The regulation of membrane fluidity in *Schistosoma mansoni*

By

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Abbreviations

5AF 5'-N-Octadecanoyl aminofluorescein

cAMP, Cyclic adenosine 3'5' monophosphate

D_L, Lateral diffusion coefficient

DTBP, Dimethyl 3,3'-dithiobispropionimidate. 2HCl

DTSSP, 3,3' Dithiobis sulphosuccinimidylpropionate

EDTA, Ethylene diamine tetraacetic Acid

EGTA, Ethylene glycol bis(β-aminoethyl Ether) N,N,N',N'-Tetraacetic Acid

f, Mobile fraction

FITC, Fluorescein isothiocyanate

FRAP , Fluorescence recovery after photobleaching

FRET, Fluorescence resonance energy transfer

GMEM, Glasgow modification of Eagles medium

NBD, 7-nitrobenz-2-oxa-1,3-didiazol-4yl

PBS, Phosphate buffered saline

PC, Phosphatidylcholine

PE, Phosphatidylethanolamine.

WGA, Wheat germ agglutinin

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SUMMARY

The effect of serotonin on the fluidity of the tegumental membranes of adult male *Schistosoma mansoni* was assessed by the Fluorescence Recovery After Photobleaching technique .

It is demonstrated that the translational diffusion of 5'-N-octadecanoyl aminofluorescein is reduced by a mechanism involving G-protein coupled activation of adenylate cyclase and lowering of intracellular calcium concentration. Furthermore, the lateral diffusion coefficient and the mobile fraction appear to be controlled by calcium and cAMP dependent pathways respectively. No change in the diffusion of the fluorescent phospholipid N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phosphatidyl choline was observed, suggesting the two probes used here partition into two different domains that are under independent control. An increase in the amount of protein associating with a membrane cytoskeleton is also demonstrated. Part of this increase is due to the polymerisation of actin, which is important in the regulation of membrane fluidity. In its cytoskeletal form, this actin appears as a pentamer. Confocal microscopy also reveals that the tegumental actin radiates out from the tubercles, which are shown to have a hedgehog like arrangement of actin spines. It is also demonstrated that changes that occur in the cytoskeleton can have consequences for molecules that are expressed on the outer surface of the tegument, such as lipids, lectin receptors and antigens of host origin. One possible connection with the surface may occur via two proteins of molecular weights 48 and 52 kilo daltons.

The regulation of membrane fluidity in *Schistosoma mansoni*.

1.1. Introduction

The membranes of parasites form the barrier between parasite and host. At present, the interactions that occur at this interface are poorly understood, as are the general functions of parasite membranes. For this reason, a brief introduction to mammalian membranes shall be given, to help in the understanding of parasite membrane processes.

1.1.1. Membranes and Membrane functions

Membranes define the outer limits of a cell or an organism, and biomembranes therefore have many important functions, especially those of unicellular organisms , where division of labour is not possible. The roles played by membranes include the maintenance of an electrochemical gradient between intra and extracellular compartments [Sweadner and Goldring, 1985], selecting which molecules can enter the cell [Collander, 1949], transmitting environmental information to the cell [Berg and Purcell, 1972], providing a medium in which enzymes can function [Farias et al, 1975] and allowing cell locomotion [Bretscher,1984].

1.1.2. Membrane proteins

The proteins found in membranes are of a diverse nature, and include enzymes [Zlotnick et al, 1987], transporters [Carafoli, 1987], receptors [Levitzki, 1984], ligands [Wilson and Hardin, 1988], translocaters [Klingenberg,1985] and structural components [Bennett, 1990].

Classically, membrane proteins are divided into peripheral and integral proteins based on their behaviour when subjected to various salt or pH conditions [Findlay, 1987]. This author defines peripheral proteins as those that

are removed from the membrane by brief exposure to ice cold NaOH at pH 11.0 . These peripheral proteins are mainly on the cytoplasmic face of the membrane [Alberts et al, 1983], and those that are found on the cell surface are usually subunits of larger complexes, such as $ß_2$ microglobulin [Bjorkman et al , 1987]. Integral proteins can be identified by the degree to which they can be labelled with hydrophobic photosensitive probes [Wells and Findlay, 1980], or the ease with which they partition into a detergent rich phase [Bordier, 1981]. Such proteins are subclassified according to the number of times the polypeptide chain crosses the bilayer, and further subdivided according to the relative positions of the N and C terminus with respect to the surfaces of the bilayer [Jennings, 1989]. The number of times a polypeptide chain crosses the membrane varies from one in monotopic proteins, such as cytochrome b5 to twenty four in the sodium gate in nerve axons [Noda et al, 1984]. The nature of these transmembrane segments is presumed to be largely alpha helical [Henderson and Unwin, 1975], with the possibility of hydrophilic connector loops which are positive on cytoplasmic sequences, and negative on extracellular sequences [Von Heijne, 1988].

Another important class of membrane proteins are those which are inserted into the membrane by lipid tails. This linkage is of a covalent nature [Hantke and Braun, 1973]. Myristoylation via an amide link has been demonstrated in many viral and eukaryotic proteins [Grand,1989]. This modification, which requires protein synthesis [Magee and Courtneidge, 1985], is probably via the N-terminus, but the possibility of linkage via lysine has not been ruled out. Palmitoylation is another means by which proteins may be modified for membrane insertion, and attachment is thought to be via an ester or thioester linkage. It also does not rely completely on protein synthesis, and is therefore a late or turnover process [O'Brien et al 1987]. Proteins may also be attached to membranes by a phosphatidyl inositol based anchor [Cross, 1990].

1.1.3. Membrane lipids

Membrane lipids of animal cells provide a matrix for the functioning of membrane proteins, and provide a permeability barrier [Collander, 1949]. These lipids fall into several classes: the glycerides, which are glycerol derivatives, the sterols and the sphingolipids, (which are fatty acid derivatives of the amino alcohol sphingosine). The glycerides are usually in the form of 1,2-diacylglycerides, and comprise the phosphoglycerides (phospholipids), the glycolipids and the plasmalogens, where one of the fatty acid chains is in a normal ester linkage at the 2-C position of glycerol, and the other is a cis alpha, beta ether linkage at the 1-C position [Hawthorne and Ansell, 1982]. Of the phospholipids, the general order of abundance in plasma membranes is Phosphatidylethanolamine, Phosphatidylcholine, Phosphatidylserine, Phosphatidylinositol and phosphatidic acid in decreasing order [Datta, 1987].

1.1.4. Membrane function in signal transduction

Transduction of information across membranes usually starts with the binding of a ligand to a receptor [Bradshaw and Frazier, 1977], although some signals such as steroids permeate the membrane and bind to an intracellular receptor [Yamamoto and Alberts, 1976]. After the receptor binds the ligand, one of several things may happen. Firstly, the receptor ligand complex may be internalised as in the Epidermal Growth Factor (EGF) receptor [Carpenter, 1987]. This receptor is a tyrosine kinase, which means that it phosphorylates tyrosine residues within various recognition sequences. Upon ligand binding, this receptor autophosphorylates, which increases the tyrosine kinase activity. Two of these sites, Thr^{669} and Ser^{671} appear to be required for receptor internalisation and substrate interaction [Heiserman et al 1990]. Another possibility is that the receptor also functions as an ion channel, and ligand binding causes the opening of these receptor operated channels,an example of such a receptor is the nicotinic

acetylcholine receptor [Stroud, and Finer-Moore, 1985]. A receptor may also be methylated, and the nature of the signal modulated that way [Hazelbaur, and Harayama, 1983]. The final thing that can happen when a receptor binds a ligand is that second messengers are produced. To date, those second messengers that have been identified are calcium ions [Irvine and Moor, 1986], inositol 1,3,4 trisphosphate, Diacylglycerol [Berridge, 1987] and the cyclic nucleotides cyclic Adenosine monophosphate (cAMP) [Sutherland, 1972] and cyclic Guanosine monophosphate (cGMP) [Garbers, 1989].

The sequence of events that leads to the production of second messengers begins with the occupation of a receptor, which facilitates the interaction of the receptor with a heterotrimeric GTP binding protein (G-protein). These proteins transmit information from an occupied receptor to an effector such as a membrane bound enzyme, and stimulation persists until the bound GTP is hydrolysed [Gilman, 1987]. The conventional view is that upon the receptor binding to a ligand, the G-protein binds GTP, releasing its GDP, and this brings about the dissociation of the protein into the GTP binding alpha subunit and a dimer consisting of the beta and gamma subunits [Lo and Hughes, 1987]. The alpha unit conveys the information to an effector in the membrane, and stimulation persists until the G-protein hydrolyses its bound GTP [Gilman, 1987], the whole process being diffusion dependent [Rimon et al 1978]. However, there have been reports that some receptors co-purify with their G-proteins, the implication being that they form a complex in the membrane [Niznik et al1986]. The beta gamma dimers have been implicated in the inhibition of atrial muscarinic potassium channels [Okabe et al 1990].

The effectors that these proteins couple to are membrane bound enzymes such as adenylate cyclase, guanylate cyclase, phospholipase C and phospholipase D [Downes, 1989, Anthes et al, 1989, Sutherland, 1972 and Garbers, 1989].

1.1.5. Membrane structure

The plasma membranes of cells when studied by electron microscopy are typically four to five nanometer thick structures. The first evidence that the lipids formed a bilayer came in 1925, when the surface area of erythrocyte lipids was measured on water, and the surface area was shown to be twice that of the original membranes the lipid was extracted from [Gorter and Grendel, 1925]. Proteins were also suggested to form a part of membranes by the surface tension experiments of [Stein and Danielli, 1956], and with the advent of freeze fracture electron microscopy, they were demonstrated to penetrate the bilayer [Pinto da Silva and Branton, 1970]. This gave rise to the current model of simple membrane structure, the fluid mosaic model [Singer and Nicholson,1972].

1.2. Membrane organisation

1.2.1. Vertical membrane asymmetry

The lipids of biological membranes are not evenly distributed between the bilayers, although there is not an absolute asymmetry in distribution, there is a preference for negatively charged and aminophospholipids to occupy the cytoplasmic face. These lipids also tend to take up conical configurations [Datta, 1987]. Maintenance of this asymmetry is thought to be due to specific ATP dependent translocases, of Mr 32 kDa whose function depends on the integrity of a sulphydryl group [Seigneurt and Devaux, 1984, Connor and Schroit, 1990]. Once in the inner monolayer, phosphatidylserine can be kept at this face by direct interaction with spectrin [Haest, 1982]. This ordering of lipids may however be disrupted by local changes in cell shape and curvature [Thomson et al, 1974].

1.2.2. Lateral membrane asymmetry

One may expect lateral asymmetry of membrane component distribution from the specificity of some lipid-protein interactions [Sankaram et al, 1989], which could provide a lipid annulus surrounding the protein. Such annuli vary from 20 lipid molecules (bacteriorhodopsin) to 685 (band 3) [Hayward and Stroud, 1981]. Electron microscopy also provides further evidence for such microdomains [Severs and Robenek, 1983], as does the presence of enzymes whose activities do not correlate with the fluidity of the bulk phase [Hockman and Zakim, 1983]. Liposomes containing simple lipid mixtures also show the ability to undergo lateral phase separations [Eklund et al, 1988]. Lateral inhomogeneities can also be detected by the staining pattern of rat hepatocyte membranes stained with cis or trans parinaric acids [Schroeder, 1983].

There is also evidence for the existence of macroscopic (micrometer scale) domains in membranes, where components of the domain may be free to diffuse within its boundary [Yechiel and Edidin, 1987]. These are typically divided by barriers which prevent proteins and possibly lipids from exchanging with other domains. Some examples of such domains are given below: The apical and basal membranes of polarised epithelial cells have distinct compositions, and molecules such as gangliosides are not free to diffuse between them [Spiege] et al,1989]. The tight junctions probably serve as the barrier between these poles, as disruption of its structure allows trans-pole diffusion of molecules [Van Meer and Simmons, 1986]. The membrane of the mammalian spermatozoon is also highly polarised into distinct membrane domains, which prevent trans-domain diffusion [de Curtis et al, 1986]. Proteins can also be separated into domains too; an example of this is to be found in the myelinated axon, where the sodium channels are located at the nodes of Ranvier, and the potassium channels are to be found in the membrane underlying the myelin sheath, which presumably forms the diffusion barrier [Waxman and Ritchie, 1986]. Proteins can themselves form domains, one example

is the purple patches in the membrane of *Halobacterium halobium*. Here the purple patches are due to semicrystalline arrays of the protein bacteriorhodopsin [Hayward and Stroud, 1981].

1.3.1. Molecular motions in membranes

There are two broad types of motions that molecules in membranes can undergo, which are intramolecular movements such as bond stretching and rotation around bonds, and there are the movements that involve the whole molecule, which can be either translational, rotational or translocational (flip-flop).

Intramolecular mobility of phospholipids can be measured by ^{13}C NMR spectroscopy. Levine and coworkers [1972] have shown by varying the position of the ^{13}C label along a dipalmitoyl phosphatidylcholine molecule that there is a mobility gradient in the bilayer, with the glycerol backbone showing restricted mobility, with motion around the C-C bonds increasing towards the terminal methyl group of the aliphatic chains. The head group shows some restricted mobility, and results using Atomic Force Microscopy suggest that lipid head groups form ordered structures in liposomes [Egger et al, 1990]. It would also appear that phosphatidylcholine head groups do not interact strongly with proteins such as the acetylcholine receptor, the lipid carbonyl groups do however become increasingly hydrogen bonded to the protein [Bhushan and McNamee, 1990].

The two major forms of motion involving whole molecules are rotation and translational motion. Translocation also occurs, but is probably a rare event [Kornberg and McConnell, 1971]. When one considers the rotation of a molecule such as a protein, it is convenient to think of a molecule rotating as a simple cylinder around a single axis perpendicular to the plane of the membrane. Proteins vary greatly in their abilities to rotate, with molecules like rhodopsin that are free to rotate, with a rotational relaxation time ($\phi_{||}$) of 20 μsec at one end of the spectrum, and cytoskeletally tethered proteins such as band 3 at the

other end ($\tau_{||}$= 0.1-1 msec) [Cherry, 1979].

Translational motion is the lateral diffusion of molecules in the plane of a membrane. In biological membranes, molecules can be free to diffuse such as lipids in mammalian cells and some proteins such as rhodopsin, or they can show restricted abilities to diffuse such as the acetylcholine receptor [Edidin and Famborough, 1973], or lipids in specialised membranes [Foley, 1986, Yechiel and Edidin, 1987].

One source of restriction to diffusion is the cytoskeleton. This is a structure that can tether proteins, and some lipids too, and can restrict their diffusion [Peters et al, 1974,]. Band 3 for example diffuses more rapidly in spectrin deficient erythrocytes [Sheetz et al, 1980], and lipids and proteins are free to diffuse where membrane blebs are induced in myotubes [Wu et al, 1980]. This interaction with the cytoskeleton is not absolute, and is in fact dynamic, with proteins being able to dissociate from it and diffuse randomly [Sheetz et al, 1989].

The cytoskeleton is not the only source of restriction to diffusion, The extracellular matrix whether connective tissue [Nackache et al 1985] or oligosaccharides attached to membrane components [Wier and Edidin, 1988, Zhang et al, 1991] can retard diffusion. Hydrogen bonding between lipids has also been suggested as a force that can affect lateral mobility between lipids [Diomede et al, 1992].

1.3.2. Lipid-protein interactions

Lipids can interact with proteins by specific head group interactions, such as band 4 and phosphatidyl inositol [Sheetz et al, 1982], or myelin basic protein, which shows a sequence of specificity of interaction with phospholipids, with negative ones preferred, but the size of the charge is not important [Sankaram et al, 1989]. Transmembrane segments of proteins can also interact hydrophobically

with lipids, and are generally surrounded by an annulus of lipids, which can interchange with the bulk pool, this diffusion rate is of the same order, but slower than bulk diffusion [Sachse et al, 1987], although the M13 phage coat-protein has a four-fold lower lipid exchange rate in the ß-sheet conformation [Marsh, 1990].

1.3.3. Fluidity and membrane function.

Fluidity in hydrodynamic terms is defined as the inverse of viscosity, which is a measure of the frictional resistance encountered between two adjacent layers of fluid moving with different velocities. For biological membranes, the term fluidity is more qualitative, and is probably best thought of as a resistance to motion, and is a sum of all the types of motions described in the previous two sections.

Fluidity is a central feature of biomembrane function and cellular defects can be correlated with abnormal fluidity [Dominiczak et al, 1991], and organisms attempt to maintain their membrane fluidity under environmemtal stress [Shinizky, 1989]. Fluidity is important in regulating the rate of second messenger production [Atlas et al, 1980] and enzyme activities such as allosteric enzymes [Farias et al, 1975] and adenylate cyclase [Rimon et al, 1978] In *S. mansoni*, adenylate cyclase activity in cercariae increases upon transformation [Bennett and Bueding, 1973], at the same time as fluidity is changing [Foley et al, 1988]. The nondiffusing acetylcholine receptor aggregates in muscle membranes are essential for producing a wave of depolarisation [Hauser and Reese, 1977]. Changes in fluidity also correlate with activation of various cells [Mountford and Wright, 1988] and ova upon fertilization [Wolf et al, 1981]. The rate of phagocyte attachment to haptenated liposomes is also fluidity dependent [Lewis et al, 1980], as may be complement mediated damage [Shin et al, 1979].

1.4.1. The cytoskeleton

The cytoskeleton is a proteinaceous arrangement of filaments that is found exclusively in eukaryotic cells . Classically , it is described as the proteinaceous residue that remains after the lipids and the soluble proteins of a cell have been extracted with the non ionic detergent Triton X 100 [Yu et al, 1973]. The cytoskeleton is organised into groups of filaments such as stress fibres, which provide a framework that gives cells their shapes [Alberts et al, 1987]. It is also arranged into other structures such as cilia and flagella, and is not merely a static framework structure, but provides the basis for most of the dynamic processes occuring in cells . Some of these functions include the generation of force in the form of muscle contraction or the beating of cilia and flagella [Summers and Gibbons, 1971], and cell movement, organising the processes of mitosis and meiosis [Alberts et al, 1987]. The cytoskeleton also extends the cellular boundary to the environment, whether by the transduction of information, for example by regulating the activities of GTP-binding proteins [Raesnick and Wang, 1988] or by forming links with the extracellular matrix, which may give positional cues during development [Alberts et al, 1987]. Other functions include the transport of substances through cells, such as the transport of neurotransmitters along axons via microtubules to synaptic vesicles [Fulton, 1982], or the engulfment of receptors, such as the low density lipoprotein receptor [Goldstein et al, 1979].

The cytoskeleton is also an unusual source of non genetic inheritance [Aufderheide et al, 1977]

1.4.1.1. The arrangement of the cytoskeleton

The cytoskeleton can conveniently be divided into three main subdivisions on the basis of their appearance in the electron microscope, and are those structures that are based on microfilaments, those that are based on

microtubules, and those based on intermediate filaments. It is not uncommon for more than one structure to be present in different cell types [Alberts et al, 1987].

1.4.1.2. The microfilament system

The microfilament system is based on arrangements of actin filaments. This protein exists as multiple isoforms, and to date eight isoforms encoded by different but highly conserved genes have been described in mammalian and avian cells [Obinata et al, 1981]. These isoforms exist in a tissue specific manner [Storti and Rich,1976]. The two main isoforms are described as muscle and non muscle actin which are further classified according to their isoelectric point, and are classed as α , β or γ in order of increasing basicity. α actin is the muscle isoform.

At a molecular level, these isoforms show 93% homology in their primary amino acid structure, but can be differentiated on the basis of the amino acid sequence of their N terminal tryptic peptides, and their DNA sequences [Vandekerckhove and Weber, 1978]. These genes encode peptides that are larger than the mature 40 Kd peptide, which is shortened post translationally by a stepwise acylation dependent process. For example, non muscle actin genes encode a methionine, asparagine, (glutamine) N terminus. The N terminal methionine is then acetylated, and removed. After cleavage, the terminal asparagine is acetylated and removed, to leave a mature protein containing a glutamine at the N terminus. Muscle actin genes encode a methionine, cysteine, asparagine, (glutamine) N terminus which is modified as above to leave a terminal glutamine residue [Solomon and Rubenstein, 1985].

4.1.3. Microfilament structure

Actin filaments are composed of two strands of globular F-actin molecules of approximately four nanometers diameter, that are twisted into a helix containing 13.5 molecules per turn [Alberts et al, 1987]. These filaments have polarity which can be visualised in the electron microscope by staining the filaments with myosin S1 fragments (a papain digestion product that contains the actin binding site) [Woolrum et al, 1975]. This polarity is essential for the function of these filaments in terms of transport and muscle contraction [Taylor and Amos, 1981].

4.1.4. Microtubules.

The microtubular system is based on a 50 Kd GTP binding protein called tubulin [Dustin, 1987], which assembles into tubular structures of about 28 nannometers in diameter. In cross section, each tubule is composed of 13 protofilaments made up of of α and β tubulin heterodimers, each of which is encoded for by different genes [Dustin, 1987]. X ray diffraction studies suggest that these protofilaments have a staggered arrangement, such that in tubule cross sections α and β tubulins lie adjacent to each other. [Roberts and Hyman, 1979].

In cells, these microfilaments originate from specific microtubule organising centres such as centromeres or basal bodies, the latter of which organise the formation of cillia and flagella [Heidermann and Kirschner, 1975]. In most cell types, the centromere is the microtubule organising centre, from which microtubules radiate out into the cell periphery. This structure is found near the nucleus as a centriole pair, and has a cylindrical morphology, which is 0.1 μm in diameter by 0.5 μm long, that is generated by a nine fold array of triplet microtubules [Osbourne and Webber, 1976].

Basal bodies are only found in cells that contain cilia and flagella, and are structurally similar to centromeres, and in some organisms, such as

Chlamydomonas, they can migrate to the nucleus where they act as centromeres during mitosis, by organising the spindle. Furthermore, basal bodies injected into *Xenopus* oocytes can induce the formation of mitotic asters [Heidermann and Kirschner, 1975].

1.4.1.5. Intermediate filaments

These are probably the most poorly characterised elements of the cytoskeleton, and appear as gently curving arrays of filaments of some eight to ten nanometers diameter, and are therefore intermediate in size between microtubules and microfilaments [Lazerides, 1980]. They are also particularly prominent in areas of cells that are under stress and therefore require mechanical support, such as tight junctions and desmosomes [Franke et al, 1978] the intermediate filament structure is unclear, but the fibres may be organised in triplets, that are similar to the triple helix structure that is found in collagen, thus accounting for their great tensile strength [Sun et al, 1979].

The proteins themselves include the keratins, neurofilaments and the vimentins; such as vimentin, glial fibrillary acidic protein and desmin [Franke et al, 1978], as a result, they contain a wide range of molecular weights.

1.4.2. The main cytoskeleton associated proteins

The cytoskeleton is not merely composed of arrays of filaments, but composed of different proteins that form interconnecting networks. These networks are usually arranged by cytoskeleton associated proteins which may modulate functions. Tubulin for example is bundled by the microtubule associated proteins (MAPS). These include MAP 1, MAP 2 and the Tau protein [Kim et al, 1979]. The erythrocyte skeleton is also only possible because of the interaction between actin and spectrin [Bennet, 1985].

Spectrin is perhaps the most studied of the cytoskeleton associated proteins, and it exists in many cell types as a dimer composed of two parallel non covalently linked subunits of Mr 240 and 225, which are termed α and β spectrin respectively [Bennett, 1990]. Association of the subunits is via an α-β head to tail linkage. Dimers then form tetramers, which form a cylindrical molecule of approximately 100 nm diameter length [Bennet, 1985].

Actin binding occurs via lateral binding sites, and is enhanced in the presence of Band 4.1a and band 4.1b [Sheetz et al, 1982].

Other cytoskeleton associated proteins, to be discussed below, include talin, α actinin, vimentin and glial fibrillary acidic protein [Otto, 1990].

14.3. Membrane-cytoskeleton and extracellular matrix interactions

The interaction of the cytoskeleton with the plasma membrane is particularly important in terms of tethering the plasma membrane to the cell body, and modification of this interaction by high ionic strength buffers [Tank et al, 1982] or polyamines and polyphosphates [Schindler et al, 1980] disrupt the structure and function of biological membranes. The association of cells with the extracellular matrix, via the cytoskeleton, also allows them to generate the force necessary for locomotion [Alberts et al, 1987]. A short discussion of these interactions will therefore follow.

There are two main mechanisms that can be employed by the cytoskeleton to allow interactions with the plasmalemma. The first is that components of the cytoskeleton can bind directly to phospholipid species present in the membrane [Sankaram and Brophy, 1989]. One such example is spectrin, which binds directly to phosphatidylserine and plays a critical role in the maintainence of cell shape and lipid asymmetry [Haest,1982]. Another important lipid-cytoskeleton interaction is that between glycophorin,

phosphatidylinositol and band 4.1. This requires the specific binding of phosphatidylinositol to band 4.1, which then facilitates the binding to this complex to the cytoplasmic portion of glycophorin A [Sheetz et al, 1982]. This is particularly important as it allows the association of the cytoskeleton with the extracllular matrix. Another important interaction that occurs between the cytoskeleton and the outside of the cell is to be found in the multilaminate myelin sheath; where the cytoskeletally tethered myelin basic protein associates with acidic phospholipid headgroups [Sankaram and Brophy, 1989]. This may be particularly relevant to schistosomes where two closely opposed membranes exist.

Elements of the cytoskeleton can also bind directly to integral membrane proteins. Such a family of proteins has been described, and is characterised by an extensive C terminal α helical domain, which interacts with the F-actin network. There is also a membrane associated N terminal domain. Proteins of this family include Band 4.1, ezrin, radaxin, moesin and talin [Suto et al, 1992]. Some of these proteins such as talin bind to the integrins and vinculin, thereby forming a contact with the extracellular matrix [Otto, 1990].

1.4.4. The regulation of intermolecular associations in the cytoskeleton.

Many of the functions of the cytoskeleton such as cell movement, phagocytosis and shape change require the reorganisation of cytoskeletal components, and this section shall concentrate on some of the mechanisms underlying organisational changes in the cell skeleton.

The dynamics of the cytoskeleton are closely related to signal transduction pathways, and receptor ligation can bring about ultrastructural changes in the cytoskeleton. The neutrophil formyl peptide receptor is an example of a receptor that rearranges the cytoskeleton, by stimulating actin

polymerisation and microfilament reorganisation [Howard and Oresajo, 1985]. This is a process that requires a pertussis toxin sensitive GTP binding-protein and cellular acidification [Molsky and Sha'afi, 1987].

Changes in cytoskeletal organisation are often brought about by proteins that transiently associate with existing filaments. Many of these proteins are sensitive to cell signalling pathways. Examples of this are phospholipase C sensitive interactions that occur between band 4.1 and glycophorin A. This is an interaction that regulates erythrocyte shape, and phospholipase C mediated hydrolysis of phosphatidylinositol lowers the affinity of band 4.1 for glycophorin A, with the result that these two species dissociate and bring about changes in cell shape [Anderson and Marcheti,1984].

Phosphatidylinositol hydrolysis is important in other cell types too. For example, phosphatidylinositol has been implicated in the functioning of α actinin and gelsolin. Both these proteins compete for the same binding site on F-actin [Way et al, 1992], and unlike other actin binding proteins such as fimbrin and filamin which only bind F-actin, Gelsolin can also bind to the monomeric G-actin [Yin, 1988]. Activation of gelsolin comes about by rises in intracellular calcium concentration, that results from Phosphatidylinositol derived inositoltriphosphate. gelsolin binds to F-actin via lateral binding sites, and brings about filament depolymerisation [Bryan, 1988].

These lateral binding sites are common to F-actin binding proteins, such as dystrophin, gelsolin, filamin, Fimbrin, α actinin and spectrin , and are characterised by 255 amino acid domains, that are divided into two 125 amino acid repeats [de Arruda et al, 1990]. The first repeat appears to be the most important for binding to F-actin [Karinish et al, 1990].

The association of some proteins occurs via post translational modifications. One such group of proteins includes the myristoylated alanine rich C-kinase substrates (MARCKS) and the synapsins [De Camilli, 1990]. Both these proteins are found in synaptic vesicles, and have been implicated in

membrane fusion and secretion [De Camilli, 1990]. Both proteins interact with actin, [Rosen et al, 1990], and are cytoskeletal binding proteins, regulated by phosphorylation. Synapsin 1 can also bind spectrin and microtubules [De Camilli, 1990]. However, it is the phosphorylated synapsin and the dephosphorylated myristoylated alanine rich C-kinase substrates that associate with membranes, and they are substrates for different kinases : calcium/calmodulin/ A kinases and C kinases respectively [Rosen et al, 1990]. These proteins also bind to membranes by different mechanisms: the synapsins bind to phospholipids and proteins, whereas the MARKCS bind via an N-terminal myristoylated domain. [Rosen et al, 1990]

1.4.5 5 hydroxytryptamine (serotonin) receptors

Serotonin receptors are usually associated with neuromuscular systems, and are classically subdivided into subtypes based on their ligand binding profiles. To date, four major receptor subtypes denoted 5HT1 through to 5HT4 have been described. The ligands that define the subtypes are: for 5HT1 , sumatriptan, metergoline, 5 carboxyamidotryptamine and metitepine; for 5HT2 ketanserin and cyproheptadine; for 5HT3 2-methyl-5ht, MDC72222 and IC5205930. The latter is also a weak 5HT4 antagonist. 5HT1 is also subdivided into 5HT1A 5HT1B 5HT1C, 5HT1Dα and 5HT1Dβ according to their relative order of inhibition or activation by a defined range of ligands [Hoyer and Milderum, 1989]. There is also very little homology between receptor subtypes at the amino acid sequence, and species level [Hen, 1992].

All of these receptors except the 5HT3 subset (which is an ion channel [Maricq et al, 1991]) function via interactions with G-proteins, and for some subtypes, the interacting G-protein species is known: mammalian receptor types 1A,1B and D, and the *Drosophila* 2A and 2B interact with the pertusis toxin sensitive Gi protein, where as the mammalian 5HT4 and *Drosophila* type 1 receptors interact with the cholera toxin sensitive Gs protein, and mammalian type 2 and 1C receptors couple to the Gq protein [Julius,1991]. Structurally, the G-protein coupled serotonin receptors share some common features, which include seven transmembrane α-helical domains. It is in these domains that most of the inter subtype sequence homology is to be found. These homologous domains are joined by connecting loops that show little homology. The first loop at the N terminus is intracellular. There is also a short cytoplasmic C terminal tail. It is the second and third intracellular domains that are believed to confer G-protein specificity [Jackson, 1990]. Ligand binding occurs on the extracellular side of the receptor, with the area around asn^{351} of transmembrane domain seven being important in the type 1 receptor [Gauss et al, 1992].

1.5. *Schistosoma mansoni.*

1.5.1. Life cycle

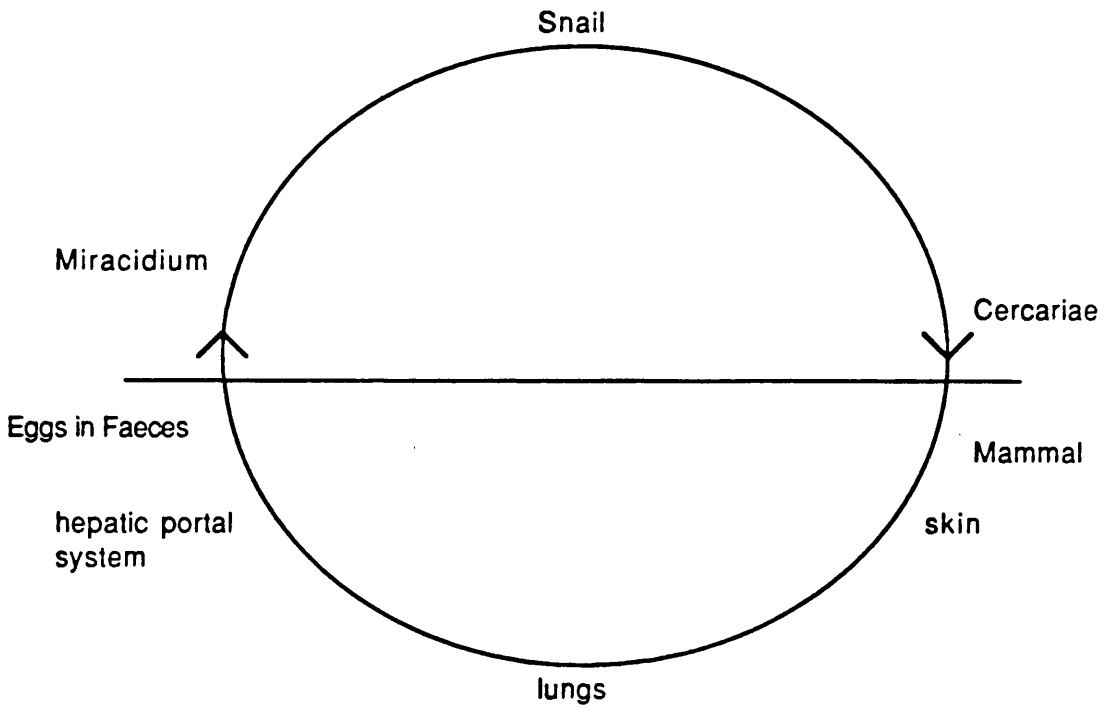
The schistosomes are unusual in their taxonomic group, the Digenea, in that they are dioecious and contain no redial or metacercarial stage. The adults live in copulo in the hepatic portal system of the mamalian host, and the female deposits eggs in the tissues of the gut. Eventually, the eggs make their way to the environment via the faeces, and a larval stage called the miracidium hatches. This will then attempt to locate the intermediate host, which is a freshwater snail of the genus *Biomphalaria*. The miracidium will then penetrate the snail and develop into a sporocyst, which will then produce daughter sporocysts and ultimately cercariae which are liberated into water, which is the larval stage that infects the definitive host, which are primates or rodents. Once this host is contacted, the cercariae penetrate the tegument and become schistosomula which migrate to the lungs by day six post infection, and from here they migrate to the liver by three weeks post infection, then by four weeks they appear in the hepatic portal system, where they grow and become adults [Noble et al 1989]. The life cycle is shown in figure 1.1.

1.5.2. Structure

The major features of schistosome structure have been outlined by Yamaguti (1971), and are as follows:

Adult male: the forebody is short, approximating a cylinder, with the hind body widened throughout, with lateral edges inrolled ventrally to form the gynaecophoric canal. The tegument may or may not contain tubercles, which may or may not posess spines. The testes normally number less than ten and are located anterior to the gynaecophoric canal in one or two rows, with the seminal vesicle pretesticular. There is no cirrus-sac and the genital pore is immediately

Figure 1.1. the life cycle of Schistosoma mansoni



posterior to the ventral sucker.

The adult female is filiform and longer than the male. The ovaries are elongate and occupy the middle third of the body. There is no Laurer's canal and vitelline follicles extend along the entire length of the common caecum. The excretory vesicle is very short and tubular with a terminal pore.

1.5.3. Phylogeny and evolution

Schistosomes are members of the order Digenea, which means they need more than one host species to complete their life cycle. Members of this taxon have two to four hosts, and are usually endoparasitic [Barnes, 1982]. It is believed that the ancestors of this group were free living turbellarians, with the dalyellioid rhabdocoels being particularly favoured, as some of these are ectocommensal on molluscs and echinoderms. Other similarities include the organisation of the reproductive system and the redial stage of the digeneans is similar in structure to the dalyellioid rhabdocoels [Hyman, 1951].

1.5.4. Schistosomiasis

Schistosomiasis is estimated to affect some 200 million people in 75 tropical and subtropical countries, with over ten percent of cases being seriously disabled [Wright, 1972]. Most pathology is due to the deposition of eggs in the host tissues, and and the resulting granuloma produced around them, with the liver and gut being most affected in mansoniasis [Von Lichtenberg, 1987]. Single sex infections or infections where the adult have been chemically sterilised cause only trivial pathology [Warren, 1970]. Millions of eggs are released over the period of infection, where the adults may live in excess of 30 years [Vermund et al, 1983]. Pathology may also be caused by systemic cellular immune responses, such as lymphoreticular proliferation and lesions due to immune complexes [Von Lichtenberg, 1987].

1.5.5. Parasitism: Host resources and problems

The ecological niches that parasites occupy; namely the tissues of other organisms, is not as unusual as it may first seem. It is indeed a rather popular niche as most species have parasites, some of which are specific to a species, and others which are more catholic in their host range [Noble et al, 1989]. The complexities of metazoan structure also leads to the existence of multiple ecological niches in one animal, which ultimately means that one organism can be infected by several different species of parasite. Man for example has parasites specific to the circulatory systems, the stomach, the small intestine, the large intestine and most other tissues [Noble et al, 1989]. Even parasitic species can have parasites of their own [Guilbride et al, 1992]. So, parasitism is not an just an ecological curiosity, but may instead be the most common strategy employed in the animal kingdom for perpetuating genes. Like any animal,there are many problems a parasite has to solve, before it can reproduce, not least being parasites occupy an environment that is actively hostile. Some of these problems are described below for *S. mansoni.*

1.5.6. Location and Infection of the definitive host

It is the cercariae that are responsible for the location and infection of the definitive host, and stimuli such as turbulence, shadow and skin substances all seem to alter cercarial behaviour [Wilson, 1987]. The normal behaviour exhibited by unstimulated cercariae consists of bursts of upward swimming followed by passive sinking [Carter, 1978]. Stimulation increases the length of time spent swimming, but does not induce a tactic response [Wilson, 1987]. Locating the host is therefore a chance event, but the increased swimming activity does increase the probability of host contact [Carter, 1978]. Once contact is made, penetration then ensues, this consists of three stages: (1) attachment; (2)

creeping over the surface and exploring for entry sites; and (3) penetration of the epidermis [Haas and Schmidt, 1982]. The physiological stimulants which induce this are probably free fatty acids on the skin. Linoleic acid for example has been shown to induce emptying of the acetabular glands by a process involving protein kinase C [Samuelson et al, 1991]. This chemoreception is thought to be via putative antero-lateral multicilliated pits [Nuttman, 1971].

1.5.7. The environment of the definitive host

When a cercaria penetrates the mamalian host, it enters an environment that differs in temperature, pH and osmolarity from the water system it came from. The parasite also enters an environment that is a rich source of nutrients and growth factors. In addition, the host also posseses a large repertiore of specific and innate immune effector mechanisms that must be overcome.

1.5.8. Parasite adaptation to its new environment

This discussion will concentrate on the morphological and biochemical adaptations that occur upon the entry of the definitive host; immunological aspects will be discussed in section 1.7.

Upon entry into the definitive host, the first morphological changes to be noticed is the shedding of the cercarial glycocalyx and the synthesis of a double tegumental membrane, which results from the fusion of multilaminate bodies with the surface [McLaren and Hockley, 1977]. This is believed to be an adaptation to a sanguinous environment [McLaren, 1982].

The biochemical and physiological changes that occur upon transformation include an increased sensitivity to water, the ingestion of materials, the uptake of amino acids, a change in the metabolism from energy production to synthesis [Smyth and Halton, 1983]. There is also a change from an aerobic form of respiration involving the Krebs cycle, to a predominantly anaerobic form of metabolism

involving glycolysis, with lactate as the end product [Rumjanek, 1987]. After transformation, there is also an increase in the serotonin activated adenylate cyclase [Estey and Mansour 1987], which will presumably affect energy metabolism. The surface also begins to take up components from the host. Triglycerides and cholesterol are probably taken up by direct interaction of lipoproteins with lipoprotein receptors [Rumjanek and McLaren 1981]. Purines are also taken up [Levy and Read, 1975], as is glucose [Rumjanek, 1987] and amino acids [Cornford, 1985].

1.6.1. The tegumental membrane of the adult male schistosome

The tegumental membrane of *S. mansoni* is a rather interesting organ in that it is the site of interaction between two organisms that are in conflict. On the one hand, there is the mammal, whose interest in this relationship is to maintain the purity and functional integrity of its tissues in order to efficiently utilise its resources to increase its reproductive fitness. On the other hand, the host provides an environment suitable for the parasite to propagate its own genome. These two strategies are in conflict, and as a result, the host has defences to the parasite, and the parasite must combat these defences. This conflict has lead to the evolution of very complex interactions between these organisms, and it is the tegumental membrane that is principally involved in the parasites part of this symbiosis.

1.6.2. Biochemical components

The composition of the adult male schistosome's tegument does not have any strikingly unusual features. All the standard lipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol as well as fatty acids and cholesterol have been reported [McDiarmid et al, 1983]. The standard protein types are present too, such as the integral membrane proteins [Schoemaker et al,

1992], the peripheral membrane proteins and lipid linked proteins such as those containing phosphatidylinositol glycan tails [Havercroft et al, 1990] and myristoylated proteins [Pearce et al, 1991]. The schistosome also inserts molecules; usually glycolipids, of host origin into the membrane [Smithers et al, 1969].

1.6.3. Structure

Schistosomes are unusual in that the adults are surounded by a double outer membrane, a structure which seems to be an adaptation to parasitism in the vertebrate blood stream [Mclaren, 1980]. This double bilayer is secreted shortly after infection [Hockley and McLaren, 1973], and it is continually renewed, by membraneous vesicles fusing at pits on the surface [McLaren, 1980]. The surface of the adult male is an ornately sculptured structure with ridges and pits. there are also tubercles present which contain spines of paracrystaline actin.

Podesta (1987) has on the basis of compositional studies suggested that the inner bilayer is more closely related to the plasmalemma of eukaryotic cells and the outer bilayer is more closely related to the envelopes of bacteria. This view can not be reconciled with the fact that this surface can maintain an electrochemical gradient [Fetterer et al, 1977]. Other problems with this model includes the number of proteins found in the outer membrane; Podesta claims that there are only four; two glycoproteins and two nonglycosylated species. This contradicts the extensive literature on surface antigens reviewed by Simpson (1990). This is also inconsistent with the spatial distribution of the intramembranous particles [McLaren et al, 1978]. McDiarmid and co workers (1983) also claim that their technique of membrane isolation, which utilises saponin, yields inner and outer bilayers with only ten percent cross contamination. This fact coupled with the low abundance of intramembranous particles [McLaren et al, 1978] and the heterogeneity of the membrane, to be discussed below, could explain why

McDiarmid and co workers only found four membrane proteins.

These membranes have been shown to possess some unusual fluid properties; in that the lateral diffusion of fluorescent lipids and lipid analogues, especially those composed of two aliphatic chains, is greatly restricted in the outer monolayer, and shows even greater restriction in inner monolayers [Foley et al, 1986]. It has therefore been suggested that the surface of this parasite may be composed of domains of heterogeneous composition, biochemical, immunological and physical properties [Kusel and Gordon, 1989]. Obviously, if membrane proteins are associating with lipid in an unusual manner, then saponin treatment may not fully dissociate the two membranes, and this may lead to the results of McDiarmid and coworkers.

1.7. Signal transduction and host signals in the parasite life cycle

The tegumental membranes of *Schistosoma mansoni* have the important function of the transmission of information from the environment to the parasite, whether this is by the direct generation of second messengers at the surface [Podesta et al, 1987], or the transport of signalling molecules across the surface [Bennett and Bueding, 1973]. Various mammalian host signals such as glucagon, serotonin, histamine, melanocyte stimulating hormone and the prostaglandins E1, E2 and B1 are capable of activating adenylate cyclase in various tissues of *S. mansoni* [Higashi et al, 1973]. The complement component C3b has been implicated in the regulation of membrane synthesis via a calcium dependent pathway [Zhou and Podesta, 1989]

Serotonin is an example of a ligand which stimulates adenylate cyclase, thus raising cyclic AMP concentration in the tegument. This activation involves a GTP-binding protein that couples the occupancy of a type 1 like receptor to the activation of adenylate cyclase [Estey and Mansour, 1987]. This serotonin activated adenylate cyclase activity is developmentally regulated, and rises to a

maximum value in schistosomula at around day five post transformation [Kasschau and mansour, 1982]. It has also been reported in adult males, that concomitant with elevation in cyclic AMP concentration, there is a reduction in the ^{45}Ca flux across the membrane [Podesta et al, 1987].

Recently, Tumor Necrosis Factor alpha has been implicated in stimulating oviposition by the female [Amiri et al, 1992], and transferrin has been shown to play a role in early schistosomular development [Clemens and Basch, 1989]. The G-protein mediated hydrolysis of phosphatidylinositol has also been reported in *S.mansoni*, but no stimulatory ligands have been identified [Wiest et al, 1992].

There is evidence for both A and C kinases: the A kinases appear to be predominately concerned with metabolic regulation [Mansour 1982], and the C kinases appear to be responsible for the induction of developmental processes [Samuelson et al, 1991]. In addition, there is also some poor evidence for the existence of tyrosine kinases, since there is a 25 Kd protein with the capacity to autophosphorylate [Podesta, 1987], and recently a DNA sequence showing homology to the human and *Drosophila* epidermal growth factor receptor, containing a tyrosine kinase domain has been identified [Shoemaker et al, 1992].

1.8. The role of the tegumental membrane in immune evasion

This section shall concentrate on the role that the tegumental membrane plays in evading the mammalian immune response, and will not concentrate on non membrane dependent processes. The most obvious adaptation of the parasite surface is its double bilayer structure, which could provide resistance to immune attack [McLaren, 1980]. This is a structure that is capable of renewal, ensuring damaged membrane does not accumulate. The membrane is renewed with a half life of three hours [Kusel and Mackenzie, 1975; Wilson and Barnes,

1977]. It has however been suggested elsewhere [Podesta, 1987], that the inner and outer membranes turn over at different rates, with respective half lives of 6 and 3 hours. It has also been suggested that the two bilayers have different origins: the outer is formed by the fusion of multilamellar bodies with the membrane, and the inner membrane is synthesised by fusion of discoid bodies with the tegument [McDiarmid et al, 1982]. Membrane turnover can also be increased by the host factors serotonin and C3b [Zhou and Podesta, 1989]. The surface is also capable of adsorbing host molecules on to its surface [Smithers et al, 1969]. This covering is laid down soon after infection, and is relatively dense by day four post-infection. [McLaren et al, 1973]. This covering also inhibits the binding of specific host antibody to parasite antigens [Harnett et al, 1985]. This covering cannot be the only source of resistance, in lung worns at least, because haptenated parasites show resistance to immune killing [Mosser et al, 1979]. This led to the idea that the membrane in this stage has an intrinsic resistance to host effector mechanisms. The presence of glycosaminoglycans on the parasite surface may inhibit the formation of the membrane attack complex [Tschopp and Masson, 1987], as will LDL receptors, by homology with the C9 aggregation sites [Rumjanek et al, 1983 ; Stanley et al, 1985]. The surface can also respond to immunological insult by increasing the activity of ion pumps in the membrane [Pearce et al, 1986]. This will prevent the breakdown of the surface electrochemical gradient [Breternitz et al, 1991].

Of a more speculative nature, lysophosphatidylcholine secreted by the parasite may affect the functioning of host effector cells [Caulfield et al, 1986], as may the biophysical properties of the surface. The physical organisation of the surface may prevent divalent antibody binding, and space immunoglobulins at such a distance as to prevent the classical activation of complement [Oi et al 1984]. The immobile nature of surface lipid [Foley, 1986], may also be expected to inhibit complement fixation, which requires fluid lipid [Shin, 1979]. Some indirect evidence for this is presented by Breternitz and co

workers (1992), who show that membrane properties may affect the size and the half-life of pores formed by the action of *Pseudomonas aeruginosa* cytotoxin on the surface. The hydrophobic nature of the male dorsal surface has also been implicated in preventing the adhesion of effector cells [Podesta et al, 1987]. Finally, the existence of an extracellular oligosaccharide matrix, which is capable of retarding the diffusion of molecules next to the surface has been proposed [Kusel and Gordon, 1989]. This could perhaps retard the diffusion of molecules with short half lives, such as activated C4 ($T_{1/2}$= 60 μsec) [Alberts et al, 1983]. This could also inhbit complement formation.

1.9. Aims of the project

The surface membrane of adult male *Schistosoma mansoni* has some unusual fluid properties (section 1.5.3.), and this project was aimed at understanding this phenomenon in molecular terms and to understand the adaptive significance of immobile lipid in the parasite membrane.

The strategy employed was to attempt to manipulate membrane fluidity physiologically and, by characterising the changes that occur to gain an insight into the components of the membrane that are involved in this unusual organisation of lipid. Changing membrane fluidity could then be exploited to identify membrane functions that are affected by fluidity.

Chapter 2

Materials and Methods

2.1 Chemicals

Reagents:5'N-Octadecanoyl aminofluorescein and the acetoxymethyl esters of Fluo-3 and Diazo-2 were purchased from Molecular Probes Inc, Eugene, Oregon, USA, as were the 7-nitrobenz-2-oxa-1,3-didiazol-4yl derivatives of phosphatidylcholine and phosphatidylethanolamine. The acetoxymethyl esters of adenosine-3',5'-cyclic monophosphate,P-1-(2-nitrophenyl)ethyl ester (caged cAMP) and NITR 5 were from Calbiochem, Nottingham (UK), Sulpho NHS biotin and Triton X-100 were from Pierce Chester (UK). Antisera and conjugates were from Sigma, Poole (UK) or the Scottish Antibody Production Unit, Law Hospital, Carluke. All other reagents were from Sigma, Poole (UK) and Aldrich, Gillingham (UK) and were of AR grade.

2.1.1 Buffers

The following buffers were used in this study:

Biotinylation buffer: 1.9 mM Ca Cl_2 : $2H_2O$, 5.4mM K Cl, 1.7 mM Mg SO_4 :$7H_2O$, 100 mM Na Cl, 5 mM D-glucose and 3.3 mM carbonate buffer pH 8.0.

Crosslinking buffer: 1mM Mg Cl_2, 100mM D-glucose and 50 mM triethanolamine pH8.

Horse radish peroxidase substrate buffer:0.02 % luminol (w/v), 0.015M Na Cl, 2mg 4'Iodophenol, 0.015M tris pH8 and 0.0065 % (v/v) 100 volsH_2O_2, or 0.03 % Diaminobenzidine and 0.01% (v/v) 100 vols H_2O_2, dissolved in PBS pH 7.4.

SDS Page sample buffer: 0.1M tris, 2% SDS (w/v), 0.05% bromophenol blue, pH 6.8.

2.2 Parasite maintenance

The life cycle of *S. mansoni* (Puerto-Rican strain) is maintained in the departments of Zoology and Biochemistry, University of Glasgow. The parasites and their intermediate hosts (*Biomphalaria glabrata*) were originally obtained from the National Institute of Medical Research Mill Hill. There have also been several introductions of material from the University of York. Adult *S. mansoni* were obtained from BALB/c mice by portal perfusion, [Smithers and Terry, 1965] using Glasgow modification of Eagles Medium and 5% Foetal calf serum at 37^0C as perfusate.

2.3 Fluorescent techniques

2.3.1 Labelling of the surface membrane of *S. mansoni*

2.3.1.1 Lipids

The lipid phase of the membrane was labelled by incubating adult male *S.mansoni* with either 10 µl of a 1 % solution of 5 AF for 10 minutes, or with fluorescent lipids in the form of liposomes. The lipids treated this way were NBD PC and NBD PE, which were made into liposomes by mixing 1mg of them with 10 mg of unlabelled lipid of the same species. The mixtures were dissolved in 100 µl of ethanol, which was then added slowly to 1ml PBS at 50^oC and swirled; this allowed the ethanol to evaporate. 10 µl of this suspension were added per ml of GMEM. After labelling, the worms were washed 3 times in GMEM.

2.3.1.2 Labelling of proteins in the parasite surface

Attempts were made to label surface proteins with FITC at pH8 using 0.1M carbonate buffer and 0.05 M D- glucose. FITC Maleimide conjugated dextrans were also used under the same conditions.

2.3.2.1 Purification of anti BALB/c erythrocyte IgG from rabbit serum

IgG was prepared from the serum of a NZW rabbit immunised against BALB/c erythrocytes by the method described in Johnstone and Thorpe (1982).

2.3.2.2 Preparation of Fab fragments from anti BALB/c erythrocyte IgG

Purified anti BALB/c erythrocyte IgG was made into Fab fragments by the method described in Johnstone and Thorpe (1982).

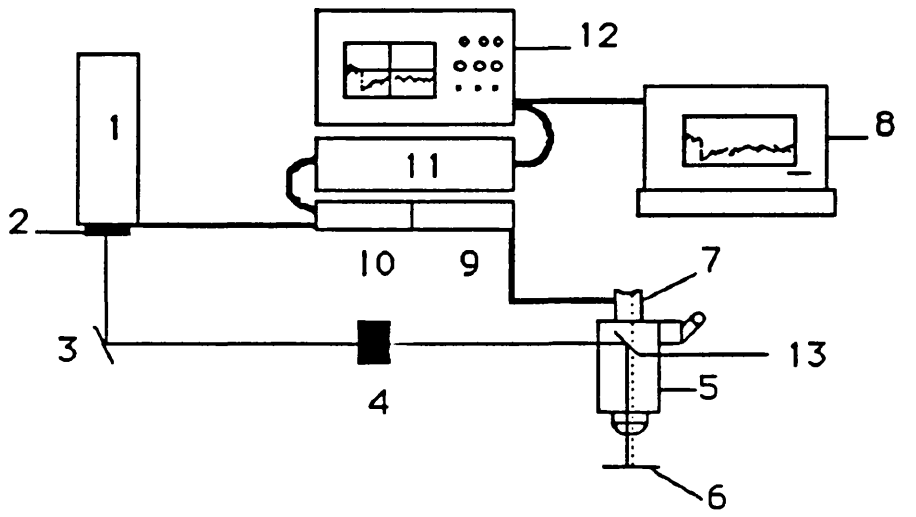
2.3.3 Fluorescent Resonance Energy Transfer

The depth of probe insertion can be determined using the technique of Fluorescent Resonance Energy Transfer (FRET). The membrane impermeant molecule trypan blue is used to as a tool to measure the depth of probe insertion in the membrane. This technique relies on the ability of 0.25% (w/v) trypan blue to absorb the emitted radiation from fluorescein and NBD based probes. This quenching relies on an energy donor, the fluorophore,having an emission spectrum that overlaps the absorption spectrum of the acceptor, trypan blue, and the efficiency of energy transfer is distance dependent [Stryer 1979].

2.3.4. Fluorescence Recovery After Photobleaching (FRAP)

The apparatus used is shown in diagrammatic form in figure 2.1. During a FRAP experiment, light is deflected from an argon ion laser (number 1, figure 2.1) to the spatial filter (number 4, figure 2.1). This consists of a 5 μm diameter pin hole and a X40 microscope objective, which functions to remove higher spatial frequencies from the Gaussian beam profile, and improves the contrast ratio of the modulated beam approximately 25 fold. From here, the beam is directed through a microscope (number 5, figure 2.1) on to a sample from which emitted

Figure 2.1
Fluorescence recovery after photobleaching apparatus



1 1.0 W Argon ion laser (lexel model 85)

2 Acousto-optic modulator (model 304D, Coherent Associates, Conneticut)

3 Adjustable mirror (Oriel model 1450)

4 Spatial filter (Photon Control, Cambridge)

5 Ortholux 2 microscope (Leitz)

6 Heating stage (Leitz)

7 Photomultiplier Tube (Thorn E.M.I.model 9924b)

8 Personal computer (Hewlett Packard model 82927A)

9 Photomultiplier tube powerpack (Brandenburg)

10 Acousto- optic modulator powerpack (Coherent Assosciates Danbury Conneticut)

11 FRAP laser controller GUED 729 (Glasgow University Electronics Department)

12 Digital storage oscilloscope (Gould type 4035)

13 Dichroic mirror (Leitz)

fluorescence is focussed on to the photomultiplier tube (number 7, figure 2.1). From here, the signal is sent to the oscilloscope (number 12, figure 2.1). For a FRAP experiment, the output intensity of the laser must be kept constant before and after the bleach., a task which is performed by the Acousto-Optic Modulator (AOM) (number 10, figure2.1). The AOM attenuates the average intensity of the laser beam by varying the on-off duty cycle. The monitoring beam is attenuated 10000 times, which means the beam is delivered as 10 μs pulses at 10 Hz, whereas the bleaching pulse is generated by an uninterrupted pulse of 10-100 μs [Garland, 1981]. After bleaching, the trace is stored on the computer (number 8, figure 2.1) , and the data fitted by a Yguarabide fitting program.

The recovery of fluorescence is due to the diffusion of bleached molecules out of the experimental area, and the diffusion of unbleached molecules into this area [Axelrod et al 1976]. This gives us two values of fluidity, the D_L value, which is the average speed of diffusion, and the mobile fraction, which is a measure of the percentage of molecules that are free to diffuse. The D_L value is calculated from the equation of Axelrod et al 1976:

$$D_L = (w^2/4t^{0.5})y \qquad (1)$$

Where w is the bleaching area at $1/e^2$ intensity, $t^{0.5}$ is the half time of fluorescence recovery, and y is a constant that depends on the beam profile and the bleach parameter K, which depends on the % bleach. This requires the accurate determination of the beam radius and for this purpose, the method of [Thompson et al 1980] was used. This method involves the photobleaching of a thin film of FITC conjugated bovine serum albumin in 95 % glycerol, and the spot size was calculated using data from the recovery kinetics. From this, the spot size was determined to be 1 μm in diameter.

2.3.4.1 Practical considerations of FRAP

When performing a FRAP experiment, the question of dye sensitized photodamage

must be addressed [Sheetz and Koppel 1979]. Experiments have been performed where the effect of repeated photobleaching was investigated [Jacobson et al 1978], and no damage was reported. Foley (1986), has performed similar experiments on *S.mansoni*, and also detected no damage. More convincing evidence was presented by Koppel and Sheetz (1981), where the D_L values of dichlorotriazinylaminofluorescein labelled erythrocytes were compared using the techniques of Fluorescence Recovery after Photobleaching and Fluorescence Redistribution after Fusion. The data obtained showed no difference in the observed D_L values.

2.3.4.2. Heating

The problem of heating during photobleaching was addressed by Axelrod (1977), who calculated the temperature increase produced by illumination by a beam of Gaussian profile. This was found to be a function of time after the start of illumination. It was shown that the rapid conduction of heat away from the illuminated spot in the intra and extracellular aqueous medium leads to a thermal steady state in a time much shorter than characteristic bleaching times. The steady state temperature increase has the maximal value

$$T_S = (\pi/2)^{1/2} q w/4K \qquad (2)$$

in the centre of the beam, where q is the rate of heat production per unit area in the centre of the beam, w is the $1/e^2$ radius of the Gaussian laser profile in the plane of the membrane, and K is the thermal diffusivity of the medium. For conditions typical of protein measurements, T_S is less than 0.03°C. There appears to be no photo-induced damage when one measures the diffusion of lipids in the schistosome surface, as repeated photobleaching does not change the fluidity of membranes [Foley, 1986].

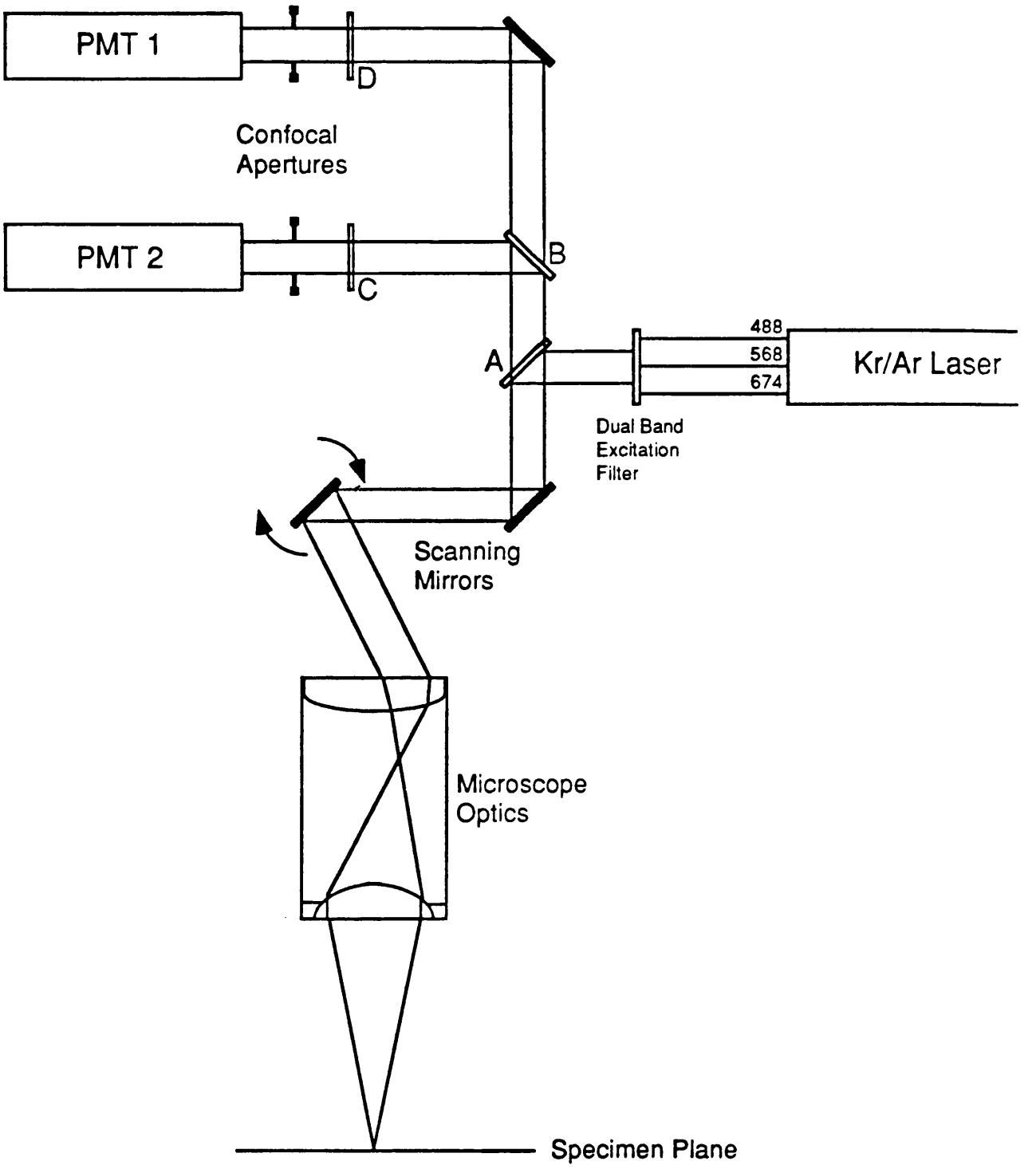
For FRAP studies, worms were immobilised in GMEM containing 25 mg/ml carbamoyl choline.

2.3.5. Confocal microscopy

In conventional fluorescence microscopy, the sample is illuminated and emitted fluorescent light from the entire specimen is viewed through the eyepiece. The outstanding feature of confocal laser fluorescence microscopy is that both illumination and fluorescence detection are confined to a very small spot within the specimen. The lateral xy resolution of this spot can approach the limits of diffraction, but the main advantage of confocal microscopy is that the image is gathered exclusively from a narrow optical section of the specimen in the z dimension. Regions above or below the "focal plane" appear black and do not contribute to the image.

The principle of confocal microscopy was originally described in 1957 [Minsky, 1957], and the emergence of today's powerful confocal laser fluorescence microscopes is a direct result of the development of laser technology and the increasing sophistication of computer and electronic equipment [BioRad, 1992].

The epifluorescence optical design of the MRC 600 Confocal Microscope is shown in figure 2.2. The excitation wavelength(s) from the Kr/Ar laser are selected by means of exchangeable excitation filters on a filter wheel. The light beam is then reflected by a dual dichroic mirror (A) onto the two oscillating mirrors which scan the coherent light in the x and y directions. As the light beam passes through the microscope optics, it is focussed to a scanning point on the specimen plane. Fluorescent (or reflected) light follows precisely the reverse pathway through the microscope optics and the scanning unit (epifluorescence microscopy) up to the dual dichroic mirror. This will transmit the fluorescent light to a second dichroic mirror and in turn through selected filters to either one of the two photomultiplier tubes. The variable confocal apertures can be used to increase the signal intensity, albeit at the expense of a thicker optical section in the z dimension. A number of other electronic and computational means of enhancing the scanned image are available. Their application is discussed at length in the



A = K1 Double Dichroic Mirror (transmits ≈ 500-550 & >590 nm; reflects 488 and 568 nm lines)
B = K2 Dichroic Mirror 560 LP (transmits >560 nm; reflects < 560 nm)
C = 522 DF 32 Filter (transmits ≈ 510 - 550 nm)
D = 585 EF LP Filter (transmits > 585 nm)

instrument manual [BioRad, 1992].

2.3.6. Fluorescence quantitation

Quantitation was performed using a Leitz MPV compact, consisting of a photomultiplier tube and a Leitz steady current power pack, which kept the voltage constant.

During quantitation, at least three background readings were taken which were then used to correct fluorescence values from the sample, which were taken from a fixed photon collecting area on the microscope slide.

2.3.7. Calcium determination

The concentration of free calcium ions can be determined using a variety of probes whose spectral properties change upon the binding of free calcium ions [Haughland, 1992]. Fura-2 is the probe of choice, as it can be ratio immaged [Grynkiewicz et al 1985]. For this study, the behaviour of the tegumental syncytium is important. However, *S. mansoni*, is multicellular, and other tissues will take up the probe and some other probe must be used. The fluorescent probe Fluo-3 is the probe that was determined to be most suitable for the study as it can be excited by the 488 nm line of the FRAP apparatus, and take advantage of the apparatus' narrow depth of focus [Wolf, 1989]. There are also other advantages of this probe, such as its high quantum yield and its low buffering ability [Eberhard and Erne, 1989]. Labelling of the worms was with the membrane permeant acetoxymethyl ester.

2.4. Flash photolysis

Flash photolysis of the compounds NITR 5, Diazo-2 and caged cAMP was by brief exposure, about 1 second, to light of 340 nm, from a mercury vapor lamp. This results in the intracellular release of calcium (from NITR 5), a calcium buffer

which chelates intracellular calcium (from Diazo-2) and cyclic AMP (from caged cAMP) . It has been shown by Foley (1986) that repeated photobleaching does not alter the fluid properties of the tegument . This means that fluidity can be monitored on the same spot of membrane over time.

2.5. Membrane Isolation and cytoskeleton preparation

Membranes were removed by freezing and thawing [Roberts et al, 1983]. After removal, the membranes were solubilised by 0.5% Triton X-100 (v/v) in 50 mM Tris NaCl,5mM EDTA, pH 8 , containing 50 μM sodium metavanadate, 10mM phenylmethylsulphonylfluoride , 6μM Na-p-Tosyl-L-Lysine chloromethyl ketone, 2μM N-Tosyl-L-phenylalanine chloromethyl ketone and 2μM Pepstatin A. After 30 min at 4 ^{0}C, the samples were separated into soluble and pellet fractions, by a 20 min centrifugation (60000 g min). The pellet was washed 3 times in the above buffer, and then solubilised in 0.7 % Triton X-100 (v/v) and 2% SDS (w/v).

2.6. Biotinylation

The labelling of surface proteins with biotin was performed by the addition of 0.32 mg of the sulpho N-hydroxysuccinimide ester per ml of biotinylation buffer (1.9 mM Ca Cl_2 : $2H_2O$, 5.4mM K Cl, 1.7 mM Mg SO_4 :$7H_2O$, 100 mM Na Cl, 5 mM D-glucose and 3.3 mM carbonate buffer pH 8.0) to intact worms at room temperature for half an hour.

2.7. Crosslinking

The chemical cross linkers Dimethyl 3,3'-dithiobispropionimidate. 2HCl (DTBP), which is membrane permeant, and the impermeant 3,3' Dithiobis (sulphosuccinimidylpropionate) (DTSSP) were used to study protein nearest neighbour interactions. Intact worms or membranes were incubated in 1mM Mg Cl_2, 100mM D-glucose and 50 mM triethanolamine pH8, to which crosslinker

was added to a final concentration of 0.5% (w/v) [Wang and Richards, 1974]. The incubation took place at 4°C for 1 hour. The crosslinker was renewed twice during the incubation, and the reaction was stopped by the addition of 50µl of 1M ammonium ethanoate.

2.8. Electrophoretic techniques

2.8.1. One dimensional SDS Polyacrylamide gel electrophoresis

One dimensional Polyacrylamide gel electrophoresis was performed according to the method of Laemli (1977), using the BioRad miniprotean II system. Gels were run for approximately 90 min at 35 mA per gel; the running conditions were set using a Pharmacia EPS 400/500 electrophoresis power supply.

2.8.2. Two dimensional SDS Polyacrylamide gel electrophoresis

After the first dimension, the gel was disassembled, and the relevant areas were cut out, and boiled in equilibration buffer (0.1M tris, 2% SDS (w/v), 0.05%, (w/v) pH 6.8) for 10 min. The sample was then placed on top of another slab gel and run as described above. For reducing conditions, 0.02% v/v ß-mercaptoethanol was added to the equilibration buffer.

2.8.3. Western blotting

The procedure of [Towbin et al 1979] was used for transferring proteins to nitrocellulose. The BioRad mini trans blot system. Blots were developed using horse radish peroxidase conjugates. Substrates consisted of 0.02 % luminol (w/v), 0.015M Na Cl, 2mg 4'Iodophenol, 0.015M tris pH8 and 0.0065 % (v/v) 100 vols H_2O_2 where sensitivity was needed, or 0.03 % Diaminobenzidine and 0.01% (v/v) 100 vols H_2O_2, dissolved in PBS pH 7.4.

2.8.3.1. Antibodies

A murine IgM monoclonal antibody specific for chichen gizzard actin was purchaced from Amersham UK, and used at a titre of 1:500.

Horse radish peroxidase and fluorescein isothiocyanate conjugated antibodies specific to mouse IgM and rabbit IgG wereobtained from the Scottish Antibody Production Unit, Law hospital, carluke, and were used at a titre of 1:500.

2.9. Protein determination

Since the determination of protein concentrations in samples containing detergents was necessary, the Pierce micro BCA kit was used through out. Samples containg Tris buffers, were dialysed for 90 minutes against Phosphate Buffered Saline using the Pierce microdialysis kit [Pierce and Warriner , 1992].

Chapter 3

General results: the labeling of the tegument.

3.1. Labelling of the surface

In order to study the surface by FRAP, it is first necessary to label it. For a probe to be useful, it must satisfy certain criteria: it must insert into the membrane, and not label the cytoplasm; ligands must be specific for the receptor of interest; lipid probes must insert in the correct manner and not attach to the surface in the form of micelles; the probe must not cause any damage to the membrane, and lipid probes must constitute less than one percent of the total lipid. All probes must also show first order bleaching kinetics and fluorescence must not recover in the absence of diffusion [Wolf, 1989]. The lipid dyes used would on energetic grounds insert as a lipid [Axelrod, 1979]; the 5AF dye would also not fluoresce if it was attached to the surface in the form of micelles [Gordon, 1989]. The depth of probe insertion can also be assessed by trypan blue quenching (FRET), where only surface fluorescence is quenched.

It is the aim of the experiments in this chapter to identify probes which satisfy the above criteria in order to use them as probes for membrane structure.

3.1.1. The Lipid phase

The lipid probes chosen for this study were NBD PC, NBD PE and 5AF. It was found that the best labelling for the outer monolayer with 5AF was a ten minute incubation in a 0.1% solution of the probe. For the NBD probes sufficient labelling was obtained using 10µl (0.1% final concentration) of the micellar suspension and a ten minute incubation. The NBD PE shows translocation to the inner leaflets. In all cases, the contribution of background noise ratio was negligible. The data is shown in table 3.1.

Table 3.1: labelling with lipid analogues.

The depth of insertion of lipid probes and their signal/ noise ratio was determined by quantitative fluorescence microscopy, using a X40 objective. 0.25% (w/v) trypan blue was used to quench surface fluorescence.

Worms were immobilised in GMEM containing 25 mg/ml carbamoyl choline, and readings were performed on 25 worms, before and after trypan blue treatment.

Table 3.1: labelling with lipid analogues.

Probe	concentration	incubation time	% quenching	signal/noise ratio
5AF	0.5%	10 min.	60.2	15.5
5AF	0.1%	10 min.	89.8	16.9
5AF	0.05%	10 min.	85.4	14.3
NBD PC	0.5%	10 min.	71.4	5.4
NBD PC	0.1%	10 min.	91.2	5.8
NBD PC	0.05%	10 min.	88.2	4.2
NBD PE	0.5%	10 min.	61.6	3.6
NBD PE	0.1%	10 min.	75.3	3.2
NBD PE	0.05%	10 min.	77.1	4.1

3.1.2. Labeling of membrane proteins

In the past, workers have used fluorescently labeled lectins and antibodies to measure the diffusion of proteins in the surface of parasites [Foley, 1986] and [Kusel and Gordon 1989]. Such an approach is unsuitable for two reasons: it is likely that the lectin binds to glycolipids in addition to glycoproteins, and neither of these probes are monovalent. Labelling with multivalent ligands can cause artifacts ranging from simple crosslinking of receptors [Wolf, 1977] to a global surface modulation of areas of the membrane not even in contact with the ligand [Edelman, 1976]. Divalent antibodies have also been shown to diffuse slower than their monovalent Fab fragments [Edidin and Famborogh, 1973]. Others, [Schlessinger et al 1977], have demonstrated no difference upon Fabulation, which presumably reflects the structure of the membrane around the antigen. It was therefore necessary to assess the value of other probes for use in protein diffusion studies.

3.1.3. Fluorescein isothiocyanate (FITC)

An attempt to covalently label the outer tegumental membrane proteins using fluorescein isothiocyanate was made, and its depth of insertion was assessed. Table 3.2 shows that good levels of labelling can be achieved but the low quenching by trypan blue indicates that the FITC is very membrane permeant and very low concentrations can permeate the membrane after 10 sec. This probe is therefore unsuitable for the measurement of surface protein lateral mobility.

3.1.4. Maleimide containing probes

Proteins containing accessible sulphydryl groups can be covalently modified by maleimide containing probes. The use of these probes have the added advantage that

Table 3.2: labelling of membrane proteins with FITC.

The depth of insertion of FITC, and the signal/ noise ratio was determined by quantitative fluorescence microscopy, using a X40 objective. 0.25% (w/v) trypan blue was used to quench surface fluorescence, and conjugation with FITC was carried out at pH8 using 0.1M carbonate buffer and 0.5 M D- glucose.

Worms were immobilised in GMEM containing 25 mg/ml carbamoyl choline, and readings were performed on 25 worms, before and after trypan blue treatment.

Table 3.2: surface labelling with FITC.

Probe	incubation	% quenching	signal/noise
0.1% FITC	10min	8.4	2.6
0.1% FITC	1min	15.4	3.4
0.01% FITC	1min	15.9	2.8
0.01% FITC	0.5 min	20.5	3.1

radiolabelled maleimide is available with high specific activity and the proteins being studied by FRAP could be identified by SDS PAGE followed by autoradiography [Connor and Schroit, 1990].

Table 3.3 shows the intensity of labelling of the surface (which is low) and the depth of probe insertion for a variety of maleimide probes. All these probes, regardless of size or charge appear to penetrate the outer monolayer. The concentrations of maleimide seemed to damage the surface, as did concentrations as low as 0.1 μM, which caused blebbing and occasional strips of membrane to fall off (figure 3.1).

3.1.5. Host antigens

FITC Fab fragments were made from Ig G purified from a rabbit antiserum raised against murine (BALB/c) erythrocytes. This would recognise the host antigens on the schistosome surface (section 1.7) and allow their diffusion to be studied. Unfortunately, figure 3.2 shows that the antiserum from which the Fab fragments were produced were unable to inhibit Fab binding. Fab binding is therefore non specific and these fragments are therefore unsuitable as a probe. Serum also tends to increase the amount of probe bound, which may reflect a change in the worms' motility (figure 3.3).

3.2. Conclusions and discussion

All attempts to label membrane proteins were unsuccessful, as the probes did not restrict themselves to the inner monolayers, and some probes such as the maleimide containing probes proved to be very damaging. How this damage occurs is unknown, but one possible explanation is that the probe is reacting with structural components that are involved in tethering the membrane to the worm, and thereby causing membrane release. Radioactive maleimides could when used with SDS PAGE, identify such putative tethering proteins.

Table 3.3: labelling of membrane proteins with FITC maleimides.

The depth of insertion of FITC maleimides, and the signal/noise ratio was determined by quantitative fluorescence microscopy, using a X40 objective. 0.25% (w/v) trypan blue was used to quench surface fluorescence, and conjugation with maleimide was carried out at pH8 using 0.1M carbonate buffer and 0.5 M D- glucose.

Worms were immobilised in GMEM containing 25 mg/ml carbamoyl choline, and readings were performed on 25 worms, before and after trypan blue treatment.

Table 3.3: labelling membrane proteins with maleimide containing probes.

Probe	incubation	% quenching	signal/noise
0.1% FITC maleimide	1Hr	2.6	2.1
0.1% FITC maleimide dextran cationic (Mr 50Kd)	1Hr	12.4	2.9
0.1% FITC maleimide dextran cationic (Mr 75Kd)	1Hr	19.8	1.4
0.1% FITC maleimide dextran anionic (Mr 50Kd)	1Hr	15.9	1.7
0.1% FITC maleimide dextran anionic (Mr 75Kd)	1Hr	17.5	2.1

Figure 3.1. The effects of N-Ethylmaleimide on membrane integrity.

The effect of N-ethylmaleimide on the integrity of the adult males' tegument was assessed using the membrane impermeant probe Hoescht 333282. Adult worms (25 per group)were incubated for 1hour, in medium containing various concentrations of NEM. After three washings in medium, there was a 10 minute incubation in Hoescht 333282 (1mg/ml in GMEM). Excess dye was washed away, and fluorescence was quantitated by fluorescence microscopy.

The data shows the mean $\pm$ 1 S.D. of triplicate experiments.

Figure 3.1.

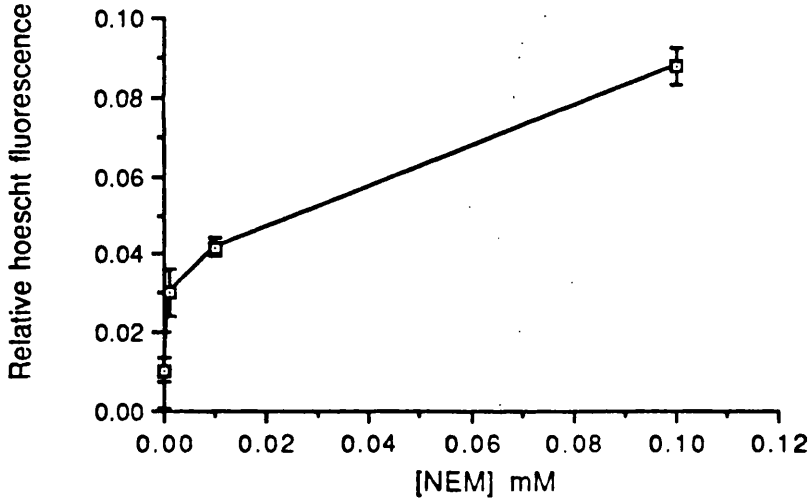

Relative hoescht fluorescence
0.10
0.08
0.06
0.04
0.02
0.00
0.00
0.02
0.04
0.06
0.08
0.10
0.12
[NEM] mM

Figure 3.2. The specificity of anti BALB/c erythrocyte Fab Fragments.

Anti BALB/c erythrocyte Fab Fragments were tested for their specificity of interaction with the male tegument by immunofluorescence microscopy.

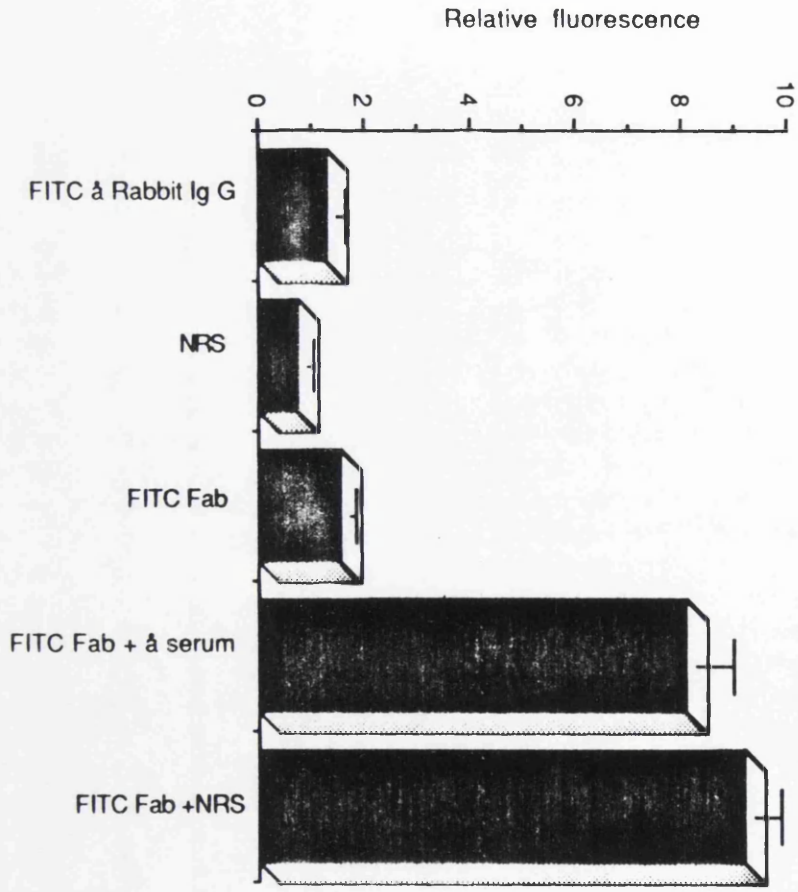
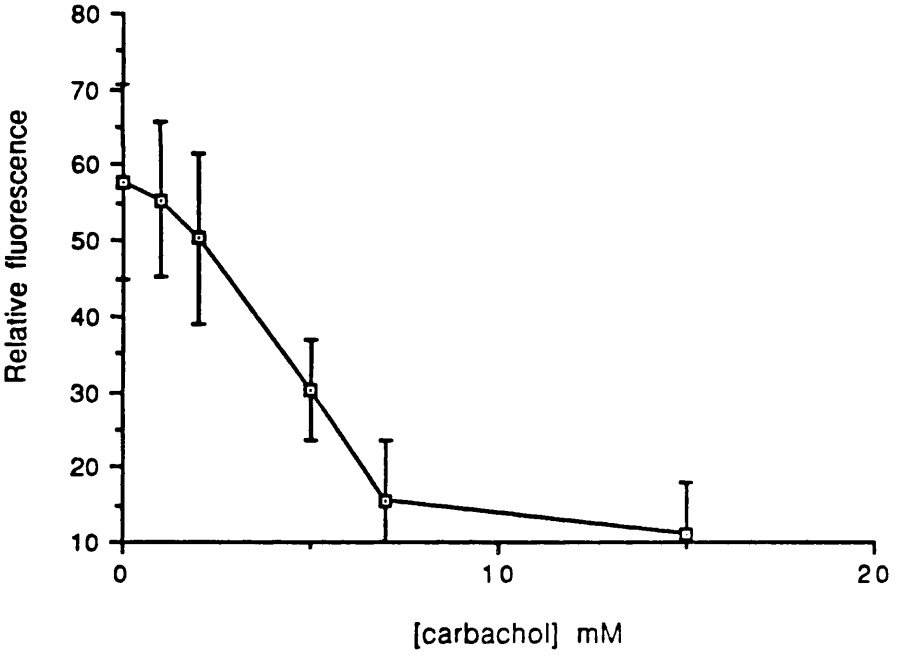

Relative fluorescence
0
2
4
6
8
10
FITC å Rabbit Ig G
NRS
FITC Fab
FITC Fab + å serum
FITC Fab +NRS

Figure 3.3. The effect of worm motility on 5AF uptake

Adult worms were labeled with 10μg ml^{-1} 5AF in the presence of various concentrations of the paralysing agent carbachol. The uptake of 5AF was then quantitated by fluorescence microscopy.

Figure 3.3. The effect of worm motility on 5AF uptake



Host derived antigens in the membrane could not be studied because the production of a host antigen specific fluorescent Fab fragment was not possible. A possible reason could be that the antigen binding site was modified during conjugation. Non specific binding was also increased when serum was added to the worms (figure 3.2.), presumably due to increased motility. This therefore suggests that serum contains factors that stimulate worm motility. Such factors could be host hormones, complement components or cytokines.

The lipid probes 5AF and NBD PC were found to be suitable for studying the fluid properties of membranes, as they could be introduced into membrane in such a way that they remained in the outer monolayer. The fluorescent lipid NBD PE was shown to translocate to the inner layers of the tegumental membranes, and therefore have potential for lipid translocational studies (section 4.1.2.) or fluorescence studies on the inner monolayers.

In mammalian cells, aminophospholipid translocation occurs through the activity of specific translocases [Connor and Schroit 1990], which can be inhibited by N-ethyl maleimide [Seigneurt and Devaux, 1984]. The translocation of NBD PE therefore suggests that such molecules exist in schistosomes. These molecules would be of value in terms of understanding the basic biology of the schistosome (nutrient uptake). They could also be of value as a chemotherapuetic or immunological target, especially if they are the molecules involved in tethering the membrane to the worm.

Chapter 4: The regulation of membrane fluidity

4.1. The regulation of membrane fluidity

In this chapter, the effect of serotonin on the structure of male dorsal membrane was studied, and the changes that occur in the membrane was also studied in an attempt to identify the membrane components responsible for its structure.

4.1.1. The effect of serotonin on fluidity

Using the FRAP technique, the effect of various ligands on the number of lipid molecules free to diffuse mobile fraction, (f), and their average speed of diffusion (D_L) was assessed on the outer monolayer of the male's dorsal surface. Table 4.1 demonstrates that 1μM serotonin is capable of reducing both the D_L value and the mobile fraction ; that is fewer molecules move, and their average rate of diffusion is reduced. The serotonin type 1 receptor antagonist methiotheipin blocked the effect of serotonin on fluidity. When the G-protein activator fluoride was used, the same effect as serotonin was observed on fluidity. The diterpene forskolin (an adenylate cyclase activator [Seamon and Daly, 1981]) however, only reduced the mobile fraction while raising the D_L value; the inactive derivative dideoxyforskolin had no effect. In contrast no effect of serotonin was observed on the two parameters of fluidity using NBD PC as a probe.The values of the fluidity parameters obtained using both probes did not follow a normal distribution, and there was great variability between groups, indicating the heterogeneous nature of the surface.

4.1.2. The effect of serotonin on probe translocation

It is possible that the probes are being translocated across the outer monolayer to the inner leaflets of the tegumental membranes which are less fluid [Foley et al, 1986]. Translocation of the probe to these leaflets would reduce both D_L and

Table 4.1. The effect of serotonin signalling on membrane fluidity

Worms were immobilised in GMEM containing 25 mg ml-1 carbamoyl choline and various compounds were assessed for their ability to change fluidity by fluorescence recovery after photobleaching. 100 were used per group, and 4 readings were taken per worm.

*, $P < 0.001$ (Wilcoxon signed rank test).

Treatment (probe)	n	Mobile fraction (%) Before	after	% pretreatment value	n	DL($\times 10^{-10}$ cm^2/sec) Before	after	% pretreatment value
none								
(5AF)	100	28.1± 21.4	30.1± 11.5	107.1	100	83.9± 26.39	69.4± 41.2	83
Serotonin (1μM)								
(5AF)	105	47.9± 19.1	27.1± 14.2	56.2*	105	118.8± 106	62.5± 104	52.6*
(NBD PC)	109	51.2± 28.3	57.9± 30.8	113	109	39.4± 19.1	62.4± 60	159.6
Methiotheipin (10μM)								
(5AF)	95	56± 11.5	53± 12.8	94.6	95	97.9± 27.4	82.2± 42.4	83.9
Fluoride (1mM)								
(5AF)	104	63.5± 9.2	45.4± 14.6	71.5*	104	187± 116	153.7± 91.2	82.8*
Forskolin (20μM)								
(5AF)	110	40.5± 18.7	28.4± 14.8	70.3*	110	44.1± 40	67.5± 38.2	153*
Dideoxyforskolin (20μM)								
(5AF)	100	37.4± 12.8	34.4± 18	92.1	100	68.1± 45	59.8± 52.1	87.9

Figure 4.1: The translocation of lipid analogues from the outer monolayer of adult *S. mansoni* .

To assay for probe translocation, labelled adult worms were treated with 1 μM serotonin, and the fluorescence at the surface was quenched by 0.25 % Trypan blue. The traces are (A) NBD-PE; a positive control that shows translocation since its fluorescence increases; (B) 5AF and (C) NBD-PC, both of which do not show translocation.

This is a representative trace from twenty readings on twenty different worms.

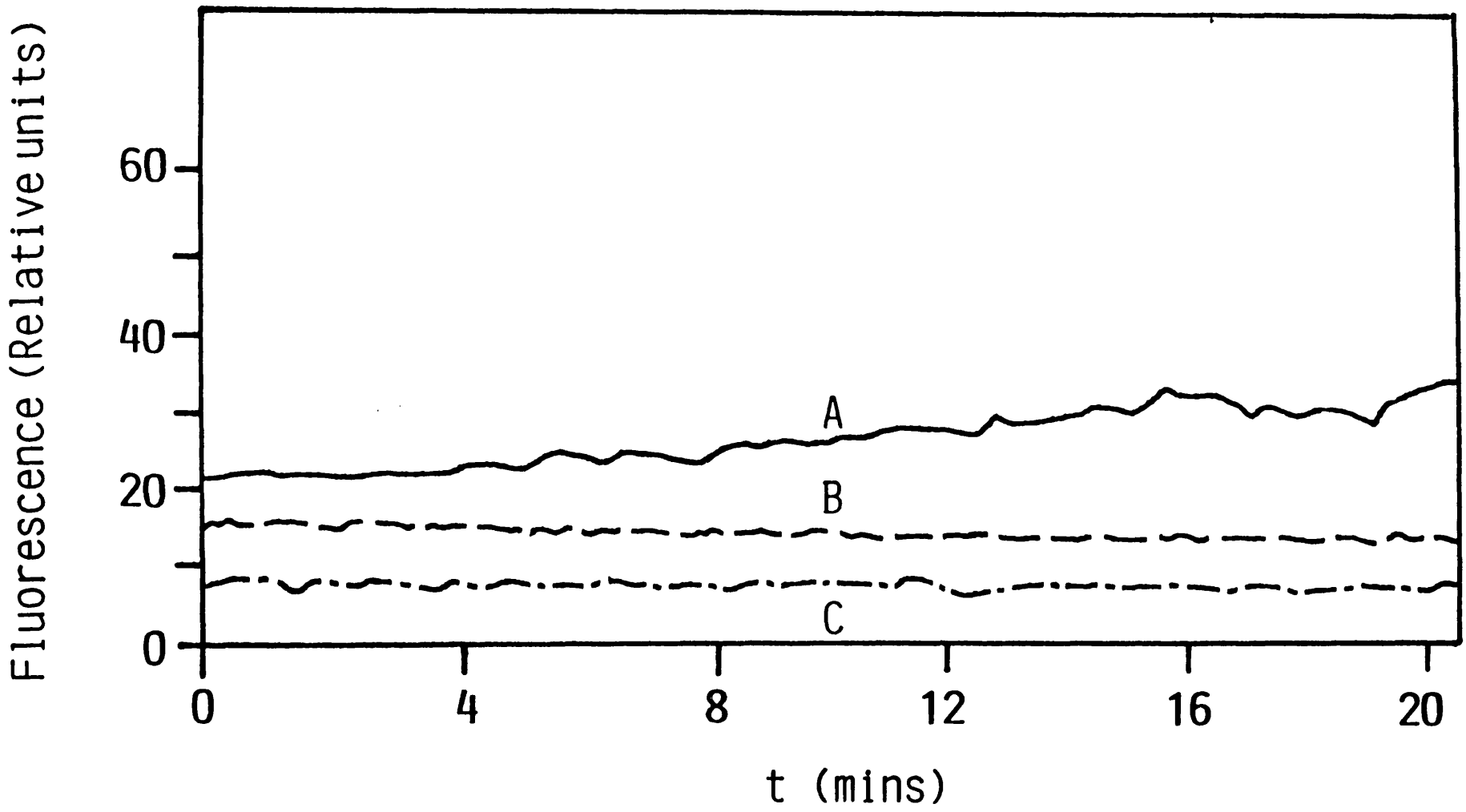

Fluorescence (Relative units)
60
40
20
0
A
B
C
0
4
8
12
16
20
t (mins)

Figure 4.2: The effect of caged cyclic AMP on membrane fluidity

The effect of cyclic AMP released from caged cAMP by photolysis on the lateral diffusion and mobile fraction of 5AF in the surface membrane of *S. mansoni*. The worms were loaded up with 20 μM of the acetoxy methylester of caged cAMP. Fluidity was measured 4 times on a spot of dorsal membrane, and the signal was averaged. The compound was then photolysed (arrow) and fluidity was monitored as before on the same spot of membrane.

The measurements were taken on 40 worms that were immobilised in GMEM containing 25mg/ml carbamoyl choline.

□, Mobile fraction (f); ◆, lateral diffusion coefficient

Figure 4.2: Effect of caged cyclic AMP on membrane fluidity

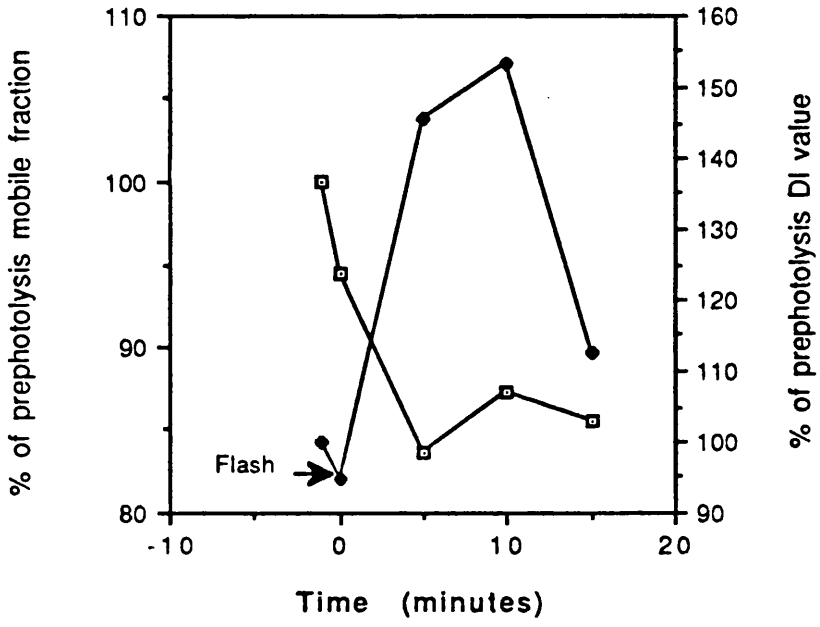


Figure 4.3; The effect of serotonin on free calcium concentration as measured by fluo-3.

The point at which 1 μM serotonin was added is indicated by the arrow. The recording was performed using a laser beam focussed just under the tegumental membrane; the signal is therefore from the underlying syncitium.

This is a trace representative of 20 worms.

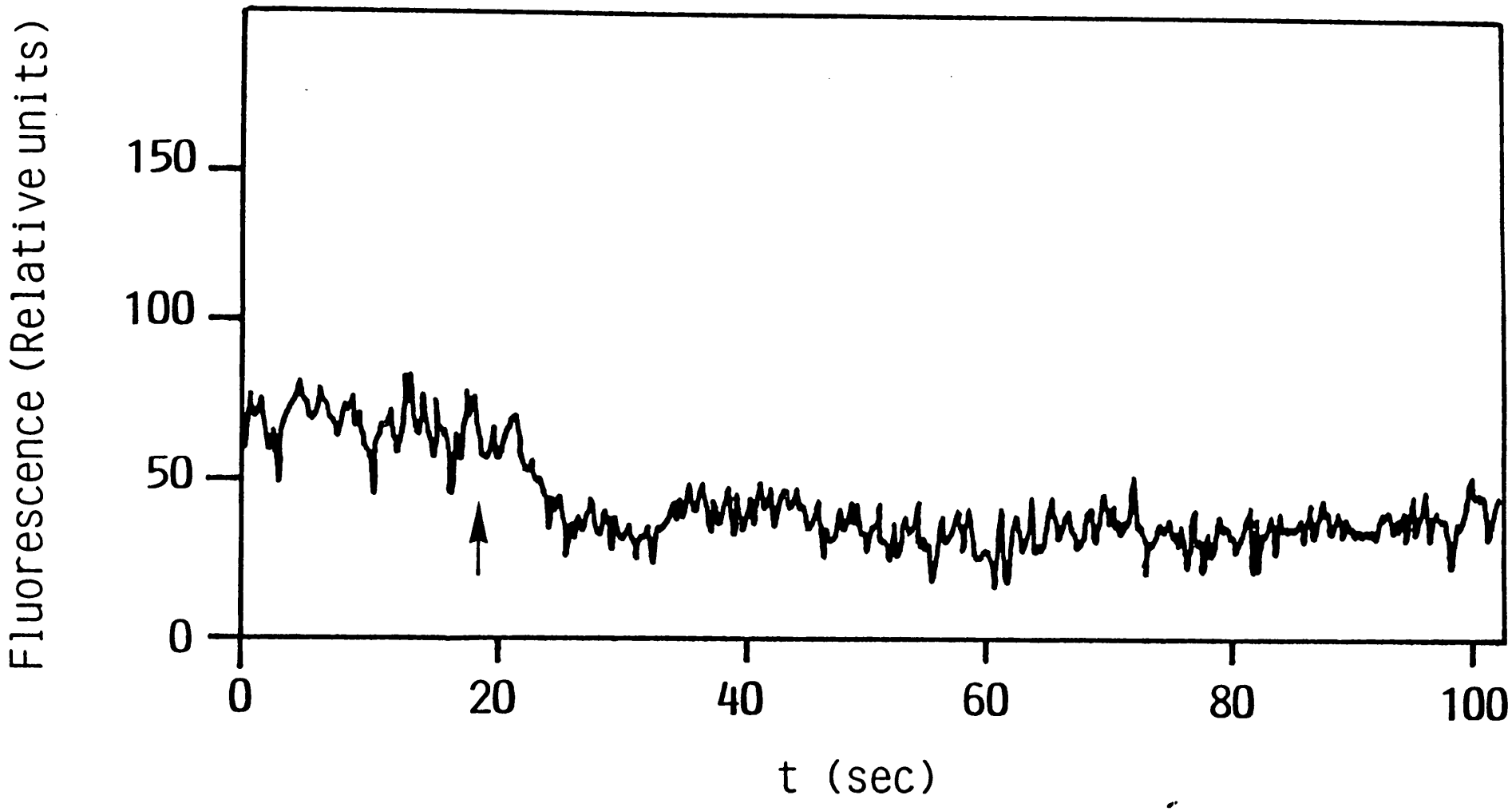
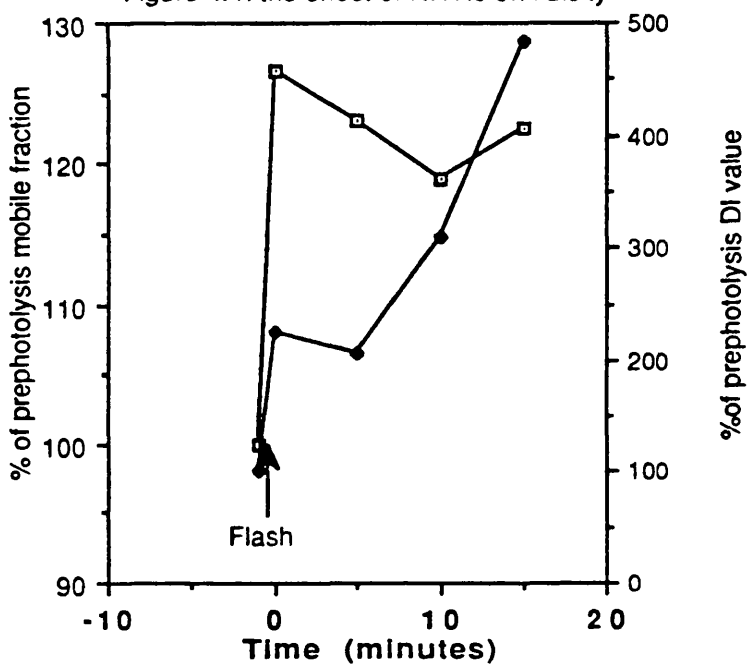

Fluorescence (Relative units)
150
100
50
0
0
20
40
60
80
100
t (sec)

Figure 4.4; The effect of NITR 5 on fluidity

the effect of calcium released from caged calcium (NITR 5) by photolysis on the lateral diffusion and mobile fraction of 5AF in the surface membrane of *S. mansoni*. The worms were loaded up with 100 μM of the acetoxy methylester of NITR 5. Fluidity was measured 4 times on a spot of dorsal membrane, and the signal was averaged. The compound was then photolysed (arrow) and fluidity was monitored as before on the same spot of membrane. The measurements were taken on 40 worms that were immobilised in GMEM containing 25mg/ml carbamoyl choline.

□, Mobile fraction (f); ◆, lateral diffusion coefficient

Figure 4.4: the effect of NITR5 on fluidity



mobile fraction. The fluorescence resonant energy transfer experiment of figure 4.1 indicates this is not the case, as no increase in fluorescence is observed, as would occur in the event of translocation or any form of endocytosis.

4.1.3. The effect of cyclic AMP on fluidity

The data in table 4.1 indicates a role for cAMP in the observed changes. To verify this, caged cAMP was employed. As can be seen from figure 4.2, following photolysis to release the c AMP, there is an increase in D_L, and a decrease in mobile fraction, on the dorsal surface, followed by a change towards pre-photolysis values. The fact that the D_L value did not decrease as when serotonin was employed, prompted us to look at the changes in free calcium levels in the tegument using the calcium sensitive fluorophore Fluo 3. As shown in figure 4.3, calcium concentration decreases upon addition of 1μM serotonin. This is in agreement with data presented by Podesta (1987), and the effect of free calcium ions on membrane fluidity was then studied.

4.1.4. The effect of free calcium ion concentration on fluidity

Worms were loaded up with Diazo-2 (a "caged" calcium chelator), and after photolysis, the D_L value was lowered, with a slight, but significant increase in mobile fraction (figure 4.5). NITR 5 ("caged" calcium") had the reverse effect, raising both D_L and mobile fraction on the dorsal tegument (figure 4.4). The decrease in the free calcium ion concentration of the tegumental syncytium that accompanies serotonin treatment could therefore account for the observed decrease in D_L value.

4.1.5. The effect of serotonin on isolated membranes.

It is possible that some membrane properties may be under the control of the neuromuscular system [Kusel and Gordon, 1989]. To determine whether this is

Figure 4.5: The effect of Diazo-2 on membrane fluidity

The effect of a calcium binding agent released by photolysis of Diazo-2 by photolysis on the lateral diffusion and mobile fraction of 5AF in the surface membrane of *S. mansoni*. The worms were loaded up with 50 µM of the acetoxy methylester of Diazo-2. Fluidity was measured 4 times on a spot of dorsal membrane, and the signal was averaged. The compound was then photolysed (arrow) and fluidity was monitored as before on the same spot of membrane.

The measurements were taken on 40 worms that were immobilised in GMEM containing 25mg/ml carbamoyl choline.

□, Mobile fraction (f); ◆, lateral diffusion coefficient

Figure 4.5: the effect of Diazo - 2 on membrane fluidity

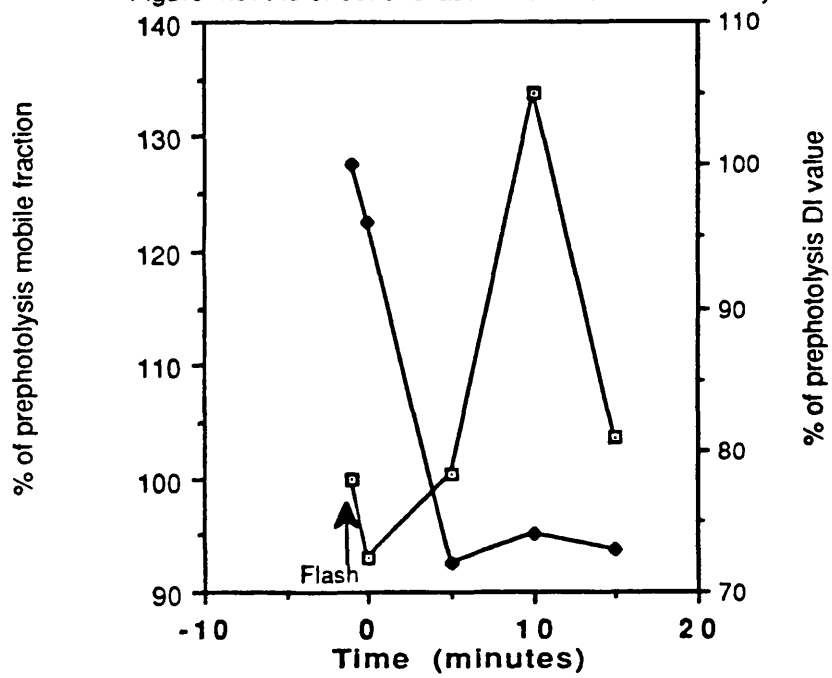


Figure 4.6: Studies on isolated membranes

The effect of serotonin on the lateral diffusion and mobile fraction of 5AF in isolated membranes from *S. mansoni*. Worms were labelled with 5AF, and either treated with serotonin, and the membrane removed (◆), or the membrane was removed and then treated with serotonin in the absence of calcium (1mM EGTA (■)), or the presence of 1mM calcium (□). 3 μM phalloidin was also added in the absence of calcium (1mM EGTA (◇)).

Thirty two readings were taken at each point, and are representative of three different experiments.

Figure 4.6: studies on isolated membranes

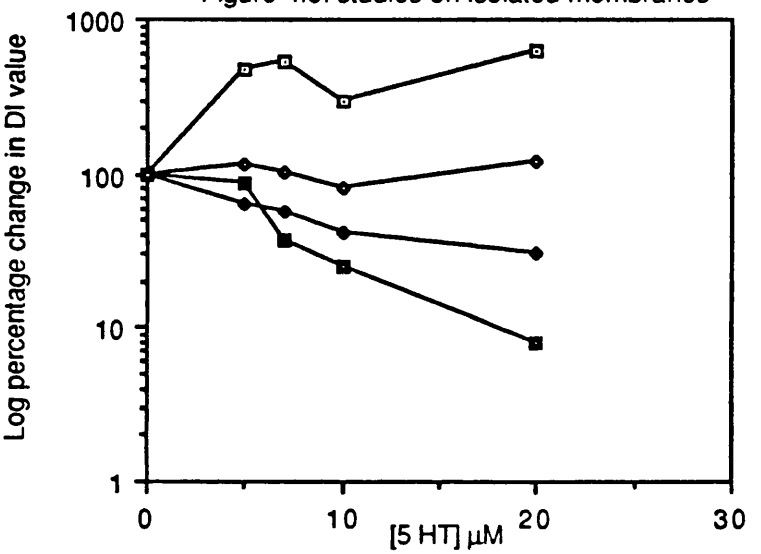


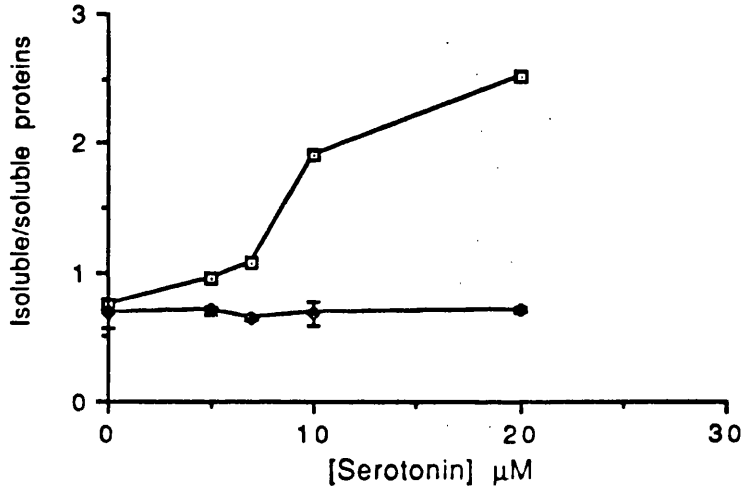
Figure 4.7: The effect of serotonin on "membrane cytoskeletal" content

The incoporation of membrane proteins into the "membrane cytoskeleton". Membranes were removed from 55 worms treated with a range of serotonin concentrations, and the ammount of protein associating with the Triton X-100 soluble and insoluble phases was measured using the peirce micro BCA kit. The graph shows the means and standard deviations of triplicate determinations, and is representative of four experiments.

(□) Normal worms

(◆) Worms pretreated with 10 μM methiotheipin

Figure 4.7: The effect of serotonin on the membrane cytoskeleton



true of fluidity, the D_L value of isolated membranes was measured (heterogeneity of membrane size ruled out measurements of mobile fraction, as this assumes an infinite reserviour of fluorescence [Axelrod, 1977]). It is clear from figure 4.6 that membrane removed from the worms before treatment with serotonin, could respond to treatment of this ligand by lowering their D_L value, only in the absence of calcium (1mM EGTA). Otherwise, in the presence of calcium, the D_L value increased (See forskolin table 4.1). Membranes from worms treated with serotonin before removal show a progressive decrease in D_L value. Phalloidin, which prevents changes in F-actin levels, was able to inhibit changes in the D_L value from occurring, suggesting cytoskeletal involvement in the maintenance of surface lipid domains. The fact these fluidity changes can occur in isolated membranes also means that fluidity changes cannot be due to changes in lipid composition.

4.2. Discussion

In this chapter, it is demonstrated that serotonin can decrease both the mobile fraction and D_L value on the intact worm tegument when 5AF was used as the fluorescent membrane probe. This means that some factors in the parasite cytosol can act to decrease the amount of lipid which can diffuse as well as decrease its rate of translational movement. The values for mobile fraction and D_L can be altered independently from each other and are likely to be under separate control. Thus release of intracellular cAMP by forskolin treatment or from the photolysis of "caged" cAMP caused an increase in D_L value but a decrease in mobile fraction, while an increase in calcium ions by photolysis of NITR 5 results in an increase in both parameters. Fluoride, an activator of G-proteins, can also decrease both parameters in the same way as does serotonin, and it is therefore likely that both responses are mediated via G-proteins. This fluidity change may be mediated by cAMP dependent kinases, which by phosphorylating the cytoskeletal proteins of the tegument may change their

conformation and or physical properties, and as a result change their associations with lipid or other proteins [Powell et al, 1987; Furuhashi and Hatano, 1990].

Phosphorylation in schistosomes is inhibited by calcium [Podesta, 1987], which might influence cytoskeletal interactions with the membrane when the calcium ion concentrations alter. Calcium is capable of interacting directly with acidic phospholipid head groups, and bringing about lateral phase separation [Eklund et al, 1988]. This would be expected to decrease both mobile fraction and D_L value, which is not observed, as an increase in calcium concentration is associated with an increase in mobile fraction and a decrease in D_L (figure 4.4). One possible effect of calcium may be that it is binding to proteins with higher affinity than lipid, and modulating their interactions with other species in the membrane [Datta, 1987]. An example of a structural protein which alters its conformation uppon binding calcium is Troponin C [Murray and Webber, 1974]. The finding that the fluidity of the membrane domains accessible to NBD PC is unaltered by serotonin is interesting, because it further argues for membrane heterogeneity. This leads to the possibility that the parasite may separate its proteins into different domains depending on its needs. It may partition some proteins into domains that are sensitive to environmemtal change, and others into more stable domains. This may be a mechanism to regulate the function of enzymes under different conditions in the host. Ion pumps for example [Rumjanek, 1987] may be in what shall be called environmentally sensitive domains, to allow rapid changes in activity when subjected to environmental stress. Proteins requiring a more stable fluid environment may be sequestered in environmentaly insensitive domains.

Chapter 5: The effect of serotonin on the molecules of the tegumental membrane

5.1. The effect of serotonin on the tegumental cytoskeleton

The finding that phalloidin can inhibit fluidity changes prompted the study of the membrane cytoskeleton and its behavior when serotonin is used.

5.1.1. The effect of serotonin on the cytoskeletal content

One hypothesis is that serotonin acts to increase the amount of cytoskeletal protein interacting with the tegumental membrane,and we would predict that the cytoskeleton would also change in such a way as to restrict diffusion. Figure 4.7 shows such a serotonin dependent association of membrane proteins with a "membrane" cytoskeleton. This means that the cytoskeletal content of the membrane has increased. The cytoskeleton could then form a matrix which can retard probe diffusion.

5.1.2. Actin polymerization

Since phalloidin can inhibit fluidity changes (figure 4.6), the state of actin in the membrane was assesed in two ways. The first was to remove the surface and label it with FITC phalloidin, and extract the phalloidin in methanol. The fluorescence was then quantitated and corrected for protein concentration. Figure 5.1 shows that 7μM serotonin is capable of causing actin polymerisation and this is inhibited by pretreating the worms with methiotheipin.

Actin polymerisation was also monitored by western blotting and figure 5.2 shows a serotonin concentration dependent increase in G-actin content in the membrane (Triton X-100 soluble). When one looks at the F-actin concentration (Triton X-

Figure 5.1: The effect of serotonin on FITC phalloidin binding to tegumental membranes.

Seventy five worms were treated with 7 μM serotonin, and the membranes removed and incubated for 1hr with 1mM FITC phalloidin. The samples were then washed free of the phalloidin with EMEM without calcium. The FITC phalloidin was then extracted from the membranes by methanol, and quantitated and corrected for protein concentration.

Column 1 normal worms, colum 2 normal worms and 10 μM methiotheipin, column 3, worms pretreated for 15 min with 10 μM methiotheipin and then 7 μM serotonin, column 4 worms treated 7 μM serotonin.

The data shows the mean ± 1 S.D. of triplicate experiments.

Figure 5.1: the effect of serotonin on FITC phalloidin binding to tegumental membranes.

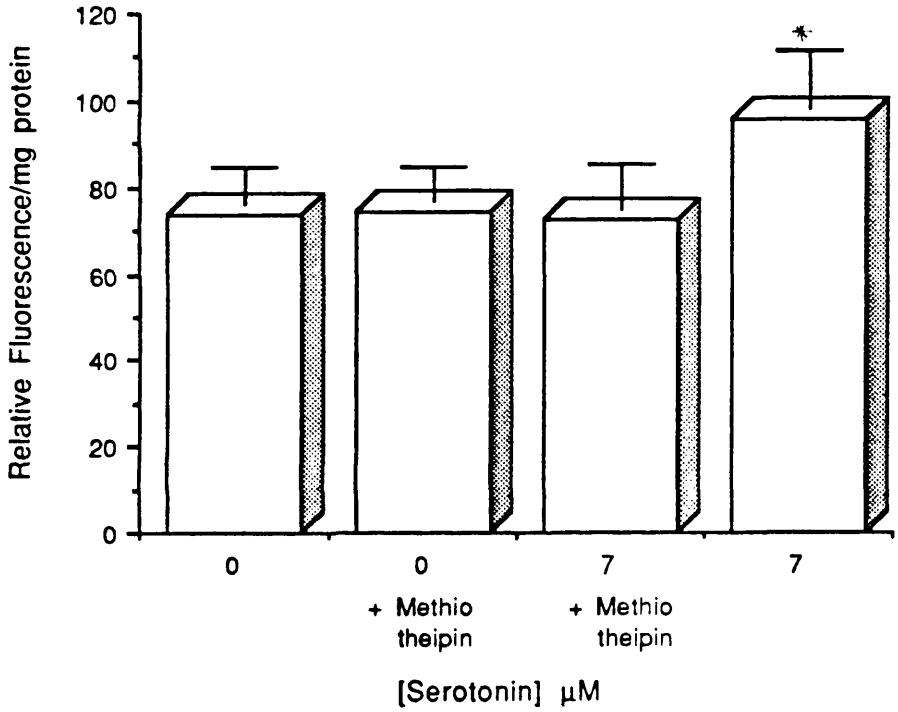


Figure 5.2. The effect of serotonin on actin polymerisation in the tegumental membranes by western blotting.

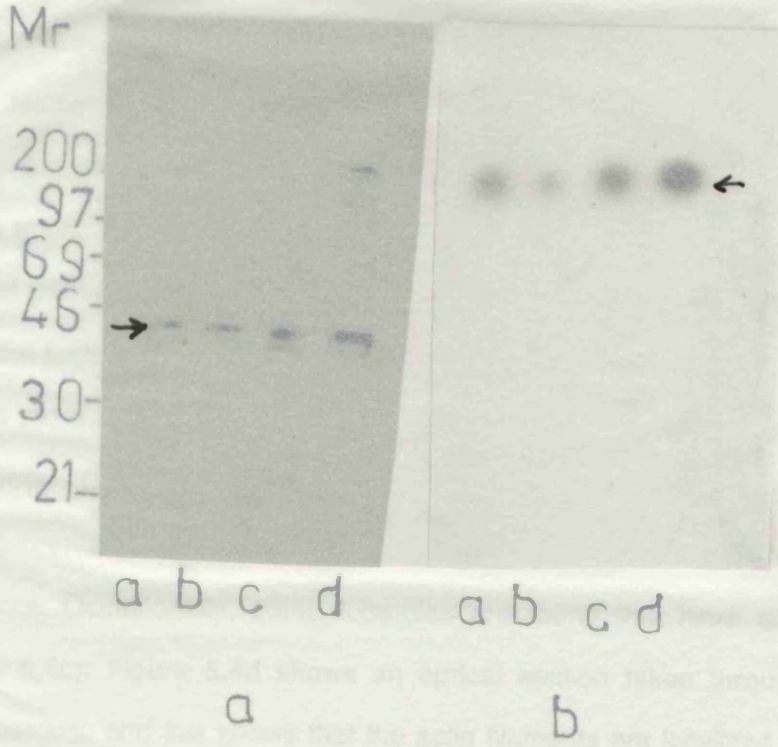
Worms were treated with a range of serotonin concentrations, and the membrane removed. Figure 5.2 a shows actin in a western blot of the Triton soluble proteins, and figure 5.2 b shows actin in a western blot of the Triton insoluble proteins. In both parts of the figure, serotonin concentrations are : lane a, 0μM; lane b, 5μM; lane a, 10μM; lane a, 20μM.

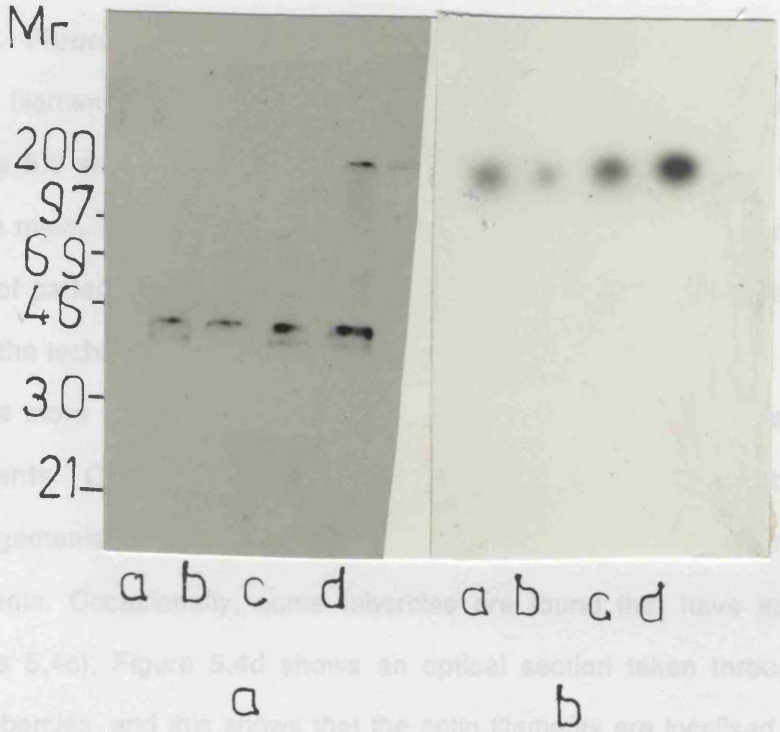
Actin was detected using a murine IgM monoclonal antibody to chicken gizzard actin (Amersham) and used at a titre of 1:500. The second antibody was a horseradish peroxidase conjugated goat ant mouse IgM (SAPU) and used at a titre of 1:500.

Horseradish peroxidase activity was visualised by luminol.

Monomeric and pentameric actins are indicated by arrows.

This is representative of 3 experiments.


Mr
200
97
69
46
30
21
a b c d
a b c d
a
b


Mr
200
97
69
46
30
21
a b c d
a b c d
a
b

100 insoluble), there is a dose dependent increase in content, but the molecular weight has shifted; it now appears to be pentameric (220 kDa versus 44 kDa). This band does not appear in the Triton soluble fraction except when a high (20 μM) concentration of serotonin is added.

5.1.3. Fluorescence studies of actin in the tegument

Actin filaments can be studied using fluorescent phalloidin [Haughland, 1992]. Figure 5.3 shows a normal epifluorescence micrograph of actin in the tegument of the male. The spines in the tegument are clearly visible, and form a hedgehog type of pattern and the tegumental cytoplasm is only slightly labelled. When one uses the technique of confocal microscopy to look at the tegument, one can see the spines more clearly (figure 5.4a), and one can also see the presence of F-actin filaments. Closer inspection of the tubercles (figure 5.4b) shows the arrangements of spines, which radiate from a central core, formed by smaller filaments. Occasionally, some tubercles are found that have actin fibre stalks (figure 5.4c). Figure 5.4d shows an optical section taken through the base of the tubercles, and this shows that the actin filaments are localised around the base of the tubercles, and they extend to neighbouring filaments, but do not interdigitate with filaments from neighbouring tubercles.

The association of the spines with the membrane can be studied by labelling the membrane with octadecanoyl rhodamine (a membrane specific lipid probe [Foley, 1986])and the spines with FITC phalloidin. Figure 5.5 shows confocal images taken after such labelling. Figure 5.5a shows the spines and figure 5.5b shows the membrane, showing the holes from which the spines emerge. When one superimposes these two images (figure 5.5c) the holes are larger than the spines, suggesting that the spines either penetrate the bilayer, or the lipid in this area of membrane is unusual and excludes octadecanoyl rhodamine. In order to determine which of these two possibilities is the case, the surface of the living

Figure 5.3: Fluorescent microscopy of tegumental actin

Worms were prepared for microscopy by a 15 min fixation in 4% (v/v) formaldehyde and 0.9% (w/v) Na Cl (formyl saline). Fixed worms were washed free of fixative by PBS, and incubated for 1hr with 3 μM FITC phalloidin.

Figure 5.3 is the tegument at a magnification of X400.

Figure 5.4a-d: Confocal microscopic studies of tegumental actin

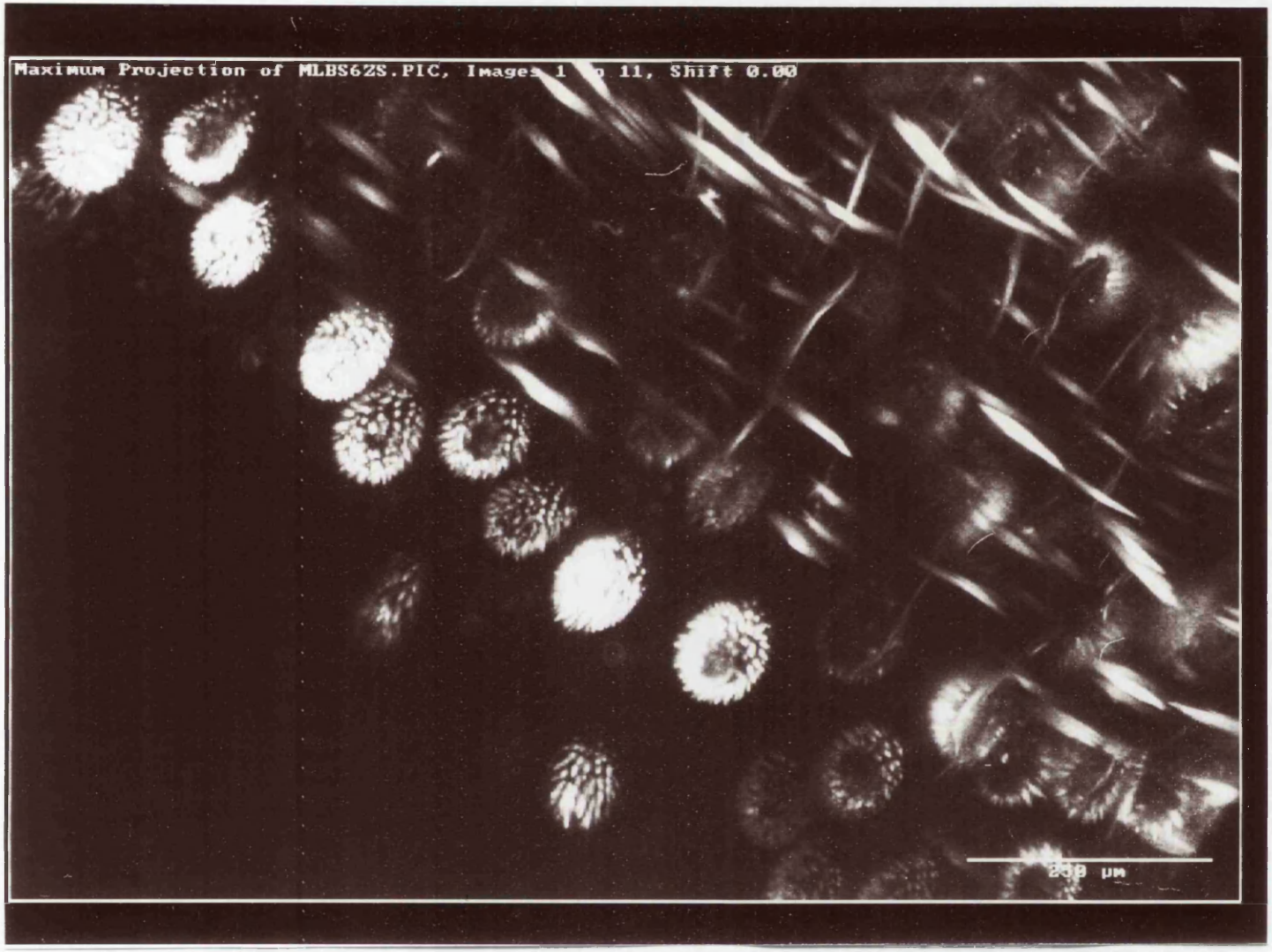
Worms were prepared for microscopy by a 15 min fixation in 4% (v/v) formaldehyde and 0.9% (w/v) Na Cl (formyl saline). Fixed worms were washed free of fixative by PBS, and incubated for 1hr with 3 μM FITC phalloidin.

Figure 5.4a: Reconstruction of the spines from 6 Kalman averaged sections

Figure 5.4b: Longitudinal section through the spines.

Figure 5.4c: Longitudinal section through the spines showing stalk.

Figure 5.4d: Transverse section through the base of the tubercles.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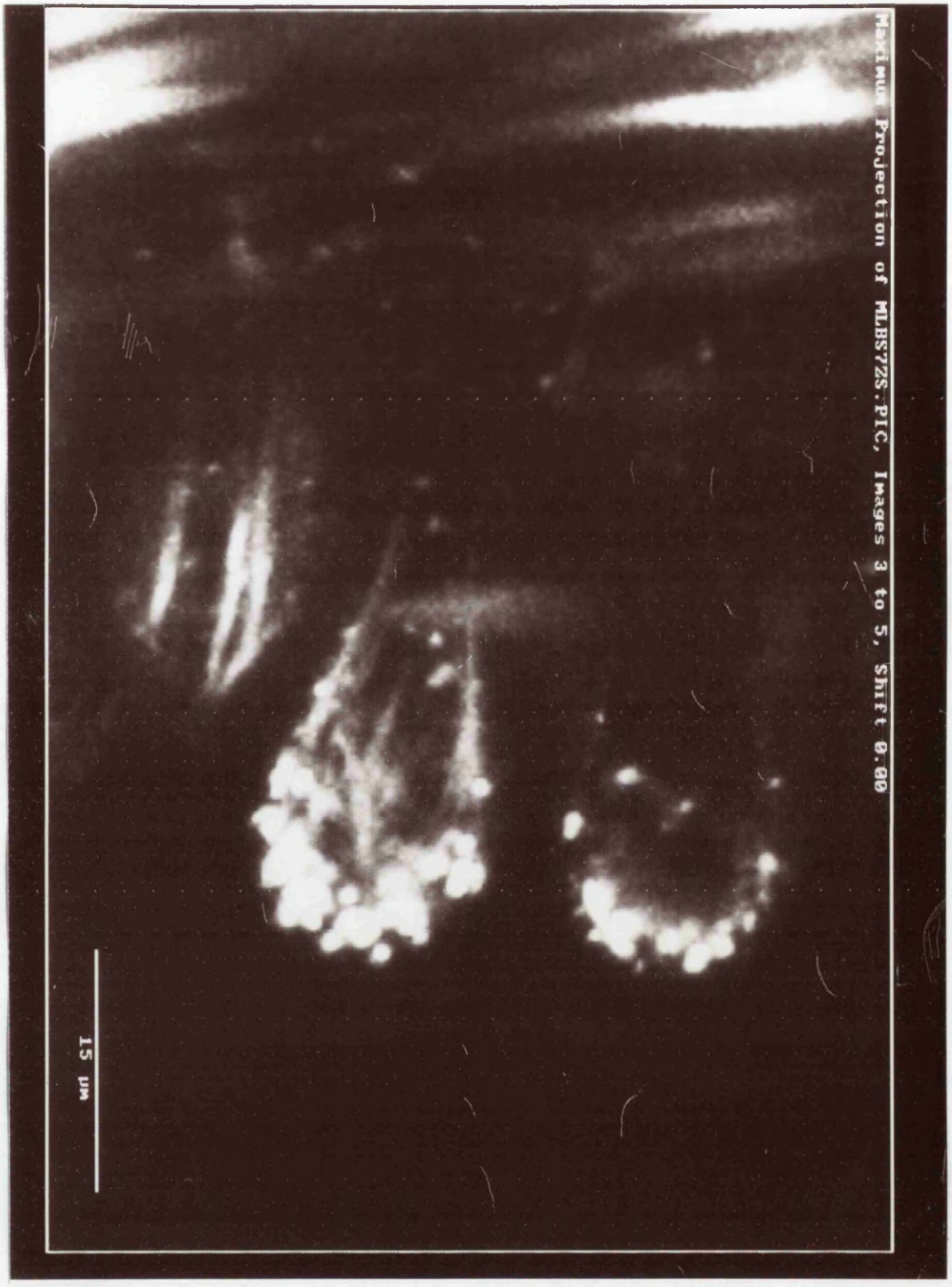
250 µm

b

C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15 µm

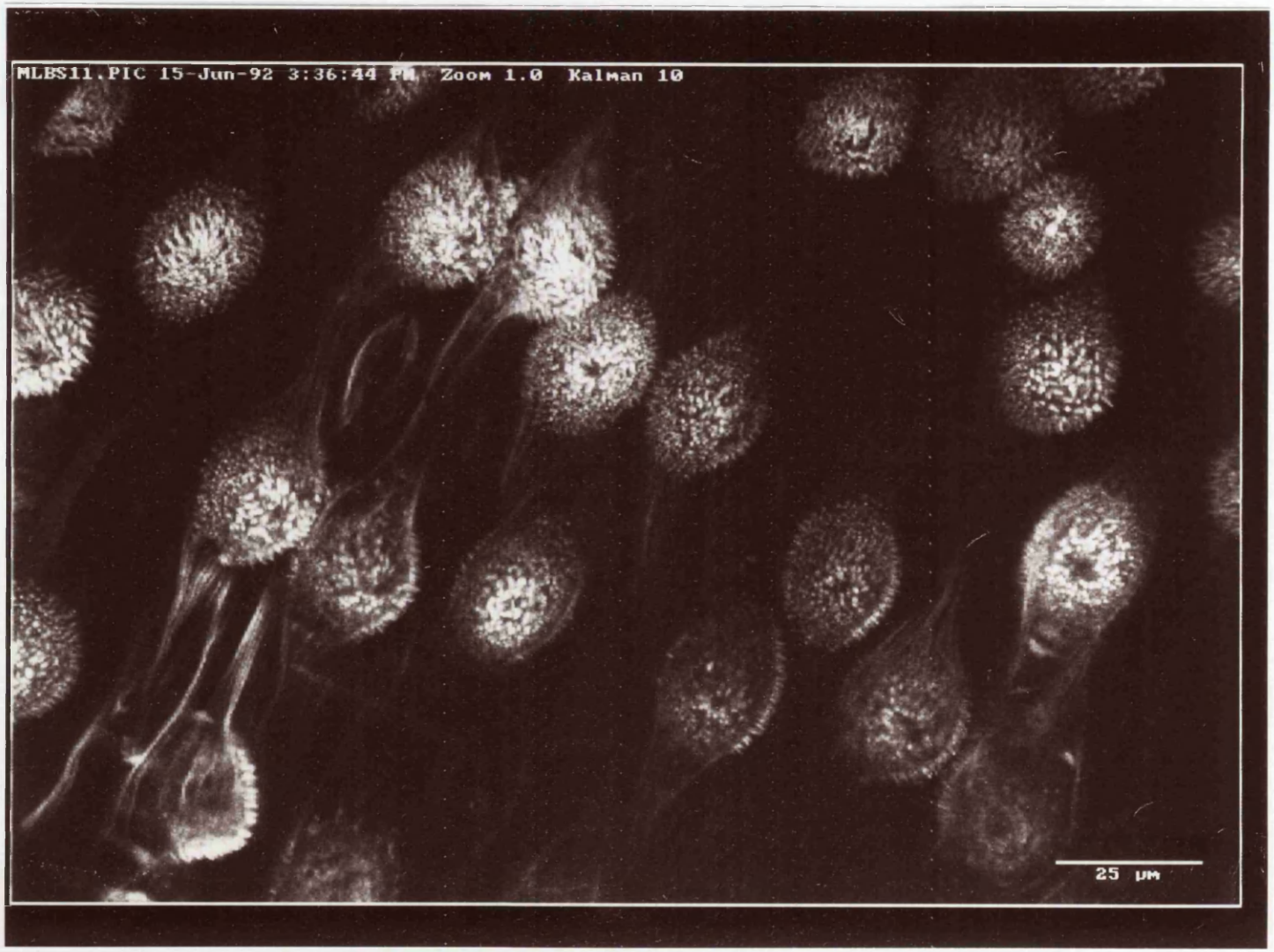

MLBS11.PIC 15-Jun-92 3:36:44 PM Zoom 1.0 Kalman 10
25 µm

Figure 5.5a-c: The association of actin with the membrane

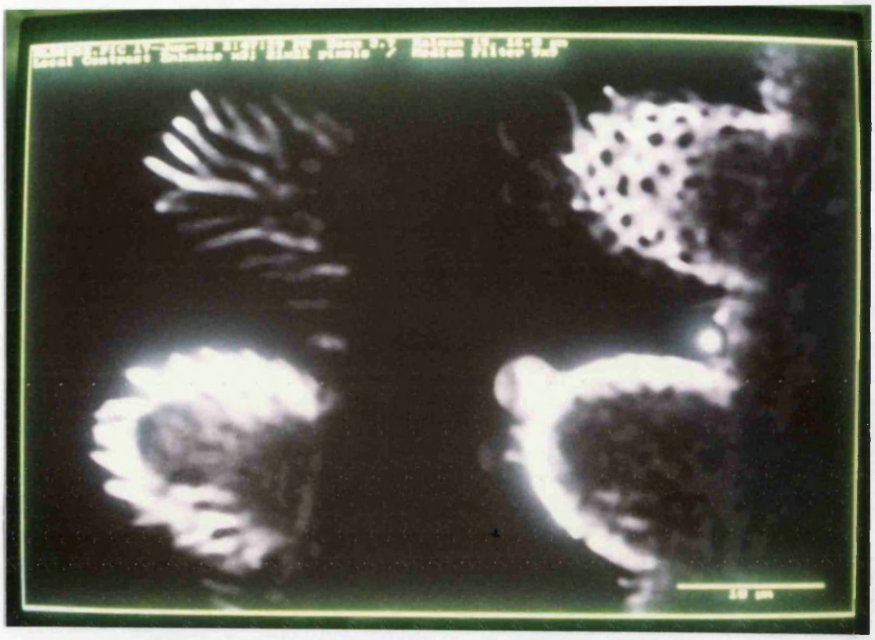
Worms were prepared for microscopy by a 15 min fixation in 4% (v/v) formaldehyde and 0.9% (w/v) Na Cl (formyl saline).Normal worms were incubated for 10 min with 1 $\mu g\ ml^{-1}$ of the membrane probe octadecanoyl rhodamine.Following this, the worms were fixed and washed free of fixative by PBS, and incubated for 1hr with 3 μM FITC phalloidin.

Figure 5.5a shows the spines labelled with FITC phalloidin.

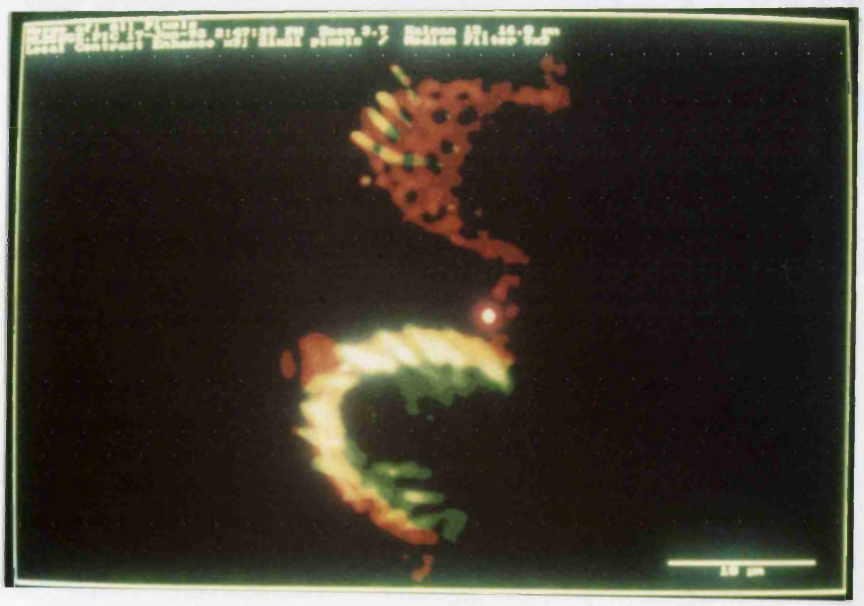
Figure 5.5b shows the tegumental membrane labelled with octadecanoyl rhodamine.

Figure 5.5c shows the superimposition of figures 5.5a and 5.5b.

a b



c



parasite was labelled with phalloidin. Figure 5.6 shows that the spines can label with phalloidin. This could however mean that this area of the surface has some unusual permeability properties. Another strategy was therefore employed, using a monoclonal antibody to actin to label the surface. This works on the assumption that a large molecule, in this case Ig M will be unable to cross the surface, and label actin internally. Any fluorescence would therefore be due to actin being exposed at the surface. Figure 5.6 shows that the spines do not label with this antibody, and the actin spines must therefore be covered with membrane. There is a diffuse general staining of actin in the fixed worms, and this is higher than the intact worms surface fluorescence.

When one looks at the effect of 7 μM serotonin on. actin, there appears to be more filaments around the base of the membrane figure 5.7, and there appears to be a thickening of the spines.

5.2. Crosslinking studies of specific membrane protein interactions

In the past,the geometrical arrangement of membrane proteins in the plane of the membrane of schistosomes has been studied by freeze fracture electron microscopy [McLaren et al, 1978]. While the overall resolution of this technique is good for investigating the global distribution of polypeptides in the membrane, it provides no information at the level of individual polypeptide chains, and a technique of higher resolution is required. Chemical crosslinking is a technique which can be used to approach this problem. It is a technique that can detect peptide associations at a distance of 5 to 15 Å [Wang and Richards, 1974].

In this study, two crosslinkers; Dimethyl 3,3'-dithiobispropionimidate. 2HCl (DTBP), which is membrane permeant, and the impermeant 3,3' Dithiobis (sulphosuccinimidylpropionate) (DTSSP) were used to study protein nearest neighbour interactions. These crosslinkers have cleavable disulphide bridges [Wang and Richards, 1974], a property which can be used to detect associating

Figure 5.7: The expression of actin at the tegumental surface

Column a shows the fluorescence of intact worms treated with a murine anti actin monoclonal antibody (IgM, Amersham, used at a titre of 1:500, and visualised by an FITC labelled rabit anti mouse IgM at a titre of 1:20).

Column b shows the fluorescence of a murine anti actin monoclonal antibody (IgM, Amersham, used at a titre of 1:500, and visualised by an FITC labelled rabit anti mouse IgM at a titre of 1:20). on worms fixed in formyl saline.

column c shows the presence of actin by the binding of phalloidin to the intact worm.

The data shows the mean $\pm$ 1 S.D. of triplicate determinations that are representative of 3 experiments.

The expression of actin at the surface of *S. mansoni.*

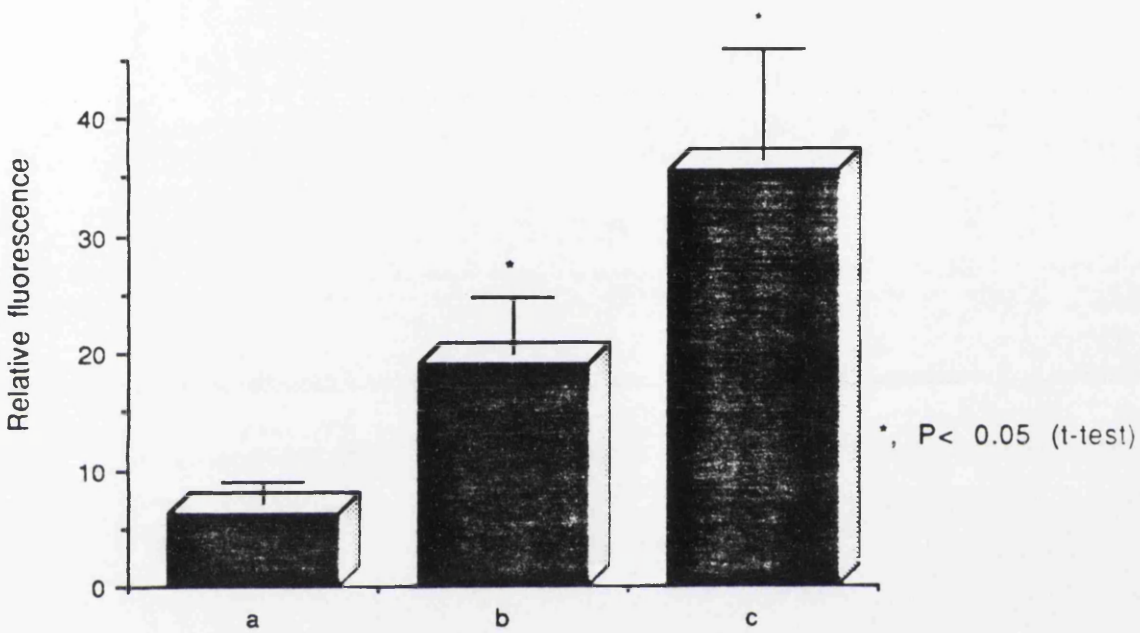
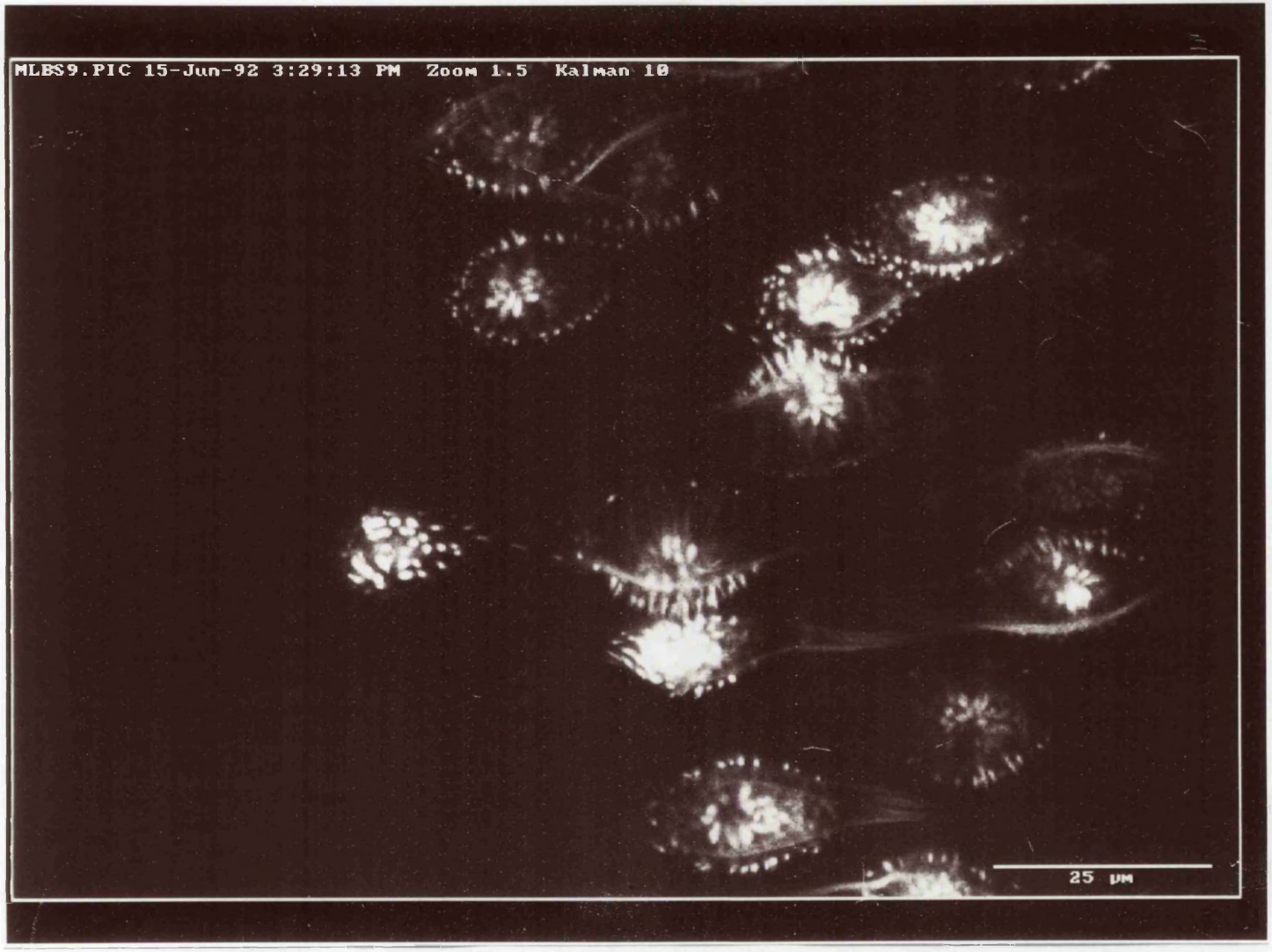


Figure 5.7. The effect of serotonin on tegumental actin.

Figure 5.7 a shows a confocal micrograph of the surface associated tegumental actin of normal worms. Optical sections were taken at the base of the tubercles, and actin filaments can be seen radiating from their bases.

Figure 5.7 b shows a confocal micrograph of the surface associated tegumental actin of worms pretreated with 7 μM serotonin. Optical sections were taken at the base of the tubercles, and actin filaments can be seen radiating from their bases.


MLBS9.PIC 15-Jun-92 3:29:13 PM Zoom 1.5 Kalman 10
25 µm

b

peptides.

Associating polypeptides can be identified by electrophoresing crosslinked proteins on a slab polyacrylamide gel, and cutting out the lane, which is subjected to reducing conditions, and placed on an other gel and electrophoresed. This gives a diagonal line, with proteins that were crosslinked running below the diagonal line [Wang and Richards, 1974].

It is possible that during the transfer to the second dimension, that some proteins particularly glycoproteins and those where disulphide bonds have been cleaved, may run above or below the diagonal line [Bretscher, 1971], due to anomalous migration. It is therefore necessary to control for this to allow the correct intepretation of the data. One approach to this problem is to electrophorese normal membrane without reducing agents in the first dimension, followed by reduction before the second dimension. Figure 5.8a and 5.8b shows such experiments for 7.5 % and 5% polyacrylamide gels respectively. From these figures it can be seen that no bands run off the diagonal line, leaving no problem with data interpretation. Figures 5.9a and 5.9b show that the crosslinkers work because of the appearance of high molecular weight bands, usually in the stacking gel, which disappear after reduction. The impermeant (DTSSP) was not effective at crosslinking, but became more effective when sagatal was added (figure 5.10), this fluidises the tegument (table 5.1) and does not appear to disrupt its integrity as an impermeant form of the nuclear stain bisbenzimide does not gain access to the nuclei (figure 5.11) after sagatal treatment.

When one runs DTBP crosslinked samples on the two dimensional system (figure 5.12a) a protein of approximate molecular weight 48 kD was found to run below the diagonal line. If this protein ran on the diagonal line, it would have an estimated Mr of 96, and may therefore occur as a dimer in the membrane. The overlay indicates the position of the crosslinked protein. Because of the nature of this technique; the application of a slab of gel to the top of another, it is

Table 5.1. The effect of sagatal on membrane fluidity

Worms were pretreated with sagatal for 15 min and membrane fluidity was assessed by FRAP.

n is the number of worms, and four readings were taken per worm.

Table 5.1: the effect of sagatal on membrane fluidity

Treatment	n	D_L value (X 10-10cm/sec)	Mobile fraction	significance
None	49	57 ± 29.8	45.6 ± 42.4	
7 µM serotonin	51	24.5 ± 12.3	29.6 ± 6.4	*P < 0.01
1µM sagatal	50	73.2 ± 31.3	64.9 ± 21.8	*P < 0.01

* Mann-Whitney U test

Figure 5.8: The testing of the two dimensional gel system.

The gels were run in the first dimension and reduced by boiling the cut out piece of gel for 10 min with 0.05% (v/v) ß mercaptoethanol in the presence of 2% (w/v) SDS and 0.001% bromophenol blue, the pH being kept at 6.8 by 0.1 M tris. The gel was then overlaid on top of another gel of the same concentration.

Figure 5.8a: The first and second dimension gels are both 7% acrylamide.

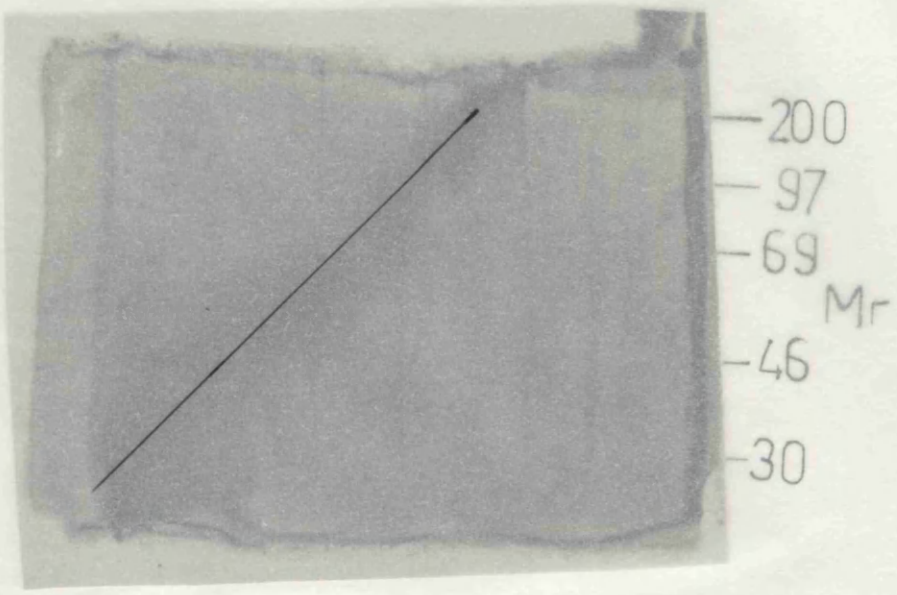
Figure 5.8b: The first and second dimension gels are both 5% acrylamide.

Proteins were stained with 0.1% coomassie blue.

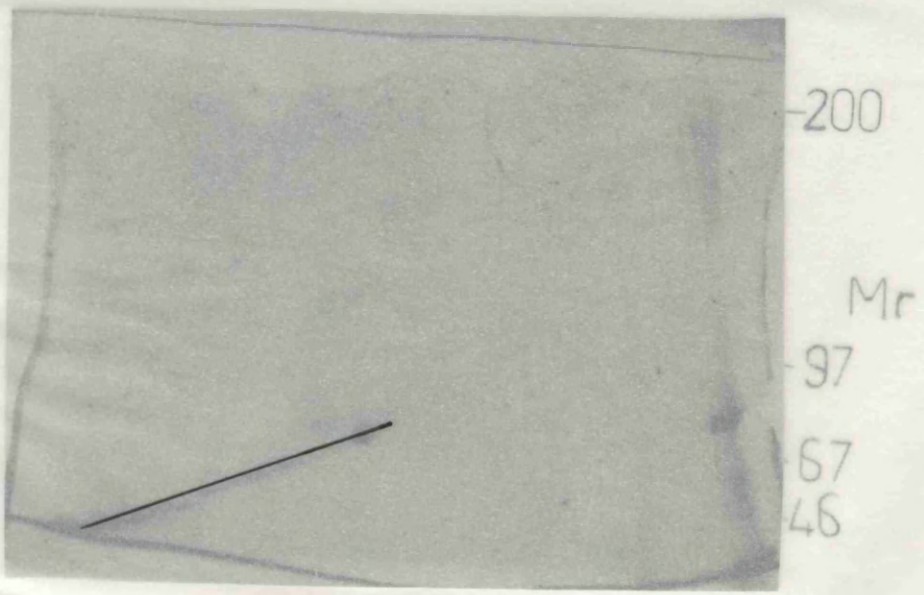
This gel is representative of 4 experiments.

The overlay shows the position of the crosslinked complexes.

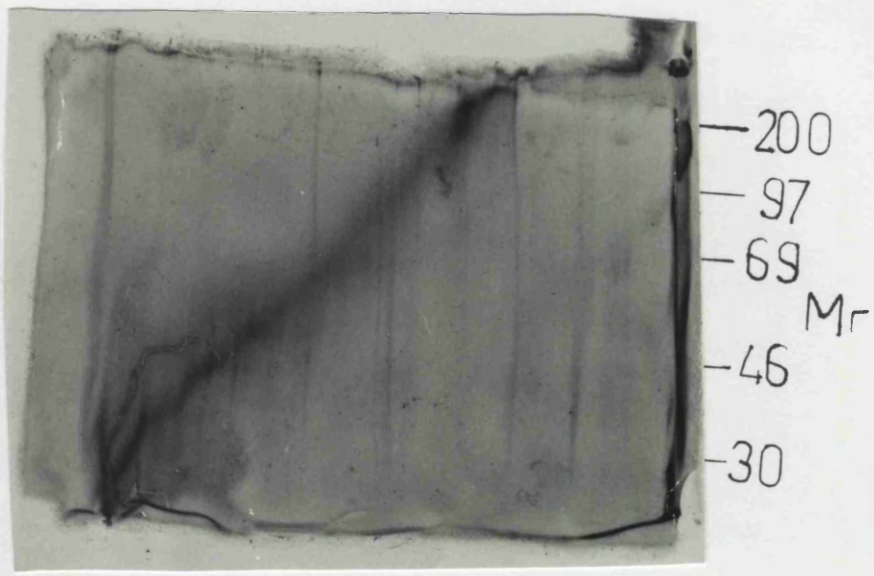
a



b



a



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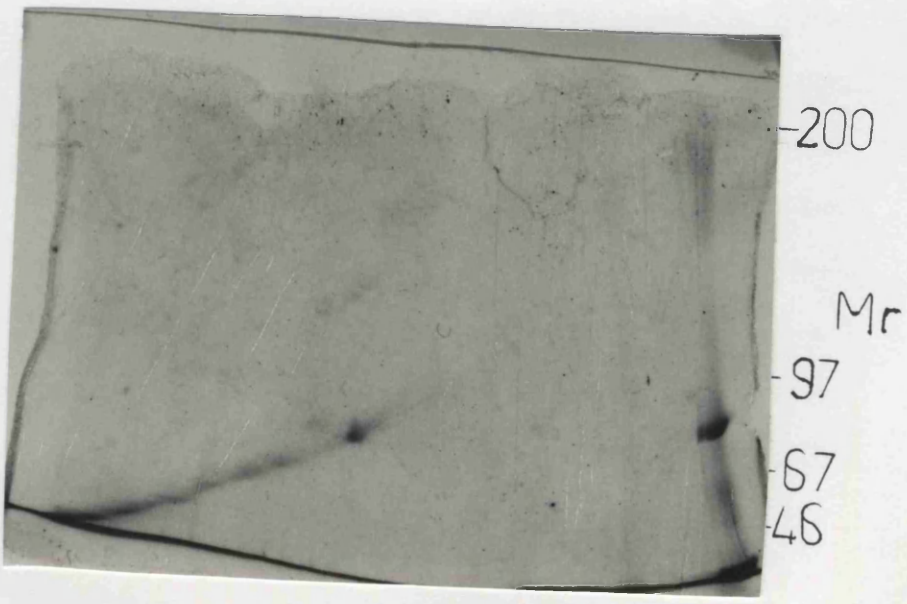


Figure 5.9: The testing of the crosslinking reagents

Figure 5.9a: The membrane impermeant crosslinker DTSSP

Lane a: molecular weight markers

Lane b: normal BALB/c erythrocytes

Lane c: crosslinked BALB/c erythrocytes

Lane d: crosslinked and reduced BALB/c erythrocytes

Lane e: normal male tegumental membranes

Lane f: crosslinked male tegumental membranes

Lane g: crosslinked and reduced male tegumental membranes

Lane h: molecular weight markers

Figure 5.9b:The permeant crosslinker DTBP

Lane a: molecular weight markers

Lane b: normal BALB/c erythrocytes.

Lane c: crosslinked BALB/c erythrocytes.

Lane d: crosslinked and reduced BALB/c erythrocytes.

Lane e: normal male tegumental membranes.

Lane f: crosslinked male tegumental membranes.

Lane g: crosslinked and reduced male tegumental membranes.

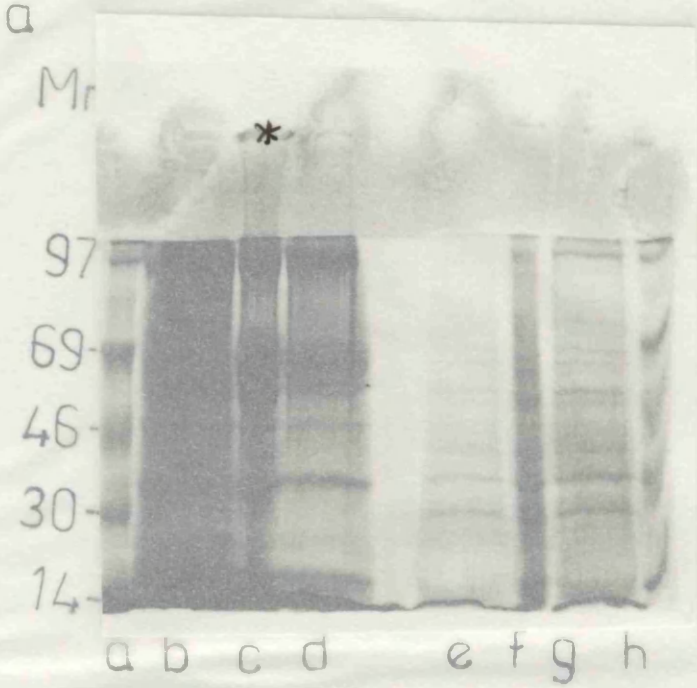
Lane h: molecular weight markers.

The overlay indicates the crosslinked proteins.

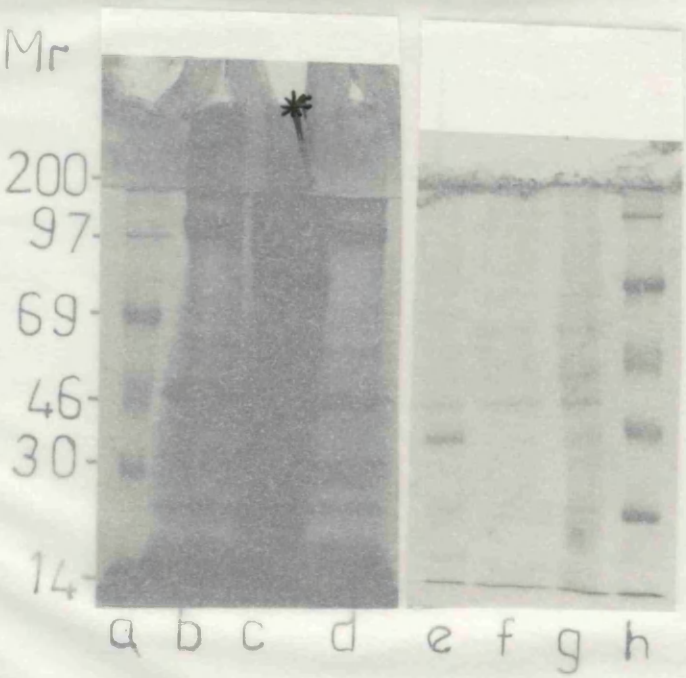
Proteins were stained with 0.1% coomassie blue.

This gel is representative of 4 experiments.

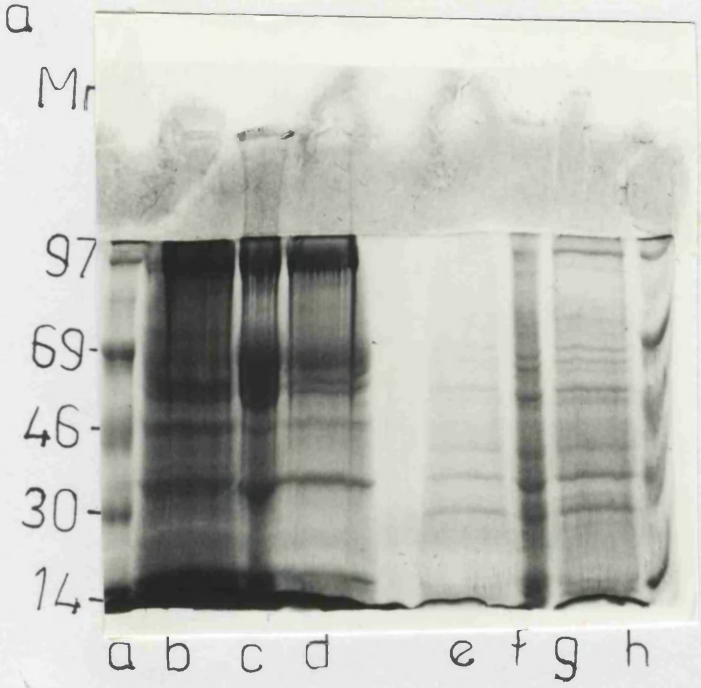
a



b



a



b

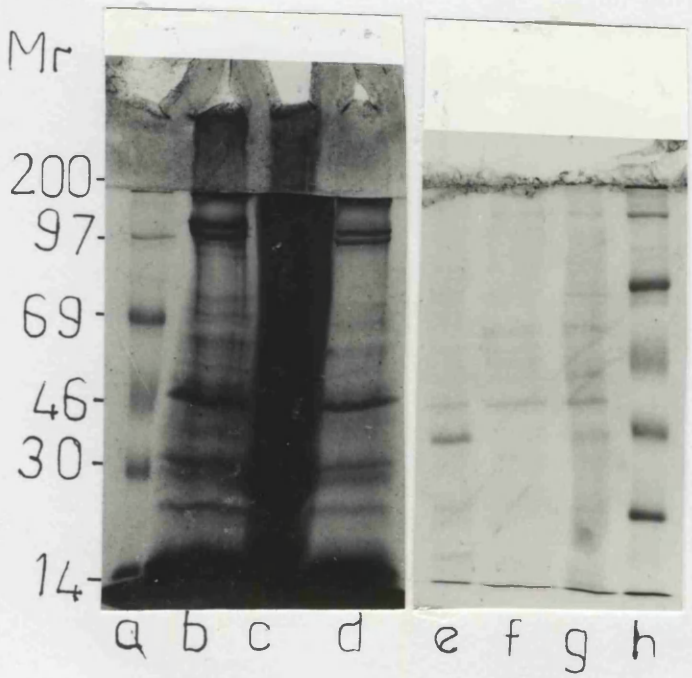


Figure 5.10: The effect of membrane fluidity on the ability of DTBP to crosslink membrane proteins

Lane a: fluidised and crosslinked membranes (1mM sagatal).

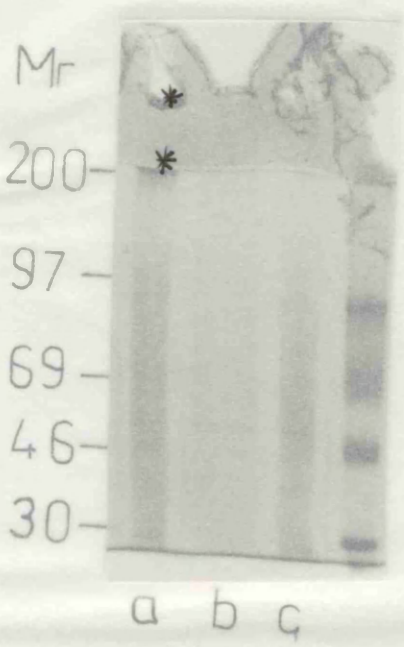
Lane b: rigidified and crosslinked membranes (1μM serotonin).

Lane c. Normal membranes.

Proteins were stained with 0.1% coomassie blue.

This gel is representative of 4 experiments.

The overlay indicates the proteins which are crosslinked.

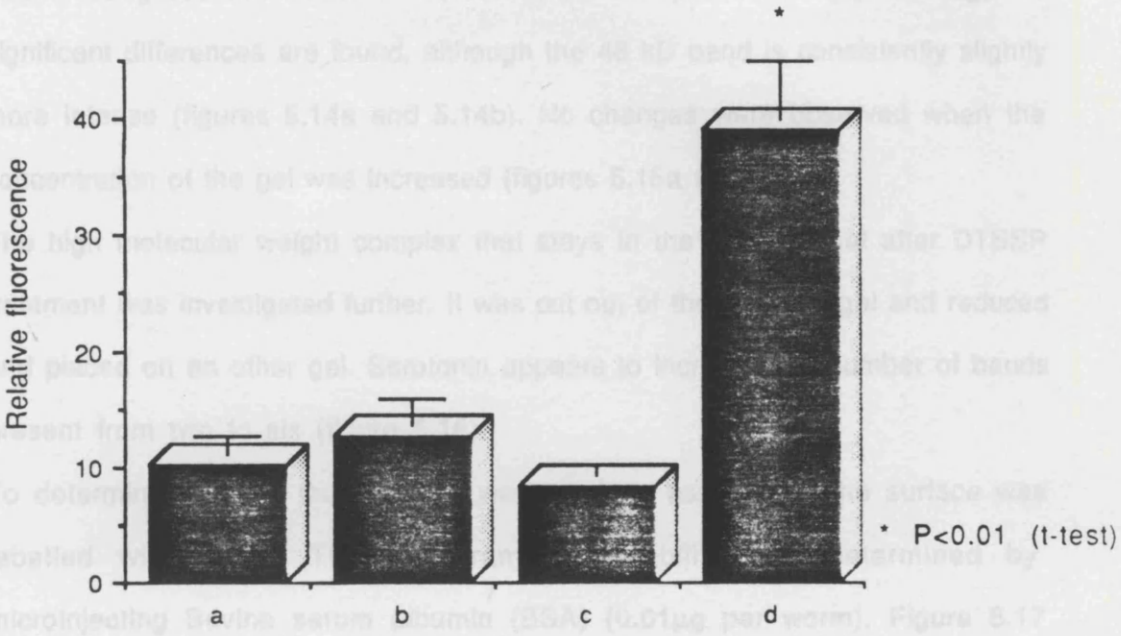

Mr
200
97
69
46
30
a b c


Mr
200
97
69
46
30
a
b
c

Figure 5.11: The effect of sagatal on membrane integrity

Normal worms (column a) or worms treated with either1mM sagital (column b)or 1μM serotonin (column c) or 0.5 % SDS (column d) were labelled with the membrane impermeant nuclear stain Hoescht 33285 to assess membrane damage. This shows the mean ± 1 S.D of triplicate readings on 25 worms per group, and is representative of three experiments.

Figure 5.11: the effect of sagatal on membrane integrity



possible that the electrophoresis of proteins could be retarded, and the real molecular weight of the 46 kD band could be lower. This could then make it an actin candidate. A similar gel was then run and blotted onto nitrocellulose and probed with a monoclonal raised to actin. Figure 5.13 however shows that there is only one actin band that has an Mr of 43, which runs on the diagonal line. When one investigates the effect of serotonin on the pattern of crosslinking, no significant differences are found, although the 48 kD band is consistently slightly more intense (figures 5.14a and 5.14b). No changes were observed when the concentration of the gel was increased (figures 5.15a and 5.15b).

The high molecular weight complex that stays in the stacking gel after DTSSP treatment was investigated further. It was cut out of the stacking gel and reduced and placed on an other gel. Serotonin appears to increase the number of bands present from two to six (figure 5.16).

To determine whether these bands were surface associated, the surface was labelled with biotin. The membrane permeability was determined by microinjecting Bovine serum albumin (BSA) (0.01µg per worm). Figure 5.17 shows that there is no labelling of BSA. It can also be seen that serotonin can in a dose dependant manner, allow biotin to gain increasing access to the noncytoskeletal surface components (figure 5.18a). The cytoskeletal components do not label (figure 5.18b), which is further evidence for the impermeability of biotin.

From labelling the membrane with biotin, it appears that two of the proteins of the high molecular weight complex, a 48 and a 52 KDa polypeptides are surface associated (figure 5.19).

5.3. The expression of cytoskeletal changes at the surface

For the cytoskeleton to retard diffusion at the surface, it must be able to interact with surface components, presumably by linking to transmembrane proteins. To investigate whether the cytoskeleton influences what happens at the surface,

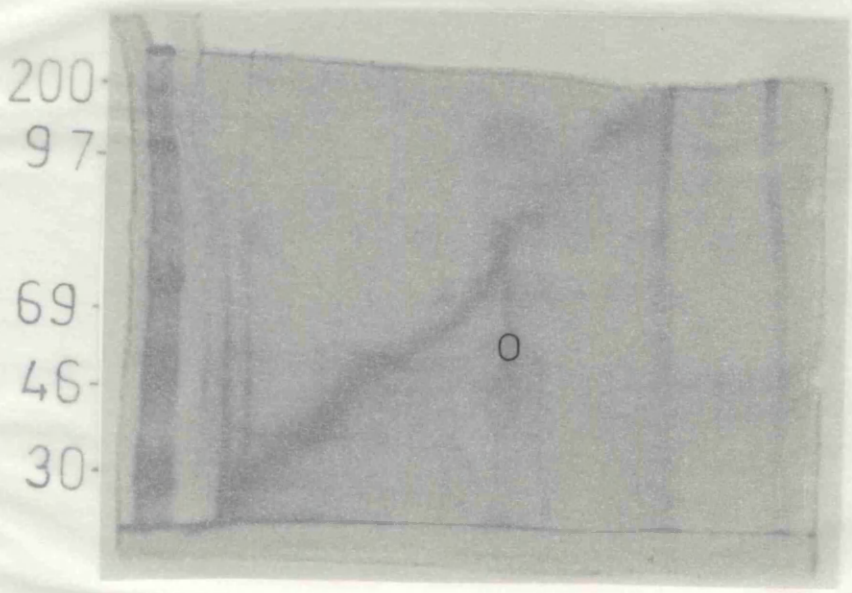
Figure 5.12: Two dimensional analysis of crosslinked proteins

Figure 5.12: Crosslinked membranes were dissolved in sample buffer by leaving it at room temperature for 30 min. The sample was then run in the first dimension (5% acrylamide), and once run, the track was cut out and reduced (10 min. boiling in 2% (w/v) SDS, 0.5 % (v/v) ß-mercaptoethanol and 0.1M tris, pH 6.5. After reduction, the gel was laid on top of an other 5% gel.

Proteins were stained with 0.1% coomassie blue.

This gel is representative of 3 experiments.

The overlay indicates the position of the crosslinked proteins.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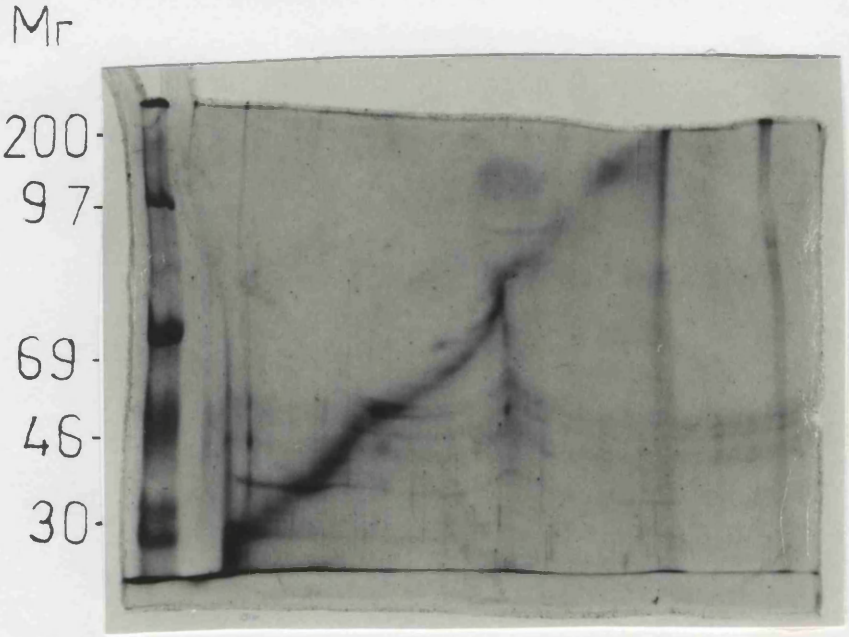
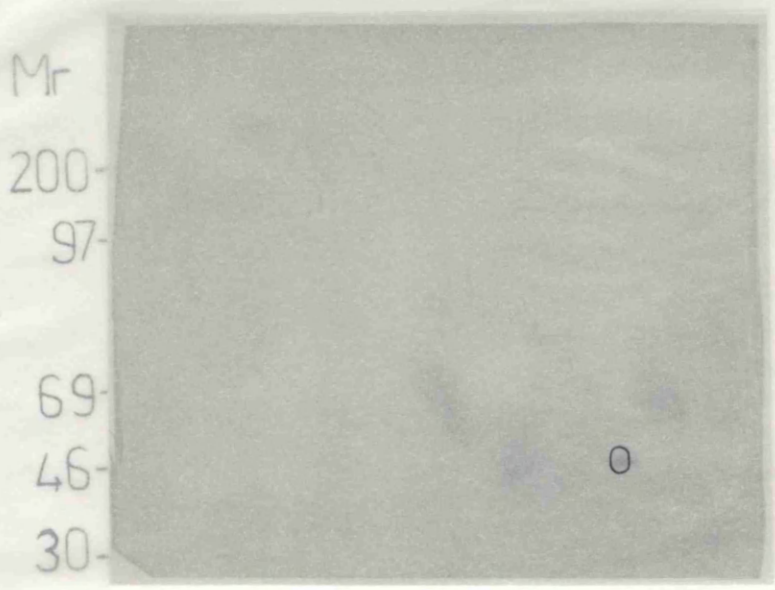

Mr
200
97
69
46
30

Figure 5.13: The detection of actin in the two dimensional system

A two dimentional gel of crosslinked (DTBP) was blotted onto nitrocellulose, and probed for actin, using a murine anti chicken gizzard anti actin monoclonal antibody (Ig M, Amersham) and used at a titre of 1:500. Horseradish peroxidase activity was detected using Diaminobenzidine.

The overlay indicates the position of the actin band.

This is representative of three experiments.


Mr
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69-
46-
30-
0

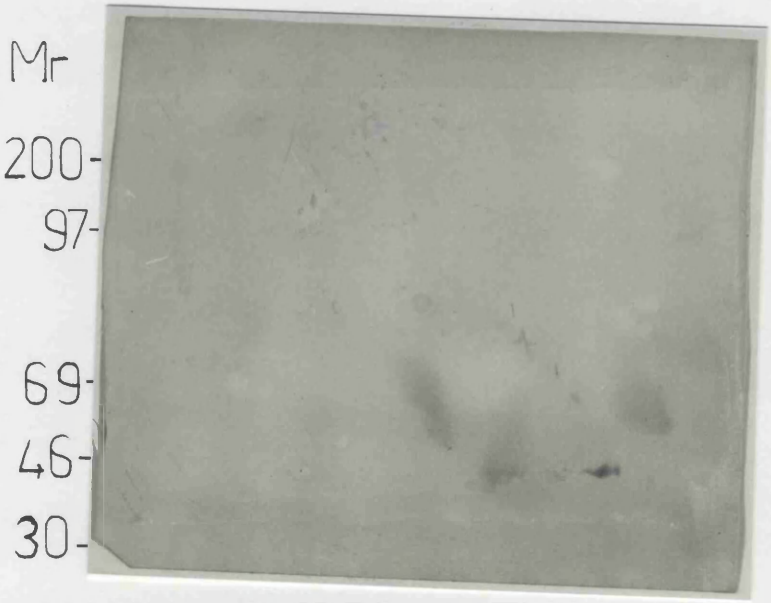

Mr
200-
97-
69-
46-
30-

Figure 5.14: The effect of serotonin on protein-protein interactions

Figure 5.14 Crosslinked membranes were disolved in sample buffer by leaving it at room temperature for 30 min. The sample was then run in the first dimension (5% acrylamide), and once run, the track was cut out and reduced (10 min. boiling in 2% (w/v) SDS, 0.5 % (v/v) ß-mercaptoethanol and 0.1M tris, pH 6.5. After reduction, the gel was laid on top of an other 5% gel.

Figure 5.14a: this shows normal worm membrane protein-protein interactions.

Figure 5.14b: this shows the membrane protein-protein interactions of worms treated with 7 µM serotonin.

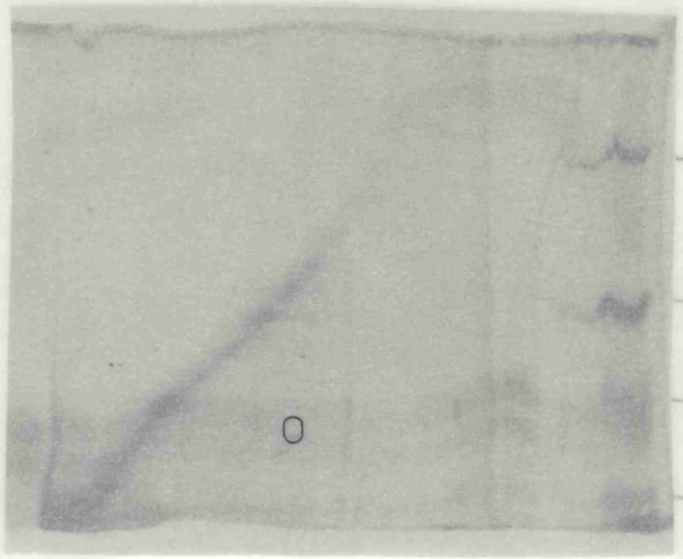
The same amount of protein was run on both gels.

The overlay indicates the crosslinked protein.

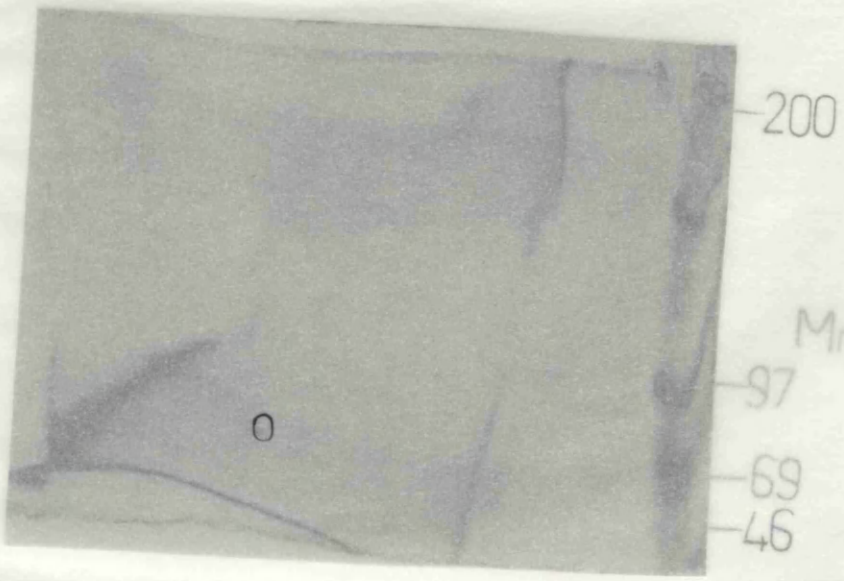
Proteins were stained with 0.1% coomassie blue.

This gel is representative of 4 experiments.

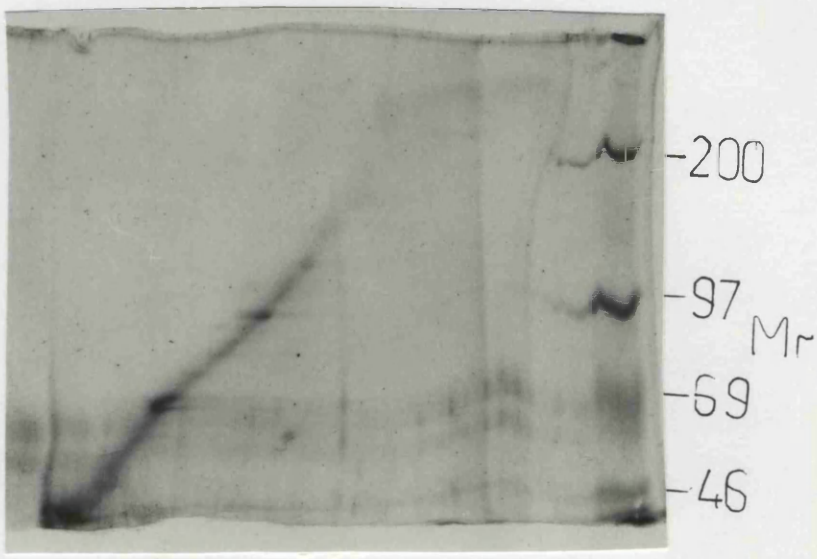
a



b



a



b

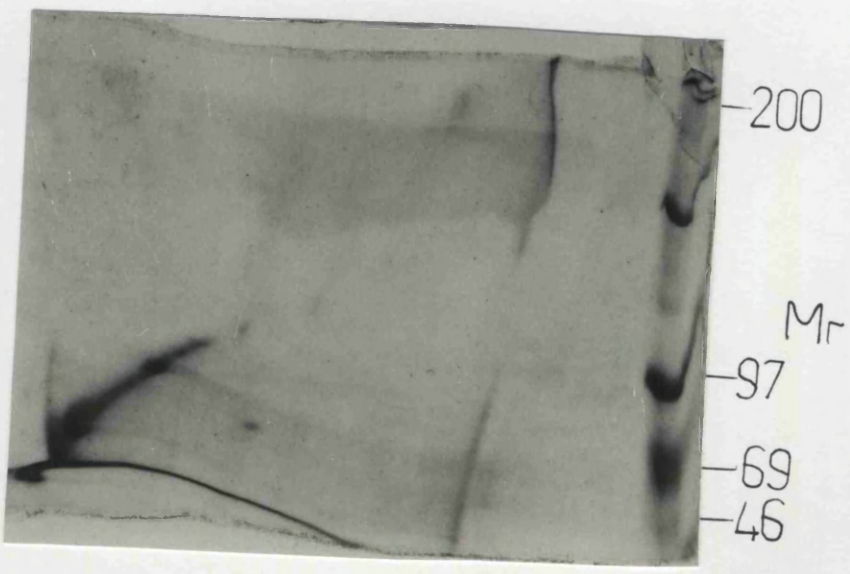


Figure 5.15: The effect of serotonin on protein-protein interactions

Figure 5.15 Crosslinked (DTBP) membranes were disolved in sample buffer by leaving it at room temperature for 30 min. The sample was then run in the first dimension (7% acrylamide), and once run, the track was cut out and reduced (10 min. boiling in 2% (w/v) SDS, 0.5 % (v/v) ß-mercaptoethanol and 0.1M tris, pH 6.5. After reduction, the gel was laid on top of an other 7% gel.

Figure 5.15a: this shows normal worm membrane protein-protein interactions.

Figure 5.15b: this shows the membrane protein-protein interactions of worms treated with 7 µM serotonin.

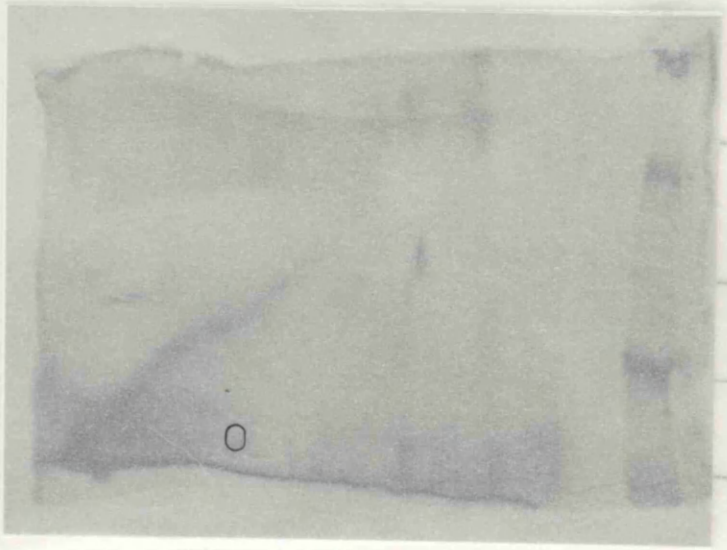
The same amount of protein was run on both gels.

The overlay indicates the crosslinked protein.

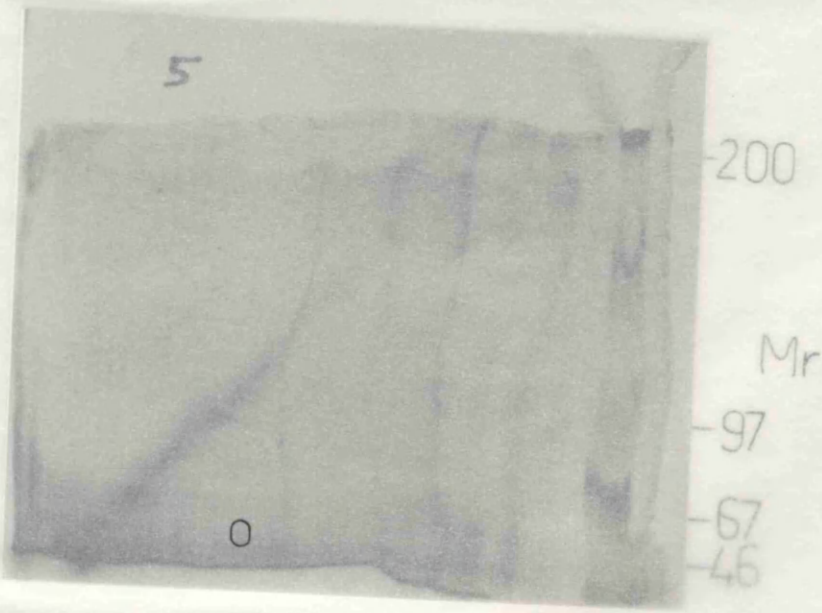
Proteins were stained with 0.1% coomassie blue.

This gel is representative of 4 experiments.

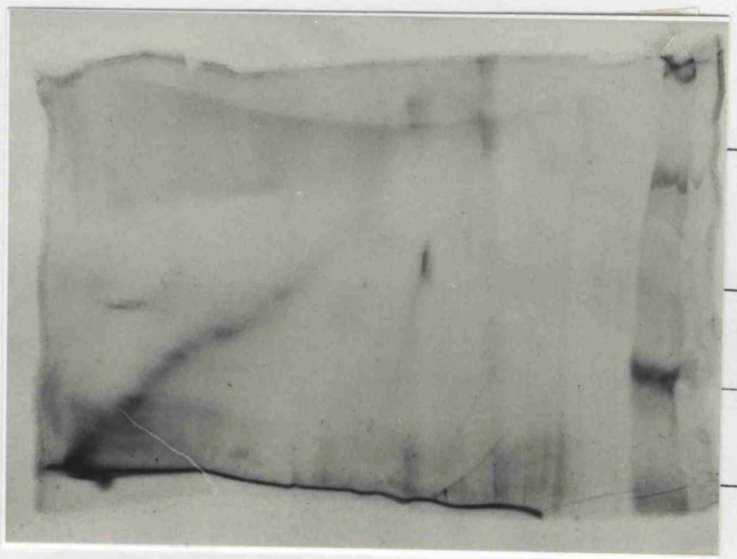
a



b



a



b

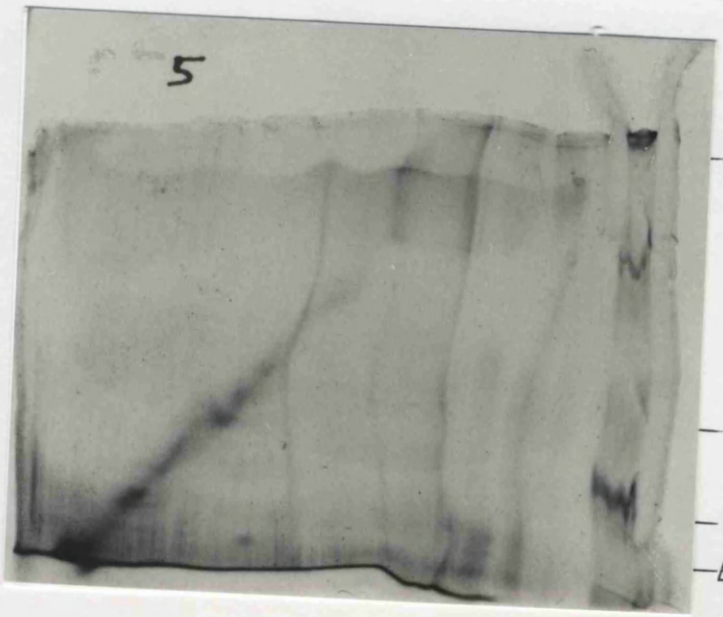


Figure 5.16: The effect of serotonin on the DTSSP crosslinked high molecular weight complex.

Figure 5.16 Crosslinked membranes were disolved in sample buffer by leaving it at room temperature for 30 min. The sample was then run in the first dimension (7% acrylamide), and once run, the top of the track was cut out and reduced (10 min. boiling in 2% (w/v) SDS, 0.5 % (v/v) ß-mercaptoethanol and 0.1M tris, pH 6.5. After reduction, the gel was laid on top of an other 7% gel.

Lane a: Molecular weight markers

Lane b: Crosslinked membrane from normal worms

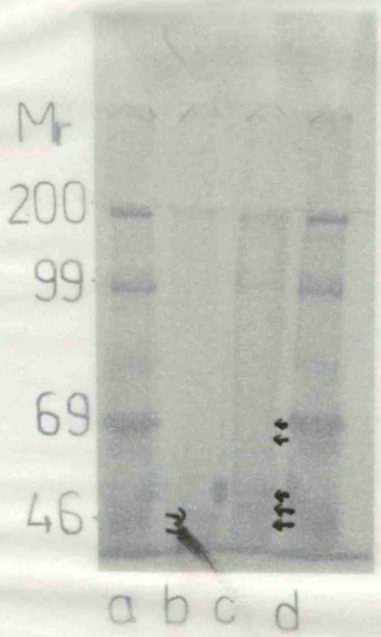
Lane c: Crosslinked membrane from worms treated with 7 µM serotonin

Lane d: Molecular weight markers.

The overlay indicates the positions of the bands.

Proteins were stained with 0.1% coomassie blue.

This gel is representative of 4 experiments.


Mr
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a b c d

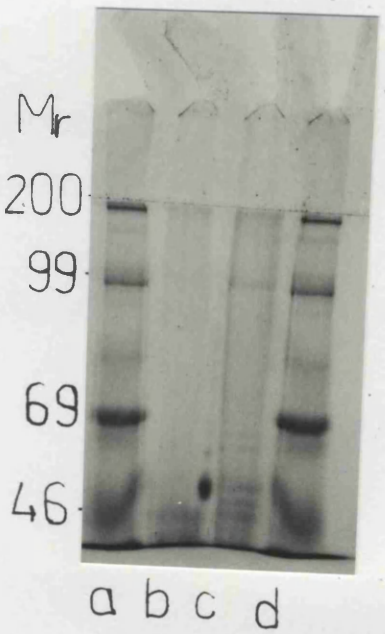
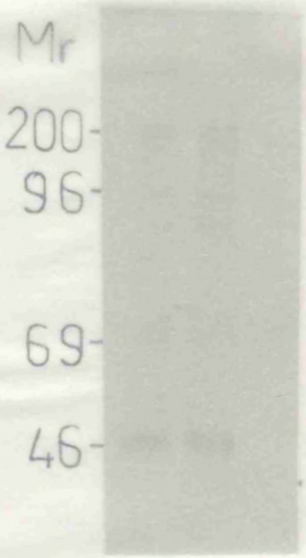

Mr
200
99
69
46
a b c d

Figure 5.17: The membrane permeability of sulpho-NHS biotin.

This is a western blot of adult male worms that were were either injected with 0.01μg of Bovine serum albumin per worm (lane a), or an equivalent volume of saline and were allowed a three hour recovery period at 37^0C in GMEM, following which they were labelled with biotin (lane b)(30min. at 20^0C in a buffer containing 0.5 mg sulpho-NHS biotin ml^{-1}, 1.9 mM Ca Cl_2, 5.6 mM K Cl, 1.7 mM Mg SO_4, 100 mM Na Cl and 60 mM D-glucose. The pH was made to 8 with 3.3 mM carbonate buffer).

Biotinylated proteins were detected using horse radish peroxidase conjugated streptavidin, at a titre of 1:200

Any biotinylated BSA would run at Mr 67 k Da.



a b

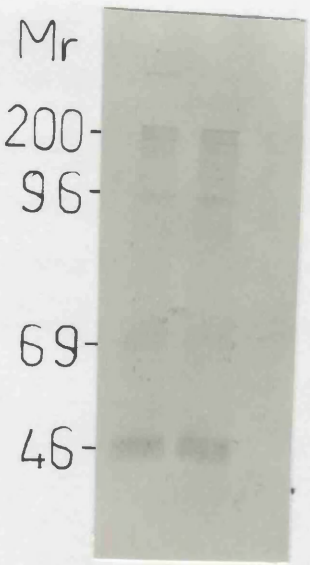

Mr
200-
96-
69-
46-
a b

Figure 5.18: The labelling of the surface with biotin.

Figure 5.18a shows a western blot of biotin labelled f Triton- X100 soluble (non cytoskeletal) membrane proteins: equal ammounts of protein was added to each lane.

Lane a: Molecular weight markers

Lane b: Membrane from worms treated with 0 μM serotonin

Lane c: Membrane from worms treated with 5 μM serotonin

Lane d: Membrane from worms treated with 7 μM serotonin

Lane e: Membrane from worms treated with 10 μM serotonin

Lane f: Membrane from worms treated with 20 μM serotonin

Lane g: Molecular weight markers.

Figure 5.18b shows the labelling of Triton- X100 insoluble (cytoskeletal) membrane proteins: equal ammounts of protein was added to each lane.

Lane a: Molecular weight markers

Lane b: Membrane from worms treated with 0 μM serotonin

Lane c: Membrane from worms treated with 5 μM serotonin

Lane d: Membrane from worms treated with 7 μM serotonin

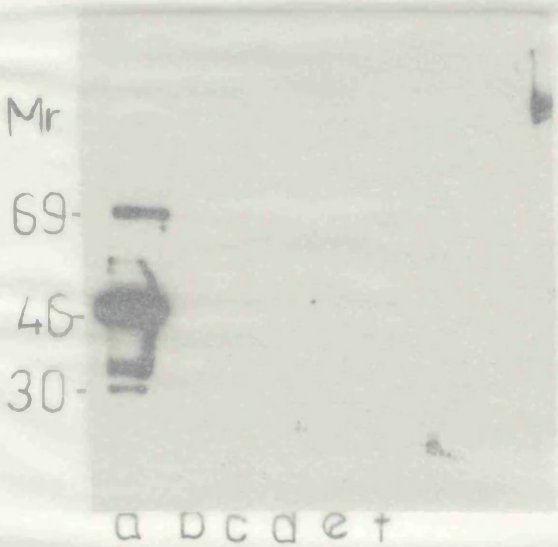
Lane e: Membrane from worms treated with 10 μM serotonin

Lane f: Membrane from worms treated with 20 μM serotonin

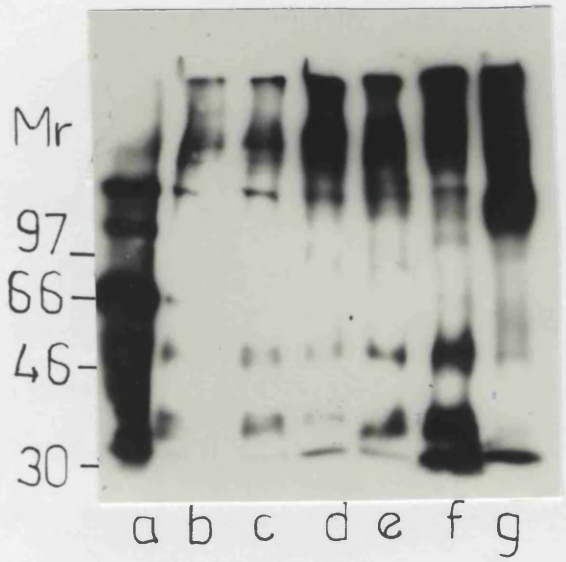
Biotinylated proteins were detected using horse radish peroxidase conjugated streptavidin, at a titre of 1:200.

This is representative of 3 experiments.

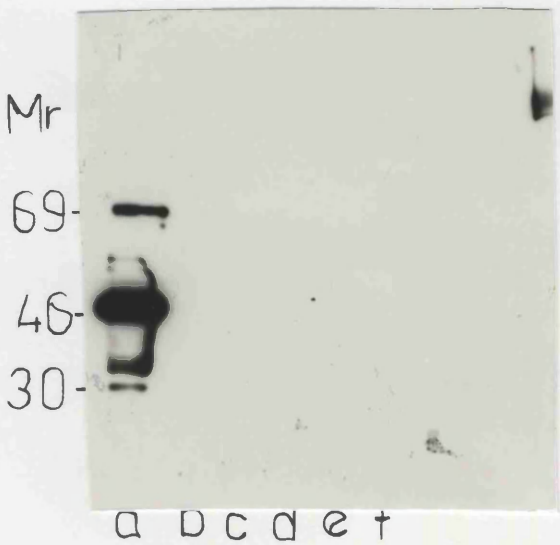
a



b



a



b

Figure 5.19: The surface exposed molecules of the DTSSP high molecular weight complex.

Biotinylated membranes were crosslinked and disolved in sample buffer by leaving it at room temperature for 30 min. The samples were then run in the first dimension (7% acrylamide), and once run, the top of the track was cut out and reduced (10 min. boiling in 2% (w/v) SDS, 0.5 % (v/v) ß-mercaptoethanol and 0.1M tris, pH 6.5. After reduction, the gel was laid on top of an other 7% gel. The blots were developed using Horse raddish peroxidase conjugated streptavidin and diaminobenzidine.

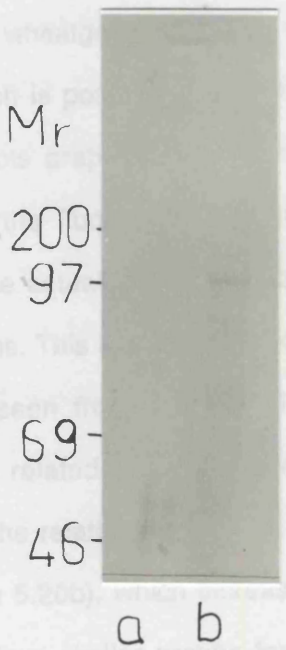
Lane a: The high molecular weight complex.

Lane b: Whole biotinylated membrane.

This is representative of 3 experiments.

The overlay indicates the positions of the surface associated bands.


Mr
200
97
69
46
a b


Mr
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a b

worms were labelled with the membrane impermeant labels FITC wheat germ agglutinin, FITC anti BALB/c erythrocyte antiserum and 5AF. The labelled worms were extracted with triton X-100, and the remaining surface fluorescence quantitated. The effect of serotonin was also assesed on the amount of bound fluorescence. Figure 5.20a (upper line) shows the effect of serotonin on the fluorescence of FITC wheatgerm agglutinin, which causes a dose dependent decrease in fluorescence which is possibly due to membrane renewal [Podesta, 1989]. This line therefore corrects graph 5.20b for this source of ligand loss. Figure 5.20a (lower line) shows the fluorescence of the triton X-100 extracted worms, it is worth noting that the actual values of fluorescence in some instances are higher than in normal worms. This appears to be due to a fluorescence enhancing effect of the detergent, as seen from figure 5.21, which also shows that fluorescence intensity is linearly related to fluorophore concentration. Subtracting the lines in figure 5.20a gives the relative amount of FITC wheatgerm agglutinin bound to the cytoskeleton (figure 5.20b), which increases in a dose dependent manner. Figures 5.20c and 5.20d show similar graphs for 5AF and FITC anti BALB/c erythrocyte antiserum respectively. These graphs show that FITC wheatgerm agglutinin, 5AF and FITC anti BALB/c erythrocyte antiserum all have ligands on the surface of *S. mansoni* that are capable of interacting with the underlying cytoskeleton after serotonin treatment, and these associations can be inhibited by the serotonin antagonist methiotheipin.

5.4. Lipid protein interactions

From the data presented above, it would suggest that fluidity can be regulated by altering the affinity of lipid for protein (section1.3.2.). The experiment below examined the possibility of this occurring, by labelling worms with ^{14}C-palmitate. The membranes were removed after labelling and extracted in Triton X-100 and washed five times. Figure 5.22 shows the amount of radioactivity left

Figure 5.20: The expression of cytoskeletal changes at the surface

Worms were labelled with FITC wheat germ agglutinin, and then treated with a range of serotonin concentrations, and the fluorescence quantitated (◆). At the same time, another batch of worms were treated with serotonin and extracted in Triton X100 (○) (figure 5.20a). These two lines were subtracted to give (□) in figure 5.20b. Line (◆) in figure 5.20b are worms pretreated for 15 min with 10 μM methiotheipin. This line was constructed as above.

Figure 5.20c Worms were labelled with FITC anti BALB/c erythrocyte serum, and treated as above. Line (◆) is the serotonin treated worms Line (□) are worms pretreated for 15 min with 10 μM methiotheipin.

Figure 5.20d Worms were labelled with 5AF, and treated as above. Line (□) is the serotonin treated worms Line (◆) are worms pretreated for 15 min with 10 μM methiotheipin.

Figure 5.20a: the effect of serotonin on "membrane cytoskeleton " WGA receptors.

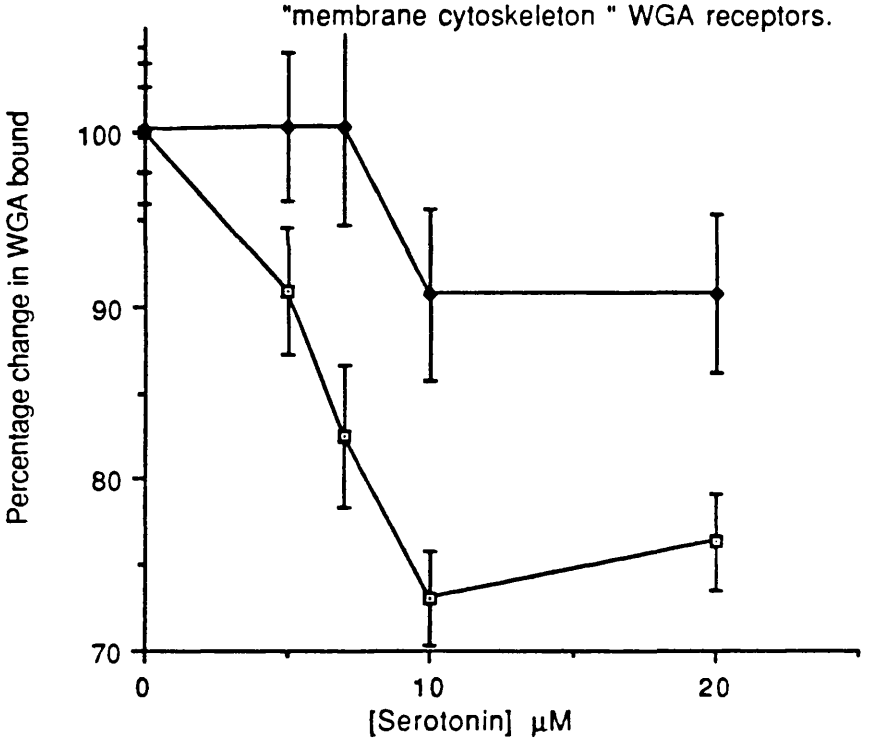


Figure 5.20b: the effect of serotonin on the ability of Triton X100 to extract tegumental WGA receptors

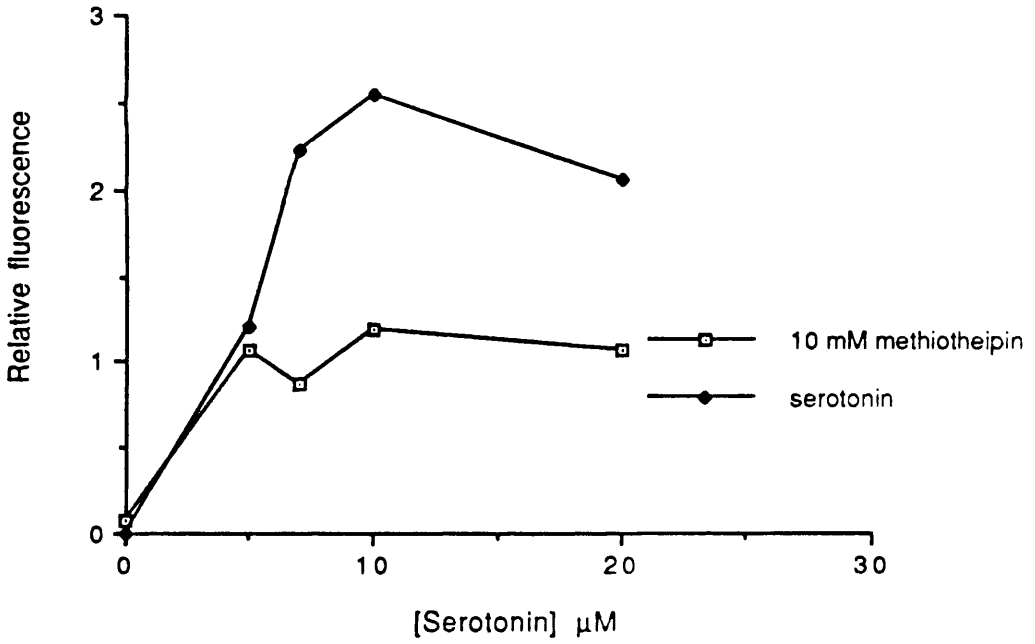


Figure 5.20c: the effect of serotonin on the ability of Triton X100 to extract host molecules from the tegument

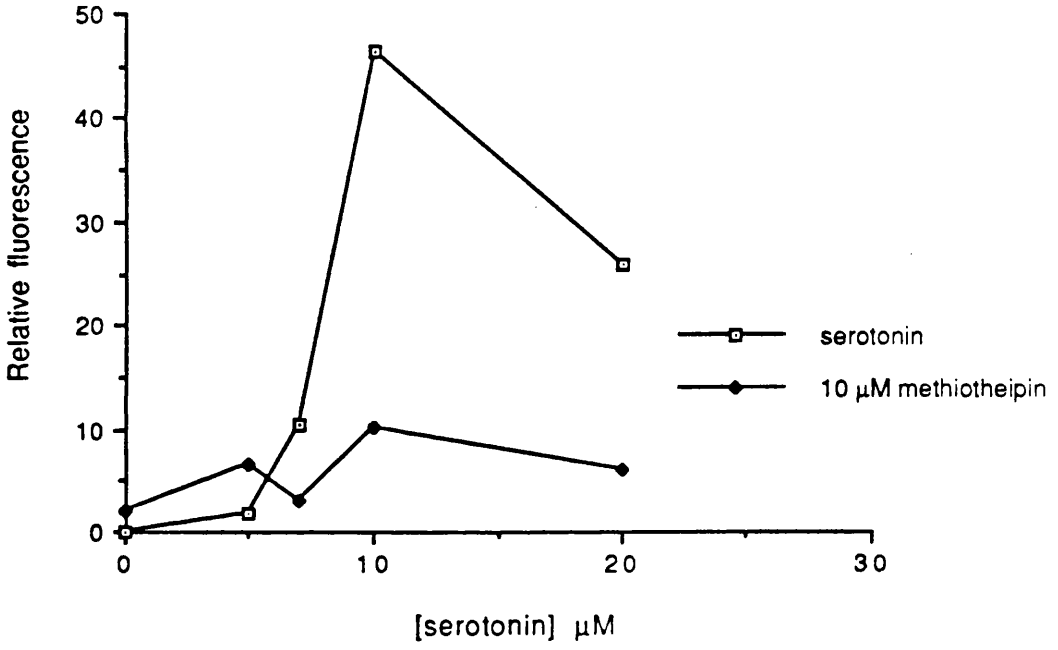


Figure 5.20d: The effect of serotonin concentration on 5AF extractability in the tegument

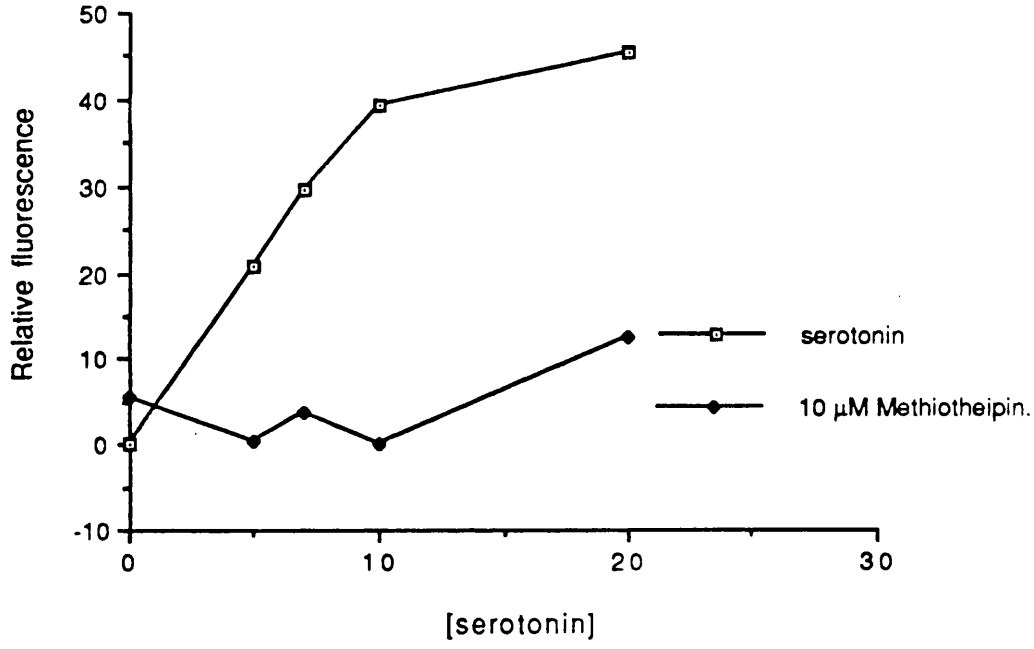


Figure 5.21: The effect of Triton X100 on the quantum yield of FITC WGA.

The fluorescence intensity of fluorescein was measured by fluorimetry with detergent concentration (○), and probe concentration at constant detergent concentration

(◆).

Figure 5.21: the effect of triton X100 on the quantum yield of FITC

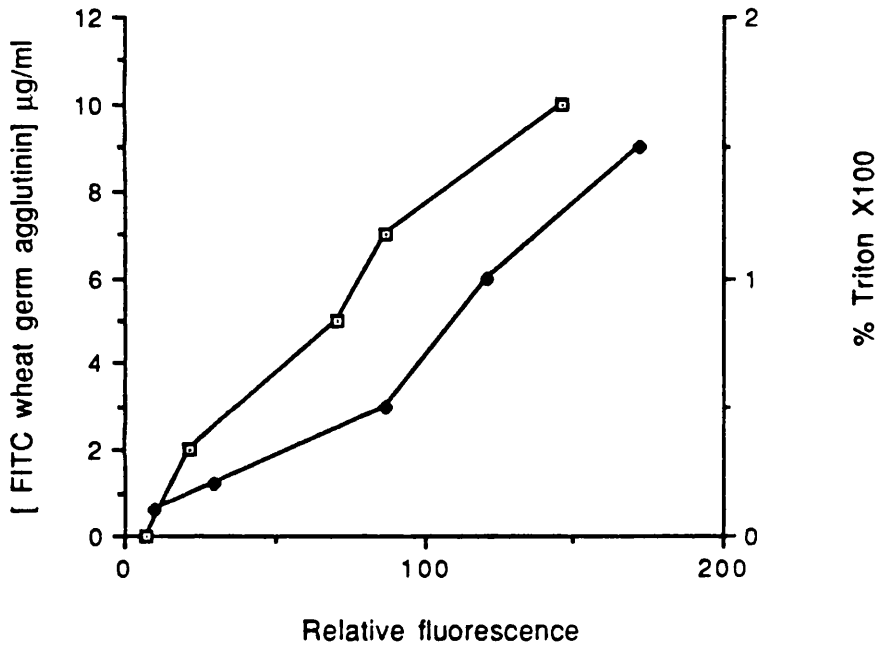


Figure 5.22: Lipid cytoskeletal Interactions.

The efficiency of extraction of membranes in Triton X100 is shown for normal membranes (columns W1-W5), and 7 μM serotonin treated worms (columns W1-W5). The graph shows the ammount of ^{14}C palmitate left in the supernatant of membranes after 30 min extractions and washing in Triton buffer. Extraction

is complete after the fourth wash.

The graph shows the mean ± 1 S.D of triplicate readings that are representative of 3 experiments.

Figure 5.22: lipid cytoskeletal interactions

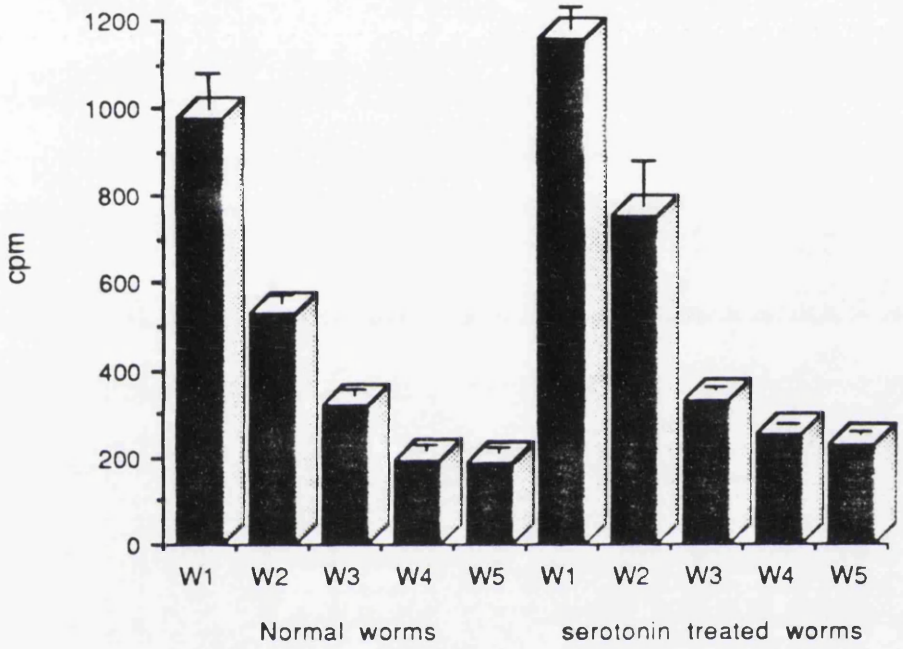


Table 5.2. The effect of serotonin on the amount of lipid associating with the tegumental cytoskeleton.

Adult male schistosomes were incubated with ^{14}C-Palmitate for 3 hours. After this time, the worms were either treated with 7μM serotonin for 15 min, or given no treatment. The membranes were removed, and extracted in triton X-100, and the protein content and the amount of bound lipid was measured.

* $P < 0.05$ (Student's t-Test)

Table 5.2.

Treatment	n	(A) ^{14}C-Palmitate incorporation (cpm)	(B) [protein]µg	(A)/(B)
None	3	206.61 ± 41.2	2.1 ± 0.5	98.38
7µM serotonin	3	398.27 ± 61.4	2.5 ± 0.4	159.3*

in the Triton X-100 washing buffer after subsequent washes, and shows extraction is complete after the fifth wash. The remaining pellet was resuspended in Triton buffer and three 2µl samples removed, and extracted in 50µl of chloroform methanol (2:1) and centrifuged to pellet out the cytoskeletal proteins. The supernatant was removed and the radioactivity was measured, and the protein concentration was also assayed. Following this procedure, those worms that were treated with 7 µM serotonin, had more radioactivity associated with the cytoskeleton (table 5.2).

5.5. Discussion

This chapter has investigated the molecular changes that occurs in tegumental membranes when serotonin is added to worms. The results follow up the finding of chapter 4 that phalloidin can inhibit serotonin induced fluidity changes. The results of this chapter indicate that actin polymerisation occurs in response to serotonin. This polymerisation can be visualised by the use of FITC labelled phalloidin as an increase in the binding of this probe to the membrane, or as an increase in the thickness of bundles of actin filaments at the base of the tubercules. Polymerisation of actin can also be demonstrated by western blotting. This technique shows the presence of a novel cytoskeletal pentameric form of actin in the tegument. Whether this pentameric actin is forming a skeletal network (a structure that has remained elusive in schistosomes [Cain and Abbas, 1987] such as that of the erythrocyte [Bennet, 1985] or forms part of the paracrystalline tegumental spines is unknown. It was however not possible to demonstrate the polymerisation of actin by crosslinking. This may be due to steric Hindrance of the probes [Pierce and Warriner , 1990], and any future studies could employ crosslinkers of different flexibilities.

It was however shown that crosslinking can produce a high molecular weight complex in the tegumental membrane, and that serotonin can increase the number

of peptides associating with it. These may allow cytoskeletal polymerisation, and may be phosphorylated after receptor ligation [Podesta, 1987], which may allow polymerisation (see section 4.2) [Furuhashi and Hatano, 1990]. Two of these proteins are surface expressed, one of Mr 48 KDa and another of Mr 52 KDa. It is possible that these proteins play an important role in the development of serotonin induced changes at the tegumental surface (increased lipid, host antigen and glycoconjugate binding to the Triton X-100 extracted surface).

Crosslinking did however, reveal the existence of a dimer (monomer molecular weight 48 KDa). Nothing can be said of what the function of this molecule may be, but it is interesting to note that ion transporters in erythrocytes are dimeric and can be crosslinked [Kopito and Lodish, 1985].

Another interesting finding revealed by crosslinking is that for the impermeant DTSSP, membrane fluidity correlates with the ability of this agent to crosslink. This supports the idea that proteins are arranged in the membrane in such a way that collisions between them are rare events [Kusel and Gordon, 1989], and this could mean that there is only one protein per domain.

Chapter 6

6.1. General discussion

In biological membranes, fluidity is important in regulating the rate of second messenger production [Atlas et al, 1980], enzyme activities such as allosteric enzymes [Farias et al, 1975] and the enzyme adenylate cyclase [Rimon et al, 1978], which increases its activity in cercariae upon transformation [Estey and Mansour, 1987], at the same time as fluidity is changing [Foley et al, 1988]. Changes in fluidity also correlate with activation of various cells [Mountford and Wright, 1988] and ova upon fertilization [Wolf et al, 1981]. The rate of phagocyte attachment to haptenated liposomes is also fluidity dependent [Lewis et al, 1980], as may be complement mediated damage to membranes [Shin et al, 1979]. It is therefore essential to understand fluidity of the schistosome surface and its modification if a full understanding of its role in host parasite interactions is to be achieved. Further understanding may lead to new targets for antiparasite drugs.

It is demonstrated in this thesis, that serotonin can induce a decrease in the fluidity of those areas of membrane accessible to the probe 5AF. This serotonin is of host origin, as the parasite lacks the ability to synthesise serotonin, due to the absence of the enzyme tryptophan hydroxylase [Benett and Bueding, 1973]. This raises the interesting philosophical question of whether the parasite is an extension of the host genome. In a sense, it probably is since it uses products of host genes, in this case serotonin, which was produced by host enzymes encoded by host genes. However, the host could be considered an extension of the parasite genome since it produces a compound needed by the parasite, which has lost its machinery for producing serotonin. This however is a host dependent phenomenon, which was the selection against the possession of serotonin synthetic enzymes in the presence of an environment rich in serotonin. With

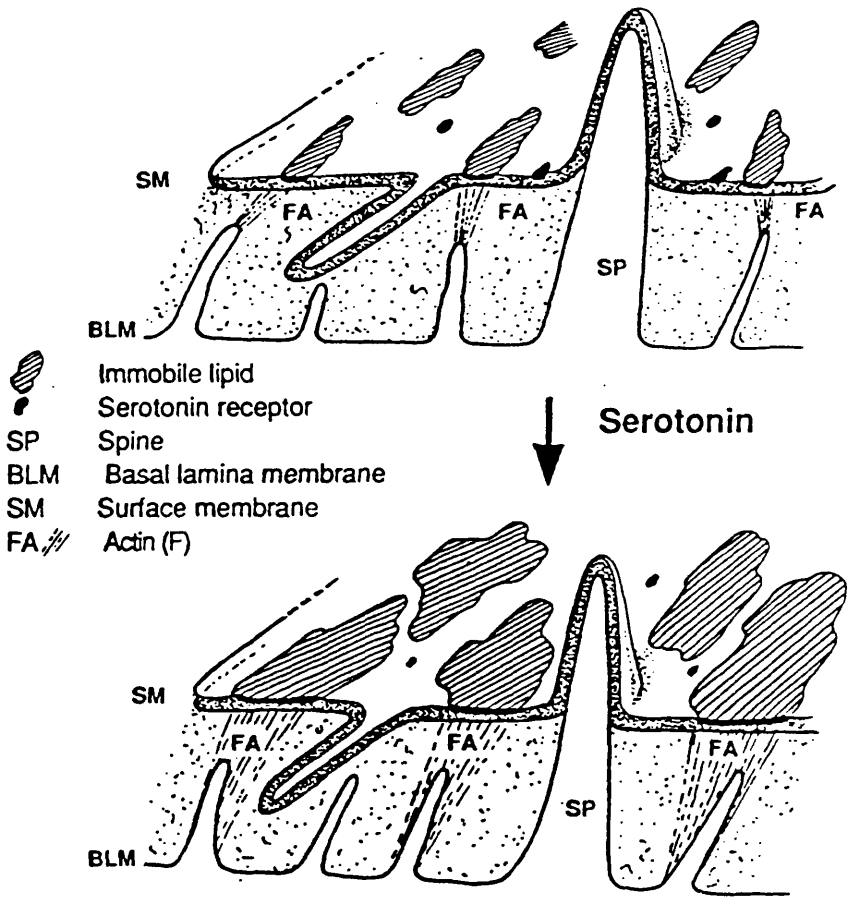
respect to serotonin, the parasite is probably not an extension of the host genome, since it is of no selective advantage to the host to be parasitised. When one considers the juvenile parasite in the snail, it is clear that the parasite contains genes that affect host reproduction [Thornhill, 1987]. These must therefore be considered to be genes for parasite reproduction, as it ensures a new generation of susceptible snails. In this case then, the snail is an extension of the parasite genome.

The involvement of the cytoskeleton, in particular actin, has been implicated in playing a central role in organising the changes in fluidity that serotonin induces. As discussed in chapters four and five, phosphorylation is a possible post translational modification which may bring about the observed changes in cytoskeletal content. Calcium binding proteins could also be involved in the organisation of cytoskeletal polymerisation. Recently, such calcium binding proteins have been demonstrated to be present in schistosomes [Havercroft et al 1990], one of which is gelsolin [Siddiqui et al 1991], a calcium activated actin fragmentor. This could allow actin polymerization to occur when the calcium levels decrease in the tegument in response to serotonin.

Figure 6.1 shows a hypothetical model of the tegumental membrane, and how it may be arranged into domains. The figure shows the possible interactions of these domains with actin filaments. It is shown that serotonin receptor ligation causes an increase in the size and the number of immobile environmentally sensitive domains in the membrane, and that there is an increase in the number of actin filaments associated with them. This polymerisation somehow causes changes at the surface, and how these are mediated is unknown. It is possible that actin filaments interact with the two surface exposed proteins described in figure 5.19. These may also be the putative membrane tethering proteins of section 3.1.4. It would be of value to attempt to clone these proteins, and obtain information about their possible function from sequence data.

Figure 6.1

Hypothetical model of the surface of adult *Schistosoma Mansoni*



Many of the measurements in this thesis have been carried out using 5AF as the membrane lipid analogue. When NBD PC was used no change in membrane properties were seen upon addition of the ligand. This suggests that a very heterogeneous surface exists in the schistosome, as does the fluidity dependence of crosslinking, with some regions of this surface not being greatly altered by ligand binding. It is unlikely that the fluidity dependence of crosslinking is due to conformational changes in the proteins, as decreasing fluidity makes membrane proteins more accessible to biotin (section 5.2.)

Finally, it is clear that the observed changes are intrinsic to the membrane, and do not require changes in lipid composition, as isolated membranes are quite capable of reacting to serotonin in a manner similar to whole worms.

In this thesis we have shown that membrane fluidity of regions of the surface accessible to 5AF can be regulated by altered levels of serotonin and other ligands all of which affect metabolism. Such changes are rapid (fig 4.2), and if they occur continually in vivo in response to environmental change, they may be crucial for the functioning of receptors in a hostile environment. These changes may also prevent antibody from binding divalently, [Oi et al, 1984]or reduce complement damage [Shin et al, 1979].

The finding that serotonin can alter membrane fluidity leaves the interesting possibility that other host factors such as insulin may affect fluidity. This could be a way of regulating glucose uptake by the parasite, by altering fluidity and as a result glucose transporter activity. This would allow the regulation of glucose uptake at times when glucose is abundant in the hepatic portal vein.

6.2. Future work

The logical continuation of this work would investigate the biological significance of membrane fluidity changes, and would concentrate on the following areas: membrane transport, resistance to specific and non specific immune mechanisms

and the effect on surface enzymes and receptors.

A change in membrane fluidity might be expected to affect transport processes, and techniques are available to measure the uptake of ^{14}C aminobutyric acid and ^{3}H deoxyglucose [Breternitz et al, 1992]. The uptake of the fluorescent lipids NBD-ceramide and NBD- dodecanoic acid can also be studied [Moffat and Kusel 1992].

Concerning resistance to specific and non specific immune mechanisms, decreases in fluidity could also cause increased resistance to damage [Shin 1979]. The extent of damage by complement and *Pseudomonas aeruginosa* cytotoxin could be assesed by immunofluorescence studies of the amount of C9 or cytotoxin that inserts into the membrane. The relative sizes and longevity of the pores can be assessed using molecules of varying sizes as rulers. Electrophysiological techniques can also be employed to study membrane damage [Breternitz et al, 1992].

The effect of serotonin on membrane enzymes and receptors can be studied by looking at the effects of serotonin on the binding of labelled ligands such as low density lipoprotein and its effects on the activities on membrane bound enzymes such as alkaline phosphatase, glutathione-S-transferase and non specific esterase.

Other lines of pursuit could be followed, including the investigation of the lipid protein interactions in the membrane. Characterisation of these and how they are modified by serotonin could yield interesting and unusual information on how schistosome proteins can interact with lipid as these must be unusual since vectorial labelling with photoactivatable nitrenes cannot be acheived with any great success [Kusel and Gordon, 1989]. Initial experiments could measure the accessibility of lipids for the proteins by measuring the transfer of energy from protein tryptophan residues to parinaric acids [Kindman , 1979], or measuring the quenching of tryptophan fluorescence by bromylated

phospholipids [Kindman, 1979]. More specific studies on proteins, in particular phosphoproteins could be made, comparing their behaviour in liposomes, and how this is changed by the action of phosphatases and kinases on them. The techniques of Fourier transform infra red spectroscopy and differential scanning calorimetry could also be used here to measure the stoichiometry of the lipid-protein interaction, the temperature and the enthalpy change of the lipid phase transition, as well as the lipid cooperativity of the transition. Biophysical studies on the affinity of proteins for lipids, and annular lipid exchange rates with the bulk phase could be measured using electron spin resonance spectroscopy [Sankaram et al, 1989]. The interactions that occur between proteins could also be studied using similar techniques.

In addition to lipid-protein interactions, actin binding proteins could be studied, by running worm homogenates down F-actin columns, and the effect of serotonin induced phosphorylation on then could be studied, by looking to see how this affects their binding to the columns.

Finally, it would be worthwhile extending these studies to other stages of the life cycle, and trying to correlate them with major developmental changes, such as synthesis of the double membrane [McLaren, 1973], the developmental increase in adenylate cyclase activity [Estey and Mansour, 1987], and the onset of intrinsic resistance [Moser et al, 1979], in an attempt to assign functions to the biophysical properties of molecules in vivo instead of hypothesising about function from unrealistic model systems that ignore the compositional and behavioral complexities of living material as in work with liposomes.

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