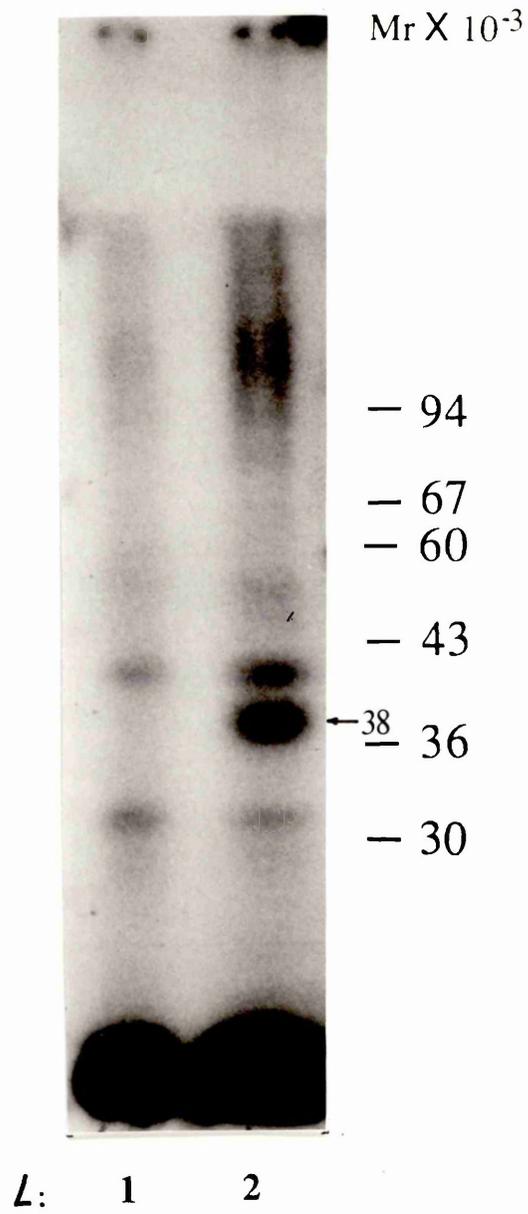— 36

— 30

L: 1   2   3

Fig.5.5. Effect of irradiation on phosphorylation of proteins in schistosomula.

The cercariae were divided into three groups. Sample one (lane 1) and sample two (lane 2) were transformed into schistosomula and labelled with $^{32}PO_4$ for 3hr. After labelling, sample one was kept at -70°C. Sample two was cultured in GMEM medium plus 5 % FCS for 24 hr. Sample three (lane 3) was irradiated for 3min and transformed into schistosomula. Irradiated schistosomula were labelled with $^{32}PO_4$ for 3hr and cultured in GEME medium (5 % FCS) for 24hr. All labelled parasites were dissolved in sample buffer and run on SDS - PAGE.

L: Lane.

(a). The gel was not treated with KOH.

(b) The gel was treated with 1N KOH for 1.5 hr after running on SDS - PAGE.

a.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

— 36

— 30

L: 1   2   3

b.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

— 36

— 30
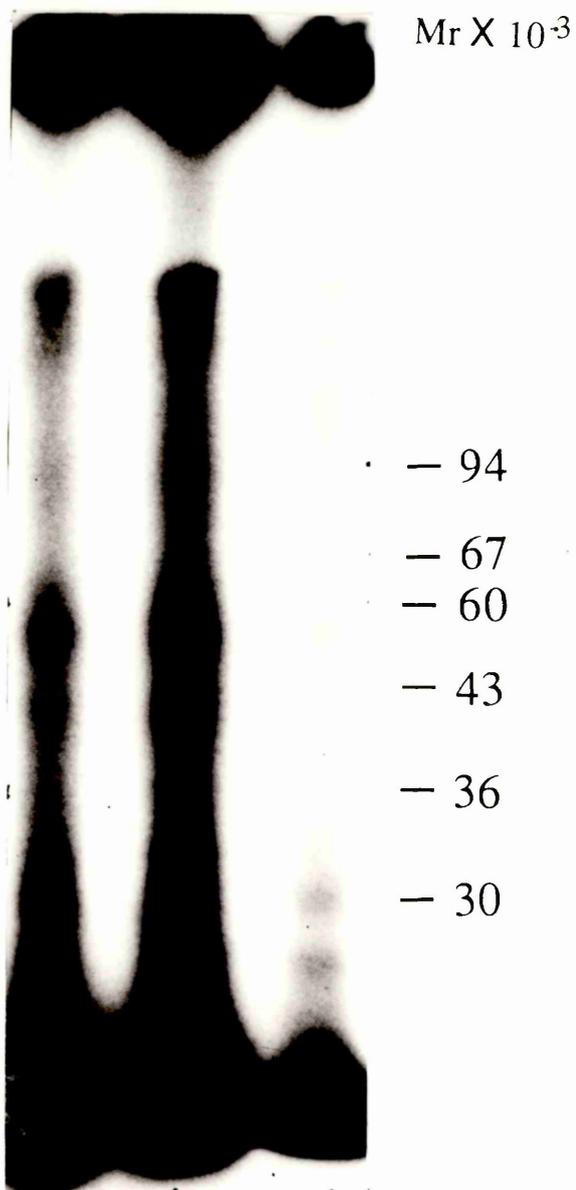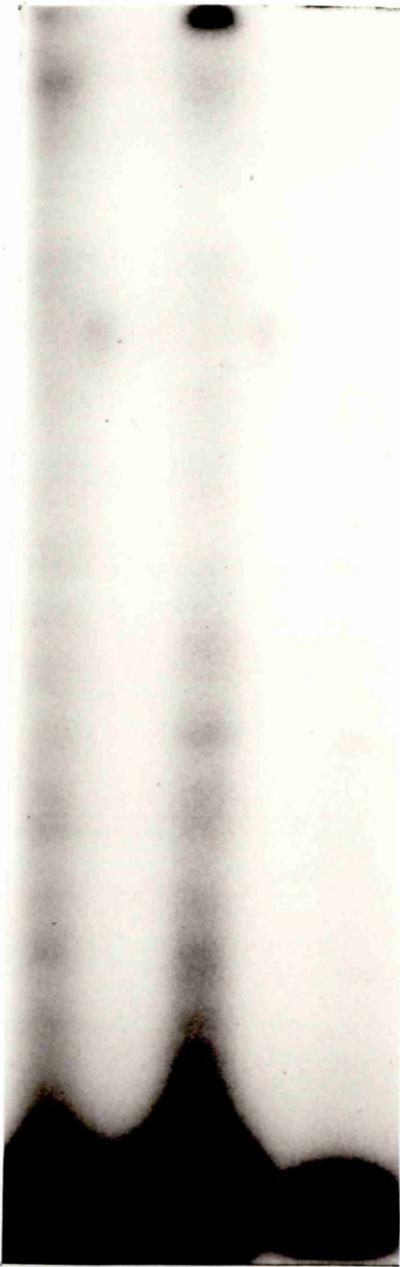
$\angle$:   1   2   3

Fig.5.6. Effect of irradiation on phosphorylation of proteins in schistosomula.

The cercariae were divided into four groups. Sample one (lane 1) and sample two (lane 2) were irradiated for 3min and transformed into schistosomula. Irradiated schistosomula were labelled with $^{32}PO_4$ for 3hr. Sample two was kept at -70 ^oC. Sample one was cultured in GMEM medium (5 % FCS) for 24 hr. Sample three (lane 3) and sample four (lane 4) were transformed into schistosomula and labelled with $^{32}PO_4$ for 3hr. Sample three was cultured in GMEM medium (5 % FCS) for 24 hr. Sample four was kept at - 70 ^oC. All labelled parasites were dissolved in sample buffer and run on SDS - PAGE.

L: Lane.

(a). The gel was not treated with KOH for 1.5hr.

(b). The gel was treated with 1 N KOH for 1.5 hr after running on SDS - PAGE..

159

a.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

— 36

— 30

L: 1   2   3   4

b.

Mr X 10^{-3}

— 94

— 67
— 60
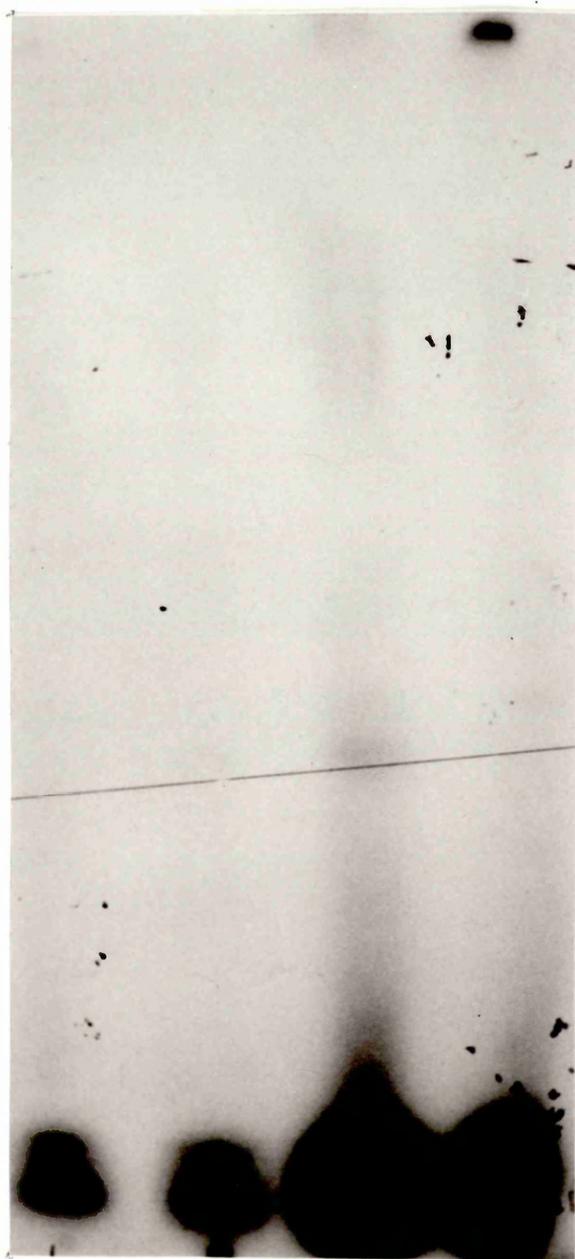
— 43

— 36

— 30

L: 1 2 3 4

Fig.5.7. Effect of irradiation on phosphorylation of proteins in schistosomula

The cercariae were divided into four groups. Sample one (lane 1) and sample three (lane 3) were normal schistosomula. The parasites in sample one were labelled with $^{32}PO_4$ for 3hr and kept at -70 ^oC. The parasites in sample three were labelled with $^{32}PO_4$ for 3hr and cultured in GMEM medium (5 % FCS) for 24hr. Sample two (lane 2) and sample four (lane 4) were irradiated schistosomula. The parasites in sample two were labelled with $^{32}PO_4$ for 3hr and kept at -70 ^oC. The parasites in sample four were labelled with $^{32}PO_4$ for 3hr and cultured in GMEM medium (5 % FCS) for 24hr. All labelled parasites were dissolved in sample buffer and run on SDS - PAGE.

L: Lane.

(a). The gel was not treated with KOH for 1.5hr.

(b). The gel was treated with 1 N KOH for 1.5 hr after running on SDS - PAGE.

a.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

— 36

— 30

L: 1   2   3   4

b.

Mr X 10^{-3}

— 94

= 67
= 60

— 43

— 36

— 30

L: 1   2   3   4
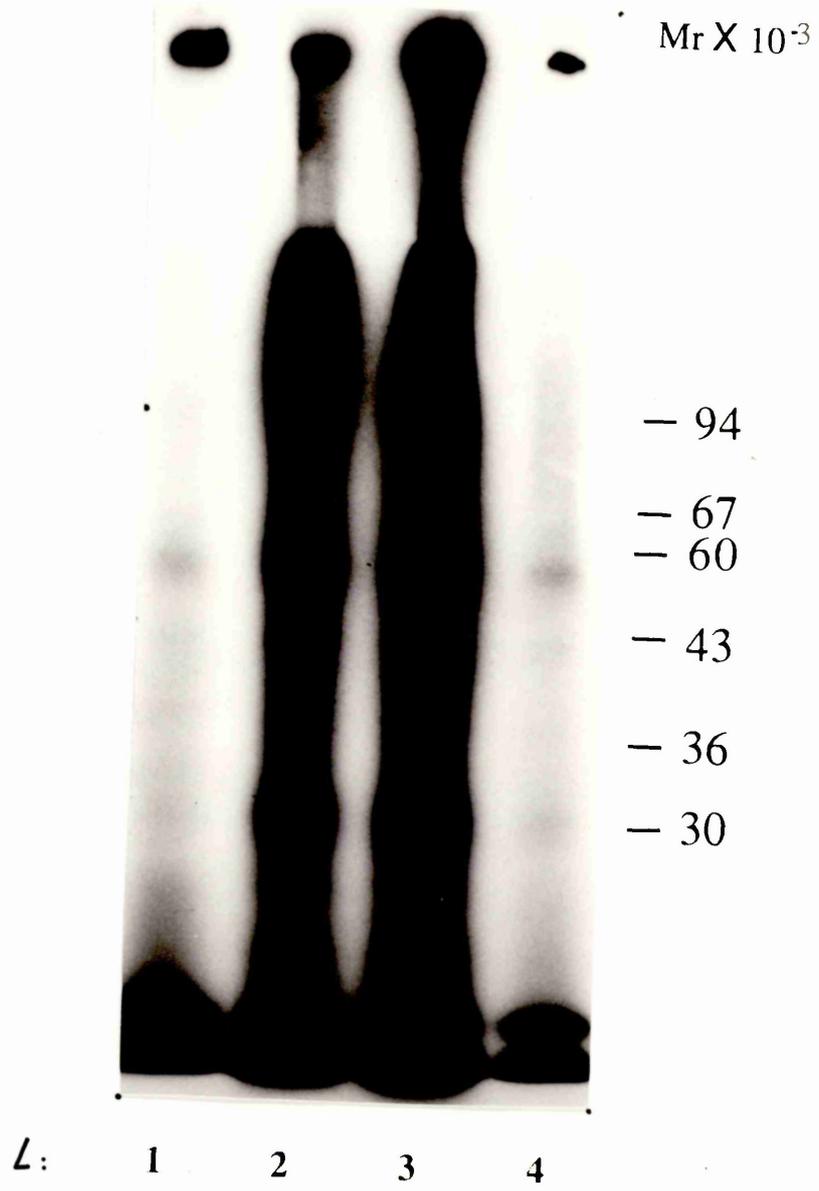
Fig.5.8. Effect of human serum on protein phosphorylation in schistosomula.

The mechanically transformed schistosomula were divided into two groups. One group (lane 2) was incubated with 10% human serum for 30 min and labelled with $^{32}PO_4$ for 3hr. The other group as control (lane 1) was incubated in GMEM medium alone after for 30 min and labelled with $^{32}PO_4$ for 3hr. The labelled parasites were dissolved in sample buffer and run on SDS - PAGE.

L: Lane.

(a). The gel was not treated with KOH for 1.5hr.

(b). The gel was treated with 1 N KOH for 1.5 hr after running on SDS - PAGE.

a.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

— 36

— 30

∠:   1   2

b.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

— 36

— 30

L :   1   2
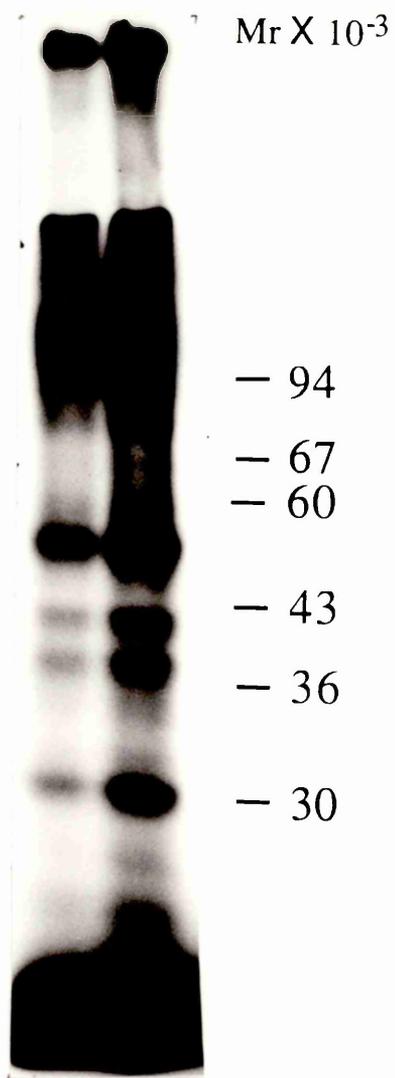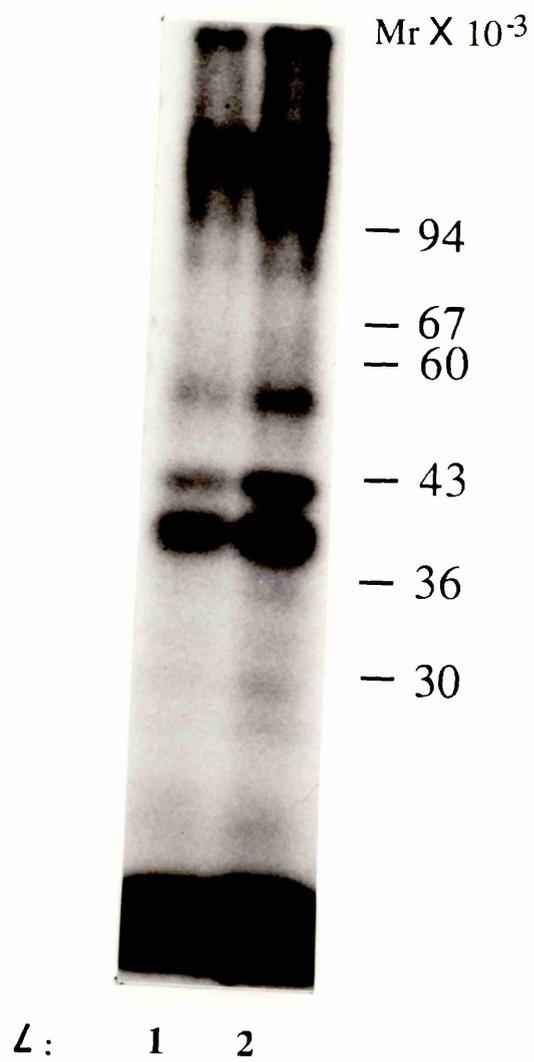
glycosylation. The effect of TM on protein phosphorylation has therefore also been measured. However, we find that there is no effect of TM on protein phosphorylation in schistosomula, whether the schistosomula were treated with TM for 24hr and then incubated with $^{32}PO_4$ label for 3hr or the schistosomula were labelled with $^{32}PO_4$ for 3hr and then treated with TM for 24hr. (see Fig. 5.9.a.b.).

5.7. Discussion

The results from this chapter show that large numbers of proteins are phosphorylated after cercariae are transformed into schistosomula. The greatest amount of ^{32}P was incorporated into low molecular weight molecules at the dye front. There was no significant difference between mechanical and skin transformed schistosomula. When the gel is treated with KOH for 1.5 hr, it seems that KOH removes all the phosphorylated proteins in cercariae, However, some phosphorylated protein bands are still present in schistosomula. They are found at 43-36kDa, 30kDa, at high molecular weight and at the dye front. Thus, during the transformation, phosphorylated proteins that are stable to KOH can be detected. The function of these proteins is not known.

In order to study changes in phosphorylated proteins during the development of schistosomula, I used two different protocols. (a). ^{32}P labelled schistosomula were cultured for 21hr. No ^{32}P labelled proteins were released from schistosomula during culture (data not shown) and also no changes in stability of phosphorylated proteins to KOH treatment were seen, compared with uncultured labelled schistosomula. Only one new phosphorylated protein appeared during the culture. The molecular weight of this protein is about 38 kDa. This protein can not be detected in U.V. irradiated schistosomula. It is known that U.V. irradiation can inhibit new protein synthesis in schistosomula (Wales 1989). This protein may be newly synthesized and phosphorylated, or this protein is synthesised before transformation, but is not phosphorylated in irradiated parasites. However, there is variability between experiments in the detection of this protein. The results from five experiments were analysed(Table 5.1.). This protein can be found in three experiments, not in two other

Fig.5.9. Effect of TM on protein phosphorylation in schistosomula.

The schistosomula were divided into two groups. One group (lane 2) was incubated with TM (10 μg / ml) for 24 hr and labelled with $^{32}PO_4$ for 3hr. The other group (lane 1) was incubated in GMEM medium alone for 24hr and labelled with $^{32}PO_4$ for 3hr. All labelled parasites were dissolved in sample buffer and run on SDS - PAGE.

L: Lane.

(a). The gel was not treated with KOH for 1.5hr.

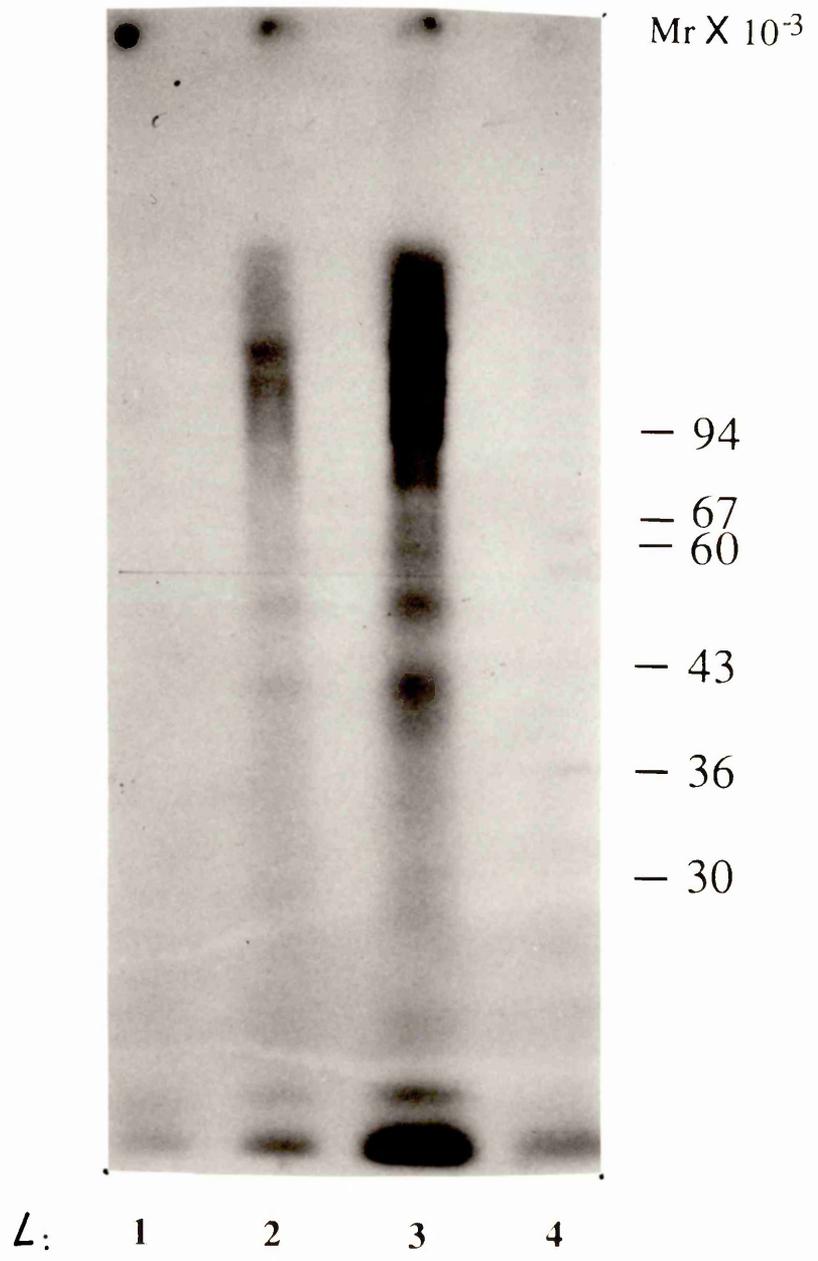(b). The gel was treated with 1 N KOH for 1.5 hr after running on SDS -PAGE.

a.

Mr X 10⁻³

— 94

= 67
= 60

— 43

— 36

— 30

L: 1   2

b.

Mr X 10^{-3}

— 94

— 67
— 60

— 43

— 36

— 30

L: 1   2
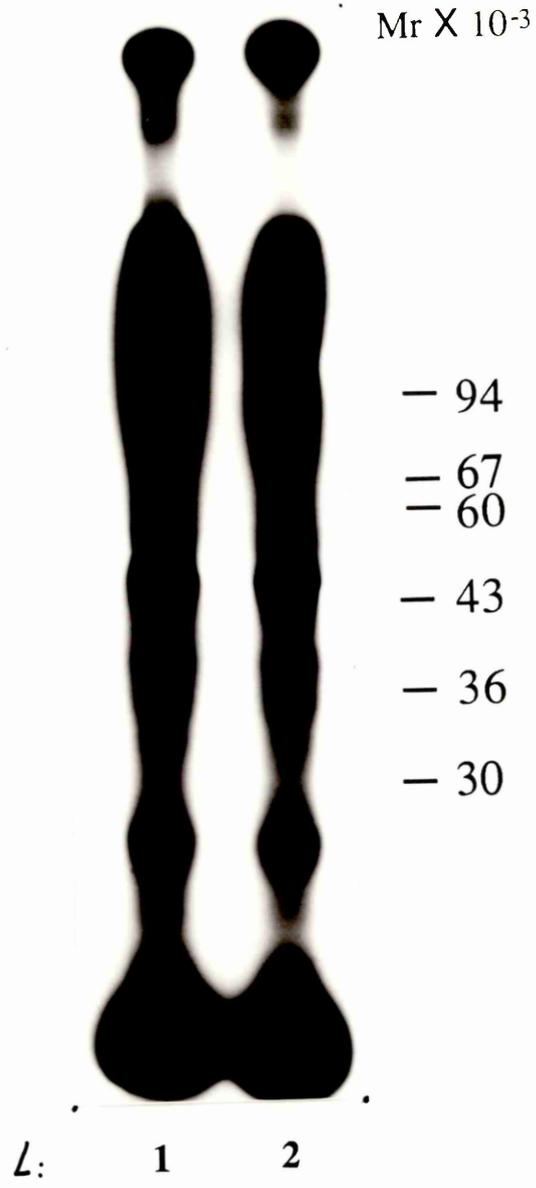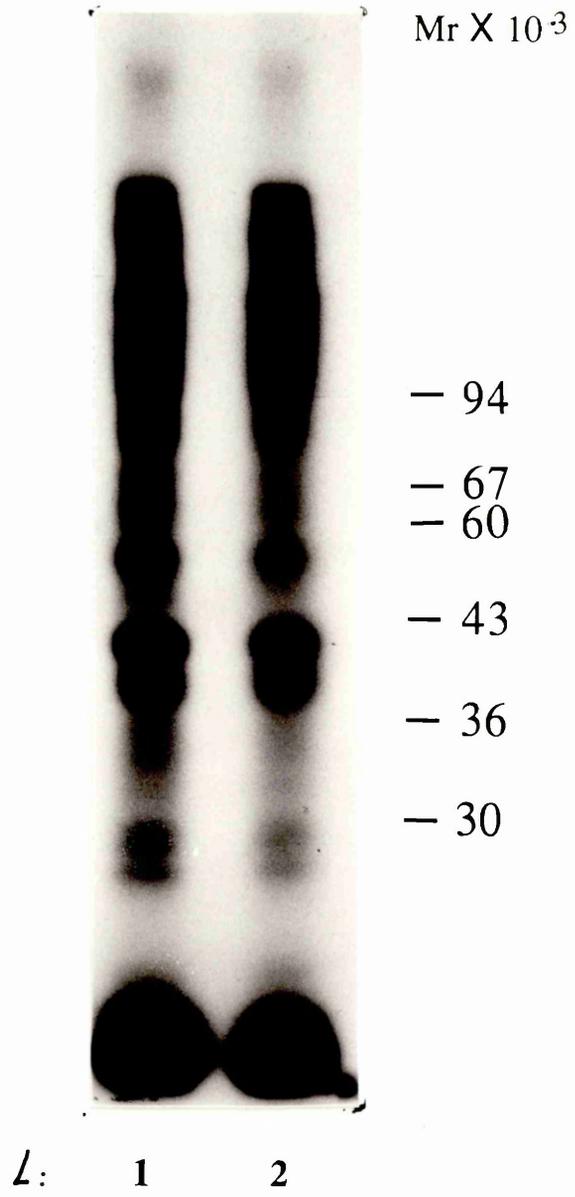
experiments. In protocol (b), the schistosomula were cultured for 21hr first, and then labelled with ^{32}P for 3hr, under these conditions there appears to be an increase in protein phosphorylation after KOH treatment. This may be due to an increase in activity of protein kinase with the development of schistosomula.

The parasite possesses an adenylate cyclase that is modulated by neurotransmitters, eg. serotonin, and drugs, eg. ergotamine.(Mansour. 1979). The co-operation in the surface membrane of receptors and adenylate cyclase lead to activation of GTP-binding proteins, that, in turn, are substrates for phosphorylation by protein kinases. 5HT and complement C_3 have been shown to stimulate synthesis and turnover of apical syncytial plasma membrane (APM) and overlying envelope (En). The work of Mansour and co-workers (1984) led Podesta et al (1987) to consider that the 5HT-sitmulated APM synthesis could be explained through a cAMP second messenger system.   However, Podesta et al. (1987) recently observed that C3b-dependent synthesis of the overlying envelope in schistosomes is a Ca^{2+} mediated event and 5HT-stimulated apical syncytial plasma membrane synthesis in schistosomes may be operating through a different second messenger system, since the in vitro protein phosphorylation reactions in response to 5HT and cAMP were quantitatively and qualitatively different. The results from chapter 4 show that the inositol phosphate metabolism pathway is present in S. mansoni. Is this pathway related to protein phosphorylation ? In mammalian cells, the primary products of this receptor-linked PI breakdown are identified as diacylglycerol and inositol phosphate. Diacylglycerol interacts with protein kinase C causing its activation, possible translocation to the plasma membrane, and the phosphorylation of various target proteins within the cell (Nishizuka, 1983). When the schistosomula were stimulated by 10% human serum for 30 mins and labelled with ^{32}P for 3hr, no difference in the number and size distribution of phosphorylated proteins was apparent. However, there was an increase in the phosphorylation of most of the proteins, especially in the high molecular weight area. When the gel was treated with KOH for 1.5 hr, no significant differences in

phosphorylation could been seen between control and human serum stimulated samples.

When cercariae were irradiated with U.V, then transformed into schistosomula and labelled with ^{32}P for 3hr, despite some variability, the results from three experiments showed that U.V.treatment of S.mansoni increased the sensitivity of phosphorylated proteins to KOH treatment. Treatment of gels with KOH, seemed to remove most of the labelled protein and decrease the heavy labelling of very low molecular weight proteins. If labelled U. V. irradiated schistosomula were cultured for 24hr, most of the labelled proteins showed decreased phosphorylation, and the remaining labelled proteins were also sensitive to KOH treatment.

The mechanisms by which human serum and U. V. irradiation affect protein phosphorylation seem to be different. Both may influence different protein kinases. The increase in phosphorylation with human serum could be a result of increased Thr- and Ser- phosphorylation. However, because the protein chemistry of the enzymes and substrates involved in protein phosphorylation in S. mansoni is not known in detail, it is not possible to say whether human serum affects protein kinase C or protein kinase A. Protein kinase C has a broad substrate specificity. Protein kinase C and A can often use the same phosphate acceptor proteins.(Nishizuka 1983.). For example, both kinases phosphorylate, to various extents, histone, protamine, myelin basic proteins microtubule associated proteins and many membrane-bound proteins. Although analysis in vitro of the phosphorylation sites indicates that protein kinase C and A show different catalytic properties, each appears to recognize the respective seryl and threonyl residues in common substrate proteins. Protein kinase C reacts with neither its own tyrosyl residues nor those in any substrate protein tested. U.V. irradiation may also affect Tyr - phosphorylation. It has been known that there is a large population of Thr- and Ser- phosphorylated protein in cells. The changes in Thr- and Ser- phosphorylation are easy to recognize. However, the population of Tyr - phosphorylated protein is very small, therefore, the changes in this type of

phosphorylation are impossible to detect without KOH treatment.

Another interesting possible reason for U. V irradiated schistosomula showing a decrease in phosphorylation during culture may be changes in protein conformation. Wales (1989) has found that U.V. irradiation can increase exposure of SH- residues. The changes in conformation of protein structure could affect the stability of phosphorylated proteins and thus increase the rate of dephosphorylation.

Chapter 6:   General discussion, conclusion and future works

6.1. General discussion:

The results presented here show that TM can inhibit some glycoprotein synthesis in S. mansoni. They indicate that schistosomes can synthesise N - linked glycoproteins and it appears that the biosynthesis pathways of N - linked glycoproteins in S. mansoni may be similar to that in mammalian cells. In mammalian cells, the pathway of N-glycosylation of proteins involves the reactions of the lipid-linked pathway leading to the formation of Glc_3Man_9 (GlcNAc)$_2$ - pp - dolichol. TM inhibits the first step in the lipid - linked saccharide pathway, i.e. the transfer of GlcNAc - 1 - p to dolichyl - 1 - p, thus preventing the formation of any lipid intermediates and inhibiting N - linked glycosylation. Thus the lipid - linked saccharide pathway may be present in S. mansoni. The degree of effect of TM on glycosylation in S. mansoni is not as high as that in lymphocyte cells. This may mean that the pathway for synthesis of N - linked glycoprotein in S. mansoni is not very active. Most of the glycoproteins which are present in S. mansoni contain O - linked oligosaccharides. Recently, studies from Nyame. et al (1975 , 1988a, 1988b) have reported that schistosomula and adult worms both synthesise O - linked and N - linked glycoproteins. O - linked glycoproteins in S. mansoni represent a high proportion of its total glycoproteins. (Nyame et al, 1987, 1988b). In addition, of particular interest is the observation that there are few qualitative differences found between schistosomula and adults with regard to the extent of inhibition of ^3H - mannose incorporation into glycoproteins with a variety of molecular weights after incubation of the parasites with this drug for 24 hr. This result supports the finding by Nyame that there is developmental regulation in the expression of S. mansoni glycoproteins. Although the functions of the N - linked glycoproteins in schistosomes are not known, N - linked glycoproteins together with O - linked glycoproteins seem to play quite important role in maintaining the stability of a microenviroment created by carbohydrates on the surface membrane of S. mansoni. Parasites treated with this drug (TM) show changes in several kinds of properties in surface membrane of S. mansoni. Inhibition of N - linked glycosylation might be

expected to disrupt the external glycocalyx, increasing in the exposure of some residues on the schistosome surface. Moreover, the carbohydrate species associated with proteins appear to be important in determining the sites of protease action and incorporation of a carbohydrate moiety - a mannose - 6 - phosphate residue - seems to play an important role in directing denatured material to lysosomes for degradation in mammalian cell.(Rothman and Lenard. 1984). Inhibition of glycosylation may prevent attachment of these messages to molecules that would normally turnover. In addition, TM binding to the surface membrane of schistosomes may also affect the membrane turnover. All of these would be expected to lead to an accumulation of macromolecules on the surface membrane and result in changes in the properties of surface membrane. These changes in the surface membrane could affect the membrane functions involved in receiving and processing information from its environment and inhibit schistosomula development in mice.

Finally, the results present in chapter 4 and 5 show that signalling systems are present in S. mansoni. The level of inositol phosphates is greatly increased with the development of S. mansoni. After transformation of cercariae into schistosomula, many proteins are phosphorylated and some phosphorylated proteins seem to be stable to KOH treatment. These proteins might be phosphorylated on tyrosine. Phosphotyrosine (P.Tyr) was first detected in hydrolysates of viral transforming protein labelled by incubation of immunoprecipitates with radioactive ATP. Further studies have demonstrated that many growth factors have tyrosine kinase activity suggesting the proteins phosphorylated on tyrosine might be likely candidates as involved in intracellular signaling. The complex migratory, site and host - finding behavior of flatworm parasites provides clear evidence that these organisms are capable of responding to ambient signals. The marked increase in the activation of inositol phospholipid during development and many of proteins phosphorylated during the transformation might be required for the development of parasite in its mammalian host. However, other mechanisms involving G protein - adenylase cyclase have been shown to be operative in S. mansoni. (Mansour and Mansour, 1989)

6.2. Conclusions :

Glycosylation in S. mansoni has been studied by tunicamycin, the inhibitor of N- linked glycoprotein synthesis. The results indicate that: (1). TM has some effect on glycosylation in S. mansoni. After long term incubation with this drug, TM inhibited some kinds of glycoprotein synthesis in S. mansoni. The biosynthesis pathways of N - linked glycoproteins in S. mansoni might appear to be similar to that in mammalian cells. (2). Although TM did not show damage to the structure of the double membrane, some properties of the membrane were changed after parasites were incubated with TM for different times. Incubation with TM also results in an increase in lectin binding, binding of antibody from rabbits infected with irradiated cercariae and a change in rate of lateral diffusion of lipid analogues in schistosomula. TM also affected the membrane turnover, reducing glycoprotein shedding from schistosomula. (3). TM inhibited the development of schistosomula in mice.

Finally, the studies from inositol phosphate metabolism and protein phosphorylation demonstrated that both PI turnover and protein phosphorylation systems are present in S. mansoni. The activation of inositol phospholipid metabolism was greatly increased with development of the parasite and this pathway can be affected by praziquantel. After transformation, many proteins have been phosphorylated and some seem to be stable to KOH treatment. Human serum and U.V irradiation appear to affect protein phosphorylation by different mechanisms.

6.3. Further work

There is still much of interest in glycosylation and signal transduction. More and more studies have demonstrated that at each stage, surface, or secreted carbohydrates and glycoproteins appear to play an important role in the parasite 's interaction with its environment. Recently, Nyame et al (1988 a, b) have made an important contribution with the first systematic studies of the structures of N-linked oligosaccharides derived from schistosomula and adult worm glycoproteins. However, the functions of these

glycoproteins are not known. The biosynthesis of the oligosaccharide chains of the N - linked glycoproteins and the structural diversity for the carbohydrate moieties should be studied. The function and subcellular location of N - linked glycoproteins in schistosomes also needs to be investigated. In addition, PI turnover has been shown to occur in schistosomes and an increase in turnover has been demonstrated during praziquantal treatment. However, precise relationships between ligand binding to intact membranes, IP3 production and calcium ion release should be carried out. Finally, the effect of human serum and U. V. irradiation on protein phosphorylation in schistosomula should be studied in great detail. Human serum and U.V. irradiation may affect different protein kinases. Thus, the protein chemistry of enzymes and substrates involved in protein phosphorylation will be studied. The variation in susceptibility of schistosomula to U. V irradiation will be also worth investigating.

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