The cell biology of the surface membrane of *S. mansoni*: a fluorescence based study.

by

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Abbreviations

BSA	Bovine serum albumin
Con A	Concanavalin A
EM	Electron microscopy
EMS	Eagle's media without serum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
HDL	High density lipoprotein
IMP	Intramembraneous particle
LDL	Low density lipoprotein
Lyso-PC	Lysophosphatidyl choline
MHC	Major histocompatibility complex
NMR	Nuclear magnetic resonance
NRS	Normal rabbit serum
NSS	Normal sheep serum
VLDL	Very low density lipoprotein
WGA	Wheat germ agglutinin

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Summary

Fluorescent lipid analogues are known to intercalate into the lipid phase of the surface membrane of S.mansoni and report on their properties. A variety of such analogues have been employed to gain insight into the structure and function of the surface membrane of both the schistosomula and adult stages of the parasite.

An analysis of the extent of uptake of the dye aminofluorescein C18 by the parasite at various time points during the first three hours of schistosomula development after transformation was carried out. A sharp increase in the relative fluorescence of the surface membrane during the first 60 minutes was observed. The rate of increase declined between 60 to 90 minutes and levelled out thereafter. This pattern of temporal variation was also observed for several other fluorescence linked parameters of this dye. These included the vertical distribution of these molecules within the leaflets (i.e. the percentage of molecules at the surface) and the mobility of the molecules as measured by FRAP. These results were discussed in relation to the formation of the outer bilayer. It was proposed that these changes accurately reflect the formation of the outer bilayer and that the saturable increase in aminofluorescein C18 can in fact be used as a fluorescent based assay to monitor the development of the outer bilayer in early < 3 hr schistosomula. Using this technique preliminary studies were carried out which demonstrate that the presence of colchicine 1 mg/ml markedly inhibits the formation of the outer bilayer indicating a possible requirement or role for microtubules. In contrast U.V. irradiation had no effect.

A qualitative difference in the labelling pattern of two lipid analogues aminofluorescein C18 and DiI C18 was observed in the newly transformed (45 mins) schistosomula. Aminofluorescein C18 labelled all regions of the parasite. DiI C18 labelling was extremely polarised and restricted to the anterior region. This polarised pattern of labelling decreased with time and was absent by three hours post transformation. It was postulated that restricted DiI C18 labelling reflected the localised loss of cercarial glycocalyx. Biophysical studies utilising FRAP and quantitative fluorescence on the more uniformly distributed analogue aminofluorescein C18 were carried put in parallel using DiI C18 as a marker for glycocalyx loss. A time course experiment was undertaken in which these biophysical properties of aminofluorescein C18, in both the anterior and posterior regions of the early schistosomula, were carried out as the polarised labelling of DiI developed shortly after transformation and receded at two hours.

The results observed were that the anterior region of the membrane exhibited both higher fluorescence and greater mobility. This contrast was maximal at around 45 minutes, the time at which DiI C18 polarisation was also maximal, but did persist to three hours. Also during this time period there was a direct correlation between membrane fluidity as measured by FRAP and dye uptake as measured by quantitative fluorescence. This correlation did not persist past 60 minutes. These results were discussed in terms of the influence of a glycocalyx on underlying membrane, the disordered nature of newly exposed surface membrane derived from membraneous bodies, and the existence of differences in membrane properties and functions along the length of the parasite.

It was observed that schistosomula labelled with aminofluorescein C18 lost their probe to media containing low concentrations of serum. This prompted an investigation of the possible interaction between LDL and the parasite surface. It was shown that it was possible to transfer lipid analogues and H^3 cholesterol from LDL to the schistosomula. The kinetics of this transfer exhibited saturable transfer at approximately 80 - 100 ug/ml LDL. An investigation of LDL binding to schistosomula, in the absence of serum, using I^{125} labelled LDL was undertaken. This showed a non-specific pattern of LDL binding which had two components. It was suggested that one of these may be incorporation of LDL into the membrane and the other binding to it. There was no evidence of lysosomal like degradation of I^{125} LDL indicating the absence of endocytosis under these conditions. This conclusion was further supported by the observation that FITC dextran beads Mol. wt. 20,000 failed to be taken up into the tegument of either the schistosomula or adult parasite. Indirect immunofluorescence studies indicated that LDL or LDL epitopes bound uniformly to the schistosomula surface membrane but in the adult binding was localised at the membrane overlying the tubercles. In schistosomula binding of LDL as measured by indirect immunofluorescence could be strongly enhanced by exposure of the parasite to Con A or anti-schistosomula human IgG.

These results were discussed in relation to possible non specific binding mechanisms dependant on membrane surface hydrophobicity and also cholesterol metabolism <u>in vivo</u> with reference in particular to a possible role for the enzyme LCAT.

Some remarkable changes in the morphology of the surface membrane of both schistosomula and adult parasites were observed upon cooling parasites previously labelled with aminofluorescein C18, rhodamine C18 and DiI C18. In adults the normally entirely fold covered dorsal surface of the adult male changes upon cooling to expose a surface consisting of alternating regions of tubercle rich folded membrane and tubercle free smooth membrane. The effect is to make the surface appear striped as the transverse folded regions stand out against the smooth background. This effect does not occur with cooling of DiI labelled adult parasites. In this case cooling induces widespread disruption of the membrane.

Similarly in schistosomula cooling of DiI labelled parasites in association with bleaching of the dye chromophore group itself causes damage. However in contrast to the adult the membrane does not disintegrate rather detatches from the parasite body to form a large balloon like bleb of membrane. This preferentially occurs at the anterior region of the schistosomula.

These observations are discussed with reference to the known biophysical and immunological differences exhibited between these two stages. The implications of the striping effect on current ideas concerning the adult surface membrane morphology and function are also discussed.

Finally, resonance energy transfer between the aminofluorescein C18 probe and DiI C18 or Merocyanine has been demonstrated to occur in the schistosomula membrane. A theoretical strategy whereby this phenomenon associated with the fluorescent analogues can be exploited in conjunction with the FRAP technique to investigate the nature of domain structure of the parasite's surface membrane is advanced.

INTRODUCTION

The cell biology of the surface membrane of S. mansoni: a fluorescence based study.

1.1. General Introduction

1.1.1 Disease and Life Cycle.

Schistosomiasis is a water borne disease known to have infected man since the ancient civilisations of Egypt [Ruffner 1910] and is still an immense public health problem today. The disease is endemic in 75 developing countries in the tropics and subtropics. Over 200 million people are believed to be infected with the disease and of that total approximately ten percent are seriously disabled by it. [Wright 1972]

The causative agent of the disease is a parasitic trematode worm. Three species belonging to the genus Schistosoma are responsible for the vast majority of human infection. Other species of the genus can parasitise a wide range of mammals and birds. The three species responsible for human infection are: Schistosoma haematobium, Schistosoma japonicum and Schistosoma mansoni. Each of these species has a similar complex life cycle, involving two free living and two parasitic developmental stages. Reproduction is sexual in man, the definitive host, asexual in the intermediate host which is a species of freshwater snail. The important differences of concern to man lie in their differing geographical distributions and differences in the pathology of the disease that each induces. Most experimental work has concentrated on S. mansoni which is the most simple to maintain experimentally in non-endemic regions. This reason has determined the species of investigation in this study. Its life cycle will be described in some detail as it involves a complex series of

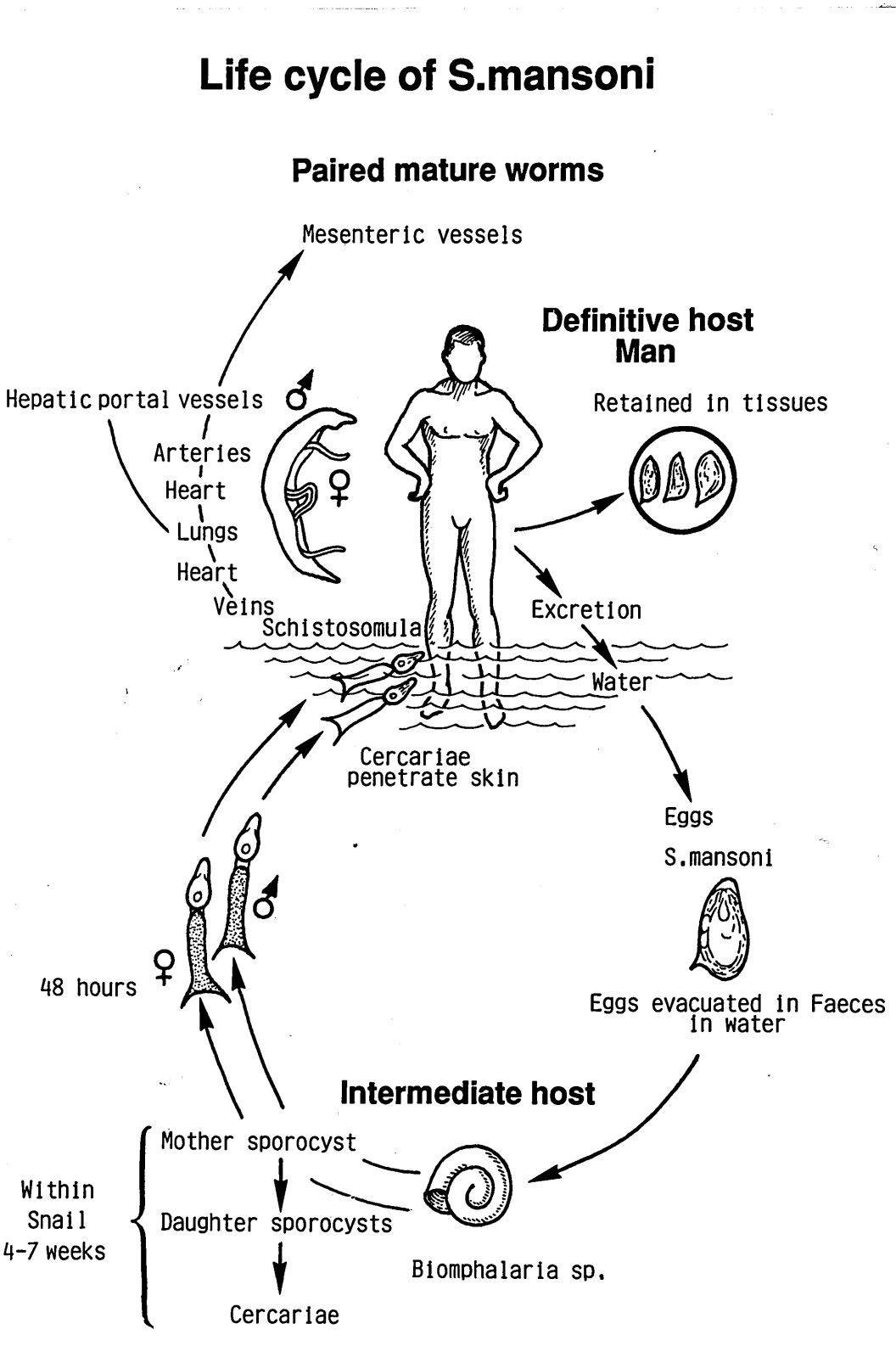
adaptations to different environments and different hosts. This adaptive process occurs within the definitive host and will be a major feature of this work. A diagrammatic representation of the life cycle is shown in Figure 1.1.

The final developmental form of the organism is the "adult" stage of the worm in man. This stage is sexually differentiated and inhabits the mesenteric veins of the liver. Male and female worms are associated in pairs, the female being held within a specialised groove in the adult male termed the gynaecophoric canal. The adult male parasite is approximately 10mm in length, the female up to 17mm in length but appreciably thinner. Both sexes have ventral and oral suckers which assist in the attachment of the parasites to the vascular endothelium.

Each paired adult female produces between 100 and 300 immature eggs per day, roughly equivalent to its own body weight. Since adult parasites survive usually between 5 - 7 years in man [Butterworth 1987] an infected host is exposed to enormous numbers of eggs. These are spherical in appearance approximately 150um long with a diameter of 50um. From the female lodged in the liver mesentery the eggs are circulated around the body in the bloodstream. The majority of eggs escape the bloodstream by breaching the blood vessel walls around the intestine. These enter the lumen of the intestine and are eventually excreted in the faeces.

However a significant number of eggs do not leave the host in this manner but become entrapped in other tissues of the body notably the liver. Inflammatory immune reactions to such eggs initiates the formation of granulomata in the liver and clinical development of the disease [Von Lichtenberg 1987].

Figure 1.1


Life cycle of S.mansoni
Paired mature worms
Mesenteric vessels
Definitive host
Man
Hepatic portal vessels
Retained in tissues
Arteries
Heart
Lungs
Heart
Veins
Schistosomula
Excretion
Water
Cercariae
penetrate skin
Eggs
S.mansoni
48 hours
Eggs evacuated in Faeces
in water
Intermediate host
Mother sporocyst
Within
Snail
4-7 weeks
Daughter sporocysts
Biomphalaria sp.
Cercariae

Introduction of the excreted eggs into water under suitable environmental conditions results in their hatching and the release of the first free living larval stage, the miracidium. The miracidium is equipped to find and penetrate the soft tissues of the intermediate host, a fresh water snail, Biomphalaria glabrata. Following penetration the miracidium transforms into a mother sporocsyt which replicates asexually to produce daughter sporocysts. These migrate to the liver or ovotestes of the snail and undergo a further period of asexual reproduction. This eventually gives rise to the second free living stage the cercaria, which is shed by the snail into water. The cercaria is the stage of the life cycle which is infective to man. Cercariae are endowed with a locomotory tail and are photosensitive. Upon shedding they swim through the water towards the surface of the water body in which the snails reside. On contact with human skin the cercaria can attach to and penetrate the skin very quickly. Penetration through the epidermis and dermis is accomplished by a combination of physical abrasion, lytic secretions and mechanical disruption [Bruce et al 1970], [Stirewalt and Dorsey 1974]. Upon penetration the tail is shed and the parasite form termed the schistosomulum. At this stage the parasite adapts very quickly from a freshwater environment to a mammalian environment of physiological osmolarity. This adaptive process is termed transformation and is marked by the parasite gaining the ability to survive in saline or serum [Stirewalt 1963] and becoming incapable of survival in freshwater [Clegg and Smithers 1968]. Schistosomula move from the skin into the bloodstream via capillaries and migrate through heart, lungs and liver to the hepatic portal vein.

This migration occurs over a period of several weeks during which time the parasite shows many developmental changes, the most obvious of these being an increase in size. In the portal vein maturation is completed, mating between male and female occurs, followed by movement to the final habitat in the mesenteric veins of the liver.

1.1.2. The Schistosomulum and its outer double membrane.

The general morphology of all schistosome stages is similar. It consists of a gut or intestine bounded by a caecal membrane that runs the length of the parasite. Surrounding this are two layers of longitudinal and circular muscles in addition to parenchymal cells and other specialised cell types such as flame cells which mediate excretion of waste products. Bounding this region is another membrane, the basal lamina. Around the exterior of the parasite there is a continuous thin layer of cytoplasm 2um in depth forming a tegument. This is bounded by a limiting membrane between the parasite and the host. The tegument consists of a continuous syncytium of cell cytoplasm. Cellular nuclei are contained in cell bodies which reside immediately beneath the basal lamina but are connected to the syncytial cytoplasm by numerous narrow microtubule lined cytoplasmic connections.

The limiting membrane of the tegument and organism is the surface membrane which overlies the tegumental cytoplasm. This has a unique double membrane structure which develops shortly after transformation. This is due to the fusion of multilamminate membraneous bodies from the tegument with the original single bilayer of the cercarial membrane. This structure appears to be the major adaptation adopted by blood dwelling flukes to their sanguinous environment [McLaren and Hockley 1977]. Formation of this structure takes about three hours <u>in vitro</u> and as monitored by electron microscopy shows a characteristic transition from a trilaminate to a heptalaminate membrane section. However formation of the outer double membrane, which will be

denoted the surface membrane, represents only the genesis of the structure. The surface membrane shows significant changes subsequently as the parasite develops from schistosomulum to adult.

The term "cell biology" of the surface membrane will be used to collectively describe biochemical or biophysical features of the membrane and the functions dependant on them. This term is a useful and descriptive shorthand as it imparts some sense of the scale and nature of the investigations carried out. Much of this study has involved the application of techniques evolved from cell biology to parasitology. The scale of this definition of cell biology ranges from substructures such as "lipid domains" within the membrane to superstructures such as pits and folds formed by it.

The main function of the surface membrane of S. mansoni is to form a limiting membrane around the organism. The most important property of this surface membrane is its ability to resist or avoid immune attack by the host's immune system. An understanding of the molecular basis of this property has been the main stimulus for many studies including this one. The immunological events occurring at the surface membrane and the immunology of schistosomiasis infections will now be discussed in some detail.

1.2 Schistosome Immunology

1.2.1. Introduction.

The salient feature of schistosome immunology is that although the host can mount an immune response to the parasite, this response in vivo is only partially effective. This is true even of a secondary challenge infection in an animal sensitised by carrying an infection itself or immunised by one of several putative "vaccines". It is clear then that in both primary and secondary infections evasion of, or resistance to, the host's immune response occurs. Research in this field has concentrated

on three topics; investigation of immune effector mechanisms capable of damaging and killing the parasite; elucidation of its defensive mechanisms against these effectors and the development of vaccines to enable the former to totally overcome the latter *in vivo*.

Since the surface membrane forms the interface between the parasite and the host it is very likely to be the site of both effector and evasion mechanisms. The outcome of this contest will be determined largely by on the one hand,the main features determining the cell biology of the surface membrane and on the other by attributes of the cellular immunology of the host. Studies that could be classified under these two headings have created a huge literature, over the past 25 years, most of it pertaining to *S. mansoni*. Not only has this provided a large body of experimental data but more importantly a number of distinct models concerning membrane function have been formulated.

This accumulated experimental evidence and the models derived from it will be reviewed in terms of: immune effector mechanisms; models of resistance and evasion; the influence of the cell biology of the surface membrane on each. This is preceded by some discussion of the nature of immunity to *S. mansoni* exhibited by infected animals in particular the phenomena of concomitant immunity.

1.2.2. Innate and Acquired Immunity.

Innate immunity is that level of resistance displayed by a naive host that has never been exposed to the parasite. In human schistosomiasis innate immunity is illustrated by the range of non-human hosts that different species of schistosome can infect and in which the parasite can be maintained under laboratory conditions. Thus *S. haematobium* is poorly adapted to non-human hosts and can be maintained only in certain rodents and primates. In contrast *S. mansoni* can be maintained in a number of

experimental animal models [Smithers and Terry 1969]. Innate immunity is also apparent in variation in the degree of susceptibility to infection exhibited by different hosts to the same species of parasite. For example only 10% of invading S. mansoni schistosomula in an exposed hamster are damaged crossing the skin of hamsters whereas the equivalent percentages for mice and rats are 30% and 50% respectively. Innate immunity to S. mansoni appears to be largely dependent on the effectiveness of the epidermis and dermis of the skin in acting as a barrier. [Clegg and Smithers 1968].

Acquired immunity is that level of damaging or lethal resistance gained by a host as a result of prior sensitization of the host. This immunity can be humoral or cell mediated, specifically induced by the parasite or non-specifically by unrelated organisms. Acquired immunity to S. mansoni can be induced in experimental animals either by infection with the viable parasite itself or an irradiation attenuated parasite or by innoculation with parasite derived antigen preparations. These two types of immunity are commonly distinguished as concomitant or vaccine immunity. Comcomitant in this context refers to infection and describes the paradoxical situation whereby the presence of an active infection induces high levels of immunity directed against parasites of subsequent infections but not against those parasites comprising the initial infection itself [Smithers and Terry 1969]. This concept has been extremely influential in that it directed research towards investigation of the mechanisms underlying the ability of the initial infection to apparently resist the host's immune response and conversely the basis of the susceptibility of parasites of the challenge infection. There is however marked variability in the ability of different experimental animal models exhibiting concomitant immunity to tolerate the primary infection. The rat exhibits self cure in which the adult worm burden of the

primary infection is eliminated between weeks four and five of infection. In contrast the mouse , a permissive host, allows the initial infection to mature and commence egg laying. There are also striking differences in the immunology of infection with S. mansoni between mice and rats. It has been suggested that the rat is particularly suitable for studies on antibody dependant cellular cytotoxic ADCC immune mechanisms and that there are parallels in such immunity between rats and man [Capron and Capron 1986, Capron et al 1987]. It has been suggested that concomitant immunity occurs in man [Bradley and McCullough 1973]. Man also exhibits the development of age related, naturally occurring acquired immunity [Butterworth 1987]. The relationship of these types of immunity with one another has been the subject of much recent research and an explanatory model recently proposed [Butterworth 1987]. However the basis of acquired immunity in humans in terms of immune effector mechanisms responsible for it is at present unclear. Extrapolation from rodent models to the human situation is difficult and can be subject to great error. However there have recently been important advances made using rodent models in elucidating the immune effector mechanisms that may be of most importance to humans. The result of this has been the emergence of a consensus view regarding immune effector mechanisms which may be plausibly extended to human infection [Smithers et al 1987].

1.3 Immune Effector Mechanisms

1.3.1. *In vitro* studies: general.

The action of various effector mechanisms against *S. mansoni* has been shown directly by reconstitutive *in vitro* killing assays or inferred by *in vitro* data, in particular radiolabelling tracking studies on migrating schistosomula. Reconstitutive *in vitro* experiments involved the isolation of the immune components of interest typically specific antibody complement and a particular immunologically active cell type such as a neutrophil. These would then be incubated *in vitro* in microtitre plates with juvenile schistosomula, lung worms or adults and their efficacy of killing estimated by various criteria notably motility and exclusion of vital dyes such as methylene blue. The most informative experiments correlated killing efficacy, as monitored by such killing assays with investigations, mainly by electron microscopy, of the mode of action of various cell types.

An exhaustive review of the experimental data concerned with this type of assay, particularly killing efficacy, is reviewed in the following sources [McLaren 1980, Smithers and Doenhoff 1982]. Of more interest with respect to the cell biology of the surface membrane is the mode of action of these effector mechanisms rather than their efficacy. Detailed analysis and comparison of the variable modes of action of the important effector mechanisms will take place later, particularly in section 1.3.

1.3.2 Efficacy of *in vitro* killing.

The data derived from *in vitro* killing assays is of significance for two reasons. Firstly it produced an *in vitro* rank order of immune effector mechanisms active against schistosomula. At the time of McLaren's review of this subject in 1980 this would have been roughly as follows. Cytotoxic T-cells would be ranked as the least effective mechanism [Vadas et al 1979] below complement and

complement plus specific antibody [Clegg and Smithers 1972, Tavares et al 1978] which are partially effective against newly transformed schistosomula. Antibody dependant cytotoxicity mechanisms would be ranked higher again with neutrophils and macrophage killing [Dean, Wistar and Murrel 1974, Perez and Smithers 1977] markedly less efficient than eosinophil mediated killing [Anwar 1979, McLaren et al 1977]. At this time antibody dependant eosinophil killing mediated by complement and both IgE or IgG was demonstrably the most efficient immune mechanism in vitro and it was postulated to be the most important mechanism in vivo [McLaren and Terry 1982]. Accordingly there was much interest in the mode of action of this cell type at the parasite surface. This was found to be primarily due to the action of locally secreted high concentrations of basic cationic protein. Sequential damage of the outer bilayer preceded damage to the inner surface membrane and was followed by tegumental vacuolation and disruption leading to parasite death [Butterworth et al 1979, McLaren et al 1977, Glauert et al 1978]. Damage by this immune effector mechanism is directed against the surface membrane.

The primacy of the eosinophil as the most effective agent of immune attack in vitro was subsequently challenged by a prime role postulated for the activated macrophage. [James et al 1982, McLaren and Boros 1983]. The mode of action of these effector mechanisms is crucially different from that of the eosinophil in that damage is directed at the subtegumental structures rather than the surface membrane [McLaren and James 1985]. The significance of this difference will be expanded upon in section 1.3.4.

The second significant contribution of cellular killing assays came from the measurement of the efficacy of killing in standardised assays such as MacKenzie 1977. This provided a means for the determination of the relative levels of resistance or susceptibility to immune attack

exhibited by different developmental stages of the parasite. Such developmental studies established that the 4 day old lung worm stage of the parasite was, relative to the 3 hour schistosomula and the adult parasite, extremely resistant to immunological attack [Ramalho-Pinto, McLaren and Smithers 1978, Incani and McLaren 1981]. These authors showed that marked immune resistance developed relatively quickly at an early stage in development.

1.3.3 *In vivo* killing.

In vivo studies investigating the ability of the host's effector mechanisms to destroy the parasites have relied on tracking experiments to determine physiologically where parasite attrition occurs. Alternatively they have utilised animal strains defective in some immune effector to infer its importance. Specific genetic deficiencies in the immune response of experimental animals have been exploited to yield reliable information of this type.

Autoradiographic studies on animals infected with cercaria labelled with very high activity radioactive markers such as Se^{75} have been widely employed. In mice, strain dependant differences in the site of attrition of challenge infections have emerged with some workers implicating the skin as the main site of attrition [Smithers et al 1987] and others the lungs [Dean et al 1984, Crabtree and Wilson 1986]. In vaccinated rats the main site is also the lungs [McLaren et al 1985, Ford et al 1984] and in the vaccinated guinea pig the lungs and the liver [McLaren and Rogers 1986]. Collectively, what these studies indicate is that there are two phases in development and migration at which schistosomula are susceptible to immune attack. An early phase in the skin and a late phase at the lungs and liver. These studies confirm the findings of Smithers and Gammage 1980 and validate their two phase killing hypothesis. Good evidence that the macrophage activity is responsible for resistance to challenge infection has come from utilising

inbred strains of mice with characterised defects in macrophage activation and delayed hypersensitivity, the P and A strains. Neither of these strains of mice develop significant levels of resistance after immunisation procedures that induce significant levels of resistance in non-defective mouse strains [James 1986]. However, other *in vivo* evidence in which a particular immune mechanism was rendered defective by the selective killing of that particular immune cell type by specific antiserum, points to a role for the eosinophils. In the paper of Mahmoud et al 1975 infected mice were treated with specific anti-sera against eosinophils, lymphocytes, macrophages and neutrophils. Anti-eosinophil serum completely abrogated resistance to reinfection in both infected and passively immunised mice, while antisera to other cell types had no effect. Thus conflicting *in vivo* evidence implicating the activated macrophage and the eosinophil has been produced. In addition these two *in vivo* studies are to an extent exclusive since genetically defective mice were defective in macrophage function not in eosinophil function. Conversely, the antisera experiments showed abrogation only in the case of the anti-eosinophil sera and not in the case of the anti-macrophage sera. The recent consensus on the primary role of the activated macrophage compared to the eosinophil has only come about due to a synthesis of *in vivo* and *in vitro* data.

1.3.4 Eosinophil or activated Macrophage.

In vitro studies in mice concerned with ADCC eosinophil killing and activated macrophage killing indicate that both cellular mechanisms can effectively destroy juvenile 3 hour schistosomula. [Pearce and James 1986, McLaren and Terry 1982] Where they differ crucially is in their respective abilities to kill at the 2 - 3 week stage. Activated macrophages can kill at this developmental stage whereas eosinophil ADCC does not. However from shortly afterwards at 3 weeks *S. mansoni* is susceptible to eosinophil ADCC

once more. In relation to in vivo killing as determined by tracking studies these in vitro studies imply that either eosinophil ADCC or activated macrophages could be responsible for the early phase of killing in the skin. Late phase killing in the lung probably is a result of activated macrophage killing but there remains some doubt about killing after three weeks in the liver in vivo. The absence of typical ADCC reactions involving cell adherence and degradation onto the parasite surface, has been reported from in vivo studies, on cutaneous or pulmonary tissues of resistant animals. This has been taken to indicate that such ADCC reactions are not important in vivo [Smithers 1987]. This argument is not conclusive because it is based on negative results and assumes that "typical ADCC" reactions against schistosomula are the same both in vitro and in vivo. Comparative in vitro studies investigating activated macrophage killing and eosinophil ADCC killing have indicated that in peritoneal exudates from infected mice both cell types are present.
These indicated that activated macrophage killing is more efficient than eosinophil ADCC [James et al 1982, McLaren and Boros 1983] and that abrogation of eosinophil specific anti sera does not abrogate killing by other cell types in the peritoneal exudate [Kassis et al 1979]. One could argue that these studies indicate that in vivo eosinophil ADCC killing and activated macrophage killing can take place concurrently.

A conclusive argument cannot be made for the primacy of either eosinophil ADCC or activated macrophage CMI killing mechanisms on the basis of available evidence. It would appear that immunity varies markedly with the species of the host, in rats the evidence points to eosinophil ADCC killing and in mice activated macrophage CMI [Capron and Capron 1986]. The situation in man at present is unclear. When considering the cell biology of the surface membrane in relation to its susceptibility to immune attack, similarities and differences in the action of immune effector mechanisms are important as they strongly direct research.

The hypothesis postulated by Smithers et al 1987 for schistosomula killing involving the combined effects of the cytotoxic effects of activated macrophages and the inflammatory response of the mouse represents a consensus view of killing in the mouse. What is proposed is that a synergistic cellular response to secreted antigens impedes the normal development and migration of the parasite. The release of mediators from activated macrophages leads to damage to the parasite which in turn results in greater local inflammation. This cycle eventually leads to death of the parasite. An important role for T-lymphocytes, primed by soluble antigens released by the parasite, in providing lymphokines to activate the macrophages is envisaged in this model. This model is attractive as it explains in particular a mechanism for the killing of the highly resistant lung stage of the parasite.

Acceptance of this synergistic cellular inflammation hypothesis as a model for parasite attrition would direct research on the cell biology of the surface membrane towards particular questions:

1. What is the nature of both the "priming" parasite antigens and the mechanism of their release from the membrane?
2. What features on the surface membrane of the parasite mediate the transient adherence of activated macrophages, which precedes their damaging effects? [McLaren and James 1985].
3. Most importantly from a biophysical perspective how are these damaging effects able to be transmitted across the surface membrane and tegument, to damage subtegumentally leaving the membrane and tegument unaffected?

Consideration of the means of action of eosinophil ADCC would prompt a set of broadly similar questions which differed in small but significant details. Eosinophil ADCC killing has been shown to come about due to the very close apposition of eosinophil and parasite membranes. This is mediated by IgE or IgG bound to the surface of the parasite and the effect is for the parasite surface to form a template to which the cell moulds [McLaren 1980]. Into the very small space between the parasite and the cell, the eosinophil discharges the contents of its secretory granules. These contain hydrolytic enzymes and a unique major basic protein which has been shown to induce damage to the surface membrane of S. mansoni [Butterworth et al 1979 b]. The process of damage is of interest as it contrasts so markedly with activated macrophages. Damage is induced at the surface membrane itself in a sequential manner beginning with damage to the outer bilayer followed by its local separation from the inner bilayer. Subsequently the contents of the eosinophil secretory granules are deposited on the inner bilayer resulting in tegumental vacuolation. This is due to permeability

changes induced in the membrane and this eventually results in the formation of small lesions in the membrane through which eosinophils migrate. These cells then strip away the tegument from the parasite body killing the parasite. This leads to the further degradation of the muscle layer by macrophages [McLaren, Ramalho-Pinto and Smithers 1978]. The main questions prompted by this cellular effector mechanism would be:

1. What primes the host to produce antibodies against the surface membrane that mediate opsonisation?
2. What features of the surface membrane do these antibodies recognise?
3. How do the contents of the eosinophil secretory granules damage and disorganise the structure of the surface membrane. In particular, what causes local separation of inner and outer bilayers and lesion formation in the inner bilayer?

1.4 Immune Evasion

1.4.1 Susceptibility and Resistance.

Analysis of the main questions listed above reveals three common elements important to explaining susceptibility of the parasite. These are: lymphocyte recognition and activation; immune recognition at the level of the parasite surface membrane itself; and structural features of the surface membrane capable of being damaged. Since successful operation of these three elements appears to be the key feature essential for killing the parasite, it follows, that modulation of these three elements by the parasite as it develops in the host would provide a means to avoid destruction by the host's immune system. In each case this has proved to be the case. In many ways studies which elucidated these mechanisms have contributed more to an understanding of the cell biology of the surface membrane than any others. These mechanisms fall neatly under the headings described, that is; modulation of the lymphocyte response, evasion of recognition at the surface membrane and the intrinsic resistance of the membrane to immunological attack.

1.4.2 Evasion by modulation of the lymphocyte response.

Comparison of the nature of the humoral immunity induced by vaccination with irradiated cercaria or non-attenuated cercaria, "vaccine" and concomitant immunity has been informative and has lead indirectly to the concept of blocking antibodies as a mechanism of immune evasion. The targets for antibodies of concomitant immunity have been shown to be mainly carbohydrate epitopes on the surface of schistosomula. These antibodies are directed against carbohydrate epitopes found on both schistosomula and the egg therefore could have been elicited by either. In contrast the antibodies of vaccine immunity are targetted against polypeptide epitopes of the schistosomular surface not found on eggs. [Omer-Ali et al 1986] These results

provided an explanation of both the previously observed close correlation of concomitant immunity with parasite eggs and its lack of species specificity. Concomitant immunity elicited against S. mansoni will protect against infection by S. haematobium also [Smithers and Doenhoff 1982]. This explanation was that in essence concomitant immunity is directed against commonly occurring carbohydrate epitopes on the schistosomula which are shared both between schistosomula of differing species and between schistosomula and eggs within species. Concomitant immunity appears to be a non-specific egg elicited immunity. Vaccine immunity on the other hand is elicited against polypeptide antigens occurring on the schistosomula itself. These conclusions were derived from data from experiments investigating differences in antibody specificity from sera of mice exhibiting concomitant and vaccine immunity. This was only possible due to recent advances in vaccination techniques which provided a means of inducing vaccine immunity that was demonstrably effective as measured by passive transfer of immunity [Smithers et al 1987].This provided a source of vaccine serum with definite immunological protective activity which could be analysed alongside concomitant immune sera. As has been seen such comparative analysis can link immunocytochemical differences to biological immunity.

The key findings underlying the concept of blocking antibodies in mice are summarised in list form below.

1. In the mouse binding of antibodies to the schistosomula surface derived from concomitant immune sera is up to three times greater than that of binding of vaccine sera antibodies. This is despite the fact that the same set of antigens is recognised by both sera.
2. Treatment of schistosomula to remove carbohydrate residues on their surface antigens removed this relative difference in binding. This indicated that the difference was due to the presence of carbohydrate binding antibodies present in concomitant immune sera but absent in vaccine sera. [Omer ali et al 1986, Simpson, James and Sher 1983]
3. In mice the presence of eggs elicits the production of high titres of antibodies which are directed against carbohydrate epitopes which cross-react strongly with schistosomula.
4. In mouse anticarbohydrate antibodies are of IgM isotype and the removal of this immunoglobin fraction from infected serum results in an increase in the relative killing efficacy as measured by <u>in vitro</u> killing assays. Similar fractionation of vaccine sera has no effect on killing efficacy. [Yi et al 1986 a, Yi et al 1986 b]

Thus in the mouse it would appear that carbohydrate epitopes of the eggs elicit production of high titres of IgM class antibody which can avidly bind to the schistosomula surface and inhibit killing. This inhibition has been proposed to be due to a blocking effect of IgM binding. Binding of non-lethal IgM to carbohydrate epitopes on glycoprotein surface antigens is likely to sterically inhibit binding of lethal IgG antibodies directed against polypeptide epitopes on the same antigen.

A similar line of enquiry has been pursued by Capron's group investigating the nature of humoral immunity in the rat. This group exploited hybridoma technology to produce two monoclonal antibodies directed against the same highly immunogenic parasite antigen of 38kD molecular weight. These monoclonals had the same specificity but were of different isotypes IgG2a or IgG2c. These authors showed that the IgG2a monoclonal could mediate eosinophil ADCC in vitro and be used to passively transfer immunity in vivo. Monoclonal antibodies of IgG2c isotype caused inhibition of such killing in vivo and in vitro [Gryzch et al 1984, Khalife et al 1985]. Thus it appeared that a major surface protein antigen on the schistosomula surface could elicit IgG of antagonistic activity depending on isotype. That IgG2c isotype could have a more general inhibitory role in the rat model was indicated by in vivo serum fractionation experiments in which removal of that isotype from serum, increased the relative killing efficacy of that serum. Moreover isotype status could be correlated respectively to resistance and susceptibility of rats to reinfection. [Khalife et al 1985].

Blocking antibodies appear to exist in both the rat and mouse models and the basis of their activity to lie in their isotype. Evidence has been produced which indicates that blocking antibodies are produced in human infection. This reflects similarities with both the mouse and the rat models in that the blocking isotype is IgM which could exert its effect on the 38kD molecular weight surface antigen [Capron 1987]. The demonstration of blocking antibodies in vitro and in vivo has lead to the hypothesis that such blocking effects underlie the observed age dependence of acquired immunity of human populations in endemic areas [Butterworth 1987]. This hypothesis proposes that in man, like mice, blocking IgM antibodies can be elicited by egg carbohydrate epitopes and potentially lethal IgG can be elicited by parasite proteins. Thus in

man there will be a balance of lethal and blocking antibodies. This balance is likely to reflect the extent of sensitization of the host by either egg or parasite antigens. Since the physical mass of the eggs is so much greater than the parasite itself, this balance is likely to favour the production of blocking IgM antibodies. This will be particularly true of trickle infections associated with childhood in endemic areas and progressively less true of older populations. These older populations will have been exposed more often to the parasite antigens themselves therefore are able to produce more antibodies directed against its surface proteins. This hypothesis is attractive as it explains both age dependant acquisition of immunity and the widespread occurrence of and susceptibility to reinfection after pharmacological cure.

1.4.3 Evasion via modulation of lymphocyte function.

Modulation of the lymphocyte function in the sense of modulating the humoral immune response has been well characterised and clearly represents a major mechanism of immune evasion. There is also some evidence for modulation of the lymphocyte function through immunosuppression of T-cell activity. Again carbohydrate parasite epitopes are involved, in this case the glycocalyx of the cercaria, which is deposited in the skin during transformation and has been shown to mediate inhibition of T-cell proliferation induced by parasite antigens in vitro. [Vieira et al 1987]. Whether immunosuppression of T-cell activity occurs in vivo to any great extent is not known but it is possible that soluble released proteins of schistosomula could mediate such an effect.

1.5 Evasion of recognition at the surface membrane

1.5.1. Host antigen uptake: in vivo experiments

The first evidence that schistosomes could acquire host antigens came indirectly from the classic transfer experiments of Smithers, Terry & Hockley 1969. The experiments indicated that adult S. mansoni could acquire host antigens from their host, exchange these with antigens of another host and that such uptake of antigens may confer protection. These conclusions were drawn from a key experiment in which groups of adult parasites from mice, "mouse worms", were transferred to rhesus monkeys. Two groups of monkeys were used one of which had been primed with mouse red blood cells and the other was a control group. "Mouse worms" transplanted to non-primed rhesus hosts adapted to the new host and resumed egg laying after transplantation. In addition they also exchanged mouse antigens for monkey antigens at their surface over an extended period of three to seven days. In contrast "mouse worms" transplanted into rhesus monkeys primed against mouse erythrocytes were rapidly destroyed.

Evidence that masking of parasite antigens was the means whereby host antigens conferred protection was presented by McLaren et al 1975. These authors utilised immunocytochemical methods in conjunction with electron microscopy to show that lethal antibodies from immune rhesus monkeys will bind to 3 hour schistosomula but not at all to 4 day old worms removed from mouse lungs. Moreover three hour schistosomula totally failed to bind anti-mouse erythrocyte antibodies, whereas 4 day lung worms gave a very strong reaction for the presence of host antigens on the surface of the schistosomula. This inability of antibodies from an immune monkey to bind to the lung worm surface was taken to indicate that acquired host antigens could protect the parasite membrane against antibody mediated damage. Subsequent studies by two groups using

independent methods found that all stages of the parasite stained positively for the presence of host antigens. However all stages with the sole exception of the lung worm could also bind lethal antibody from an immune host albeit weakly [Goldring et al 1977]. However this conclusion, that lung worms do not expose detectable antigens at their membrane surface, has been challenged by Bickle & Ford 1982 who demonstrated adherence of a macrophage cell line P388D via Fc receptors after exposure to serum from chronic infected and irradiated vaccine serum. This implied that IgG from the two types of sera bound to exposed antigens on the parasite surface membrane.

1.5.2 Host Antigen Uptake: *in vitro*.

It is clear then from the *in vivo* evidence that uptake of host antigens correlated well with the development of protection. Numerous *in vitro* studies have shown directly that the parasite surface is capable of acquiring host antigens from both serum and cell membranes themselves. This line of evidence began with experiments which are the *in vitro* equivalent of the *in vivo* transfer experiments. Clegg et al 1971 showed that *in vitro* incubation of schistosomula in media supplemented with serum and erythrocytes of a particular blood group rendered these schistosomula highly susceptible to killing upon transfer into a monkey primed against that blood group. Thus growth in A^+ or B^- erythrocytes and serum, *in vitro*, followed by appropriate homologuous transfer into monkeys primed against A^+ or B^- resulted in the destruction of the parasites. Transfer of such parasites into a non-primed animal did not affect the viability of the schistosomula and in addition heterologous transfer to a monkey primed against a blood group other than that of the initial culture resulted in high levels of survival. Clegg 1971 interpreted these results as indicating that schistosomula can acquire glycolipid blood group antigens *in vitro*.

These in vitro culture experiments led to subsequent experiments which showed that both blood group antigens and gene products of the major histocompatibility complex of the mouse could be acquired. Subsequent analysis of the uptake of blood group substances indicated that the antigens acquired were glycolipid in nature and included A, B, H and Lewis b+ antigens. Significantly glycoprotein constituents of the erythrocyte membrane were not detected in these experiments. [Goldring et al 1976, Goldring, Kusel and Smithers 1977]. In contrast the MHC gene products shown to be acquired, the K and I gene products, are known to be integral membrane glycoproteins [Sher et al 1978].

These glycoproteins were acquired from host cells, therefore some mechanism of antigen transfer from membrane to membrane must occur. Such a mechanism would also need to explain the apparent selectivity for certain glycoproteins derived from mouse lymphocytes [Sher et al 1978] and human lymphoblasts, tonsil cells and platelets [McLaren 1979] but not erythrocyte glycoproteins. Further demonstration of a capability of the very young schistosomula to acquire integral glycoproteins from cell membranes was produced by Smith, McQueen and Kusel 1977. These authors reported specific uptake of intercellular substance ICS antigens from mouse skin in vivo and in vitro.

1.5.3. Mechanisms of Uptake.

The uptake of host blood group glycolipids is readily explicable by transfer and interdigitation of the hydrophobic portion, the ceramide group, of these structures into the surface membrane. [Clegg 1972]. A similarly convincing explanation for the transfer of integral membrane proteins from cell to schistosomula membranes has only recently been proposed [Golan et al 1986]. This proposes fusion of the cell membrane and the parasite membrane as being the mechanism of both transfer

and intercalation. This is a more convincing explanation of uptake of glycoproteins than receptor mediated uptake of MHC antigens by binding of B_2 microglobulin [Torpier et al 1979] as it is less specific, and therefore could apply to ICS glycoprotein uptake as well as MHC antigen uptake. Moreover this explanation describes a mechanism to account for transfer from cell to parasite that could apply more readily to the lung worm stage.

1.5.4 Antigen masking as a mechanism of immune evasion.
Uptake of host molecules by the parasite surface membrane certainly does occur and the masking of parasite antigens that it engenders provided an attractive and conceptually simple hypothesis to explain immune evasion by the parasite in vivo [Smithers, Terry and Hockley 1969]. In particular the close correlation between the total lack of lethal binding to the lung stage and its extreme resistance to immune attack in vitro suggested that such a mechanism could operate in vivo. Developmentally, transition from schistosomula to lung worm was proposed to lead to increasing parasite antigen masking and resistance [Smithers, Terry and Hockley 1969].

This simple and attractive model was subsequently challenged by several groups of workers who showed that resistance to immune attack in vitro could develop in the absence of host molecules (i.e. serum) [Dean 1977 and Dessein et al 1981]. This lead to the concept that schistosomula could develop intrinsic resistance to immune attack due to some developmental change in the tegument or surface membrane. This theory was supported by Moser, Wassom and Sher [1980]. These workers exposed parasites to immune attack under conditions in which antigen masking was by-passed due to labelling the surface membrane with the hapten Trinitrophenyl (TNP) and exposing them to anti-TNP antibody. These experiments were extended to show that schistosomula and lung worms treated in this way and

exposed to an in vitro killing assay showed differential susceptibility, schistosomula were killed while lung worms remained resistant [Moser, Wassom and Sher 1980].

An attempt to define the relative contributions of the two main mechanisms of immune defence identified at that time, antigen masking and intrinsic resistance, was made by McLaren and Terry [1982]. In this paper the experimental approach was to monitor in vitro killing of parasites of varying ages from 5 days to adults in a system in which masking was abrogated by directing the immune attack against the host antigens absorbed onto the parasite rather than parasite antigens themselves. It was shown that 5 day, 2 week and small 3 week parasites were resistant to such attack. Larger 3 week and adult parasites were susceptible. These authors concluded that the parasites employed two mechanisms of defence. Masking by acquired host antigens which is operative throughout development and some form of intrinsic resistance mechanism which is operative only until the third week of development. Thus the main mechanism of defence of adult parasites was concluded to be parasite antigen masking by host molecules. This confirmed the classic hypothesis of Smithers and Terry drawn from their transfer experiments in vivo. The validity of extending this hypothesis to earlier developmental stages particularly the lung worm is more uncertain due to their possession of a second major defence mechanism. It would appear from the in vitro experiments described that protection by antigen masking of the parasite between approximately 5 and 20 days into development is superfluous. Until some way is found of abrogating the intrinsic resistance mechanism of the lung worm the relative contributions of each mechanism cannot be distinguished. The most important contribution of this paper of McLaren and Terry is in its identification of an implied developmental transition in mechanisms of immune defence employed by the parasites at around three weeks in

development. Large scale changes in parasite growth, morphology and membrane properties also take place at this time. Therefore this paper provides good circumstantial evidence of a link between the structure of the membrane and its function in terms of immune defence. This developmental link between surface membrane cell biology and function will be explored in detail in section 1.7.2.

1.6 Mechanisms of Intrinsic Resistance

1.6.1. Importance of intrinsic resistance.

The concept of the intrinsic resistance of the surface membrane of S. mansoni has been of great importance to this project. In summary the aims of this project have been to devlop or apply new techniques to investigate the structure of the surface membrane and to relate such knowledge to its function. At the outset of this project it was clear that such a biophysical approach could be most usefuly applied to the investigation of the basis of the intrinsic resistance of the parasite surface membrane. Although over the course of this project important progress has been reported in this area by other workers, this rationale still applies at the time of writing.

1.6.2 An immunologically inert surface.

An alternative mechanism of resistance has been postulated for lung worms by Pearce et al [1986]. These authors proposed that the reduced surface antigenicity of developing schistosomula is due to antigen shedding rather than host molecule acquisition. They demonstrated that schistosomula cultured in vitro exhibit markedly greater expression of parasite antigens than parasites grown in vivo for an equivalent time period after transformation. This was ascertained by quantitative indirect immunofluorescence or quantitative fluorescence of FITC labelled lectins. This differential is apparent at 24 hours maximal at 6 days and persists up to 10 days at which point binding of anti-parasite antibody can still be

demonstrated on schistosomula cultured in vitro whereas in vivo insignificant binding is monitored from 6 days post transformation.

When radiolabelled 6 day old schistosomula cultured in vitro are injected intravenously into mice and recovered from the lungs of mice at various points thereafter there appeared to be a rapid loss of both lectin binding sites and anti-parasite antibody binding sites. These authors proposed that surface antigen shedding promoted by some unidentified host stimulus accounts for the reduced surface antigenicity of schistosomula. They concluded that such shedding occurs naturally in vivo and contributes to the immune resistance of the parasite.

These results are in conflict to some extent with those of Bickle and Ford [1982]. One of the main conclusions of Pearce et al [1986] is that there are exposed parasite antigen epitopes expressed at the lung stage surface membrane at a very low density. Therefore the disparaties in detection of antibody binding at this stage can be explained by the relative sensitivities of the assays employed by various workers. Clearly reduced antigenicity at the lung stage will render the surface relatively immunologically inert, however this seems to occur in parallel with the acquisition of some other form of membrane intrinsic resistance. This is because by-passing this reduced antigenicity by exploiting absorbed host antigens as a target for immune attack [McLaren & Terry, 1982] or using artificial haptens such as TNP [Moser, Wasson and Sher, 1980] does not result in killing. Therefore reduced surface antigenicity cannot be the sole explanation for the intrinsic resistance exhibited by lung worms.

In the adult stage an analogy between the structures and functions of the outer bilayer and the envelope of Gram negative bacteria has been drawn by Podesta et al [1987]. These authors propose that the thick relatively hydrophilic

carbohydrate on the outer leaflet of the adult functions similarly to the coat of wild type S. typhinurium and renders the parasite relatively resistant to hydrophobic dependent binding of immune effector cells. This is an example of an immunologically inert surface relying on a biophysical inhibition of binding rather than inhibition of recognition.

1.6.3 Accelerated membrane turnover.

Some important contributions to an understanding of the cell biology of the surface membrane of S. mansoni have come from the collaborative work of Samuelson and Caulfield. In a series of informative papers they have described three potential mechanisms to explain aspects of intrinsic resistance and in the case of two of these a common molecular mechanism, which may also explain uptake of integral glycoproteins by the parasite membrane.

The demonstration of the rapid loss of surface glycoprotein to the media surrounding in vitro newly transformed schistosomula within a half time of 8-10 hours for Con A labelled glycoproteins [Samuelson, Caulfield, David 1982] or 5-6 hours for surface antigens and C3 acceptor sites [Samuelson, Sher, Caulfield 1980] is indicative of rapid membrane shedding. This was shown to be shedding of the entire membrane. This suggests a means whereby developing schistosomula could evade recognition due to the shedding of antigen plus attached antibody, or the pre-shedding of potential antibody or complement binding sites prior to this. These rates of shedding indicate that any immune effector mechanism dependent on recognition of antigens specific to the newly transformed schistosomula has only 24 hours to operate in vitro and probably much less in vivo. McLaren et al [1978] have provided evidence that the large scale turnover of membrane, implied by these experiments, does occur. They demonstrated that shed membrane could be replaced by fresh membrane derived from the fusion of large

numbers of tegumental membraneous bodies to the apical region of the tegument. In these studies this mechanism could be overcome by the damaging effects of eosinophils.

Evidence for accelerated membrane turnover in response to immune attack has also been presented for the adult stage of the parasite [Perez and Terry 1973]. An interesting mechanism of membrane shedding involving the aggregation and flow of intra-membraneous particles off the spines of adult parasites has been implicated by several groups [Torpier and Capron 1980, Wilson and Barnes 1977]. Such shedding implies either mass flow of membrane within the surface membrane of the adult parasite or that the IMP recognised are mobile in a fluid membrane. As a mechanism of immune defence accelerated turnover clearly has limitations in its effectiveness [Perez and Terry 1973, McLaren et al 1978]. This point has been taken up by Saunders, Wilson and Coulson [1987]. These authors showed using a sensitive radiometric assay that the loss of erythrocyte antigens is relatively slow with a half life of up to 45 hours. This was taken to indicate that the membrane itself had a similarly slow rate of turnover. This would be insufficient in itself to mediate immune evasion. However they also showed that exposure of the adult membrane to cationised ferritin increased the rate of turnover markedly as reported in previous papers [Wilson & Barnes, 1977, Roberts et al 1983]. This increase was interpreted as reflecting a capacity of the surface membrane for rapid but limited repair of damage. This property was proposed to account for earlier reports listed above indicating that the parasite membrane turned over rapidly thereby contributing to immune evasion. Therefore accelerated membrane turnover per se is not a continuous, ongoing, general mechanism of immune evasion in the adult However it appears that a localised limited increase in turnover in response to damage is. Such a distinction is important since it implies that the parasite surface is

sufficiently differentiated that it can detect and respond to local changes in its environment in a relatively restricted area. A further distinction could be drawn between the schistosomula where a new membrane is developing relatively rapidly and the adult which is fully differentiated and in which the membrane appears to be relatively stable until damaged. In cells turnover of cell membrane lipids and proteins is achieved by endocytosis. Shedding of membrane in the manner described may well represent the parasites alternative to this. This alternative it would seem may also be beneficial to some extent as a mechanism of immune evasion either at the surface membrane itself or by releasing immunomodulating agents into the bloodstream.

1.6.4 Neutrophil Inhibition.

Neutrophils have been shown to be one of the less efficient immune effectors of schistosomula killing [Anwar, Smithers and Kay 1979]. The mechanism of both neutrophil and eosinophil interaction with the schistosomula membrane has been investigated by Caulfield et al [1980]. These authors produced evidence for a series of events during interaction between schistosomula, opsonised with specific antibody, and neutrophils which culminated in the fusion of the neutrophil membrane with that of the schistosomula it recognised. In this system parasite cell attachment was mediated by receptor ligand interactions, the ligand being antibody on the parasite surface, the receptor Fc receptors on the neutrophil. The comparison of neutrophil and eosinophil attachment by electron microscopy showed that the neutrophil attached by several discrete foci between cell and parasite, in contrast the eosinophil showed greater areas of more closely opposed membrane attachment. Closer examination showed that the means of neutrophil parasite attachment was due to fusion of small areas of the neutrophil cell membrane with the outer bilayer of the schistosomula to form structures similar to occludens tight

junctions. This was not the case with eosinophils. Agitation of schistosomula with adherent eosinophils had no effect on their adherence. In contrast agitation of schistosomula with adherent neutrophils lead to the breakdown of the tight junction structure and the release of the neutrophil from the parasite. As a consequence small foci of neutrophil lipid and integral membrane proteins derived from the neutrophil outer membrane were left in the parasite membrane. Prior to the fusion event neutrophils were observed to endocytose small areas of schistosomula membrane including lipids and proteins of the parasite [Caulfield, Korman & Samuelson 1982]. There are three important consequences of this endocytotic, leading to fusion, sequence of events. Most importantly endocytosis of ligand receptor complexes by the neutrophil could inhibit exocytosis of the granulocytic enzymic and protein contents of the neutrophil thereby inhibiting its damaging action. Secondly, fusion of neutrophil membrane with the parasite outer membrane provides a mechanism for the observed uptake of integral membrane protein, such as MHC antigens by the parasite. Thirdly, endocytosis of the parasite membrane by neutrophils could provide a means of promoting membrane turnover by the parasite itself.

1.6.5 The action of lyso PC.

The most recent work of these authors extended the previous studies and importantly provided a molecular explanation of neutrophil and parasite membrane fusion [Golan, Caulfield et al 1986]. In this paper it was reported that the lipid lyso PC was shed from the surface membrane of schistosomula at relatively high concentrations compared with its contribution to total worm lipid. It was also shown that changes in the membrane properties of adherent red blood cells were consistent with changes induced by lyso PC itself. These changes, essentially an increase in the immobility of erythrocyte lipids and proteins, eventually resulted in the lysis of the RBCs. The rate of lyso PC

release by the schistosomula *in vitro* was calculated to be sufficiently high to attain lytic concentrations of lyso PC at the surface of the parasite. The fusogenic and lytic properties of this molecule were proposed as the molecular basis for a number of important cellular immunological effects observed at the surface membrane of *S. mansoni*. These included lysis of eosinophils prior to degranulation [Caulfield, Korman and Samuelson 1982], fusion between neutrophils and schistosomula surface membrane [Caulfield et al 1980] and also inhibition of mast cell degranulation [Mio et al 1985]. The detergent properties of lyso PC were postulated as providing an explanation for both the shedding and endocytosis of parasite surface proteins by neutrophils without damage to the cells themselves. Three general roles were proposed for lyso PC in the parasite's surface membrane. Neutralisation of attacking host immune cells by lysis and modulation of cell membranes; fusion between cells and parasites leading to the uptake of host membrane components; modulation of antigen presentation and immune recognition of parasite antigens. Locally high concentrations of shed lyso PC at the surface of the parasite membrane may underly the operation of several processes or mechanisms which contribute to the intrinsic resistance of the surface membrane to immune attack. The production and release of lyso PC at the surface membrane could therefore represent the central unifying mechanism of intrinsic resistance. This hypothesis is attractive in its simplicity as a unifying explanation of a range of different processes occurring at the surface membrane. However, its very wide ranging nature itself raises doubts as to whether this mechanism could account for intrinsic resistance in totality. Assuming that lyso PC does mediate all the processes attributed to it by Golan, Caulfield et al at the surface membrane of schistosomula, the question arises whether there is enough of it produced to activate all these various effects. In addition, all of these effects have been demonstrated *in vitro*. *In vivo* two

important factors are likely to affect the central point of the hypothesis namely that at the surface of the schistosomula the absolute concentration is high enough to cause its lytic and other effects. Firstly, in vivo the parasite will exist in the bloodstream which is an environment in which the medium will be constantly renewed as it flows past the schistosomula. Although diffusion very close to the surface membrane and hence the relative concentration of lyso PC will not be greatly affected by plasma flow it would be expected to have some effect in increasing removal of lyso PC from around the parasite. Related to this point removal of lyso PC from the parasite surface by constituents of the plasma in vivo such as albumin and lipoproteins which bind lyso PC may be expected to occur. In the in vitro experiments only RPMI media with 1% BSA was used therefore essentially all the lyso PC produced would have been available to the adherent cells. Taken together the effect of a constant flow of plasma containing potentially non-cellular membrane competitors may be synergystic and could act to reduce the relative concentration of lyso PC at the schistosomula surface. In support of the hypothesis, however, it is very significant that the plasma protein most likely to bind lyso PC competitively, serum albumin, does not bind to the surface of schistosomula [Sher, Hall & Vadas 1978]

To return to the original question of whether enough lyso PC is produced. Leaving aside any potential competition from plasma in vivo, Golan and Caulfield estimate that enough lyso PC is produced to lyse 4-5 erythrocytes a day in vitro. Given that eosinophils are much larger than erythrocytes and that lyso PC appears to act by reaching a lytic condition with the cell membrane itself then one would expect that perhaps one eosinophil could be lysed per day. Translating such in vitro rates to the in vivo situation, is lysis of one eosinophil per day enough to confer protection? The critical information required here

is how many eosinophils adhere to the schistosomula in a naive or immune host. Some indication of this can be gained from in vivo experiments involving intraperitoneal injection of schistosomula into naive or immune hosts. Initially moderate numbers of cells adhere to such schistosomula particularly to those injected into immune animals [Chao 1984]. Moreover since the measure of intrinsic resistance is exemplified by the ability of lung worms to resist attack in vitro under conditions capable of mediating eosinophil cytotoxicity of schistosomula it is pertinent to ask how many eosinophils attach to parasites under these conditions. In such an assay relatively few cells, approximately 10-30, attach to lung worms and significantly such cells are often observed to lyse. However not all of them do and in these assays in vitro conditions apply [McLaren and Terry 1982]. Under these conditions many eosinophils are observed to degranulate, therefore intrinsic resistance could be due to some other property of the membrane.

It is of course possible to argue the opposite case by stressing the fact that the importance of the in vitro experiments of Golan, Caulfield et al is not the rate of lyso PC production in vitro measured but the fact that the schistosomula can produce such a potent potential modulator of membrane function at all. This is likely to be due to the action of phospholipase enzyme, this being the case the activity in vivo may be much greater than the in vitro measurements which may not provide optimal conditions for such enzymatic activity. The ability to argue both sides of this in vivo - in vitro question indicates that the case for lyso PC being a central mechanism of intrinsic resistance is at present not proven. Nevertheless it does represent an important recent contribution to the elucidation of the mechanisms of intrinsic resistance exhibited by the surface membrane of S. mansoni.

1.7. The biophysics of the surface membrane

1.7.1 Fluorescence Recovery after Photobleaching (FRAP).

The FRAP based study of the surface membrane by Foley [1986] provided information on two of the important topics alluded to in this introductory discussion. Firstly it suggested or supported particular mechanisms of immune evasion directly from the data accumulated. Secondly, it provided further evidence of developmental change, in this case in the biophysical structures of juvenile and adult parasites. Moreover this evidence could be related to the developmental switch in mechanisms of immune defence described in McLaren and Terry [1982]. The main findings of Foley's study could be summarised as follows:-

Lipids

1. In adult S. mansoni differential quenching of two lipid probes DiI C18 and aminofluorescein C18 indicated that the aminofluorescein resided in the outermost monolayer of the surface membrane, DiI C18 in one or more of the three inner monolayers. Quenching in this context refers to the quenching of the fluorescence of a membrane bound lipid probe by the membrane impermeable dye trypan blue. Lipid attached fluorophores located at the surface of the membrane ,that is, at the interface of the membrane and the media, will be preferentially quenched. In schistosomula both probes resided in the outermost monolayer the E1 leaflet (Fig 1.2).
2. Measurement of fluidity of these two probes in adult membranes revealed a striking difference in mobility as measured by % recovery and rates of lateral diffusion (DL) values. The DiI C18 was extremely immobile, the aminofluorescein moderately mobile. These results were interpreted as indicating a vertical fluidity gradient in the surface membrane and it was postulated that this could be maintained by differences in the phospholipid composition of the various monolayers. Such differences have been extensively reported in cellular surface membranes [Schroeder 1980].

3. Such a differential localisation and fluidity gradient using these two probes was not found to occur in schistosomula or lung worms. However both probes located in the outer leaflet of these juvenile forms reported substantial immobility. This was interpreted as indicating that differentiation horizontally in the plane of the membrane into fluid and gel phase domains takes place in schistosomula and lung worms. A profound developmental change in membrane organisation between schistosomula and lung worms on the one hand and liver worms and adults on the other was indicated by the contrast in probe's mobility and location between schistosomula and adults. This interpretation was further supported by striking differences in the effect of bleb formation, induced by hypertonic saline on each stage. In adults both lipids and glycoproteins (next section) that were previously highly immobile became totally mobile on blebbing. In addition the fluidity gradient broke down. In contrast blebbing had no effect on the mobility of either lipids or glycoproteins in the surface membrane of schistosomula.

4. These developmental differences produced two conclusions:
   (a) The structural organisation of the two bilayers changes markedly during development.
   (b) Two separate mechanisms exist to mediate immobility. The behaviour of the adult membrane on blebbing was considered consistent with the presence of an immobilising cytoskeleton, the behaviour of schistosomula on blebbing indicated some other mechanism and the action of lyso PC was proposed.

Glycoprotein and Complement Mobility

1. The lateral diffusion of glycoprotein receptors in the outer membrane of all developmental stages is highly restricted with over 75% of receptors being completely immobile over the time scale of the experiment. Blebbing in adults released this restriction whereas in schistosomula it did not.

2. In schistosomula both C3 and C9 molecules are totally immobile whereas C3 is 50% mobile on the adult surface.

1.7.2. Changes in mechanisms of resistance during development.

The developmentally linked loss of intrinsic resistance by the parasite at somewhere around three weeks implied in McLaren and Terry [1982] is very important. It follows from this argument that the biophysical features of the membrane, underlying the mechanism of intrinsic resistance, may be expected to be lost and others more important to the function of the adult membrane gained. Clearly the FRAP based data and conclusions of Foley are very relevant in this respect. On face value the biophysical change from horizontal lipid asymmetry in the schistosomula and lung worm to vertical asymmetry in the adult correlates well with a developmental switch in defence mechanisms. By this analysis adult membranes structured by the presence of a cytoskeleton are not able to mediate intrinsic resistance. Conversely the lipid domain structure thought to exist in schistosomula and lung worms would be a prime candidate as a basis for intrinsic resistance. Two questions follow from this line of thought, firstly how can a differentially mobile surface membrane engender intrinsic resistance and secondly how is such a structural arrangement of lipids maintained.

1.7.3. Schistosomula lipid domains: maintenance.

Foley [1986] has suggested that since incorporation of lyso PC into schistosomula membranes and lung worms is up to eight times greater than incorporation into adult membranes, differences in lyso PC levels may be the distinguishing feature in mechanisms of lipid immobility between juvenile and adult forms. This particular argument, for the role of lyso PC, is open to question because in the study cited [Vial et al 1985] measurement of the lyso PC was that of the whole worm lipid not of the surface membrane, the area of interest. Furthermore the levels of incorporation of labelled oleate into lyso-PC was considered negligible by these authors and at the limit of the resolution of their measuring technique. In postulating a role for lyso PC in promoting immobilisation of the surface membrane the critical considerations must be the concentration of that lipid species in relation to the other lipids in the membrane. In this respect the reports of Young and Podesta [1984] in which lyso PC comprised one third of phosphatidyl choline lipids in the adult further suggests that lyso PC is an important lipid component in the adult. Of more relevance is the percentage lipid composition of lyso PC reported by [Golan et al 1986]. These authors monitored lyso PC as consisting of 2% of total schistosomula phospholipid. The fact that this phospholipid species comprised 34% of lipid shed into the media indicates that the 2% lyso PC may well be concentrated in the surface membrane. That being the case lyso PC might be expected to comprise upwards of 5% of schistosomula and lung worm phospholipids. At this sort of percentage level its proposed role in promoting the observed levels of 50% immobilisation of the outer monolayer of schistosomula and lung worm, becomes a reasonable indeed likely proposition.

As with the fusogenic and lytic roles proposed for lyso PC the answer hinges on the amount of the lipid available and

its concentration in the surface membrane. The variation in its measurement between various groups [Golan et al 1986, Vial et al 1985, Young and Podesta 1984, Rogers and McLaren 1987] makes confirmation of its role at present difficult. However its demonstrated ability to cause immobilisation of erythrocyte membrane lipids and glycoproteins and its yet undefined association with the surface membrane make it an ideal candidate mechanism for the similar type of immobility observed in the schistosomula itself. Indeed it is difficult to imagine how the parasite membrane which sheds this lipid could itself escape its effects. In erythrocytes incorporation of lyso-PC has been implicated in the induction of lipid domain formation, which causes membrane protein and lipid immobilization. Should lyso PC turn out to be the cause of schistosomula and lung worm immobility its contribution to mechanisms of intrinsic resistance will be twofold. Firstly it will mediate processes of intrinsic resistance such as cellular lysis at the surface membrane. Secondly its membrane immobilising effects are likely to mediate a more inert structural mechanism of intrinsic resistance. Such immobility could effect resistance in two ways, by inhibiting the action of immune components such as complement and by decreasing the efficiency of cellular recognition at the parasite surface.

1.7.4. Membrane Fluidity and Complement Fixation.

Both pathways for complement activation and damage to cell membranes act through the formation of lesions formed by the membrane attack complex (MAC). The crucial stage of its action is the formation of pores following the insertion of the terminal components of the complement cascade C5b and C9 [Bhakdi and Tranum-Jensen 1983]. There is considerable evidence that for this complex to integrate into target membranes rapid lateral diffusion of lipids is required [Parce et al 1978, Taylor 1983]. In schistosomula the large immobile functions indicated by FRAP would in

themselves mitigate against MAC formation. In addition the apparent partition of C3 and C9 complement components exclusively into the immobile or gel phase domains in schistosomula and lung worms further reduces the likelihood of MAC formation. Large scale immobility of the lectin glycoprotein receptors also indicates that antibody dependent complement fixation by the classical pathway is likely to be inhibited. This is because in this pathway an initial step is the binding of two antibody molecules by the C1q component of the complement cascade. There is an optimal membrane fluidity at which such antibody paired binding can occur. As approximately 75% of all lectin receptors are immobile at all stages of the parasite and presumably a proportion of these are important antigens, then complement fixation by this method must be inhibited due to the membrane structure.

In comparison to schistosomula the lipids and bound complement C3 components of adults are extremely mobile. Both juvenile forms and adults can activate complement on their surface, leading to the deposition of C9 without toxicity or ultrastructural damage [Ruppel et al 1983, Ruppel et al 1984, Ruppel and McLaren 1986]. This could indicate that immobility of the surface membrane is not the whole explanation for intrinsic resistance to complement damage and by extension immune damage generally. Although lung worms are distinguished from both earlier and later parasite stages by their extreme intrinsic resistance to immune attack there has been no similar distinguishing biophysical feature from the FRAP studies which correlates with this. There are only very slight changes in DL values and percentage recovery measured in the outermost monolayer as the parasite develops from schistosomula to lung worm. Absolute levels of membrane fluidity as measured by FRAP do not correlate with the intrinsic resistance of lung worms to immune attack. Biophysical or biochemical parameters other than mobility would appear to be important to the development of intrinsic resistance.

1.7.5. Other Biophysical Studies - Freeze Fracture.

The only other directly biophysical studies on the surface membrane, other than FRAP, have been electron microscopy studies. Of most interest to this discussion of intrinsic resistance is the freeze fracture study described in McLaren [1980 Chapter 5]. These studies showed that the occurence of integral membrane proteins varied markedly within the four monolayers of the parasite and over time as the parasite developed. Particularly striking was the unusually large and evenly distributed IMP's on the outermost leaflet of the lung worm. These were postulated as representing aggregations of acquired host molecules accumulated at the site of parasite glycoproteins. Such a mechanism could be the basis of the lack of binding of lethal antibody at this stage. Acquisition of host molecules in this manner would be likely to mask parasite antigens fully. The implication of this work is that in addition to having a highly developed intrinsic resistance mechanism, lung worms also exhibit a membrane specialised to mediate antigen masking very efficiently or alternatively expose no detectable antigens [Pearce, Basch & Sher 1986].

Indirect biophysical studies in which the approach was to observe damage to the membrane integrity itself due to the action of some perturbing agent have also been carried out. The most obvious conclusion to be drawn from these types of study is that the surface membranes of schistosomula are markedly different as judged from their relative susceptibility to agents such as retinol [Kusel, Stones and Harnet 1981] or bacterial toxins [Ruppel, Breternitz and Lutz 1987]. This latter study is of interest in that it exploits a pore forming cytotoxin from Pseudomonas aeruginosa. The efficacy of this toxin at different stages is remarkable. The sensitivity of the parasite to the damaging action of this toxin increases from very low levels at exactly 27 days to very high levels at 28 days.

This change correlates with a length threshold of 1.8mm and is thought to represent a critical developmental change associated with switching of immune defence mechanisms [McLaren and Terry 1982], elimination of schistosomes from rats (self-cure) [Cioli, Blum and Ruppel 1978] and pharmacological sensitivity to the drugs oxaminiquine and hycanthone [Sabah et al 1986]. This spectacular and rapid change in susceptibility to the damaging effects of this toxin could be due to the rapid acquisition of a specific receptor at this time of marked developmental change in membrane morphology [Mclaren 1980]. Alternatively it could represent the loss of a damage limitation mechanism associated with the intrinsic resistance of the more juvenile parasites. Ruppel has speculated that the combination of demonstrable low levels of cytotoxicity of this toxin at lung worm to 3 week worm stages coupled with an apparent lack of ultrastructural damage indicate two important facts. Firstly pores are formed in the surface membrane by the toxin and secondly the parasites are able to withstand their damaging effects. Since this toxin is a model for complement it follows that the lung and post lung worm stages of the parasite have some mechanism for tolerating the damage and more importantly permeability changes associated with low levels of pore formation caused by complement activation in the outer membrane. This mechanism was proposed to be the membrane pump/carrier which functions to compensate for immune damage induced ion imbalances in developing schistosomula [Pearce et al 1986]. The implications of such a hypothesis for the cell biology of the surface membrane are of significance. They imply that in addition to being able to deflect immune attack due to failure of recognition as in antigen masking or to neutralise immune attack via lyso PC lysis, the surface membrane can absorb and tolerate a certain amount of structural damage.

1.7.6. T-cell recognition and the surface membrane

Recent advances in immunology have elucidated both the structure of the T-cell receptor [Schwartz 1985] and important aspects of its function at the molecular level [Allen 1987]. Recognition is associative in the sense that a particular feature of the secondary structure of the antigen, an alpha helical region of protein, mediates recognition. These structures are thought to provide both the epitope, which is recognised by the T-cell receptor and the agretope which binds within a cleft in the MHC antigen.

Recognition is then dependent on the secondary structure of the antigen protein. Most, but not all, antigens require some processing in order to expose such secondary structures. This entails a process of intracellular denaturation by antigen presenting cells. Antigens can be classified according to their processing requirements into three groups; type 1 which require no processing, type 2 which require unfolding of the protein structure, and type 3 which require cleavage and unfolding [Allen 1987].

In S. mansoni absorption of MHC antigens Ia,k and d determinants has been reported by Sher, Hall and Vadas [1978]. This observation is paradoxical in that it could render the parasite susceptible to T-cell recognition via MHC restricted recognition of parasite antigens. However the recent advances in the immunology of T-cell recognition place considerable constraints on such recognition at the surface of the parasite itself. Firstly the MHC antigens absorbed would have to have the correct orientation in order to mediate binding of antigen. The fact that B_2 microglobulin is not accessible at the surface of schistosomula [Torpier, Capron & Ouassi, 1979] indicates that this may not be the case for class 1 molecules. Secondly the secondary structure of such parasite antigens that are exposed would have to be appropriate for coupling to MHC antigens. Clearly the type of antigen most likely

to be recognised at the parasite surface would be the S. mansoni equivalent of type 1 antigens - that is antigens capable of mediating T-cell recognition without any kind of processing. It is possible however that some kind of processing of parasite antigens could occur by default as the parasite sheds antigens. This could result in the unfolding of their structure enough to expose T-cell recognition epitopes and MHC agretopes. Virtually nothing is known about the secondary structure of S. mansoni surface antigens therefore the direct MHC restricted T-cell recognition at the parasite surface remains theoretically possible but less likely than once thought due to the restrictions described above. Experimental evidence regarding T-cell recognition at the parasite membrane surface is both limited and ambiguous. Stimulation of primed T-cells directly by the parasite surface itself in vitro has been suggested by James [1981]. However, Butterworth et al [1979] concluded that cytotoxic T-cells did not adhere to or damage schistosomula. In this work the authors utilised immune peritoneal exudate cells rich in cytolytic T-lymphocytes (CTL) with specificity for alloantigens (k and d) by immunisation of mice with appropriate tumour cells. These CTL were subsequently exploited in in vitro killing assays directed against lung worms which had passively acquired alloantigens of the same specificity as the CTL's. These cells were shown to be adherent to schistosomula and their specific CTL activity was demonstrated by the fact that cells that had adhered to schistosomula were subsequently able to lyse an appropriate tumour target cell. These authors claimed that such CTL were unable to damage lung stage schistosomula even after prolonged periods of culture. This claim was also made for skin transformed schistosomula. In this case adherence was promoted by using Con A as a non-specific binding agent, again adherence but not damage was recorded. The conclusion that the parasites were not damaged in such experiments is

crucially dependant on the sensitivity of the assay for damage employed. The criteria for damage applied to the lung worm experiments were entirely based on light microscopy, dependent on morphological change, motility, and the ability of the parasite to take up the vital stain toludine blue in an intense and granular fashion. The assay for damage to schistosomula was similar but also included a Cr^{51} release assay at eighteen hours after exposure to T-cells. The examinations by microscopy continued for up to 66 hours in culture. Significantly immotility of schistosomula by itself in the absence of a granulated appearance associated with toludine blue uptake, does not appear to have been considered a parameter of parasite damage in these assays. Thus this assay was primarily dependant on the uptake of vital dye which is sensitive to the integrity of the surface membrane and tegument. Cr^{51} release is more sensitive to surface membrane damage but still dependant on this type of change in membrane integrity. [Kusel,Stones & Harnett 1982].

Comparison of this paper with that describing ultrastructural studies of the killing of schistosomula by activated macrophages is instructive [McLaren & James 1985]. These authors showed that damaging and eventually lethal subtegumental damage could occur, causing large scale disorganisation of the subtegumental muscle cells and mitochondria visualised by E.M., leaving the tegument intact and the surface membrane with complete integrity. Uptake of vital dye such as toludine blue will not be sensitive to this type of damage, furthermore the morphological events associated with it such as subtegumental vacuolation clearly shown by E.M. will be difficult to monitor by light microscopy. This would be particularly true if only toludine blue staining parasites were closely examined. It is likely that if such subtegumental damage was the mode of action of cytolytic T-cells, it would not have been noticed in Butterworth's

study of cytotoxic T-cells. There is a parallel here in the study of activated macrophages in that Perez & Terry [1973] relying on vital dye uptake as a measure of damage did not fully appreciate the action of activated macrophages.

There are significant similarities in the mode of action of CTL's and activated macrophages in cell biology. In particular, neither cell type is required to constantly adhere to its target in order to inflict damage. Transient adherence administering a "lethal hit" in the case of CTL contrasts with more extended but finite adherence lasting up to ten minutes or more for macrophages [James et al 1981]. In neither case is damage necessarily directed against the membrane itself [Martz 1976]. Both cell types require some cell to target period of contact and both are capable of subsequently moving on to kill other target cells.

Therefore on the basis of this paper the possibility that cytotoxic T-cells are mediators of subtegumental damage cannot be ruled out. Indeed a subsequent report by a group working with T-cells derived from human donors who had not been exposed to S. mansoni has reported that T-cells can kill schistosomula of S. mansoni in vitro [Ellner et al 1982]. This activity was dependent upon activation of the T-cells by mitogens, soluble antigens or alloantigens. The assay for schistosomula death in this case was again the uptake of a vital dye (methylene blue) but in addition the infectivity of schistosomula exposed to activated T-cell attack was compared with a control group of schistosomula exposed to non activated T-cells. It was demonstrated that activated T-cells could kill up to 40% of target schistosomula as adjudged by methylene blue uptake. Significantly, the damaging effect of activated T-cells may have been even greater than this since the effect of treatment with activated T-cells was to reduce infectivity

by 57%. This may suggest that cytotoxic T-cells could damage the schistosomula in a manner not sensitive to methylene blue uptake such as subtegumentally as suggested above. Clearly the question of whether cytotoxic T-cells are important immune effectors against S. mansoni remains to be definitively proven and with this the mechanism(s) of damage. In both cases however the T-cells were activated and adherence to the parasite was observed but damage only occurred with the human derived cells. The discrepancies between the two papers cited above could be a species dependent difference between the murine and human host. This could reflect either a qualitative or quantitative species dependent difference in the damaging action of this cell type which could further understanding of how cytotxic T-cells act. As far as the membrane structure is concerned the most important factors would be how the cytotoxic T-cell damages the parasite. The similarities between this cell type and macrophages suggest that this may be via inducing subtegumental damage although the uptake of methylene blue dye reported by Ellner suggests that direct damage of the membrane may occur.

1.8.1 S. mansoni: summary

As has been discussed, immunological studies represent the bulk of experiments carried out concerning the surface membrane of S. mansoni. Several immune effector mechanisms, in particular eosinophil ADCC killing and activated macrophage killing have been elucidated and researched in detail. Similarly, rather more immune defence mechanisms have been proposed and investigated. Their role in vivo has necessarily been rather more difficult to define than that of immune effector mechanisms. Nevertheless, in schistosome immunology there has emerged a general picture of a number of well characterised immune effector mechanisms ranged against a number of rather less well defined immune defence mechanisms. What is also clear is that the relative importance of the different elements on

either side of this "contest" change markedly with the developmental status of the parasite and the immune status of the host. The site of this "contest" is generally agreed to be at the surface membrane, the interface between the host and parasite. Therefore the properties and functions of this membrane will determine both the susceptibility and the resistance of the parasite. Most of the useful information relating the cell biology of this surface membrane to its immunologically related effects has been derived from electron microscopy. However very recently another biophysical investigative technique, FRAP, has been employed by two groups and the data derived proved pertinent to an understanding of the function of the surface membrane of S. mansoni. This technique, like electron microscopy, utilises immunocytochemical methods. However in addition it utilised fluorescent lipid analogues, as a probe to determine the nature of membranes. This project is primarily concerned with the exploitation of these fluorescent lipid analogues to gain a greater understanding of the cell biology of the surface membrane. The most important, but not exclusive stimulus, to this course of action has been the immunological perspective of the function of the surface membrane that has been selectively described above.

Convention for naming the lipid leaflets of the surface membrane of S. mansoni.

After McLaren 1980.

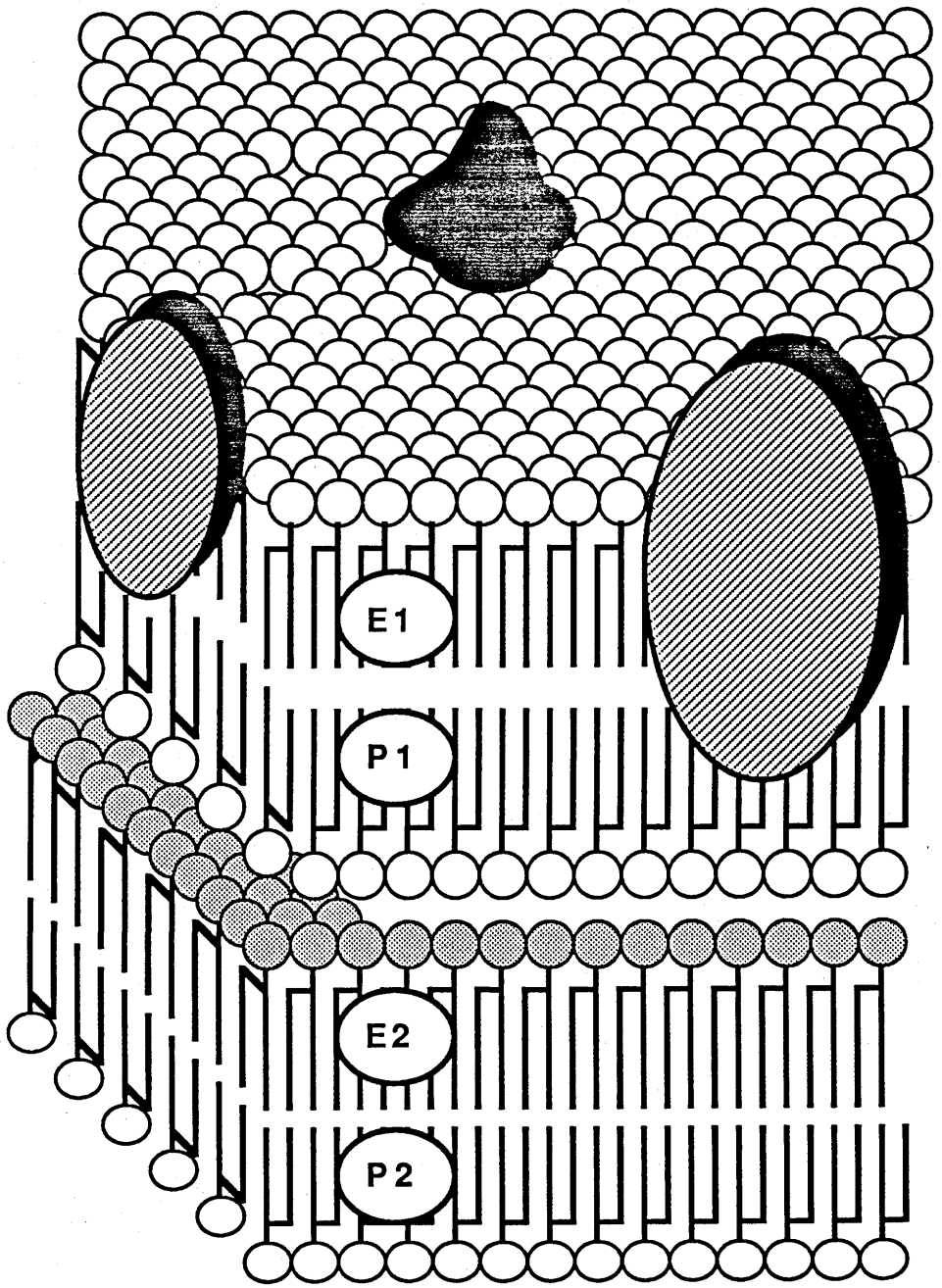


Figure 1.2

Section 2. Membranes

2.1.1. The fluid mosaic model.

Biological membranes perform a wide variety of functions including: secretion, transport, endocytosis and signal transduction. These functions all occur in the plasma membranes of cells which are the most appropriate analogous membranes to the surface membrane of S. mansoni. The variety and flexibility of function exhibited by cell plasma membranes indicates the dynamic nature of membranes and their structure.

The major paradigm concerned with lipid structure is that of the "fluid mosaic model" [Singer & Nicholson 1972]. This model postulated that biological membranes comprise an extremely flexible and dynamic basic structure, consisting of a lipid bilayer containing transmembrane or peripheral proteins inserted into the lipid bilayer or attached to it. Both lipid and proteins were proposed to be free to diffuse within the plane of the membrane in the absence of any constraint. Since first published this model has received abundant experimental support and its general principles have been widely accepted.

Recent biophysical research has both modified and extended knowledge of both membrane structure and function. Considering membrane structure, the original simplistic "protein icebergs floating in a sea of lipid" analogy that embodies the fluid mosaic model has been modified to take account of subsequent discoveries. Firstly, that the proteins can be subject to constraints in mobility and secondly, that the nature of the lipid bilayer milieu surrounding these proteins is not homogenous in character.

In addition to advances in knowledge concerning the biophysical structure of membranes, investigation of membrane function has demonstrated that the structure and

behaviour of cell plasma membrane allows both transmembrane signalling and transport to occur.

The particular advances and additions to the "fluid mosaic model" based membrane biology of interest to this work have been: lipid asymmetry both vertical to and in the plane of the membrane bilayer, transmembrane signalling via phosphatidyl inositol, transmembrane transport via receptor mediated endocytosis. These subjects are representative of recent trends in membrane research but more importantly are directly relevant, though not necessarily applicable to, an understanding of the relationship between structure and function in the surface membrane of S. mansoni. Prior to considering these particular features and functions of membranes the assumption implicit in this discussion "that the plasma membranes of cells and hence the fluid mosaic model" are appropriate models for the surface membrane of S. mansoni will be examined.

The fact that S. mansoni exhibits a double bilayer complex as its surface membrane, while representing the major difference between the plasma membrane of cells and the parasite's surface membrane, does not by itself render the fluid mosaic model invalid. The functions of the surface membrane of S. mansoni although not as well characterised in terms of mechanism as cellular membranes are largely similar. Transport of metabolic substrates [Podesta & Dean 1982], purine and pyrimidine transport and amino acids have all been reported as has the presence of an electrical potential across the membrane [Fetterer Pax & Bennett 1980, Pearce et al 1986]. The schistosome surface membrane also carries out the fundamental limiting and interface forming roles that the plasma cell membrane performs. In addition there is circumstantial evidence to suggest that the external environment of the parasite can influence the behaviour of the cytoplasmic tegument across the surface membrane. Such phenomena as accelerated membrane turnover in response to immune attack suggest that transmembrane

signalling of some sort takes place. Shared functions do not however necessarily imply similar structure. The biophysical evidence of McLaren [1980] utilising freeze fracture studies and the FRAP evidence presented by Foley is more relevant.

The freeze fracture studies of Mclaren indicated that the bilayer leaflets of the surface membrane of S. mansoni contained proteins embedded within them and this was generally compatible with a fluid mosaic model. The measurements of Foley on lateral diffusion were also broadly similar to the rates of lateral diffusion of both lipids and proteins in cell membranes. However as described above, the extent of lipid and protein mobility was markedly reduced compared with cell membranes. From these studies it would appear that the bilayers comprising the surface membrane of S. mansoni conform to the basic pattern of membrane structure described by the fluid mosaic model. However in cell biology it has been demonstrated that a wide range of cell types with varying plasma membrane functions and biophysical structures exist and all of these broadly conform to the fluid mosaic model. More instructive analogies between plasma cell membranes and schistosome surface membranes require consideration of more specific structural and functional features such as those described above.

2.1.2. PI Signalling.

Phosphatidyl inositol (PI) is found in cellular membranes and makes up about 6% of total phospholipid content. It also makes up a similarly minor fraction of schistosome membrane lipids [Furlong & Caulfield 1988] In cells quantitatively minor phospholipids also exist: phosphatidyl inositol 4 phosphate (PIP), phosphatidyl inositol 4, 5-biophosphate (PIP_2) and protein bound glycosyl-phosphatidyl inositol (PI-glycan) [Low et al 1986]. Binding of certain hormones, neurotransmitters and

growth factors to their specific cell surface receptors leads to the hydrolytic cleavage of PIP_2 to generate inositol 1,4,5-triphosphate IP3 and Sn-1,2, diacylglycerol (DAG). These two molecules are both second messengers: IP-3 stimulates the release of Ca^{2+} from intracellular stores and DAG is the physiological activator of protein kinase C [Berridge and Irvine 1984].

The relevance of this mechanism to schistosomes is threefold. Firstly, it could provide a means of transmembrane signalling affecting the tegument function that conserves the integrity of the parasite membrane. However, while not affecting the integrity of the membrane the operation of such PI signalling could affect the fluidity of the parasite membrane due to the fusogenic effects of DAG. The effect of receptor binding of its agonist is relayed to the site of PI breakdown, via phospholipase enzymatic activity, in the inner bilayer by G proteins.

This last form, PI-glycan, is the means whereby VSG proteins in trypanosomes are attached to their membrane. Significantly these VSG proteins have recently been shown, using FRAP, to be 100% mobile [Bulow, Overath & Davoust 1988]. In this case the lateral mobility of the lipid determines the lateral mobility of a protein attached to it. One of the puzzles of schistosome membrane organisation is how immobile proteins in the outer bilayer in adults are constrained. The transmission of the fettering effect of a tegumental cytoskeleton across the inner bilayer has been proposed [Kusel & Foley 1986]. However it is not clear how this could come about. Amphitropic proteins which anchor the cytoskeletal proteins to cell plasma membranes have been reported. These act as linking molecules to integral membrane proteins. This occurs at either the membrane cytoplasm interface or with the hydrophobic core of the lipid bilayer itself [Burn 1988]. One problem with the hypothesis that a tegumental

cytoskeleton is responsible for immobility of lipid and protein in the outer bilayer of S. mansoni is to explain how the restraining effect of a tegumental cytoskeleton could be transmitted across the inner bilayer. The properties of the amphitropic proteins and their interactions with cellular cytoskeletal protein filaments reported to date are consistent with the concept that a cytoskeleton could immobilise proteins in the inner bilayer but not directly those of the outer bilayer. For this to occur directly due to the cytoskeleton one would have to propose that cytoskeletal filaments actually pierce the inner bilayer. This is unlikely therefore some other indirect mechanism of transmission is required.

PI-glycan linkages between PI inserted in the outer leaflet of the inner bilayer and attached proteins spanning the outer bilayer may be such a mechanism whereby transmission of the restraining effect of cytoskeletal elements in the tegument is transmitted across the inner bilayer. The fact that the inner leaflets are immobile is consistent with such a mechanism, since PI-glycan structures in the inner bilayer may be expected then also to be immobile [Foley 1986]. Moreover the very small gap between the two bilayers is sufficient to allow the ethanolamine and glycan linkages that intervene between the inositol group on the lipid and the C terminal amino acid of the protein [Ferguson, Low & Cross 1985]. An electron lucent substance deposited between the inner and outer bilayers of S. mansoni has been demonstrated which could represent the linking glycans of PI-glycan protein complexes. Therefore, in addition to signalling, PI in the surface membranes of S. mansoni could also have an integrating role in the double bilayer structure. To summarise, recent research in cell biology has indicated that PI although comprising only a small percentage of membrane lipid may have important roles in both the function (signalling) and the structure of cell membranes. This whole area awaits further investigation in S. mansoni.

2.1.3 Receptor mediated endocytosis.

Receptor mediated endocytosis is the process whereby ligands such as LDL and transferrin are taken up by cells. This involves attachment of ligand to receptors, the receptors diffuse in the membrane and aggregate in coated pits, which bud off to form membrane invaginations termed endosomes which deliver the receptor and its bound ligand to the cell lysosomes. The receptor is recycled and is reinserted into the membrane to maintain the cycle [Goldstein & Brown 1977 & 1984]. This process is of interest in relation to the schistosome membrane in this study for two reasons. Firstly it provides a well characterised model of membrane function which can mediate transport, signalling and membrane turnover. Endocytosis can be thought of as a type of signalling in the sense that uptake of extracellular ligand can affect intracellular function. More specifically this mechanism has strongly featured in recent research on LDL cholesterol metabolism. This aspect will be discussed in the next section.

In contrast to PI signalling, receptor mediated endocytosis appears to represent an aspect of membrane function drawn from cell biology which is not applicable to the surface membrane of S. mansoni. Several workers have produced evidence to suggest that endocytosis does not occur. Labelling with fluorescent Con A does not result in internalisation as expected if endocytosis were occurring in a manner analogous to cells [Samuelson, Caulfield & David 1984]. Similarly killing by Ricin, which in cells is endocytosis dependent, does not occur at any stage of S. mansoni when tested in vitro [Cushley & Kusel 1987]. In addition transmission E.M. studies have never shown structures analogous to those occurring during endocytosis in cells. The balance of evidence would suggest that endocytosis, and hence receptor mediated endocytosis, is not a mechanism that can occur in the surface membrane of S. mansoni. A small measure of caution is appropriate,

however, since the evidence against endocytosis in S. mansoni is heavily dependent on monitoring lectins bound to the surface. Less formal work has been done on lipid trafficking although again the indications from labelling experiments with fluorescent lipid analogues indicate that endocytosis does not occur. The experience in cell biology where the realisation that endocytosis occurs did not take place until cells were exposed to particular, somewhat artificial, culture conditions also gives cause for some scepticism of the claim that endocytosis-like phenomena do not occur in S. mansoni. Leaving aside such residual doubt acceptance that endocytosis like mechanisms do not occur at the surface membrane of S. mansoni profoundly influences one's view of the biology of the parasite. Lack of endocytosis and demonstration of membrane loss by shedding indicates that the parasite differs fundamentally from cells in that it does not turnover and renew its membrane internally but simply continually sheds and replaces it.

2.1.4 Lipid asymmetry (vertical).

An asymmetric distribution of phospholipids has been shown to exist between the two halves of many different cell plasma membranes. The human erythrocyte membrane has been the best characterised cell type in this regard. Numerous studies using chemical labelling [Bretscher 1972] and phospholipases [Verkleij et al 1973] indicate that in this membrane phosphatidyl choline and sphingomyelin are mainly located in the outer leaflet while phosphatidyl ethanolamine and phosphatidyl serine locate in the inner leaflet. This pattern has been observed in most cell plasma membranes. Maintenance of such asymmetry is thought to be due to the operation of a selective ATP dependent, protein mediated, transfer of aminophospholipids from the outer to the inner leaflet of erythrocytes [Tilley et al 1986], reticulocytes [Zachowski et al 1985], lymphocytes [Zachowski 1986] and cultured fibroblasts [Martin & Pagano 1987].

Another possibility suggested is that membrane curvature and lipid asymmetry are intimately connected thus changes in cell shape might produce local changes in membrane composition [Thomson, Huany & Litman 1974]. This is strongly supported by studies on erythrocytes which showed that addition of small fractions of exogenous lipids into one erythrocyte leaflet produces a modification of the lipid balance in between inner and outer leaflets and is the cause of well documented changes in the shape of these cells [Seigneuret & Devaux 1984]. Therefore changes in lipid composition may also influence membrane curvature and cell shape.

In schistosomes there is evidence to suggest that such asymmetry in lipid composition [Foley 1986] and function [Mcdiarmid, Dean & Podesta 1983] exists. The demonstration of a fluidity gradient in adult S. mansoni and the fact that PE preferentially locates within an inner leaflet suggests that membrane asymmetry in adult S. mansoni could influence both enzymatic activity and membrane curvature. The relatively high fluidity of the outer leaflet of adults and schistosomula indicates that this bilayer could be, as one might expect, biochemically the most active. The correlation between development of a fluidity gradient and a large increase in the extent of membrane curvature engendered by the morphological changes leading to the characteristic folding pattern of the adult stage may also either reflect or determine vertical lipid asymmetry in surface membrane.

2.1.5. Lateral membrane asymmetry.

As described earlier in this discussion there is good evidence to suggest that the bulk lipid structure of membranes, both cell plasma membranes and the surface membrane of S. mansoni, is not homogeneous but is differentiated into domains of differing "fluidity". Prior

to discussing the evidence for this concept of "domain structure" in membranes some definition of the term "fluidity" as applied to membranes is required. The term "membrane fluidity" has been widely applied in describing both model phospholipid bilayers and natural membranes. It combines in a single term the effects of lipid dynamics and acyl chain order. Dynamic processes include lateral and rotational diffusion of the whole molecule as well as rotation around single carbon-carbon bonds. Acyl chain order or lipid packing refers to the average orientation of each carbon atom along the chain. In a more disordered membrane, the acyl chains do not pack as tightly therefore holes or gaps can form between the chains.

The physical definition of fluidity is, the inverse of viscosity in an isotropic homogeneous liquid. This definition cannot be directly applied to an anisotropic system such as a bilayer. Nevertheless, fluidity has been a useful concept for assessing the bulk lipid structure of the membrane. However it is important to stress that the parameter described as fluidity is technique dependent with most techniques reporting on changes in lipid dynamics or lipid order. This is illustrated in this discussion where the data presented concerning the presence of lipid domains in biological membranes has been derived largely from two techniques, FRAP which measures lipid dynamics and fluorescence polarisation studies which measure lipid order.

Lateral phase separation resulting in the formation of domain structures has been reported in many model membranes using a variety of techniques including electron microscopy, X-ray defraction and magnetic resonance spectroscopy [Grant et al 1974, Tardieau et al 1973, Griffin 1981]. These non-invasive techniques demonstrate that lipid domains do form in model membranes containing heterogeneous mixtures of lipids. Domain structure has also been inferred from studies in which fluorescent lipid

probes are introduced into model bilayers and fluidity monitored by FRAP [Schlessinger & Elson 1982]. In most biological membranes demonstration of such phase separations has been more difficult. This is because biological membranes are relatively more heterogeneous in composition containing both proteins and cholesterol. This would be expected to result in a membrane structure in which domains are: less absolute due to the presence of cholesterol, small and fragmented due to the heterogeneity in composition and possibly less stable due to the dynamic properties of the membrane. However one group of workers has presented evidence to suggest that lipid domains do occur in a variety of cell plasma membranes [Klausner et al 1980, Karnovsky et al 1982]. The significance of these workers approach was that a direct link between the demonstration of "domain structure" in model membrane liposomes and cell plasma membranes was made. This was due to the fact that the technique used to demonstrate the presence of domains in liposomes, fluorescence polarisation, was employed to demonstrate domains in cell membranes.

Measurement of the fluorescence decay of DPH (diphenylhexatriene) incorporated into homogeneous single phase liposomes and heterogeneous mixed phase liposomes produced a single monoexponential decay in the single phase system but a three component decay in the mixed phase system. This was interpreted as reflecting contributions from the gel phase, the fluid phase and an interface region existing in the mixed phase liposome. The system used was a mixture of dilauryl and dimyristroyl phosphatidylcholine which coexists in fluid and gel phases at 25^{o}C [Shimshick & McConnell 1973]. Having shown that it was possible to detect the heterogeneity of sites into which DPH can partition in model membranes the technique was applied to cell surface membranes. With the single exception of the erythrocyte membrane, a number of cell types including mouse lymphocytes, WTS lymphoma, BHK cell plasma membranes

and bovine aortic endothelial plasma membranes were tested. These were shown to have heterogeneous binding sites for DPH indicating that some form of domain structure existed in these cell surface membranes. Thus this technique, which monitors acyl chain order indicates that domains of relatively rigid gel like lipid coexist with more fluid regions in a variety of cell membranes.

These workers also produced evidence to suggest that free fatty acids partitioned into different domains, depending upon their structure. Incorporation of free fatty acids affected both DPH polarisation and the direction of phase temperature shift. This was dependent on the structures of the fatty acids employed. Unsaturated fatty acids incorporated into plasma membranes reduced DPH polarisation and increased ANS polarisation while trans unsaturated fatty acids and saturated fatty acids had no effect on DPH but did increase ANS polarisation. The presence of the different exogenous fatty acids induced differential opposite shifts in mid part of transition temperatures of homogeneous liposomes as monitored by polarisation. That is, incorporation of cis unsaturated free fatty acids decreased transition temperature whereas trans unsaturated free fatty acids increased transition temperature. This was interpreted as indicating that the cis unsaturated fatty acids partitioned into fluid phase domains and the trans unsaturated and saturated into gel phase domains. This was further evidence for the differentiation of the membrane into domains.

The use of the same thermodynamic property, the change in the temperature of transition from gel to fluid phase, has been used to measure perturbations in the phase transition temperature of model membranes induced by fluorescent lipid analogues. This approach has been exploited to show that DiI C18, one of the fluorescent analogues utilised in this study, preferentially partitions into gel phase regions. This study showed that fluorescent carbocyanine probes of

approximately the same acyl chain length as the membrane into which they inserted partitioned preferentially into gel phase domains, those with longer or shorter acyl chain length than the membrane into fluid domains [Klausner & Wolf 1980]. This partition preference was related directly to lateral diffusion by FRAP and biologically to observations of dramatic changes in the rates of lateral diffusion of DiI C18 labelled sea urchin eggs upon fertilisation [Wolf et al 1981]. These observations were interpreted as reflecting an initial preferential partitioning of probes according to their acyl chain length into lipid domains present in the membrane. Fluidity changes associated with fertilisation were postulated as a reordering of these domains, leading to a change in partitioning of the dyes and changes in both fluorescence and mobility of the dye. Strong support for this interpretation has recently been produced by Dictus et al [1984] where Xenopus egg cell plasma membrane prelabelled with aminofluorescein C18 dye exhibited remarkable segregation between large scale gel phase and liquid crystalline macro-domains associated directly with the animal or vegetal hemispheres of the fertilised egg. This study is of particular interest because the dynamic characteristics of the FRAP measurements of the mobility of aminofluorescein C18 were very similar to those monitored in the schistosome membrane. In each case recovery was only about 50-60%. The only other reports of such high levels of immobility, as measured by FRAP, are from studies on rat spermatozoa [Wolf 1988]. These are of interest since a cytoskeletal mechanism involving actin filaments has been postulated to immobilise the lipids of the spermatozoa membrane.

To summarise, two lines of evidence based on independent techniques monitoring lipid order and dynamics indicate that phenomena analogous to phase separations, that are known to occur in model membranes, also occur in biological

membranes. Thus in biological membranes immiscible domains of relatively fluid or gel like fluidity may coexist in the plane of the membrane. Two of the dyes used in the present study, aminofluorescein C18 and DiI C18, have clearly shown the presence of large scale occurrence of such domain structure in poikilothermic and spermatozoa cells. The high levels of immobility of these dyes reported in these specialised cells are similar to those monitored for these dyes in schistosomes, particularly the schistosomula surface membrane. The surface membrane of S. mansoni may therefore exhibit differentiation into fluid and gel phase domains to a greater degree than occurs in most plasma cell membranes but similar to certain specialised cell types.

2.1.6. FRAP of endogenous lipids in plasma cell membranes.

A recent FRAP study utilising endogenous fluorescently labelled phospholipids in Chinese Hamster Ovary cells, has challenged the view that domain structure exists in cell membranes [Dupou et al 1988]. The results of this investigation impinge directly on the interpretation of the FRAP results derived by Foley et al [1986]. The main findings and conclusions of Dupou et al could be summarised as follows:

1. Intrinsic fluorescent probes introduced metabolically exhibit a certain degree of immobility (25%) that is not expressed by extrinsic probes whether such extrinsic probes are structurally similar or dissimilar to the intrinsic anthracene probes. One of the extrinsic probes used was aminofluorescein C16.

2. The fluorescence of the anthracene probe employed self quenches upon strong illumination and can be used to monitor fluorescence decay. When such probes are introduced into the membrane as extrinsic probes via an ethanolic suspension or intrinsically to the cell via metabolic labelling and fluorescence decay monitored both show a single second order reaction. This is

indicative of a membrane of undifferentiated bulk phase fluidity, that is one without domains. Three main conclusions were drawn from this work, firstly extrinsic probes do not gain access to all areas of the plasma membrane, secondly coexistent fluid and gel phase domains do not exist in this plasma membrane and thirdly in the absence of "phase" domains as postulated by Klausner the lipid immobilisation must be due to some other mechanism, probably an interaction with protein.

This paper contradicts that of Klausner in two important respects, firstly the structurally dissimilar aminofluorescein C16 and anthracene-9-PC probes did not partition into different domains as measured by FRAP. This contrasts with the results presented by Klausner where different free fatty acids located in particular environments. This suggests that such differing environments or domains do not exist. The observation of only a single monoexponential decay curve on bleaching of both extrinsic and intrinsic anthracene probes supports this and suggests that the CHO cell plasma membrane has a uniform bulk membrane fluidity i.e. there are no domains.

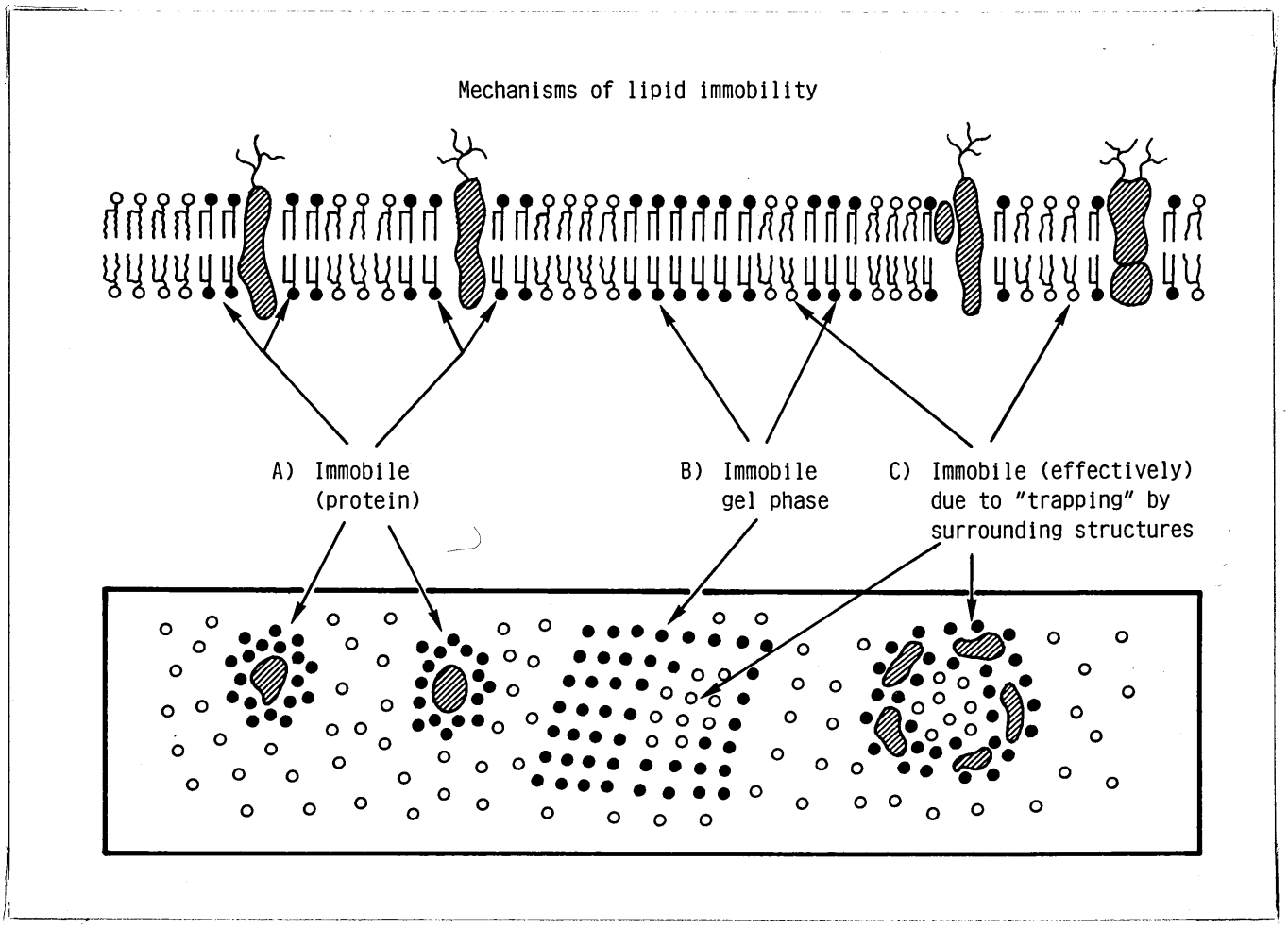
The observation that aminofluorescein C16 is excluded from partially immobile regions of the plasma membrane amenable to metabolic labelling but not extrinsic labelling is of some significance. Much of the FRAP data on schistosomes was obtained utilising this analogue, albeit of C18 chain length. It is possible that the other extrinsic probes utilised to measure lipid mobility in this and Foley's study notably DiI C18 and aminofluorescein C18 would behave in a similar way in CHO plasma membranes. One would expect a possible consequence of this to be that the extent of lipid immobility in schistosomes as measured by FRAP to date may be underestimated. Moreover Dupou et al put forward two mechanisms whereby proteins in the membrane may immobilise lipid. The first is a direct cytoskeleton to

intramembraneous-protein to lipid interaction leading to immobilisation of lipid around cytoskeletally restrained membrane proteins. The second is an indirect patching effect whereby small pools of mobile lipid are held within a protein latticework. This mechanism was proposed to explain the inability of extrinsic probes to label the immobile regions labelled by intrinsic metabolic probes. This immobility however, is only apparent and dependent on the limitations of the FRAP technique which requires diffusion to take place over relatively long distances. It is proposed that these areas of lipid are in the liquid crystalline or fluid state but on bleaching in FRAP cannot exchange bleached non-fluorescent lipid molecules with non-bleached fluorescent lipid molecules due to the effect of the protein lattice. Such lipid areas, although in a fluid state, would appear as immobile by FRAP.

2.1.7. Lipid immobility in schistosomes.

The evidence for lipid domains in cell plasma membranes is at present conflicting and varies with technique and the particular cell type used. However there are several means whereby lipid immobilisation could be achieved. These are domain structure due to phase formation, direct immobilisation via interaction with intramembraneous cytoskeletally linked proteins and indirect patching of lipid in protein lattices. (See Fig 1.3)

Figure 1.3


Mechanisms of lipid immobility
A) Immobile (protein)
B) Immobile gel phase
C) Immobile (effectively) due to "trapping" by surrounding structures

Immobilisation of integral membrane proteins by cell cytoskeletons has been reported to occur in a number of cell types. It has been estimated that around each such immobilised protein a region of lipid 50 angstroms in diameter is also immobilised [Jain & White 1978]. If this analogy is applied to schistosomes it is clear from McLaren's freeze fracture studies of the surface membrane that after three weeks there are insufficient intramembraneous particles (IMP's) occurring within all four leaflets of the membrane that could mediate significant immobilisation directly by cytoskeletal-protein interaction. This is because there can only be a few, if any, intramembraneous proteins spanning all four leaflets. However not all proteins within a membrane appear as IMP's, proteins smaller than 5000 KD will not. Therefore it is possible that other proteins are present within the membrane and are able to interact with lipids causing immobilisation. In this respect the case of the rat spermatozoa where a meshwork of actin filaments is thought to cause the large scale immobilisation of membrane lipid, detected by FRAP, may be significant [Wolf et al 1988]. There is some evidence for the presence of actin filaments within the tegument of schistosomes [Matsumoto et al 1988]. In the light of this it is possible that actin filaments may extend to the surface membrane also.

The "patching" hypothesis of Dupou et al may be relevant here as this would, if operable, amplify the ability of proteins within membranes to immobilise lipid.

As has been discussed at some length all the evidence for differentiation of the surface membrane of S. mansoni into regions of varying fluidity comes from FRAP data. Interpretation of the data derived from this technique, which uses extrinsic lipid probes, must be viewed with some caution in the light of the Dupou et al findings. This technique may not probe all areas of the membrane and could potentially underestimate the immobility due to incomplete

reporting on all areas or overestimate immobility if patching of small areas of mobile lipid by proteins occurred. This "patching" effect could also conceivably occur if small areas of mobile lipid were surrounded by gel phase domains (see Figure 1.3).

These qualifications aside the extensive immobility displayed in S. mansoni is clearly significant and represents a fundamental difference between the plasma membrane of the host's cells and the surface membrane of the parasite. The simplest explanation of this immobility would be the presence of some type of domain structure. The observation of similar levels of immobility in poikliothermic cells and spermatozoa provided some precedent for this. Significantly they also may provide models of mechanism as the spermatozoa with an actin meshwork may immobilise primarily via a cytoskeleton and the poikilothermic cells via phase separation. It is important to stress that these two mechanisms are not mutually exclusive. They provide the two most likely hypotheses to explain the biophysical structure of the surface membrane of S. mansoni. At the present time it is impossible to distinguish between them and it may well be that both operate and vary with the developmental stage of the parasite.

2.1.8. Background to the project.

The field of fluorescence based cell biological studies has made great advances in recent years and is currently an area of rapid development. Two factors have been primarily responsible for this: the increasing development and accessibility of a wide range of fluorescent analogues of proteins, lipids and other biological probes (Molecular probes 1988). Secondly advances in technology have greatly increased the scope for such a fluorescence based approach utilising fluorescent analogues and probes to address problems in cell biology . Currently the introduction of

commercially available systems for confocal laser scanning microscopy is underway and promises to provide greatly enhanced image formation combined with computer aided image analysis. Such systems promise to allow detailed investigations of such dynamic cellular processes as lipid trafficking in cells. The ability to utilise the wide range of fluorescent probes now available is directly determined and also limited by the technology available.

For the main part of this project the technology available was a good fluorescent microscope with a photomultiplier attachment which gave a digitised quantitation of fluorescence in a defined area. The photomultiplier was extremely sensitive and therefore allowed accurate quantitation from defined areas of the parasite membrane. Data generation was therefore extremely accurate, its handling was not as good since the system was not computerised and all data was accumulated manually and required separate statistical analysis.

Several classes of fluorescent lipid analogue notably the indocarbocyanine and the aminofluorescein analogues had been utilised in this laboratory to study the properties of the surface membrane of S. mansoni. This was done to greatest effect in a close collaboration between this group and another group [Foley & Garland] in Dundee University which applied the FRAP technique and utilised these dyes. These investigators established that the fluorescent dyes used in this study did behave in a manner which was largely analogous to endogenous lipids. They also provided evidence for the presence of differentiation of the surface membrane into domains. The application of the FRAP technique to the study of the surface membrane established the use of fluorescent lipid analogues as a valid and informative approach to the study of the surface membrane. This technique, FRAP, although conceptually simple and informative represented only one rather specialised way of exploiting the properties of these fluorescent probes. At

the outset of this project it was clear that, these fluorescent lipid probes did interact with the parasite membrane in a manner similar to endogenous lipids. It was also apparent that the full potential of these dyes had not been realised. The basic technology (quantitative fluorescence microscopy) was available in this laboratory to exploit further the properties of these dyes by techniques complementary and additional to FRAP.

2.1.9 Dyes: justification.

The list below sets out the arguments which support the basic assumption concerning the use of fluorescent lipid analogues, that they locate and behave in a manner analogous to and representative of endogenous phospholipids and free fatty acids.

The dyes used in this study were aminofluorescein C18, Rhodamine C18, DiI C18 and Merocyanine 540, the first three of these are close analogues to phospholipids in the sense that they possess acyl chains eighteen carbons long. These are believed to insert alongside endogenous lipids in the membrane for the following reasons.

1. The fluorescence observed after labelling with a 1% ethanolic solution of these dyes, as routinely employed in this study, cannot be due to fluorescent micelles adhering to the surface of the parasite. This is because the aminofluorescein and carbocyanine dyes self-quench in micelles. This indicates that fluorescence is from the membrane itself.

2. Fluorescence microscopy of parasites labelled with these lipid analogues revealed the same general staining pattern as that seen on lectin labelled parasites visualised by electron microscopy. These lectins locate at the surface membrane, therefore the correlation in staining patterns indicates a location at the surface membrane for the fluorescent dyes [Torpier & Capron 1980]. This argument could also be

extended to scanning studies of the parasite itself particularly the adults. Surface membrane features such as transverse folds and tubercles are clearly distinguishable by fluorescence microscopy of labelled parasites. Morphologically these structures appear similar whether observing by S.E.M., fluorescence microscopy or light microscopy.

3. Fluorescence quenching data indicates that both these probes locate in the outer leaflet of the surface membrane in schistosomula but only aminofluorescein locates there in adult membranes [Foley 1986]. The intense rim pattern of labelling with these dyes indicates both a lack of internalisation and a probable surface membrane location.
4. One would expect on energetic grounds insertion of the hydrocarbon chain of fluorescent lipids into the membrane. Consistent with such an interpretation the conjugated bond of DiI C18 has been shown to align parallel to the plane of the membrane when it binds to erythrocytes [Axelrod 1979]
5. The rate of lateral diffusion of these dyes in schistosomes is consistent with that calculated for phospholipids in biological membrane. This strongly implies that these probes are aligned parallel to the phospholipid molecules of the membrane itself.

Section 3. LDL and Cholesterol Metabolism

3.1.1. Nutritive requirements.

Investigation of the interaction between the surface membrane of S. mansoni and plasma lipoproteins is warranted for the following reasons. The parasite S. mansoni cannot synthesise either cholesterol or long chain fatty acids de novo and is therefore dependent on the uptake from the host to supply its requirement for these lipids [Meyer et al 1970]. Two potential sources of these molecules are available to the parasite in the bloodstream. Cholesterol and long chain fatty acids could either be obtained via the surface membrane interacting with plasma lipoproteins or the parasites lipid requirement could be met by digestion of erythrocyte membranes or possibly lipoproteins in the gut. This option may not be available to earlier stages of the parasite as in schistosomula younger than three days the gut is not fully functional. At present no study has been done to distinguish or quantitate the relative contributions of uptake via the gut or tegument. This study is restricted to the investigation of lipoprotein interaction with the surface membrane.

There have been several reports indicating that this does occur and that the composition and function of the surface membrane is altered as a result. In addition there have been reports implicating interactions of free cholesterol with the adult stage of the parasite. Since lipoprotein function and cholesterol metabolism are intimately related in plasma it is inappropriate to consider cholesterol uptake and metabolism by the parasite separately from lipoprotein interaction. Therefore this discussion will proceed by reviewing the limited data concerning LDL and cholesterol interaction with or function in S. mansoni. This data will be related to the principal functions and metabolic reactions of the various lipoprotein classes which collectively control both cholesterol metabolism and transport in the plasma.

3.1.2. Studies on S. mansoni.

Rumjanek and McLaren [1981] have monitored serum induced changes in the lipid composition of schistosomula and reported that mechanically transformed 3hr schistosomula exposed to foetal calf serum exhibit a loss of monoglycerides and diglycerides from their surface membrane. They also become resistant to complement mediated in vitro damage, relative to non-serum treated controls. Incubation of such schistosomula with 10% human serum also lead to a loss of monoglycerides and diglycerides but in addition resulted in a marked uptake of both triglycerides and cholesterol ester by the parasite. These schistosomula treated with human serum were subsequently shown to be relatively resistant to cell mediated immune attack. This line of research was continued with the demonstration of a 45 kD dimer protein induced by exposure to dialysable component of serum [Rumjanek, McLaren & Smithers 1983] which appeared to bind specifically to immobilised low density lipoproteins immobilised in sepharose [Rumjanek et al 1985]. Thus there is good evidence indicating that there is an LDL receptor induced by human serum. The induction of this receptor correlates well with human serum associated uptake of cholesterol ester and the appearance of putative LDL protein fragments within the surface membrane of schistosomula exposed to human serum and LDL. This last observation has been suggested as representing the degradative products of some kind of endocytosis like mechanism operating within the parasite tegument which internalises and degrades LDL resulting in the acquisition of cholesterol ester by the parasite [Rumjanek, McLaren & Smithers 1983]. Interestingly high levels of cholesterol ester and triglyceride also appear to be a characteristic of the highly resistant lung stage of S. mansoni. The data implicating interaction of cholesterol with the membrane of the adult stage is rather sparse and indirect. Haseeb et al 1983, have shown that cholesterol can be acquired by the adult worm and accumulates relatively specifically within

the tubercles of the male worm and in the vitellaria of the female. Moreover these authors also demonstrated the transfer of radioactively labelled cholesterol and products between male and female worms. Soares et al 1987 have also demonstrated the transfer of hormone products which appear to be derived from cholesterol which control the maturation and egg laying of the female. Indirect evidence of an LDL interaction or effect on the adult parasite has also been presented by Harnett & Kusel [1987]. These authors provided evidence that incubation of adult parasites in LDL or HDL significantly increased their antigenicity and this was proposed to be due to cholesterol transfer to the membrane.

In summary the limited number of studies on S. mansoni concerned with lipoprotein and cholesterol metabolism and interaction provide evidence that lipoproteins may induce immunologically important compositional changes in the surface membrane of juvenile forms of the parasite. In the adult there are strong indications that cholesterol uptake and metabolism may be of fundamental importance to development. Two points are worth making in respect of this data. Firstly a direct interaction between lipoprotein and the parasite surface membrane, although strongly implied by the data, has not been directly demonstrated. Secondly the biochemical or cell biological mechanisms operative in plasma that mediate cholesterol transfer between lipoproteins and membranes have not been fully discussed in any of the papers cited above, with respect to their possible interactions with the surface membrane of S. mansoni.

3.2.1. Lipoproteins and cholesterol metabolism.

Lipoproteins function to transport cholesterol and lipids within the plasma between the liver and non-hepatic tissues. Lipoproteins consist of complexes of lipid and apoproteins which are, with the exception of Apoprotein-B of LDL, extremely dynamic. That is, both the lipid and

protein composition of lipoproteins can change very quickly with lipid and apoproteins exchanging easily within different lipoprotein classes and membranes. The concepts concerning the structure of lipoproteins have changed recently as it has become apparent that the core of lipoprotein of all classes, usually cholesterol ester and triacylglycerol, are in equilibrium with the surface of the lipoprotein. Previously it was thought that the outermost regions of lipoprotein consisted of a monolayer of phospholipid and apoprotein which shielded the contents of the core to some extent. It is clear however that the core of lipoprotein is easily and very rapidly rendered accessible to enzymic reactions occurring outwith the lipoprotein itself [Miller & Small 1983, Lund-Katz & Phillips 1984].

The nature of the enzymic reactions to which the lipid and sterol contents of lipoproteins are subjected is determined by the particular components associated with them. Apoproteins act as cofactors and ligands, effectively labelling the lipid and sterol contents and directing their subsequent metabolic fate. There are four main classes of lipoprotein, each of which will be described in some detail. Classification of lipoproteins is by lipid content density and apoprotein composition. However classification of the various classes is not absolute as each of the parameters mentioned, particularly buoyant density and lipid content, represent a continuum. The term "LDL" represents lipoproteins covering a range of lipid composition and buoyant densities that fall between a certain range and have particular apoproteins associated with them. The four main classes of lipoproteins, chylomycrons, VLDL, LDL and HDL, although intimately connected metabolically to each other, have different functions. Chylomicrons function primarily to transport dietary fatty acids from the gut, VLDL and LDL transport cholesterol from the liver to extrahepatic tissues and HDL mediates return of excess cholesterol to the liver.

3.2.2. Chylomicrons.

Chylomicrons are triacylglycerol rich lipoproteins consisting of complexes of cholesterol ester and triacylglycerol stabilised by Apoprotein B48. Their lipid contents are derived from the gut and the apoproteins from the mucosa of the gut. Upon formation they are secreted into the intestinal lymph and thence into the circulation. This class of lipoprotein functions to transport dietary fatty acids and cholesterol. It does not interact with the other lipoprotein classes due to its short lifetime, T1/2 = 3 - 4 minutes. Therefore there is little opportunity for it to exchange its lipid content by exchange with other lipoprotein classes. In addition the particular type of Apoprotein B that associates with it, does not interact with the Apoprotein B/E receptor which mediates receptor mediated endocytosis of VLDL and LDL.

Chylomicrons are metabolised on the surface of endothelial cells by the action of the enzyme lipoprotein lipase (LPL). This enzyme is not secreted by the endothelial cells themselves but is produced by underlying parenchymal cells. It is thought to bind ionically at the luminal end of the endothelial cell surface glycosaminoglycans containing heparin. The enzyme binds the triacylglycerol rich chylomicron to the surface of the cell and hydrolyses the triacylglycerol and a small amount of cholesterol ester. Due to the fact that the lipoprotein and cell membrane are contiguous with one another the free fatty acids and free cholesterol transfer from lipoprotein to cell. In effect the enzyme acts to briefly adsorb the lipoprotein onto the cell surface, effect hydrolysis and transfer takes place due to diffusion of the product down a local concentration gradient. This is a multistep process at the end of which approximately 80% of the triacylglycerol and 5% of the cholesterol ester are hydrolysed and transferred. The remaining cholesterol ester rich remnants are subsequently cleared by the liver. The limiting factor on hydrolysis appears to be the presence of apoprotein Apo C2 which is

recycled between the chylomicrons and an HDL fraction. [Fielding & Fielding 1982].

This lipoprotein/enzyme system represents a major pathway for the transport of dietary fat within the bloodstream. It is pertinent to point out that S. mansoni has access to chylomicrons due to its habitat within the blood vessels. In addition there is evidence to suggest that glycosoaminoglycans are present on the surface of the parasite [Robertson & Cain 1985]. This may well be a means whereby the parasite could satisfy its requirement for the exogenous free fatty acids which act at the parasite surface membrane.

3.2.3. Very low density lipoproteins (VLDL).

VLDL is secreted by the liver into the plasma where its main characteristics are a low buoyant density of < 1.006, a high triacylglycerol content and an association with Apoprotein B100. It is similar physically and functionally to chylomicrons in that it can also act as a substitute for LPL acting at the endothelial surface membrane. Like chylomicrons it loses a large part of its triacylglycerol due to the activity of lipoprotein lipase.

In contrast to chylomicrons the cholesterol ester rich remnants of VLDL are not immediately cleared by the liver. A large fraction of the remnants proceed by a process involving further triacylglycerol and apoprotein loss, but cholesterol ester gain, to form LDL. It may be more accurate to say that these remnants acquire the characteristcs of LDL. The relatively long life time of VLDL in the plasma (T1/2 = 90 minutes) relative to chylomicrons allows this maturation process to take place due to its greater ability to exchange lipid and apoprotein. Also the particular type of Apoprotein B, Apoprotein B100, that characterises VLDL allows it to interact with cellular Apoprotein B/E receptors and deliver its contents to cells via receptor mediated endocytosis.

3.2.4. Low density lipoproteins (LDL).

LDL is characterised by the Apoprotein B100, largely to the exclusion of other Apoproteins, and a buoyant density intermediate between that of VLDL and HDL (<1.096). Both VLDL and LDL function to transport cholesterol from the liver to sites of extrahepatic utilisation. There are two mechanisms by which this lipoprotein to cell transfer of cholesterol contents can take place. The best known and to some extent best characterised of these mechanisms is the process of receptor mediated endocytosis elucidated by Goldstein & Brown [1979]. In the context of schistosome surface membrane this is of some interest as it has been suggested as a possible mechanism by which the 45 KD doublet LDL receptor identified in schistosomula may operate [Rumjanek, McLaren & Smithers 1983]. If this were the case it would have profound implications for the understanding of the cell biology of the surface membrane of S. mansoni. The existence of such a mechanism would indicate that the double bilayer structure was much more dynamic than previously supposed.

3.2.5. Receptor mediated endocytosis.

Receptor mediated endocytosis of LDL occurs in many cell types and is dependent on recognition of Apoprotein B or Apoprotein E. The receptor responsible has been characterised as a glycoprotein of molecular weight 120.000. This receptor has a much greater affinity (x 23) for Apoprotein E compared with Apoprotein B. The biological basis of this is thought to be that acquisition of Apoprotein E by LDL directs them towards the liver. Thus LDL early in its lifetime contains only Apoprotein B, but that fraction of LDL which is not endocytosed is redirected towards the liver.

Internalisation of LDL into cells requires binding of LDL to the receptor followed by localisation of the receptors plus bound LDL into coated pits. These structures are

clathrin coated invaginations in the membrane which pinch off from the plasma membrane as endosomes thus internalising both receptor and ligand. The contents of these endosomes are eventually delivered to lysosomal compartments in which the cholesterol ester core of LDL is hydrolysed releasing free cholesterol and free fatty acids for utilisation by the cell. Prior to this however the receptors themselves are separated from LDL itself and redirected towards the plasma membrane surface and recycled. Further metabolism of the cholesterol derived from endocytosed LDL leads to the formation of oxidised derivations of cholesterol, oxysterols, which inhibit cellular cholesterol synthesis at the level of the enzyme HMG-CoA reductase.

At one time it was thought that the LDL receptor mechanism was the rate limiting factor determining cholesterol synthesis in cultured cells and in vivo. However, several observations notably that patients with homozygous familial hypercholesteremia (an inherited disease with partial or complete deficiency of LDL receptors) had levels of whole body cholesterol synthesis similar to normal subjects, contradict this view. In addition in cells depleted of cholesterol in lipoprotein deficient serum induction of HMG-CoA reductase, indicative of endogenous cholesterol synthesise, preceded induction of lipoprotein receptors. It has also been shown that, under conditions pertaining in vivo the net amount of cholesterol derived from LDL due to diffusion of free cholesterol down its concentration gradient is similar to that derived from receptor mediated endocytosis. The balance of experimental evidence now suggests that cellular cholesterol content is normally maintained by local cholesterol synthesis to balance any transient inequities between molecular (non-receptor) pathways of cholesterol influx and efflux [Stange & Deitschy 1983]. This option of method of control, cholesterol synthesis, is not however available to

schistosomes. However the alternative mechanism of cholesterol uptake, namely net transport due to the flux of cholesterol down local concentration gradients between lipoproteins and membrane is. In the absence of any S. mansoni equivalent of receptor mediated endocytosis this would be the most likely mechanism by which the surface membrane of S. mansoni could acquire free cholesterol from lipoproteins.

3.2.6. Net Cholesterol Flux.

It has been demonstrated that the low solubility of free cholesterol in aqueous media is sufficient to allow net movement of cholesterol down a concentration gradient between lipoprotein and membrane or between lipoprotein classes [Fielding et al 1984]. These workers have shown that flux rates of cholesterol calculated from theoretical consideration of diffusion rates, as well as measured cholesterol flux rates between cholesterol labelled synthetic vesicles in the absence of protein receptors, are very similar to those observed between living cells and lipoproteins and cell membranes in native plasma. This indicates two important points, firstly physical contact between lipoprotein and membrane is not required for transfer of cholesterol to take place. The major part of cholesterol flux is due to a rate-limiting desorption and diffusion between the acceptor and donor surfaces [Fielding 1984]. Therefore although transfer will take place in the absence of collision or contact the closer the donor lipoprotein and acceptor membrane are the more efficient such diffusion dependent transport will be. In respect of S. mansoni its inability to synthesise its own cholesterol and free fatty acids presumably renders it relatively cholesterol poor compared with plasma lipoproteins. Thus it may be reasonably expected that such cholesterol flux may take place between the parasite and the lipoprotein. Three factors would determine the extent to which this would occur, the closeness of contact between lipoprotein

and parasite, the surface area of membrane exposed by the parasite and its cholesterol concentration relative to the lipoproteins. Some mechanism whereby free cholesterol was removed from the outer bilayer such as esterification within the tegument would maximise net cholesterol flux. In this respect the ability of cholesterol to flip flop rapidly between the two leaflets of plasma membranes [Lange & Steck 1985] may be relevant. Were cholesterol similarly mobile within the double membrane of S. mansoni esterification of free cholesterol within the cytoplasm of the tegument could act to maintain the membrane itself in a state of relatively low cholesterol concentration thereby maintaining the flow of cholesterol from lipoprotein to parasite surface membrane.

3.2.7. High density Lipoprotein and LCAT.

High density lipoprotein has a buoyant density between 1.063 and 1.21 and is characterised by Apoproteins A1 and A2. It functions to some extent in opposition to LDL and VLDL in that it transports cholesterol from non-hepatic tissues to the liver where it is catabolised to form bile salts. This process termed "reverse cholesterol transport" is necessary since cholesterogenesis occurs in a number of peripheral non-hepatic tissues notably endothelial cells. This mechanism allows them to transfer excess cholesterol thereby limiting the cellular cholesterol content. This efflux of excess free cholesterol to HDL is mediated by the enzyme lecithin cholesterol acyl transferase (LCAT) which esterfies free cholesterol. It can use free cholesterol derived from plasma cell membranes but also from VLDL or LDL. It has however a strong preference for membrane cholesterol in that if equal amounts of membrane cholesterol and lipoprotein cholesterol are available to it it will esterify the membrane cholesterol first. The enzymatic activity is associated with a particular subfraction of HDL which in contrast to the bulk of HDL is smaller and contains only Apoprotein A1 as opposed to

Apoprotein A1 and A2. This HDL fraction represents a "catalytic" fraction in which the Apoprotein A1 acts as a cofactor to the LCAT enzyme. In plasma this small Apoprotein A1 HDL fraction represents an active complex which rapidly turns over and provides the majority of cholesterol esters contained within the plasma lipoproteins.

The basis of this small Apoprotein 1 rich HDL fraction's ability to act as a specific mediator of LCAT activity may reside in its small size which allows this type of HDL to approach membranes more closely that the larger bulk fraction of HDL. In the plasma this HDL/LCAT fraction functions to esterify free cholesterol largely because the substrates namely free cholesterol and phosphatidyl choline exist at locally higher concentrations than product cholesterol ester. This is because the cholesterol synthesised by most cells is greater than their requirements for membrane production therefore cholesterol is in excess. The HDL/LCAT complex is also dependent on two plasma transfer proteins TP1 which transfers phosphatidyl choline from VLDL or chylomicrons to HDL Apoprotein 1 and TP2 which transfers the product cholesteryl ester to acceptor lipoproteins either HDL, LDL or VLDL. With regard to schistosomes both these transfer proteins are potential sources of exogenous lipid either as phosphatidyl choline or cholesterol ester or even triacylglycerol which is exchanged by TP2 in place of cholesterol ester. [Tall et al 1983, Albers et al 1984].

3.3.1. Summary and Perspectives.

The paragraphs above have briefly described the major types of lipoproteins and their function. The common denominator that characterised the function of each of the four classes is that they act at and upon exposed plasma cell membranes within the bloodstream. The membrane surface exposed by S. mansoni appears from E.M. studies, from FRAP studies and

in composition to be fundamentally similar to that of plasma cell membranes. It is likely therefore that some or even all, of the lipoproteins associated enzymatic activities and their related transport of sterol and lipid species described above, take place on the surface of S. mansoni. The ability of free cholesterol to equilibrate between lipoprotein species and with cell membranes strongly suggests that it will equilibrate with this parasite's surface membrane also.

The operation of such enzymatic and transport activities on the surface membrane may or may not be sufficient to supply the whole parasite with its total requirement for exogenous lipid. This would however certainly affect the composition and structure of the surface membranes and therefore influence its function. This preliminary study of the cell biology of lipoprotein interactions with the surface membrane of S. mansoni has been directed towards answering two general questions.

How does plasma lipoprotein interaction affect the surface membrane of S. mansoni in terms of structure and function?

Is there evidence of cholesterol or fatty acid transport from lipoproteins across the surface membrane?

Thus there is both a "nutritive" aspect and a "local" aspect to this study concerned respectively with the nutrition of the whole organism and the structure and function of its surface membrane.

4.1. Aims of the project.

The fundamental aim of this project was to exploit the properties of fluorescent lipid probes and our quantitative fluorescence facility in order to investigate the cell biology of the surface membrane of S. mansoni.

From this rather general aim a number of more specific investigations were developed. These focussed on aspects of the development and structure of the surface membrane as well as an investigation of its interaction with host molecules. These are listed below.

Development

To utilise fluorescent lipid analogues to monitor and characterise the early (<3hr) formation and development of the double bilayer structure of S mansoni schistosomula.

Host Interaction

To investigate the nature of the interaction between LDL and the surface membrane of S mansoni.

Gross structure

To examine the gross structural changes in membrane morphology in the surface membrane of both juvenile and adult stages of S mansoni induced by cooling and relate these to the structure and function of the membrane.

Fine structure

An investigation of the occurrence of sub-membraneous domain structure within the plane of the membrane with a view to develop techniques that would allow biophysical characterisation of such structures.

CHAPTER 2
MATERIALS AND METHODS

2.1 Biological Materials

2.1.1 The parasite

The life cycle of a Puerto Rican strain of Schistosoma mansoni is maintained in the Department of Zoology, University of Glasgow by Mrs J. Thornhill, Department of Biochemistry. The parasite and the intermediate host, Biomphalaria glabrata, were originally obtained from the National Institute for Medical Research, Mill Hill, London. During the period of this study these stocks of both parasite and intermediate host were largely renewed by acquisition of stock from the University of York.

2.1.2 Animals

Mice of predominantly Balb/c strain with lesser numbers of Parkes mice have been used to maintain the life cycle and provide adult worms.

2.1.3 Reagents

All general chemical stock were obtained from British Drug House Ltd., Poole, Dorset or Northumbrian Biochemicals Ltd. Other reagents were derived from various sources listed below.

Chloroform, methanol, Sagatal	May & Baker Ltd., Dagenham, England.
Sephadex G.25	Pharmacia (Great Britain).
(I^{125}) NaI H^3 Cholesterol	Radiochemical Centre, Amersham, England.
Penicillin Streptomycin Heparin	Vestric & Co. Ltd, Glasgow
ConA BSA Trypan Blue Carbachol Colchicine	Sigma Chemical Co., Poole, Dorset.

2.1.4. Plasticware & Glassware

All plasticware from Sterilin, Teddington, Middlesex. Microscope slides and cover slips from Chance Proper Ltd., Warley, England.

2.1.5 Anti-sera

FITC anti rabbit IgG	Sigma
FITC anti human IgG	Sigma
FITC anti sheep/donkey IgG	Scottish Antibody Production Unit

Anti ApoB(sheep). A kind gift from Dr C. Packard, Glasgow Royal Infirmary.

2.1.6 Fluorescent Molecules and Stains

Fluorescein Isothiocyanate (FITC)

Merocyanine 540

1,1'-Di(octadecyl)-3,3,3',3'tetramethyl indocarbocyanine perchlorate; DiI C18 (see Figure 2.1 a)

5-N-(octadecanoyl)aminofluorescein; aminofluorescein C18 (see Figure 2.1 b)

Octadecyl rhodamine B, chloride ; rhodamine C18 (see Figure 2.1 c)

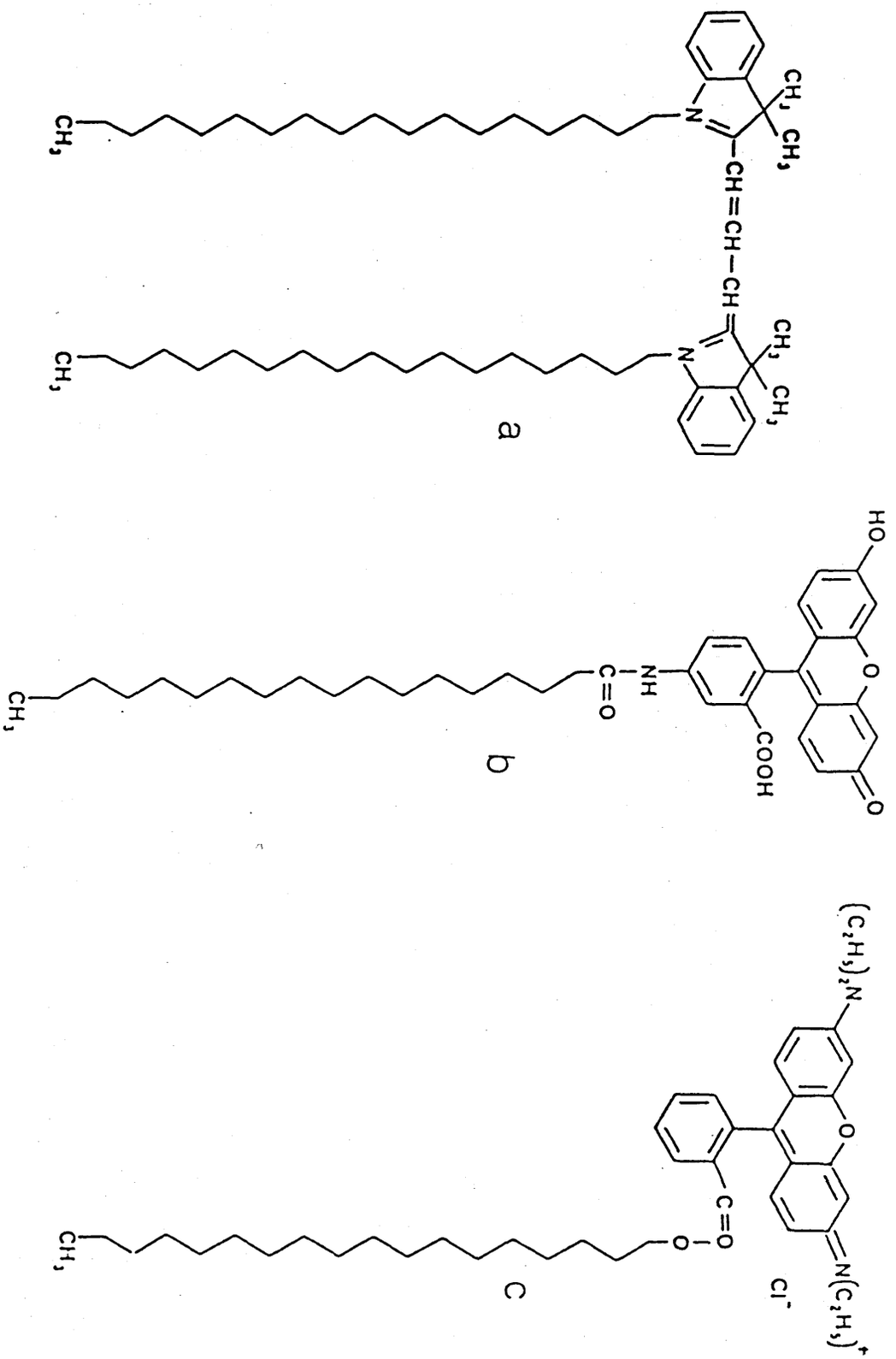
Oil Red O

NBD Cholesteryl ester

All of the above were obtained from Molecular Probes Inc., Oregon 97448, U.S.A.

Nile Red — Nile red was isolated from the histological stain Nile blue (May & Baker) by the method of Greenspan et al 1985. 5g of Nile blue were dissolved in 0.5% H_2SO_4 and boiled under reflux for two hours. After cooling, Nile red was separated by repeated extraction into Xylene. The organic solvent was evaporated under nitrogen.

Figure 2.1 Molecular Structures of principal probes



2.1.7. Standard Solutions

i) Eagles Medium.

This was made up by the addition of the following volumes of stock solution(s) to 425 ml sterile H_2O.

a) 50 ml X10 Glasgow's Modification of Eagle's Media.

b) 15 ml $NaHCO_3$ Final concentration 2.5%.

c) 5 ml Glutamine stock.

d) 5 ml Penicillin/Streptomycin stock. Final conc. 10,000 units/litre and 100 mg/litre respectively.

Prior to use pH was adjusted to 7.4 by the addition of 5M NaOH. On occasion HEPES buffer 20 mM was utilised rather than bicarbonate.

Formulations for this medium can be found in the FLOW laboratories manual.

ii) Citrate-Saline.

Citrate-saline is prepared by adding 17 g NaCl and 30 g sodium citrate to 2 litres of H_2O.

iii) Phosphate buffered saline, pH 7.2 or 7.4 (PBS)

Solution A: 500mM-$Na_2H\ PO_4/NaH_2\ PO_4$, pH 7.2 or 7.4

Solution B: 140mM-NaCl

PBS consists of 10% by volume Solution A and 90% by volume Solution B

2.2 Handling of the parasite

2.2.1 Perfusion of animals for recovery of adult worms (Smithers & Terry, 1965).

6 - 8 week infected animals were killed by giving them an injection of sagatal (1 ml/100 gm body weight) intraperitoneally. The animals were dissected to expose the abdominal and thoracic cavities and then held in place by two thick rubber bands against a vertical perspex stand such that both cavities faced outward. An incision was made in the portal vein and then warm EMS or citrate-saline (2.1.7) was pumped into the heart using a 50 ml disposable syringe (Plastipak - B - D, Becton, Dickinson and Co., Ireland). The flow of fluid through the circulation leads to worms being washed out through the incision in the portal vein. The worms were collected in a glass bowl, washed in EMS and employed in various experiments as described elsewhere.

2.2.2 Preparation of schistosomula by mechanical agitation (Colley and Wikel, 1974).

Cercariae freshly shed from snails were transferred to a universal bottle and concentrated on ice. Water was removed until approximately 1 ml remained and then 9 ml of EMS pre-incubated at 37°C was added. The cercariae were sucked up and down using a 10 ml syringe (Plastipak - B - D, Becton, Dickinson & Co., Ireland) 5 or 6 times in order to separate the bodies (i.e. mechanically transformed schistosomula) from the tails. The mixture was then transferred to a 10 ml test tube and centrifuged (100 g for 5s) to sediment the schistosomula (the tails remain in the supernatant). Schistosomula were then washed (x 3) before being employed in the various labelling procedures.

2.3 Microscopy and Fluorescence Quantitation Apparatus

2.3.1 Leitz Microscope and Photographs

Fluorescence observations and photomicrographs were obtained with a Leitz Orthulux II fluorescence microscope, a standard camera attachment, and Ektachrome 800-1600 ASA professional film. Exposures were between 20 and 90s. Appropriate filter combinations were used to discriminate between the various fluorescent probes used.

2.3.2 Leitz MPV Compact

This apparatus consists of a photomultiplier tube and control panel which allows quantitation of fluorescence from a defined area. This was fixed at 400 um^2. For each group of readings an average background reading from a non fluorescent region of the slide was subtracted. The voltage across the photomultiplier was fixed at 700V and the mains current held constant by a Leitz steady current power pack.

2.3.3 Fluorescence Quenching

The depth of insertion of lipid probes in the membrane was examined by measuring the ability of a non-permeant molecule, trypan blue (0.25%, w/v), to quench probe fluorescence as described in Chapter 3.

2.3.4 Fluorescence Recovery After Photobleaching (FRAP).

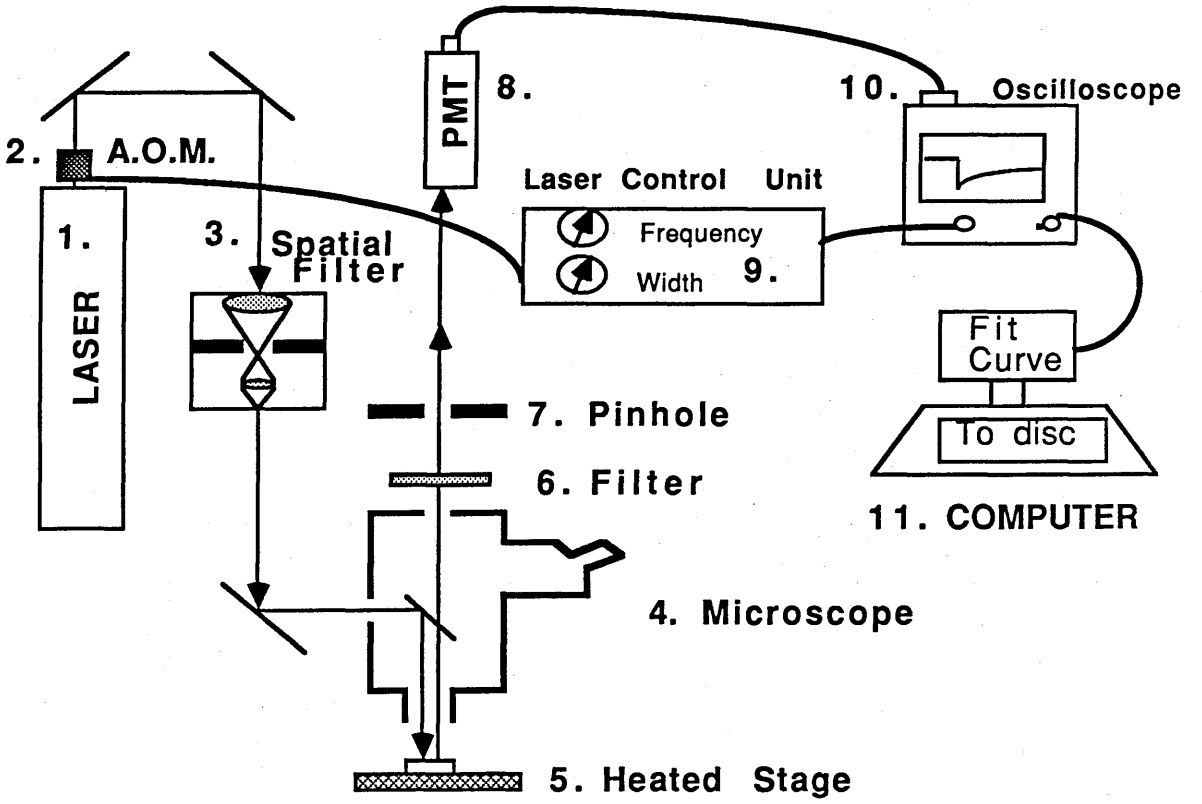
A highly attenuated laser beam was directed into the side of a microscope and focussed down through the objective lens onto a small spot (approximately 1 um in radius) on the surface of the specimen (Figure 2.2). The fluorescent molecules within this spot were excited, the fluorescence was detected by a photomultiplier tube and the signal recorded. The laser beam was briefly unattenuated for approximately 100 ms which served to increase the laser power by 10^4 fold and provide a bleach pulse. As a result of this pulse a proportion of the fluorophores (30-70%) in the spot were irreversibly bleached. This produced a reduced fluorescence intensity when examined, immediately

after the bleach, with the attenuated laser beam. The intensity of fluorescence in the illuminated spot was followed as a function of time. Any subsequent increase (recovery) of fluorescence occurred by lateral diffusion of unbleached molecules from the surrounding area into the spot. From the resultant recovery curve one can discriminate between two different types of molecular movement; diffusion and flow. Using the value obtained for the half time of recovery i.e. the time taken for recovery to proceed to one half of the final recovery, one can calculate the lateral diffusion coefficient. The fraction of molecules free to move in the plane of the membrane (and thus also the immobile fraction) can be determined from the percentage recovery relative to the initial fluorescence.

Figure 2.2

Fluorescence Recovery after Photobleaching

Layout of apparatus Glasgow 1987



1. LEXEL MODEL 85 Argon ion laser HERTS. U.K.
2. Acousto Optic Modulator 304D COHERENT U.K.
3. Spatial Filter PHOTON CONTROL , CAMBRIDGE U.K.
4 Microscope LEITZ (Ortholux) , LUTON, BEDS. U.K
5. Heated Stage LEITZ.
6. Filters Leitz K530 , k590 .
7. Pinhole 0.5mm Dept Nat. Phil. Workshop Glasgow Univ.
8. PMT Photomultiplier tube Model 9924b THORN EMI U.K.
9. Control Unit Dept. Electronic engineering Glasgow Univ.
10 Oscilloscope DIGITAL STORAGE MODEL 4035 GOULD U.K.
11. Computer Hewlett Packard Model 82927A

2.4 Labelling procedure for fluorescent lipid probes

2.4.1 General procedure

1. To 1 ml volume of media (usually EMS only) was added 10 ul of (1 mg/1 ml) solution of dye in ethanol (Analytical grade). Parasites were agitated and capped test tube (Sterilin 144AS) placed in water bath for twenty minutes.
2. Parasites were washed three times in excess EMS.
3. After last wash aqueous media was removed and parasites concentrated in a loose pellet (schistosomula) or sedimented by gravity (adult parasite).
4. A fraction of these are added to a microscope slide with a chamber pre-formed with silicone grease. To this chamber the appropriate volume of a 10 mg/ml carbochol solution was added so that final concentration of carbochol was 500 ug/ml for fluorescence quantitation, 2 mg/ml (schistosomula) or 5 mg/ml (adult worm) for FRAP measurements.
5. If possible readings were taken immediately after mounting of parasites on a glass slide. Otherwise slides were stored in a moist plastic container at room temperature.

2.5.1 Isolation of lipoproteins

See Figure 2.3.

Standard buffer: 0.15M NaCl, 10mM Tris , 0.01% EDTA
Standard density solutions.
1.063g/ml 9.2g KBr/100ml standard buffer 4°C
1.21g/ml 29.6g KBr/100ml standard buffer 4°C

Human venous blood was taken from a healthy, fasted donor and added to 10% sodium citrate. The plasma was isolated by centrifugation (400g for 10mins) in an MSE bench centrifuge then spun in a Beckman 250-5B (200,000g for 24hr 15°C ultra-centrifuge to isolate very low density lipo--proteins (VLDL). This lipoprotein class is present as a

white turbid floating fraction. The orange coloured protein containing infranate was then collected by pasteur pipette and its density adjusted to 1.063g/ml by the addition of 83.4mg KBr/ml infranate. This was layered onto a standard density KBr solution of 1.063g/ml and ultra-centrifuged at 200,000g for 24 hrs at 15°C as before. The yellow floating fraction containing low density lipoproteins was then harvested. The density of the remaining infranate was adjusted to 1.21g/ml by the addition of 236mg/ml KBr layered over a standard density KBr solution of 1.21mg/ml and centrifuged as above. The floating fraction recovered was high density lipoprotein HDL and the remaining infranate now comprises delipidated serum. All recovered fractions were subject to exhaustive dialysis against standard buffer and with the exception of delipidated serum sterilised by passage through a Millipore filter (0.2um pore size) prior to use. Concentration of lipoproteins is described in terms of milligrams of protein per ml. (Diagram of isolation procedure Fig 2.3). This was determined by Lowry assay [Lowry 1951].

Figure 2.3 Isolation of lipoproteins.

Standard buffer

Plasma

Spin 400g 10mins

VLDL Harvest Dialyse Sterilise

Infranate

KBr Density 1.063g/ml

Infranate adjusted to 1.063g/ml

Spin 200,000g 24hrs

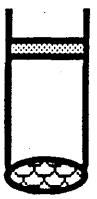
LDL Harvest Dialyse Sterilise

Infranate

KBr Density 1.210g/ml

Infranate adjusted to 1.210g/ml

Spin 200,000g 24hrs



HDL Harvest Dialyse Sterilise

Infranate is De-lipidated serum Dialyse

2.5.2. Iodination of LDL

After McFarlane 1958
Typical iodination reaction as below.

1) 1ml LDL solution (5mg/ml).
2) Add 250ul 1M glycine PH 10.
3) Add "cold" ICl at ratio 2.5uM : 1 uM LDL.
   (7.8ul stock solution 2.5uM/ml ICl in 1M NaCl PH 7.4)
4) 1-2 mCi I^{125} added in volume of 10-20 ul.

Solutions rapidly mixed and allowed to react for 5 minutes. Free iodine removed from iodinated LDL by passage through PD10 column (Sephadex G25). Labelled LDL fractions pooled and protein concentration redetermined by Lowry assay.

Typical specific activity in the experiments reported in chapter 4 was 60 cpm/ng LDL.

2.5.3. Fluorescent labelling of LDL.

a. DiI-LDL. To 1ml of purified LDL (5mg/ml) 10ul of an ethanolic solution of DiI C18 1mg/ml added. Dye and LDL incubated for 45 minutes at 37°C prior to use.

b. Aminofluorescein C18-LDL (AF-LDL). To 1ml of purified LDL (5mg/ml) 10ul of an ethanolic solution of Aminofluorescein (C18 1mg/ml) added. Mixture of dye and LDL incubated for 45 minutes 37°C prior to use. At these concentrations of dye and LDL all aminofluorescein molecules bound to LDL i.e. there are insignificant numbers of aminofluorescein molecules in the aqueous phase Sklar et al 1980.

c. AF/H ^{3} cholesterol-LDL. To 250ul LDL (5mg/ml) 20ul (1mg/ml) of H ^{3}cholesterol in DMSO added. Mixture incubated for 30 minutes at 37°C prior to use. The specific activity of the fraction was 98,000 cpm/ug LDL protein.

d. FITC-LDL. To 1ml of LDL(5mg/ml) dialysed against 0.25M phosphate buffer 200ug of fluorescein isothiocyante added and the mixture incubated overnight with continuous stirring at 4°C. FITC-LDL was separated from free FITC by passage of mixture through a Sephadex G-25(PD-10) column. the concentration of FITC-LDL was redetermined by Lowry assay prior to use.

2.6.1 Isolation of human IgG with QAE - Sephadex

1. Column K9/15 (Pharmacia) half filled with swollen QAE sephadex - and washed with Tris (10mM) HCL buffer PH 6.3.
2. 2 ml fraction of serum layered onto sephadex QAE.
3. Flow initiated through column, chased by Tris HCL buffer PH6.3.
4. O.D. of collected fractions measured at 280 nm.
5. Most concentrated samples pooled and concentration of protein determined by Lowry assay [Lowry 1951].

CHAPTER 3
MONITORING THE MEMBRANE

3.1.1. Introduction

The early development of the schistosomula surface membrane immediately after transformation is a period of rapid and fundamental change. The single glycocalyx covered bilayer of the cercaria which is adapted for an aqueous environment transforms and gives rise to the double bilayer structure which is adapted for a blood dwelling parasitic existence. A great deal is known about this process from electron microscopy studies [McLaren & Hockley 1976].

Within this laboratory it has been perceived for some time that fluorescent lipid probes could be used to monitor the dynamic properties of the parasite membrane. In this respect their use is more flexible than electron microscopy which can only provide information from fixed parasites at a static point in their development. The use of fluorescent lipid probes has the potential of providing a relatively quick and simple method of investigating dynamic changes in the membrane of live developing parasites.

The best example to date of using fluorescent lipid probes to monitor the dynamic nature of the membrane has been that of Foley [1986]. This worker used the FRAP technique to gain information on the mobility of the dyes in the membrane of a living parasite. Clearly the dynamic properties of the parasite membrane such as mobility cannot be measured by electron microscopy. This FRAP study utilised the fluorescence emitted by lipid probes within the membrane in a particular specialised way. However, it is worth noting that the FRAP technique is in essence dependant on quantitation of a fluorescent signal from a membrane probe.

In this present study, a less sophisticated fluorescence quantitation system, the Leitz MPV-Compact has been used to monitor the absolute levels of fluorescence in labelled parasites. A number of workers in this laboratory have observed that this fluorescence parameter, that is the brightness of the parasite membrane after labelling, was modulated in an apparently meaningful way by a large number of agents or treatments. These agents include drugs, macromolecules such as lectins, models of immune damaging agents such as toxins and cationic proteins, "synthetic" damaging agents such as detergents and variation in culture conditions. These were found to alter membrane labelling by the dye compared to control parasites not exposed to the particular agent or treatment.

It was clear that the fluorescence of aminofluorescein C18 dye was very probably sensitive to induced changes in the membrane. What was not clear however was what such increases or decreases in fluorescence relative to control values in these experiments actually meant. For example did such changes reflect a structural change in the membrane, such as increased folding, or a change in the lipid environment in which the aminofluorescein C18 resided? Interpretation of the data attained by quantitative fluorescence using aminofluorescein C18 and other lipid analogues presented a fundamental problem. In this study this problem was addressed for a particular stage of the parasite, the early schistosomula up to 3 hours post transformation.

The initial approach was to investigate whether these analogues could effectively monitor the development of the double membrane structure itself. This was for two reasons. Firstly a developmental study on the early (<3 hr) membrane was of great interest in its own right. Secondly both the rate of membrane change and the contrast between initial and final states are maximal in this stage of the parasite. Therefore this stage represented a test

case of whether these probes could be usefully employed. The rationale behind this approach was that if such major membrane changes could not be monitored via fluorescent probes it was unlikely that more subtle changes in structure associated with intrinsic resistance would be detected.

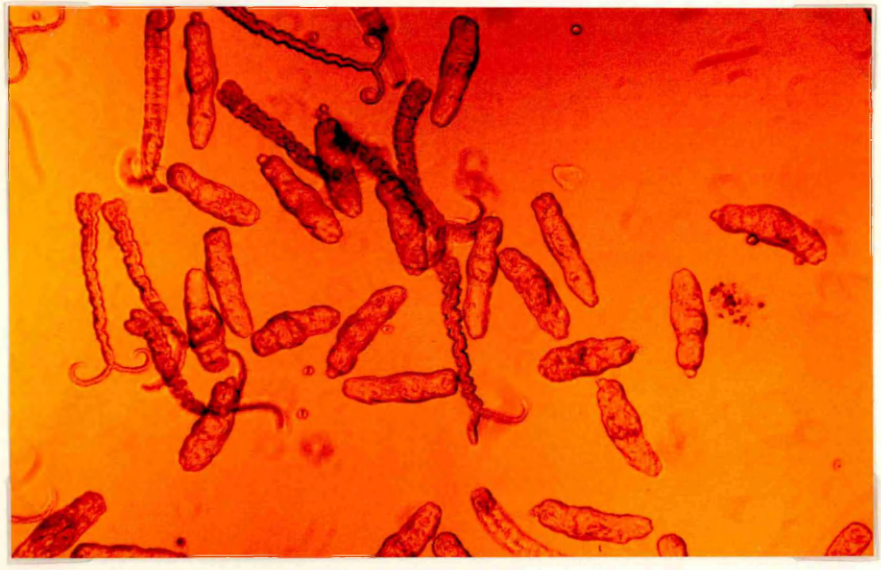
This chapter is concerned with the early development of the parasite membrane, that is, up to 3 hours post-transformation. The experiments presented in the following pages were all done using mechanically transformed schistosomula cultured in Eagle's media. The experiments described below were carried out in chronological order. The data presented represents the results of a series of interrelated experiments concerned with early development and is discussed as a whole at the end of this chapter.

3.1.2 Contrasting labelling with DiI C18 and aminofluorescein C18.

Some very simple experiments using aminofluorescein C18 and DiI C18 to label schistosomula shortly after transformation have proved to be informative. Immediately after transformation schistosomula can be labelled with aminofluorescein C18 whereas DiI C18 will not label . This is the situation in cercaria also. However if labelling with either dye is attempted at 40 minutes after transformation a different pattern is achieved (Plates 3.1 - 3.8). Aminofluorescein still gives a relatively uniform pattern of labelling whereas DiI C18 gives a strongly polarised pattern with labelling restricted to the anterior portion. At 3 hours after transformation DiI also exhibits a generally uniform pattern of labelling (Plate 3.9).

Plates 3.1 & 3.2

Labelling of schistosomula with aminofluorescein C18 at 40 minutes after transformation in EMS.



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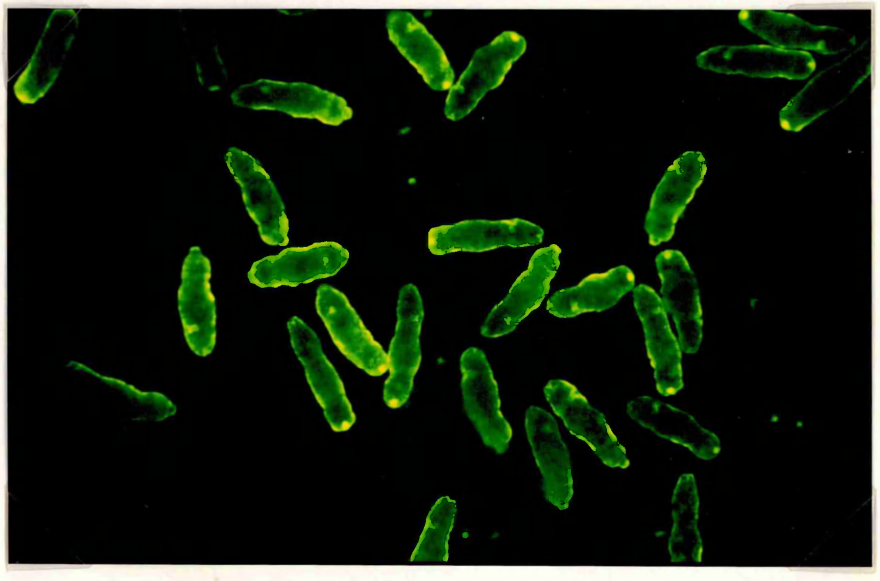

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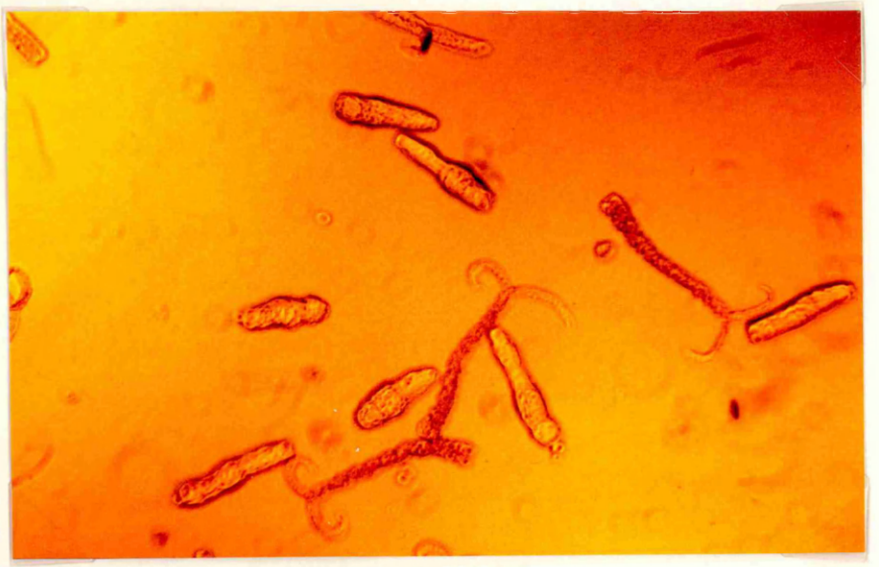
Plates 3.3 & 3.4

Labelling of schistosomula with DiI C18 at 40 mins after transformation in EMS.



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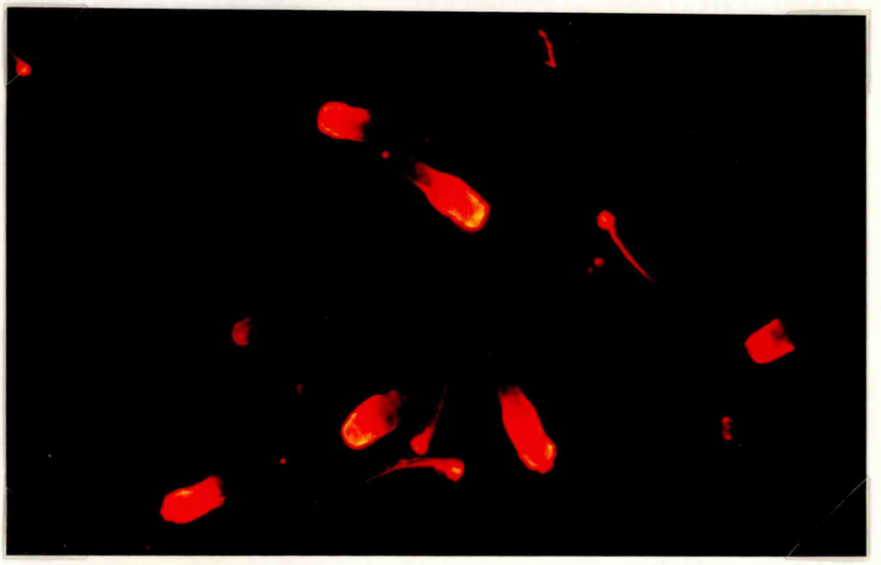

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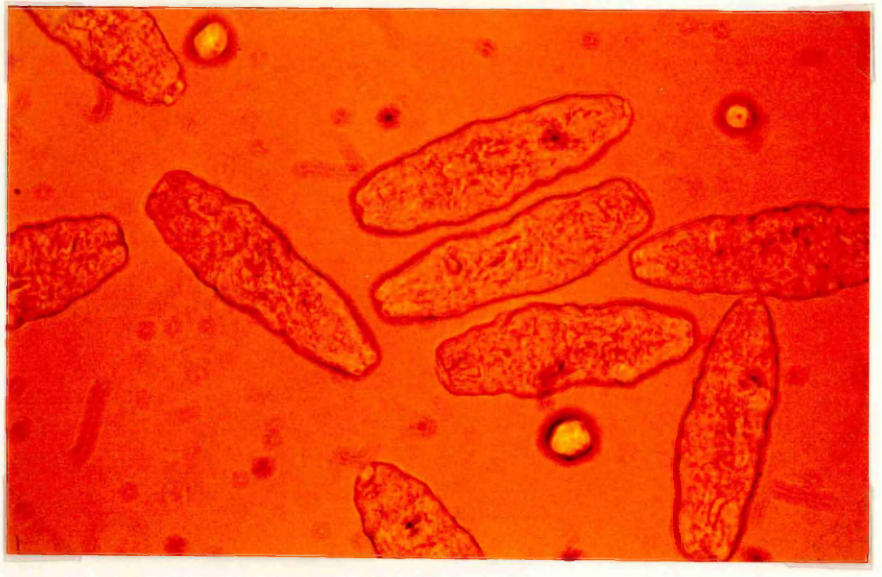
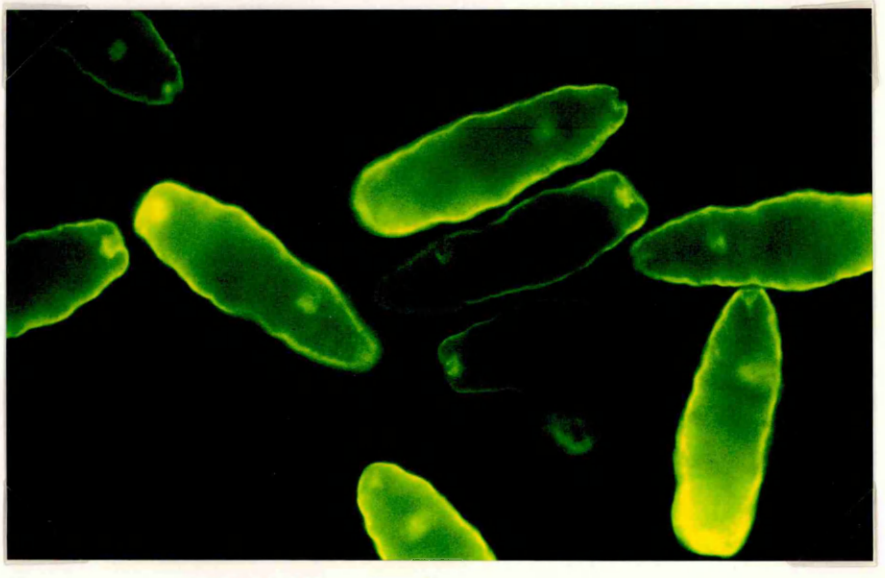
Plates 3.5 & 3.6

Labelling of schistosomula with aminofluorescein C18 at 40 minutes after transformation in EMS.



40 um



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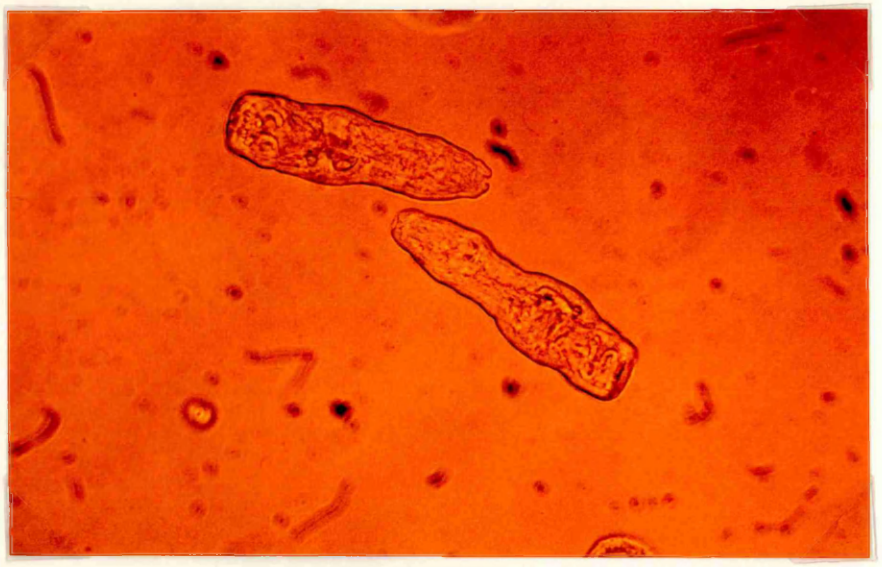
Plates 3.7 & 3.8

Labelling of schistosomula with DiI C18 at 40 mins after transformation in EMS.



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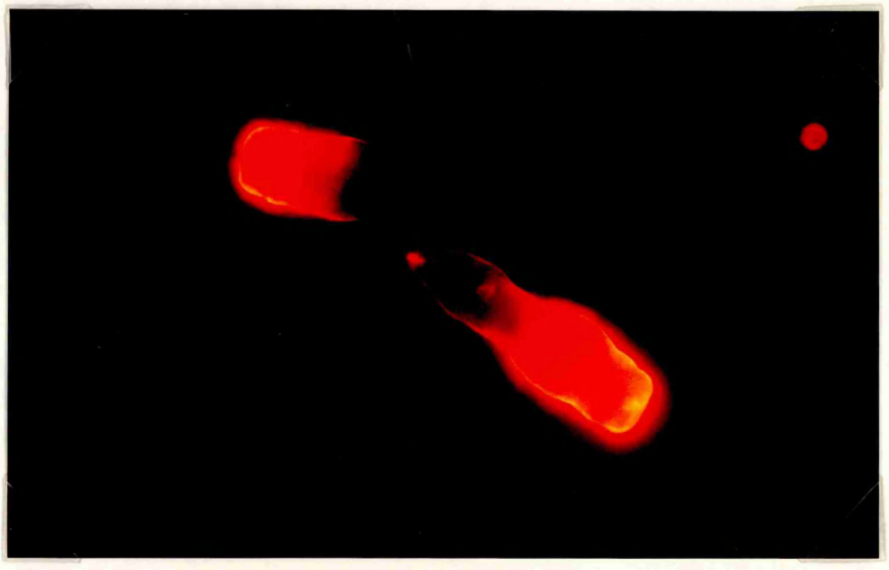

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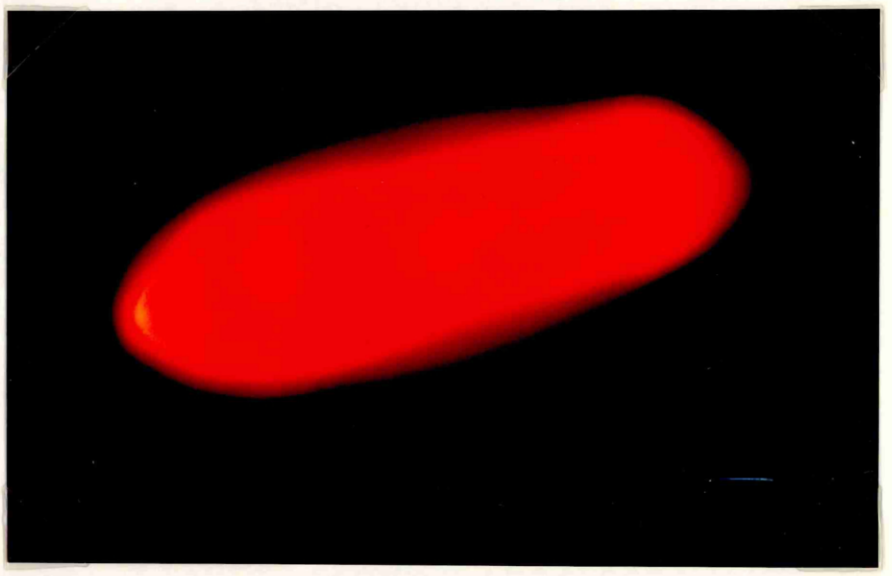
Plate 3.9

Labelling of schistosomula with DiI C18 at 3 hrs after transformation in EMS.



25 um

The transition in the polarised pattern of labelling exhibited at 40 minutes after transformation to a more uniform pattern is shown in Figure 3.1. In this experiment the absolute level of fluorescence on the anterior, central and posterior areas of the parasite were monitored after an initial exposure to label.

Some general conclusions can be drawn from these experiments. The first is that different lipid analogues can behave markedly differently in the same stage of parasite. The second is that some restriction of DiI C18 labelling is present in cercaria and newly transformed schistosomula which is lost sequentially from the front to the rear on the parasite's membrane. In addition lipid molecules appear to be able to diffuse from the anterior to the posterior surface membrane as the parasite develops. This pattern of labelling exhibits close similarities to the sequence of events concerned with loss of cercarial glycocalyx reported by Samuelson and Caulfield [1985]. The restriction on DiI C18 labelling observed may well be the presence of a glycocalyx overlying the membrane. Due to this restricted non-uniform pattern of labelling DiI C18 was not judged to be a suitable dye for monitoring overall developmental changes representative of the whole surface membrane of the early schistosomula.

3.1.3. Aminofluorescein C18 time course experiments.

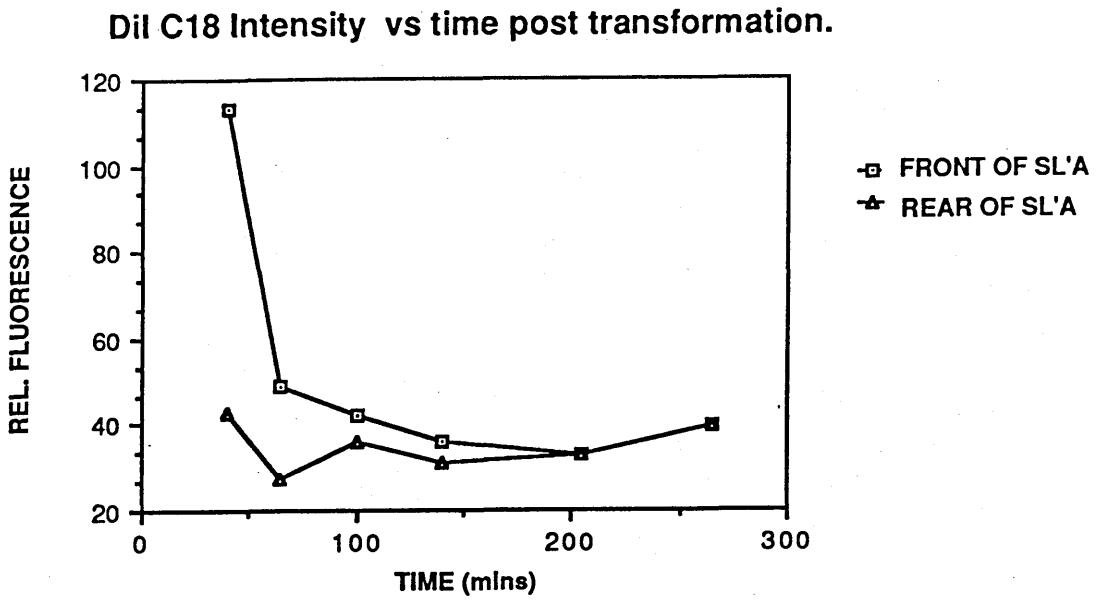
Aminofluorescein appears to gain relatively unrestricted access to the cercarial and newly transformed schistosomula membrane. It does not exhibit strong polarisation of labelling to a particular region of the parasite. For these two reasons it was chosen as a probe to investigate whether the early development of the parasite, encompassing the formation of the double bilayer could be followed by quantitative fluorescence microscopy.

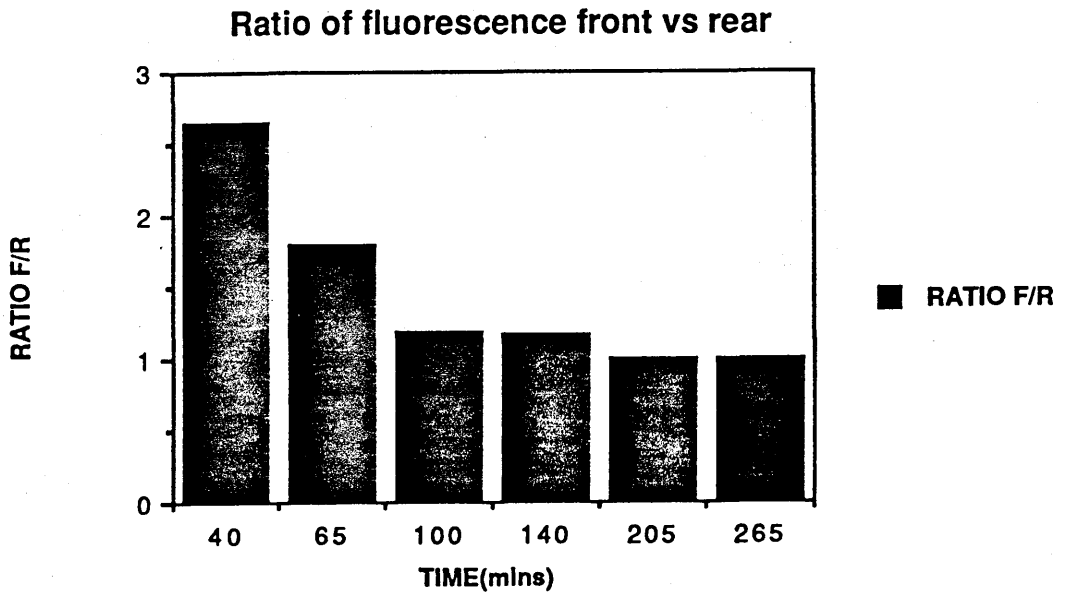
Figure 3.1

The development of a polarised pattern of labelling in schistosomula labelled with DiI C18.

Cercaria were transformed in E.M.S. at 4°C and labelled with, 10ug/ml DiI C18 in E.M.S., immediately afterwards. Labelling took place at 37°C.

The schistosomula were washed three times in E.M.S. to remove excess label. Samples were removed and their membrane fluorescence quantitated at various time points after transformation and labelling. Quantitation of fluorescence took place in two areas of membrane, at the front and rear, in each of 20 parasites per time point. The mean of 20 readings taken on individual schistosomula are shown opposite plotted against time in Figure 3.1a, Figure 3.1b represents the ratio of front to rear fluorescence at the given time points, derived from 3.1a.


Dil C18 Intensity vs time post transformation.
REL. FLUORESCENCE
120
100
80
60
40
20
0
100
200
300
TIME (mins)
FRONT OF SL'A
REAR OF SL'A


Ratio of fluorescence front vs rear
RATIO F/R
3
2
1
0
40
65
100
140
205
265
TIME(mins)
RATIO F/R

The approach adopted was to carry out a series of time course experiments in which sub-populations of schistosomulae from a single population of cercaria were labelled at various points after transformation. The transformation event was carried out at 4°C in order to minimise membrane development before labelling. The incubation temperature during labelling was at 37°C and storage after washing at 4°C to prevent further change in the membrane.

When this type of time course experiment was carried out a consistent pattern of labelling shown in Figure 3.2 (microscope) was achieved in many different experiments. Quantitative fluorescence microscopy on groups of parasites labelled at various time points after transformation always showed an increase in fluorescence of 2 to 5 times the initial fluorescence. This increase occurred predominantly in the first two hours and subsequently levelled off.

In the particular experiment shown in 3.2 the relationship between the observed increase of fluorescence in the membrane and the uptake of dye molecules by the parasite was investigated. In other words, did the observed increase in fluorescence monitored by quantitative fluorescence microscopy reflect an increased number of dye molecules incorporated into the membrane? Alternatively, did the number of dye molecules within the membrane remain constant but their intensity of fluorescence increase?

In experiment 3.2 samples of parasites were take from the same population and thereafter labelled at various time points after transformation. At each time point the labelled parasites were split into groups. The first group had fluorescence of its membrane measured by quantitative fluorescence microscopy. The second group had the amount of dye in its membranes measured by fluorimetry, after chloroform/methanol extraction. The results in Figure 3.2a

indicate that as the parasite appears to become brighter by fluorescence microscopy there is indeed an increase in uptake of the fluorescent lipid analogue by the parasite. This correlation between the two measured parameters is extremely close as shown in Figure 3.2b.

This was an important experiment because it established for the first time in this context a link between the physical uptake of the dye by the parasite and its subsequent level of fluorescence quantitated by microscopy. Therefore during the first three hours of development, at least, it can be said that an increase in the fluorescence of the parasite membrane labelled by aminofluorescein C18 indicates that the membrane has taken up more molecules of the dye. Due to the fact that the increase in fluorescence observed in Figure 3.2a is temporally coincident with the development of the outer bilayer the use of aminofluorescein C18 as a putative marker of membrane development was pursued further.

Figure 3.2

The correlation of aminofluorescein C18 fluorescence with uptake in the newly transformed schistosomula membrane

Schistosomula were transformed in E.M.S. at 4°C and cultured subsequently at 37°C. At various time points after transformation subpopulations of schistosomula were labelled with the dye aminofluorescein C18. This involved a 15 minute exposure of the parasites to 10 ug/ml aminofluorescein in E.M.S. Parasites were then washed three times in E.M.S. prior to quantitation. Following this the sample was split into two groups. The smaller of these was used to monitor the fluorescence of the dye in the living parasite's membrane by quantitative fluorescence microscopy (designated microscope on graph).

The majority of the parasites labelled at a given time point were subjected to chloroform/methanol (2:1) extraction overnight at 4°C. The fluorescence of this extract was subsequently measured directly by fluorimetry with excitation wavelength 488 and emission at 525 nm.

Figure 3.2b

In order to test the hypothesis that the intensity of fluorescence observed in the membrane by quantitative microscopy reflects the number of dye molecules within the membrane i.e. the uptake of dye by the membrane. The degree of correlation between the two methods of measuring fluorescence was investigated statistically. Figure 3.2b shows a scatter graph of paired data points at the various time points shown in Figure 3.2a. The coefficient of correlation was 0.97. This is a significant degree of correlation at the level of > 0.01.

Figure 3.2a

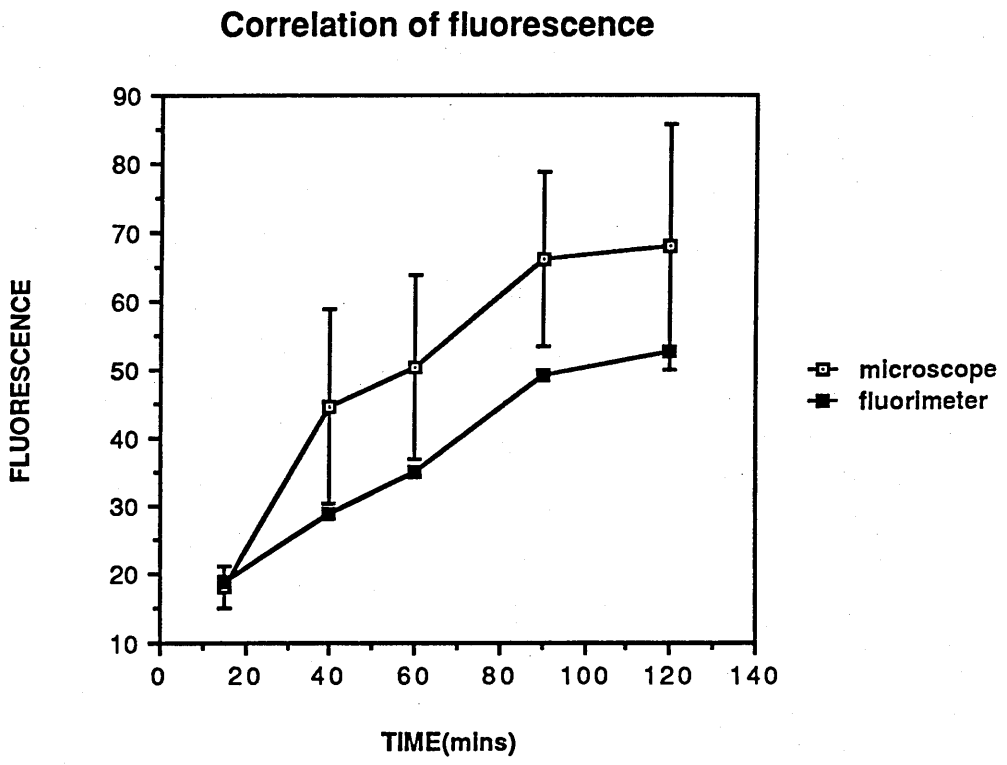
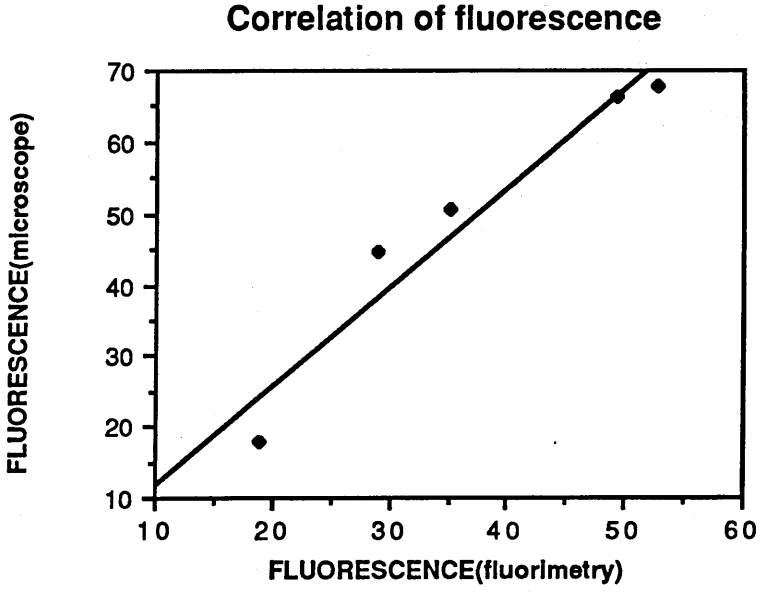


Figure 3.2b



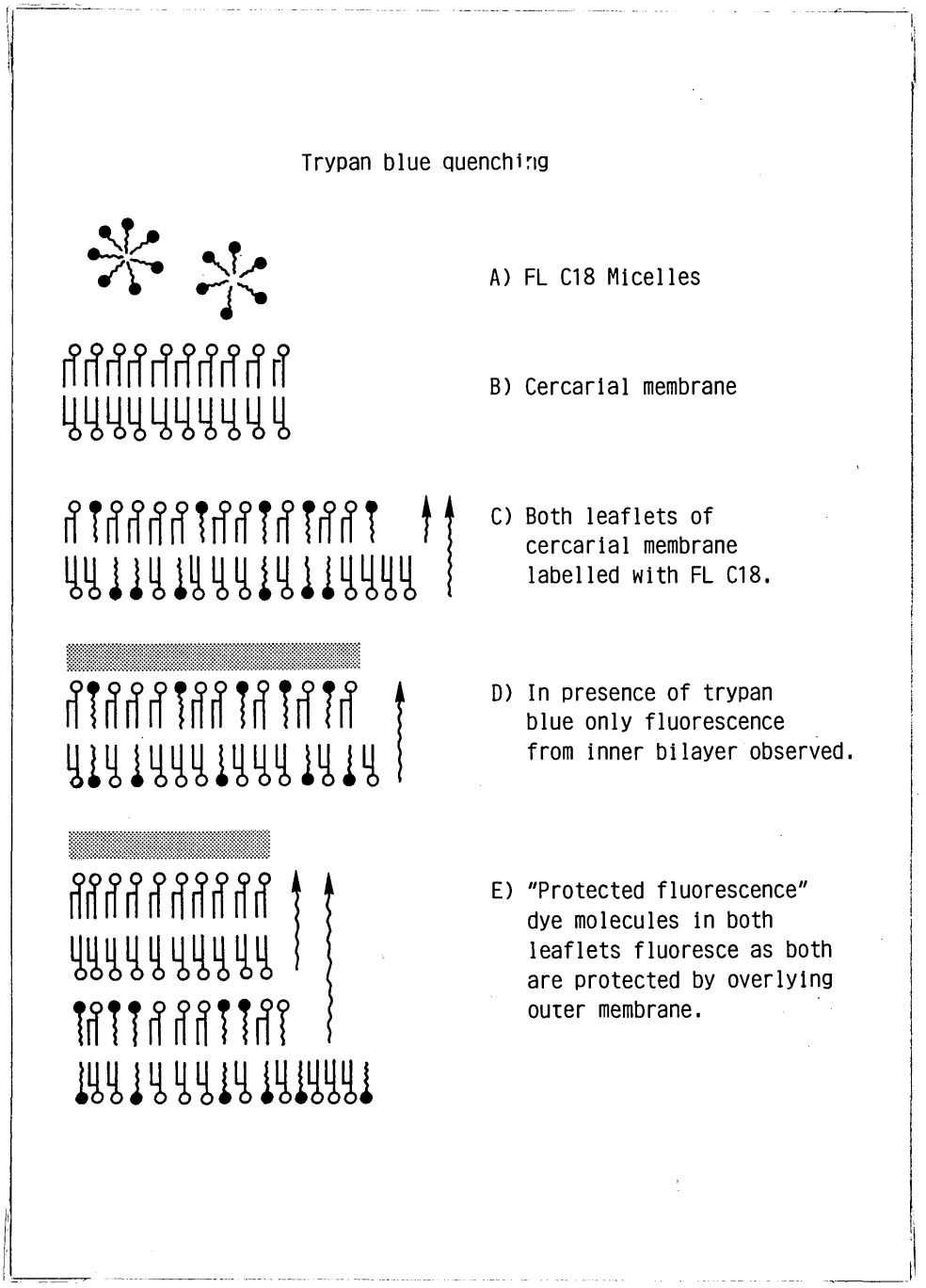
3.1.4 Fluorescence quenching experiments using Trypan Blue

The use of aminofluorescein C18 labelling as a monitor of the outer bilayer clearly implies that the dye itself locates within one of the two leaflets of the developing outer bilayer. The vertical location of the aminofluorescein C18 dye in terms of individual leaflets of the double bilayer can be determined to some extent by the technique of fluorescence quenching with Trypan Blue [Foley et al 1986].

Trypan Blue quenches the fluorescence of aminofluorescein C18 due to the phenomena of resonance energy transfer. The spectral overlap between the emission spectra of aminofluorescein C18 and the absorption spectra of Trypan Blue is such that if the two molecules are sufficiently close, less than Ro value 3.8 nm, energy (fluorescence) will be transferred from one to the other (for further explanation of resonance energy transfer see chapter 6). This transfer is exhibited as quenching of the donor (aminofluorescein C18) fluorescence. It is strongly distance dependent and falls off very rapidly above the Ro value. Thus resonance energy transfer manifest in this case as quenching of fluorescence can be used effectively as a spectroscopic ruler [Stryer 1978]. This is the case where schistosomula are surrounded by 0.25% Trypan Blue in the aqueous media, only aminofluorescein C18 chromophore moieties 3.8 nm and closer will be quenched. In practice since the aminofluorescein C18 chromophore group locates in a position parallel with the head groups of the membrane phospholipid only those moieties at the aqueous interface will be quenched. That is, only aminofluorescein C18 in the outer leaflet of the outer bilayer, the E_1 face under McLaren's terminology (see Fig 1.2) will be quenched. This technique therefore effectively determines whether the dye is in the E_1 face or one of the other three innermost leaflets (Fig 3.3).

Figure 3.3

Trypan blue quenching of fluorescence in the early schistosomula membrane.

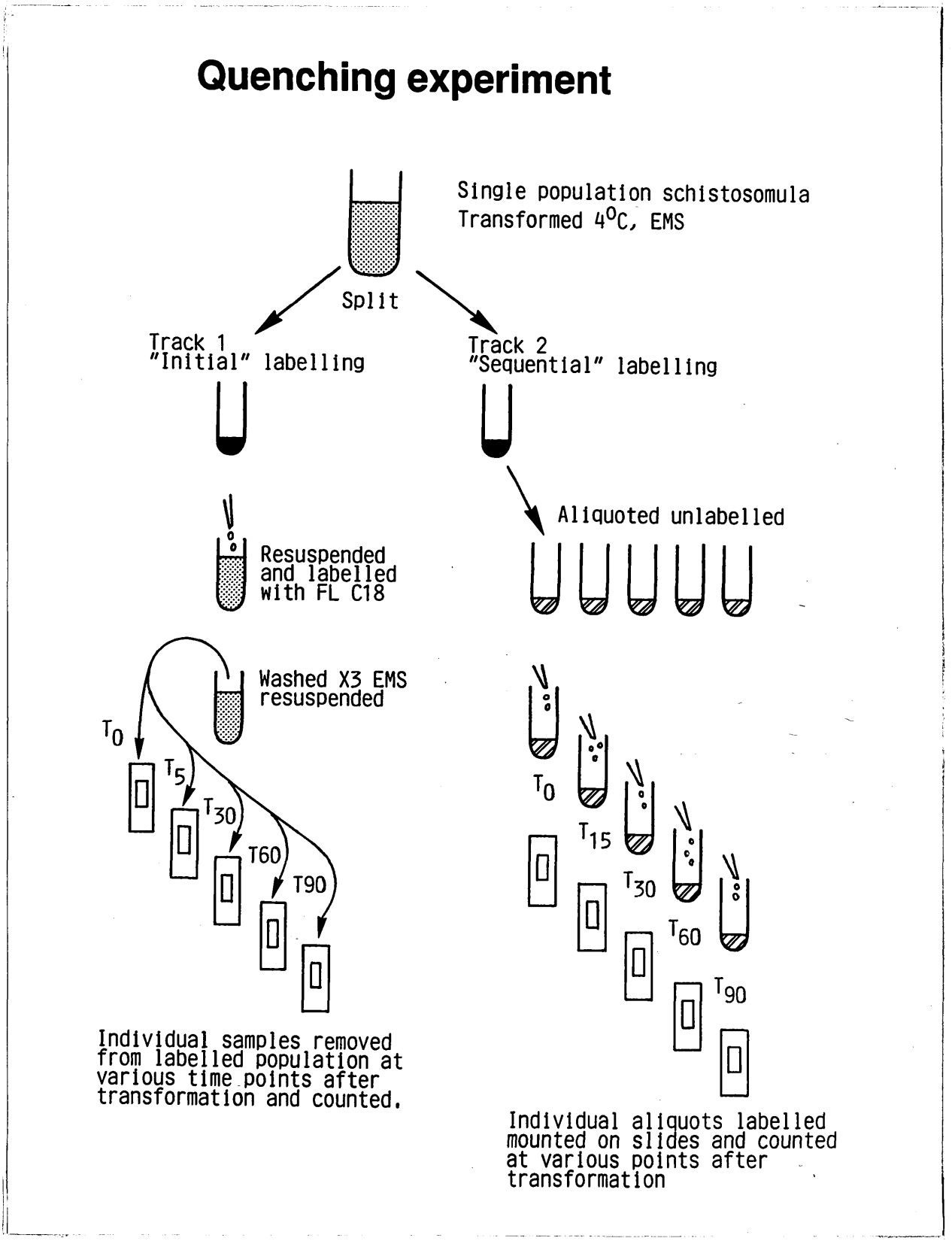


Using this technique two questions concerning the ability of aminofluorescein C18 to monitor the formation of the outer bilayer were addressed.

Firstly, where did the dye molecules responsible for the increase in fluorescence observed by microscopy in Figure 3.2a reside? It was known that in 3 hour schistosomula, lung worms and adults aminofluorescein C18 located predominantly in the E_1 face of the parasite [Foley 1986]. This implied that aminofluorescein C18 locates in the first membrane leaflet that it encounters when the label is applied externally. In the context of a monitor of outer membrane development this property would be advantageous in that the outer bilayer would be labelled as soon as it appeared.

Secondly, could the effect of the formation of the outer bilayer over and above the underlying inner bilayer be observed utilising this quenching technique? The rationale behind this approach was based on the knowledge that the outer bilayer is likely to be formed by the extrusion of the contents of membraneous bodies over and above the inner or cercarially derived membrane [McLaren & Hockley 1977]. This being the case it was reasoned that this inner bilayer should become, during the first three hours of schistosomula development, protected from the effects of membrane impermeable agents. An attempt was made to demonstrate this hypothesis using aminofluorescein C18 and Trypan Blue. The cercarial/inner membrane was labelled by labelling with aminofluorescein C18 immediately after transformation and excess dye in the aqueous media washed away. The ability of the dye Trypan Blue, which is membrane impermeable, to quench the fluorescence of this labelled inner membrane was monitored as the parasite developed (Figure 3.3).

Figure 3.4


Quenching experiment
Single population schistosomula
Transformed 4°C, EMS
Split
Track 1
"Initial" labelling
Track 2
"Sequential" labelling
Aliquoted unlabelled
Resuspended
and labelled
with FL C18
Washed X3 EMS
resuspended
T0
T5
T30
T60
T90
T0
T15
T30
T60
T90
Individual samples removed
from labelled population at
various time points after
transformation and counted.
Individual aliquots labelled
mounted on slides and counted
at various points after
transformation

Figures 3.5 - 3.7

Trypan Blue quenching experiments in early schistosomula

In the presence of 0.25% trypan blue aminofluorescein C18 molecules residing in the outermost leaflet of the schistosomula double membrane will have their fluorescence quenched. This property was exploited in two ways. Firstly, to determine the location of the dye responsible for the increase in membrane fluorescence observed by microscopy in Figure 3.2a. Secondly, to investigate the stability of the location of dye inserted into newly transformed schistosomula. Each investigation employed a slightly different labelling approach. The first investigation employed a protocol whereby samples were drawn from a main population of unlabelled parasites and labelled sequentially at various points after transformation. This group of data points is referred to as "sequential". The other track of this experiment was done in parallel with the "sequential" approach. In this parasites were labelled immediately after transformation, washed and the fluorescence of these "initially" labelled parasites observed over time.

Figures 3.5 and 3.6 represent two examples of this type of experiment. The suffix Q distinguishes readings taken in the presence of trypan blue. Each data point represents the average of twenty readings on individual parasites. Standard deviations were omitted for clarity, but these were comparable or smaller in extent than those shown in Figure 3.2.

Figure 3.7

In this experiment the "initial" track of data points is derived in the same manner as those in the other two examples. However the "continuous" data points represent readings from samples of schistosomula taken from a main population of schistosomula continuously exposed to aminofluorescein C18 dye.

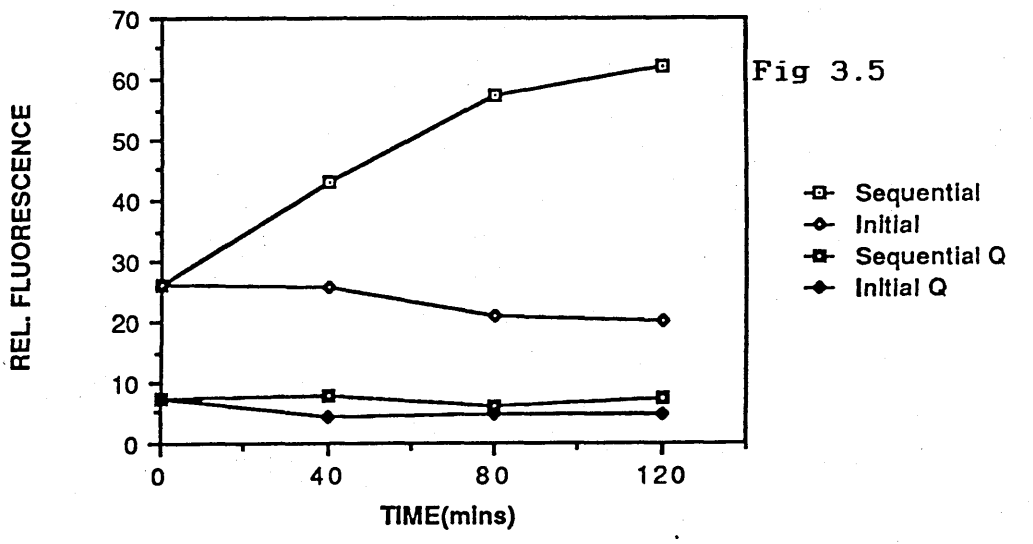


Fig 3.5

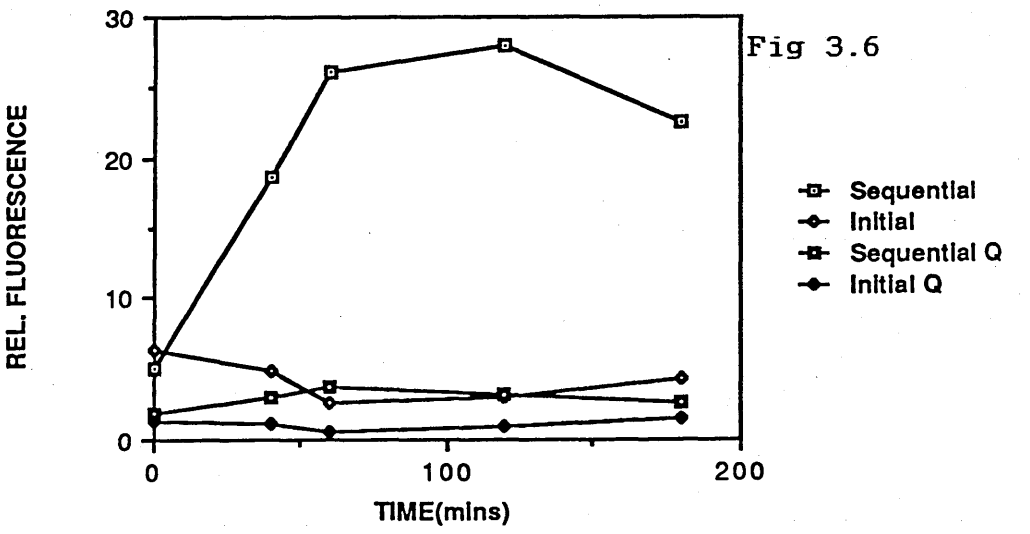


Fig 3.6

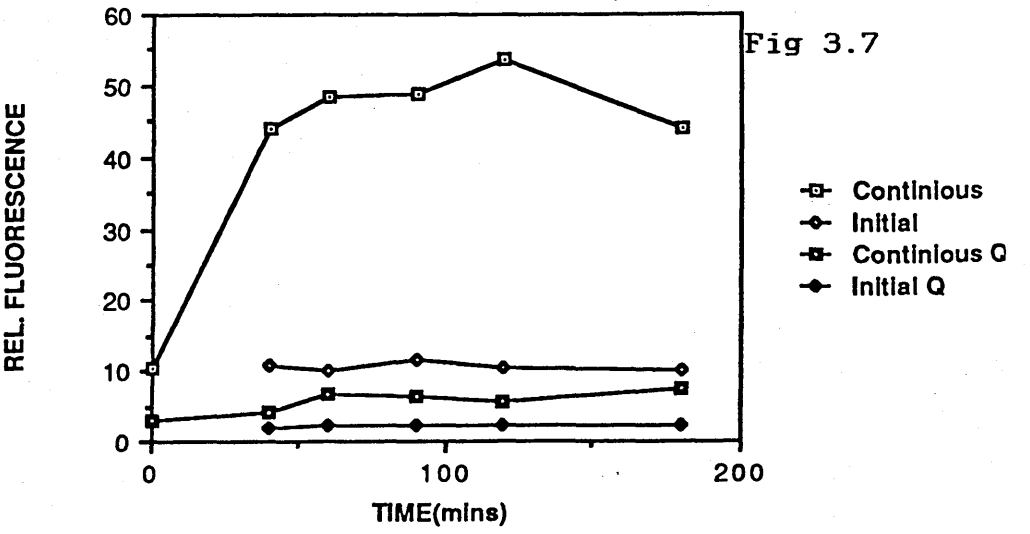


Fig 3.7

Figure 3.8

Variation in quenching of aminofluorescein C18 inserted into the schistosomula membrane at various points during its early development.

Figure 3.8a represents in tabular form the data of Figures 3.5 and 3.6 as a ratio of fluorescence after quenching with 0.25% Trypan blue divided by fluorescence prior to quenching. This is expressed as % fluorescence (remaining) after quenching and the ratio will be referred to as the "Qr" ratio in the text.

Figure 3.8b

This represents the pooled "Qr" ratios derived from the three experiments 3.5 to 3.7 plotted against time. The difference in Qr ratios was significantly different at all three time points 40, 80 and 120 minutes after transformation. The levels of significance were determined by T test and were as follows.

0	no significant difference at 0.1
40	> 0.001
80	> 0.01
120	> 0.1

Figure 3.8a

Data from quenching experiments

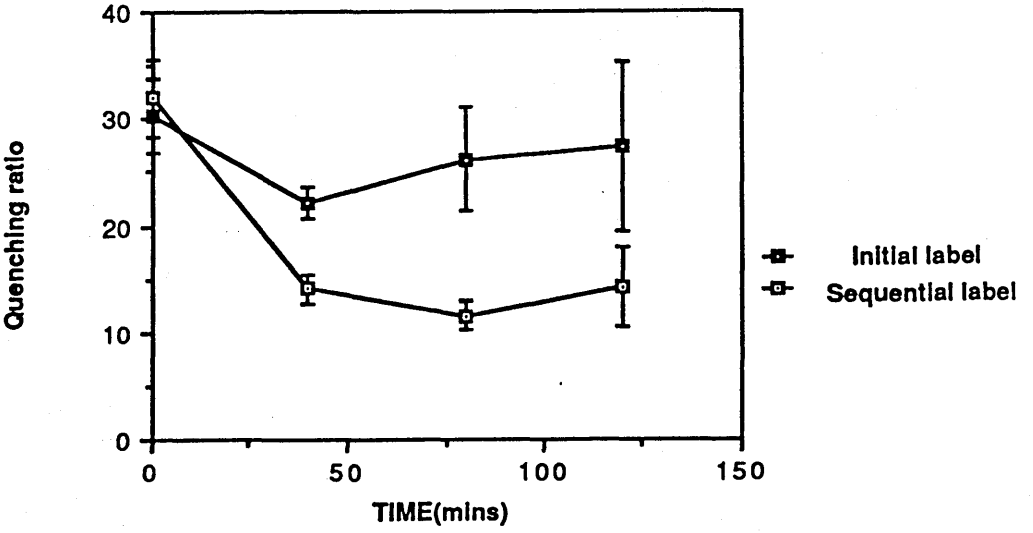
% Fluorescence after Quenching

Experiment	Time (mins)			
	0	40	80	120
3.13 Initial	30	18	22.5	22.5
3.13 Sequential	32	19	9.5	12.5

	Time (mins)			
	0	60	120	180
3.14 Initial	34	34	33	33
3.14 Sequential	34	14.2	11	11.5

Figure 3.8b

Pooled quenching data vs time



Experimentally these two questions were addressed in parallel which accounts for the "twin track" nature of the experiments shown in Figures 3.4 - 3.8. This was done in order to correlate the type of fluorescence increase observed in Figure 3.2a to changes in quenching mediated by Trypan Blue. These experiments took a "twin track" form whereby the quantitative measurements were carried out on samples derived from the same population. The application of the label was identical but its timing was different (see protocol in legend for figures page 17). Figure 3.8 presents in graphical form the pooled quenching ratio data derived from these three experiments.

3.1.5 Explanation and discussion of quenching experiments

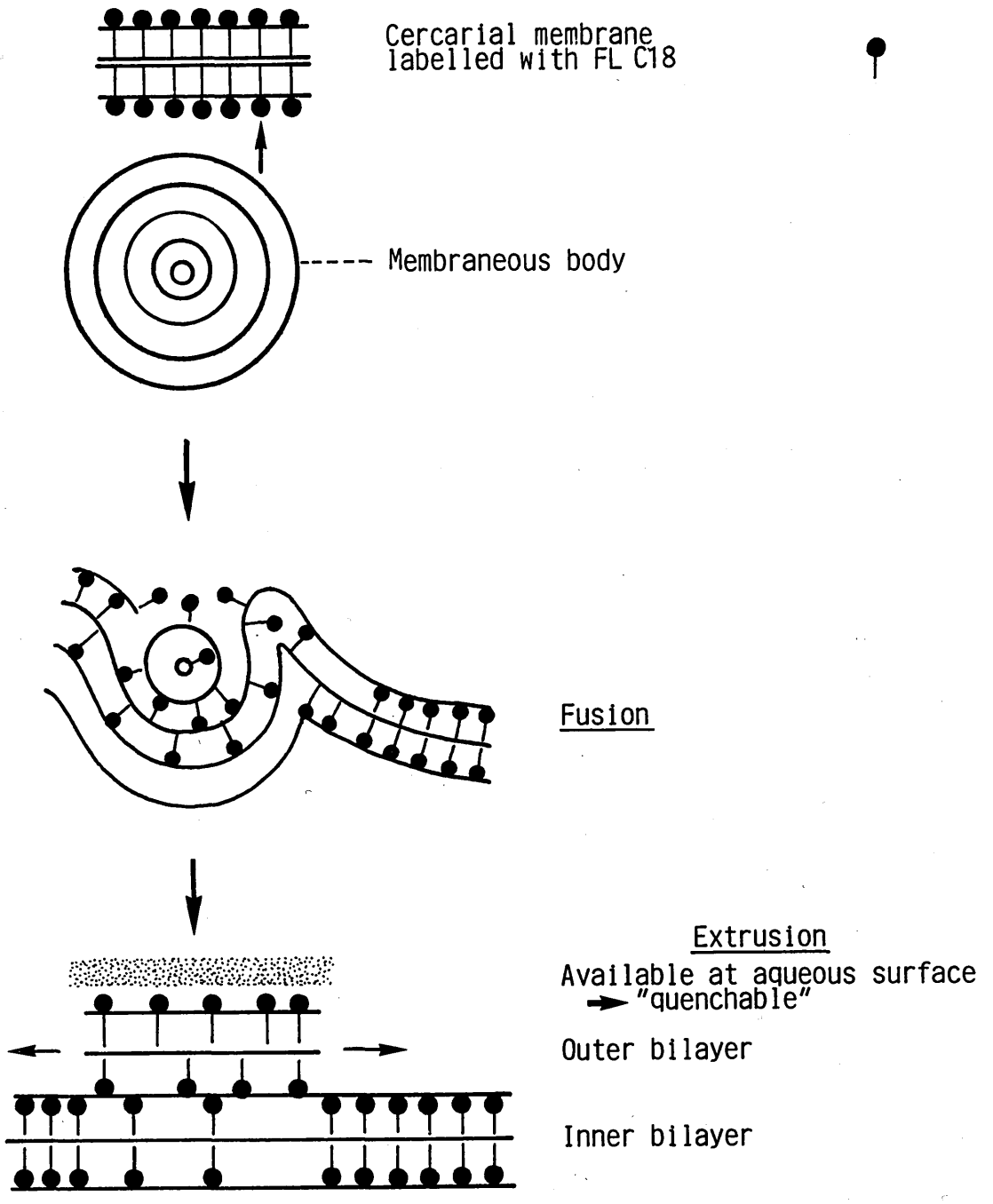
Several conclusions can be drawn from this data. Firstly, and most importantly in view of the role proposed for aminofluorescein C18, the great majority of the aminofluorescein C18 molecules responsible for the observed absolute increase in levels of fluorescence reside in the external leaflet of the schistosomula membrane. As can be seen in both the graphs and tables of figure 3.8 schistosomula labelled at 2 hours and later utilising the "sequential" labelling protocol exhibit very low quenching ratios in the order of 11-12%. This means that approximately 90% of the dye molecules reside at the membrane aqueous interface and are available for quenching. Therefore the sharp absolute increase in fluorescence levels observed in this period reflects the properties of the limiting surface membrane of the parasite. At two hours this membrane would be the outer bilayer itself. The increase observed prior to this would be most simply explained by attributing it to the formation of the outer bilayer. This hypothesis will be examined in detail in section 3.2.2.

Secondly, the pattern of change in quenching ratios of schistosomula labelled under either protocol is similar and correlates temporally with the observed increase in absolute levels of fluorescence. This change takes the form of an initial rapid fall in quenching ratio which levels off one hour after transformation. It indicates that immediately after transformation the availability of aminofluorescein C18 at the aqueous interface changes. This implies a redistribution of the dye within the various leaflets of the surface membrane or membranes. Surprisingly this was true of the parasites which were labelled immediately after transformation, washed and then monitored (initial group) as well as the "sequential" groups which represent fluorescence "snapshots" in time. Clearly the particular hypothesis that the formation of an outer bilayer over a previously labelled inner bilayer would lead to the protection of that bilayer from the effects of Trypan Blue is not fully applicable during this period.

In the "initial" group the early decline in the quenching ratio indicates that some of the dye, which at time zero immediately after transformation was most probably residing within the inner leaflet of a single "cercaria" like membrane, is by three hours now located at the surface. This could be the surface of either a single bilayer or the E_1 face of a newly formed double bilayer. This latter case is perhaps the most likely explanation since one could envisage that in the process of outer bilayer formation involving as it does the fusion of membraneous bodies with the inner bilayer that the whole structure would be in a state of flux. At the region where the membraneous body fuses with the inner bilayer and effectively bursts through it one might expect that some of the aminofluorescein C18 molecules from the inner bilayer would get caught up in the process and mixed with the extruded contents (see Figure 3.9).

Figure 3.9

Mixing of aminofluorescein C18 (FL C18) between outer and inner leaflets during early development of schistosomula.



This could explain the observed decrease in quenching ratios. If true it would have important consequences since it implies that components of the inner/cercarial derived membrane can become incorporated into, and exposed at, the surface of the schistosomula double membrane. One would expect that this would alter the immunogenicity of the surface as a whole.

Thirdly, the fact that the quenching ratios of the "sequential" and "initial" labelled groups although both declining nevertheless do not level out at the same value is worth comment. The "initial" group remains, with some variation, higher than the "sequential" groups. This implies that the original hypothesis, concerning the behaviour of an "initial" labelled inner membrane, had some validity. This is seen if you compare the distribution of dyes labelled immediately after transformation, then washed, and incubated for two hours ("initial" group) with the distribution in parasites labelled at two hours ("sequential" group) there is a clear and significant difference. Proportionally less dye molecules in the early labelled ("initial" group) reside after two hours at the surface. To some extent this could reflect the protective effect of the formation of the outer bilayer as postulated above. This difference is however only a relative effect as it is dependent on a comparison of the proportion of dye molecules at the aqueous interface, elicited by varying the application of label. This partially protective effect could be of some practical importance as a means of labelling all the membrane leaflets of the outer bilayer of three hour schistosomula with a common dye. This could allow a comparative investigation of the biophysical properties of all the different leaflets by biophysical techniques such as FRAP. To date detailed FRAP studies have only been carried out on the properties of the external leaflet of the outer bilayer in schistosomula [Foley 1986]. This has largely been due to the lack of a suitable technique for labelling the inner bilayers.

3.1.6. The effect of colchicine on the uptake of aminofluorescein C18 by developing schistosomula.

The rationale behind these experiments was that if the developmentally linked increase in aminofluorescein C18 fluorescence represented the formation of the outer bilayer, agents such as colchicine, which might be expected to block development by affecting microtubule assembly would inhibit this effect. A concentration of colchicine B of 1 mg/ml was found to be non-toxic to the parasites as judged by motility and the integrity of the outer membrane as indicated by the labelling pattern of aminofluorescein C18 itself. Damaged schistosomula can be distiguished from undamaged schistosomula by the fact that in damaged parasites the dye molecules can be clearly seen to internalise into tegumental and other internal membranes. In undamaged parasites only the surface membrane is labelled and this appears as a clear edge. As shown in the experiments shown in Figures 3.10 to 3.12 the presence of colchicine in the aqueous media reduced the extent of labelling compared with non-exposed controls. In the experiments shown the labelling process was reversed from a steady increase to a steady decline. This effect occurred in the presence or absence of serum. The effect of colchicine on the quenching ratio has not been investigated.

Figures 3.10 - 3.12

The effect of colchicine on the development of the newly transformed schistosomula membrane.

Time course experiments involving a "sequential" mode of labelling were carried out in the presence and absence of 1 mg/ml colchicine in the incubating media. Figures 3.10 and 3.11 were carried out in 10% guinea pig serum, Figure 3.12 in the presence of E.M.S. only. Colchicine is present in the incubating media from the outset that is immediately after transformation. In each case the membrane fluorescence in the presence of colchicine was significantly (> 0.05) different to membrane fluorescence in the control. This difference was apparent from approximately 30 minutes onwards.

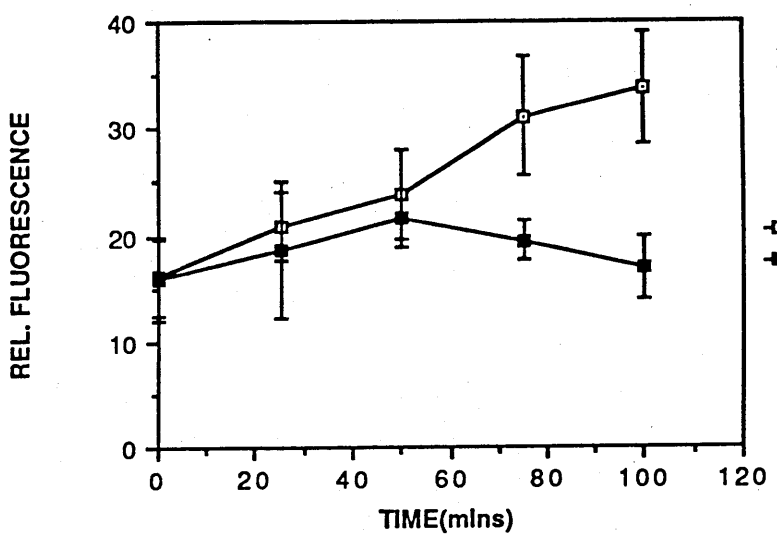


Fig 3.10

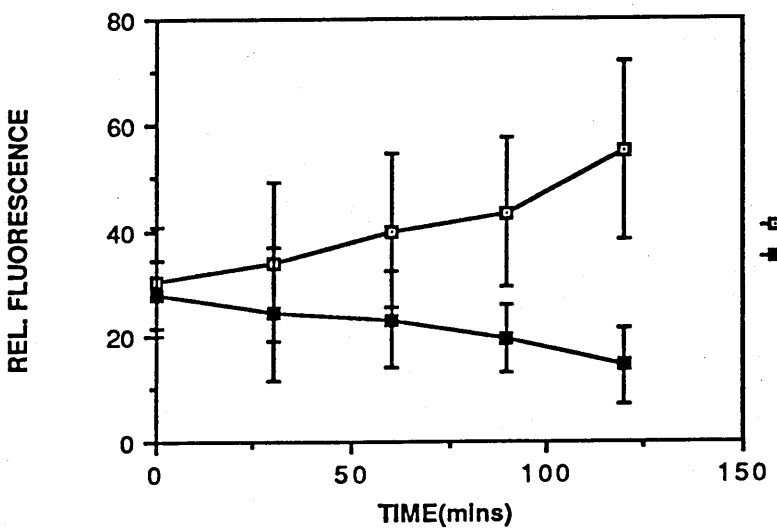


Fig 3.11

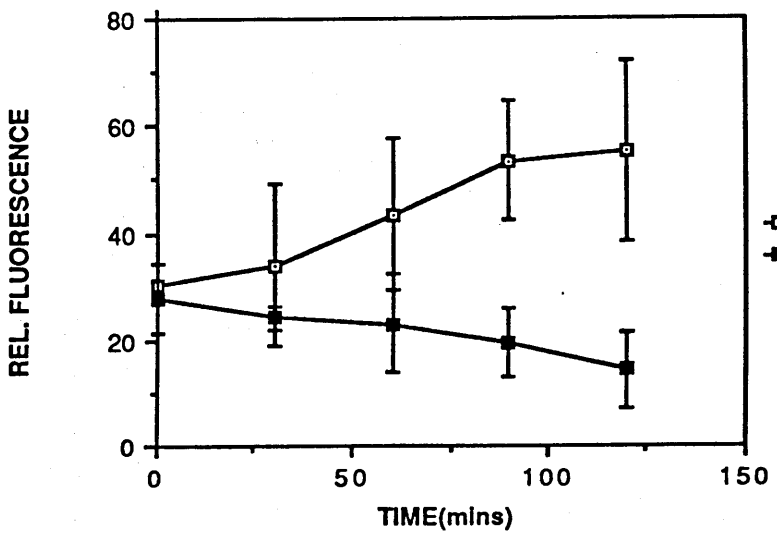


Fig 3.12

3.1.7 FRAP Experiments

The availability of FRAP apparatus allowed the differing labelling characteristics of DiI C18 and aminofluorescein C18 to be exploited to investigate the biophysics of the early development of the membrane. Of particular interest was the effect of the presence of a "cercarial" glycocalyx on the mobility of the underlying membrane. The early schistosomula at approximately 45 minutes provided a good model system since at this time the anterior part of the surface membrane is free of glycocalyx whereas its posterior portion is still covered [Samuelson & Caulfield 1985].

DiI C18 was used as a marker. This dye as described earlier exhibits extreme polarisation in labelling at 45 minutes, indicating a difference between the two regions, probably the absence of glycocalyx. Aminofluorescein, in contrast, gains access to both regions and appears to be unaffected by the presence of glycocalyx, since cercaria label well with it. The question addressed was whether there were longitudinal differences in the absolute levels of aminofluorescein C18 labelling and the percentage recovery of this label by FRAP. In this context "longitudinal" means simply "along the the length of the parasite". The figures shown in Table 3.1 indicate that there is a clear difference in the relative fluorescence between the anterior and posterior regions in the developing schistosomula. This difference develops after transformation and stabilises at a point where fluorescence in the posterior region is approximately 75% that of the anterior.

A series of experiments investigating longitudinal variation in the membrane mobility (% recovery) in schistosomula 45 minutes after transformation were also undertaken Table 3.2. Most of these experiments were done at room temperature for two reasons. Firstly, the original quantitation work presented earlier in this chapter was

Tables 3.1 and 3.2

Differential fluidity along the length of the early schistosomula membrane.

A series of experiments were carried out to investigate whether parasites at the developmental stage which exhibited polarised DiI C18 labelling showed longitudinal differences in membrane fluidity as measured by aminofluorescein C18. Groups of schistosmula were labelled with either DiI C18 or aminofluorescein C18. In each experiment listed DiI C18 labelling was strongly polarised. FRAP and fluorescence quantitation were carried out on two separate spots, either in the anterior or posterior region, on the parasite. The pairs of data from a number of different individual parasites were compiled and statistical analysis by students T test carried out. The null hypothesis was that there was no difference in either parameter of fluorescence.

Table 3.1

Table 3.1 shows data regarding the differential in fluorescence intensity that exists at 45 minutes post transformation. Figures in brackets are the standard deviations of the groups.

Table 3.2

Table 3.2 shows the data generated by FRAP in a number of experiments at either 37°C or a slightly variable "room temperature". Figures in brackets after the average percentage recovery are the standard deviations of the data groups.

Table 3.1

Exp	Time (mins)	Temperature °C	no. pairs readings	Fluorescence Front	Fluorescence Rear	Significance
1	45	25	10	48.8 (11.8)	33.5 (7.5)	< 0.05
2	45	25	10	68.0 (16.8)	48.0 (14.5)	< 0.05

Table 3.2

Exp	Time (mins)	Temperature °C	no. pairs readings	% Recovery Front	% Recovery Rear	Significance
1	45	28	13	69.6 (10.3)	60.8 (9.4)	< 0.05
2	35	25	6	40.0 (10.2)	24.1 (6.1)	< 0.001
3	35	23	10	35.4 (10.6)	24.0 (4.7)	< 0.01
4	45	37	8	82.9 (7.0)	64.0 (15.0)	< 0.002
5	60	37	7	79.1 (3.1)	73.9 (5.5)	< 0.05

done at room temperature, due to the absence of a heating stage. Some of these experiments were carried out at 37°C and the results are presented. However it was observed that the parasites themselves developed during the period that the quantitative measurements were taken on the microscope. This was clearly demonstrated by the loss of the polarised labelling of DiI C18 labelled schistosomula. To reduce this effect and to provide comparability the bulk of these experiments were done at room temperature.

These experiments demonstrated that there was a clear difference between the front and rear areas of the parasite membrane at this time point. Subsequent time course experiments sought to answer two questions: firstly did this difference persist after 45 minutes and secondly was there any correlation between the absolute levels of fluorescence emitted by aminofluorescein C18 in the membrane and the mobility of that membrane as measured by its percentage recovery by FRAP?

Figure 3.13 presents a preliminary experiment of this type. In this experiment both percentage recovery and absolute fluorescence were measured using the FRAP apparatus in the anterior and posterior areas of individual parasites, over time. The data percentage recovery and absolute fluorescence for each region on each particular parasite at the various time points were paired and the degree of correlation between them analysed. This correlation test was done in addition to an investigation, via T tests, of any contrast between the anterior and posterior measurements. The data as presented graphically in Figure 3.14 clearly shows that the anterior region takes up significantly more aminofluorescein C18 than the posterior region and that its mobility is significantly greater. A very significant degree of correlation between the two parameters was observed at 45 minutes at the anterior region and a rather less significant correlation at the

posterior region. Such significant levels of correlation did not persist to two hours after transformation, although it is noteworthy that in all cases both percentage recovery and absolute levels of fluorescence were greater at the anterior region at two hours and beyond. This time course experiment, using a different, more accurate means of measuring fluorescence gave a broadly similar result to the earlier time course experiments in that there was a general increase which began to level off at 3 hours. A similar general increase in percentage recovery values, which levelled off at or before 90 minutes, was also observed.

Figure 3.13 and 3.14

Parallel time course experiment - Fluidity and Uptake.

This experiment involved a time course experiment involving a "sequential" labelling protocol. Both percentage recovery and relative fluorescence were measured utilising the FRAP apparatus. Both types of reading were done in parallel at the front and rear regions of the parasite. This was essentially similar to experiment 3.1.

Approximately 10 paried readings, percentage recovery and relative fluorescence, from individual parasites in each data point represented. Figures 3.13a and 3.13b show the average and standard deviations of each group.

Figures 3.14a and 3.14b show scatter graphs of the two paired elements of the readings on individual parasites at the time points 45 and 120 minutes. This addressed the question of whether there was a relationship between the fluidity of the membrane and its ability to take up the dye. The degree of correlation and the level of significance is as follows:

45 minutes	Correlation Coefficient	0.82
	Degree of significance	< 0.001
120 minutes	Correlation Coefficient	0.16
	Degree of significance	> 0.05

Figure 3.13a

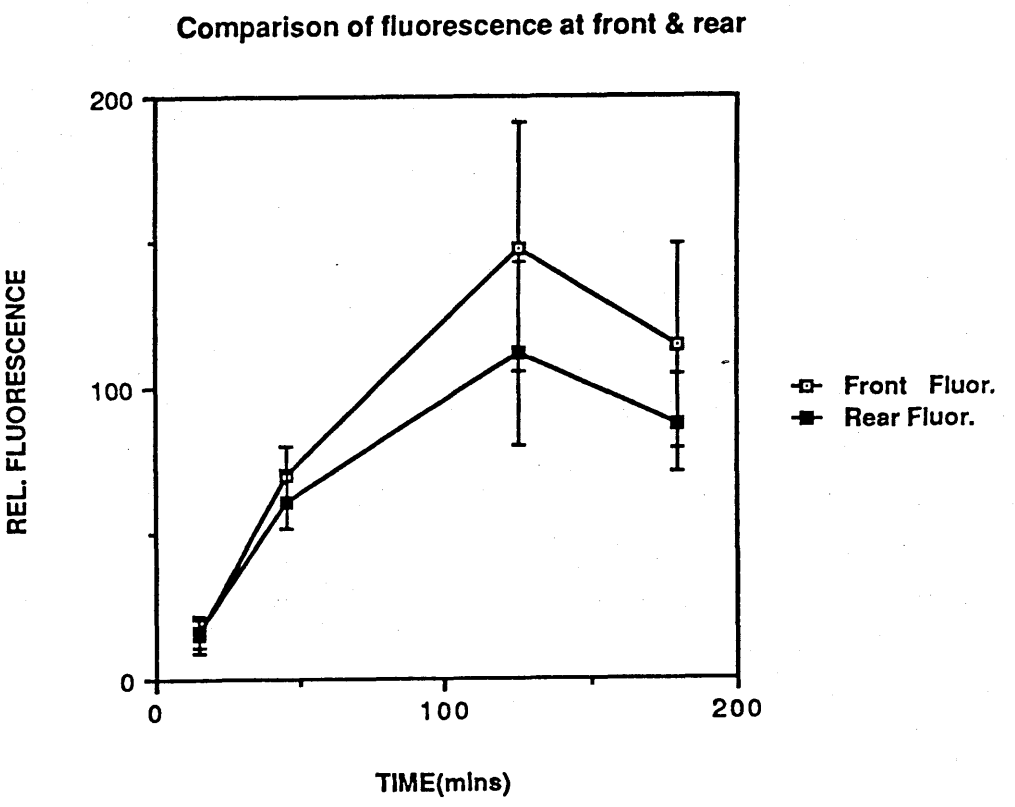


Figure 3.13b

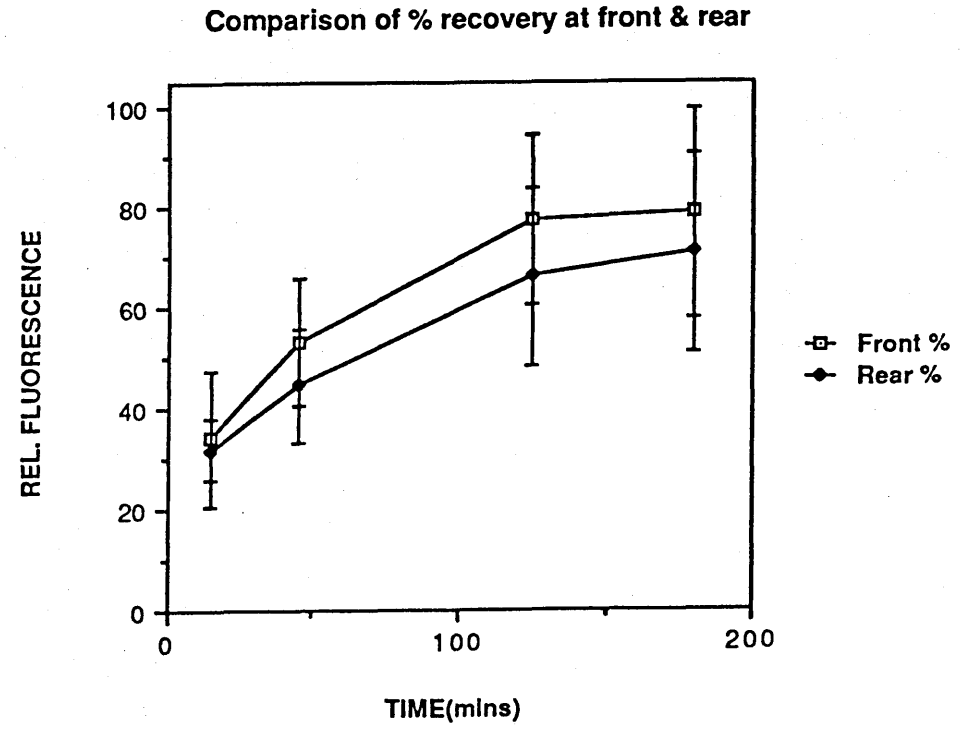


Figure 3.14a

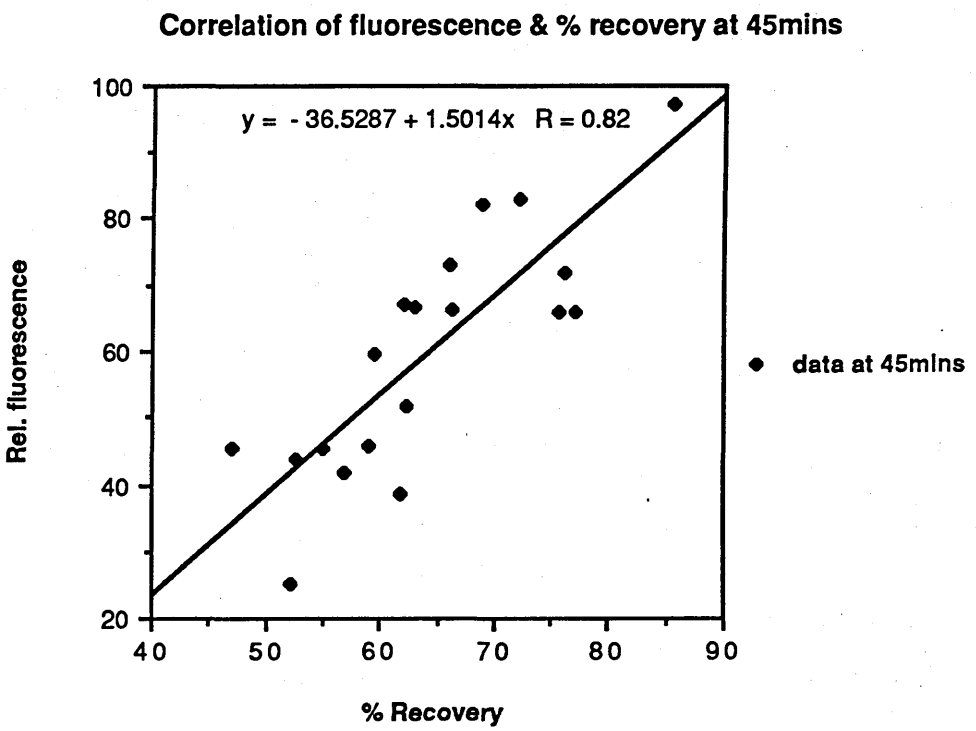
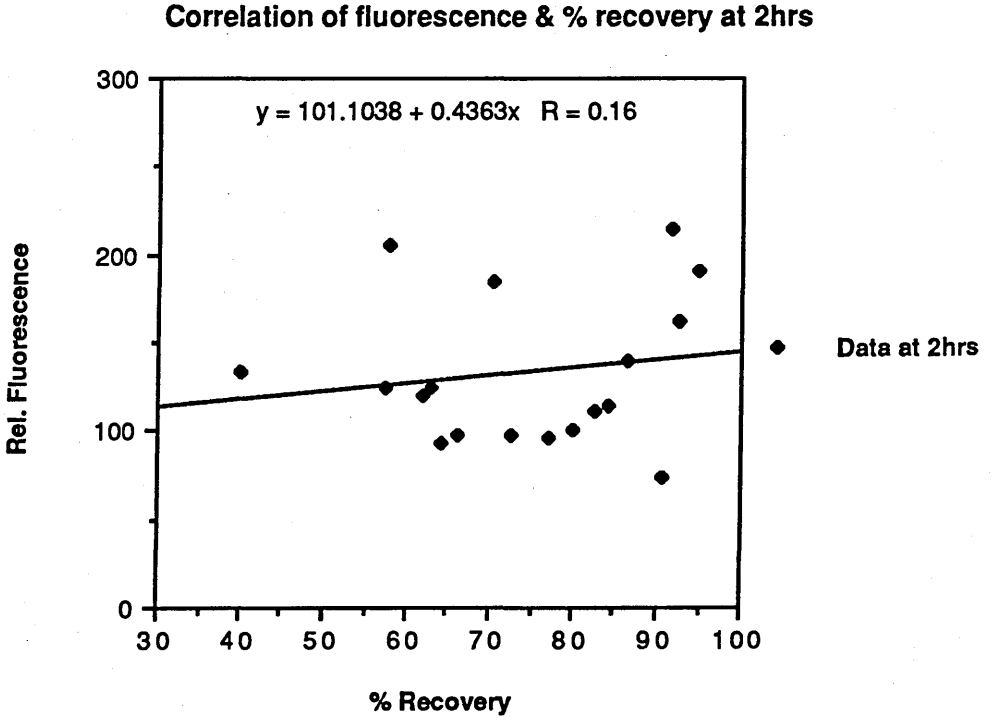


Figure 3.14b



3.2. Discussion

3.2.1 Analysis of data

The fundamental experimental observations reported in this chapter are the general increase in aminofluorescein C18 fluorescence up to 2 hours post-transformation and the observation of polarised uptake of fluorescent lipid analogues. This discussion is primarily concerned with determining what these observations represent and what they can tell us about membrane development.

The salient features of the increase in aminofluorescein C18 uptake are:

1. The increase correlates temporally with the development of the outer bilayer.
2. The analogue molecules responsible for this increase locate in an external exposed membrane leaflet.
3. The increase in absolute values of fluorescence correlates temporally with both an increase in membrane fluidity and in lipid analogue distribution in the membrane.

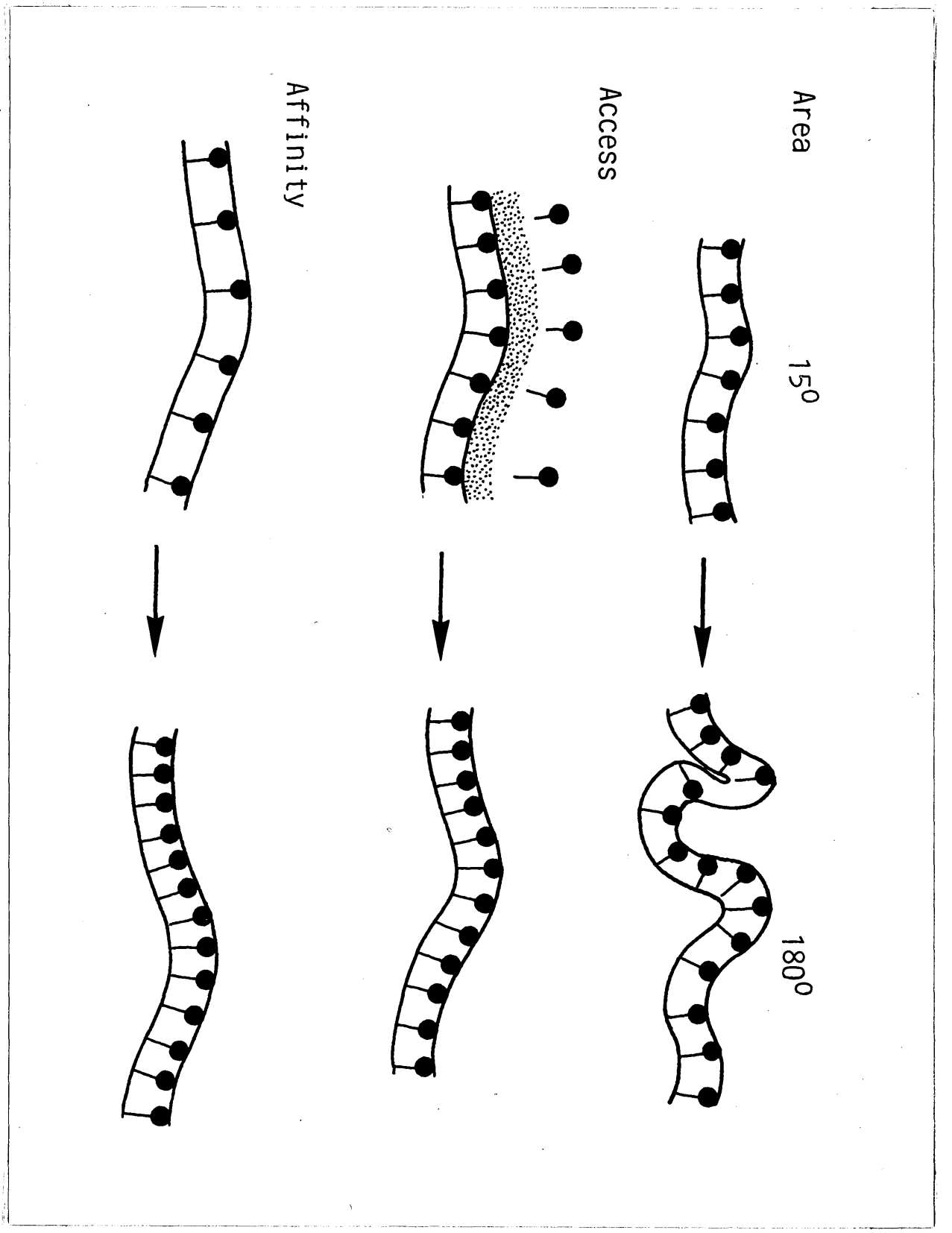
A comparison of the properties of the schistosomula membrane labelled at twenty minutes after transformation with that labelled at ninety minutes is revealing. The latter membrane is more mobile, has an apparently higher affinity for aminofluorescein C18 which distributes differently between different membrane leaflets. Proportionately more dye molecules reside in the outermost leaflet. Thus the membrane surface labelled at ninety minutes has markedly different properties from that labelled at twenty minutes. During this time period the double membrane structure becomes fully developed. [Hockley & McLaren 1977]. The most simple and likely hypothesis consistent with these observations, is that literally a different membrane, the outer bilayer, is labelled at ninety minutes after transformation. Aminofluorescein fluorescence could then be considered a marker for the outer bilayer. Consideration of the kinetics of the various

aminofluorescein C18 linked parameters measured, absolute fluorescence, percentage recovery and quenching ratio, further supports but refines this conclusion. In all cases the rate of change is maximal between 40 - 80 minutes and by ninety minutes is declining or level. This is consistent with the interpretation that changes in fluorescence reflect the formation of the outer bilayer. However consideration of these experiments shows that the fluorescent analogue is being used as a monitor of membrane change rather than as a marker for a particular structure itself. It is the relative change in the parameters that is informative rather than the particular level of fluorescence itself. Data displayed in the form of Quenching ratios and percentage recovery can be compared between experiments, but similar comparison of the absolute levels of fluorescence between groups in different experiments is difficult due to great variation within different populations of parasites.

Assuming that aminofluorescein C18 fluorescence is acting as a monitor of outer bilayer development what features associated with this development is it monitoring? This question applies particularly to the measurement of absolute fluorescence itself, since clearly percentage recovery reflects mobility and Quenching ratio distribution of dye between lipid leaflets.

Fig 3.15

Access, Area, Affinity : Membrane properties that could affect uptake of dye during early development.



3.2.2 Explanation of increased aminofluorescein C18 uptake

Three factors associated with the parasite's membrane development during this period could be advanced to explain an increase in aminofluorescein C18 uptake (Figure 3.15). These are:

a) **Access**

Some inhibition of access to the membrane by the fluorescent analogue, notably the glycocalyx is removed during this period.

b) **Area**

The surface area of membrane is increased. This could result in a higher reading being returned since the quantitatiion apparatus quantitates fluorescence from a given two-dimensional target area on the parasite. It does not take into account the extent of folding on that surface.

c) **Affinity**

The affinity of the membrane for the dye. If this were to increase during development, a given unit area of membrane, would return a higher fluorescence reading due to the presence of more chromophore groups.

Access

In this context the most important influence on the degree of access of a fluorescent lipid analogue to the membrane is the presence of a glycocalyx overlying the membrane itself. In cercaria which are totally covered by a glycocalyx, aminofluorescein C18 can gain access to the entire underlying membrane. The extent of this access appears to be uniform since there is no significant difference in aminofluoresein fluorescence between the anterior and posterior regions of cercaria and newly transformed schistosomula (Figure 3.13a). These two observations support the view that the level of aminofluorescein C18 uptake is not affected by the presence of glycocalyx.

However, at forty five minutes when there is a striking contrast between the glycocalyx free anterior schistosomula membrane and a glycocalyx rich posterior region there is a significant difference in aminofluorescein C18 uptake. This indicates that the absence of a glycocalyx may increase aminofluorescein C18 labelling via increased access. However an alternative view is that the difference in uptake is due to some biophysical difference between the membrane in the two regions. This could be due to a difference in the composition of the membrane or an "access independent" biophysical effect of the presence or absence of the glycocalyx. At forty-five minutes there is a very strong correlation between percentage recovery and absolute fluorescence. This supports the view that the difference in aminofluorescein C18 fluorescence could be due to a qualitative change in the biophysical properties of the membrane. In addition, significant differences between aminofluorescein C18 fluorescence persist at 3 hours long after the polarity in glycocalyx cover between the anterior and posterior regions has disappeared. This further supports the view that the level of aminofluorescein C18 uptake is not determined by a glycocalyx dependent access effect. Differences in aminofluorescein C18 fluorescence on different areas of the parasite membrane appear more likely to be due to some feature of the membrane other than the presence of glycocalyx. This is not the case for all fluorescent probes since DiI C18 fluorescence clearly appears to be influenced by the presence of glycocalyx.

Area

One such feature could be the surface or to be more exact the three-dimensional complexity of the membrane. However the observed changes in absolute fluorescence in the first 3 hours of development do not appear to be due to this factor. There are several lines of evidence to suggest this.

Firstly, the permanent increase in surface area during this period, that is excluding a transient contribution due to the formation of microvilli, can only be of the order of 10%. In contrast the increases in observed absolute fluorescence ranged from 200 to 500%. This estimate has been made by extrapolating the results from the most accurate measure of surface area, that published by [Samuelson, Caulfield & David 1982]. These authors calculated the change in parasite surface area over the first four days of development. Two parameters were taken into account, a volume related measure treating the parasite as a cylinder and monitoring changes in length and width. In addition a complexity factor, taking into account the extent to which the surface was convoluted by folding, was also estimated from transmission electron micrographs. These authors showed a fairly linear increase in surface area of 325% in the first four days. Extrapolating backwards for the time points presented in this study produces the figure of approximately 10% quoted earlier. This may in fact be an overestimate since the complexity factor increased with time. This complexity factor or the extent of folding is of most interest in this context. It is significant that the most important structural feature of the early schistosomula that contributes to this complexity, the spines, is relatively constant in occurrence being present in both cercaria and schistosomula. Therefore, developmental change in neither the gross surface area of the surface membrane nor the morphological surface complexity of that membrane appear to be sufficient to account for the changes in aminofluorescein C18 uptake observed during the first hours of parasite development. However one other aspect of the surface area of the parasite membrane also changes during this time. This is the surface area or percentage cover of the outer bilayer itself. If this membrane had a markedly greater affinity for the dye aminofluorescein C18 than the inner "cercarial" membrane then the observed changes in

absolute fluorescence could be explained. This "affinity" hypothesis proposes that the newly formed outer bilayer had both a different lipid composition and altered biophysical properties in comparison to the inner bilayer. These differences result in this outer bilayer having a greater affinity for the aminofluorescein C18 analogue relative to the inner bilayer, during this period. Affinity in this context means the extent to which aminofluorescein C18 molecules can intercalate or partition into a given unit area of membrane.

Affinity

The process of labelling a membrane with aminofluorescein C18 involves the partitioning into or intercalation of an aminofluorescein C18 molecule into the endogenous lipids of the membrane. Existing lipid-lipid interactions are broken and new ones formed. These are primarily ionic interactions between the charged head groups themselves, hydrogen bonding between the head groups and the aqueous media and Van der Waals interactions between the lipid tails. The collective strength of these bonds is determined by the composition of the membrane and reflected in the biophysical properties of the membrane. Generally a more disordered fluid membrane would present less of a barrier on energetic grounds to analogue intercalation. Therefore on theoretical grounds one would expect a more mobile membrane to take up more aminofluorescein C18.

The fluidity of the newly formed outer bilayer in schistosomula is clearly greater than that of the newly transformed schistosomula and cercaria as measured by FRAP. This could be due to a tethering effect of the glycocalyx on underlying membrane lipid or the particular composition of the membrane itself. This will be addressed in the following section. If one assumes that the schistosomula inner bilayer, being at least partially derived from the cercarial membrane, retains its characteristics one might expect there to be differential fluidity between the outer

and inner bilayers of the schistosomula. Such a phenomena has clearly been implicated in the adult parasite where a fluidity gradient between outer and inner monolayers has been proposed. Recent work on schistosomula has indicated that a similar situation occurs in schistosomula also in which lipid in the E_1 leaflet is mobile whereas lipid in one or more of the inner leaflets is immobile. This was done by carrying out FRAP analysis of fluorescence from an aminofluorescein C18 labelled schistosomula after Trypan blue quenching [Kusel unpublished]. There is then evidence to suggest that in the schistosomula the outer bilayer is relatively more fluid than the inner bilayer. The appearance during early development of this more fluid outer bilayer could, in theory, explain the observed increase in aminofluorescein C18 uptake during this period.

In practice, at around forty-five minutes post-transformation there is indeed a strong correlation between fluidity as measured by percentage recovery and the absolute levels of aminofluorescein C18 fluorescence. Furthermore the extent, or slope, of this correlation is sufficient to account for changes in the levels of absolute fluorescence of the order observed. These increase by a factor of 2 to 5 times in the first 3 hours of development. Overall fluidity increases from around 20% to around 70%. At forty-five minutes a 10% increase in percentage recovery results in an increase in absolute fluorescence of 60-70%. This particular time point is highly significant because it is representative of the period in which production of outer bilayer is maximal. At later time points (2hrs) this correlation is markedly reduced (Figure 3.14b). However the biophysical situation is entirely different at this point, the double bilayer structure is developing rather than forming. One of the ways in which it does develop is by the appearance of intramembraneous particles in the E_1 and P_1 faces as observed by freeze fracture E.M. [Mclaren, 1980]. This developmental process after

formation is likely to result in the stabilisation of the membrane lipids due to the stabilising effects of protein-lipid interactions . This may well account for the loss of correlation between the two parameters observed at ninety minutes. Thus in a newly formed fluid and disordered membrane aminofluorescein C18 uptake behaves as though in an ideal situation and intercalates according to the entropy of the membrane. As this is reduced due to the ordering effect of inserted proteins the degree to which intercalation is dependent on lipids alone, as reflected in percentage recovery values, declines and the association is lost.

In summary we have observed consistently an increase in the fluorescence of schistosomula labelled with aminofluorescein C18, which together with changes in the mobility and vertical distribution of the dye correlates temporally with the formation of the outer bilayer. Neither the effect of residual glycocalyx nor changes in the surface area and morphology appear to be sufficient to explain the extent of this increased fluorescence. There is evidence to suggest that the outer bilayer of the schistosomula may be more fluid than the inner bilayer. Partition theory predicts that this should result in a greater uptake of analogue. At a critical period of development we observed in this outer bilayer a strong association between fluidity and aminofluorescein C18 fluorescence, sufficient to explain the observed increases in fluorescence. Thus it is proposed that aminofluorescein C18 can be exploited to directly monitor the formation of the outer bilayer of the early schistosomula double membrane structure. This is done using quantitative fluorescence microscopy.

3.2.3 Colchicine Experiments

Clearly, applying the analysis outlined above implies that the effect of colchicine in the experiments shown in Figures 3.10 - 12 is to inhibit the formation of the outer bilayer. Microtubules are known to exist within the tegument where they form a ring around the thin neck of cytoplasm which joins the subtegumental cell bodies, containing cell nuclei, with the bulk of the sycitial cytoplasm of the tegument. In the context of outer bilayer production this is significant since the subtegumental bodies produce the lipid, in the form of membraneous bodies, which ultimately forms the outer bilayer. This process begins with production of membraneous bodies by the subtegumental cell bodies, followed by transport via the microtubule lined cytoplasmic necks to the tegument where they fuse with the inner/cercarial membrane and extrude their contents which flow over the inner bilayer to eventually form the outer bilayer [Hockley 1973]. Colchicine could inhibit this process by disrupting the microtubules surrounding the connecting cytoplasmic neck. This would result in the "choking" off of the site of production from the site of fusion or consumption of membraneous bodies. The net effect would be a relative decline in outer bilayer cover in colchicine treated parasites compared to controls.

This is a reasonable explanation of events because it is not entirely based on the fluorescence observations alone. It is consistent with what is known of the pharmacological effects of the drug and the "cellular" physiology of the parasite's tegument. This interpretation could be independently confirmed by E.M. studies. Several clear predictions can be made. One would expect to see, in colchicine treated parasites, a loss of microtubules and an accumulation of membraneous bodies in sectioned transmission micrographs. Furthermore the action of this drug may also independently confirm the basic premise of

aminofluorescein C18 as a monitor of outer bilayer formation. In addition to the above E.M. predictions one would expect to see a reduction in the occurrence of heptalaminate membrane structures in the treated group.

The colchicine based experiments represent preliminary experiments to exploit this technique. A number of similar projects are planned. These included monitoring the effect of "cercarial" irradiation on the subsequent formation of the outer bilayer. Other drugs which may also pharmacologically attenuate the parasite either at the D.N.A. level actinomycin D, or by inhibition of phospholipid synthesis, fenfluramine [Brindley et al 1978] are also being investigated. The main advantages of this technique are that it is relatively quick and simple, compared with E.M. and it can be done on living parasites. In practice its greatest potential is as a quick screening technique prior to a follow up investigation by electron microscopy or controlled animal infections.

Some representative data from this type of approach is shown in Figure 3.16. This implies a requirement for de novo phospholipid synthesis in the production of the outer bilayer and also that formation of the outer bilayer is not affected by irradiation at these levels. Irradiation at this level will inhibit protein synthesis by the schistosomula up to 5 days post transformation [Wales unpublished]. This indicates that enzymes sufficient to meet the newly transformed schistosomula's requirement for phospholipid synthesis exist in the cercaria. How their activity interacts with the membraneous bodies which contribute to outer membrane formation remains to be determined. One possibility is that the flow across the tegument to their site of fusion at the membrane reported by McLaren & Hockley 1976 is dependent on de novo synthesis in the subtegumental cell bodies to "drive" membrane formation to completion.

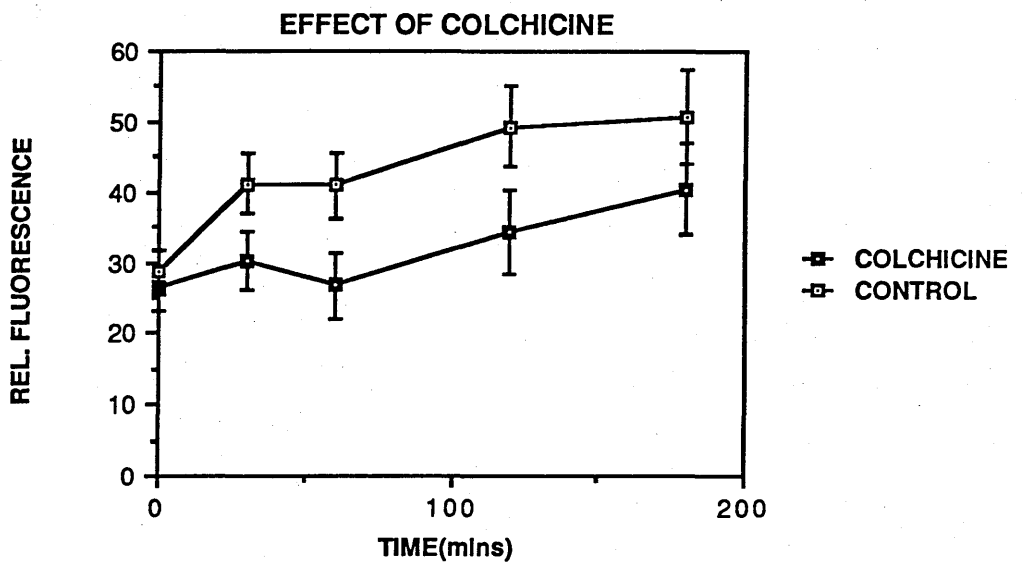
Figure 3.16

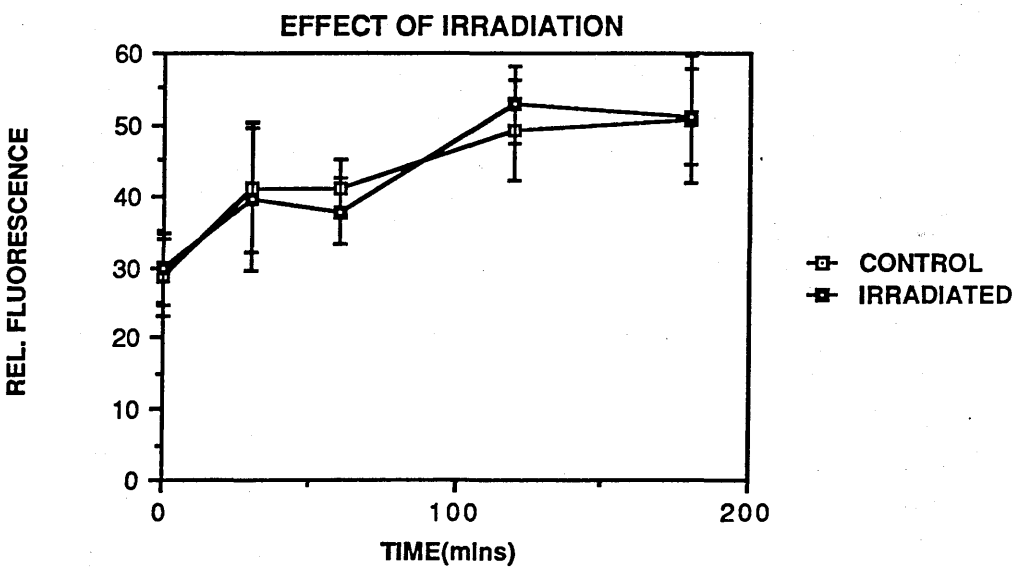
Multiple assay of membrane development using aminofluorescein C18

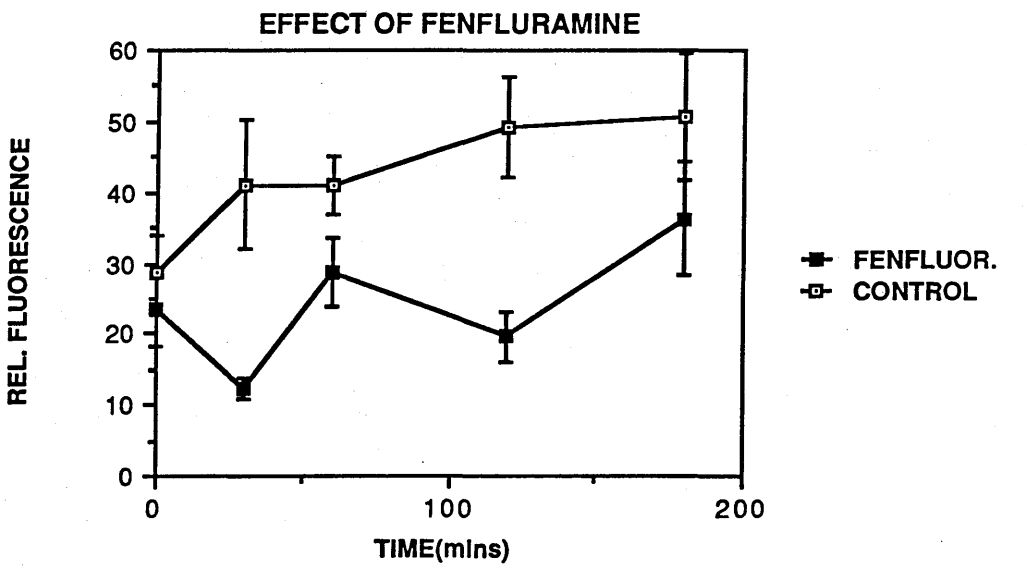
In this experiment all groups of schistosomula were derived from a common population of cercaria. This was split into 4 groups.

1. Control: Cultured in EMS only.
2. Colchicine: Cultured in the presence of 1 mg/ml colchicine.
3. Irradiated: Exposed to U.V. irradiation (400 mW mins/cm^2).
4. Fenfluramine: Cultured in the presence of 0.5 mM Fenfluramine.

Cercaria were transformed at 4°C and schistosomula cultured in EMS at 37°C. At various time points after transformation aliquots of schistosomula were removed, labelled for 15 minutes at 37°C with 10 ug/ml aminofluorescein C18, washed 3 times in EMS and mounted for fluorescence quantitation. Each data point opposite represents the mean and standard deviation quantitation of 20 parasites.


EFFECT OF COLCHICINE
REL. FLUORESCENCE
60
50
40
30
20
10
0
0
100
200
TIME(mins)
COLCHICINE
CONTROL


EFFECT OF IRRADIATION
REL. FLUORESCENCE
60
50
40
30
20
10
0
0
100
200
TIME(mins)
CONTROL
IRRADIATED


EFFECT OF FENFLURAMINE
REL. FLUORESCENCE
60
50
40
30
20
10
0
0
100
200
TIME(mins)
FENFLUOR.
CONTROL

3.2.4 Polarised labelling with DiI C18

The same considerations of access, surface area and affinity are valid when seeking to explain the nature of DiI C18 labelling. In this case the factor most clearly implicated is access or rather an inhibition of access due to the glycocalyx. In glycocalyx covered cercaria there is no uptake, in glycocalyx free 3 hour schistosomula there is an overall uniform uptake. Interestingly T spiralis which resemble cercaria in that their membrane is covered by a thick glycocalyx show an identical pattern or selectivity of labelling well with aminofluorescein C18 but not with DiI C18 [Kennedy et al 1987].

The most interesting facet of DiI C18 labelling is in its morphologically polarised pattern at forty-five minutes. This represents an intermediate pattern between a uniform absence of labelling in cercaria to its uniform presence in schistosomula. This appears to proceed in a wave down the length of the parasite from anterior to posterior. This is clearly a developmentally linked phenomenon which implies that development, is not uniform in this respect, longitudinally over the parasite surface. Such longitudinal differences in development and biophysical structure have been reported in both schistosomula [Samuelson and Caulfield 1985] and adult schistosomula [Foley 1986]. The first authors cited reported the occurrence of a "polarised" reticular pattern of labelling with fluorescent ConA at forty-five minutes post-transformation which is very similar to the DiI C18 pattern reported here. Similarly, Foley has reported polarised fluorescence in the anterior region of prelabelled schistosomula immediately after transformation. In this study parallels were drawn between the transformation event in cercaria and fertilisation in Sea Urchin eggs [Freidus et al 1984]. In both cases highly localised changes in lipid order from a gel state to a liquid crystalline one were observed after a developmental signal. Further work

in adult schistosomes revealed differences in percentage recovery by FRAP along the length of the parasite. Longitudinal differences along the length of the parasite are therefore not unknown.

The experiment shown in Figure 3.13 was an attempt to exploit the polarised pattern of labelling of DiI C18 at forty-five minutes to determine whether the presence of a glycocalyx affected the biophysical properties of the membrane underneath. This was done by labelling with the glycocalyx independent dye aminofluorescein C18 in parallel with DiI C18. Clear parallels were observed at the time of greatest polarisation in DiI C18 labelling, T_{45} minutes, there were significant differences in percentage recovery and absolute fluorescence between the anterior and posterior regions of membrane. As the extent of polarised labelling declined so did the difference in percentage recovery but not relative fluorescence. Generally the anterior portion was more mobile and took up more aminofluorescein C18 than the posterior region even if these differences were not stastistically significant. The implication of the pattern of DiI C18 labelling and aminofluorescein C18 measurements is that the anterior region is more dynamic and developmentally advanced. At forty-five minutes therefore, the membrane of the posterior region which is glycocalyx covered, is significantly less mobile than the glycocalyx free membrane on the same parasite. This implies that the presence of an overlying glycocalyx restrains the mobility of the lipid molecules in the membrane underneath it.

This "restraining" effect of the glycocalyx could explain both the decreased percentage recovery of aminofluorescein C18 and its decreased level of aminofluorescein C18 uptake in the posterior region. In addition its localised presence could result in a polarised pattern of label. The polarised pattern of DiI C18 labelling could be the result of either the differential retention of glycocalyx on a membrane developing uniformly over the parasite surface. Alternatively it could represent relatively accelerated development of the double bilayer structure on the anterior region. These two possibilities represent two extreme views but have important implications since the former implies a uniform "global" development of the double membrane structure and the latter a localised accelerated development.

Previous E.M. studies show transmission E.M. micrographs of heptalaminate and trilaminate membrane structures co-existing in the plane of section [Hockley & McLaren 1973]. Moreover Samuelson and Caulfield [1985] have also shown both types of membrane side by side but in addition have shown the retention of at least residual glycocalyx over a fully formed double membrane structure. These observations showing localised Con A labelling at the anterior region of the schistosomula is in the light of this not convincing proof that there are more "schistosomula" outer membrane antigens in this region. Such antigens could be present but masked by residual glycocalyx in the posterior region. The question of the rate at which the double membrane structure develops across different morphological regions of the schistosomula during early development awaits further clarification, particularly with respect to possible membrane development "underneath" the glycocalyx shortly after transformation.

The balance of evidence from fluorescence based studies of the lipid in particular [Foley, 1988] but also this DiI C18 based one indicate that membrane development may be relatively accelerated in the anterior. This makes sense biologically since it is this region that is first exposed to the host at the skin and possibly to changes within the host such as penetration of the vascular endothelium. Uptake of skin derived antigens also exhibits a polarised pattern which is present which is present at two hours post transformation [Smith, McQueen & Kusel 1977]. This may indicate a possible biological function of differential membrane development along the length of the early schistosomulum. This could be a specialisation of the membrane that allows efficient uptake of host antigens initially at the anterior region which later spread more evenly around the parasite surface as the parasite develops.

Until the question of whether double membrane development takes place uniformly or differentially across the parasite surface is answered the question of what the presence of DiI C18 labelling represents cannot be settled. Clearly the possibility exists that it too can be used in these circumstances as a marker of double bilayer production.

3.2.5 Summary of discussion

In summary, DiI C18 and aminofluorescein C18 longitudinal studies on the nature of the parasite surface have revealed clear differences. The anterior region appears relatively more dynamic exhibiting a greater ability to take up aminofluorescein C18 which is more mobile. These differences are greatest during early development indicating either a relatively accelerated development of the double membrane in these areas or a restraining effect of residual glycocalyx in the posterior regions. They do persist at three hours indicating that as in the adult more permanent stable differences exist in the membrane along the length of the schistosomula.

CHAPTER 4

THE INTERACTION OF LDL WITH S. MANSONI

4.1.1. Introduction

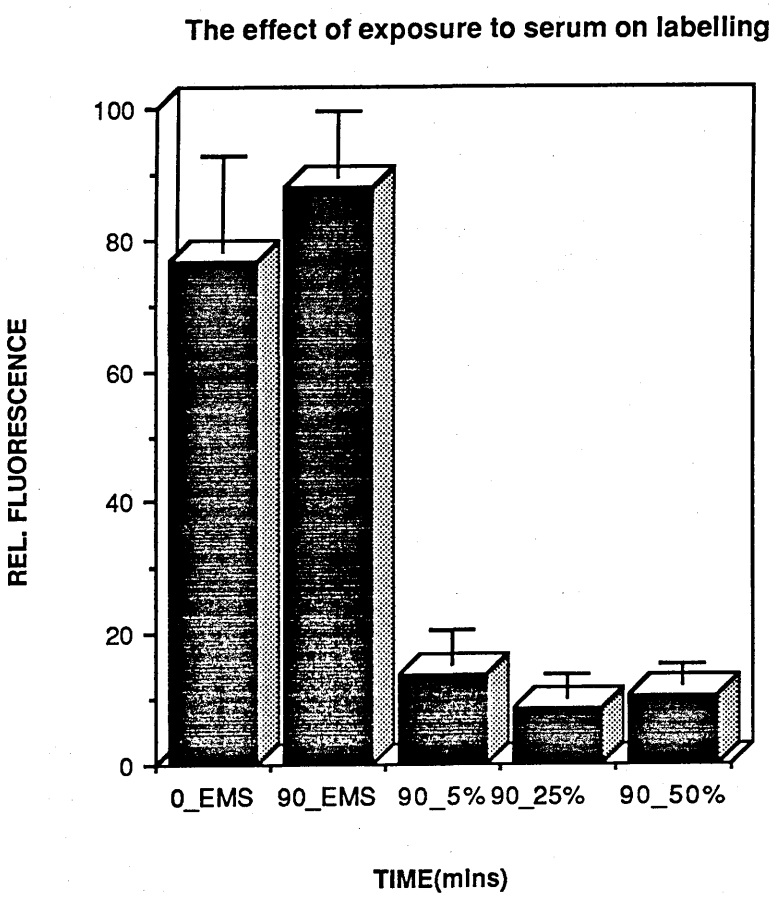
During the course of the experiments outlined in the previous chapter it became apparent that the presence of serum could affect the extent of labelling of the schistosomula membrane by aminofluorescein C18. This effect could have come about either by a change induced in the membrane by the serum or a change in the efficacy of the labelling process itself. This raised the possibility that serum components may be interacting with the membrane. This line of thought was also supported by the observation that if aminofluorescein C18 labelled parasites were exposed to even low concentrations of human serum they lost their label from the membrane to some serum component (Fig 4.1). It was thought that the most likely and interesting possibility was that such aminofluorescein loss could have been to serum lipoproteins. There have been a series of reports by Rumjanek and co-workers suggesting that lipoproteins particularly LDL interact with the membrane [Rumjanek & McLaren 1981 ; Rumjanek,Mclaren & Smithers 1983]. The other serum component most likely to account for the observed loss of dye from the parasite membrane, serum albumin, has in contrast generated reports which indicate a lack of interaction with the parasite membrane [Sher, Hall & Vadas 1978].

Figure 4.1

Loss of Aminofluorescein C18 to serum

3 hr schistosomula were labelled with Aminofluorescein C18 in EMS for 30 minutes at 37°C. They were then washed twice in EMS at 37°C prior to quantitation by MPV-Compact. One group was counted immediately after labelling (T_0) whereas four other samples were incubated for 90 minutes in EMS, plus 5, 25, or 50% whole human serum. These groups had their average fluorescence quantitated at ninety minutes.

Twenty parasites in each group were counted and the results presented opposite as mean of group and standard deviation.


The effect of exposure to serum on labelling
REL. FLUORESCENCE
100
80
60
40
20
0
0_EMS
90_EMS
90_5%
90_25%
90_50%
TIME(mins)

These fluorescence based observations were the initial experimental observation which prompted an investigation into the interaction between LDL and the surface membrane of S. mansoni which is described in this chapter.
The theoretical justification, based on reports in the literature, for such an investigation is summarised below.

a) Meyer et al 1970 have shown that the parasite is unable to synthesise its own cholesterol and is therefore dependent on cholesterol derived from the bloodstream. Cholesterol metabolism and transport in the bloodstream is controlled by lipoproteins and enzymes associated with them [Cryer 1983].
b) Rumjanek has shown that acquisition of the contents of lipoproteins namely cholesterol and cholesterol esters from serum occurs and significantly affects the composition and immunological resistance of the membrane [Rumjanek & McLaren 1981].
c) Such acquisition is correlated with the induction of a specific LDL receptor[Rumjanek et al 1985]. This raised the possibility that its biological effect could be demonstrated on living parasites in vitro.
d) Although strongly implied in Rumjanek's work a direct demonstration of an LDL parasite membrane interaction in living parasites has not been shown.
e) The mechanism of transfer of the contents of this proposed LDL membrane interaction is unknown and the possibility existed that a process analogous to receptor mediated endocytosis in cells was occurring. This, if it occurred, would profoundly alter existing perceptions of the structure and function of the outer membrane of S. mansoni

4.1.2 Fluorescent labelled LDL experiments

Figure 4.1 showed loss of aminofluorescein C18 dye to unknown serum components. Rather than characterise the nature and final destination(s) of this loss, the interaction of analogue labelled LDL with the membrane was investigated directly.

The first experiments in this investigation followed on naturally from the fluorescence experiments and involved labelling the LDL itself with fluorescent lipid analogues and observing whether dye was transported to the parasite membrane. The first experiment of this type used DiI C18 labelled LDL in a competition assay. The extent to which a given amount of DiI C18 labelled LDL could label the parasite membrane was measured in the presence of increasing amounts of unlabelled LDL. The extent of labelling of the different schistosomula samples derived from the same population exhibited a linear relationship with the amount of competing unlabelled LDL (Figure 4.2). Also shown in the legend of this figure is data pertaining to the extent of labelling using the standard "ethanolic" labelling procedure. This solution was effectively (x50) greater in terms of concentration of DiI C18 added to the sample than the concentration applied via DiI C18-LDL. However the extent of labelling was comparable indicating that the efficacy of labelling may be increased by applying dye "carried" by LDL molecules. More importantly the linear nature of the competition with unlabelled LDL indicates that LDL is interacting with the membrane and transferring dye.

An alternative approach was employed using aminofluorescein C18 labelled LDL. Aminofluorescein C18 is known to become incorporated into LDL molecules near their surface [Sklar et al 1980]. Samples of schistosomula from a given population were subjected to increasing concentrations of aminofluorescein C18 labelled LDL (AF-LDL). This results in an apparently saturable increase in relative fluorescence. The extent of this increase could be modulated by pre-exposure of the parasites to human serum dialysable components. This has been reported to be sufficient stimulus to induce expression of an LDL receptor on schistosomula [Rumjanek, McLaren & Smithers 1983]. Schistosomula exposed in such a way showed increased levels of aminofluorescein C18 compared with controls (Fig 4.3).

Figure 4.2

DiI C18 labelled LDL competition experiment.

In this experiment LDL pre-labelled with DiI C18 was used to label 3 hr schistosomula. The LDL itself was labelled by the addition of 10 ug of DiI C18 in 10 ml ethanol (1ug/ml) to 1 ml of purified LDL (5 ug/ml protein). The assay was to test whether the labelling of this DiI-LDL could be competed out by unlabelled LDL. To this end the extent of labelling by a given amount of DiI-LDL was monitored in the prescence of increasing amounts of unlabelled LDL. The total volume of media in which the groups of schistosomula were labelled was kept fixed at 1 ml. This is expressed on Figure 4.2 as % competition whereby

100% represents	5 ml DiI-LDL	+	0 ml unlabelled LDL
50% represents	5 ml DiI-LDL	+	5 ml unlabelled LDL
33% represents	5 ml DiI-LDL	+	10 ml unlabelled LDL
16% represents	5 ml DiI-LDL	+	25 ml unlabelled LDL
10% represents	5 ml DiI-LDL	+	45 ml unlabelled LDL

After exposure to label the schistosomula parasites were washed twice in EMS. Data points represent mean and standard deviation of 30 parasites at each time point.

Effect of competition on LDL-DiI C18 labelling

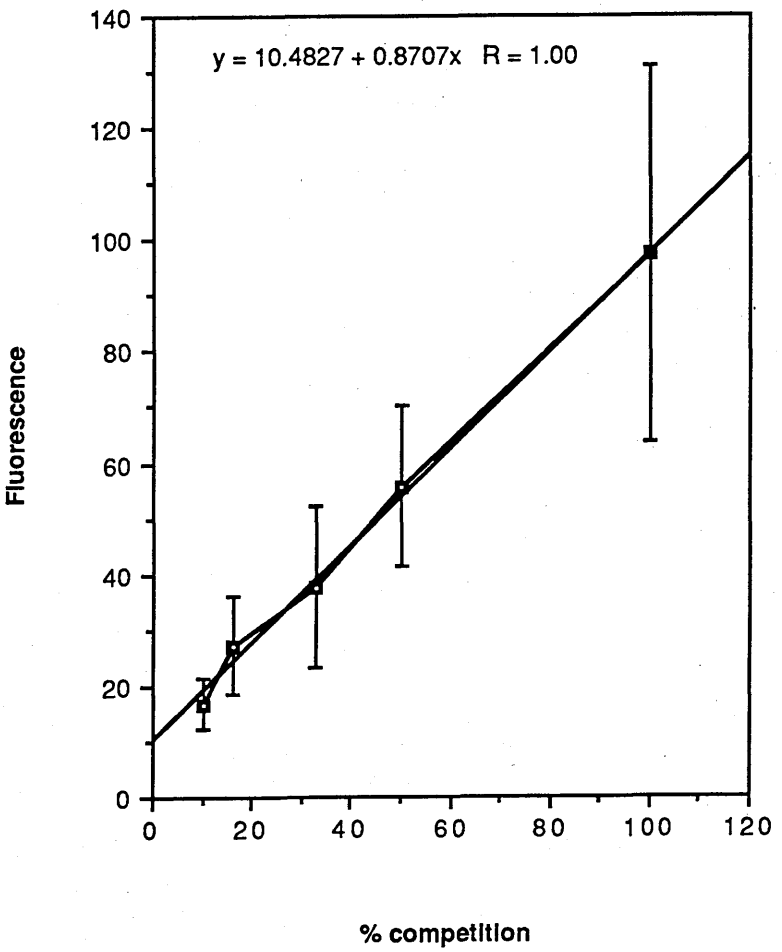


Figure 4.3

The effects of serum dialysable components on uptake of AF-LDL.

Aminofluorescein C18 labelled LDL was used to investigate the effect of pre-exposure of developing schistosomula to serum dialysable components. To do this two groups of schistosomula from the same population were employed. One group was cultured for the first 3 hrs post transformation in EMS only (S-), the other was cultured in a 25 ml Universal container into which a loop of dialysis tubing containing whole human serum (S+). The samples of schistosomula were subject to labelling with increasing concentrations of AF-LDL. The total volume and number of parasites were kept constant at 0.2 ml and approximately 500 respectively. After labelling in the presence of AF-LDL for 20 minutes the schistosomula were washed twice in EMS and their fluorescence quantitated by MPV-Compact. Data points represent the mean and standard deviation readings taken from 25 schistosomula per sample.

A time course experiment utilising the same approach during the early development of the schistosomula is shown in Fig. 4.3C. Aliquots of schistosomula are removed at various time points after transformation labelled and quantitated.

The effect of pre-exposure to serum on dye uptake from LDL

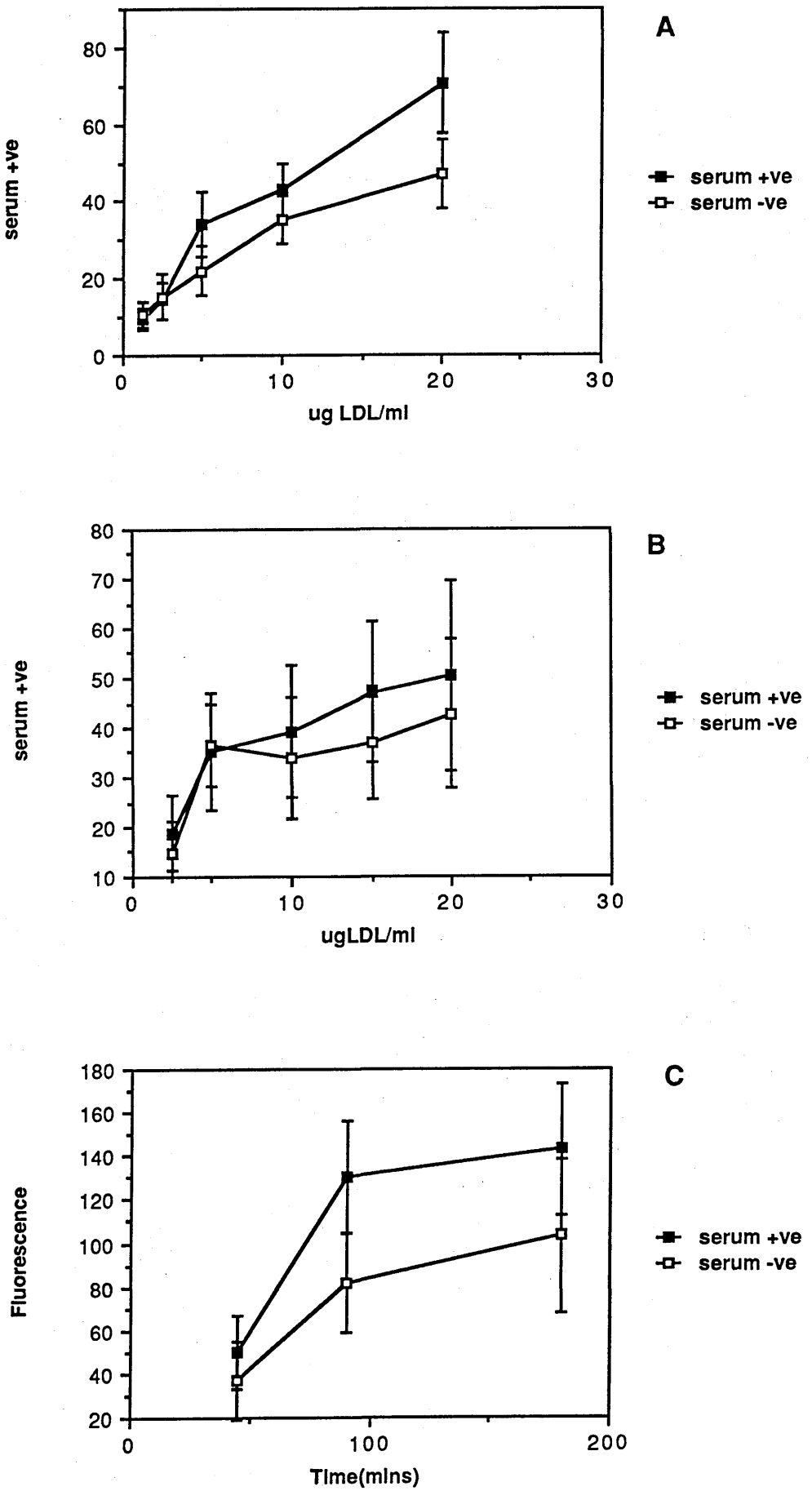


Figure 4.4

The effect of colchicine on early membrane development using AF-LDL as a monitor

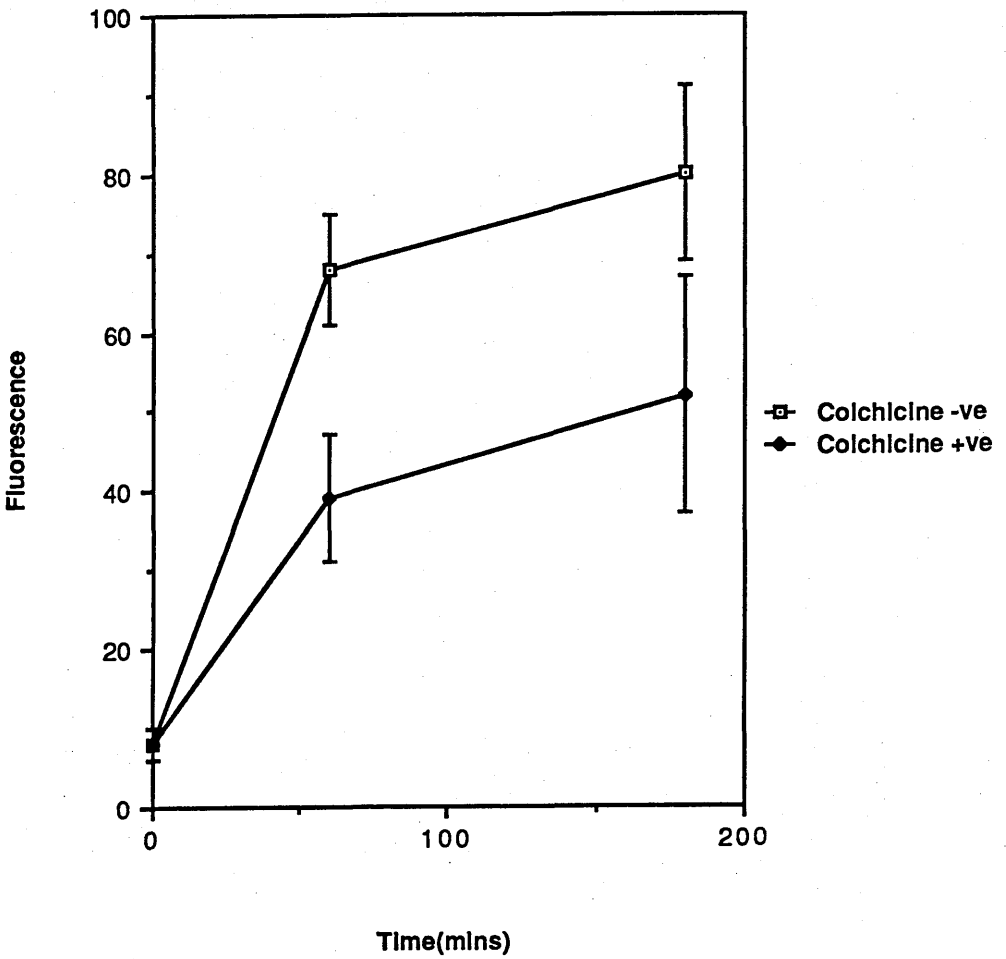
LDL labelled with aminofluorescein C18 was used to monitor the early development of the parasite membrane in the presence and absence of 1 mg/ml colchicine in the surrounding aqueous media.

AF-LDL represented 1 ml LDL at 5 mg/ml to which 10 ul of a 1 mg/ml ethanolic solution of aminofluorescein was added.

Groups of schistosomula at T_0 (immediately after transformation), T_{60} minutes and T_{180} minutes post transformation were exposed to labelling with 10 ul AF-LDL to 1000 ul aqueous media containing approximately 500 schistosomula. Labelling occurred at 37°C for 20 minutes after which the schistosomula were washed twice in EMS and their fluorescence quantitated.

The data points represent the mean and standard deviation of thirty individual parasites. There were significant differences in the fluorescence between colchicine positive and negative groups at 1 and 2 hours. This was determined by students T test and levels of significance were < 0.01.

The effect of colchicine on dye uptake from LDL



This AF-LDL was also used in a time course experiment similar in design to those reported in the last chapter involving colchicine [Figures 3.10 to 3.12]. Schistosomula were transferred and exposed to colchicine in the media. Quantitation of the aminofluorescein fluorescence of the parasite membrane was then carried out. As can be seen in Figure 4.4 the results were broadly comparable to those obtained when aminofluorescein C18 was applied in ethanolic form (Figures 3.10 -3.12).

These experiments utilising fluorescent lipid analogues to label LDL suggest that:

a) LDL does interact with the membrane.
b) It can transfer lipid analogues from LDL to the membrane.
c) The extent of this interaction of transfer may be increased by the induction of a surface membrane receptor as indicated by Fig 4.3 a & b or alternatively due to some other direct effect of exposure to serum components such as better membrane development Fig 4.3c. This latter alternative would in essence be a nutritive effect.
d) Labelling with fluorescent lipid analogues gives comparable results to those achieved using ethanolic solutions of the dye but the efficacy of labelling is markedly increased.

However one important consideration has prevented the more widespread utilisation of LDL as a carrier for fluorescent lipid analogues in labelling parasite membrane in this study. This is the location of the fluorescent lipid analogue under these circumstances. Does the fluorescence come from the lipid molecules intercalated into the membrane of lipid analogues residing with LDL molecules binding to the surface of the membrane? This problem could by resolved by FRAP since the DL value in each situation should be different.

The most likely explanation is that the analogue molecules reside in both locations, that is, within the membrane but also within LDL attached to the membrane. This assumption underlies the conclusions listed above. The colchicine experiment 4.4 does suggest that the aminofluorescein fluorescence is sensitive to change in the membrane.

4.1.3 H^3 Cholesterol Labelling.

The next step was to try and confirm the conclusions indicated by fluorescence based LDL study above with a radioactive marker of LDL. The initial approach was to undertake a double labelling experiment labelling LDL with H^3 cholesterol and aminofluorescein C18. Saturable labelling was achieved with both labels (Figure 4.5.). This repeated the results using fluorescent lipids alone by another means and further supports the conclusion that LDL is directly interacting with the schistsomula membrane and transferring its contents. They also show the effect that even low concentrations of serum have on monitoring LDL interactions by this particular method. Numerous attempts were made to examine whether pre-exposure to serum in solution or via serum dialysable components would result in a greater interaction of the contents of LDL, as measured by cholesterol transfer. This could not be achieved. This was probably due to several factors. One of these is that H^3 cholesterol equilibrated with other lipoprotein fractions of the serum thereby dispersing the radioactive label in serum components other than LDL. The presence of serum could in addition directly compete with LDL for binding to the surface membrane as well as changing the nature of that surface membrane due to lipid exchange. These attempts to accurately monitor the transfer of LDL contents to the parasite, by this method, were unsuccessful. They did show, however, that H^3 cholesterol transfer did occur.

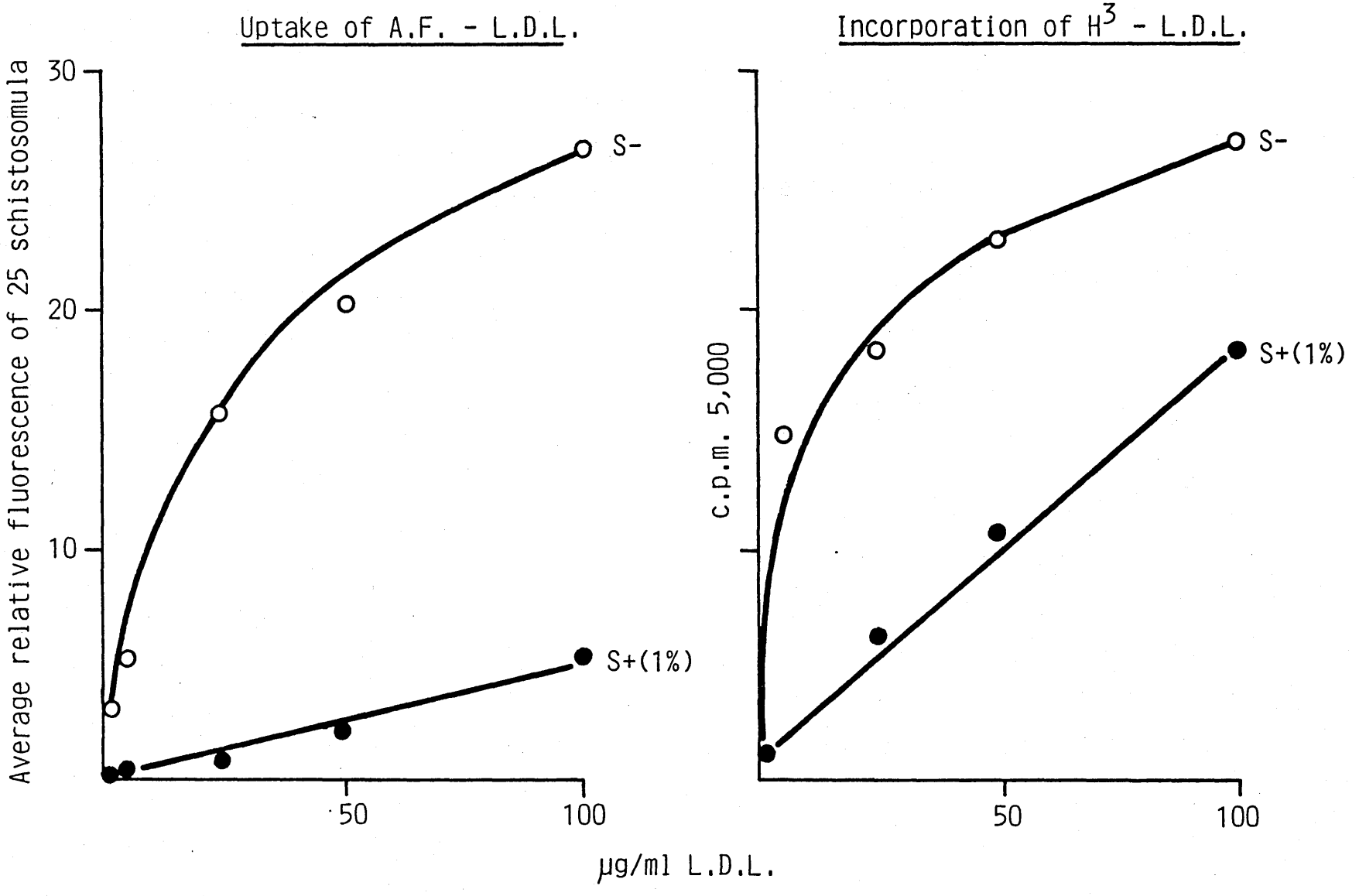
Figure 4.5

H^3 cholesterol and aminofluorescein C18 double labelling.

1 ml LDL (5 mg/ml) was double labelled with aminofluorescein C18 and H ^{3}cholesterol. Samples of a population of 3 hrs schistosomula cultured in EMS only were subjected to labelling with increasing concentrations of this double labelled LDL solution. The samples were washed three times and the uptake of aminofluorescein C18 and H^3 cholesterol determined in the presence or absence of 1% whole human serum (S+, S-). From each sample point ten per cent of the sample of double labelled schistosomula was removed and the fluorescence counted by MPV compact. Each sample point therefore represents the mean of twenty five readings taken on individual parasites shown in Figure 4.5a.

The remaining 90% of schistosomula approximately 900 in a volume of 45 ml were subjected to extraction in 45 ml PPO scintillent overnight at 4^{o}C. The radioactivity was quantitated on a Beckman B counter model for 2 minutes. These determinations of the radioactivity are directly comparable to the samples in which aminofluorescein C18 fluorescence was quantitated. They are shown alongside them in Figure 4.5b.

Uptake of double labelled L.D.L. by schistosomula at 37°C



4.1.4. I^{125} labelling of LDL

Due to the instability of LDL labelling with H^3 cholesterol and the difficulties associated with interpreting results a more stable method of labelling was sought. The following series of experiments shown in Figures 4.6 to 4.9 utilised the Iodine monochloride labelling method of McFarlane 1958. This method labels irreversibly a structural component of the LDL, its major apoprotein, Apoprotein B. This covalently attaches an I^{125} atom to the apoprotein. This irreversibly labels the protein component of the lipoprotein and allows the fate of the lipoprotein molecule itself, as opposed to the lipid contents of the lipoprotein, to be followed. The protocol for these experiments was adapted from the techniques used by Goldstein and Brown in a series of papers which demonstrated the phenomenon of receptor mediated endocytosis of LDL in human fibroblasts. These experiments are essentially binding assays using I^{125}-LDL as the ligand. They were all carried out on fully developed schistosomula cultured in EMS only. The first experiment, Figure 4.6, shows an apparently biphasic pattern of LDL binding at 37°C. The two components of this labelling are separable by labelling in the presence of excess "cold" LDL (Figure 4.7). This resolves the binding into two portions, a high affinity binding which accounts for the marked increase in binding at low < 20 ug/ml LDL concentrations and a lower affinity binding which still occurs in the presence of excess LDL. This pattern of labelling occurs at both 37°C and 4°C (Figure 4.8).

Some investigation into the specificity of the binding was carried out and the results shown in Figure 4.9. Bovine serum albumin does not compete with LDL for binding to the membrane whereas HDL at high concentration, and LDL the positive control, both compete for binding.

Figures 4.6 to 4.9
I^{125}-LDL labelling of schistosomula

These experiments had the same general protocol.

3 hr schistosomula, 500 in a volume of 500 ml, were subjected to an incubation period of 30 minutes in the presence of increasing concentrations of I^{125} labelled LDL. In some experiments excess unlabelled LDL (Figures 4.7 & 4.8) or competition with BSA 1 ug/ml and excess HDL (Figure 4.9) was employed. After the labelling period the schistosomula were washed three times in EMS and the radioactivity in each sample quantitated in a LKB Gamma counter. The counting period was 60 seconds.

The specific activity of the LDL ranged between 14 cpm/ug and 60 cpm/ug. For comparability between experiments and to ascertain % cover by LDL, the measure of uptake employed is the unit picogram I^{125} bound per schistosomula.

Figure 4.6

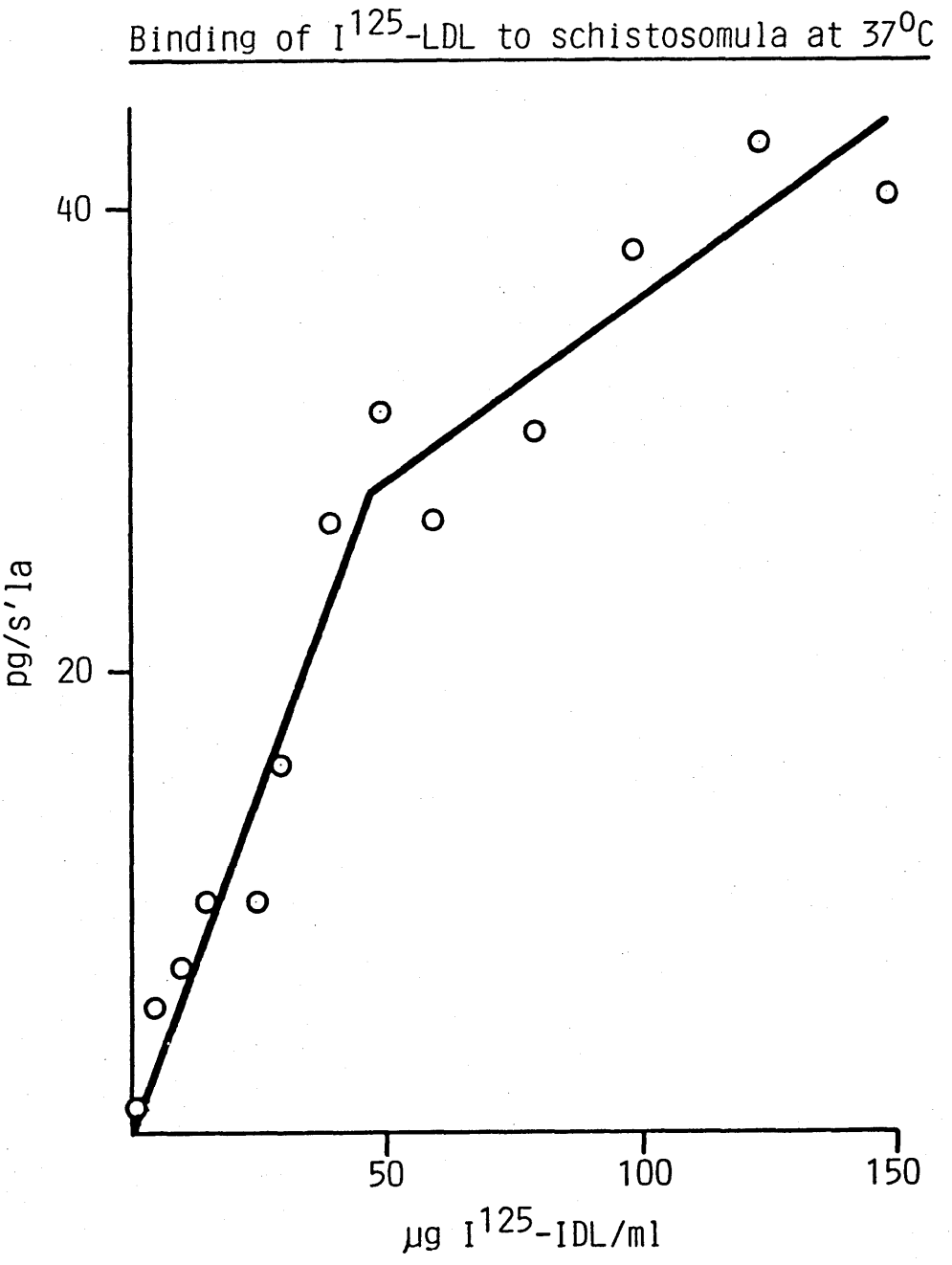
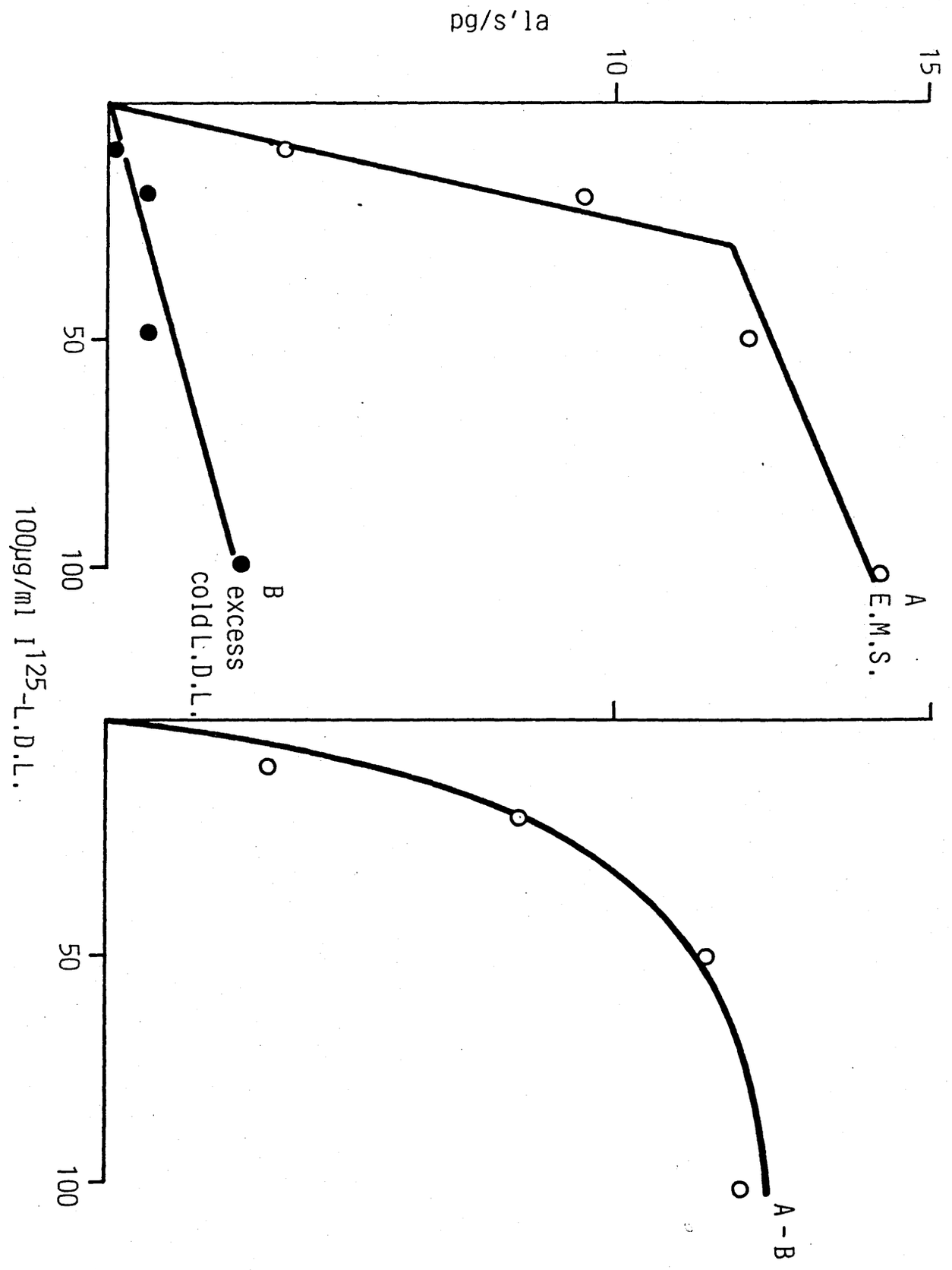
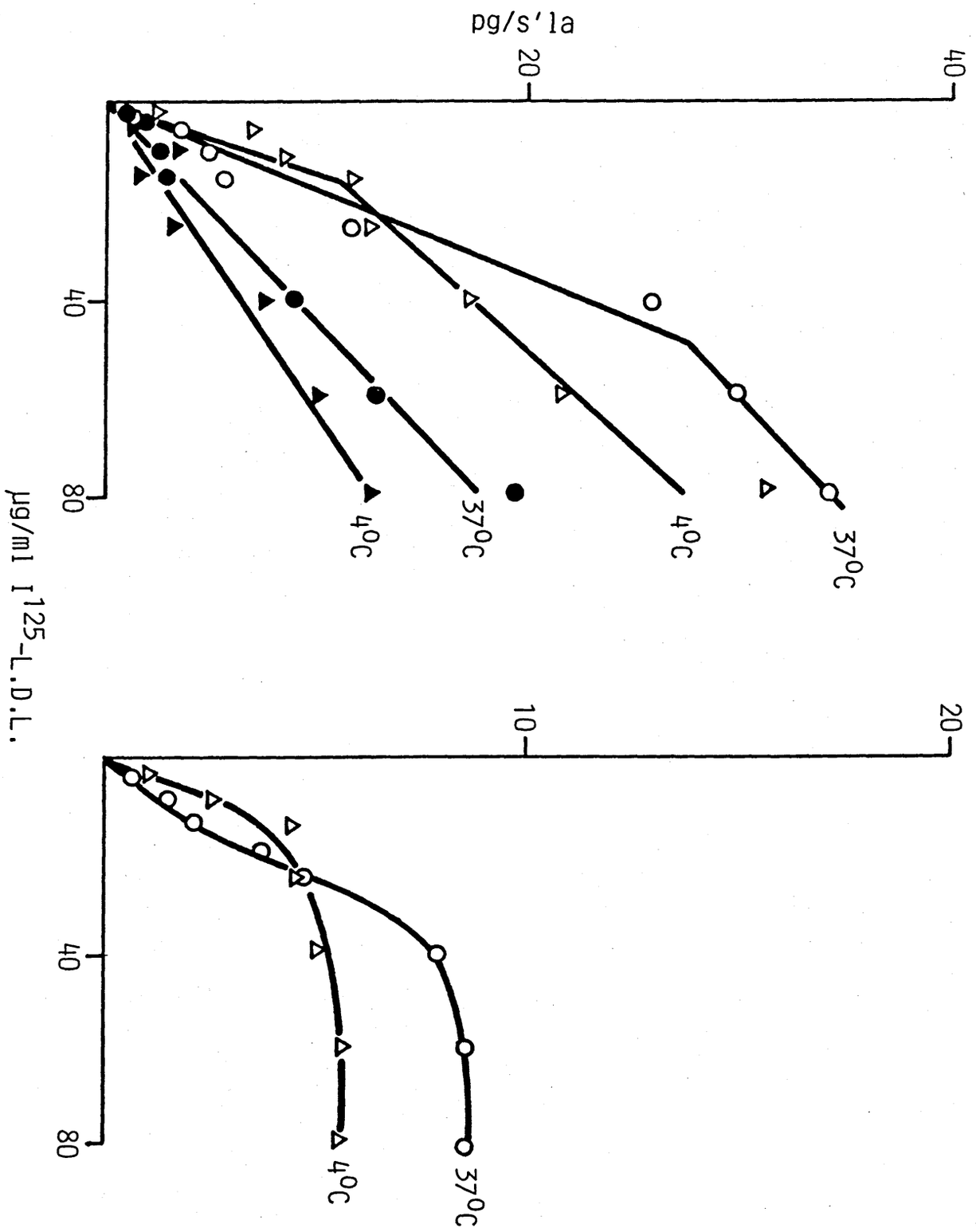

Binding of I125-LDL to schistosomula at 37°C
40
20
pg/s'la
50
100
150
µg I125-IDL/ml

Figure 4.7



Binding of I^{125}-L.D.L. by schistosomula cultures at 37°C

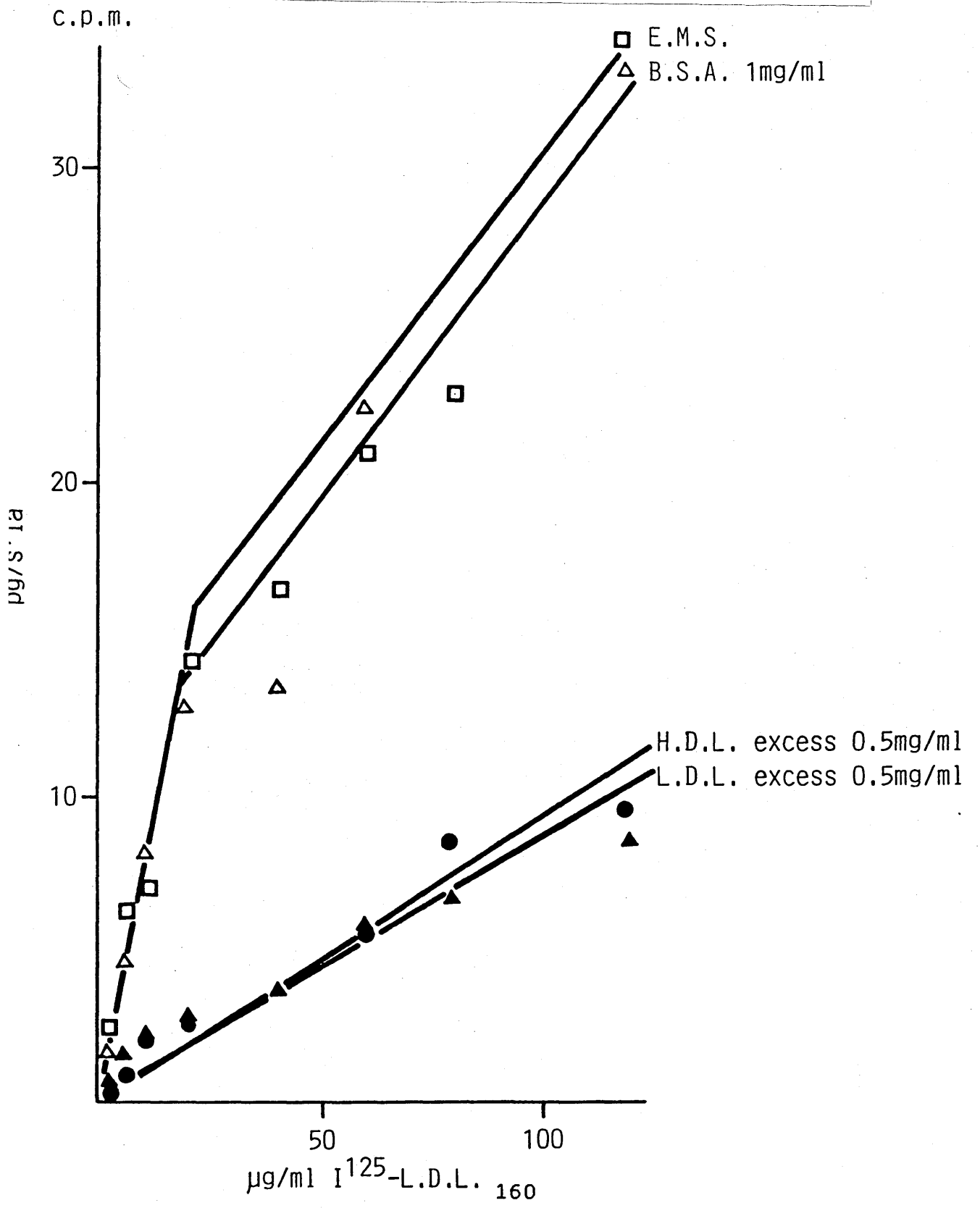


Binding of I^{125}-L.D.L. to schistosomula at 4°C and 37°C

Figure 4.8

Figure 4.9

The competitive effects of B.S.A., L.D.L. and H.D.L. on L.D.L. binding.



What do these experiments indicate about LDL parasite membrane interactions?

a) Firstly that LDL does interact, confirming the earlier fluorescence based results.
b) The biphasic nature of the labelling indicates that two types of interaction may be occurring.
c) The Km values of LDL binding to the parasite membrane are remarkably close to those attained for LDL binding to fibroblasts.
d) HDL can compete with LDL in binding to the parasites surface. Therefore by implication HDL may be binding to the parasite's surface also. In contrast bovine serum albumin does not compete as is consistent with other reports which state that it does not bind [Sher, Hall & Vadas 1978].

4.1.5. Receptor Mediated Endocytosis

The biphasic binding and closeness of Km values to those found in fibroblasts raised the possibility that receptor mediated endocytosis was occurring at the parasite surface. This analogy with fibroblasts was explored by looking for the degradation products of this phenomenon. In a culture of fibroblasts exhibiting receptor mediated endocytosis of I^{125} LDL, LDL is first bound to the surface via a receptor which is internalised via clathrin coated pits into vesicles which deliver the LDL to the lysosomes. The receptors themselves are recycled prior to this (Figure 4.10). Once in the lysosomes both the lipid and protein compnoents of the LDL are degraded. The degradation products of the I^{125} labelling Apoprotein B of LDL are small I^{125} peptides and amino acids which are recovered in the aqueous media of the cell culture. These act in effect as a marker of receptor mediated endocytosis which can be observed via I^{125} labelling of LDL [Goldstein & Brown 1974]. This approach was applied to cultures of schistosomula and the results shown in Figure 4.11.

Figure 4.10

Diagrammatic representation of endocytosis.

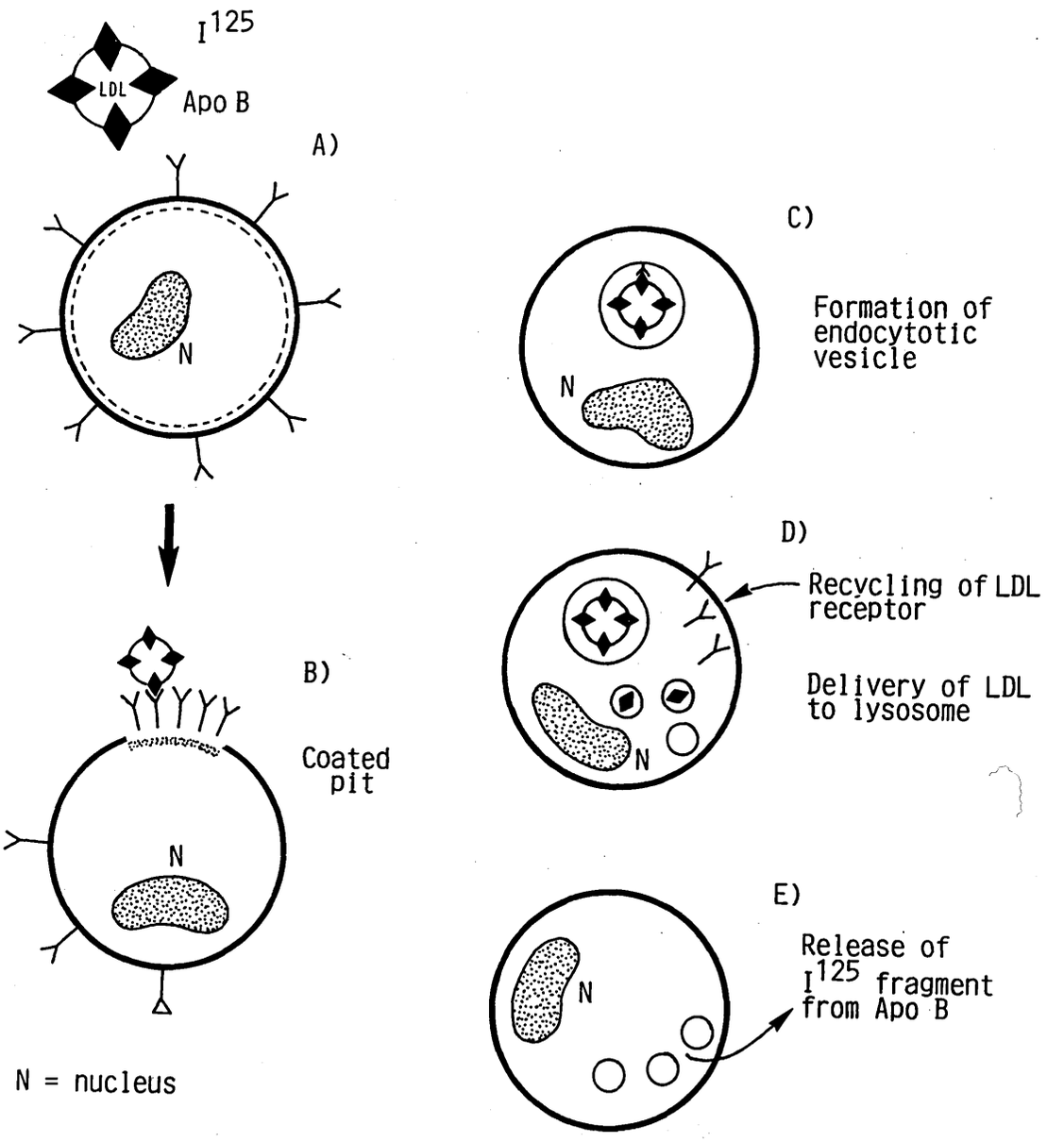
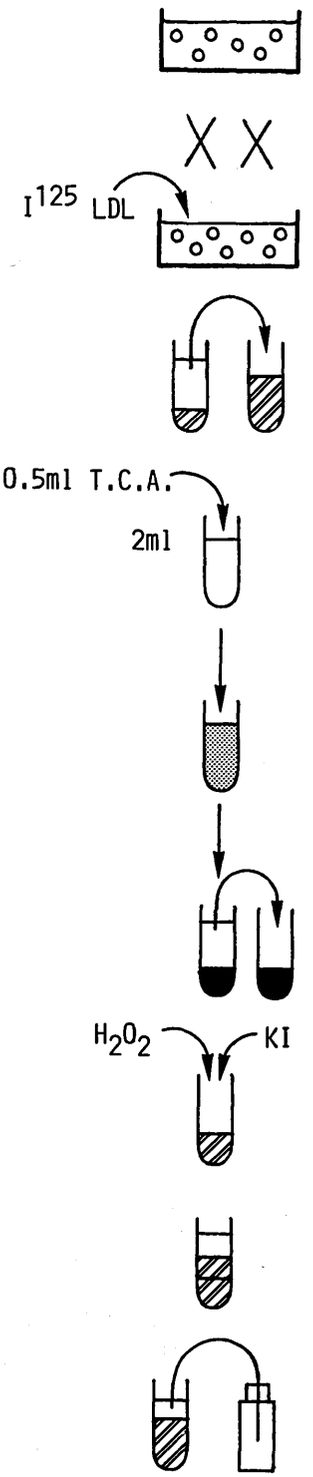


Figure 4.11

I^{125}–LDL degradation product assay.



Sla + 5% human serum (pulse 1 hr.)

Washed X2 in EM

Sla + 100µg/ml I^{125} LDL O/N 37°C

Sla sedimented and aqueous media removed

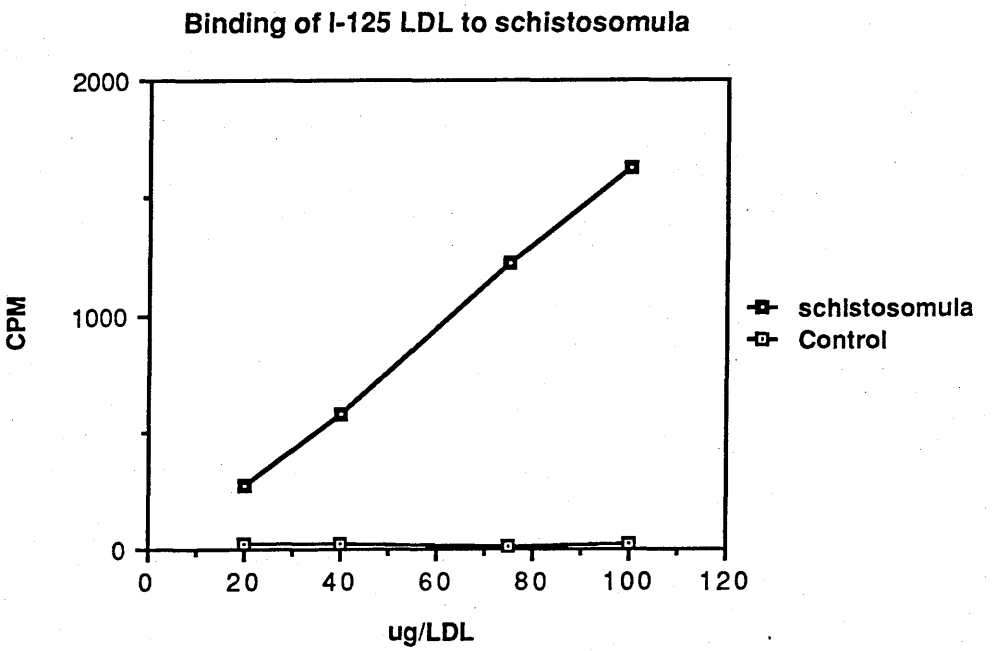
To 2ml fraction aqueous media 0.5ml 50% TCA added incubated for 30° at 4°C

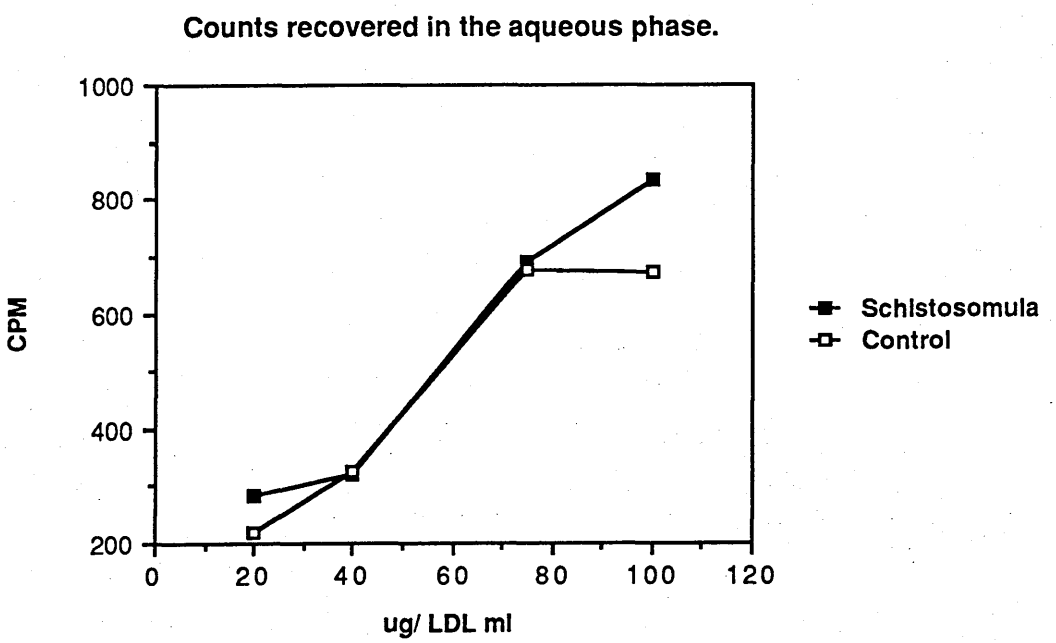
TCA precipitate spin down and 1ml fraction of aqueous media removed

To this 10µl 40% W/V KI and 40µl 30% H_2O_2 added mixed and incubated at room temp. for 5 mins.

Mixture extracted with 2ml $CHCl_3$

0.5ml fraction of extracted aqueous media removed and counted in gamma counter


Binding of I-125 LDL to schistosomula
2000
1000
0
CPM
0
20
40
60
80
100
120
ug/LDL
schistosomula
Control


Counts recovered in the aqueous phase.
1000
800
600
400
200
CPM
0
20
40
60
80
100
120
ug/ LDL ml
Schistosomula
Control

Degradation products of I^{125} LDL could not be observed. This negative result is in agreement with other studies directed at this question of whether receptor mediated endocytosis occurs in schistosomes [Cushley & Kusel 1987]. These authors used killing of the parasite by Ricin as a test for endocytosis. The action of this toxin is dependent on receptor mediated endocytosis mediated by the lectin like properties of the B chain of this toxin. This allows internalisation of the A chain, which irreversibly inactivates eukaryotic ribosomes. Both schistosomula and adults clearly bind this toxin but are unaffected by it suggesting that endocytotic vesicles are not found at the surface of the parasite membrane.

A further test of endocytosis was carried out using FITC dextran beads which accumulate in the phagocytotic vesicles of macrophages due to endocytosis but do not accumulate in the tegument of schistosomula.

The binding of LDL appears to be non-specific since if the saturating concentrations of approximately 10 picograms of LDL bound per schistosomula are correct then the surface area of the membrane covered by LDL exceeds comfortably the area of the schistosomula membrane at this point in development. The calculations and assumptions behind this conclusion are shown in Figure 4.12.

Figure 4.12

Projection of bound LDL onto schistosomula surface

Molecular Wt of LDL = 64,000 kD

Saturation level / schistosomula = 10pg / schistosomula

Diameter of LDL molecule = 250 angstroms

$$\text{No. of molecules bound per schistosomula} = \frac{10 \times 10^{-12}}{6.4 \times 10^{3}} \times \text{Avogarde's No.}$$

$$= 1.5625 \times 10^{-15} \times 6.02 \times 10^{23}$$

$$= 9.41 \times 10^{8}$$

Surface area of 1 LDL molecule: $r^2 = 3.14 \times (125A)^2$

$$= 4.9 \times 10^{4}\ A^2$$

$10^8 (A) = 1\ um^2$

Therefore projected surface area = $4.62 \times 10^5\ um^2$

1 schistosomula surface area treated as a cylinder 25 um x 150 um has surface area of $D \times h + 2(\pi r^2)$

$$= 11{,}775 + 2(490)$$

$$= 12{,}756\ um^2$$

$$= 1.2 \times 10^{4}\ um^2$$

Therefore surface area of LDL exceeds surface area of parasite by factor of approximately 38.

4.2.1. Indirect Immunofluorescence studies

Quantitative indirect immunofluorescence, utilising as first antibody an antiserum specifically directed against the major protein component of LDL Apoprotein B and as second antibody FITC anti rabbit or sheep IgG was also carried out to further investigate the LDL interaction. Quantitation of fluorescence was carried out comparing the fluorescence of schistosomula incubated in the presence of LDL with that of schistosomula cultured only in EMS. The results are shown in Figure 4.13. Clearly pre-incubation or pre-exposure to LDL results in a significant increase in the binding of this antisera relative to controls. It was observed that the parasites were uniformly labelled. This result indicates that Apoprotein B epitopes are present at the surface of the membrane of schistosomula pre-incubated in LDL.

An attempt was made to see if antibodies directed against the schistosomula membrane could block LDL binding. The reasoning behind this was that if LDL binding was dependent to some extent on a serum inducible receptor an immune sera containing IgG directed against it may serve to block LDL binding. To do this two human sera were used one from an individual with a high anti-schistosome antibody titre, the other from an individual with a low titre. After pre-incubation in these sera and thorough washing the amount of LDL bound to the schistosomula exposed to the "high" antibody titre sera repeatedly showed significantly greater binding of LDL than both the "low" titre sera and control parasites cultured in EMS only. This effect was shown to be due to the IgG component of these two sera by isolating the IgG and repeating the experiment (Figure 4.14). Such an effect could also be produced by pre-incubation with the lectin Con A (Figure 4.15). Clearly, binding of such macromolecules enhances rather than inhibits LDL binding.

Figure 4.13

Indirect Immunofluorescence with LDL

3 hr schistosomula pre-cultured in EMS only were split into two groups. Group one was exposed to a 30 minute incubation at 37°C in 500 ug/ml LDL in EMS, group two to a 30 minute incubation at 37°C in EMS only.

The parasites were washed twice and indirect quantitative immunofluorescence carried out. The first antibody used was a specific Anti-Apoprotein B raised in rabbit. The second antibody was FITC anti-rabbit IgG. Exposure to each antisera was for thirty minutes followed by two washes in EMS. The anti-Apo B antisera was used at a dilution factor of 1/10 the FITC anti-rabbit at 1/20. The binding of the antisera was quantitated by indirect immunofluorescence. The columns and standard deviations shown in figure 4.13 are derived from quantitation of groups of ten readings on individual parasites (A, B, C, D) or twenty individual readings (groups E and F). The incubation protocol for each column is shown below.

	Exposure to LDL (0.5 mg/ml)	1st Antisera (Rabbit)	FITC 2nd Antibody
A	−	Anti-Apo B	+
B	−	−	+
C	+	−	+
D	+	Normal Rabbit	+
E	+	Anti-Apo B	+
F	+	Anti-Apo B	+

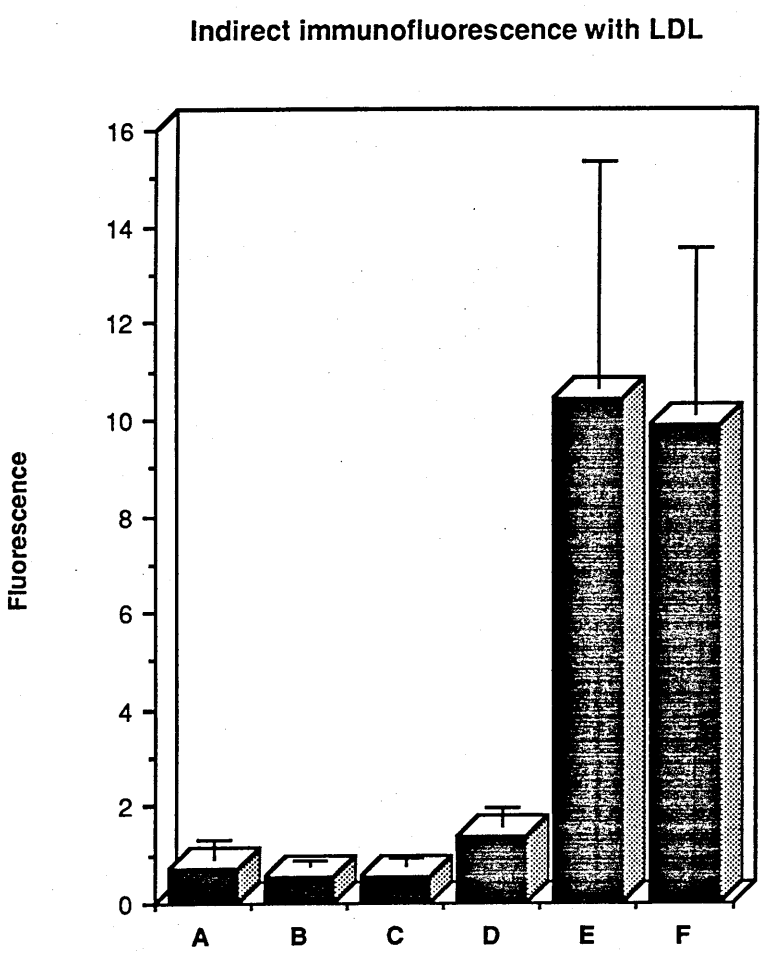

Indirect immunofluorescence with LDL
Fluorescence
16
14
12
10
8
6
4
2
0
A
B
C
D
E
F

Figure 4.14

The effect of human IgG on LDL binding to schistosomula

The protocol used was similar to 4.13 and involved;

a) Incubation of 3hr schistosomula at 37°C for 1 hour in the presence of EMS only or 0.5 mg/ml human IgG isolated from heat inactivated sera. Two sources of sera were employed. One from a non-exposed individual with a low antibody titre to S mansoni and the other from an individual previously exposed to S mansoni with a high antibody titre.

b) Incubation of schistosomula for 1 hour at 37°C in EMS in the presence or absence of LDL (20 ug/ml or 50 ug/ml).

c) Indirect immunofluorescence consisting of sequential exposure to anti Apo B antisera or normal rabbit serum followed by labelling with FITC anti rabbit IgG (30 minutes at 37°C).

d) Binding of antisera was monitored by quantitative fluorescence on a Leitz MPV Compact. Data presented represent the mean and standard deviation of groups of 25 individual parasites. The non-specific background binding monitored by the use of normal serum was approximately 10% of that obtained with anti Apo B antisera and has been subtracted from these readings. Between each component of this protocol, a, b, and c (both parts), schistosomula were washed in EMS. These data are representative of four experiments.

In each case pre-exposure to "high titre" IgG increased LDL binding significantly (<0.01 by Student's T test) whereas exposure to "low titre" IgG had either no effect or decreased binding as in this example.

Effect of pre-incubation with low and high affinity IgG (0.5µg/ml) on subsequent L.D.L. availability on surface membrane.

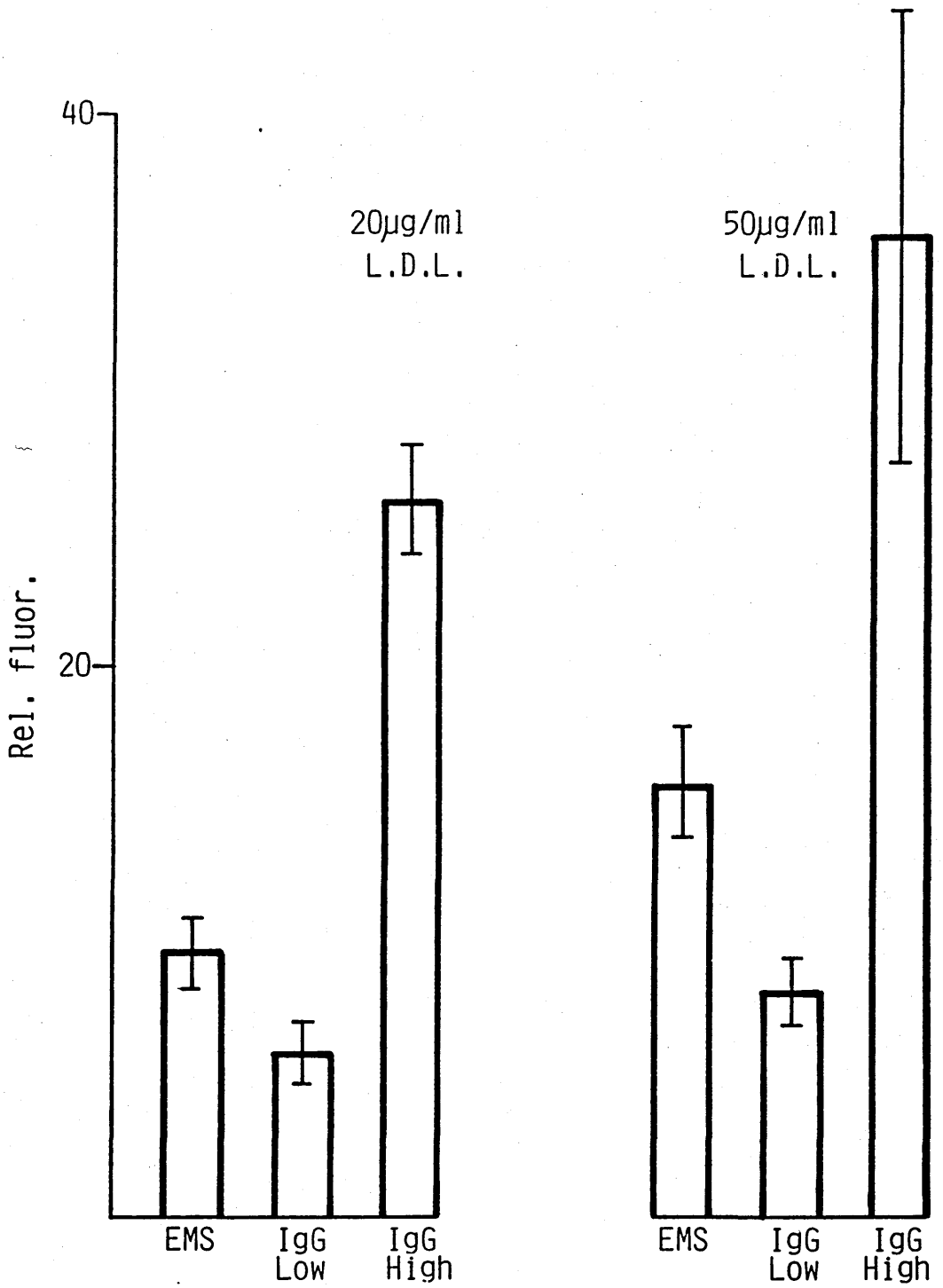


Figure 4.15

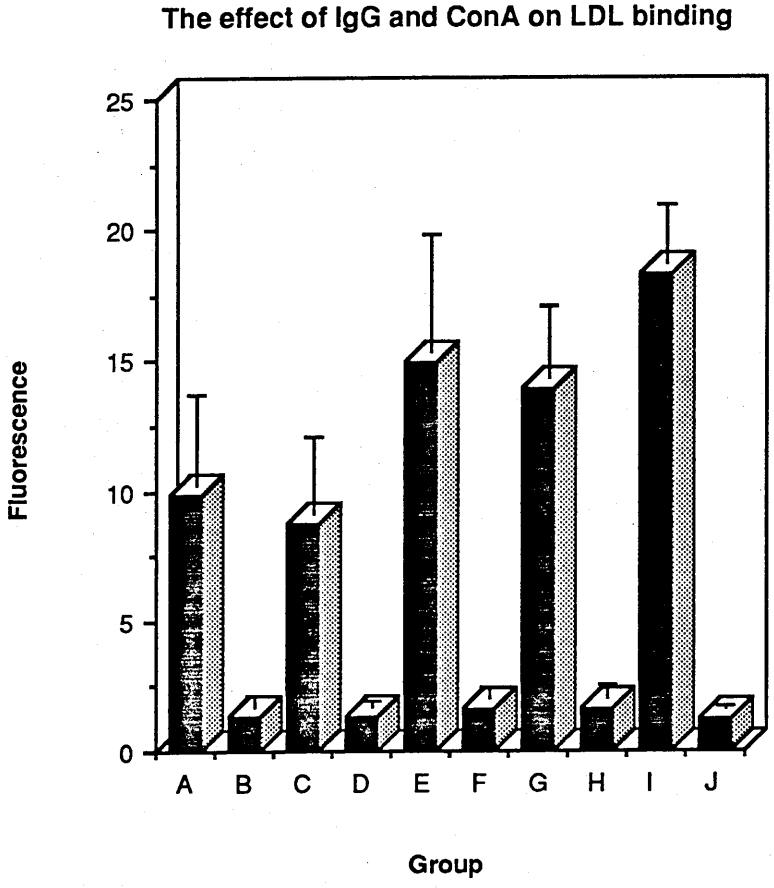
The effect of low concentrations of human IgG and Con A on LDL binding to schistosomula

This experiment is similar to 4.14 in its protocol but in this case the stimulating IgG and Con A molecules are at low concentrations (100 ug/ml or less) and the LDL at a high saturating concentration (500 ug/ml). The protocol consisted of exposure of 3 hr schistosomula to EMS, "low" IgG, "high" IgG, Con A (20 ug/ml or 50 ug/ml). This was followed by an incubation at a saturating concentration of LDL and binding was measured by indirect immunofluorescence as in Figure 4.14. The individual columns represent the mean and standard deviation of 20 individual readings. The labelling protocols for each column are as below.

	1st exposure	0.5 mg/ml (sheep) LDL	1st antisera 2nd antisera (Sheep)	FITC 2nd antibody
A	EMS	+ve	Anti Apo B	+ve
B	EMS	+ve	normal sheep	+ve
C	100 ug/ml low IgG	+ve	Anti Apo B	+ve
D	100 ug/ml low IgG	+ve	normal sheep	+ve
E	100 ug/ml high IgG	+ve	Anti Apo B	+ve
F	100 ug/ml high IgG	+ve	normal sheep	+ve
G	20 ug/ml Con A	+ve	Anti Apo B	+ve
H	20 ug.ml Con A	+ve	normal sheep	+ve
I	50 ug/ml Con A	+ve	Anti Apo B	+ve
J	50 ug/ml Con A	+ve	normal sheep	+ve

Statistics: T tests were done on each experimental group paired against the EMS control. The results are tabulated below. Background values derived from the normal sheep sera were substituted prior to calculations in each case.

A (EMS) vs	Significant (< 0.05)	p value
C low IgG	No	0.15
E high IgG	Yes	0.44
G 20 ug/ml Con A	Yes	< 0.001
H 50 ug/ml Con A	Yes	< 0.001


The effect of IgG and ConA on LDL binding
Fluorescence
25
20
15
10
5
0
A
B
C
D
E
F
G
H
I
J
Group

4.2.2 LDL Binding to the adult membrane in vitro

The same approach, employing indirect immunofluorescence, was used to investigate LDL binding on the adult surface. Unlike the schistosomula binding was not uniform and appeared to concentrate at the tubercles of the parasite, particularly those on the dorsal surface on the male (Plates 4.1 & 4.2). This apparently localised labelling was further explored by utilising LDL labelled directly by FITC (Plate 4.3).

This observation of localised LDL binding at the tubercles is of significance when considered in the light of Haseeb's paper which showed localised uptake of C14 cholesterol at the tubercles by adult parasites incubated in serum containing media [Haseeb et al 1981]. This last point is important because under the conditions employed by Haseeb the radioactive label was likely to equilibrate with lipoproteins of the serum supplemented media. The conjunction of these two observations suggested that cholesterol transport from LDL to the parasite may be occurring locally at the tubercles. This hypothesis lead to an attempt at a "histological" like investigation of the tubercles using fluorescent analogues. That is a variety of fluorescent and histological stains were used to stain the living parasite with the aim of observing patterns of labelling that were tubercle specific. In particular, evidence of localised accumulation of lipid or sterols at the tubercles was sought. This approach did indeed yield a number of observations that provide some insight into the properties and possible function of the tubercle itself.

Firstly when the parasites were labelled with low concentrations of the dye Merocyanine 540 the area of membrane overlying the tubercles was apparently specifically labelled (Plates 4.4 to 4.10). This dye has been reported to be sensitive to the nature of lipid packing of the membrane and has been utilised to distinguish leukemia lymphocytes from normal lymphocytes [Schlegel et al, 1980]. The implication of this observation is that the membrane overlying the tubercles is relatively cholesterol poor and loosely packed [Humphries & Lovejoy 1983] in terms of its lipid organisation than parasite membrane on other regions of the parasite.

Further evidence to suggest that the tubercles contain fairly large bodies of lipid, possibly neutral esters of cholesterol is presented in the following pages. These are a series of plates showing the results of labelling the adult parasite with a variety of dyes that selectively stain fat bodies. These are :

Oil red O	Plates 4.10 and 4.11
NBD Cholesterol ester	Plates 4.12 to 4.17
Nile Red	Plates 4.18 to 4.22

List of plates.

Plates 4.1 & 4.2

Indirect immunofluorescence on the adult dorsal membrane of parasite preincubated in 5mg/ml LDL for 3hrs at 37°C. Fig 4.1 shows labelling pattern on parasite where 1st anti-sera was 10% sheep anti Apo B (30mins) and 2nd anti-sera 5% FITC anti-sheep IgG (30 mins). Fig 4.2 is the control 1st incubation was in normal sheep serum.

Plate 4.3

FITC-LDL binding at tubercle after incubation of adult parasite for 3hrs in 500mg/ml FITC-LDL. (Oil immersion)

Plates 4.4 to 4.7

Localised labelling of tubercles with Merocyanine 540 applied via diffusion from prelabelled Dowex beads.

Plates 4.8 to 4.9

Localised labelling of tubercles acheived by using a very low concentration of the dye Merocyanine 0.01 ug/ml in 1% ethanol in EMS.

Plates 4.10 & 4.11

Localised accumulation of the histological stain Oil Red O in the lipid bodies of the tubercle. This stain is a general histological stain for accumulations of fat.

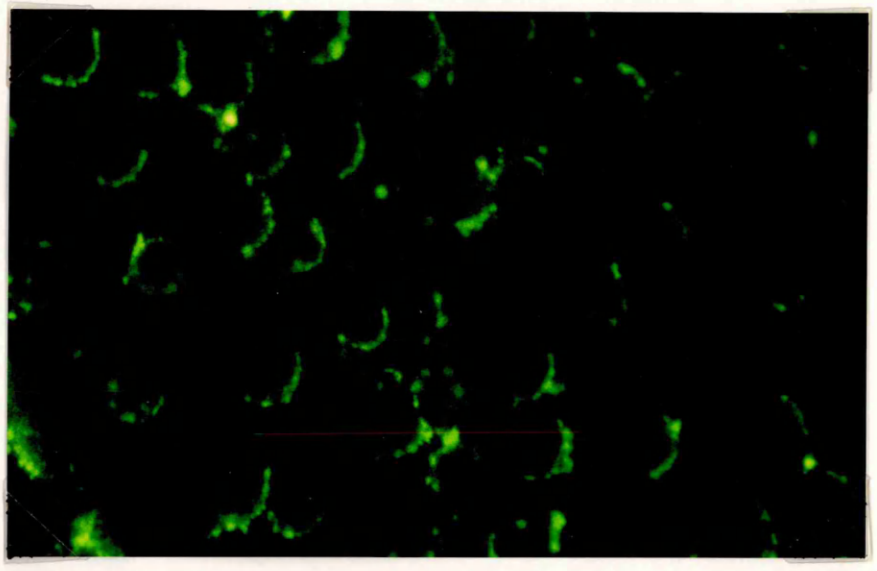
Plates 4.12 to 4.17

Labelling of lipid bodies of tubercle with NBD-cholesterol ester. This was done by briefly exposing the dorsal surface of the parasite to droplets of 2:1 chloroform methanol containing 1mg/ml NBD cholesteryl ester. Parasites were then removed in the aqueous phase mounted and photgraphed.

Plates 4.18 to 4.22

Labelling of tubercle lipid bodies with the dye Nile Red (10ug/ml in 1% ethanol in EMS). This stain is specific for accumulations of neutral lipids and exhibits fluorescence,in both the green and red, according to the hydrophobicity of its enviroment.

Plates 4.1 & 4.2



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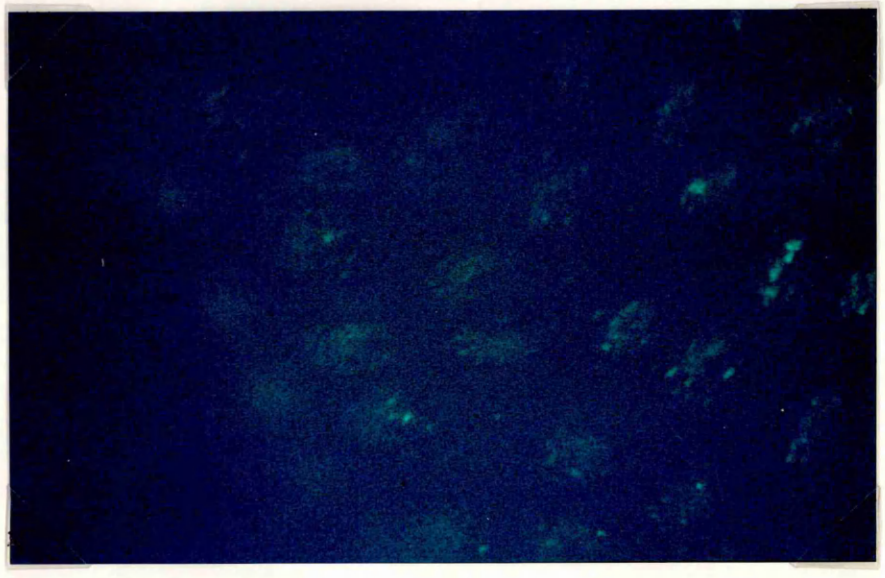

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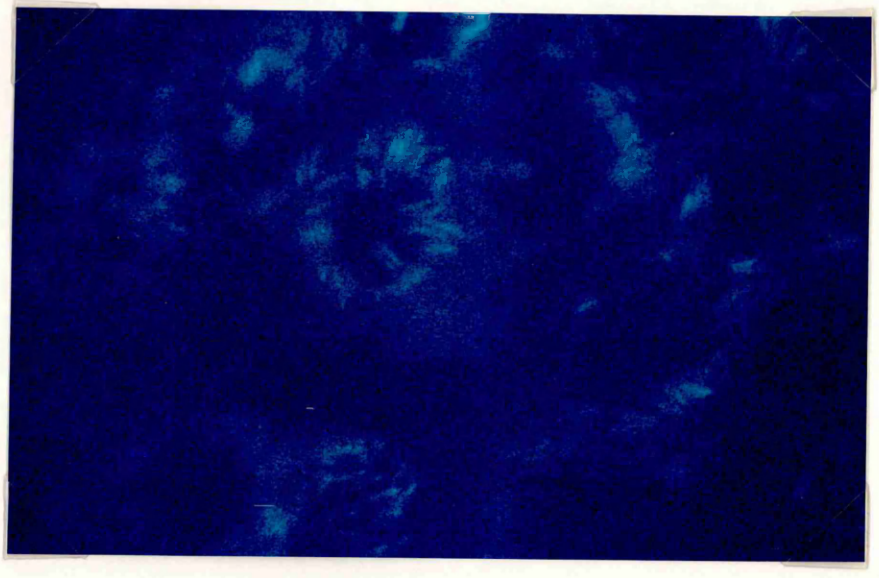
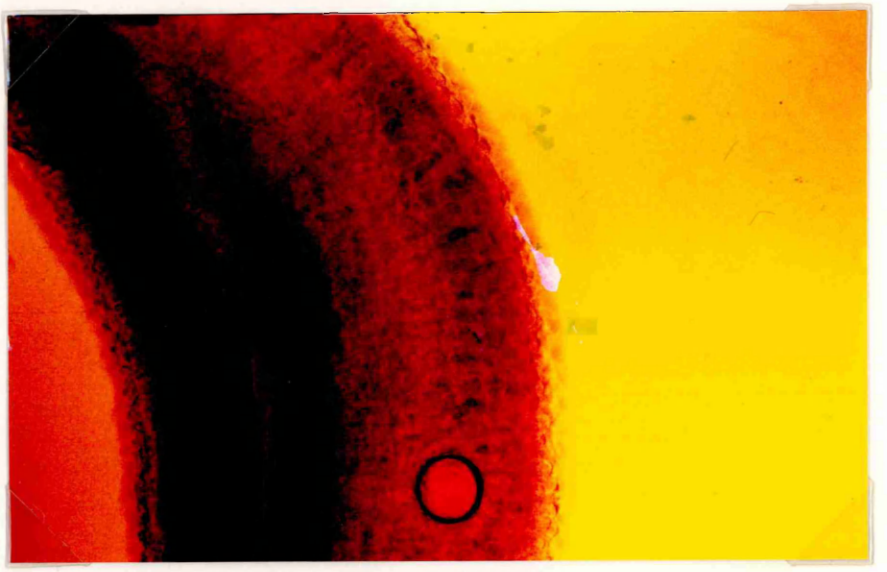
Plate 4.3



10um

Plates 4.4 & 4.5



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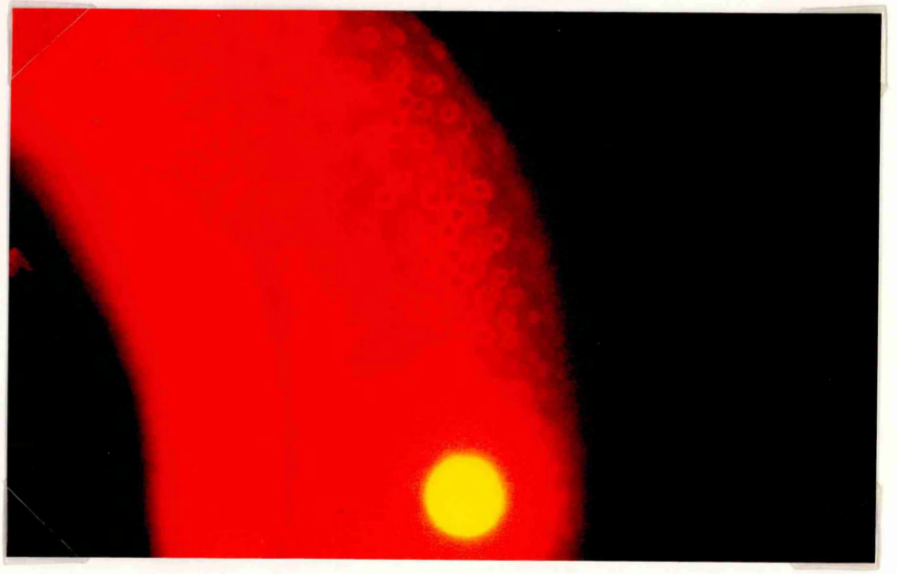

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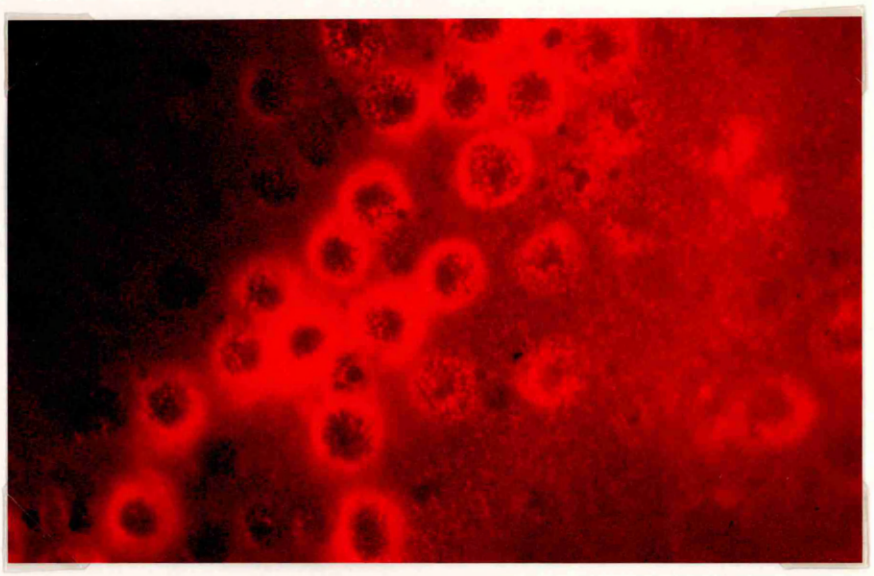
Plates 4.6 & 4.7



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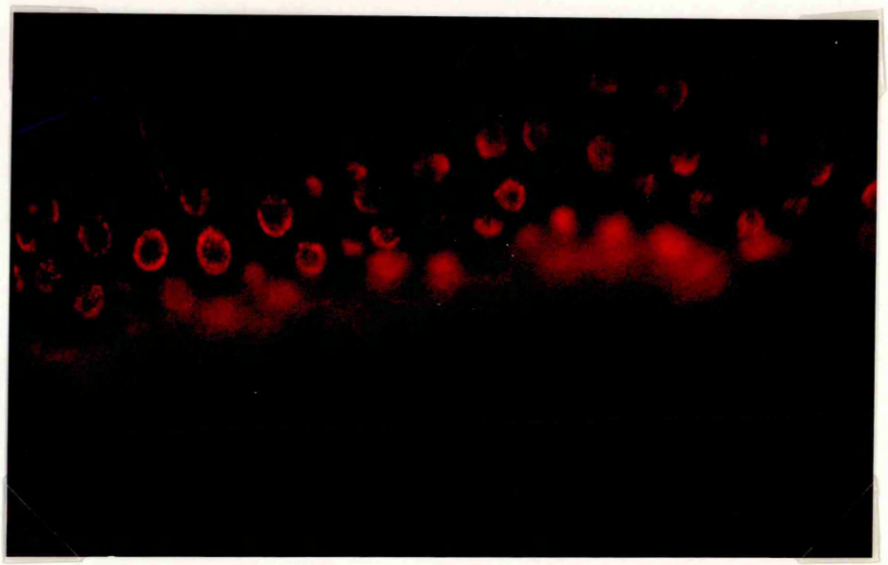

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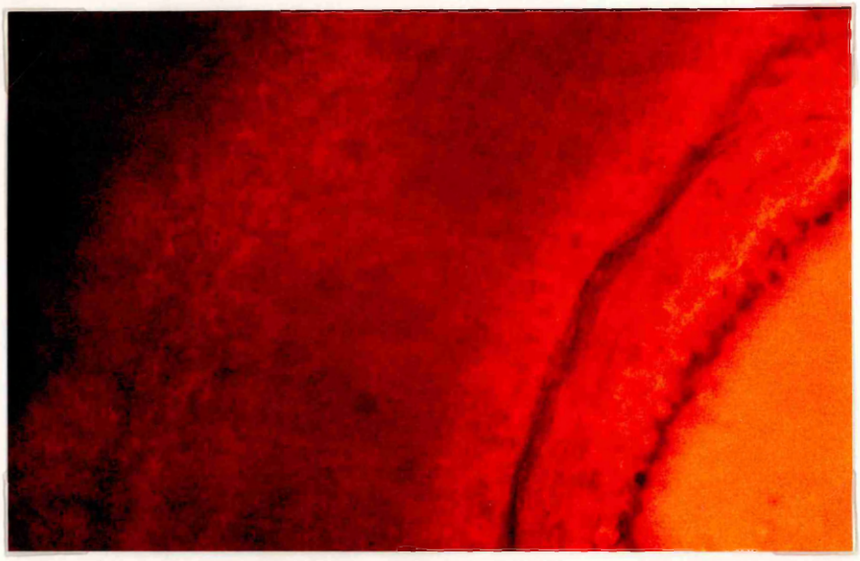
Plates 4.8 & 4.9



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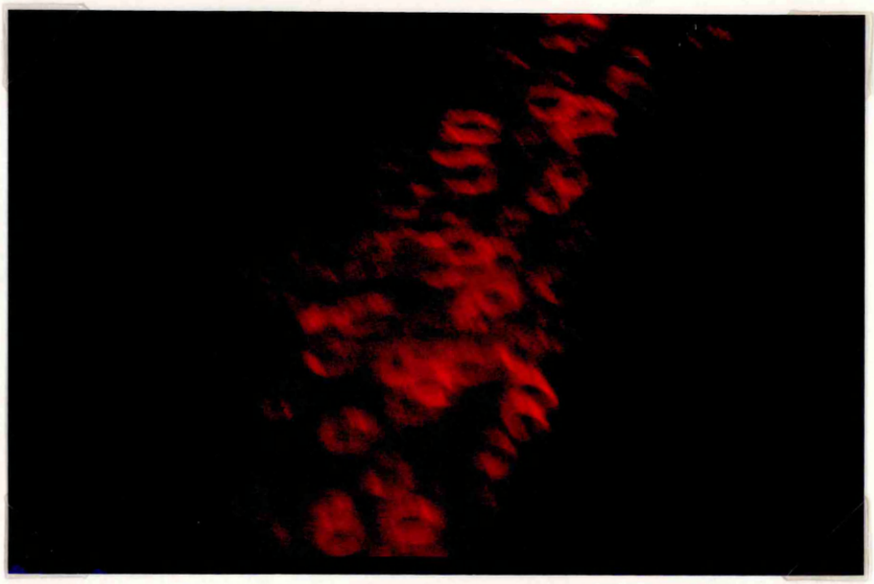

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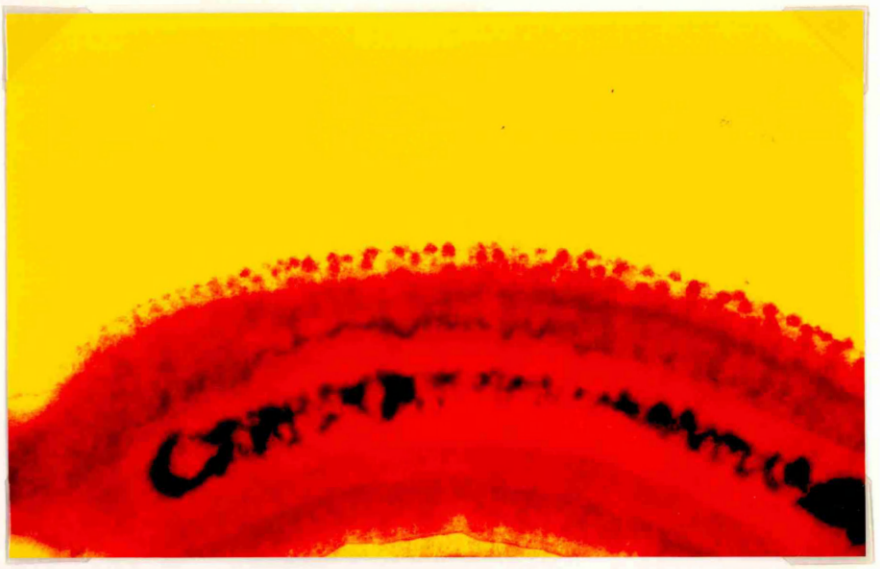
Plates 4.10 & 4.11



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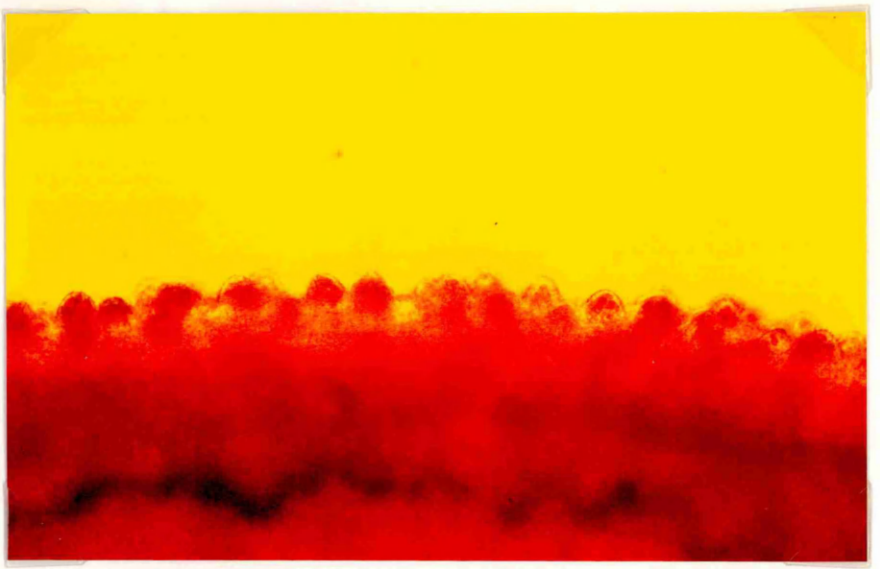

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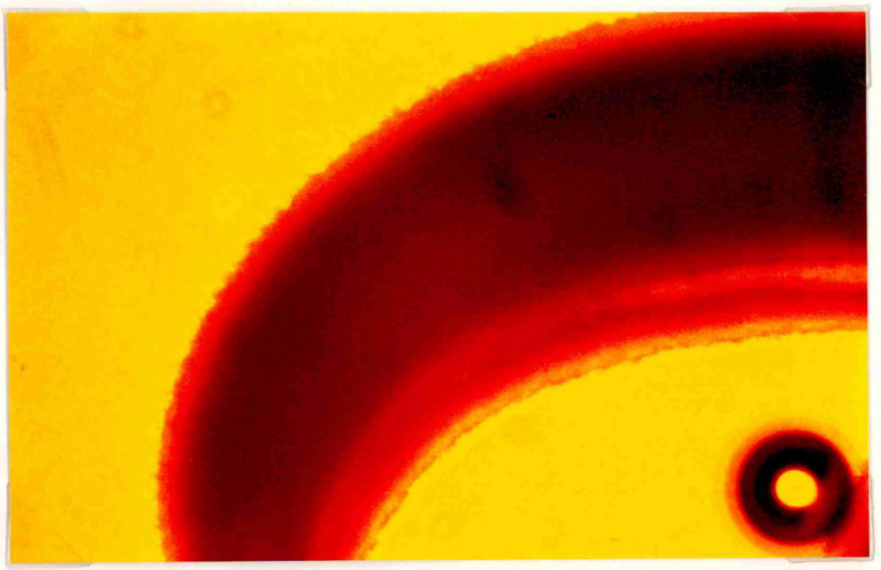
Plates 4.12 & 4.13



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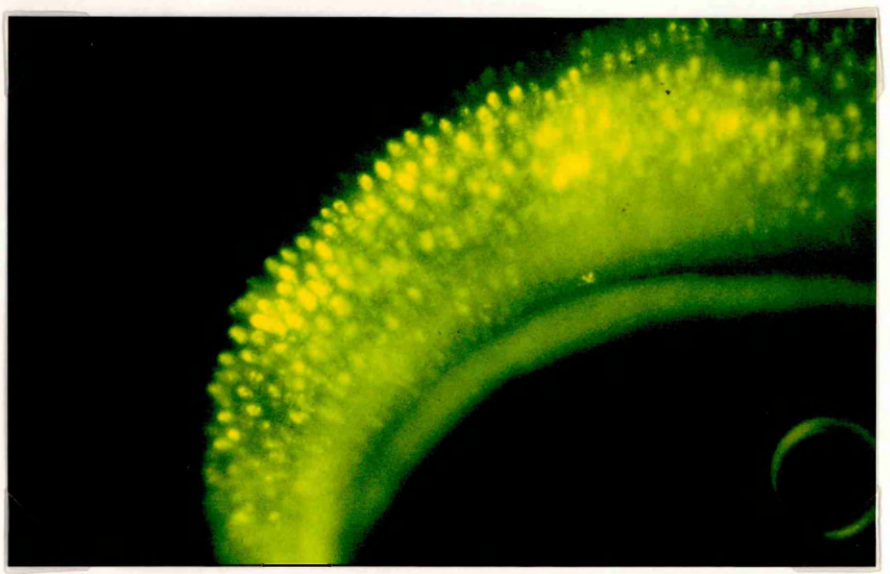

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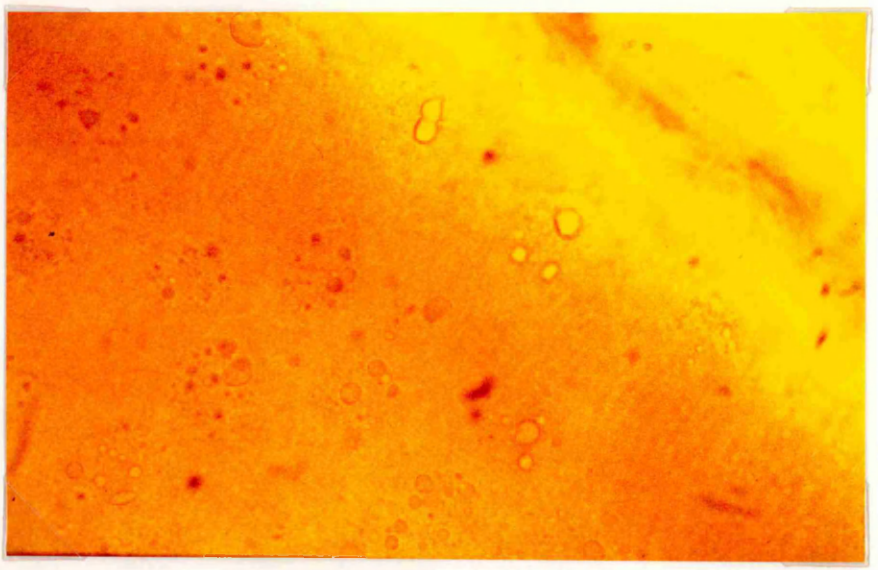
Plates 4.14 & 4.15



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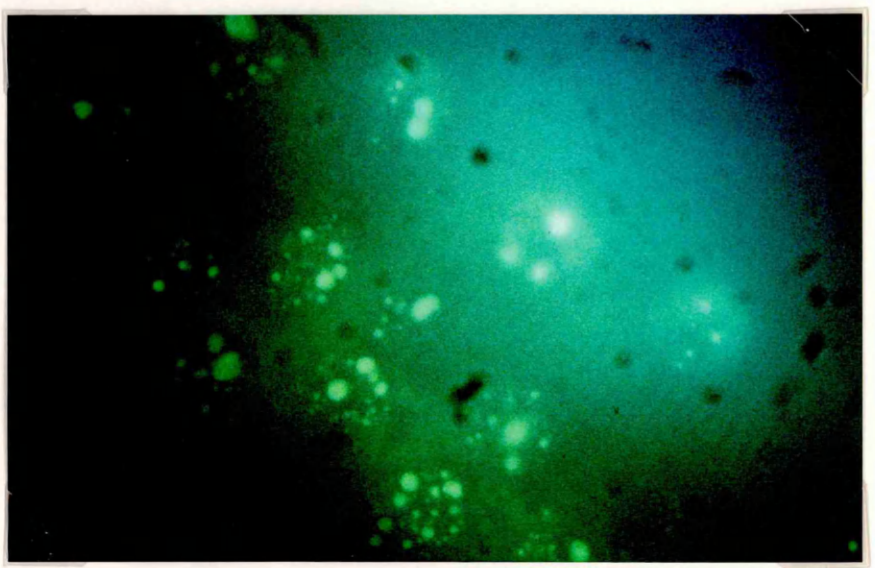

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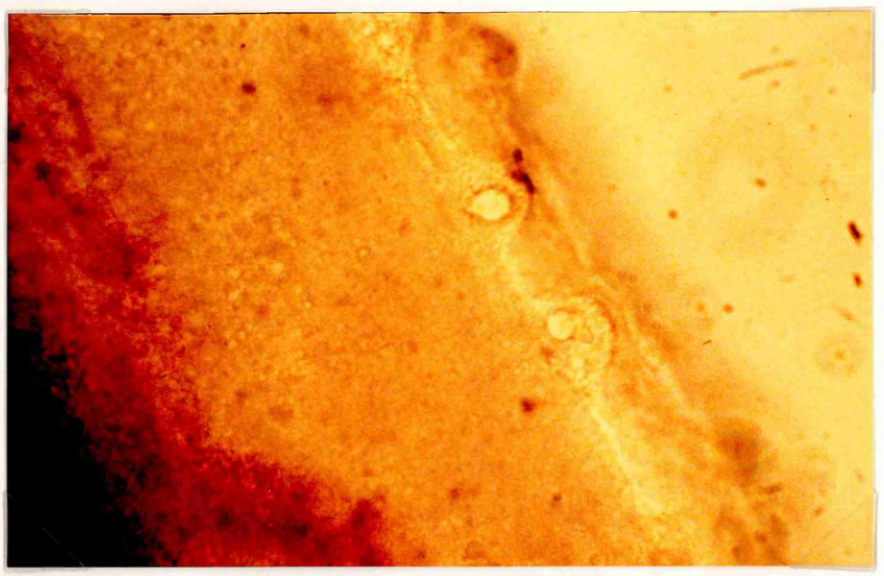
Plates 4.16 & 4.17



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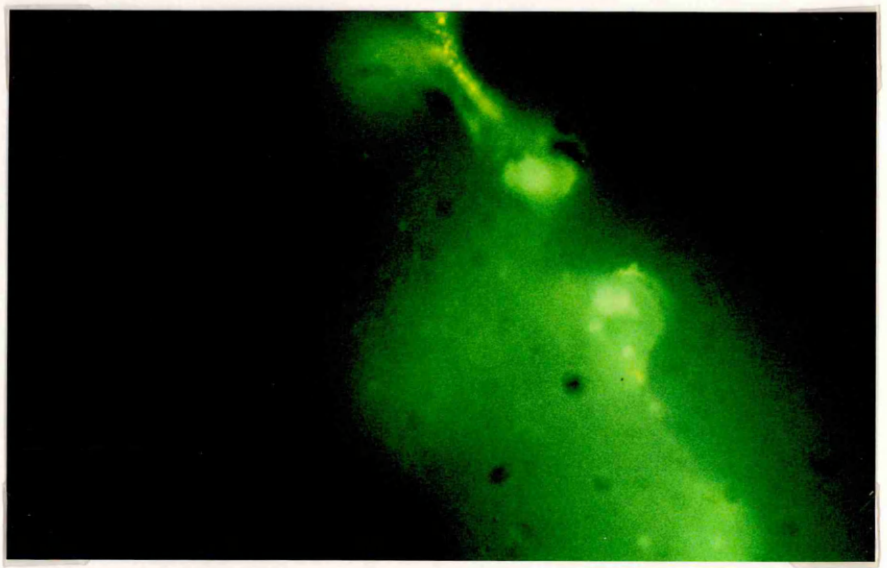

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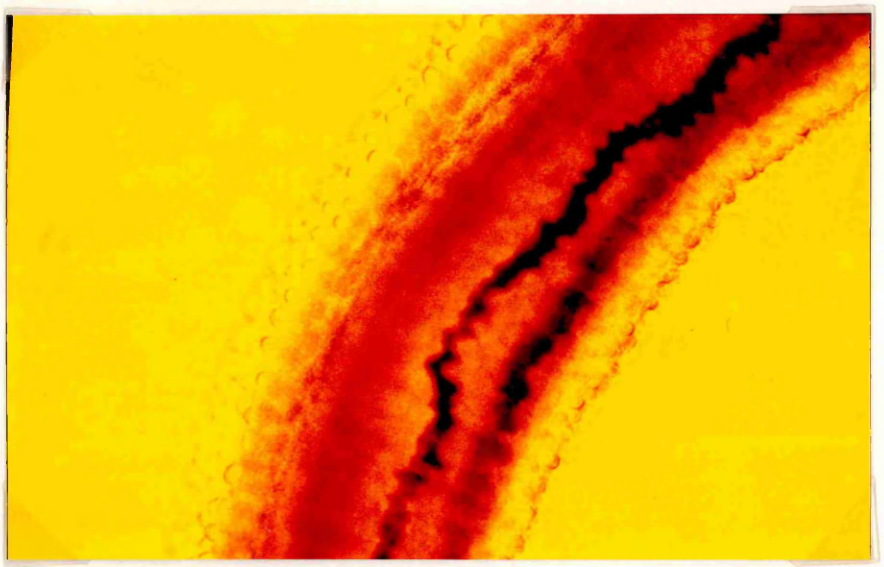
Plates 4.18 & 4.19



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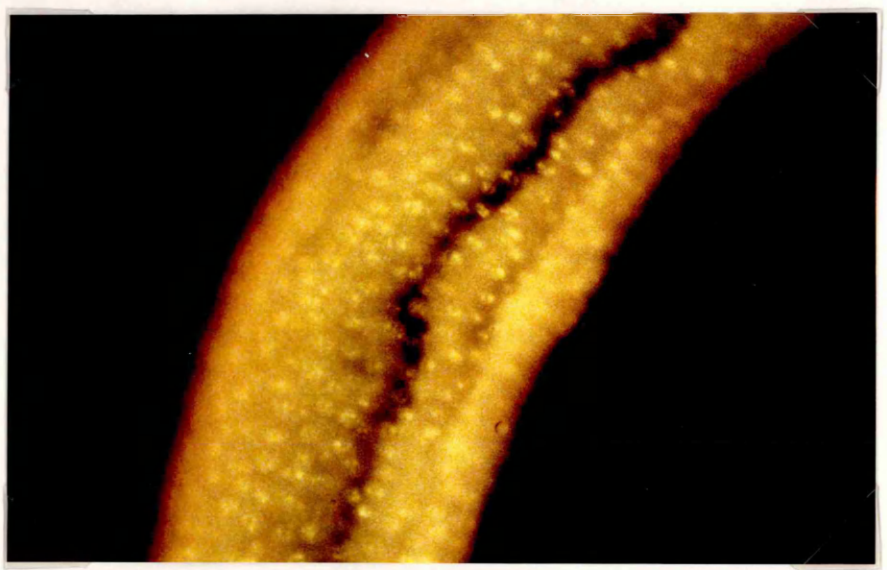

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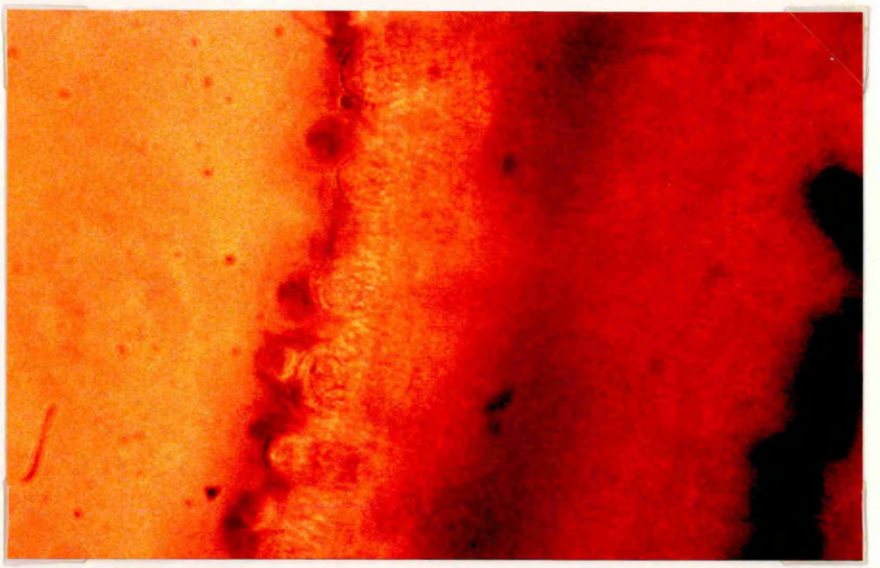
Plates 4.20 to 4.22



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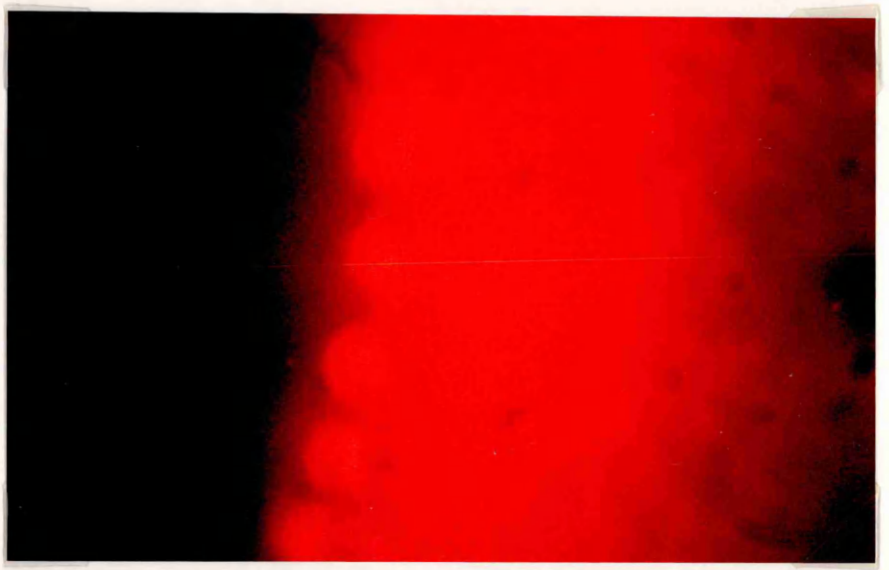

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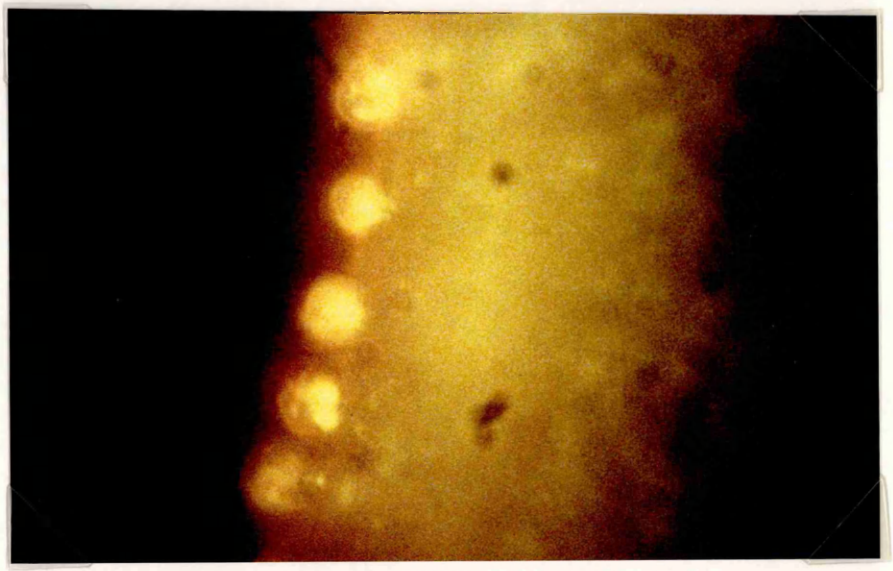

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4.2.3 Summary of results

Prior to discussion the major conclusions and features derived from the above presentation of data will be briefly summarised.

a) LDL will bind to the surface membrane of schistosomula as determined by transfer of fluorescent lipid analogues and H^3 cholesterol, binding of I^{125} labelled LDL and indirect immunofluorescence.

b) LDL binds locally to the tubercles of adult male S. mansoni. This was determined primarily by indirect immunofluorescence and localised labelling of FITC-LDL.

c) Binding of human IgG or Con A to the surface of schistosomula significantly increased the ability of that surface to bind LDL.

d) Transfer of fluorescent lipids and H^3 cholesterol showed saturation at high levels of LDL (100 ug/ml) exposure. This transfer was markedly decreased by the presence of low amounts of serum. However exposure to a dialysable fraction of human serum slightly increased the ability of the parasite's membrane to obtain aminofluorescein from LDL.

e) Binding of I^{125} LDL to schistosomula showed a biphasic pattern remarkably similar to that of human fibroblasts. One component of this binding exhibited saturation comparable to that observed by using aminofluorescein labelled or H^3 cholesterol labelled LDL.

f) The area of membrane overlying the tubercles of the adult male exhibited, relative to other areas of surface membrane, a high affinity for the fluorescent lipid analogue Merocyanine 540.

g) Lipid bodies were observed within the tubercles using several fluorescent lipid probes.

4.3.1 Discussion

Several interrelated features concerning the interaction of LDL with the surface membrane are raised by the information summarised above. This discussion is concerned with examining the nature of LDL binding to the parasite and the means whereby its sterol and lipid contents are transferred to the parasite. Such an examination must be set in the context of the host's cholesterol and lipid, transport and metabolism within the bloodstream.

There are two aspects to this, the first being that this discussion is prompted by observations made in vitro and their extension to the in vivo situation must be subject to some caution. For example, LDL isolated from plasma clearly binds to S. mansoni in vitro however there may well be other lipoproteins competing for binding to the surface in vivo.

The second aspect is that of the specificity of any observation or hypothesis presented here, to the parasite itself. When considering a blood dwelling parasitic organism such as S. mansoni it is pertinent to consider the extent to which the organism could simply utilise the reactions of the host's cholesterol/lipoprotein related metabolism to meet its requirements. This metabolism consists, in essence, of the action of a small number of enzymes on the sterol and lipid constituents of lipoproteins and cell plasma membranes. Indeed the most important of these enzymes, LCAT, will preferentially use cholesterol from cell membranes as a substitute over lipoprotein cholesterol[Fielding & Fielding 1982]. Clearly it is possible that the schistosome membrane could act as a substitute for this or related enzymes. This returns to the question of specificity posed earlier. This is, are the lipoprotein linked metabolic events occurring on the surface membrane of S. mansoni in vivo in any way different? That is, are they qualitatively or

quantitatively different from those occurring on, for example, nearby endothelial or lymphocyte plasma membranes? Such differences would be important as they could form the basis of immunological or pharmacological attack on the parasite's lipid metabolism. Several variations of this strategy have been suggested involving blocking of an LDL receptor immunologically [Rumjanek, McLaren & Smithers 1983] or interfering with the sexual maturation of females by inhibiting cholesterol derived hormone production by the male [Soares, Friche and Rumjanek 1986].

4.3.2 The nature of LDL binding.

The biphasic nature of I^{125} LDL binding indicates that two types of interaction may be occurring at the surface of schistosomula. One mechanism that could explain these observations is if a fraction of LDL (Apo B epitopes) bound to the surface was in some way incorporated into the surface membrane itself. This could occur via the incorporation of whole LDL macromolecules or after some partial degradation which disrupted the integrity of the LDL molecule to some degree, short of lysosomal degradation, leaving either Apoproteins or Apoprotein fragments available to incorporate into the membrane. In either case the sterol and lipid contents of the LDL would be made available to the parasite. This hypothesis is also consistent with the monophasic pattern of labelling obtained with aminofluorescein C18 and H ^{3}cholesterol labelled LDL. The I^{125} labelling method labels the non transferable (Apo B) protein structural components of the LDL the aminofluorescein C18 and cholesterol their relatively easily transferable constituents.

When considering the total amount of label measured on parasites labelled with I^{125}LDL there are two sources: those molecules incorporated into the membrane or; those bound to it. However the label measured on schistosomula labelled with aminofluorescein C18 and cholesterol represents the contribution of probably a small number of

LDL molecules incorporated into the membrane, a larger number bound to it and a very much larger number of labelled molecules that have interacted with the membrane. These would have bound briefly transferred some label then detached. The contribution to the total extent of labelling from the LDL molecules actually incorporated into or bound to the membrane would under these circumstances be relatively small. This could explain the lack of a biphasic pattern of labelling observed when using these molecules to label LDL.

The lack of degradation fragments under these circumstances would then not necessarily reflect a lack of degradation of LDL. Partial degradation leading to incorporation of protein fragments actually into the membrane oculd also explain this observation. Rumjanek has reported the appearance of putative LDL protein bands on SDS-PAGE gels of extracts of Iodogen labelled schistosomula. These proteins are therefore located at the surface of the membrane. They were correlated with the appearance of an LDL receptor but not positively identified as being derived from LDL [Rumjanek, McLaren & Smithers 1983].

This interpretation of the results proposes therefore the partial or complete incorporation of LDL molecules into the membrane. Such a mechanism would provide a method of delivery of LDL sterol and lipids with the added advantage to the parasite of incorporating host antigens into the membrane.
How could an intact LDL particle or a partially degraded lipoprotein containing a cholesterol ester core be incorporated into a lipid bilayer?

There have been recent reports of the demonstration of particles closely resembling lipoproteins occurring within the lipid bilayer of a wide variety of cells [Mountford & Wright]. These structures are inserted within a normal bilayer configuration of phospholipid molecules of the plasma membrane due to the formation of areas of non

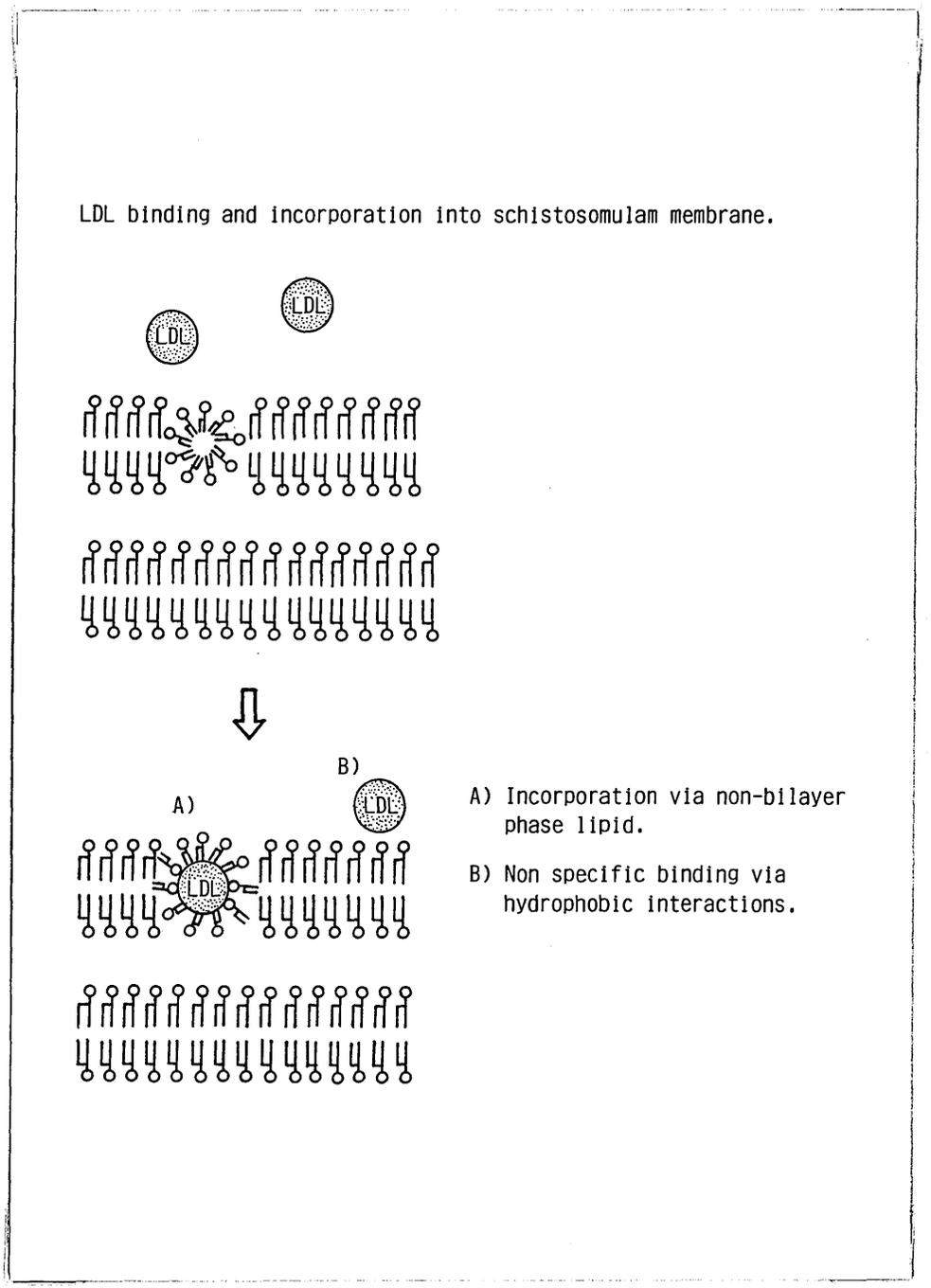
bilayer phase lipid. The occurrence of such non bilayer hexagonal phase (H_{II}) lipid is dependent on the concentration of calcium ions available at the surface and on the lipid composition of membrane. If such areas existed within the parasite surface membrane they could act in effect as large scale "macro" membrane receptors.

Interestingly such non bilayer structures are relative to normal lipid bilayers disordered loosely packed areas of lipid. Thus the observation that the areas of membrane overlying the tubercles, where LDL seems to preferentially locate, preferentially take up a marker of loosely packed lipid, Merocyanine 540 may be significant. Very little is known about the occurrence of non bilayer phase in the schistosome surface membrane. The FRAP studies of Foley indicate a large immobile fraction in the E_1 face of schistosomula but a more mobile E_1 face in adults. As was suggested in chapter 3 the schistosomula outer membrane soon after formation may be highly disordered. Some of this may persist at later points in development and be due to non bilayer phase lipid.

Another means whereby lipoprotein may be partially degraded and incorporated into the schistosomula membrane could be due to the action of lyso-phosphatidyl choline on the LDL. This has been implicated in promoting the fusion of cell membranes, including their integral membrane proteins, with the surface membrane of S. mansoni [Golan et al 1986]. The observation that after incubation in human serum the schistosomula membrane has a high cholesterol ester [Rumjanek & McLaren 1981] content would also be consistent with the incorporation of the cholesterol ester core of LDL into the membrane. This interpretation proposing the hypothesis that LDL is incorporated into the membrane is shown in diagrammatic form in Figure 4.16. Clearly it is a speculative hypothesis. However elements of it could be tested by several means. The first would be to determine conclusively whether the putative LDL bands correlated with

Figure 4.16

Incorporation of LDL into the membrane.



the LDL receptor by Rumjanek actually were derived from LDL. This could be done by immunoblotting with the anti LDL serum used in this study. The suggestion that LDL molecules or partially degraded remnants are incorporated into the membrane could be tested by an NMR study on the appropriate membranes. The lipoprotein like particles reported in cell membranes were discovered by an NMR investigation [Mountford & Wright 1988]. An NMR study of the schistosome surface membrane would be of immense value generally. Several fundamental questions concerning the membrane including; the existence of lipid domains in the plane of the membrane, the composition of the ruthenium red staining carbohydrate layer between the bilayers as well as the incorporation of LDL particles, could be addressed.

4.3.3 LDL binding without incorporation

The properties of the interface between the parasite membrane and the aqueous environment will control the extent of interaction between the membrane and other surfaces such as cells or LDL [Schurch, Gerson & McIver 1981, Gerson 1980]. The adhesiveness of the parasite membrane for LDL will be determined by the surface energies, that is the degree of hydrophobicity or hydrophilicity, of the two contacting surfaces. Hydrophobic surfaces have a high interfacial free energy in an aqueous environment whereas hydrophilic surfaces have a low interfacial free energy. Binding can be seen as an event controlled by thermodynamic considerations. That is binding will occur if the total free energy of the exposed interfaces is reduced after binding and will not occur if the end result would be an increase in the total. The formation of lipid micelles of aminofluorescein C18 in a 1% ethanolic solution or of liposomes after rehydration of dried lipid are good examples of this principle. In each case the formation of these structures minimises the hydrophobicity of the interface by adopting a vesicular or bilayer configuration of lipid molecules. The formation of such structures is energetically favourable.

When considering the energetics of LDL binding to the parasite we are dealing with a similar but not directly comparable situation. The membrane and the LDL molecules individually are already in their most thermodynamically stable form. Both the parasite membrane and the LDL molecules have surfaces exposed to the aqueous environment each of which has an interfacial free energy associated with it. Both LDL and a lipid bilayer have relative to an aqueous environment relatively hydrophobic surfaces. Therefore the binding of LDL to a membrane would be thermodynamically favourable since the total collective interfacial surface energy of all the LDL molecules and the membrane would be reduced upon binding. This would certainly be the case for a lipid bilayer, however the presence of carbohydrate and proteins in cellular and parasite membranes along with accumulation of macromolecules of an extracellular matrix such as fibronectin or glycosaminoglycans can alter this degree of hydrophobicity. In the case of schistosomula cultured in a defined media for 4.5 hours as used in these experiments there would not be for example a thick glycocalyx derived from membrane proteins. Some glycocalyx elements may have been retained from the cercarial membrane. Overall the surface presented would be fairly hydrophobic. The avid uniform binding of LDL to these schistosomula observed is therefore readily explained.

In contrast, the dorsal surface of the male has associated with it a thick glycocalyx consisting of carbohydrate which endows the parasite with a relatively hydrophilic surface similar to gram negative bacteria [Podesta et al 1987]. These authors have suggested that this surface would inhibit the adhesiveness of cells on energetic grounds and the same principle would apply to hydrophobic molecules such as LDL. It would be interesting to know if this glycocalyx was uniformly thick at the membrane over the tubercles. One would expect from the localised binding of

LDL in the adults that it was not. This author also reported developmentally linked changes in the thickness of this glycocalyx on the ventral surfaces of the male and the dorsal surface of the female which mediated pairing. This implies that variation in the properties of the membrane can occur. Therefore it is not unreasonable to propose localised variation in the dorsal surface itself. Studies monitoring the mobility of fluorescent lipids on the adult surface [Foley 1986] and the absolute levels of dye uptake have repeatedly shown large scale variation along the length of the adult male dorsal surface. Therefore the patterns of LDL binding exhibited by each stage of the parasite are most simply explained by proposing that whereas schistosomula expose a fairly uniform glycocalyx free, hydrophobic membrane surface which promotes LDL binding such a surface is only locally exposed at the tubercles on the adult dorsal surface. (See Figs 4.17 & 4.18)

Figure 4.17

LDL binding to the schistosomula membrane.

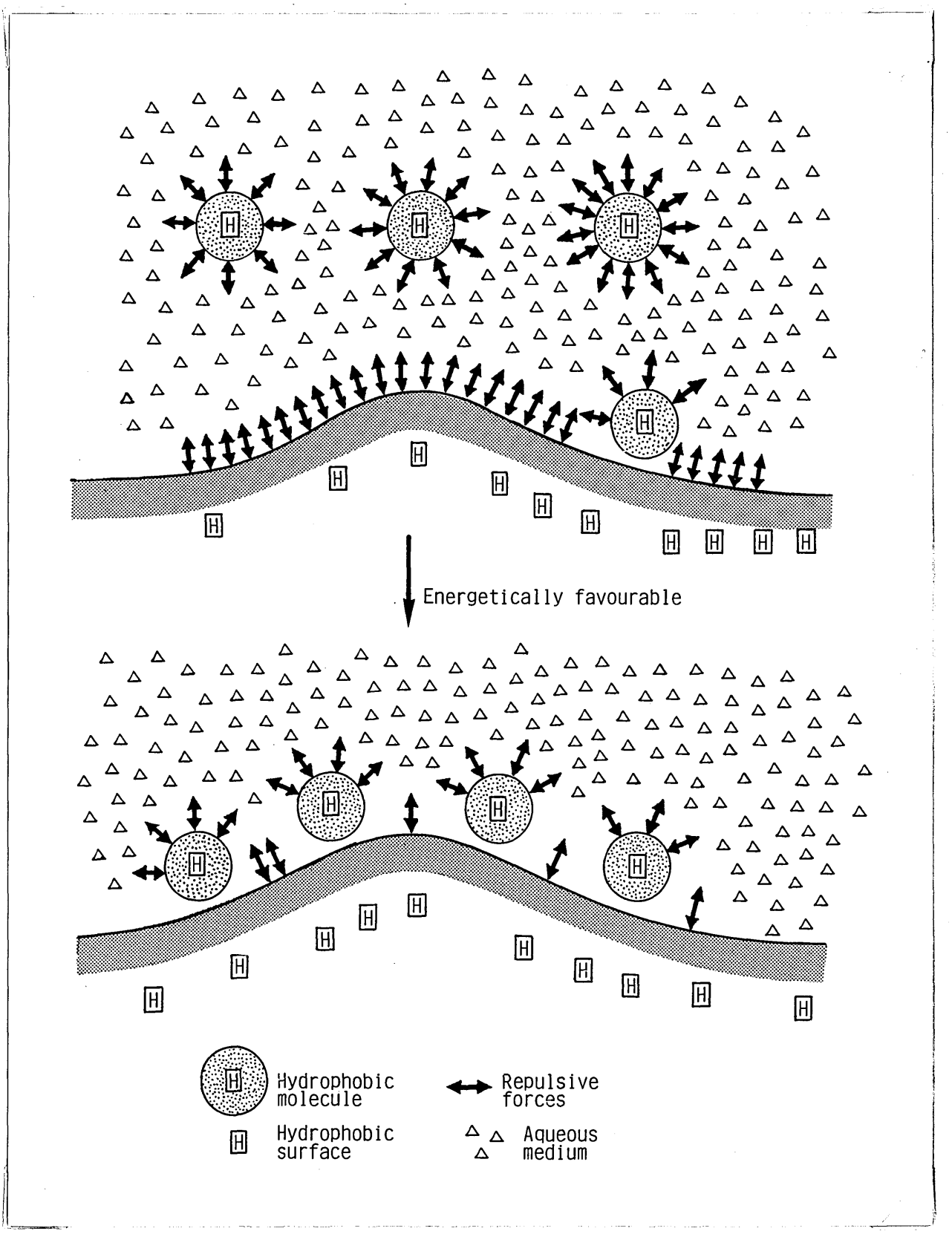
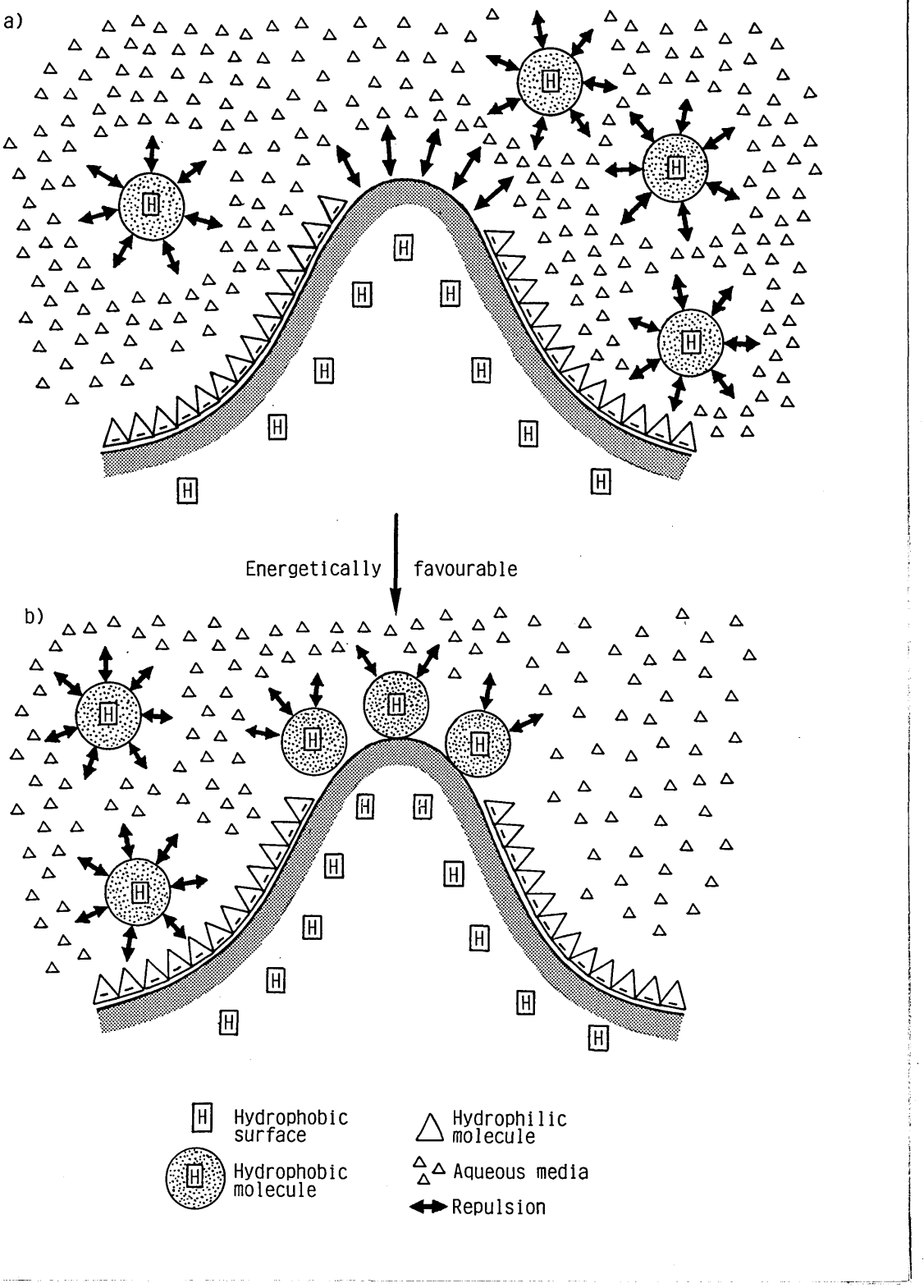


Figure 4.18

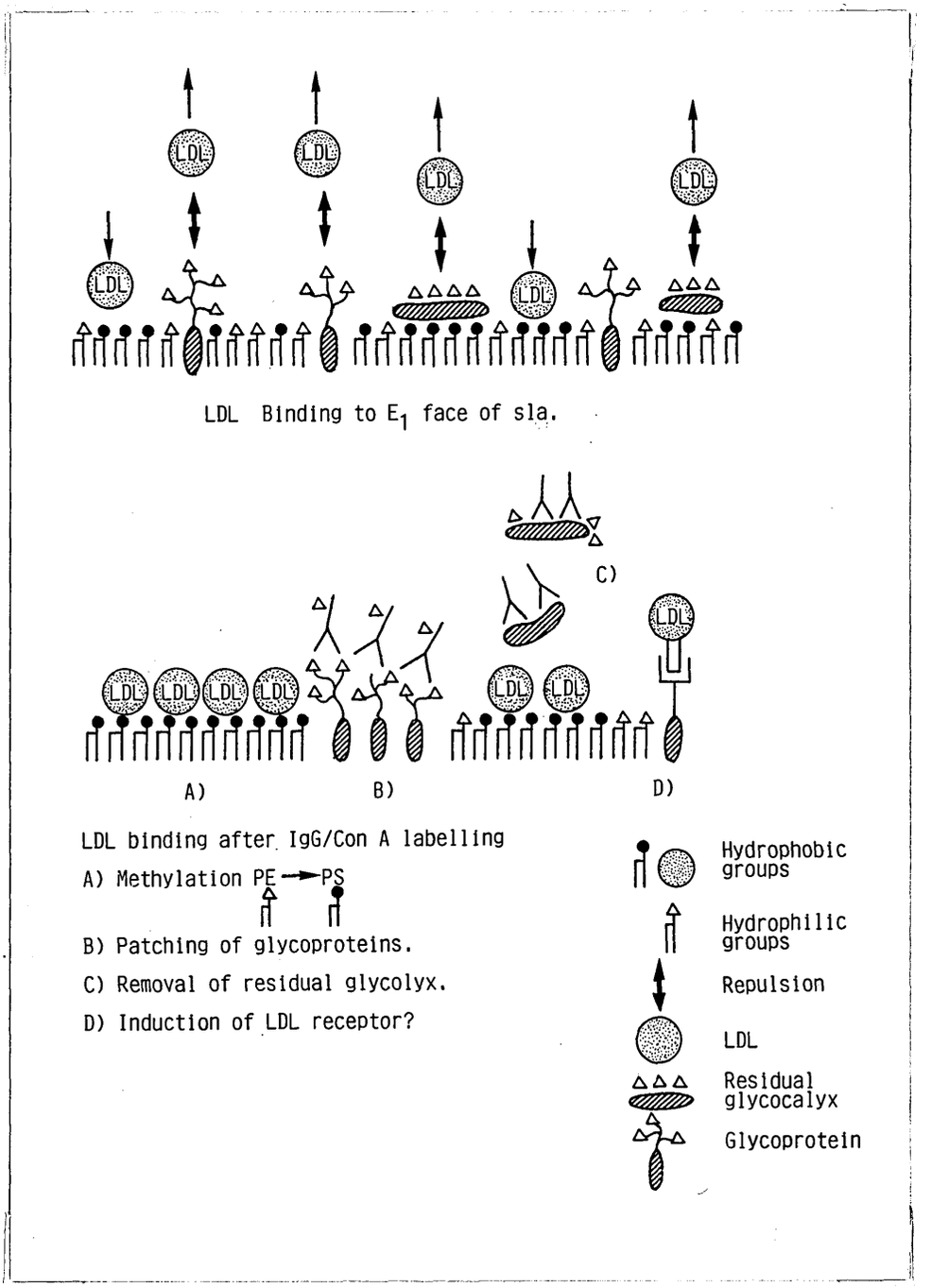
LDL binding to the adult membrane at the tubercle.



Does this explanation hold good for the IgG and Con A effect on LDL binding to schistosomula reported above? the direct effect of molecules of lectin acid and immunoglobin binding at the surface could be to increase the hydrophilicity of the surface thereby decreasing LDL binding. They also however have indirect effects which could increase hydrophobicity enough to counterbalance this direct effect and produce a net increase in hydrophobicity. Capping or patching of both integral membrane proteins and cercarial glycocalyx elements could in effect clear the surface of carbohydrate hydrophilic molecules. This could be promoted by both Con A and IgG which are divalent molecules. In addition Con A has been reported as promoting methylation of membrane PE to PS which would also render the membrane more hydrophobic [Parra et al, 1986]. Another possible indirect effect would be if binding of Con A or IgG at the surface induced the intra-membrane or transmembrane stimulus for the induction of the LDL receptor reported by Rumjanek, Mclaren & Smithers 1983. These various possibilities are shown in diagrammatic form in Figure 4.19. The mechanism underlying this Con A/IgG effect remains to be elucidated. The observation itself, particularly concerning Con A, may be another aspect of how the binding of potentially damaging agents to the membrane appears to promote increased resistance of the membrane [van Pijkeren, Tavares & Gazzinelli 1982].

Figure 4.19

The possible effects of IgG & Con A binding on LDL binding.



4.3.4. Biological significance of LDL binding patterns on schistosomula and adults.

What is the explanation for the difference in the pattern of LDL binding between the adult stage and the schistosomula stage of the parasite? Neither is able to synthesise its own long chain fatty acid or sterols, therefore both must derive them from the host. The schistosomula at three hours is changing rapidly, its lipid metabolism is relatively high and it is actively acquiring host molecules. It's gut is not functional therefore acquisition of nutrients must occur via the tegument. It is also developing some type of "intrinsic" immune resistance. Under these conditions it makes sense that the parasite would avidly bind lipoproteins to its surface to satisfy its nutritive requirements to acquire host antigens and possibly to contribute to immune resistance. Acquisition of cholesterol and cholesterol esters by schistosomula has been correlated to the development of resistance to attack by eosinophil mediated ADCC [Rumjanek and McLaren 1981].

The adult parasite exhibits important differences. Firstly it has a functional gut which contributes to its nutrition. Its lipid metabolism in the adult male is relatively lower than the schistosomula although the female has a requirement for cholesterol and its derivatives for egg production and female maturation. [Soares, Friche & Rumjanek 1986]. Its membrane appears to have lost the intrinsic resistance characteristic of earlier stages and to be more dependent on presenting a disguised [McLaren & Terry 1982] or inert face to the host [Podesta 1987]. The biophysical properties of its outer bilayer have changed becoming more fluid [Foley 1986] and the double bilayer is generally more easily damaged by a number of agents including bacterial toxin [Ruppel, Breternitz & Lutz 1987] and lectins [Simpson & McLaren 1982]. It appears as though the integrity of the adult double membrane is crucially dependent on an interface of "inert" host antigens and

parasite glycocalyx to protect an underlying membrane that is relative to the schistsomula susceptible to damage. Clearly the ability of LDL to freely interact with the adult membrane would prejudice its integrity because such a property would also allow interaction of the immune system particularly its cellular arm. In these circumstances it is not surprising that LDL does not interact very much with the bulk of the surface, rather the existence of apparently localised interaction at the tubercles appears to be the case (Fig 4.18).

The above discussion implies that interaction between the parasite membrane and host molecules for nutritive purposes in the adult is inimical to the ability of the membrane to resist immune attack. This does not appear to be the case to the same extent in the lung worm. This difference may be accounted for by some intrinsic resistance of the lung worm membrane lost by the adult. Assuming that opening up the adult membrane to nutritive molecules such as lipoproteins renders that area of membrane susceptible to immune attack how would the parasite carry out some perhaps low level but essential uptake of nutrients or equally excretion of essential hormones.

One solution could be to develop specialised areas of membrane adapted for interaction that could either be shielded by some means other than the passive protection of an inert layer and that if damaged could be shed quickly and replaced. The tubercles on the adult male of S. mansoni may well have such a role and the following section is devoted to exploring this hypothesis.

4.4.1 The function of the tubercles?

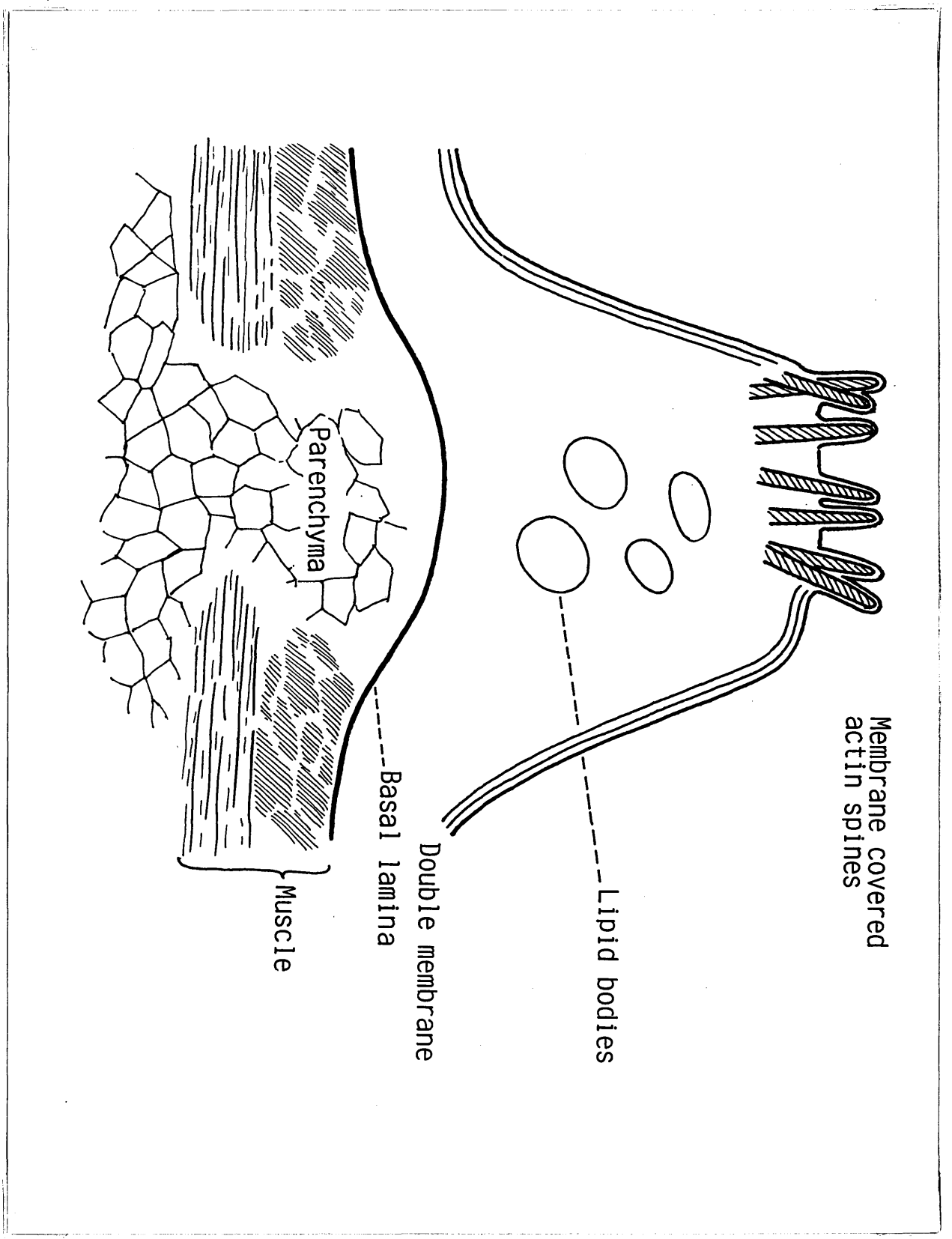
The biological function of the tubercles on adult male S. mansoni parasites is unclear. It has been suggested that the spines serve to anchor the parasite to the endothelium. However the parasite is endowed with two large suckers on its head for this purpose. There have been reports of

shedding of protein complexes from the membrane overlying the tubercle spines [Wilson & Barnes 1977] also of release of lipid droplets through the membrane at this region [Haseeb et al 1985]. These two observations might suggest that the tubercle functions as a specialised region responsible for the shedding of potentially damaging proteins from the membrane or lipid soluble hormones from the tegument.

Localised accumulations of glycogen and neutral lipids within the tegument of the tubercle have been reported in older histological studies [Smith et al 1969]. In addition it is known that the outer membrane can transport ions and sugars across it [Podesta 1983]. These studies support the concept that the tubercle could function as a specialised region of absorption of nutrients within the membrane. There are other reasons concerned with the morphology of the tubercle that suggest that it could be an adaptation concerned with trans-membrane transport. These are shown in diagrammatic form in Figure 4.20 and summarised below;

a) The protuding shape and the presence of the spines increase the surface area of this region and would be expected to increase the efficacy of any transmembrane transport occurring.
b) The tubercle has direct access to the parenchymal cells of the parasite due to the absence of intervening muscle layers immediately underlying it.
c) There have been reports of shedding of damaged membrane from the tips of the actin spines within the tubercle [Wilson & Barnes 1977]. This may endow the tubercle membrane with a means of damage limitation in that if the mechanism of transport exposed the membrane to damage it could be rapidly shed and replaced.
d) The localised binding of Merocyanine indicates that the tubercle is overlaid with a relatively loosely packed lipid membrane. One reason for this could be a locally low level of membrane cholesterol.

Figure 4.20
The structure of the tubercle.



The observations reported in this study, namely localised binding of LDL containing cholesterol esters at one side of a loosely ordered possibly cholesterol poor membrane with an accumulation of neutral lipids probably cholesterol esters on the other side is consistent with the hypothesis that localised transport of cholesterol is occurring at the tubercles. This could come about by a sequence of events involving hydrolysis of cholesterol esters of LDL on the external face of the membrane, the insertion of free cholesterol liberated into a cholesterol deficient membrane, transfer of cholesterol across this double membrane and re-esterification into lipid droplets at the other side. How could such events occur?

Cholesterol is known to flip flop rapidly between the leaflets of cell plasma membranes [Lange & Steck 1985]. Unless the carbohydrate layer that is known to exist between the two bilayers in S. mansoni exerted some inhibitory effect it is reasonable to assume that cholesterol entering the E_1 face of the schistosome double membrane would equilibrate between the four leaflets. The action of a tegumental acyl transferase to re-esterify such cholesterol the tegument could result in a net transfer of cholesterol and keep the E_1 face relatively cholesterol poor. Thus a chemical potential gradient may exist at the parasite surface and mediate net transport of cholesterol from plasma to parasite. Recently, a protein of 27,000 mol weight with esterase activity which is localised at the tubercle has been reported [Doenhoff, Modha and Lambertucci 1988]. This protein antigen was detected by indirect immunofluorescence only after treatment of the parasites with Praziquantel which is known to induce damage to the membrane. Therefore this protein could represent a surface antigen not normally accessible to IgG at the tubercle membrane surface or alternatively it could be a tegumental enzyme. This antigen is of importance because it has been implicated as a target antigen responsible for the immune

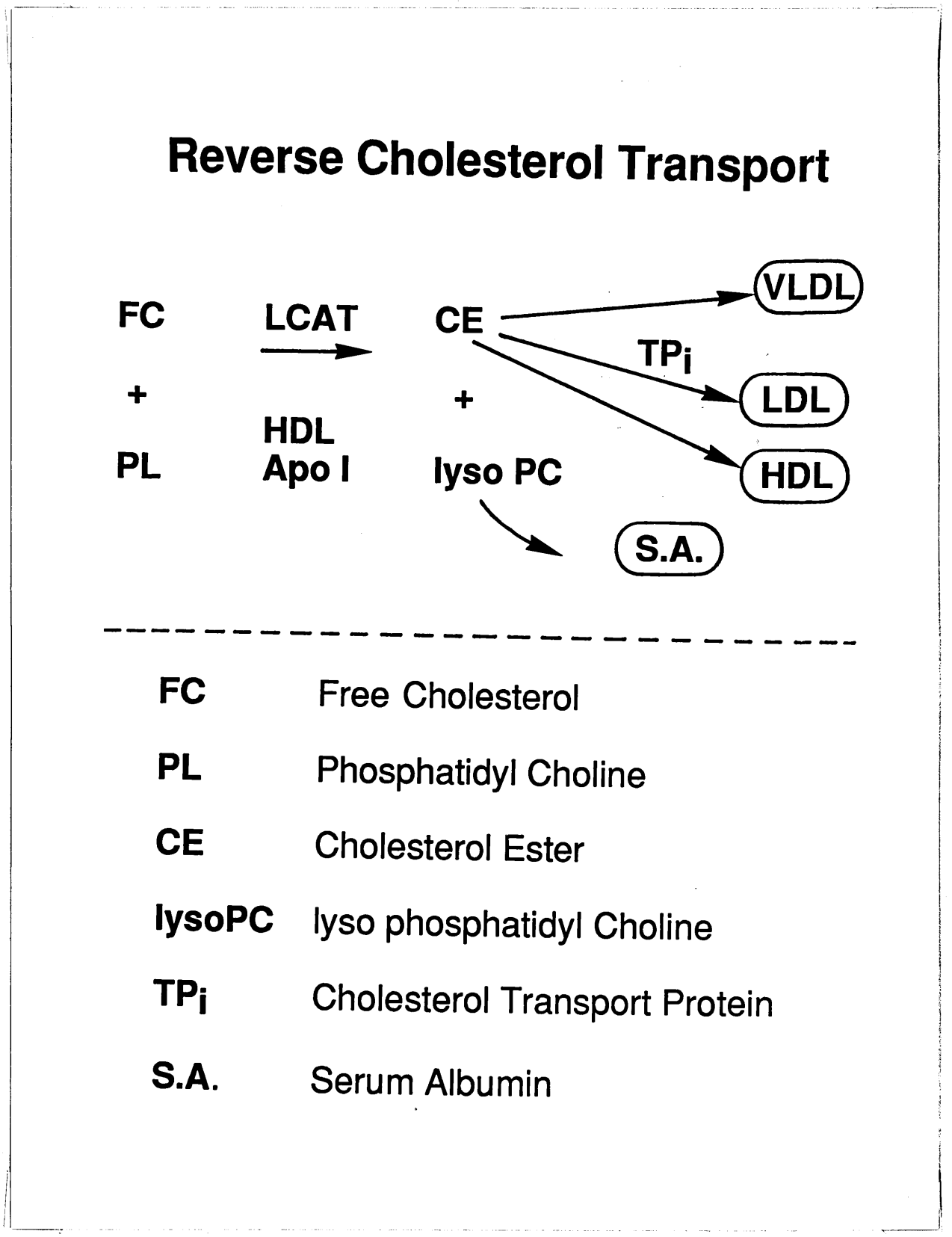
dependence of Praziquantel. It has been identified on the basis of it's non specific esterase activity. Clearly this enzyme represents a promising candidate for the role of tegumental acyl transferase suggested above. This depends on determination of it's physical location in the tubercle.

This leaves the question of how free cholesterol could be generated on the external surface. Two possibilities exist, either the parasite could produce its own endogenous cholesterol esterase at the surface, again the protein reported by Doenhoff et al is a candidate. A more likely alternative given the organism's parasitic lifestyle is that it utilises the action of the enzyme, LCAT, which controls the host's cholesterol transport and balance between lipoproteins and cell membranes.

Lecithin choline acyl transferase, LCAT, is a plasma enzyme bound to a specific fraction of "small" Apo I bearing HDL that is responsible for the phenomenon of reverse cholesterol transport. Most cells in the body have a net positive balance of cholesterol. This excess cholesterol is redirected back to the liver for catabolism, via LDL and HDL. This is due to the action of LCAT which having a preference for cholesterol from membrane catalyses the esterification of free cholesterol in the membrane to cholesterol ester. The cholesterol ester so produced is transferred to the LDL and VLDL via cholesterol ester transfer proteins [Fielding & Fielding 1983].

There is reason to believe that this reaction which normally goes to the right in the plasma (see Figure 4.21) could locally at the surface of the tubercle go to the left. This is because in this region it is probable that one of the products of the reaction lyso PC is locally high in concentration whereas the substrate free cholesterol is low. The parasite has a net requirement for cholesterol so its availability at the membrane surface, assuming the operation of some pathway tegumental esterification such as

Figure 4.21
Reverse cholesterol transport.



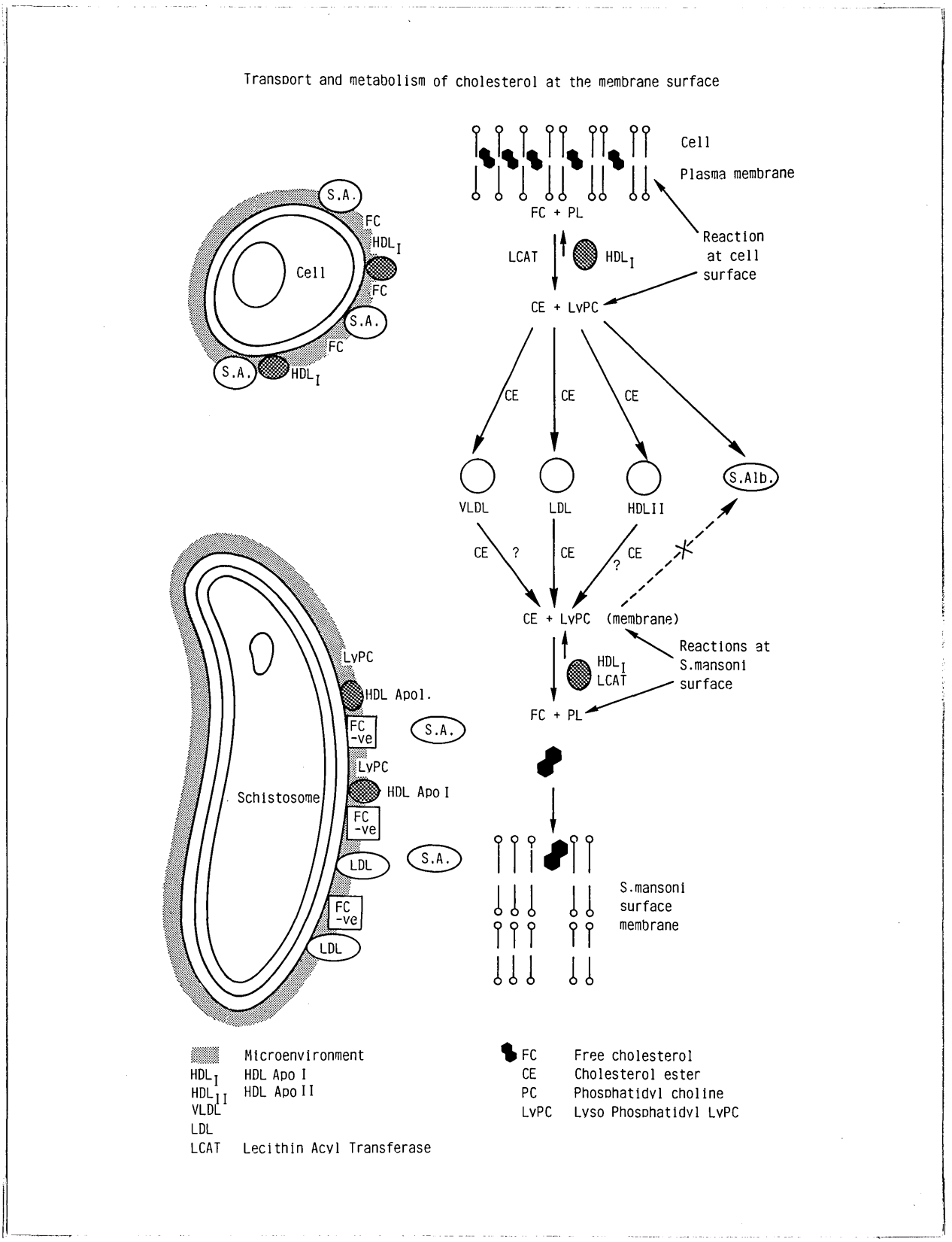
suggested above, will be low. Lyso PC on the other hand may be locally high since it has been reported that lyso PC is produced and shed into the aqueous media by schistosomula [Golan et al 1986] and is also present in the adult [Furlong et al 1988]. Furthermore serum albumin, which removes lyso PC formed after esterification of free cholesterol by the LCAT HDL Apo I complex, does not bind to the surface of S. mansoni [Sher, Hall & Vadas 1978]. Clearly in these circumstances the reaction could go in reverse. Cholesterol ester from LDL or VLDL near the surface could be hydrolysed to free cholesterol which partitions into the membrane as would the phosphatidyl choline the product of lyso PC from the membrane and the free fatty acids from the ester. This would provide therefore a means whereby the parasite could acquire both exogenous cholesterol and long chain fatty acid. This hypothesis is shown in diagrammatic form in Figure 4.22.

This mechanism could operate at either the schistosomula or the adult membrane. It may be particularly suited to the adult however since localised transfer of cholesterol and fatty acid could take place literally under the cover of non-immunological host molecules. It does imply an additional role for lyso PC to that suggested by Golan et al [1986], namely as a local modulator of the hosts cholesterol metabolism in a localised microenvironment on the parasite's surface.

This concept of microenvironment is important in this respect. The unstirred layer of aqueous solution which lies immediately adjacent to any membrane represents an environment where diffusion of molecules is intrinsically an order of magnitude less that in the bulk of the aqueous media and this inhibition may be further enhanced due to the effects of macromolecular structures external to the membrane itself [Kusel & Gordon 1989]. In other words a parasite conditioned "microenviroment" exists at the membrane surface.

Figure 4.22

Cholesterol transport within a parasite microenviroment.



This would allow localised changes in concentration of lyso PC and cholesterol to occur. Interestingly one of the reasons that Apo I HDL is thought to efficiently catalyse LCAT activity is that its small size allows it to adhere closely to the cell membrane and enter to a greater extent than other lipoproteins this diffusion boundary layer. Clearly this ability in the context of a lyso PC rich, cholesterol poor microenvironment surrounding the parasite is significant.

There are then grounds for believing that free cholesterol could be liberated from lipoproteins due to the action of LCAT at the membrane surface of S. mansoni, that this would flip-flop between the leaflets of the membrane and therefore be made available for re-esterification in the tegument. Whether net transfer of cholesterol into the lipid bodies of the tegument occurs by this type of mechanism remains to be determined.

Haseeb et al [1985] has reported that the contents of these lipid bodies are transported through the double membrane in vitro by E.M. and has provided evidence to suggest that this occurs in vivo also. He postulated that such lipids could act as carriers for lipid soluble hormones which could act as a signal to control male to female pairing. This being the case the scheme outlined above and shown in Figure 4.22 may act to incorporate exogenous cholesterol and free fatty acids into the outer membrane only. The rest of the parasites exogenous sterol and lipid requirements could be derived from the metabolism of the membranes of erythrocytes or lipoproteins ingested by the gut.

The significance of the loosely ordered lipid membrane on the tubercle is not clear. It could be either a feature mediating efficient uptake of nutrients such as cholesterol or a consequence of lipid excreted across the membrane.

It is possible that absorption of lipid and sterol into the membrane could coexist with excretion of lipid droplets through it. However net transfer of cholesterol into the lipid bodies as postulated in Figure 4.22, along with excretion of lipid derived from it seems unlikely . This could only occur if the lipid bodies themselves were differentiated into separate compartments one of which accumulated exogenous lipid and the other provided the source of lipid for excretion. Haseeb has provided evidence for both processes [Haseeb, Eveland & Fried 1985 Haseeb et al 1985]. The former paper showed evidence that radiolabelled cholesterol applied to the aqueous media surrounding the adult S. mansoni accumulated within the tegument particularly at the tubercles. Significantly the radiolabelled cholesterol was applied in a media containing serum therefore it is likely that much of this cholesterol would locate in lipoproteins within this serum. The accumulation of this label in the tubercles may reflect the prior localised binding of LDL at the tubercles before transport of their contents. On the other hand the latter paper [Haseeb et al 1985] showed evidence for transfer of the contents of the lipid bodies through the membrane and into the external environment.

In the former paper radiolabelled cholesterol accumulated in the tubercles. Given the hypothesis advanced above, the accumulation of C^{14}-cholesterol could reflect the accumulation of cholesterol in esters within lipid bodies in the tubercles. The question of whether net transfer of lipid via the tegument takes place and supplies the parasite with a significant part of its lipid and sterol requirements, as postulated in Figure 4.22, is dependent on the contribution of the gut to these requirements. It is not known to what extent, if any, cholesterol and lipids are metabolised from erythrocytes and lipoproteins digested in the gut. The whole field of lipid transport or traffic in cell biology is relatively new and is only now emerging

from the more developed field of protein trafficking. Fluorescent lipid analogues are widely exploited in this area; therefore the same techniques could be applied to parasites.

At present the experimental evidence from electron microscopy and Haseeb's studies of the lipid bodies suggest that movement of lipid in the tegument of S. mansoni is all one way namely externally towards the surface membrane. However the sheer size of the organism and the presence of a rich source of lipid nutrients bathing the surface membrane raises the possibility that the lipid and sterol requirements of the tegumental syncytium may be most efficiently met by absorption through the immediately adjacent outer membrane rather than absorption then transport from the gut. The answering of such basic fundamental questions concerning how and where the parasite gets its exogenous lipid in vivo must be of importance particularly to further developments in chemotherapy.

4.4.2 Cholesterol transport and the surface membrane

The types of interactions between the host's lipoprotein metabolism and the parasite membrane are important even if it is eventually found that the lipid requirements of the tegument are met totally by gut absorbed nutrients. This is because this lipoprotein related metabolism of the blood plasma will undoubtedly affect the properties of the membrane itself. That is lipids and sterols will be introduced to or taken from the parasite's membrane due to the action of lipoproteins and associated enzymes. There is clear evidence from studies in which schistosomula were incubated in sera of various kinds that the lipid composition of the membrane is altered by such a mechanism [Rumjanek & McLaren 1981]. In addition biophysical studies employing FRAP have indicated that incubation in serum alters the properties of the membrane [Foley 1986]. In the former study by Rumjanek the acquisition of cholesterol and

cholesterol esters by schistosomula after incubation in human serum was correlated to increased resistance to ADCC while loss of diglycerides to foetal calf serum was correlated to increased resistance to lethal antibody. It is not clear how such foetal calf serum induced differences in lipid composition affect the biophysical properties of the membrane and how in turn such biophysical changes mediate resistance. Increased levels of cholesterol and cholesterol esters in the surface membrane after incubation in human serum is interesting since it may reflect either incorporation of LDL into the membrane or of cholesterol and cholesterol esters derived from it. Parallels have been drawn between certain immunologically resistant tumour cell lines which have a high cholesterol to phospholipid ratio [Ohanian, Schlager & Saha 1982] and the surface membrane of schistosomula [Rumjanek & McLaren 1981]. How the presence of such high sterol content could inhibit cellular attack is unclear but two possibilities can be advanced. The first is the direct transfer of these sterols to the membrane of the attacking immune effector cells such as macrophages, which inhibits their action [Chapman & Hibbs 1977]. The second is that the presence of such sterols renders the membrane more resistant to attack. The presence of increased cholesterol itself will modulate the bulk phase or general properties of the membrane [Yeagle 1985]. However the acquisition of large amounts of exogenous sterol and sterol esters may cause the formation of cholesterol rich domains within the surface membrane. This would have the effect that the biophysical properties of the membrane will become more heterogenous over its surface area. One effect of this differentiation of the membrane into heterogeneous environments or domains may be to spatially segregate elements of the immune response, such as parasite antigens thereby inhibiting the efficacy of immune recognition and attack. This concept of the differentiation of the schistosome surface membrane into lipid domains will be the subject of chapter 6.

4.5.1 Summary of Discussion

This discussion has been concerned with explaining the experimental observations (summarised in section 4.2.3) of this study and relating these to the wider issues of the interaction of the schistosome with the host's cholesterol and lipoprotein metabolism and also to the properties of the surface membrane in particular the function of the tubercle.

Two factors are proposed to explain the nature of LDL interactions observed. These were the non-specific interaction of the LDL with the parasite membrane governed by hydrophobic surface energy dependant interactions. Secondly that LDL or LDL remnants could become incorporated into the membrane.

It is proposed that transfer of cholesterol from LDL cholesterol ester occurs via the "reverse" action of HDL bound LCAT within an aqueous microenvironment immediately adjacent to the parasite surface membrane. It was also proposed that in the adult male stage this microenvironment was localised at the tubercles and that this mediated a specialised nutrient gathering role of the tubercle.

CHAPTER 5

The effects of cooling the surface membrane of S. mansoni

5.1 The Adult Membrane

5.1.1 Introduction

The concept of investigating the nature of the surface membrane by subjecting it to low temperatures (4°C - 10°) appears to be rather paradoxical. Nevertheless a number of studies on the effect of cooling schistosomula or adults, labelled with aminofluorescein C18 or DiI C18, have revealed some striking changes in membrane morphology associated with cooling. Clearly these observations are artefacts in the sense that in vivo the parasite will never be exposed to such conditions. However observing the way in which the membrane morphology reacts to such low temperatures has proved to be an informative and novel approach.

Storage of biological samples at 4°C is routine in cell biology. Manipulation of cellular or membrane function can be achieved in this manner. This is particularly true of studies, involving fluorescent lipid probes, on lipid trafficking within epithelial cells. Labelling at 4°C allows the apical membrane of the epithelial cell monolayer to be labelled. This provides a convenient starting point for following subsequent lipid trafficking by microscopy. On warming the lipids redistribute within the cell into various membrane compartments characteristic of that particular lipid. This approach has allowed the successful mapping of intracellular transport of important constituents of membranes such as sphingomyelin and gangliosides [Lipsky & Pagano 1985].

Used in this way low temperatures are exploited as an inhibitor of cellular metabolism and function. A more common use is in membrane biology, whereby investigation of membrane transition temperature is carried out as a

means of indirectly investigating membrane composition and biophysical structure. The best example of this is the Arrenhius plot in which a membrane function (in this case an enzymatic activity) is monitored while temperature is varied between, typically, 6°C and 37°C.

The observations described in the second part of this chapter, pertaining to schistosomula, stemmed from attempts to carry out "Arrenhius plot" type of experiments. However in the adults dramatic changes in the morphology of the surface membrane were also observed upon cooling of parasites labelled with aminofluorescein C18 or rhodamine C18 lipid analogues. This was a result of a fortuitous observation.

5.1.2 Changes in the morphology of the adult membrane upon cooling

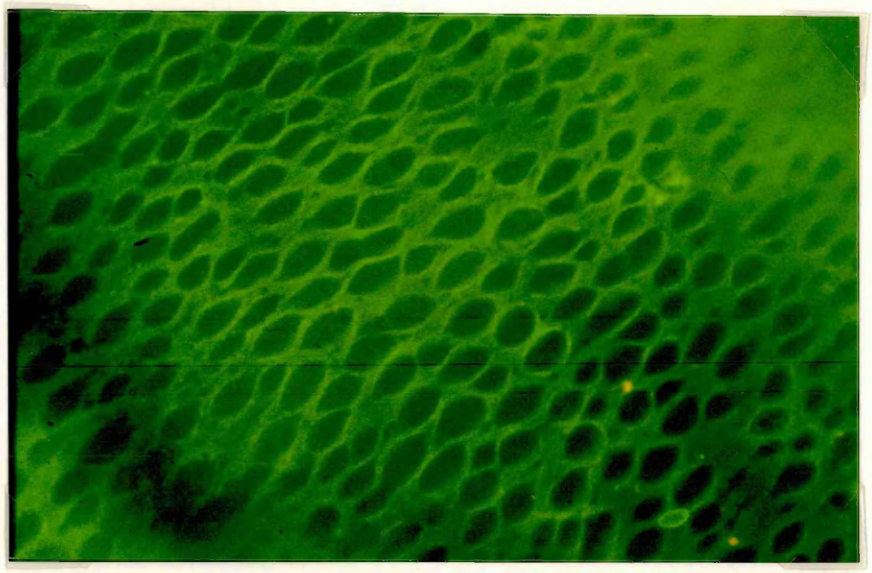
Adult S. mansoni labelled with aminofluorescein C18 or rhodamine C18 lipid analogues and observed at 37°C or room temperature exhibit a surface membrane morphology similar to that of Plate 5.1. However if such labelled parasites are kept at 4°C for several hours and then observed at low temperatures the surface morphology is markedly different. (Plates 5.2 - 5.4) The same effect can be produced with the label rhodamine C18 (Plates 5.5 & 5.6). It has a striped appearance due to the intercalation of folded "tubercle rich" areas of membrane with "smooth" non-folded regions. These two types of membrane morphology will be termed respectively "stripes" and "gaps" and the process itself "striping". This pattern of overall "striped" morphology will only be retained so long as the parasite is observed at low temperatures (10°C). Cooled parasites exhibiting such a striped appearance lose these features when rewarmed to room temperature or 37°C. That is the process is reversible and causes no damage to the parasite. Significantly parasites labelled with DiI C18 and subject to the same cooling and rewarming procedure show extensive

damage to the membrane (Plate 5.7 & 5.8). This damage is due to the effects of cooling and rewarming rather than simply due to the dye itself because it is possible to achieve a "normal" unstriped undamaged pattern of labelling with DiI C18 at room temperature or 37°C.

Two further points can be made concerning "striped" labelling by aminofluorescein C18 and rhodamine C18. Firstly there is great variation in the extent to which the phenomenon occurs. After three hours cold (4°C) incubation of labelled adult parasites in EMS some of the parasites will exhibit striping to a greater or lesser extent. After overnight incubation the phenomenon is marked in all parasites but again the extent is variable. Interestingly pre-incubation of the parasite with serum inhibits the rate of "stripe" formation but appears to enhance the eventual contrast between folded and non-folded regions. Generally this contrast is greatest at the anterior or head region of the parasite, so much so, that it has been possible to observe similar cold induced striping on adult males labelled with FITC WGA rather than fluorescent lipid (Plate 5.9).

List of Plates

Plates 5.1 & 5.2



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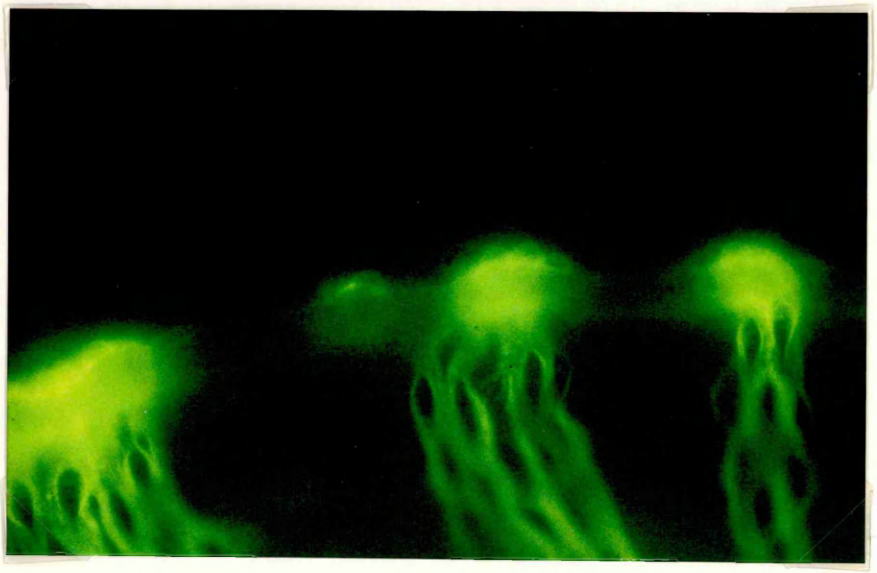

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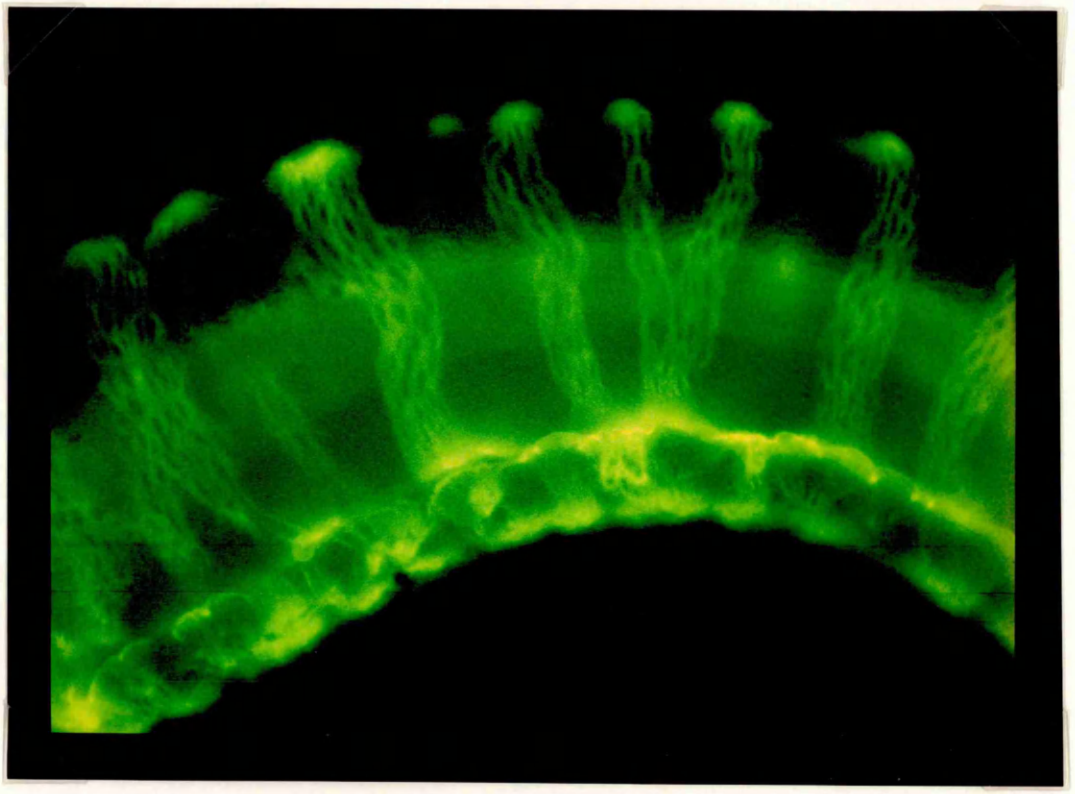
Plates 5.3 & 5.4



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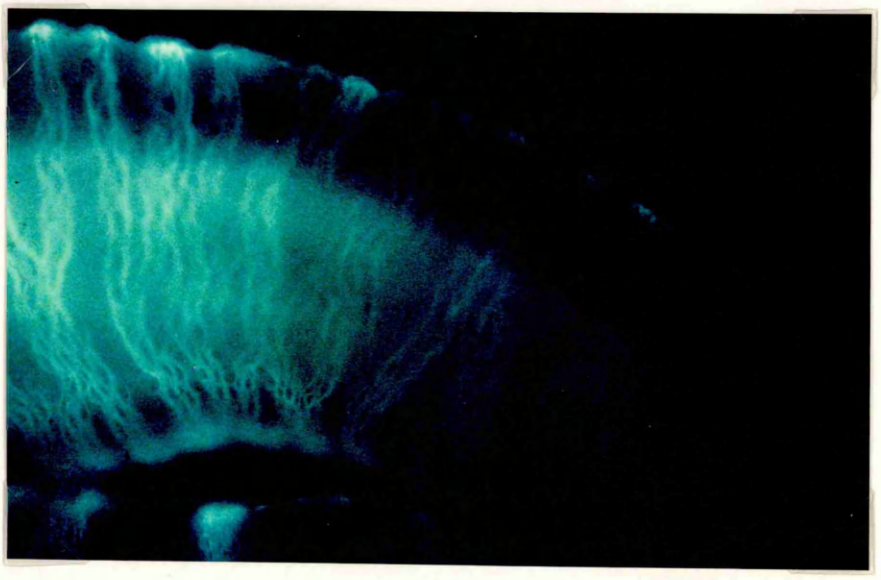

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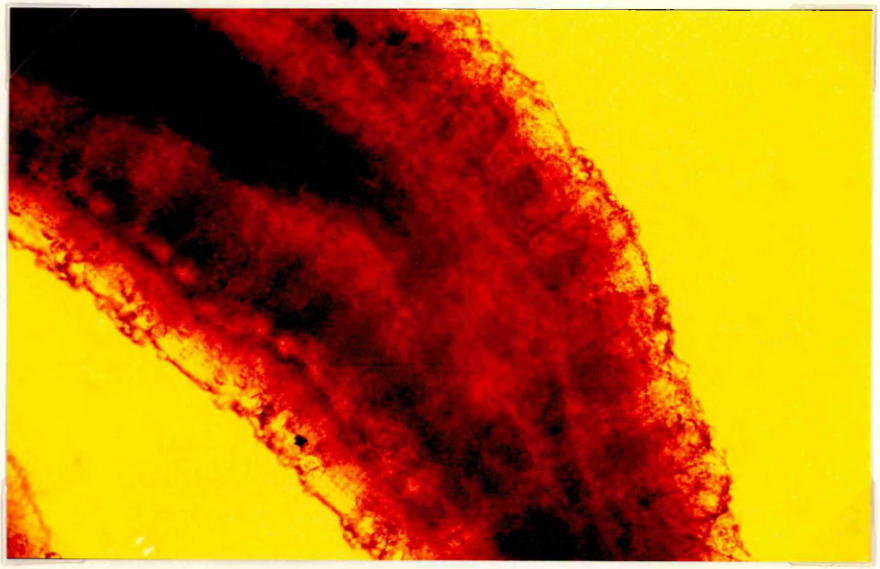
Plates 5.5 & 5.6



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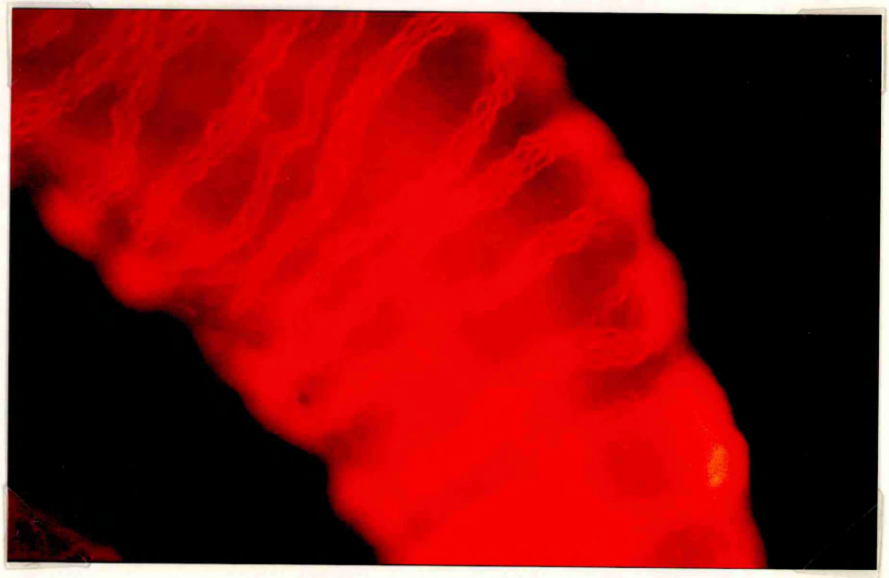

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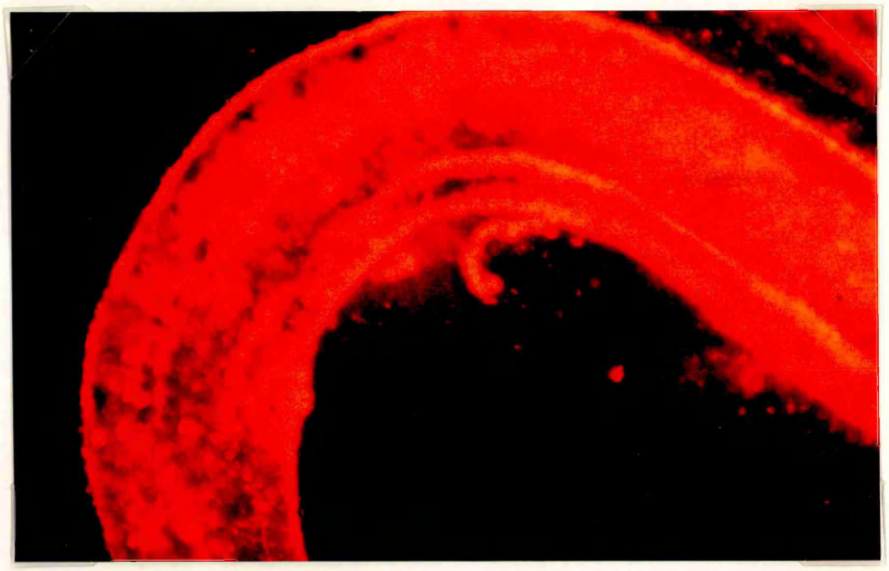
Plates 5.7 & 5.8



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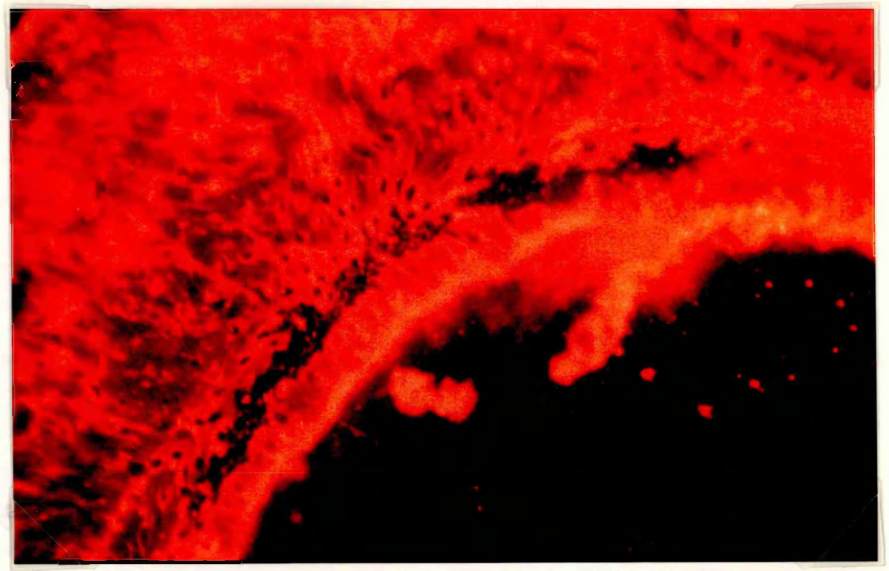

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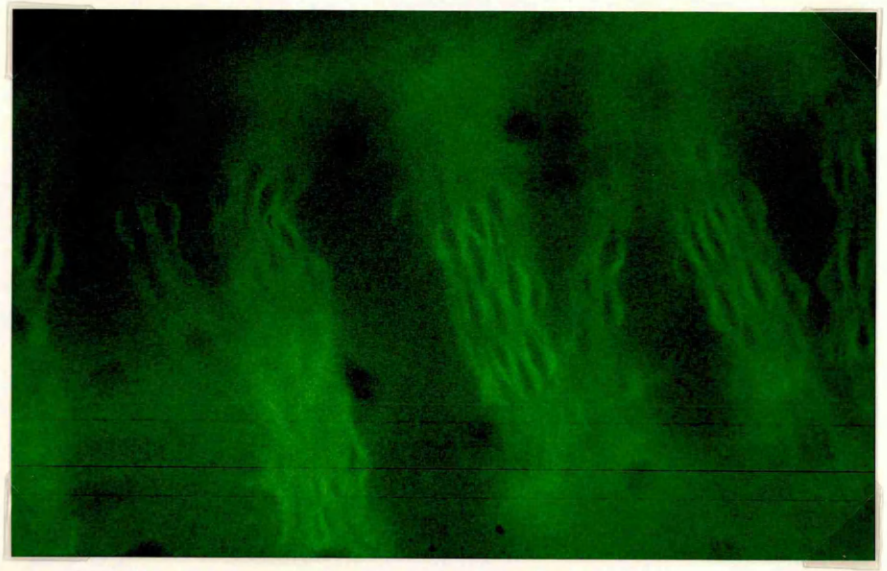
Plates 5.9 & 5.10



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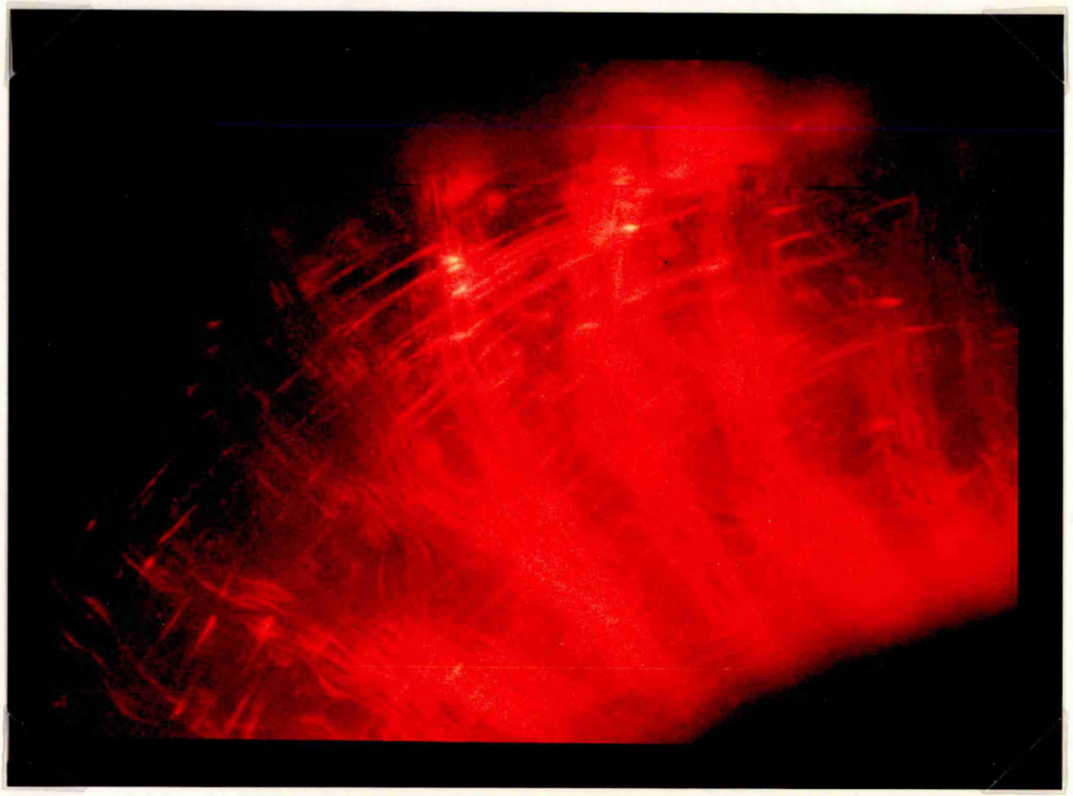

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5.1.3 Mechanism(s) of striping

Two questions could be asked concerning these observations. Firstly how do such striking changes in morphology come about? Secondly what does this process tell us about how the surface membrane functions?

The salient feature of this event is the appearance of transverse regions of smooth as opposed to folded membrane on the parasites dorsal surface upon cooling. The effect is to provide a contrast with the areas of "normal" transversely folded membrane which appear as relatively brighter areas of labelled membrane.

Two explanations can be advanced to explain the appearance of these smooth membrane regions upon cooling. Their appearance could reflect the emergence in a highly visible form of smooth areas of membrane which exist within adult schistosomes at 37°C or room temperature. Such areas could, at these temperatures, remain oriented nearly vertical to the plane of view by fluorescence microscopy. In these circumstances their existence would not be readily apparent.

Alternatively the cooling could induce the unfolding of regions of membrane in a regularly spaced transversely oriented manner. These two alternatives are shown in diagrammatic form in Figures 5.1 & 5.2.

5.1.4 A Role for circular muscles

These two alternatives cannot at present be distinguished but other evidence obtained in collaboration with Dr J. R. Kusel suggests that these cooling events are controlled ultimately by the muscles of the parasites. There are two main sets of muscles within the parasite one which runs longitudinally along the length of the parasite and another set of regularly spaced circular muscles. There is evidence to suggest that the stripes are regions of folded

circular muscles. That is directly underlying a stripe there is a region of contracted muscle. This observation implies a direct link between bands of circular muscle underlying the tegument and the surface membrane itself. This is because the degree of physical coincidence between muscle labelled with fluorescent phalloidin and the aminofluorescein C18 labelled membrane "stripe" is such that it suggests that the folded, tubercle rich, areas of membrane are directly linked and synchronised with the unit pattern of repetitive circular muscle blocks (Plate 5.10). Phalloidin labels the actin filaments of the muscle.

The most likely candidate for such a physical link would be some type of cytoskeletal element. Three main types of cytoskeletal structures exist, microtubules, actin and intermediate filaments of various types. The close association between regions of folded membrane and tubercles suggests that the actin spines of the tubercles may play a role in this transmission of trans tegumental, restraining or supportive force from muscle to membrane. This may be most plausibly suggested as being due to the presence of actin filaments since both the tubercles and the longitudinal muscles are composed of actin. In addition there is some evidence for the presence of unpolymerised actin in the tegument itself in adult parasites [Matsumoto et al 1988].

Other experimental evidence produced by Dr. Kusel some time before this study is relevant to this discussion. The phenomenon of anisotropic diffusion of the fluorescent lipid dye DiI C18 has been demonstrated to occur within the surface membrane of S. mansoni. Again this process was most evident on the dorsal surface of the adult male It was produced by introducing a discrete local transfer of DiI C18 from dowex beads to the adult membrane. This was done at room temperature. Initially the dye formed a discrete spot around the region where the dye made contact. Of most significance to this discussion was the observation

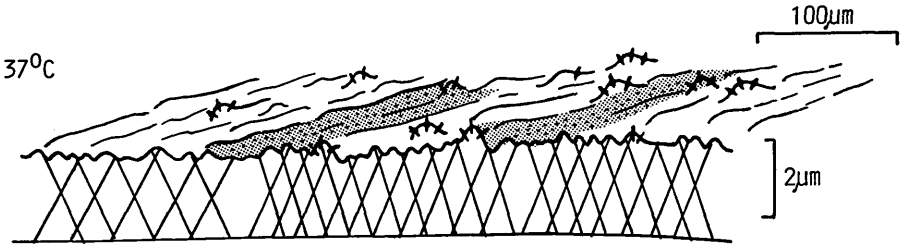
that as the dye diffused within the membrane from this initial spot it did not move equally in all directions but dispersed anisotropically and transversely across the width of the parasite. The end result was a labelled strip of membrane remarkably similar in appearance and dimensions to the stripes reported above. The significant difference is that this process occurred at room temperature not 4°C. These results could be explained by postulating some kind of barrier to longitudinal diffusion of lipid. Alternatively the process could be an optical artefact produced by the presence of alternating smooth, fold free, transverse areas of membrane in deep clefts between areas of tubercle rich folded membrane. This hypothesis is of course the same as the first mechanism advanced to explain the aminofluorescein C18 stripes (Figure 5.1). Effectively it proposes that the surface morphology of the adult schistosome membrane/tegument is similar to a contracted concertina or bellows and that only when extended is the true differentiated nature of the surface apparent (Figure 5.2).

There is no published corroborative evidence from E.M. studies which reports the occurrence of differentiation of the surface membrane of adult S. mansoni into large alternating regions of folded and non-folded regions of membrane [Hockley 1976]. However this author did not cool the parasites before fixation. There has however been a report presenting E.M. scanning photomicrographs of adult parasites showing a similar striped pattern of alternating folded and unfolded regions of membrane. This was induced by exposure of the parasites to praziquantel. One of the noticeable effects of Praziquantel is to induce contraction of the parasite musculature in response to an influx of calcium ions through the permeabilised surface membrane [Bricker et al 1983]. This reinforces the link between musculature and membrane morphology.

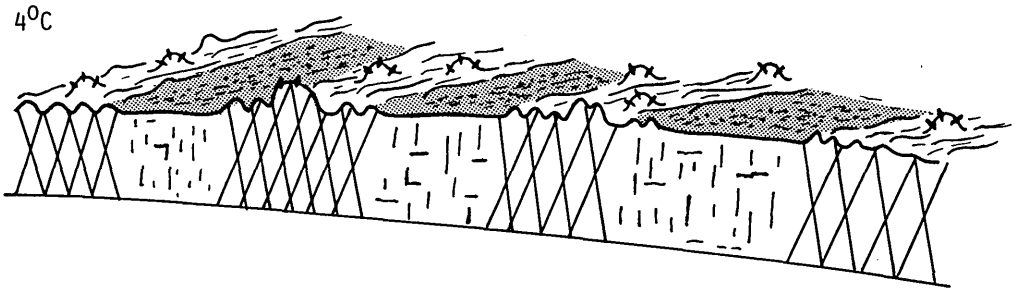
Figure 5.1

Striping mechanism 1 : cytoskeletal depolymerisation.

A ..... "Stripe" formation due to depolymerisation of cytoskeleton.



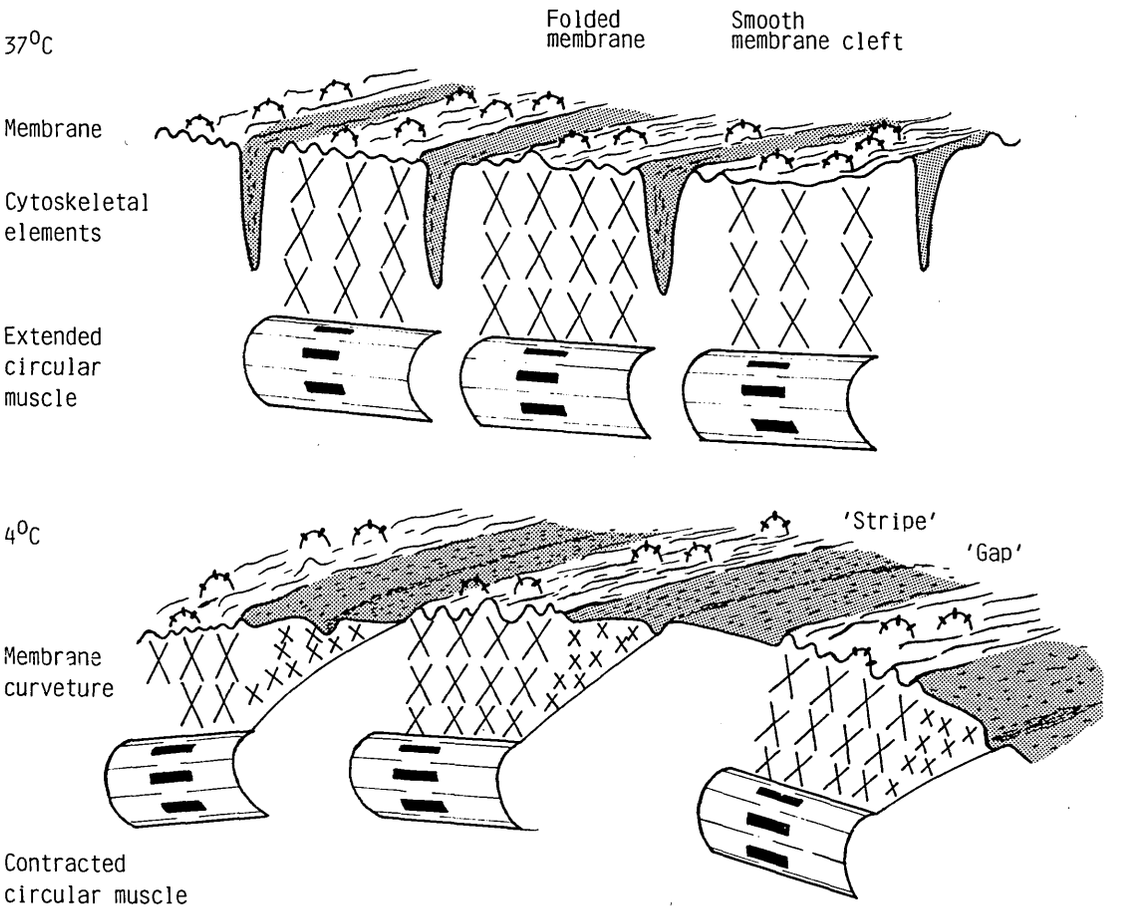
Folds underlain by cytoskeletal elements which maintain folded appearance of surface.



Gaps form under regions of membrane where cytoskeleton is depolymerised due to cooling.

Figure 5.2
Striping mechanism 2 : Muscle linked clefts.

B Stripe formation due to exposure of vertically orientated smooth membrane in clefts between blocks of folded tubercle rich membrane linked to underlying circular muscle.



Formation of smooth "gap" areas due to stretching out of smooth membrane cleft. Muscle blocks at 4°C should be shorter than those at 37°C and membrane appearance "curled".

5.1.5 Biological implications

In this discussion the possible biological implications of accepting the first hypothesis to explain this striping phenomenon are discussed. That is that the cold induced striping and the anisotropic pattern of DiI C18 diffusion reported by Kusel are both manifestations of a fundamental feature of the surface membrane morphology. This feature represented by the "concertina" model in Figure 5.2 is that the surface membrane of adult S. mansoni is differentiated into regions of tubercle rich folded membrane and tubercle deficient unfolded membrane. These regions are oriented transversely across the width of the parasite, are 50 - 100 um wide, and alternate consecutively. There are three areas in which these observations and hypothesis described above may have significant import to an understanding of the surface membrane and tegument.

These concern the transverse orientation of surface morphology; the effect of musculature organisation on membrane morphology and the specificity of DiI C18 induced damage associated with cooling.

5.1.6 The transverse orientation of membrane morphology

It is clear that the predominant orientation of the surface membrane morphology of adult parasites is transversely across the width of the parasite. This is true of the main feature of morphology that is the folds on the surface. Within the gynaecophoral canal there are no folds similar to those occurring on the dorsal surface but the membrane surface is divided into sections by structures that appear like grooves. The results presented above reporting transverse stripes and anisotropic diffusion reinforce and extend this concept.

This suggests that the surface is to some extent divided into transverse sections alternating along the length of the parasite. The basic unit structure of such sections would consist of a transverse area of folded tubercle rich membrane flanked by smooth vertical regions of membrane (see Figure 5.2). Three possible advantages could acrue to the parasite from such a structural organisation of the membrane surface.

5.1.7 Transverse transport

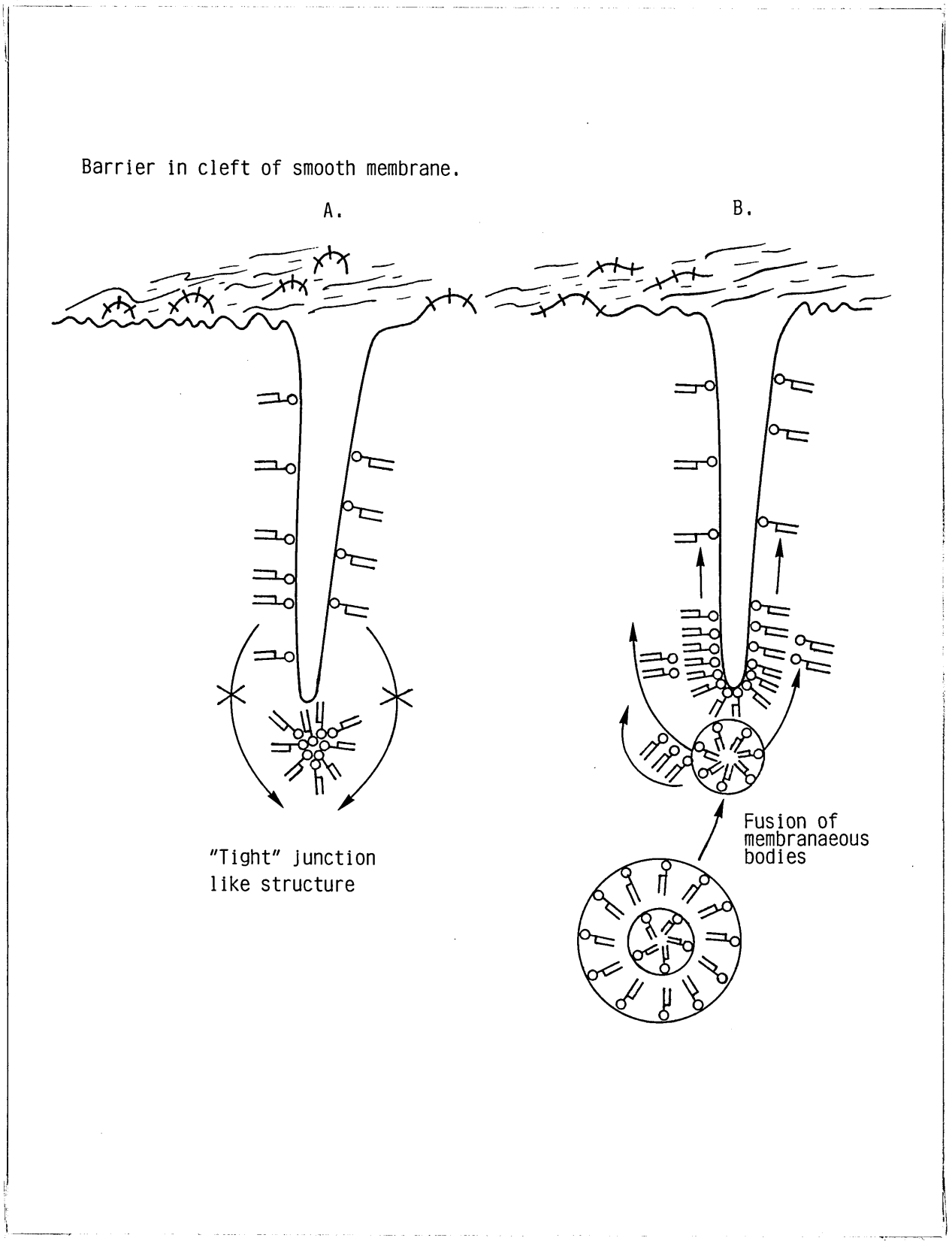
Transport of molecules within or even on the membrane around the dorsal surface to and into the gynaecophoral canal could most efficiently occur under these circumstances. The observed anisotropic diffusion of DiI C18 serves as a model for such transport. Removal of a substance from the membrane of the gynaecophoral canal by the female could "drive" the diffusion of membrane soluble nutrients. This would be movement from a site of absorption on the dorsal surface of the adult male, along transverse folds in the membrane round the dorsal surface and into the gynaecophoral canal. One such nutritive substance could be cholesterol since the female has a high requirement for sterols to produce eggs but exists in a relatively enclosed environment within the groove of the male's gynaecophoral canal. This location may inhibit direct absorption of nutrients from the bloodstream. Such a hypothetical arrangement as shown in diagrammatic form (Fig 5.4) would provide a mechanism of nutrient absorption by the female while retaining the advantages of a protective environment in the gynaecophoral canal. The tranverse orientation of the surface morphoplogy could then provide a biophysical link between a specialised site of absorption on the dorsal surface of the male and the female in the gynaecophoral canal.

In order for the membrane surface to work most effectively some block or inhibition on longitudinal diffusion would be advantageous in that it would increase the efficacy of net transverse movement. The organisation of the surface into a basic unit structure consisting of separate blocks or segments of alternating folded and smooth regions of membrane could ensure this. The site of inhibition of transverse diffusion would in this scheme be the smooth "gap" like region more specifically a structure or mechanism running along its length. One such feature could be a structure analogous to the "tight junction" (fig 5.3a)that effectively separates the apical and basolateral membrane of epithelial cells while maintaining a continuity of membrane itself [van Meer & Simons 1986]. Another possibility would be a transverse region in which fusion of membraneous bodies took place selectively (Fig 5.3b) thus generating a barrier to movement across this region.

5.1.8 Accelerated membrane turnover in response to damage.
The rate of turnover of adult membrane has recently been shown to be lower than previously thought [Saunders et al 1987]. However this author suggested that a mechanism for relatively fast turnover of membrane in response to irritation or damage to the membrane may also exist within a general environment of low membrane turnover [Wilson & Barnes, 1977]. Interestingly this fast membrane turnover in response to irritation took the form of membrane flow from the base of the pits and shedding from the tubercles. This was postulated as being due to an ability of the adult parasite to undergo limited accelerated membrane turnover in response to irritation. This was via an enhanced rate of fusion of tegumental membraneous bodies. In order for such fusion to result in a flow of membrane over and off tubercle spines insertion of extra membrane must take place some distance from it. Random insertion of membrane at all points in the membrane would be an inefficient process of accelerated membrane turnover. This is because it will be less likely to generate a directional flow of membrane,

Figure 5.3

Possible barriers to diffusion at the base of cleft.



from the tubercles. In addition it would not be an efficient way of totally replacing a damaged region of membrane. This is because random insertion of membrane would result in shedding of both damaged and undamaged membrane. A more efficient means of rapidly replacing an area of damaged membrane entirely with new undamaged membrane would be to replace an entire section of damaged membrane with undamaged membrane. One way in which this could come about is suggested by the striping pattern of labelling. This model, shown diagrammatically in Figure 5.3b, proposes localised linear insertion of membraneous bodies along two channels or lines of fusion on either side of a damaged region of folded membrane. Insertion of membrane in this manner would lead to directed flow up the smooth sides of membrane block which would result in shedding of membrane from the points at which the forces of membrane flow generated meet namely at the tubercles. Such a system is an extension of the phenomena of enhanced fusion in the pits and flow of membrane onto the tubercle reported by Wilson and Barnes. It would have three advantages.

a) Shedding at the tubercles.
   It provides an explanation of why irritation induced shedding occurs at the tubercles.

b) Efficacy of replacement.
   It provides an extremely efficient means of replacing damaged membrane as it ensures that mainly damaged membrane is shed and replaced efficiently with purely undamaged membrane.

c) Damage limitation.

It provides an inbuilt "damage limitation" mechanism . That is if the membrane was organised into such unit structures proposed in Figure 5.2, upon damage the means of shedding only that particular damaged strip or stripe of membrane is available. For example, if the head region is locally irritated or damaged by Ferritin or complement only a transverse strip of membrane in the head region needs to

be repaired in order to ensure the viability of the organism. Overall shedding of the entire membrane in response to irritation would not be necessary.

Two less likely consequences of such a morphology could be:

d) The deep cleft could be an adaptation for absorption as in epithelial cells.

e) Such a structure could also promote the sequestration or the physical inaccessibility of some antigens on the surface.

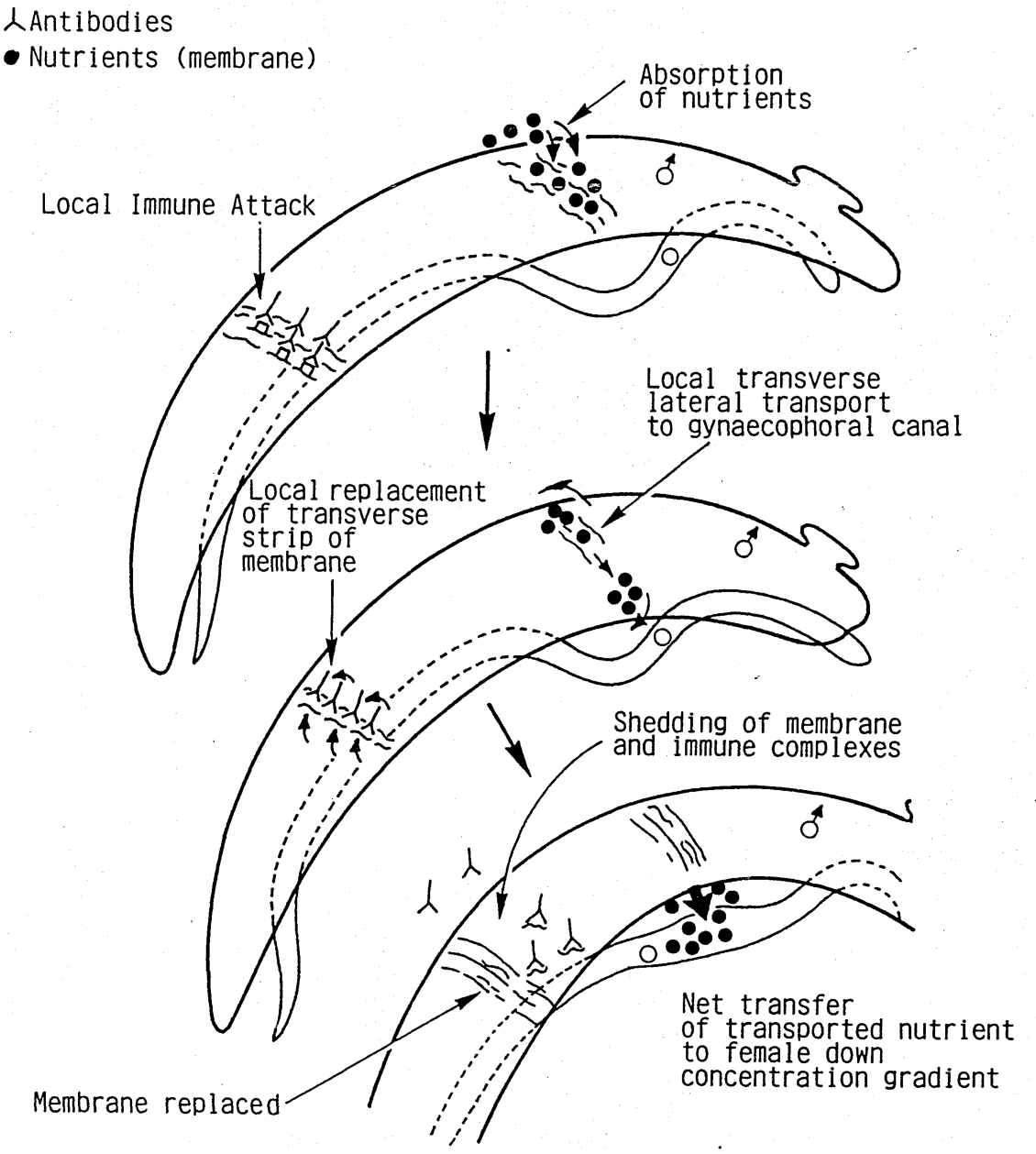
A diagrammatic representation of these concepts is shown in Figure 5.4.

5.1.9 Adult S. mansoni and stripes: summary

Clearly these hypotheses regarding transverse transport of nutrients and a mechanism to explain accelerated membrane turnover in response to damage are speculative. However they are entirely consistent with the observations reported above. They are of value in that they provide some clear models of membrane morphology and function which can be tested. In particular they predict that a specialised region of membrane running along the length of the smooth gap regions of the "stripes" described above exists and functions to prevent diffusion of lipid across it. These observations also highlight the need to undertake investigation of the biological features of the parasite at the level of the "macro" tegumental level. That is looking for differences in biochemical function between different regions of tegument and membrane within S. mansoni particularly the adult stage. For example an E.M study examining local variation in the nature and extent of membrane body fusion with the tegument may reveal the sort of transversely organised localised regions of fusion postulated above. Another study of this type could be a comparison of the tegumental function in the gynaecophoral canal with the dorsal surface of the male parasite.

Figure 5.4

Possible functions of stripe induced segmentation of the adult membrane.



5.2 DiI C18 cold induced damage to S. mansoni

5.2.1 The Adult S. mansoni

The data presented in this section of chapter 5 refers mainly to schistosomula. However one of the striking features of the "striping" phenomena reported in section 5.1 was that while striping could be induced by cooling of aminofluorescein C18 and rhodamine C18 labelled adult parasites this was not the case when DiI C18 was used.

Labelling with DiI C18 at 37°C or room temperature gives a pattern of labelling essentially similar to that attained by the other two dyes. However when such DiI C18 labelled adults are cooled rather than forming "stripes" the membrane shows severe disruption and shedding (Plates 5.7 & 5.8). An important difference between DiI C18 and aminofluorescein C18/rhodamine C18 labelling in adult membranes is the vertical location of the dye within the four leaflets of the double membrane structure. The latter two dyes locate predominantly (90%) locate in the E_1 face whereas DiI C18 locates within one of the inner leaflets. Moreover DiI C18 is largely immobile whereas aminofluorescein C18 and rhodamine C18 are largely mobile. These observations have been interpreted by Foley et al [1986] as suggesting a vertical fluidity gradient within the double membrane of adult S. mansoni. They suggest that the outer bilayer is relatively fluid whereas the inner bilayer is immobile. These biophysical observations accord well with evidence produced by other workers which suggests that the properties of the two membranes are significantly different [McDiarmid, Podesta & Dean 1983].

5.2.2 Possible mechanisms of damage.

The implication of this contrast between the effect of cooling DiI C18 labelled adult parasite membrane and aminofluorescein C18 or rhodamine labelled membranes is that the presence of DiI C18 in a cooled inner membrane causes disruption of the double membrane structure itself.

It does not appear that cooling in itself will cause damage since parasites can be cooled and rewarmed either labelled with aminofluorescein C18 or unlabelled without inducing damage. It is not clear how this damage is achieved but it is likely that reduction of temperature below the transition temperatures of the membrane itself could be involved. The transition temperature of the S. mansoni double surface membrane as a whole is not known for this stage. Most cell membranes have a transition temperature of around 28°C. It is likely that cooling the surface membrane to 4°C results in a decrease in temperature from above to below its transition temperature. It has been reported in model membranes that DiI C18 can alter the transition temperature of model membranes in a chain length dependent manner [Klausner & Wolf, 1980]. The presence of DiI C18 molecules could be viewed as an exogenous lipid component which may have the ability to either form new immiscible domains in the membrane upon cooling or perhaps more likely it could intercalate and alter the existing domains within the membrane. Phase transition itself involves long range cooperative interactions between the lipid constituents of the lipid bilayer. The lipid bilayer cannot be treated as a bulk phase [White 1978] but as a number of cooperative phases which are more or less miscible with the other. At higher temperatures these differences in miscibility between the different domains of the S. mansoni may be relatively slight due to the general fluidity of the membrane itself. On cooling below transition temperature segregation and contrast between domains will be maximal. During transition itself the permeability of the membrane is increased. The presence of exogenous lipid domains of DiI C18 or altered DiI C18 containing domains could increase this transient permeability to the extent that the integrity of the membrane is destroyed leading to damage.

An alternative mechanism of damage could be via disruption of membrane cytoskeletal links. There is evidence to

suggest that the immobility of the DiI C18 containing inner bilayer is maintained by cytoskeletal links [Foley et al 1986]. This may be mediated by amphipathic proteins which insert into the membrane [Burn 1988] and immobilise the membrane around them. In adult parasites the DiI C18 locates predominantly within an immobile inner leaflet. The DiI C18 may preferentially disrupt the immobile region of membrane around cytoskeletal membrane links. Disruption of this leaflet by a DiI C18 phase change associated mechanism could then result in breaking or weakening of cytoskeletal protein membrane connections leading to structural damage to the membrane as observed.

Two of the most effective schistosomocidal drugs, Praziquantel and Cyclosporin A, appear to act at the level of the membrane in S. mansoni. Praziquantel has been shown to induce phase changes in membranes resulting in the formation of non-bilayer H_{II} phase within the bilayer [Harder, Goosens & Andrews 1988]. Cyclosporin A will kill S. mansoni in vivo by an as yet undefined mechanism [Chappel & Walker 1982] which is thought to be a direct effect on the membrane rather than an effect of the host's immune system. The incorporation of Cyclosporin A into model membranes markedly alters the biophysical changes associated with transition [O'Leary et al, 1985] in a way that shows some parallels with DiI C18 alteration of transition [Klausner & Wolf, 1980]. These two groups observed these effects in model membranes of the same lipid composition namely dipalmitoylphosphatidylcholine.

To summarise, there is good reason to believe that cooling and alteration of the lipid phase by exogenous molecules is required to induce such DiI induced damage. (Plates 5.7 & 5.8). Of more significance perhaps is the indication that the immobile inner bilayer may be uniquely susceptible to damage caused by insertion of exogenous lipid or other components.

5.2.3 DiI C18 induced damage to schistosomula.

The damaging effect of DiI C18 observed in the adult parasite shows parallels with the situation in the schistosomula. When these are labelled with DiI C18 and chilled at 4°C, for a period of several hours then rewarmed under illumination at 540 nm (epifluorescence) on a heated (37°C) microscope stage large scale decoupling of the surface membrane from the parasite body was observed (Plates 5.11 to 5.16 , Plates 5.17 to 5.19). The first set of plates shows the effect of illumination and chilling on schistosomula labelled with both aminofluorescein C18 and DiI C18. The second set were labelled with DiI C18 only. Simply rewarming chilled DiI C18 labelled schistosomula caused localised blebbing in a proportion of parasites. Large scale membrane uncoupling was not observed in parasites labelled with only aminofluorescein C18.

There are several interesting features of this process:

1. It requires a viable undamaged schistosomula well labelled with DiI C18 in its surface membrane. It will not occur in damaged schistosomula, where the dye moves into internal membranes, or in partly labelled schistosomula.
2. It is enhanced by pre-incubation of the parasites in serum.
3. The dissociation step involves the retraction of the parasite body from the membrane rather than the expansion of the membrane away from the parasite body. This is illustrated by the fact that the volume of the organism enclosed by the membrane after decoupling remains the same. This can be seen in Plates 5.17 to 5.19 by comparing the damaged schistosomula with the undamaged schistosomula which was not subject to intense fluorescence illumination.
4. It occurs preferentially at the anterior portion of the schistosomula.

List of Plates

Plates 5.11 & 5.12
Schistosomula double labelled with aminofluorescein C18 and DiI C18 (observed at room temperature prior to cooling).

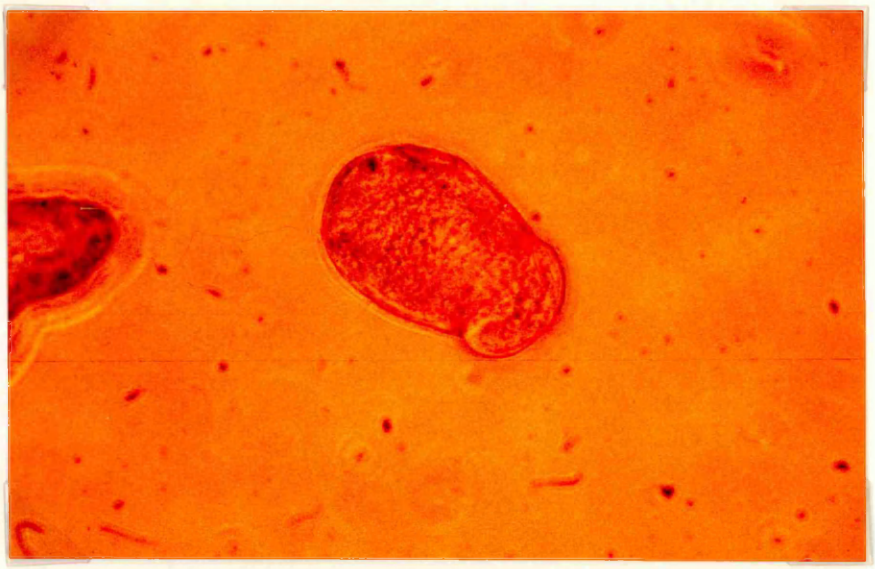
Plates 5.13 & 5.14
Same parasite after cooling to 4°C and rewarming to 37°C under bleaching levels of illumination at 540nm with Mercury Lamp (HBO Wotan). Excitation at 490 nm.

Plates 5.15 & 5.16
Same parasite as 5.13 - illuminated at 540 nm.

Plates 5.17 & 5.19
Schistosomula labelled with DiI C18 only and subjected to cooling and rewarming under bleaching levels of illumination at 540 nm focussed on left hand schistosomula.

Plate 5.17 Bright field illumination.
Plate 5.18 Bright field and 540 nm fluorescence.
Plate 5.19 Illumination at 540 nm only.

Plates 5.11 & 5.12



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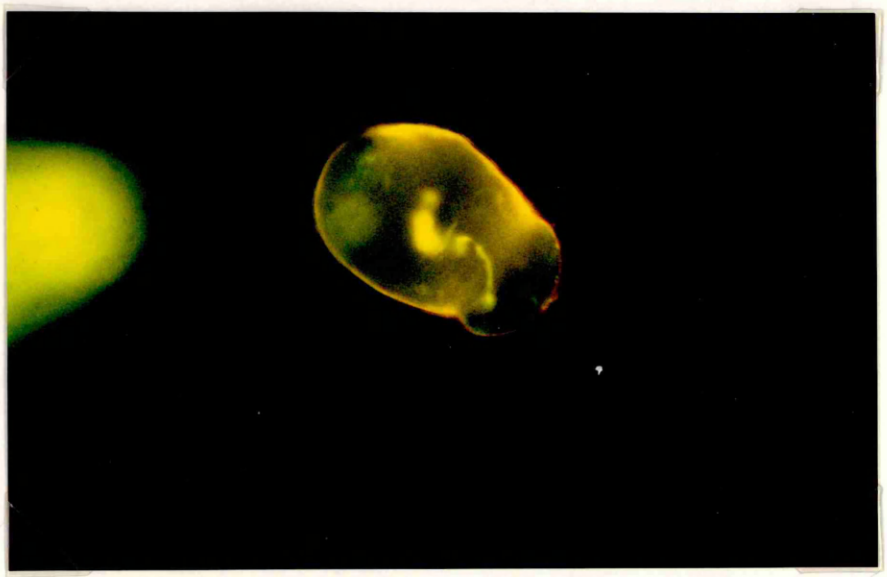

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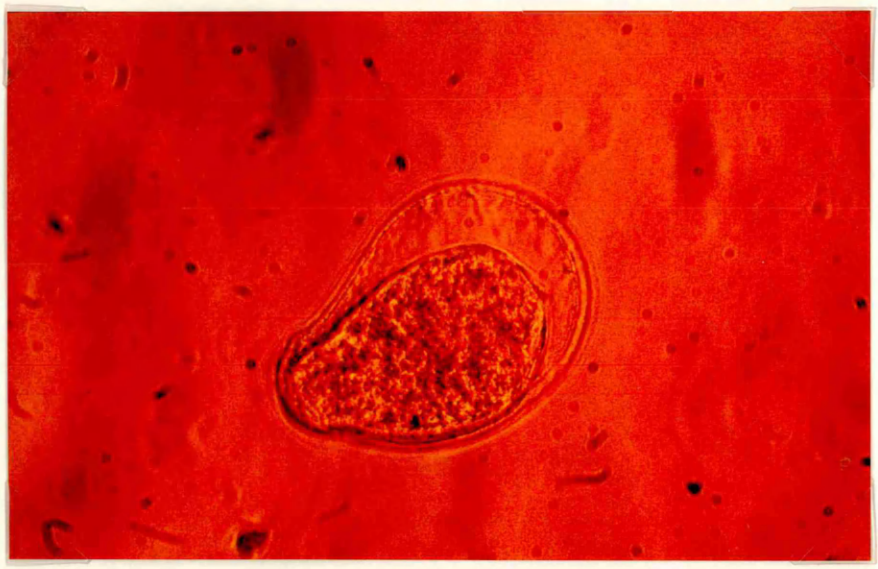
Plates 5.13 & 5.14



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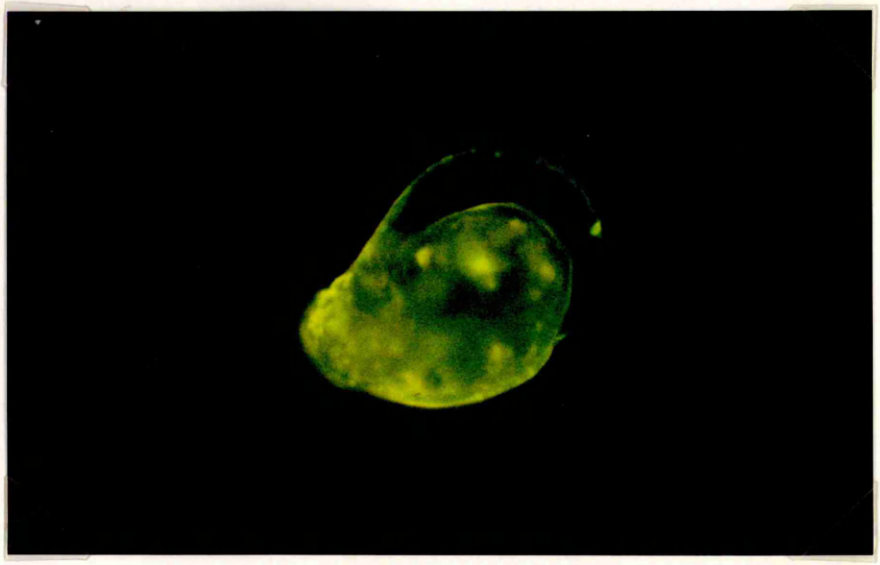

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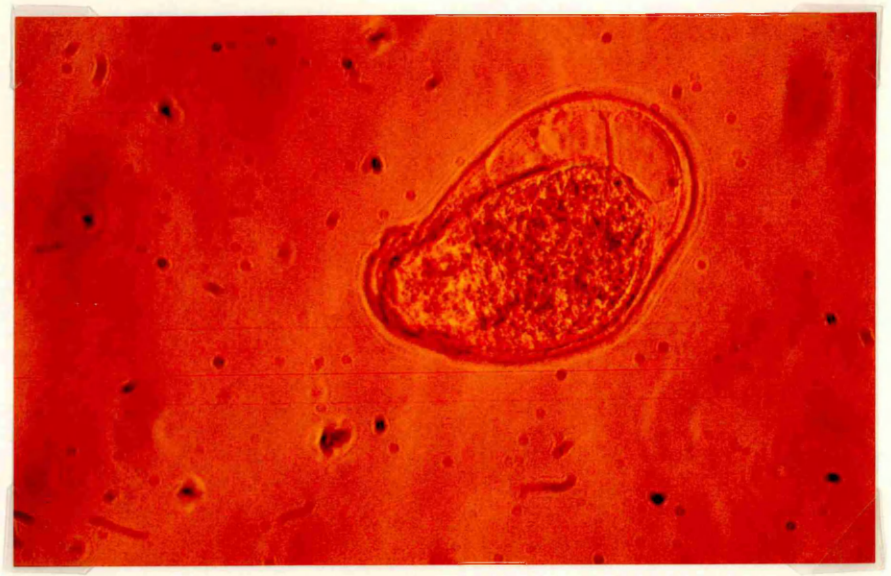
Plates 5.15 & 5.16



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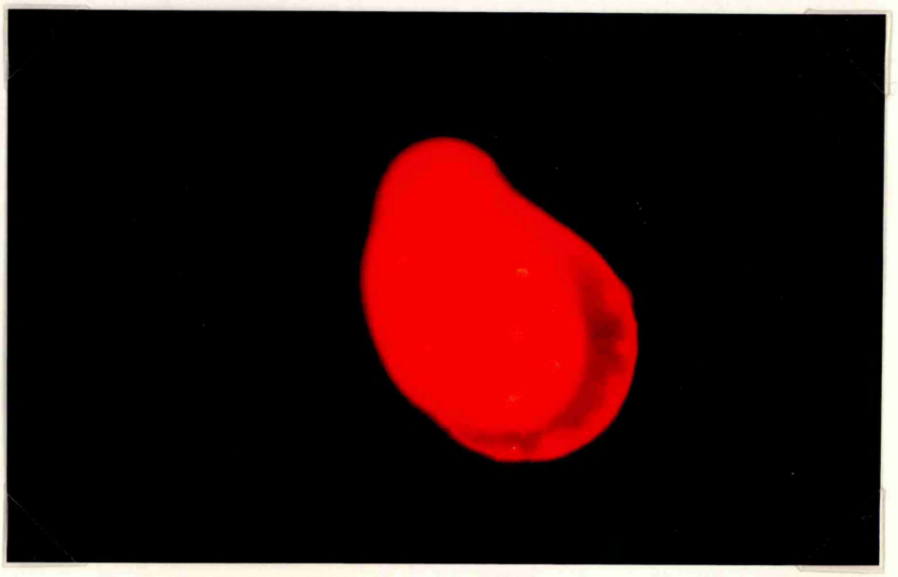

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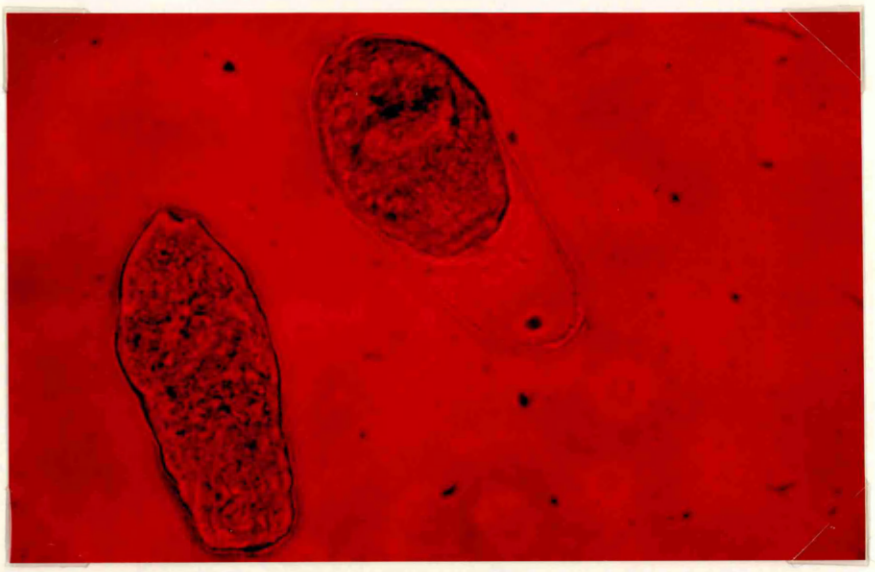
Plates 5.17 to 5.19



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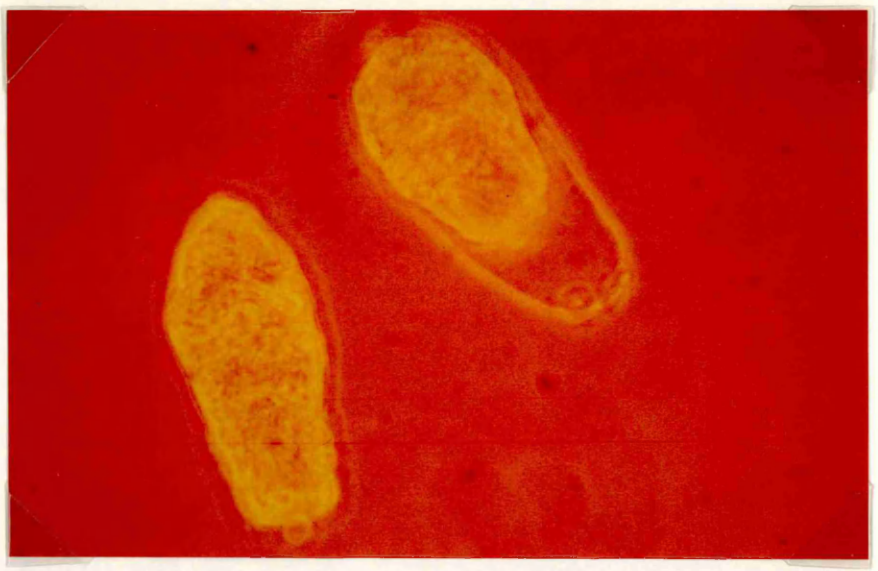

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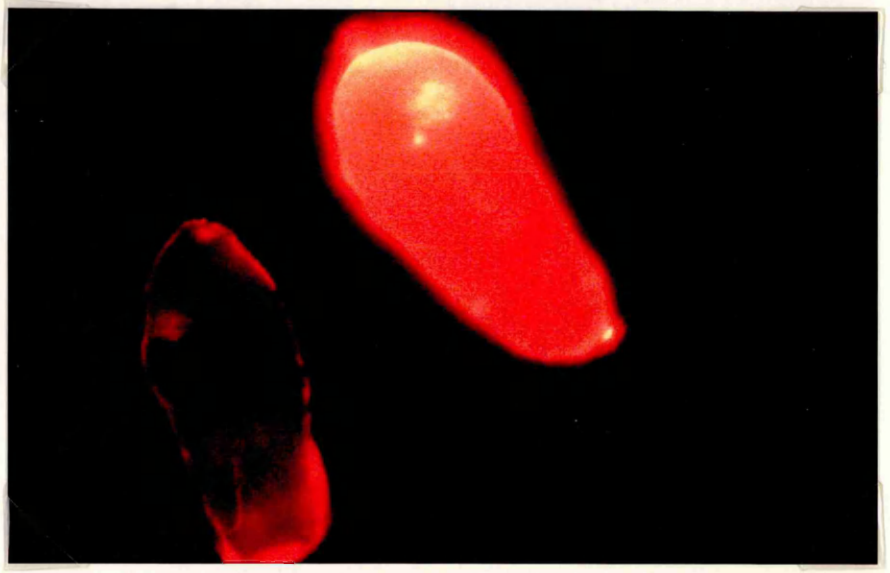

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This process is dependent on the illumination, up to the point of total bleaching, of the labelled schistosomula membrane. The two schistosomula on Plates 5.17 to 5.19 show similarly labelled parasites on the same slide and subject to the same changes in temperature. Only the parasite subjected to intense "bleaching" illumination shows blebbing. The process appears to be promoted by DiI C18 excited at 540 nm. Excitation at the "wrong" fluorescence wavelength does not produce the blebbing effect.

5.2.4 Possible mechanisms of DiI C18 damage to schistosomula

Large scale blebbing and uncoupling of membrane from schistosomula similar to this has been reported by several authors under a variety of conditions. McLaren et al 1981 has reported large scale blebbing in response to eosinophil basic protein. A technique involving chilling and rewarming of dead "high salt" treated schistosomula has been developed by Kusel et al 1984. The same author showed that treatment of schistosomula with the detergent Saponin will result in the large scale detachment of surface membrane leaving the parasite body intact in a manner similar to that shown here [Kusel 1972].

These three studies show different ways in which blebbing can be induced. Major Basic Protein from eosinophils appears to damage the integrity of the membrane allowing breakdown of osmotic control and swelling resulting in blebbing. In this respect it is similar to the high salt treatment which also induces osmotic shock killing the parasite. However it has the additional elements of cooling and rewarming indicating the changes to the lipid phase of the membrane induced by moving through two membrane transitions is important. In this respect the high salt treatment resembles the DiI C18 induced damage, it also shows similarities in that pre-incubation in serum promotes formation of vesicles which in turn form preferentially from the anterior portion of the membrane.

The decoupling of the membrane via Saponin indicates that both breakdown of osmotic control by the membrane and expansion of the membrane could contribute to blebbing.

From these studies three features can be listed which appear to be involved in membrane blebbing or decoupling. These are breakdown of the osmotic barrier of the membrane, lipid phase transition and membrane expansion.

It is impossible to say to what extent these three mechanisms are operating in the DiI C18 situation. There are obvious parallels with the other techniques but also some important differences. The integrity of the membrane as an osmotic barrier is obviously debilitated to some extent by this process since the very first thing that happens when the labelled parasite is subjected to fluorescent light for more than about 20 seconds is that the parasite contracts. This suggests that an influx of Ca^{++} is taking place. In addition uptake of the hydrophilic dye Hoëcht after formation of large scale blebs has been observed (not shown). However neither the surface membrane nor the parasite body appears to swell up in response to such ion influx. This apparent stability of the surface membrane under conditions in which its osmotic integrity must be damaged is puzzling and illustrates the most important difference between DiI C18 induced damage to the adult membrane and DiI C18 induced damage to the schistosomula membrane.

Lipid phase transition associated changes are clearly implicated due to the requirement for cooling. In this respect the DiI C18 induced damage is similar to the "high salt" treatment of vesicle production of Kusel. However this DiI C18 decoupling and blebbing takes place on intact viable parasites rather than swollen dead parasites in high salt. Indeed this type of DiI C18 blebbing will not occur on dead parasites.

There may be some scope for membrane expansion due to a heating effect of DiI C18 absorption of fluorescence light energy. Alternatively degradation products of DiI C18 bleaching could induce damage. This might be due to the formation of short lived products such as free radicals rather that a Saponin like detergent effect.

5.2.5 Biological significance of the effect

It is likely that the DiI C18 induced blebbing is a multifunctional pheneomenon involving all three factors to some extent. Its precise mechanism would be extremely difficult to elucidate, however this apart its relevance lies in the following areas.

1. The surface membrane can be physically uncoupled from the tegument and parasite body leaving both the surface membrane and the basal lamina membrane enclosing the body of the parasite physically intact.
2. There is a developmental difference between the type of damage induced by DiI C18 labelling and cooling between adults (membrane destruction) and schistosomula (membrane uncoupling).
3. In spite of this developmental difference in effect there is evidence to suggest that the site of action in each case is DiI C18 containing immobile regions of membrane.

There is evidence to suggest that in the schistosomula DiI C18 has a preference for immobile regions of membrane (section 6.1) in any case FRAP analysis from schistosomula indicates that there is a large immobile fraction of lipid in schistosomula labelled with DiI C18.

Therefore in both schistosomula and adults it could be argued that the presence of DiI C18 in immobile regions (domains) of the surface membrane undergoing phase transition by cooling and rewarming differentially disrupts the integrity of membrane leading to its destruction in adults and uncoupling in schistosomula. This uncoupling in

schistosomula appears to require some additional factor perhaps localised heating or free radical production in the schistosomula membrane to cause uncoupling.

Clearly whatever is holding the surface membrane to the parasite body is broken in the schistosomula (figure 5.5). The physical or mechanical force responsible for this breakage appears to be derived from muscle contraction in the parasite body and must in terms of tegumental forces be considerable. Nevertheless the surface membrane itself retains its integrity. This is evidence to suggest that membrane integrity and perhaps immobility in the schistosomula membrane is not maintained by trans-tegumental cytoskeletal links whereas it is in adults. This conclusion was drawn by Foley who observed that high salt blebbing of adult membranes resulted in release of constraints on mobility of the adult membrane protein and lipids but that blebbing or vesiculation of schistosomula membrane did not have any effect on the large scale immobility of lipid probes inserted into the membrane [Foley et al, 1986]. No data concerning the mobility of lipids in DiI C18 induced blebs is available at present. This author [Foley] suggested that the immobility of such high salt treated schistosomula surface membrane lipids was maintained by the immobilising or gelling effect of lyso PC molecules after Golan et al [1986]. This argument could be extended to cover the physical integrity of the membrane as a whole. That is the schistosomula membrane's integrity could be intrinsic to the membrane composition itself rather than dependant on some external support such as cytoskeleton. This hypothesis, however, does not preclude a role for trans-tegumental cytoskeletal elements which tie the surface membrane to the rest of the parasite body. This idea is attractive as it proposes that as in the adult membranes (Figure 5.6) transition induced changes in immobile lipid regions could result in a disruption of cytoskeletal membrane links crucial to the attachment of the schistosomula membrane to the parasite body.

Figure 5.5
Blebbing in the schistosomula induced by cooling and DiI C18.

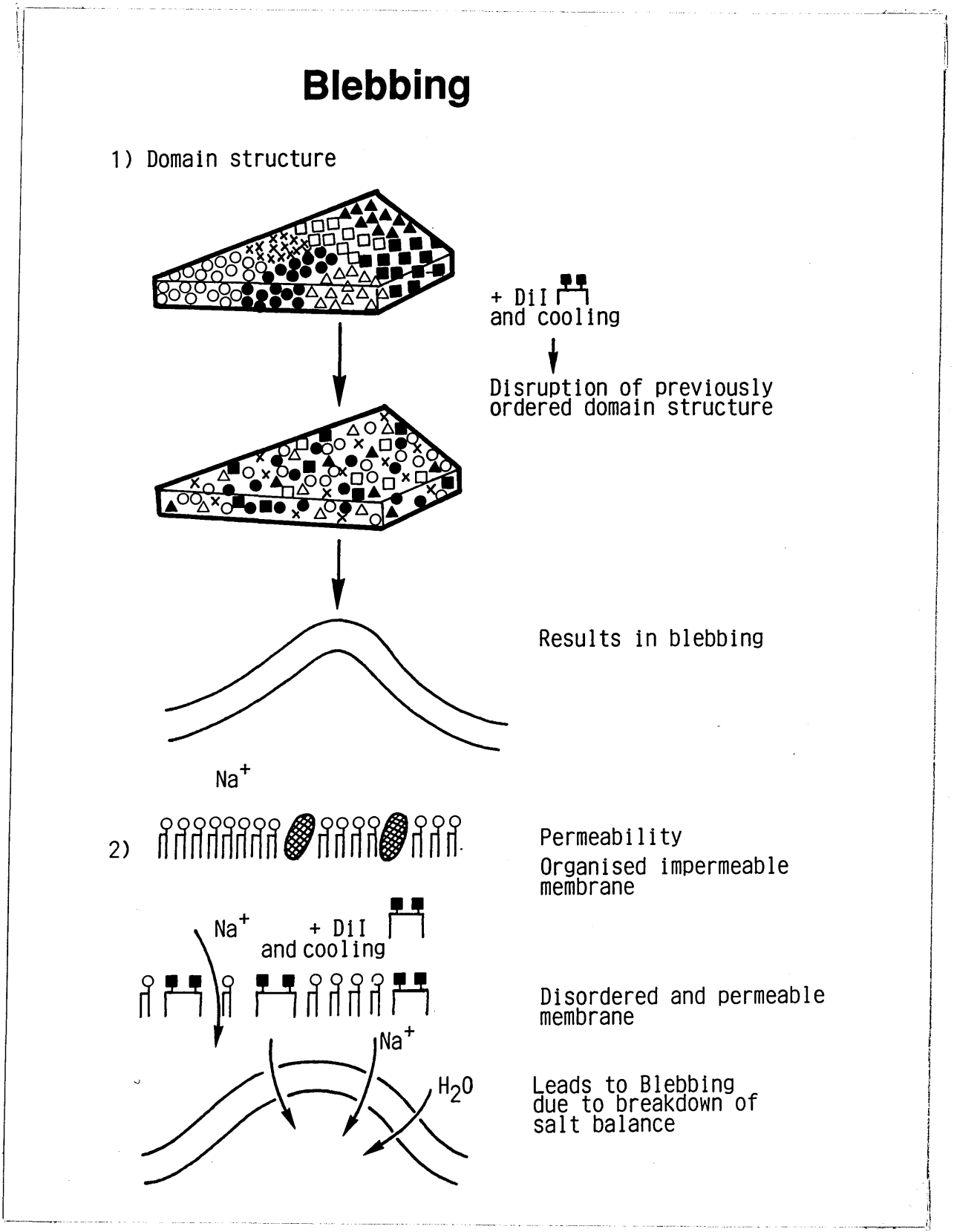
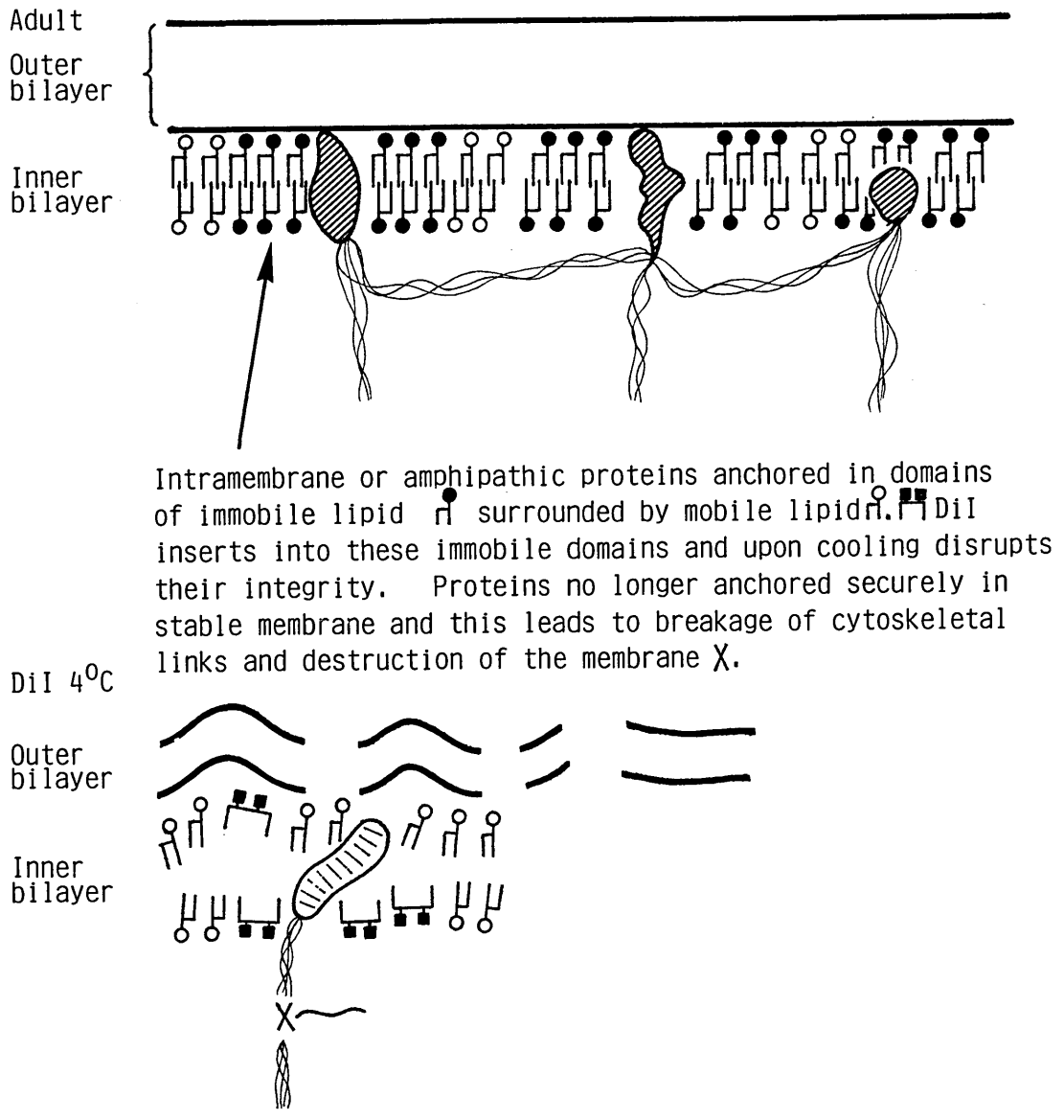


Figure 5.6

Blebbing in the adult induced by cytoskeletal disruption via cooling and DiI C18



Thus this same mechanism of DiI C18 induced destruction of cytoskeletal membrane anchoring could result in the destruction of the adult membrane which, is dependant for its integrity on cytoskeletal interactions, but only the uncoupling of the schistosomula membrane which has an intrinsic integrity due to its composition. These hypotheses are shown in model form in Figures 5.5. & 5.6.

5.2.6 Practical application of the effect

One possible use of this or related blebbing/vesiculation effects would be to develop a technique for producing a parasite attenuated at the membrane level. Irradiated vaccines at present are produced by inducing lesions at the D.N.A. level, the effect of this being to produce a viable parasite, the behaviour and or surface of which is sufficiently altered to allow greater recognition by the host and thereby to confer partial protection. Typically the irradiated schistosomula are viable enough to last until the lung stage of their migration where they die. It is not known whether death is due primarily to the irradiation induced lesions in their D.N.A. or enhanced immunogenicity and recognition by the host.

It is possible that the same principle of irradiation induced alteration could be employed using the membrane as the site of lesion formation. Clearly this could be approached by "irradiating" the membranes of schistosomula or even cercaria with one of the dyes used in this study. The DiI C18 damaging effect manifest as large scale decoupling of the membrane represents an extreme case of such "fluorescence irradiation" damage. It may well be possible to damage a fluorescently labelled schistosomula sufficiently to allow it to remain viable to the lung stage. One possible advantage of such an approach could be that an altered membrane surface, in which for example previously immobile antigens due to low level dissociation of the membrane were mobilised, was produced. Development of such an approach would allow correlation of biophysical

membrane properties to immunogenicity of membrane surface and antigens. In the long term such knowledge could potentially be very important in determining or designing an effective means of presenting recombinant D.N.A. technology derived vaccine antigens to the host in for example liposomes with particular biophysical properties.

5.2.6 Summary

Cooling of adult S. mansoni labelled with aminofluorescein C18 and Rhodamine C18 results in a striped pattern of labelling which may reflect a macromolecular level of membrane organisation. Cooling of DiI C18 labelled schistosomula and adults followed by rewarming, in contrast to the dyes mentioned above, results in damage to the surface membrane. The nature of this damage is stage dependant. In adult DiI C18 associated cooling results in the destruction of the membrane while in schistosomula DiI C18 associated cooling in conjunction with fluorescence illumination results in uncoupling of the membrane from the body of the parasite. In each case there is reason to believe that he DiI C18 resides in immobile regions of lipid and that these regions would be disrupted by cooling. A hypothesis linking the nature of damage to the effect of DiI C18 induced destruction of cytoskeletal membrane links is advanced.

Chapter 6

An investigation of domain structure using resonance energy transfer as an investigative technique.

6.1. Introduction.

6.1.1 The biological function of domains.

As has been described earlier in this study there is evidence to suggest that the lipid phase of the surface membrane of S. mansoni exists in a complex state differentiated into regions of differing biophysical characteristics (domains). This hypothesis rests entirely on the observations of Foley 1986 who showed large scale immobility of several fluorescent lipid analogues within the surface membrane. This was interpreted as indicating differentiation of the membrane into gel phase and liquid crystalline domains. That is into regions of membrane lipid that were relatively mobile or fluid in their biophysical properties and other regions which were gel like and immobile.

As described in the introduction [section 2.1.5 Chapter 1] such differentiation into domains is clearly seen in certain specalised cell types such as spermatozoa and ova and may be an important fundamental feature of all cell membranes. The biological importance of this phenomena remains to be fully elucidated but clearly domain formation in the plasma membrane would allow lateral compartmentalisation of membrane function in the plane of the membrane. An analogy can be drawn with the intracellular role of membranes which physically compartmentalise the different metabolic and enzymic activities of the cell and allow it to function efficiently. For example the potentially harmful effects of acid hydrolysis are contained within the lysosomes and physically separated from protein production in the endoplasmic reticulum.

Similarly lateral compartmentalisation in plane of the surface cell plasma membrane would allow potentially conflicting processes such as trans membrane signalling, membrane fusion or excision and surface membrane enzymic activity to function in parallel alongside each other in the same physical structure of the plasma membrane. There is some evidence for this interpretation of the biological function of domains.

This includes the requirement of particular membrane enzymes for an annuli of specific lipid in order to function. Adhesion of cells to one another as in myoblast fusion or to substrates as in focal adhesion plaques , the formation of coated pits during endocytosis, lectin induced capping are all examples where differentiation of the cell plasma membrane into specialized regions is clearly related to biological function. These examples are somewhat extreme since generally they represent a response to some stimulus.

Karnovsky et al showed domain structure was implicated in a variety of cell types while in a more unstimulated or steady state condition. This can be related to the examples cited above by proposing that differentiation of the plasma cell membrane is a fundamental and intrinsic property dictated by the fact that it's molecular constituents; lipids, sterols and proteins are not perfectly miscible and will tend to segregate to some extent thus forming domains. Domain structure could therefore, be considered an intrinsic property of the plasma membrane and the specific examples cited above represent the biological expression of this property.

6.1.2 Domain function in the surface membrane of S. mansoni

Clearly this analysis can be extended to the surface membrane of S. mansoni since it also would be expected to have specialisation of its membrane into regions of differing function. The observations described in this study concerning the longitudinal differences in the properties of the surface membrane of S. mansoni and localised differences at the tubercle support this contention.

The domain size implicated by Foley's study must be in the order of hundreds of square angstroms rather than square microns in area since the area monitored by FRAP is 1um in radius. However, variation in the membrane at this level of domain structure could well be the biophysical basis of the larger scale regional changes over microns described above. For example a localised reduction in the occurrence of immobile domains at the anterior region of all stages of the parasite could explain the finding that the anterior membrane is generally more mobile relative to the rest of the parasite membrane. Similarly the increased uptake of merocyanine 540 at the tubercle may reflect a locally more disordered fluid domain structure.

Investigation of the domain structure of the surface membrane of S. mansoni is then of interest in terms of its probable contribution to membrane function generally and as a mechanism of generating differentiation in the properties of parasite membrane locally across its surface.

6.1.3 Domain differences between parasite and host

There is a further reason for investigating the membrane at this level of organisation. The properties of the two lipid analogues used by Foley 1986, aminofluorescein C18 and DiI C18, differ markedly in behaviour within the schistosomula and lung worm stage surface membranes compared with most cell plasma membranes. The immobile

fraction of these dyes in S. mansoni is markedly greater. This indicates that the biophysical properties of the parasite surface membrane differ significantly from those of the cells surrounding it in the bloodstream of the host. This difference is likely to be a relatively greater fraction of immobile lipid molecules at the lipid aqueous interface. It is also possible that in addition to this absolute difference in fraction of immobile lipid expressed at the surface there is also a qualitative difference in extent to which differentiation into domains occurs. That is the relatively greater immobile fraction in S. mansoni as compared to cells could take the form of a limited number of fairly large immobile domains. Alternatively the extra 25% or so immobile fraction could exist in the form of numerous small domains. This rather abstruse distinction has important consequences for the nature of the surface exhibited by the parasite. To adapt and paraphrase Singer & Nicholson's "icebergs" analogy. In this instance the term "iceberg" more appropriately represents gel phase immobile lipid rather than intramembraneous protein particles. To make the analogy with the above discussion concerning an increase in the immobile fraction in a FRAP spot of roughly 25%. Consider an area of sea with 25 million tons of ice floating in it. This description could apply equally as well to an area where one or two large icebergs float in a largely uniform surface of unobsructed seawater or to an area totally covered with broken "ice floe". The surface presented in each case is very different, the latter exhibiting a more subdivided differentiated surface which affects the sort of activities that can occur there. This analogy can be applied to the parasite and cell surface membranes. It is known that within a spot of 2um diameter on the surface membrane of S. mansoni 50% of aminofluorescein C18 molecules introduced will be immobile whereas the comparative figure for a lymphocyte will be around 20%. In neither case is it possible to relate such gross measures

of mobility / immobility to any structural properties concerning the "texture" or degree of "differentiation" of the domain structure as illustrated by the simple analogy outlined above.

6.1.4. Domain differentiation: function

There is no generally accepted terminology for this concept. In this study the term domain differentiation will be used. The convention adopted is that a highly differentiated domain structure describes a membrane in which the immiscible domains, fluid/gel, are numerous and fragmentary. This would be analogous to the "broken pack ice" in the above example. A poorly differentiated domain structure would consist of markedly fewer large domains. It is worth stressing that immiscible domains could occur within either a wholly gel or liquid crystalline phase and that the gel liquid crystalline distinction although the most relevant to this discussion is not necessarily the only type of domain difference.

To give a hypothetical example of how domain differentiation may affect membrane function in S. mansoni. It has been reported in model membranes that membranes with a high cholesterol to phospholipid ratio exhibit a segregated structure whereby long strips of cholesterol rich membrane interdigitate with parallel strips of cholesterol poor membrane [Rectenwald & McConnel 1981]. It is possible that the same type of differentiation occurs in for example the dorsal surface of the adult male. This may also have a high cholesterol to phospholipid ratio due to the interaction of lipoproteins with the membrane as discussed in chapter 4. If organised in this manner the domain structure of the membrane itself may facilitate transverse anisotropic diffusion across the membrane surface towards the gynaecophoral canal as postulated in chapter 5 (Figure 5.4).

The biological function of this relatively high immobile fraction in S. mansoni remains unknown. Foley 1986 has suggested that the increased immobility may act to inhibit immunological reactions which are dependant on the diffusion and coming together of molecules in the membrane. This would apply to phenomena such as complement activation to form the membrane attack complex and to antibody mediated complement activation. [Parce et al 1978 , De Lisi & Wengel 1983].

It is important to note that while increased immobility per se will tend to inhibit these reactions increased immobility associated with increased differentiation as discussed above will have an even greater effect on these immune reactions.

6.1.5. The possible effect of domain differentiation on antibody binding to S. mansoni

This is particularly true of IgG binding. IgG is essentially a divalent molecule and therefore the avidity of binding is determined almost entirely by whether one or both binding sites attach to the antigen. There is up to a thousand fold increase in avidity upon binding of both valencies of an IgG molecule as compared to monovalent binding [Roitt, Brostoff & Male 1985].

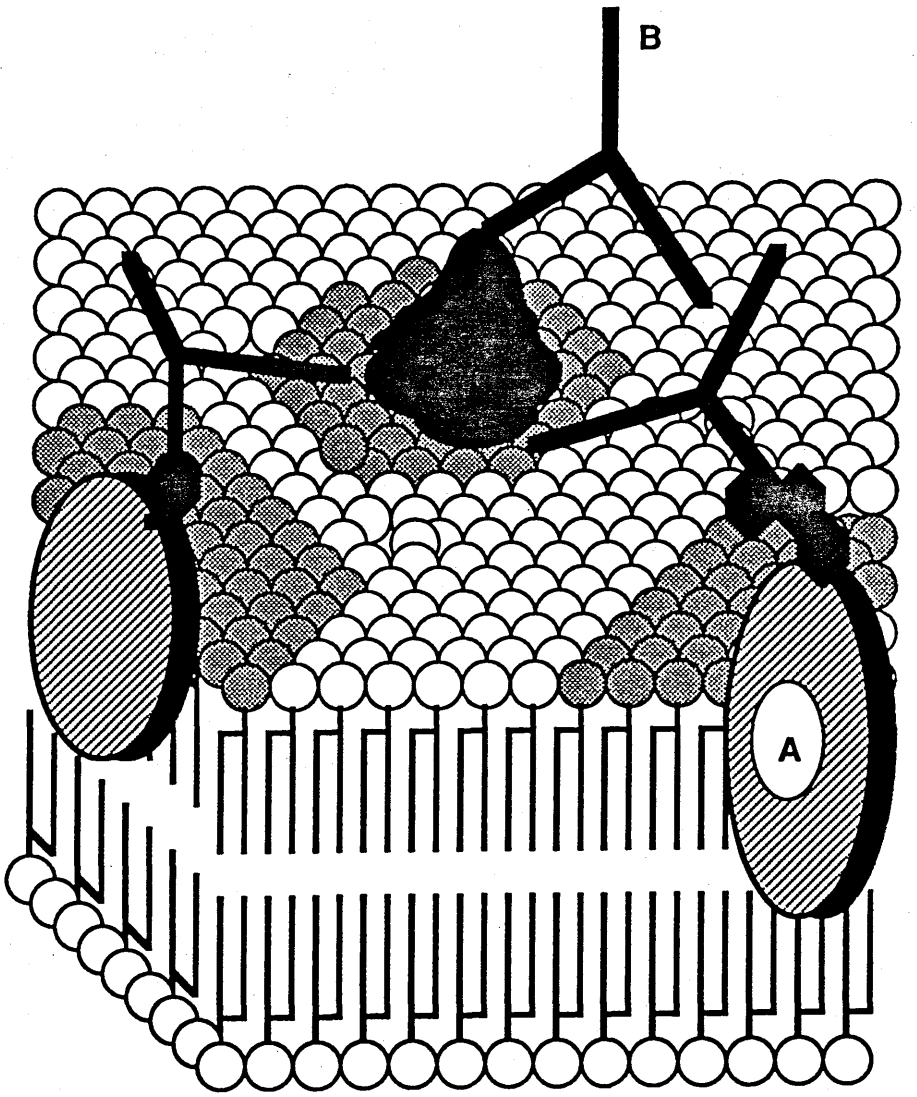
There is some dispute as to the level or more pertinently the density of parasite antigens exposed at the lung worm stage [Bickle & Ford 1982, Pearce et al 1986]. This is most plausibly accounted for by assuming that there is a very low level of parasite antigen exposed which is at the limit of the methods of detection used. Moreover, there is good reason to suppose that the majority of such exposed epitopes are within proteins located in immobile domains. This is because FRAP analysis of lung worm glycoprotein mobility revealed almost total immobility [Foley 1986]. Clearly then, the ability of IgG to bind avidly to such a

surface will depend on whether both binding sites on the immunoglobin can bind to one epitope and stretch across the domain structure sufficiently to attach to another thus effecting divalent binding. The actual physical separation of the two binding sites in either arm of the IgG molecule is between 100 - 140 angstroms [Valentine & Green 1967].

In a situation where there is a low density of recognisable epitopes exposed, differentiation of the membrane into immmobile domains of dimensions only slightly greater than this could totally debilitate the effects of any low level antibody binding (See Fig 6.1). Divalent binding of antibody to two antigenic determinants on separate immobile domains would be inhibited as shown on the diagram. However this same mechanism could also abrogate divalent binding between determinants in immobile and mobile regions, if the "islands" of immobile lipid comprising the immobile domains were sufficiently large i.e greater than 100 angstroms in radius. Antibody dependent cellular killing mechanisms and antibody dependent complement fixation would both be abrogated. Thus the intrinsic resistance of the lung worm membrane could be genuinely intrinsic to the biophysical structure of the membrane itself. By this analysis a combination of factors including; the mobility, density, spatial distribution and minimal potential separation of any antigen exposed on the parsite's surface will determine the extent to which an antibody will exhibit divalent binding to that surface. This hypothesis that domain structure determines the avidity and hence the effectiveness of bound antibody is an important reason for developing methods to determine domain structure and also the biophysical parameters listed above associated with particular antigens.

Figure 6.1

The effect of domain structure on the avidity of IgG binding



A Integral membrane glycoprotein

B Immunoglobin molecule (IgG)

Mobile lipid molecule

Immobile lipid molecule

Glycosidic residues

Another reason for this approach was that the FRAP results of Foley cleary indicated that the domain structure of the surface membrane of juvenile S. mansoni is different from most cell membranes. This difference may represent a fundamental biophysical difference between the surface membrane of the juvenile parasite and its host's cell surface membranes. This raises the question of why such a difference is not recognised by the host and the related hypothesis described above that this very difference actually inhibits recognition. This chapter is concerned with the use of the fluorescent lipid probes to investigate both the occurrence and nature of the lipid domains postulated by Foley et al 1986. It has used the same probes but attempted to develop and employ fluorescence techniques other than FRAP but complementary to it to address these questions. The approach adopted in this study was to investigate whether the phenomena of resonance energy transfer between FRAP characterised lipid probes could be demonstrated and then exploited to gain information concerning the presence and differentiation of domains within the surface membrane of schistosomula. Prior to discussing this particular application a short description of the theory and application of the phenomena of fluorescence resonance energy transfer follows.

6.2.1 Resonance energy transfer

The exploitation of the phenomena of resonance energy transfer between fluorescently labelled analogues of biological molecules was pioneered by Stryer 1979. In essence the technique measures the separation of a suitable pair of fluorescent chromophore groups over distances in the range of 10 to 100 angstroms. The technique exploits the fact that with a suitable donor and acceptor pair one in which the absorption spectra of the acceptor overlaps the emission spectra of the donor (see fig 6.2) it is possible to measure the indirect excitation of the acceptor chromophore molecule after the direct excitation of the donor (see fig 6.3).

Figure 6.2

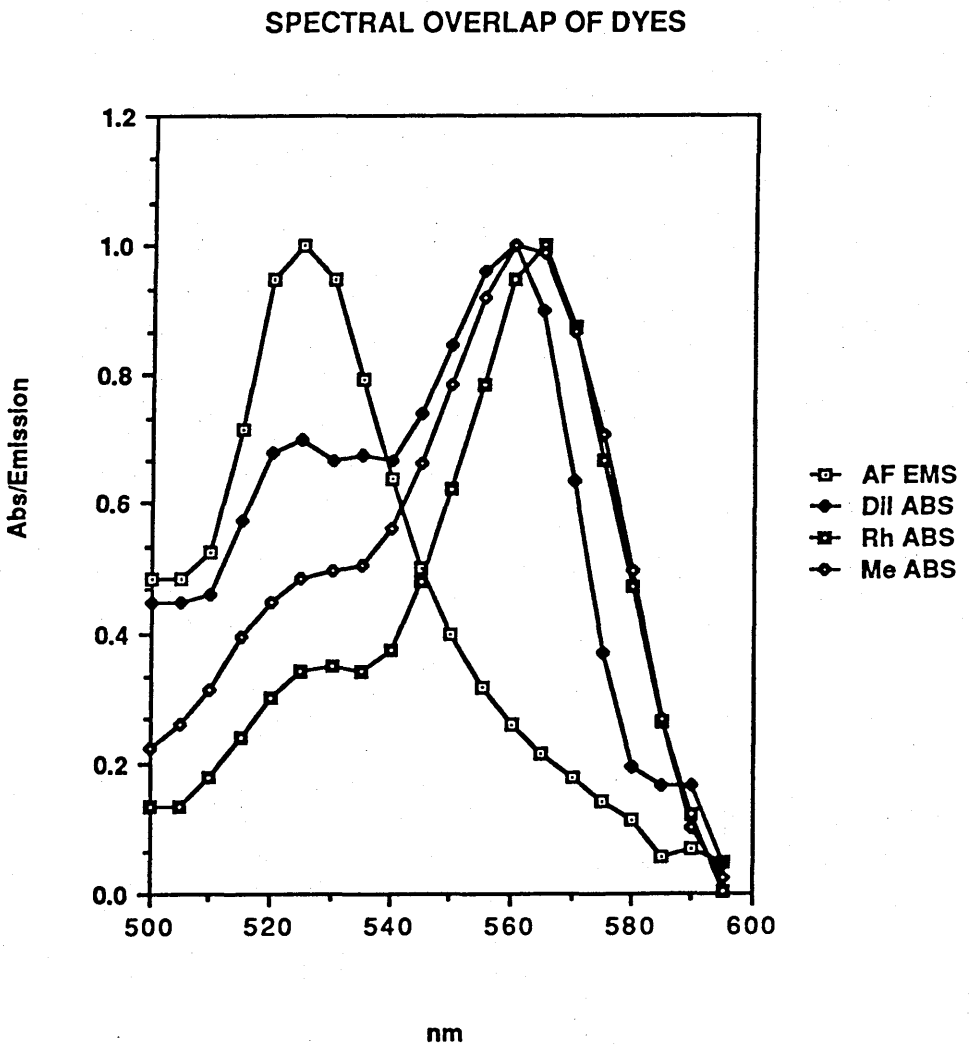
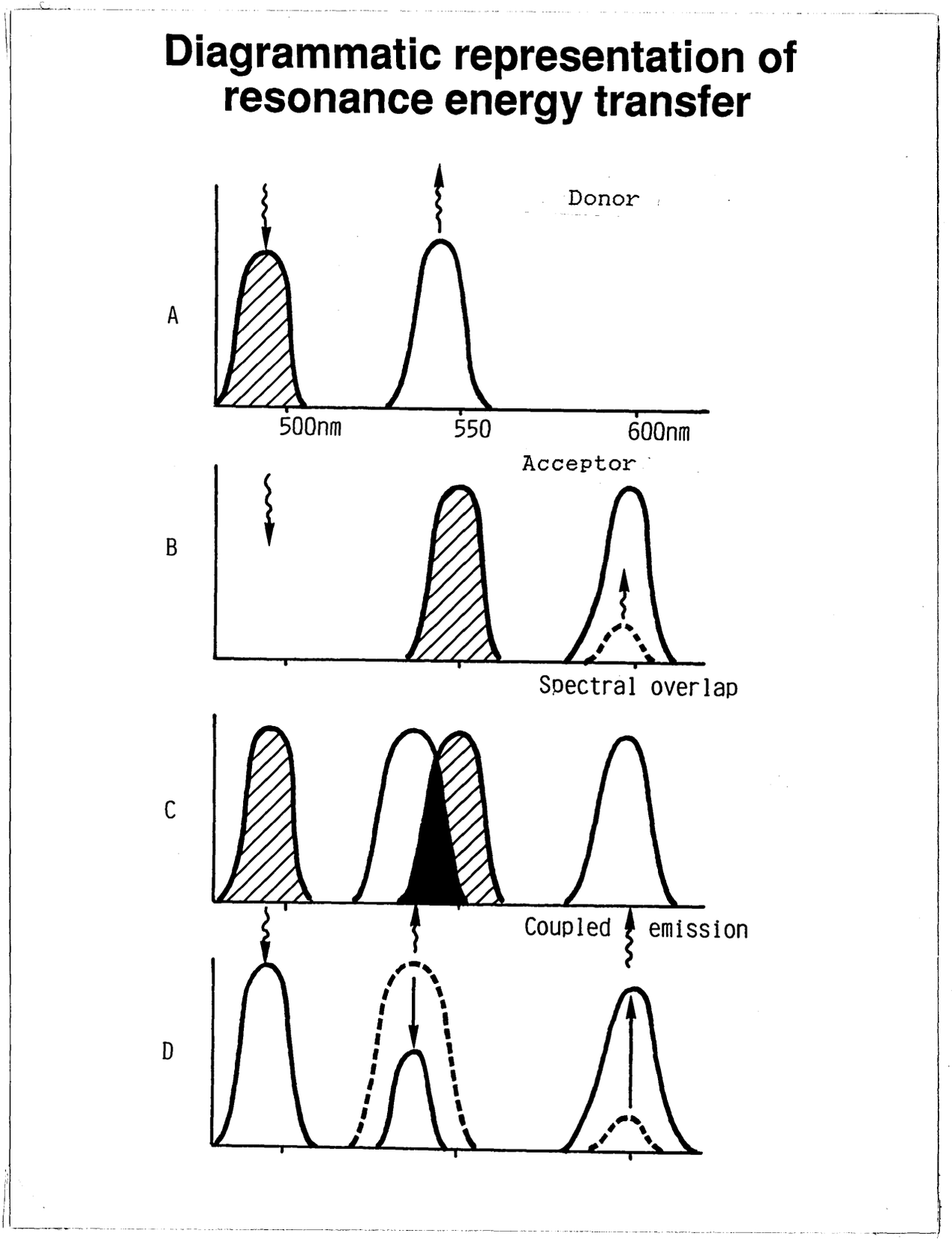

SPECTRAL OVERLAP OF DYES
Abs/Emission
1.2
1.0
0.8
0.6
0.4
0.2
0.0
500
520
540
560
580
600
nm
AF EMS
DII ABS
Rh ABS
Me ABS

Figure 6.3


Diagrammatic representation of resonance energy transfer
Donor
A
500nm
550
600nm
Acceptor
B
Spectral overlap
C
Coupled
emission
D

This transfer of energy from donor to acceptor can also be monitored by measuring quenching of the donor signal in the presence of acceptor. This is accompanied by a concomitant enhancement and depolarisation of the acceptor signal. This transfer of energy is strongly distance dependant and falls off rapidly above the Ro value, a value that is defined as the separating distance at which direct excitation of the donor equals indirect excitation of the acceptor. In other words in such a situation if the donor chromophore is excited 50% of the energy absorbed will be emitted as fluorescence by the donor and 50% of the energy will be transferred to the acceptor. Clearly if only 50% of the donor's energy is emitted in the presence of an acceptor at Ro, then effectively the signal of the donor will be quenched by 50%. This represents an experimental definition of the Ro convention. That is in the situation where the fluorescence emission of a donor probe (aminofluorescein C18) is reduced by 50% due to the presence of a suitable acceptor (DiI C18) then one can say that the average separation of these two molecules is equal to the Ro value of that donor/acceptor pair. In this way it is possible to relate quantitative measurements of fluorescence to the average separation of two probes within a membrane.

This technique has been widely used in two ways. Firstly in a semi qualitative manner in which the occurrence rather than the absolute extent of resonance is monitored. An example of this is intracellular tracking studies which are aimed at determining whether different plasma membrane constituents are processed within the same intracellular compartments [Uster & Pagano 1987]. In this study a fluorescent microscope with the appropriate filters and photography was employed. The extent of resonance was not quantitated in any way but simply used as a monitor of acceptor and donor mixing and association. The same principle has been employed as a qualitative monitor of cellular or liposome fusion .

In addition, resonance energy transfer has been employed in a strictly quantitative empirical way as a spectroscopic ruler in order to determine the spatial separation of donor and acceptor chromophores, usually on differing molecules or parts of molecules [Stryer 1978].

These examples indicate how the technique can be used to measure in essence either linear separation between chromophores on a molecular structure or the vertical separation between different elements at a membrane surface. Of more interest to this study other workers have employed the technique to measure the lateral separation of molecules within the plane of the membrane [Fernandez & Berlin 1976]. This is essentially what has been attempted in this study using fluorescent lipid analogues rather than fluorescent labelled lectins.

6.2.2. Fluorescence resonance energy transfer as applied to the surface membrane of *S. mansoni*.

This section summarizes the arguments for studying domain structure and describes the features of the fluorescent analogues employed that render them suitable for such an investigation. The domain structure of the membrane represents a fundamental level of membrane organisation which may underly important features of the membrane's function. These could include; localised differences within a single parasite's membrane; developmental differences between the membrane of different stages and related to this the molecular basis of "intrinsic" resistance. The high levels of lipid immobility exhibited by juvenile stages of the parasite represent both an important host parasite difference and a membrane feature which may be associated with the development of "intrinsic" resistance. It is suggested that this feature could be the degree of "differentiation" of the surface membrane into domains rather than the extent of lipid immobility per se. At present there is no way of estimating the number, size or shape of such domains.

The exploitation of the phenomena of resonance energy transfer as an analytical technique to investigate this problem was attempted for the following reasons.

a) The probes used routinely for FRAP analysis form suitable donor pairs. In particular the analogue aminofluorescein C18 could be used as a common donor and a range of other dyes DiI C18, Merocyanine 540 and Rhodamine C18 used as acceptors. These latter three dyes exhibit a range of mobilities as monitored by FRAP.
b) FRET is highly distance dependant and measures association or spacing of molecules within the range 10 to 100 angstroms. Thus it can be used in quantitative studies on domains.

The important feature of this technique is that it has the potential to relate the levels of fluorescence emitted by these probes, of known mobility, to their lateral separation across the membrane. This is a prerequisite for any estimation of the size of domains. Moreover the range in which this technique is sensitive, 10 to 100 angstroms, is ideal. This facet of the technique, as an empirical measure of lateral distance, has not been approached experimentally in this study. However a theoretical framework utilising FRET between two such probes in the membrane as a scalar against which domain size could be estimated is outlined in section 6.6. A more immediate objective was to use the knowledge gained from FRAP, concerning the preference for immobile or mobile regions of membrane exhibited by these probes, and relate this to FRET. This was done in a predictive manner. Essentially the rationale was that there should be a strong correlation between the degree to which the partition preference of the donor acceptor pair resemble each other and the efficacy of FRET exhibited by such molecules introduced into the parasite membrane. In other words if the donor molecule was 100% mobile and the acceptor 100% immobile then FRET

between this particular pair would be expected to be very poor. There is an important qualification to this. This will only be the case if the size of the domains formed is greater than the Ro value of the acceptor donor probe. The Ro value is the threshold distance at which efficient FRET will take place (see section 6.2.1). Conversely an acceptor donor pair which both showed 100% mobility, that is partitioned into the fluid domain, would be expected to exhibit efficient FRET, assuming that no fluid/fluid immiscible domains existed within the membrane.

The concept underlying this approach is that of acceptor or donor redundancy. Consider the former example cited above. In this situation where donor and acceptor partition preferentially into immiscible domains some energy transfer could still occur if for example the acceptor partitioned into spherical domains 200 angstroms across. They could accept energy indirectly from the donor at the border between the liquid crystalline and gel phase regions. The average separation would be comparable to the Ro value of that pair say 60 angstroms. However, within the centre of this spherical domain i.e. > 100 angstroms from the nearest donor molecule there would be no appreciable resonance energy transfer and these molecules could be considered "redundant" in terms of FRET. The same situation would apply in reverse to donor molecules within the heart of the mobile domain.

This approach allowed a fairly crude assessment of whether domains greater than, X2 Ro value, exist within the membrane. It was facilitated by the fact that the probes most commonly used in FRAP analysis could form suitable donor acceptor pairs. In particular it was possible to utilise aminofluorescein C18 as a common donor of fairly neutral gel/fluid preference and compare FRET between it and a number of FRAP characterised acceptors exhibiting a range of percentage recovery. This ranged from DiI C18 which shows a strong preference for immobile regions,

Rhodamine C18 which is similar to aminofluorescein C18 and Merocyanine 540 which is completely mobile in the membrane. These values along with the appropriate Ro values for the aminofluorescein C18 donor pair are shown below. The other important factor that allowed this type of analysis was the knowledge that in the schistosomula all of these dyes locate within the same lipid leaflet, the E_1 face. Therefore FRET between pairs of such probes reflects lateral separation of molecules in the plane of the membrane not vertical distribution between the leaflets. This is further enhanced by the fact that the fluorescent chromophore groups of all the analogues locate at or near the aqueous interface of the membrane.

Table 6.1

Biophysical properties of commonly used dyes.

	FRAP	**FRET**
	% recovery after photo--bleaching	Ro value (Angstroms) aminofluorescein C18 as donor to dye.
Aminofluorescein C18	55%	----
Rhodamine C18	62%	50 A
DiI C18	30%	56 A
Merocyanine	100%	56 A

These values (% recovery) pertain to mechanically transformed schistosomula at >3hrs post transformation. The Ro values were calculated assuming a quantum yield for Aminofluorescein C18 equal to 0.6.

To summarise briefly, this "predictive" approach is based on the known mobility characteristics of the analogues and the fact that they made suitable acceptor donor pairs of specific Ro values. It is based on the premise that if immobile domains exist and are greater than a particular size, X2 Ro values approximately 100 angstroms, then their effects may be observable by the relative efficacy of FRET between particular donor acceptor pairs. This approach

appeared to hold out the promise of indirect corroborative support for the hypothesis that domains existed and the possibility of estimating their minimum size. The "scalar" approach outlined in section 6.6 has not been developed as yet, it outlines a longer term strategy for developing a "model" of the membrane which would allow estimation of the probable size, number and shape of the domain structure. This could form the basis of a computerised simulation or model of the membrane structure.

6.3.1 FRET experimental development.

The theoretical framework underpinning this approach was not formulated in the detail described above at the start of this project. This is reflected in the nature and order of the data described below. However the basic premise of the approach was appreciated from the start. This was that the separation of two potentially resonant probes such as aminofluorescein and DiI C18, Rhodamine C18 or merocyanine 540, introduced into the membrane will be affected by the structural properties of that membrane and that this can be measured via the extent of FRET between them. The long term objective of the work described in this chapter is to develop techniques to do this and relate these measurements to structure. More immediately the objective was to gain corroborative evidence i.e. derived from techniques other than FRAP for the existence of differentiation of the schistosomula into domains. The experimental data presented below reflect these concerns and describe the first steps towards fufilling these goals.

6.4.1. The effect of temperature on fluorescence emission by lipid analogues in the schistosomula membrane.

An important stimulus to the investigation of domain structure via FRET was the observation that the fluorescence of the lipid analogues was differentially sensitive to changes in temperature. These experiments were undertaken to investigate the influence of cold

induced changes in membrane. In particular, it was hoped that variation in the fluorescence emission would give some indication of the transition temperature of the membrane. The fact that such a fundamental property of the membrane is not known indicates the dearth of biophysical studies on the parasite's membrane. In the event there was some evidence to suggest that the transition temperature of the schistosomula membrane was about 28°C (Fig 6.4). This preliminary data will be followed up by monitoring change in the FRAP parameters % recovery and DL value against temperature. It does suggest that the transition temperature of the schistosomula surface membrane is very similar to that of most "natural" cell plasma membranes. This possible sensitivity to transition temperature was obtained with only one lipid analogue DiI C18 the others fluorescence varied with temperature but did not reveal any change that resembled a transition breakpoint. The data shown in figure 6.4 was derived by monitoring fluorescence intensity on a given area of schistosomula membrane over time as temperature was varied.

The effect of bleaching of the analogue was resolved by taking intermittent readings every 15 seconds and by attenuating the excitatory beam partially. This process was repeated for a number of dyes and the results shown in figure 6.5. This pattern of results was achieved irrespective of the direction of temperature change.

The notable feature of this data is the relative sensitivity of the dye DiI C18 to temperature. This is remarkable on two counts. Firstly in its scale and secondly in its orientation or trend. All the other dyes exhibited only a slight variation in % change in intensity with temperature whereas the change in DiI intensity almost doubled over the same range. Moreover while the emission of DiI markedly decreased as temperature increased emission from the other dyes increased.

Figure 6.4

Schistosomula (3 hrs mechanically transformed) were labelled with DiI C18 (10 ug/ml) for 20 minutes at 37°C and washed three times in E.M.S.

The fluorescence intensity of an area of labelled schistosomula in the centre of the parasite 400 um^2 was monitored as the temperature was varied. Individual readings were taken every 15 seconds, a measure which eliminated bleaching caused by fluorescence illumination. Each spot represents an individual reading. This diagram is a representative trace obtained for a single parasite in a group.

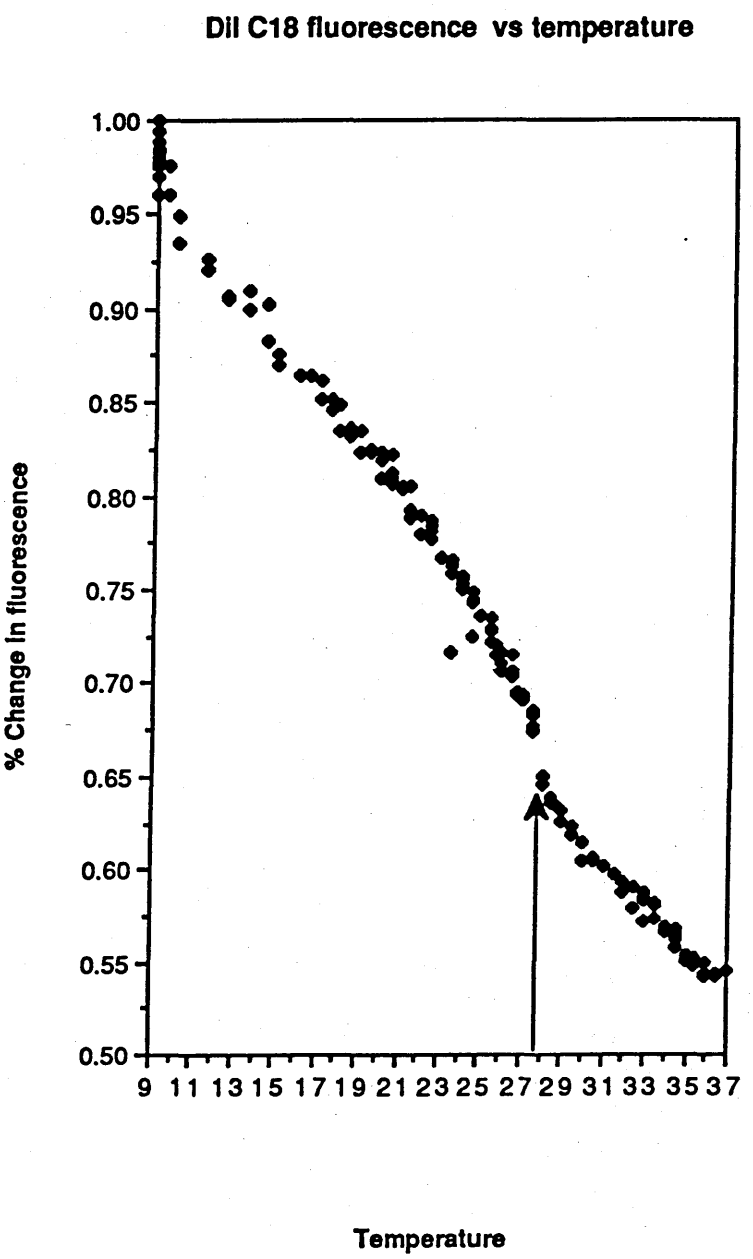
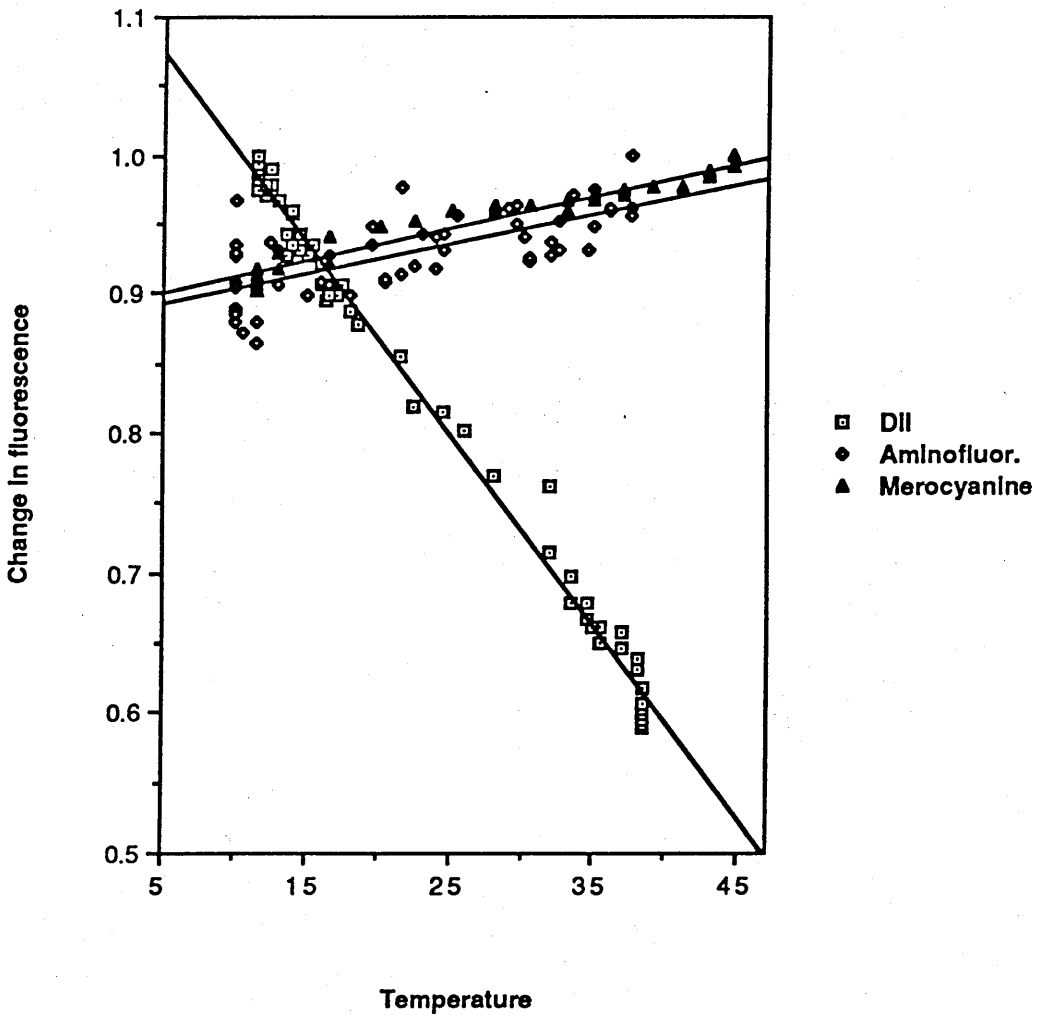

DiI C18 fluorescence vs temperature
% Change in fluorescence
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
9 11 13 15 17 19 21 23 25 27 29 31 33 35 37
Temperature

Figure 6.5

A population of 3 hr mechanically transformed schistosomula was split in three and labelled with 10 ug/ml of aminofluorescein C18 (Aminofluor), DiI C18 (DiI) or Merocyanine 540 (Merocyanine) for 20 minutes at 37°C in E.M.S. All groups were washed three times prior to quantitation. Fluorescence readings were taken every 15 seconds as the temperature was slowly increased. Each spot represents the average of 2 or 3 readings taken on a fixed area on an individual parasite at a particular temperature as temperature was slowly increased.

These traces are representative of each labelled group of parasites. The effect is reversible, that is the same trend in fluorescence increase or decrease is seen whether the temperature is increased from 10°C or decreased from 37°C.

Fluorescence intensity vs temperature



The nature of this temperature sensitive behaviour of the dye DiI C18 in the schistosomula surface membrane exhibits strong similarities with a study utilising the same dye in model membranes [Klausner & Wolf 1980]. These authors reported a similar decrease in DiI C18 emission upon warming an artificial membrane. This was correlated to the phase transition temperature of that membrane. Their explanation of this result was that DiI C18 had a preference for gel phase lipid and when forced out of this preferred enviroment formed small aggregates of DiI molecules which self quenched. The decrease in DiI emission in the schistosomula could reflect the formation of such aggregates within the parasite membrane. This interpretation reinforces the view derived from FRAP that DiI C18 has a strong preference for gel phase domains. This represents further indirect evidence that such domains do exist within the surface membrane of the schistosomula. In addition it supports the contention that these will be segregated within gel phase domains at physiological temperatures.

Aminofluorescein C18, Rhodamine C18 and to a lesser extent Merocyanine 540 appear to be phase neutral, that is, they appear to partition fairly equally into gel and liquid crystalline phases as adjudged by FRAP. They exhibit little change in fluorescence emission with change in temperature indicating that as the phase characteristics of membrane change they are not forced from a preferred enviroment but remain in a stable monomeric state. Merocyanine is different in that there is little change in emission with temperature change while % recovery is at 100% at both room and physiological temperatures. These characteristics probably reflect the fact that within the schistosomula membrane the dye occupies a position parallel to the glycerol backbone of the membrane. This position has been implicated by studies carried out on artificial membranes [Lelkes & Miller 1980]. In such a position the dye, which does not have a long acyl chain, would not be

subject to the constraints imposed by the acyl chain core of the membrane. As a consequence of this it would be expected to be relatively insensitive to phase changes within the membrane. This interpretation is consistent with the behaviour of the dye in the parasite membrane monitored to date. It has important implications for its use as an acceptor molecule in FRET, notably that it may not distinguish between the immobile/mobile domains at all but is free to move within and between either of them.

6.4.2 Enhanced emission of acceptor: initial experiments. Due to technical limitations the first experiments on FRET in the schistosomula concentrated on measuring resonance energy transfer via the enhanced acceptor fluorescence of the dyes Merocyanine 540 and DiI C18 using aminofluorescein C18 as the donor. The results are shown in Figure 6.6.

These are shown in the form of a ratio whereby the sum of the fluorescence readings derived from the two control single labelled populations of schistosomula is compared with the reading derived from a double labelled population. Statistical analysis was carried out in such a way as to maximise the variance. This was done by ranking the two sets of control readings such that the highest in one population was paired with the highest in the other population and similarly the lowest with the lowest. Thus the maximum range of ratio values is generated. This composite set of readings was compared with the experimental population which was double labelled, by students T test. This analysis indicated that schistosomula labelled with both aminofluorescein and Merocyanine showed a synergistic increase in fluorescence in the red (acceptor emission) significantly greater than the sum of the two control populations. This was not the case for the population of schistosomula double labelled with aminofluorescein and DiI C18.

These experiments indicate that efficient excitation of Merocyanine via aminofluorescein is occurring in such double labelled populations. The results for aminofluorescein and DiI did not at this stage look promising.

However these experiments represented the earliest measure of resonance which concentrated only on one factor, enhanced fluorescence of the acceptor in the presence of the donor. However these results are of some interest in that even with this limited measure of resonance there were some indications that manipulation of culture conditions by addition of serum or affecting the phase composition of the membrane by cooling could influence the extent of resonance between two probes introduced into the membrane.

6.4.3 Concentration dependent quenching.

The acquisition of a specialised narrow band pass filter allowed the effect of quenching of the donor (aminofluorescein C18) to be carried out. This allowed the effect of increasing the concentration of acceptor molecules (DiI C18) on samples of an aminofluorescein C18 labelled population of schistosomula to be monitored. The results are shown in Figure 6.7 and indicate that saturable quenching of aminofluorescein fluorescence with DiI C18 can be achieved. This is consistent with a mechanism of quenching via resonance energy transfer by the DiI C18.

6.4.4 Concomitant donor quenching & acceptor enhancement

The definitive proof that resonance energy transfer is occurring is the demonstration of quenching of the donor (aminofluorescein) concomitant with an increase in acceptor fluorescence. This is demonstrated for both Merocyanine and DiI C18 in Figures 6.8 & 6.9. Clearly the efficacy of the effect is greater with Merocyanine, however it is still significant with DiI C18 also. This relatively increased efficacy of Merocyanine probably reflects the fact that

Merocyanine due to its intercalation at the glycerol backbone of the membrane can very closely approach the chromophore group of the aminofluorescein molecule which resides at the membrane aqueous interface.

The nature of the DiI induced quenching described in Figures 6.8 & 6.9 is interesting in that there appears to be fairly efficient quenching of the donor compared to the control which was labelled only with aminofluorescein but relatively poor enhancement of the acceptor fluorescence. Again this was relative to control values and in this respect was similar to the early experiments described in figure 6.6 which focussed only on enhanced fluorescence. An attempt was made to estimate the relative levels of the two acceptor dyes used in this experiment by monitoring the levels of fluorescence emission in both the double labelled experimental group and the appropriate singly labelled control by exciting the acceptor directly. In this situation most of the fluorescence in the experimental group will come from the acceptor molecule since aminofluorescein emission is minimal and can be subtracted from the total. When this was done it was clear that while uptake of the acceptor Merocyanine by both control and experimental group was equivalent and exhibited no significant difference in emission, this was not the case for DiI C18. When this difference is taken into account as in Figures 6.8 & 6.9 it is clear that some resonance energy transfer leading to "enhanced" levels of acceptor (DiI C18) fluorescence occurs.

Two reasons could be advanced to explain this decrease in uptake of the DiI C18 by parasites pre labelled by aminofluorescein C18. The presence of aminofluorescein C18 in the membrane could inhibit the uptake of DiI by the membrane or alternatively allow uptake but cause the formation of self quenching aggregates of DiI C18. This latter suggestion would neatly account for the apparent discrepancy between apparently efficient DiI associated

quenching but poor enhancement. This is because such aggregates which show an absoption maxima at 512 nm could effect quenching of the aminofluorescein signal but would not themselves fluoresce. The effect of this would be to give good quenching coupled with anomalous levels of acceptor fluorescence.

An investigation was carried out to examine whether attenuation by U.V. irradiation of the parasite as cercaria prior to transformation induced any change in the levels of FRET between aminofluorescein C18 and DiI C18 or Merocyanine 540. No significant differences in either the relative quenching of the donor or the enhancement of the acceptors was observed (Fig 6.10). However there was an interesting pattern in the fluorescence intensity of the schistosomula labelled with the different dyes. Irradiation resulted in increased fluorescence in Merocyanine labelled schistosomula but decreased fluorescence with DiI C18 labelling. It had no effect on aminofluorescein labelling (Fig 6.12). This may indicate that the irradiated parasite has a more fluid disordered membrane structure into which relatively more Merocyanine, a marker for cholesterol free disordered membrane, can intercalate. Conversely such a membrane would have less immobile domain(s) and be less able to take up DiI C18 which is associated with immobile gel phase regions. Aminofluorescein C18 with no particular preference is as one might expect not affected by irradiation. This conclusion that irradiation induces some increase in fluidity of the membrane is supported by recent FRAP investigations (not shown) indicating a small but significant increase in aminofluorescein C18 fluidity in irradiated schistosomula as compared with non irradiated schistosomula. This area of the irradiation induced biophysical changes in the surface membrane is one where the techniques developed in this study can clearly be applied to relate membrane biophysics to immunological function(s) and effects.

Figures 6.6 to 6.10

In all these resonance transfer experiments the rationale has been to compare the enhanced fluorescence of a group of schistosomula double labelled with acceptor and donor with the sum of the fluorescence of two control groups singly labelled with either donor or acceptor. For the quenching parameter the comparison is with the level of fluorescence of the singly labelled donor control group (aminofluorescein C18).

When measuring enhanced fluorescence the singly labelled control groups are added in such a way as to maximise the variance of the composite control group termed AF + DiI or AF + Me derived from them. This is done by ranking the readings in each of the two groups and adding them together such that the highest and lowest values are paired. A single composite set of values is thus produced and this is used for statistical comparison. Students T tests are carried out comparing the range of fluorescence of these composite groups with that of the double labelled groups and the level of significance assessed.

This approach is shown in its most simple form in Figure 6.6.

Figure 6.6

Three hour mechanically transformed schistosomula were subject to a labelling regime which involved splitting the population into 5 groups. These groups were then subject to the following labelling protocol.

	Donor	Washed	Acceptor	Washed
1 (AF/DiI)	+	+	+ve (DiI C18)	+ve
2 (AF/ME)	+	+	+ve (Merocyanine 540)	+ve
3 (AF only)	+	+	-ve	+ve
4 (DiI only)	-	+	+ve (DiI C18)	+ve
5 (Mero only)	-	+	+ve (Merocyanine 540)	+ve

In each group 20 readings from individual parasites were taken where excitation is at 490 nm and emission at > 590 nm.

On the graph opposite
Group 1 is shown as AF/DiI + standard deviation.
Group 2 is shown as AF/ME + standard deviation.
Group 3 and Group 4 are combined to give Group AF + DiI.
Group 3 and Group 5 are combined to give Group AF + ME

The folowing Students T tests were carried out.
(AF + ME) vs (AF/ME) Significant at < 0.0001
(AF + DiI) vs (AF/DiI) Not significant at < 0.05

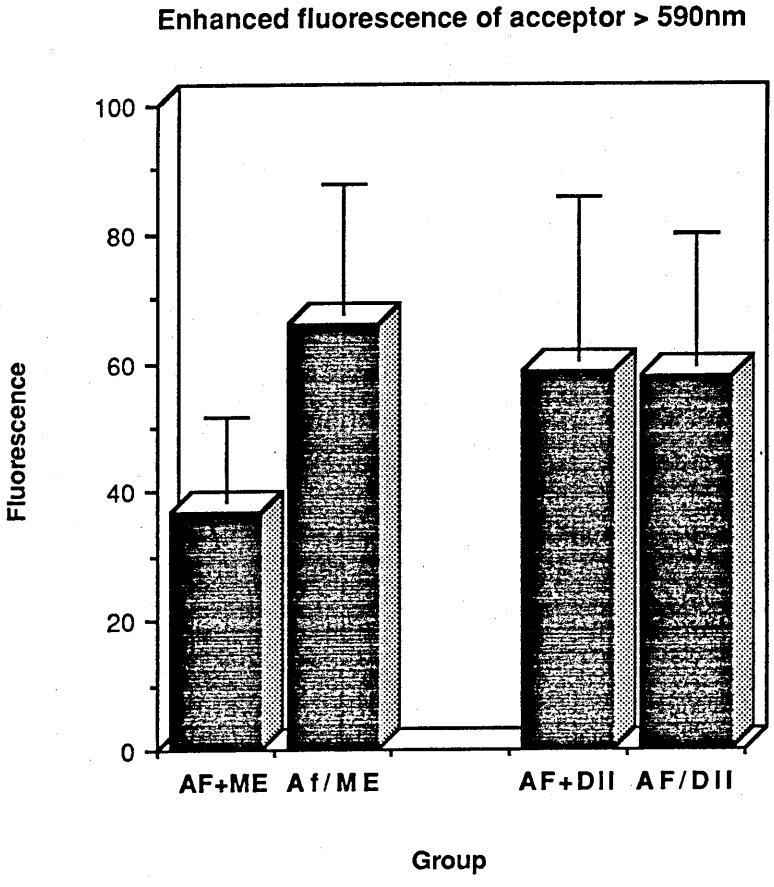
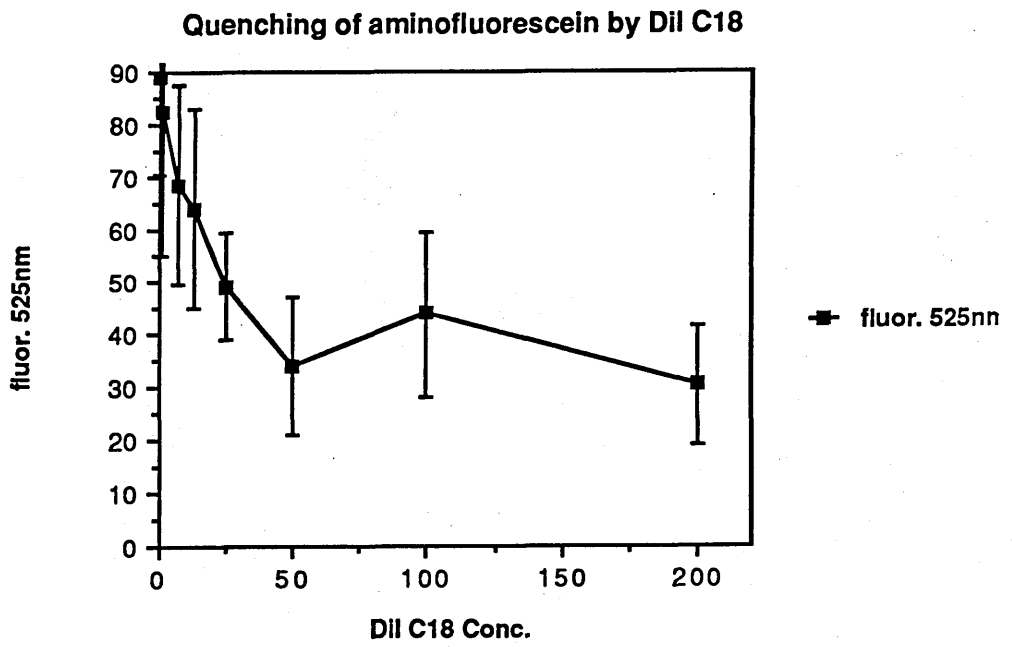

Enhanced fluorescence of acceptor > 590nm
Fluorescence
100
80
60
40
20
0
AF+ME
Af/ME
AF+DII
AF/DII
Group

Figure 6.7

3 hr mechanically transformed schistosomula were labelled with aminofluorescein C18 (10 ug/ml) for 20 minutes at 37°C. The population was then washed 3 times in excess E.M.S. and subdivided in aliquots of 1 ml containing approximately 400 schistosomula. These were then further labelled with increasing concentrations of DiI C18 for twenty minutes, washed three times and their fluorescence at 525 nm measured. Each spot represents the mean and standard deviation of 20 readings on individual parasites. The Y axis DiI concentration is in (ug DiI C18/ml).


Quenching of aminofluorescein by DiI C18
fluor. 525nm
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
DiI C18 Conc.
fluor. 525nn

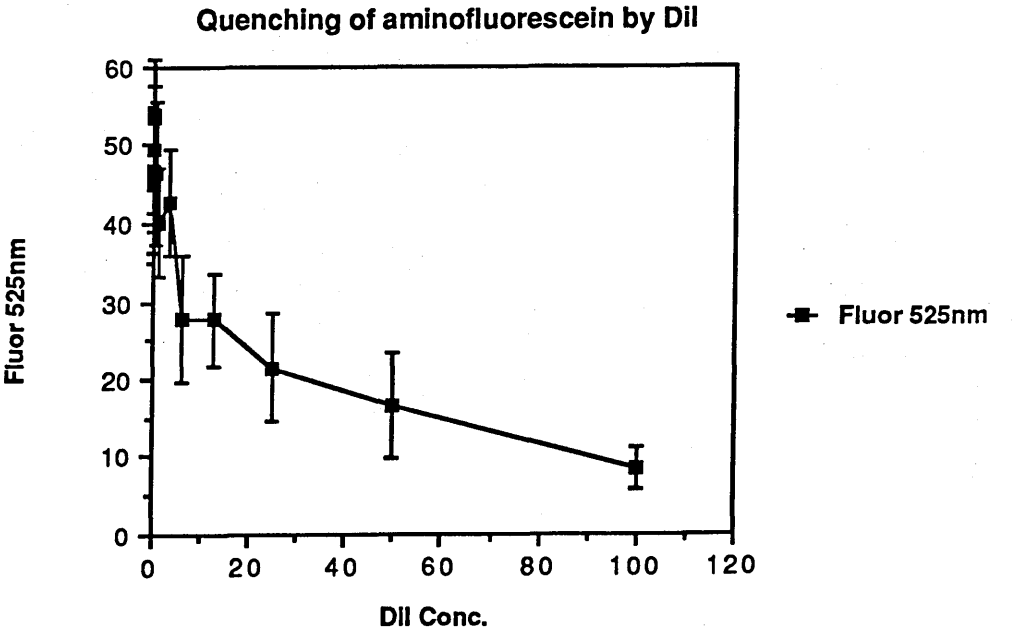

Quenching of aminofluorescein by DiI
Fluor 525nm
60
50
40
30
20
10
0
0
20
40
60
80
100
120
DiI Conc.
Fluor 525nm

Figure 6.8

3 hr mechanically transformed schistosomula cultured in E.M.S. and 5% FCS were split into 5 groups and labelled with either donor(aminofluorescein C18) or acceptor dyes for 20 minute periods at 37°C according to the following protocol.

	Donor	Washed	Acceptor	Washed
1 (AF/DiI)	+ve	+ve	(DiI C18)	+ve
2 (AF/ME)	+ve	+ve	(Merocyanine 540)	+ve
3 (AF only)	+ve	+ve	-ve	+ve
4 (DiI only)	-ve	+ve	(DiI C18)	+ve
5 (Mero only)	-ve	+ve	(Merocyanine 540)	+ve

After the 2nd set of washes fluorescence was quantitated at 525 nm and > 610 nm in each group (20 readings on individual parasites). The values obtained from groups 3 and 5 were combined to give AF + DiI and from groups 3 and 5 to give AF + ME.

Statistics: Student T tests.

Quenching of Fluorescence at 525 nm (Upper Diagram).

Group 1 AF only vs Group 2 AF/DiI Sig. at <0.0001

Group 1 AF only vs Group 3 AF/ME Sig. at <0.0001

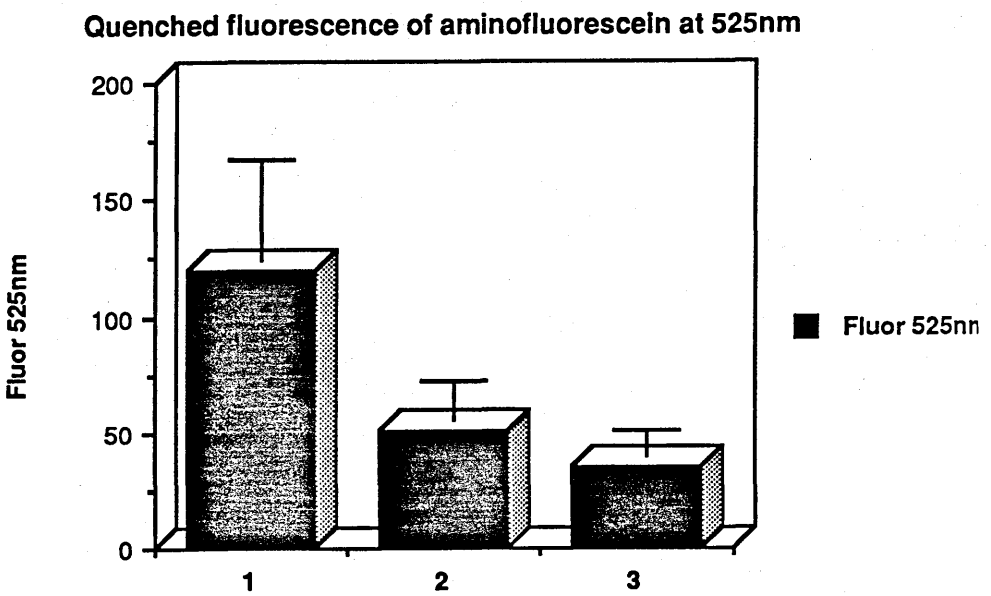
Enhanced fluorescence at > 610 nm (Lower Diagram).

AF/DiI (Double labelled) vs AF + DiI (sum of controls)

Significant at < 0.05 p = 0.031

AF/ME (double labelled) vs AF + ME (sum of controls)

Significant at < 0.0001


Quenched fluorescence of aminofluorescein at 525nm
Fluor 525nm
200
150
100
50
0
1
2
3
Fluor 525nm

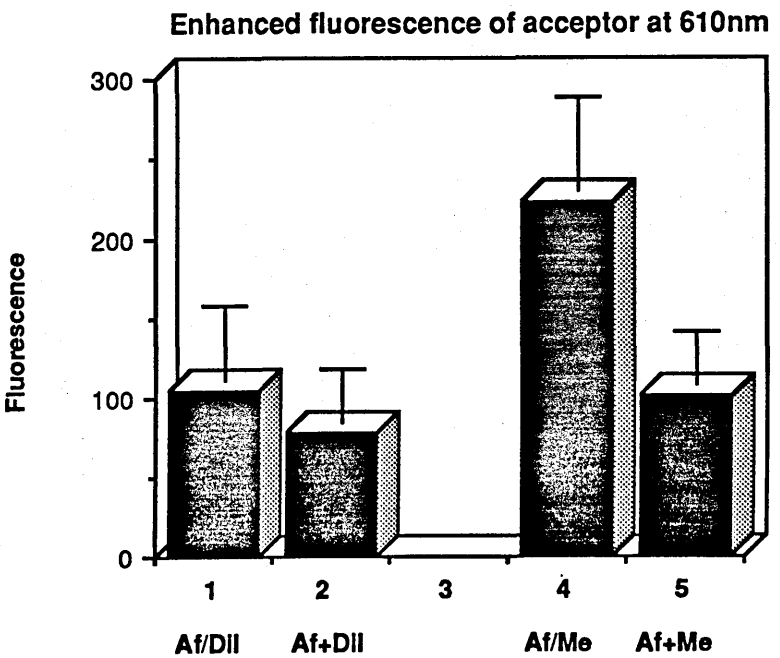

Enhanced fluorescence of acceptor at 610nm
Fluorescence
300
200
100
0
1
2
3
4
5
Af/DII
Af+DII
Af/Me
Af+Me

Figure 6.9

In this experiment a population of cercaria was split prior to transformation and half subjected to U.V. irradiation 400 mW mins/cm^2. After transformation both irradiated and non-irradiated schistosomula were cultured in E.M.S. for 6 hours. Both groups were then further split into 5 groups and subjected to exactly the same labelling as described for figure 6.8. The results of this are shown in Figure 6.9. In addition the levels of fluorescence for each of the singly labelled control groups were also quantitated and shown in Figure 6.10. Statistics - Student T tests.

Figure 6.9a Quenching of fluorescence at 525 nm

Non-irradiated

Group 1 AF only vs Group 2 AF/DiI	Sig. at < 0.0001
Group 1 AF only vs Group 3 AF/ME	Sig. at < 0.0001

Irradiated

Group 5 AF only vs Group 6 AF/DiI	Sig. at < 0.0001
Group 5 AF only vs Group 7 AF/ME	Sig. at < 0.0001

Figure 6.9b Enhanced fluorescence > 610 nm

Non-irradiated

Group 1 AF/DiI vs Group 2 AF + DiI	Sig. at < 0.05 p = 0.026
Group 7 AF/ME vs Group 5 AF + ME	Sig. at < 0.0001

Irradiated

Group 4 AF/DiI vs Group 5 AF + DiI	Not sig. at < 0.05 p = 0.073
Group 10 AF/ME vs Group 11 AF + ME	Sig. at < 0.0001

Figure 6.9c Relative fluorescence of irradiated vs non-irradiated parasites.

Group 1 vs Group 2 (aminofluorescein C18)
not significant at < 0.05 p = 0.93

Group 3 vs Group 4 (DiI C18)
not significant at < 0.05 p = 0.2

Group 5 vs Group 6 (Merocyanine 540)
significant at < 0.05 p = 0.047

Figure 6.9a

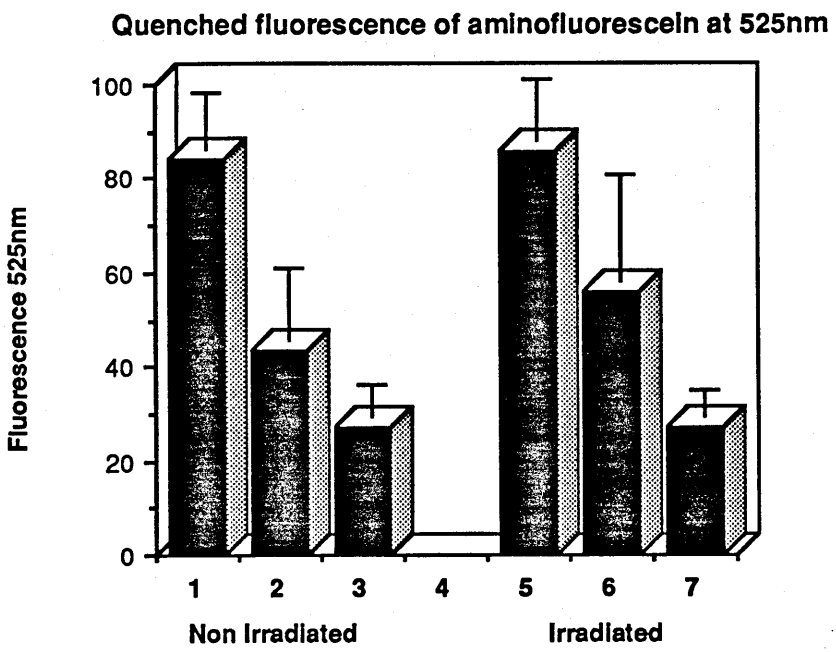


Figure 6.9b

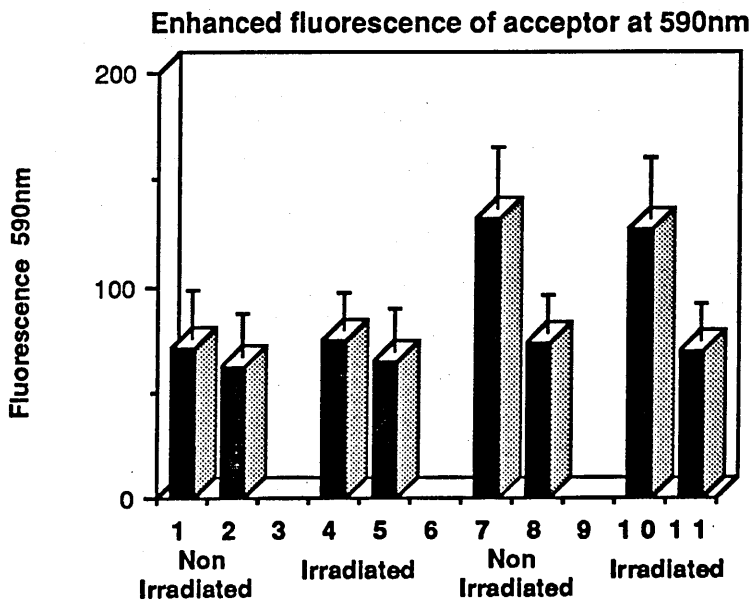
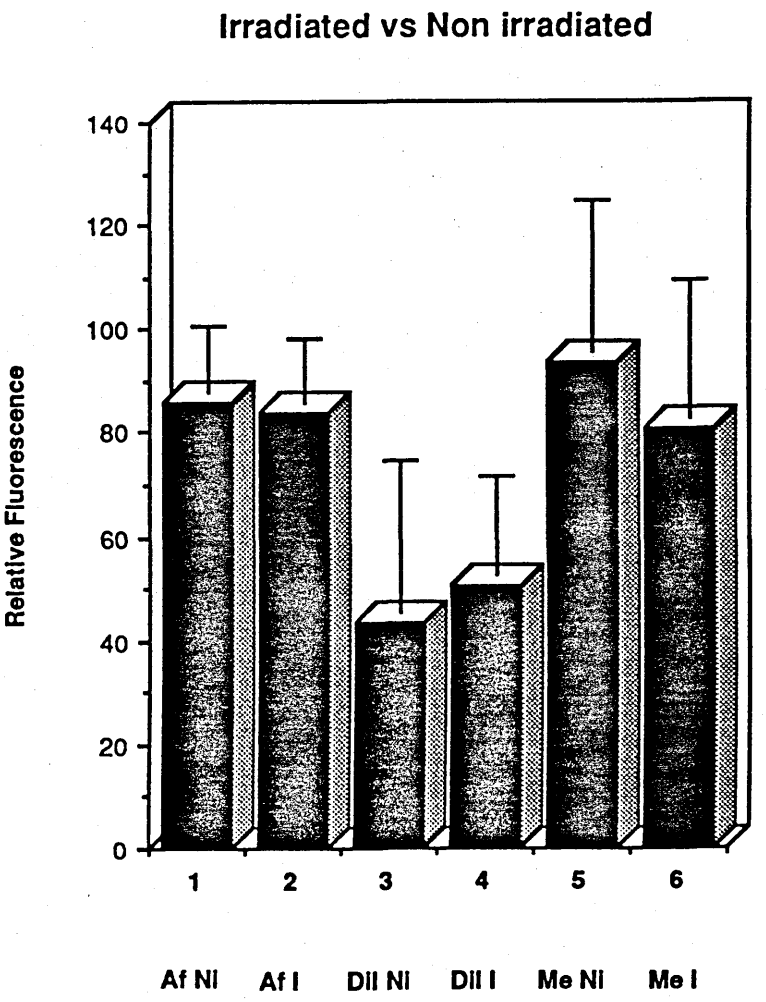


Figure 6.9c


Irradiated vs Non irradiated
Relative Fluorescence
140
120
100
80
60
40
20
0
1
2
3
4
5
6
Af NI
Af I
DII NI
DII I
Me NI
Me I

6.5.1 Conclusions

The following conclusions can be drawn from this data:

a) Fluorescence energy transfer between pairs of exogenous fluorescent probes can be demonstrated to occur in the surface membrane of schistosomula.

b) This can be quantitated using the available apparatus the Leitz MPV compact.

c) When the analogues aminofluorescein C18 and Merocyanine 540 are introduced into the surface membrane of the schistosomula there is efficient resonance energy transfer between them indicating that they locate on average within Ro angstroms of one another. This is consistent with the hypothesis that there is little or no lateral segregation in the plane of the membrane between the molecules.

d) When the analogues aminofluorescein C18 and DiI C18 are introduced into the membrane there is sufficient mixing between them to allow resonance energy transfer to occur. That is at least some of the molecules come within Ro angstoms of one another.

e) The behavior of the fluorescence emitted by the various probes upon cooling the membrane below the presumed transition temperature of the membrane indicates that the behavior of DiI C18 is markedly different to that of the other probes employed. This has been interpreted as indicating that the analogue DiI has a strong preference for gel phase lipid and can form self quenching aggregates when forced out of this enviroment. The other analogues employed do not show this sensitivity and are in contrast relatively enviroment or "phase" neutral.

6.5.2 Interpretation of data

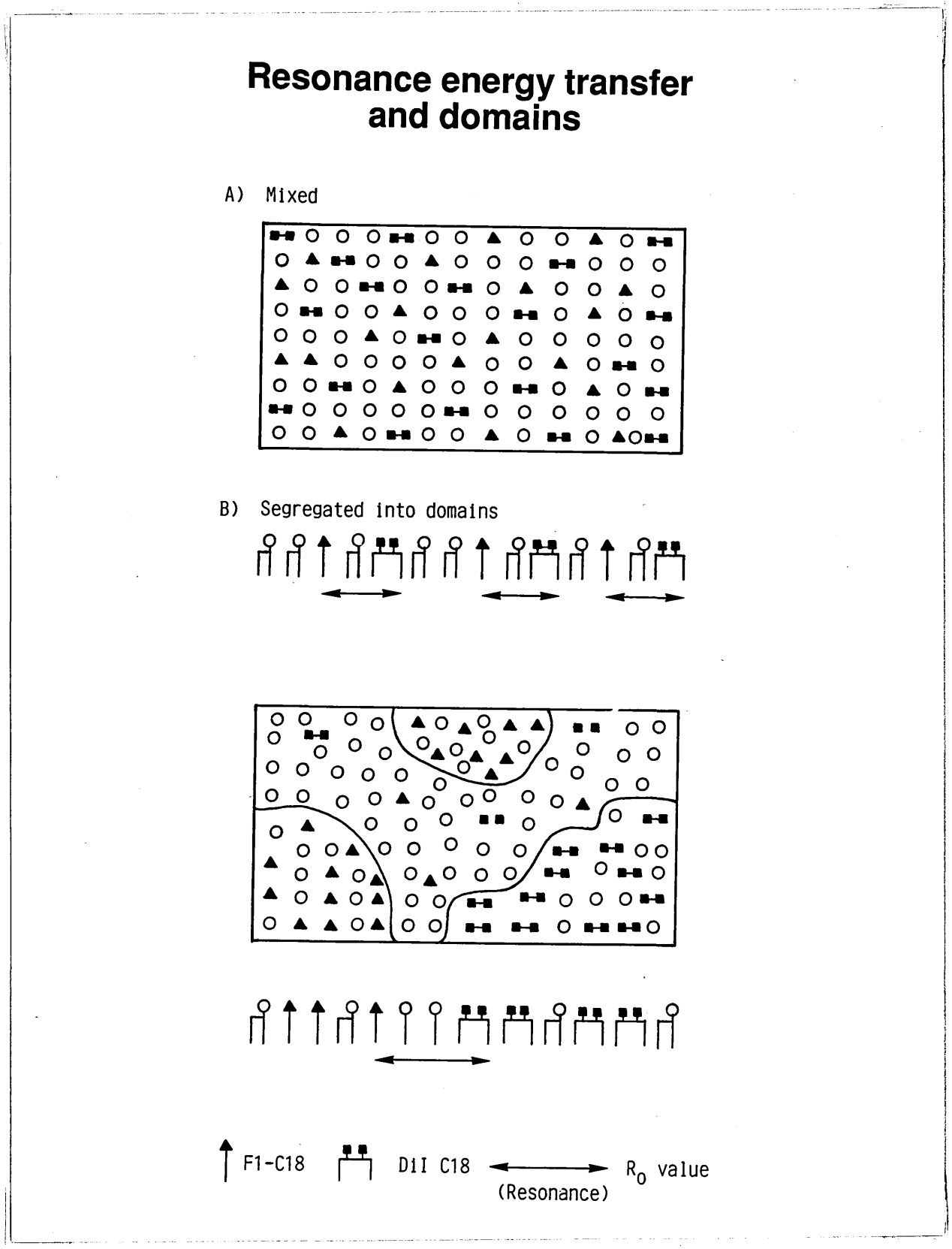
These results show a rough correlation with "predictive " strategy in as much as aminofluorescein C18 exhibits efficient transfer to the mobile phase neutral acceptor merocyanine but an apparently more complex less efficient transfer to DiI C18 which has a marked preference for gel phase. However it is clear that this qualitative approach provides little information on domain differentiation other than providing indirect circumstantial evidence for their existence within the plane of the membrane.

Consideration of the data pertaining to DiI C18 highlights the problems of interpreting this data. On the one hand the temperature sensitivity of the dye and the apparently anomolously low levels of enhanced resonance of the DiI C18 are consistent with the formation of aggregates of dye segregated to some extent from the rest of the lipid membrane. On the other hand there is clear evidence of resonance and hence mixing between aminofluorescein and DiI C18. This illustrates the main problem in relating the demonstration of FRET within the membrane to domain differentiation. It is that such resonance could come from one or more of the following types of donor acceptor interactions. These are:

a) Resonance between donor and acceptor molecules located wholly within the liquid crystalline phase.
b) Resonance between molecules located within the gel phase.
c) Resonance between donor and acceptor across the immobile/mobile lipid interface. This could be between molecules at the edge of a large domain or between all the molecules in a domain of Ro radius.

These possibilities are shown in diagrammatic form in figure 6.10

Figure 6.10


Resonance energy transfer and domains
A) Mixed
B) Segregated into domains
F1-C18
DiI C18
(Resonance)
R_0 value

These considerations concerning the site of resonance are particularly relevant to the DiI experiments. It is possible that in this case mixing and segregation of acceptor and donor could coexist alongside one another in the same membrane. The most likely way in which this could come about would be for mixing to occur in the fluid liquid crystalline region and segregation within the gel phase. Clearly a fluid enviroment is inherently more capable of effecting mixing whereas a rigid gel phase is less so. It is probable that the resonance observed between aminofluorescein and DiI C18 in the schistosomula membrane is derived predominantly from mixing of the dyes in a common bulk fluid phase while the gel remains relatively segregated. This hypothesis could only be verified by a technique which dissected the fluorescence derived from resonating analogues in an area of membrane into its immobile and mobile phase component parts. One way in which this could be approached could be to combine the techniques of FRAP which is sensitive to mobility with FRET. This approach forms an integral part of the theoretical approach to using FRET between membrane probes as a molecular scalar against which domain size could be estimated. The final sections of this chapter are devoted to a brief descrition of this concept which can described as a process of "membrane mapping by constraint".

SECTION 2.

6.6.1 Modelling domain differentiation.

The major problem with both the FRAP and the FRET techniques lies in relating these measurements to the physical structure of the domains that exist in the membrane. FRET indicates that two molecules are interacting over a defined range within the membrane but the site of this interaction (in terms of domains) is unknown. FRAP indicates the relative distribution of a molecule between two domains but the relationship between this measure of relative distribution and the relative area of the two domains depends on two assumptions. The first and most important of these is that the probe used is indeed a lipid analogue and therefore the behavior of this dye is analogous to the behavior of the majority of the endogenous lipids These are predominantly the phospholipids phosphatidylcholine C18 and phosphatidyl serine C18. In other words how representative of properties of the unlabelled endogenous lipid is the exogenous labelled lipid analogue introduced into the membrane.

If it can be established that a probe is representative it is reasonable to assume that in a phospholipid bilayer if a particular proportion of lipid molecules are immobile a similar proportion of the surface area of the membrane will be immobile. This is particularly true of a relatively protein free membrane such as the E_1 face of the S. mansoni surface membrane.

These assumptions are implicit in the argument advanced above, derived from Foley's studies [Foley 1986 ,Foley et al 1987], that the relatively low % recoveries recorded for a number of lipid analogues in the surface membrane of S. mansoni indicate the presence of large scale restriction of lipids within immobile domains. It is reasonable to expect that such immobile domains comprise a similarly large proportion of the surface area of the parasite

membrane. Two relationships have been implicitly assumed when interpreting FRAP data. These are, that % recovery of the analogue is related to the % mobility of endogenous lipid and that this in turn correlates with surface area of domain. However, they have never been directly tested. This has largely been due to the absence of a method to do this. It is proposed that a combination of the techniques of FRAP and FRET incorporated into a membrane model can be used to test both these assumptions. Moreover by doing this it will be possible to construct a model or "map" of domain differentiation which describes the number, size and shape of the immobile domains. This approach is based on the correlation of data derived from a number of fluorescence analogue dependent measurements currently possible with the FRAP/FRET combination technique. In addition several constants and basic assumptions are incorporated in the model. The paragraphs below describe in detail the development of this mathematical model which attempts to describe domain differentiation from first principles. Constants, experimental inputs and theoretical assumptions are clearly stated as appropriate. The underlying rationale of this approach was to use the combination of FRET and FRAP to determine the overall size of the immobile domain and the extent to which it is subdivided.

6.6.2 % recovery from two resonant probes

The starting point for this approach was the realisation that the FRAP parameter % recovery, when derived from a membrane double labelled with two probes that could exhibit resonance energy transfer, was not simply determined by the distribution of the probes between the two phases but reflected, in addition, the extent to which those probes interacted within a particular domain. Consider the equation describing the parameter % recovery from a normal FRAP trace in which there is only one dye molecule, for example aminofluorescein C18, which distributes between the two domains, the mobile liquid crystalline and the immobile

gel. In this case every individual aminofluorescein C18 molecule will fluoresce equally brightly therefore the recovery of the fluorescence signal will directly reflect how many molecules are able to diffuse into the bleached spot and are therefore mobile and also the proportion that remain bleached and are immobile. This is shown in equation 1 below:

6.7.1. % Recovery as applied to FRET.

Equation 1.

$$\% \text{ Recovery} = \frac{F_{lc}}{F_{lc} + F_{g}} \times 100\%$$

Where F ..... Fluorescence

lc.....Liquid crystalline domain (mobile)

g .....Gel domain (immobile)

However, in the situation where the fluorescence signal from a FRAP spot is derived from a membrane in which resonance between donor and acceptor molecules is occurring the assumption that every donor or acceptor is equally fluorescent cannot be made. The fluorescence of a particular donor or acceptor molecule will be determined by its propinquity to another complementary donor or acceptor molecule. Thus the fluorescence of every aminofluorescein C18 donor molecule in a membrane double labelled with aminofluorescein C18 and rhodamine C18 will vary according to how close a quenching molecule of rhodamine C18 was. Conversely if the rhodamine acceptor molecules were excited indirectly via the donor aminofluorescein their fluorescence would vary according to how close the nearest available donor was. In this situation where the enhanced fluorescence of the acceptor was monitored the total fluorescence from the spot could be described by equation 2.

Equation 2.

Total F = **Flc**+ **Fg** + **Fint** + (**Findirect**)

Where F = Fluorescence emitted from:

lc = Liquid crystalline domain

g = Gel phase domain

int = Interface between gel & liquid crystalline

indirect = Indirect excitation of acceptor

This last component can easily be estimated and allowed for leaving equation 3.

Equation 3.

Total FRET**da** = **Flc** + **Fg** + **Fint**

Where FRET**da** is Fluorescence energy transfer from donor to acceptor and F denotes fluorescence from particular components of the membrane.

This introduces two new factors into the % recovery equation, FRET dependent fluorescence in each domain and also FRET dependent fluorescence across the interface between domains. These novel elements have been exploited to introduce a distance dependent factor or fluorescence scalar element which allows fluorescence to be related to area. In addition a differentiation dependent factor is also introduced since the extent of FRET across the interface is dependent on the length of this interface. This enables fluorescence to be related to the extent of differentiation, since a highly differentiated, fragmented domain structure similar to the "broken ice floe" in the earlier analogy will have a much greater interface than a poorly fragmented "iceberg" like pattern of differentiation.

The scalar element can be introduced due to the fact that in a membrane labelled with aminofluorescein C18 which exhibits 50% quenching relative to the control levels of aminofluorescein C18 fluorescence then one can conclude

that the average separation between these two molecules is Ro(56) angstroms. Experimentally this can readily be achieved by taking samples of a population of schistosomula labelled "identically" with aminofluorescein and subjecting them to labelling with increasing concentrations of donor as in Figure 6.4. The sample exhibiting 50% quenching can then be subjected to FRAP analysis in which the fluorescence signal monitored is that of the indirectly excited acceptor molecule. This can be done by using the appropriate filters. This would allow FRAP analysis in which only the fluorescence of the acceptor is monitored. This would be predominantly due to resonance energy transfer. The % recovery in this situation can be described by equation 4 below. This is derived directly from equation 3.

Equation 4.

$$\%\ \text{recovery of donor \& acceptors} = \frac{F\ \text{mobile phase}}{F\ \text{mobile} + F\text{immobile} + F\ \text{interface}}$$

Expressed below using the subscripts

lc..... Liquid crystalline
g ..... Gel
int ... Interface
% FRET**da**.. % recovery of FRET derived fluorescence (F) via indirect excitation of acceptor by donor.

Equation 5. FRAP/FRET.

$$\%\ \text{FRET}_{da} = \frac{F_{lc}}{F_{lc} + F_g + F_{int}} \times 100\%$$

In practice, this could be done by exciting the donor aminofluorescein C18 at 490 nm and monitoring the emission of the indirectly excited acceptor at 610 nm before and after a bleaching pulse at 490 nm. This would preferentially bleach the donor molecules although

bleaching of acceptor would also be significant. The relative efficacy of donor and acceptor bleaching could be estimated by bleaching singly labelled controls. In the situation where most of the fluorescence emitted was due to resonance energy transfer this differential would not cause appreciable problems of interpretation. The equation above would still hold good since bleaching would bleach the signal within the immobile domain and also the signal due to energy transfer from "excess" donors within the immobile domain to "redundant" acceptors in the mobile domain.

6.7.2. Excess & Redundant molecules.

The concept of "excess" donors and "redundant" acceptor molecules derives from the fact that two different probes will partition differently between the two domains. For example DiI C18 exhibits a % recovery of only 30% in the outer membrane of mechanically transformed schistosomula whereas aminofluorescein C18 is 55% mobile. Clearly in the immobile domain there will be relatively more DiI C18 molecules than aminofluorescein C18. Therefore in a situation where FRET between these two probes was being measured, the distribution of the two dyes in each domain would be strongly asymmetric. In the mobile liquid crystalline domain the donor aminofluorescein C18 will be in excess while in the immobile gel domain there will be more acceptor molecules than there are donors to excite them. There will not be pairing on a one to one basis and it could be said that in this sense these acceptors are "redundant". The same considerations apply less forcibly when considering FRET between aminofluorescein C18 and rhodamine C18. The more dissimilar the distributions of the two probes the greater the potential contribution of the component **Fint** of equation 5.

The important feature of this approach is that the contribution to total FRET from an area of membrane under these conditions could be dissected between that occurring

in the mobile phase and elsewhere. This would show up concentrations of dye molecules within a particular domain. For example in the case where rhodamine C18 was used to quench aminofluorescein C18 fluorescence within a membrane by 50% one can say that the average separation between the donor and acceptor will be Ro(56 angstroms). However this could mask wide differences in the average separation within either domain, fluid or immobile. For example there could be a very small area of immobile lipid in which rhodamine C18 and aminofluorescein C18 molecules are concentrated with an average separation much less than Ro and a larger bulk fluid phase in which the molecules are more spaced out. The average separation would be Ro angstroms but the bulk of the resonance energy transfer could be taking place in only a small fraction of it.

This returns to the question of how representative the % recovery of FRAP derived from a particular analogue is of the % mobility of the endogenous lipid. An estimate of how representative a probe is can be gained by comparing the value of FRET % recovery returned by the FRAP/FRET technique represented by equation 5 with a prediction of what this value, the % recovery of indirectly excited acceptor molecules, is expected to be. This expected value is based on the extent to which donors and acceptors are in excess or redundant. This kind of analysis is carried out by assuming that in the situation where 50% quenching of donor is achieved the average separation of donor and acceptor is Ro angstroms, the donor to acceptor ratio is equal to one and that each pair occupies a discrete area of membrane (equivalent to two hexagonal areas Ro in width). That is the components **Flc** and **Fg** are directly proportional to the area of the liquid crystalline and gel phase domains respectively.

The total fluorescence in this situation comprises these two components plus a contribution from resonance across the interface between them. The contribution of each

component will be determined by the number of resonant pairs within them. The potential fluorescence of these components can be estimated from the % recoveries exhibited by each probe when used singly to label the parasite membrane. Thus for the donor acceptor pair aminofluorescein C18 and rhodamine C18 the % recoveries are 55% and 62%. It is assumed that in a double labelled membrane the mobile fraction of the membrane has 55% of the aminofluorescein donor molecules in it and 62% of the acceptor molecules. Within this domain there will be an excess of redundant acceptor (rhodamine C18) if the donor to acceptor ratio is one to one. Similarly in the immobile domain there will be an excess of acceptor molecules of 7% the difference between the % recoveries returned by the dyes in the singly labelled controls. This value determined by the extent to which the two dyes differ can then be used to estimate the extent to which FRET between redundant molecules could take place across the interface. This level of fluorescence is termed **Fint**.

The two extremes of this potential contribution are the situation in which domain size is less than Ro in which all redundant molecules will be able to transfer energy by FRET and coversely when domain size is appreciably bigger than Ro and little or no resonance occurs across the interface.

The fluorescence contributions of **Fg** and **Flc** can be similarly estimated by calculating the maximal overlap in % recoveries in each domain under the idealised conditions listed above. Therefore in the case of aminofluorescein C18 and rhodamine C18 these would be 55% (**Flc**) the mobile domain and 38% in the immobile domain(**Fg**).

The convention adopted for this discussion is shown below:

Flc = % recovery donor (55)
Fg = (1-% recovery acceptor) (38)
Fint = % recovery acceptor - % recovery donor (7)

If the % recovery of the donor > than % recovery acceptor this process would be reversed. This is because its function is to calculate the maximum proportion of resonant donor and acceptor pairs that can exist in firstly either domain, liquid crystalline or gel, and then the number of pairs that could potentially exist in resonance across the interface. This has been done by treating each domain as a separate entity in which either donors or acceptors are the limiting factor in pair formation. The calculation of the Fint factor estimates the maximum proportion that these "excess" donors and "redundant" acceptors could possibly contribute to the fluorescence signal FRET**da**EXP. This represents the expected range of values returnable by FRET % recovery , under the conditions described above in which quenching of the donor signal is 50%. The expected range of values takes into account the maximum and minimum contribution of **Fint** and assumes the idealised conditions described above.

Equation 5. FRAP/FRET.

$$\%\ \text{FRET}\mathbf{da}\text{EXP} = \frac{\mathbf{Flc}}{\mathbf{Flc} + \mathbf{Fg} + \mathbf{Fint}}$$

Where the subscript EXP denotes an expected value.

The range of values expected for the pair aminofluorescein and rhodamine FRET**da**EXP

$$= \frac{55}{7 + 55 + 38} \text{ to } \frac{55}{55 + 38}$$

FRET range expected = 55% to 59%

An averge value taken as the midpoint of this range, which in effect represents the expected value where 50% of the potential resonance energy transfer across the interface will be used as a standard for future calculations. This will simply be termed % FRET**exp**. In this case the expected value for this particular donor acceptor pair at 50% quenching is 57%.

How can this value be exploited ?

The main assumption inthe calculation of this value is that in each of the two domains the average separation of donor and acceptor molecules is equal to Ro. Deviation of the observed value outwith this range is taken to indicate that in one domain or other the average separation of donor and acceptor molecules is not equal to Ro. The direction and scale of this deviation are informative. In the hypothetical example described above where the majority of FRET between the donor and acceptor occurred in a small immobile domain the effect would be that the contribution to total FRET fluorescence from the immobile domain would be increased. Due to this the % recovery of FRET observed would be less than that predicted FRET**exp**.

Conversely, if concentration of donor and acceptors occured in a small, mobile, liquid crystalline phase the % recovery value observed would be greater than that predicted.

In essence the expected value presumes that donor acceptor separation is equal to Ro and that because of this the % area of fluid is proportional to the % recovery of FRET from a membrane exhibiting 50% quenching. An allowance is made for the infuence of resonance across the interface.

Experimentally observed deviation of % recovery of FRET from this expected value over and above the margin estimated and allowed for **Fint** indicates that the average separation is not Ro in each phase. The direction of deviation indicates the domain in which deviation from the assumption that average separation takes place. The magnitude of the deviation is interpreted as indicating the extent to which the presumed relationship between % recovery of FRET and % surface area of mobile lipid varies from a direct correlation. An estimate of this variance is made and incorporated in the term % **Fsam** which is derived from equation 6 shown below. This value is taken to be an

estimate of the % of the surface area of the membrane covered by mobile lipid i.e the extent of the liquid crystalline phase.

Equation 6. An estimate of % area of the surface membrane that is covered by the fluid liquid crystalline domain.

$$\%\ F_{sam} = \frac{\%\ FRET_{exp} \times \%\ FRET_{exp}}{\%\ FRET_{obs}}$$

Where % FRET**exp** the expected value derived from equation 5 by substituting known values into the equation .

Where % FRET**obs** is the % recovery observed after subjecting that double labelled membrane to FRAP.

6.8.1 Estimating domain fragmentation.

The next section outlines how this estimate can reincorporated into the original % recovery FRET equation along with other fluorescence derived data to estimate a value Nd the number of individual domains.

To reiterate, the fundamental problem with the FRAP data concerning the schistosome surface membrane is relating the measurements of the analogues within the membrane to the structure of that membrane. This is of particular relevance to the schistosomula surface membrane which returns relatively low values for % recovery with a number of analogues which in cell plasma membranes return significantly higher values. This difference has been interpreted as indicating that the parasite has a larger immobile fraction of lipid within it's surface membrane organised into some form of domain structure, than cell plasma membranes. A method whereby the domain structues of cell and parasite membranes could be modelled "mapped out" and compared is clearly required. This would enable the original assumption that the difference in mobility of these dyes between cells and parasite's represented an important host parasite difference in domain

differentiation expressed at the host parasite interface or simply a difference in the affinity of the immobile domain of the parasite for the analogues employed. This latter alternative would be of some interest in that it would indicate that the immobile domains of the parasite had a different composition to those of cell plasma membranes. To summarise the alternatives, the low recoveries in the schistosomula indicate that:

There is, relative to the cell plasma membrane, larger or more numerous immobile domains within the parasite surface membrane.

or alternatively,

That the immobile domains are similar in nature but the parasite immobile domain simply has a greater affinity for the dyes which acts to reduce the % recoveries returned.

Both of these alternatives imply a host/parasite difference. The former is of most interest since it would imply that at this fundamental level of membrane structure the parasite surface is markedly different to that of the host's cells.

The aim of this membrane model building approach is to attempt to answer this question of what a difference in % recovery of a probe actually represents.

Constructing a model to map out domain differentiation resolves down to two interrelated questions - how big are these domains and how many of them are there?

An assumption based on thermodynamic grounds can reasonably and conveniently be made about their shape. That is they will be circular since this would minimise the entrophy of the system. In other words like will join with like.

The approach used to estimate the variable N_D the number of domains, is outlined below. Basically this utilised the range of measurable parameters associated with a membrane double labelled with a donor and acceptor pair ideally aminofluorescein C18 and Rhodamine C18 in the state of 50% quenching of the donor.

The following range of measurements could be made in such a situation:
Density of acceptor molecule/um^2
Density of donor molecule/um^2
% recovery of donor
% recovery of acceptor
% recovery of FRET between donor and acceptor (FRET/FRAP)
In addition the following constants are known:
The average separation between donor and acceptor molecules Ro.
The FRAP apparatus spot size.

Also the assumption that domains are either gel phase or liquid crystalline and circular, i.e. there is no differentiation within phase into immiscible domains.

Given this range of measurements, constants and assumptions it is possible to create a mathematical model describing the behaviour of fluorescence under defined conditions incorporating the unknown variable ND and the value % Fsam which is the "estimated" value of the actual proportion of membrane surface area that is covered by mobile lipid.

The key concept to this approach is that covered by the equation describing the fluorescence of FRET/FRAP. Each of the assumptions and measurements made places constraints on the distribution that the donor and acceptor molecules can adopt. All of these factors plus the two unknown variables can be incorporated within the FRAP/FRET equation. This in turn could form the basis of a set of algorithims for a computer model. This could be used to substitute into the

equation derived form a series of experimental observations a range of values for the unknown variables. In this way the values for % **Fsam** and N_D for a given set of experimental observations could be generated. The key element in this approach is the equation describing the components of fluorescence in a double labelled parasite membrane where the donor molecule (aminofluorescein) is quenched by 50% and enhanced fluorescence is monitored before and after bleaching. This is once again equation 3.

FRET Total = **Fg** + **Flc** + **Fint**

g = gel phase
lc = Liquid crystalline
int = interface

the fluorescence of the **Flc** component and the **Fg** component will be determined by the number of pairs of acceptor and donor molecules in each phase and the average separation between them. The degree of FRET, **Fint** across the interface between these two phases will be determined by the number of redundant molecules in each phase and the average separation between them. This will be determined by the domain size. A method for estimating each of these components using the measurable parameters and constraints listed above follows.

Each component is treated as a separate entity. That is the number of resonating pairs is calculated for **Fg** and **Flc** and the assumption is made that only the remaining excess or redundant donor molecules in each phase contribute to **Fint** This process occurs in two stages.

Stage 1

Calculate the number of "resonant" donor acceptor pairs for each component. This can be estimated by where % recovery of donor > % recovery of acceptor by

Equations 7 & 8. The number of donor acceptor pairs/domain.

Nlc = (% recovery donor) x donor density um^2 x spot size um^2

Ng = (1-% recovery acceptor) x donor density um^2 x spot size um^2

Equation 9. The potential number of donor acceptor pairs across the interface.

Nint = (% recovery donor - % recovery acceptor) x donor density um^2 x spot size um^2 + ((1-% recovery acceptor) - (1-% recovery donor)) x donor density um^2 x spot size um^2

Nint simply calculates the sum of the differences in percentage recovery between the donor and acceptor molecule in each phase.

Step 1 returns an estimate of the number of resonant donor acceptor pairs in each of the two phases, gel and liquid crystalline, and an estimate of the number of redundant or excess pairs that could potentially be in resonance across the interface between these regions.

Only 4 factors are required:

% recovery of the donor
% recovery of acceptor
FRAP spot size um^2
Surface density of donor/um^2 membrane

This last factor can be determined by extracting the labelled membrane, converting to molecules bound per schistosomula and dividing the surface area of the membrane by this value to yield donor density per um^2 of parasite membrane.

Step 2

Each component of fluorescence Fg+ Flc + Fint will have N number of paired molecules in a particular area. The fluorescence resonance energy transfer of each component will be:

Equations 11 & 12 The fluorescence from each domain.

$$Fg = Fg \times \frac{Ro}{Av.\ Sep.g}$$

$$Flc = Nlc \times \frac{Ro}{Av.\ Sep.lc}$$

Where Av. Sep. **x** = the average separation of the donor and acceptor molecules in a given domain x.

These equations illustrate that if the average separation of the two dyes is less than Ro, i.e. is concentrated within one phase, the fluorescence of that component will be increased and conversely if the average separation is greater the fluorescence component will be decreased.

The **Fint** component will be influenced by the domain size of the mutually immiscible phases. This can be taken into account by the formula below, where **Fint** is multiplied by a factor derived from Ro/R_D to give **FintC** defined as the fluorescence across the interface adjusted to reflect the effect of domain size (R_D) on fluorescence.

Equation 13 An estimate of resonance across the interface.

$$FintC = \frac{Ro}{R_D} \times \frac{Nint \times Ro}{Aver.\ Sep.int}$$

Where R_D is the radius of a domain.

The term average separation is in this case the "modelled" average separation in that it is dependant upon generating a value % **Fsam** for the "real" % area of immobile lipid on the surface. This can be described thus:

It is assumed that each molecule of the donor acceptor pair occupies a circular area of lipid of radius equal to half of the average separation of the donor and acceptor pair for that particular domain. Therefore, the distance between the two centre spots of the adjacent areas of lipid occupied by a pair of molecules will be equal to the distance of one Average Separation. The best way to model this situation is to assume that the molecules pack together in an array of hexagonal shapes the centres of which are one Average Separation distance apart. Under these conditions each molecule will occupy an area of:

$(0.865 \times \text{Aver Sep})^2$ square angstroms.

The method of calculating the average separation of the donor and acceptor pair has been to divide the estimated area of that domain by the number of molecules within it. This returns the average area of membrane lipid occupied by a donor or acceptor molecule which is presumed to be hexagonal in geometry. Assuming that within a given domain, donor and acceptors are randomly and therefore on average fairly evenly distributed then:

Equation 14. The average separation of donor and acceptor within domains.

$$\text{Av. Sep.lc} = \sqrt{\frac{(\%\ \mathbf{Fsam} \times \text{Ss um}^2)}{(0.865 \times \mathbf{Nlc} \times 2)}}$$

$$\text{Av. Sep.g} = \sqrt{\frac{(100 - \%\ \mathbf{Fsam} \times \text{Ss um}^2)}{(0.865 \times \mathbf{Ng} \times 2)}}$$

The average separation of redundant molecules across the interface starts from the assumption that potentially any of these molecules could interact with the others. This would be the case if the domains were sufficiently numerous, small and spread evenly across the membrane. An estimate of is seen in equation no. 15.

Equation 15. An estimate of the average separation of excess and redundant molecules.

$$\text{Aver. Sep.int} = \sqrt{\frac{Ss}{(0.865 \times \mathbf{Nint} \times 2)}}$$

This means that in effect there are a certain number of pairs which are not restricted but distribute over the whole area of membrane.

An estimate of R_D the radius of the domains proceeds by deciding which phase forms the domain and which the bulk phase. The convention that will be adopted is that the smaller fraction of the donor mobility will be considered as from circular domains within a bulk phase. In other words if the donor exhibits 60% observed recovery immobile domains will be considered as forming in a fluid background. This convention holds true for aminofluorescein C18. Therefore, the following procedure applies:

An estimate of "actual" domain size is generated by the equation where DA represents the area of a domain.

Equation 16. The area of the immobile domain.

DAg (gel) = ((100 - % **Fsam**) x Ss) um^2

Ss = FRAP spot size um^2

It is assumed that the domains are circular in shape. Therefore it can be taken that:

Equation 17. The radius of a circular domains of area DAg.

$$R_D = \sqrt{\frac{DAg}{\pi \times N_D}}$$

Where:

R_D is the radius of an immobile domain.

N_D is the number of circular domains of R radius.

To return to the original equation, **Equation 5 FRAP/FRET.**

$$\%\ FRETda = \frac{Flc}{Flc + Fg + Fint}$$

This can be expanded to the following by incorporating the equations describing it,s individual components where **Fint** is modified to **FintC** by multiplying by Ro/R_D.

Equation 18. Expanded form of 5. FRAP/FRET.

$$\%FRETda = \frac{((Ro \times Nlc) / (Av.\ Sep.lc))}{\frac{Ro \times Nlc}{Av.Sep.lc} + \frac{Ro \times Ng}{Av.Sep.g} + \frac{Ro}{R_D} \times \frac{Nint \times Ro}{Aver.Sep.int}}$$

The value R_D can be derived by solving for R_D in the the equations above.

Equation 19. The radius of an immobile domain R_D

$$R_D = \frac{(1/Fint) * ((\%\ FRETda/Flc) - (Flc + Fg))}{Ro}$$

Where **Fint** = (**Nint** * Ro)/ Aver.Sep.**int**

Once calculated the estimated radius of the domain can be substituted into the following equation to deduce N_D.

Equation 20. The number of individual immobile domains

$$N_D = \frac{(100 - \%\ \mathbf{Fsam}) \times Ss}{\pi \times (R_D)^2}$$

The value % **Fsam** is common to both the above equations.

Once the number of circular domains hs been estimated along with their radius are known a simple diagram of domain differentiation within the FRAP spot (Ss um^2) can be drawn assuming that the domains are randomly distributed.

Two approaches to computer modelling directed towards obtaining information concerning domain structure are possible. The first is a simple one based on this mathematical model. Put simply it is to write a simple computer program with the equations above as its alogrithms and generate values of % **Fsam** and N_D until a fit with experimental readings is achieved. From these two values a simple map of domain differentiation could be drawn.

A more powerful approach would be actually to model the distribution of the various donor and acceptor molecules. An image constructed by plotting the inferred separating distance of donors and acceptors could be generated and inferences drawn concerning domain structure. This would require a more powerful computer and sophisticated software involving image analysis. It would have the advantage that some of the limitations of the above approach notably the assumption of evenly spread circular domains could be dispensed with.

Both of these approaches rely on the premise that each of the linked experimental observations on the membrane place a constraint of some kind on the pattern of the donor and acceptor molecules distribution. A further basic

assumption is that ultimately the configuration of these molecules within the plane of the membrane is determined by domain differentiation. Therefore the basic principle employed is that each experimental observation places a constraint on the possible configurations of donor and acceptor distribution available. Together it is expected that they could be employed to reduce the possible values of R_D and N_D to a single pair.

The equations presented above are not the definitive solution to this problem. They are presented as an intial attempt to use the fairly wide range of fluorescence analogue based assays available or in development in an integrated manner. It is expected that using this integrated approach with a number of parameters there would be sufficient synergism generated to allow a particular domain differentiation structure to be generated from a given data set.

CHAPTER 7

Conclusions and perspectives

7.1.1. The fundamental objective and general conclusions.

The fundamental objective of this project was to further the exploitation of fluorescent lipid analogues as an analytical tool for investigation of the cell biology of the surface membrane of S. mansoni. Each of the experimental chapters illustrates a method whereby this objective has been achieved. It is clear that these dyes provide an extremely flexible and versatile tool which can yield significant insight into membrane structure and behaviour at a number of different levels. At the level of the gross structural properties of the membrane organisation these include: the development of the double bilayer itself; transverse fold organisation; longitudinal variation within an individual parasite and developmental differences between parasite stages concerning their binding of lipoproteins and susceptibility to damage induced in the inner bilayer via lipid phase changes. These observations and inferences all concern membrane structure at the macromolecular level of organisation, that is over a scale of microns or above. However collectively they imply that a lower level of organisation in the order of angstroms or tens of angstroms also exists and that this is ultimately responsible for these higher order effects. This applies in particular to variation in membrane properties within and across a parasite's surface membrane.

A good deal of data concerning this lower angstrom based level of membrane organisation has already been obtained via the FRAP technique by Foley and a central paradigm concerning membrane organisation in the schistosomula advanced. This hypothesis, the differentiation of the surface membrane into domains of differing biophysical properties, may well be the foundation on which the ability

of the parasite to vary its membrane properties over distance, within a parasite and over time as it develops, is based. As discussed in chapter 6 there are some fundamental problems in relating FRAP based data to the actual physical structure of the domains within the membrane.

The central problem is that the relationship assumed to exist between the mobility of exogenous lipid analogues such as aminofluorescein C18 and the unlabelled endogenous lipid molecules is unknown. This is particularly true of the parameter of FRAP, % recovery on which the hypothesis of immobile domains rests. It may well be the case that the value of % recovery returned by a particular dye via FRAP accurately represents the actual percentage of endogenous lipid within that membrane. This would be most likely with a probe that can easily partition into all environments within the membrane. Aminofluorescein C18 and rhodamine C18 are two such putative probes. The importance of chapter 6 lies in the fact that it advances both the means (FRET) and the method for tackling this problem and establishing this critical experimental relationship. Once achieved the way lies open to construct a computer model which accurately reflects the dynamics of this extraordinary membrane. The potential of this approach lies in elucidating the biophysical basis of intrinsic resistance thereby establishing a model of membrane structure in a "living" biological membrane which exhibits extreme resistance to immunological attack. Such a model would be of general interest particularly in some areas of developmental and cellular biology where phenomena similar to "intrinsic" resistance are found. The membrane of trophoblasts and certain resistant tumour cell types are two relevant examples.

The points raised above could be summarised as follows:

a) Fluorescent lipid probes are an extremely versatile tool for investigating the structure and function of the parasite's surface membrane.

b) They indicate that the properties of the surface membrane can vary markedly, with distance, over the surface of an individual parasite.

c) The properties of the membrane vary markedly with time during the development of the parasite.

d) The major difficulty in the use of fluorescence probes as a tool is in relating the data to the structure of the membrane . This is true of all techniques but it can be resolved to some extent by combining several fluorescence based assays as illustrated in chapter 1 and devloped in chapter 6.

7.1.2. Specific conclusions and future work.

The various chapters of this study are devoted to a specific line of investigation. Each chapter was associated with a particular combination of experimental technique, data and hypotheses derived from these. To conclude this document the main themes of this study are summarised and the hypothesis derived from them evaluated in terms of their scope for future development by experimental testing.

The data presented in chapter 1 supported the contention that an increase in the uptake of the dye aminofluorescein C18 by the early schistosomula directly reflected the formation of the outer bilayer itself. This conclusion was only able to be drawn due to the corroborative support of several techniques.

This illustrates a general point that reliance on measuring only one fluorescence based parameter is often insufficient to allow meaningful interpretation of results. A combination of several techniques can be most informative. In this particular instance the very close correlation between % recovery of aminofluorescein C18 and its absolute levels of fluorescence in a localised area of membrane in a distinct period immediately after transformation illustrates the point. It was proposed that this combination of features represents an extremely disordered area of membrane produced immediately after fusion of membraneous bodies with innner "cercarial" membrane of the newly transformed schistosomula. This data is in good agreement with observations by Foley et al 1988 concerning localised uptake of merocyanine 540 which also reflect a discrete area of disordered membrane at the anterior region of the schistosomula. This may reflect a specialisation on the part of the parasite to facilitate the uptake of host antigens. Thus this phenonema of disordered lipid may be of general relevance to all stages of the parasite. Two features of this work are of interest and worth developing. The first is to test the hypothesis that the close correlation between aminofluorescein C18 uptake and mobility in this highly specific manner is due a locally disordered area of membrane. This is currently being addressed by constructing an adaptation to the FRAP apparatus to allow measurement of the steady state anisotropy of fluorescent probes. This is essentially a measure of the extent to which the probe can deviate from a fixed vertical orientation within the membrane. It is a measure of the degree to which the membrane is ordered since in a disordered membrane the lipid is free to rotate and tumble to a greater extent off and around its vertical axis. The expectation would be that the anterior portion of the early schistosomula would exhibit high levels of anisotropy or disorder. A similar prediction could be made

concerning the membrane immediately overlying the tubercles. The relative degrees of order of the male dorsal membrane and its gynaecophoral canal also warrants investigation on the grounds that membrane disorder may correlate with trans membrane transport.

The second feature of this chapter worth developing futher is simply to use the assay developed to monitor the effect of various agents on the formation of the outer bilayer. This has already been done to some extent and the studies into the effect of cytoskeletal disrupting agents, phospholipid metabolism inhibitors and irradiation will be extended.

The studies on the interaction of LDL with the surface membrane have, in conjunction with data from other workers, allowed the formulation of a hypothesis which integrates the whole question of cholesterol metabolism at the surface membrane into a testable hypothesis. The emphasis of future work in this area will switch away from studying the interaction of LDL with the membrane towards investigating whether the HDL_I fraction can mediate cholesterol uptake at the surface membrane. It is clear that LDL binds at the membrane surface and could provide the parasite with it's cholesterol requirements either by passive diffusion from LDL. Alternatively a more active and efficient process of free cholesterol generation from LDL, due to the action of HDL_I bound LCAT, as proposed in figure 4.22 could occur.

This hypothesis can be tested by examining whether HDL_I is responsible for the metabolism of radiolabelled cholesterol in the form of LDL cholesterol ester at the membrane.

This could be done by monitoring the ability of purified fractions of lipoproteins, in particular mixtures rich or deficient in HDL_I to mediate cholesterol uptake. A further test would be to investigate this enzyme's role by

inhibiting the activity of the LCAT at the membrane/aqueous interface of the parasite by the drug dinitrotreo benzimide DNTB. If it were shown that LCAT was responsible for cholesterol uptake it would be possible to correlate the effects of inhibiting cholesterol metabolism on membrane biophysics. Some attempts utilising DNTB and delipidated serum towards this end have already been attempted. In each case the effect was to fluidise the membrane as adjudged by % recovery of aminofluorescein C18 in treated and control groups.

The damaging effects of DiI C18 associated with cooling are of interest in that the nature of the damage in the adult would appear to be due to phase change induced disruption to the inner membrane. This indicates that this particular membrane may be particularly susceptible to this type of damage. This may be of more general relevance since Praziquantel is thought to act by inducing phase changes within the lipid of the membrane. At the present time the action of these agents is manifest in gross structural damage in the membrane. It is possible that the initial lesion induced by Praziquantel is in the inner bilayer. This hypothesis could be tested by undertaking biophysical studies in DiI C18 labelled adult parasites treated with Praziquantel and other drugs. A further advantage of using DiI C18 is that it is sensitive to membrane potential. Therefore the generation of drug induced phase changes could be investigated at the biophysical level with FRAP and their effect on membrane permeability monitored in parallel.

The DiI C18 induced damage to the schistosomula is of interest on similar grounds. Many immunological and pharmacological agents active against schistosomula induce blebbing at the surface membrane. Several questions are of interest in this regard. The mechanism of blebbing is

unknown in particular the involvement, if any, of a tegumental cytoskeleton remains to be determined. The DiI C18 related observations point again to an involvement of lipid phase changes and they also indicate that the schistosomula surface membrane has some property, lost by the adult, which allows it to stay intact upon dissociation of the membrane from the parasite body. This raises a final question which is, does the process of blebbing affect the way in which schistosomula surface antigens are presented to the host in vivo? This could be either at the membrane surface directly or at later stages such as the macrophages which recognise and attack drug damaged parasites. Moreover, the drug RO11 which can induce immunity in an animal cleared of its worm burden also induces large scale blebbing of the parasites surface [Bickle & Andrews, 1985]. Finally a greater understanding of the mechanisms of DiI C18 induced damage as well as giving an oblique view on some aspects of drug activity could itself be exploited to produce an interesting variant on the irradiation vaccine approach. By utilising these dyes it may be possible to attenuate the parasite by inducing controlled lesions directly in the membrane itself and to monitor the type of immunity, if any, induced.

The striping effect is an interesting phenomenon associated with cooling. It is not clear whether it is an artefact induced within the membrane by cooling or simply the exposure of a large scale feature of membrane organisation intrinsic to the membrane. Although many scanning and transmissiom electron microscopy studies have been done this effect has not been reported. However it is possible that the surface morphology of the chilled parasite has not been investigated at this scale. The main question to be addressed is whether the "major transverse folds" reported here exist in the membrane at physiological temperature. This would be most easily achieved by constructing a 3D image of the surface morphology using a confocal scanning

microscope apparatus. This would have the advantage that an investigation of the living parasite could be undertaken. Alternatively some rather difficult and painstaking transmission E.M. studies, looking to chart the occurrence of very deep clefts running deep into the membrane, could be attempted.

Biophysical studies on the surface membrane of S. mansoni utilising fluorescent probes have proved to be extremely informative and provided insight into some very basic properties of the membrane. The approach outlined in chapter 6 would take analysis based on the fluorescence emission close to it's limits in terms of the amount of information extracted. The only other major advance on this envisaged at present concerns the technology of the microscopy rather than the exploitation of the dyes. This would be to acquire scanning and computer aided imaging facilities. It is appreciated that total reliance on analysis of fluorescence from these probes to investigate the biophysics of the membrane is by itself too narrow an approach. This approach has been extremely informative but some other technique independent of it is required to test and corroborate the main conclusions derived. The development of the facility to measure the steady state anisotropy of the fluorescence polarisation presently underway represent a response to this problem based on making imaginative use of existing apparatus. This technique still involves the use of exogenous probes and as is clear from this study the problems of how representative such analogues are of their endogenous models and the extent to which such probes themselves actually alter the membrane under investigation, are always present. The ideal corroborative method would be a non invasive one such as differential scanning calorimetry or nuclear magnetic resonance. These have one important drawback in that the membrane on the living parasite cannot be analysed directly.

For the study of the schistosomula membrane it is possible that the high salt technique of Kusel et al 1974 which produces isolated membrane vesicles may be exploited. Neither the biophysical properties of the membrane [Foley 1986] nor the protein and lipid composition of the membrane appear to change significantly upon vesiculation. Therefore one may be able to corroborate the conclusions concerning domain differentiation generated by the fluorescence approach in the membrane of the living parasite with the proporties of these vesicles analysed by an independent non invasive technique such as NMR. This problem of confirming the conclusions derived from fluorscence based study represents a major challenge to further development of biophysical analysis of the surface membrane of S. mansoni.

REFERENCES

Albers, J.J., Tollefson, J.H., Chen, C.H. & Steinmetz, A.(1984) Arteriosclerosis 4, 49-58.

Allen, P.M. (1987) Immunology Today 8, 270-287.

Anwar, A.R.E., Smithers, S.R., Kay, A.B. (1979) J. Imm. 122, 628-637.

Axelrod, D. (1977) Biophys. J. 26, 557-574.

Berridge, M.J. & Irvine, R.F. (1984) Nature 312, 315-321.

Bhakdi, S., & Tranum-Jensen, J. (1983) Biochim. Biophys. Acta. 737, 343-372.

Bickle, Q.D., & Ford, M.J. (1982) J. Immunol. 128, 2101-2106.

Bickle, Q.D., & Andrews, P. (1985) Parasitology 90, 325-328

Bradley, D.J., & McCullough, F.S. (1973) Trans. R. Soc. Trop. Med. Hyg. 67, 491-500.

Bretcher, M.S. (1972) J. Mol. Biol. 71, 523-528.

Bricker, C.S., Depenbusch, J.W., Bennet, J.L. & Thomson, D.P. (1983) Z. Parasitkund. 69, 61-67.

Brindley, D.N., Bowley, R., Stirton, G., Pritchard, P.H., Burditt, S.L., & Cooling, J. (1978) in Central Mechanisms of Anorectic Drugs. Eds. Garattin, S. & Samanin, R.

Bruce, J.I., Pezzlo, F., McCarty, J.E. & Yajima, Y. (1970) Am. J. Trop. Med & Hyg. 19, 959-981.

Bulow, R., Overath, P., & Davoust, J. (1988) Biochemistry. 27, 2384-2388.

Burn, P. (1988) TIBS. 13, 79-83.

Butterworth, A.E. (1987) Acta Tropica. 44, Suppl. 12, 31-40

Butterworth, A.E., David, J.R., Franks, D., Mahmoud, A.A.F., David, P.H., Sturrock, R.F., & Houba, V. (1977) J. Exp. Med. 145, 136.

Butterworth, A.E., Vadas, M.A., Martz, E. & Sher, A. (1979) J. Immunol. 122, 1314-1321.

Butterworth, A.E. (1982) Immunol. Rev. 61, 5-39.

Butterworth, A.E., Capron, M., Cordingley, J.S., Dalton, P.R., Dunne, D.W., Kariuki, H.C., Keoch, D., Magambi, M.,

Ouma, J.H., Prentice, M.A., Richardson, B.A., Siongok, T.K., Sturrock, R.F., & Taylor, D.W. (1985)
Trans. Roy. Soc. Trop. Med. Hyg. 79, 393.

Capron, M., & Capron, A. (1986)
Parasitology Today. 2, 69-73.

Capron, M., Capron, A., Khalife, J., Butterworth, A.E., & Gryzch, J.M. (1987) Acta Tropica. 44, Suppl. 12, 55-62.

Caulfield, J.P., Korman, G., Hogan, M., & David, J.R. (1980) J. Cell. Biol. 86, 46-63.

Caulfield, J.P., Korman, G., & Samuelson, J.C. (1982)
J. Cell. Biol. 94, 370-378.

Chao, L.P. (1984) Ph.D. Thesis. University of Glasgow.

Chapman, H.A., & Hibbs, J.B. (1987) Science. 197 282-284.

Chappell, L.H., & Walker, E. (1982)
Comp. Biochim. and Physiol. 63, 250-258.

Chappell, L.H., Thomson, A.W., Barker, G.C., & Smith, S.W.G. (1987) Antimicrobial Agents and Chemotherapy. 5, 1567-1571.

Cioli, D., Blum, K., & Ruppel, A. (1978)
Exp. Parasit. 45, 74-80.

Clegg, J.A., & Smithers, S.R. (1968)
Parasitology. 58, 111-128.

Clegg, J.A., Smithers, S.R., & Terry, R.J. (1970)
Parasitology. 61, 87-94.

Clegg, J.A. (1972) In Symposia of the British Society for Parasitology. Parasitology 10, 23-40.

Clegg, J.A., & Smithers, S.R. (1972)
Int. J. Parasit. 2, 79-98.

Cogan, U. & Schachter, D. (1981) Biochemistry. 20, 6396-6403.

Colley, P.G. & Wikel, S.K. (1974) Exp. Parasitol. 35, 44-51.

Crabtree, J.E., & Wilson, R.A. (1986)
Parasit. Immunol. 8, 265-285.

Cryer, A. (1983) in Biochemical Interactions at the Endothelium Surface. pp. 245-274. Ed. Cryer, A. Elsevier, Amsterdam.

Cushley, W. and Kusel, J.R. (1987)
Mol. Biochem. Parasitol. 24, 67-71.

Dean, D.A., Wistar, R., & Murrell, K.D. (1974)
Am. J. Trop. Med. & Hyg. 23, 420-428.
Dean, D.A. (1977) J. Parasitol. 63, 418-426.
Dean, D.A., Mangold, B.L., Giorgi, J.R., & Jacobson, J.H.
(1984) Am. J. Trop. Med. & Hyg. 33, 89-96.
Dessein, A., Samuelson, J.C., Butterworth, A.E., Hogan,
M.E., Vadas, M.A., Sherry, B.A. & David, J.R. (1981)
Parasitology, 82, 357-374.
De Lisi, C., & Wengel, F.W. (1983)
J. Theor. Biol. 102, 307-322.
Dictus, W.G.A., van Zoelen, E.J.J., Tetteroo, L.G.J.,
Tertoolen, S.W., de Latt, S.W., & Bluemink, J.G. (1984)
Dev. Biol. 101, 201-211.
Doenhoff, M.J., Modha, J., & Lambertucci, J.R. (1988)
Immunology. 65 (in press)
Dupou , L., Teissie, J., & Tocanne, J.F. (1988)
Eur. J. Biochem. 171 669-674.
Ellner, J.J., Olds, G.R., Lee, C.W., Kleinhertz, M.E.,
& Edmonds, K.L. (1982) J. Clin. Invest. 70 369-378.
Ferguson, M.A.J., Low M.G.,& Cross A.M. (1985).
J. Biol. Chem. 260 14,547-14,555
Fernandez, S.M., & Berlin, R.D. (1976)
Nature 264 411-415
Fetterer, R.H., Pax R.H., & Bennett J.L. (1980)
Exp. Parasit. 49 353-365
Fielding, C.J., & Fielding P.E. (1982)
Meds. Clinics. N. Am. 66 363-373
Fielding, C.J., & Fielding, P.E. In
Biochemical interactions at the endothelium.
Elsevier, Amsterdam.
Fielding, C.J. (1984) J. Lipid Res. 25 1624-1628
Fielding, C.J., Reaven, G.M., Lia, G. & Fielding P.E.
(1984) Proc. Natl. Acad. Sci. U.S.A. 81 2512-2526
Foley, M. 1986 Ph.D. Thesis University of Dundee.
Foley, M., MacGregor, A.N., Kusel, J.R., Garland, P.B.,
Downie, T., & Moore, I. (1986) J. Cell. Biol. 103 807-818.

Foley, M., Kusel, J.R., & Garland, P.B. (1988)
Parasitology 100 85-97.
Ford, M.J., Bickle, Q.D., Taylor, M.G. & Andrews B.J. (1984)
Parasitology 89 461-482.
Fraya, G.H., & Smyth, J.D. (1983)
Adv. Parasitol. 22 309-387.
Freidus, D.J., Schlegel, R.A., & Williamson P. (1984)
Biochim. Biophys. Acta. 803 191-196.
Furlong, S.T., & Caulfield, J.P. (1986) Fed. Proc. 45 623.
Furlong, S.T. & Caulfield, J.P. (1988)
Exp. Parasit. 65 222-231.
Gerson, D.F. (1980) Biochim. Biophys. Acta. 602, 269.
Gitter, B.D., McCormick, S.L., & Damian, R.T. (1982)
J. Parasit. 68, 513-518.
Gitter, B.D., & Damian, R.T. (1982)
Parasite Immunol. 4, 383-393.
Glauert, A.M., Butterworth, A.E., Sturrock, R.F. & Houba, V. (1978). J. of Cell Sci. 34, 173-192.
Golan, D.E., Brown, C.S., Cianci, C.M.L., Furlong, S.T., & Caulfield, J.P. (1986) J. Cell Biol. 103, 819-828.
Goldring, O.L., Clegg, J.A., Smithers, S.R., & Terry, R.J. (1976) Clin. Exp. Immunol. 26, 181-187.
Goldring, O.L., Sher, A., Smithers, S.R., & McLaren, D.J. (1977) Trans. R. Soc. Trop. Med. Hyg. 71, 144-148.
Goldring, O.L., Kusel, J.R., & Smithers, S.R. (1977)
Exp. Parasit. 43, 82.
Goldstein, J.L., & Brown, M.S. (1974)
J. Biol. Chem. 249, 5153-5162.
Goldstein, J.L., & Brown, M.S. (1984)
J. Lipid Res. 25, 1450-1461.
Grant, C.W.M., Wu, S.H., & McConnell, H.M. (1974)
Biochim. Biophys. Acta. 363, 151-158.
Griffin, R.G. (1981) Methods Enzymol. 72, 108-174.
Greenspan, P., Mayer, E.P., & Fowler, S.D. (1985)
J. Cell Biol. 100, 965-973.
Gryzch, J.M., Capron, M., Dissous, C. & Capron, A. (1984)
J. Immunol. 133, 998.

Harder, A., Goosens, J., & Andrews, P. (1988)
Mol. Biochem. Parasitol. 29, 55-60.
Harnett, W., & Kusel, J.R. (1986)
Exp. Parasit. 61, 146-150.
Haseeb, M.A., Eveland, K.K., & Fried, B. (1985)
Comp. Biochem. Physiol. 82A, (2), 421-423.
Haseeb, M.A., Eveland, L.K., Fried, B., & Hayat, M.A. (1985) Int. J. Parasit. 15, 49-53.
Hockley, D.J., & McLaren, D.J. (1973)
Int. J. Parasit. 3, 13-25.
Humphries, G.M.K., & Lovejoy, J.P. (1983) BBRC. 111, 768.
Icani, R.N., & McLaren, D.J. (1981)
Parasite Immunol. 3, 107.
Jain, M.K., & White, H.B. (1978) Adv. Lipid Res. 15, 1-60.
James, S.L. (1981) Parasitology. 83, 147.
James, S.L., Sher, A., Lazdus, J.K., & Meltzer, M.S. (1982)
J. Immunol. 122, 1535.
James, S.L. (1986) Immunologic Research 5, 139.
Karnovsky, M.J., Kleinfeld, A.M., Hoover, R.L., & Klausner, R.D. (1982) J. Cell Biol. 94, 1-6.
Kassis, A.I., Aikawa, M., & Mahmoud, A.A.F. (1979)
J. Immunol. 122, 398-405.
Kennedy, M.W., Foley, M., Kuo, Y.M., Kusel, J.R., & Garland, P.B. (1987) Mol. Biochem. Parasitol. 22, 233-240.
Khalife, J., Capron, M., Gryzch, J.M., Bazin, H., & Capron, A. (1985) J. Immunol. 135, 2780.
Klausner, R.D., Kleinfeld, A.M., Hoover, R.L., & Karnovsky, M.J. (1980) J. Biol. Chem. 255, 1286-1295.
Klausner, R.D., & Wolf, D.E. (1980)
Biochemistry. 19, 6199-6203.
Kusel, J.R., Gazzinelli, G., Colley, D.G., De Souza, C.P.S., & Cordeirio, M.N. (1984) Parasitology. 89, 483-494.
Kusel, J.R., Stones, L., & Harnett, W. (1981)
Biosci. Rep. 1, 253-261.
Kusel, J.R., Stones, L., & Harnett, W. (1982)
Mol. Biochem. Parasitol. 5, 147-163.
Lange, Y, & Steck, T.L. (1985) J. Biol. Chem. 260, 15592-97

Low, M.G., Ferguson, M.A.J., Futerman, A.H., & Silman, I. (1986) TIBS. 11, 212-215.
Lelkes, P.I., & Miller, I.R. (1980) J. Membr. Biol. 52, 1-15.
Levy, M.G., & Read, C. (1975) J. Parasit. 61, 627-632.
Lipsky, N.G., & Pagano, R.E. (1985) Science 288, 745-747.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
Lund-Katz, S., & Phillips, M.C. (1984) Biochemistry 23, 1130-1138.
Mackenzie, C.D., Ramalho-Pinto, F.J., McLaren, D.J. & Smithers, S.R. (1977) Clin. Exp. Immunol. 30, 97-104.
Mahmoud, A.A.F., Warren, K.S., & Peters, P.A. (1975) J. Exp. Med. 142, 805-815.
Martin, O., & Pagano, R.E. (1987) J. Biol. Chem. 262, 5890-5898.
Martz, E. (1976) J. Immunol. 117, 1023-1029.
Matsumoto, Y., Perry, G., Levine, R.J.C., Blanton, R., Mahmoud, A.A.F., & Aikawa, M. (1988) Nature (London) 333, 76-80.
Matzinger, P. (1981) Nature (London). 292, 497-501.
Meyer, F., Meyer, H., & Bueding, E. (1970) Biochim. Biophys. Acta. 210, 256-266.
Miller, K.W., & Small, D.M. (1983) J. Biol. Chem. 258, 13772-13784.
Mio, M., Ikeda, A., Akagi, M., & Tasaki, K. (1985) Agents Actions 16, 113-117.
Moser, G., Wassom, D.L., & Sher, A. (1980) J. Exp. Med. 152, 41-53.
Mountford, C.E., & Wright, L.C. (1988) TIBS. 13, 172-177.
McDiarmid, S.S., Dean, L.L., & Podesta, R.B. (1983) Mol. Biochem. Parasitol. 7, 141-157.
McFarlane, A.S. (1958) Nature (London). 182, 53.
McLaren, D.J., Clegg, J.A., & Smithers, S.R. (1975) Parasitology. 70, 67-76.
McLaren, D.J., & Hockley, D.J. (1977) Nature (London). 269, 147-149.

McLaren, D.J., Ramalho-Pinto, F.J., & Smithers, S.R. (1978) Parasitology. 77, 313-324.

McLaren, D.J. (1980) Schistosoma mansoni: The surface in relation to host immunity. Research Studies Press, Chichester.

McLaren, DJ., McKean, J.R., Olsson, I., Venge, P., & Kay, A.B. (1981) Parasite Immunol. 3, 359-373.

McLaren, D.J., & Terry, R.J. (1982) Parasite Immunol. 4, 129-148.

McLaren, D.J., & Boros, D.L. (1983) Exp. Parasit. 56, 346.

McLaren, D.J., & James, S.L. (1985) Parasite Immunol. 7, 315-331.

McLaren, D.J., Pearce, E.J., & Smithers, S.R. (1985) Parasite Immunol. 7, 29-44.

McLaren, D.J., & Rogers, M. (1986) Parasite Immunol. 8, 307-318.

Ohanian, S.H., Schlager, S.I. & Saha, S. (1982) Molecular Immunology. 19, 535-542.

O'Leary, T.J., Ross, P.D., Lieber, M.R., & Levin, I.W. (1986) J. Biophysical Soc. 49, 795-801.

Omer-Ali, P., Magee, A.I., Kelly, C. & Simpson, A.J.G. (1986) J. Immunol. 137, 3601.

Parce, J.W., Henry, N., & McConnell, H.M. (1978) Proc. Natl. Acad. Sci. (USA). 75, 1515-1518.

Parra, J.F.C., Franca, R.C.S., Kusel, J.R., Gomez, M.V., Figueriedo, E.A., & Mota-Santos, T.A. (1986) Mol. Biochem. Parasitol. 21, 151-159.

Payares, G., McLaren, D.J., Evans, W.H., & Smithers, S.R. (1985) Parasitology. 91, 83-99.

Pearce, E.J., Basch, P.F., & Sher, A. (1986) Parasite Immunol. 8, 74-94.

Pearce, E.J., & James, S.L. (1986) Parasite Immunol. 8, 513-520.

Pearce, E.J., Zilberstein, D., James, S.L., & Sher, A. (1986) Mol. Biochem. Parasitol. 21, 259-267.

Perez, H., & Terry, R.J. (1973) Int. J. Parasit. 3, 499-503

Perez, H., & Smithers, S.R. (1977)
Int. J. Parasit. 7, 315-320.
Podesta, R.B., & Dean, L. (1982)
Mol. Biochem. Parasitol. 5, 353-360.
Podesta, R.B. (1983) J. Exp. Biol. 106, 195-204.
Podesta, R.B., Boyce, J.F., Schurch, S., & McDiarmid, S.S. (1987) Cell Biophysics. 10, 23-31.
Ramalho-Pinto, F.J., McLaren, D.J., & Smithers, S.R. (1978)
J. Exp. Med. 147, 147-156.
Rectenwald, W., & McConnell, H.M. (1981)
Biochemistry. 20, 4505.
Roberts, S.M., Aitken, R., Vojrodic, M., Wells, E., & Wilson, R.A. (1983) Mol. Biochem. Parasitol. 9, 129-143.
Robertson, N., & Cain, G.D. (1985)
Comp. Biochem. Physiol. 82B, 299-306.
Rogers, M.V., & McLaren, D.J. (1987)
Mol. Biochem. Parasitol. 22, 273-288.
Roitt, I.M., Brostoff, J., & Male, D.K. (1985) Immunology. Churchill Livingstone, Edinburgh.
Rumjanek, F.D., & McLaren, D.J. (1981)
Mol. Biochem. Parasitol. 1, 31-42.
Rumjanek, F.D., McLaren, D.J., & Smithers, S.R. (1983)
Mol. Biochem. Parasitol. 9, 337-350.
Rumjanek, F.D., Capana-Pereira, M.A., & Vidigal-Silviera, M. (1985) Mol. Biochem. Parasitol. 14, 63-73.
Ruffer, M.A. (1910) Brit. Med. Jour. 1, 16.
Ruppel, A., Rotter, U., Vongenchter, H., & Diesfeld, H. (1983) Parasitology. 87, 75-86.
Ruppel, A., McLaren, D.J., Diesfeld, H., & Rotter, U. (1984) J. Immunol. 14, 702-708.
Ruppel, A., & McLaren, D.J. (1986) Exp. Parasit. 22, 273-88
Ruppel, A., Breternitz, R..B., & Lutz, L. (1987)
Parasitology. 95, 241-251.
Sabah, A.A., Fletcher, C., Webbe, G., & Doenhoff, M.J. (1986) Exp. Parasit. 61, 294-303.
Samuelson, J.C., Sher, A., & Caulfield, J.P. (1980)
J. Immunol. 124, 2055-2057.

Samuelson, J.C., Caulfield, J.P., & David, J.R. (1982)
J. Cell. Biol. 94, 355-362.
Samuelson, J.C., & Caulfield, J.P. (1985)
J. Cell. Biol. 100, 1423-1434.
Saunders, N., Wilson, R.A>, & Coulson, P.S. (1987)
Mol. Biochem. Parasitol. 25, (2), 123-31.
Schlegel, R.A., Phelps, B.M., Waggoner, A., Terada, L., & Williamson, P. (1980) Cell. 20, 321-328.
Schlessinger, J., & Elson, E. (1982)
Methods Exp. Phys. 20, 197-227.
Schroeder, F. (1980) Eur. J. Biochem. 112, 293-307.
Schurch, S., Gerson, D.F., & McIver, J.L. (1981)
Biochim. Biophys. Acta. 640, 577.
Schwartz, R.H. (1985) Prog. Clin. Biol. Res. 172B, 333-341.
Seigneuret, M., & Devaux, P.F. (1984)
Proc. Natl. Acad. Sci. (USA) 81, 3751-3755.
Sher, A., Hall, B.F., & Vadas, M.A. (1978)
J. Exp. Med. 148, 46.
Shimshick, E.J., & McConnell, H.M. (1973)
Biochemistry. 12, 2351-2360.
Simpson, A.J.G., & McLaren, D.J. (1982)
Exp. Parasit. 53, 105-116.
Simpson, A.J.G., James, S.L., & Sher, A. (1983)
Infectious Immunology. 41, 591.
Sims, P.J., Waggoner, A.S., Way, C.H., & Hoffman, J.F. (1974) Biochemistry. 13, 1315-1330.
Singer, S.J., & Nicholson, G.L. (1972)
Science. 175, 720-731.
Sklar, L.A., Doody, M.C., Gotto, A.M., & Powmill, H.J. (1980) Biochemistry. 19, 1294-1301.
Smith, H.V., McQueen, A., & Kusel, J.R. (1980)
Proc. R. Soc. Edinburgh. 79B, 173-181.
Smith, J.H. , Reynolds, E.S. & Lichtenberg, F. von. (1969)
Am. J. Trop. Hyg. 18 28-49.
Smithers, S.R., & Terry, R.J. (1965)
Parasitology. 55, 695-701.

Smithers, S.R., & Terry, R.J. (1969) In Advances in Parasitology. Ed. B. Dawes. Academic Press, London.
Smithers, S.R., Terry, R.J., & Hockley, D.J. (1969) Proc. Roy. Soc. B. 171, 483-494.
Smithers, S.R., & Gammage, K. (1980) Parasitology. 80, 289-300.
Smithers, S.R., Simpson, A.J.G., Yi, X., Omer-Ali, P., & Kelly, C. (1987) Acta Tropica. 12, 21-38.
Soares-Silveira, A.M., De Lima Friche, A.A>, & Rumjanek, F.D. (1986) Comp. Biochem. Physiol. 85B, 851-857.
Stange, E.F., & Dietchy, J.M. (1983) Proc. Natl. Acad. Sci. (USA) 80, 5739-5743.
Stirewalt, M.A. (1963) Parasitology. 13, 395-406.
Stirewalt, M.A., & Dorsey, C.H. (1974) Exp. Parasit. 35, 1-15.
Storch, J.S., & Kleinfield, A.M. (1985) TIBS (Nov), 418-424.
Stryer, L. (1978) Ann. Rev. Biochem. 47, 819-846.
Tall, A.R., Abreau, E., & Shuman, J. (1983) J. Biol. Chem. 258, 2174-80.
Tardieau, A., Luzzati, V., & Reman, F. (1973) J. Mol. Biol. 75, 711-733.
Tavares, C.A.P., Gazzinelli, G., Mota-Santos, T.A., & Dias da Silva, W. (1978) Exp. Parasit. 46, 145-151.
Taylor, P.W. (1983) Microbiol. Rev. 47, 46-83.
Thomson, T.E., Huang, C., & Litman, B.J. (1974) in Ed. A.A. Moscona. John Wiley & Sons, Chichester.
Tilley, L., Cribler, S., Roelofsen, B., Opdenkamp, J.A.F., & Van Deenen, L.L.M. (1986) FEBS Lett. 194, 21-27.
Torpier, G., Capron, A., & Ouassi, M. (1979) Nature (London) 278, 447.
Torpier, G., Ouassi, M., & Capron, A. (1979) J. Ultrastructural Res. 67, 276.
Torpier, G., & Capron, A. (1980) J. Ultrastructural Res. 72, 325-335.
Torpier, G. (1982) Nature (London) 296, 810.
Uster, P.S., & Pagano, R.E. (1986) J. Cell Biol. 103 1221-1234.

Vadas, M.A., David, J.R., Butterworth, A.E. Pisani, N. Siongok, T.A. (1979) J. Immunol. 122 1228-1236.
Valentine, R.C., & Green, N.M. (1967)
J. Mol. Biol. 1967 27 615-617.
Van Meer, G., & Simmons, K. (1986)
EMBO Journal 5 1455-1464.
Van Pijkeren, T.A., Tavares, C.A.P., & Gazzinelli, G. (1982) Parasitol. 84 239-252.
Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurious, P., Kastelijn, D., & Van Deenan, L.L.M. (1973)
Biochim. Biophys. Acta. 323 178-193.
Verkleij, A.J. (1984) Biochim. Biophys. Acta. 779 43-63.
Vial, H.J., Torpier, G., Ancelin, M.L., & Capron, A. (1985)
Mol. Biochem. Parasitol. 17 203-218.
Viera, L.Q., Colley, D.G., De Souza, C.P.S. & Gazzinelli, G. (1987) Am. J. Trop. Med. Hyg. 36 378-386.
Von Lichtenberg, F. (1987) In The biology of Schistosomes.
Eds. Rollinson & Simpson.
Academic Press, London.
Waggoner, A.S. & Stryer, L.
Proc. Natl. Acad. Sci. U.S.A. 67 579-589.
Watts, T.H. & McConnell, H.M. (1986)
Proc. Natl. Acad. Sci. U.S.A. 83 9660-9664
White, S.H. (1978) Ann. N.Y. Acad. Sci. 303 243.
Wolf, D.E., Kinsey, W., Lennartz, W., & Edidin, M. (1981)
Dev. Biol. 81 133-138.
Wolf, D.E., Lipscomb, A.C. & Maynard, V.M. (1988)
Biochemistry 27 860-865.
Wilson, R.A. & Barnes, P.E. (1977)
Parasitol. 74 61-71.
Wright. W.H. (1972) Bull. W.H.O. 47 559-566.
Yeagle, P.L. (1985) Biochim. Biophys. Acta. 822 267-287.
Young, B.W. & Podesta, R.B. (1984)
J. Parasit. 70 447-448.
Yi, X., Simpson, A.J.G. De Rossi R., Smithers, S.R. (1986a)
J. Immunol. 137 3955-3946.

Yi, X., Simpson, A.J.G. De Rossi R., Smithers, S.R. (1986b) J. Immunol. 137 3946-3955.

Zachowski, A., Fellman, P., Devaux, P.F. (1985) Biochim. Biophys. Acta. 815 510-514.

Zachowski, A., Favre, E., Cribier, S. Heve, P. & Devaux P.F. (1986) Biochemistry 25 2585-2990.

Kusel J.R. & Gordon J.F. (1989) Parasite. Immunol. (September)