SCHISTOSOMA MANSONI : LOCALISATION OF SCHISTOSOME ANTIGENS USING MONOSPECIFIC ANTIBODIES RAISED AGAINST ADULT WORM TEGUMENT MEMBRANES

VOLUME 1 (2 VOLS.)

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TO MY PARENTS AND FAMILY

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ABSTRACT

The distribution and stage-specificity of Schistosoma mansoni antigens have been investigated, using a library of monoclonal antibodies (50 MABs) to study their location in worm tissues. The MABs were raised by immunizing mice with highly enriched tegument fractions of adult worm. Various stages in the life cycle of S. mansoni were used as antigenic targets in the indirect immunofluorescence technique. Polyclonal infection sera and normal sera were also used as positive and negative controls, respectively. Sixteen MABs reacted with the adult worm tegument. Eleven MABs reacted with the muscle of the parasite. Two MABs reacted with larval stages of the parasite only. Four MABs reacted with larval and adult stages, but not adult tegument. Seventeen MABs did not react with any stage of the parasite tested.

Distribution of the target epitopes of the 16 anti-tegument MABs within the adult worm was investigated. Five different staining patterns for sections of adult tegument could be distinguished. Stage-specificity was examined using sections of cercariae, 3h schistosomula and adult worms; eleven staining patterns were observed.

Only two anti-tegument MABs reacted with the surface of intact cercariae. Five anti-tegument MABs bound to the entire surface of intact 3h schistosomula; others MABs reacted strongly with the apical area alone. None of the 50 MABs reacted with the surface of intact 5-day-lung worms and 21-day-liver worms. Rabbit anti-mouse erythrocyte ghost serum was used to demonstrate host antigens on the surface of lung and liver worms. Vaccinated rabbit serum was used to demonstrate parasite antigens on the lung worms's surface. The results from the present study indicate that the schistosome tegument antigens are conserved throughout development within the vertebrate host and present at the parasite surface. Some antigens are expressed on the worm surface only after transformation from cercaria to schistosomulum; these antigens are preformed in the cercarial body. Antigens detectable on the surface of newly transformed schistosomula are present in the same location in intact adult worms, but not accessible to antibody.

Cross-reactivity between S. mansoni and S. japonicum tegumental antigens was examined. Cross-reactivity of muscle antigens between schistosome, insect and vertebrate was also investigated. Finally, localisation of schistosome tegumental antigens at the electron microscope level was studied using the cryoultramicrotomy technique. Cryosections were visualised using secondary antibodies conjugated with colloidal gold. The results showed that the gold particles could be observed in the tegument and in the cytoplasmic connections between tegumental cell bodies and the tegument.

LIST OF ABBREVIATIONS

Sera

AMS	Acute mouse serum
GAR/Ig/FITC	Goat anti-rabbit Ig conjugated with fluorescein isothiocyanate
MAB	Monoclonal antibody
MABs	Monoclonal antibodies
NGS	Normal goat serum
NMS	Normal mouse serum
NRS	Normal rabbit serum
NS-1	P3-NS1-1-Ag4-1 Myeloma cell ascites fluid
RAM/Ig/FITC	Rabbit anti-mouse Ig conjugated with fluorescein isothiocyanate
VRS	Vaccinated rabbit serum

Adult Worm Tegument Fractions

D	Digitonin pellet
G, GP	Gradient pellet
M	Microvesicular fraction.

Miscellaneous

Ac	Acetone
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CHR	Cercarienhullenreaktion
DB	Discoid bodies
FITC	Fluorescein isothiocyanate
Glut	Glutaraldehyde
h	Hour
Ig	Immunoglobulin
IIF	The indirect immunofluorescence technique
kDa	Kilodalton
K4M	Lowicryl K4M resin
MB	Membraneous bodies
MC	Methyl cellulose
MEM	Eagle's Minimal essential medium
min	Minute
Mr	Relative molecular weight
ms	Mechanically transformed schistosomula

MW	Molecular weight
PBS	Phosphate buffered saline
PF	Paraformaldehyde
PLT	Progressive lowering of temperature
RFU	Relative fluorescence unit
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA	Soluble egg antigen
sp	Skin penetrated schistosomula (in vitro)
SPIT	Solid phase isolation technique
TEM	Transmission electron microscope
TNP	Trinitrophenyl
UA	Uranyl acetate
UV	Ultraviolet

LIST OF ABBREVIATIONS

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A	Apical end or anterior end
B	Body
BI	Basal invagination
BL	Basal lamina
C	Cytoplasmic connections
CM	Circular muscle fibres
d	Duct
D	Digestive tract
DB	Discoid body
ER	Endoplasmic reticulum
F	Female worm
g	Postacetabular gland
G	Gland cells
GA	Golgi apparatus
GD	Gland duct
H	Head capsule
Hb	Haemoglobin
I	Internal tissues
L	Lamellae
LM	Longitudinal muscle fibres
M	Muscle layer
MF	Muscle fibres or myofilaments
MT	Mitochondria
n	Nephridia
N	Nucleus
p	The point where the cercarial tail detached from the body
P	Packets
PE	Posterior end
PM	Plasma membrane
PT	Parenchymal tissues
SB	Secretory body
sp	Surface pit
T	Tail
TM	Tegument
V	Vacuole

CHAPTER 1

GENERAL INTRODUCTION : MORPHOLOGY AND ANTIGENS OF SCHISTOSOMA MANSONI

1. INTRODUCTION

Schistosomiasis,or Bilharziasis is one of the most important human parasitic diseases in the tropical world. It is a chronic disease widespread in Africa, South America, and the Far East. About 200 million people are infected. It is caused by parasitic helminths of the genus Schistosoma.

During the last two decades, several control programmes have been devised. Treatment by mass chemotherapy is one of the methods which can lead to a prolonged reduction in incidence rates, mean intensities of infection (Jordan, Bartholomew, Grist & Auguste, 1982; Pugh & Teesdale, 1984) and also a reduction in the prevalence of morbidity (Sleigh, Hoff, Mott, Maguire & Da Franca Silva, 1986). In recent years, the introduction of praziquantel as a safe and very effective treatment, usually given as a single dose for all schistosome species, has increased optimism about the possibility of control based on mass chemotherapy. However, its application is limited and has potential future problems. Although there is an overall reduction in the prevalence and intensity of infection soon after mass chemotherapy, reinfection in areas of intense transmission can often be extremely rapid especially in younger children (Butterworth, Capron, Cordingley, Dalton, Dunne, Karinki, Doech, Mugambi, Ouma, Prentice, Richardson, Siongok, Sturrock & Taylor, 1985; Wilkins, Blumenthal, Hayes & Tulloch, 1987). Some individuals are predisposed to heavy infections (Anderson & Medley, 1985). Finally, parasite strains resistant to praziquantel will probably develop. As a result of these problems chemotherapy is likely to become less effective. Therefore, the development of an effective vaccine is necessary for the long term control of schistosomiasis.

1.1 The Life Cycle

The life cycle of this blood fluke involves asexual larval reproduction in an intermediate snail host, giving rise to the infectious free-swimming fork-tailed cercaria. Cercariae penetrate human skin and transform into schistosomula.

From the skin, schistosomula migrate to the lungs and then to the hepatic portal system and liver where they eventually mature as adult male and female worms and come to reside in the mesenteric veins (Schistosoma mansoni, Schistosoma japonicum) or veins of the urinary bladder (Schistosoma haematobium).

Eggs produced by the female S. mansoni worm penetrate the intestinal wall to be voided in the feces; a proportion of the eggs are carried in the portal system to the liver where they are retained.

Immunopathological host responses to egg secretions involving granuloma formation and subsequent fibrosis in the liver are the manifestations of chronic infection in schistosomiasis mansoni and schistosomiasis japonicum (Andrade & Warren, 1964).

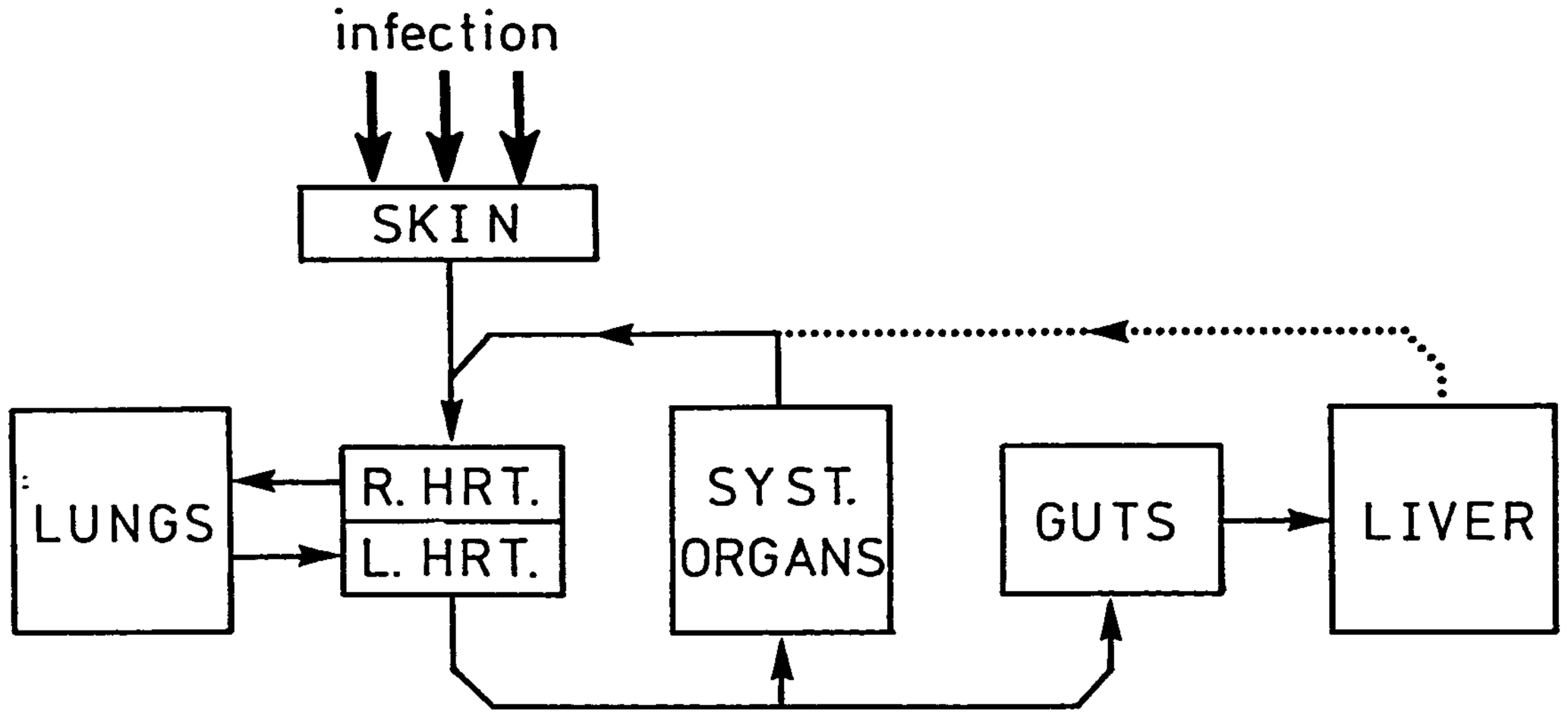
1.2 Migration of the Parasite Through the Vascular System of the Host

The initial phase of migration is entry into the dermis either directly across the basal lamella of the epidermis, via hair follicles, or both (Gordon & Griffiths, 1951; Stirewalt, 1959; Stirewalt & Dorsey, 1974). In the mammalian host, schistosomes take an entirely intravascular migration route from the skin (epidermis and dermis) to the hepatic portal system (Text Figure 1.1) (Miller & Wilson, 1978, 1980; Wheater & Wilson, 1979). After 3 - 4 days in the dermis, the majority of parasites leave the skin via the venous drainage, only 10 - 15% via the lymphatics. They are carried passively with the flow of blood via the right side of the heart and pulmonary artery to the lungs where they undergo a phase of elongation which is not accompanied by an increase in mass (Wilson, Draskau, Miller & Lawson, 1978). This process lasts several days and adapts them for onward migration through vascular beds (Wilson et al., 1978; Crabtree & Wilson, 1980).

Histological (Wheater & Wilson, 1979) and experimental evidence (Miller & Wilson, 1980) indicates that schistosomula leave the lungs via the pulmonary vein and pass through the left side of the heart to the aorta to be distributed to the systemic organs of the body. Those parasites entering splanchnic arteries (coeliac, anterior mesenteric and posterior mesenteric) will gain access to the hepatic portal vein after passage through a capillary bed. The majority of parasites reaching the liver by this route are sequestered and begin to grow and develop. Those schistosomula distributed by arteries other than the splanchnic are apparently able to traverse vascular beds to the venous compartment and travel back to the lungs (Miller & Wilson, 1980). An individual schistosome could make several circuits of the pulmonary and systemic vasculatures before being distributed by chance to a splanchnic artery which leads to the hepatic portal system.

1.3 Morphology of Various Stages of S. mansoni

The schistosomes differ from typical trematodes in their thin elongated shape and separate sexes. The larger grayish male has a cylindrical anterior end, and its bigger body is folded to form a long ventral gynaecophoral canal in which the darker slender female is held



Text Figure 1.1

Diagrammatic representation of the route of migration of S. mansoni from the skin to the hepatic portal system. The majority of parasites entering the liver are trapped; the dotted line from the liver represents the small fraction which escape back to the general circulation (From Wilson & Coulson, 1986).

during copulation.

The cercaria is the first stage in the life-cycle of the parasite that involves infected humans or definitive hosts. Thus, the cercaria is described first and then followed by other stages of the parasite.

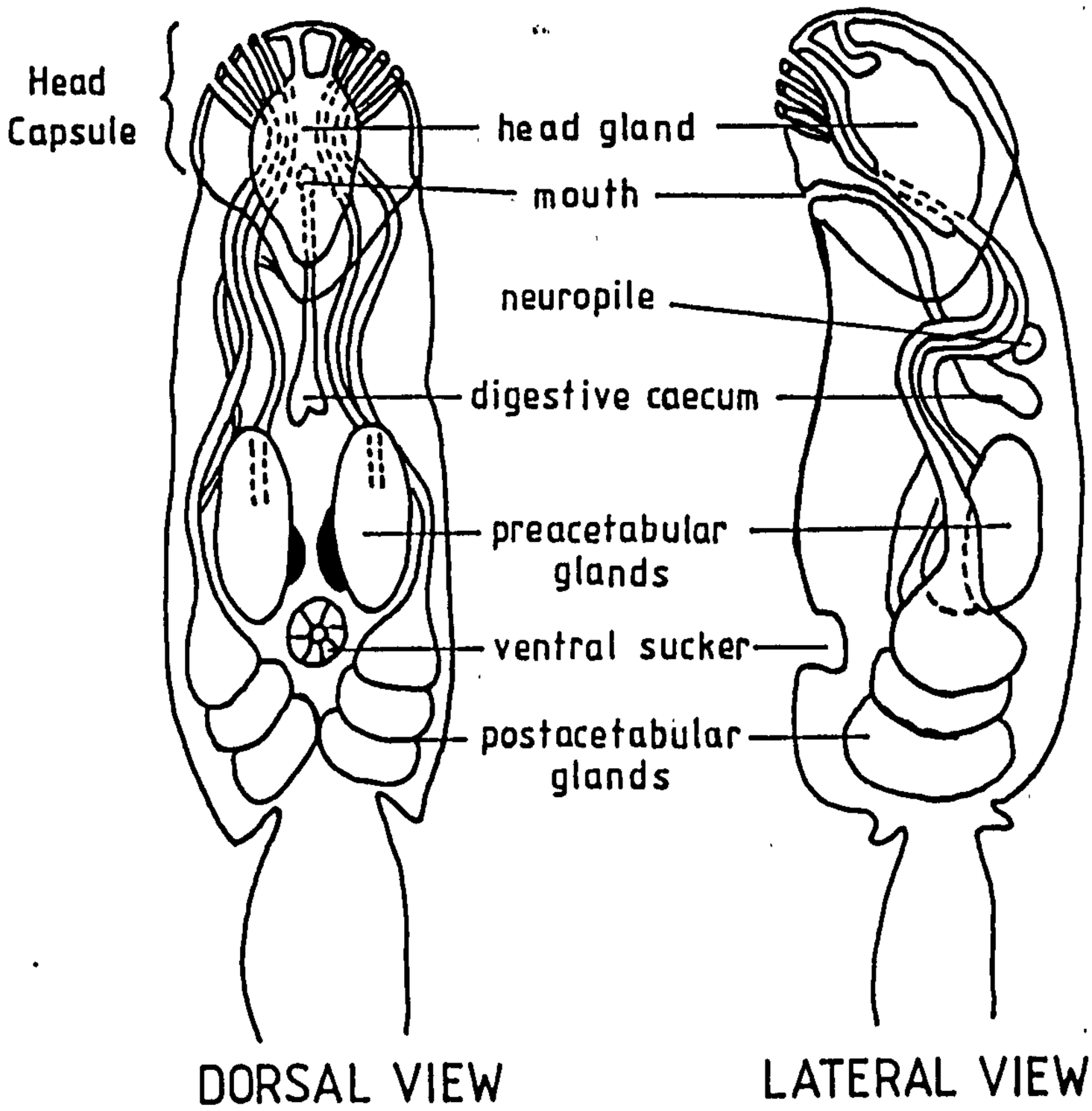
1.3.1 The Cercaria

Schistosomes enter the vertebrate host by direct penetration through the skin. The infective stage, the cercaria escapes from the snail intermediate host into the surrounding water. The cercaria are not able to survive for long in this free-living stage; within a few hours many have lost their infectivity and all die within a day if a vertebrate host is not contacted (Olivier, 1966). After entering host skin, cercariae transform into schistosomula by the loss of tail, glycocalyx, resistance to water, and penetration gland contents.

The cercaria is a highly motile organism which has an elongated, cylindrical body (120 - 190 μm in length and 25 μm in diameter) and a long bifurcate tail (300 μm long). The entire surface of the cercaria is covered with backwardly-directed spines about 1.0 μm in length (Hockley, 1968). The cercarial body contains excretory, nervous, and digestive systems and secretory glands, all of these are surrounded by two muscle layers (Text Figure 1.2) (Stirewalt, 1974). The anterior of the body is called the "head capsule" and the extreme anterior is called the "apical area" (Crabtree & Wilson, 1980).

1.3.1.1 The Tegument

Hockley & McLaren (1973) showed that the cercaria is covered by a syncytial tegument about 0.5 μm thick on the body and 0.2 μm thick on the tail. The tegument is connected to cell bodies beneath the musculature by thin strands of cytoplasm. The cercarial tegument is bounded by a typical trilaminate plasma membrane about 8.5 nm in thickness which bears a 1 - 2 μm thick surface coat or fibrillar glycocalyx (Hockley, 1973; Hockley & McLaren, 1973). The fibres are about 15 Å thick and orientated at right angles to the surface membrane. They are branched and interconnected to form a diffuse network (Morris, 1971). Histochemically the surface coat is known to give a strong periodic-acid-schiff (PAS) reaction indicating the presence of mucopolysaccharide (Stirewalt, 1963; Smith, Reynolds & Lichtenberg, 1969; Kemp, 1970). The cercaria begins to lose its surface coat within the first 15 minutes after penetration into the skin of the vertebrate host (Hockley, 1970) and becomes a schistosomulum. This surface coat is thought to control the permeability of the membrane (Stirewalt, 1963; Morris, 1971).



Text Figure 1.2

Diagrams from the dorsal and lateral views of the body and proximal tail area of the cercaria of S. mansoni (Redrawn from Stirewalt, 1974).

In serum from an immune host a thick envelope forms around cercariae; this is the cercarienhullenreaktion (CHR) of Vogel and Minning (1953). Hockley (1970) showed that this envelope is due to combination of antibody and the surface coat. Although the surface coat is not entirely removed during penetration, schistosomula no longer give a CHR envelope in immune serum (Stirewalt, 1963).

The basal plasma membrane is also trilaminate. Inclusion bodies within the cercarial tegument are mitochondria, spines, discoid bodies and typical cercarial bodies. They arise from the tegumental cell bodies which are situated below the musculature of the body wall (Hockley, 1973). The cercarial bodies, present only in the tegument of the body, are spherical, about 100 nm in diameter, and have a trilaminate membrane. They contain a peripheral electron-lucent region with a dense granular core (Hockley, 1973).

The discoid bodies (100 nm by 20 nm), found in both the body and tail tegument, are less numerous than the cercarial bodies and have dense granular contents. The spines lie completely within the tegument, covered by plasma membrane.

The tegument overlies a thick layer of interstitial material which consists of irregularly arranged fibres (Hockley, 1973). Beneath the interstitial material are two muscle layers. The outer layer is composed of circular muscle fibres, the inner layer of longitudinal muscle fibres (Hockley, 1970; Nuttman, 1975). The nature and function of the musculature will be dealt with fully in Chapter 3.

1.3.1.2 The Secretory Glands

These glands are generally called "penetration gland cells" because their contents are discharged during penetration into the host. Their function may serve for attachment, lubrication or protection of the penetrating larva.

There are three types of unicellular glands within the cercarial body; the preacetabular, the postacetabular and the head glands (Ebrahimzadeh, 1970; Ebrahimzadeh & Kraft, 1971; Morris, 1971; Dorsey & Stirewalt, 1971) which all function during penetration (Text Figure 1.2). Each gland consists of an enlarged fundus containing secretion granules, a nucleus and cytoplasm, and ducts directed towards the apical end of the cercaria. There are two pairs of preacetabular glands and 3 - 4 pairs of postacetabular glands. Ducts arising from these glands are grouped together into two bundles and open directly to the exterior at the apical area. The acetabular glands are storage sacs of elaborated secretion (Stirewalt, 1974).

The "postacetabular gland" secretion contains mucin (Stirewalt & Walters, 1973) which may help to promote attachment to the skin

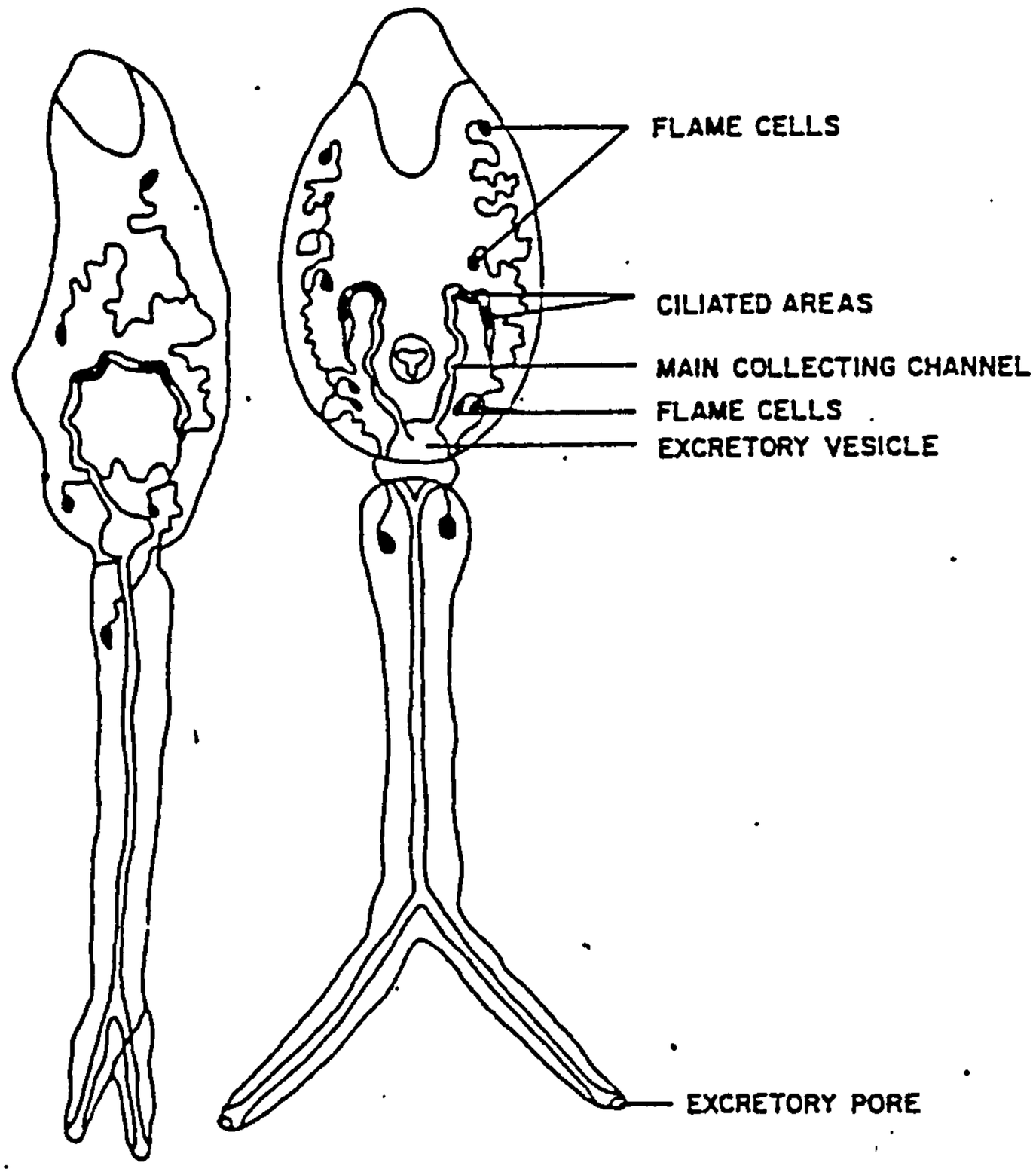
surface, to protect the tegument during the invasion stages and to aid penetration of the stratum corneum and cellular layers of the epidermis (Stirewalt, 1974).

The "preacetabular gland" secretion contains calcium (Stirewalt & Kruidenier, 1961; Dresden & Edlin, 1974) and various enzymes (Stirewalt & Austin, 1973; Stirewalt, 1974) which are important for degradation of keratin at penetration (Stirewalt & Hackey, 1956; Stirewalt, 1974). It may also be involved in schistosomular transformation (Howells, Ramalho-Pinto, Gazzinelli, de Oliveira, Figueiredo & Pelligrino, 1974). Secretion by the postacetabular glands precedes but overlaps that of the preacetabular glands. One hour after penetration the gland cells and ducts are empty and shrunken (Cousin, Stirewalt & Dorsey, 1981).

The "head gland" lies within the muscular head-capsule (Ebrahimzadeh, 1970). It is a single cell with multiple, short, microtubule-lined ducts which open into the tegument at the apical area rather than to the exterior. The head gland contains small, membrane-bound secretion granules which change their morphology after entering the tegument (Morris, 1971; Torpier, Capron & Capron, 1977). Torpier et al. (1977) reported that the typical cercarial inclusion bodies were formed in certain lobes of the head gland, while other lobes secreted two different types of membraneous bodies that play a role in membrane changes associated with transformation to the schistosomulum stage. The tegument cell bodies beneath the muscle layers also release their secretions into the tegument of the cercarial body.

1.3.1.3 Excretory System

This system (in the cercaria) comprises peripherally placed flame cells, primary and secondary collecting tubules, a pair of main collecting tubes in the body and a single tube in the tail, an excretory bladder, an excretory atrium and excretory pores (Text Figure 1.3) (Gordon, Davey & Peaston, 1934; Kuntz, 1950; Kruidenier, 1959; Ebrahimzadeh & Kraft, 1971). In the protonephridial system of the cercaria, four flame cell pairs are located in the body and one pair in the tail (Gordon et al., 1934). The flame cells are irregularly shaped cells lying between parenchymal cells. They are connected by primary collecting tubules (protonephridial tubules) to larger collecting tubules (excretory ducts) which empty into the excretory bladder at the posterior end of the body. Before the tail is shed, the bladder wall is cellular and attached to the surface tegument and to the two main collecting tubes of the body by septate desmosomes. After the tail is shed, the bladder wall becomes



Text Figure 1.3

Diagrams from the lateral and ventral views of cercariae of S. mansoni showing the parts of the excretory system (From Gordon, Davey & Peaston, 1934).

continuous with the surface tegument (Powell & Sogandares-Bernal, 1970).

In the tail, one pair of flame cells and their short collecting tubules, a proximally bifurcated single main collecting duct with its furcal branches and two excretory pores are present. After the tail is shed, the excretory bladder pore becomes the terminus of the excretory system (Kuntz, 1950).

1.3.2 Transformation from Cercaria to Schistosomulum

Cercariae penetrating a host undergo a set of changes called "transformation". This is an adaptation process from a free-living organism in water to a parasitic form living in the blood vessels of the vertebrate host.

The surface coat or glycocalyx acts to waterproof the free swimming cercaria and control the permeability of the surface membranes (Stirewalt, 1963; Morris, 1971). The loss of the glycocalyx and the formation of a new membrane at the surface of the tegument after skin penetration presumably facilitates free exchange of solutes between the parasite and host tissue fluids.

During transformation, the cercarial membrane is lost through the formation and shedding of microvilli (Hockley & McLaren, 1973; McLaren & Hockley, 1976). Schistosomula recovered 30 minutes after skin penetration still have a trilaminate limiting membrane but the surface coat is reduced in thickness. Numerous tegument cell bodies connect to the tegument and synthesize a large number of small membraneous vesicles (100 - 150 nm in diameter). These vesicles pass from the tegument cell bodies into the tegument cytoplasm via microtubule-lined cytoplasmic connections (Hockley & McLaren, 1973). These connections are present in the cercaria but devoid of vesicles (Cousin et al., 1981).

The membraneous bodies are limited by two closely apposed trilaminate membranes, and have either a central lucent region, or a central region filled with membraneous material. The fused bodies form larger membraneous vacuoles in the tegument and move towards the apical surface of the tegument. The function of the vesicles is to participate in the formation of the multilaminate tegument surface of the schistosomulum.

By 20 minutes after skin penetration numerous microvilli up to 4 um long appear on the parasite surface (McLaren & Hockley, 1976). The microvilli are evenly distributed over the entire body or show patchy distribution. Most of them arise from the interspinal regions, others originate from the sides or the apical tips of the spines.

By 60 minutes post penetration some of the larger membraneous vacuoles are connected to the trilaminate tegumental membrane and open to the exterior. The membranes of the vacuole become part of the tegumental outer membrane, and the contents of the vacuole spread over the surface of the schistosomulum.

The microvilli are limited by a trilaminate membrane, invested with a thin surface coat and are eventually detached from the parasite surface and eliminated. Samuelson and Caulfield (1985) have reported that microvilli are shed after 40 - 60 minutes. The formation and elimination of the microvilli coincides with the incorporation of membrane from the membraneous bodies into the plasma membrane of the cercarial tegument.

The purpose of microvillus formation appears to be elimination of the cercarial trilaminate membrane and glycocalyx, prior to the formation of a new tegumental surface membrane consisting of two closely apposed lipid bilayers (MaLaren & Hockley, 1976). Samuelson & Caulfield (1985) suggested that some new membrane as well as the original cercarial membrane was incorporated into the microvilli.

Thirty to 50% of the glycocalyx is lost during the time that microvilli are formed and shed (Hockley & McLaren, 1973). Residual glycocalyx (less than 40%) was present on the surface of 3 hour schistosomula transformed in culture medium at 37°C (Samuelson & Caulfield, 1985), or in rodent skin (Stirewalt, 1963; Hockley & McLaren, 1973).

Transformation begins immediately after the cercaria has penetrated the vertebrate host and is completed within about 3 hours. The parasites are then called "schistosomula". Transformation can also be performed in vitro by penetration of excised skin (Stirewalt, Mannick & Fregean, 1966) or by incubation of cercarial bodies in physiological media (Ramalho-Pinto, Gazzinelli, Howells, Mota-Santos, Figueiredo & Pelligrino, 1974; Cousin, Stirewalt & Dorsey, 1981).

1.3.3 The Lung Worm

Four to five days postpenetration, the parasites begin to arrive in the lungs via the pulmonary artery. They are retained in the lungs for a minimum of 2 - 3 days (Miller & Wilson, 1980). They become longer and thinner than skin schistosomula (Crabtree & Wilson, 1980). This change is believed to facilitate migration along the lumina of blood vessels. On day 4 and 7 post-infection schistosomula are located only within the vasculature. Newly arrived day 4 schistosomula are highly convoluted and completely occlude vessels. By day 7 parasites are located in pulmonary capillaries causing considerable distension (Crabtree & Wilson, 1986a). Only the anterior

and posterior extremities are covered with spines, the rest of the body is ridged transversely and each ridge is finely pitted (McLaren, 1980). The pits disappear completely and the tegument surface is almost smooth in fully extended regions of the body (Crabtree & Wilson, 1980, 1986a).

The subtegumentary fibrous interstitial layer is absent in elongated lung schistosomula. The tegument is in close apposition to the vascular endothelium. The tight fit of a lung worm in the pulmonary capillaries causes slow migration (Crabtree & Wilson, 1986a).

The tegument exhibits surface folds and pits and contains homogeneous inclusions (Hockley & McLaren, 1973; McLaren, Hockley, Goldring & Hammond, 1978). Homogeneous bodies are spherical (150 nm in diameter), limited by a trilaminate membrane and have dense, finely granular contents; they are formed in the tegumental cell bodies. The homogeneous bodies are present at day 4 in worms recovered from the skin and lung, and in in vitro cultured schistosomula. These inclusions are unique to the lung stage and disappear after the worms arrive in the liver (McLaren, 1980).

1.3.4 The Liver Worm

The majority of parasites which enter hepatic portal system are trapped in the liver, begin to feed on blood, and increase in size. The worms primarily lodge within branches of the hepatic portal vein (Wheater & Wilson, 1979). As they grow they move upstream into progressively larger vessels. Two to three days after arrival in the liver, the parasites are shorter and there is an accompanying marked loss of motility (Wilson et al., 1978; Crabtree & Wilson, 1980). The tegument surface of a 10 day liver worm is highly ridged, and small numbers of spines reappear in the mid-body region. The anterior ventral region begins to form the oral sucker around the sub-terminal mouth. The ventral sucker is enlarged and protruded from the ventral body surface. Feeding on blood commences at this stage. New sensory nerve endings also appear which persist through to the adult worm (Crabtree & Wilson, 1980).

1.3.5 The Adult Worm

The male and female worms are slender, elongated parasites, about 1.0 cm in length. They live in the hepatic portal and mesenteric veins of infected hosts. The female is longer and more slender than the male. The lateral margins of the male worm curve ventrally and overlap, to form a gynaecophoral canal which holds the female; only

the head and tail of the female are exposed (McLaren, 1980). A mature female worm releases more than 300 eggs per day into the blood stream. The eggs, when oviposited, are immature but within 10 days contain a miracidium.

1.3.5.1 Surface Topography

The dorsal surface of the male worm bears numerous large tubercles, each of which is invested with pointed spines. There are 50 to 250 spines per tubercle (Miller, Tulloch & Kuntz, 1972). The spines are directed towards the apex of the tubercle. The tubercles are limited to the region behind the ventral sucker. They become fewer at the posterior end and at the lateral margins of the dorsal surface (McLaren, 1980). The surface between the tubercle has a characteristic pitted appearance, and bears ciliated sensory organelles. The ventral surface of the male is characterised by the oral and ventral suckers and the gynaecophoral canal. The female is cylindrical in shape. A few spines are present at the posterior end. The entire surface is pitted (McLaren, 1980).

1.3.5.2 Surface Morphology

The adult worm is invested with a syncytial tegument about 4 - 5 um in thickness, some 10 times thicker than the tegument of the cercaria (Smith et al., 1969). The spines are enclosed within the outer tegumental membrane and the basal plasma membrane of the tegument (Text Figure 1.3).

The tegumental cytoplasm consists of an electron dense granular, ribosome-free matrix. Three major types of inclusion can be seen in the tegument cytoplasm. There are mitochondria, discoid bodies (DB) and membraneous bodies (MB). Discoid bodies are numerous in the adult tegument. They measure about 40 nm x 200 nm, with a trilaminate limiting membrane and dense granular contents. Burton (1966) suggested that the discoid bodies of other species of Digenea may contribute to the dense ground substance of the tegumental cytoplasm. It has also been suggested that the discoid bodies may break down to form the tegumental spines (Smith et al., 1969)

Membraneous bodies measure about 150 - 200 nm in diameter and consist of concentric whorls of membrane, limited by a single lipid bilayer (Smith et al., 1969). The membraneous bodies are less numerous than the discoid bodies. They are distributed throughout the tegument and are sometimes in close contact with the outer tegumental membrane lining the surface pits. The membraneous bodies contain carbohydrate (Stein & Lumsden, 1973) and phospholipids but not neutral

lipids (Wilson & Barnes, 1974a).

The tegumental nuclei are located in the tegumental cell bodies beneath the muscle layers of the body wall (Morris & Threadgold, 1968; Smith et al., 1969; Silk, Spence & Gear, 1969) as shown in Text Figure 1.4. The cell bodies are irregular in shape, sometimes multinucleate, and contain Golgi bodies, ribosomes and a few mitochondria. They also contain discoid bodies and membraneous bodies. The cell bodies are joined to the tegument by the microtubule-lined cytoplasmic connections (Text Figure 1.4). These cells synthesize the tegumental inclusion bodies. The inclusions are transported to the tegument via cytoplasmic connections. The discoid bodies and the membraneous bodies are believed to arise from different Golgi bodies within the same subtegumental cells (Wilson & Barnes, 1974a).

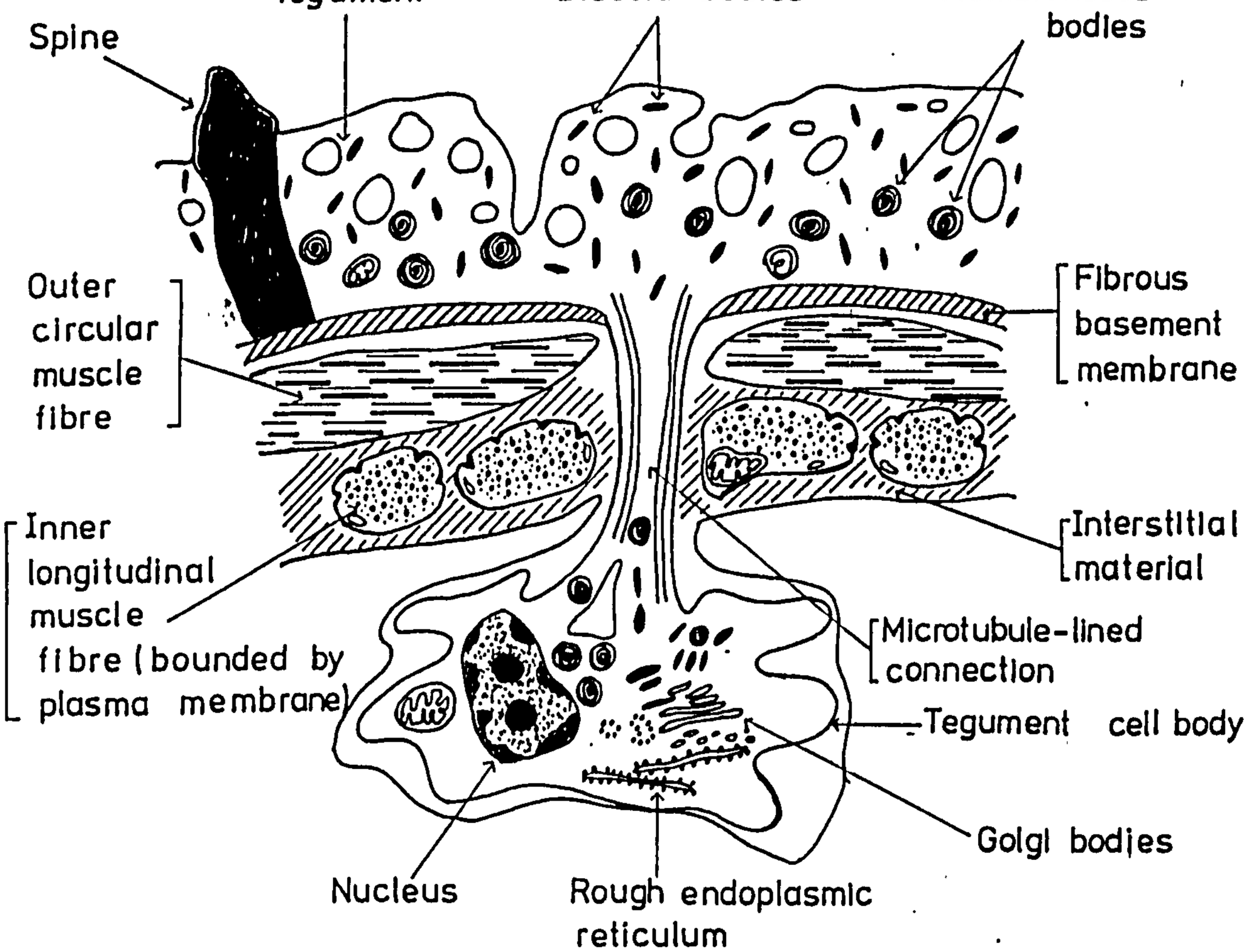
The outer tegumental membrane consists of two closely applied bilayers. The inner bilayer is equivalent to the plasma membrane. The outer bilayer is an overlying secretion. The two bilayers measure about 17 nm in thickness (McLaren & Hockley, 1977). The double bilayer surface is a characteristic feature of the blood flukes (McLaren & Hockley, 1977)

1.3.5.3 The Digestive Tract

The alimentary canal is well developed, consisting of the foregut (mouth, pharynx and oesophagus) and the paired intestinal caeca (Smyth & Halton, 1983). The posterior oesophageal lining is highly folded and contains two forms of secretory body. They are dense, membrane-bound bodies containing an ordered array of tubules, and acid phosphatase-positive vesicles. Both are synthesized by the tegumental cell bodies which form the oesophageal gland (Bogitsh & Carter, 1977; Dike, 1971; Ernst, 1975).

The caecal wall consists of a lining epithelium (gastrodermis) which is supported by a thin, basal lamina and a layer of circular and longitudinal muscle fibres (Morris, 1968; Spence & Silk, 1969). The syncytial epithelial surface bears irregular projections (Morris, 1968). Plate-like invaginations of the basal plasma membrane partly partition the cytoplasm in the gastrodermis and separate the nuclei. The gut epithelium contains large basal nuclei, ribosomes, Golgi, numerous mitochondria and an extensive endoplasmic reticulum. Numerous lipid-like droplets are present in the gut lumen and within the epithelial cytoplasm. Haemoglobin degradation products are also found in the gut lumen (Morris, 1968).

Text Figure 1.4 TEGUMENT OF ADULT WORM (♂ VENTRAL)



Text Figure 1.4

Diagram illustrating the connection of a tegument cell body to the tegument. The cell bodies are situated beneath the muscle fibres (circular and longitudinal); they contain a nucleus, rough endoplasmic reticulum, Golgi bodies and inclusion bodies. Membraneous bodies and discoid bodies pass up into the tegument through microtubule-lined (cytoplasmic) connections. The spines, although projecting beyond the surface of the tegument, are completely enclosed within the outer tegumental membrane and the basal plasma membrane of the tegument. Interstitial material (extracellular) is in the spaces between the muscle cells. The cell bodies are irregular in shape (Redrawn from Hockley, 1973).

1.3.5.4 Parenchymal Cells

The parenchyma of S. mansoni is a conglomerate of stellate cells, muscle fibres and nerve axons in which the digestive tract and the reproductive system are embedded (Reissig, 1970).

Parenchymal cells are structurally related to the tegument, gut, muscle, and excretory and reproductive systems by means of gap junctions (Morris, 1968). These junctional complexes are sites of intercellular communication which facilitate the transport of substances (e.g. ions, small metabolites and excretory products) throughout the body in the absence of a circulatory system.

1.3.5.5 Fibrous Interstitial Material

This is a connective tissue of collagen-like fibres in a granular carbohydrate matrix which ramifies between the parenchymal cells and around organ systems (Threadgold & Gallagher, 1966).

It is skeletal in function and, together with the fibrous basal lamina of the tegument and gastrodermis, provides anchorage for the muscle fibres against which they can exert a force during contraction. The muscles are attached to this fibrous "skeleton" by desmosome-like plaques (hemidesmosomes) or dense bodies (see Smooth Muscle in Chapter 3 for structural detail). Hemidesmosomes may also be seen along the basal surface of the tegument (Smyth & Halton, 1983).

1.4 Schistosome Surface Antigens

In schistosomiasis, the surface antigen expression changes during development and maturation. The utilization of fluorescent antibody-binding techniques and in vitro cytotoxicity assays have demonstrated that schistosomula, immediately after their transformation from cercariae, express antigens and are vulnerable to a variety of killing mechanisms whereas older parasites are generally less antigenic and more resistant to immune killing (Smithers & Doenhoff, 1982).

Polypeptide antigens on the parasite surface can be detected using surface-labelling techniques and immunoprecipitation, or Western blotting. By these methods, the target antigens of monoclonal and polyclonal antibodies which bind to the parasite surface can be identified (Dissous, Grzych & Capron, 1982; Simpson, James & Sher, 1983; Payares, McLaren, Evans & Smithers, 1985b). The schistosome surface is a target for the host's immune response. The identification and characterization of schistosome surface antigens, as well as their expression, are very important in studying parasite immunity and the mechanism by which the schistosome avoids the immune

response. It is possible that the study of schistosome surface antigens involved in protective immunity will eventually provide important information for the development of a vaccine. Hence, surface antigen of various stages of the parasite will be described in the following sections.

1.4.1 Cercarial Surface Membrane Antigens

To date, very little has been discovered about cercarial surface membrane antigens. Only the glycocalyx which covers the surface of cercaria has frequently been studied. The cercarial glycocalyx is antigenic (Samuelson & Caulfield, 1985) and binds antibodies from humans and laboratory animals infected with S. mansoni (Vogel & Minning, 1949; Standen, 1952; Stirewalt & Evans, 1955; Kemp, 1972; Kemp, Damian & Greene, 1973).The surface of the cercaria activates complement by the alternative pathway (Culbertson, 1936; Dias da Silva & Kazatchkine, 1980; Ramalho-Pinto, McLaren & Smithers, 1978).

The cercarial membrane and the glycocalyx are lost during transformation (Hockley & McLaren, 1973; McLaren & Hockley, 1976; Samuelson & Caulfield, 1985). The loss of the glycocalyx is a major change in antigen expression on the cercarial surface. It may coincide with a decrease in antibody binding and complement fixation and may account for the increasing resistance of developing schistosomula to immune attack (Dean, 1977; Ramalho-Pinto et al., 1978; Samuelson, Sher & Caulfield, 1980; Dessein, Samuelson, Butterworth, Hogan, Sherry, Vadas & David, 1981). The binding of concanavalin A (Con A) to schistosomula but not to cercariae suggests there are surface components expressed only after transformation (Samuelson & Caulfield, 1985).

There are common surface antigens present on cercariae, schistosomula and adult worms (Shah & Ramasamy, 1982). The major surface antigens described on the cercariae are glycoproteins of molecular weight (M.W.) 32 - 38 kilodaltons (kDa) which are probably associated with the glycocalyx (Payares et al., 1985b). Some of the cercarial surface antigens are also expressed on the newly transformed schistosomular surface. The persistence of the cercarial antigens is probably due to remnants of the glycocalyx persisting on the surface of newly transformed schistosomula (Brink, McLaren & Smithers, 1977; McLaren, 1980; Samuelson et al., 1980; Incani & McLaren, 1983; Stirewalt, Cousin & Dorsey, 1983). There is thus antigenic continuity between the cercarial and schistosomulum surfaces. Monoclonal antibodies raised against cercarial glycoproteins react with the antigens exposed on the surface of schistosomula up to 72 hours post-transformation (Hazdai, Levi-Schaffer, Brenner, Horowitz, Eshhar &

Arnon, 1985). Some new antigens are expressed after transformation from cercaria to schistosomulum (Payares et al., 1985b). This may be influenced by the loss of cercarial tail, glycocalyx and the formation of a new membrane.

1.4.2 Schistosomular Surface Membrane Antigens

After transformation the antigens exposed on the surface of the newly transformed (3 h) schistosomulum bind anti-parasite antibodies. It is uncertain whether these antigens are present in the tegument plasma membrane and/or the overlying secreted bilayer. Some original cercarial membrane antigens migrate to the new surface membrane of the schistosomulum during transformation (McLaren & Hockley, 1976). Some low molecular weight antigens (15, 17 & 20 kDa) are only expressed after transformation (see Table 1.1 from Payares et al., 1985b). Antisera from mice immune to S. mansoni or from mice exposed to highly irradiated cercariae can be used to investigate changes in antigen exposure at the parasite surface, using techniques such as immunoprecipitation or Western blotting (Dissous, Dissous & Capron, 1981; Simpson et al., 1983).

The antibody-binding to the schistosomular surface is reduced with time in culture. Schistosomula cultured in defined medium for 2 or 6 days cannot bind antibodies on their surface (Dean, 1977). The loss of antibody and complement C3 binding occur during 24 - 48 h in culture (Dessein et al., 1981). Some antigen i.e. the 17 kDa antigen which is recognized specifically by chronic mouse serum have essentially disappeared from 48 h cultured schistosomula (Payares et al., 1985b). The majority of the antigenic components of freshly transformed schistosomula disappear within the first 24 h in culture (Samuelson et al., 1980; Dessein et al., 1981; Samuelson & Caulfield, 1982).

The exposure of proteins or polypeptides at the parasite surface can be studied by iodination, catalysed either by lactoperoxidase or Iodogen (Phillip & Rumjanek, 1984; Simpson & Smithers, 1985). Lactoperoxidase-catalysed iodination techniques have been used to label adult worms and larval stages (Ramasamy, 1979; Snary, Smith & Clegg, 1980; Dissous et al., 1981; Shah & Ramasamy, 1982; Simpson, James & Sher, 1983). Electron microscope autoradiography of sections of iodinated worms has shown that the label is essentially restricted to the tegument of both stages (Shah & Ramasamy, 1982; Roberts, Aitken, Vojvodic, Wells & Wilson, 1983). Two different experimental approaches have shown that several lactoperoxidase-labelled larval components are exposed on the worms' surface. Labelled antigens were immunoprecipitated with antibodies bound to iodinated living

Table 1.1 Changes Detected in the Surface Antigens of *S. mansoni* During Maturation (from Payares *et al.*, 1985b).

	Schistosomula					5-day-old lung worms	3-week-old liver worms	Adult membranes
Antigens MW (kDa)	Cercaria	3h (ms)	24h (ms)	48h (ms)	24h (sp)			
>100	++					+	+	
92-98						++	++	++
65						+	++	
40							++	
38	++	++	+					
32	++	++	++	++	++	+	+	++
25						+	+	++
20		++	++	++	+	+	+	++
17		++	+	+	+			
15		++	++	++	++	+	+	+
10			+	+	+	+		+

++ Dominant
+ Less dominant
+ Faint
ms = Mechanically transformed schistosomula
sp = Skin penetrated schistosomula (*in vitro*)
By immunoprecipitation and SDS-PAGE analysis of labelled antigens.

schistosomula prior to detergent-solubilization of the tegument (Simpson et al., 1983), and in other experiments, antigenic components were shown to be released when intact radiolabelled larvae were incubated with trypsin (Dissous et al., 1981). However, both the number and the apparent molecular weight of surface components reported by different authors are dissimilar. This might be due to different strains of S. mansoni expressing different sets of surface molecules, the intrinsic fragility of the adult worm tegument, or the different portocols used (Smith et al., 1969; Simpson, Schryer, Cesari, Evans & Smithers, 1981). The tegument of the schistosomulum is more resistant to ultrastructural damage than that of the adult worm under in vitro culture conditions, and more consistent surface labelling results than with adult worms. Iodogen, another reagent for protein iodination, gives similar results to those obtained with lactoperoxidase, but lipid components are also labelled (Hayanga, Murrel, Taylor & Vannier, 1979). It appears that the 15 - 38 kDa molecules represent the most prominent polypeptide antigens exposed on the schistosomular surface (Simpson et al., 1983; Knight, Simpson, Payares, Chaudri & Smithers, 1984).

Several monoclonal antibodies (MABs) have been raised against the surface polypeptides of newly transformed schistosomula. They are monoclonal anti-38 kDa (Grzych, Capron, Bazin & Capron, 1982; Dissous et al., 1982), anti-32 kDa (Bickle, Andrews & Taylor, 1986), anti-24 kDa (Taylor & Butterworth, 1982), and anti-20 kDa (Tavares, de Rossi, Payares, Simpson, McLaren & Smithers, 1984). These MABs all react with the surface antigens of freshly transformed schistosomula by an immunofluorescence technique.

The 32 kDa antigen which is present on the cercarial membrane is also present on the schistosomular surface and persists through to the adult stage (Table 1.1, from Payares et al., 1985b). This indicates the presence of common antigens in all developmental stages in the vertebrate host. An antigen with identical M.W. (32 kDa) was also shown to be present on the schistosomular surface (Dissous et al., 1981) and adult worm tegument (Dissous & Capron, 1983) but not the cercarial surface (Bickle et al., 1986). This indicates some antigen is present only after transformation. Similar results were also shown by Payares, McLaren, Evans & Smithers, (1985a). They used anti-membrane antisera from rabbits, rats and mice to identify common antigens of relative M.W. 32 and 20 kDa on the surface from young schistosomula up to the adult stage.

Some schistosomular surface antigens are present on cercariae but not lung worms as has been reported by Dissous et al. (1982). They used a rat MAB which recognized a 38 kDa antigen to identify the surface antigen on cercariae and mechanically transformed

schistosomula up to 24 h of incubation. This indicates there is some antigenic continuity between the cercarial and schistosomular surfaces.

There are changes in schistosomulum polypeptide antigen expression during early maturation. Simpson, Payares, Walker, Knight & Smithers (1984) have demonstrated the expression of schistosomulum surface antigens during *in vitro* culture. Three distinct groups of schistosomulum surface antigens can be defined : those that are quickly lost from the parasite surface, those that persist, and those that appear only after a further period of maturation after transformation. They suggested that those antigens that are lost are not masked by host-derived molecules nor shed from the parasite, but are sequestered to a site within the parasite where they are no longer available to surface-directed reagents.

Some new antigens are expressed on the surface of the parasite only after a period of incubation. A receptor for host lipoprotein (a 45 kDa molecule) becomes exposed on the surface membranes of schistosomula after contact with human serum. The antigen is pre-synthesized by cercariae (Rumjanek, McLaren & Smithers, 1983; Rumjanek, Pereira & Silveira, 1985). Therefore, lipid uptake by direct transfer to the schistosomular surface may mask the parasite antigens (Rumjanek *et al.*, 1985).

1.4.3 Lung Worm Surface Antigens

Schistosomula change or mask their original surface antigens during development and migration from the skin to the lungs. Ramalho-Pinto *et al.* (1978) have shown that 4 day lung worms no longer activate complement via the alternative pathway and do not bind specific anti-schistosome antibody to their surface. The complement dependent lethal antibody kills 3 h schistosomula *in vitro* but does not kill 4 day-lung worms and 4 day-cultured schistosomula in the presence of serum or serum and erythrocytes (Clegg & Smithers, 1972). Changes in surface antigenicity of 3 h and 4-day-old schistosomula which express parasite and host antigens respectively have been reported (McLaren, Clegg & Smithers, 1975). Young schistosomula recovered from the skin of infected animals at 3 h and 20 h, and adult worms, have parasite as well as host antigens on their surfaces, but 5-day-old schistosomula recovered from the lung demonstrate only host antigen (Goldring, Sher, Smithers & McLaren, 1977). The expression of host antigen on the surface of lung worms is increased with the time that the parasite spends in the host (Imohiosen, Sher & von Lichtenberg, 1978).

It has been difficult to demonstrate parasite antigens on the surface of lung-stage schistosomula by immunofluorescence microscopy. Chronic mouse serum does not bind to the surface of lung worms as has been demonstrated by many workers (McLaren et al., 1975; Goldring et al., 1977; McLaren et al., 1978; Payares et al., 1985a). Antibody from rabbit raised against purified adult worm membranes can bind to the surface of 5 day lung worms as well as 3 h and 18 h schistosomula and adult worms (Payares et al., 1985a). This indicates some of the exposed antigen on the surface of lung worms is similar to that on schistosomula and adult worms; the antigen can be detected by immunofluorescence methods.

Some antigens such as the 32 kDa molecule, although present on the surface of the intact lung worm, are not recognized by antibody but can be detected by radioiodination followed by immunoprecipitation (Payares et al., 1985a). This indicates that the parasites share identical epitopes between various stages, but on the lung worm's surface the epitopes are masked by host molecules.

During development of the parasite in the mammalian host a number of new antigens are expressed. Some surface antigens can only be detected from the lung stage through adult stage, for example a 65 kDa antigen which has been demonstrated previously to be alkaline phosphatase (Payares, Smithers & Evans, 1984), a 25 kDa antigen and a 92-98 kDa polypeptide complex (Table 1.1), a 97 kDa antigen may be of host origin (Payares et al., 1985b). The resistance of 5 day old parasites to humoral and cellular cytotoxicity in vitro has been related to a number of evasive stratagems, including disguise and intrinsic changes in the tegumental outer membrane (McLaren, 1984) which will be described in Sections 1.4.5 and 1.4.6

1.4.4 Adult Worm Surface Antigens

During maturation from liver worm to adult worm schistosomes are not highly antigenic; only low amounts of antigen can be detected by immunofluorescence techniques using immune sera (Goldring et al., 1977; Smith, Clegg & Snary, 1984). In contrast, they give a very strong reaction for the presence of host blood-group antigens (McLaren et al., 1975, 1978). Adult worms obtained from mice have antigens on their surfaces which cross-react with surface antigens of mouse erythrocytes (Smithers, Terry & Hockley, 1969; Clegg, Smithers & Terry, 1970). Though these antigens are firmly bound to the tegument of the worms, they are lost a few days after worms are surgically transferred to Rhesus monkeys.

Adult worms can release membrane antigens into the culture medium by a rapid secretory process and a slower membrane turnover (Kusel &

Mackenzie, 1975). These membrane antigens have molecular weights which are identical to those of antigens in the surface of the schistosomulum (Kusel, Mackenzie & McLaren, 1975). An indication of common epitopes can be shown by radio-iodination of tegumental membranes. MAB that recognized an antigen (20 kDa) in schistosomular surface membrane also recognize antigens (20 & 32 kDa) in adult worm membranes. This suggests that antigens, associated with the adult surface membrane although not exposed on the surface, are cross-reactive with the schistosomulum surface antigens (Tavares et al., 1984; Payares, Kelly, Smithers & Evans, 1985c). It has been suggested that the masking of the adult epitopes by incorporation of host antigen prevents antigen expression in this stage (Smithers & Doenhoff, 1982).

1.4.5 Host Antigens on the Schistosome Surface

The expression of parasite antigens as a target for the immune response occurs on the schistosomulum surface. As the parasite matures these antigens become undetectable. Smithers et al. (1969) have suggested that the loss of antigenicity of the parasite surface results from the acquisition of host antigens which become firmly attached to, or form part of, the tegumental outer membrane. They are not synthesized by the parasite. There is evidence for host antigen acquisition by schistosomula even during skin penetration (Smith & Kusel, 1979).

Host antigens can be acquired in the form of human blood group antigens (Goldring, Clegg, Smithers & Terry, 1976; Dean, 1974; Dean & Sell, 1972). All of those determinants which are glycolipids (ABH, Lewis substances) are acquired by the parasite (Goldring et al., 1977). Antigens in the form of glycoproteins (MN, Rhesus and Duffy antigens and Thy-1, Ly-1 and C3) are not acquired by the parasite (Goldring et al., 1976; Sher, Hall & Vadas, 1978). Clegg (1972) proposed that host antigens are taken up by the intercalation of exogenous lipids within the surface membrane of the schistosome. Extensive interchange of surface lipids between the schistosome and its environment can occur (Rumjanek & McLaren, 1981).

The major histocompatibility complex antigens can be acquired by the surface of schistosomula and adult worms recovered from mice (Sher et al., 1978; Gitter & Damian, 1982; Gitter, McCormick & Damian, 1982). There is some controversy about acquired glycoprotein by the parasite. Simpson, Singer, McCutchan, Sacks & Sher (1983) have reported that H-2K glycoprotein antigen is acquired by lung-stage schistosomula. In addition, immunoglobulins (IgG1, IgG2, IgG3, IgA and IgM) have been found on the surfaces of adult schistosomes

(Damian, Greene & Hubbard, 1973; Campbell, 1973; Sogandares-Bernal, 1976; Kemp, Damian & Greene, 1976; Kemp, Merritt, Bogucki, Roster & Seed, 1977; Kemp, Merritt & Roster, 1978). These antibodies are not specific for the worm's tegument (Kemp et al., 1977). The immunoglobulin binds via the Fc portions to Fc receptors on the parasite surface (Kemp, Brown, Merritt & Miller, 1980). Surface receptors (Fc) for IgG and human beta2-microglobulin have been shown on the surface of newly transformed schistosomula (Torpier, Capron & Ouassi, 1979).

Anti-schistosome antibody can bind to schistosomes in the presence of host antigens whereas lung worm surface antigens may be masked by acquired host antigens (McLaren et al., 1975; McLaren et al., 1978). Host antigen acquired during schistosome development may protect the parasite's membranes from antibody mediated damage (Smithers et al., 1969; McLaren & Terry, 1982). Alternatively, the loss of antigenicity of the lung worm's surface might not result solely from acquisition of host antigen disguise as has been reported by Moser, Hall & Sher (1979). Changes in the schistosome surface, independent of host antigen acquisition may be responsible for ensuring parasite survival from immune attack (Sher, 1979).

Although there is evidence that host glycolipids, glycoproteins and immunoglobulin are acquired by schistosomes, there is no verification that such host molecules can protect the parasites against the immune response. Moser, Wassom & Sher (1980) demonstrated that freshly transformed schistosomula are killed by labelling their surface with trinitrophenyl (TNP) groups and exposing them to anti-TNP antibody plus complement or eosinophils; the same treatment does not kill lung-stage schistosomula. Moreover, exposure to anti-host red blood cell antisera and eosinophils lead to adherence and degranulation of the cells but do not cause death in lung worms (McLaren & Terry, 1982); the same procedure kills adult worms. Therefore, acquired host antigens on the parasite surface are not necessary to facilitate immune evasion of lung-stage schistosomula but they may protect older worms.

1.4.6 Antigen Masking or Shedding

It has been well documented that over the first few days in vivo, S. mansoni schistosomula lose their capacity to bind anti-schistosome antibodies (McLaren et al., 1975; Goldring et al., 1977; Imohiosen et al., 1978). It is assumed that this reduction in surface antigen expression constituted an evasive strategy against immune effector mechanisms directed against the parasite surface (McLaren, 1984).

Two major hypotheses have been proposed to explain the reduction of surface antigenicity. The first hypothesis is proposed by Smithers, Terry & Hockley (1968) that parasite antigens persist at the surface, but are hidden or masked from the host immune system by acquired host molecules. This hypothesis is based largely on circumstantial evidence which has inversely correlated host antigen acquisition with decreasing parasite antigen expression (McLaren et al., 1975; Goldring et al., 1976, 1977; Sher et al., 1978; McLaren, 1984). The second hypothesis implicates parasite surface antigen shedding as the major explanation for reduced anti-schistosome antibody binding to older worms. This proposal is based on studies in which schistosomula cultured in vitro in defined host molecule-free media spontaneously lose their ability to bind anti-schistosome antibodies (Samuelson et al., 1980; Dessein et al., 1981; Samuelson & Caulfield, 1982).

Samuelson et al. (1980) and Dessein et al. (1981) demonstrated that the loss of antigenicity of the parasite surface during schistosomula development results from the spontaneous membrane changes without acquisition of host components. Later in 1982, Samuelson & Caulfield demonstrated that the loss of labelled proteins and lipids from cultured schistosomula is similar to that observed with Concanavalin A, which is shed intact with a halftime of 8-10 h (Samuelson, Caulfield & David, 1981). Antischistosomal antibodies and complement are also lost at a similar rate (Samuelson et al., 1980). It appears that molecules which bind to the surface of schistosomula are being lost at about the same rate as the parasite surface itself. The parasite may defend itself in part by shedding antibody, complement and parasite molecules to which they are bound as part of the normal turnover of its surface (Samuelson & Caulfield, 1982).

The relationship between parasite surface antigen expression and host molecule acquisition have also been investigated by Pearce, Basch & Sher (1986). They demonstrated that schistosomula recovered from mice at 24 h and 5-10 days post-infection bound low or insignificant amounts of a variety of anti-schistosome antibodies including those from chronically infected mice, mice vaccinated with irradiated cercariae, a vaccinated rabbit and rabbits hyper-immunized with non-living larval and adult schistosome antigen preparations. In contrast, parasites maturing in vitro continued to bind highly significant levels of each of these antibody preparations until at least 10 days post transformation.

Six-day-cultured (in the presence of human serum) parasites that had been injected intravenously into mice and recovered from the lungs after 30 minutes in vivo, exhibited a significantly decreased capacity to bind anti-parasite antibodies and Concanavalin A (Con A), and by 16

hours had lost their binding sites for fucose binding protein as well. The reduced ability of these parasites to bind anti-parasite antibodies coincided with the loss of ^{125}I-labelled surface proteins. This observation led Pearce *et al.* (1986) to suggest the reduction in antigenicity was due to shedding of surface antigens. Furthermore, unlike 6 day schistosomula which had developed wholly *in vivo*, 6-day-cultured parasites recovered after 30 minutes *in vivo* failed to bind anti-host antibodies suggesting that parasite antigens were not masked by host molecules (Pearce *et al.*, 1986).

1.5 Egg Antigens

During schistosome (*S. mansoni*) infection, egg deposition in the liver commences between 5 and 6 weeks post-infection and eggs accumulate at a linear rate thereafter. The amount of eggs in the liver is directly related to the number of mature paired worms (Wilson, Coulson & McHugh, 1983). Schistosome eggs which lodge in the liver stimulate a delayed-type hypersensitivity reaction (Warren, Domingo & Cowan, 1967). As a consequence, a granulomata is formed around each egg. Disruption of hepatic tissue organization, portal hypertension, and hepatic fibrosis occur. In addition, porta-systemic anastomoses shunting a fraction of hepatic portal blood to the vena cava can also occur (Cheever, 1965).

The granulomatous-inducing antigens are secreted through ultramicroscopic pores in living eggs (Race, Michaels Martin, Larsh & Matthews, 1969). Lyophilized eggs can also mediate the circumoval precipitin (COP) reaction with schistosomiasis sera. Soluble egg antigen (SEA) is a water-soluble extract of homogenized schistosome eggs that has been centrifuged at 100,000 g (Boros & Warren, 1970).

The SEA contain proteins, glycoproteins and polysaccharides. Carbohydrate-containing molecules of *S. mansoni* eggs are major antigens (Andrade & Barka, 1962; Smithers & Williamson, 1961). *S. mansoni* SEA can induce and elicit granulomatous hypersensitivity in the mouse lung (von Lichtenberg, 1962). An intraperitoneal injection of SEA (from *S. mansoni* eggs) will sensitize mice to produce an accelerated and enhanced granulomatous response to an intravenous injection of *S. mansoni* eggs trapped in the lungs (Warren & Domingo, 1970a). Sensitization is stage-specific. In addition, the antigens are relatively schistosome species-specific (Warren & Domingo, 1970b). SEA of *S. mansoni* is able to induce a delayed cellular response in the absence of humoral antibody (Boros & Warren, 1970). SEA has been demonstrated from 5 weeks post-infection onwards in Kupffer cells in the liver and from 4 weeks post-infection onwards in macrophages of the spleen. It cannot be detected in kidney glomeruli (El-Dosoky, Van

Marck & Deelder, 1984).

1.6 Circulating Schistosome Antigens

The detection of schistosome antigens in the blood or urine of an individual should signify the presence of the living parasite. Antigens can be detected in the urine of S. japonicum patients (Okabe & Tanaka, 1958), and in the serum and urine of mice and hamster heavily infected with S. mansoni (Berggren & Weller, 1967). On subsequent characterization, this antigen was shown to be a negatively charged polysaccharide which gives a characteristic anodic precipitation in immunoelectrophoresis (Deelder, Klappe, Van den Aardweg & Van Meerbeke, 1976) and it is therefore designated "Circulating Anodic Antigen" (CAA). Colley (1983) reported that an antigen fraction callled "GASP" (gut associated proteoglycan), purified by Nash, Lunde & Cheever (1981) from a crude TCA-soluble polysaccharide fraction of adult S. mansoni is identical to CAA. CAA has an relative molecular weight (M_r) greater than 200 kDa.

CAA contains large amounts of carbohydrate, primarily N-acetylgalactosamine and D-glucuronic acid residues with a minor amount of amino acids. Therefore, the antigen has the characteristics of a proteoglycan (Nash, Nasir & Jeanloz, 1977). CAA is highly immunogenic both in infected and in immunized animals, specific antisera against this circulating antigen have been raised for use in immunodiagnostic and immunohistological studies (Nash et al., 1977; Deelder, Kornelis, Van Marck, Eveleigh & Van Egmond, 1980). CAA has been demonstrated in the epithelium lining the oesophagus and gut of adult worms (von Lichtenberg, Bawden & Shealey, 1974; Nash, 1974). It is a gut-specific antigen produced by various life cycle stages of S. mansoni (De Water, Fransen & Deelder, 1986). It is present in primordial gut cells of schistosome cercaria (Andrade & Sadigursky, 1978; Deelder et al., 1980). It can be found in many organs of infected mice i.e. Kupffer cells of liver, kidney glomeruli (3 weeks post-infection onwards), and spleen macrophages (Van Marck, 1975; Van Marck, Deelder & Gigase, 1977; Deelder et al., 1980; El-Dosoky et al., 1984).

A second circulating antigen was originally found in the urine and serum of S. mansoni patients and in the milk of infected mothers (Carlier, Bout, Bina, Camus, Figueiredo & Capron, 1975; Carlier, Bout & Capron, 1978; Santoro, Borojevic, Bout, Tachon, Bina & Capron, 1977). It is called "antigen M" (Carlier et al., 1975). This antigen has the characteristics of a polysaccharide with a cathodic migration at pH 8.2, it has been called "Circulating Cathodic Antigen" (CCA) (Deelder et al., 1976). Its M_r less than 30 kDa. It is present only in adult worms (Carlier et al., 1978).

Deelder et al. (1980) and Carlier, Bout & Capron (1980) have demonstrated the presence of CCA in the kidneys of infected hamsters. Deelder, El-Dosoky, Van Marck & Qian (1985) have used monoclonal antibodies directed against CCA to localise CCA in tissue of S. mansoni infected mice. They have found that CCA is present in Kupffer cells in the liver, in macrophages in the spleen and in kidney glomeruli from 2 weeks, 3-4 weeks, and 8 weeks post-infection onwards, respectively. The immunofluorescence reactions on CCA in kidney glomeruli is relatively weak. These anti-CCA mouse monoclonal antibodies are raised against gut-associated polysaccharide antigens of S. japonicum. Therefore, Deelder et al. (1985) have proved that CCA is a gut-associated antigen, and that CCA from S. mansoni and S. japonicum are partially immunologically identical, as has been earlier observed by Qian & Deelder (1983).

Indirect immunofluorescent studies have shown that CAA and CCA are derived from the epithelial cells of the schistosome gut (Nash, 1974; Carlier, Bout, Strecker, Bebray & Capron, 1980; Deelder et al., 1980). It appears that the gut associated proteoglycan (GASP) is secreted by the epithelial cells of the gut and is regurgitated into the bloodstream.

CCA is normally excreted into the urine by infected mice and hamsters, while CAA is not (Deelder et al., 1980). Both CCA and CAA are immunogenic in infected animals and in man, and can form the immune complexes which are deposited in the mesangium of the kidney glomeruli, as a consequence of glomerulonephritis (Deelder & Van den Berge, 1981; Van Marck, Deelder & Gigase 1981).

1.7 Aims of the Study

The study of schistosome surface membrane antigens has been motivated by the well established concept that tegumental components play an important role in the host-parasite interaction (Smithers, McLaren & Ramalho-Pinto, 1977). In schistosomiasis, the adult worms are resistant to an immune response mounted by the vertebrate host and can survive for several years. The mechanisms of this immune evasion are not clearly understood.

It is assumed that the antigens responsible for eliciting a protective response, in hosts immune to S. mansoni, are situated either in the tegument plasma membrane (inner bilayer) or in the secreted outer bilayer. This assumption is supported by numerous in vitro studies on mechanisms of immune killing of schistosomes (reviewed by McLaren, 1980; Butterworth, Taylor, Veith, Vadas, Dessein, Sturrock & Wells, 1982).

The aim of my study was to use a library of monoclonal antibodies (MABs) which were already characterized, to locate the antigens in situ in schistosome tissues. The MABs were raised by immunizing mice with highly enriched tegument fractions of adult worm. Therefore, they should react predominantly with tegument structures.

Parallel studies carried out by Vojvodic (unpublished) have identified the molecular weight of target antigens of many of MABs under investigation. The ability of these MABs to protect against infection, after administration to mice was also assessed by Vojvodic. The purpose of the present study was to relate the data on molecular targets, protective properties, and antigen location in worm tissues. Indirect immunofluorescence techniques were used to investigate a number of questions about schistosome antigens.
These were :

1). Within which worm tissues were the target antigens of each MAB located ?

2). Were any antigens stage-specific or were they present in all stages from the cercaria to adult worm ?

3). Was there any evidence for the redistribution of antigens during cercaria to schistosomulum transformation, as indicated by morphological studies ?

4). Were the epitopes recognized by a MAB confined to the same tissues or cells in all parasite stages ?

5). Was there cross-reactivity between the antigens of S. mansoni and other schistosome species ?

During the course of my study I also investigated the following question : Can host antigens be detected on parasite surfaces using polyclonal antiserum to host erythrocytes ?

Indirect immunofluorescence is a highly sensitive and specific method for localisation of schistosome antigens (see Section 2.1.2 in Chapter 2). Frozen sections of various stages in the life cycle of the schistosome i.e. cercaria, 3h schistosomulum and adult worm were used to examine the distribution of antigens within internal tissues (work described in Chapters 2 & 3). A test for stage-specificity and surface binding of MAB to live and paraformaldehyde-fixed intact parasites (cercaria, 3h schistosomulum, 5-day-lung worm and 21-day-liver worm) was also performed in the present study (see Chapter 2).

The most promising MABs showing highest specificity, were then used to localise the target antigens at the ultrastructural level by electron microscopy. The approaches attempted were the fixation and embedding of tissue, using low temperature water soluble resin (K4M, see Chapter 4), and the cryoultramicrotomy technique (see also Chapter 4). The sections prepared in one of the above ways were reacted with individual anti-schistosome MABs or control sera. The binding sites of antibody were then visualised using secondary antibodies conjugated with colloidal gold for electron microscopy.

CHAPTER 2

MONOCLONAL ANTIBODIES REACTING AGAINST TEGUMENT ANTIGENS

2.1 INTRODUCTION

2.1.1 The Immunofluorescence Techniques

A fluorescent antibody test was first introduced by Sadun, Williams & Anderson (1960) for the serodiagnosis of schistosomiasis in humans. Immunofluorescence combines histochemical and immunological methods to pinpoint specific antigen-antibody complexes present in tissue sections or cellular smears, with the aid of fluorochrome conjugated to the antibody. Fluorochromes and ultraviolet light sources are the mainstays of the technique. A fluorochrome is a substance which emits light of longer wavelength than that of the excitation absorption. Fluorescence is defined as the emission of light by a substance whilst it is being excited. Fluorescent substances can be tagged or conjugated to the antibody globulin without interfering with its immunological specificity and its ability to combine with antigen.

I. The Fluorescein Isothiocyanate System

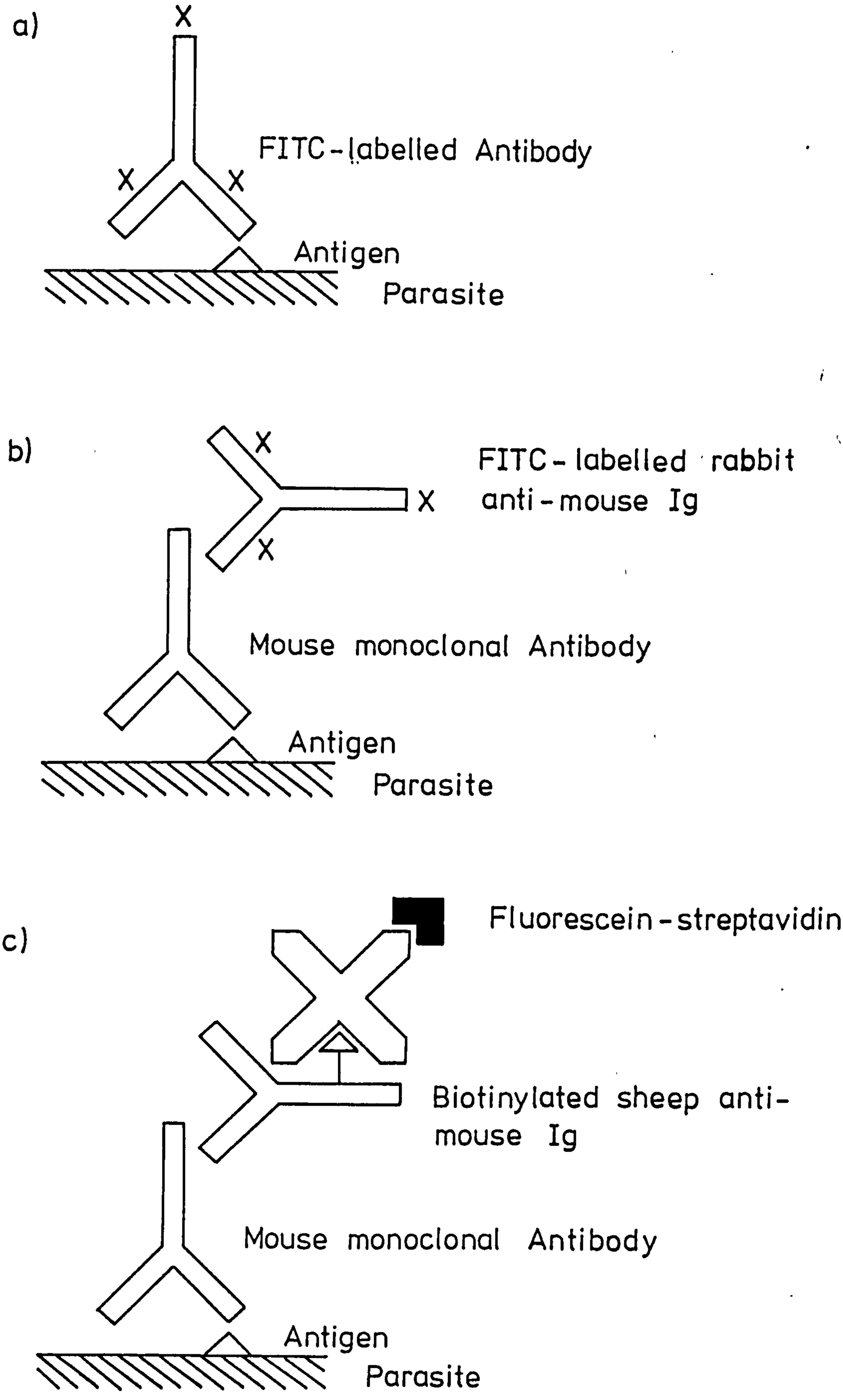
The fluorochrome used in the present study was Fluorescein Isothiocyanate (FITC). The dye exhibits greenish-yellow fluorescence and has a molecular weight of 389.4; maximum wavelength of absorption 490 mu, maximum wavelength of emission 520 mu. FITC is the best dye available for the fluorescent antibody technique in terms of fluorescence efficiency, stability, and combining capacity with protein. Coons, Creech & Jones (1941) were the first to use a conjugate of an antibody with a fluorescent dye for the localisation of an antigen in a tissue section.

There are two variants of the immunofluorescence technique.

a). Direct Method

When tissues or cells containing a particular antigen, e.g., influenza A2 virus, are stained with a specific fluorescein-conjugated antibody and examined under a fluorescence microscope, only those cells containing or infected with influenza A2 virus fluoresce brilliantly. Cells infected with influenza B virus will not fluoresce at all if the fluorescent antibody has been carefully checked for specificity and is appropriately diluted. This is a one-step staining method described by Coons & Kaplan (1950); see Text Figure 2.1a.

Text Figure 2.1 IMMUNOFLUORESCENCE TECHNIQUES



b). Indirect Method

This is a two-step staining method adapted by Coons, Leduc & Connolly (1955); see Text Figure 2.1b. First, the primary antibody (unconjugated) is reacted with an antigen. In the second step, the antigen-antibody complex formed is stained with an antibody which is raised to the immunoglobulin of the species which donated the first antibody. For example, rabbit anti-mouse immunoglobulin (Ig), is conjugated with FITC. The first antibody, mouse anti-antigen, which is bound to antigenic sites in the tissue section, then acts as an Ig antigen for the second, fluorescent antibody.

II. The Biotin-Streptavidin System

An alternative antibody detection system in indirect immunofluorescence is streptavidin conjugated with FITC (Text Figure 2.1c). The interaction of the egg white protein avidin with the coenzyme biotin has simplified the problem of linking tracers to biological probes. Avidin binds biotin with one of the strongest non-covalent bonds discovered ($K_D = 10^{15}\ M^{-1}$) (Green, 1975), allowing rapid reactions with diluted biotinyl- or avidin conjugates.

Biotin is a small, water-soluble vitamin (molecular weight 244) found in egg yolk. Biotinylation can be achieved to a high degree without damage to the antigen binding ability or other physical properties. Streptavidin is a protein (molecular weight 60,000) (Chaiet & Wolf, 1964) with four high affinity sites for biotin. It is isolated from the bacterium Streptomyces avidini and has the same biotin-binding properties as the egg white protein, avidin. Biotinylated antibody is reacted with the unconjugated primary antibody, which is bound to antigenic sites in the tissue section. Then the fluorescein-labelled streptavidin is added to visualise the antigen-antibody complex.

2.1.2 Tissue Localisation of Schistosome Antigens at the Light Microscope Level

The immunofluorescence technique has been used for immunodiagnostic purposes in patients with schistosome infection by many workers. The indirect immunofluorescence (IIF) test suffers the disadvantage of cross-reactivity with antisera from other helminthic infections resulting in false positive diagnoses (Sadun, Williams & Anderson, 1960; Cookson, 1963; Sadun & Biocca, 1962; Moore, Kaiser, Lawrence, Putnam & Kagan, 1968; McCarten, Nzelibe, Simonton & Fife,

1975). An adaptation of this test is the use of minute amounts of dried blood smears on filter paper for the serodiagnosis of schistosomiasis (Anderson, Sadun & Williams, 1961; Sadun, Anderson & Williams, 1961). Anderson et al. (1961) have also used preserved cercariae (cercariae stained with rhodamine bovine albumin and fixed with 10% formalin for several weeks) in the fluorescent antibody test for schistosomiasis. Many other antigens such as adult worm particles (Camargo, Hoshino & Silva, 1965), sections of adult worms or liver containing worms and eggs (Coudert, Garin, Ambroise-Thomas & Pothier, 1967) have been used routinely. Later, Wilson, Sulzer & Walls (1974) showed that the sensitivity and specificity of the IIF test with frozen sections of adult S. mansoni antigen were 91.7% and 95.8% respectively. Moreover, the IIF test with adult worm antigen was more sensitive than, and as specific as, the complement fixation test (Wilson, Fried, Mcquay & Sulzer, 1977).

From 1975 onwards investigators began to study the location of antigen in the parasite or host tissue, recognized by antibody, rather than looking for anti-schistosome antibodies in serum. In 1975, Van Helden, Terpstra, Okot-Kotber & Eyakuze used adult worm sections and the IIF technique for serodiagnostic purposes in human schistosomiasis infections. They also reported distinctive patterns of immunofluorescence, possibly associated with the stage of infection. Two years later Goldring, Sher, Smithers & McLaren (1977) used the IIF technique to demonstrate the presence of either mouse host antigens or parasite antigens on the surface of S. mansoni schistosomula and adult worms. The IIF technique has been used to demonstrate the presence and location of antigens sharing determinants with circulating schistosomal antigens (CSA) in host tissue, using specific antiserum to CSA (Nash, Prescott & Neva, 1974) and goat anti-rabbit globulin/FITC (Andrade & Sadigursky, 1978). The IIF test has also been used with sections of Rossman's-fixed paraffin-embedded adult worms to demonstrate the presence of specific human IgM and IgG antibodies to a polysaccharide in the epithelial cells of the schistosome gut (Nash, 1978). The investigator claimed that this test was highly specific, and easy to perform because of the availability and stability of the adult worm sections.

Direct fluorescence techniques have been used to localise schistosome antigens, antibodies and antigen-antibody complexes in host tissues e.g. liver, spleen and kidney. As with the indirect immunofluorescence technique, the early work was for diagnostic purposes, later work for localisation of parasite antigens in tissue. Andrade, Paronetto & Popper (1961) detected schistosome antigens directly with fluorochrome-conjugated immunoglobulin from serum of

human patients infected with S. mansoni. Other schistosome antigens [i.e. the circulating anodic antigen (CAA), the circulating cathodic antigen (CCA) and the soluble egg antigens (SEA)] can be localised on frozen sections of liver and spleen of infected animals. The CAA and CCA are also found in the kidneys (Van Marck, 1975; Van Marck, Deelder & Gigase, 1977; Deelder, Kornelis, Van Marck, Eveleigh & Van Egmond, 1980; Deelder, El-Dosoky, Van Marck & Qian, 1985; El-Dosoky, Van Marck & Deelder, 1984; Byram & Von Lichtenberg, 1977). The gut-associated proteoglycan (GASP), a specific excretory-secretory antigen has been detected in the Kupffer cells of the livers of infected mice (Nash, 1982). De Brito, Shimizu, Yamashiro & Da Silva (1985) have studied the distribution of schistosome antigen, immunoglobulins and complement C3 by direct immunofluorescence tests on cryostat and paraffin sections of human liver biopsies in schistosomiasis mansoni. Fluorochrome-conjugated monoclonal antibodies have also been used to identify protein antigens in the liver of mice infected with S. mansoni (Salama, Aronstein, Weiss & Strand, 1984).

2.1.3 Surface Antigens as the Target of Host Immunity

Adult schistosomes persist for many years in the vertebrate host. In vivo evidence suggests that there is a gradual development of immunity to reinfection despite the continuing presence of a healthy population of adult worms. This phenomenon was called "concomitant immunity" (Smithers & Terry, 1969), and it is now recognized as an established characteristic of schistosome infections in baboons, mice and almost certainly occurs in man (Bradley & McCullough, 1973).

It is not clear which schistosome antigens are important in eliciting protective immunity in laboratory hosts or man. In vitro, antibody-dependent cellular cytotoxicity assays (ADCC) and complement-fixing antibodies implicate surface antigens of 3h schistosomula as being involved in protective immune responses (Clegg & Smithers, 1972; Butterworth, Sturrock, Houba, Mahmoud, Sher & Rees, 1975; Capron, Bazin, Desaint & Capron, 1975; Perez & Smithers, 1977).

However, autoradiographic tracking experiments have shown that in previously infected mice, as in naive mice, very few S. mansoni schistosomula are eliminated in the skin (Dean & Mangold, 1984). By day 6 after challenge infection, essentially identical numbers of schistosomula had migrated from the skin to the lungs in control and 6- or 25-week-infected mice (Dean & Mangold, 1984). A possible interpretation of the mechanism of immunity in hosts with a chronic infection is that it results from a leaky hepatic portal system caused by schistosome eggs lodged in the liver (egg-induced pathology), not

immune killing (Wilson, 1980; Wilson, Coulson & McHugh, 1983). Wilson et al (1983) suggested that in consequence there was a progressive reduction in the efficiency with which migrating schistosomula were sequestered in the liver.

In contrast, the resistance induced by irradiated cercariae or schistosomula (the vaccination model) differs from that induced by a chronic infection because of the absence of adult worms of both sexes and the failure to produce eggs. Therefore, no liver pathology develops. Autoradiographic tracking studies by Dean, Mangold, Georgi & Jacobson (1984) found that migration of schistosomula from skin to lungs of vaccinated mice was delayed for several days but there was no evidence of skin elimination. The challenge parasites were lost after arrival in the lungs and before their development into adult worms in the hepatic portal system. Dean et al (1984) also found greater numbers of schistosomula in vaccinated mouse lungs 10-21 days after challenge. This suggested that schistosomula reached the lungs of vaccinated mice in normal numbers but their further migration was obstructed. Crabtree & Wilson (1986b) found that intravascular schistosomula in the lung of vaccinated mice attracted foci of host leucocytes (85% or more mononuclear, containing both lymphocytes and macrophages) whereas parasites in normal mice did not. They suggested that pulmonary inflammation, by impeding the movement of schistosomula through the vasculature effectively terminated migration in the lungs.

Many groups have reported the production of MABs which bind to the schistosomulum surface and also mediate immunity in passive transfer experiments in vivo against schistosomes (Smith, Clegg, Snary & Trejdosiewicz, 1982; Zodda & Phillips, 1982; Grzych, Capron, Bazin & Capron, 1982; Harn, Mitsuyama & David, 1984; Horowitz, Brenner & Arnon, 1985; Bickle, Andrews & Taylor, 1986). MABs were given around the time of infection (within 24 h before or after infection). Delivery of antibodies at later times did not result in protection. So it is likely that passive immunization with the MABs mediates some skin-phase immunity in vivo. These passive transfer experiments demonstrate that if one or more epitopes are sufficiently densely expressed on the schistosomulum surface they can act as targets for immune killing. Moreover, the presence of a high enough concentration of antibody against certain antigens can protect against subsequent infection.

It is presently difficult to reconcile the results of parasite tracking studies in vivo with those obtained with monoclonal antibodies.

2.1.4 Monoclonal Antibodies Raised Against Adult Worm Tegument Surface Membrane

Monoclonal antibodies (MABs) used in the present study were raised by Vojvodic & Boot, following vaccination of CBAxBALB/C mice with adult worm tegument fractions i.e.

a). Total tegument surface membrane fraction (gradient pellet, G).

b). Microvesicular fraction (M) containing discoid granules.

Gradient pellet and microvesicular fraction were prepared according to the method of Roberts, Macgregor, Vojvodic, Wells, Crabtree & Wilson (1983) as shown in Text Figure 2.2.

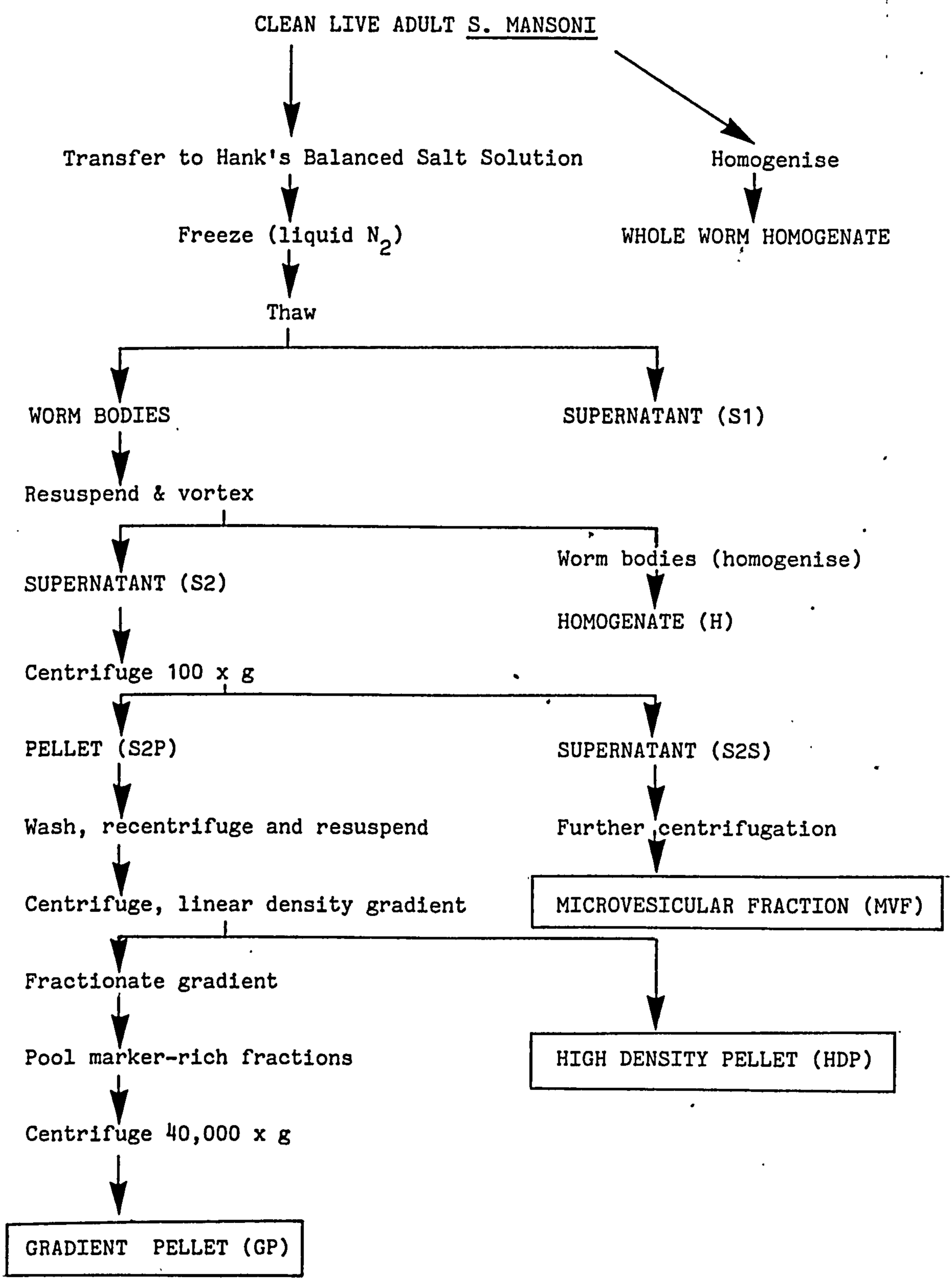
c). Digitonin pellet containing surface membrane was prepared as described by McDiarmid, Dean & Podesta (1983).

The logic of the use of adult worms as starting material for membrane isolation was that

1). Adult worm membranes were readily isolated in our laboratory.
2). Adult tegument structure and function were better understood than that of larval stages and this made it easier to interpret the results.
3). Large amounts of membrane material can be obtained from adult worms compared to schistosomula.

Approximately 1200 adult worm pairs (the burden of 50 mice or 6 hamsters) are equivalent in mass to 2,000,000 newly transformed schistosomula (Lawson & Wilson, 1980). Spleen cells from those mice vaccinated with tegument fractions gave a higher number of clones producing antibodies against surface membrane constituents than spleen cells from mice vaccinated with adult worm homogenate (Hackett, Simpson, Knight, Ali, Payares & Smithers, 1985) or from hosts infected with S. mansoni cercariae (Verwaerde, Grzych, Bazin, Capron & Capron, 1979; Taylor & Butterworth, 1982). Hybridoma cell lines were generated by fusing NS-1 myeloma cells with splenocytes from vaccinated mice.

Text Figure 2.2 SCHEME FOR ISOLATION OF TEGUMENT MEMBRANES (Roberts et al., 1983).



NS-1 Myeloma Cell Line (NS-1)

NS-1, abbreviated from P3-NS1-1-Ag4-1, is a cell line derived from a BALB/C myeloma cell line. NS-1 does not produce the heavy chain; it synthesizes the light chain but internally degrades it (synthesizes but does not secrete light chain). The advantage in using NS-1 as the fusion myeloma parent cell line, rather than other myeloma cell lines (such as P3-X63-Ag8), is its inability to produce a heavy chain. Antibody-producing hybrid cell lines derived with this fusion partner will produce mixed molecules with only the light chain; the molecules will not have both light and heavy chains derived from the myeloma cell (Kohler & Milstein, 1976). NS-1 ascitic fluid was used as a negative control in the present study.

2.1.5 Properties of Monoclonal Antibodies (MABs)

A total of 50 MABs were produced and the isotypes and target antigens of these MABs were also defined by Miss M. Vojvodic and Mr. C. Boot in our laboratory. The properties of 16 MABs which were selected on the basis of fluorescent staining the tegument on adult worm sections are shown in Table 2.1 (Vojvodic, Boot, Riengrojpitak & Wilson, 1987).

2.1.6 The Objectives of the Study

The objective of the work described in this Chapter was to investigate the distribution and the stage-specificity of tegumental antigens. The monoclonal antibodies were ideal for this purpose because of their specificity for a single epitope. Monoclonal antibodies were used as antigen-detecting probes to localise schistosome antigens at the light microscope level in various stages of the life cycle of the parasite. My first task was to screen all 50 MABs against frozen sections of adult worm in order to characterize their specificity for particular tissues. A total of 16 MABs which reacted predominantly with the tegument were selected for detailed study. The parasite preparations subsequently used were frozen sections of cercariae, 3h schistosomula and adult worms, and intact cercariae, 3h schistosomula, 5-day-lung worms and 21-day-liver worms. The indirect immunofluorescence technique (IIF) was employed. The cross-reactivity of the 16 MABs reacting with the adult worm tegument was also investigated using Schistosoma japonicum adult worm sections. Anti-host antibody was used to examine the host antigen on the surfaces of intact lung worms and liver worms. The loss of host

Table 2.1

The MAB isotypes have been identified by isotype specific ELISA using culture supernatants. Molecular weights obtained by Western blotting are the means of 5 or more experiments, bracket ( ) shows bands seen in less than 50% of the experiments. Antigens have also been identified by solid phase isolation technique (SPIT) on unreduced membrane preparations. The results are marked with an asterisk (*). The spot blots have been performed on untreated membrane preparations spotted onto nitrocellulose; " + " and " - " represent positive and negative staining respectively.

Table 2.1 MAB Isotypes and Target Antigens Identified in Adult Worm Tegument Preparations.

Group	Clone	Isotype	Dot blot	Molecular weights (kilodaltons)		% Protection
				Unreduced	Reduced	
1.	G6.7	G1	+	32, 50	-	-2.2
	M7.4	G1	+	32, (50)	-	32.6
2.	G6.1	G3	+	*25, 39, 55	25	13.5
	D7.2	G1	+	*25, *38, (*54)	25	6.48
3.	D7.4	G3/M	+	*29, 39, 49	29, 39, 49	13.1
	G6.2	G3	+	29, (39), 49	29, (38), 48	-11.3
	M7.3	G1	+	29, 38, 49	29, (38), 49	33.7
4.	G6.6	M	+	66, 123	66, 123	-3.7
5.	D7.1	G1	+	*18, *29, (39), 49	18, 29, (39), 49	14.2
	D7.3	G3/M	+	*18, *29, (38), 49	18, 29, (39), 50	-19.4
6.	G3.12	G1	+	*18, *29, 39	18, 29, 38	6.5
7.	G5.3	M	-	-	-	9.7
8.	M7.5	M	+	19, *21, 22, *29	18, 20, 21, 29	-4.1
9.	G5.10	E	-	-	-	2.9
10.	G6.4	M	+	-	-	7.8
11.	M7.6	M	+	-	-	5.7

antigens from the liver worm surface after culture in vitro for 24 hours was also investigated. The expression of schistosome antigens by the parasite in each developmental stage is discussed

2.2 MATERIALS AND METHODS

Different stages in the life cycle of S. mansoni within the vertebrate host were used with the indirect immunofluorescent antibody test (IIF) to localise schistosome antigens.

2.2.1 Preparation of Biological Materials for Use as Target Antigens

The life-cycle of a Puerto Rican strain of S. mansoni was maintained in the laboratory using LACA strain white mice and the snail Biomphalaria glabrata as intermediate hosts.

A). Preparation of Cercariae

Cercariae were collected from snails infected 6 weeks previously with S. mansoni miracidia. The methods for obtaining cercariae were as follows :-

Preparation of Miracidia

Miracidia were prepared for infecting snails by trypsin digestion of homogenized livers and intestines of mice which had been exposed to approximately 200 cercariae 6 weeks previously. The eggs were separated from the digest by sedimentation, washed 4 times in 0.9% saline and then transferred to dechlorinated tap water (aerated tap water). The eggs were left under a light for the miracidia to hatch for a period of 1 hour.

Snails Exposure to Miracidia

Uninfected snails, shell diameter 6-8 mm were exposed to miracidia (8 - 10 miracidia per snail) in a plastic-well plate. The snails were left for 1 hour to allow the miracidia to penetrate. The infected snails were pooled in a container filled with dechlorinated tap water, fed with lettuce and kept in a 12 h light, 12 h dark regime in a constant temperature room set at 25°C. Six weeks after infection cercariae were shed from the snails under a bright light for 2 hours. They were then used intact, or for infecting mice, or for cryostat sections.

B). Preparation of Skin Transformed Schistosomula "in vitro"

By definition, a schistosomulum is the stage of a schistosome into which a cercaria transforms after penetration of skin (Faust & Meleney, 1924). Schistosomula, transformed by penetration of an isolated skin preparation in vitro are considered to resemble most closely in vivo transformed individuals (Clegg & Smithers, 1972).

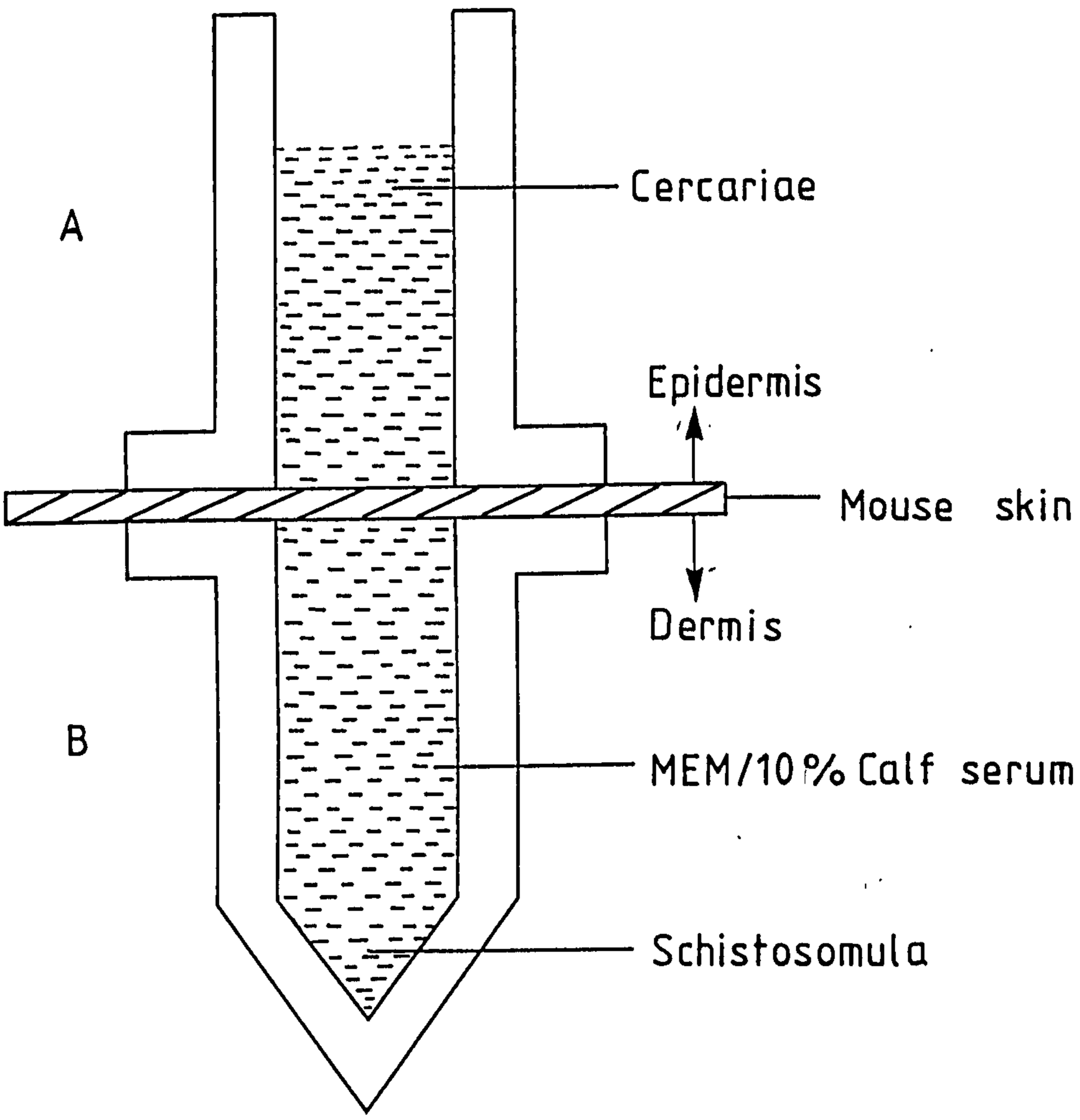
Hair was removed with clippers from the abdominal skin of freshly-killed old male mice and an area of skin about 2 cm x 2 cm excised. Most of the gel-like dermal tissue was removed by rubbing of the undersurface of the excised piece of skin with pads of gauze soaked in 0.9% normal saline. The upper surface (epidermis) of the skin was briefly wetted with dechlorinated tap water to remove any saline. Then the piece of thinned skin was mounted in the glass penetration assembly shown in Text Figure 2.3.

Before mounting the skin, the lower tube of the penetration apparatus (B) was completely filled with warm Eagle's medium (MEM; Wellcome Ltd.) containing 10% (v/v) heat-inactivated calf serum (Gibco Ltd.) and 20 mM HEPES buffer (Gibco Ltd.). The prepared skin was placed, dermal side downwards over the MEM and held in place by tube A. The joints were held together by 2 metal clips. The internal diameter of the tubes used was 7 mm and 10 mm. The apparatus was then placed in a rack in a water bath at 37°C so that the MEM/10% calf serum was held at this temperature.

Approximately 2,000 cercariae in about 2-3 ml of dechlorinated tap water were pipetted into tube A. Three hours after application of cercariae (after incubation at 37°C), schistosomula were harvested from tube B. Incubation in 37°C culture medium for 2 hours or more stimulates the cercaria-to-schistosomulum transformation (Stirewalt, Cousin & Dorsey, 1983). The schistosomula were combined (usually 10 assemblies were used at one time to obtain a large number of schistosomula) and washed 3 times by slow centrifugation in MEM containing 1% heat-inactivated calf serum to prevent the schistosomula sticking in the centrifuge tube.

The percentage of cercariae which penetrate through the skin preparation and could be collected as schistosomula depends on a number of factors, chiefly the amount of dermis removed, the time allowed for penetration and the age and sex of the donor mice (old male mice were preferable). In my hands the technique was very reproducible and the yield of schistosomula was 20 - 30 per cent of the cercariae applied. The skin transformed schistosomula obtained thus were ready for use as antigenic targets either as cryostat sections or as live/fixed intact schistosomula for reacting with

Text Figure 2.3 PENETRATION APPARATUS



monoclonal antibodies.

C). Recovery of Schistosomula from the Lungs (Lung Worms)

The technique used for the recovery of schistosomula from mouse lungs was adapted from the procedure described by Clegg (1965). Briefly, lung-stage S. mansoni schistosomula were obtained from the lungs of LACA mice infected 5 days previously with approximately 2,000 cercariae. The infected mice were anaesthetized by intraperitoneal (i.p.) injection with 10% Sagatal (May & Baker) at a dose of 0.1 ml per 10 g body weight. The body was wetted with tap water and the body cavity opened to expose the heart and lungs. The left ventricle was punctured with a pair of scissors to allow the perfusate to flow away with the blood from the lung. Minimal Eagle's medium (MEM) 10-15 ml, pH 7.4 containing 4 international units (I.U.) of heparin (Sigma) per ml MEM was injected into the right ventricle of the heart by means of a perfusion pump fitted with a 21-gauge needle. This procedure perfuses blood from the lungs and expands them for easy dissection. The schistosomula were held firmly inside the lung capillaries and could not be perfused out. This was checked by examining the perfusate under the dissecting microscope. The perfused lungs became clear and were removed from the animal placed on a piece of filter paper for blotting the excess medium. They were transferred to a clean, dry petri dish and chopped into small fragments approximately 1.5-2.0 mm^3 with a pair of sharp, fine-pointed scissors.

The pieces of lung were transferred to a 50 ml conical flask containing 10 ml of MEM with 10% heat-inactivated calf serum. The flask was covered with parafilm and incubated in a shaking water bath at 37°C for 4 hours. This procedure was repeated on each mouse. During the incubation period, schistosomula crawled out from the lung capillaries.

After incubation, the contents of each flask were filtered through a wire mesh sieve glued to a glass tube (the tube is about 5.5 cm long, 1.5 cm diameter, the sieve 100 mesh, 42 gauge) into a 15 ml centrifuge tube, which allowed the schistosomula to pass through but retained most of the pieces of lung. The resulting filtrate was centrifuged for 45 seconds at lowest speed in a bench centrifuge to sediment the worms, and the supernatant was drawn off. The schistosomula were washed several times with MEM and transferred to a new petri dish. They were separated from the contaminating lung tissues using a very fine Pasteur pipette whilst viewing under a dissecting microscope.

D). Recovery of Schistosomula from the Liver (Liver Worms)

Schistosomula were recovered from the liver by the method of Smithers & Terry (1965). They were perfused from the liver of mice infected 21 days previously with 5,000 cercariae via the abdominal skin. The infected mice were anaesthetized by i.p. injection with 10% Sagatal. The body of each animal was wetted with tap water and the body cavity opened to expose the liver. The hepatic portal vein was punctured with a pair of sharp-pointed forceps to allow the perfusate to emerge. MEM 10-15 ml, pH 7.4 containing 4 I.U. of heparin per ml was injected into the aorta or the left ventricle of the heart by means of a perfusion pump fitted with a 21-gauge needle. The liver was perfused again via the hepatic vein. At this stage the liver became clear (pale) and enlarged. The perfusate containing both worms and erythrocytes was collected and transferred to a 25 ml centrifuge tube and centrifuged in a bench centrifuge at the lowest speed for 30 seconds. The supernatant which contained mostly the mouse red blood cells was removed. The schistosomula were washed several times with fresh Eagle's medium and recentrifuged until most of the red blood cells had been removed and a clean suspension of liver schistosomula remained at the bottom of the centrifuge tube. They were ready to use as live or fixed intact worms.

E). Preparation of the Adult Worms

LACA mice body weight between 25 to 35 g were anaesthetized with 10% Sagatal (0.1 ml per 10 g body weight). The abdominal skins were shaved and they were infected percutaneously with approximately 200 cercariae of S. mansoni, according to the method of Smithers & Terry (1965). Six to seven weeks after infection, the adult worms were recovered from mice by hepatic perfusion after the mice had received anaesthetic doses of Sagatal. The perfusing medium was heparinised (4 I.U./ml) minimal Eagle's medium (MEM). Worms were washed several times with this medium without heparin to remove extraneous materials. They were then transferred to a glass petri dish. By using a dissecting microscope and a Pasteur pipette, the worms were removed to a cleaned centrifuge tube and washed again in MEM. They were ready for cryostat sectioning.

2.2.2 Preparation of Antibodies

a). Monoclonal Antibodies (MABs) : MABs were raised against adult worm tegument surface membrane (see Section 2.1.4).

b). Acute Mouse Serum (AMS) : AMS was obtained from LACA mice infected with 200±20 cercariae of S. mansoni via the abdominal skin and bled 6 weeks later.

c). Normal Mouse Serum (NMS) : NMS was obtained from uninfected LACA mice of the same age.

d). Rabbit Anti-Mouse Erythrocyte Ghost Serum : Rabbit anti-mouse erythrocyte ghost serum was kindly supplied by Dr. N. Saunders (serum obtained from a rabbit vaccinated with mouse erythrocyte ghosts (Saunders, 1986).

e). Normal Rabbit Serum (NRS) : NRS was obtained from an uninfected or unimmunized rabbit from the animal house of the department.

f). Vaccinated Rabbit Serum (VRS) : VRS was kindly supplied by Dr. Q.D. Bickle (serum obtained from a rabbit vaccinated 5 times with irradiated cercariae).

2.2.3 Preparation of Parasite Antigens

a). Frozen (cryostat) Sections : Cercariae, 3h schistosomula and adult worms of S. mansoni were fixed in 1% paraformaldehyde (PF) in 0.1 M phosphate buffer, pH 7.4 for 15-20 min at room temperature (see Appendix). They were washed 3 times in 0.1 M phosphate buffer. Fixed and unfixed parasites were mixed with embedding compound (Bright CRYO-M-BED, Raymond A. Lamb, London) on the specimen holder and frozen by spraying Aerosol freezer (RS Components Ltd.) onto them. Sections were cut at 6-8 um thickness and picked up on microscope slides. The sections were used for immunofluorescent staining immediately or were stored frozen at -80°C, until ready for processing for examination.

b). Intact Parasites for Surface Antigen Detection : The cercariae, 3h schistosomula, 5-day-lung worms or 21-day-liver worms were fixed in 1% PF and washed in 0.1 M phosphate buffer as previously described.

2.2.4 The Indirect Immunofluorescence Test of MABs on Frozen Sections of Cercaria, 3h schistosomulum and adult worm

2.2.4.1 The Fluorescein Isothiocyanate (RAM/Ig/FITC) System

The reaction was carried out as follows. The slides bearing cryostat sections were :

a). removed from the freezer and allowed to thaw at room temperature (RT) for 30 minutes.

b). fixed in acetone for 10 min at RT and washed 3 times with 0.01 M phosphate buffered saline (PBS), pH 7.4, 5 min each wash (this step was not applied to PF-fixed specimens).

c). covered with 10% normal rabbit serum in PBS (10% NRS/PBS) for 30-60 min at RT. This step was to block the non-specific binding (reactive sites) on the section. Because the non-specific adsorption reactions were likely to be of low affinity the serum was not washed off, merely drained.

d). covered with the appropriate dilution of the primary antibody (MAB 1:60; AMS 1:200; NMS 1:200; NS-1 1:60) and incubated for 1 h at RT.

e). washed 3 times in PBS, 10 min each wash.

f). covered with the appropriate dilution of the secondary antibody (RAM/Ig/FITC) and incubated for 1 h at RT.

g). washed 3 times as above.

h). mounted in buffered glycerol, pH 8.6 (see Appendix) or Citifluor mounting medium (Citifluor Ltd.) and a coverslip.

i). sealed with nail varnish (Nail Shine 17).

j). examined under a Nikon fluorescence microscope with a blue filter (495 nm). Photomicrographs were taken using Kodak Ektachrome (ASA 400) colour slide film and Plus X black & white film to record the results.

Positive and negative controls were carried out simultaneously, by replacing the MAB with AMS, NMS, NS-1 and PBS during the initial incubation period. AMS was used as a positive control; NMS, NS-1 and PBS were used as negative controls. Scoring was based on lack of specific fluorescence (negative) or specific diffuse fluorescence of any intensity (positive).

2.2.4.2 The Biotin-Streptavidin System

The protocol for immunostaining was similar to the one for RAM/Ig/FITC system as described previously. The different steps were :

i). Biotinylated second antibody (anti-mouse Ig, whole antibody from sheep; purchased from Amersham) was used instead of RAM/Ig/FITC.

ii). Fluorescein-streptavidin (from Amersham) at an appropriate dilution was added to label the antigen-antibody reaction in the tissue section.

iii). 10% sheep serum (Sera-Lab) in PBS was used for diluting all antisera and for blocking non-specific binding.

2.2.5 Detection of Surface Antigens of Intact Parasites

The fluorescein isothiocyanate (RAM/Ig/FITC) system was used. The reaction was carried out as follows.

The parasites were :

a). resuspended in 10% NRS/PBS or 10% NRS/MEM for fixed or live parasites respectively (live, unfixed schistosomula were also tested for comparison). These solutions were also used as diluents and washing media.

b). distributed in 100 ul aliquots containing 20-30 organisms into Eppendorf tubes, incubated for 1 h at RT.

c). centrifuged in a microfuge, speed 10,000 g (approximate) to remove supernatant (cercaria and 3h schistosomulum only).

d). incubated with 100 ul of MAB at a dilution of 1:60 for a further 1 h at RT. Dilutions 1:200 of AMS and NMS were used

as positive and negative controls respectively. NS-1 ascites at a dilution of 1:60 was also used as a negative control.

e). washed 3 times in washing medium.

f). incubated a further 1 h at RT in 100 ul of RAM/Ig/FITC at a dilution of 1:60.

g). washed 3 times as above, transferred to a microscope slide, and covered with a cover slip.

h). The parasites were examined under the Nikon fluorescence microscope. At least 10 parasites on each slide were examined and fluorescence was recorded on an arbitary scale from negative, in which there was no detectable surface fluorescence, to strongly positive (4+).

i). Fluorescence photomicrographs were also taken to record the results.

2.2.6 Titration of Antisera

A high dilution of the antiserum results in reduction of non-specific binding of antibody to a section. Therefore, the highest possible dilution of antibody which still gives a good specific antibody binding is required in immunocytochemistry work. In order to find the optimum dilutions of antisera to be used, they were titrated with the frozen sections of adult worm and intact 3h skin transformed schistosomula.

2.2.6.1 Titration of the RAM/Ig/FITC System

It was necessary to titrate 2 antisera i.e. the primary antibody and the secondary antibody.

i). Titration of acute mouse serum (the first Ab) by keeping the second Ab, RAM/Ig/FITC dilution 1:25 constant. Two fold dilutions (1:8, 1:16, 1:32, ...1:4096) of AMS were reacted with frozen sections of adult worm and skin transformed schistosomula (fixed intact worms). From this titration, the optimum dilutions of AMS were obtained.

ii). Titration of RAM/Ig/FITC by using a constant dilution of the AMS at 1:50 and 1:128 for frozen sections of adult worm and skin transformed schistosomula, respectively. The optimum dilutions of RAM/Ig/FITC for both antigenic targets were obtained.

2.2.6.2 Titration of the Biotin-Fluorescein-Streptavidin System

There were 3 components to be titrated i.e. the primary antibody, the secondary antibody (biotinylated anti-mouse Ig), and the detection system (fluorescein labelled streptavidin).

i). Titration of AMS (the first antibody) by keeping the second antibody (biotinylated anti-mouse Ig) and the fluorescein labelled streptavidin dilution 1:100 constant, the optimum dilution of AMS was obtained.

ii). Titration of biotinylated anti-mouse Ig by keeping the AMS dilution 1:60 and the fluorescein-streptavidin dilution 1:100 constant, the optimum dilution of biotinylated antibody was obtained.

iii). Titration of fluorescein-streptavidin by keeping the AMS dilution 1:60 and the biotinylated antibody 1:150 constant, the optimum dilution of fluorescein-streptavidin was obtained.

The amount of fluorescence exhibited by skin schistosomula and sections of adults was measured with a Vickers fluorimeter (courtesy of Dr. H. Leese) to permit a titration curve to be plotted. Background readings obtained by measuring a field with the same area adjacent to the parasites were substracted from each measurement. A single reading was taken on each of 20 individual larvae or 10 different areas on sections of adult worm. The mean value was calculated (mean ± S.E.).

2.2.7 Maximum Antigenicity of Skin Transformed Schistosomula

This assay was carried out in order to find out at which incubation time the skin-transformed schistosomula showed the highest amount of target antigen when reacted with the antibody.

The live skin transformed schistosomula were incubated in MEM containing

- 10% (v/v) heat-inactivated calf serum (Gibco Ltd.)
- 20 mM HEPES buffer (2ml/100ml MEM, HEPES buffer 1M, pH 7.3;

Gibco Ltd.).
- 100 units/ml penicillin
- 100 µg/ml streptomycin

They were incubated at 37°C water bath for various time periods i.e. 1, 3, 6, 9, 24 h. Samples from each incubation period were reacted with the AMS at a dilution of 1:128, and the RAM/Ig/FITC dilution 1:25. Normal mouse serum dilution 1:128 was used as a control. The amount of fluorescence was measured using the Vickers fluorimeter.

2.2.8 Detection of Host Antigens and Parasite Antigens on the Surface of Intact Parasites

2.2.8.1 Reaction of 3h Schistosomulum With Rabbit Anti-Mouse Erythrocyte Ghost Serum and AMS

Sera from a rabbit vaccinated with mouse erythrocyte ghosts and from acutely infected mice were used to detect host antigen and parasite antigen, respectively. Skin transformed schistosomula (3h) were prepared and immunofluorescence-stained as previously described. The antisera used to block the non-specific binding or used as diluents were different depending on the species in which the second antibodies were raised. 10% Normal goat serum in PBS (10% NGS/PBS) and 10% normal rabbit serum in PBS (10% NRS/PBS) were used with goat anti-rabbit Ig/FITC (GAR/Ig/FITC) and RAM/Ig/FITC, respectively. Sera from normal rabbit (NRS) and normal mouse (NMS) respectively, were used as negative controls.

2.2.8.2 Reaction of Lung Schistosomula With Rabbit Anti-Mouse Erythrocyte Ghost Serum and Vaccinated Rabbit Serum (VRS)

Rabbit anti-mouse erythrocyte ghost serum and serum from a rabbit vaccinated with irradiated cercariae (VRS) were used to detect host antigen and parasite antigen respectively. Lung worms (5-day-old) were obtained and immunostained as previously described. Sera from normal rabbit, normal mouse, normal goat and acutely infected mouse were also included in the test. The appropriate secondary antibodies were used with the appropriate primary antibodies i.e. GAR/Ig/FITC was used for rabbit serum and RAM/Ig/FITC for mouse serum.

2.2.8.3 Reaction of Liver Schistosomula With Rabbit Anti-Mouse Erythrocyte Ghost Serum and AMS

Liver schistosomula (21-day-old) were obtained from infected mice as previously described. They were resuspended in 10% NGS/PBS or 10% NRS/PBS for the detection of host antigen or parasite antigen respectively. Rabbit anti-mouse erythrocyte ghost serum or AMS was used as a primary antibody in the IIF technique. GAR/Ig/FITC and RAM/Ig/FITC were used as second antibodies against rabbit anti-mouse erythrocyte ghost serum and AMS, respectively. Normal sera from rabbit, mouse and goat were also tested as negative controls.

Cultured liver worms were also investigated. The liver worms (21-day-old) were incubated *in vitro* for 24 hours in order to examine whether host antigens were lost during the culture period. The parasites were cultured for 24 h in a 95% air/5% CO_2 incubator at 37°C in MEM containing

- 10% (v/v) heat-inactivated calf serum (Gibco)
- 20 mM HEPES buffer (Gibco)
- 100 units/ml penicillin
- 100 µg/ml streptomycin (1 ml/MEM 50 ml; Sigma; see Appendix)

The worms were then fixed in 1% PF in 0.1 M phosphate buffer and immunostained as described previously.

2.2.9 Cross Reactivity with Schistosoma japonicum

The 16 MABs which reacted with *S. mansoni* adult worm tegument were also tested with *S. japonicum* adult worm sections in order to examine any cross-reactivity. Adult worms of *S. japonicum* were fixed in 1% PF in 0.1 M phosphate buffer, frozen sections were taken and stained for immunofluorescence as previously described (infected *Oncomelania hupensis hupensis* snails were supplied by Dr. N.A. Moloney). Sera from *S. mansoni* infected mouse and normal mouse were used as positive and negative controls respectively. NS-1 ascites was also used as a negative control.

2.3 RESULTS

2.3.1 Titration of Antisera in the RAM/Ig/FITC System

The optimum dilution of each antiserum was the highest dilution that gave maximum fluorescence and lowest background staining. The optimum dilution of AMS was 1:200 for both frozen sections of adult worm, and intact skin-transformed schistosomula. The results are shown in Text Figure 2.4 and 2.5 respectively. The optimum dilution of RAM/Ig/FITC was 1:64 for both sections of adult worms and intact skin-transformed schistosomula as shown in Text Figure 2.6 and 2.7 respectively.

2.3.2 Titration of Antisera in the Biotin-Streptavidin System

For frozen sections of adult worm, the optimum dilution of AMS was 1:200, biotinylated anti-mouse Ig was 1:150 and fluorescein-streptavidin was 1:150 (titration curves not shown). During the course of my experiments I found the biotin-streptavidin system showed high background staining or non-specific binding of avidin conjugates. Despite a very high affinity of avidin for biotin, problems of non-specific binding have been attributed to the stickiness of the protein caused by its carbohydrate moiety (Hoffman, Wood, Brinton, Montibeller & Finn, 1980). Therefore, the biotin-streptavidin system was not used regularly in this study. As far as could be judged the fluorescence staining patterns obtained from the RAM/Ig/FITC and the biotin-streptavidin systems were similar.

2.3.3 Maximum Antigenicity of Skin Transformed Schistosomula

The amount of fluorescence measured at each time interval is plotted in the graph (Text Figure 2.8).

- 1h schistosomula were very variable. The range was from very bright fluorescence (4+) to negative (no fluorescence binding). Only the positive schistosomula were measured for fluorescence binding so the value in Text Figure 2.8 is biased at this time.

- 3h schistosomula showed highly positive fluorescence (4+) and more consistency of binding.

- 6h schistosomula showed slightly lower fluorescence intensity

(3+) when compared to the 3h schistosomula. The pattern of binding was somewhat inconsistent.

- 9h schistosomula showed a range of fluorescence intensities, from 2+ to 4+.
- 24h schistosomula showed very patchy binding of fluorescence. The intensity varied from 1+ to 3+.

Conclusion : 3h skin-transformed schistosomula were considered to be the best in terms both of antigenicity and consistent fluorescence binding on their surfaces. Therefore, they were used in all subsequent investigations.

Text Figure 2.4

Graph showing the two-fold dilutions of acute mouse serum (1:8 to 1:4096) with frozen sections of adult worm. The dilution of RAM/Ig/FITC was 1:25. Results are presented as mean fluorescence in relative units (RFU)±S.E. The dashed-line represents the mean value for normal mouse serum at a dilution of 1:40 (mean±S.E. = 3.4±0.20). The optimum dilution of acute mouse serum was 1:200.

Text Figure 2.5

Graph illustrating the titration of acute mouse serum with skin transformed schistosomula. The dilution of RAM/Ig/FITC was 1:25. Results are presented as mean fluorescence in relative units (RFU)±S.E. The dashed-line represents the mean value for normal mouse serum at a dilution of 1:2 (mean±S.E. = -0.73±0.15). The optimum dilution of acute mouse serum was 1:200.

Text Figure 2.6

Graph illustrating the titration of RAM/Ig/FITC with frozen sections of adult worm. The dilution of acute mouse serum was 1:200. Results are presented as mean fluorescence in relative units (RFU)±S.E. The dashed-line represents the mean value for normal mouse serum at a dilution of 1:50, RAM/Ig/FITC dilution 1:25 (mean±S.E. = 4.0±0.21). The optimum dilution of RAM/Ig/FITC was 1:64.

Text Figure 2.7

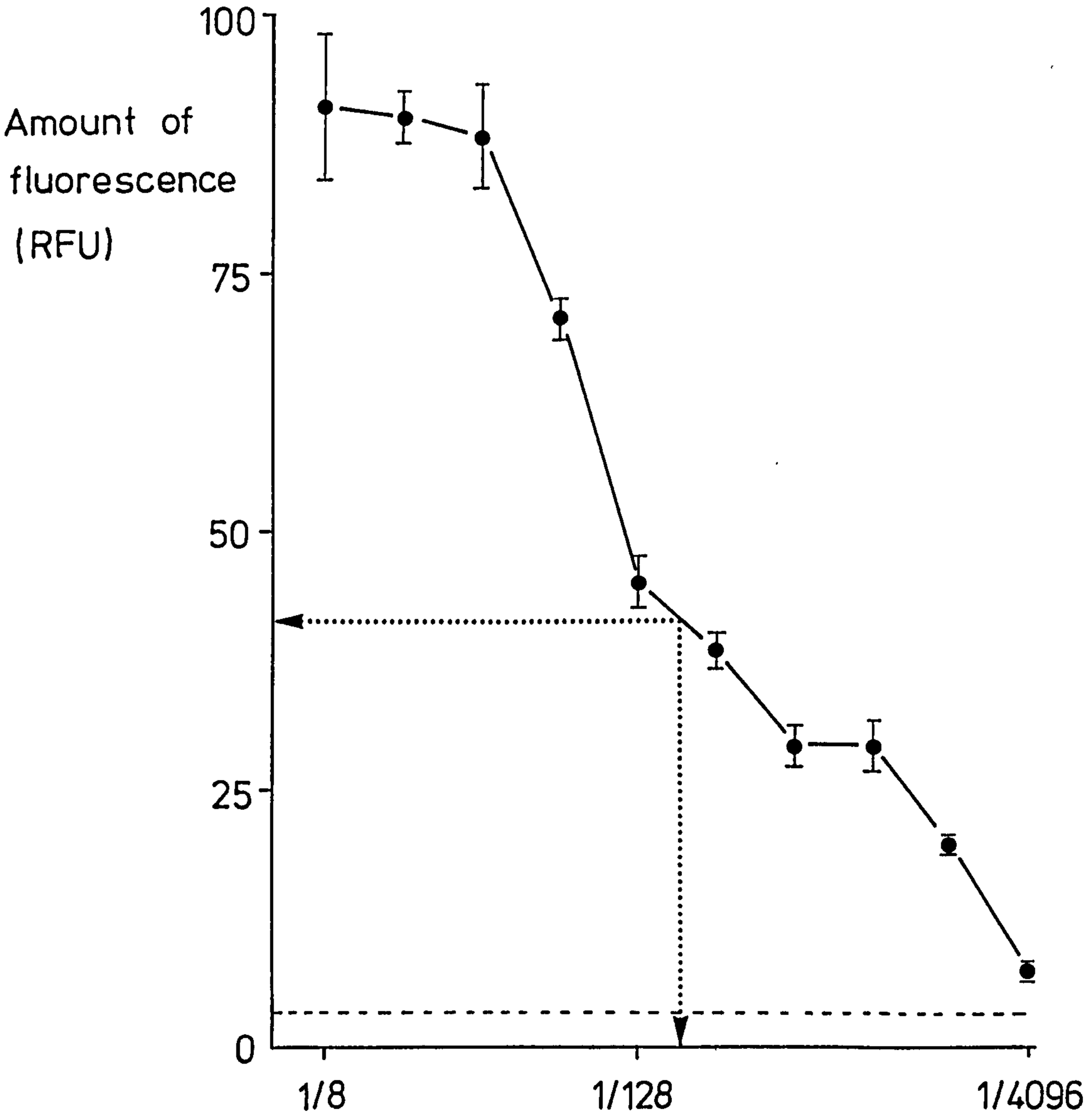
Graph showing the titration of RAM/Ig/FITC with skin transformed schistosomula. The dilution of acute mouse serum was 1:200. Results are presented as mean fluorescence in relative units (RFU)±S.E. The

dashed-line represents the mean value for normal mouse serum at a dilution of 1:2, RAM/Ig/FITC dilution 1:8 (mean±S.E. = 0.445±0.28). The optimum dilution of RAM/Ig/FITC was 1:64.

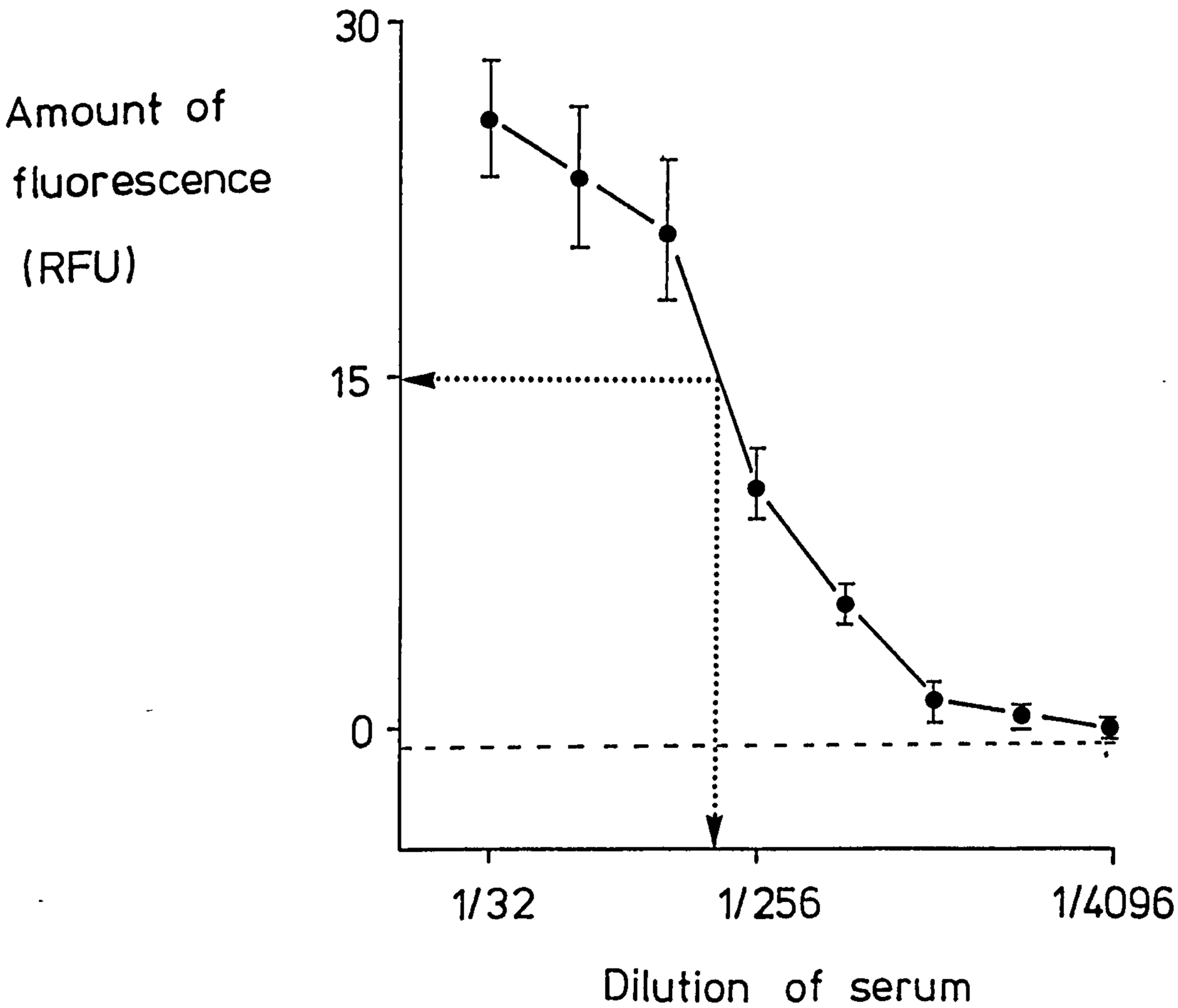
Text Figure 2.8

Graph showing the maximum antigenicity of skin transformed schistosomula. The relative fluorescence units of the skin transformed schistosomula decrease with incubation time. 3h skin transformed schistosomula were considered to be the best in terms both of positive fluorescence and yield of parasites.

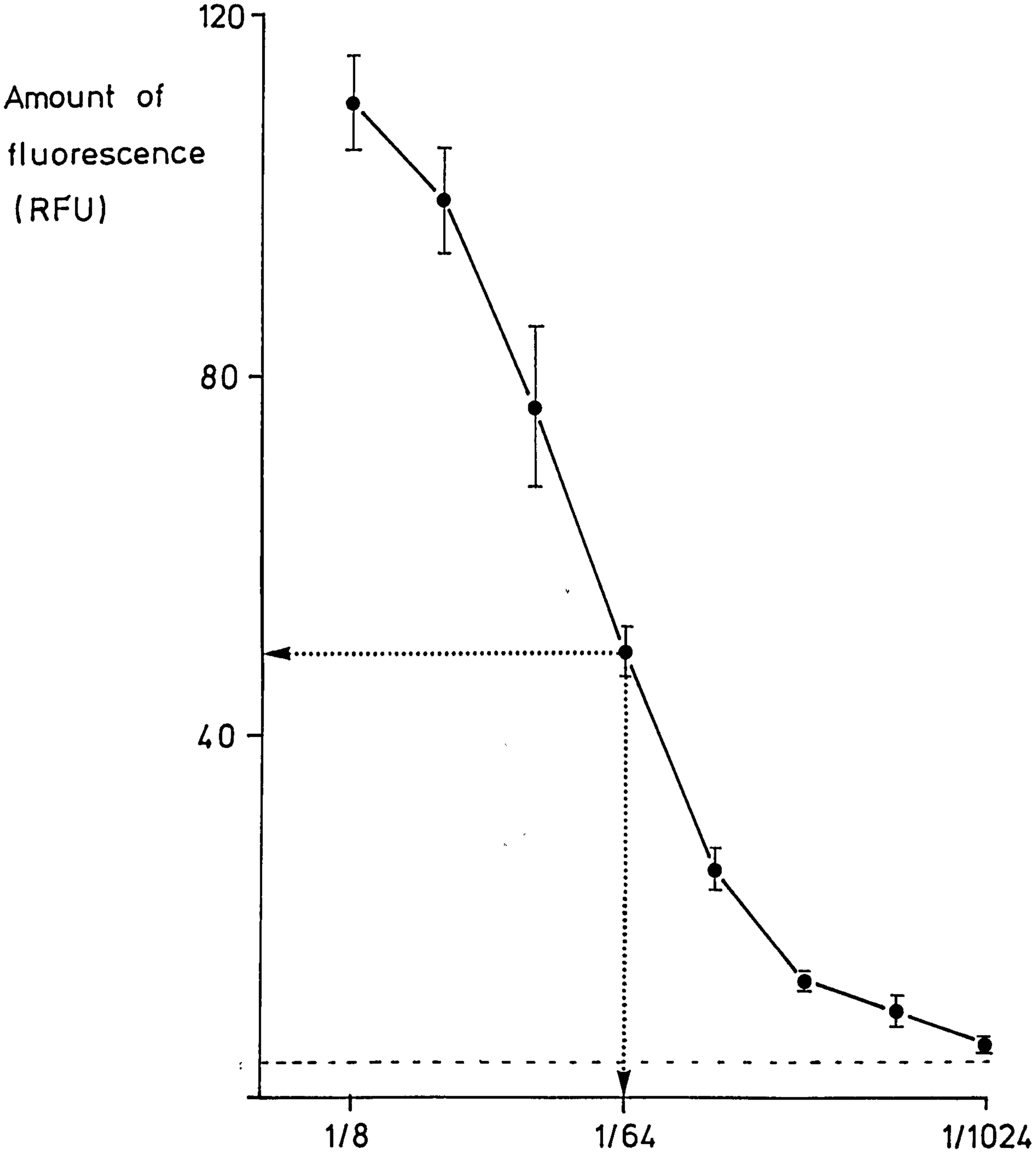
Text Figure 2.4 TITRATION OF ACUTE MOUSE SERUM WITH FROZEN SECTIONS OF ADULT WORM



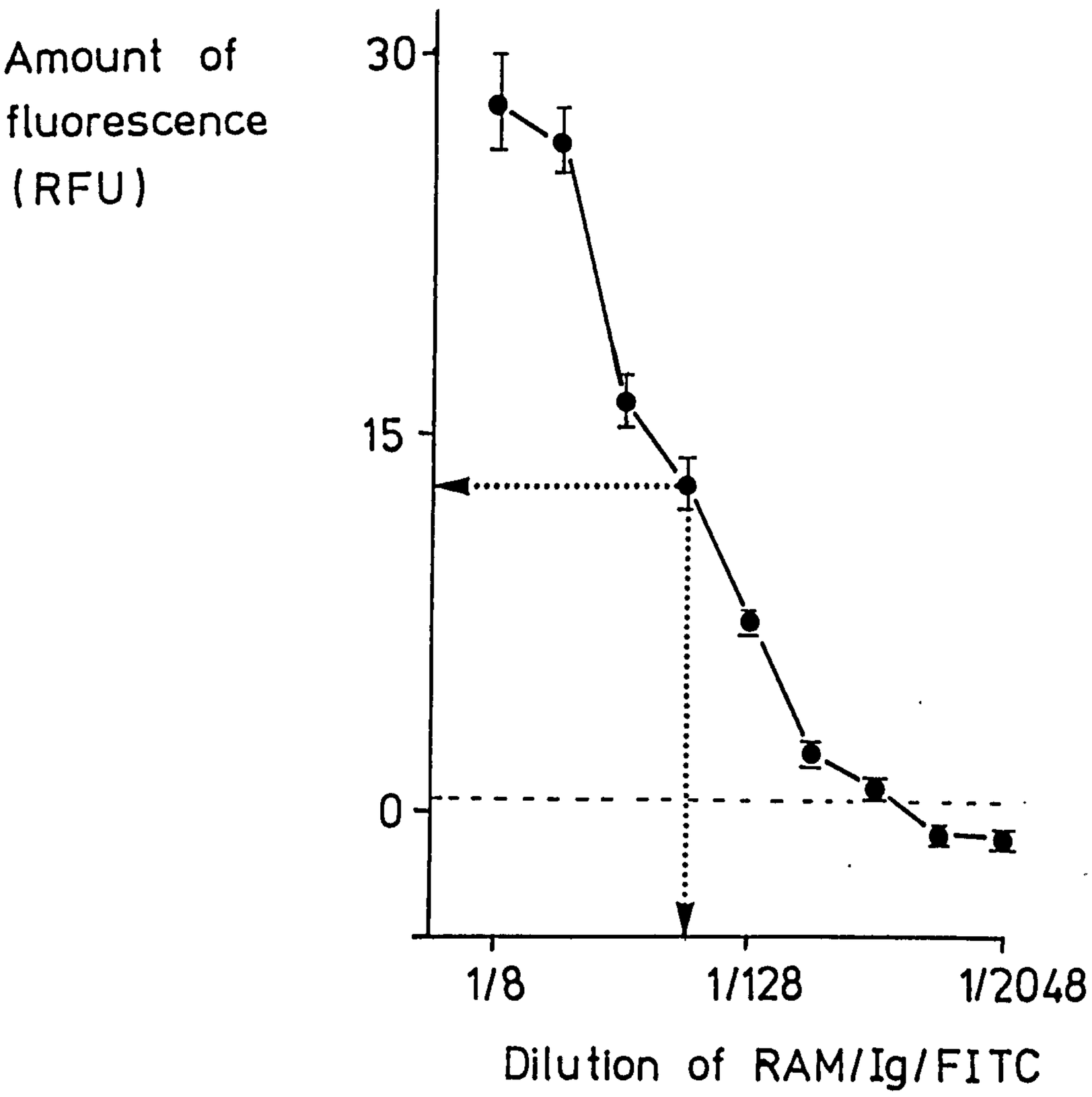
Text Figure 2.5 TITRATION OF ACUTE MOUSE SERUM WITH SKIN TRANSFORMED SCHISTOSOMULA



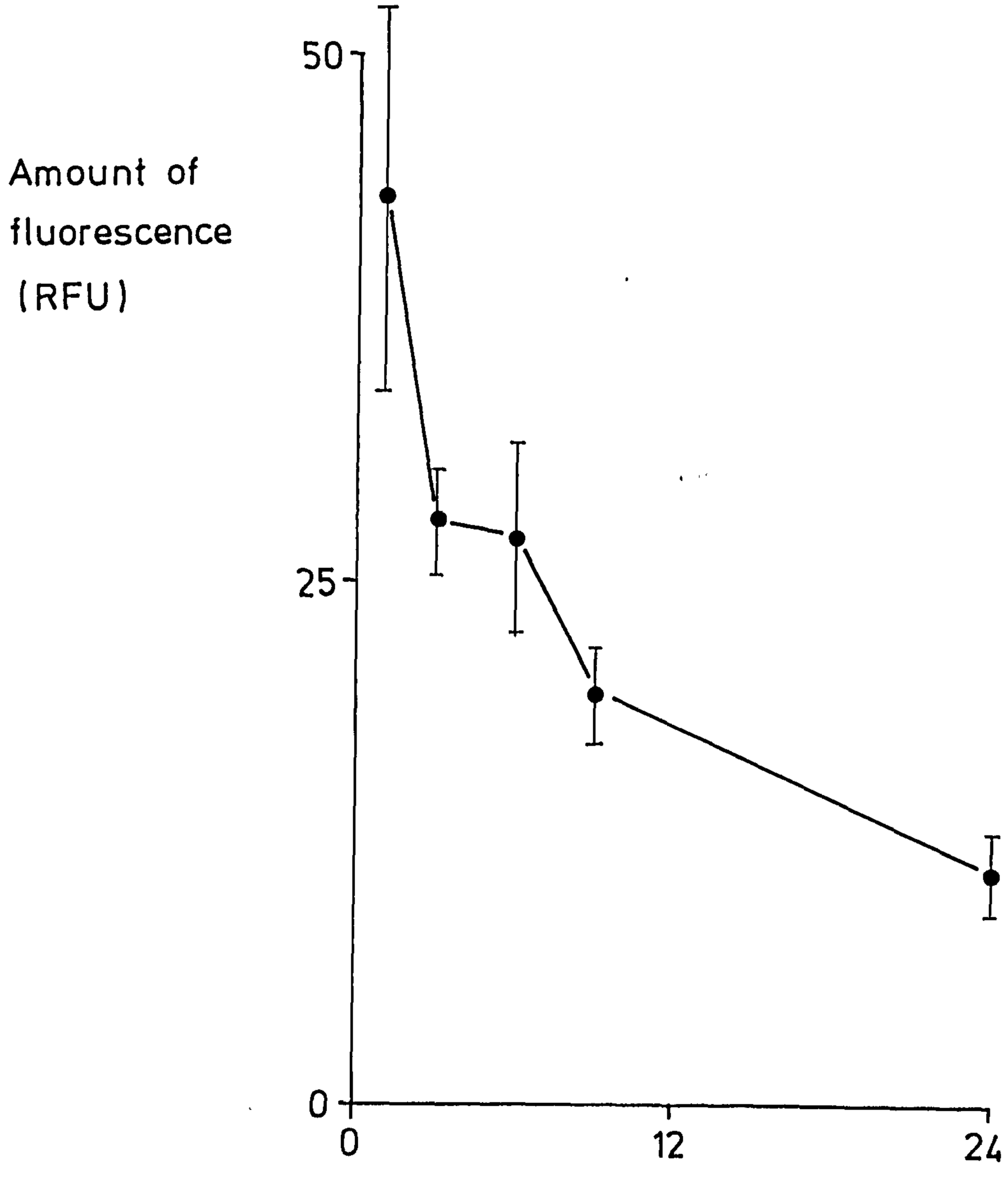
Text Figure 2.6 TITRATION OF RAM/Ig/FITC WITH FROZEN SECTIONS OF ADULT WORM



Text Figure 2.7 TITRATION OF RAM/Ig/FITC WITH SKIN TRANSFORMED SCHISTOSOMULA



Text Figure 2:8 MAXIMUM ANTIGENICITY OF SKIN TRANSFORMED SCHISTOSOMULA



2.3.4 Localisation of Schistosome Antigens with Antisera

The indirect immunofluorescence results on frozen sections and intact parasites, reacted with various antisera, are described in this Chapter. The fluorescence observed under the microscope was recorded on an arbitrary scale from negative (-), in which there was no detectable fluorescence, to strong positive (4+), in which there was a very bright fluorescence.

2.3.4.1 Control Sera

I). Positive Control (Acute Mouse Serum)

a). Frozen Sections of Parasites

The adult worm (Fig. 2.1), cercaria (Fig. 2.2) and 3h schistosomulum (Fig. 2.3) all showed a high intensity of fluorescence (4+), a bright, localised, yellow-green positive staining, on whole sections. The tegument was strongly labelled and was particularly bright towards the apical surface. The spines on the tubercles were also positive. Acetone and paraformaldehyde-fixed sections of cercariae and 3h schistosomula gave similar results. In adult sections, acetone fixation resulted in brighter fluorescence than paraformaldehyde (PF) fixation.

b). Surface Expression on Intact Parasites Fixed in 1% PF

cercaria : Fluorescent antibodies bound to the entire surface of cercariae. The staining pattern of some parasites exhibited a patchy appearance. The cercarial tail was also labelled (Fig. 2.4).

3h schistosomulum : Fluorescence (4+) was seen diffusely over the surface of schistosomula. The fluorescence labelling was of uniform appearance (Fig. 2.5).

5-day-lung worm : No detectable fluorescence was observed on the surface of lung schistosomula (Fig. 2.6).

21-day-liver worm : Positive fluorescence (2+) of patchy appearance was seen over the surface of 21-day-liver worms (Fig. 2.7).

II). Negative Controls

a). Normal Mouse Serum (NMS)

No detectable fluorescence was observed on any of the parasite preparations tested (Fig. 2.8).

b). NS-1 Myeloma Cell Ascites Fluid (NS-1)

Similar results to those obtained with NMS were observed. No specific fluorescence was noted.

c). Absence of Primary Antibody

Negative results were obtained on all parasite preparations reacting with FITC conjugated secondary antibody only.

2.3.4.2 MABs Raised Against Adult Tegument Membrane

A total of 50 MABs were screened against various stages in the life cycle of S. mansoni. The parasite preparations were frozen sections of adult worms, cercariae, 3h schistosomula, and intact parasites i.e. cercariae, 3h schistosomula, 5-day-lung worms and 21-day-liver worms. The reactivities of all 50 MABs were then divided as follows :

- 16 MABs reacting with the adult worm tegument. These 16 MABs will be described in the subsequent sections (Section 2.3.5).

- 11 MABs reacting with the muscle of the parasite (see Section 3.2.1 in Chapter 3).

- 2 MABs reacting with larval stages of the parasite only (Section 3.2.3 in Chapter 3).

- 4 MABs reacting with larval and adult stages, but not adult tegument (Section 3.2.3 in Chapter 3).

- 17 MABs which did not react with any stage of the parasite tested.

2.3.5 Monoclonal Antibodies Against the Schistosome Adult Tegument

Sixteen MABs were selected on the basis of staining the adult worm tegument. They were given a simplified code for ease of description. MABs were obtained from mice vaccinated with gradient pellet (G), microvesicular fraction (M), and digitonin pellet (D). The name of each MAB begins with the letter G, M or D followed by the fusion and clone numbers, separated by a point.

The 16 MABs were :-

G3.12	G6.2	M7.3	D7.1
G5.3	G6.4	M7.4	D7.2
G5.10	G6.6	M7.5	D7.3
G6.1	G6.7	M7.6	D7.4

2.3.5.1 Distribution of the Target Epitopes of the 16 MABs Within the Adult Worm

After acetone fixation, 5 different patterns of tegument staining were observed on frozen sections of adult worm (Table 2.2). PF-fixed sections gave different results with some of the MABs (see later).

I). The MABs giving specific fluorescence positive only in the tegument of the parasites were MABs M7.5, G6.2, D7.4, M7.3 & G5.3 (Figs. 2.9 & 2.10).

II). The MABs giving positive reactions with the tegument and the tegument cell body were MABs G3.12 & G5.10 (Figs. 2.11 & 2.12).

III). The MABs showing positive fluorescence in the tegument, interstitial material around the muscle cells and tegument cell bodies were MABs G6.1, D7.1, D7.2 & D7.3 (Figs. 2.13 & 2.14).

IV). The MABs giving positive fluorescence in the tegument, interstitial material around the muscle cells and all internal membranes of the adult worms were MABs G6.6, G6.7 & M7.4 (Figs. 2.15 & 2.16).

Table 2.2 Distribution of Epitopes Within the Adult Worm.

-Tegument	- Tegument - Cell body	- Tegument - Cell body - Interstitial material around muscle fibres	- Tegument - Internal membranes - Interstitial material around muscle fibres	- Tegument - Cell body - Interstitial material around muscle fibres - Nephridia
M7.5	G3.12	G6.1	G6.6	G6.4
G6.2	G5.10	D7.1	G6.7	M7.6
D7.4		D7.2	M7.4	
M7.3		D7.3		
G5.3				

V). The MABs reacting with the tegument, interstitial material around the muscle cells, tegument cell bodies and nephridia were MABs G6.4 & M7.6 (Figs 2.17 & 2.18).

2.3.5.2 Distribution of the Target Epitopes Between Stages in the Life Cycle of the Parasite

No specific fluorescence was observed on the surface of intact 5-day-lung worms and 21-day-liver worms with any of the 50 MABs tested. According to the fluorescence staining patterns of various stages in the life cycle of the parasites, I divided these 16 MABs which reacted with sections of adult worm tegument into 11 different groups (Table 2.3). The staining pattern of each group will be described in turn below. The results are summarised in Table 2.5.

Group 1 MABs G6.7 & M7.4

Adult worm sections : Frozen sections of adult worm fixed in acetone and reacted with undiluted culture supernatant of MAB G6.7 showed positive fluorescence (3+) over the tegument and the muscle plasma membranes of muscle fibres of the body wall. In the muscle layers (circular & longitudinal) beneath the tegument, muscle fibres are bounded by plasma membrane and surrounded by interstitial material. At the light microscope level it was sometimes very difficult to differentiate which of these structures were fluorescently labelled. In cases of uncertainty the term "interstitial material " will be used to describe the staining pattern in the following results.

When ascites was used at a dilution of 1:60 (most MABs ascites were used at a dilution of 1:60 unless otherwise stated) on the PF fixed sections, the tegument was strongly positive (4+). The plasma membranes of internal cells were also positive but showed less fluorescent label (3+); the muscle cytoplasm was unstained. The muscle layers showed a honey comb appearance (Figs. 2.19 & 2.20)

M7.4 ascites was used at dilutions of 1:10 and 1:60 on both acetone and PF fixed sections. A homogeneous fluorescence was observed over the entire section of adult worm fixed in acetone. On PF-fixed sections the results were similar to those obtained with MAB G6.7. An intense fluorescence was visible over the tegument and to a lesser extent in the internal tissues. The fluorescent labelling of the plasma membranes of parenchymal cells was stronger than their cytoplasm (Figs. 2.21 & 2.22).

Table 2.3 Specificity of MABs and Control Sera By Immunofluorescence.

Group	MAB	Intact cercaria	Intact 3h sch'lum	Cercarial section	3h sch'lum section	Adult section
1.	G6.7 M7.4	+	+	+	+	+
2.	G6.1 D7.2	-	+	+	+	+
3.	G6.2 D7.4 M7.3	-	-	+	+	+
4.	G6.6	-	+	+	+	+
5.	D7.1 D7.3	-	-	+	+	+
6.	G3.12	-	-	-	-	+
7.	G5.3	-	-	+	+	+
8.	M7.5	-	-	+	+	+
9.	G5.10	-	-	-	+	+
10.	G6.4	-	-	+	+	+
11.	M7.6	-	-	+	+	+
Controls :						
	AMS	+	+	+	+	+
	NMS	-	-	-	-	-
	NS-1	-	-	-	-	-

Cercarial sections : Specific fluorescence was observed in the tegument and the periphery of the body beneath it, on sections reacted with MABs G6.7 & M7.4. The peripheral staining pattern appeared as packets of fluorescence. The head glands were also positive (Fig. 2.23). Sections of tail fixed in acetone were unlabelled. Similar results were obtained on sections of cercarial body fixed in acetone and PF. The fluorescence obtained on PF-fixed sections was brighter than that on acetone-fixed ones (Fig. 2.24). The muscle membranes of sections of tail were fluorescently labelled with MAB G6.7, but not with M7.4 (Figs. 2.25 & 2.26).

3h Schistosomular sections : On longitudinal sections the tegument and the peripheral areas beneath the tegument of the parasites were strongly labelled. The oesophagus was also positive (Fig. 2.27). Both MABs gave similar results on acetone and PF-fixed sections. The fluorescent label of the head capsule with MAB G6.7 was stronger than that with M7.4 on acetone-fixed sections.

Intact cercariae : Fluorescent antibodies bound to the entire surface of the cercaria. The binding was greater on the head capsule and the area where the tail detached than the cercarial body. The intensity of fluorescence with MAB M7.4 & G6.7 was not as great as that observed with AMS because the monoclonals presumably bind to only one out of several antigens recognized by antibodies present in immune serum. The cercarial tails were fluorescence-negative. MAB G6.7 gave a stronger reaction (3+) than MAB M7.4 (2+) (Fig. 2.28).

Intact 3h schistosomula : The antibodies bound uniformly to the surface of the schistosomula and gave a diffuse fluorescent staining pattern (4+) (Fig. 2.29). Both MABs G6.7 & M7.4 gave stronger surface binding on schistosomula than on cercariae. The intensity of fluorescence with MAB M7.4 was greater than that with MAB G6.7.

Intact 21-day-liver worms : MABs G6.7 & M7.4 did not bind to the surface of freshly perfused liver worms. After 24 h culture in medium most liver worms' surfaces were rough, irregular and contained numerous pores. Uniform tegument was also observed on the same parasite. The positive fluorescence around rough areas was observed with most MABs tested. MAB G6.7 did not bind to the surface of cultured liver worms (Table 2.4). The binding of MAB M7.4 to the worms' surface was very weak and patchy (Fig. 2.30). The smooth area on the parasite surface was negative (similar to that shown in Fig. 2.7). At high magnification, false positive staining of the muscle

Table 2.4 Specificity of MAB and Control Sera By Immunofluorescence.

Group	MAB	Cultured (24 h) 21-day-liver worms (fixed in 1% PF)
1.	G6.7	-
	M7.4	2+
2.	G6.1	1+
	D7.2	2+
3.	G6.2	-
	D7.4	-
	M7.3	1+
4.	G6.6	wk+, -
5.	D7.1	1+
	D7.3	2+
6.	G3.12	1+
7.	G5.3	-
8.	M7.5	-
9.	G5.10	wk+
10.	G6.4	3+
11.	M7.6	2+
Controls :	AMS	2+
	NMS	-
	NS-1	-
	NRS	-

N.B. Primary antibody 1:40, secondary antibody 1:25.

layer was seen in the area in which the tegument was damaged (Fig. 2.31).

Group 2 MABs G6.1 & D7.2

Adult worm sections : Targets for antibodies in the adult worm were located in the worm tegument, the interstitial material around the muscle fibres and tegumental cell bodies beneath the muscle layers; the tegument was brightest (Fig. 2.13). At high magnification, a reticulate pattern was seen on the tegumental surface (Fig. 2.14). Both acetone and PF-fixed sections gave similar results.

Cercarial sections : On acetone and PF fixed sections of cercariae reacted with MABs G6.1 & D7.2, discrete packets of specific fluorescence were seen inside the body beneath the thin muscle layers (Fig. 2.32). Packets of fluorescence obtained with MABs G6.1 & D7.2 were more distinct than those observed with MABs G6.7 & M7.4 (Fig. 2.23). These packets probably correspond to the tegumental cell bodies of cercariae, in which tegumental inclusions are synthesized. These inclusions are later translocated into the tegument. Packets of fluorescence on PF-fixed sections were stronger than those on acetone-fixed sections (Fig. 2.33). The tegument of the cercarial body was unlabelled. Sections of tail were also negative.

3h Schistosomular sections : The surface syncytium of the tegument was strongly labelled. The oesophagus was also positive. Interestingly, the packets of fluorescence beneath the muscle layers were not observed (Fig. 2.34). Similar results were obtained using acetone and PF-fixed sections.

Intact cercariae : MABs G6.1 & D7.2 did not bind to the cercarial body and tail. Apical ends of some parasites were occasionally observed to be weakly fluorescent (1+) where gland ducts opened to the exterior. The posterior end of the body (at the points of attachment between the body and the tail) was also positive (1+) (Fig. 2.35). The strong fluorescence at this location was occasionally observed with most antisera.

Intact 3h schistosomula : Fluorescence was seen covering the entire parasite surface. The staining pattern was similar to that with MAB in Group 1 (Fig. 2.29). Some surface binding showed a patchy appearance.

Intact cultured 21-day-liver worms : The binding of MAB G6.1 to the worm surface was very weak (1+) and appeared as a dotted pattern over the rough areas and around the ventral sucker. MAB D7.2 bound to the parasite surface in a patchy appearance (2+). The staining pattern was similar to that with MAB M7.4 (Figs.2.30 & 2.31). The fluorescent label with MAB D7.2 was slightly stronger than that with G6.1.

Group 3 MABs G6.2, D7.4 & M7.3

Adult worm sections : The expression of these epitopes was confined to the tegument only which was brightly fluorescent (4+). Internal tissues were unlabelled. Acetone and PF-fixed sections gave similar results (Figs. 2.9 & 2.10).

Cercarial sections : Fluorescent label of MABs in Group 3 was mainly associated with the tegument and the periphery of the body. Some internal structures were also weakly labelled with MAB G6.2. Sections of cercarial tail were negative (Fig. 2.36). No differences in staining patterns on sections fixed in acetone and PF were observed.

3h Schistosomular sections : Positive fluorescence was observed in the tegument on schistosomular sections. MABs G6.2 & D7.4 gave weaker (2+) reactions than M7.3 (3+). Internal structures and the oesophagus of some sections of schistosomula were also weakly (1+) labelled with MAB G6.2. The staining pattern was similar to that shown in Fig. 2.37. Both fixatives gave similar results.

Intact cercariae : Negative results were obtained on the surfaces of the cercarial body and tail. MAB G6.2 gave slight positive fluorescence at the posterior end of the body where the tail detached, and also at the anterior end of the tail.

Intact 3h schistosomula : The antibodies in Group 3 did not bind to the schistosomular surface.

Intact cultured 21-day-liver worms : MABs G6.2 & D7.4 did not bind to the surface of 21-day-liver worms. The binding of MAB M7.3 to the parasite surface was very weak (1+) and patchy in appearance.

Group 4 MAB G6.6

Adult worm sections : The tegument, the parenchyma and the gut epithelium were all brightly labelled on PF-fixed sections. The membranes of the parenchymal tissues were brighter than the cytoplasm. The muscle cells were unstained (Figs. 2.38 - 2.40). On acetone fixed sections, the parasite showed an overall fluorescence which was brightest on the tegument (Figs 2.15 & 2.16).

Cercarial sections : Acetone and PF- fixed sections showed positive fluorescence on the entire section with the strongest reactivity at the periphery (4+) (Fig. 2.41). The staining patterns and the fluorescence intensity with MAB G6.6 were similar to those observed with AMS. In some of the longitudinal sections, the gland cells within the body were unlabelled.

3h Schistosomular sections : The entire section was brightly labelled on both acetone and PF-fixed sections. Some internal tissues exhibited strong fluorescence. The tegument of the parasites on PF-fixed transverse sections was fluorescently labelled and appeared as a very bright thin line. Internal structures were also strongly positive (Figs. 2.42 & 2.43).

Intact cercariae : No detectable fluorescence was observed on the surfaces of the cercarial body and tail. Fluorescent label was slight at the apical area and the posterior end of the body where the tail detached (similar staining pattern as shown in Fig. 2.34).

Intact 3h schistosomula : The parasite surface was diffusely fluorescent with the anterior of the body appearing brightest.

Intact cultured 21-day-liver worms : MAB G6.6 bound weakly to the parasite surface. Some liver worms were unlabelled.

Group 5 MABs D7.1 & D7.3

Adult worm sections : On acetone-fixed sections, the staining pattern of MABs D7.1 & D7.3 was similar to that observed in Group 2. Specific fluorescence was seen in the tegument, cell bodies and the "interstitial material " around the muscle fibres (Figs. 2.13 & 2.14); the tegument was brightest (4+). On PF-fixed sections, with both MABs, the reactivity was similar to that on acetone-fixed ones,

except the parenchymal tissues were also fluorescently labelled. Parenchymal fluorescence was stronger with D7.3 (3+) than with D7.1 (2+). The tegument cell bodies of adult worms, in PF fixed sections reacted with MAB D7.3, were not clearly defined because of the diffuse but strong reaction of the parenchyma (Fig. 2.44). Tubercles were also fluorescently labelled but the gut epithelium was negative.

Cercarial sections : MAB D7.3 gave positive fluorescence within the tegument (4+) and internal structures (3+) on the cercarial body. The tegument was very bright and appeared as a very thin line (Fig. 2.45). Positive fluorescence on the entire section, brightest at the edge, was also observed. In sections of tail, the tegument (3+) and internal tissues (2+) were also labelled. The binding of MAB D7.1 to sections of cercarial body and tail was very weak at the periphery giving a dotted appearance. Internal tissues were unlabelled. MABs D7.1 & D7.3 gave similar results on sections fixed in acetone and PF.

3h Schistosomular sections : The tegument fluorescence was very strong (4+) with MAB D7.3 and appeared as a thick band covering the entire section. Internal structures were all negative. Some parasites were fluorescently labelled (3+) on the entire section. MAB D7.1 gave a weak reaction in the tegument only; internal tissues were unstained. Similar results were obtained with both fixatives.

Intact cercariae : No detectable fluorescence was observed on the surfaces of the cercarial body and tail.

Intact 3h schistosomula : MABs D7.1 & D7.3 did not bind to the surface of 3h schistosomula.

Intact cultured 21-day-liver worms : MABs D7.1 & D7.3 bound to the parasite surface. The binding was very weak and patchy with both MABs. MAB D7.3 (2+) gave slightly stronger reaction than D7.1 (1+) (Table 2.4).

Group 6 MAB G3.12

Adult worm sections : Fluorescent label was seen in the tegument and tegumental cell bodies. The reactivity was stronger in the former than in the latter (Figs. 2.11 & 2.12). Similar results were obtained with both fixatives.

Cercarial sections & 3h schistosomular sections : Negative results were obtained on sections of cercariae and 3h schistosomula.

Intact cercariae & 3h schistosomula : MAB G3.12 did not bind to the surface of intact cercariae and 3h schistosomula.

Intact 21-day-liver worms : The binding of MAB G3.12 to the parasite surface was very weak (1+) and showed a patchy appearance.

Group 7 MAB G5.3

Adult worm sections : The antigenic determinants were located in the tegument only. The fluorescent label was very bright (4+). Internal tissues were unstained (Figs. 2.9 & 2.10). PF gave a stronger reaction than acetone fixation (2+). The gut was also positive.

Cercarial sections : Specific fluorescent label was seen in the tegument of the cercarial body and tail. The tegument was strongly labelled and appeared as a very thin line on sections of the body. The membranes of the head capsule and the oesophagus were also brightly fluorescent (Figs. 2.46 & 2.47). Internal structures were occasionally reactive in some sections. Both fixatives gave similar results but the reactions were stronger after treatment with PF.

3h Schistosomular sections : The staining pattern resembled closely that observed on cercarial sections. The tegument and the oesophagus were brightly labelled (Fig. 2.48). The membranes of the head capsule were also positive. Some sections showed fluorescence at the periphery only. Weak labelling of internal structures was also occasionally observed in some sections.

Intact cercariae & 3h schistosomula : No specific fluorescence was noted on the surface of cercariae and 3h schistosomula. The apical and posterior ends of some cercarial bodies were slightly positive (weak +). The staining pattern was similar to that shown in Fig. 2.35.

Intact cultured 21-day-liver worms : MAB G5.3 did not bind to the surface of 21-day-liver worms.

Group 8 MAB M7.5

Adult worm sections : The fluorescent staining pattern was similar to that observed in Group 7 (MAB G5.3) (Figs. 2.9 & 2.10). Only the tegument was fluorescently labelled on sections fixed in acetone and PF. All internal tissues were negative.

Cercarial sections : In contrast to adult worms, fluorescent staining was restricted to the muscle of the cercarial body and tail on PF-fixed sections (Fig. 2.49). The thin muscle layers of the body wall were fluorescently labelled. The muscle fibres which surround the gland ducts within the head capsule were also positive (see " musculature of cercaria " in Chapter 3). Fluorescent label was also observed in internal structures. Striations of longitudinal muscle on sections of cercarial tail were clearly defined (Fig. 2.50). The tegument was unlabelled. Negative results were obtained on acetone-fixed sections.

3h Schistosomular sections : The epitope recognized by MAB M7.5 was also present in the muscle in sections of 3h schistosomula. An individual muscle block of the body wall could be easily recognized in transverse sections (Fig. 2.51). The epitope was also destroyed after acetone fixation.

Intact cercariae & 3h schistosomula : No specific fluorescence was observed on the surface of cercariae and 3h schistosomula.

Intact cultured 21-day-liver worms : MAB M7.5 did not bind to the surface of 21-day-liver worms.

Group 9 MAB G5.10

Adult worm sections : The target epitope was located in the tegument and the tegument cell bodies on acetone-fixed sections (Figs. 2.11 & 2.12). These epitopes were destroyed after PF fixation; weakly positive fluorescence was observed over the whole section. Some sections were negative.

Cercarial sections : Internal structures were weakly labelled on sections of cercariae fixed in both fixatives. Fluorescence was slightly stronger on acetone (1+) than that on PF-fixed sections (weak+). The tegument was unlabelled. Some sections were negative.

3h Schistosomular sections : Only the tegument was labelled (2+) on acetone-fixed sections. Internal tissues were negative. Weak positive fluorescence on the entire section and negative reactions were obtained after PF fixation.

Intact cercariae & 3h schistosomula : MAB G5.10 did not bind to the surface of cercariae and 3h schistosomula. The apical end of some schistosomula was slightly labelled.

Intact cultured 21-day-liver worms : The binding of MAB G5.10 to the surface of 21-day-liver worms was very weak.

Group 10 MAB G6.4

Adult worm sections : Strong specific fluorescence was seen in the tegument (4+) and weaker reactions in the interstitial material around the muscle fibres (3+), tegument cell bodies (2+) and nephridia (3+) on both acetone and PF-fixed sections. Nephridia (N) were seen as thin rod-like structures in the parenchyma. The fluorescent staining patterns were similar to those obtained with MAB M7.6 (Figs. 2.17 & 2.18). Fluorescence was brighter on sections fixed in acetone than in PF. Nephridia were also easily recognized on acetone-fixed sections. The gut and parenchymal tissues were negative.

Cercarial sections : Specific fluorescence was seen in the tegument and the periphery on sections fixed in both fixatives. Internal tissues were negative. Similar staining patterns were obtained on sections of tail. Nephridia could not be observed. Both fixatives gave similar results.

3h Schistosomular sections : At low magnification, the staining patterns were similar to those obtained on sections of cercariae. Only the tegument was fluorescently labelled; internal tissues were negative. At high magnification the tegument was strongly labelled (4+) and appeared as a thin line on sections of 3h schistosomula. Nephridia were also fluorescent labelled (2+) and appeared as curly structures on some sections (Fig. 2.52). Other internal tissues were unlabelled. Positive fluorescence was also observed at the periphery of sections. Similar results were obtained on sections fixed in both fixatives.

Intact cercariae & 3h schistosomula : No fluorescent label could be detected on the surface of cercariae and 3h schistosomula,

except for slight positive staining at the apical area of schistosomula and the posterior end of cercariae.

Intact cultured 21-day-liver worms : MAB G6.4 bound to the surface of 21-day-liver worms in a patchy pattern. Fluorescent label was strong (3+) in the rough areas.

Group 11 MAB M7.6

Adult worm sections : The staining pattern on acetone-fixed sections was similar to that observed in MAB G6.4 (Group 10). The tegument was brightly fluorescent (4+). The interstitial material around muscle fibres, tegument cell bodies and nephridia were all labelled to the same degree (3+). The gut epithelium was also positive, but parenchymal tissues were negative (Figs. 2.17 & 2.18). The target epitope of this MAB was destroyed after PF fixation, a negative result being obtained.

Cercarial sections : On acetone-fixed sections the tegument and some internal structures were weakly labelled. Fluorescence was stronger on sections fixed in PF than in acetone. The tegument was brightly labelled whilst the internal structures were less antigenic in both the cercarial body and tail. Nephridia were also labelled on sections of the body and the tail, especially in the centre of the latter (Figs. 2.53 & 2.54). The gland cells within the body were also positive.

3h Schistosomular sections : The staining pattern closely resembled that observed in cercarial sections in this group and schistosomular sections in Group 10 (Fig. 2.52). The tegument and nephridia were brightly fluorescent. Similar results were obtained on sections with both fixatives.

Intact cercariae & 3h schistosomula : No detectable fluorescence was observed on the surfaces of cercariae and 3h schistosomula. Only weak fluorescence was seen at the anterior and posterior ends of cercarial bodies.

Intact cultured 21-day-liver worms : The binding of MAB M7.6 to the parasite surface was patchy in appearance. Fluorescent label was moderate (2+) in the rough areas.

Table 2.5 Distribution of Epitopes Between Stages in the Life Cycle of the Schistosome.

Group	MAB	Intact cercaria	Intact 3h schistosomulum	Cercarial section
1.	G6.7 M7.4	Body surface (2+) Tail surface (-)	Surface (4+)	Ac & PF - Tegument & periphery of the body section
2.	G6.1 D7.2	(-)	Surface (3+)	Ac & PF - Discrete packets inside the body
3.	G6.2 D7.4 M7.3	(-)	(-)	Ac & PF - Tegument & periphery of the body section - oesophagus
4.	G6.6	(-)	Surface (2+)	Ac & PF - Tegument was brighter than internal structures
5.	D7.1 D7.3	(-)	(-)	Ac & PF - Tegument was brighter than internal structures
6.	G3.12	(-)	(-)	Ac & PF Negative (-)

3h schistosomular section	Adult worm section
Ac & PF - Tegument & periphery of the section - oesophagus	Ac - Tegument - Interstitial material PF - Tegument - Interstitial material - Internal membranes
Ac & PF - Tegument & periphery of the section - Oesophagus	Ac & PF - Tegument - Interstitial material - Cell bodies
Ac & PF - Tegument	Ac & PF - Tegument - Interstitial material - Cell bodies
Ac & PF - Whole section	Ac - Positive allover, brightest fluorescence in the tegument PF - Tegument (4+) - Interstitial material - Internal membranes (3+)
Ac & PF - Whole section, brightest fluorescence in the tegument	Ac & PF - Tegument - Interstitial material - Cell bodies - Internal membranes (PF)
Ac & PF - Negative (-)	Ac & PF - Tegument - Cell bodies

N.B. Ac = Acetone-fixed. PF = Paraformaldehyde-fixed.
(-) = Negative.

Table 2.5 continue

Group	MAB	Intact cercaria	Intact 3h schistosomulum	Cercarial section
7.	G5.3	(-)	(-)	Ac & PF - Tegument - Oesophagus
8.	M7.5	(-)	(-)	Ac - Negative PF - Muscle
9.	G5.10	(-)	(-)	AC & PF - Negative
10.	G6.4	(-)	(-)	Ac & PF - Periphery or tegument
11.	M7.6	(-)	(-)	Ac & PF - Tegument - Nephridia
	AMS	Body & tail surface (4+)	Surface (4+)	Ac & PF - Whole section
	NMS	(-)	(-)	(-)
	NS-1	(-)	(-)	(-)

3h schistosomular section	Adult worm section
Ac & PF - Tegument - Oesophagus	Ac & PF - Tegument
Ac - Negative PF - Muscle	Ac & PF - Tegument
Ac - Tegument PF - Negative - Weak positive fluorescence the entire section	Ac - Tegument - Cell bodies PF - Negative - Weak stained allover
Ac & PF - Tegument - Nephridia	Ac & PF - Tegument - Interstitial material - Cell bodies - Nephridia (Ac)
Ac & PF - Tegument - Nephridia	Ac - Tegument - Interstitial material - Cell bodies - Nephridia PF (-)
Ac & PF - Whole section (4+)	Ac & PF - Whole section (4+)
(-)	(-)
(-)	(-)

2.3.6 Cross Reactivity with S. japonicum

The 16 MABs which reacted with the adult worm tegument of S. mansoni were also used to test the cross-reactivity with the adult worm sections of S. japonicum. Eleven of these 16 MABs did not react with PF-fixed sections of adult S. japonicum. They were MABs G3.12, G5.3, G6.1, G6.2, G6.7, M7.3, M7.4, M7.6, D7.1, D7.2 & D7.4. Only 5 MABs showed a positive fluorescence reaction i.e. MABs G5.10, G6.4, G6.6, M7.5 & D7.3. The specificities of these 5 MABs for sections of adult S. mansoni are compared with those for S. japonicum in Table 2.6.

MAB G5.10 : The entire section was fluorescently labelled (3+), some regions of tegument were very bright.

MAB D7.3 : The entire section was brightly labelled, except the tegument which was negative.

MAB G6.4 : The antibody bound to the entire section of adult worm (2+) with brightest fluorescence in the tegument (3+).

MAB G6.6 : The MAB bound to the parenchymal tissues of the parasite; some tegument was also positive (Fig. 2.55).

MAB M7.5 : The epitope recognized by MAB M7.5 was located in the muscle fibres of the worm. The staining pattern was very weak (2+) with a dotted and filamentous appearance.

"S. mansoni" infected mouse serum (AMS) : AMS from S. mansoni infected mice gave a strong reaction on the entire section of the S. japonicum adult worm. The staining pattern was similar to that on sections of adult S. mansoni (Fig. 2.1).

Normal mouse serum & NS-1 ascites : NMS and NS-1 ascites were used as negative controls. No detectable fluorescence was observed with both sera.

2.3.7 Host Antigens & Parasite Antigens

Intact 3h skin-transformed schistosomula, 5-day-lung worms and 21-day-liver worms were used for an examination of host antigens on the parasite surface. The presence of host antigens and/or parasite antigens on the schistosomes' surface is shown in Table 2.7.

Table 2.6 Cross-Reaction of MABs Against S. mansoni Adult Tegument With Adult S. japonicum

MAB	Fluorescent staining patterns	
	S. mansoni	S. japonicum
G5.10	Ac - Tegument, cell bodies PF - Weak positive allover	PF - Whole section (3+), some regions of tegument were very bright
D7.3	Ac - Tegument, cell bodies, interstitial material PF - Tegument, interstitial material, internal membranes	PF - Whole section (3+), tegument was negative
G6.4	Ac & PF - Tegument, cell bodies, interstitial material - Nephridia (Ac)	PF - Tegument (3+), Parenchyma (2+)
G6.6	Ac - Positive all over with brightest fluorescence in the tegument PF - Tegument - Interstitial material - Internal membranes	PF - Whole section (4+) - Some regions of tegument were negative
M7.5	Ac & PF - Tegument	PF - Muscle
AMS	Ac & PF - Whole section (4+)	PF - Whole section (4+)
NMS	Ac & PF (-)	PF (-)
NS-1	Ac & PF (-)	PF (-)

Ac = Acetone fixed. PF = Paraformaldehyde fixed.
(-) = Negative.

3h Schistosomula : Freshly transformed schistosomula recovered from isolated mouse skin (in vitro) bound AMS (anti-parasite antibody) (Fig. 2.5), but did not bind rabbit anti-mouse erythrocyte ghost antiserum (anti-host antibody) (Figure not shown). This indicated that parasite antigens, but not host antigens, were exposed on the surface of in vitro transformed 3h schistosomula.

5-day-lung worms : 5-day-old schistosomula recovered from the lungs of infected mice showed positive fluorescence with rabbit anti-mouse erythrocyte ghost serum (Fig. 2.56), but not with AMS (Fig. 2.6). This indicated that host antigens were present on the surface of parasites which were recovered in vivo.

Serum from a rabbit vaccinated with irradiated S. mansoni cercariae (VRS) also bound to the surface of lung worms. Specific fluorescence was seen over the entire surface of the parasites (Fig. 2.57). Fluorescent label was slightly stronger with anti-host antibody (4+) than with anti-parasite antibody (3+). No detectable fluorescence was observed using AMS, NMS and NS-1 ascites.

21-day-liver worms : Anti-host antibody bound to the surface of 21-day-liver worms freshly recovered from mice. The surface of the worms appeared uniformly bright (3+) (Fig. 2.58). A patchy appearance was observed on the surface of some parasites. AMS also bound to the liver worms' surface. The binding was very patchy (1+) and weaker than that with anti-host antibody (Fig. 2.7). The results obtained in these experiments indicated that both host antigens and parasite antigens were present on the surface of the worms which were recovered in vivo.

Cultured 21-day-liver worms : The binding of anti-host antibody and anti-parasite antibody on the surface of 21-day-liver worms, cultured for 24 h in vitro, was also investigated. The purpose of culturing the parasites for 24 h in MEM was to examine whether host antigens were lost during the culture period. The liver worms, immediately after recovery from mice, reacted more strongly with anti-host antibody (3+) than with AMS (1+) (Table 2.7).

In contrast, after culture in medium for 24 h at 37°C the parasite antigens were slightly more exposed than on freshly perfused worms, but the host antigens were not decreased (Table 2.8 & Fig. 2.59). The staining pattern of cultured liver worms with AMS was similar to that with MABs. The parasite surface was very patchy and irregular (Fig. 2.60).

Control sera (NMS, NRS & NGS) gave no specific fluorescence associated with the surfaces of lung schistosomula and liver worms (Table 2.7 & 2.8).

Table 2.7 Host Antigens and Parasite Antigens.

Antisera	Intact parasites (fixed in 1% PF)	Fluorescence intensity
Rabbit anti-mouse RBC ghost	3h skin-transformed schistosomula	(-)
" "	5-day-lung worms	4+
" "	21-day-liver worms	3+
Acute mouse serum (AMS)	3h skin-transformed schistosomula	4+
" "	5-day-lung worms	(-)
" "	21-day-liver worms	1+ patchy
Vaccinated rabbit serum (VRS)	5-day-lung worms	3+
Normal mouse serum (NMS)	3h skin-transformed schistosomula	(-)
" "	5-day-lung worms	(-)
" "	21-day-liver worms	(-)
Normal rabbit serum (NRS)	3h skin-transformed schistosomula	(-)
" "	5-day-lung worms	(-)
" "	21-day-liver worms	(-)
Normal goat serum (NGS)	5-day-lung worms	(-)
" "	21-day-liver worms	(-)

N.B. (-) = Negative

Table 2.8 Fluorescence Intensity of Host Antigens and Parasite Antigens on the Surface of 21-Day-Liver Worms Before and After Culture.

Antisera	21-day-liver worms	
	Freshly recovered from mice	Culture in MEM (24 h, 37°C)
Rabbit anti-mouse RBC ghost	3+	3+ patchy
AMS	1+ patchy	3+ patchy
NMS	(-)	(-)
NRS	(-)	(-)
NGS	(-)	(-)

N.B. Primary antibody dilution 1:10
Secondary antibody dilution 1:25

(-) = Negative

2.4 DISCUSSION

2.4.1 Monoclonal Antibodies Against the Schistosome Adult Tegument

The MABs used in the present study were derived by vaccinating mice with highly enriched fractions of worm tegument i.e. GP, D and M fractions. The spleen cells from these mice were fused with hybridoma cells. Sixteen anti-adult worm tegument MABs were developed. Molecular targets of 12 monoclonals of these 16 MABs were defined using the gradient pellet fraction. According to the immunofluorescent staining pattern, these 16 MABs were divided into 11 groups. Discussion points common to all 16 MABs are dealt with first. The distributuion of the target epitope of each group from cercaria to adult worm is then summarised.

General Points

No fluorescent label could be detected by any of the 16 MABs over the surface of intact lung and liver worms but the antigen could be detected in adult worm sections. These observations indicated that the epitope was still present but masked in the tegument of lung and liver worms. Therefore, it was not accessible for localisation in intact older parasites. Two possibilities could be advanced. Firstly, host antigens are taken up at the surface of the developing schistosomula and serve to protect the membrane of the worm against the immune response of the host by masking susceptible parasite antigens (Smithers et al., 1969; McLaren et al., 1975; McLaren & Terry, 1982). However, some workers have claimed that the unsusceptibility of the worms does not depend on the acquisition of host antigens nor does it correlate with the binding of antibodies to the parasite surface (Dean, 1977; Tavares et al., 1980; Levi-Schaffer & Smolarsky, 1981; Moser et al., 1980; Levi-Schaffer et al., 1982). Secondly, although the antigens are present there is steric hindrance that prevented antibody access or the amounts of these antigens exposed on lung worms and liver worms are too small to be detectable by fluorescent labelling techniques. Host and parasite antigens will be discussed later in this Section.

MABs in Group 4-11 did not confer protection in passive transfer experiments.

Group 1 MABs G6.7 & M7.4

The target antigens recognized by MAB G6.7 & M7.4 were located in the tegumental surface membrane of the parasites. The MABs reacted strongly with the surface of intact 3h schistosomula and more weakly with cercariae. The target was not the surface coat glycocalyx but the tegument of cercariae as shown in sections.

In sections of the cercarial body the tegument and the head gland were labelled. Packets of fluorescence were seen beneath the tegument. This probably corresponds to sub-tegumental cells which will be dealt with in more detail in MABs of Group 2. The intensely staining head gland contents might be secreted onto the cercarial surface after skin penetration. It has been shown that on penetration of host skin the inclusion granules of the head gland are secreted into the tegument (Morris, 1971; Dorsey, 1976; Torpier et al., 1977; Cousin et al., 1981). In the present study the surface of 3h schistosomula was brighter than that of cercariae. This might be due to the distribution of the contents of the sub-tegumental cells and head gland into the tegument.

In sections of 3h schistosomula, the epitope was confined to the tegument and the membrane of the oesophagus. The oral cavity and the oesophagus are lined with a thin layer of tegument, which is syncytial and continuous with that of the body wall (Ebrahimzadeh & Kraft, 1969). In sections of adult worm, the antigen appeared to be present in the plasma membranes of most schistosome cells including the gut at low density and the tegument plasma membrane at high density. These observations indicated that the epitope recognized by MABs G6.7 & M7.4 was a membrane antigen and ubiquitous from cercaria to adult worm.

MABs G6.7 & M7.4 recognized targets of 32 and 50 kDa on unreduced adult membrane preparations, the 32 kDa band was dominant. The epitope was destroyed by reduction (Vojvodic unpublished). MAB M7.4 conferred on mice protection against challenge ranging from 26-39% when given intravenously around the time of infection. In contrast, MAB G6.7 was not protective.

Payares, McLaren, Evans & Smithers (1985b) reported that the major surface antigens on the cercaria were glycoproteins of MW 32-38 kDa which were probably associated with the glycocalyx. The results obtained in the present study suggested that the antigen was located in the tegument, not the glycocalyx, as shown in cercarial sections. Payares et al. (1985b) also found that the 32 kDa glycoprotein persisted on the tegument after in vivo maturation and was conserved (Table 1.1). An antigen with identical MW (32 kDa) was also shown to

be present on the schistosomular surface (Dissous, Dissous & Capron, 1981; Bickle, Andrews & Taylor, 1986) and adult worm tegument (incubation products) (Dissous & Capron, 1983) but not the cercarial surface (Bickle et al., 1986).

The 32 kDa molecule although present on the surface of the intact lung and liver worms was not recognized by MABs M7.4 or G 6.7 using immunofluorescence technique. The result was consistent with the finding of Payares, McLaren, Evans & Smithers (1985a). They could not show the surface location of the fluorescence using rabbit anti-32 kDa (rabbit antiserum raised against glycoprotein of 32 kDa MW, partially purified from adult worm tegumental membranes) on the surface of 5-day-lung worms. However, they could detect the 32 kDa molecule on the surface of 5-day-old lung worms and 3-week-old liver worms by radioiodination followed by immunoprecipitation (Payares et al., 1985b).

The properties of MAB M7.4 were similar to those reported by Bickle et al. (1986). They found that a MAB (M22H12C) against the 32 kDa antigen which bound to the surface of in vitro-derived schistosomula and schistosomula recovered from mouse skin up to 3 days after infection conferred significant passive protection to mice. Simpson et al. (1984) demonstrated that five low MW polypeptide antigens (38, 32, 20, 17 & 15 kDa) were expressed on the surface of freshly transformed schistosomula and were reproducibly identified by surface labelling with ^{125}I by using IODOGEN and immunoprecipitation with immune mouse sera. The 32, 20 & 15 kDa antigens remained exposed on the schistosomulum surface for up to 2 days of in vitro culture.

Group 2 MABs G 6.1 & D7.2

The epitope recognized by MABs G6.1 & D7.2 is present in discrete packets in the cercaria but not exposed on its surface. Positive fluorescence was seen on the surface of the intact 3h schistosomulum and the discrete packets were not observed in sections of 3h schistosomulum. These observations indicated that during transformation the material in the packets (which probably corresponds to tegument cell bodies) contributed to the newly formed schistosomulum surface. The target molecule appeared to be a differentiation antigen.

Hockley & McLaren (1973) and McLaren & Hockley (1976) have shown that the tegument is connected to cell bodies beneath the two muscle layers by microtubule-lined connections. The cell bodies are filled with membraneous vesicles. During transformation, the cercarial membrane is lost through the formation and shedding of microvilli.

Membraneous vesicles enter the tegumental cytoplasm from the cell bodies. The contents of vesicles are then discharged onto the tegumental surface.

The target antigen of MABs G6.1 & D7.2 may be located in the inner bilayer of the tegument. The fact that the MABs could gain access to the antigen on the 3h schistosomulum surface may be because the inner bilayer was not completely covered by the outer bilayer. In contrast, the outer bilayer of lung and liver worms is possibly covered with host antigens as shown by the binding anti-host antibody which will be discussed later. Therefore, MABs cannot gain access to the inner bilayer of older parasites.

In sections of adult worm, the epitope appeared to be confined to the tegument, tegument cell bodies and interstitial material around the muscle fibres (which contains cytoplasmic connections between the tegument and tegument cell bodies). A possible interpretation is that the antigen was continually being synthesized by tegument cell bodies of the parasite and translocated and distributed into the tegument via the microtubule-lined cytoplasmic connections (Hockley, 1973; Hockley & McLaren, 1973). Antigenic conservation during schistosome development is demonstrated by MABs in Group 1 & Group 2. In other words, schistosomula share epitopes with adult worms.

The cross-reactivity of adult antigens with schistosomular epitopes has been shown by many workers. Dissous, Dissous & Capron (1981) used competition experiments, in which materials released from adult worms inhibited binding of specific antibodies to the surfaces of newly transformed larvae. Similarly, Dissous & Capron (1983) have shown that a 38 kDa MW molecule from schistosomula seemed to cross-react with a 115 kDa MW antigen from adult worms. Tavares, De Rossi, Payares, Simpson, McLaren & Smithers (1984) raised a monoclonal antibody against adult worm tegumental components that was able to immunoprecipitate a 20 kDa MW antigen from surface labelled schistosomula.

The isotypes of MABs G6.1 & D7.2 are IgG3 & IgG1 respectively. Both MABs recognized targets of 25, 38 & 55 kDa on unreduced adult membrane preparations, the 25 kDa band was dominant. When reduced material was used, the MABs stained only a single band of 25 kDa. MABs G6.1 & D7.2 did not confer protection in passive transfer experiments (Vojvodic unpublished).

An antigen with identical MW (25 kDa) which was immunoprecipitated by rabbit anti-membrane serum (purified membranes from adult schistosomes) and chronic mouse serum (20 normal cercariae, 15 week chronic infection) was obtained by surface-labelling of 5-day-old lung worms and 3-week-old liver worms (Payares et al., 1985b).

The 25 kDa antigen was also detected on purified membranes from adult worms (Payares et al., 1985b). Payares et al. (1985b) suggested that the 25 kDa antigen was conserved from lung stage through to the adult worm but was not expressed. This agrees with the observation in the present study that negative fluorescence was seen on the surface of intact lung and liver worms. The epitope was expressed on the schistosomular surface as shown by positive fluorescence results in the present study, but Payares et al. (1985b) could not detect it by immunoprecipitation of surface-labelled schistosomula. However, a 24 kDa MW protein from a detergent extract of schistosomula was recognized by 2 different monoclonals (MSM1.29 & MSM1.37) (Taylor & Butterworth, 1982).

Group 3 MAB G6.2, D7.4 & M7.3

MABs of this group did not bind to the surface of cercariae, 3h schistosomula, lung worms and liver worms. These observations indicated that the antigens were not exposed on the parasite surface but could be detected in sections of cercariae, 3h schistosomula and adult worms. The tegument cytoplasm was positive in sections of cercariae, 3h schistosomula and adult worms. The oesophagus was also labelled in sections of larval stages. It is known that the tegument covers the entire cercaria with a cytoplasmic sheath and is continuous with the epithelium of the oral cavity, oesophagus, excretory bladder and some portions of the excretory ducts (Smith et al., 1969; Powell & Sogandares, 1970; Ebrahimzadeh & Kraft, 1969, 1971).

It seems likely that the target antigens detected by MABs in Group 3 were preformed in cercaria and were not shed during transformation, but incorporated into the new membrane of schistosomula and retained. There is morphological evidence to support such a conclusion (McLaren & HOckley, 1976; Torpier et al., 1977). It is possible that the antigens were present in the trilaminate membrane of cercariae but not detected by immunofluorescence because the epitope was not accessible to the antibody. The antigen could reside within the membrane or on the cytoplasmic face. Alternatively the target antigen may be present in the tegument cytoplasm.

MAB M7.3 was a protective monoclonal. It has proved consistently protective when administered to mice, resulting in up to 33.7 % resistance. In contrast, MAB G6.2 & D7.4 which produced the same Western blot staining pattern as M7.3 did not confer any protection (Vojvodic unpublished).

The target epitope of MAB in Group 3 has a dominant band of MW 29 kDa on Western blots. An antigen with similar MW (28 kDa) was shown by MAB M.1 which was raised by immunizing mice with a surface membrane enriched extract of mechanically transformed schistosomula (Harn, Mitsuyama, Huguenel, Oligino & David, 1985). M.1, an IgG1, bound to the surface membranes of cercariae and young (0-24 h post-transformation) schistosomula but did not bind to older schistosomula or cultured lung worms. M.1 immunoprecipitated a similar 27-28 kDa antigen from membrane-enriched extracts of miracidia, lung and adult worms as well as from schistosomula.

The results obtained from the present study showed that MABs in Group 3 did not bind to the surface of intact parasites, but could be detected in sections of cercariae, 3h schistosomula and adult worms. Furthermore, antigens of identical molecular weights had been shown to be surface membrane components of young schistosomula using sera from infected patients or animals (Taylor, Hayunga & Vannier, 1981; Taylor, Cordingley & Butterworth, 1983). There is further evidence that this antigen may be present in the inner bilayer (Taylor et al., 1981). Taylor et al. (1981) immunoprecipitated a 28 kDa antigen with immune mouse serum from iodinated surface membrane antigens of cultured (18h) schistosomula. They noted that their larvae only had a single bilayer, not the usual heptalaminate one (a complete heptalaminate membrane was apparent only after 36-48 h).

Recently the gene from adult S. mansoni coding for a 28 kDa molecule has been cloned in E. coli (Balloul, Sondermeyer, Dreyer, Capron, Grzych, Pierce, Carvallo, Lecocq & Capron, 1987). Balloul et al. (1987) immunized rats, hamsters and monkeys with a recombinant protein inducing a strongly cytotoxic antibody response and protection against a natural challenge infection with live cercariae. They also showed that the 28 kDa antigen was present in extracts of other schistosome species (S. bovis, S. haematobium and S. japonicum). In the present study MABs in Group 3 did not cross-react with S. japonicum. Therefore, the epitope recognized by MABs in Group 3 could be a different molecule from that reported by Balloul et al. (1987) even though the MW of the target was quite similar.

Group 4 MAB G6.6

MAB G6.6 bound to the surface of 3h schistosomula but not cercariae, lung or liver worms. The antigen could be detected in sections of cercariae and 3h schistosomula. In sections of adult worm, the distribution of the target epitope was similar to MAB M7.4 through all cell plasma-membranes, but with high intensity in the

tegument. These observations indicated that the target epitope was present in all developmental stages from cercaria to adult worm, and expressed on the parasite surface after transformation. The antigen was associated with the plasma-membranes of schistosomes.

MAB G6.6 reacted with proteins of molecular weight 66 and 123 kDa on both reduced and unreduced gels. After treatment of Western blots with sodium periodate, G6.6 was the only anti-tegument MAB which no longer stained any bands, all the other banding patterns were unaltered. This suggested that G6.6 recognized a carbohydrate epitope and that the other MABs recognized protein epitopes (Vojvodic unpublished).

The distribution of antigens recognized by MAB G6.6 as shown by the immunofluorescent staining pattern in the present study as well as the molecular targets on immunoblots suggested that MAB G6.6 was an antibody to alkaline phosphatase.

Payares et al. (1984) have shown that a molecule of 65-66 kDa is alkaline phosphatase. They reported that the purified enzyme consisted of a single glycosylated polypeptide MW 65 kDa on reduced SDS-PAGE. They also located this enzyme in the tegument of intact adult worm using surface radio-labelling reagents followed by purification. The enzyme was radio-iodinated only with difficulty in adult worms. They were not able to detect this well-characterized component of the adult worm surface, earlier than day 21. They concluded that the enzyme was not exposed at the schistosome's surface, and was probably buried in the tegumental membrane network. Their conclusion was contrary to the immunofluorescence result obtained using MAB G6.6 in the present study if the target was alkaline phosphatase. The antibody reacted with the surface of intact 3h schistosomula, strongly with the tegument of adult worms and also the entire section of cercariae.

Ebrahimzadeh (1970) and Hockley (1970) showed that alkaline phosphatase activity was localised within the tegumental cytoplasm of the cercaria, and not in association with any inclusion bodies. Alkaline phosphatase was present in tissues of S. mansoni capable of active transport (Cesari, 1974). A histochemical study has shown that the alkaline phosphatase enzyme in male and female S. mansoni is distributed in the epidermis, gastrodermis and genitodermis (Sidkey et al., 1985). In the present study the gut epithelium of adult worm was also brightly labelled. Cytochemical staining showed that there were several different alkaline phosphatases in the tegument of the worm (Nimmo-Smith & Standen, 1963; Bogitch & Krupa, 1971; Wheater & Wilson, 1976). Electron microscopy revealed that these enzymes were present over the whole surface of the female tegument, but were

restricted to the outer membrane of the dorsal tegument in the male worm (Morris & Threadgold, 1968; Bogitch & Krupa, 1971).

Alkaline phosphatase has been used as a marker enzyme for tegument membranes of schistosome parasites (Halton, 1967; Cesari, 1974; Simpson et al., 1981; McDiamid et al., 1983; Roberts et al., 1983; Taylor & Wells, 1984). Cesari et al. (1981) demonstrated that the great abundance of alkaline phosphatase enzymes in the tegument of the parasite, together with its antigenic properties (shown by partial inhibition of the enzymatic activity by immunoglobulin G from chronically infected mice) made it an important constituent of the tegument.

Group 5 MABs D7.1 & D7.3

MABs D7.1 & D7.3 did not bind to the surface of intact parasites. The tegument was labelled in sections of cercariae, 3h schistosomula and adult worms. Internal tissues were also positive. These observations indicated that target antigens were not exposed but resided within the tegument throughout development in the vertebrate host. In sections of adult worms, tegumental cell bodies and parenchymal membrane were also positive. The observation in the present study also indicated that adult worm surface membranes and internal membranes contained similar antigenic components. This result was consistent with the unpublished observation of Kelly (1985) that adult worm surface membranes and internal membranes appeared to contain the same major polypeptide antigens.

MABs D7.1 & D7.3 had molecular targets of 18, 29 & 49 kDa on both reduced and unreduced gels with a dominant band of MW 18 kDa. Payares et al. (1985b) demonstrated that an antigen of MW 17 kDa was found in the surface of 3-24 h schistosomula but not cercariae or later stages of the parasite. Samuelson & Caulfield (1982) labelled freshly prepared skin or mechanical schistosomula with galactose oxidase followed by reduction with $NaBH_4$. They found that a protein of MW 17 kDa was labelled and lost with a halftime of 10-15 h in culture in defined medium. An antigen of 19 kDa was found to be present in a cercarial extract, by immunoprecipitation and immunoblotting (Horowitz & Arnon, 1985).

Group 6 MAB G3.12

MAB G3.12 bound to sections of adult worm only. The tegument and tegumental cell bodies were fluorescently labelled. It is possible that the target epitope was a stage-specific antigen actively

synthesized from cell bodies and distributed into the tegument of adult worms. Another interpretation of the failure to detect the target in sections of larval stages could be that the epitope was present at very low density in the cercaria and 3h schistosomulum, or was more rapidly destroyed by fixation than in the adult worm.

MAB G3.12 has molecular targets of 18, 29 & 39 on immunoblots. The molecular weight of the dominant band was 18 kDa. However, the pattern of bands on Western blots and also the fluorescent staining are different from those in Group 5. Therefore, it is probably a different 18 kDa molecule.

Group 7 MAB G5.3

The staining pattern of MAB G5.3 was similar to that in Group 3. The antibody did not bind to the surface of intact parasites. In sections of cercaria, 3h schistosomulum and adult worm, the tegument and the gut were positive (see Group 3). A molecular target of MAB G5.3 could not be identified by blotting or immunoprecipitation. The failure to detect the molecular target using these techniques might be due to denaturation of the antigenic determinants (Vojvodic unpublished).

Group 8 MAB M7.5

MAB M7.5 did not bind to the surface of intact parasites. In sections of cercariae and 3h schistosomula, the target antigen was located in the muscle fibres. In contrast to larval stages, fluorescent staining was seen only in the tegument of adult worms. The epitope was shared between the tegument and the muscle cells of the parasite.

M7.5 recognized targets of 19, 21, 22 & 29 kDa on Western blots with a dominant band at 19 kDa. It is known that the myofibrillar proteins are the major constituents of muscle cells i.e. myosin (thick filaments) and actin (thin filaments) (see also Introduction in Chapter 3). It has been shown that there are three light chains in fast-twitch skeletal muscle myosin. Two of these are the alkali light chains with molecular weights of 21 & 16 kDa and the third is the 5',5'-dithiobis (2-nitrobenzoate) (DTNB) light chain with a MW of 18 kDa. Antibodies have been prepared against each of these (Holt & Lowey, 1975a, 1975b, 1977; Lowey, Siberstein, Gauthier & Holt, 1979; Obinata, Masaki & Takano, 1979). It is possible that MAB M7.5 recognized one of the myosin light chains.

Myosin has been isolated and characterized from some non-muscle cells i.e. cloned mouse fibroblasts, human blood platelets and polymorphonuclear leucocytes (Adelstein, Conti, Johnson, Pastan & Pollard, 1972; Adelstein, Pollard & Kuehl, 1971; Stossel & Pollard, 1973). The present study showed that there was cross-reactivity between the tegument and the muscle as detectable by MAB M7.5. To date, this is the only observation to suggest that the schistosome tegument may contain myosin.

Although the schistosome tegument contains many spines which consist of hexagonally packed actin filaments (Cohen, Reinhardt, Castellani, Norton & Stirewalt, 1982). It is highly unlikely that MAB M7.5 recognized actin. This molecule has a MW of 42 kDa (Kuroda & Masaki, 1980). M7.5 did not recognize a parasite component of this MW.

Group 9 MAB G5.10

MAB G5.10 did not bind to the surface of intact parasites. Internal tissues were weakly labelled in sections of cercaria. The tegument was positive in sections of 3h schistosomulum and adult worm. Tegumental cell bodies of adult worms were also fluorescently labelled. These observations indicated that schistosomular antigen was pre-synthesized in the cercaria and was not exposed on the schistosomular surface but could be detected in sections. The antigen persisted through to the adult stage. A molecular target of MAB G5.10 could not be identified by Western blotting or immunoprecipitation.

Group 10 MAB G6.4

MAB G6.4 did not bind to the surface of intact parasites. The tegument was positive in sections of cercaria, 3h schistosomulum and adult worm. Nephridia were also labelled in sections of 3h schistosomulum and adult worm. Tegumental cell bodies were positive in sections of adult worm. These observations indicated that the target epitope was not exposed on the parasite surface but conserved in the same structure throughout developmental stages. It is possible that this antigen is a secretory or excretory macromolecule. The epitope was shared between the tegument and nephridia of the parasite. It has been shown that the nephridial wall of the cercaria is cellular and attached to the tegument by a septate desmosome. After tail loss, the nephridial wall becomes continuous with the tegument cytoplasm (Powell & Sogandares-Bernal, 1970). This could explain the presence of shared antigen between the two epithelia.

A molecular target of MAB G6.4 could not be identified by blotting or immunoprecipitation, but positive result was obtained on the dot blots.

Group 11 MAB M7.6

The target epitope was not exposed on the surface of intact parasites. In sections of adult worms, the tegument and cell bodies were positive as well as the nephridial canals. It is possible that the antigen is a secretory molecule as described in Group 10. A molecular target of MAB M7.6 could not be identified by blotting or immunoprecipitation but a positive result was obtained on the dot blots.

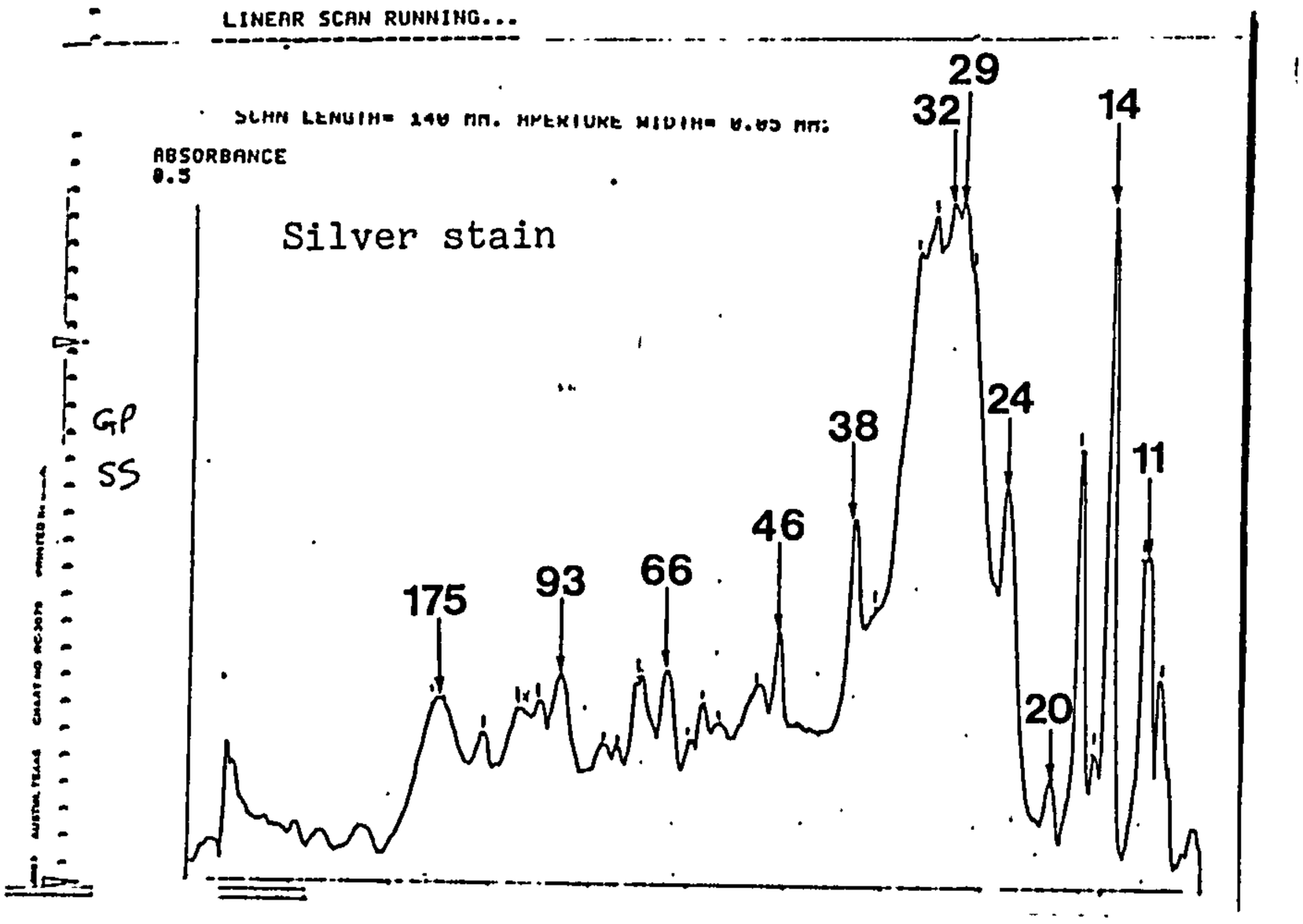
2.4.2 Protein Composition of Adult Worm Tegument Membrane

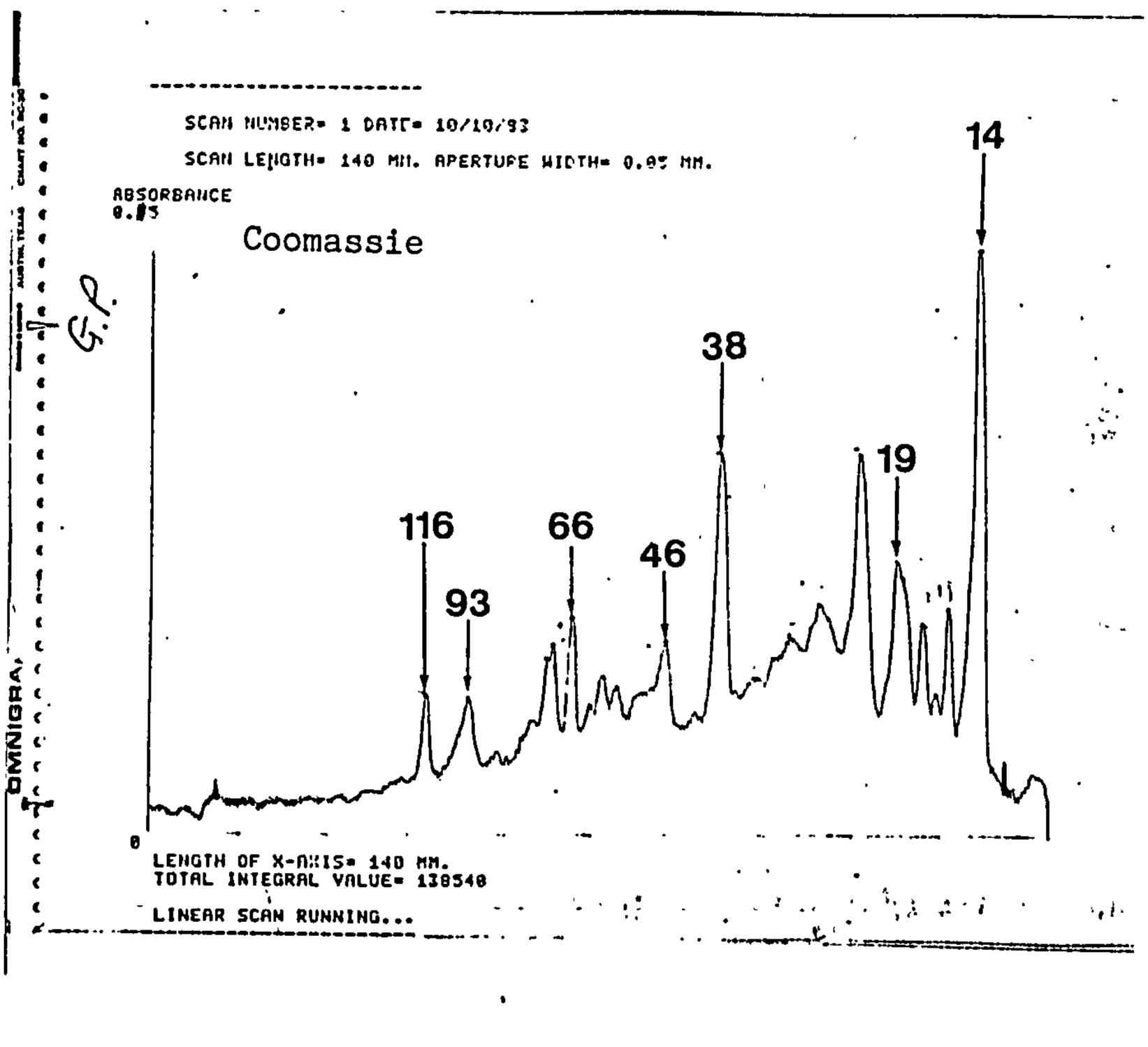
The protein composition of the gradient pellet (GP) was studied by Roberts (unpublished). A scan of the GP fraction after electrophoresis and the relative molecular weights of bands from staining of gels and Western blots are shown in Text Figure 2.9. There were 15 major proteins (Silver stain) with molecular weights as follows :- 175, 100, 93, 73, 70, 66, 56, 50, 46, 38, 37-24, 20, 16.5, 14 & 11 kDa. Proteins at 116 & 19 kDa stained strongly using Coomassie but only weakly with Silver stain.

MABs used in the present study recognized targets of MW 18 (MABs D7.1, D7.3 & G3.12), 19 (M7.5), 25 (G6.1 & D7.2), 29 (G6.2, D7.4 & M7.3), 32 (G6.7 & M7.4) and 66 kDa (G6.6) in Western blots (Vojvodic unpublished). The 18 & 25 kDa antigens were probably the same bands as the 19 & 24 kDa proteins identified by Roberts (unpublished). Only the targets of 25, 32 & 66 kDa were extrinsic protein components of S. mansoni as shown by positive fluorescence on the surface of intact 3h schistosomula. Other molecules were probably intrinsic proteins contained within the two bilayers or associated with the cytoplasmic face of the membrane. Therefore, the epitopes were not accessible to the antibodies as shown by negative fluorescence on the surface of intact parasites.

2.4.3 Cross-Reactivity of MABs with S. japonicum

There was cross-reactivity between S. mansoni and S. japonicum. The tegument of S. mansoni shared common antigenicity with the tegument and/or parenchymal tissue of S. japonicum as demonstrated by MABs G5.10, G6.4, G6.6 & D7.3. This agreed with the observation of





Text Figure 2.9

A scan of the gradient pellet (GP) fraction after electrophoresis showing the relative molecular weights (kDa) of the major proteins (Roberts et al., 1987).

Beisler *et al*. (1984b) that the tegument of *S. japonicum* reacted strongly with *S. mansoni*-adult immune serum by immunofluorescence technique. The cross-reactivity was also detected in the gut and egg of *S. japonicum* using this serum.

Surprisingly, the epitope recognized by MAB G5.10 on *S. mansoni* adult worm was destroyed by paraformaldehyde fixation, but on *S. japonicum* was not. The fluorescence results suggested that antigenic components which occurred on the tegument of the parasite of both species were similar. MAB M7.5 recognized the epitope in the muscle of *S. japonicum* adult worm and larval stages of *S. mansoni* and also the tegument of adult *S. mansoni*. These observations suggested that the shared epitope between the tegument and the muscle was found in more than one schistosome species. Evidence of cross-reactivity was also reported by Schinski, Clutter & Murrell (1976) using sera of patients infected with heterologous schistosome species. In the present study only 5 of 16 (31.25 %) MABs were cross-reactive with *S. japonicum*.

Some of the surface membrane antigens of *S. mansoni* were species specific as shown by 11 MABs that did not react with *S. japonicum* adult worm sections. Bickle, Andrews, Doenhoff, Ford & Taylor (1985) have reported that species-specific antigens were necessary for the induction of protection. They found that significant levels of resistance against *S. mansoni* challenge were developed by mice exposed to highly irradiated (20 krad) cercariae of the homologous species (53-67 %), whereas vaccination with *S. bovis*, *S. haematobium* or *S. japonicum* failed to confer significant levels of resistance to reinfection (-5 to 12 %). Similarly, Cheever, Hieny, Duvall & Sher (1983) reported that immunization of mice with highly irradiated *S. mansoni* cercariae failed to protect against *S. japonicum* challenge. Thus demonstrating the specificity of the immunizing procedure.

2.4.4 Host Antigens

In vitro skin-transformed schistosomula lacked mouse host antigens but bound antibody from infected mouse serum (AMS) whilst 5-day-lung worms (recovered from mice) possessed mouse red blood cell antigens and did not bind antibody from AMS. These observations suggested that host antigens were present on the parasite surface. These results corroborated the finding of McLaren *et al*. (1975), they used an antibody-enzyme bridge technique to localise antigen on 3h *in vitro* and 4-day *in vivo* schistosomula. They concluded that the presence of host antigen served to protect the surface membrane of the schistosomula against antibody-mediated damage. Similar result was

also reported by Goldring et al. (1977).

In the present study, strong binding of anti-schistosome antibody (AMS) was found on cryostat sections of adult worm as shown by an intense fluorescent staining in the tegument. Intact 21-day-liver worms were strongly positive for host antigens (rabbit anti-mouse RBC ghost) and weakly positive for parasite antigen (AMS). These observations indicated that parasites which possessed host antigens could also bind anti-schistosome antibody. These results were similar to those of other workers. Adult worms bind little or no anti-schistosome antibody compared to anti-host antibody (McLaren & Terry, 1982; Harnett, Kusel & Barrowman, 1985). The results also corresponded to the observation of Goldring et al (1977) that live or 10% paraformaldehyde-fixed adult worms were strongly positive for host antigen and weakly positive for parasite antigen. They found that the binding of anti-parasite antibody was associated with the dorsal tubercles of the worm and occurred in scattered patches limited to about 20% of the worm's surface. They also demonstrated that schistosomula recovered from the skin of normal or immune mice 3 & 20 hours after cercarial penetration were positive for host antigens but also bound immune antibody.

The loss of host antigen from the surface of 21-day-liver worms after culture in vitro for 24 h was very slight as judged by fluorescent antibody staining. This observation resembled the finding of Saunders (1986) that whichever medium was used the loss of host antigen from the adult worm surface during incubation in vitro was very slight. He found that there was a significant loss of host antigen corresponding to mean half-times of 30.1 hours from worms incubated in rabbit anti-mouse RBC ghost serum, and 61.9 hours from worms incubated in NRS (normal rabbit serum). The results from the present and Saunders' study disagreed with the observation of Wilson & Barnes (1977) that surface labelling with cationised ferritin caused rapid sloughing of the outer bilayer, with a half-life of 2-3 hours.

The results from the present study also showed that AMS did not bind to the surface of lung worms. It is possible that parasite antigens were present on the schistosome surface but they were masked by host molecules as shown by rabbit anti-mouse RBC ghost serum. Another explanation of the dramatic developmental change in the immunochemical behavior of the worm surface was that parasite antigens could be cleared from the worm's surface as part of its normal development (Dean, 1977). However, this hypothesis did not apply to the observation in the present study because the loss of host antigen from cultured parasites was very slight.

2.4.5 Fixatives

Frozen sections fixed in acetone gave comparable results to those of paraformaldehyde-fixed specimens. However, some epitopes were destroyed after paraformaldehyde treatment such as epitopes recognized by MABs M7.6 & G5.10. From the results obtained throughout the present study I suggest that the use of frozen (cryostat) sections is necessary for examination of tegument antigenicity. Tegument antigens were labile antigens, they could be easily destroyed by glutaraldehyde fixation (see Chapter 4). Beisler *et al*. (1984b) similarly demonstrated a distinctly stronger tegument reaction in cryostat sections than in formalin-fixed paraffin embedded sections.

Intact parasites were fixed in 1% paraformaldehyde (PF) in 0.1 M phosphate buffer in the present study. They were fixed prior to immunofluorescent staining for the following reasons :-

i). To prevent cercarial transformation to the schistosomulum during incubation with antisera while performing the test. I found that false positive results were obtained on the cercarial surface when live or unfixed parasites were used. In the absence of fixation the remnant of the surface coat glycocalyx was seen detaching from the cercarial surface.

ii). To prevent parasite death from mechanical damage during the course of the test. False positive results were obtained when live or unfixed 3h schistosomula were used. Dead parasites were intensely fluorescent.

iii). To prevent the internalisation of the antibody by the parasites.

Fixation of tissue sections or cells containing antigen is indispensable to subsequent staining for two reasons. Firstly, it prevents the tissue from being pulled off the glass slide, and second, it facilitates the formation of the antigen-antibody complexes by eliminating agents (for example, lipid) which prevent the antibody from gaining access to the antigen and/or inhibit the antigen-antibody reaction. The treatment should not, however, enhance non-specific fluorescence due to the antigen or tissue.

CHAPTER 3

MONOCLONAL ANTIBODIES REACTING WITH MUSCLE AND NON-TEGUMENTAL STRUCTURES OF *SCHISTOSOMA MANSONI*

3.1 INTRODUCTION

Eleven monoclonal antibodies (see Section 2.3.4.2 in Chapter 2) of the 50 MABs used in the present study which were raised against highly enriched fractions of worm tegument (GP & M fractions) reacted with the muscle cells of the parasite. These 11 MABs might also recognize epitopes in the cytoskeletal elements of other schistosome tissue. Therefore, the "cytoskeleton" will be described first in this Chapter in order to understand the components and the structures of protein filaments in muscle and nonmuscle cells, followed by the musculature of S. mansoni, and what is known as nonmuscle cytoskeletal elements.

3.1.1 The Cytoskeleton

The "cytoskeleton" is the organized system of protein filaments that is responsible for cell movement and contributes to cell shape in eucaryotic cells (Alberts, Bray, Lewis, Raff, Roberts & Watson, 1983; Avers, 1986).

I. Cytoskeletal Protein Filaments

The two most important types of protein filaments in the cytoskeleton are microfilaments and microtubules. Both filaments can assemble and disassemble rapidly in the cell. A third type of cytoskeletal protein consists of intermediate filaments.

"Microfilaments" (actin filaments) are composed of globular actin (G actin) molecules that are polymerized into strands of filamentous actin (F actin). Each microfilament consists of two strands of F actin twisted around each other in a helix conformation. Although actin is the major component of the microfilament, several other proteins are also present.

"Microtubules" are long, hollow, unbranched cylinders. The average diameter of a microtubule is 25 nm, of which about 15 nm represents the hollow core. The cylinder wall is made up of 13 protofilaments lined up side by side in a circle. Each protofilament consists of a long chain of heterodimer subunits made up of monomers of the globular protein "tubulin" (alpha & beta tubulins).

"Intermediate filaments" are found in most animal cells; they are made of fibrous protein subunits. Intermediate filaments are the most stable of the cytoskeletal protein filaments, so called because their diameter of 10 nm is intermediate between the 6-nm-wide microfilaments

and the 25-nm-wide microtubules. Each type of intermediate filament is composed of a characteristic set of subunit proteins, and any one kind of cell usually contains only one of the many different types of intermediate filament. For example, epithelial cells contain tonofilaments called "keratin" filaments; fibroblasts contain "decamin" filaments; muscle cells contain "desmin"; mesenchymal cells contain "vimentin"; neurons contain neurofilaments; glial cells contain "glial" filaments.

In addition to the three major types of protein filaments, the cytoskeleton also contains many different accessory proteins that either link the filaments to one another or to other cell components such as the plasma membrane, or influence the rate and extent of the filament polymerization. Specific sets of accessory proteins interact with protein filaments to produce movement. For example the contraction of muscle which depends on actin filaments, and the beating of cilia which depends on microtubules. Both processes require ATP hydrolysis and the sliding of one protein filament against another.

II. Muscle Fibres

Striated (skeletal) muscle has a distinctive appearance due to a pattern of cross-striations or bands of alternating dark and light material in its individual muscle fibres. The muscle fibre is long, thin, multinucleate and made up of "myofibrils" which extend the entire length of the cell.

The contractile units of the myofibrils are composed of repeating assemblies of thick and thin filaments. Each of the regular repeating units called "sarcomeres", is about 2.5 um long. In a longitudinal section viewed at high magnification, a series of light and dark stripes can be seen in each sarcomere. The darker stripes are called "A bands", the lighter stripes "I bands"; the dense line in the centre of the I band that separates one sarcomere from the next is called the "Z line" (or Z disc).

Each sarcomere contains two sets of parallel and partly overlapping protein filaments : thick filaments (1.6 um long and 15 nm in diameter), extending from one end of the A band to the other; and thin filaments (1.0 um long and 8 nm in diameter), extending across the I bands and part way into the A band. A cross-section of muscle in the region of the A band where thick and thin filaments overlap shows that the thick filaments are arranged in a regular hexagonal lattice, with the thin filaments placed in a regular manner between them.

"Actin filaments" consist of two strands of globular molecules about 4 nm in diameter twisted into a helix. Actin filaments have been identified as the principal components of thin filaments of skeletal muscle both by X-ray diffraction pattern and by fluorescent anti-actin antibodies that stain the I-band region of the sarcomere.

"Myosin filaments" are polymers of the protein myosin about 15 nm in diameter. Myosin filaments are found in many kinds of cells, but they are most abundant and prominant in skeletal muscle. The myosin molecule has a molecular weight of 500 kDa and it consists of 6 polypeptide subunits. Two heavy chains of about 200 kDa, are coiled around each other to form the long rod-like tail, and each chain terminates in a globular head portion of the molecule. Two pairs of light polypeptide chains of about 20 kDa and 16 kDa each, are associated with the heads of the molecule; one chain of each pair is present on each head. The myosin molecules associate by their tails. The body of the thick filament is composed of hundreds of myosin tails packed together in a regular staggered array from which the myosin heads project in a repeating pattern. The structure is bipolar, with a bare central region where two oppositely oriented sets of myosin tails come together. The globular heads of the myosin molecules interact with actin and form the cross-bridges between the thick and thin filaments of muscle. The globular head is also the site of ATPase catalytic activity in ATP hydrolysis leading to fibre movement or to skeletal muscle contraction.

Other protein components of the myofibril are "troponin" and "tropomyosin" which regulate muscle contraction, and "alpha-actinin" and "desmin" which have a structural role.

"Alpha-actinin" and "desmin" are found in the region of the Z disc. The former determine the arrangement of filaments in each sarcomere and the latter tie adjacent sarcomeres together. At the end of each sarcomere, parallel actin filaments are embedded in one side of the Z disc. It is thought that alpha-actinin binds to the ends of actin filaments and anchors them in the Z disc. Desmin, an intermediate filament protein, is found in high concentration in the regions between Z discs of adjacent myofibrils and is thought to link Z discs together.

"Tropomyosin" is a rigid, rod-shaped protein, which lies in the long pitched grooves on either side of the actin filament, thereby stiffening the filament. Tropomyosin has cooperative effects during regulation of actin-myosin interaction (2 sites per actin).

"Troponin" is a protein composed of three polypeptides (troponins T, I, and C). Troponin T has a binding site for tropomyosin and is thought to be responsible for positioning the troponin-tropomyosin

complex along an actin filament. Troponin I inhibits the actin-myosin interaction. Troponin C binds calcium ions. A troponin complex is attached at one specific site on each tropomyosin molecule. Troponin and tropomyosin mediate the Ca^{+2} regulation of muscle contraction.

III. Smooth Muscle (Rhodin, 1977)

In vertebrates, smooth muscles are composed of long spindle like cells, each with a single centrally located elongated nucleus, and cross-striations are absent. These muscle cells contain actin and myosin of normal type. Smooth muscle tissue is often referred to as a visceral muscle since it is responsible for the movement of the internal organs. It is particularly abundant in the uterus.

The smooth muscle fibre is a slender, fusiform, cylindrical, slightly flattened, and occasionally branched cell. The smooth muscle cell is bounded by a sarcolemma and is filled with myofilaments. Attached to the cytoplasmic aspect of the sarcolemma at varied intervals are areas of high electron density called "dense bodies". The contractile sarcoplasm of the smooth muscle fibre is made up of three sets of myofilaments : thick, intermediate, and thin filaments. They are oriented parallel to the long axis of the smooth muscle fibre. The thick filaments contain myosin. The intermediate filaments form lattice-like bundles between the thick filaments. Two or three rows of thin filaments may surround a thick filament to form rosette-like configurations; they contain actin. The thin filaments are held closely together by structures called dense bodies which are distributed widely among the filamentous parts of the sarcoplasm. The thin filaments attach to the dense bodies along the sarcolemma.

IV. Actin Filaments and Actin-Binding Proteins in Nonmuscle Cells

Actin is also found in many different structures in the cell, and associates with a large number of different actin-binding proteins. Actin filaments serve two functions in nonmuscle cells :

They form cross-linked bundles which provide mechanical support for various cellular structures, and together with myosin they form the contractile systems which response for cellular movements. Bundles of actin filaments are present in the core of microvilli. Actin filaments associated with nonmuscle myosin are found in cells where contractions are needed, such as the contractile ring of a dividing cell, the belt desmosomes at the apical region of an epithelial cell, and the stress fibres of a tissue culture cell.

In addition, less well-organized networks of actin filaments are found throughout the cytoplasm, concentrated in a cortical layer located just beneath the plasma membrane. By interacting with nonmuscle myosin and with proteins that anchor them to the plasma membrane, these networks are responsible for cell-surface movement and cell locomotion.

3.1.2 The Musculature of S. mansoni

The musculature of schistosomulum stage parasite has not been reported; only the adult worm and the cercaria have been studied extensively.

A). Adult Worm Musculature

The musculature of schistosomes is non-striated and fairly typical of invertebrate, slow-acting smooth muscle (Lumsden & Foor, 1968; Silk & Spence, 1969). The orientation of myofibres is arranged in circular, longitudinal and radical fashion (Silk & Spence, 1969). The female musculature is not as well developed as that of the male and sometimes only one circular and one longitudinal layer can be identified (Silk & Spence, 1969).

The muscle cells are surrounded by fibrillar interstitial connective tissue and the precursors of this material are probably formed in dilated cisternae of the sarcoplasmic reticulum which discharge their contents to the exterior at the sarcolemma (Silk & Spence, 1969).

The tegument is separated from the musculature and interstitial tissue by a basement membrane which evaginates into the tegumentary matrix as a system of microtubular canals. The basement membrane invests the lower part of each spine which is anchored to the sarcolemma of a longitudinally orientated muscle fibre (Silk, Spence & Gear, 1969).

The contractile elements of the myofibril consist of thick, tapered myofilaments, each surrounded by an irregular array of 8-14 thin filaments in transverse section. In longitudinal section the thick myofilaments are parallel and discontinuous with tapered ends. Branching and cross-linking of the thin filaments occur occasionally (Silk & Spence, 1969).

The thin filaments are attached to fusiform dense bodies which are equivalent to the Z disc of striated fibres of vertebrate muscle. These dense bodies are important in the co-ordination of myofilament movement; they represent sites of adhesion of intermediate filaments.

These bodies are found at the periphery of the myofibril bundles and attached to the sarcolemma. In longitudinal section they are continuous with the thin myofilaments (Silk & Spence, 1969).

The nuclei of the muscle cells show prominent nucleoli and clumped chromatin. The sarcoplasmic reticulum is rough and poorly defined. Numerous mitochondria are distributed peripherally in contact with the sarcolemma and along the borders of each myofibril. Golgi apparatus and glycogen are present in the sarcoplasm. The muscle cells form junctional complexes between themselves and adjacent tegumental cells (Silk & Spence, 1969).

B). Cercarial Body Musculature

The organization of the cercarial body muscle has been described by many authors (Kruidenier & Vatter, 1960; Pan, 1965; Smith, Reynold & von Lichtenberg, 1969; Ebrahimzadeh & Kraft, 1969; Dorsey & Stirewalt, 1971; Stirewalt & Dorsey, 1973; Stirewalt, 1974). It can be catalogued as follows :

- a subtegumental system comprising one outer circular and one or more subjacent longitudinal layers of muscle fibres.

- deeper diagonally oriented muscle fibres which traverse the parenchyma.

- the conical and diagonal head capsule musculature.

- muscle sheaths around internal organs such as the digestive tract, the unicellular acetabular and escape glands including their ducts.

- the small discrete muscle cells of the parenchymal network.

A cercarial muscle cell (myocyte) consists of a perikaryon and long tortuous cell processes or myofibres, the whole embedded in the intercellular matrix. Myofibres appear coarse as in the musculature of the body wall, tail and head capsule, or fine as in the parenchymal network.

The body wall musculature is situated immediately below the interstitial layer. The structural pattern is similar to that of the adult worm, with outer circular fibres overlying inner longitudinal fibres (Kruidenier & Vatter, 1960; Lumsden & Foor, 1968; Nuttman, 1975). The circular fibres in the mid-region are wider than those in

the anterior and posterior of the body. The longitudinal fibres vary in size and number in transverse sections because they are branched (Nuttman, 1975).

The muscle fibres are non-striated. The myofibrils contain thick and thin myofilaments and possess dense bodies, similar to the adult muscle fibres. The dense bodies are randomly distributed, attached to the thin filaments, and fuse with the sarcolemma at the periphery of the cell. Mitochondria are most frequently located along the periphery of the myofibril, surrounded by glycogen particles (Lumsden & Foor, 1968). Flattened cisternae of the sarcoplasmic reticulum lie immediately beneath the sarcolemma, but a transverse tubule system (T-tubule, tubular invaginations of the sarcolemma) for rapid transfer of stimuli to within the myofibril is absent (Nuttman, 1974). Myocyte nuclei are large and irregularly-shaped with darkly staining chromatin in dense clumps (Kruidenier & Vatter, 1960; Pan, 1965; Stirewalt & Dorsey, 1973).

The muscle of the head capsule and the ventral sucker is also attached to a layer of fibrous interstitial material which is continuous with that of the body wall (Nuttman, 1975). Nuttman (1975) refers to this layer in the head capsule as the "diaphragm" and his detailed reconstruction of the musculature is illustrated in Text Figure 3.1. On the posterior surface of the fibrous layer are radially directed fibres. The fibres on the anterior surface (anterior retractor fibres) are circularly orientated in the central region, but at the periphery become longitudinally aligned and interdigitate with the body wall fibres, before displacing inwards and attaching to the apical area (Nuttman, 1975). The anterior retractor muscles work antagonistically against the muscles lying on the interstitial layer, to control the extension and retraction of the apical area (Robson, 1974; Nuttman, 1975).

The musculature of the head capsule is specialized in shape and in the arrangement of its myofibres. A conical, caudally-directed musculature bounds this region aborally. It consists of a circular muscle layer positioned between two longitudinal layers. Coarse oblique muscle fibres are prominent. The longitudinal and oblique muscle fibres are coarser than the circular ones. The ventral sucker is provided with muscle fibres which radiate outward, beyond which are circularly arranged muscle fibres. Coarse fibres from the ventral sucker traverse the parenchyma to anchor in the body wall (Pan, 1965; Stirewalt & Dorsey, 1973).

Myocytes of the parenchymal net and of the sheaths of the digestive tract and secretory cells are all similar in structure, but the arrangement of the myofibres varies (Stirewalt, 1974). The mouth

Text Figure 3.1

Diagrammatic reconstruction of the musculature of the head capsule of the cercaria, shown in longitudinal and transverse sections (from Nuttman, 1975).

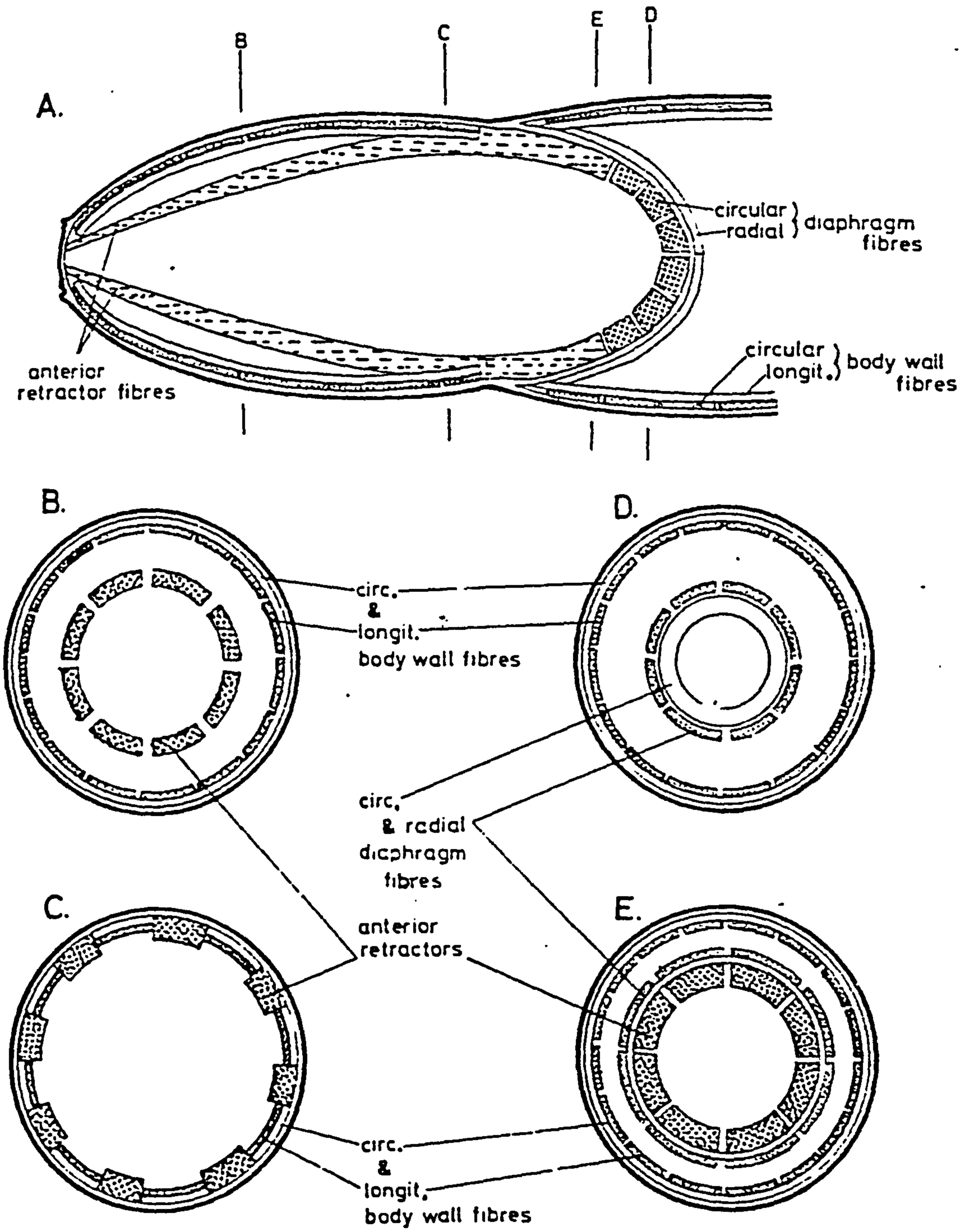
A. Longitudinal section of head capsule to show relative position of body wall muscle fibres and those of the diaphragm and anterior retractors. Although the latter are shown in longitudinal section, they are shaded for clarity. The locations of the following transverse sections are indicated as B, C, D & E.

B. Transverse section in central region of head capsule.

C. Transverse section just anterior to the periphery of the diaphragm. Note interdigitation of anterior retractor and body wall muscle fibres.

D. Transverse section of posterior end of head capsule, which passes through the prominent, circular muscle fibres of the diaphragm.

E. Transverse section near the periphery of the diaphragm showing the anterior retractors adjacent to the diaphragm radial muscle fibres.


A.
B
C
E
D
circular
radial
diaphragm fibres
anterior retractor fibres
circular
longit.
body wall fibres
B.
D.
circ.
&
longit.
body wall fibres
circ.
& radial
diaphragm fibres
anterior retractors
C.
E.
circ.
&
longit.
body wall fibres

and oral cavity have an especially heavy sheath. Those myofibres adjacent to the epithelial syncytium of the oral region of the digestive tract are circularly oriented; longitudinal fibres lie next to the parenchyma. Radial myofibres are also present (Stirewalt, 1974). The oesophagus is well endowed in this respect, but the number, compactness and coarseness of the myofibres decrease nearer to the digestive cecae, which have little of a muscular sheath (Ebrahimzadeh & Kraft, 1969; Dorsey & Stirewalt, 1971; Stirewalt & Dorsey, 1973).

The muscular ensheathment of the acetabular glands is variously structured at different levels of the funduses and ducts. It is markedly sparse around the ducts as they near the pores. Short longitudinal muscle fibres are also present under the tegumentary folds around these duct openings (Ebrahimzadeh & Kraft, 1969, 1971; Dorsey & Stirewalt, 1971; Stirewalt & Dorsey, 1973). Therefore, muscular contraction extrudes the secretions out of the glands. Post-acetabular gland ducts have a larger diameter and a greater number of surrounding muscle fibres within the head capsule. These allow the secretion of the postacetabular glands prior to the preacetabular glands (Robson, 1974).

C. Cercarial Tail Musculature

The shape and poise of the body and the tail, and the position of the two caudal forks of the tail determine whether cercaria swim backward or forward. Cercaria larvae of the forked-tail species undergo some shape changes and high speed tail movements of 20-24 tail oscillations per second during swimming (Graefe, Hohorst & Drager, 1967). When cercaria swim backward, the two forked branches of the tail are spread stiffly laterally and perpendicularly to the long axis of the tail, while in forward swimming they are extended caudally and parallel to the longitudinal axis of the tail.

The muscle fibres present along the entire length of the cercarial tail are arranged in outer circular and inner longitudinal layers as in the (cercarial) body. Circular muscle fibres of the tail are smaller than those of the body, and lack transverse striation; they are smooth muscles (Lumsden & Foor, 1968; Nuttman, 1974; Reger, 1976).

There are three muscle cell types in the forked tail of Schistosoma sp. cercarial larvae (Reger, 1976).

i). A longitudinally directed fast, cross-striated muscle functioning in rapid tail movements.

ii). A slow, smooth muscle at the body-tail junction functioning in body and tail shape changes. Alternate constriction and relaxation occurs in this region when the cercaria shifts from backward to forward swimming movement (Graefe et al., 1967).

iii). A slow, smooth muscle in the tail forks functioning to maintain fork rigidity during backward swimming movements.

The longitudinal myofibres of the tail appear stronger, more compact and contain more myofilaments than those of the body. They differ structurally from the body fibres in :

a). the orientation of the dense bodies into distinct cross-bands (striations). The striations are the result of transversely aligned Z densities interspersed with branched elements of subsurface sarcoplasmic reticulum cisternae.

b). the division of myofibrils into sarcomeres with typical A and I bands reflecting overlapping thick and thin myofilaments.

c). a more extensive sarcoplasmic reticulum which extends into the myofibril and forms a network among the striations.

d). larger and more numerous mitochondria.

All these features characterise fast, cross-striated fibres, and are consistent with the more rapid contraction of tail muscles in cercarial movement (Lumsden & Foor, 1968; Reger, 1976; Smyth & Halton, 1983).

There are six distinct blocks of longitudinal muscle in the tail stem, lying immediately beneath the circular muscle layer. They are four lateral blocks, one mid-dorsal block and one mid-ventral block. The four main dorso- and ventro-lateral longitudinal muscle blocks appear in transverse section consisting of up to five fibres. Each fibre lies at an angle of approximately 10-12° to the longitudinal axis of the tail (Text Figure 3.2 from Nuttman, 1974). Transverse striations are present in the four main longitudinal muscle blocks; they are absent from dorsal and ventral longitudinal fibres.

The smooth muscle cells at the body-tail junction are composed of two sets of cells, each perpendicularly arranged with respect to the

Text Figure 3.2

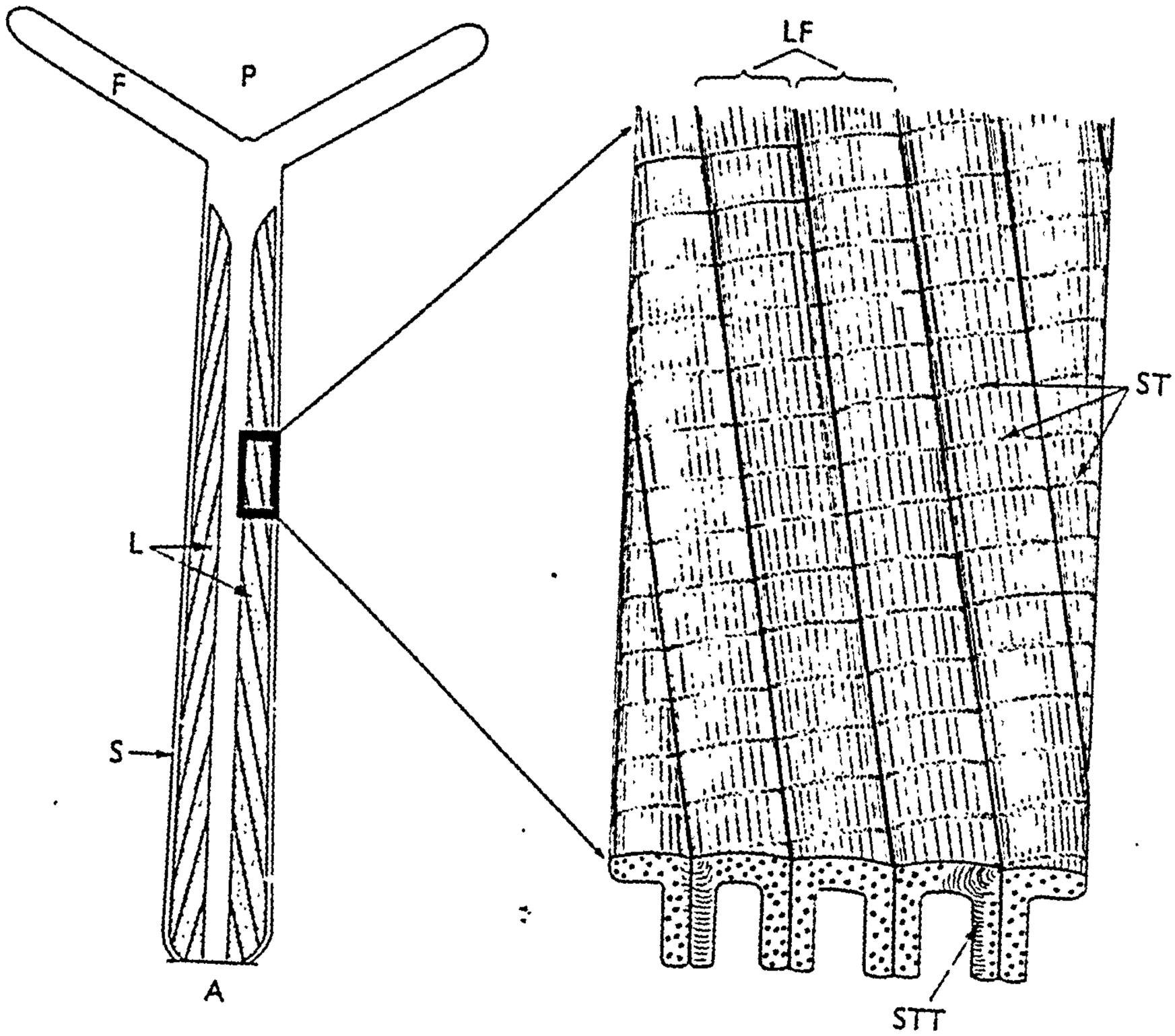
A diagrammatic reconstruction of the organization of the tail musculature of S. mansoni (from Nuttman, 1974).

A. Dorsal view of tail to illustrate location of dorsolateral muscle blocks and orientation of myofibres (not all myofibres shown, for clarity). Also shown is an enlarged segment of one of these blocks, showing the contractile regions of myofibres in surface view and in transverse section.

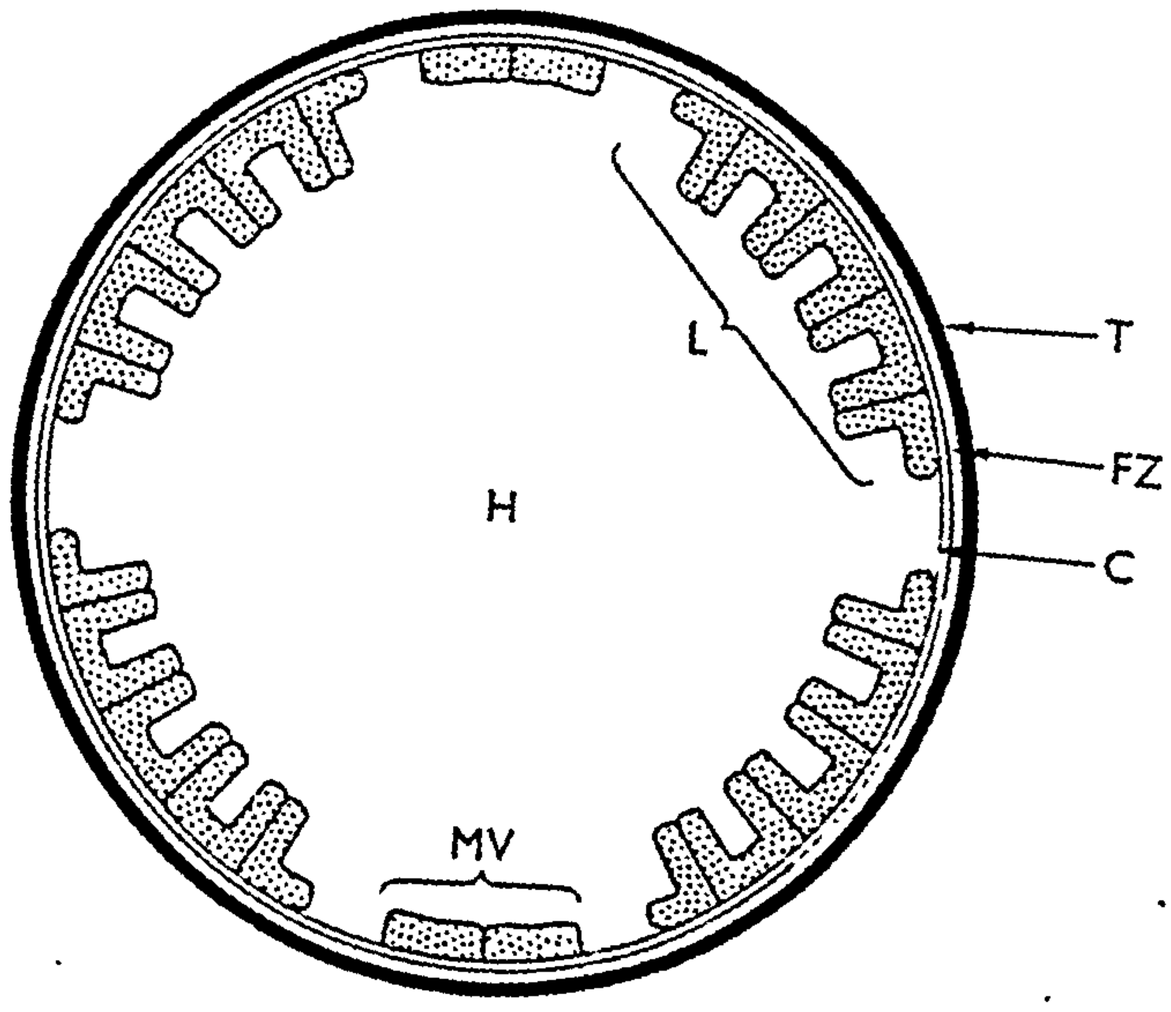
B. Transverse section of central region of tail stem (sarcoplasmic regions of myofibres, excretory system etc. are omitted).

A	=	Anterior
C	=	Circular muscle
F	=	Furca
FZ	=	Subtegumentary fibrous zone
H	=	Tail "core", the hydrostatic skeleton
L	=	Lateral longitudinal muscle block
LF	=	Longitudinal fibre
MV	=	Mid-ventral muscle block (longitudinal)
P	=	Posterior
S	=	Tail stem
ST	=	Striation of myofibre (surface view)
STT	=	Striation of myofibre in transverse section
T	=	Tegument

A



B



other. They contain irregularly arranged Z densities, overlapping thick and thin myofilaments, and small amounts of subsurface sarcoplasmic reticulum cisternae. The smooth muscle cells of the two forked terminal portions of the tail are also composed of two sets of muscle cells, perpendicularly arranged to each other. The thick filaments exhibit a repeating cross-period striation of 350 to 400 Å .

3.1.3 Cytoskeleton of the Schistosome Tegument

Scanning electron microscopy has revealed that the surface of the schistosome is irregular, spiny and has a characteristic pitted appearance (ridged and convoluted) (Miller, Tulloch & Kuntz, 1972). It is likely, though not proven, that the schistosome's tegument contains cytoskeletal structures. It has been shown that the effects of drug cytochalasin on cells was to inhibit action on contractile microfilament systems (Wessels, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn & Yamada, 1971) and endo- and exocytosis (secretion) (Allison, Davies & de Petris, 1971; Allison, 1972). Wilson & Barnes (1974b) incubated adult worms S. mansoni for 5h in medium containing cytochalasin [10 µg/ml cytochalasin in 1% DMSO (dimethylsulphoxide)]. They found that the cytochalasin induced collapse of the surface channels of the schistosome tegument. Their observations indicate that a microfilament system may be responsible for maintaining surface configuration.

The spine is the only tegumental structure that definitely contains cytoskeletal protein. The surfaces of cercariae and adult schistosomes are covered with spines. Their spines lie entirely within the tegument cytoplasm. The spines have been described on cercariae as recurved and dagger-shaped (Robson & Erasmus, 1970), long, thin, apically pointed, and basally rounded (Morris, 1971) about 1 um long (Hockley, 1968). The SEM has shown that spines are present over all the body surface with the following exceptions : around the mouth and the disc at the oral tip of the body bearing the lateral crescents with the openings of the acetabular ducts; the areas surrounding the oral sucker sensory papillae (Robson & Erasmus, 1970); on the aboral body collar (Hockley, 1968; Race, Martin, Moore & Larsh, 1971); and around the excretory duct pores at the furcal tips (Hockley, 1968). The spines are longer and more concentrated on the ventral sucker than on the body (Hockley, 1968). Schistosomula also have spines. On migrating stages, in addition to damaging host tissue, these surface projections probably ensure that locomotion through tissue is only in a forward direction (Robson & Erasmus, 1970;

Rifkin, 1971).

Smith et al. (1969) showed by TEM that the spines of adult schistosomes are closely associated with discoid granules, and paracrystalline in structure. Schmidt & Roberts (1981) reported that the spines consist of a "crystalline protein". The size of the male worm (1-1.5 cm long and 1 mm thick) and the size of the spines (2.5-3.0 μm in length) make it feasible to examine these structures by negative staining (uranyl acetate) in the electron microscope (Cohen, Reinhardt, Castellani, Norton & Stirewalt, 1982). Cohen et al. (1982) found that schistosome spines comprised actin filaments aligned in register axially and spaced laterally in a regular array. Optical diffraction patterns of selected areas of the spines showed strong reflections lying on a set of layer lines characteristic of the helical symmetry of actin. Similar features have been described in a large variety of actin bundles (De Rosier, Mandelkow, Silliman, Tilney & Kane, 1977; De Rosier & Tilney, 1982). Thin sections (TEM) of the spines showed the hexagonal packing of the actin filaments cross-linked with an additional protein (Cohen et al. 1982).

The surface spines of schistosomes appear to be similar to those of other parasitic flukes (trematodes) such as Fasciola hepatica which have closely related tegument morphology and development. The tapeworms (cestodes) are covered with very small, slender, flexible microvilli (microtriches) which also contain hexagonally packed actin filaments (Lumsden & Specian, 1980).

3.1.4 The Objectives of the Study

The objective of the work described in this Chapter was to investigate the specificity of schistosome antigens of MABs not reacting with adult worm tegument. The work consists of 3 parts. Parasites at various stages of development (cercaria, 3h skin-transformed schistosomulum, 5-day-lung worm, 21-day-liver worm and adult worm) were used with the indirect immunofluorescence technique. In the first part, the antigens in the schistosome muscle were examined using specific (muscle) MABs. In the second part, the cross-reactivity of the (muscle) MABs with the muscle of insects (fly & Bumble bee) and vertebrate (rabbit) was studied. In the final part, those MABs which reacted neither with tegumental nor muscle antigens of adult worm S. mansoni were investigated.

3.2 MATERIALS & METHODS

3.2.1 Monoclonal Antibodies Reacting with the Muscle of Schistosomes (see Section 2.3.4.2 in Chapter 2)

The indirect immunofluorescence technique was used to localise target antigens in schistosome muscle at the light microscope level (see Sections 2.2.4 & 2.2.5 in Chapter 2). Frozen sections of cercariae, 3h skin-transformed schistosomula and adult worms, and intact parasites were used as antigen preparations (see Materials & Methods in Chapter 2). MABs were used as primary antibodies. The secondary antibody was RAM/Ig/FITC.

The 11 MABs which recognized the muscle of the parasite were :

G3.1	G3.4	G3.5	G3.8	G5.1	G5.2
G5.4	G5.7	G5.12	M7.8	M7.9	

3.2.2 Cross-Reactivity of MABs With Muscle of Insect, and Rabbit Uterus

All 11 muscle MABs were also tested with frozen sections of Bumble bee (Bombus terrestris) dorsal longitudinal flight muscle (kindly supplied by J. Molloy), rabbit uterus (kindly supplied by Dr. H. Leese) and glycerol-extracted fibrillar flight muscle of blowfly (Calliphora erythrocephala) (kindly supplied by Dr. J. Sparrow). Glycerol extraction (-20°C in 50% glycerol for 24 h) removes most of the sarcoplasmic material e.g. mitochondria, T-system and reticulum, and muscle fibre membrane systems, leaving only the contractile structures (Ashhurst, 1969). These experiments were carried out in order to examine the cross reactivity of muscle antigens between schistosome, insect and vertebrate using the immunofluorescence technique (see Section 2.2.4 in Chapter 2).

The muscles of S. mansoni are largely of the smooth type (Silk & Spence, 1969). Uterus contains abundant smooth muscle (Rhodin, 1977). Mouse uterus could not be used because it contains mouse immunoglobulins which give false positive results since the MABs under investigation were raised in mice.

3.2.3 Larval Specific MABs and MABs Reacting with Internal Antigens (see Section 2.3.4.2 in Chapter 2)

Two MABs (G3.3 & G3.10) reacted with larval stages of the parasite only. Four MABs (G5.8, M7.1, M7.2 & M7.7) reacted with larval and adult stages, but not adult worm tegument or muscle. Frozen sections and intact parasites were used to localise the antigens by the indirect immunofluorescence method (see Materials & Methods in Chapter 2).

3.3 RESULTS

3.3.1 Monoclonal Antibodies Reacting With the Muscle of Schistosomes

None of the 11 muscle MABs reacted with the surface of intact parasites (cercaria, 3h schistosomulum, lung worm & liver worm). Positive fluorescence was detected on the muscle cells of acetone-fixed sections of cercaria, 3h schistosomulum and adult worm. The results are summarised as follows :-

Adult worm sections : Fluorescent label was observed in the cytoplasm of the muscle cells beneath the tegument and in the parenchymal tissue as shown by most MABs (G3.1, G3.4, G3.5, G3.8, G5.1, G5.2, G5.4, G5.7, G5.12 & M7.8) (Figs. 3.1-3.5). MAB M7.9 was an exception, reacting with the muscle membrane of the muscle fibres; the cytoplasm of the myofibres was unlabelled (Fig. 3.6). In the female worm the muscle surrounding the gut was distinctly labelled by most of the MABs (Fig. 3.7). Immunofluorescent label was also observed over the nuclei which were scattered throughout the worm parenchymal tissues as shown particularly by MAB G5.2 (Fig. 3.8).

Cercarial sections : Fluorescent antibodies bound to the muscle of cercariae in different patterns. For examples, MAB G5.1 bound to the subtegumental muscle layers of the body wall and appeared as a thick line in longitudinal section (Fig. 3.9). The muscles in the head capsule and parenchyma were also labelled. In transverse sections of tail, fluorescent label was seen in the four lateral longitudinal muscle blocks and had a filamentous appearance (Fig. 3.10). In longitudinal sections of tail, distinct striations of the peripheral muscle was fluorescently labelled (Fig. 3.11).

MAB G3.8 bound to the muscle cells of the body wall and the head capsule, and the muscle appeared as individual blocks (Fig. 3.12). MAB M7.8 reacted strongly with the myofibres of the cercarial body, which had a filamentous appearance (Fig. 3.13). Cross-striated muscle was clearly seen in the longitudinal sections of tail (Fig. 3.14).

MAB M7.9 reacted with the muscle membrane of the adult worm binding to the muscles of the body wall and to the small discrete muscle cells of the parenchyma. The binding appeared as fine dots. Strong specific fluorescence was also seen in the four lateral longitudinal muscle blocks in transverse sections of the cercarial tail (Fig. 3.15).

3h Schistosomular sections : Fluorescent staining patterns in sections of 3h schistosomulum were as varied as those observed in cercarial sections. For example, the muscle cells were fluorescently labelled and appeared as a thick layer at the periphery of the schistosomular body in longitudinal section (Fig. 3.16). In transverse sections, the muscle cells at the periphery of the body appeared as a thick band; individual myofibres were also observed (Fig. 3.17). The muscle sheath around the digestive tract of the parasite was positive (Fig. 3.18). MAB M7.8 bound to the muscle of schistosomular body in an irregular dotted pattern. The myofibres in the head capsule were clearly labelled (Fig. 3.19). MAB M7.9 bound to the muscle cells giving a dotted and filamentous appearance (Fig. 3.20).

No detectable fluorescence was observed on sections of cercaria, 3h schistosomulum and adult worm when NMS and NS-1 ascites were used.

3.3.2 Cross-Reactivity of MABs With Muscle of Insect, and Rabbit Uterus

The specificities of the 11 MABs for muscle of insect and rabbit (vertebrate) are compared with those for S. mansoni (Table 3.1).

The blowfly (Calliphora erythrocephala) : All the 11 MABs reacted with sarcomeric proteins of isolated myofibrils from flight muscle which had been previously extracted with glycerol. Three different staining patterns were observed. Nine MABs (G3.1, G3.4, G3.5, G3.8, G5.1, G5.2, G5.4, G5.7 & G5.12) gave similar staining patterns as shown in Fig 3.21. Sharp, bright bands of fluorescence was seen periodically along the myofibrils. MAB M7.9 gave similar staining periodic pattern but the distance between bands was wider than those described above (Fig. 3.22). MAB M7.8 gave a different staining pattern. The bands were wider and the distances between bands were narrower than those obtained with other MABs (Figure not shown). NS-1 ascites gave negative result.

Bumble bee (Bombus terrestris) : Six MABs (G3.1, G5.1, G5.2, G5.4, G5.7 & G5.12) reacted with proteins in the sarcomere of myofibres of Bumble bee flight muscle. Fluorescent staining was in a periodic pattern (Fig. 3.23). MAB G3.5 reacted with protein in the membrane of myofibrils. Periodic staining of bands was not observed (Fig. 3.24). Four MABs (G3.4, G3.8, M7.8 & M7.9) did not react with frozen sections of bee flight muscle. NS-1 ascites gave negative result.

Table 3.1 Cross-Reactivity of MABs With Fibrillar Flight Muscle of Blowfly (Calliphora erythrocephala), Bumble Bee (Bombus terrestris), and Rabbit Uterus.

MABs	Fluorescent Staining Pattern			
	S. mansoni	Blowfly flight muscle (glycerol-extracted)	Bumble bee flight muscle (frozen section)	Rabbit uterus (frozen section)
G3.1	Myofibre	Sarcomeric protein	Sarcomeric protein	Myofibre
G3.4	"	"	(-)	(-)
G3.5	"	"	Muscle membrane	(-)
G3.8	"	"	(-)	Myofibre
G5.1	"	"	Sarcomeric protein	"
G5.2	"	"	"	"
G5.4	"	"	"	"
G5.7	"	"	"	"
G5.12	"	"	"	Glandduct epithelium
M7.8	"	Thick or thin filament	(-)	Myofibre
M7.9	Muscle membrane	Sarcomeric protein	(-)	(-)
NS-1	(-)	(-)	(-)	(-)

Rabbit uterus : Seven MABs (G3.1, G3.8, G5.1, G5.2, G5.4, G5.7 & M7.8) reacted with the myofibres of frozen sections of rabbit uterus (smooth muscle) (Fig. 3.25). Surprisingly, MAB G5.12 predominantly recognized the gland duct epithelium of the rabbit uterus. The membrane of myofibres was also positive (Fig. 3.26). Three MABs (G3.4, G3.5 & M7.9) gave negative results (Figure not shown). No detectable fluorescence was observed when NS-1 ascites was used as a primary antibody.

3.3.3 Larval Stage-Specific MABs

Two MABs reacted only with larval stages of the parasite i.e. MABs G3.3 & G3.10. These MABs bound to the sections of cercaria and 3h schistosomulum, but not the adult worm (Table 3.2).

Cercarial sections : Negative and weak positive results were obtained using MAB culture supernatants. MABs G3.3 & G3.10 ascites gave positive fluorescence in the tegument of the cercarial body and tail. The tegument (4+) appeared as a very bright thin line (Fig. 3.27 & 3.28). Internal structures of the body (2+) were also fluorescently labelled but weaker than the tegument. Internal tissues of sections of tail were negative. Both MABs gave similar staining patterns on acetone and PF-fixed sections.

3h Schistosomular sections : MABs G3.3 & G3.10 bound to sections of 3h schistosomula. Positive fluorescence (3+) was confined to the tegument and the periphery of most parasites. Internal tissues were weakly labelled or negative. The patterns of fluorescence obtained were the same with both fixatives.

Intact cercariae & 3h schistosomula : MABs G3.3 & G3.10 did not bind to the surface of cercariae and 3h schistosomula. Some cercariae exhibited positive fluorescence at the apical and posterior ends, similar to those in Fig. 2.35. Some apical ends of 3h schistosomula were also brightly fluorescent.

Intact 21-day-liver worms : The binding of MABs G3.3 & G3.10 to the parasites' surface was very weak and showed a patchy appearance which was similar to that observed with most MABs.

Table 3.2 Specificity of MABs Recognizing Schistosome Antigens, But not Adult Worm Tegument By Immunofluorescence.

MABS	Intact cercaria	Intact 3h sch'lum	Cercarial section	3h Sch'lar section	Adult worm section
G3.3 G3.10	(-)	(-)	Tegument	Tegument	(-)
G5.8 M7.1 M7.2 M7.7	(-)	(-)	Whole section except gland cells	Internal structure	Parenchyma

3h sch'lum = 3h schistosomulum

3.3.4 MABs Reacting with Internal Structures

There were 4 MABs which reacted with internal tissues of parasites but not the adult worm tegument or muscle (see Section 2.3.4.2). They were MABs G5.8, M7.1, M7.2 & M7.7.

Adult worm sections : All MABs gave weak positive fluorescence on the entire section of adult worm fixed in acetone. The parenchymal tissues of the worm were fluorescently labelled with MAB M7.2 on PF-fixed sections. The parenchyma in the tubercles was also positive. The tegument and the muscle layers were unlabelled. The interstitial material around the muscle fibres was weakly positive. PF-fixed sections reacted with MABs G5.8, M7.1 & M7.7 were not examined.

Cercarial sections : Weak positive reactions with these MABs were obtained on the entire section fixed in acetone. Stronger reactions were obtained on PF-fixed sections. Some internal structures were very bright, but the gland cells were negative. Sections of tail were also positive.

3h Schistosomular sections : Fluorescent label (3+) was seen in internal structures; the tegument was negative. Weak labelling on the entire section was also observed. All MABs gave similar results with both fixatives.

Intact cercariae & 3h schistosomula : Negative results were obtained on the surface of intact parasites except the apical and posterior ends of some cercarial bodies which were slightly positive.

Intact cultured 21-day-liver worms : A similar staining pattern was observed, as described for most other MABs. Antibodies bound to the parasites' surface in a patchy pattern. The reactivity was strongest with MAB M7.1 (3+) and weaker with M7.2 (1+), M7.7 (2+) and G5.8 (weak+).

3.4 DISCUSSION

3.4.1 Monoclonal Antibodies Reacting With the Muscle of Schistosome

The MABs used in the present study were derived by vaccinating mice with highly enriched fractions of worm tegument i.e. membrane (G) and microvesicular fraction (M).

The fact that the 11 MABs reacted with the muscle of the schistosome implies 2 possibilities. Firstly, the membrane preparations contained a significant quantity of muscle proteins. Secondly, the tegument contained cytoskeletal protein shared with muscle cells. The results obtained in the present study showed that none of the 11 MABs reacted with the tegument. It seems unlikely, therefore, that the tegument possessed a cytoskeleton. On the other hand, there may be cytoskeletal components in the tegument, but the proteins are present in much higher concentration in the muscle. Therefore, cytoskeletal proteins could not be detected by the immunofluorescence technique. Alternatively, the cytoskeleton may not have been preserved by the fixatives used. Since the results obtained from these 11 MABs did not provide evidence of cytoskeleton in the tegument, it is concluded that the membrane preparation contained significant quantities of muscle protein.

MAB G5.2 stained the nuclei of the worm parenchyma. This may indicate that a cytoskeleton supported the nuclei in the centre of the cell. Wolosewick & Porter (1979) have shown that the microtrabecular lattice is the nonrandom structure of the cytoplasmic ground substance of living cultured cells. They have suggested that this lattice supports or contains the organelles of the cell with the possible exception of mitochondria.

In the present study, most of the MABs stained the cytoplasmic components of the myofibrils (the proteins in the myofibrils) in the sections of adult worm. The cytoplasmic components (contractile elements) of the myofibril could be actin, myosin or other proteins. In contrast, MAB M7.9 stained the membrane of the myofibres.

It has been shown that the musculature of schistosomes is non-striated (Lumsden & Foor, 1968; Silk & Spence, 1969) except in the cercarial tail in which the longitudinal, caudal fibres possess transverse striations. These striations reflect the presence of regular transverse arrays of sarcoplasmic tubules, interdigitating with transversely extended dense bodies, which are continuous with the thin myofilaments. Most of the sarcomere area contains both thick and thin filaments (Nuttman, 1974). There are up to 10 or 12 thin

filaments surrounding each thick filament (Nuttman, 1974). Dense bodies are the functional equivalent of the Z disc of vertebrate muscle. No true transverse tubule (T) system has been found in. S. mansoni (Nuttman, 1974).

MAB M7.9 reacted strongly with the four lateral muscle blocks in the tail. In the longitudinal section of tail, striations could be seen using MAB M7.8. Nuttman (1974) has shown that there are six distinct blocks of longitudinal muscle in the tail stem, lying immediately beneath the circular muscle layer. These do not pass directly through the tail base into the body. Transverse striations are present in the four lateral longitudinal muscle blocks; they are absent from the mid-dorsal and mid-ventral blocks. The lateral fibre contractile regions are distinctive in the structural periodicity.

The muscle in the body of cercariae and 3h schistosomula is non-striated (smooth muscle). MABs which gave positive fluorescence in an individual muscle block as shown by MAB G3.8 were probably antibodies to thick filament (myosin). MABs which gave a filamentous staining appearance were probably antibodies to thin filament (actin). MAB M7.9 gave a fine dotted pattern in both sections of cercarial body and 3h schistosomula. In the larvae, the target antigen could be a membrane protein in the myofibres, as observed in the adult worm. Unfortunately, the muscle fibres of cercaria and 3h schistosomulum are very small, and so the location of fluorescent label within the muscle structure could not be resolved.

Throughout the course of my study and the work in our laboratory, our major interest was the schistosome tegumental antigens which would play an important role in protective immunity. Consequently, the molecular targets of the 11 muscle MABs were not studied by immunoblotting. All I can conclude from the results obtained in the present study is that the 11 muscle MABs possibly react with the contractile protein i.e. actin, myosin or other proteins in the myofibrils of schistosome.

3.4.2 Cross-Reactivity of MABs With Muscle of Insect and Vertebrate

There was cross-reactivity between the muscle of S. mansoni, insect and vertebrate (rabbit). The muscle of S. mansoni shared common antigenicity with the muscle of the Blowfly as demonstrated by all the 11 MABs and the Bumble bee (7 MABs). It has been shown that all insect flight muscles are cross-striated (similar to vertebrate striated muscle) (Ashhurst,1967). For example, comparable transverse striation patterns with A-, I-, and H-bands and Z- and M-lines are

present. The I-band is much narrower (16-18% of sarcomere length) and less obvious than in vertebrate or other insect flight muscle types. Z- and M-lines and an H-band are usually clearly visible.

Myofibrillar proteins of asynchronous insect flight muscle contain all the major proteins of a striated muscle and a large number of other components as well. Myosin is the major component of insect flight muscle thick filaments (Zebe, 1966; Bullard & Reedy, 1973). Insect myosin has a molecular weight similar to that of rabbit myosin and has the same complement of heavy and light chains. Two heavy chain peptides of approximately 200 kDa each plus light chains, to make a 490 kDa molecule (Bullard & Reedy, 1973). The light chains have a different molecular weight from the rabbit ones as judged by SDS-PAGE; the heaviest has a molecular weight of 30 kDa (Bullard, Dabrowska & Winkelman, 1973).

The arrangement of myosin molecules in the natural filaments is different from that in vertebrate striated muscle. The filaments are thicker (Reedy, 1968) and contain more myosin per unit length, nearly twice that present in the vertebrate striated muscle thick filament (Tregear & Squire, 1973). The enzymatic properties appear qualitatively similar to those of rabbit smooth muscle myosin. The thin filaments of insect flight muscle have not been studied in detail. They contain actin in the usual double-helical conformation (Miller & Tregear, 1972) and probably also tropomyosin and troponin (Lehman, Kendrick-Jones & Szent-Gyorgyi, 1972). The Z line (of insect) contains alpha-actinin and Zeelin (Bullard & Sainsbury, 1977).

The results obtained from the present study showed that most MABs reacted with sarcomeric proteins of insect flight myofibrils. The fluorescent staining pattern showed a distinct structural periodicity of transverse striations. The striations could possibly be the Z disc or M-line of the sarcomere. MAB M7.8 gave a different staining pattern to that obtained with other MABs. The fluorescent staining bands were wider than the unstained bands in between. The target antigen of MAB M7.8 could possibly be in the thick (myosin) or thin (actin) filaments.

In the present study, the myofibrils were examined with epifluorescent optics, but not phase contrast microscopy. Therefore, it was difficult to specify the exact location of the target epitopes of the MABs.

Most of the MABs reacted with the cytoplasmic components of myofibres of the rabbit uterus. In contrast, MAB G5.12 reacted with the uterine gland duct epithelium and the nuclei of the myofibres. The muscle of the uterus is of a smooth type. Therefore, no cross-striations could be observed in the sections of uterus. The

fluorescent staining was diffuse throughout the entire section. The nuclei appeared as open circles in sections of uterus stained with MAB G5.12 similar to those described with MAB. G5.2 in sections of schistosome adult worm. This study demonstrated that the MABs cross-reacted with the muscle of schistosomes, insects and a vertebrate.

Myofibrillar proteins make up the major portion of the proteins in muscle cells. Muscle proteins have been studied using the fluorescent antibody technique. Pepe (1968) studied vertebrate skeletal muscle myofibrils using antimyosin antibody. He found that the fluorescent staining was restricted to the A band. Marshall, Holtzer, Finck & Pepe (1959), in addition to using myosin, also used the myosin fragments, heavy meromyosin (HMM) and light meromyosin (LMM) as immunogens. They found that whereas antimyosin stained the entire A band, anti-HMM stained more brightly in the middle of the A band (nonoverlap region), and anti-LMM stained more brightly at the edges of the A band near each A-I junction.

Pepe (1966) showed that the M band was made up of proteins other than myosin, actin, or tropomyosin. The first M protein to be isolated from skeletal muscle fibrils was termed "M-substance" (Masaki, Takaiti & Ebashi, 1968). Antibody to this protein stained only the M band. Preparation of antiactin has presented problems because of its poor antigenicity. Fluorescent antiactin has been shown to stain the I band of skeletal myofibrils without staining the Z band (Groschel-Stewart, Ceurremans, Lehr, Mahlmeister & Paar, 1977; Herman & Pollard, 1979; Jockusch, Kelly, Meyer & Burger, 1978). In general, the staining appeared to be restricted to the I band and did not extend into the region of overlap between myosin and actin filaments. Fluorescent antitropomyosin and antitroponin have been shown to stain the I band of skeletal muscle myofibrils (Endo, Nonomura, Masaki, Ohtsuki & Ebashi, 1966; Pepe, 1966).

Alpha-actinin was first localised in the Z band by Masaki, Endo & Ebashi (1967) with fluorescent antibody staining. The cross-reactivity of protein in the Z disc of insect flight and vertebrate striated muscle has been shown by Bullard, Pringle & Sainsbury (1981). They found that there is a protein in the vertebrate (rat) Z disc that is antigenically similar to insect zeelin. Pringle (1972) showed that asynchronous flight muscles have an unusually large Z disc with a thickness twice that of the vertebrate Z disc.

3.4.3 Larval Specific MABs and MABs Reacting With Internal Antigens

As stated earlier, all 50 MABs used throughout the course of my study were prepared against adult worm tegument membrane. MABs G3.3 & G3.10 reacted with the tegument of frozen sections of cercariae and 3h schistosomula but not the adult worms. This observation indicated that the target antigens were present in the adult tegument but in lower concentration than in the larval stages. The amount of antigen molecules present in the adult could be insufficient to give a positive signal. Therefore, they could not be detected by the immunofluorescence technique. Alternatively, the antigens in the adult worms were less stable to fixation. Thus they may have been destroyed during preparation of specimen.

MABs G5.8, M7.1, M7.2 & M7.7 reacted with the internal structures of frozen sections of cercaria, 3h schistosomulum and adult worm. The results indicated that the schistosome membrane preparations were contaminated with internal structures or the antigens which were present in the tegument were also present at higher concentration in the parenchyma.

3.4.4 Muscle Proteins and Protective Immunity to S. mansoni

It has been shown by Pearce, James, Dalton, Barrall, Ramos, Strand & Sher (1986) that mice immunized intradermally with extracts of S. mansoni in combination with the adjuvant BCG are significantly protected against subsequent infection with living cercariae. These vaccinated animals produce antibodies predominantly against a single parasite protein of molecular weight 97 kDa (Sm-97). A complementary DNA that encodes about half of the Sm-97 molecule has been cloned and sequenced by Lanar, Pearce, James & Sher (1986). They found that the amino acid composition of the cloned gene and several properties of the native protein are similar to that of paramyosin (an alpha-helical protein that forms the core for myosin filaments in invertebrate muscle). They also found that antibodies to Sm-97 reacted with paramyosin isolated from S. mansoni adult worms as well as with a known paramyosin from molluscan muscle.

Paramyosin has been isolated from many invertebrate muscles (Winkelman, 1976) and in particular from asynchronous flight muscle of four different insect species (Bullard, Luke & Winkelman, 1973).

My investigations have shown that schistosome muscle proteins contain similar epitopes to those in insect and vertebrate. The antigens are tissue-specific rather than species-specific. This

suggests that it could be dangerous to use schistosome muscle protein e.g. paramyosin as a vaccine immunogen in man. Autoimmunity might develop because of the cross-reactivity between antigens in schistosome and vertebrate muscle. An example of the development of anti-parasite immunity producing cross-reactivity with self components has been reported in Chagas' disease. It has been shown that sera from patients with Trypanosoma cruzi infection contain antibodies that recognize mammalian vascular endothelial cells (Wilkin, Woodhams & Ribiero dos Santos, 1982), a component of peripheral nerve cells, possibly Schwann cells (Khoury, Ritacco, Cossio, Laguens, Szarfman, Diez & Arana, 1979), the plasma membrane of striated muscle and endothelial cells (Cossio, Diez, Szarfman, Kreutzer, Candiolo & Arana, 1974) and certain peripheral and central neurones (Ribeiro dos Santos, Marquez, Von Gal Furtado, Ramos de Oliveira, Martins & Koberle, 1979). Wood, Hudson, Jessell & Yamamoto (1982) have reported that a MAB, generated against mammalian dorsal root ganglia, exhibits a unique specificity for subpopulations of central and peripheral neurones and also recognizes an antigenic determinant on the protozoan Trypanosoma cruzi.

Pearce, James, Dalton, Barrall, Ramos, Strand & Sher (1986) used MAB 1A6 (MAB against paramyosin) to localise Sm-97 in sections of adult schistosomes. They found that regions of brightest specific fluorescence were just below the surface of the parasite, either in subtegumental cells or possibly within the muscle layers or tegument itself, and also in a region below the gut syncytium of both male and female worms. The results in the present study showed that all the 11 muscle MABs reacted with the muscle and not the tegumental surface of the parasite.

How an antibody to muscle proteins could produce protective immunity in the host is still unclear. However, Pearce et al. (1986) demonstrated that vaccination of mice intradermally (i.d.) with purified Sm-97 elicited delayed-type hypersensitivity. They suggested that the Sm-97 molecule was capable of evoking cell-mediated immune (CMI) responses, and that the CMI rather than antibody provides the effector mechanism of resistance in i.d. vaccinated mice. This does not resolve the question of how an immune response directed against muscle antigens, not expressed at the parasite surface, could mediate protective immunity.

CHAPTER 4

LOCALISATION OF SCHISTOSOME ANTIGENS AT THE ULTRASTRUCTURAL LEVEL

4.1 INTRODUCTION

4.1.1 Immunolabelling for Electron Microscopy

The localisation of tissue antigens by electron microscope immunocytochemistry is a compromise between retaining sufficient antigen in the specimen to bind the antibody and maintaining adequate ultrastructural detail of the tissue for identification of the site of the immune reactions. The immunolabelling can be done before or after the tissue has been embedded; these are the so-called pre-embedding and post-embedding techniques, respectively.

A). Pre-Embedding labelling Techniques

The tissue is usually fixed with an aldehyde to prevent internalization of the reagents, and then incubated with antibody followed by a marker which renders the antibody visible at the electron microscope level. Thus the distribution of antigen can be visualised. The tissue is further fixed and contrasted with osmium tetroxide (a highly effective membrane contrasting agent) and uranyl acetate. It is embedded in resin and then sectioned and prepared for electron microscopical examination using standard procedures. This technique permits the retention of good ultrastructure and good contrast, but it is limited to the localisation of external cell surface antigens (de Waele, 1984). Localisation of internal antigens by this technique needs permeabilization of the outer cell membranes with detergents such as saponin (Bohn, 1978). or Triton X (de Mey, Moeremans, Geuens, Nuydens & De Brabander, 1981; Yang, Lieska, Goldman & Goldman, 1985) to allow antibody and probe access to cellular components.

The pre-embedding approach also has two important advantages. Firstly, antigens may be destroyed or obscured by the dehydration and embedding processes required for post-embedding techniques. So the pre-embedding procedure is useful when an antigen is present in low concentrations or an antigen is sentitive to embedding. Secondly, many antigens are very sensitive to osmium tetroxide. In post-embedding procedures, osmium is normally avoided, but it can be added after pre-embedding immunostaining prior to dehydration and embedding. Therefore, the pre-embedding procedure is the method of choice when osmium is necessary in order to obtain adequate membrane fixation and contrast (Priestley, 1984).

Cells generally contain a high concentration of protein. If the protein molecules are rigidly cross-linked, antibodies will not be

able to penetrate into the cell interior. Therefore, the fixative must be in an appropriate dilution to enable a large portion of the cytosolic protein to be extracted. In pre-embedding ultrastructural immunocytochemistry, immunostaining is carried out on relatively thick tissue sections. There is little penetration of antibodies into thick fixed tissue slices, because the fixative induces cross-linking. Membranes can be made permeable by freeze-thawing (Morgan, Hsu, Rifkind, Knox & Rose, 1961), detergent treatment (Bohn, 1978; Ohtsuki, Manzi, Palade & Jamieson, 1978) or vibratome sectioning (Pickel, Joh & Reis, 1975) of the fixed material. However, all these procedures can alter or disrupt the cellular structure and introduce non-uniform penetration of immunological reagents into cells or allow antigen to diffuse out. False negative results can be obtained (Beesley, 1985). The other problem with the pre-embedding immunostaining method is that the secretory granules and the dense area of intercellular junctions are not accessible to antibodies, unless they have been sectioned first. These are disadvantages with pre-embedding staining.
There are some variables in pre-embedding immunocytochemistry :-

i). Tissue Fixation

Fixatives inducing intra- and inter-molecular cross-links will decrease antigenicity and inhibit antibody penetration. This is particularly a problem with bifunctional fixatives such as glutaraldehyde. Higher concentrations of glutaraldehyde can be used for post-embedding staining than are used in pre-embedding methods. This suggests that the fixative has an effect on antibody access rather than on antigenicity. Various model systems have been used to assess the effects of the fixative on antigenicity (Kraehenbuhl & Jamieson, 1974; Larsson, 1981; Schipper & Tilders, 1983) but there is no evidence for assessing the effects of fixation on antibody penetration. When higher concentrations of glutaraldehyde (1-5%) are used, the sections should be washed thoroughly to remove unreacted fixative. This is to prevent non-specific staining. Most workers have used a mixture of glutaraldehyde and paraformaldehyde for pre-embedding immunocytochemistry. A particular antigen may require different proportions of fixative in different tissues or even in different areas of the same tissue (Priestley, Somogyi & Cuello, 1982; Somogyi, Priestley, Cuello, Smith & Bolam, 1982).

ii). Tissue Sectioning Penetration of Immunoreagents

The vibratome is the most suitable instrument for cutting tissue

slices with minimal damage to ultrastructure. Detergent (Triton X-100) is commonly used to enhance penetration. This causes more extensive destruction of membranes than saponin (Ohtsuki et al., 1978). Pre-embedding immunocytochemistry can be varied depending on the particular antigen, primary antibody and biological system under study.

B). Post-Embedding Labelling Techniques

These are the most popular of all the immunolabelling techniques. Ultrathin sections of tissue are mounted on nickel or gold grids and immunostained with antibody and probe. Every antigen that is exposed on the cut surface of the section, whether internal or external to the cell, is accessible to the antibody. Post-embedding methods also have some problems. Tissue must be fixed otherwise extraction will take place during processing and embedding. Post-fixation in osmium tetroxide is avoided because it denatures many antigenic determinants. Therefore, more reactive aldehydes, higher concentrations of fixative, or longer times of fixation are required. The cross-linking of protein has to be minimised, leaving them recognizable to antibodies.

Labelling technique on thin sections of embedded material have been developed using different plastics for embedding (Leduc, Scott & Avrameas, 1969; Sternberger & Cuculius, 1969; Kawarai & Nakane, 1970). Some example of embedding materials are polyethylene glycol (Mazurkiewicz & Nakane, 1972), glutaraldehyde cross-linked serum albumin (McLean & Singer, 1970) or concentrated sucrose solutions for frozen thin sections (Tokuyasu, 1973; Tokuyasu & Singer, 1976).

4.1.2 Resins for Immunocytochemistry

The choice of resins for electron immunocytochemistry is important in order to preserve antigenicity of the tissue and access of the antibody to the antigen. The chemical reactivity of the cured resin, the method of curing and the degree of cross-linking have to be considered (Causton, 1984). Investigators must adjust the embedding techniques to suit their tissues and staining methods so that minimum process times and optimum degrees of cross-linking can be achieved.

Epoxy resins and acrylic resins give the best results and flexibility of technique. However, antigens can interact with the epoxy resin. The epoxy group is very reactive, capable of ring opening addition reactions to many organic moieties, and this might be the cause of reduces antigenicity. The commercial acrylic embedding medium lowicryl (Lowicryl is registered Trade Mark of Chemische Werke

Lowi GmbH, D-8264, Waldkraiburg, West Germany) is a hydrophilic resin. The tissue is embedded at a temperature of -30°C or below. Since this minimises the denaturation and extraction of protein during dehydration and embedding, the procedure is particularly suitable for immunocytochemistry (Carlemalm, Garavito & Villiger, 1982).

The acrylic-methacrylic ester class of resins have specific characteristics (Carlemalm et al., 1982).

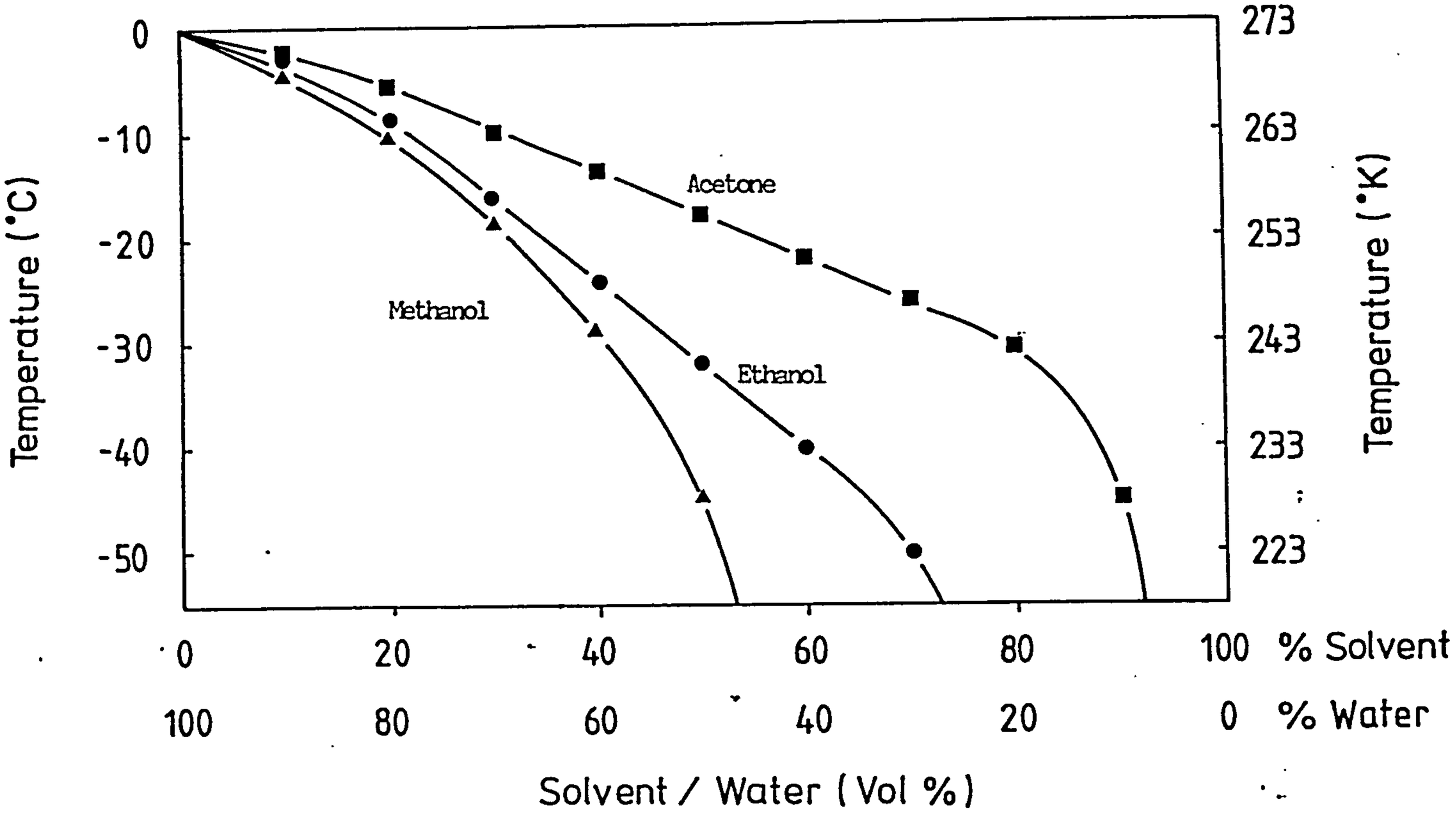
- low monomer molecular weights
- low freezing points
- relatively temperature-independent polymerization by UV-irradiation.

4.1.3 K4M : Low Temperature Embedding Technique

Lowicryl K4M and HM20 are highly crosslinked acrylate- and methacrylate-based embedding media, specially formulated to provide low viscosity at low temperatures.

K4M is a polar (hydrophilic) resin which can be used at -35°C. HM20 is non-polar (hydrophobic) and can be used at temperature as low as -70°C. Both resins are photopolymerized by long wavelength (360 nm) ultraviolet (UV) light at low temperatures or can be chemically polymerized at +60°C. The hydrophilic properties of K4M provide some advantages. During dehydration and infiltration the specimens can be kept in a partially hydrated state. K4M can be polymerized with up to 5% (w/w) water in the block (Carlemalm et al., 1982; Kellenberger, Carlemalm, Villiger, Roth & Garavito, 1980). Furthermore, K4M is useful for immunolabelling of sections using specific antisera or lectins (Roth, 1982; Roth, Bendayan, Carlemalm, Villiger & Garavito, 1981). K4M gives better structural preservation (Roth et al., 1981), impoves preservation of antigenicity (Bendayan & Shore, 1982; Roth, 1982; Roth & Berger, 1982) and gives significantly lower background labelling than HM20.

There are two different ways for embedding in these resins. One way is to fix the specimen at room temperature and gradually decrease the temperature during dehydration, as the specimen is exposed to an ascending series of concentrations of the dehydrating agent (Text Figure 4.1) (Armbruster, Carlemalm, Chiovetti, Garavito, Hobot, Kellenberger & Villiger, 1982). The other way is to use freeze-substitution. This is the rapid freezing of specimens followed by substitution of ice in the tissue by an organic solvent at temperatures (180°K) which inhibit ice-crystal damage. The substituted specimen is subsequently infiltrated with resin and



Text Figure 4.1

Freezing point of dehydrating agents as a function of concentration (Redrawn from Carlemalm, unpublished).

polymerized at low temperature (230°K) (Hunziker, Herrmann, Schenk, Mueller & Moor, 1984; Hunziker & Schenk, 1984).

The progressive lowering of temperature (PLT) technique involves stepwise reductions in temperature, concomitant with increasing concentration of dehydrating agent (Armbruster et al., 1982; Armbruster, Garavito & Kellenberger, 1983; Carlemalm et al., 1982). A temperature is selected at each step which is just above the freezing point for the concentration used in the previous stage. This corresponds to the concentration of the dehydrating agent actually in the tissue block as it is introduced into the next higher concentration in the series (Text Figure 4.1).

This class of resins includes acrylate and methacrylate compounds which form a vinyl type of carbon-carbon backbone during polymerization. The methacrylate resins are formed from a free radical addition reaction to the monomers (Text Figure 4.2a) to themselves in a unidirectional manner (Text Figure 4.2d). In mixed systems, acrylate compounds (Text Figure 4.2b) can also participate in the polymerization reaction but with different rate constants. The side groups (R) can be varied to give a number of different properties to the liquid mixture of monomers and the fully polymerized resin.

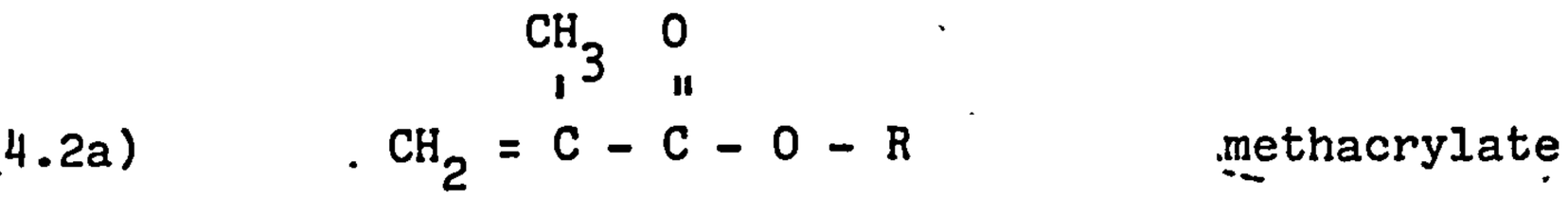
The mixtures of the K4M resin that I used consisted of the following components.

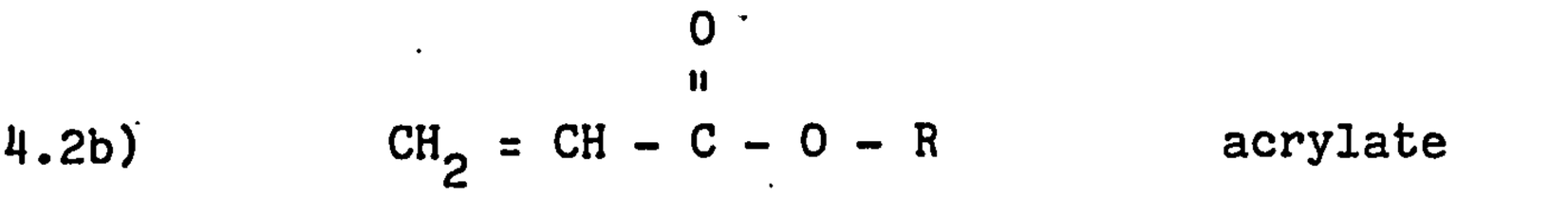
- Hydroxypropyl methacrylate, the main component.
- Hydroxyethyl acrylate to balance the brittleness.
- n-Hexyl methacrylate to remove excess hydrophilicity.
- Triethylene glycol dimethacrylate, crosslinker gives the resin more physical integrity which improves the sectioning properties of the resin, and electron beam stability.
- Benzoyl methylether as an initiator.

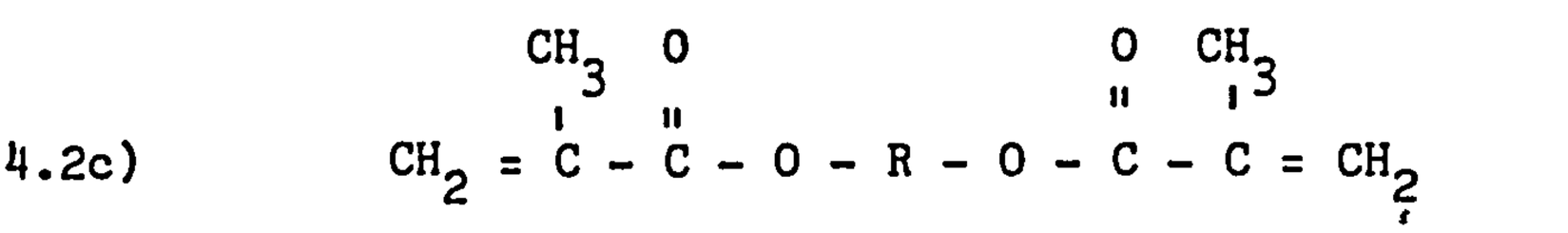
The free radical reaction initiated by UV-irradiation runs uniformly and quickly at temperatures between 303 and 223°K (40 to -50°C). The resin is very mobile even at low temperatures and is very suitable for low temperature antigen preservation. The disadvantages appear to be the instability of these resins in the electron beam and specimen damage (Carlemalm et al., 1982).

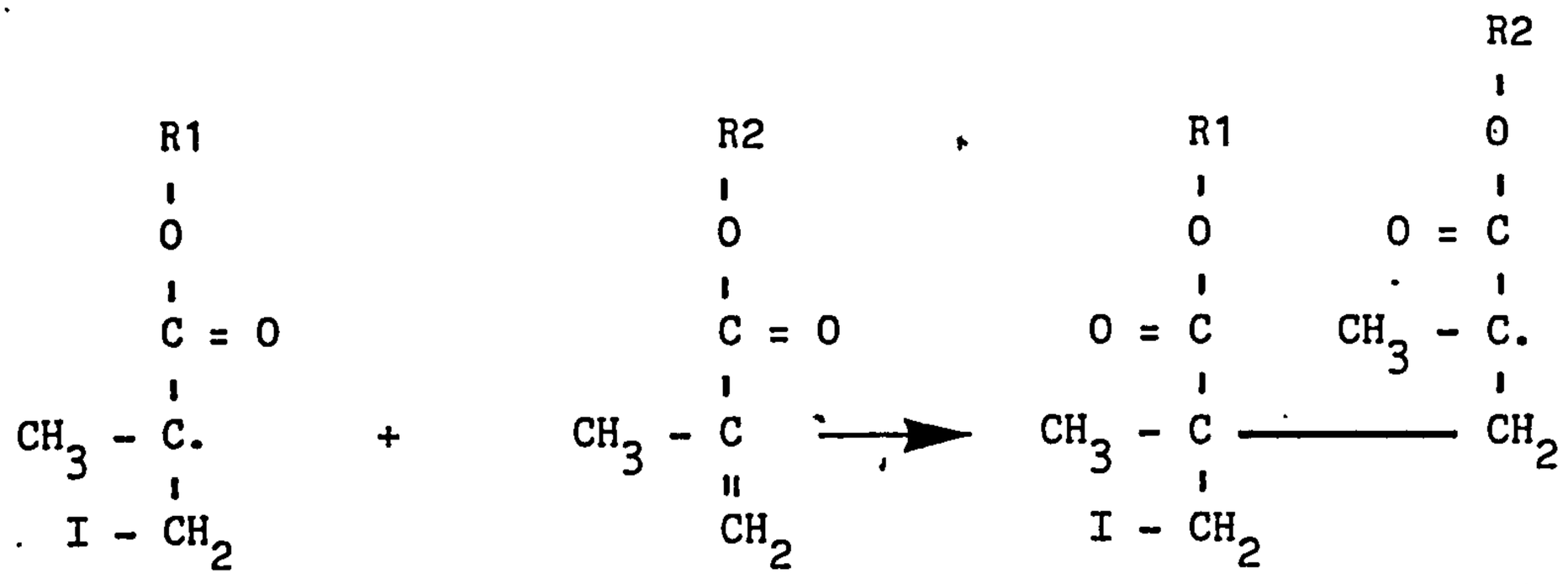
4.1.4 Application of Cryoultramicrotomy to Immunocytochemistry

Chemical fixation, dehydration and embedding cause distortion of bilogic activity, lead to extraction or dislocation of many soluble components, and limit to a great extent chemical and immunologic

4.2a) 
methacrylate

4.2b) 
acrylate

4.2c) 
dimethylacrylate crosslinker

4.2d) 

Text Figure 4.2

A schematic formula showing the basic monomers and the reaction pathways for polymerization. R (in 4.2a, 4.2b) represents the side group in the monomer. R (in 4.2c) represents a dialcohol linker between the two methacrylate groups. In 4.2d, a methacrylate monomer has been activated with an initiator (I) to form an unpaired electron at C2. This radical now attacks another monomer to begin the growth of the polymer chain (Carlemalm et al., 1982).

reactions. Cryoultramicrotomy was developed to minimise these undesirable effects. Frozen ultrathin sections of unfixed specimens are best for the study of the localisation of diffusible substances by immunocytochemistry. However, chemically fixed and frozen sections have provided significant information in the demonstration of intracellular antigens. Prefixation and cryoprotection prior to freezing allow localisation of intracellular antigens.

Specimens are subjected to rapid freezing to minimise the size of ice crystals. Visible ice crystals will be absent when the specimen is smaller than 0.3 mm in diameter (Bachmann & Schmitt, 1971). However, it is not always practical to obtain unfixed tissue blocks smaller than 0.3 mm in diameter without extensive mechanical damage. The smaller the size of the tissue block, the longer the proportion of the block showing the mechanical damage.

In cryoultramicrotomy, ice serves as the embedding material by providing the rigidity that is required for cutting tissues or isolated cells into ultrathin sections. Sections of the frozen specimen regain their original hydrated state and are immunostained after thawing at room temperature. This method generally provides a better accessibility of antigens to antibodies and also allows the examination of the specimens more quickly than pre- and post-embedding methods using resins (Tokuyasu,1984; Tokuyasu, 1986). This approach has been reviewed by Tokuyasu (1980), Singer, Tokuyasu, Dutton & Chen (1982), Griffiths, Simons, Warren & Tokuyasu (1983), Slot & Geuze (1983), Ivanov, Pleskin, Sabatini & Rindler (1984), and Tokuyasu (1984).

4.1.4.1 Concept of Cryoultramicrotomy (Tokuyasu, 1986)

A). Fixation

Fixation should preserve structures of interest and immobilize the target antigen but it should not destroy antigenicity or render the antigen inaccessible to the antibody. An overall preservation of cell structure does not necessarily mean the immobilization of a specific antigen (Tokuyasu, Dutton, Geiger & Singer, 1981). Many specimens fixed with 0.1-2% glutaraldehyde or in combination with 2-4% formaldehyde for 0.5-1 hour are well preserved. However, the antigenicity of some proteins may be destroyed after a short fixation with 0.1-0.2% glutaraldehyde. In this case, a solution of formaldehyde should be used (Kyte, 1976a; Geuze, Slot, Strous, Hasilik & Von Figura, 1984).

Concentration of formaldehyde up to 8% can be used and the fixation period can be from several hours to overnight. The cross-linked condition may be reversed when the fixed specimen is exposed to buffer for a long time (Tokuyasu & Singer, 1976). Therefore, formaldehyde 0.3-1% is added in the post-fixation solution, the sucrose-infusion solution and the sucrose solution used to recover frozen sections (Tokuyasu & Singer, 1976; Kyte, 1976a). Excess formaldehyde is removed and the remaining reactive aldehyde groups quenched with glycine immediately before immunostaining.

B). Sucrose-Infusion and Freezing

Cryoprotectants are substances which allow cells to be frozen with the formation of smaller ice crystals and with less damage than would otherwise occur (Nash, 1966; Skaer, 1982). Pre-freezing infusion of sucrose into specimen blocks of 0.5-1 mm^3 is used to overcome the problem of ice-crystal damage and also to provide the plasticity which is required for smooth ultrathin sectioning of the specimen (Tokuyasu, 1973).

The standard procedure is to infuse 2-2.3 M sucrose pH 7-7.4 into the specimen block and then freeze the specimen by plunging it into liquid nitrogen at -196°C (77°K) and to section at -90°C to -120°C (183-153°K). The high concentration range of 1.6-2.3 M provides excellent cryoprotection but some specimens are too soft to cut ultrathin sections at or above -80°C (193°K) (Tokuyasu, 1973). Geuze, Slot, Scheffer & van der Ley (1981) successfully used 2.3 M sucrose for sectioning at -90°C (183°K).

Monosaccharides e.g. 4-5.5 M fructose solution can also be used for infusion. Polyethylene glycol of MW 300-500 is used in cases where sugar must be avoided. However, a long exposure of specimens to polyethylene glycol will damage the membranes (Tokuyasu, 1984). Glycerol is known to be an excellent cryoprotectant but when infused into specimens it does not improve the plasticity of the frozen tissue. If fixation is essential, it should be carried out as gently and as mildly as possible (Glauert, 1974; Hayat, 1981). When the fixation is very light and the specimen is highly hydrated, a high sucrose concentration will provide the plasticity necessary for sectioning.

C). Sectioning

The standard temperature for ultrathin sectioning is between -90°C and -120°C (183-153°K). The lower the temperature, the harder the specimen will be. Specimen blocks infused with 2-2.3 M sucrose and then frozen are softer than resin-embedded blocks. Slow sectioning is essential as in conventional microtomy to get the best sections. Fast sectioning may provide a section with each cutting stroke but it creates an irreversible compression in the sections and also a build up of static electricity results in curling or jumping of sections. Slow sectioning can cause wrinkling of sections if specimens are soft. However, when sections are brought to room temperature and expanded on a sucrose droplet they will often regain a flat form.

The sharpness of the glass knife is very important for successful cryosectioning. Even for a good knife, the central third of the edge will be the best portion for cutting the sections (Tokuyasu, 1980). The closer the final fracture line to 45° the sharper the resultant knife edge (Tokuyasu & Okamura, 1959) [see Procedure for Making the Glass Knife (Appendix)].

D). Section Recovery

To recover the sections, a droplet of 2.3 M sucrose on a wire loop of 1.5-2.0 mm in diameter is inserted quickly into the cryochamber. The sections on the knife are gently touched onto the bottom of the sucrose droplet before it is completely frozen; they are brought out from the cryochamber, and allowed to expand on the thawed sucrose droplet at room temperature (Tokuyasu, 1973). To recover very fragile sections, a mixture of 2 M sucrose and 0.75% gelatin which has a lower surface tension than 2 M sucrose is used (Tokuyasu & Singer, 1976). A mixture of 2 M sucrose and 1-2% formaldehyde may be used to recover sections of formaldehyde-fixed specimens. A droplet of this mixture remains in a liquid state for a longer time in a cryochamber than a droplet of 2.3 M sucrose and may be routinely used.

4.1.5 Markers for Antigen Detection at Electron Microscope Level

The marker of choice for post-embedding immunocytochemistry is colloidal gold. Faulk & Taylor (1971) first described the application of colloidal gold as a specific immunocytochemical marker in transmission electron microscopy (TEM). They used a complex of antibodies and colloidal gold particles (rabbit anti-**Salmonella** serum

absorbed onto colloidal gold) as a direct immunocytochemical labelling probe to identify surface antigens on **Salmonella.** Since then, gold particles have been used more and more as markers in immunocytochemistry. Gold particles can be accurately made to particular sizes and firmly linked to specific molecules such as proteins (protein A, immunoglobulin, enzymes, lectins, lipoprotein), toxins, hormones, polysaccharides and glycoproteins.

Colloidal gold particles are useful markers for cell surface antigens because of their relatively large particle sizes (3-20 nm). The ability to penetrate into tissue is restricted and therefore, colloidal gold use in pre-embedding methods is also limited. Nevertheless its has been successfully used to localise intracellular antigens (Willingham, 1983; De Mey et al., 1981).

4.1.5.1 Properties of Colloidal Gold (Roth, 1982)

Colloidal gold is a hydrophobic colloid and the gold particle carries a net negative surface charge. The auro-complex $H[AuCl_2]$ on the particle surface dissociates into H^+ and $AuCl_2$ and is responsible for this electrical charge (Pauli, 1949). The stability of colloidal gold in water is maintained by electrostatic repulsion. Goodman, Hodges & Livingstone (1980) demonstrated that the addition of electrolytes in a concentration above 7 mM caused flocculation of the particles into secondary aggregates which was accompanied by a change in colour from red to violet or blue and by formation of a precipitate. Hydrophobic colloidal gold can be stabilized against this electrolyte-induced flocculation by electrically charged hydrophilic substances such as proteins. Proteins are adsorbed onto colloidal gold particles by electrostatic Van der Waals forces resulting in the formation of a stable complex. Geoghegan & Ackerman (1977) observed that the adsorption of a protein to colloidal gold particles is pH dependent.

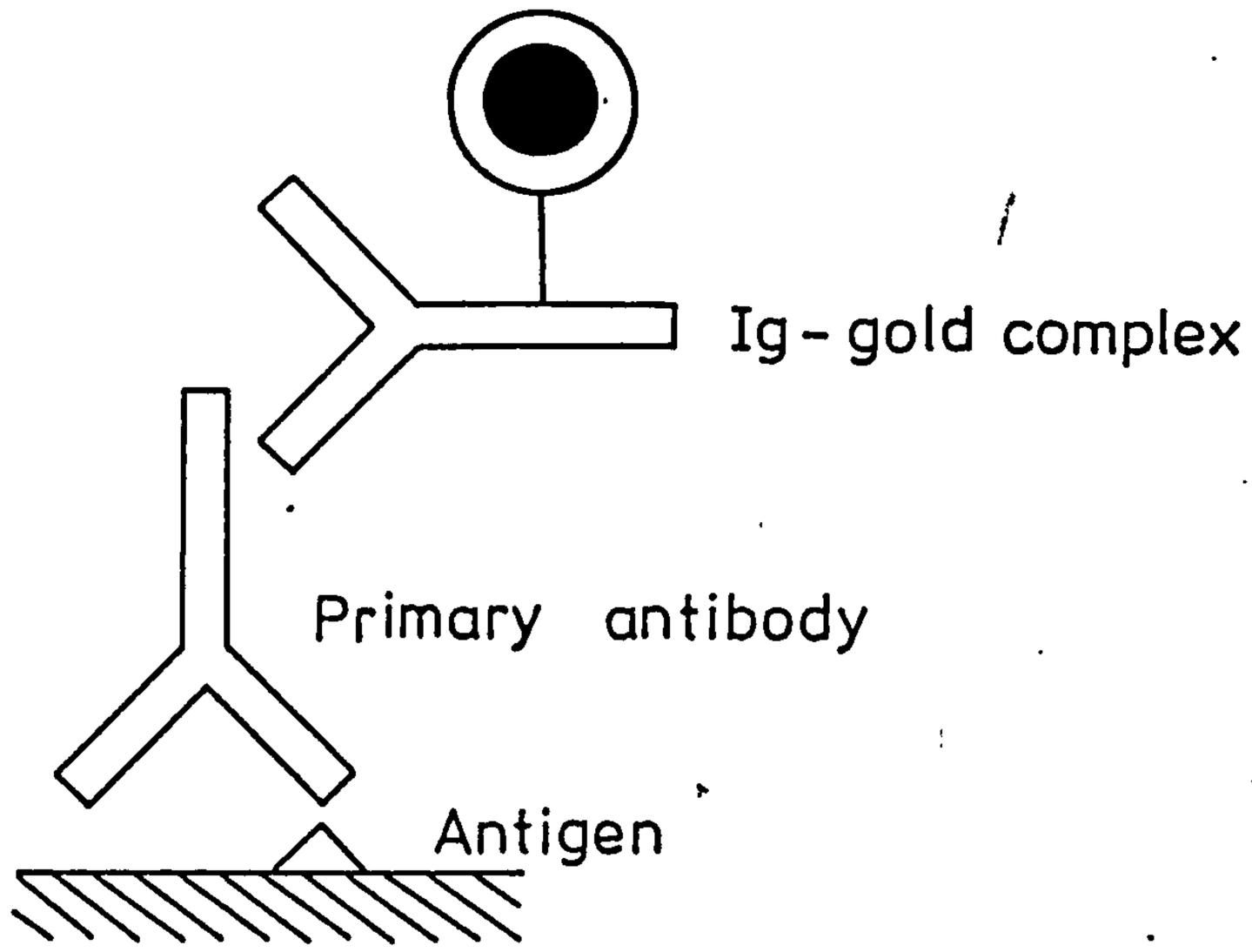
4.1.5.2 Applications of Colloidal Gold in Electron Microscopy

The gold particles exhibit high density (contrast) when viewed in the TEM. Due to their electron opacity and characteristic shapes, gold particles of about 3 nm in diameter can be easily recognized and can be clearly differentiated from cellular constituents, even on post-stained sections. Roth, Bendayan & Orci (1978, 1980) developed a post-embedding staining technique on plastic thin sections using protein A-gold complexes to localise antigenic material with high specificity and high resolution.

Protein A (pA), a staphylococcal cell wall protein has a specific affinity for the Fc portion of IgG from many mammalian species (Forsgreen & Sjoquist, 1966; Kronvall & Williams, 1969). One molecule of Protein A contains four highly homologous Fc region-binding sites (Hjelm, Sjodahl & Sjoquist, 1975; Sjodahl, 1976; Wright, Willan, Sjodahl, Burton & Dwek, 1977) and is able to bind two IgG molecules (Sjoquist, Meloun & Hjelm, 1972; Hjelm et al., 1975). The binding of protein A with IgG does not affect the antigen-antibody reaction (Langone, Boyle & Borsos, 1978). Romano & Romano (1977) were the first to use protein A-gold complexes for immunolabelling. Roth (1983) used pA-gold for intracellular localisation studies of sections from resin-embedded tissue. Geuze et al. (1981) used pA-gold for double labelling studies on ultrathin frozen sections.

Colloidal gold-labelled secondary antibodies can substitute for protein A (Garaud, Eloy, Moody, Stock & Grenier, 1980; Probert, De Mey & Polak, 1981; Garaud, Doffoel, Stock & Grenier, 1982). The immunoglobulin-gold technique (Ig-gold) is a two-step post-embedding method carried out on thin sections. In the first step the specific primary antibody interacts with the antigen exposed at the surface of the section. The antigen-antibody complex is then revealed in the second step by the Ig-gold complexes. Indirectly, the gold particle enables the localisation of the antigen on the tissue section (Text Figure 4.3).

Antibodies and gold probes do not penetrate resin sections (Beesley & Adlam, 1982), Therefore, the use of ultrathin frozen sections is advantageous. The larger probes, which are easily identified in the EM, are often used for low magnification studies to assess the overall distribution of antigen, or for antigen localisation in large-sized organelles such as secretory granules. The smaller probes are used for higher magnification studies of small structures such as viruses, bacterial pili (Beesley, 1985), for localisation of membrane constituents, or membrane-associated antigens. The size of gold particles is also important in the quantitative evaluation of immunolabelling. The sensitivity and resolution become inferior with increasing particle size. In TEM, 5-20 nm particles are commonly used. The selection of the size is based on a compromise between the degree of magnification necessary and the density of the marking obtained. In other words, the density of marking increases when the particle size decreases (Horisberger, 1984). The protein-gold complexes show only a very low degree of nonspecific interaction on thin resin-embedded sections or frozen materials (Horisberger & Rosset, 1977; Roth et al., 1978; Garaud et al., 1980; Bendayan, 1981; Geuze et al., 1981). This has allowed



Text Figure 4.3

Diagram illustrating the principles of the labelling Ig-gold technique. In a first step, the primary antibody interacts specifically with the antigen on the surface of a thin section. In a second step, the Ig-gold complex reacts with the Fc portion of the primary antibody. With this indirect method, the gold particles of Ig-gold complex permit the localisation of antigenic sites.

the localisation of a variety of constituents in different cellular compartments.

4.1.6 Aims of the Study

The advantages of a low temperature in preserving native protein structure have been shown by Carlemalm et al. (1982). Therefore, low temperature embedding techniques using K4M resin and cryoultramicrotomy (according to Tokuyasu's technique) were used to localise **Schistosome** antigens in the present study.

The objective of the work described in this Chapter was to localise **Schistosome** antigens at the electron microscope level. The work consists of three parts. The first part was to find the optimal fixation condition, using the indirect immunofluorescence technique on frozen sections of adult worms. The second part was to use lowicryl K4M resin to embed the adult worms; the K4M-embedded sections were then immunofluorescent stained. The final part was to use the cryoultramicrotomy technique; the cryo-thin sections were immunostained with MAB and secondary antibody conjugated with colloidal gold particles (GAM/Ig/gold).

4.2 MATERIALS AND METHODS

4.2.1 Screening of Fixatives on Frozen Section of Adult Worms

Before embarking on an electron microscopical study, various fixatives were screened by light microscopical immunocytochemistry. The indirect immunofluorescence technique was used to find the optimal fixation conditions in order to ascertain which were compatible with the particular antigen and primary antibody under investigation.

LACA mice were infected with approximately 200 cercariae of S. mansoni. Six weeks later adult worms were recovered from the hepatic portal system. The parasites were fixed in the following fixatives.

Group 1. Fixatives Containing Low Concentrations of PF and Glutaraldehyde

-1% PF + 0.02% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4
-1% PF + 0.04% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4
-2% PF + 0.01% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4
-2% PF + 0.02% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4

The parasites were fixed for 1 h at room temperature (RT). After fixation, the parasites were washed 3 times in 0.1 M phosphate buffer, 10 min each wash. Those parasites fixed in the fixative containing glutaraldehyde were treated with 0.5 M NH_4Cl in phosphate buffer for 30 min to 1 h at RT in order to block free aldehyde groups (Roth et al., 1981). They were washed again 3 times in buffer and embedded in Tissue-Tek (O.C.T.) for frozen sectioning. The cryostat sections were immunostained with primary antibodies (MABs D7.1, D7.2, M7.4, CMS, NMS, and NS-1) and fluorescein labelled secondary antibody (RAM/Ig/FITC). The same method was used as described in Chapter 2.

These results showed that the best fluorescent staining was observed on sections fixed in 2% PF + 0.01% glutaraldehyde (Table 4.1). Therefore, fixatives in Group 2 were tried.

Group 2. Fixatives Containing PF Alone or With 0.2% Glutaraldehyde

- 2% PF in 0.1 M PO_4 buffer, pH 7.4
- 4% PF in 0.1 M PO_4 buffer, pH 7.4
- 2% PF + 0.2% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4

The adult worms were fixed for 1 h and 10 min at RT and processed as described above. The results from this group showed that PF between 2 to 4 percent gave good immunofluorescent staining (Table 4.2). Consequently, fixatives in Group 3 were used.

Group 3. Fixatives Containing PF and 0-0.2% Glutaraldehyde

- 3.5% PF in 0.1 M PO_4 buffer, pH 7.4
- 3.5% PF + 0.05% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4
- 3.5% PF + 0.1% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4
- 3.5% PF + 0.2% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4

The adult worms were fixed for 2 h at RT and processed as described. From these experiments fixatives in Group 3 were used to preserve the parasites for K4M embedding (see Section 4.2.3).

4.2.2 Preparation of Lowicryl K4M Resin

The chemicals were supplied by Agar Aids and were provided as highly purified 3 component systems.

A). Cross-linker
B). Monomer
C). Initiator

Mixtures for UV-polymerization

Component	Amount (g)
Cross-linker A	2.70
Monomer B	17.30
Initiator C	0.10

The above mixtures produced blocks of average hardness. The hardness can be varied by incorporating more or less cross-linker in the resin mixtures (the more cross-linker the harder the blocks). For K4M, the cross-linker concentration can be varied from 4 to 18% (w/w, 0.8 to 3.6 g per 20 g resin).

The cross-linker and the monomer were weighed in a tared brown stoppered bottle. The mixtures can be measured by volume. They were mixed gently by one of the following methods for 3-5 min.

a). Bubbling a stream of dry nitrogen gas into the mixture with a Pasteur pipette. This allowed mixing of the resin and prevented incorporation of oxygen into the resin.

b). Mixing gently with a glass rod.

c). Slowly rocking the covered vial from side to side, avoiding the formation of air bubbles.

The initiator was added and mixing continued until the initiator was completely dissolved in the resin.

The resin mixture was prepared in brown glass containers to protect it from direct light. All the components are readily miscible with each other. Excessive stirring results in the incorporation of too much oxygen into the mixture. This prevents complete polymerization. Gloves were used to avoid skin contact, because methacrylates would cause skin reaction (eczema). The resin was mixed in a fume hood so that the vapour would not be inhaled. The resin mixture was precooled at -35°C in a refrigerated chamber before use for infiltration and embedding.

4.2.3 Lowicryl K4M Embedding Procedure

Schistosoma mansoni adult worms were recovered from the hepatic portal system of mice 6 weeks post infection. They were processed as follows :-

I). Fixation

The fixatives used were chosen from the results of fluorescence screening (Section 4.2.1). The adult worms were fixed in the following fixatives :

- 3.5% PF in 0.1 M PO_4 buffer, pH 7.4
- 3.5% PF + 0.05% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4
- 3.5% PF + 0.1% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4
- 3.5% PF + 0.2% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4

The parasites were fixed for 2 h at RT and washed 3 times in 0.1 M phosphate buffer. 0.5 M NH_4Cl in phosphate buffer was used to block free aldehyde groups after fixation in glutaraldehyde. The worms were washed again in buffer.

II). Dehydration

The parasites were dehydrated with ethanol by the progressive lowering of temperature (PLT) technique (Text Figure 4.1).

Ethanol (vol% in H_2O)	Temperature (^oC)	Time (min)
30	0	30
50	-20	60
70	-35	60
95	-35	60
100	-35	60
100	-35	60

All apparatus was precooled and the experiments were performed in the cold in order to minimise the condensation of water and the crystallization of ice on the specimen vials and on the capsules.

The dehydration processes were carried out in glass stoppered vials. An aluminium block with drilled holes was used to accommodate the specimen vials in order to minimise temperature gradients. The metal block was first placed in the ice bath and allowed to equilibrate at a particular temperature before the specimen vials were placed in it. Each change of solution was preceded by removal of the supernatant. At temperatures below 0^oC, the residual water in the specimen should not be allowed to freeze during the dehydration steps.

III). Infiltration

After dehydration in absolute ethanol, the specimens were infiltrated with the K4M resin. The schedule was as follows :-

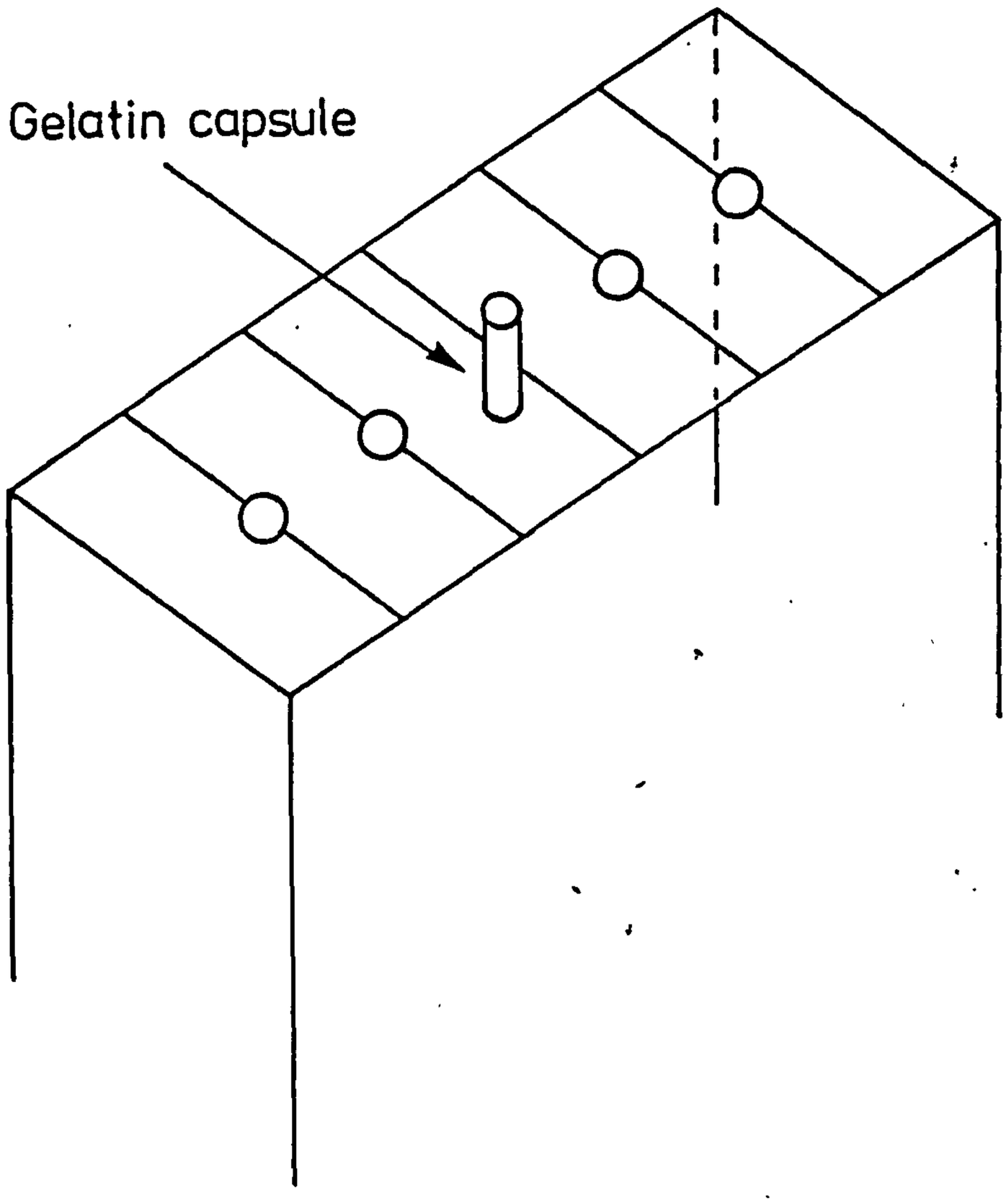
Resin : Absolute ethanol (v/v)	Temperature (^{o}C)	Time
1 : 1	-35	60 min
2 : 1	-35	60 min
Pure resin	-35	60 min
Pure resin	-35	overnight

Infiltration with K4M resin was performed at the final dehydration temperature (-35^{o}C). During dehydration and infiltration, the specimen was periodically agitated by gently swirling the specimen vials by using the rotator to ensure uniformly infiltrated specimens.

IV). Polymerization

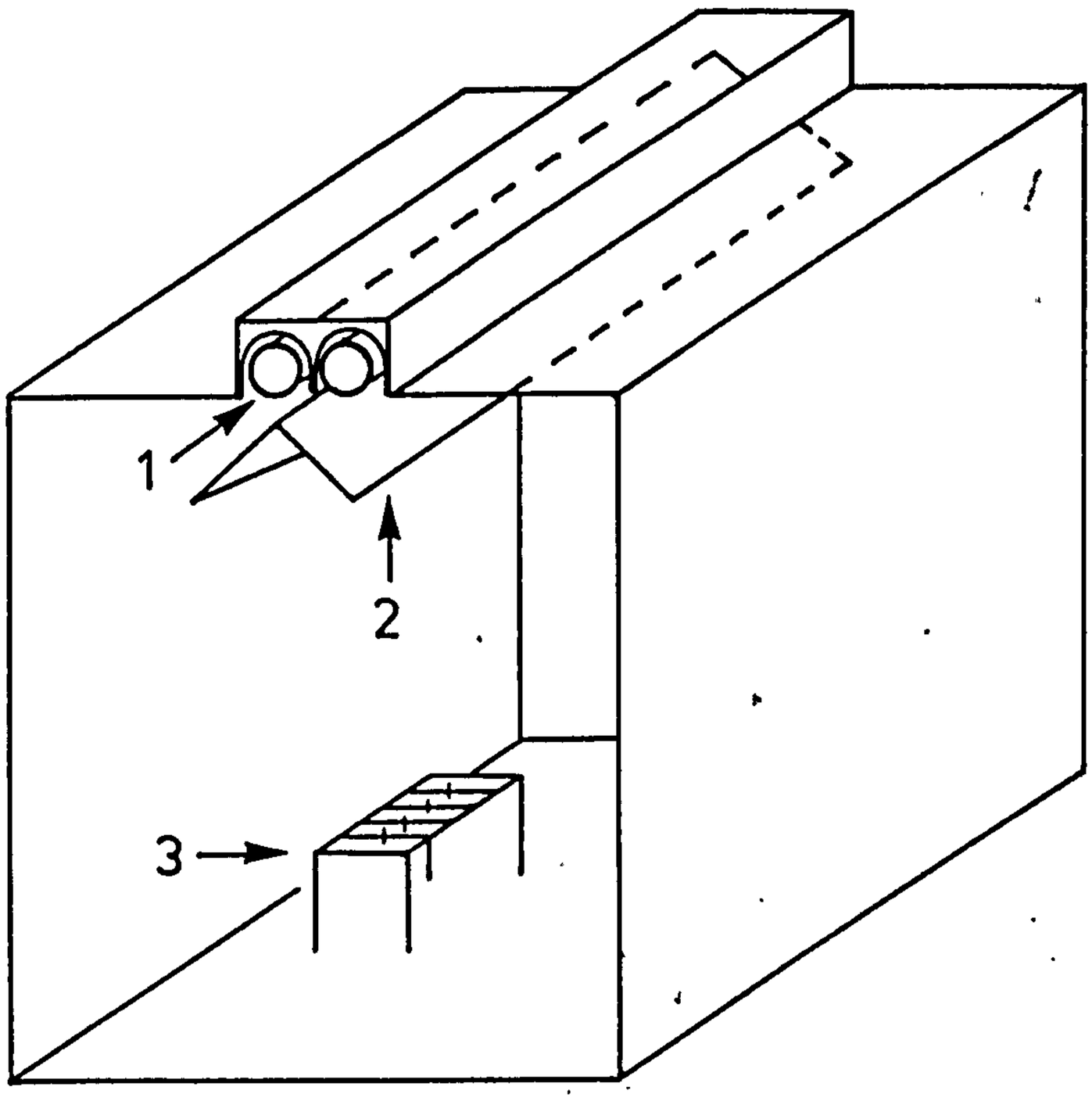
After infiltration in pure resin at -35^{o}C overnight, the specimens were transferred with Pasteur pipette into gelatin capsules filled with fresh precooled resin. The gelatin capsules were placed in a cold aluminium block with predrilled holes. The capsules were filled to the top, to minimise the dead space of air over the resin. The capsules were closed, transferred to the capsule holder (Text Figure 4.4) and the temperature was allowed to equilibrate at -35^{o}C for 10-15 min.

Polymerization was achieved with long-wavelength (360 nm, preferably two 15-watt fluorescent tubes, Philips TLD 15 W/05) indirect UV-irradiation at -35^{o}C for 24 hours (Text Figure 4.5). The capsule were then irradiated for another 2-3 days at room temperature or were further cured by exposure to sunlight for 1-2 weeks in a sealed petridish. The dish was lined with aluminium foil. The purpose of irradiation at a higher temperature was to shorten the total polymerization time thereby improving sectioning quality. More than 95% of the curing reaction occurs at the low temperature and the rest of the curing reaction can be done at room temperature without any disadvantages (Carlemalm, Villiger, Hobot, Acetarin &



Text Figure 4.4

A wire capsule holder for UV polymerization at low temperature.



Text Figure 4.5

A polymerization chamber for indirect UV irradiation. The UV source (1) is diffused by a right-angle reflector (2). The capsule holder (3) is placed 30-40 cm below the UV source (Redrawn from Carlemalm, unpublished).

Kellenberger, 1985).

Specimens could be polymerized in BEEM or unstained gelatin capsules. A capsule holder stand was required so that the capsules received UV irradiation from all sides. A stand was made of heavy gauge wire, and finer gauge twisted wire loops were soldered onto it to hold the capsules.

A polymerization box was fitted in a cryostat chamber (Text Figure 4.5). All six inner surfaces, as well as the reflector, were lined with aluminium foil to provide diffuse illumination so that each specimen was subjected to uniform irradiation. A right-angle reflector was suspended below the fluorescent tubes. The whole box should not be too tightly made; ventilation from the top and bottom provides air circulation to minimise temperature gradients in the chamber. A small UV lamp can be used, Provided it emits at 360 nm. For a smaller UV source, the size of the polymerization chamber is reduced, and the lamp-to-capsule distance is also reduced to 10-15 cm. If weak UV sources are used, radiation from the bottom of the chamber will help because the light will have less resin to traverse before polymerizing the resin in the specimen. The polymerized specimen blocks were kept in the dessicator to prevent absorption of moisture.

V). <u>Sectioning</u>

The specimen block was viewed and trimmed under a dissecting microscope using a razor blade. The final pyramid was trimmed with a glass knife on the microtome or on a trimming apparatus. The sides and the face of the block must be clean, and under illumination it should be clear and transparent. The sides of the pyramid were trimmed at an angle of 28-30^{o} from the cutting face. Sections were cut with glass knives on a Reichert OM U3 Ultramicrotome.

K4M is a hydrophilic resin. Therefore, precautions must be taken to ensure that the block face does not wet during sectioning. The level of liquid in the trough was filled to slightly below normal. In these circumstances the reflection from the trough fluid along the knife edge was slightly darker than the normal bright silver colour. However, the trough fluid must not be so low that the knife edge becomes dry. Because of the hydrophilic property of this resin, the sections were collected as soon as possible after they had been cut. Sectioning speeds of 2-5 mm/second were used. Sections were transferred onto glass microscopic slides, which had been coated with Poly-L-lysine to prevent them detaching from the slides. They were examined by light microscopy.

VI). Immuno-Staining

The K4M-embedded sections were immuno-stained by the indirect immunofluorescence technique (as described in Chapter 2) to examine whether the antigens remained intact after K4M embedding. Briefly, the sections were incubated with 10% normal rabbit serum in PBS for 30 min at RT to block the non-specific binding sites. The slides were drained. An appropriate dilution of monoclonal antibody was then added to the sections and they were incubated for 1 h at RT. The slides were washed 3 times in PBS, 5 min each wash. Rabbit anti-mouse Ig labelled with fluorescein isothiocyanate (RAM/Ig/FITC, from Nordic) at a dilution of 1:64 was added to the sections which were incubated for a further 1 h at RT. The sections were washed again as above and mounted with buffered glycerol and cover slips. They were examined under a Nikon Fluorescence Microscope.

The polymerization process was checked in the absence of specimens in trial experiments. Shrinkage and deformation along the sides of the block indicated that the polymerization was too rapid. When this occurred the distance between the lamps and the capsules was increased.

4.2.4 Cryoultramicrotomy

Adult worms were processed for cryo-sectioning for electron microscopy according to the technique of Tokuyasu (1973, 1976, 1986).

I). Fixation

Two and 4% paraformaldehyde in 0.1 M PO_4 buffer, pH 7.4 were used to fix the parasites. They were fixed for 3.5-4 h at RT and were washed 3 times with the same buffer. The cross-linking using these fixatives is partially reversible (Tokuyasu & Singer, 1976). Therefore, freshly fixed specimens were used on each occasion.

II). Infusion

The specimen was transferred to 2.3 M sucrose in 0.1 M PO_4 buffer for at least 1 h (usually overnight).

III). Freezing & Mounting

The sucrose-infused specimen was mounted on a specimen holder in a small amount of the sucrose infusion solution. For thin sections

the tissue piece should be small (1-2 mm) and pre-trimmed or mounted in such a way as to reduce trimming in the microtome. A specimen holder that has a small hole drilled into the top was used to hold the worm for cutting transverse sections. Placing the specimen on the specimen holder pin takes a few minutes. Small specimens are quickly dehydrated during this stage. The sectioning quality of frozen specimens can seriously deteriorate because of the supersaturation of sucrose or formation of minute sucrose crystals resulting from the dehydration.

When the specimen has been mounted on its holder it should be frozen as quickly as possible by plunging rapidly into liquid nitrogen. The liquid nitrogen was filled to the top of the container to minimise the depth of dense, cold gas above the liquid surface which would pre-cool and freeze the specimen at very slow cooling rates.

IV). Mounting Specimen on the Ultramicrotome

The pin with frozen tissue was transferred to the cold chamber (-100°C) of the ultramicrotome as quickly as possible using a small insulated container of liquid nitrogen to prevent condensation of moisture or momentary thawing. The holder with specimen was picked up using a pair of pre-cooled forceps (dipped in liquid nitrogen) and inserted into the chuck of the ultramicrotome. The specimen was allowed to come to thermal equilibration with its holder chuck before sectioning commenced. The temperature of the specimen, the knife and the chamber were also equilibrated. This took 30 min to 1 hour.

V). Sectioning

Cryosectioning was carried out in the Reichert OM U4 Ultracut E Ultramicrotome with the attachment of the Reichert-Jung Cryochamber FC4 (Provided Courtesy of Mr. David Robinson & Mr. Peter Walsh. The Institute of Pathology, Leeds University).

The cryochamber is an insulated box. It is cooled by liquid nitrogen or cold nitrogen gas. It completely surrounds the specimen and knife areas. The temperature in the cryochamber should remain stable at -100°C because this temperature affects the surface temperatures of the specimen and the knife. The temperature of the cryochamber should be monitored during cryosectioning.

It is of critical importance to select the correct temperature for sectioning and to ensure that this temperature is accurately maintained. With 2.3 M sucrose, I used a temperature between -90°C

and -100°C and a very slow cutting speed (0.4-0.6 mm/second). Sections were cut with the automatic advance of the specimen and with a dry glass knife without trough fluid. The section thickness was about 0.11 um. Frozen thin sections tend to roll up during sectioning and curl over the cutting edge and also become electrostatically charged causing strong repulsive forces during collection.

VI). Section Collection

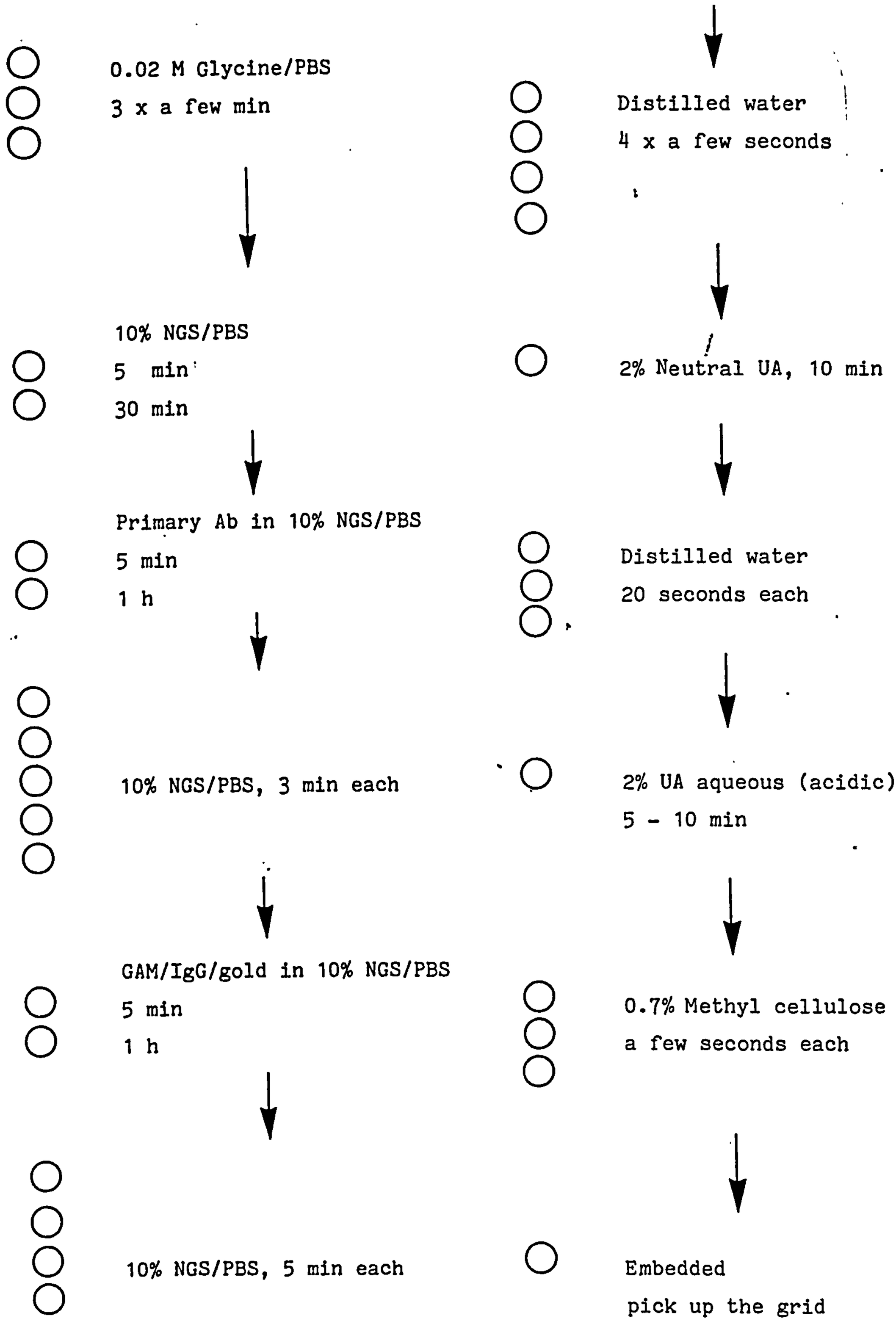
An eyelash probe precooled with liquid nitrogen was used to move sections away from the knife edge and to ensure that sections were not lying on top of each other. A wire loop (2 mm in diameter) was used to pick up the sections. The loop was dipped into 2.3 M sucrose in phosphate buffer and the drop of sucrose was brought quickly into the cryochamber. As the droplet cools it becomes very viscous. The sections were touched onto the bottom surface of the drop. This is a critical and often difficult step. The sucrose should still be in a liquid state when the sections are touched, otherwise they would not flatten onto the sucrose surface. At -100°C, the operator has only 1-2 seconds to touch the sections before the sucrose freezes. The droplet was removed from the cryochamber and melted in a stream of warm air; the section expanded. Thawed sections which had spread on the bottom surface of the sucrose droplet were then put onto a Formvar/carbon-coated Nickel, 200 mesh, hexagonal-lattice grid. When the sections touched the grid they immediately attached.

Grids with sections were placed face down on a shallow layer of PBS on top of 2% gelatin in phosphate buffer cooled by ice. They were left for 15-30 min to enable the sucrose to diffuse out of the sections. The gelatin was then warmed to room temperature for 15 min and the grids became coated as it melted. The gelatin coating prevents non-specific background staining. Immuno-staining should be done immediately but the grids can be stored on this plate on ice for a few hours if necessary. Some sections were brought back to York for immuno-staining. A vacuum flask was used to transport the sections (from Leeds to York by train).

VII). Immuno-Staining

The following procedures were performed at room temperature. Phosphate buffered saline (PBS) containing 0.02 M glycine, pH 7.4 was gently added to the gelatin plate to quench remaining reactive aldehyde groups from the fixative. Sections were processed in droplets of reagents arranged on a Parafilm sheet (Text Figure 4.6).

Text Figure 4.6 Protocol for immunostaining the cryosection on grids



All antisera had been centrifuged before use. A pair of anticapillary tweezers was used for transferring the grids from one solution to another to minimise the volume of solution carried with the grid. The grids were placed on 3 droplets of PBS-glycine, a few minutes each droplets.

The sections were then placed on two droplets of 10% normal goat serum in PBS (10% NGS/PBS, from species supplying second antibody) for 5 min and 30 min respectively, to block non-specific binding sites. During incubation in antisera, the grids were covered with a petridish in a moist atmosphere.

The sections were transferred next to the first drop of specific primary antibody (MAB) at a dilution of 1:30 in 10% NGS/PBS for 5 min. Then transferred to the second drop of the same antibody and left for 1 hour (MABs giving strong reaction on adult worm tegument were used i.e. MABs D7.2, D7.4, M7.4). The grids were washed in 5 droplets of 10% NGS/PBS (per grid), 3 min in each droplet.

Following this, the sections were immunolabelled with 2 droplets of the gold-labelled secondary antibody [Goat anti-mouse IgG, gold 10 nm, (GAM/IgG/gold), EM Grade, Janssen Pharmaceutica, Belgium] at a dilution of 1:25 in 10% NGS/PBS for 5 min and 1 hour respectively. The GAM/IgG/gold was centrifuged prior to use to remove micro-aggregates of gold particles which accumulate on storage and was diluted just before use. Subsequent washes were performed in the same manner, 5 min in each droplet. Finally, the grids were washed on 4 droplets of distilled water, a few seconds in each droplet, in preparation for staining or contrasting with heavy metal compounds.

VIII). <u>Positive Staining</u>

- The grids were placed on a drop of 2% neutral uranyl acetate (UA, 2% UA in 0.15 M oxalic acid; Tokuyasu, 1980) for 10 min. This step serves the purpose of stabilising the membranes.

- After brief washing with 3 droplets of distilled water, 20 seconds in each droplet, the sections were stained with an acidic UA solution (2% UA aqueous) for 5-10 min.

- The grids were passed quickly through 3 droplets of 0.7% methylcellulose (MC, 400 Centipoise, cps, Sigma) before being transferred to the surface of large droplets of 0.7% MC solution. Since MC is more soluble in cold than in warm water, a petridish on ice was used for embedding.

- The grids were floated on a drop of MC. Each grid was picked up on a loop (diameter > diameter of grid) by placing the loop under the grid.

- After removing the excess embedding medium with a piece of filter paper, the grids were dried in air. The dried film of the embedding medium showed a silver-gold to gold-blue colour.

- When the grids were dry, they were removed from the loop and put in a grid box, ready for examination under the transmission electron microscope (TEM).

4.3 RESULTS

4.3.1 Indirect Immunofluorescence on Frozen (Cryostat) Sections

Table 4.1 shows the results of experiments using fixatives in Group 1. When MAB D7.2 was used as a primary antibody at a dilution of 1:50, the secondary antibody (RAM/Ig/FITC) was used at a dilution of 1:64. Some areas of the tegument showed positive fluorescence (1+), whilst others were negative on the same section. The fixative used was 1-2% paraformaldehyde in the presence of 0.02-0.04% glutaraldehyde. The tegument was weakly fluorescence-positive (wk+, 1+) on the sections fixed in 2% PF + 0.01% glutaraldehyde. Similar results were obtained when MAB M7.4 was used as a primary antibody.

Acute mouse serum (AMS) at a dilution of 1:200, was used as a positive control. The sections fixed in 2% PF with 0.01-0.02% glutaraldehyde, gave better positive fluorescence than those fixed in 1% PF plus 0.02% or 0.04% glutaraldehyde.

In the negative controls, NMS at a dilution of 1:200 and NS1 at a dilution of 1:50 were used. False positive results were obtained from most fixatives. Only 2% PF plus 0.01% glutaraldehyde gave reliable results.

From these experiments, the most acceptable fixative for the parasite was 2% PF + 0.01% glutaraldehyde. Glutaraldehyde gives good ultrastructural preservation but destroys antigenicity. Therefore, other fixatives (in Group 2) were tried.

The results of experiments using fixatives in Group 2 are shown in Table 4.2. The antigens recognized by MABs D7.1, D7.2, & M7.4 were not destroyed after fixation in 2% or 4% PF. If 0.2% glutaraldehyde was included in 2% PF, this destroyed the antigens. Therefore, false negative results were obtained. No significant difference was observed when AMS was reacted with those sections. AMS is a polyclonal antibody. The positive reaction was very strong even though some of the epitopes had been destroyed by the fixative. When NMS and NS1 were used as negative controls, a false positive reaction occurred on the paraformaldehyde-glutaraldehyde fixed sections. This might be due to the free aldehyde groups from the glutaraldehyde solution even though the specimens had been blocked with NH_4Cl. The other reason for nonspecific fluorescence comes from improper fixation of the tissue. The results from this group of experiments showed that 2% to 4% PF could be used to fix the parasites for these particular antigens.

The results from previous experiments suggested that up to 4% PF could be the optimum concentration used to preserve antigens. Further

Table 4.1 Intensity of Immunofluorescence Staining on Frozen Sections of Adult Worms Using Various Fixatives (Group 1).

Primary antibody	Fixatives (1h, RT)	Tegument	Internal structure
D7.2 (1:50)	1% PF + 0.02% Glut.	+/-	-
	1% PF + 0.04% Glut.	+/-	-
	2% PF + 0.01% Glut.	wk+/+	-
	2% PF + 0.02% Glut.	+/-	-
M7.4 (1:50)	1% PF + 0.02% Glut.	wk+/+	-
	1% PF + 0.04% Glut.	wk+/-	-
	2% PF + 0.01% Glut.	++	-
	2% PF + 0.02% Glut.	+/-	-
AMS (1:200)	1% PF + 0.02% Glut.	++	++
	1% PF + 0.04% Glut.	++	+
	2% PF + 0.01% Glut.	+++	+++
	2% PF + 0.02% Glut.	++, +++	++
NMS (1:200)	1% PF + 0.02% Glut.	++/-	+/-
	1% PF + 0.04% Glut.	++/-	-
	2% PF + 0.01% Glut.	+/-	-
	2% PF + 0.02% Glut.	++/-	-
NS1 (1:50)	1% PF + 0.02% Glut.	+/-	-
	1% PF + 0.04% Glut.	-	-
	2% PF + 0.01% Glut.	-	-
	2% PF + 0.02% Glut.	+/-	-

RAM/Ig/FITC dilution 1:64 was used for the secondary antibodies in all experiments.

PF = Paraformaldehyde
Glut. = Glutaraldehyde
\+ = Positive fluorescence
wk = Weak positive fluorescence
\- = Negative (no fluorescence)

Table 4.2 Intensity of Immunofluorescence Staining on Frozen Sections of Adult Worm Using Various Fixatives (Group 2).

Primary Antibody	Fixatives (70 min, RT)	Tegument	Internal structure
D7.2 (1:50)	2% PF	++++	-
	4% PF	+++	-
	2% PF + 0.2% Glut.	+/-	-
M7.4 (1:50)	2% PF	++++	+++
	4% PF	+++	++
	2% PF + 0.2% Glut.	+/-	+
D7.1 (1:50)	2% PF	++++	-
	4% PF	+++	-
	2% PF + 0.2% Glut.	+/-	-
AMS (1:200)	2% PF	++++	++++
	4% PF	++++	++++
	2% PF + 0.2% Glut.	+++	+++
NMS (1:200)	2% PF	-	-
	4% PF	-	-
	2% PF + 0.2% Glut.	+/-	-
NS1 (1:50)	2% PF	-	-
	4% PF	-	-
	2% PF + 0.2% Glut.	+/-	-

experiments (Group 3) were carried out. The results are illustrated in Table 4.3. Fixatives containing 3.5% PF and 3.5% PF + 0.05% glutaraldehyde gave similar results to 2% PF and 4% PF respectively in the tegumental antigens which were recognized by MABs D7.2 & D7.1. The degrees of positive fluorescence were quite strong (4+, 3+). When higher concentrations of glutaraldehyde (0.1 - 0.2%) were added to the fixative. the amount of fluorescence was dramatically decreased to 2+ and 1+. With the AMS, the positive fluorescence was seen on the whole section of the adult worm; no significant differences in the amount of fluorescence were observed on sections when fixatives in Group 3 were used. On sections of adult worm reacted with NMS or NS1, no positive fluorescence results were obtained. However, some false positive fluorescence was seen on sections fixed in 3.5% PF + 0.2% glutaraldehyde. All the results in Table 4.3 were quite similar to the results in Table 4.2.

4.3.2 Indirect Immunofluorescence Results on Sections Embedded in K4M

The results from Table 4.3 gave satisfactory immunofluorescence staining on frozen sections of adult worms. Therefore, fixatives in Group 3 were used to fix the parasites for K4M embedding and sectioning. The results are shown in Table 4.4.

MAB D7.2 gave a weakly positive result on the tegument of adult worm sections fixed in 3.5% PF. Non-specific staining was seen in the resin background. The fixatives containing glutaraldehyde gave negative results.

AMS at a dilution of 1:100 was used as a positive control. The immunofluorescence results were not so strongly positive as those in the frozen sections. (see Table 4.3) even though the dilution of AMS was lower. High background staining was observed. NMS at a dilution of 1:100 was used as a negative control. Negative results were obtained after fixation with PF or PF containing glutaraldehyde.

In order to find out whether the decrease in immunofluorescent staining was due to the destruction of antigens or the lack of penetration of the antibodies lower dilutions of the antibodies were used (see Table 4.5). Weak positive fluorescence was obtained in the sections fixed in 3.5% PF and reacted with MAB D7.2 at a dilution of 1:5. Also in the positive control (AMS 1:50) the staining was not improved.

The results from Table 4.4 and 4.5 suggested that K4M embedded sections were not suitable for localisation of schistosome antigens because labile antigens were destroyed by dehydration and embedding

Table 4.3 Intensity of Immunofluorescence Staining on Frozen Sections of Adult Worm Using Various Fixatives (Group 3).

Primary Antibody	Fixatives (2 h, RT)	Tegument	Internal structure
D7.2 (1:50)	3.5% PF	++++	-
	3.5% PF + 0.05% Glut.	+++	-
	3.5% PF + 0.1% Glut.	++	-
	3.5% PF + 0.2% Glut.	+/-	-
D7.1 (1:50)	3.5% PF	++++	-
	3.5% PF + 0.05% Glut.	+++	-
	3.5% PF + 0.1% Glut.	++	-
	3.5% PF + 0.2% Glut.	+	-
AMS (1:200)	3.5% PF	++++	++++
	3.5% PF + 0.05% Glut.	++++	++++
	3.5% PF + 0.1% Glut.	+++	+++
	3.5% PF + 0.2% Glut.	+++	+++
NMS (1:200)	3.5% PF	-	-
	3.5% PF + 0.05% Glut.	-	-
	3.5% PF + 0.1% Glut.	-	-
	3.5% PF + 0.2% Glut.	+/-	-
NS1 (1:50)	3.5% PF	-	-
	3.5% PF + 0.05% Glut.	-	-
	3.5% PF + 0.1% Glut	-	-
	3.5% PF + 0.2% Glut.	+/-	-

Table 4.4 Intensity of Immunofluorescence Staining on K4M-Embedded Sections of Adult Worm Using Various Fixatives (Group 3).

Primary Antibody	Fixatives (2 h, RT)	Tegument	Internal structure
D7.2 (1:20)	3.5% PF	wk+	-
	3.5% PF + 0.05% Glut.	-	-
	3.5% PF + 0.1% Glut.	-	-
	3.5% PF + 0.2% Glut.	-	-
AMS (1:100)	3.5% PF	+	++
	3.5% PF + 0.05% Glut.	wk+	++
	3.5% PF + 0.1% Glut.	wk+	+
	3.5% PF + 0.2% Glut.	-	+
NMS (1:100)	3.5% PF	-	-
	3.5% PF + 0.05% Glut.	-	-
	3.5% PF + 0.1% Glut.	-	-
	3.5% PF + 0.2% Glut.	-	-

Table 4.5 Intensity of Immunofluorescence Staining on K4M-Embedded Sections of Adult Worm Using Various Fixatives (Group 3). The Dilutions of Primary Antibodies Used Were Reduced.

Primary Antibody	Fixatives (2h, RT)	Tegument	Internal structure
D7.2 (1:5)	3.5% PF	wk+	-
	3.5% PF + 0.05% Glut.	-	-
	3.5% PF + 0.1% Glut.	-	-
	3.5% PF + 0.2% Glut.	-	-
AMS (1:50)	3.5% PF	+, wk+/-	-
	3.5% PF + 0.05% Glut.	wk+	+
	3.5% PF + 0.1% Glut.	wk+	+
	3.5% PF + 0.2% Glut.	-	-
NMS (1:50)	3.5% PF	-	-
	3.5% PF + 0.05% Glut.	-	-
	3.5% PF + 0.1% Glut.	-	-
	3.5% PF + 0.2% Glut.	-	-

processes. Therefore, cryoultramicrotomy was used.

4.3.3 Cryoultramicrotomy Results

Thirteen experiments using the cryoultramicrotomy technique were performed over a period of 4 months. Although all of the sections were cut on the cryoultramicrotome in Leeds, some had to be transferred to York for immunostaining. This affected the quality of the sections.

Staining performed in Leeds or York is marked with an asterisk (*) or asterisks (***) respectively.

* Experiment 1

Cryosections were collected with 2.3 M sucrose and floated in distilled water to allow the sucrose to diffuse from the sections. They were then stained with UA and embedded in 1.5% methyl cellulose. Parts of the sections were detached or dispersed when the grid was directly floated on a water surface. This was because of the high surface tension of the sucrose droplet. It was thought that this might be overcome by adding 0.5 - 2% gelatin to the sucrose solution (see Experiment 2).

* Experiment 2

A solution of 2 M sucrose with 0.75% gelatin was used to collect the cryosections from the knife, instead of 2.3 M sucrose as above. The sections were stained with UA and embedded in 1.5% methyl cellulose as above. The sections showed ice-crystal damage.

Some sections were too thick and showed poor ultrastructure. Others tore, and showed high background staining. It was difficult to focus under the electron microscope because the sections were embedded in 1.5% methyl cellulose. This concentration appeared to be too high, and left a thick film covering the sections which blurred the image.

* Experiment 3

The sections were prepared and immunostained as Experiment 2 except that they were embedded in 0.7% methyl cellulose instead of 1.5% methyl cellulose. The sections did not show any better ultrastructure than in the previous experiments, but the focus of the specimen under the microscope was improved.

* Experiment 4

Paraformaldehyde 2% was used in Experiments 1-3 to fix the specimen in order to maximise antigenicity. However, the ultrastructure was not well preserved.

Since the ultrastructure needed to be improved, the fixative used had to be reconsidered. The parasites were fixed using 4% PF in 0.1 M phosphate buffer, pH 7.4 for 3.5 h at room temperature. The sections were collected, stained with uranyl acetate and embedded as in Experiment 3. The sections showed good ultrastructure without ice-crystal damage.

The tegument (syncytial epithelium) comprises an outer, anucleate layer of cytoplasm connected by cytoplasmic strands to nucleated portions of cytoplasm, called tegumental cell bodies, located in the parenchyma beneath the fibrous basal lamina and superficial muscle layers. This can be seen in Figs. 4.1 - 4.3. The outer plasma membrane of the tegument extends over the entire pitted surface of the parasite. There are two muscle layers immediately beneath the tegument. The outer layer consists of circular muscle fibres, the inner layer of longitudinal muscle fibres. The parenchymal cells and fibres of interstitial material occupy most of the space beneath the muscle layers. Numerous mitochondria are seen in the parenchymal cells (Figs. 4.1-4.3).

The tegumental cytoplasm consists of an electron dense granular, but ribosome-free matrix. Discoid bodies are numerous in the adult tegument and comprise a trilaminate limiting membrane and dense granular contents. These bodies exhibit a variety of shapes in thin sections. Membraneous bodies are not seen in Fig. 4.5.

The tegument cell bodies are irregular in shape. These cells have a narrow rim of very basophilic and electron-dense perinuclear cytoplasm and irregular nuclei with the chromatin distributed in clumps. They have one or several small but very distinctive Golgi complexes which appear well outlined by osmium impregnation (Morris & Threadgold, 1968). These cells contain characteristic cytoplasmic inclusion bodies called "discoid bodies" and "membraneous bodies" which are similar to those found in the tegument cytoplasm (Fig. 4.5). They are also found in clusters in varicose swellings of the cytoplasmic connections that connect the tegument with its cell bodies (Fig. 4.4). In these clusters the membraneous bodies generally predominate over the discoid bodies. Each nucleated cell body is connected to the tegument at several points by slender cytoplasmic

extensions (Fig. 4.3). The diameter of the connecting stalks at their thinnest point is barely larger than that of the membraneous bodies. Just below the basal membrane of the tegument these connections contain several microtubules which run parallel to the longitudinal axis and extend from the basal membrane of the tegument towards the cell bodies.

The parenchyma of the adult worm examined by electron microscopy shows several different cell types. There are tegument cell bodies, muscle cells and nerve cells (Reissig, 1970). The muscle cells give rise to muscle fibres. The nerve cells contain electron-dense droplets as shown in Fig. 4.4. Fig. 4.6 shows that the caecum is well preserved in the specimen. The caecal wall consists of a lining epithelium or gastrodermis which is supported by a thin, basal lamina and a layer of circular and longitudinal muscle fibres.

The gastrodermis of S. mansoni is a syncytial epithelium. Plate-like invaginations of the basal plasma membrane partly partition the cytoplasm in the syncytial gastrodermis and thus separate the nuclei (Fig. 4.7). The gastrodermis contains large basal nuclei, ribosomes and mitochondria. In addition, the bulk of the cytoplasm is occupied by RER (rough endoplasmic reticulum), Golgi stacks and dense, membrane-bound secretory bodies (Fig. 4.7). In Fig. 4.8, haemoglobin and haematin can be seen in the luminal area. The gastrodermis is characterised by considerable surface amplification which increases absorptive capacity. The surface extensions are in the form of pleomorphic lamellae. The microvilli generally form a regular and extensive border to the gastrodermis. The lamellae are broad and sheet-like structures (Fig. 4.8).

Some sections were collected using 2 M sucrose with 0.75% gelatin. These sections showed ice-crystal damage. Therefore, 2.3 M sucrose was used to collect the cryosections in the following experiments.

*, *** Experiment 5

The specimens were prepared and cryosectioned as in Experiment 4. The sections were immunostained with MAB D7.2 or D7.4 and GAM/Ig/gold (both in Leeds and in York). The sections immunostained in York showed extensive ice-crystal damage, and the tegument appeared highly vacuolated (Figures not shown). The morphology of the sections immunostained in Leeds was better. Less ice-crystal damage was seen in internal tissue, but vacuoles still appeared in the tegument (Figs. 4.9 & 4.11). However, the gold particles could be seen in the tegument and in the cytoplasmic connections between the tegument and

tegumental cell bodies (Figs. 4.12 - 4.14).

*, *** Experiments 6-8

The parasites were prepared and immunostained with MAB and gold Ig as in Experiment 5. The results obtained from the sections immunostained in Leeds were similar to the results in Experiment 5. The ultrastructure was still very poor even though the sections were perfectly cut. However, some areas of the sections showed ice-crystal damage. The sections stained in York tore and because the proteins leached from the damaged cells there was high background staining.

In all the experiments described above (Experiments 1-8), the adult worms were obtained from the infected mice by portal perfusion. They were washed in Eagle's medium prior to fixation in paraformaldehyde.

When the tegument of a worm was given a brief wash in Eagle's medium, after removal from the mouse and before fixation, the spaces within the basal invaginations expanded to form large extracellular vacuoles. This is also reported by Wilson & Barnes (1974b). Therefore, in the following experiments, the adult worms were collected without portal perfusion.

* Experiments 9-13

The adult worms were recovered from infected mice by incision of the hepatic portal vein after the mice had received anaesthetic doses of Sagatal (from May & Baker). The worms were handled as little as possible to minimise cell damage and placed directly in 4% paraformaldehyde for 4 h, at room temperature. Ultracryosections were immunostained with MAB and RAM/Ig/gold. Again there was only a little improvement in the ultrastructure. The gold particles could be seen only in the tegument of sections reacted with MAB D7.2 and RAM/Ig/gold (Fig. 4.15). When AMS was used as a positive control, many gold particles were seen in the tegument and in the internal tissue of the worm (Fig. 4.16).

From the distribution of gold particles, it would seem that the epitopes recognized by MAB D7.4 were located in the tegument cytoplasm and the cytoplasmic connections. The intense labelling was found in the tegument cytoplasm rather than the tegumental surface (Fig. 4.17).

With MAB M7.4, the antigenic determinant was located on the tegument. Gold particles were seen in association with the membranes of the inclusion bodies. Some particles were distributed over the cytoplasm of the tegument (Figures not shown).

When NMS and NS1 were used as negative controls, no gold particles could be detected on any sections (Figs. 4.18 & 4.19).

The large vacuoles formed by basal invaginations were not observed in most sections of worms recovered by dissection. Some ice-crystal damage still occurred. However, the gut and the parenchymal cells were well preserved.

4.4 DISCUSSION

4.4.1 Schistosome Antigens at Ultrastructural Level

To date, there has been only one publication on the ultrastructural localisation of schistosome antigens within the parasite. De Water, Fransen & Deelder (1986) localised the circulating anodic antigen in the digestive tract of S. mansoni using monoclonal antibodies and the immunogold technique on paraformaldehyde (4%) fixed and K4M-embedded sections.

McLaren, Clegg & Smithers (1975) have used an antibody-enzyme bridge technique involving labelling with horseradish peroxidase and histochemical localisation of the enzyme on worm surfaces. No problems of permeation of the antibodies occur. They demonstrated the binding of antibodies in immune rhesus serum to the surface membrane of 3 h skin-transformed schistosomula at the ultrastructural level. They also showed the binding of mouse erythrocyte antigens on the surface of 4-day schistosomula by using the same technique. These surface antigens could survive fixation in 1.25% glutaraldehyde. This pre-embedding immunostaining method is particularly useful for localisation the surface antigens. It is not an appropriate technique for localisation of internal antigens, because the penetration of antibodies into thick, fixed specimens is slight, so I did not attempt to use it.

In contrast to the report of De Water et al. (1986), the results described in Tables 4.4 & 4.5 showed that low temperature (K4M) embedding of adult worms did not preserve schistosome tegument antigens. This is surprising as some authors have suggested that low temperature embedding (below -20°C) will enhance the stability of the biological material (Fernandez-Moran, Oda, Blair & Green, 1964; Bullivant, 1970) and immobilize molecules by decreasing thermal vibration during solvent exchange (Frauenfelder, Petsko & Tsernoglou, 1979).

MABs D7.1 & D7.2 are antibodies that recognize antigens in the tegument of the adult worm (see Tables 4.2 & 4.3, and also Chapter 2). It thus appears that tegument antigens are heat sensitive. Beisler, Matsuda, Nakao & Tanaka (1984b) reported that the tegument lost its cross-reactivity with anti-egg antibodies after formalin fixation and paraffin embedding, in contrast to strong tegument fluorescence on frozen acetone-fixed sections.

As mentioned earlier De Water et al. (1986) could localise the gut-specific antigen of S. mansoni on K4M-embedded sections. Other authors have also reported the reactivity of schistosome gut antigen

using paraffin sections. Beisler, Matsuda, Nakao & Tanaka (1984a) demonstrated that the tegument lost its reactivity with S. japonicum infected rabbit serum after formalin fixation and paraffin embedding, but antigens in the gut epithelium and in the egg-vitelline area remained unchanged.

Nash (1978) measured specific IgM and IgG antibodies to the schistosome gut antigen by the IIF technique, using sections of Rossman's fixed and paraffin embedded adult worms. Furthermore, antibody to the gut epithelial cells of schistosomula was also detected in the paraffin sections of cold ethanol-fixed specimens using immunized rabbit serum (Moore,1967). The antigens of the schistosome gut and egg were regarded as heat stable glycoproteins (Kamiya & Kamiya, 1980; Nash, 1978). Other reasons for failure of the K4M embedding technique are discussed below.

4.4.2 Fixation

Chemical fixation is important in preserving cellular structures. Prior to cryoultramicrotomy, fixation is required to enable thawing of sections without loss of ultrastructural integrity due to the effects of surface tension. Glutaraldehyde and formaldehyde are the two most commonly used fixatives for biological specimens. The advantages of formaldehyde in immunocytochemistry are the retention of antigenicity and the accessibility of antibody.

Frozen sections of formaldehyde fixed specimens are commonly used in immunofluorescence microscopy as shown in Tables 4.2 & 4.3, and also in Chapter 2. The reactions were strongly positive. The results were comparable with frozen sections fixed in acetone (see Chapter 2). Formaldehyde fixation is not always appropriate in immuno-electron microscopy. This is because the cross-linking is not strong enough to withstand the processes of dehydration and embedding.

In the present study the concentration of paraformaldehyde (2% or 4%) used in cryoultramicrotomy seems to be insufficient to preserve the ultrastructure of the schistosome. The digestive tract (Figs. 4.6-4.8) and the parenchymal cells were well preserved. But the inclusion bodies in the tegument cytoplasm were not easy to recognize (Figs. 4.15-4.17). Higher concentrations (6-8% PF) or increased fixation time should be used in future experiments in order to get better ultrastructural preservation. If the morphology is improved then studies could be carried out to determine whether the antigenicity is still retained or not. Unfortunately, these concentrations of fixative have not been tried due to the time limitation imposed, and the limited availability of the

cryoultramicrotome.

Formaldehyde reacts with proteins but there is less cross-linking than with glutaraldehyde. The reaction is also slow and reversible if the fixed specimen is exposed to buffer for a long time (Hayat, 1970; Tokuyasu & Singer, 1976). Therefore, formaldehyde-fixed adult worms were stored in 0.4-1% formaldehyde in phosphate buffer at 4°C to prevent the reversal of cross-linking. However, the leaching of protein from the sections still occurred. This is probably because 2 or 4% paraformaldehyde used to fix the parasite was insufficient to crosslink the protein.

Glutaraldehyde reacts very rapidly with proteins. It is a dialdehyde which stabilises structures by cross-linking before extraction by the buffer has occurred. The schistosome antigens that are recognized by the MABs D7.1, D7.2 & M7.4 are protein or glycoprotein in nature. These epitopes were destroyed by glutaraldehyde fixation even at concentrations as low as 0.01-0.2% as shown by the weak positive or negative results in the immunofluorescence technique (Tables 4.1 & 4.2). Glutaraldehyde can affect the antigenic properties of different proteins to a varying degree down to complete abolition of antigenicity (Kyte, 1976a, 1976b; Kraehenbuhl, Racine & Jamieson, 1977). This problem can be overcome by fixing in a solution of formaldehyde alone (Kyte, 1976a; Geuze et al., 1984). As a consequence, glutaraldehyde was not used to fix the adult worms for the cryoultramicrotomy study.

4.4.3 Lowicryl K4M Resin

It is pertinent to ask why sections of worm tissues embedded in K4M resin showed minimal reactivity with antisera. Fixatives in Group 3 gave good fluorescence staining on frozen sections of adult worm (Table 4.3) especially 3.5% PF plus 0-0.05% glutaraldehyde. In K4M embedded sections, the same fixatives gave weak positive and negative reactions when MAB D7.2 was used (Tables 4.4 & 4.5) even though the dilution of MAB was as low as 1:5. K4M sections reacted with AMS also showed weak fluorescent staining (Tables 4.4 & 4.5). It seems very likely that the reduction in antigenicity was due to embedding in K4M resin, not to the formaldehyde fixation. This provides a second indication that schistosome surface antigens are labile and cannot resist dehydration and embedding processes even at low temperatures.

A second explanation for failure of K4M sections of worm to stain could be the inability of antibody to penetrate the resin. This seems unlikely because the non-specific staining of the background was very high. This result was contrary to that reported by Roth et al.

(1981). They found that low background was due to reduction of non-specific adsorption of the immunoglobulins to the K4M-embedded tissue. If higher dilutions of antibody had been used in order to decrease background staining, it is likely that positive staining would not have been detectable. All these observations suggest that antibody was able to penetrate K4M resin.

The results from Tables 4.4 & 4.5 showed that the K4M-embedding technique did not preserve the antigenicity of schistosomes as screened by the indirect immunofluorescence method. Therefore, no attempt was made to localise schistosome antigen using K4M sections for electron microscopy. An alternative technique of immunostaining on ultrathin frozen sections (Tokuyasu, 1973, 1976, 1986) was used.

4.4.4 Cryoultramicrotomy

The cryoultramicrotomy technique used today was largely developed by Tokuyasu (1973, 1976, 1978) and it has now become an accepted technique for immunocytochemistry. Since each investigator has to experiment to find the appropriate protocol for his own specimens there are various methods available.

The results outlined in this chapter took several months of effort with this technique, and a great deal of patience and perseverance was necessary.

From the results obtained, immunostaining with MAB and colloidal gold was shown to be highly specific to those antigens synthesized in the tegument cell bodies and distributed to the tegument via cytoplasmic connections (Figs. 4.12 - 4.14).

No gold particles were seen on cryosections reacted with NMS or NS-1 and RAM/Ig/gold (Figs. 4.18 & 4.19). This indicated that low background staining was achieved with cryosections and colloidal gold complexes.

Cryosections of adult worms fixed in 4% PF showed good ultrastructure when viewed at low magnifications (Figs. 4.1 - 4.3). At higher magnifications, the morphology of tegumental organelles was not well preserved. This made it very difficult to identify the cytoplasmic inclusions to which the gold particles were attached. To improve ultrastructure higher concentrations of PF should be used in future experiments. In some appropriately fixed specimens, the ultrastructure obtained from ultrathin cryosections was comparable to that obtained from conventional ultrathin sections.

Freezing damage to specimens with high water content can be prevented by treatment with a cryoprotectant (Nash, 1966; Skaer, 1982). 2.3 M sucrose was used to prevent ice-crystal formation when

freezing the parasites. However, some ice crystal damage was observed. This could be due to the freezing process rather than to the cryoprotectant used; 2.3 M sucrose has been successfully used by other workers (Tokuyasu, 1973, 1984, 1986; Beesley 1984, 1986).

Inappropriate freezing can cause ice-crystal damage. The paraformaldehyde-fixed and sucrose-infused adult worms were frozen by plunging them into liquid nitrogen. Although it has been suggested that melting occurs during sectioning, recent studies have shown that the energy liberated during cutting section does not lead to any significant change in the structure of ice crystals (Frederik & Busing, 1981; Karp, Silcox & Sombyo, 1982). Neither section melting nor growth of ice crystals occur during cryosectioning. Therefore, the ice crystals must be produced by the freezing process.

Unfortunately, there is no cryoultramicrotome in York. The instrument used to obtain the results outlined in this chapter is situated in Leeds. The only method available in Leeds to freeze the specimen was to immerse it into liquid nitrogen. The method of specimen insertion is very important if reproducible results are to be obtained. The depth of a cryogen into which a specimen is plunged has an important effect on cooling velocity, because it influences the exposure to new cold liquid and allows the specimen to keep moving at the initial entry velocity. The shape of the specimen, the angle of insertion, and the velocity (the entry rate) have effects on the cooling rate. The renewal of the coolant around the specimen must be sufficiently rapid to prevent the formation of a gaseous layer which would retard heat dissipation. The specimen should be the first part to contact the coolant.

Liquid nitrogen at its equilibrium boiling point (-196°C) is not a good coolant. It would be impossible to achieve reproducible cooling rates in this cryogen. Liquid nitrogen "slush" (a mixture of solid nitrogen and the sub-cooled liquid) is a better coolant than liquid nitrogen for rapid cooling. This mixture is not in a state of stable equilibrium because of the uncertain locations of solid and fluid phases. Even sub-cooled liquids such as this have substantial temperature gradients throughout the liquid depth. There can be significant variation in cooling rates between runs when using this method as well. It is possible that use of liquid nitrogen slush to freeze the specimens for the experiments described earlier in this chapter would have reduced the problem of ice-crystal damage. Unfortunately, when the work was carried out, this technique was not available in Leeds.

Other factors can influence the quality of sections. It is very difficult to cut frozen sections to a precise predetermined thickness.

Since there is a difference of refractive index between cryosections and sections in conventional ultramicrotomy, interference colours do not provide a good indication of the section thickness. The ultrastructure is poor in thick sections.

The hardness (brittleness) of frozen specimens depends on the temperature, the nature of the specimen, and the size of the ice-crystals (Sleytr & Robards, 1977). Specimens with small ice crystals are easy to cryosection whereas specimens containing very large ice crystals are difficult to cut because the sections crumble.

In contrast to specimens stained in Leeds immediately after sectioning, the sections immunostained in York showed very poor ultrastructure. Some sections tore apart, and this caused the leaching of proteins from the cells. Many organelles appeared as very large vacuoles. The grids with the attached sections were floated upside down on a thin layer of PBS on solid 2% gelatin in phosphate buffer cooled by ice. This method for transporting the sections seemed to be unsuitable. In future experiments, I suggest the grids with the attached cryosections float face down in Eppendorf tubes containing PBS, if transportation is necessary.

Cryoultramicrotomy is becoming a widely used technique for localisation of antigen at the electron microscope level. The disadvantages of cryoultramicrotomy are its requirement of special techniques and equipment to obtain satisfactory results and the high cost of the instrument. However, successful results can be obtained provided the specimen is treated and frozen in the proper way. The skill and investment of time by the operator must be considerably more than for conventional ultramicrotomy. A large number of papers have been published showing the successful use of cryoultramicrotomy for immunolabelling. Most of the antigens studied can resist glutaraldehyde fixation.

From the results obtained in this chapter, I conclude that schistosome surface antigens were destroyed by fixation in glutaraldehyde (even at very low concentrations) and by embedding in K4M resin. Prefixation in paraformaldehyde solution, and the cryoultramicrotomy technique, should be used to localise the schistosome surface antigens in any future study.

A large number of problems have been overcome using the cryoultramicrotomy technique on schistosome parasites even though a final solution has not been reached. The basic technique has therefore been established and if the appropriate instruments and time become available it should be possible to obtain good, meaningful results.

CONCLUDING DISCUSSION

This thesis has been concerned mainly with the location of schistosome antigens *in situ* using monoclonal antibodies raised against tegument fractions of adult *S. mansoni*. The aims of my study were :

firstly, to localise schistosome tegumental antigens in worm tissues in order to interrelate the data on molecular targets and protective properties.

secondly, to investigate host antigens and parasite antigens on the worm surface.

finally, to examine the cross-reactivity between *S. mansoni* and *S. japonicum* tegumental antigens, and cross-reactivity of muscle antigens between schistosome, insect and vertebrate.

In this concluding discussion, I wish to consider these 3 important points.

I). Schistosome Tegument Antigens

a). Some schistosome antigens are conserved throughout development within the vertebrate host. The conserved antigens can be within the same structure, such as the tegument. Antigens detected on the surface of the cercaria and schistosomulum are present in the same location on the adult worm but not accessible to antibodies (as shown by MABs in Group 1).

b). There is antigenic continuity between the surfaces of the cercaria and schistosomulum (as shown by MABs in Group 1). It is possible that cercarial membrane antigens are not shed during transformation, but incorporated into the new membrane of the schistosomulum and retained. There is morphological evidence to support such a conclusion (McLaren & Hockley, 1976; Torpier *et al.*, 1977). It is equally possible that the newly formed schistosomulum surface membrane contains some antigens which are identical to those of the cercarial membrane.

c). Some conserved antigens are exposed on the parasite surface only after transformation from cercaria to schistosomulum (as shown by MABs in group 2 & 4). It seems likely that these antigens are

preformed inside the cercarial body and secreted onto the schistosomular surface during transformation. Surface components expressed only after transformation have been reported by Samuelson & Caufield (1985) using concanavalin A (Con. A). Other antigens not exposed on the larval surfaces, could be detected on sections (as shown by MABs in Groups 3, 5, 10 & 11).

d). Very few surface membrane antigens were only synthesized by the adult worm (as shown by MAB G3.12).

e). There are common membrane antigens between larval and adult stages and also within the adult worm. Examples of the former were shown by MABs in Groups 1, 2, 3, 5, 7, 9, 10 & 11. Examples of the latter were shown by MABs in Groups 1 & 5 suggesting that adult worm surface membrane and internal membranes contained similar antigenic components.

Epitopes are shared between the tegument and the oesophagus of the cercaria and 3h schistosomulum (as shown by MABs in Groups 1, 2, 3 & 7). Epitopes are also shared between the tegument and nephridia of the parasites (as shown by MABs in Groups 10 & 11), and between the tegument and the muscle cells (MAB M7.5). These observations indicated the similarity of antigenic epitopes on different membranes.

f). It appears in the present study that schistosome tegument antigens are destroyed after glutaraldehyde fixation. Only paraformaldehyde could be used to fix the parasites. The conventional epoxy resins which polymerized at +60°C could not be used to embed the parasite. In addition, low temperature embedding resin (Lowicryl K4M) which polymerized at -35°C also destroyed the antigenic determinants of the schistosome. It seems very likely that the reduction in antigenicity was due to the dehydration and embedding processes even at low temperatures and not due to the inability of antibody to penetrate the resin. This conclusion was reached because the non-specific staining of the background was very high. This was not the experience of Roth et al. (1981) who reported low background adsorption of immunoglobulins to K4M-embedded tissue.

From the results obtained in Chapter 4, I suggested that schistosome tegument antigens could be preserved by fixation in paraformaldehyde (6-8%), and that the cryoultramicrotomy technique should be used to localise the antigens at the electron microscope level.

II). Host Antigens and Parasite Antigens

Host molecules could be demonstrated in the present study by rabbit anti-mouse erythrocyte ghost serum. The antibodies bound to the surfaces of intact lung and liver worms but not 3h schistosomula. Antibody from infected mice (AMS) bound to the surface of newly-transformed (3-hour) schistosomula but not to 5-day-lung worms. These observations indicated that host antigens are acquired by developing schistosomula. The acquisition of host antigens coincides with the loss of reactivity of intact parasites to anti-schistosome sera. Thus it is possible that the host antigens mask the parasite antigens. The results in the present study showed that both AMS and rabbit anti-mouse erythrocyte ghost serum bound to the surface of intact 21-day-liver worms (freshly recovered from mice). These observations indicated that immune serum could bind to the worm surface in the presence of host antigens. The loss of host antigen from the surface of 21-day-liver worms after culture *in vitro* for 24 h was very slight as judged by fluorescent antibody staining. This observation confirmed the finding of Saunders (1986) using an indirect radioimmunoassay.

III). Cross-Reactivity of Schistosome Antigens

There was cross-reactivity between *S. mansoni* and *S. japonicum* (5 of 16 MABs, 31.25%). The tegument of *S. mansoni* shared common antigenicity with the tegument and parenchymal tissues of *S. japonicum*. Such cross-reactivity has been reported by other workers (Beisler *et al.*, 1984b).

There was cross-reactivity between the muscles of *S. mansoni*, insects (blowfly and bumble bee flight muscle), and vertebrate (rabbit uterus). Myofibrillar proteins of schistosomes, insects and vertebrates contain similar major proteins of striated muscle i.e. myosin, actin and other components. Protective immunity has been reported by Pearce *et al.* (1986) in mice immunized with extracts of *S. mansoni* identified as muscle protein, in combination with the adjuvant BCG. Since there is cross reactivity between schistosome, insect and vertebrate muscle, the idea of using schistosome muscle protein (paramyosin) as a vaccine in man, needs to be treated with caution. It is possible that autoimmunity could develop as a result of the demonstrated cross-reactivity. The development of anti-parasite immunity producing cross-reactivity with self components has been reported in Chagas' disease where there are shared antigenic

determinants between mammalian neurones, cardiac muscle and *Trypanosoma cruzi* organisms.

APPENDIX

I. Reagents

1). Phosphate Buffered Saline (PBS) pH 7.4 (10x stock)

Di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4)	23.875	g
Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$)	5.793	g
Double-distilled water	1.0	l

Adjus to the required pH (7.4). Add 175.325 g Sodium chloride (NaCl) and make up to a total volume of 2 litres

Dilute stock 1:10 prior to use, resulting in a final buffer of 0.01 M Phosphate and 0.15 M NaCl.

2). Buffered Glycerol Mounting Medium

Glycerol	9	parts
PBS pH 7.4	1	part

pH should be about 8.6, an acid medium will lessen fluorescent staining. 1,4-diazobicyclo-(2-2-2)-octane 25 mg/ml may be added to the mounting medium to prevent fading (Johnson et al., 1982).

3). Eagle's Medium (MEM)

Eagle's medium (Wellcome Ltd.)	10.0	g
Sodium hydrogen carbodrate ($NaHCO_3$)	2.2	g
Double-distilled water to make up	1.0	litre

For perfusion mice (to obtain adult worms), 4 units heparin per ml MEM is added.

4). Eagle's Medium for Culture Liver Worms

Eagle's medium (MEM)	45.0	ml
Heat-inactivated calf serum	5.0	ml
20 mM HEPES buffer	1.25	ml
Penicillin/Streptomycin	1.0	ml

5). 0.02 M Glycine in PBS, pH 7.4

Glycine (M.W. 75.07)	0.03	g
PBS to make	20.0	ml

6). 8% Paraformaldehyde (PF) in Distilled Water

Paraformaldehyde (powder, from Fisons)	1.6	g
Distilled water to make	20.0	ml

Heat to 65°C with stirring. The resultant solution is cloudy. Add a few drops of 1N NaOH, with continued stirring, until the solution becomes clear. Allow the solution to cool.

7). 2% Neutral Uranyl Acetate (UA)

2% Neutral UA was made by mixing equal volumes of aqueous solutions of 4% UA and 0.3 M Potassium oxalate and adjusting the pH of the mixture to 7-7.4 by adding a small volume of 10% Ammonium hydroxide (Tokuyasu, 1980b).

8). 0.7% Methyl Cellulose (MC)

0.7% MC was made by suspending MC powder in hot water (60°C) and then cooling the solution to 4°C and stored at 4°C.

II. To Prevent Sections Becoming Detached from Slides

Poly-L-lysine Hydrobromide (M.W. 150,000-300,000, Sigma) concentration of 1 mg/ml, about 10 ul was applied on clean glass slides at one end and spread over the slide with the end of another slide, as making a blood film. The coated slides were marked because the spread film was not visible. Vials of Poly-L-lysine solution was stored frozen (Polak & Van Noorden, 1984).

III. Procedure for Making the "Optimal" Glass Knife (Griffiths et al., 1983)

The LKB Knife-Maker Bromma 7800 was used for preparing the glass knives for cryosectioning.

1). The method of symmetrical breaking is used to make a rectangular piece of glass with the length exactly twice as

long as the width and then breaks it symmetrically to produce two square plates. The corner edge is perpendicular to the square (Text Figures 6a & 6b).

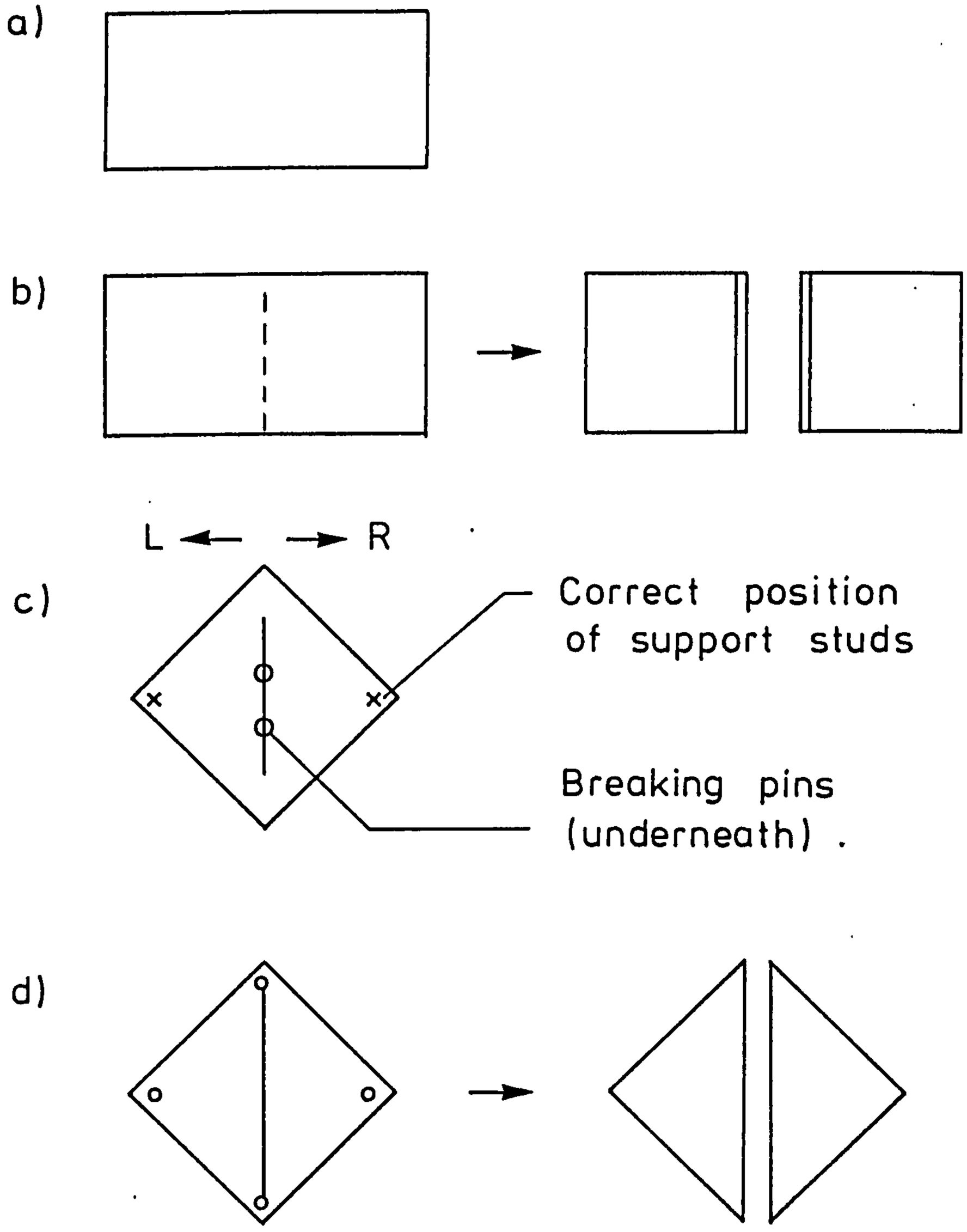
2). Examine which corner produced by the previous step is the better right angle and place the glass between the corner holders. Position it so that the studs are arranged symmetrically along the diagonals, scored (used the longest setting) and break slowly (Text Figures 6c & 6d).

3). Remove the two knives and examine them under a low power stereomicroscope or in the microtome with a portable fibre-optic illuminating system.

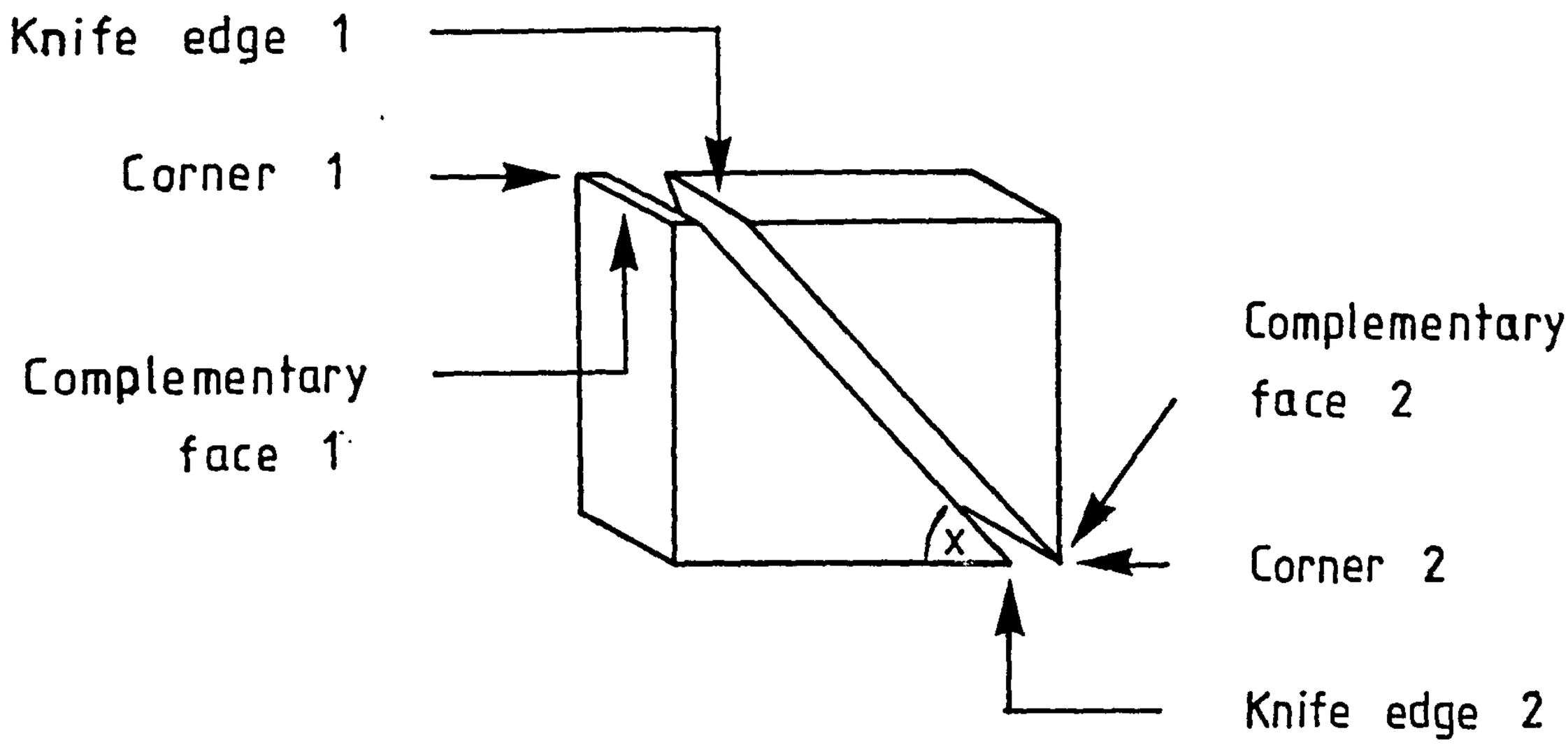
To obtain good (sharp) knives, the break of a square glass plate should occur very close (within 0.1-0.2 mm) to the diagonal line (Tokuyasu & Okamura, 1959), in other words, the width (w) of the complementary face must be minimised (Text Figure 6e). The sharpness of the glass knife is a very important factor in the quality of the section. The central third of the edge (X region in Text Figure 6f), the sharpest part of the knife is the best part for cutting the sections. The smaller the complementary face, the fewer the surface marks and the sharper the knife will be. F region in Text Figure 6f. produces knife-marks when use for sectioning. The B region is the bluntest part of the knife

When the complementary face is > 0.2 mm, the surface faults extend to halfway across the knife. As the width of the complementary face decreases, the surface faults decreases. In an optimal knife they are almost completely absent.

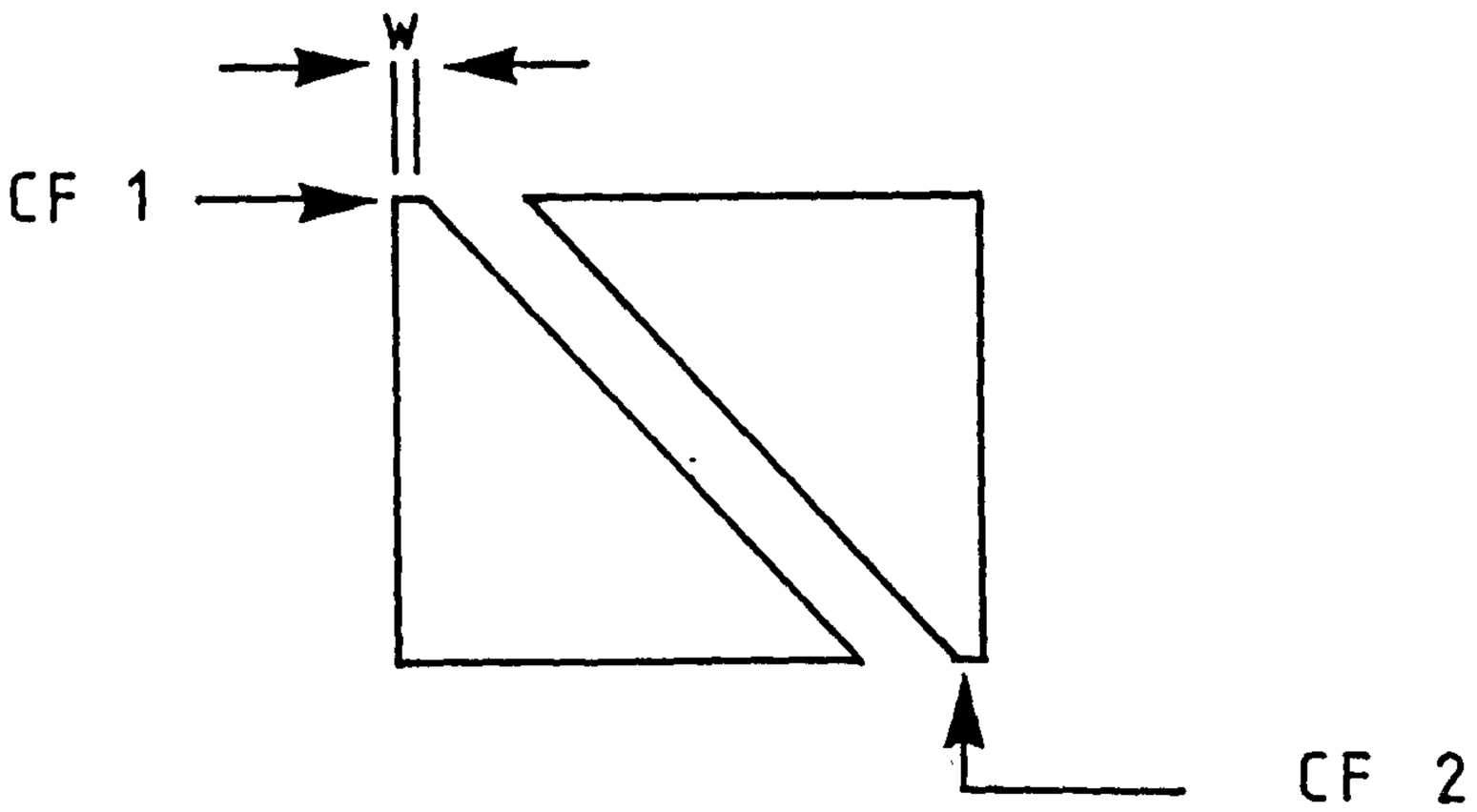
Text Figure 6. Procedure for making the "optimal" glass knife



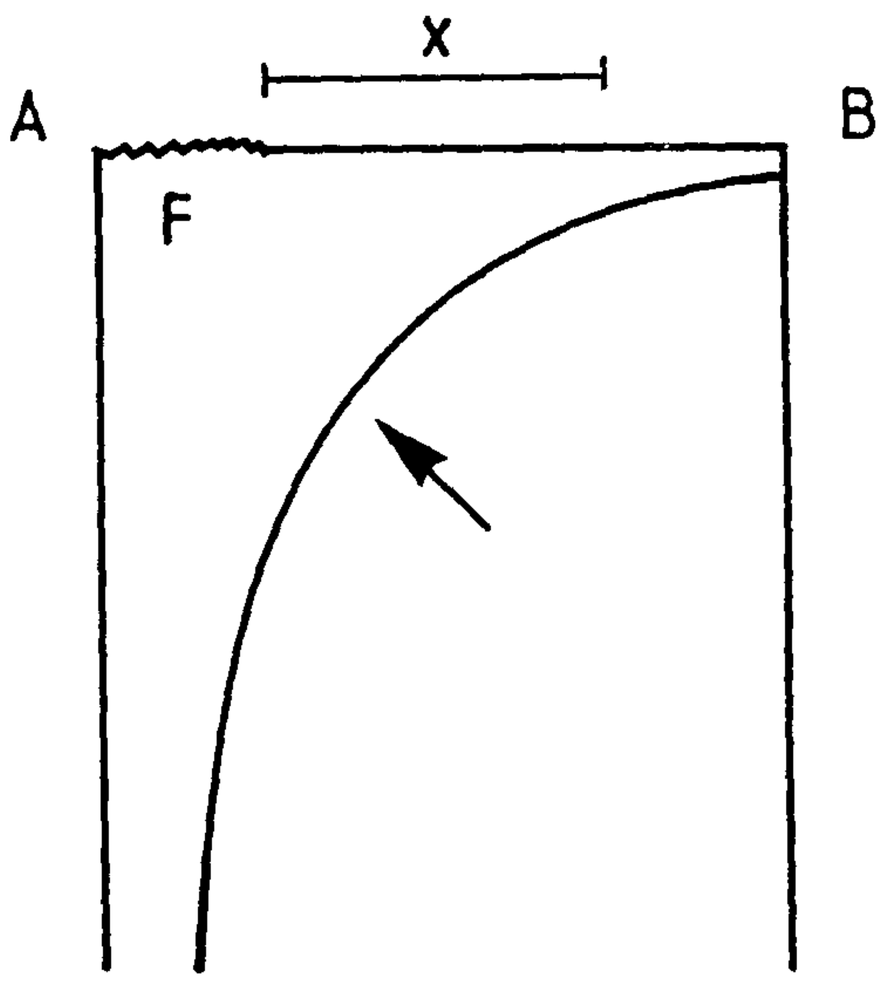
e)


Knife edge 1
Corner 1
Complementary face 1
Complementary face 2
X
Corner 2
Knife edge 2

f)


W
CF 1
CF 2

g)


X
A
B
F

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